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The determination of *in vivo* envelope-specific cell-mediated immune responses in equine infectious anemia virus-infected ponies

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Abstract

Distinct from human lentivirus infection, equine infectious anemia virus (EIAV)-infected horses will eventually enter an inapparent carrier state in which virus replication is apparently controlled by adaptive immune responses. Although recrudescence of disease can occur after immune suppression, the actual immune correlate associated with protection has yet to be determined. Therefore, EIAV provides a model for investigating immune-mediated protective mechanisms against lentivirus infection. Here, we have developed a method to monitor EIAV-envelope specific cellular immunity *in vivo*. An EIA carrier horse with no clinical signs infected 7 years ago and 4 related experimental ponies infected 6 months previously were used in this study. Forty-four 20-mer peptides, representing the entire surface unit protein (gp90) of EIAV, were combined into 14 peptide pools and intradermally injected into the neck of EIAV-infected horses. An identical volume of saline alone was injected into a fifteenth site as a negative control. After 48 h, those sites with palpable infiltrations were measured prior to the collection of 2 mm and 4 mm punch biopsies. Total RNA was extracted from each 2 mm biopsy for determination of CD3 and interferon- γ (IFN- γ) mRNA expression by real-time PCR. The 4 mm skin biopsies were formalin-fixed and paraffin-embedded for immunohistochemistry (IHC) staining for CD3, CD20, CD25 and MAC387 (macrophage marker). Peripheral blood mononuclear cells (PBMC) were obtained prior to the injection and tested for *in vitro* reactivity against the same peptides. Histological examination showed that some of the envelope peptides elicited a lymphocytic cellular infiltration at the injection site, as evidenced by positive staining for CD3. Gp90 peptide-specific increases in CD3 and IFN- γ gene expression were also detected in the injection sites. Furthermore, differences were found between *in vivo* and *in vitro* responses to gp90 specific peptides. These results demonstrate a novel method for detecting *in vivo* cell-mediated immune responses to EIAV-specific peptides that is readily applicable to other host/pathogen systems.

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Conflict of interest statement

The authors declare that no conflict of interest exists.

Keywords

EIAV; Lentivirus; Cellular immune responses; *In vivo*; Envelope; DTH

1. Introduction

In contrast to lentivirus infections in humans, equine infectious anemia virus (EIAV) replication is eventually controlled in most infected horses (Leroux et al., 2004). Following initial infection, equids may exhibit recurring febrile viremic episodes associated with high viral loads. After 12–24 months the frequency of disease episodes begins to diminish and eventually the infected animal becomes free of overt clinical signs and enters an inapparent carrier state that may last for many years (Craig and Montelaro, 2010). Maintenance of this carrier state is dependent on active immune responses as evidenced by the fact that immunosuppressive drugs can induce virus replication and the recurrence of disease (Craig et al., 2002; Tumas et al., 1994). Inapparent carriers are resistant to re-infection by other strains of EIAV, indicating that they have acquired a certain degree of cross-reactive protective immunity (Montelaro et al., 1993). Therefore the EIAV/horse system provides an opportunity for investigating mechanisms of protective immunity against lentiviruses.

Both humoral and cellular immune responses appear to play important roles in controlling EIAV infection. Virus-specific cytotoxic T cells (CTL) and neutralizing antibodies are detected after the resolution of the acute phase of infection (Leroux et al., 1997; McGuire et al., 2004). There is a progressive maturation of envelope-specific antibody responses in EIAV infected horses, as characterized by the continuing increase in titer, avidity and breadth of epitope reactivity throughout the first year of infection (Hammond et al., 1997). However, little is known regarding the evolution of cellular immune responses during EIAV infection. Until this study, characterization of cellular immunity to EIAV infection has been investigated using *in vitro* assays (Chung et al., 2004; Mealey et al., 2005; Tagmyer et al., 2007, 2008). However, these *in vitro* assays may not detect alterations in cellular immune responses occurring *in vivo*. While methods have been developed to monitor *in vivo* immune responses in mice (Ashbridge et al., 1992; Nishino et al., 1994), guinea pigs (Estrada et al., 1992; Mackall et al., 1993) and humans (Sitz et al., 1997a); similar approaches have not been used to monitor cellular immune responses in horses. Here, we have developed a method capable of defining epitope-specific cell-mediated immune responses in EIAV-infected horses. Using this approach, specific differences in gp90 epitope recognition between acutely infected and inapparent carrier were identified. There were also differences between *in vivo* and *in vitro* responses to the gp90 peptides.

