

Correlation between the induction of Th1 cytokines by an attenuated equine infectious anemia virus vaccine and protection against disease progression

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The equine infectious anemia virus (EIAV) donkey-leukocyte attenuated vaccine (DLV) has been used to protect against equine infectious anaemia (EIA) disease for several decades in China. The attenuated mechanism and immunological protective mechanisms remain to be elucidated. To identify responses that correlate with the protection against disease, we immunized horses with DLV, followed by challenge with an EIAV wild-type strain LN. All vaccinated horses were asymptomatic and had a low level of virus replication (< 10 copies ml^{-1}). The expression level of cytokines including gamma interferon, interleukin 2 and 12 in DLV immunized horses was 5–100-fold higher than that in non-vaccinated controls ($n=4$, $P<0.01$). After challenge with virulent LN, horses vaccinated with DLV showed lower viral loads ($< 10^3$ copies ml^{-1}) with no temperature increase, except for one transient febrile episode in one animal. In contrast, horses in the non-vaccinated control group experienced much higher viral loads ($> 10^7$ copies ml^{-1}) and intermittent febrile episodes. Cytokine production in the DLV-vaccinated horses increased and attained a plateau level at approximately 50 days post-vaccination, and exceeded 10^7 copies per 10^7 peripheral blood mononuclear cells (PBMCs) 1–3 months post-challenge. However, non-vaccinated control horses died after several fever episodes (≥ 39 °C), which coincided with higher viral load (10^6 – 10^7 copies ml^{-1}) and lower cytokine production ($< 10^4$ copies per 10^7 PBMCs). The results indicate that high levels of EIAV-specific cytokines induced by the attenuated EIAV vaccine may contribute to the protective immune response against EIA disease.

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INTRODUCTION

Equine infectious anemia virus (EIAV) is a lentivirus related to *Human immunodeficiency virus* (HIV). EIAV donkey-leukocyte attenuated vaccine (DLV) was produced by serial *in vitro* passages of an EIAV homologous wild-type strain LN in donkey-leukocyte cells in the 1970s. Previous studies showed that DLV could induce protective immunity against challenges at fatal doses of virulent LN and effectively protects against disease in horses and donkeys (Shen *et al.*, 1979). DLV is the only retrovirus attenuated vaccine used on a large scale in the world to date. It provides a unique natural model system. Elucidation of the DLV attenuated

mechanism and immunological protective mechanisms may aid in the development of vaccines for other retroviruses, including HIV. In previous studies, we have identified several key mutation sites in the DLV sequence which are likely to contribute to the attenuation (Liang *et al.*, 2006; Shen *et al.*, 2006). However, the immunological protective mechanism of EIAV attenuated vaccine DLV was not clear.

It was shown that neutralizing antibody responses developed about 30–40 days post-infection and matured over a period of several months (Kono *et al.*, 1973; Rwambo *et al.*, 1990; Hammond *et al.*, 1997). The period of immune system maturation after vaccination with live-attenuated vaccine is at least 6 months in horses (Shen *et al.*, 1979; Li *et al.*, 2003). The neutralizing antibodies are primarily directed toward the EIAV envelope. Given the relatively slow development of neutralizing antibodies, cellular immune responses are likely to be important in combating viruses in the early stage of EIAV infection. Studies showed that cellular immunity

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Supplementary tables showing the primers used for this study are available in JGV Online.

could significantly contribute to the control of lentivirus infection (Jin *et al.*, 1999; Ogg *et al.*, 1999; Schmitz *et al.*, 1999). The cytotoxic T-lymphocytes (CTL) response is considered to play a critical role in the control of HIV replication (Goulder & Watkins, 2004). EIAV-specific CD8⁺ and CD4⁺ CTL have been identified in horses recently infected with EIAV and memory CTL are present in EIAV-infected healthy carrier horses (McGuire *et al.*, 2004), indicating that the CTL response may associate with the control of virus replication. However, it remains unknown whether this is the case in our attenuated EIAV vaccine model.

