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Dual infections of equine herpesvirus 1 and equine arteritis virus in equine respiratory mucosa explants

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ABSTRACT

Equine herpesvirus 1 (EHV-1) and equine arteritis virus (EAV) induce respiratory problems and abortion in horses and are considered as two serious threats to equine industry. Both EHV-1 and EAV misuse patrolling leukocytes in the upper respiratory tract to breach the basement membrane (BM) and to migrate to blood vessels. So far, the behavior and impact of a double infection in the respiratory mucosa of a horse are unknown. In the present study, the outcome of double infections with EHV-1 and the low virulent EAV strain 08P187 (superinfection with an interval of 12 h or co-infection) were compared with single infections in fully susceptible RK-13 cells and equine upper respiratory mucosa explants. When RK-13 cells were inoculated with either EHV-1 or EAV 12 h prior to the subsequent EAV or EHV-1 inoculation, the latter EAV or EHV-1 infection was clearly suppressed at 24 hpi or 36 hpi, respectively, without EHV-1 and EAV co-infecting the same RK-13 cells. After simultaneous infection with EHV-1 and EAV, higher numbers of EAV infected cells but similar numbers of EHV-1 infected cells were found compared to the single infections, with a low number of EHV-1 and EAV co-infected RK-13 cells at 48 hpi and 72 hpi. In the upper respiratory mucosa exposed to EAV 12 h prior to EHV-1, the number and size of the EHV-1-induced plaques were similar to those of the EHV-1 single infected mucosa explants. In nasal and nasopharyngeal mucosae, EAV and EHV-1 pre-infections slightly reduced the number of EHV-1 and EAV infected leukocytes compared to the single infections and co-infection. In double EAV and EHV-1 infected explants, no co-infected leukocytes were detected. From these results, it can be concluded that EAV and EHV-1 are only slightly influencing each other's infection and that they do not infect the same mucosal leukocytes.

1. Introduction

Equine herpesvirus 1 (EHV-1) and equine arteritis virus (EAV) are two important pathogens of horses, which have significant economical impacts on the equine breeding industry worldwide (Allen and Bryans, 1985; Bażanów et al., 2014; Timoney and McCollum, 1990). EHV-1 is an important cause of respiratory distress, abortion, neonatal foal death and myeloencephalopathy (Lunn et al., 2009; Pusterla et al., 2009). Likewise, a clinical infection with EAV is characterized by influenza-like illness in adult horses, abortion in pregnant mares and interstitial pneumonia in young foals (Timoney, 2000). EHV-1 is a member of the subfamily *Alphaherpesvirinae* with a 150 kilobases double stranded DNA genome (Telford et al., 1992), whereas EAV is a single-stranded, positive-sense RNA genome of approximately 12.7 kilobases that belongs to the family *Arteriviridae* (Snijder and Meulenberg, 1998).

Different EHV-1 strains induce different clinical signs in the field. Studies indicated that a single nucleotide polymorphism (A2254/G2254) in the EHV-1 DNA polymerase gene (ORF30) is associated with the neuropathogenic potential of naturally occurring strains (Nugent et al., 2006; Van de Walle et al., 2009). Although no difference exists in the cell tropism between the neurological and abortigenic strains of EHV-1, it has been reported that the former infect a higher number of CD172a$^+$ monocytic cells than the latter in the upper respiratory mucosa (Vandekerckhove et al., 2010). Although EAV has only one known serotype, geographical and temporal distinct strains of EAV differ in the severity of clinical signs they induce and in their abortigenic potential (Stadejek et al., 1999; Zhang et al., 2007).

For both EHV-1 and EAV, primary replication takes place in the mucosa of the upper respiratory tract. Subsequently, both viruses disseminate via a leukocyte-associated viremia, which enables both viruses to reach endothelia of capillaries in multiple secondary organs (Bryans and Allen, 1989; Kydd et al., 1994). Viral replication in clinical organs lead to abortion for both viruses and central nervous disorders for certain strains of EHV-1 (Balasuriya, 2014).

