

Selection of a Rare Neutralization-Resistant Variant following Passive Transfer of Convalescent Immune Plasma in Equine Infectious Anemia Virus-Challenged SCID Horses[∇]

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Vaccines preventing HIV-1 infection will likely elicit antibodies that neutralize diverse strains. However, the capacity for lentiviruses to escape broadly neutralizing antibodies (NAbs) is not completely understood, nor is it known whether NAbs alone can control heterologous infection. Here, we determined that convalescent immune plasma from a horse persistently infected with equine infectious anemia virus (EIAV) neutralized homologous virus and several envelope variants containing heterologous principal neutralizing domains (PND). Plasma was infused into young horses (foals) affected with severe combined immunodeficiency (SCID), followed by challenge with a homologous EIAV stock. Treated SCID foals were protected against clinical disease, with complete prevention of infection occurring in one foal. In three SCID foals, a novel neutralization-resistant variant arose that was found to preexist at a low frequency in the challenge inoculum. In contrast, SCID foals infused with nonimmune plasma developed acute disease associated with high levels of the predominant challenge virus. Following transfer to an immunocompetent horse, the neutralization-resistant variant induced a single febrile episode and was subsequently controlled in the absence of type-specific NAb. Long-term control was associated with the presence of cytotoxic T lymphocytes (CTL). Our results demonstrate that immune plasma with neutralizing activity against heterologous PND variants can prevent lentivirus infection and clinical disease in the complete absence of T cells. Importantly, however, rare neutralization-resistant envelope variants can replicate *in vivo* under relatively broad selection pressure, highlighting the need for protective lentivirus vaccines to elicit NAb responses with increased breadth and potency and/or CTL that target conserved epitopes.

Development of an effective vaccine will be critical in the efforts to control the human immunodeficiency virus type 1 (HIV-1) pandemic. Unfortunately, vaccines evaluated in completed human efficacy trials have shown moderate to no protective effects, and, clearly, much more work is needed to define the correlates of lentivirus immune protection. Although these correlates are still not entirely known, vaccine strategies that elicit antibodies with broad neutralizing activity are currently of considerable interest, and it is widely believed that HIV-1 envelope glycoproteins that induce broadly neutralizing antibodies (NAbs) will be critical components of a protective vaccine (21, 28, 53, 63).

Equine infectious anemia virus (EIAV) is a macrophage-tropic lentivirus that causes persistent infection in horses worldwide and serves as an important large-animal translational model in which to dissect basic correlates of protective lentiviral immunity (9, 31, 33, 38, 57). EIAV is a naturally occurring lentivirus, and infection results in a predictable course of recurrent episodes of plasma viremia and clinical disease. As with HIV-1 and simian immunodeficiency virus (SIV), EIAV infection is not cleared. However, infected horses eventually control viral replication and clinical disease to remain persistently infected inapparent carriers.

Adaptive immune responses, including NAbs, are required for EIAV control since young horses (foals) with severe combined immunodeficiency (SCID), unlike normal foals, fail to eliminate the initial viremia following challenge (46). Equine SCID is caused by a frameshift mutation in the gene encoding the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) (55, 60) and has an autosomal recessive mode of inheritance (47). The equine SCID defect is more severe than its murine counterpart in that SCID foals are incapable of forming either coding or signal joints (55). Adoptive transfer of EIAV-specific T and B lymphocytes to a SCID foal results in functional cytotoxic T lymphocytes (CTL) and NAb activity and is protective against homologous EIAV challenge (33).

During acute EIAV infection, each recurrent episode coincides with the emergence of an antigenically distinct EIAV variant as defined by type-specific NAb, which neutralizes virus isolated during early disease episodes but not virus isolated during subsequent disease episodes (2, 20, 22, 43, 52). Amino acid variation primarily occurs within hypervariable regions V1 to V8 of the envelope gp90 surface unit (SU) and particularly within the V3/principal neutralizing domain (PND) region (1, 19, 24, 25, 57). Our work with EIAV-infected SCID foals indicates that significant envelope diversification does not occur in the absence of NAbs but that rapid envelope diversification occurs when adaptive immune responses are reconstituted (35). Thus, adaptive immunity, including NAb, drives selection of EIAV envelope variants during acute infection. Amino acid changes occur primarily within the V3 to V7 hy-

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pervariable SU regions, and many changes affect potential N-linked glycosylation sites (PNLGS) (35). Importantly, however, CTL also target the SU, and variants that escape CTL recognizing an EIAV V3/PND epitope have been identified (37, 38). Thus, both NABs and CTL are capable of contributing to the selection of EIAV SU variants, but the relative contributions of each to such selection are not known.

Recently, SU variation was evaluated in an immunocompetent pony experimentally inoculated with the virulent wild-type Wyoming strain of EIAV (57). Seventy-one distinct V3 variants that partitioned into five major nonoverlapping groups were identified and designated PND1 to PND5. Neutralization assays using chimeric infectious molecular clones containing these PNDs suggested a transition from type-specific NAB responses toward more broadly reactive immune responses during the course of infection and indicated that genetic changes conferring resistance to broadly NABs lead to recrudescence of clinical disease following a lengthy clinically quiescent period (57). Thus, the NAB response broadens significantly during long-term persistent EIAV infection, and broadly NABs play a critical role in EIAV immune control.

Studies of nonhuman primates have provided important information regarding the protective effects of NABs. Passive immunization of macaques with purified immunoglobulin from chimpanzees infected with several different HIV-1 isolates results in complete protection from homologous chimeric simian/human immunodeficiency virus (SHIV) infection when the immunoglobulin is given 24 h prior to challenge (54). Passive transfer of a triple combination of broadly neutralizing human monoclonal antibodies directed against the envelope of a primary HIV-1 isolate results in complete protection against SHIV infection in some macaques while others become infected but exhibit decreased plasma viremia (29). The contribution of T cells to partial protection in these studies is not clear, and the presence or absence of viral escape variants in the unprotected macaques has not been evaluated. In neonatal macaques, various combinations of broadly neutralizing human monoclonal antibodies directed against conserved HIV envelope epitopes administered before and after SHIV challenge result in protection against persistent systemic infection in some animals, but clinical disease develops in others (12–14). Virus-specific T-cell proliferative responses are detected in some of the protected animals, indicating that cellular immune responses occur and likely contribute to protection by eliminating infected cells (13).

Despite the fact that NABs can block experimental SHIV infection, selection pressure exerted by NABs plays a critical role in HIV-1 and SIV envelope evolution during infection, and evasion of NAB responses is an important mechanism of HIV-1 and SIV persistence (11, 16, 27, 48, 59). The maturation of a type-specific NAB response in SIV-infected rhesus macaques significantly correlates with diversification in the V1/V2 region of the SIV envelope (50). In HIV-1, NABs are detectable within the first 2 months postinfection and result in an early and significant selection force on the virus population (49). Escape from NABs involves many amino acid substitutions with little cross-neutralization between closely related strains, and NAB responses drive the diversification of the HIV-1 envelope during the early stages of infection (16). The early appearance of NABs in patients with acute HIV-1

infection results in the replacement of neutralization-sensitive virus by successive populations of resistant virus, and virus escape primarily involves changes in N-linked glycosylation (59). Thus, overcoming neutralization escape constitutes a significant barrier to the ultimate efficacy of any NAB-eliciting HIV-1 vaccine.

Because the SCID defect occurs naturally in the horse, it provides a powerful and unique opportunity to finely dissect the protective effects of immune interventions against a naturally occurring lentivirus independent of other *de novo* adaptive immune responses. This level of dissection is not possible in other lentivirus model systems. The goal of the current study was to determine if broadly NABs could protect against lentivirus challenge in the complete absence of T lymphocytes and other adaptive immune responses. We hypothesized that convalescent immune plasma from a long-term persistently infected inapparent carrier horse containing antibodies capable of neutralizing homologous and several heterologous EIAV SU PND variants would provide complete protection when infused into SCID foals before experimental virus inoculation. This plasma was administered to four SCID foals 24 h prior to challenge, and four control SCID foals received normal horse plasma. Clinical outcome, plasma viral load, and serum neutralization activity were analyzed in all foals. Although complete protection was achieved in one treated foal, infection occurred in the others. In foals that became viremic, the SU sequence and neutralization phenotype of the breakthrough virus were determined. As part of these experiments, blood containing this virus was inoculated into a naive immunocompetent horse, and the adaptive immune responses associated with its control were further evaluated.