Proinflammatory cytokines are the central mediators of both innate and adaptive immunity and modulation of these cytokines has been shown to alter anti-HIV-1 reactivity *in vitro* (Connolly *et al.*, 2005). Because the Th-1 cytokines gamma interferon (IFN- γ), interleukin (IL)-2 and -12 have been widely used to gauge the activities of cellular immunity, methods were established to measure the production of these cytokines in horses (Wang *et al.*, 2005) to determine cellular immunity.

In this study, horses were immunized and then challenged with EIAV wild-type strain LN. The resulting cytokine production and viral load levels in peripheral blood mononuclear cells (PBMCs) of vaccinated and non-vaccinated control groups were compared.

METHODS

Virus stock and cell culture. Chinese EIAV attenuated vaccine DLV was developed by a series of passages *in vitro* from EIAV wild-type LN strain. The vaccines were developed in two stages: (i) a carefully selected field isolate LN was passaged 133 times *in vivo* to generate the highly virulent and pathogenic strain D510, which kills animals within a few weeks; (ii) D510 went through 135 passages *in vitro* to obtain DLV vaccine strains. The vaccine strains are very stable in that no virulent reversion has been observed in numerous tests involving several hundred horses and donkeys. Vaccinated animals resist challenge from homologous and heterologous virulent EIAV strains, and safety and efficacy have been repeatedly confirmed in over 70 million vaccinated horses for more than 20 years use in China. Both DLV and LN were stored at -80°C as stock at Harbin Veterinary Research Institute. Fetal donkey dermal cells (FDD) and donkey primary lymphocyte (DL) culture systems were described previously (Shen *et al.*, 1979). DL cell cultures were prepared from heparinized peripheral blood as equine MDM (monocyte/macrophage differentiation-maturation system) cells. The minimal infectious dose of DLV was determined to be 1×10^4 TCID₅₀ by limiting dilution assay with DL cells.

Horses, clinical evaluation and sample collection. All horses used in these studies were thoroughbreds. Prior to the experiment, serum samples from all horses were tested twice by the agar gel immunodiffusion test (VMRD) to ensure that they were seronegative for EIAV infection (Pare & Simard, 2004). They were housed in screened box stalls to exclude haematophagous insects and all animal handling protocols were approved by the Animal Management Committee of Chinese Harbin Veterinary Research Institute. Rectal temperature and clinical status were recorded twice daily. Samples of plasma and whole blood were collected every 2 weeks in the first 3 months and then every month thereafter.

Samples were also collected during each febrile episode (rectal temperature $\geq 39^{\circ}\text{C}$). The plasma samples were stored at -80°C for viral load detection. Clinical episodes of equine infectious anaemia (EIA) were defined when thrombocytopenia ($< 100\,000$ platelets μl^{-1}) occurred with fever, concurrent with detectable plasma viral RNA.

Vaccination procedures. The animals were randomized into four groups: naive healthy horses (A2 and A5) inoculated with 3 ml supernatant of normal DL culture as negative controls (Group I), DLV vaccination followed by a virulent EIAV challenge (A3, A6, A7 and A8) (Group II), virulent EIAV challenge without DLV vaccination (A1, A4, A10 and A12) (Group III) and naturally EIAV-infected horses (C1, C2, C65 and C66) as positive controls (Group IV). The Group IV horses were discovered when the horses were screened for EIAV infection by agar gel immunodiffusion test prior to the study. We arbitrarily selected four seropositive horses for EIAV infection that had had no clinical presentations and fever episodes in the last 3 months. The important regions of the viral genome, including *gag*, *pol* and *env*, were sequenced. Sequencing data showed that the EIAV strain naturally present in horses had nearly 70% similarity with LN strain in amino acid sequence. The dose of DLV vaccination was 4×10^5 TCID₅₀ and that of virulent EIAV LN challenge was 10^5 TCID₅₀. All animals were subcutaneously injected with vaccine or challenge strain.

