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The role of secreted glycoprotein G of equine herpesvirus type 1 and type 4 (EHV-1 and EHV-4) in immune modulation and virulence

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ABSTRACT

Equine herpesvirus type 1 and 4 (EHV-1 and EHV-4) are important pathogens of horses worldwide. Infection with EHV-4 usually remains restricted to the upper respiratory tract, whereas infection with EHV-1 can generalize after leukocyte-associated viremia. Here we examined whether differences in the immunomodulatory glycoprotein G (gG) between the two viruses determine EHV-1's ability to cause systemic infection. To this end, mutant viruses were constructed based on the neurovirulent EHV-1 strain OH-03, in which the entire gG gene or parts thereof were exchanged with EHV-4 gG sequences. *In vitro* chemotaxis assays showed that supernatants of cells infected with the various gG mutant viruses interfered to variable degrees with neutrophil migration. More specifically, supernatants of cells infected with the gG deletion virus (vOH-ΔgG1) or OH-03 expressing EHV-4 gG (vOH-gG4) were unable to interfere with chemotaxis. Re-insertion of the predicted chemokine-binding region of EHV-1 gG in the vOH-gG4 mutant (vOH-gG4hyp1) did not completely restore the ability to inhibit neutrophil migration, whereas insertion of the hypervariable region of EHV-4 gG into vOH-03 (vOH-gG1hyp4) did not lead to a complete loss of chemokine-binding function. Very similar results were obtained in an *in vivo* study where the amount of neutrophils present in bronchioalveolar lavages (BALs) of mice infected with the different mutants was analyzed by flow cytometry. Taken together, our results show that, in a virus background, the hypervariable region is not solely responsible for the immunomodulatory potential of EHV-1 gG.

1. Introduction

Equine herpesviruses types 1 and 4 (EHV-1 and EHV-4) are endemic in horse populations worldwide and are both members of the *Varicellovirus* genus of the *Alphaherpesvirinae*, a subfamily of the *Herpesviridae*. Other members of the genus include human varicella zoster virus (VZV) and important animal pathogens such as bovine herpesvirus type 1 (BHV-1) and pseudorabies virus (PRV) (Allen and Bryans, 1986; Roizmann et al., 1992; Telford et al., 1992, 1998). Until 1981, EHV-1 and EHV-4 were considered subtypes of one virus species, causing either rhinopneumonitis or abortion. Restriction endonuclease analyses, however, clearly identified the two viruses as different species with collinear genomes and a highly conserved genetic structure (Studdert et al., 1981; Whalley et al., 1981). EHV-1 and EHV-4 are shed from the nasal cavity of infected horses and transmitted to neighboring horses by virus containing droplets or through direct smear infection.

Primary infection with EHV-1 and EHV-4 and following replication in the upper respiratory tract results in the clinical syndrome of rhinopneumonitis. In addition, EHV-1, but not EHV-4, regularly reaches the regional lymphoid tissues and infects leukocytes, which then results in a leukocyte-associated viremia (Allen and Bryans, 1986). From infected leukocytes, EHV-1 is transferred to and replicates in endothelial cells of end vessels supplying the pregnant uterus or the central nervous system, which can cause vasculitis and thrombosis, ultimately leading to abortions or myeloencephalopathy, a condition which is characterized by paralytic symptoms (Allen and Bryans, 1986; Edington et al., 1986; Patel et al., 1982; Pusterla et al., 2009).

Secreted glycoprotein G (gG) of EHV-1, which is encoded by open reading frame (ORF) 70, was shown to bind to chemokines of human and mouse origin and to act as a viral chemokine binding protein (vCKBP) by blocking interaction of chemokines with both chemokine specific receptors and glycosaminoglycans (GAGs) (Bryant et al., 2003). More recently, it was found that a gG deletion mutant based on the EHV-1 strain RacL11 showed a more pronounced inflammatory response when compared to wild-type virus (von Einem et al., 2007). Further, *in vitro* chemotaxis experiments demonstrated that EHV-1 gG was indeed able to inhibit migration of equine neutrophils in response to recombinant equine IL-8 (Van de Walle et al., 2007). Interestingly, and despite the fact that the gG of EHV-4 is also secreted into the medium of infected

