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Differences in replication kinetics and cell tropism between neurovirulent and non-neurovirulent EHV1 strains during the acute phase of infection in horses

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ABSTRACT

Equine herpesvirus 1 (EHV1) replicates in the respiratory tract of horses, after which infected leukocytes transport virus throughout the body, resulting in abortion or nervous system disorders. Two EHV1 strains circulate in the field: neurovirulent and non-neurovirulent. To investigate differences in replication in the upper respiratory tract (URT), an experimental inoculation study in ponies was performed with both strains. Two groups of six ponies, were inoculated intranasally with $10^{6.5}$ $TCID_{50}$ of either strain. Clinical signs, nasal shedding and viremia were evaluated. At early time points post-inoculation (pi), one pony of each group was euthanized. Tissues were collected for titration and immunostainings. Number and size of EHV1-induced plaques were calculated, and individual EHV1-infected cells were quantified and characterized. Inoculation with either strain resulted in nasal shedding and replication in several tissues of the URT. Both strains replicated in a plaquewise manner in epithelium of the nasal mucosa, but replication in epithelium of the nasopharynx was largely limited to non-neurovirulent EHV1. Plaques were never able to cross the basement membrane, but individual infected cells were noticed in the connective tissue of all examined tissues for both strains. The total number of these cells however, was 3–7 times lower with non-neurovirulent EHV1 compared to neurovirulent EHV1. $CD172a^+$ cells and $CD5^+$ lymphocytes were important target cells for both strains. Interestingly, in lymph nodes, B-lymphocytes were also important target cells for EHV1, irrespective of the strain. Viremia was detected very early pi and infected cells were mainly $CD172a^+$ for both strains. In summary, these results are valuable for understanding EHV1 pathogenesis at the port of entry, the URT.

1. Introduction

Infectious respiratory tract disease has been recognized as the cause of major health problems in horses. The most important respiratory pathogen is equine herpesvirus 1 (EHV1) and infections with this virus cause serious economic losses in the horse industry worldwide (Allen and Bryans, 1986; Ostlund, 1993; van Maanen, 2002). The upper respiratory tract is the first line of defence against respiratory pathogens and is also the primary replication site of EHV1, where it causes upper respiratory disorders (Kydd et al., 1994). In addition, EHV1 can spread via infected blood leukocytes to internal organs, for example

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the pregnant uterus, causing symptoms such as abortion and neonatal foal death (Allen and Bryans, 1986). EHV1 can also reach the central nervous system, where replication in endothelial cells results in severe nervous system disorders with frequent fatal outcome. Neurological disease in horses caused by infection with certain isolates of EHV1 is a severe condition which is poorly understood (McCartan et al., 1995; Stierstorfer et al., 2002; van der Meulen et al., 2003a). The pathogenesis for developing this devastating condition is largely unknown and full protection against these secondary and severe symptoms cannot be obtained by vaccination with the currently available vaccines (Heldens et al., 2001; Goodman et al., 2006; van der Meulen et al., 2007). Therefore, an improvement of the existing vaccines and/or the development of alternative strategies for the prevention and treatment of EHV1-induced infections are necessary. Importantly, this implies a firm notice about the pathogenesis of EHV1 during the acute phase of infection.