Viral RNA purification. All samples from the same time point were processed in parallel to minimize the difference potentially caused by the operator. Viral RNA was isolated from 140 μl EDTA-anticoagulated plasma by using a QIAamp Viral RNA kit (Qiagen) and treated with DNase I on a spin column (DNase I set; Qiagen). All procedures strictly adhered to the manufacturer's instructions. RNA was finally eluted in 60 μl AVE buffer and analysed immediately.

Quantification of plasma virus RNA. Real-time RT-PCR was used to determine plasma viral load by amplification of the EIAV *gag* gene (Liang *et al.*, 2006). Briefly, the following primers were used for real-time RT-PCR using a QuantiTect Probe RT-PCR kit (Qiagen): forward primer, 5'-CAGATTGCTGCTCAGATAAA-3'; reverse primer, 5'-GTGTCTGTCAGGAATTTAGTT-3'; and TaqMan probe (Applied Biosystems), 5'-FAM-TCAGCCGGATGTCCCTCACT-TAMRA-3'. An RNA standard template was made by cloning the partial *gag* DNA segment from pFD3 (nt 1144–1533) into pGEM-T Easy vector (Promega), followed by *in vitro* transcription. Data analysis was conducted with Sequence Detection software version 1.9 (Perkin-Elmer).

Equine cytokine production detection. Specific primers of horse IFN- γ (Curran *et al.*, 1994; GenBank accession no. D28520), IL-2 (accession no. X69393) and IL-12 IP40 subunit (accession no. Y11130) are listed in Supplementary Tables S1 and S2 (available in JGV Online).

Preparation of cytokine RNA. Total RNA was isolated from healthy and vaccinated horse PBMCs with an RNeasy mini kit (Qiagen), according to the manufacturer's instructions. Briefly, freshly isolated horse PBMCs (1×10^7) were disrupted by adding 600 μl Buffer RLT (Qiagen). The lysate was homogenized for 30 s using a rotor-stator homogenizer, and 1 vol. 70% ethanol was added. Then, 700 μl sample was transferred to an RNeasy spin column and centrifuged for 15 s at 10 000 r.p.m. (8500 g); the RNA was eluted with 50 μl RNase-free water.

After total RNA was prepared, the quality of the RNA samples was examined by agarose-formaldehyde electrophoresis and ethidium bromide staining. The respective rRNAs should appear as clear bands

or peaks. The apparent ratio of 28S rRNA to 18S rRNA should be approximately 2:1. The RNA samples were also examined by absorbance measurement to assess the RNA recovery efficiency.

RNA standard preparation. First-strand cDNA was primed in a 10 µl DEPC H₂O solution containing 1 µg total RNA isolated from healthy horse PBMCs, 50 pmol oligo(dT) primer and 1.5 U reverse transcriptase. Reaction conditions were as follows: 65 °C for 5 min, then 25 °C for 10 min. Full-length DNA fragments of IFN-γ (521 bp), IL-2 (632 bp) and IL-12 P40 subunit (669 bp) were separately amplified by PCR in 20 µl reactions containing 4 µl PCR reaction buffer (5×), 0.4 µM forward primer, 0.4 µM reverse primer, 0.5 mM dNTP, 4 units *Taq* DNA polymerase (Qiagen) and 1 µl cDNA. Reaction conditions were as follows: 95 °C for 2 min, followed by 30 cycles at 94 °C for 20 s, 56 °C for 20 s and 72 °C for 30 s, and finally 72 °C for 5 min. The PCR products were purified and cloned into pBluescript II SK vector (Stratagene) individually for RNA transcription *in vitro*. The RNA transcripts were used as RNA standards.

