Discerning an Effective Balance between Equine Infectious Anemia Virus Attenuation and Vaccine Efficacy

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Among the diverse experimental vaccines evaluated in various animal lentivirus models, live attenuated vaccines have proven to be the most effective, thus providing an important model for examining critical immune correlates of protective vaccine immunity. We previously reported that an experimental live attenuated vaccine for equine infectious anemia virus (EIAV), based on mutation of the viral *S2* accessory gene, elicited protection from detectable infection by virulent virus challenge (F. Li et al., J. Virol. 77:7244–7253, 2003). To better understand the critical components of EIAV vaccine efficacy, we examine here the relationship between the extent of virus attenuation, the maturation of host immune responses, and vaccine efficacy in a comparative study of three related attenuated EIAV proviral vaccine strains: the previously described EIAV$_{UK}$ΔS2 derived from a virulent proviral clone, EIAV$_{UK}$ΔS2/DU containing a second gene mutation in the virulent proviral clone, and EIAV$_{PR}$ΔS2 derived from a reference avirulent proviral clone. Inoculations of parallel groups of eight horses resulted in relatively low levels of viral replication (average of 10^2 to 10^3 RNA copies/ml) and a similar maturation of EIAV envelope-specific antibody responses as determined in quantitative and qualitative serological assays. However, experimental challenge of the experimentally immunized horses by our standard virulent EIAV$_{PV}$ strain by using a low-dose multiple exposure protocol (three inoculations with 10 median horse infective doses, administered intravenously) revealed a marked difference in the protective efficacy of the various attenuated proviral vaccine strains that was evidently associated with the extent of vaccine virus attenuation, time of viral challenge, and the apparent maturation of virus-specific immunity.

Development of effective vaccines to animal lentiviral infections is complicated by the diverse array of immune evasion mechanisms used by these viruses to circumvent host immune surveillance. Thus far, the development of vaccines to human immunodeficiency virus type 1 has relied substantially on the use of animal lentivirus models to evaluate the efficacy of various vaccine strategies. Animal lentiviral systems used as AIDS vaccine models have included simian/human immunodeficiency virus (SHIV)-monkey, simian immunodeficiency virus (SIV)-monkey, equine infectious anemia virus (EIAV)-horse, and feline immunodeficiency virus (FIV)-cat models (1). Interestingly, the greatest level of success has been realized with live attenuated animal lentivirus vaccines that are able to drive a critical maturation of virus-specific humoral and cellular immune responses (3, 4, 15, 16, 20, 29, 36–38).

EIAV, a macrophage-tropic lentivirus, causes a persistent infection in horses and a chronic disseminated disease of worldwide importance in veterinary medicine (reviewed by Montelaro et al. [26]). EIAV is typically transmitted via blood-feeding insects or iatrogenic sources such as contaminated syringe needles. The disease, EIA, is characterized by well-defined recurring cycles of viremia at irregular intervals and is associated with clinical signs that include fever, anemia, thrombocytopenia, edema, diarrhea, and lethargy. Infected horses

typically progress by 8 to 12 months postinfection to life-long inapparent carriers that lack clinical signs but continue to experience various steady-state levels of virus replication maintained by monocyte/macrophage-specific tissue reservoirs of infection (11), (13, 26). Among virulent lentiviruses, EIAV is unique in that, despite aggressive virus replication and rapid antigenic variation, >90% of infected animals progress from a chronic disease state to an inapparent carrier stage by establishing strict immunologic control over virus replication (26). The EIAV system therefore serves as a uniquely dynamic model for the natural immunologic control of lentiviral replication and disease and provides a useful and novel lentivirus system for identifying critical immune correlates of protection and ascertaining the potential for developing an effective prophylactic vaccine to protect horses from EIAV infection.

During the past 15 years, we have evaluated a number of experimental EIAV vaccines based on inactivated whole virus and on viral or recombinant envelope subunit vaccines. The results of these vaccine trials demonstrated a remarkable breadth of efficacy, ranging from protection from detectable infection and/or disease to severe enhancement of EIAV replication and disease. These data indicate that vaccine-induced immune responses are a double-edged sword that can have beneficial or deleterious effects on the outcome of virus exposure (9, 10, 12, 14, 28, 31, 32).

We previously described a genetically engineered, live attenuated proviral vaccine candidate, EIAV$_{UK}$ΔS2, containing a mutation of the accessory *S2* gene (20). In these initial experiments we reported a comprehensive analysis of the properties

of the EIAV$_{UK}$ΔS2 in horses experimentally immunized and challenged intravenously with single or multiple doses of the reference EIAV$_{PV}$ virulent strain of virus. To examine in more detail the critical parameters of attenuated EIAV vaccine efficacy, we describe here a comparative study of the virologic, immunologic, and protective efficacy of variant attenuated EIAV proviral constructs in experimentally immunized horses. The results of these studies demonstrate that vaccine efficacy is markedly affected by the specifics of the vaccine genetic engineering and reveals a critical and delicate balance between vaccine virus attenuation and the capacity to drive immune maturation sufficient to achieve optimal protective immunity. The implications for these observations with experimental attenuated EIAV vaccines for AIDS vaccine design are discussed.

MATERIALS AND METHODS

Design and production of attenuated viral vaccines. Three distinct attenuated viral vaccines were generated for evaluation: (i) the EIAV$_{UK}$ΔS2 vaccine strain containing a mutation of the *S2* gene of the reference virulent EIAV$_{UK}$ proviral clone (19), (ii) the EIAV$_{UK}$ΔS2/DU vaccine containing mutations in both the *S2* and the *DU* genes of the EIAV$_{UK}$ provirus, and (iii) the EIAV$_{PR}$ΔS2 vaccine strain produced from a reference avirulent proviral clone by mutation of the viral *S2* gene. The EIAV$_{UK}$ΔS2 proviral clone was derived from the in vivo pathogenic molecular clone, EIAV$_{UK}$ (6), by inserting two stop codons in the S2 reading frame. The EIAV$_{UK}$ΔS2/DU also derived from the in vivo pathogenic EIAV$_{UK}$ molecular clone a previously described 330-base nucleotide sequence deletion in the viral DU (dUTPase) gene (22). The EIAV$_{PR}$ΔS2 proviral clone, constructed by standard molecular biological techniques (24), was also produced from the avirulent EIAV$_{PR}$ provirus (30) by introduction of two termination codons at the beginning of the S2 gene, as described above for the EIAV$_{UK}$ΔS2 proviral clone. All of the attenuated proviral clones were sequenced to verify the required genetic mutations.

Virus stocks were prepared by harvesting the supernatant medium from Lipofectamine-mediated transfection (Gibco-BRL) of equine dermal (ED) cells (ATCC CRL 6288) with mutant proviral DNA (19). Viral stocks were assayed by a micro reverse transcriptase assay, and stock virus titers were determined in an infectious center assay in fetal equine kidney cells, as described previously (8, 22).

Experimental subjects, clinical evaluation, and sample collection. A total of 35 outbred horses of mixed age and gender were used in the various vaccine trials. All horses were clinically monitored daily and maintained as described previously (10, 14). Daily rectal temperatures and clinical status were recorded. Samples of serum, plasma, and whole blood were collected from each horse at defined intervals and daily during apparent febrile episodes (>39°C). Plasma samples were stored at −80°C for use in quantitative or qualitative reverse transcription-PCR (RT-PCR) assays to determine the levels and identity (vaccine or challenge virus) of plasma viral RNA. Serum samples were stored at −20°C for serological assays.

Experimental vaccination and virus challenge procedures. We examined the immunogenicity and protective efficacy of each vaccine construct (EIAV$_{UK}$ΔS2, EIAV$_{UK}$ΔS2/DU, and EIAV$_{PR}$ΔS2) individually in groups of eight horses experimentally inoculated with the designated attenuated virus strain. Each vaccinate was then challenged by using a low-dose multiple exposure (LDME) challenge to simulate natural exposure by horsefly bites (20). The test horses were vaccinated two times at 30-day intervals by intramuscular injection of 10^5 50% tissue culture infective dose(s) (TCID$_{50}$) of the individual vaccine stocks (20). Six months after the initial vaccine dose, the eight vaccinated horses in each group and four naive horses were challenged by the LDME procedure with the reference virulent EIAV$_{PV}$ stock. The LDME protocol consisted of three sequential intravenous inoculations, at 2-day intervals, of 10 median horse infectious doses (HID$_{50}$) of the virulent challenge virus, EIAV$_{PV}$ (HID$_{50}$ determinations were made though EIAV$_{PV}$ titrations in 15 horses; 1 HID$_{50}$ is the equivalent of ~0.1 TCID$_{50}$). The horses were monitored daily for clinical symptoms of EIA, and blood was drawn at regular intervals (weekly, daily if febrile) for assays of platelets, viral replication, and virus-specific immune responses (11, 14). The horses were observed for a total of 270 days, at which time they were euthanized.