It has been suggested that distinct isolates of EHV1, differing in pathogenic capacity, circulate in the field. Nugent et al. (2006) indicated that a single nucleotide polymorphism (SNP) in the DNA polymerase is strongly associated with neurological versus non-neurological disease outbreaks. Studies in naturally infected horses with nervous system disorders have revealed a more robust cell-associated viremia in horses infected with neurovirulent isolates of EHV1, in contrast to horses infected with non-neurovirulent isolates (Allen and Breathnach, 2006). This was further confirmed by reverse genetic experiments of EHV1 strains with a sole mutation of the DNA polymerase SNP, where experimental inoculation with such strains altered neurologic disease in horses (Goodman et al., 2007; Van de Walle et al., 2009). Moreover, *in vitro* experiments with these strains revealed differences in leukocyte tropism which could explain the difference in clinical outcome (Goodman et al., 2007). Still, the exact identity of carrier cells susceptible to EHV1 infection and hereby responsible for dissemination of infectious virus to sites of secondary replication, is a matter of debate. Blood samples from experimentally infected ponies were collected and examined for the presence of neurovirulent EHV1 (Scott et al., 1983). Hereby, T-lymphocytes were found to be the most important cell fraction to harbor virus *in vivo*. However, no full characterization of these cells was performed. Different studies with *in vitro* EHV1-infected leukocytes have also been performed to determine the identity of these cells. Hereby, mainly monocytes were susceptible to infection with non-neurovirulent EHV1 (van der Meulen et al., 2000). After stimulation with mitogens, T-lymphocytes became more susceptible, followed by B-lymphocytes (van der Meulen et al., 2001). Despite these studies, information on possible differences between neurovirulent and non-neurovirulent EHV1 strains at the port of entry, and identity of carrier cells during viremia remains limited.

To investigate this, six Shetland ponies were experimentally inoculated with a neurovirulent EHV1 strain and six with a non-neurovirulent EHV1 strain. These animals were subsequently euthanized to collect different tissues of the upper respiratory tract. Tissues that were positive for EHV1 on titration were stained with markers for different cell types to determine and quantify the carrier cells of EHV1 in the upper respiratory tract. In addition, peripheral blood mononuclear cells (PBMC) were also collected to quantify and identify the infected cell type during cell-associated viremia.

2. Materials and methods

2.1. Horses

Twelve male Shetland ponies, between the age of 6 months and 2 years, were used in this study. They were housed inside isolated stables. They were fed daily with a commercial, complete feed. Drinking water and hay were supplied *ad libitum*.

Prior to the experiment, ponies were monitored for 4 weeks. Rectal temperatures were measured daily and complement-dependent seroneutralization (SN)-tests and immunoperoxidase monolayer assays (IPMAs) were performed weekly to determine EHV-specific antibody titers.

2.2. Virus and inoculation

Ponies were divided into two groups of six. For the first group the Belgian EHV1 strain 03P37, isolated from the blood of a paralytic horse in 2003, was used for inoculation (Garre et al., 2009; van der Meulen et al., 2003a). The second group was inoculated with the Belgian strain 97P70, which was isolated from an aborted fetus (van der Meulen et al., 2006). The strains were typed in the DNA polymerase as D_{752} and N_{752} respectively in cooperation with the Animal Health Trust in the United Kingdom (Nugent et al., 2006). Based on their origin and genotyping, these strains will be referred as neurovirulent and non-neurovirulent in this paper. Virus stocks used for inoculation were at the 6th passage; 2 passages in rabbit kidney cells (RK13) and 4 subsequent passages in equine embryonic lung cells (EEL). Both viruses had virtually identical single-step growth properties in RK13 cells for intra- and extracellular virus yields at all tested time points post-infection (data not shown).

Ponies were inoculated oronasally with 20 ml of a virus suspension containing $10^{6.5}$ tissue culture infectious dose 50 ($TCID_{50}$). Ten ml of the virus suspension was administered intranasally (5 ml per nostril) using a thin probe and 10 ml was inoculated orally with a syringe. The virus titer was confirmed by titration of the inoculum.

2.3. Serological examination

For the seroneutralization (SN)-test, 2-fold dilution series of the sera were prepared in MEM. Fifty microliters of these serial dilutions were incubated for 23 hours (h) at 37 °C with a fixed number of infectious virus (300 $TCID_{50}$ of EHV1 strain Arabica in 50 μl). Hereafter, 25 μl of guinea pig complement was added. After 1 h of incubation, the mixture of serum, virus and complement was added to RK13 cells. Inoculated cultures were further incubated at 37 °C in an atmosphere containing 5% CO_2. After 7 days of incubation, the cultures were analyzed for the presence of