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Talia R. Henkle

University of Kentucky, taliahenkle@hotmail.com

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Comparison of Th1 cytokines and T cell markers gene expressions between virulent and an attenuated EIAV vaccine strain

Talia Henkle, Chong Liu, David W. Horohov.

Gluck Equine Research Center, University of Kentucky

Abstract

The equine infectious anemia virus (EIAV) is closely related to HIV and has been used as a model to identify protective mechanisms against lentivirus infection. In horses, EIA infection progresses for about a year before infected horses manage to control virus replication. This naturally-gained protection is absolutely dependent on active immune responses as evidenced by the fact that immunosuppressive drugs can induce the recurrence of disease. As the resolution of initial viremia correlates with the appearance of virus specific cytotoxic T lymphocytes (CTL), we believe that cellular immune responses play a key role in controlling EIAV in the horse. In a previous study, a modified live EIAV strain (D9) provided effective protection against homologous virus challenge without causing disease, but this vaccine could not achieve optimum protective immunity until six months post vaccination. In this study, development of cell-mediated immunity was monitored in EIAV_{D9} infected ponies over a six-month period. We hypothesized that both Th1 cytokines and CTL would be upregulated in EIAV_{D9} infected ponies. To test this hypothesis, ponies were inoculated with the attenuated EIAV_{D9} strain. Whole blood samples were collected into PAXgene™ tubes weekly during the acute infection stage (first 6 weeks) and at monthly intervals thereafter corresponding to the chronic infection phase. Total RNA was isolated from the PAXgene™ tubes and gene expression for both Th1 cytokine (IFN γ) and CTL markers (Granzyme B and Perforin) were determined using real-time PCR. At the same time, another group of ponies receiving a virulent EIAV were monitored and sampled as comparison. We found that Th1 cytokine (IFN γ) and CTL marker (perforin) genes were significantly increased four months post EIAV_{D9} infection. These results indicate that the maturation of cellular immune response in EIAV_{D9} infected horses requires at least four months.

1. Introduction

Modified live or attenuated vaccines have been proven effective at preventing viral diseases such as polio, measles, and smallpox (Mims, 1987). Since an attenuated vaccine for HIV-1 is desired, work towards this goal has focused on closely related animal lentiviruses (Craig et al., 2007).

Equine Infectious Anemia Virus (EIAV) is a lentivirus that solely infects equids. EIAV causes a globally prevalent chronic infection among equids, which makes the development of a vaccine for EIAV an active area of research in equine veterinary medicine (Capomaccio et al., 2012a; Capomaccio et al., 2012b; Craig et al., 2007).

EIAV is transmitted via blood-feeding insect vectors, or via medical procedures such as the use of virally contaminated needles (Capomaccio et al., 2012a; Capomaccio et al., 2012b). EIAV manifests itself in three stages; acute, chronic, and inapparent. Once infected with EIAV, the virus replicates within the equid for about 5 weeks before it can be detected. Viremia is usually indicated by a fever, which occurs right before the equid tests positive (Coggins Test). The acute signs of EIAV are fever, reduced platelet counts,

and a mild loss of appetite. The resolution of the acute phase is correlated with the appearance of the virus specific cytotoxic T-lymphocytes (CTLs) (Capomaccio et al., 2012a; Capomaccio et al., 2012b). After approximately 3-4 months the disease progresses into the chronic phase which is characterized by recurring fever, pronounced platelet reduction, ruptured blood vessels on mucous membranes, anemia, depression, and swelling of limbs (Capomaccio et al., 2012a; Capomaccio et al., 2012b; Craigo et al., 2007). More than 90% of horses progress into the life-long inapparent carrier phase 8-12 months post infection (Craigo et al., 2007). In this phase, viral load is tightly controlled by the horse's immune system and the horse becomes asymptomatic of EIAV infection. Unlike equids and EIAV, humans possess no natural immunological protection mechanisms against their respective lentivirus, HIV, which makes EIAV a unique and valuable model for HIV research.

Over the past twenty years, a wide range of EIAV vaccines have been developed with efficacies ranging from sterilizing immunity to profound enhancement of viral replication (Craigo et al., 2007). In a previous study, a modified EIAV strain (D9) provided effective protection against a homologous virus challenge without causing disease. However, this vaccine could not achieve optimum protective immunity until six months post vaccination (Craigo et al., 2007). This delay in the development of protective immunity indicated that a maturation of the effector mechanism needed to occur. Analysis of longitudinal serum from EIAV infected horses displayed a prolonged evolution of envelope-specific antibodies responses, which are represented by avidity index, conformational ratio, and neutralization titer, in EIAV infected horses (Hammond, 1997). Cellular immune responses also play an important role in controlling EIAV infection as the resolution of the acute phase is correlated with the appearance of the virus specific CTL (Capomaccio et al., 2012a; Capomaccio et al., 2012b). The cell-mediated immune response has not yet been thoroughly studied.