MATERIALS AND METHODS

Horses. Foals affected with SCID were obtained by selective breeding of Arabian horses and Arabian/mixed-breed pony crosses heterozygous for the SCID trait (10, 33, 46, 47) and included four experimental and four control animals. The experimental animals consisted of three Arabian SCID foals (animals A2239, A2240, and A2241) and one pony-cross SCID foal (animal H703), each of which received infusions of immune plasma prior to virus challenge. The control animals consisted of two Arabian SCID foals (A2245 and A2247) and two pony-cross SCID foals (H707 and H713), each of which received normal horse plasma prior to virus challenge. An additional pony-cross SCID foal (H719) was used as an unchallenged control to confirm that the infused immune plasma did not contain infectious virus. Initial diagnosis of SCID was based on persistent lymphopenia ($<1,000$ peripheral blood lymphocytes [PBL] per μL) (32, 33) and was confirmed by identification of the homozygous mutation in the DNA-PKcs gene sequence (55). SCID foals were maintained as described previously (33, 36, 45) with some modifications. For the first 24 to 48 h of life, SCID foals were housed in individual box stalls with their dams to allow ingestion of colostrum. After adequate passive transfer of maternal IgG was confirmed, SCID foals were moved into isolation stalls and fed a commercial mare's milk replacer. In an effort to prevent bacterial infection as well as opportunistic lung infection by *Pneumocystis carinii*, SCID foals were administered systemic antibiotics, which included one or more of the following: trimethoprim-sulfamethoxazole (20 mg/kg of body weight, administered orally [p.o.] every 12 h [q12h]), cefpodoxime (10 mg/kg, p.o. q12h), amikacin (21 mg/kg, p.o. q24h), and chloramphenicol (50 mg/kg, p.o. q8h). SCID foals were 2 weeks of age when used in this experiment.

Immunocompetent horse A2150 was 9 years old and had been infected with EIAV strain WSU5 (EIAV_{WSU5}) for 8 years (38). This horse was a source of immune plasma for infusion into SCID foals. In addition, a 1-year-old naive immunocompetent horse (A2215) was used as a recipient of a whole-blood transfer that contained a breakthrough virus from one of the experimental SCID foals.

All SCID foals were humanely euthanized at the end of the study. The timing of euthanasia varied and was determined based on the severity of clinical signs

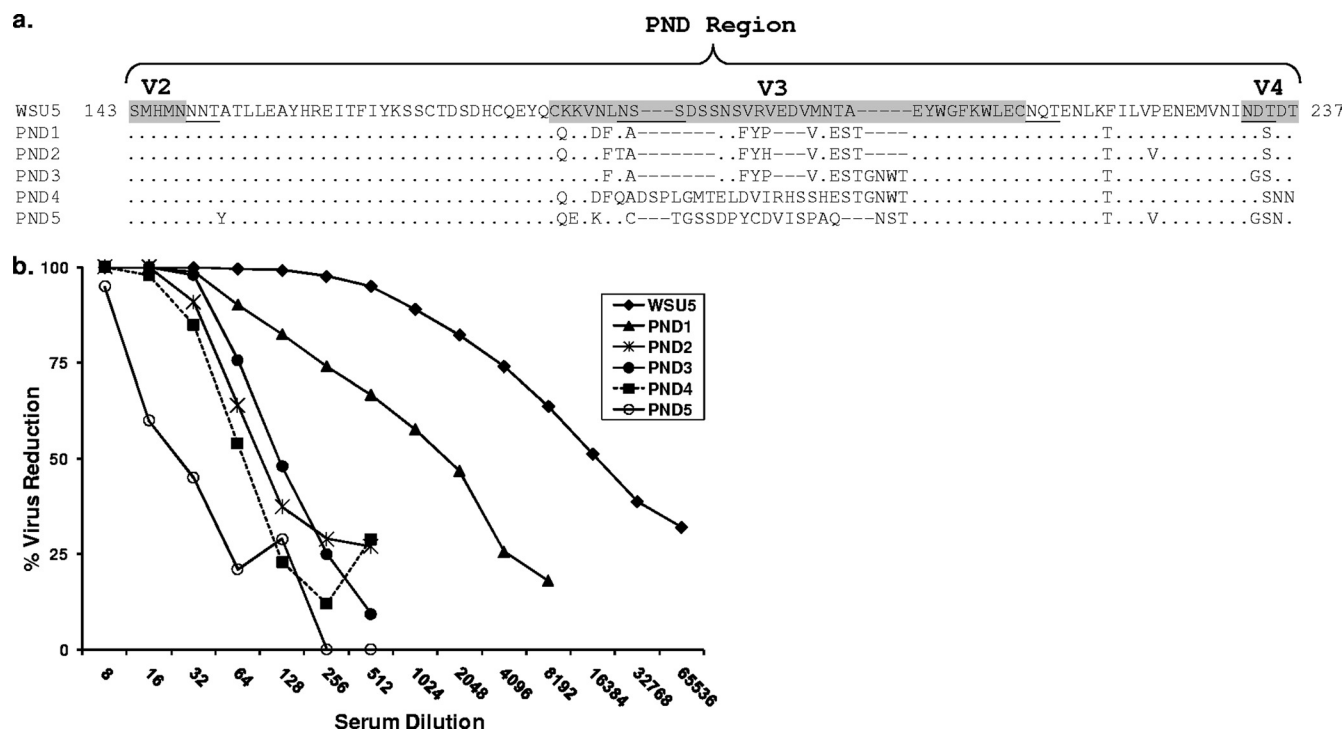


FIG. 1. (a) Deduced amino acid sequences of the SU PND region of EIAV_{WSU5} and the five heterologous PND variant molecular clones used to characterize the neutralizing breadth of A2150 immune serum. Hypervariable regions V2 to V4 are labeled and shaded. PNLGS are underlined. (b) Neutralizing activity of A2150 immune serum against homologous EIAV_{WSU5} and the five heterologous PND variant molecular clones.

associated with EIAV and/or other secondary bacterial/viral infections. All experiments involving horses and foals were approved by the Washington State University Institutional Animal Care and Use Committee.

Neutralization activity of immune plasma. Long-term EIAV inapparent carrier horse A2150 was used as the immune plasma donor in this study. Virus NAbs titers in serum from this horse were determined using a focal reduction assay on equine dermal (ED) cells (3, 57). Briefly, serum was heat inactivated to destroy complement, serially diluted 2-fold in supplemented Dulbecco's modified Eagle's medium (DMEM), and incubated at 37°C for 1 h with 550 focus forming units (FFU) of six EIAV envelope SU variant viruses (see below). Quadruplicate wells of ED cells were inoculated with the virus-serum mixture in the presence of 4 µg of Polybrene, and the culture medium was changed the following day. Cells were incubated for an additional 72 h and fixed with ice-cold methanol, and foci of EIAV-infected cells were detected by immunocytochemistry and enumerated. Results were expressed as the serum neutralization titer, which was defined as the highest serum dilution that yielded a 50% reduction in the FFU count compared with negative-control serum (3).

Viruses used in this assay included the homologous EIAV_{WSU5} inoculum (43) (GenBank accession number AF247394) and five previously described viruses generated from infectious molecular clones containing heterologous chimeric envelopes (Fig. 1a) (57). These were derived from a pony experimentally infected with the virulent Wyoming strain of EIAV (EIAV_{Wyo}), and each chimeric clone represented a predominant envelope SU genotype corresponding to a different stage of disease. The five clones were previously designated PND1 to PND5 (57) to denote differences in the PND regions among the isolates.

Preparation of immune plasma. Plasma from horse A2150 was collected aseptically and stored at -20°C. Before infusion into SCID foals, the plasma was treated with 1 µM methylene blue and white light (methylene blue-white light) as described previously (15, 40) to inactivate potential infectious virus. Briefly, plasma was thawed, and methylene blue was added, followed by exposure to white light at 2,800 lumens for 1 h. The plasma was then refrozen at -20°C until needed for infusion. The effectiveness of this method for inactivation of infectious EIAV was confirmed by spiking 1 liter of normal horse plasma with 5 × 10⁷ 50% tissue culture infective doses (TCID₅₀) of EIAV_{WSU5}, followed by virus titration (3) before and after methylene blue-white light treatment. The pretreatment virus titer was 2 × 10³ FFU/ml while the virus titer was zero after methylene blue-white light treatment. Prior to infusion into SCID foals, all plasma

treated with methylene blue-white light was confirmed negative for infectious virus by virus titration and real-time reverse-transcription PCR (RT-PCR) (37).

