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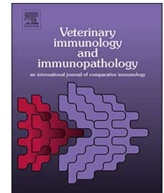
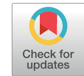


Absence of relationship between type-I interferon suppression and neuropathogenicity of EHV-1



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ABSTRACT

Equine herpesvirus-1 (EHV-1) infection is an important and highly prevalent disease in equine populations worldwide. Previously we have demonstrated that a neuropathogenic strain of EHV-1, T953, suppresses the host cell's antiviral type-I interferon (IFN) response *in vitro*. Whether or not this is unique to EHV-1 strains possessing the neuropathogenic genotype has been undetermined. Here, we examined whether there is any direct relationship between neuropathogenic genotype and the induced IFN-β response in equine endothelial cells (EECs) infected with 10 different strains of EHV-1. The extent of virus cell-to-cell spread following infection in EECs was also compared between the neuropathogenic and the non-neuropathogenic genotype of EHV-1. We then compared IFN-β and the total type-I IFN protein suppression between T953, an EHV-1 strain that is neuropathogenic and T445, an EHV-4 strain mainly associated only with respiratory disease. Data from our study revealed no relationship between the neuropathogenic genotype of EHV-1 and the induced IFN-β mRNA by the host cell. Results also indicate no statistically significant difference in plaque sizes of both genotypes of EHV-1 produced in EECs. However, while the T953 strain of EHV-1 was able to suppress IFN-β mRNA and type-I IFN biological activity at 12 h post-infection (hpi), EHV-4 weakly induces both IFN-β mRNA and type-I IFN biological activity. This finding correlated with a statistically significant difference in the mean plaque sizes produced by the two EHV subtypes in EECs. Our data help illuminate how EHV-1, irrespective of its genotype, evades the host cell's innate immune response thereby enabling viral spread to susceptible cells.

1. Introduction

EHV-1 infection is an important disease of equids that was first documented in the early 1930s (Dimock and Edwards, 1933). The viral infection is known for its clinical manifestations including respiratory disease, sporadic abortion during the third trimester of gestation, birth of weak newborns, chorioretinitis and neurological disease (Hussey et al., 2013; Jackson et al., 1977; Ostlund, 1993; Patel and Heldens, 2005; Peet et al., 1978; Reed and Toribio, 2004; Van Maanen, 2002). Like most other herpesviruses, EHV-1 has the ability to establish latency after primary infection, leading to a carrier state in infected horses. Additionally, the disease is highly contagious among horses and one of the most devastating manifestations of EHV-1 infection is the neurologic form termed equine herpesvirus myeloencephalopathy (EHM). Although EHM is not new, there has been an increase in the outbreak of the disease condition since the year 2000 (Allen, 2008; Goehring et al., 2010; Marenzoni et al., 2008; McFadden et al., 2016; Patel and Heldens, 2005). It has been suggested that distinct strains of EHV-1 differing in pathogenicity circulate in the field (Nugent et al., 2006). Strains of EHV-1 have been broadly classified neuropathogenic and non-neuropathogenic, based on the presence of a single nucleotide polymorphism ($A_{2254} \rightarrow G_{2254}$) in the viral DNA polymerase with G_{2254} associated with neuropathogenic strains (Nugent et al., 2006). Generally, the EHV-1 strains possessing the neuropathogenic genotype are involved in neurologic outbreaks while those with the non-neuropathogenic genotype are predominantly isolated from cases of sporadic abortions in pregnant mares (Allen et al., 1999; Edington et al., 1986; Mumford et al., 1994; Patel et al., 1982; Whitwell and Blunden, 1992). It is, therefore, noteworthy that some of the EHM cases are associated with the A_{2254} non-neuropathogenic genotype, and the G_{2254} neuropathogenic genotype does not necessarily lead to EHM (Paccamonti and Pycock, 2009; Perkins et al., 2009; Pronost et al., 2010; Smith et al.,