2. Materials and method

2.1. Animals, virus and experimental challenges

The overall methodology to evaluate CMI responses *in vivo* was developed and optimized using a long-term, EIA inapparent carrier horse (D64) along with four ponies (H40, H41, H42 and H43) experimentally infected within six months of the commencement of these studies. D64 had been infected with the pathogenic viral strain EIAV_{PV} (Rwambo et al.,

1990) and had not experienced a febrile episode for more than 7 years. All four ponies (H40, H41, H42 and H43) had the same sire and each was challenged with the 3 cloned EIAV viruses (EV0, EV6 and EV13). Derived from the same infectious molecular clones of EIAVPV (Craig et al., 2007), EV6 and EV13 differed from EV0 in amino acid sequences of envelope gp90 by 6% and 13%, separately. A non-infected pony (F31) was used as a negative control. All animals were handled under the Guide for the Care and Use of Agricultural Animals in Agricultural Research, U.S. Department of Agriculture, according to protocols approved by the University of Kentucky Institutional Animal Care and Use Committee.

2.2. Production of synthetic gp90 derived peptides and construction of peptide pools matrix

Initially, forty-four peptides of 20 amino acids in length, overlapping sequential peptides by 10 amino acids, and spanning the entire surface unit protein (gp90) of EIAV_{pv}, were synthesized (GenScript USA Inc., Piscataway, NJ, USA) and used to construct peptide pools 1–7 and A–G as shown in Fig. 1A. An additional 17 and 26 peptides specific for EV6 and EV13, respectively, were synthesized for the construction of a new peptide pool matrix for testing in the four infected ponies. All the peptides sequences were previously published (Tagmyer et al., 2008) and each peptide was HPLC-purified, and the purity was confirmed by mass spectrometry (Tagmyer et al., 2007).

All peptides were dissolved in 100% dimethyl sulfoxide (DMSO, Sigma–Aldrich, St. Louis, MO) at a stock concentration of 2 mg/ml. Later, the peptides were diluted in saline for determination of the peptide specific responses *in vivo*. The optimal working concentration for individual peptides was determined by serially diluting a known positive peptide (#38) and injecting each dilution intradermally into the long-term infected inapparent carrier (D64) at final concentration of 4.5, 1.5, 0.5, 0.17 and 0.06 µg in 100 µl saline. The positive peptide elicited responses at doses greater than 0.17 µg (data not shown). In contrast, there was no significant gene expression in response to the negative peptide, #2 at the highest dose tested. Based on these preliminary results, each peptide was tested at a dose of 0.5 µg both individually and when incorporated into the peptide pools.

2.3. Punch biopsy and sample processing

The neck of each horse was clipped and cleaned with 75% ethanol before intradermal injection of 0.1 ml peptide pools or single peptides. Saline alone was injected to serve as the negative control. All injection sites were marked with an indelible marker for identification. After 48 h, the area of palpable infiltrations was measured (mm²) at each injection site and a 2 mm skin biopsy was collected and stored in RNALater® (Ambion, Austin, TX) in –20 °C. A 4 mm skin biopsy sample was also collected and placed in 10% formalin for subsequent paraffin embedding. The biopsy sites were closed using a single suture.

