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An attenuated EIAV vaccine strain induces significantly different immune responses from its pathogenic parental strain although with similar *in vivo* replication pattern

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ABSTRACT

The EIAV (equine infectious anemia virus) multi-species attenuated vaccine $EIAV_{DLV121}$ successfully prevented the spread of equine infectious anemia (EIA) in China in the 1970s and provided an excellent model for the study of protective immunity to lentiviruses. In this study, we compared immune responses induced by $EIAV_{DLV121}$ to immunity elicited by the virulent $EIAV_{LN40}$ strain and correlated immune responses to protection from infection. Horses were randomly grouped and inoculated with either $EIAV_{DLV121}$ (Vaccinees, Vac) or a sublethal dose of $EIAV_{LN40}$ (asymptomatic carriers, Car). Car horses became $EIAV_{LN40}$ carriers without disease symptoms. Two of the four Vac horses were protected against infection and the other two had delayed onset or reduced severity of EIA with a lethal $EIAV_{LN40}$ challenge 5.5 months post initial inoculation. In contrast, all three Car animals developed acute EIA and two succumbed to death. Specific humoral and cellular immune responses in both Vac and Car groups were evaluated for potential correlations with protection. These analyses revealed that although plasma viral loads remained between 10^3 and 10^5 copies/ml for both groups before $EIAV_{LN40}$ challenge, Vac-treated animals developed significantly higher levels of conformational dependent, Env-specific antibody, neutralizing antibody as well as significantly elevated $CD4^+$ T cell proliferation and IFN-γ-secreting $CD8^+$ T cells than those observed in $EIAV_{LN40}$ asymptomatic carriers. Further analysis of protected and unprotected cases in vaccinated horses identified that cellular response parameters and the reciprocal anti-p26-specific antibody titers closely correlated with protection against infection with the pathogenic $EIAV_{LN40}$. These data provide a better understanding of protective immunity to lentiviruses.

1. Introduction

Among the different types of lentiviral vaccines, the live attenuated formulations demonstrated the best efficacy based on the level of protective immunity by providing complete or near-complete protection from homologous challenges (Koff et al., 2006; Tagmyer et al., 2008), controlling or dramatically reducing the replication of heterologous viral strains (Reynolds et al., 2008), and eliciting long-term protection (Yankee et al., 2009). However, live vaccines are typically not considered viable options in the development of lentiviral vaccines, especially in the context of AIDS vaccines because of the risks that are associated with genomic integration and reverse mutations. Therefore, a better understanding of the protective immune responses that are elicited by attenuated vaccines would assist with the development of vaccines against lentiviral infections (Letvin, 2006; Robb, 2008; Whitney and Ruprecht, 2004).

The equine infectious anemia virus (EIAV) is one of the least complex lentivirus and like other members of the lentivirus genus, such as HIV-1, EIAV has the capacity to change its envelope surface proteins as an immune evasion mechanism. However, after the initial progressive, recurring febrile episodes many infected horses eventually become asymptomatic EIAV carriers. Therefore, defining the nature of the immune response resulting in asymptomatic infections is critical to EIAV vaccine design. Several studies have demonstrated that reduced EIAV replication in asymptomatic horses was a consequence of host immunity and not due to EIAV attenuation over time. For example, adoptive transfer of whole blood from asymptomatic horses to EIAV-negative horses resulted

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in progression towards equine infectious anemia (EIA) (Craigo et al., 2005; Hammond et al., 1997, 2000; Harrold et al., 2000) and following dexamethasone-mediated immune suppression, plasma viremia in EIAV carriers dramatically increased (Craigo et al., 2002; Newman et al., 1991). These studies suggested that EIAV virulence remained unaltered and that asymptomatic carriage was a consequence of acquired immunity to EIAV (Perryman et al., 1988) thereby providing an ideal model for the study of protective immunity against this pathogen. Several studies characterized immune responses in EIAV-infected horses, including Env-specific antibody maturation, avidity and conformation-dependence, in addition to characterization of EIAV-specific lymphocyte proliferation and cytotoxic T lymphocyte (CTL) activity. These studies suggested that these responses played a role in controlling EIAV *in vivo* (Chung et al., 2004; Hammond et al., 1999, 2000).

However, it remains undefined what specific immune response parameter(s) are most important in mediating resistance (or conferring protection) to lentivirus infections, especially since research in this area is controversial. Some studies demonstrated that controlling EIAV replication requires specific cellular immune responses in combination with high levels of neutralizing antibodies (McGuire et al., 2002; Tagmyer et al., 2007). Conversely, high levels of CTL activity and the presence of neutralizing antibodies were absent from Rhesus macaques (*Macaca mulatta*) resistant to infection with pathogenic SHIV (simian immunodeficiency virus) strains (Mansfield et al., 2008; Reynolds et al., 2008; Yankee et al., 2009). One critical reason for these conflicting results with respect to the role of immunity in containing lentivirus infections is the lack of an appropriate infection model (Harris, 2009). In addition, most data published to date describing lentivirus immunity has been derived from infection studies not designed to examine protective immunity (Craigo et al., 2005; Hammond et al., 2000; Rosenberg et al., 1999). Recently, a series of studies on protective immunity using live attenuated EIAV vaccines were published and revealed important information on the correlation of immunity with protection (Craigo and Montelaro, 2010; Tagmyer et al., 2008).The attenuated EIA vaccine ($EIAV_{DLV121}$) was developed by Shen et al. by serially passing the pathogenic wild-type $EIAV_{LN40}$ strain in donkeys for 110 generations followed by 121 *in vitro* passages in donkey monocyte-derived macrophages (MDM). $EIAV_{DLV121}$ was utilized extensively in China between 1975 and 1990 where 30 million equines were vaccinated to control an EIA pandemic. As a result, the spread of EIA in China, which directly or indirectly (slaughtering of infected animals) resulted in the loss of at least 424,000 horses, mules and donkeys, was under control by the early 1980s as reported by the Chinese Ministry of Agriculture (Shen et al., 1984). Although this attenuated EIA vaccine was the first vaccine that demonstrated effective protection against a lentivirus related diseases, the mechanism resulting in protective immunity remains undefined. More importantly, dexamethasone-mediated immune suppression of $EIAV_{DLV121}$-vaccinated horses did not either significantly elevate plasma viral loads or trigger clinical EIA (Ma et al., 2009), implicating the difference in immune responses between induced by the vaccine strain and resulted by asymptomatic infections.