To evaluate the dynamics of the maturation and efficacy of EIAV$_{UK}$ΔS2 vaccine immunity in experimentally inoculated horses, a group of five horses was experimentally vaccinated with EIAV$_{UK}$ΔS2 as described above but then challenged at 2 months postinoculation by using the standard LDME regimen (20). The horses were monitored daily postchallenge for clinical symptoms of EIA, and blood was drawn at regular intervals (weekly, daily if febrile) for assays of platelets, viral replication, and virus-specific immune responses (11, 14). The horses were observed for a total of 170 days, at which time they were euthanized.

Quantitative and qualitative serological analyses. Detection of serum antibody reactivity to EIAV core protein p26 was conducted by using a ViraCHEK/EIA kit according to the manufacturer's instructions (Synbiotics Laboratory, Via Frontera, San Diego, Calif.). Serum samples were also evaluated for seroreactivity by the standard agar gel immunodiffusion (AGID) procedure (2) diagnostic assay for EIA. Serum immunoglobulin G antibody reactivity to EIAV envelope glycoproteins was assayed quantitatively (endpoint titer) and qualitatively (avidity index and conformation ratio) by using our standard concanavalin A enzyme-linked immunosorbent assay (ELISA) procedures as described previously (11). The statistical significance of the differences between vaccine groups was determined by a nonparametric one-way analysis of variance (Kruskal-Wallis Test) with Dunnett's post test (GraphPad InStat, version 3.0; GraphPad Software, San Diego, Calif.). Virus neutralizing activity to the challenge virus strain EIAV$_{PV}$ mediated by immune sera was assessed in an indirect cell-ELISA based infectious center assay with a constant amount of infectious EIAV$_{PV}$ and sequential twofold dilutions of serum (10). Correlations between the presence of neutralizing antibody and protection from infection were performed by using the Spearman rank correlation test with GraphPad InStat.

Quantitative and qualitative RT-PCR of plasma virus RNA. Plasma samples from all animals were analyzed for the levels of viral RNA per milliliter of plasma by using a previously described semiquantitative RT-PCR assay based on *gag*-specific amplification primers (11). The standard RNA curve was linear in the range of 10^2 molecules as a lower limit and 10^8 molecules as an upper limit. To differentiate the virion genomic RNA of the vaccine strains (EIAV$_{UK}$ΔS2, EIAV$_{UK}$ΔS2/DU, or EIAV$_{PR}$ΔS2) from the challenge virus (EIAV$_{PV}$), virion-associated genomic RNA was extracted from plasma samples and then characterized by a nested RT-PCR and restriction digestion analysis that differentiated parental and mutated *S2* gene sequences (20). This diagnostic assay is based on the introduction of a unique SpeI restriction site in a 582-bp segment of the modified *S2* gene that is not present in the wild-type *S2* gene segment (20). The generated SpeI diagnostic site is present in all three attenuated vaccine strains. The nested RT-PCR is highly specific and provides detection levels down to 20 RNA copies of *S2*. Diagnostic analyses were performed during all three periods of observation: prechallenge, postchallenge, and during immunosuppression (see Fig. 1 to 4).

RESULTS

Experimental vaccination with live attenuated EIAV vaccine candidates and challenge with virulent EIAV. To assess the role of viral attenuation in vaccine efficacy, we immunized three groups of eight horses intravenously with 10^5 TCID$_{50}$ of three different live attenuated virus stocks, EIAV$_{UK}$ΔS2, EIAV$_{UK}$ΔS2/DU, and EIAV$_{PR}$ΔS2. The vaccinated horses were monitored daily for clinical signs of EIA (fever, lethargy, petechiation, and diarrhea), and blood samples were taken at regular intervals for measurement of platelets, plasma virus, and EIAV-specific humoral immunity. To examine the protective efficacy of the immune responses elicited by the attenuated virus infection, we challenged all three groups of vaccinated horses with our reference virulent EIAV$_{PV}$ strain used in previous vaccine trials (9, 12, 20, 28, 31). To mimic the natural route of infection we utilized an LDME challenge that consists of a series of three intravenous inoculations of 10 HID$_{50}$ of virulent EIAV$_{PV}$ administered at 2-day intervals (20). A control group of four naive horses was also challenged in an identical manner.

Prechallenge profiles of EIAV$_{UK}$ΔS2, EIAV$_{PR}$ΔS2, and EIAV$_{UK}$ΔS2/DU vaccinated horses. As summarized in Fig. 1, all of the EIAV$_{UK}$ΔS2-vaccinated horses remained asymptomatic for EIA during the 7-month prechallenge period of obser-
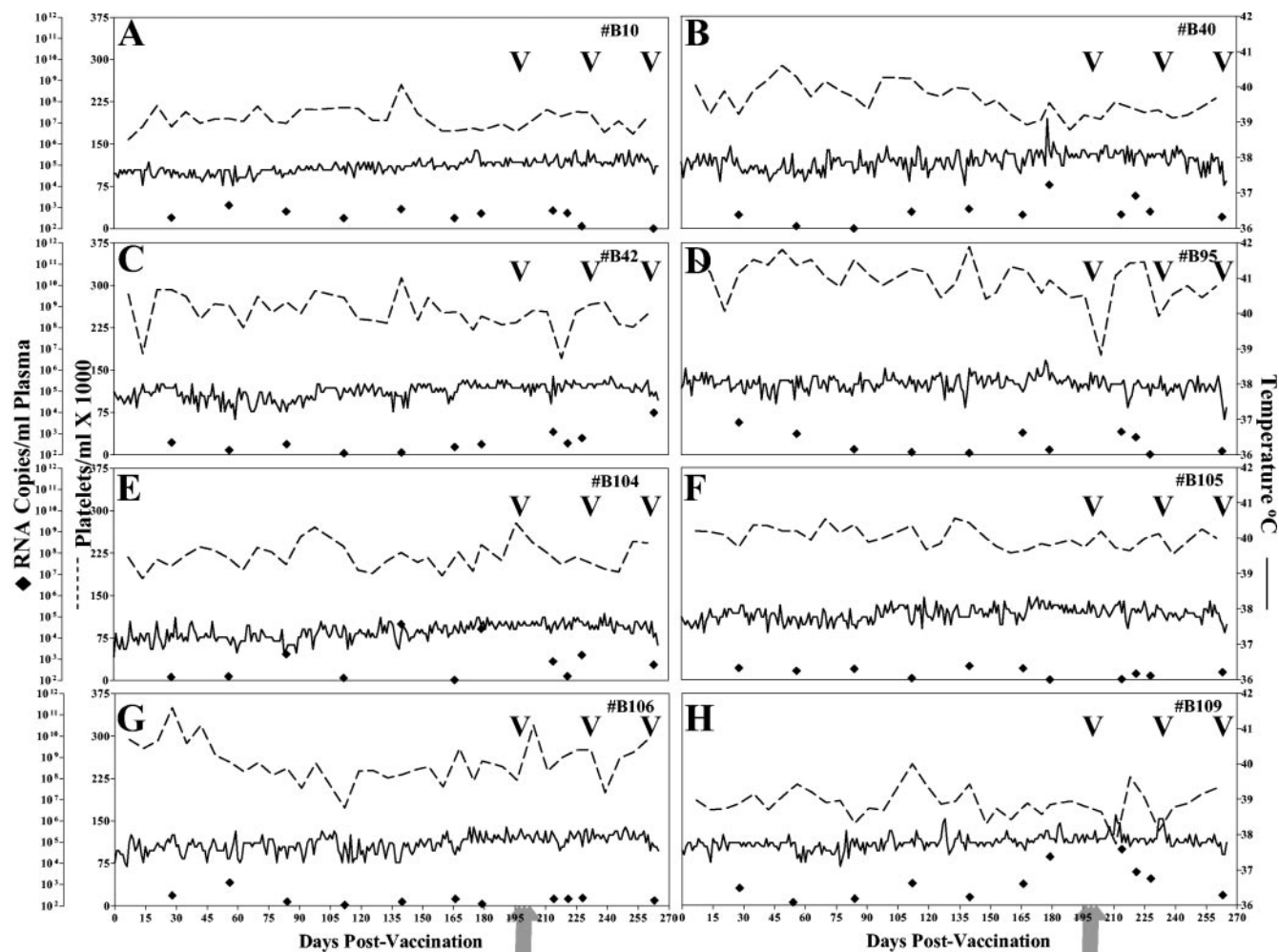
FIG. 1. Clinical and virologic profiles of EIAV$_{UK}\Delta$S2 experimental horses. Eight horses (A to H, respectively) were inoculated with EIAV$_{UK}\Delta$S2 as described in Materials and Methods. Rectal temperature (solid line, right *y* axis) and platelet counts (dotted line, first left *y* axis) were monitored daily for up to 300 days (*x* axis) after the first vaccine dose. Quantification of the virus load (◆, second left *y* axis) was performed on viral RNA extracted from plasma at periodic time points prior to and after virulent virus challenge by using the LDME protocol (arrows). S2 diagnostic results for each animal are indicated in each respective panel above the date of analysis by either a V (vaccine strain) or a C (challenge strain).