Detection of the corresponding copy numbers of cytokines. IFN-γ, IL-2 and IL-12 mRNAs from horses inoculated with EIAV virulent or attenuated strains were detected using real-time RT-PCR. The 50 µl reaction solutions contained 25 µl RT-PCR Master Mix (2×; Applied Biosystems), 0.4 µM forward primer, 0.4 µM reverse primer, 0.2 µM Taqman probe, 0.5 µl RT mix and 10 µl RNA sample or RNA standard. Reactions were performed in triplicate. Cycling conditions were 48 °C for 30 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min.

The cycle threshold (C_t) value for each sample was calculated by determining the point at which the fluorescence exceeded a threshold limit (10 times the standard deviation of the baseline as determined between cycles 3 and 15). Standard curves proportional to the log of the starting amount of nucleic acid were produced according to that C_t value. Detection and data analysis were performed with the ABI Prism 5700 SDS program.

Statistical analysis. Two-tailed *t*-test and ranks test were used for normal and abnormal distributions, respectively. An alpha level of 0.05 was used.

RESULTS

Observation of clinical symptoms and measurement of viral loads

To examine the protective efficacy of the immune responses elicited by the attenuated vaccine, horses were monitored daily for clinical signs of EIA (fever, lethargy and diarrhoea). Blood samples were taken at regular intervals as described previously for measurement of platelets, plasma virus and EIAV-specific cellular immunity and cytokine production. Eight months post-DLV vaccination the horses were challenged with virulent EIAV LN and observed for 240 days.

Group I and II horses remained asymptomatic for EIA. This was demonstrated by lack of fever episodes and thrombocytopenia, which are considered to be sensitive indicators of EIA. The absence of detectable clinical symptoms after DLV vaccination was associated with the relatively low level of plasma viral loads, ranging from undetectable to 10^3 copies (ml of plasma)⁻¹ (data not shown). The sustained low-level

virus replication may be helpful in driving the maturation of immune responses to establish enduring, broad protective immunity.

Eight months later, Group II horses were challenged with virulent EIAV LN, all horses in this group survived the observation period (Fig. 1). The horses showed no temperature increase except for one febrile episode (39–42 °C) in one animal (A3), which was concurrent with a lower platelet count ($91\,750$ platelets µl⁻¹) that returned to normal 3 days later. The others remained asymptomatic for EIA. In the first 3 months post-LN challenge, the viral loads of horses A3, A6, A7 and A8 were consistently less than 1×10^3 copies ml⁻¹, except A3 which reached 1×10^5 copies ml⁻¹ during the fever episode. Thereafter, the viral load was too low to be detectable (Fig. 2a).

All horses without pre-immunization with DLV, however, developed typical EIA episodes (fever and thrombocytopenia). Horses A1 and A12 had similar patterns of disease progress and died of EIA within 21 days and A4 died of EIA on day 141 (Fig. 1). Horse A10 died on day 219. The reason for the death of A10 remains unknown since no pathological examination was accessible at that time and may not be related to EIA since horse A10 was not febrile and did not have an obvious thrombocytopenia at the time of death.

The lowest platelet counts were about $45\,000$ platelets µl⁻¹ for A1 and A12 and $65\,000$ platelets µl⁻¹ for A4 and A10 during the fever episodes. The plasma viral RNA levels reached 10^8 copies (ml of plasma)⁻¹ during fever episodes, and were in excess of 10^3 copies ml⁻¹ even in the interval between fever episodes (Fig. 2b).

Cytokine production

As shown in Fig. 3(a), the mean baselines of IFN-γ, IL-2 and IL-12 in PBMCs were 3.7×10^4 , 4.99×10^3 and 4.03×10^5 copies per 10^7 PBMCs, respectively, for horses without any treatment; and 4.05×10^5 , 5.39×10^4 and 1.3×10^6 copies per 10^7 PBMCs, respectively, for Group IV. The absolute value of IL-12 was highest among the three cytokines in both groups. However, the magnitude increase of IL-12 (3-fold) elicited by EIAV natural infection was much lower than that