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2010; Vissani et al., 2009), indicating that other viral and host factors are involved in EHM. Equine herpesvirus-4 (EHV-4) is a different virus type but closely related to EHV-1 with nucleotide sequence identity within individual homologous genes ranging from 55 to 84 percent and amino acid sequence identity ranging from 55 to 96 percent (Telford et al., 1998; Telford et al., 1992). The virus was earlier classified as a subtype of EHV-1 until 1981 when molecular evidence became available which allowed for the differentiation between the two viruses (Studdert et al., 1981). Sequence information, therefore, substantiates the view that EHV-1 and EHV-4 are two closely related but distinct herpesviruses of the horse (Allen et al., 2004). The detailed pathogenesis of EHV-4 has not been well studied but the infection mirrors that of EHV-1 during the early onset. EHV-4 infection begins with virus replication in mucosal epithelial cells of the upper respiratory tract following inhalation of infectious aerosols or contact with infected fomites. However, the pathogenicity, extent of viral replication and tissue destruction in horses caused by EHV-4 are far lower than those observed for EHV-1 (Allen et al., 2004). It has been suggested that the biological difference in pathogenicity between EHV-1 and EHV-4 can be attributed to the difference in cellular tropism between the two viruses (Osterrieder and Van de Walle, 2010). While primary infection of EHV-4 has been reported to be limited mainly to the epithelial cells of the upper respiratory tract of a susceptible host (Ma et al., 2013), *in vivo* replication of EHV-4 in endothelial cells has also been described (Blunden et al., 1995). It has, however, been demonstrated that EHV-4 has less tropism for mononuclear cells and is less efficient in infecting these cells when compared to EHV-1 (Osterrieder and Van de Walle, 2010; Vandekerckhove et al., 2011). Consequently, leukocyte-associated viremia which is a prerequisite for the induction of abortion or neurologic disease in EHV-1 is not a common feature of EHV-4 infection (Vandekerckhove et al., 2011). The type-I IFN response is critical in restricting viral spread from infected to non-infected cells and several viruses have evolved mechanisms to evade this potent antiviral system. The action of EHV-1 on the host cell type-I IFN system during infection has been better studied than that of EHV-4. Recently, we have demonstrated in cell culture that an EHV-1 strain isolated from a neuropathogenic outbreak and possessing the neuropathogenic genotype, T953, has the ability to suppress type-I IFN response at 12 hpi when co-infected with either of the IFN inducers, Sendai virus or Poly-I:C (Sarkar et al., 2015). This virus strain was not only successful in down-regulating the type-I IFN response but was also capable of inhibiting downstream type-I IFN mediated antiviral activity *in vitro* (Sarkar et al., 2016b). Some of the mechanisms determined to be used by this EHV-1 strain to downregulate the type-I IFN response include interference with the nuclear translocation of STAT-1 protein (Sarkar et al., 2016b) and the disruption of the interferon regulatory factor-3 (IRF-3) signaling pathways (Sarkar et al., 2016a). Both STAT-1 and IRF-3 are important transcription factors that are indispensable for the sensitization and induction of type-I IFN. Thus it is clear that, in cell culture, the neuropathogenic T953 strain of EHV-1 can overcome the type-I IFN response of the host cell. For this reason, we hypothesized that neuropathogenicity is correlated with the capacity for a reduced type-I IFN response in the host cell. If true, that would suggest that low type-I IFN induction in the host cell might be a contributing mechanism for neuropathogenicity. To test our hypothesis, here we compared the type-I IFN response between EHV-1 strains possessing the neuropathogenic genotype and those with the non-neuropathogenic genotype using our established cell culture system. We also compared the type-I IFN response following infection with the two closely related but different EHV subtypes: EHV-1 and EHV-4.