In this study, we aimed to monitor the kinetics of Th1 and CTL markers gene expressions in EIAV_{D9} infected ponies over six months in order to provide new insights into cellular immunity development in EIAV_{D9} infected ponies. Perforin and granzyme B are markers for CTLs. Both are located in the secretory granules of activated CTLs. When CTLs kill their target cells, perforin, a pore-forming protein, allows granzyme B to enter the infected cell and activate the cellular apoptosis pathway (Lowin et al., 1994).

2. Materials and Methods

2.1 Experimental setup.

Eight ponies were inoculated with the EIAV_{UK} strain as the control group and six ponies were inoculated with the EIAV_{D9} vaccine. Approximately 3.0 ml of whole blood samples were collected into PAXgene[™] tubes at weekly intervals after inoculation for five weeks and then monthly, for three months, 4 months after inoculation. PAXgene[™] tubes stabilize intracellular RNA in the blood sample.

2.2 Determination of Th1 cytokine (IFN γ) and CTL markers (Granzyme B and Perforin) gene expressions.

After the samples were collected, the tubes were incubated at room temperature for approximately 24 hours and then stored at -20°C . Once ready to be processed the samples were thawed. Total RNA was extracted from the samples using the PAXgene™ blood RNA extraction kit (Qiagen, Valencia, CA) using the protocol provided by the manufacturer. The samples were then analyzed for RNA concentration using a spectrophotometer. $1\mu\text{g}$ RNA was then dissolved in $41.5\mu\text{l}$ ribonuclease free water (Qiagen, Valencia, CA) and then transcribed into cDNA using $38.5\mu\text{l}$ of reverse transcription master mix ($16\mu\text{l}$ AMV buffer [Promega], $16\mu\text{l}$ MgCl_2 [25mM , Promega], $4\mu\text{l}$ dNTP [10mM ; Promega], $1\mu\text{l}$ RNAsin [40 units/ml, Promega] $1\mu\text{l}$ oligo dT primer [$0.5\mu\text{g/ml}$; Promega], $0.5\mu\text{l}$ avian myeloblastosis virus [AMV] reverse transcriptase [20 units/ml; Promega, Madison, WI]), as previously described (Coombs et al., 2006). The reaction mixture was incubated at 42°C for 15 min and then 95°C for 5 min. The cDNA was stored at -20°C until RT-PCR was performed.

The cDNA samples were then loaded onto 384-well plates in duplicate wells. Each well contained $4.5\mu\text{l}$ cDNA and $5.5\mu\text{l}$ master mix ($0.5\mu\text{l}$ $20\times$ assay mix for primer/probe set of interest [Applied Biosystems], and $5\mu\text{l}$ Sensimix [Bioline, Taunton, MA]) The gene expressions of granzyme B, $\text{IFN}\gamma$, and perforin were then determined using RT-PCR (ABI systems 7900 RT-PCR Instrument, Foster City, CA). The primer/probe sets were all equine specific. The PCR reactions were initially incubated for 10 min at 95°C , followed by 40 cycles of 95°C for 15s and 60°C for 60s. The internal PCR amplification efficiencies were calculated in LinReg PCR (Ramakers et al., 2003). to confirm that the application efficiencies exceeded 99%. To achieve relative quantification the $\Delta\Delta\text{C}_T$ method (Schmittgen and Livak, 2008) was utilized to determine any changes in gene expression. Beta-glucuronidase (β -GUS) was used as the endogenous control. The average of the medium control samples of each horse were calculated and then used as the calibrator for each target gene. The Relative quantity (RQ) was calculated using $2^{-\Delta\Delta\text{C}_T}$ (Schmittgen and Livak, 2008). Gene expressions over 2 were viewed as positive.

3. Results

Th1 cytokines ($\text{IFN}\gamma$) and CTL markers (GrzB and Perforin) genes were significantly increased two weeks post infection in pathogenic EIAV infected group (Figure 1). This correlates with the time of appearance of CTL response in previous studies. In contrast, the EIAV_{D9} ponies (EIAV attenuated vaccine group) did not show any changes in gene expressions early after inoculation. In comparison, $\text{IFN}\gamma$ and perforin genes were significantly increased four months post infection. This result indicated that the maturation of cellular immune responses in D9 infected equines at least 4 months.