vation, as characterized by a lack of febrile episodes ($<$39°C) and normal levels of blood platelets ($\geq$105,000/μl whole blood), a documented measure of EIA disease (7, 11, 17, 21, 33, 39). The lack of clinical signs in the vaccinates was associated with a relatively low level of EIAV$_{UK}\Delta$S2 replication during the first 6 months postinoculation. Viral RNA levels in plasma measured at various times postinoculation ranged from 10^2 to 10^4 copies of RNA/ml of plasma, with an overall average of ~10^3 RNA copies/ml. We have previously reported that EIAV clinical episodes are associated with virus loads in excess of 10^6 RNA copies/ml (7, 18, 20).

Figure 2 summarizes the clinical and virologic profiles of each of the eight horses inoculated with the EIAV$_{UK}\Delta$S2/DU provirus strain. As observed above, all of the horses inoculated with the EIAV$_{UK}\Delta$S2/DU vaccine strain remained asymptomatic for EIA during the 7-month prechallenge observation period, as evidenced by a lack of febrile episodes and reduced blood platelet levels. The observed levels of replication of the

EIAV$_{UK}\Delta$S2/DU strain in the inoculated horses were consistently at a level of ca. 10^2 to 10^3 RNA copies/ml of plasma, representing an apparent 10-fold reduction in virus replication compared to the EIAV$_{UK}\Delta$S2 vaccine strain described above. This reduced replication is consistent with the previous studies that defined the role of the EIAV DU protein in promoting virus replication in equine macrophage cultures in vitro and the effects of DU mutation on virus replication and virulence in vivo (22, 35).

The final group of eight vaccinates received the genetically attenuated virus EIAV$_{PR}\Delta$S2 containing a mutated *S2* gene in the context of an avirulent viral backbone. As depicted in Fig. 3, six of the eight horses demonstrated no signs of EIA during the 7-month prechallenge observation period, as characterized by a lack of temperature increases and normal levels of blood platelets. However, two of the eight horses, #97–17 and #97–16 (Fig. 3G and H, respectively) experienced increased temperatures during the prechallenge period. The fevers oc-
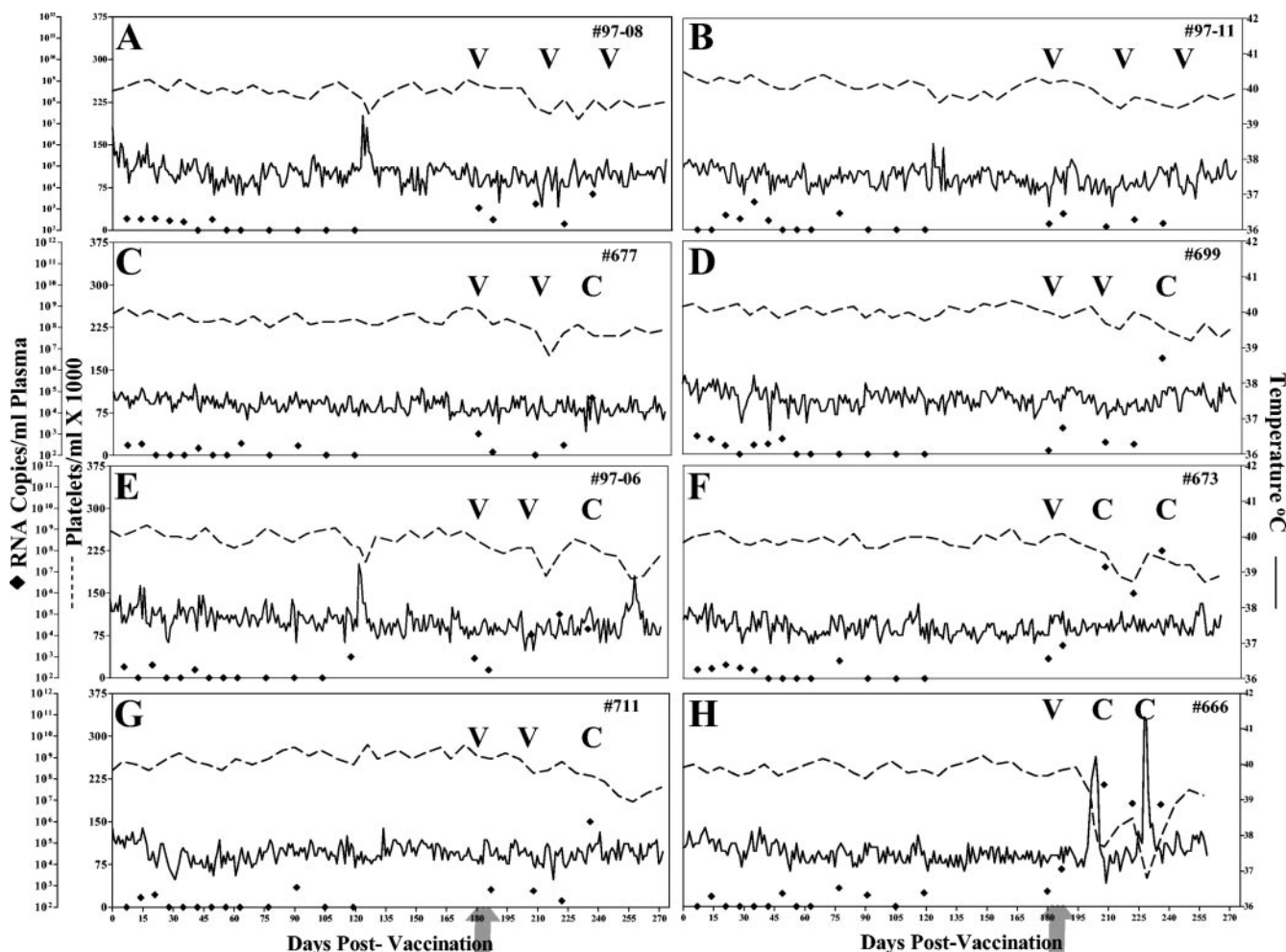
FIG. 2. Clinical and virologic profiles of EIAV$_{UK}$ΔS2/DU experimental horses. Eight horses (A to H, respectively) were inoculated with EIAV$_{UK}$ΔS2/DU as described in Materials and Methods. Rectal temperature (solid line, right y axis) and platelet counts (dotted line, first left y axis) were monitored daily for up to 300 days (x axis) after the first vaccine dose. Quantification of the virus load (♦, second left y axis) was performed on viral RNA extracted from plasma at periodic time points prior to and after virulent virus challenge by using the LDME protocol (arrows). Febrile episodes (see panel A) were defined by a rectal temperature of >39°C in conjunction with a reduction in platelets (< 100,000/μl of whole blood) and other clinical symptoms of EIA. S2 diagnostic results for each animal are indicated in each respective panel above the date of analysis by either a V (vaccine strain) or a C (challenge strain).

curred without concurrent drops in platelets. Upon further examination, these animals were diagnosed with equine herpesvirus infections, which appeared to be causing the increased temperatures. Thus, these observations were consistent with an absence of apparent virulence from the attenuated EIAV vaccine strain. Measurements of virus RNA levels in plasma in the horses inoculated with the EIAV$_{PR}$ΔS2 strain indicated a relatively consistent low level of vaccine virus replication, averaging less than 10^2 RNA copies/ml.

Taken together, these prechallenge observations demonstrated that each of the genetic modifications attenuated viral virulence such that persistent infections were asymptomatic for EIA disease, as required for an attenuated vaccine strain. In addition, the virus load measurements indicated a general trend in that the sustained replication levels of the three different proviral vaccine strains were reduced incrementally by

~10-fold in the order EIAV$_{UK}$ΔS2 > EIAV$_{UK}$ΔS2/DU > EIAV$_{PR}$ΔS2.