In this study, we first provided the historic unpublished data on the efficacy of the attenuated $EIAV_{DLV121}$ strain against infections with either homologous or heterologous EIAV pathogenic strains. The immune responses elicited following vaccination with $EIAV_{DLV121}$ or with an $EIAV_{LN40}$ sublethal dose were characterized by comparing humoral and cellular response and characterizing the relationship between the nature of the immune responses and protection. This is the first systematic evaluation of the immune responses elicited by a lentiviral vaccine that effectively prevented infections with either homologous or heterologous EIAV strains.

2. Materials and methods

2.1. EIAV strains

Five EIAV strains were utilized in this study: (i) the virulent $EIAV_{LN40}$ strain initially isolated from an EIA positive horse from the Liaoning Province of China and passed for 16 generations in horses. $EIAV_{LN40}$ infections resulted in a 100% incidence of acute EIA in infected horses when 10^4 $TCID_{50}$ EIAV was used; (ii) the live attenuated $EIAV_{DLV121}$ vaccine strain derived from the virulent $EIAV_{DV117}$ strain by successive MDM passages *in vitro* (Shen et al., 1984); (iii) $EIAV_{FDDV12}$, a strain comprising a second-generation of Chinese attenuated EIAV vaccine that was developed by further passing the $EIAV_{DLV121}$ vaccine strain in fetal donkey dermal cells (FDD) for 12 generations (Shen et al., 2006); (iv) $EIAV_{DLV34}$, a donkey MDM-adapted virulent strain that was passed for 33 generations. Like $EIAV_{LN40}$, infections with $EIAV_{DLV34}$ resulted in acute EIA incidence rates of 100% when horses were infected with 10^4 $TCID_{50}$; (v) the American $EIAV_{Wyoming}$ virulent strain, a gift from Dr. Dawei Shen, Washington State University, and was passed in horses for three generations to increase the virulence.

2.2. Experimental animals, clinical evaluation and longitudinal sample collection

To evaluate the protective efficacy of the attenuated $EIAV_{DLV121}$ vaccine strain, 116 mixed gender, outbread adult horses were randomly selected and hypodermically vaccinated with 4×10^5 $TCID_{50}$ of $EIAV_{DLV121}$ and then challenged with 1×10^4 $TCID_{50}$ of virulent strain $EIAV_{LN40}$ 3, 6, 10 or 12 months post vaccination. Twenty-two horses were immunized with $EIAV_{DLV121}$ for either 6, 9 or 15 months prior to challenge with the $EIAV_{Wyoming}$ strain. Twenty-seven and 13 unvaccinated horses (treated with saline) were infected with either $EIAV_{LN40}$ or with the American $EIAV_{Wyoming}$ strain (1×10^4 $TCID_{50}$). Rectal temperatures for each horse were measured twice daily. Acute EIA was defined as fever >39 °C, thrombocytopenia (<9×10^4 platelets/μl blood) and jaundice.

For experiments comparing immune responses, 16 horses of mixed gender were randomly grouped and inoculated with: (i) the vaccine strain $EIAV_{DLV121}$ (group Vac, four horses); (ii) sublethal dose (1×10^3 $TCID_{50}$) of the virulent strain $EIAV_{LN40}$ to develop asymptomatic carriers of EIAV pathogenic strain (group Car, three horses); (iii) lethal dose (1×10^4 $TCID_{50}$) of the virulent strain $EIAV_{LN40}$ to develop acute EIA (group Acu, three horses); (iv) the same volume of saline (Mock, three horses). Horses, except for those in the group Acu, were challenged with lethal dose (10^5 $TCID_{50}$) of the virulent strain $EIAV_{LN40}$ 23 weeks post the initial inoculation. In addition, three horses were mock inoculated and challenged with saline as healthy controls (group Hea). Serum samples prior to the start of the experiment were tested twice by the agar gel immunodiffusion assay for the presence of EIAV antiserum to confirm that each animal was uninfected at the beginning of the experiment (Coggins et al., 1972). Serum, plasma, and whole blood samples were collected from horses at indicted intervals and were stored at −20 °C until serological characterization was carried out. A plasma aliquot was stored at −80 °C until plasma viral loads were determined using real-time RT-PCR. Equine peripheral blood mononuclear cells (ePBMC) were isolated from whole blood over a discontinuous density gradient of Ficoll-Histopaque (density = 1.077 g/ml) for evaluating EIAV-specific cellular immune responses. During the course of all experimental process, all animals were clinically monitored daily as described (Zhang et al., 2007). All the above animal studies have been reviewed and approved