Intravenous infusion of immune plasma. The volume of immune plasma infused intravenously (i.v.) into each SCID foal was determined by calculating the volume necessary to completely neutralize the challenge virus, based on the neutralization titer of the immune plasma and the plasma volume of the SCID foal recipient. Briefly, previous data indicated that Arabian SCID foals inoculated with 10⁶ TCID₅₀ of EIAV_{WSU5} develop plasma virus loads between 10⁴ and 10⁵ RNA copies/ml by 2 weeks postinoculation (36). In the current study, SCID foals were 2 weeks of age at inoculation, weighing approximately 40 kg, with a plasma volume of approximately 2 liters. Although a total plasma virus burden of approximately 10⁶ virions would be anticipated in these foals immediately following i.v. challenge with 10⁶ TCID₅₀ of EIAV_{WSU5}, the plasma virus burden anticipated at 2 weeks postinoculation (approximately 5 × 10⁴ RNA copies/ml, or a total of 5 × 10⁷ virions) was used as a neutralization target. This was done to ensure that adequate doses of immune plasma were administered. Since the focal reduction assay described above indicated that a 1:32 dilution of 250 µl of A2150 immune plasma (7.8 µl) was capable of completely neutralizing 550 FFU of input EIAV_{WSU5} (100% neutralizing titer), 709 ml of A2150 immune plasma would be required to completely neutralize 5 × 10⁷ EIAV_{WSU5} virions. To be conservative, a volume of 1 liter was chosen for infusion. This was 71 times the volume required to neutralize the actual challenge dose (10⁶ TCID₅₀) at inoculation. Although the experimental pony-cross SCID foal (H703) weighed approximately 40% less than the Arabian SCID foals (with a corresponding lower plasma volume), calculations as above indicated that 1 liter of immune plasma was similarly more than sufficient to completely neutralize an anticipated 2-week-postinoculation total plasma virus burden. Thus, 1 liter of methylene blue-white light-treated A2150 immune plasma was infused i.v. into experimental SCID foals (A2239, A2240, A2241, and H703) at 24 h prior to EIAV challenge and again 7 and 14 days after challenge. Four EIAV-challenged control SCID foals (A2245, A2247, H707, and H713) received 1 liter of methylene blue-white light-treated normal horse plasma at the same time points. One control SCID foal (H719) received 1 liter of methylene blue-white light-treated A2150 immune plasma i.v. without subsequent virus challenge to further confirm that the infused immune plasma as treated did not contain infectious virus.

All SCID foals (except H719) were challenged i.v. with 10⁶ TCID₅₀ of EIAV_{WSU5} (10, 33, 35, 36, 46). Immunocompetent horse A2215 was inoculated i.v.

with 250 ml of whole blood obtained from SCID foal A2239 at 41 days post-EIAV inoculation.

Immunological and virologic monitoring. Following EIAV challenge in SCID foals (or blood transfer in immunocompetent horse A2215), rectal temperatures and clinical status were recorded daily, and complete blood counts (CBC) with platelet counts were performed on whole blood collected every 2 days. Platelet counts (per μl) at least 3 standard deviations below the prechallenge mean were considered thrombocytopenic. Plasma and serum were collected and stored at -80°C every 2 days. Whole blood was collected from immunocompetent horse A2215 at 404 to 504 days after the infectious blood transfer was received, and peripheral blood mononuclear cells (PBMC) were isolated and either placed directly into stimulation cultures or cryopreserved for later use, as described previously (37).

Virus NAb titers in serum from all challenged foals was determined weekly (or at various other time points in A2215) using the focal reduction assay described above (3, 57). Also in A2215, serum antibodies against EIAV Gag p26 were detected using both enzyme-linked immunosorbent assay (ELISA) and agar gel immunodiffusion (AGID) test kits (VMRD, Inc., Pullman, WA), according to the manufacturer's instructions.

Viral load was determined by extracting plasma viral RNA and performing real-time quantitative RT-PCR as described previously (37). This assay could repeatedly detect dilutions of EIAV RNA containing 10 copies per reaction mixture but not dilutions containing only 1 copy. Thus, the reliable detection limit in assayed plasma was 430 RNA copies/ml when 140 μl of plasma for the RNA extraction and 10 μl of the 60- μl eluate were used per reaction mixture. Assays were repeated independently at least twice to confirm negative results. For peak viremic plasma samples, single-genome amplification (SGA) was performed as described previously (51) to identify potential SU variants. Briefly, cDNA was synthesized using an iScript Select cDNA Synthesis Kit (Bio-Rad, Hercules, CA), and serial dilutions (1:20, 1:60, 1:180, and 1:540) were made. A primary PCR was performed to detect virus by amplification of a 2,353-bp segment immediately 5' of the envelope gene and including all of gp90 and all but the 3' end of gp45, with 16 reaction mixtures per dilution in a 96-well format. Primers 5321F (5'-CATGGTCAGCATCGCATTG-3') and 7674R (5'-GCGAG AGTTCCTTCTGGGC-3'), along with Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), were used in the primary reaction. The primary reaction conditions were as follows: 2 min at 95°C and 35 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 2 min 30 s. A nested PCR was then performed using primers 5366F (5'-CAACCCCTATTACCAAC-3') and 7677R (5'-TTCCTTCTGGG CTTAATGTC-3') to amplify a 2,311-bp segment of the envelope gene that began 43 bp downstream of the segment amplified in the primary PCR and included most of gp90 and gp45. The nested reaction conditions were 2 min at 95°C and 45 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 2 min 30 s. According to a Poisson distribution, the cDNA dilution that yields PCR products in no more than 30% of wells contains one amplifiable cDNA template per positive PCR more than 80% of the time (44, 51). Thus, positive nested PCR products that met a Poisson distribution were then sequenced.

To confirm the presence or absence of EIAV infection in SCID foals at the end of the study, DNA was extracted from postmortem splenic tissue, and nested PCR was performed to detect provirus by amplification of a 288-bp segment of the *gag* gene as described previously (33, 38).

An EIAV variant with a divergent SU sequence (designated EIAV_{WSU5-V55}) was identified in this study. To specifically amplify this variant, nested RT-PCR (or PCR) was performed using variant sequence-specific primers. Initial primers 5841F (5'-TCATTGTCAAGAGTATCTATGTAAAGGAC-3') and 6280R (5'-ATATATCTAGGCGACCCGTCTC-3') were used to amplify a 399-bp segment of the variant SU that included all of V3 through V6. The primary reaction conditions were 2 min at 95°C and 40 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 30 s. A nested reaction was then performed using primers 5872F (5'-GATCTTAATTCCTCTAACTCTTATTGGG-3') and 6264R (5'-CGTCTC GCAAACGTCTC-3'), which amplified a 351-bp segment of the variant SU that began 32 bp downstream of the segment amplified in the primary reaction and included most of V3 through V6. The nested reaction conditions were 2 min at 95°C and 40 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 30 s.

PBMC stimulation and CTL assays. Freshly isolated or cryopreserved PBMC from immunocompetent horse A2215 were stimulated *in vitro* for 7 days using Gag peptide pool-pulsed, EIAV_{WSU5}-infected or EIAV_{WSU5-V55}-infected autologous monocytes as described previously (34, 38, 38) with modifications. For peptide stimulation, Gag peptide pool 1, comprising 14 overlapping Gag p15 peptides containing immunodominant CTL epitopes, was used as described previously (34). The EIAV_{WSU5-V55} variant virus for use in the PBMC stimulation culture and subsequent CTL assay was isolated using methods similar to those described by O'Rourke et al. (42). Very briefly, plasma from SCID foal

A2239 (containing EIAV_{WSU5-V55} exclusively as determined by SGA above) was used to infect equine kidney (EK) cells, which were then expanded and maintained in DMEM with 5% calf serum. Cell culture supernatants were collected to make a virus stock, which was quantitated by titration. Viral RNA was then extracted from this stock and analyzed by SGA to confirm the EIAV_{WSU5-V55} SU consensus sequence. For stimulation cultures, viruses in 17% fetal bovine serum (FBS) were added to A2215 cryopreserved PBMC in amounts equal to a multiplicity of monocyte infection of 1, assuming that PBMC were 5% monocytes. Virus and PBMC were incubated for 2 h at 37°C with occasional mixing before centrifugation at $250 \times g$ for 10 min. PBMC were then resuspended at $5 \times 10^6/\text{ml}$ of RPMI 1640 medium with 10% FBS, 20 mM HEPES, 10 $\mu\text{g}/\text{ml}$ gentamicin, 10 μM 2-mercaptoethanol, and 10 U/ml recombinant human interleukin-2 ([IL-2] Roche Diagnostics, Indianapolis, IN). Tissue culture plates (24 well) were seeded with 2×10^6 PBMC/well and incubated at 37°C with 5% CO_2 for 7 days. Virus-specific CTL activity was measured in stimulated PBMC with a 17-h ^{51}Cr release assay (34, 37, 38) using homologous EK target cells infected with either EIAV_{WSU5} or EIAV_{WSU5-V55}. For Gag peptide pool 1-pulsed target cells, a final concentration of 10^4 nM for each peptide in the pool was used (34). The following formula for percent specific lysis was used: $[(E - S)/(M - S)] \times 100$, where E is the mean ^{51}Cr beta counts per min of three test wells, S is the mean spontaneous release from three target cell wells without effector cells, and M is the mean maximal release from three target cell wells with 2% Triton X-100 in distilled water. Assays were independently performed at least twice, with effector/target cell ratios from 12:1 to 50:1. For these assays, each well contained $\sim 30,000$ target cells. Only assays with a spontaneous target cell lysis of $<15\%$ were used. The standard error (SE) of percent specific lysis was calculated using a formula that accounts for the variability of E , S , and M (56). Specific lysis was considered significant if it exceeded 10% and was at least 2 SEs above that for non-peptide-pulsed (or uninfected) target cells.