Postchallenge clinical and virologic profiles of EIAV$_{UK}$ΔS2-, EIAV$_{UK}$ΔS2/DU-, and EIAV$_{PR}$ΔS2-vaccinated horses. The four EIAV-naive control horses developed, upon challenge with EIAV$_{PV}$, classic EIA signs (Fig. 4) by 40 days postchallenge, displaying concurrent fevers and declines in platelets accompanied by peaks in virus loads of 10^6 to 10^8 RNA copies/ml of plasma. In contrast, the eight EIAV$_{UK}$ΔS2-vaccinated animals remained asymptomatic for EIA after challenge with EIAV$_{PV}$, again as monitored by a lack of fluctuation in temperature and platelet levels (Fig. 1). Virus loads remained at levels similar to the prechallenge data, ranging between 10^3 and 10^4 copies of RNA/ml of plasma. Diagnostic RT-PCR analyses of the RNA populations in plasma postchallenge detected only the mutant vaccine virus *S2*. The absence of de-
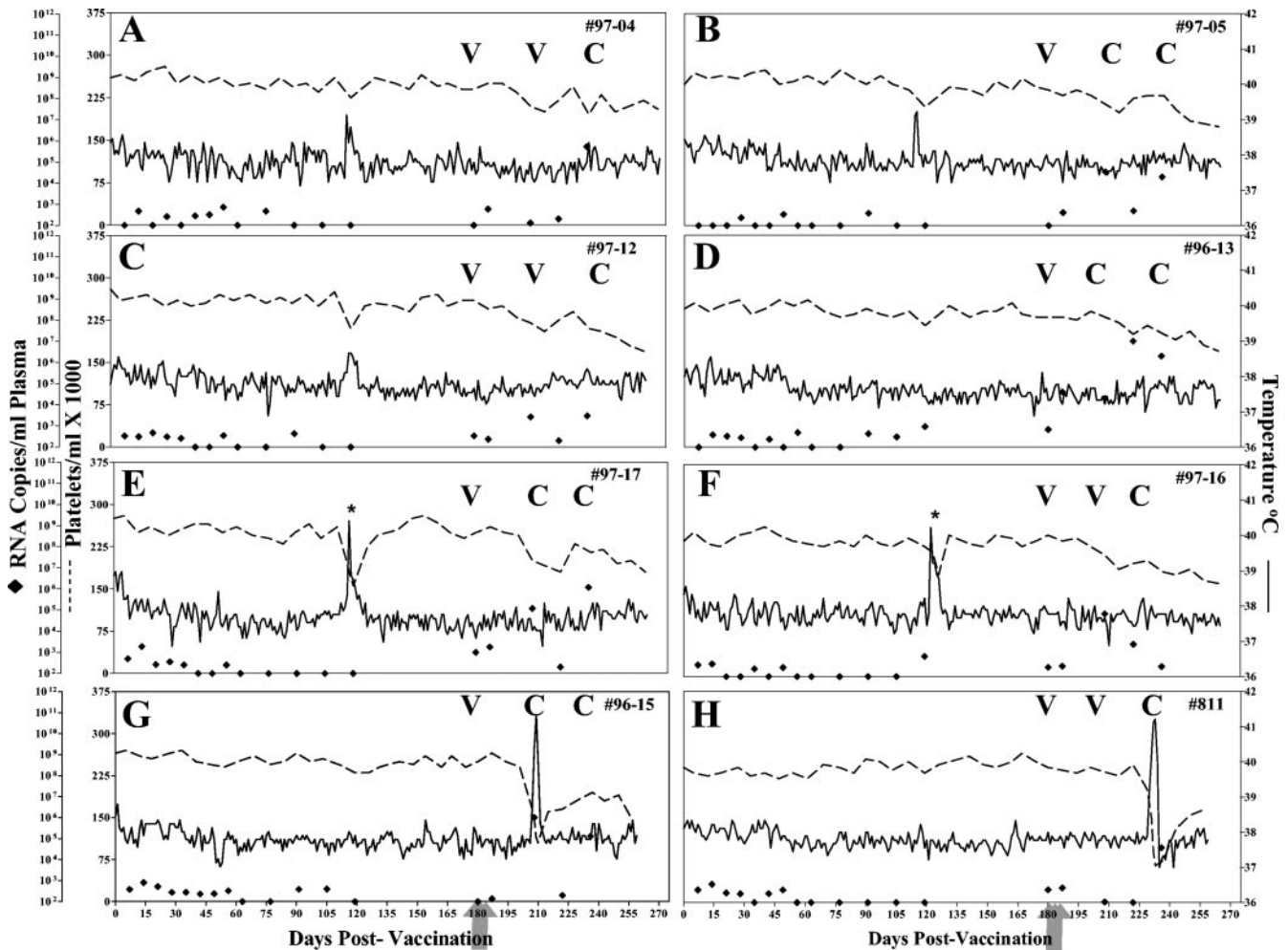
FIG. 3. Clinical and virological profiles of EIAV$_{PR}$ΔS2 experimental horses. Eight horses (A to H, respectively) were inoculated with EIAV$_{PR}$ΔS2 as described in Materials and Methods. Rectal temperature (solid line, right y axis) and platelet counts (dotted line, first left y axis) were monitored daily for up to 300 days (x axis) after the first vaccine dose. Quantification of the virus load (♦, second left y axis) was performed on viral RNA extracted from plasma at periodic time points prior to and after virulent virus challenge by using the LDME protocol (arrows). Febrile episodes (E and H) were defined by a rectal temperature of >39°C in conjunction with a reduction in platelets (<100,000/μl of whole blood) and other clinical symptoms of EIA. S2 diagnostic results for each animal are indicated in each respective panel above the date of analysis by either a V (vaccine strain) or a C (challenge strain). ✱, EHV-associated fevers.

tectable challenge virus *S2* is consistent with an apparent protection from infection by experimental challenge, thus confirming the observations reported previously with this particular experimental vaccine (20).

The postchallenge observations for the group of horses vaccinated with the EIAV$_{UK}$ΔS2/DU strain are summarized in Fig. 2. Seven of these eight vaccinates remained asymptomatic for EIA after challenge with EIAV$_{PV}$, as indicated by no increase in temperature or reduction in platelet levels after virulent viral challenge (Fig. 2). Virus load measurements postchallenge indicated that only two of the horses in this vaccine group (#97–08 and #97–11, Fig. 2A and B) appeared to control virus replication to the same level observed prior to virulent viral challenge, i.e., <10^3 RNA copies/ml. Despite the lack of clinical symptoms in the other seven vaccinates postchallenge, five of these horses did experience increases in viral loads, averaging between 10^5 and 10^6, with horse #673 peaking

at 10^8 copies RNA/ml of plasma (Fig. 2C to G). A single animal, #666 (Fig. 2H) in the trial experienced clinical EIA, having two fever episodes postchallenge that were accompanied by platelet drops and increases in viral loads that peaked in excess of 10^8 RNA copies/ml of plasma. Diagnostic RT-PCR analyses of plasma virus S2 species postchallenge revealed the presence of challenge virus in all six of these vaccinates that experienced increased viral loads postchallenge, whereas only vaccine virus S2 was detected in the two horses (#97–08 and #97–11) in which no increase in virus loads postchallenge were observed.

