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Divergence, not diversity of an attenuated equine lentivirus vaccine strain correlates with protection from disease

Jodi K. Craigo[a,b], Shannon Barnes[a,b], Sheila J. Cook[c], Charles J. Issel[c], Ronald C. Montelaro[a,b,*]

[a] Center for Vaccine Research, University of Pittsburgh, Pittsburgh, PA 15261, United States
[b] Department of Microbiology and Molecular Genetics, University of Pittsburgh, Pittsburgh, PA 15261, United States
[c] Department of Veterinary Science, Gluck Equine Research Center, University of Kentucky, Lexington, KY 40516, United States

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ABSTRACT

We recently reported an attenuated EIAV vaccine study that directly examined the effect of lentiviral envelope sequence variation on vaccine efficacy. The study [1] demonstrated for the first time the failure of an ancestral vaccine to protect and revealed a significant, inverse, linear relationship between envelope divergence and protection from disease. In the current study we examine in detail the evolution of the attenuated vaccine strain utilized in this previous study. We demonstrate here that the attenuated strain progressively evolved during the six-month pre-challenge period and that the observed protection from disease was significantly associated with divergence from the original vaccine strain.

1. Introduction

Attenuated virus vaccines are currently used for the prevention of infectious diseases such as influenza, chicken pox, and yellow fever and have previously been used effectively to control significant viral outbreaks such as smallpox, polio, and measles epidemics [2–4]. However, the use of an attenuated human immunodeficiency virus (HIV) vaccine has been controversial due to concerns that have arisen about vaccine safety [5–10]. Nonetheless, regardless of the potential commercial use of an attenuated vaccine, the model itself is to date one of the best measures of both potential vaccine efficacy and correlates of protection and remains an asset to the field of study. Thus far the development of vaccines to HIV-1 has relied substantially on the use of animal lentivirus models to evaluate the efficacy of various vaccine strategies.

EIAV, a macrophage-tropic lentivirus, causes a persistent infection in horses and a chronic disseminated disease of worldwide importance in veterinary medicine (reviewed in Craigo and Montelaro, 2008; Montelaro et al., 1993). The virus infection, which is transmitted via blood-feeding insects or iatrogenic sources such as contaminated syringe needles, occurs in three stages: acute, chronic, and inapparent. EIA is characterized during its acute and chronic stages by defined episodes of clinical disease triggered by waves of viremia and distinguished by fever, anemia, thrombocytopenia, edema, and various wasting signs. By 8–12 months post-infection horses typically progress to life-long inapparent carriers, but maintain varying steady state levels of viral replication in monocyte-rich tissue reservoirs [11–14]. Stress or immune suppression of EIAV inapparent carriers can induce an increase in viral replication and potentially a recrudescence of disease [15–17]. Among virulent lentiviruses, however, EIAV is unique in that despite aggressive virus replication and associated rapid antigenic variation, greater than 90% of infected animals progress from a chronic disease state to an inapparent carrier stage. This progression to an inapparent stage of disease is achieved by a strict immunologic control over virus replication [13,14]. The EIAV system therefore serves as a uniquely dynamic model for the natural immunologic control of lentiviral replication and disease. Thus, this model provides a novel and useful lentiviral system for identifying critical immune correlates of protection and ascertaining the potential for developing effective prophylactic lentivirus vaccines.

Over the past 20 years we have evaluated a number of experimental EIAV vaccines based on inactivated whole virus, viral or recombinant envelope subunit vaccines, and various attenuated proviruses [1,18–24]. The results of these vaccine trials demonstrate a remarkable breadth of efficacy, ranging from protection from detectable infection and/or disease to severe enhancement of EIAV replication and disease. Among our most recent vaccine work are serial studies evaluating the efficacy of an attenuated EIAV proviral vaccine containing a mutation in the viral S2 accessory gene ($EIAV_{D9}$) [1,18–20]. The results of these studies indicated that 100% horses inoculated with the $EIAV_{D9}$ viral vaccine were

* Corresponding author at: Center for Vaccine Research, 9016 Biomedical Science Tower 3, University of Pittsburgh, School of Medicine, Pittsburgh, PA 15261, United States. Tel.: +1 412 648 8869; fax: +1 412 624 4440.
E-mail address: rmont@pitt.edu (R.C. Montelaro).

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protected from disease by homologous virulent EIAV challenge. Thus, the EIAV system mirrors other animal lentivirus vaccine models that have consistently identified attenuated vaccines, among the various vaccine strategies evaluated to date, as producing the highest level of vaccine protection, typically against homologous virus challenge [25–28].

Our latest published reports of this attenuated vaccine system detail the specific effects of envelope (Env) sequence variation on vaccine protection [1,29–31]. In that study we identified for the first time a significant, inverse, linear correlation between vaccine efficacy and increasing divergence of the challenge virus Env surface gp90 protein compared to the vaccine virus gp90 protein. The vaccine study demonstrated approximately 100% protection of immunized horses from disease after challenge by virus with a homologous gp90, but only 50% protection against challenge by virus with a gp90 that was 13% divergent from the vaccine strain. Immune analysis of potential correlates of protection between the three challenge groups revealed minor associations, but were not definitive. Whether this lack of strong immune system correlations is an accurate statement on *in vivo* immune responses or a call to develop new *in vitro* assays for immune analysis remains to be determined.