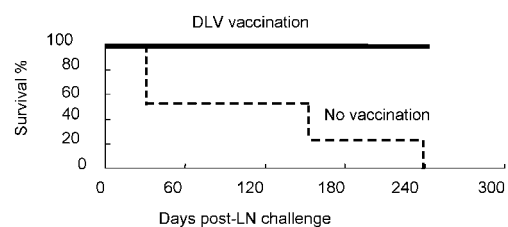


Fig. 1. Survival time of horses after LN challenge. The group of DLV vaccinated horses consists of horses A3, A6, A7 and A8, and the group of non-vaccinated horses consists of horses A1, A4, A10 and A12. The *x*-axis indicates days post-LN challenge. The *y*-axis indicates the percentage of surviving horses.

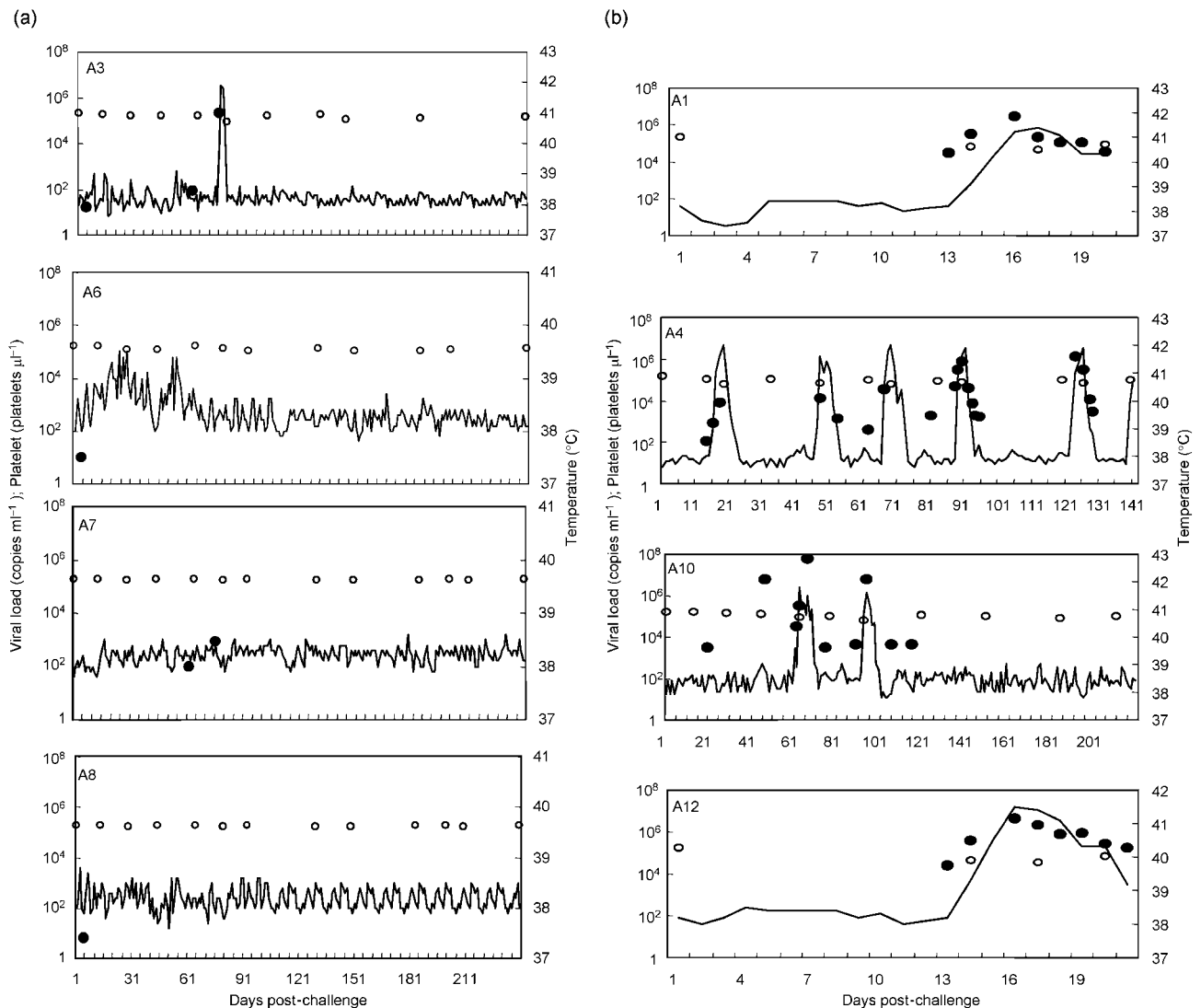


Fig. 2. Viral load after challenge with LN in horses vaccinated with or without DLV. (a) Horses A3, A6, A7 and A8 were first vaccinated with DLV (4×10^5 TCID₅₀), followed by virulent EIAV LN challenge (1×10^5 TCID₅₀). Plasma samples were collected at least once a month, but viral loads were undetectable at most time points. (b) Naive, healthy horses A1, A4, A10 and A12 were challenged with virulent EIAV LN (1×10^5 TCID₅₀). The x-axis indicates days post-challenge, the left y-axis indicates viral load (copies ml⁻¹) and platelet counts (platelets μ l⁻¹), and the right y-axis indicates rectal temperature (°C) of horses. The symbols ●, ○, and – indicate horse viral load, platelet and temperature, respectively