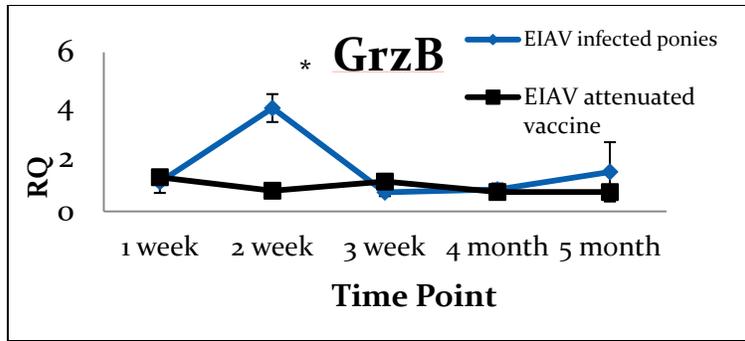


Figure 1. Expression of Granzyme B mRNA in EIAV infected and EIAV_{D9} vaccinated ponies over a five-month period.

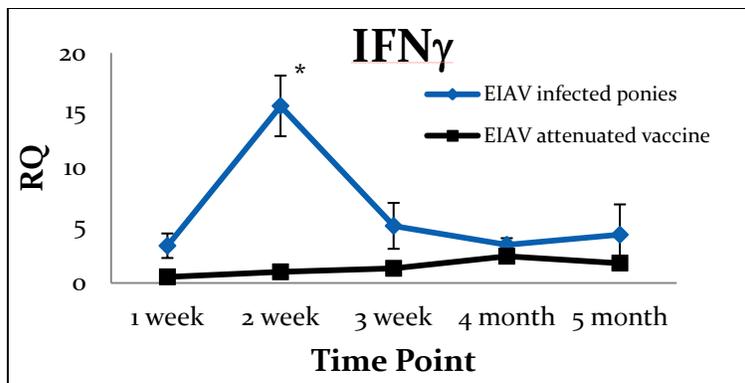


Figure 2. Expression of IFN γ mRNA in EIAV infected and EIAV_{D9} vaccinated ponies over a five-month period.

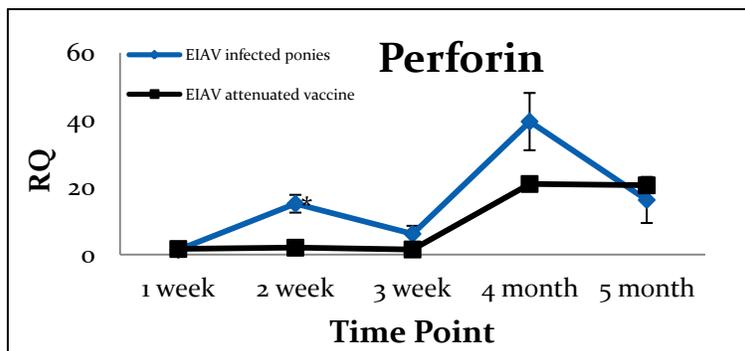


Figure 3. Expression of perforin mRNA in EIAV infected and EIAV_{D9} vaccinated over a five-month period.

Discussion:

The data indicated that the cellular immune system might play an important role in the immunological control of EIAV as previously suspected. As previously stated, more than 90% of EIAV infected equids are able to effectively control viral replication after 8-12

months. EIAV_{D9} infected ponies, show no symptoms of infection (when not immunosuppressed) and are able to effectively protect against a challenge with the parental strain of EIAV 6 months after vaccination. The EIAV_{UK} virus used in this study has greater virulence resulting in higher viral loads post challenge compared to the attenuated D9 strain. This likely resulted in greater stimulation of the immune response in the EIAV_{UK} recipients, as evidenced by earlier expression of the immune related genes. Indeed, there was increased expression of both perforin and granzyme B following infection with EIAV_{UK} before that seen in the EIAV_{D9} infected ponies. Nonetheless, the significant upregulation of perforin in both groups of ponies at the 4 month mark indicates that cellular immunity may be involved in the effective immune response to both viruses. Exactly how the cellular immune system may be involved in the control disease is still under investigation.

Understanding the immunological processes that are involved in controlling EIAV replication in the inapparent carrier phase as well as horses vaccinated with EIAV_{D9} could have global implications in the control of all lentiviral diseases, including those that are prevalent in domestic animals as well as HIV in humans. Compared to pathogenic viruses which cause recurring cycling of EIA, this attenuated D9 strain does not cause any clinical EIA and could finally reach an optimum protective immunity six months post infection. In this study, we found that EIAV_{D9} infected ponies did not show significant IFN γ and perforin gene expressions until 4 months post infection. This slow development of cellular immunity might correlate with the significantly decreased replication of this attenuated virus in horse body (Craig, 2005; Peng, 2005). Further studies underlining the mechanisms related to the development of cellular immunity in EIAV_{D9} infected ponies would provide useful information for improved lentivirus vaccine design.

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