2.4. Hematoxylin and Eosin (H&E) and immunohistochemistry (IHC) staining

Serial 6 µm sections were cut from 4 mm skin biopsy samples using Leica RM2235 Microtome and placed onto poly-L-lysine-coated glass slides for hematoxylin and eosin (H&E) as well as IHC staining. H&E staining was performed according to established

protocols. IHC staining was performed using automated staining system (Bond-MAX, Leica Biosystems, Buffalo Grove, IL). The paraffin embedded biopsies were de-paraffinated in xylene, and rehydrated with decreasing alcohol rinses, followed by heat epitope retrieval. An EDTA based buffer and surfactant (pH 8.9–9.1) was used for equine CD3, CD25 and MAC387 epitope retrieval. A citrate-based buffer and surfactant (pH 5.9–6.1) was used for equine CD20 epitope retrieval. Pan T lymphocytes were detected using a cross-reactive, primary mouse anti-human CD3 antibody (Clone: LN10) and activated T cells with a monoclonal anti-human CD25 antibody (Clone: 4C9). B cells were detected using a primary mouse anti-human CD20 antibody (Clone: 7D1). Neutrophils, monocytes, and macrophages were recognized by primary mouse anti-macrophage marker antibody (Clone: MAC387). Following primary staining, slides were incubated with a post primary blocking reagent (8 min), horse-radish peroxidase-labeled polymer (8 min) and DAB substrate (10 min). After counterstaining with hematoxylin, sections were dehydrated by increasing graded alcohols and mounted with cover slips. Between additions of each reagent, washing steps were performed using Bond Wash Solutions. All antibodies and reagents were purchased from Leica Biosystems (Buffalo Grove, IL).

2.5. Relative quantitation of gene expressions by real-time PCR

The 2 mm skin biopsy was transferred in 1.5 ml eppendorf tube containing 0.5 ml RNA-STAT 60 (Tel-Test) and one 3 mm zirconium oxide bead (Retsch, Newtown, PA). The biopsies were homogenized using a Retsch MM301 Ball Mill (Newtown, PA). All samples were then placed on ice for 30 s before a second homogenization. Afterwards, the suspended cell-lysates were transferred to another eppendorf tube with an additional 0.5 ml RNA-STAT 60 and stored at -80°C for later RNA extraction. Total RNA was isolated using the phenol: chloroform method (Chomczynski and Sacchi, 1987). One microgram of RNA was dissolved in 41.5 μl nuclease-free water (Qiagen, Valencia, CA) and reverse transcribed into cDNA, as previously described (Breathnach et al., 2006) and stored at -20°C until later use.

Equine-specific, intron-spanning TaqMan primers and probes were used for RT-PCR amplification. PCR reactions were performed as previously described (Liu et al., 2011). Amplification efficiencies were determined using Linreg (Ramakers et al., 2003) and only samples with amplification efficiencies above 99% were included for further analyses. Beta-glucuronidase (β -GUS) was used as housekeeping gene and the C_T method (Livak and Schmittgen, 2001) was used to determine relative gene expression with saline injection site for each horse used as the calibrator. Relative quantity (RQ) was calculated as 2^{-C_T} .

2.6. Stimulation and measurement of effector cells *in vitro*

Heparinized blood was collected prior to intradermal injections and peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque PlusTM (Amersham Biosciences, Piscataway, NJ) gradient centrifugation according to the manufacturer's protocol. The PBMC (3×10^6) were pulsed with 20 $\mu\text{g}/\text{ml}$ peptide pools for 4 h, then transferred to fresh RPMI-1640 (Gibco, Grand Island, NY) supplemented with 2.5% (v/v) fetal equine serum (FES, Sigma, St. Louis, MO), 2 mM glutamine (Sigma), 100 U/ml penicillin/streptomycin (Sigma), and 55 μM 2-mercaptoethanol (GIBCO, Grand Island, NY). The peptide-stimulated PBMC were incubated *in vitro* for 4 days in a 24 well plate at

37 °C in a 5% CO₂ incubator. The cells were then pelleted and re-suspended in 1 ml of RNA-STAT 60 (Tel-Test) for further analysis of gene expressions by real-time PCR, as described above.