A question that has yet to be defined in the field of lentiviral attenuated vaccines is what role potential evolution of the attenuated vaccine, and more specifically, evolution of the vaccines immunogenic target, the Env gene, plays in vaccine efficacy. To date, very little is known about attenuated viral vaccine evolution. Thorough analyses of the attenuated viral replication and interactions with the host immune response, which affect evolution, have been examined, but direct analysis of the actual genomic evolution of the quasispecies population is absent. We know that in order to achieve a protective and yet safe vaccine that the level of attenuation has to be precisely controlled to allow adequate persistent viral replication and presumably sustained viral antigen presentation necessary to sufficiently drive host immune responses to achieve protective immunity. We have also determined that a second critical parameter for achieving protective immunity with an attenuated vaccine is the length of time post vaccination with the attenuated virus [19,32–36]. However, only a few studies of attenuated lentiviral vaccine evolution have been performed, and the majority of these explored compensatory mutations but not a full quasispecies analysis of population evolution. In this current study we characterize the evolution of the attenuated viral vaccine from our most recent study [1] by examining its viral population at the day of challenge (DOC). Further, we compare it to the longitudinal evolution of its pathogenic viral strain counterpart while also examining its correlation with protective efficacy of the vaccine.

2. Materials and methods

2.1. Experimental subjects, inoculations, clinical evaluation, and sample collection

All equine procedures were conducted at the Gluck Equine Research Center of the University of Kentucky according to protocols approved by the University of Kentucky IACUC. Clinical EIA episodes were determined on the basis of rectal temperature and platelet count in combination with the presence of infectious plasma virus [14,37,38]. CBC analysis of whole blood was performed using an IDEXX QBC Vet Autoreader. Hematocrit and platelet numbers were monitored weekly using the Unopette microcollection system (Becton Dickinson, Rutherford, NJ). Plasma samples were collected at regular intervals and during each disease cycle (defined as rectal temperature > 39 °C and platelet number < 70,000/μl of whole blood) and stored at −80 °C until RNA extraction was performed. During the course of these experiments ponies that demonstrated severe disease-associated symptoms resulting in distress as outlined by the University of Kentucky IACUC were euthanized.

2.1.1. Subjects from attenuated vaccine inoculations

Twenty-four outbred Shetland ponies of mixed age and gender and serognegative for EIAV were utilized. Daily rectal temperatures and clinical status were recorded. The $EIAV_{D9}$ attenuated virus stock was produced and vaccinations performed as described [1,18,19]. All animals were vaccinated two times at 30-day intervals by intravenous injection of 10^3 $TCID_{50}$ as previously described [1]. Vaccinated ponies were challenged six months post-second inoculation with 10^3 $TCID_{50}$ of EV0, EV6, or EV13 [1]. The ponies were monitored daily for clinical symptoms of EIA.

2.1.2. Subjects from pathogenic infections

Two outbred, mixed-breed Shetland ponies were experimentally inoculated intravenously with 10^3 $TCID_{50}$ of the pathogenic viral strain $EIAV_{PV}$ [23,39,40]. Rectal temperatures and clinical status were recorded daily. The clinical and immune responses of these experimentally infected ponies during acute, chronic, and inapparent infection have been extensively described [12,17,37,41,42].

2.2. Quantitation of viral RNA, RNA purification, and RT-PCR

Plasma samples from all animals were analyzed for the levels of viral RNA per milliliter of plasma using a previously described Taqman quantitative real-time multiplex RT-PCR assay based on *gag*-specific amplification primers [43]. The standard RNA curve was linear in the range of 10^1 molecules as a lower limit and 10^8 molecules as an upper limit.

For sequence analysis, viral RNA was extracted from plasma as previously described [16,37,42]. Reverse transcription of 2–5 μl of purified viral RNA was performed with the SuperScriptII PreAmplification System (GibcoBRL, Rockville, MD) as specified by the manufacturer using the EIAV specific primer PV12AS [37]. Multiple nested amplifications of the gp90 Env gene were performed as previously described [42] using the Elongase mix (Gibco BRL, Rockville, MD) and 5 μl of the cDNA in a final volume of 50 μl.

2.3. Cloning and sequencing of RT-PCR products

Cloning and sequencing of the $EIAV_{PV}$ pathogenic isolates has been described previously [17,42]. The cloning and sequencing of the DOC attenuated plasma viral populations from all 24 $EIAV_{D9}$ vaccinated animals were performed similarly. Briefly, several independent RT-PCR products (at least 2 independent RT reactions and 2–4 independent nested PCR reactions) were generated from plasma samples, gel-purified using Qiagen's Qiaex Gel Purification System (Valencia, CA) and cloned individually into the pCR2.1-TOPO® vector from the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA). Due to the highly unstable nature of EIAV *env* sequences when associated with high copy plasmids, ligations were transformed into Stbl2 cells (Invitrogen, Carlsbad, CA). Clones were screened for the proper size insert by PCR using M13 primers from the TOPO TA Cloning kit. Plasmid DNA was extracted and purified with a midiprep kit (Qiagen, Valencia, CA). Clones (2–5 isolates from each vaccinate) were automatically sequenced with the Taq Dye Deoxy Terminator Cycle Sequencer Kit (Applied Biosystems, Foster City, CA) using internal EIAV primers (variable region 3 through variable region 7) as previously described [37]. DNA sequences were resolved with an ABI Prism 373 DNA sequencer (Applied Biosystems, Foster City, CA). Error rate associated with our nested amplification with the SuperScript and Elongase polymerases was