The postchallenge observations with the group of horses inoculated with the EIAV$_{PR}$ΔS2 strain are summarized in Fig. 3. After challenge with EIAV$_{PV}$, six of the eight EIAV$_{PR}$ΔS2-vaccinated animals remained asymptomatic for EIA (Fig. 3), again defined by a lack of fevers or thrombocytopenia after virulent virus inoculation. Two horses, #96–15 and #811 (Fig.
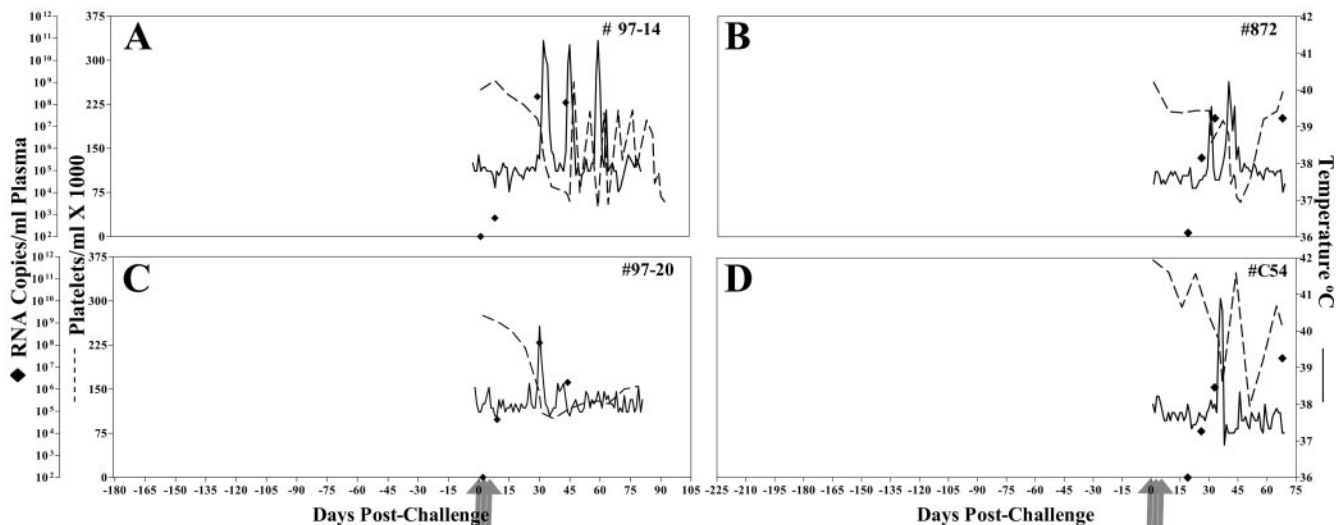
FIG. 4. Clinical and virologic profiles of control naive horses. Four horses (A to D, respectively) were subjected to challenge with virulent EIAV$_{PV}$ by using the LDME protocol (see Materials and Methods). Rectal temperature (solid line, right y axis) and platelet counts (dotted line, first left y axis) were monitored daily for up to 300 days (x axis) postchallenge. Quantification of the virus load ($\blacklozenge$, second left y axis) was performed on viral RNA extracted from plasma at periodic time points prior to and after virulent virus challenge by using the LDME protocol (arrows). Febrile episodes were defined by a rectal temperature of >39°C in conjunction with a reduction in platelets (<100,000/μl of whole blood) and other clinical symptoms of EIA.

3G and H, respectively) experienced clinical EIA as indicated by marked increases in temperature and decreases in platelets. Virus load assays postchallenge revealed that all of these horses experienced increases in virus loads postchallenge; postchallenge viral loads ranged from 10^4 to 10^6 RNA copies/ml compared to $< 10^2$ RNA copies per ml prechallenge. In addition, the challenge virus S2 gene was detected in all eight of the horses in this group postchallenge.

The combined postchallenge data from the three vaccine groups reveals a spectrum of vaccine efficacy among the three experimental vaccine strains (Table 1). The EIAV$_{UK}$ΔS2 immunization provided 100% protection from disease and detectable infection by the virulent virus challenge. The EIAV$_{UK}$ΔS2/DU immunization produced substantial protection from apparent disease (87.5%) but little protection (25%) from infection by the challenge virus. Finally, the EIAV$_{PR}$ΔS2 immunization produced a similar protection from disease (75%) but failed to protect any of the vaccinates in this group from infection by the virulent challenge virus.

Development of humoral immune responses to EIAV vaccination. In light of the range of protective efficacy observed with the panel of attenuated EIAV strains, we next sought to evaluate the properties of the antibody responses elicited by each vaccine immunization.

The immunogenicity of each vaccine strain was first characterized by reactivity testing in standard U.S. Department of Agriculture (USDA)-approved commercial diagnostic assays for EIAV infection based on detecting antibody to the virus capsid protein, p26. These diagnostic tests included two USDA-approved assays, the reference AGID test (2) and the ELISA-based ViraCHEK assay (see Materials and Methods) (20). All experimentally immunized horses were determined to be seropositive in the ViraCHEK diagnostic assay at the day of challenge (data not shown). In contrast, the same immune serum samples were uniformly determined to be negative in the less-sensitive AGID tests (data not shown). These results illustrated that horses inoculated with EIAV$_{UKΔ}$S2, EIAV$_{UKΔ}$S2/DU, or EIAV$_{PRΔ}$S2 can be seropositive in diagnostic assays that are the basis of USDA and other national regulatory policies to control EIAV infection.

A dynamic, complex, and lengthy maturation of immune responses to the viral envelope proteins during the first 6 to 8

TABLE 1. Summary of observed vaccine efficacies[a]

Vaccine strain	Dose	DOC	Plasma virus RNA at DOC	EIAV challenge strain	No. of animals protected/no. tested (%)	
					Protection from clinical disease	Protection from infection
EIAV$_{UK}$ΔS2	10^5	6	10^2–10^4	PV	8/8 (100)	8/8 (100)
EIAV$_{UK}$ΔS2/DU	10^5	6	$<10^2$–10^3	PV	7/8 (87.5)	2/8 (25)
EIAV$_{PR}$ΔS2	10^5	6	$<10^2$	PV	6/8 (75%)	0/8 (0)
EIAV$_{UK}$ΔS2	10^5	2	10^2	PV	3/5 (60)	1/5 (20)
Control	NA	NA	NA	PV	0/6 (0)	0/6 (0)

[a] DOC, day of challenge.