3. Results

3.1. Identification of gp90 derived peptide responses in an EIAV inapparent carrier

Seven of the 14 peptide pools produced measurable swelling, ranging from 49 mm² to 625 mm², at the injection site in the inapparent carrier (D64) at 48 h post injection (Fig. 1B). Furthermore, in biopsy samples from all 7 of these injection sites there were significant increases in expression of CD3 mRNA (Fig. 1C). It was predicted based on the peptide pool matrix that D64 could respond to a maximum of 12 individual peptides, as indicated by the gray boxes (Fig. 1B and C). However, subsequent experiments demonstrated these responses were restricted to five peptides (4, 12, 37, 38, and 40) (Fig. 2A). When these five sites were biopsied and processed to determine CD3 gene expression by real-time PCR, all five injection sites exhibited increased CD3 gene expression (Fig. 2B). In contrast, there was no evidence of increased CD3 gene expression at sites injected with peptides that produced no measurable lymphocyte infiltration (data not shown).

3.2. Histopathology in response to injection with an individual reactive EIAV gp90 derived peptide

Biopsy samples from saline and a positive peptide (#37) injection site were selected for histopathologic examination. H&E staining showed that injections of the peptide induced vasodilation and recruitment of lymphocytes to the injections site (Fig. 3A and B). The recruited cells were mainly activated T lymphocytes as determined by CD3 and CD25 staining (Fig. 3C and D). There were few B cells or macrophages present (Fig. 3E and F). No vasodilation or cell recruitment occurred in the sites where saline was injected (Fig. 3G and H). Likewise, CD3 and CD25 staining showed only resting T cells randomly dispersed (Fig. 3I and J) and few B cells or macrophages detected (Fig. 3K and L) in the saline injection site. Similar results were seen for the other positive peptides (data not shown).

3.3. *in vitro* and *in vivo* IFN γ gene expression following stimulation with EIAVgp90 derived peptide pools

Following intradermal injection of D64 with EIAVgp90 derived peptide pools IFN γ gene expression was only significantly up-regulated within those sites receiving pools C, E, F, G, 3, 4 and 6 (Fig. 4). Therefore, as predicted the results closely mirrored those obtained during transcriptional analysis of CD3. Stimulation of PBMC prepared from D64 with the same peptide pools produced increases in IFN γ gene expression in cells receiving pools E, F, G, 3 and 6 as found *in vivo*. However, in contrast to the *in vivo* results there was no increase in IFN γ gene expression in response to pools C and 4, although transcription of this gene was up-regulated following the addition of pools A, 1 and 7 (Fig. 4).

3.4. Determination of *in vivo* cellular immune responses in four ponies at 6 months post-infection with EIAV

Four ponies were intravenously challenged with three EIAV virus (EV0, EV6 and EV13), and all ponies seroconverted 28 days post challenge. Three ponies (H40, H41 and H42) exhibited evidence of EIAV replication as indicated by febrile episodes, thrombocytopenia and viral isolation from plasma, whereas H43 did not (data not shown). The gp90-specific cellular immune responses *in vivo* were determined at 6 month post challenge following the procedures described above. Ponies H40 (peptide pools H and 8), H41 (peptide pools D, E, H, 1, 3, 4, 8) and H42 (peptide pools A, C, D, F, I, J, 2, 8, 9) responded to gp90 peptide pools whereas H43 did not. Single peptide reactivity was predicted, as shown in gray boxes in Fig. 5, and individual peptides were injected into the neck of the corresponding ponies. Positive physical reactions to these individual peptides (indicated with * in Fig. 5) were confirmed by increased CD3 and IFN γ gene expression. As shown in Fig. 5, all the positive peptide pools contained at least one confirmed positive single peptide. Although some of the peptides from the EV0, EV6, and EV13 viruses share similar sequences (Craig et al., 2007), the ponies only recognized specific peptides from one virus. For example, H42 recognized single peptide #34 from EV6, but not single peptide #34 from EV0 or EV13 that share similar amino acid sequences, demonstrating the specificity of this *in vivo* assay (Table 1). Further, it has been shown that even a single amino acid change will abrogate (Tagmyer et al., 2008) or possibly induce T cell recognition. Such changes may alter the binding affinity of the peptide for the MHC molecule or the ability of T-cells receptor to recognize the peptide-MHC complex (Mealey et al., 2006).