of IFN- γ and IL-2, which were almost 11-fold. A similar trend was also observed in the DLV-vaccinated group. The production of all three cytokines increased steadily for 2 weeks and reached a plateau 2–3 months post-DLV vaccination. The corresponding values for IFN- γ , IL-2 and IL-12 were 7.33×10^6 , 5.84×10^5 and 2.16×10^6 copies per 10^7 PBMCs. These represent significant increases above the baseline (198-fold, 117-fold and 5-fold, respectively; $P < 0.01$) and above that of Group IV (18-fold, 10-fold and 2-fold, respectively; $P < 0.01$).

Eight months post-DLV vaccination, horses were challenged with the virulent strain EIAV LN. The production of all three

cytokines rapidly increased after infection with LN (Fig. 3b) and peaked (10^7 – 10^8 copies per 10^7 PBMCs) 1 month post-LN infection; thereafter, the cytokine production gradually declined. Interestingly, while both IFN- γ and IL-12 levels dropped to the baseline level 3 months post-LN challenge, IL-2 production retained an approximately 10-fold baseline level, suggesting that IL-2 level may be an important indicator for protection.

All horses without DLV vaccination developed acute or chronic disease after challenge with virulent EIAV. There were two different patterns of disease progression (Fig. 4). One pattern was rapid progression to EIA and death within