Statistical analysis. A nonparametric Mann-Whitney test (41) was used to compare areas under the curve (AUC) for viral load times the number of days post-EIAV inoculation (dpi) between experimental and challenged control foals using a two-tailed significance level for α of 0.05. For comparisons of the percentages of days that the plasma viral load was $>10,000$ RNA copies/ml and of the percentages of days of thrombocytopenia between experimental SCID foals and controls, a t test with Welch correction for unequal variances was used with a two-tailed significance level for α of 0.05. A viral load value of $>10,000$ RNA copies/ml was chosen for comparison because clinically apparent infection with fever and declining platelet counts associated with this viral load are consistently observed in EIAV-infected horses (36-38), and a viral load set point at this level distinguishes between EIAV progressor and nonprogressor horses (24). Statistical analyses were performed using GraphPad InStat, version 3.06, and GraphPad Prism, version 5.01 (GraphPad Software, San Diego, CA).

Nucleotide sequence accession numbers. Three amplified EIAV_{WSU5-V55} viral sequences, including a common consensus sequence and two additional sequences with minor amino acid divergence, have been submitted to the GenBank under accession numbers 1238448, 1238466, and 1238469.

RESULTS

A2150 immune serum/plasma had broad neutralizing activity against EIAV. Serum from long-term EIAV-inapparent carrier horse A2150 contained antibodies capable of neutralizing homologous EIAV_{WSU5} as well as chimeric heterologous infectious molecular clones PND1, -2, -3, -4, and -5 (Fig. 1b). As expected, the immune serum neutralized EIAV_{WSU5} more effectively than the five heterologous PND virus clones. Thus, A2150 immune serum was considered to have broad neutralizing activity although titers against heterologous PND viruses were lower than the neutralizing titer against the homologous virus.

Protective effects of broadly neutralizing immune plasma against EIAV infection and clinical disease in SCID foals. The four experimental SCID foals (A2239, A2240, A2241, and H703) received 1 liter of methylene blue-white light-treated immune plasma *i.v.*, followed by *i.v.* challenge with 10^6 TCID₅₀ of EIAV_{WSU5} 24 h later. Then, 1 liter of immune plasma was administered *i.v.* to each foal 7 and 14 dpi. These passive

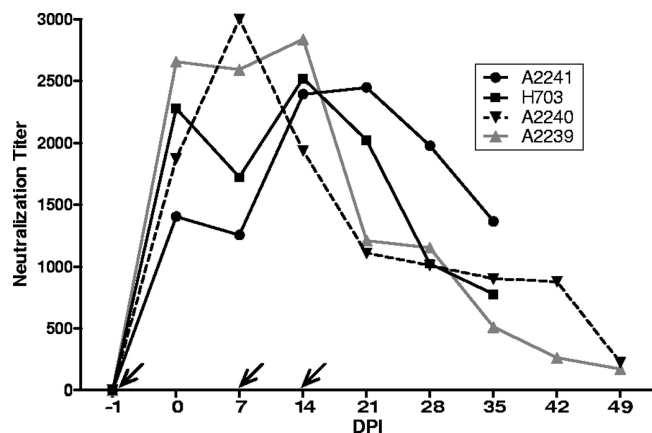


FIG. 2. Weekly 50% serum neutralization titers against homologous EIAV_{WSU5} in experimental SCID foals. Slanted arrows indicate days that immune plasma was infused.

transfers resulted in peak EIAV_{WSU5}-specific NAb titers between 2,000 and 3,000 in all foals, with similar rates of peak acquisition and subsequent decay. However, the peak titer was reached later and declined more slowly in one foal (A2241) than in the other three (Fig. 2). All four foals maintained a normal platelet count throughout the study (Fig. 3a to d). Due to the absence of immune-mediated mechanisms, fever (rectal temperature of >101.5°F) can be variable in SCID foal EIAV challenge studies. Fever can also be nonspecific because secondary bacterial, fungal, and other viral (particularly adenovirus) infections can occur. Although fever occurred at various time points in all four foals, (particularly at the end of each experiment), it was not consistently associated with EIAV infection. Foal A2241 did not develop plasma viremia, while animals A2239, A2240, and H703 became viremic at 41, 48, and 33 dpi, respectively. The viral load continued to rise in A2239, with a peak occurring at 55 dpi. These three foals also had detectable proviral DNA in postmortem splenic tissue, confirming infection. In contrast, proviral DNA was not detected in postmortem splenic tissue in foal A2241, confirming that this foal did not become infected.

Four control SCID foals (A2245, A2247, H707, and H713) received methylene blue-white light-treated normal horse plasma prior to EIAV challenge with the same infusion schedules as the experimental SCID foals. No serum neutralization activity was detected at any time point in these control foals (data not shown). All four challenged control SCID foals became viremic by 13 dpi and developed thrombocytopenia and fever (Fig. 3e to h). The unchallenged control SCID foal (H719) that received only methylene blue-white light-treated A2150 immune plasma did not develop thrombocytopenia or plasma viremia, and proviral DNA was not detected in postmortem splenic tissue. In addition to the lack of detectable virus in the treated A2150 immune plasma itself, this result further confirmed that the immune plasma infused into the foals of this study did not contain infectious virus and therefore was not a source of infection in the experimental foals that became viremic.

Experimental SCID foals had a significantly lower percentage of days of thrombocytopenia (<1%) than challenged

control SCID foals (34%; $P = 0.01$). Consistent with these findings, experimental SCID foals had a significantly lower percentage of days of plasma viral load of >10,000 copies/ml (<1%) than challenged control SCID foals (61%; $P = 0.0002$). Lastly, the median AUC value (viral load times the number of days post-EIAV inoculation) for experimental foals (4.7×10^4) was significantly less ($P = 0.0294$) than that for control foals (2.3×10^7) (Fig. 4).

Single-genome amplification and sequencing of plasma viral RNA in viremic SCID foals identified a significantly divergent envelope SU variant. Replicating virus was detected in three of the four SCID foals, and it was of interest to determine the genetic and antigenic properties of the breakthrough virus. Single-genome amplification (SGA) of plasma viral RNA at 41 and 55 dpi in SCID foal A2239 and at 48 dpi in SCID foal A2240 was performed, and the SU sequences of single amplicons were determined. An identical virus genotype, containing the same amino acid substitutions, insertions, and deletions, including a 14-amino-acid deletion in the PND region of SU and a 2-amino-acid insertion in V7, were identified in both foals (Fig. 5a and b). This variant was designated EIAV_{WSU5-V55} and was 12.6% divergent from EIAV_{WSU5} at the amino acid level within SU. Although amino acid changes primarily involved hypervariable regions V1 to V8, changes also occurred at more conserved sites between hypervariable regions. The 14-amino-acid deletion within V3 in the PND spanned two previously mapped neutralization epitopes (E_{NT} and D_{NT}) recognized by murine monoclonal antibodies (1). Importantly, amino acid substitutions in EIAV_{WSU5-V55} resulted in the creation of new PNLGS and in the shifting or deletion of existing PNLGS. The EIAV_{WSU5-V55} variant was identified in 32/32 single amplicons from foal A2239 (Fig. 5a) and in 7/7 single amplicons from foal A2240 (Fig. 5b). Although experimental SCID foal H703 developed transient plasma viremia at 33 dpi, single RNA genomes could not be amplified for sequencing. However, the EIAV_{WSU5-V55} provirus was detected by PCR (and confirmed by sequencing) in H703 postmortem splenic DNA at the end of the study using EIAV_{WSU5-V55}-specific primers (data not shown).