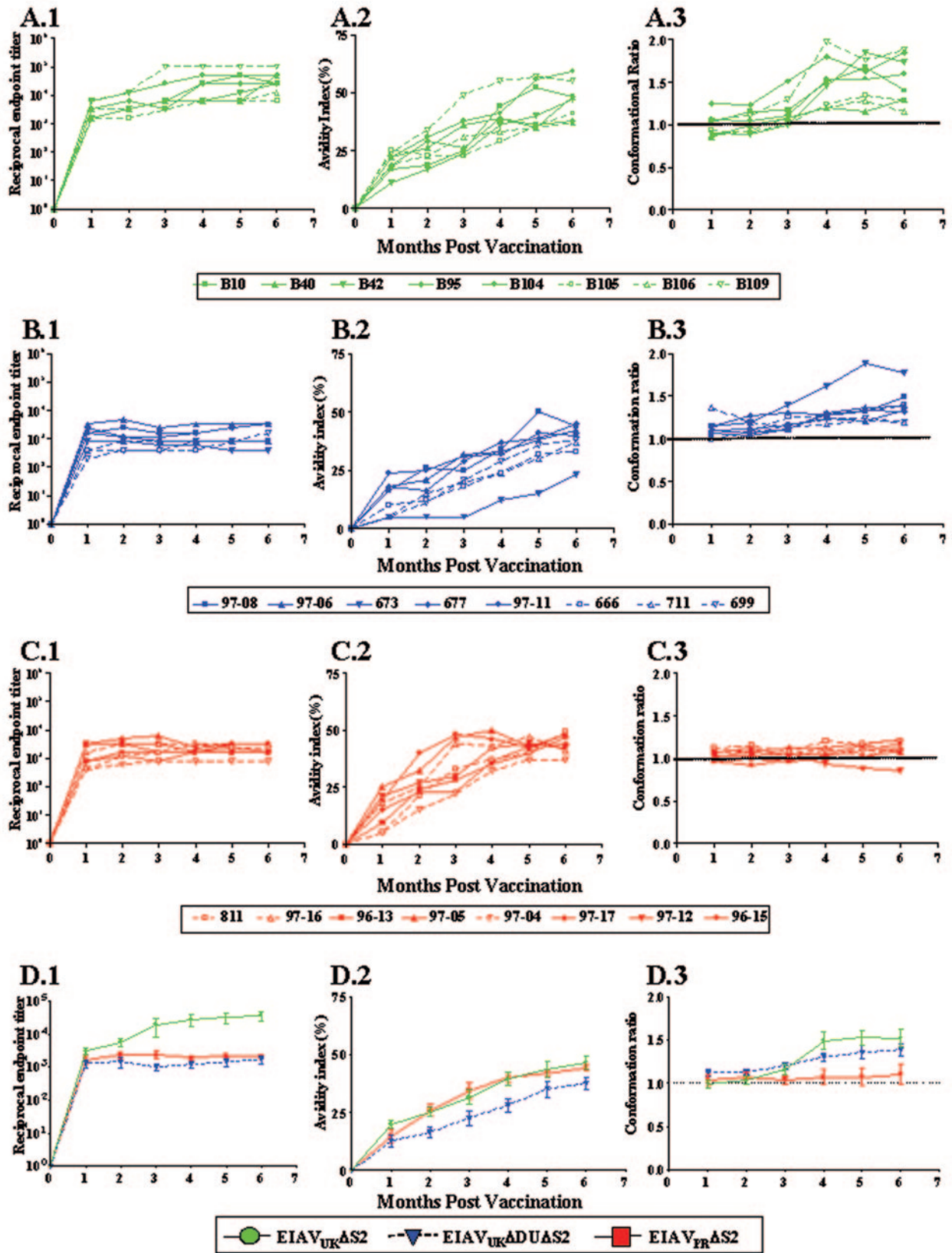FIG. 5. Development of envelope-specific antibody responses to various attenuated EIAV vaccines. Longitudinal characterization of the quantitative and qualitative properties of induced EIAV envelope-specific antibodies were conducted in concanavalin A ELISAs of endpoint titer (panels 1), avidity (panels 2), and conformational dependence (panels 3) as described in Materials and Methods. Analyses were performed on all
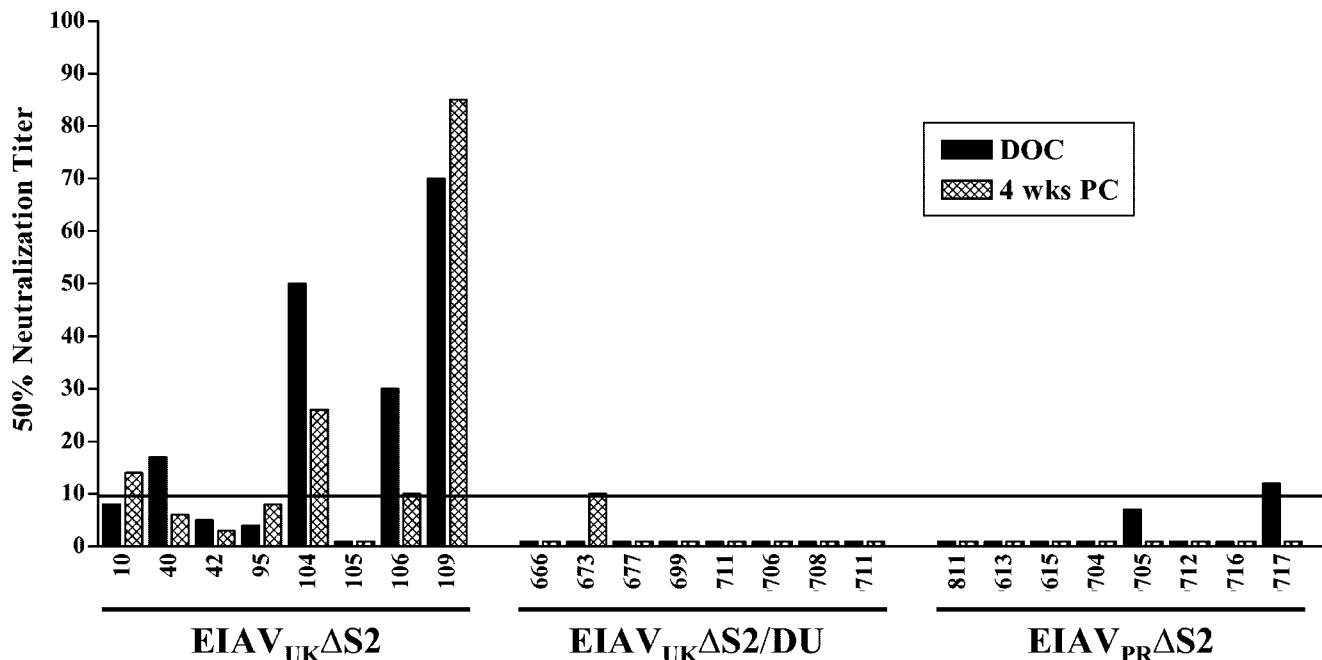
FIG. 6. Characterization of virus-specific serum neutralization in horses inoculated with EIAV$_{UK}$ΔS2, EIAV$_{UK}$ΔS2/DU, or EIAV$_{PR}$ΔS2. The mean reciprocal dilutions of serum from vaccinated horses which neutralized 50% of input EIAV$_{PV}$ as measured in an infectious center assay are presented for serum samples collected at the day of challenge (DOC) and 4 weeks postchallenge (PC), as described in Materials and Methods. The line denotes the cutoff (≥10) value for valid 50% neutralization titers.

months postinfection appears to be a distinctive feature of lentivirus infections as the persistent infection achieves steady-state viral replication and host immunity (3, 5, 27). We have previously described, by using a novel panel of quantitative and qualitative serological assays, a characteristic maturation of EIAV envelope-specific serum antibodies in experimentally infected horses that precedes immune and virologic steady state (10, 11). These mature and immature immune responses have been further correlated with protective and nonprotective or enhancing vaccine immunity in experimental EIAV vaccines (12). Therefore, we next utilized our standard panel of quantitative (titer) and qualitative (avidity and conformation) assays to examine and compare the maturation of EIAV envelope-specific antibody responses during the prechallenge observation period of observation (Fig. 5). These data revealed a similar evolution of envelope-specific antibody response in all three vaccine groups, with only minor variations among individual horses in each vaccine group. In general, envelope-specific antibody titers increased rapidly during the first couple of months postinoculation, reaching steady-state levels that averaged 1:10,000 for the EIAV$_{UK}$ΔS2 vaccinates (Fig. 5A.1) and 1:1,000 for the EIAV$_{UK}$ΔS2/DU (Fig. 5B.1) and EIAV$_{PR}$ΔS2 (Fig. 5C.1) vaccinates. Overall, the EIAV$_{UK}$ΔS2

vaccinates (Fig. 5D.1) achieved significantly higher steady-state antibody titers ($P < 0.05$) compared to the other two attenuated strains.

In contrast to the rapid establishment of steady-state antibody levels in serum, the qualitative properties of avidity and conformational dependence of the envelope-specific antibodies progressively evolved over the initial 6 months, at which time relatively steady-state levels were established (Fig. 5A.2, B.2, and C.2). In general, antibody avidity values progressed from an average of ca. 20% at 1 month postinfection to steady-state levels of ca. 40% at 6 months (Fig. 5D.2). Although the antibody avidity values observed over the first 6 months for the EIAV$_{UK}$ΔS2/DU vaccinates were consistently lower than those observed in the other two vaccine groups, these differences were not statistically significant ($P > 0.05$). Interestingly, the assays for antibody conformational dependence revealed more marked differences in the specificities of the antibodies elicited by the different attenuated vaccine strains (Fig. 5A.3, B.3, and C.3). The envelope-specific serum antibodies generated in the EIAV$_{UK}$ΔS2 vaccinates demonstrated an increase in conformational dependence from a relatively equal specificity of linear conformational envelope determinants (average ratio of 0.9) to a specificity for predominantly conformational