4. Discussion

For over a hundred years, the delayed-type hypersensitivity reaction has been used for the diagnosis of both human and bovine tuberculosis (TB). Swelling and induration become apparent between 24 and 72 h after the intradermal injection of a small amount of antigen in sensitized individuals. However, it was not until the 1940s when Landsteiner and Chase were able to clarify that the reaction was mediated by cellular instead of the humoral immune responses (Black, 1999). More recently, specific protein epitopes have been identified which further refine this method (Whelan et al., 2010). The DTH response has been used to assess either CD4 $^{+}$ or CD8 $^{+}$ T cells response to a variety of antigens (Dietert et al., 2010; Poulter et al., 1982; Waksman, 1979). However, this approach has rarely been used in horses (Hall et al., 2004). Further, only limited efforts have been made to test the specificity and sensitivity of DTH responses to small peptides in large animals. By contrast, DTH testing has been widely used in mice (Ashbridge et al., 1992; Nishino et al., 1994), guinea pigs (Estrada et al., 1992; Mackall et al., 1993), and humans (Kran et al., 2012; Sitz et al., 1997b, 1999) to evaluate epitope-specific responses using small peptides. Here, we show that small peptides from EIAV gp90 can elicit DTH responses within 48 h post injection in EIAV infected horses. In order to further characterize this DTH response in horses, we collected skin biopsies at the injection sites for histology and gene expression analysis. The histological examination showed an influx of CD3 $^{+}$, CD25 $^{+}$ T cells (Fig. 3) within 48 h post-injection. Furthermore, we found this cellular influx was directly correlated with increased CD3 and IFN γ gene expression, a phenomenon not observed at injection

sites receiving the saline control or peptides that failed to stimulate palpable reactions. By employing a peptide matrix approach, followed by injection of individual peptides, it is possible to characterize cell mediated immune responses at the level of individual protein epitopes. This provides the opportunity for comprehensive investigations of *in vivo* antigen-specific cellular immune responses in EIAV infected horses.

While DTH responsiveness is often associated with IFN γ production *in vitro* (Kapsenberg et al., 1991; Weynants et al., 1995), two gp90 derived peptide pools which induced DTH responses *in vivo* did not up-regulate IFN γ gene expression *in vitro*. This disparity between *in vivo* and *in vitro* responses has also been reported in the case of HIV (Kvale et al., 2005). There are several possibilities for these differences. By virtue of the sampling method, PBMC represents a minor fraction of the total lymphocyte population. A low frequency of antigen-specific cells thus reduces the chance of including such cells in a blood sample. Since precursor expansion is required for the *in vitro* detection of specific immune responses, both an infrequent precursor population and the likelihood that the T cells will undergo apoptosis *in vitro* (Herbein et al., 1998; Shearer, 1998) further reduces the sensitivity of this assay. By contrast, the DTH response involves the active recruitment of antigen-specific T cells to the injection site, thus increasing the likelihood of a measurable response even with a low frequency population. We also observed the situation where peptide pools were recognized *in vitro*, but did not elicit a DTH response *in vivo*. Such discordant responses may reflect a situation where the effector cell produces IFN- γ but not the other cytokines or chemokines required for a DTH response (Vestweber, 2007). Another possibility is that over time, the kinetics of development or loss of DTH compared to IFN- γ secretion differ (Black et al., 2001). The significance of this dichotomy between *in vitro* and *in vivo* responses in terms of protective immunity remains to be determined.