To determine the virus genotype associated with clinical disease in SCID foals receiving normal serum, we performed single-genome amplification and sequencing of plasma viral RNA from control SCID foal A2245 at peak plasma viremia. The EIAV_{WSU5} inoculum SU sequence was present in nine out of nine amplicons (Fig. 5c), and no variant EIAV_{WSU5-V55} amplicons were detected. Similarly, SGA of plasma viral RNA from control SCID foals A2247, H707, and H713 at peak viremia identified the same EIAV_{WSU5} inoculum SU sequence in 9/9, 17/17, and 10/10 amplicons, respectively (data not shown). Although only EIAV_{WSU5} was identified using SGA of plasma viral RNA from all challenged control SCID foals, nested RT-PCR using EIAV_{WSU5-V55}-specific primers and subsequent sequencing detected the EIAV_{WSU5-V55} variant in plasma from these control foals (data not shown).

The EIAV_{WSU5-V55} variant was present as a rare species in the EIAV_{WSU5} inoculum. Identification of the same EIAV SU variant in more than one experimental SCID foal suggested that EIAV_{WSU5-V55} preexisted in the immune plasma or virus inoculation. Therefore, EIAV_{WSU5-V55}-specific nested RT-PCR was used as above to detect the possible presence of

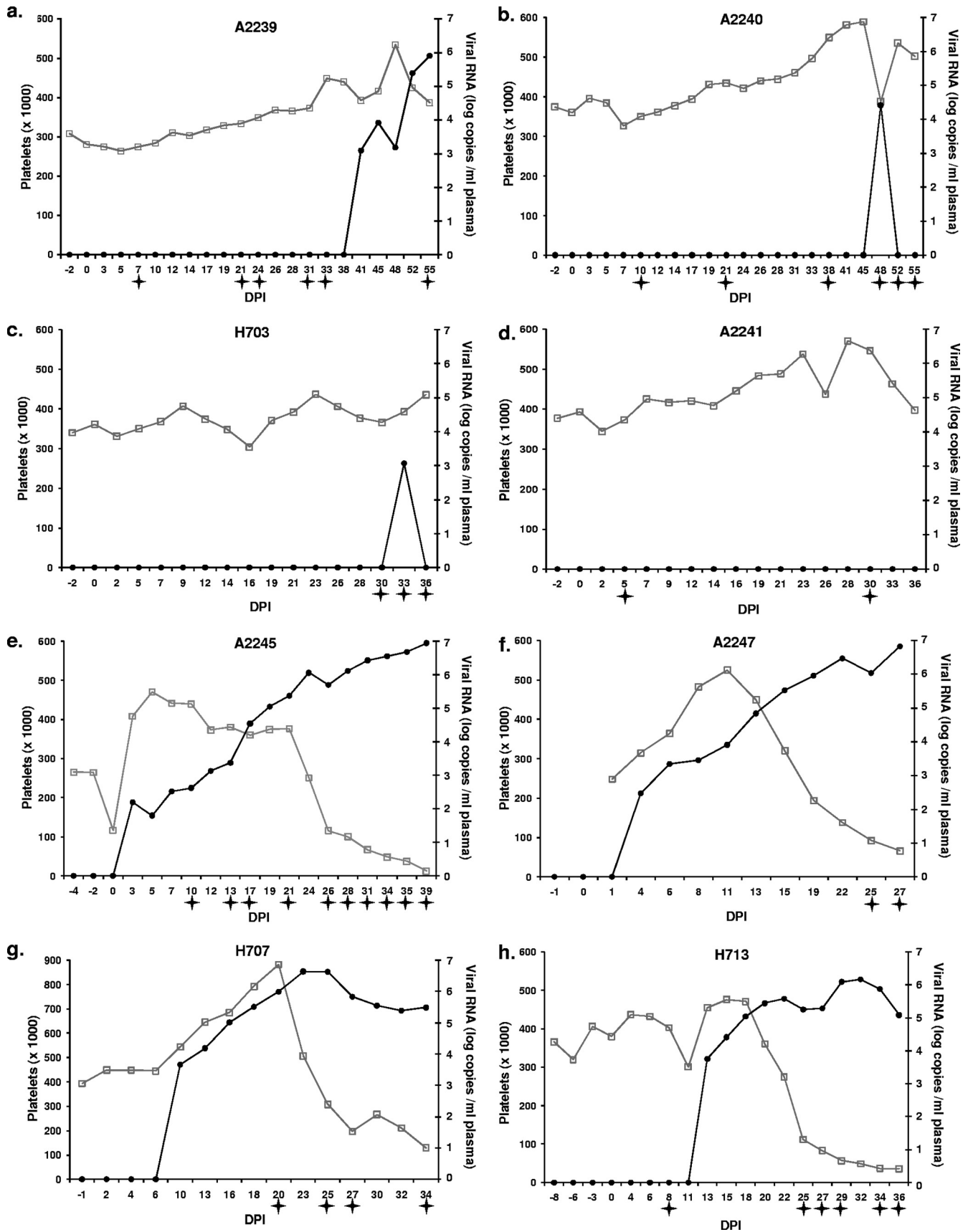


FIG. 3. Peripheral blood platelet counts (open squares) and plasma viral loads (filled circles) for SCID foals that received A2150 convalescent immune plasma (experimental) (a to d) and normal horse plasma (controls) (e to h). Stars indicate febrile days (rectal temperature of >101.5°F). Platelet counts were not obtained at missing data points.

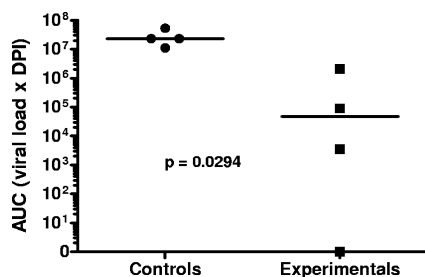


FIG. 4. Comparison of AUC values between experimental and control SCID foals challenged with EIAV_{WSU5}. Horizontal bars indicate medians.

EIAV_{WSU5-V55} RNA in the methylene blue-white light-treated A2150 immune plasma and in the EIAV_{WSU5} inoculum. For these experiments, viral RNA was extracted from 1.5 ml of plasma (or virus stock) to enhance the probability of detection. EIAV_{WSU5-V55} RNA was not detected in the treated A2150 plasma; however, EIAV_{WSU5-V55} RNA was identified in the EIAV_{WSU5} virus stock used for inoculation. To determine the relative frequency of the EIAV_{WSU5-V55} variant in the EIAV_{WSU5} virus stock, SGA and sequencing were performed. Of 25 single amplicons analyzed, 24 had the EIAV_{WSU5} SU sequence while 1 had the EIAV_{WSU5-V55} variant SU sequence (data not shown). Thus, the EIAV_{WSU5-V55} variant was present as a minor population in the EIAV_{WSU5} virus stock used to inoculate the SCID foals in this study. Since the EIAV_{WSU5} stock virus had been passed and amplified in cell culture several times over the years to prepare new stocks, it was of interest to determine if the EIAV_{WSU5-V55} variant was present in the original EIAV_{WSU5} seed stock prepared in 1988 (42). Interestingly, the EIAV_{WSU5-V55} variant was also identified in this stock by nested RT-PCR using the EIAV_{WSU5-V55}-specific primers.

The EIAV_{WSU5-V55} SU variant was resistant to neutralization. To determine the neutralization phenotype of the EIAV_{WSU5-V55} variant, virus was isolated from experimental SCID foal A2239 55-dpi plasma and propagated in cell culture. The isolate was confirmed to be EIAV_{WSU5-V55} by SGA analysis and sequencing. Serum neutralization assays against this EIAV_{WSU5-V55} isolate were then performed using A2239 55-dpi serum, A2240 48-dpi serum, and immune serum from A2150 (the source of immune plasma used for infusion). Compared to the ability of sera from these animals to neutralize EIAV_{WSU5}, the EIAV_{WSU5-V55} isolate was neutralized very poorly (Fig. 6a). Thus, EIAV_{WSU5-V55} was confirmed to be a neutralization-resistant SU variant.

The EIAV_{WSU5-V55} SU variant resulted in clinical disease in an immunocompetent horse but was efficiently controlled in the absence of type-specific NAb. Blood inoculation into a susceptible horse with subsequent monitoring for clinical disease and seroconversion is one of the oldest and most sensitive methods for confirming EIAV infection (7). Whole blood from experimental SCID foal A2239 was obtained at 41 dpi (the first day of detectable plasma viral RNA), and 250 ml was inoculated i.v. to immunocompetent horse A2215. This horse developed a fever at 15 days post-blood transfer (dpt), which correlated with plasma viral RNA and thrombocytopenia (Fig.