three trial groups: EIAV$_{UK}$ΔS2 (A), EIAV$_{UK}$ΔS2/DU (B), and EIAV$_{PR}$ΔS2 (C). (D) Average levels for each assessment were also calculated to extricate and simplify comparative analysis. (A.1 to D.1) Antibody titers in serum for each time point are presented as the log$_{10}$ of the highest reciprocal dilution yielding reactivity two standard deviations above background. (A.2 to D.2) Avidity index measurements are presented as percentages of the antibody-antigen complexes resistant to disruption with 8 M urea. (A.3 to D.3) Conformation dependence values are calculated as the ratio of serum antibody reactivity with native envelope compared to denatured envelope antigen. Conformation ratios of >1.0 indicate predominant antibody specificity for conformational determinants, whereas ratios of <1.0 indicate predominant antibody specificity for linear envelope determinants.
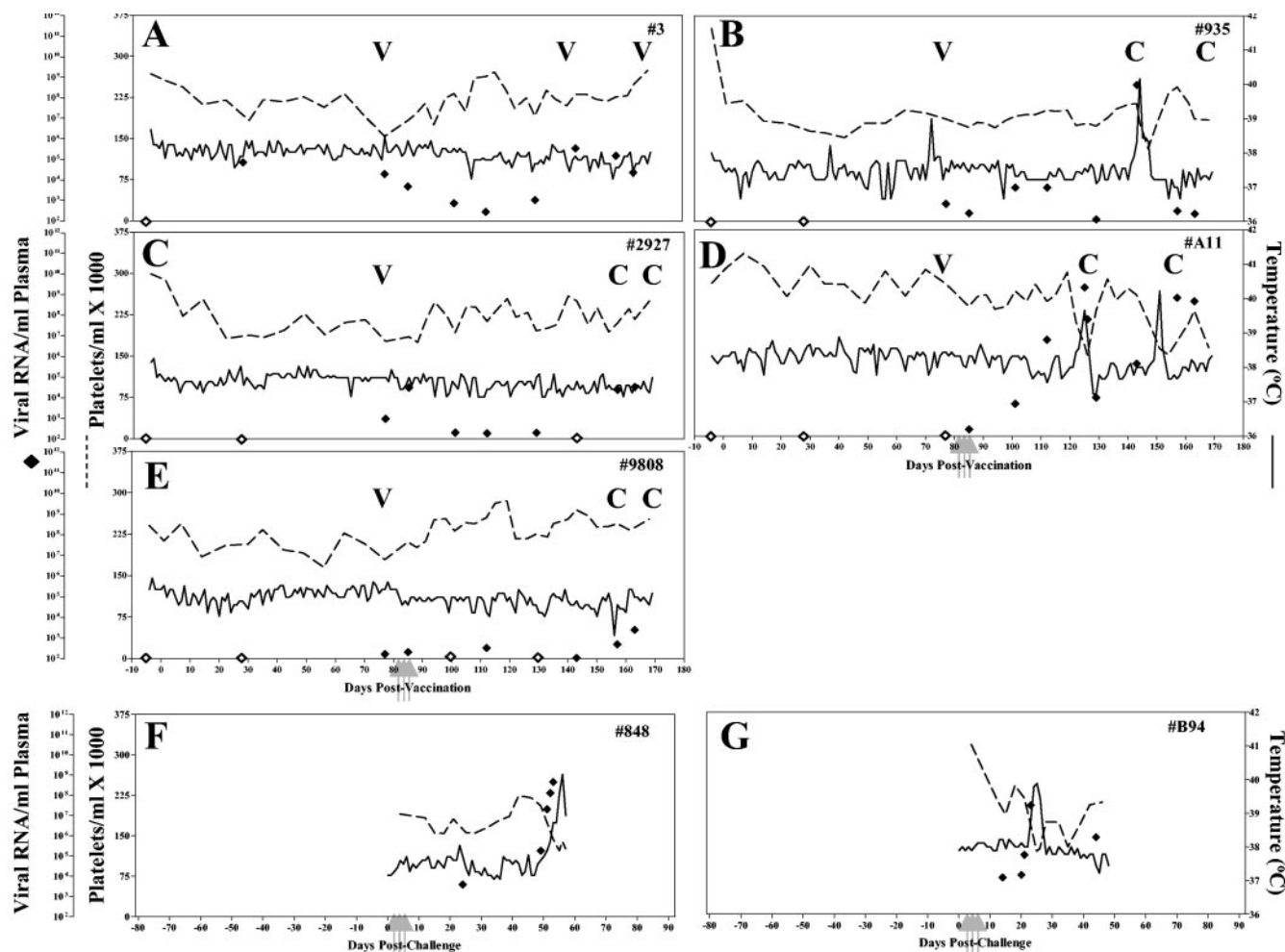
FIG. 7. Clinical and virologic profiles of EIAV$_{UK}$ΔS2 vaccinated horses and naive control horses challenged at 2 months. Five horses (A to E, respectively) were inoculated with EIAV$_{UK}$ΔS2 and challenged at 3 months after the first vaccine dose, as described in Materials and Methods. Two naive control horses (F and G) were subjected to challenge in parallel with virulent EIAV$_{PV}$ by using the LDME protocol (see Materials and Methods). Rectal temperature (solid line, right y axis) and platelet counts (dotted line, first left y axis) were monitored daily for up to 170 days (x axis) after vaccination. Quantification of the virus load (♦, second left y axis, ◇, undetectable) was performed on viral RNA extracted from plasma at periodic time points prior to and after virulent virus challenge by using the LDME protocol (arrows). S2 diagnostic results for each animal are indicated in each respective panel above the date of analysis by either a V (vaccine strain) or a C (challenge strain).

determinants (average ratio of 1.5). This progression of anti-body conformational properties was similar to the maturation observed in horses experimentally infected with the virulent EIAV$_{PV}$ (10, 11). Interestingly, the EIAV$_{UK}$ΔS2/DU (Fig. 5B.3) and EIAV$_{PR}$ΔS2 (Fig. 5C.3) inoculations were characterized by a less pronounced evolution of antibody conformational dependence compared to the EIAV$_{UK}$ΔS2 immunization (Fig. 3)5D). For example, the antibody conformational dependence values observed in the EIAV$_{PR}$ΔS2 vaccinates remained relatively constant at ~1.0 over the 6-month observation period, which is significantly different from the steady-state levels of EIAV$_{UK}$ΔS2 and EIAV$_{UK}$ΔS2/DU ($P < 0.01$), indicating a lack of the characteristic immune maturation by this particular vaccination.

We previously reported a relatively slow development of serum neutralizing antibodies over a several-month period after experimental EIAV infection with either virulent or aviru-

lent strains, with average maximum titers averaging 1:300 (11). To examine the ability of the various attenuated EIAV strains to elicit serum neutralizing antibody, we tested immune serum samples taken at 6 months postvaccination for their ability to inactivate the parental virulent EIAV$_{PV}$ infectivity in cultured fetal equine kidney cells (10), as summarized in Fig. 6. These data demonstrated that the EIAV$_{UK}$ΔS2 attenuated candidate generated serum antibody neutralizing titers that ranged from 1:10 to 1:90 at 6 months postinoculation. Although the level of neutralizing antibodies required to protect horses from infection or disease is not known, neutralizing antibodies were detectable in all eight vaccinates; however, only four horses developed titers above background levels by the day of challenge. In contrast, none of the horses in the EIAV$_{UK}$ΔS2/DU or the EIAV$_{PR}$ΔS2 groups displayed significant levels of serum neutralization in these standard assays. To evaluate statistically whether there was a correlation between protection from in-

TABLE 2. Properties of envelope-specific antibody responses in EIAV$_{UK}$ΔS2-immunized horses at 2 months

Animal	Endpoint titer	Avidity index	Conformation ratio	50% Neutralization titer
2927	3,200	22	1.09	0
9808	2,400	22	1.13	0
935	3,200	25	0.61	0
3	6,400	18	1.15	0
A-11	3,200	25	0.78	0

fection and increased levels of neutralizing antibodies, a Spearman rank correlation test was performed utilizing the data from all 24 animals. The calculated two-tailed P value was 0.0535 ($r = 0.3989$), which is considered not significant.

The combined serological assays described above indicate that the protective vaccine immunity did not require neutralizing antibody responses but that the vaccine efficacy was more closely associated with the extent of antibody maturation to the attenuated virus infection. The best protection was observed with the EIAV$_{UK}$ΔS2 inoculation, and this immunization elicited the highest values for envelope-specific antibody titer, avidity, and conformational dependence that most closely resembled steady-state antibody properties observed in long-term inapparent carriers (10, 11).

Development of vaccine protective immunity. To more directly test the correlation of antibody maturation with vaccine protective efficacy, we next evaluated the protection provided by vaccine immunity in horses inoculated with the highly effective EIAV$_{UK}$ΔS2 strain but subjected to LDME challenge at only 2 months postinoculation, instead of the 6-month interval described above. As depicted in Fig. 7A to E, three of the five vaccinates were protected from disease, whereas two experienced typical EIA symptoms by about 2 months postchallenge. Virus loads prior to challenge for all five horses were ~10^2 copies RNA/ml of plasma. Upon challenge with virulent EIAV, four of five horses displayed marked increases in virus loads. Virus levels peaked at an average level of 10^6, ranging between 10^5 and 10^8 copies of RNA/ml of plasma. In addition to the lower level of protection from disease, early challenge also resulted in four of five animals with detectable challenge virus. The two EIAV-naive control horses upon challenge with EIAV$_{PV}$ developed classic EIA symptoms (Fig. 7F and G) by 40 days postchallenge, displaying concurrent fevers and declines in platelets, accompanied by peaks in virus loads.

The properties of the EIAV envelope-specific antibody responses elicited by the EIAV$_{UK}$ΔS2 immunization by 2 months on the day of challenge are summarized in Table 2. These early antibody responses are characterized by a relatively low envelope specific antibody titer (average, 1:3,680), a lack of detectable neutralization activity, and avidity and conformational dependence values characteristic of immature immunity. Thus, these results clearly demonstrate a critical correlation between the extent of the maturation of vaccine immunity and the level of vaccine protection from virus exposure. Although the development of vaccine immunity with the attenuated virus vaccine is characterized here by a maturation of envelope-specific antibody responses, it is logical to assume a similar evolution of cellular immune responses as part of this maturation process.