Three of the newly infected ponies used in this study exhibited variable clinical signs of EIA, characterized by fever, decrease in platelets, and increased virus load in plasma. There appears to be a relationship between virus replication and the number of the peptides recognized by these ponies. Thus, H42, which recognized multiple peptides, also had multiple febrile episodes. These febrile episodes are typically associated with recurring viremia (Hammond et al., 1997). This indicates that the broader peptide recognition was associated with repeated exposure to the virus, thus likely expanding the pool of antigen-specific T cells. Since protective immunity eventually occurs in EIAV-infected horses, this approach provides the opportunity to identify the evolution of the protective mechanisms responsible for virus control in the EIAV infected ponies, and perhaps as well for other lentivirus (Addo et al., 2003). By contrast, H43, which did not show any clinical signs of EIA, did not recognize any envelope peptides tested. It has been shown that T cells recognizing other EIAV proteins, such as Rev (Mealey et al., 2003) may play roles in providing protection. Without testing other EIAV proteins, we could not exclude the possibility that H43 recognized other EIAV proteins. Additionally, we occasionally encounter this situation where a particular equid effectively controls EIAV replication while invariably having very weak antibody responses. Our current results indicate there is a similarly weak CMI reaction at least to envelope gp90 peptides in these seemingly resistant

animals. While the mechanism of this innate resistance remains unknown, it also likely involves non-immunological mechanisms based on these results.

In summary, we described a method to assess EIAV envelope specific cellular immunity *in vivo*. Histological and gene expression analysis were used to confirm the cellular nature of this response. As such, this approach offers an alternative to *in vitro* detection of antigen-specific cellular immune responses. While our work focused on the response to EIAV, this approach may be used to assess immunity in horses that have been immunized with other inactivated or recombinant antigens.

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Fig. 1.

Identification of single peptide responses by peptide pool screening. (A) The peptide components of each pool used for injection. (B) Predicted single peptide reactivity as determined by the physical response and (C) CD3 gene expression for each pool. Intersecting pools (gray shading) identify possible peptide specificities.

Fig. 2. Confirmation of individual peptide reactivity in D64. Single peptides 4, 12, 37, 38 and 40 were injected into the neck and physical responses measured after 48 h. Positive responses were indicated by measurable palpable infiltrations (A) and increased CD3 gene expression (B).

Fig. 3.

Histopathology of #37 peptide injection site from D64. Sections (A–F) and saline alone (G–L) were stained with H&E (A, B, G and H) or with CD3 (C and I), CD25 (D and J), CD20 (E and K) or MAC387 specific antibodies (F and L). Images (A and G) are shown at 4× magnification and the boxed area represents area used for subsequent imaging at 40× for each of the antibody stains.

Fig. 4.

Comparison between *in vivo* and *in vitro* IFN γ mRNA expression in response to the peptide pools. Peptide pool-induced IFN γ mRNA expression gene was detected by real-time PCR in biopsy samples (open bars) and from *in vitro* stimulated PBMC (closed bars). The “+” and “x” indicate positive responses to peptide pools *in vitro* and *in vivo*, separately.

Fig. 5.

Cellular epitope recognition in newly infected ponies. The original peptide pools (Fig. 1A) were supplemented with those peptides unique to the EV6 and EV13 viruses. Those peptides in italics are unique to EV6 and those underlined are unique to EV13. Palpable infiltrations (mm²) for each pool are shown on the bottom and left sides of each figure. Intersecting pools (gray shading) identify possible peptide specificities. Single peptides with * are the peptides which could elicit positive responses *in vivo*.

Table 1

Peptides sequence comparisons among EV0, EV6 and EV13.

	#34 Peptide sequence ^a	CD3 gene expression ^b	IFN γ gene expression ^b
EV0	PIFYTCNFTNITSCNNEPII	0	0
EV6	PIFYTCNFTNITSCNNE <u>S</u> II	4	16
EV13	<u>P</u> MFYTCNFT <u>S</u> ITSCN <u>D</u> E <u>S</u> IT	0	0

^aUnderscores indicate amino acid differences from the original EV0 peptide.

^bRelative gene expression determined using RT-PCR.