6b). The fever subsided within 4 days, and no further viremic episodes occurred during 455 days of monitoring. Gag-specific antibodies were detected in serum collected at 30 dpt by AGID assay and ELISA. SGA analysis and sequencing of viral RNA obtained from A2215 on the day of peak viremia (dpt 19) identified the EIAV_{WSU5-V55} variant exclusively (24/24 single amplicons) (Fig. 6c).

Serum obtained from A2215 at 19, 55, 111, 230, 342, and 496 dpt was assayed for neutralizing activity against both EIAV_{WSU5-V55} (A2215 inoculum) and EIAV_{WSU5} (SCID foal inoculum). As was observed previously for the A2150, A2239, and A2240 sera (Fig. 6a), serum samples from A2215 poorly neutralized EIAV_{WSU5-V55} (Fig. 6d). Although 111-dpt serum had the highest level of EIAV_{WSU5-V55} neutralization, none of the A2215 sera achieved 50% virus reduction at dilutions as low as 1:8. Quite unexpectedly, however, A2215 serum neutralized EIAV_{WSU5} with a peak titer of 1,024 on dpt 230 (Fig. 6e). Overall, these results indicated that efficient and long-term control of the EIAV_{WSU5-V55} variant occurred in A2215 but that EIAV_{WSU5-V55} infection did not elicit a significant EIAV_{WSU5-V55}-specific NAb response despite production of EIAV_{WSU5}-specific NABs.

Long-term control of EIAV_{WSU5-V55} was associated with EIAV Gag-specific CTL. During the period of prolonged control of EIAV_{WSU5-V55} in A2215 (dpt 400 to 500), CTL activity against EIAV Gag and whole virus (EIAV_{WSU5} and EIAV_{WSU5-V55}) was identified in both freshly isolated and cryopreserved PBMC. Memory CTL in PBMC stimulated with Gag peptide pool 1, which comprised 14 overlapping synthetic peptides containing immunodominant EIAV Gag p15 epitopes (34), specifically lysed Gag pool 1-pulsed homologous EK target cells (Fig. 7a). In addition, memory CTL in PBMC stimulated with EIAV_{WSU5-V55} recognized both EIAV_{WSU5-V55}- and EIAV_{WSU5}-infected target cells with similar levels of specific lysis (Fig. 7b). Moreover, memory CTL stimulated with EIAV_{WSU5} also recognized both EIAV_{WSU5-V55}- and EIAV_{WSU5}-infected targets with similar levels of specific lysis (Fig. 7b). These results demonstrated that durable control of the EIAV_{WSU5-V55} variant was associated with Gag-specific CTL and that these CTL targeted conserved epitopes as both EIAV_{WSU5} and EIAV_{WSU5-V55} were recognized.

DISCUSSION

In this study, we documented that immune plasma from a horse persistently infected with EIAV_{WSU5} contained antibodies capable of neutralizing homologous virus plus five other viruses containing heterologous SU PND regions derived from a pony persistently infected with wild-type EIAV_{Wyo}. When infused into four SCID foals prior to and after EIAV_{WSU5} challenge, this plasma resulted in clinical protection against EIAV-induced disease in all foals and prevented infection in one foal. Importantly, however, the breakthrough infections in three of the foals were caused by a neutralization-resistant SU variant that was present at low copy number in the inoculum. Durable control of this variant virus was achieved in the absence of type-specific NABs in an immunocompetent horse and was associated with the presence of Gag-specific CTL. This work demonstrates for the first time in a large animal lentivirus model that broadly active NABs can be protective in the com-

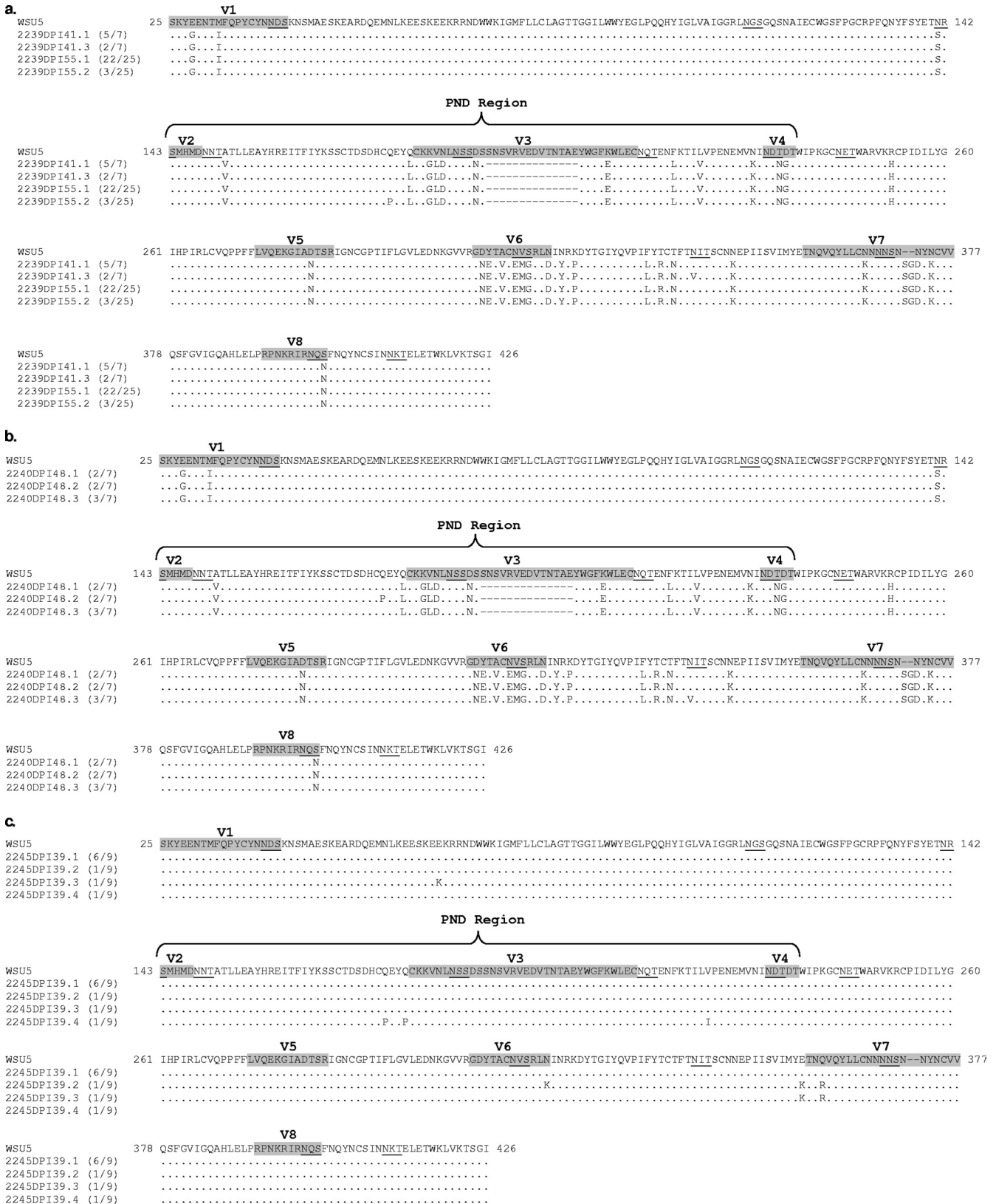


FIG. 5. Deduced amino acid sequences of single plasma viral RNA RT-PCR amplicons from A2239 at 41 and 55 dpi (a), A2240 at 48 dpi (b), and control SCID foal A2245 at 39 dpi (c). The EIAV_{WSU5} inoculum consensus sequence for comparison is shown at the top. For each unique sequence, the ratio of the number of amplicons with that sequence to the total number of amplicons sequenced is indicated in parentheses. Hypervariable regions V1 to V8 are labeled and shaded, and the PND region is indicated. PNLGS are underlined.