DISCUSSION

We previously reported that inoculation of horses with an attenuated EIAV proviral vaccine, EIAV$_{UK}$ΔS2, induced protection from detectable virus infection and disease by experimental virulent EIAV challenge (20). The present study confirms these earlier observations with EIAV$_{UK}$ΔS2, supporting the ability of this vaccine to provide effective protection from virus exposure. Our observations with an engineered EIAV proviral vaccine appear to be relatively consistent with earlier reports describing an attenuated EIAV vaccine used in millions of horses in China during the 1980s and credited with controlling EIA in that country, leading to a discontinuation of vaccination against this disease (34). The Chinese EIAV vaccine was attenuated by classical long-term serial passage in donkey leukocytes, with the final vaccine being a genetically uncharacterized attenuated strain but presumably a complex mixture of genomic quasispecies. Although there has been some speculation that the efficacy of the Chinese EIAV vaccine was attributed to special but undefined properties of this virus mixture, our evaluations of the EIAV$_{UK}$ΔS2 proviral vaccine clearly indicate that vaccine protection against EIAV can be achieved with a homogenous vaccine virus strain engineered from a molecular proviral clone. Similar vaccine protection has been observed with attenuated provirus clones in the SIV-monkey and FIV-cat lentivirus systems (15, 16, 23, 29, 36).

However, the data presented here also demonstrate that not all attenuated EIAV proviral constructs are equally effective in eliciting protective vaccine immunity in experimentally inoculated horses. In fact, relatively minor modifications in the genetic mutations used to attenuate the virus resulted in marked differences in the efficacy of protection from disease and infection by experimental challenge (Table 1). The protective efficacy of the series of attenuated EIAV strains was inversely correlated with their extent of attenuation and steady-state replication levels in inoculated horses. We have previously reported that mutation of the S2 gene of the virulent EIAV$_{UK}$ provirus clone reduced in vivo steady-state virus replication levels by ~100-fold compared to the parental virus, leading to a substantial attenuation of virulence (18). The EIAV$_{UK}$ΔS2 and EIAV$_{PR}$ΔS2 vaccine strains contain the S2 mutation but in the context of a virulent and avirulent proviral backbone, respectively. Since the EIAV$_{UK}$ and EIAV$_{PR}$ provirus clones were derived from the same parental virus stock, their genomic sequences are highly conserved, with <1.06% amino acid sequence variation in their envelope genes. However, the virulent EIAV$_{UK}$ provirus has been shown to establish steady-state replication levels in experimentally infected horses at levels that are 10- to 100-fold higher than those observed for the avirulent EIAV$_{PR}$ provirus (6). This difference in replication properties appeared to be reflected in the S2 attenuated proviruses, since the levels of EIAV$_{UK}$ΔS2 virus in plasma were consistently 10- to 100-fold higher than the virus levels in plasma observed with the EIAV$_{PR}$ΔS2 in the parallel vaccine groups. Similarly, introduction of mutations in both the S2 and DU genes of EIAV$_{UK}$ reduced steady-state replication levels of the EIAV$_{UK}$ΔS2/DU provirus by 10- to 100-fold compared to the EIAV$_{UK}$ΔS2 containing only the S2 mutation. Interestingly, an inverse relationship between vaccine protection and

proviral attenuation has also been reported in the SIV-monkey system (16, 25). These combined observations in two lentivirus systems indicate a threshold level of persistent viral replication and presumably sustained viral antigen presentation necessary to sufficiently drive host immune responses to achieve protective immunity.

In addition to adequate levels of steady-state EIAV replication, the second critical parameter for achieving protective vaccine immunity is the length of time postinoculation with the attenuated virus. In the case of the $EIAV_{UK}\Delta S2$ vaccine, 100% protection from detectable infection and disease was achieved at 6 months postinoculation, whereas at 2 months postinoculation the protective efficacy was reduced to 20% for infection and 60% for disease by the same attenuated virus construct and virus challenge modality. Thus, these data indicated a progressive development of protective immunity during the first 6 to 7 months postinfection, suggesting an evolution of virus-specific immune responses to the persistent attenuated EIAV infection. In studies with an attenuated SIV vaccine in monkeys, we have previously reported a protective efficacy of 50% at 3 months postinoculation compared to 100% at 8 months postinoculation (3–5). Thus, the combined EIAV and SIV attenuated virus vaccine data suggest that the prolonged process for the development of protective vaccine immunity may be a general and distinguishing property of lentiviruses.

Although the specifics of the evolution of these immune responses to persistent EIAV infection remain to be defined, the serological assays defining the development of EIAV-envelope specific antibody responses appear to reflect the progression to protective vaccine immunity. Thus, the extent of immune maturation was directly associated with the level of protective vaccine efficacy. For example, the most protective vaccine immunity elicited by the $EIAV_{UK}\Delta S2$ strain at 6 months postinoculation was associated with mature antibody responses characterized by high titer, high avidity, and predominantly conformational dependence. In contrast, the reduced vaccine protection observed with the $EIAV_{UK}\Delta S2$ virus at 2 months postinoculation was associated with immature antibody responses characterized by relatively low titer, low avidity, and equally linear and conformational specificity (Table 2). Similar to these early vaccine immune responses, the more severely attenuated $EIAV_{PR}\Delta S2$ and the $EIAV_{UK}\Delta S2/DU$ vaccine strains generated relatively immature antibody responses by 6 months postinoculation. It remains to be determined whether the immune responses to these latter more attenuated vaccines can achieve maturation and higher protection, even with a substantially more extended infection time.

The association of EIAV envelope-specific antibody maturation parameters with protective efficacy of vaccine immunity provides useful insights into the properties of protective immunity but cannot with this limited number of animals reveal statistically reliable immune correlates of protection. In this regard, a more detailed characterization of the evolution of cellular immune responses to attenuated EIAV vaccines is currently in progress. However, the current studies do indicate that in vitro serum antibody neutralization activity was apparently not required for the maximum vaccine protection as produced by the $EIAV_{UK}\Delta S2$ vaccine. In fact, horses immunized with this attenuated virus were equally protected regard-

less of whether low levels of neutralizing antibody titers were detected in the immune serum from the day of challenge. Correlation analysis between neutralizing antibodies and protection from infection suggested some association but failed to reach statistical significance in this data set. These observations suggest that cellular immune responses or antibody functions not measured in our in vitro assays are sufficient for protective vaccine immunity. The data do not necessarily eliminate the potential role of neutralizing antibodies in vaccine protection, if present at sufficient levels.

The achievement of protective immunity by a suitable attenuated EIAV vaccine lends support to the hypothesis that effective vaccine immunity is feasible to preclude lentivirus infections, despite the challenges that these viruses pose to the host immune system. It is important to note here that the current vaccine trials assess efficacy based on a challenge virus ($EIAV_{PV}$) that is a biological clone that is closely related to the provirus vaccine envelope species. Based on the solid protection observed here, it will be of importance in future experiments to evaluate the efficacy of the $EIAV_{UK}\Delta S2$ vaccine in protecting against a panel of EIAV envelope variants with different levels of defined envelope protein sequence variation. Thus, the attenuated EIAV vaccine model provides a unique system in which to directly test the concept that natural virus envelope heterogeneity poses a major obstacle to vaccine development, as assumed for AIDS vaccines.

Do attenuated provirus vaccines offer a practical approach to the development of a commercial EIAV vaccine that can be applied to horses worldwide? As noted above, Chinese veterinarians have reported elimination of EIAV outbreaks in that country after a compulsory inoculation of millions of horses with their attenuated EIAV vaccine (34). Due to the lack of systematic monitoring during the immunization program, however, reliable data on the current rate of EIA infections or the incidence of vaccine reversions appear to be unavailable. Although offering an important and informative lentivirus vaccine model for discerning immune correlates of protection, attenuated EIAV vaccines pose a number of practical problems for application. First, there are the theoretical concerns about potential reversion of the attenuated vaccines strain to virulence, a possibility made more likely by the persistent nature of the infection and the propensity for relentless genomic mutation during EIAV replication. To minimize the potential for reversion, attenuated vaccines could be engineered with multiple gene mutations, but the experiments described here highlight the delicate balance between the extent of attenuation and the degree of vaccine protection. Second, the persistent infection by an attenuated EIAV poses an undefined risk for in utero transmission and abortion and for virulence in immune compromised horses or foals with developing immune systems. Finally, attenuated EIAV vaccines elicit antibody responses to viral core and envelope proteins, resulting in seropositivity in current diagnostic assays used to control EIA in the United States, Europe, and other countries. In developing countries where EIAV infection is common and government regulatory policies are not defined, it is possible that the risk to benefit calculation could favor use of an attenuated EIAV vaccine for several years to break the cycle of infection and disease, as reportedly done in China.

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