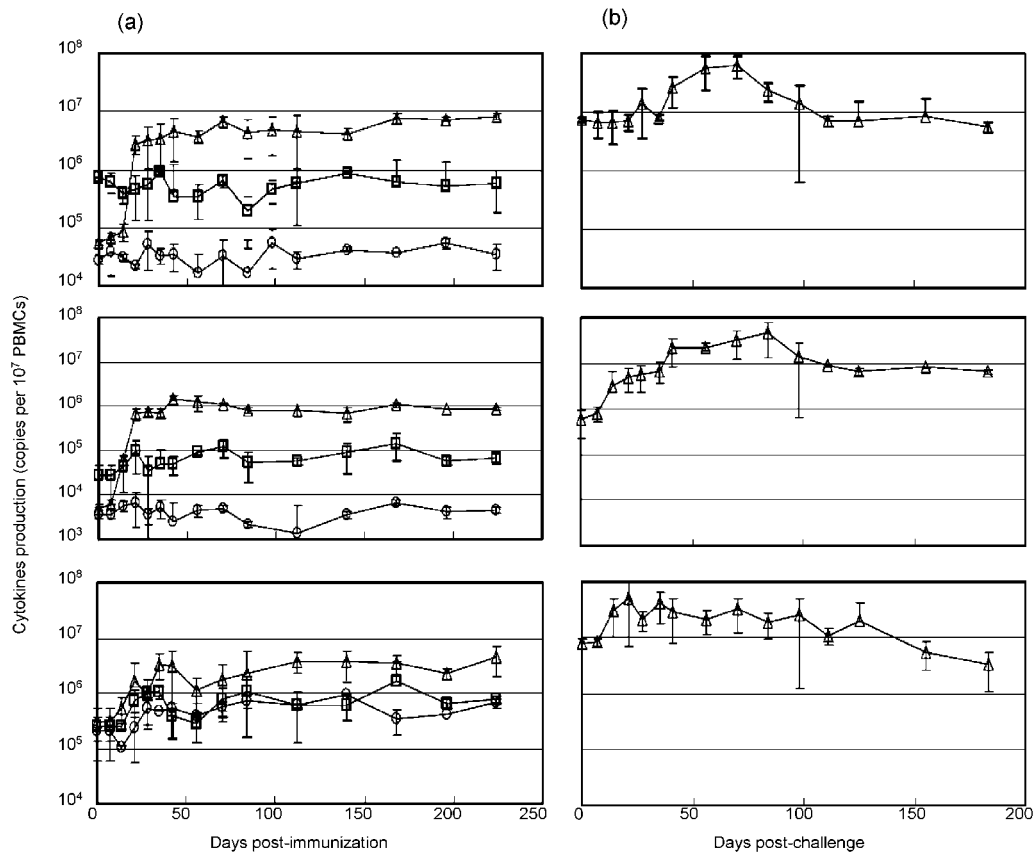


Fig. 3. Production of cytokines induced post-DLV vaccination and LN challenge. The mean level of cytokine production (copies per 10^7 PBMCs) for non-vaccinated control horses (\circ), DLV vaccinated horses post-vaccination (\triangle) and EIAV naturally infected horses (\square) are shown at left (a) and DLV vaccinated horses post-viral challenge are shown at right (b). The x-axis indicates days post-immunization (a) and challenge (b). The y-axis indicates cytokine production (copies per 10^7 PBMCs).

3 weeks, such as in horses A1 and A12; the second was slow progression as exemplified by horse A4. During the primary acute infection period, a transient increase of cytokine production of IFN- γ and IL-12 was observed in horses A1, A10 and A12 but not in horse A4. It was followed by a decrease when the fever episode occurred (2 weeks for A1 and A12 to develop the fever episode and 65 days for A10), which coincided with the absence of clinical episode within the first 65 days post-infection and prolonged survival. A gradual recovery of cytokine production in horses A4 and A10 occurred coincidentally with the decrease of viral loads and resolution of fever.

DISCUSSION

Animal experiments showed that DLV induces protective immunity against challenges of fatal doses of homologous (LN) and heterologous (Wyoming) EIAV virulent strains in horses and donkeys (Shen *et al.*, 1979). The mechanism by

which the attenuated EIAV vaccine protects against viral challenge by the diverse virulent EIAV strains remains to be determined.

In this study, the previous observation that DLV vaccination could protect against EIA disease was partially confirmed. Three out of four vaccinated horses were fully protected from EIA disease and did not show symptoms. The 100% protected horses (A6, A7 and A8) were found to maintain normal temperatures and low levels of plasma viral loads. Only one horse (A3) showed a spike of high fever and higher viral load (10^5 copies ml^{-1}). Non-vaccinated horses challenged with LN, however, developed typical EIA. A1 and A12 died after only a single clinical episode, A4 and A10 died after several fever episodes though A10 was afebrile at the time of death. To elucidate the protective mechanism, horse temperature, plasma viral loads and EIAV-specific cytokine production were monitored. Viral load was highest in the group of horses challenged with LN without DLV vaccination [about 10^7 – 10^8 copies ($\text{ml plasma})^{-1}$ during a febrile episode and greater than 10^3 copies ml^{-1} between febrile episodes].