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During the invasion process of both viruses into the deeper tissues of the upper respiratory tract, the $CD172a^+$ cells and to a lesser degree $CD3^+$ T cells function as Trojan horses (Vairo et al., 2013a; Vandekerckhove et al., 2010).

So far, clinical outcomes of EHV-1 and EAV infections are indistinguishable (Givens and Marley, 2008) and co-infection may occur in horses under field conditions. However, up till now, there is no information available on the behavior of a co-infection with EHV-1 and EAV in the equine respiratory mucosa. The research on the interaction between EAV and EHV-1 in the respiratory mucosa will allow a better understanding of the pathogenesis of dual infections (DaPalma et al., 2010). The present study was designed to assess the outcome of double infections with neurological and abortigenic EHV-1 strains and a low virulent strain of EAV in the fully susceptible rabbit kidney epithelial cells (RK-13 cells) and equine nasal and nasopharyngeal mucosa explants and to reveal that whether EHV-1 and EAV (initiated from upper respiratory) by co-infection (or superinfection) can be more prone to penetrate the upper respiratory mucosa, and facilitate infection compared with single infection.

2. Material and methods

2.1. Animals and explants

The nasal septum and nasopharynx were collected from 3 healthy horses, between 4 and 6 years old at a local slaughterhouse. Horses were seropositive for EHV-1 and EAV (Vairo et al., 2012; Vandekerckhove et al., 2010).

The collected tissues were transported on ice in phosphate buffered saline (PBS) supplemented with 10 μg/ml gentamicin (Gibco), 1 mg/ml streptomycin (Certa, Braine l'Alleud, Belgium), 1000U/ml penicillin (Continental Pharma, Puurs, Belgium), 1 mg/ml kanamycin (Sigma, Bornem, Belgium), and 5 μg/ml fungizone (Bristol-Myers Squibb, New York, USA). Nasal and nasopharyngeal mucosae were stripped from the nasal surface and divided into pieces of 50 mm^2. The explants were placed on fine-meshed gauze with the epithelium upwards for culture on an air-liquid interface at 37 °C with 5% CO_2.

2.2. Cell culture

RK-13 cells were used and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Paisley, UK) supplemented with 10% fetal calf serum (Invitrogen), 100U/ml penicillin, 0.1 mg/ml streptomycin and 1 μg/ml gentamicin.

2.3. Viruses

EHV-1 neurological strain 03P37, EHV-1 abortigenic strain 97P70 and EAV strain 08P187 were used for this study. All strains were isolated from clinical cases of horses in Belgium as described earlier (Vairo et al., 2013a; Vandekerckhove et al., 2010). Both EHV-1 03P37 and 97P70 strains were at the sixth passage, four passages in equine embryonic lung cells (EEL) and two subsequent passages in RK-13 cells, with a titer of $10^{6.5}$ tissue culture infectious dose with 50% endpoint per milliliter ($TCID_{50}$/ml). The 08P187 strain was at the fourth passage in RK-13 cells with a titer of $10^{6.5}$ $TCID_{50}$/ml.

2.4. Single and dual infections with EHV-1 and EAV

2.4.1. In RK-13 cell cultures

2.4.1.1. EHV-1 and EAV inoculation method. RK-13 cells were seeded in 24-well culture plates (Nunc A/S, Roskilde, Denmark) with inserts at a density of 2.5×10^5 cells/ml and cultured at 37 °C with 5% CO_2. The cells were divided into five different groups. Groups 1 and 2 were single infections and group 3 was a co-infection. In detail, RK-13 cells were MOCK incubated for 12 h then inoculated with 200 μl containing 7.0×10^2 $TCID_{50}$ EHV-1 (group 1-MOCK/EHV-1), EAV (group 2-MOCK/EAV) or inoculated with both 7.0×10^2 $TCID_{50}$ EHV-1 and 7.0×10^2 $TCID_{50}$ EAV [group 3-MOCK/(EHV-1 + EAV)]. Groups 4 and 5 were superinfections. In detail, we first inoculated with 200 μl containing 7.0×10^2 $TCID_{50}$ EHV-1 or EAV on RK-13 cells (Glorieux et al., 2012) for 1 h, then removed the inoculum, washed with PBS and overlaid with 1% high viscosity carboxymethylcellulose (CMC) (Sigma) medium for 12 h. Subsequently, the overlaying medium was removed and RK-13 cells were washed and inoculated with the same amount of EAV (group 4-EHV-1/EAV) or EHV-1 (group 5-EAV/EHV-1) for another 1 h and afterwards performed the same way as the inoculation for the first virus. All the viruses were inoculated at 37 °C with 5% CO_2.