2. Materials and methods

2.1. Cells and viruses

Equine pulmonary artery endothelial cells (EECs; (Hedges et al., 2001)) were cultured in Dulbecco's modified Eagle's medium (DMEM, Mediatech Inc.) with 10% iron-supplemented bovine calf serum (BCS, Hyclone Laboratories, Inc., Logan, UT), 100 U/ml penicillin-streptomycin (Life Technologies, Carlsbad, CA), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids and 200 mM L-glutamine (Life Technologies, Carlsbad, CA) in a humidified incubator at 37 °C with 5% CO_2. A total of 10 EHV-1 isolates including T953, a well-characterized, neuropathogenic strain also known as the Findlay strain isolated from a nasopharyngeal swab of a horse suffering from an EHV-1 neurologic disease (Henninger et al., 2007), were included in the study. In addition to T953, the other EHV-1 isolates were: AB4, T313, T970, TX06, T75, T186, T220, T262, and T61. An isolate of EHV-4, T445, was also included. To prepare working virus stocks, confluent EECs were infected with EHV-1 at a multiplicity of infection (MOI) of 0.1. After the virus had produced nearly 100% cytopathic effect (CPE), the tissue culture fluid (TCF) containing the virus was freeze-thawed three times and clarified at 2000 × *g* for 30 min at 4 °C, filtered through 0.45 μm cellulose acetate membrane filters (Thermo Scientific Nunc, Pittsburgh, PA) and purified by ultracentrifugation at 100,000 × *g* for 4 h at 4 °C through a 20% sucrose cushion. The virus pellet was re-suspended in DMEM with 2% BCS, sonicated briefly, aliquoted in 100 μl volumes and stored at −80 °C until further use. The infectious virus titer was determined by plaque assay in EECs as described (McCollum et al., 1962) with slight modifications. Briefly, confluent EECs propagated in 6-well plates were infected with 10 fold serial dilutions of cell culture supernatant containing virus. For each dilution of the virus, duplicate wells were infected. The virus was let to adsorb for 1 h, at 37 °C with 5% CO_2. Unadsorbed viruses were removed by washing and 0.75% carboxymethylcellulose (CMC) media (Sigma-Aldrich, St. Louis, MO) was added to each well and the plates were further incubated in a humidified 5% CO_2 atmosphere at 37 °C for 96 h. The CMC media was removed and the infected cells stained with 1% crystal violet (CV) solution in buffered formalin (10%) for plaque visualization. A total of 30 plaques were photographed for each virus strain and the plaque sizes were measured using ImageJ software (Schindelin et al., 2015). Genotyping of field isolates of EHV-1 was carried out as previously described (Smith et al., 2012) with slight modification. The viruses were grouped into either the neuropathogenic or the non-neuropathogenic genotype of EHV-1 based on the detection of either G_{2254} or A_{2254} in the viral DNA polymerase.

2.2. Viral infections

EECs were plated into 6-well culture plates (Corning, NY) 48 h prior to infection to obtain more than 90% confluency at the time of infection. Monolayer cells were infected with the various virus strains at an MOI of 5 for 1 h in a humidified incubator at 37 °C with 5% CO_2. In parallel, cells were either mock-infected with virus diluent (negative control) or treated with 80 μg/ml of Poly-I:C (positive control). The cells were then washed with D-PBS and incubated with complete growth medium for 12 h. All experiments were performed in duplicate and repeated on three independent days.

2.3. RNA extraction and real-time RT-PCR assay

Total cellular RNA was extracted using QiaAmp RNeasy plus mini kit (Qiagen Inc. Valencia, CA) from EHV-1-infected and control EECs at 12 hpi according to the manufacturer's protocol. The quantity and quality of the cellular RNA were examined by OD_{260}/OD_{280} measurement using the Synergy H1 hybrid plate reader (Biotek, Winooski, VT). One microgram of total cellular RNA was reverse transcribed as described (Breathnach et al., 2006) using 0.5 μg oligo dT primer. Equal amounts of cDNA were used for the transcription analysis of different genes by TaqMan real-time PCR using specific primers and probes in a ViiA™ 7 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). The sequences of primers and probes used in this study are listed in

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