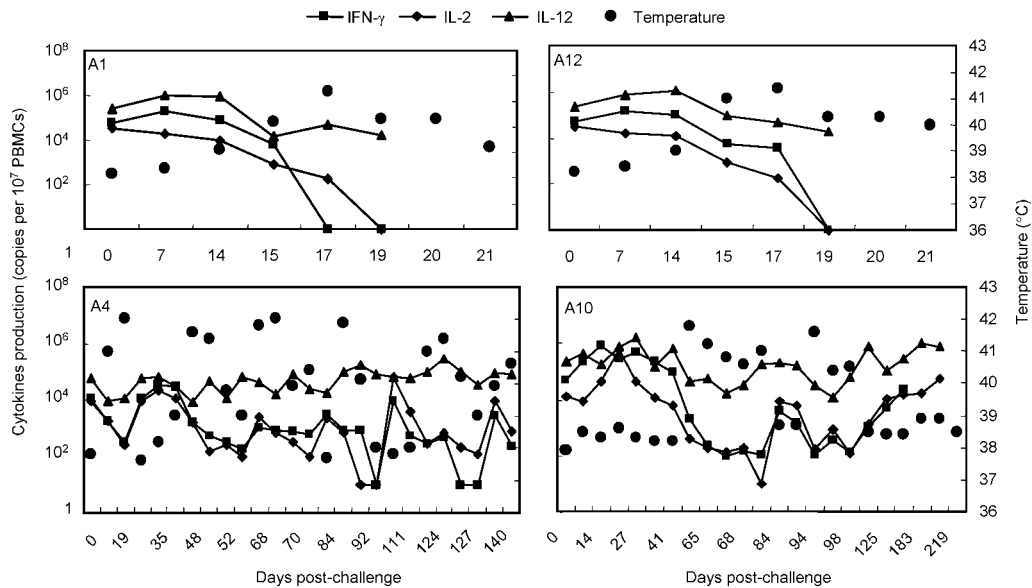


Fig. 4. Cytokine production of LN-challenged horses without vaccination. Naïve, healthy horses A1, A4, A10 and A12 were challenged with LN without prior DLV vaccination. The x-axis indicates days post-challenge, the left y-axis indicates cytokine production (copies per 10^7 PBMCs), and the right y-axis indicates horse rectal temperature ($^{\circ}\text{C}$).

Significantly increased expression of IFN- γ , IL-2 and IL-12 was observed after both DLV vaccination and the following virulent EIAV LN challenge. Overall, the cytokine data indicated that: (i) DLV vaccination resulted in a significant increase in cytokine production compared to baseline and to negative controls, which was also higher than that seen in naturally EIAV-infected horses; (ii) IFN- γ and IL-12 returned to a level similar to baseline 3 months post-LN challenge; (iii) IL-2 levels remained relatively high and constant after LN challenge in the DLV-vaccinated horses and may be an important predictor for protection; (iv) cytokine levels were either not sustained or fluctuated at a lower level in non-vaccinated horses after LN challenge.

It has been demonstrated that antibody responses mature by increasing in avidity and conformational dependence following infection with EIAV (Hammond *et al.*, 1997) and may play a role in protection from disease or infection. In the present study, neutralizing antibodies were also detected in the sera of horses vaccinated with DLV. These results suggest that neutralizing antibodies elicited by DLV may play an important role in the control of EIAV infection of LN strain (Shen *et al.*, 1979; and unpublished data). The mechanism of the humoral immune response elicited by DLV is currently under investigation.

The elucidation of these immunological protective mechanisms should provide a foundation for designing new EIAV vaccines, which may also lead to more effective vaccines for other lentiviral infections such as HIV.

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