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Research paper

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.

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 (Craigo and Montelaro, 2010). Maintenance of this carrier state is

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dependent on active immune responses as evidenced by the fact that immunosuppressive drugs can induce virus replication and the recurrence of disease (Craigo 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 $EIAV_{PV}$ (Craigo 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 (mm^2) 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