2.4.1.2. Confocal microscopy. The time point after one-hour incubation with the 2nd virus was taken as 0 h post inoculation (pi). At 0 h, 12 h, 24 h, 36 h, 48 h and 72 hpi, cells were fixed with 4% paraformaldehyde for 10 min at room temperature (RT) and permeabilized with 0.1% Triton X-100 for 5 min at RT. To detect EHV-1 positive cells, cells were incubated with equine biotinylated equine polyclonal anti-EHV-1 IgG (diluted 1:10 in PBS) (van der Meulen et al., 2000), followed by Texas Red®-labelled Streptavidin (Invitrogen, 1:200 in PBS). To detect EAV positive cells, cells were incubated with monoclonal antibody 17D3 against EAV nucleocapsid (N) protein (VMRD, 1:100 in PBS), followed by FITC®-labelled goat anti-mouse IgG antibody (Invitrogen, 1:200 in PBS). Isotype-matched control antibodies were used to confirm the specificity of each staining. All the antibodies were incubated at 37 °C for 1 h with 5% CO_2. The nuclei were counterstained with Hoechst 33342 (10 μg/ml, Molecular Probes) at 37 °C. Cells were washed with PBS and mounted with glycerin/1,4-diazabicyclo[2.2.2]octane. Samples were analyzed with a Leica TCS SP2 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany). For each group, 5 regions of interest (ROI) were chosen per well. The number of EHV-1 and EAV infected cells were counted separately. Three independent replicates were performed for each experiment.

2.4.2. In upper respiratory mucosa explants

After 24 h of culture, explants were washed twice with warm medium and transferred on top of solid agarose. The margins were filled with agarose. All the explants were divided into five different groups with the same way as described for RK-13 cells. In brief, explants were MOCK incubated for 12 h then inoculated with 500 μl medium containing $10^{6.5}$ $TCID_{50}$ EHV-1 (group 1- MOCK/EHV-1), EAV (group 2-MOCK/EAV) or inoculated with both EHV-1 and EAV (group 3-MOCK/(EHV-1 + EAV)). In addition, explants were inoculated with 500 μl medium containing $10^{6.5}$ $TCID_{50}$ EHV-1 or EAV first, then 12 h later inoculated with the same amount of EAV (group 4-EHV-1/EAV) or EHV-1 (group 5-EAV/EHV-1). All the viruses were inoculated at 37 °C for 1 h with 5% CO_2. After each inoculation, the explants were rinsed and further incubated with fresh medium (Vairo et al., 2013b). Finally, the explants were collected at 0, 12, 24, 36, 48 and 72 hpi, embedded in methocel™, and snap frozen at −70 °C.

Of each explant, 50 serial 16 μm cryosections of 8 mm^2 were made for immunofluorescence (Kydd et al.) stainings. Briefly, the cryosections were fixed with 100% methanol for 20 min at −20 °C. Subsequently, EHV-1 and/or EAV infected cells were stained following the aforementioned protocol. After staining, the cryosections were rinsed three times in PBS and mounted with glycerin/1,4-diazabicyclo[2.2.2]octane. All the results of the IF stainings were analyzed by confocal microscopy. In the lamina propria of the mucosa, two regions of interest (ROI) were chosen for analysis of EHV-1 replication. One region was underneath a plaque of EHV-1