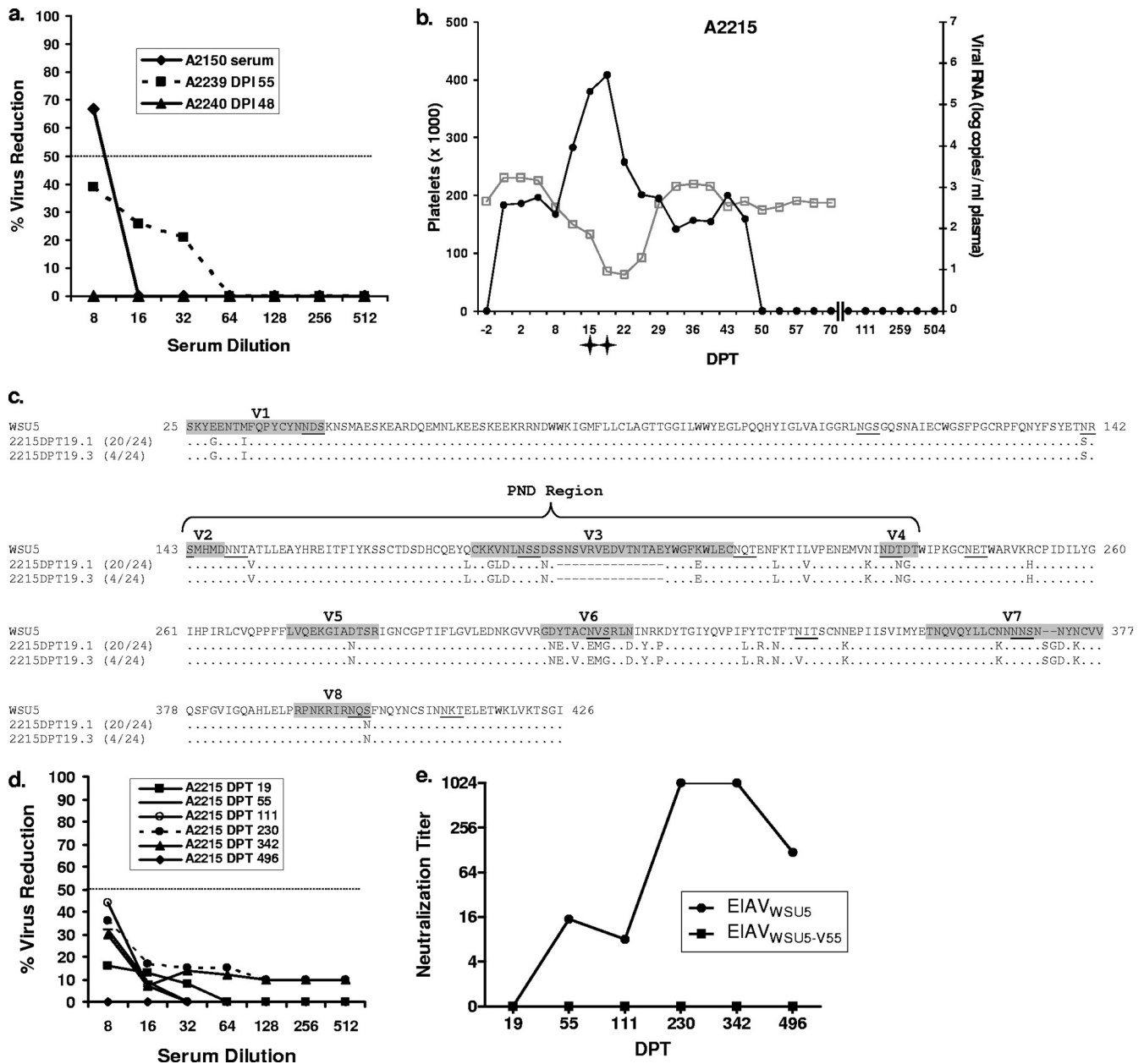


FIG. 6. (a) Serum neutralization of the EIAV_{WSU5-V55} variant by A2150 immune serum, A2239 serum at 55 dpi, and A2240 serum at 48 dpi. The dashed line represents 50% virus reduction. (b) Peripheral blood platelet counts (open squares) and plasma viral loads (filled circles) for immunocompetent horse A2215 after inoculation with blood from SCID foal A2239. Stars indicate febrile days (rectal temperature of >101.5°F). Platelet counts were not obtained at missing data points. (c) Deduced amino acid sequences of single plasma viral RNA RT-PCR amplicons from A2215 at 19 dpt. The EIAV_{WSU5} consensus sequence for comparison is shown at the top. Other annotations are the same as described in the legend of Fig. 5. (d) Serum neutralization of the EIAV_{WSU5-V55} variant by A2215 serum at 19, 55, 111, 230, 342, and 496 dpt. The dashed line represents 50% virus reduction. (e) The 50% serum neutralization titers against EIAV_{WSU5} and EIAV_{WSU5-V55} by A2215 serum at 19, 55, 111, 230, 342, and 496 dpt.

plete absence of other adaptive immune responses, but the limitations of such an approach were also evident.

Although SU epitopes recognized by equine NABs have not been precisely defined, three linear epitopes recognized by murine monoclonal NABs have been mapped, with two in the PND V3 and one in the V5 region (1). Previous work has indicated that the V3 and V4 domains are the primary determinants of EIAV SU neutralization sensitivity and resistance (19). The immune

plasma infused in this study neutralized several viruses containing SU regions V2 to V4 heterologous and contained NABs that blocked homologous EIAV_{WSU5} infection in all treated SCID foals. In three foals, however, these NABs did not prevent infection by EIAV_{WSU5-V55}, a rare variant present in the EIAV_{WSU5} inoculum. The profound amino acid variation/deletion within V3 of EIAV_{WSU5-V55} suggested that NABs in the infused plasma were targeted to this region. However,

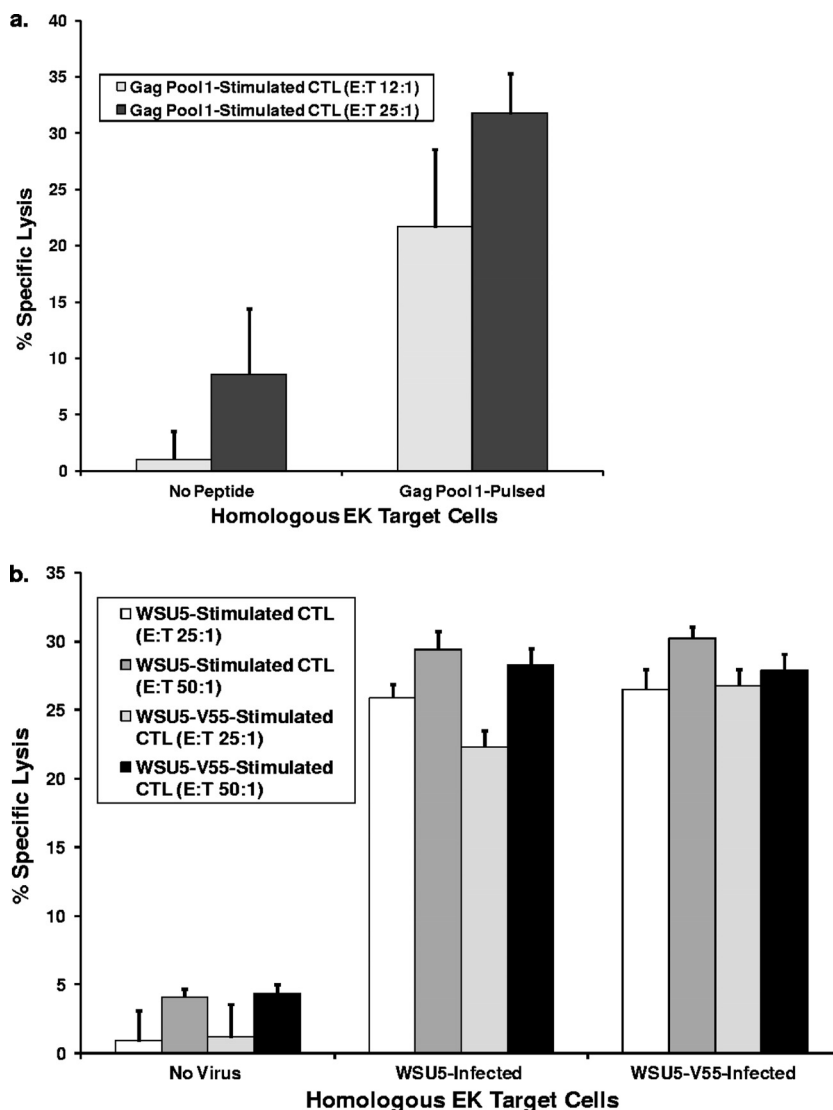


FIG. 7. (a) Percent specific lysis of non-peptide-pulsed and Gag peptide pool 1-pulsed homologous EK target cells by CTL in Gag peptide pool 1-stimulated fresh PBMC isolated from horse A2215 on dpt 404. Results of two independent assays are shown, one performed with an effector/target (E/T) ratio of 12:1 and the other with an E/T ratio of 25:1. Error bars represent SE. (b) Percent specific lysis of uninfected, EIAV_{WSU5}-infected, and EIAV_{WSU5-V55}-infected homologous EK target cells by CTL in EIAV_{WSU5}-stimulated and EIAV_{WSU5-V55}-stimulated cryopreserved PBMC isolated from horse A2215 on dpt 504. Results of two independent assays are shown, one performed with an E/T ratio of 25:1 and the other with an E/T ratio of 50:1. Error bars represent SE.

numerous amino acid changes, including those affecting PNLGS, also occurred within V4, V5, V6, and V7, with the most significant of these changes encompassing V6 and V7. Thus, the neutralization-resistant phenotype of EIAV_{WSU5-V55} may have resulted from changes that masked neutralizing epitopes within the PND and/or from variation in neutralizing epitopes located within V6/V7. Interestingly, we have observed SU changes similar to those above in immunocompetent horses following EIAV_{WSU5} infection. These included significant deletions and substitutions in V3 and substitutions in V2 and V4 (37, 38). More profound changes in V3 to V7 were observed in an EIAV_{WSU5}-challenged SCID foal that had been reconstituted with EIAV_{WSU5}-specific T and B lymphocytes (35). A significant number of these changes involved PNLGS. Although the SU changes observed in these previous studies were the result of

immune selection, with some resulting in CTL escape (37, 38), it was not known whether the predominant selection force was NAbs, CTL, or both. In contrast, the current study provided a novel means to prove that NAbs alone constitute a significant selection force on the lentivirus envelope.

Based on the spacing of conserved cysteines, the EIAV SU V3/V4 region is structurally analogous to the HIV-1 gp120 V1/V2 stem-loop (17). The V1/V2 stem comprises part of the inner domain bridging sheet of HIV-1 gp120, which forms the CD4 binding pocket, and, along with the outer domain V3 loop, also binds the chemokine coreceptor (23). These binding sites are shielded by the V1/V2 and V3 loops (23). In addition to these structural similarities, some PNLGS are conserved between EIAV and HIV-1; two of these occur within and just downstream of EIAV V4 (17, 26). In the current study, the

PNLGS within V4 (N233) was shifted two positions downstream in EIAV_{WSU5-V55} by a T235N substitution. This could have masked an NAb epitope or changed the conformation of SU such that an epitope was abolished. Unlike HIV-1, however, EIAV can utilize a single receptor, the equine lentivirus receptor 1 (ELR-1) (61). Although the EIAV SU binding site for ELR-1 remains to be defined, deletions in V4, V5, V6, V7, or V8 abolish binding while deletions in V1, V2, or V3 do not (58). Interestingly, though, a murine monoclonal antibody against an epitope in V3 did block ELR-1 binding (58). Thus, complicated and discontinuous sequences in EIAV SU mediate receptor binding, and the roles of V3, V4 and the other hypervariable regions in receptor binding and/or shielding are not completely understood. In the current study, the amino acid changes in V4 to V8 observed in EIAV_{WSU5-V55} could have contributed to its neutralization resistance just as much as, or more than, the changes in V3.

The appearance of the same neutralization-resistant variant in three separate SCID foals made it unlikely that the amino acid substitutions and deletions in EIAV_{WSU5-V55} arose *de novo* simultaneously in all three foals. Although our studies confirmed that EIAV_{WSU5-V55} was a rare variant in the EIAV_{WSU5} virus stock used for inoculation, the factors contributing to its origin and persistence in this stock are not clear. Regardless, infusion of convalescent immune plasma from a horse infected with EIAV for over 8 years exerted the selection force of a fully mature NAb response in a period of just a few weeks in foals devoid of adaptive immunity. This provided a rigorous test of the efficacy and limitations of NAbs alone in the protection against lentivirus infection. The fact that our challenge included two functionally heterologous viruses was important since humans can acquire more than one virus when infected with HIV-1 (51).

A previous study (25) detected an SU variant, designated EIAV_{ΔPND}, during the third febrile episode in a pony experimentally infected with the PV strain of EIAV (EIAV_{PV}). EIAV_{ΔPND} has changes in the V3 and V4 regions similar to EIAV_{WSU5-V55}, including a 14-amino-acid deletion in the PND. Consistent with our findings, autologous serum samples failed to neutralize the EIAV_{ΔPND} variant but displayed high levels of neutralization activity against the EIAV_{PV} inoculum (25). Interestingly, two ponies inoculated with a chimeric virus containing the EIAV_{ΔPND} SU failed to develop high viral loads and clinical disease or NAbs; however, immunosuppression with dexamethasone resulted in higher viral loads and clinical disease, followed by the appearance of variant-specific NAbs (8). The type-specific NAb response mapped to the variant V3 and V4 regions, indicating the importance of these regions for neutralization by sera from these ponies (18). In contrast to those studies, the EIAV_{WSU5-V55} variant reported here replicated to a high enough level to cause clinical disease when inoculated into an immunocompetent horse, yet it was not capable of eliciting a significant type-specific NAb response. Unlike the previously reported EIAV_{ΔPND}, however, EIAV_{WSU5-V55} also contains significant changes in V6 and V7 which may have affected V3/V4 immunogenicity via conformational changes. Moreover, one of the murine monoclonal antibody neutralization epitopes in V3 (D_{NT}) is intact in EIAV_{ΔPND} (18) but disrupted in EIAV_{WSU5-V55}, and additional amino acid changes that are not present in EIAV_{ΔPND} occurred within and

just upstream of V4 in EIAV_{WSU5-V55} (18). Regardless, critical epitopes were effectively abolished or masked by the SU changes in EIAV_{WSU5-V55} such that neutralizing B-cell responses against this variant could not be efficiently primed.

Although EIAV_{WSU5-V55} did not elicit a detectable type-specific NAb response in immunocompetent horse A2215, antibodies elicited against this virus were capable of neutralizing the parent EIAV_{WSU5} SCID foal inoculum. An intriguing explanation is that antibodies were elicited in A2215 against epitopes in the EIAV_{WSU5-V55} envelope that were shared with EIAV_{WSU5}. However, because of conformational differences in the two envelopes, these antibodies did not efficiently interfere with the complex interactions between SU hypervariable regions and the ELR-1 receptor for EIAV_{WSU5-V55} but did so for EIAV_{WSU5}. Despite similarity in epitopes, antibodies with slight differences in recognition of the HIV-1 gp120 CD4 binding site induce significant differences in gp120 conformation (4). Poorly neutralizing antibodies recognize conformational shifts that result in V1/V2 orientations that are poorly compatible with the functional viral spike. Thus, only the most accurately targeted CD4 binding site antibodies (such as b12) are able to neutralize resistant (tier 2) HIV-1 isolates (4). Whether the same holds true for EIAV receptor binding site antibodies remains to be determined. Nonetheless, identification of convalescent immune plasmas capable of neutralizing resistant heterologous lentiviruses such as EIAV_{WSU5-V55} poses a significant challenge, as will developing strategies to elicit efficient NAb responses against them.

It was not surprising that control of the EIAV_{WSU5-V55} variant in immunocompetent horse A2215 was associated with Gag-specific CTL, given our previous work indicating that the majority of EIAV-infected horses that effectively control infection mount Gag-specific CTL responses (30). Similarly, Gag-specific CTL play an important role in controlling HIV-1 replication in elite controllers (39). Although envelope SU-specific CTL are immunodominant in EIAV-infected horses, these CTL do not correlate with control of viremia due to SU variation and subsequent CTL escape (37, 38). Amino acid variation can occur in EIAV Gag (37, 62), but these changes do not lead to loss of recognition by CTL that correlate with control (37). Importantly, the p15 region of Gag targeted by A2215 CTL in the current study contains epitope clusters recognized by CTL from major histocompatibility complex class I (MHC-I)-disparate horses (5). These epitope clusters are targets for high-avidity CTL that correlate with long-term virus control (6). While the current study indicated that broadly reactive immune plasma was sufficient to control homologous virus in SCID foals, control of the EIAV_{WSU5-V55} variant was not consistently achieved. In contrast, this same variant was effectively controlled in an immunocompetent horse in the absence of EIAV_{WSU5-V55}-specific NAbs when Gag p15-specific CTL were present. These observations highlight the importance of developing lentivirus vaccines that include conserved CTL immunogens in addition to eliciting broad NAb responses.

In summary, our results provide a strong basis for further dissection and fine mapping of NAb epitopes important in protection from EIAV infection and/or clinical disease. Since EIAV SU shares structural features with the SU of HIV-1, results of these studies could have important implications for

the design of protective HIV-1 vaccines where experiments of this type are not possible.

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