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cells and shares an overall identity of 58% with its EHV-1 counterpart, no chemokine binding function nor an interference with neutrophil migration was found (Bryant et al., 2003; Crabb and Studdert, 1993; Van de Walle et al., 2007). Based on this observation, it was proposed that the immunomodulatory property of EHV-1 gG might contribute to EHV-1's ability to induce a more severe and systemic outcome of infection in contrast to EHV-4 (Bryant et al., 2003; Van de Walle et al., 2007). A closer examination of the gG of both viruses identified a hypervariable region, comprising amino acid (aa) 287–382 in EHV-4 gG and aa 288–350 in EHV-1 gG, which shared only 21% sequence identity (Crabb et al., 1995). It was hypothesized that the hypervariable region might be responsible for the potential of EHV-1, but not EHV-4 gG, to bind chemokines. This was confirmed using hybrid proteins in which the hypervariable region of EHV-1 and EHV-4 gG was exchanged. More specifically, the epitopes comprising aa 301–340 of EHV-1 gG were shown to be responsible for binding to chemokines *in vitro* (Van de Walle et al., 2009a).

The aim of this study was to further test the hypothesis that the hypervariable region of EHV-1 gG is indeed the determining factor for binding to chemokines. We therefore engineered different EHV-1 gG mutant viruses, which were evaluated *in vitro* as well as *in vivo*. Our salient findings were that, in a virus background, the hypervariable region of EHV-1 gG is not solely responsible for the observed immunomodulatory chemokine-binding function.

2. Materials and methods

2.1. Cells and viruses

Rabbit kidney (RK13) cells (169th passage) were maintained in minimal essential medium (MEM, Biochrom AG) supplemented with 5% fetal bovine serum (FBS, Biochrom AG), 100 U/ml penicillin (Fisher-Scientific) and 0.1 mg/ml streptomycin (Sigma–Aldrich) and used for EHV-1 virus amplification, $CaCl_2$ transfections and plaque purification assays. Equine fibroblasts (NBL6) cells (11th passage) were maintained in MEM supplemented with 20% FBS, 7.5 ml non essential amino acids (Biochrom AG), 7.5 ml sodium pyruvate (Invitrogen), 100 U/ml penicillin and 0.1 mg/ml streptomycin and were utilized for EHV-4 virus amplification and plaque purification assays. Both cell lines were cultured at 37 °C under a 5% CO_2 atmosphere. Equine neutrophils were isolated from heparinized blood of healthy horses, using a discontinuous, pH-adjusted Percoll gradient of 59% and 75% (Percoll™, GE Healthcare), exactly as described previously (Sedgwick et al., 1986). The neutrophils were washed twice with 1× Hank's buffered salt solution (HBSS, Biochrom AG) and used immediately for chemotaxis assays. The EHV-4 isolate KT-4 was kindly provided by Dr. Kerstin Borchers (Borchers et al., 2005). The neurovirulent EHV-1 strain OH-03 was constructed as a bacterial artificial chromosome (BAC), designated pOH-03, by insertion of the mini-F sequence and contained an egfp marker gene in lieu of gene 71, which encodes glycoprotein 2 (gp2) (Rosas et al., 2006). All mutant viruses used in this study were generated and propagated in RK13 cells (Table 1).

2.2. Plasmids

For the generation of the various gG mutants, two different transfer plasmids were constructed to insert the positive selection marker I-SceI-aphAI into EHV-4 gG or its hypervariable region. To this end, EHV-4 gG was first amplified from KT-4 using primers NT1 and NT2 (Table 2A) and cloned either into pUC19 or pcr2.1 (Invitrogen). Next, the positive selection marker I-SceI-aphAI was amplified from plasmid pEPkanS1 (Tischer et al., 2006) and cloned into EHV-4 gG or its hypervariable region (aa 287–382) (Crabb and Studdert, 1993) using AgeI or BsrGI as unique restriction sites, resulting in pUC19-EHV4-gG-Kana and pcr2.1-TOPO-EHV4gG-Kana, respectively (Table 2A). In addition, a plasmid was constructed that contained the gp2-encoding ORF71 of strain Ab4 and was used for reconstitution of gp2 expression (Rudolph et al., 2002a; von Einem et al., 2007).

Table 1
Viruses generated and used in this study.

Virus designation	Genotype
KT-4	Equine herpesvirus type 4 (EHV-4) – parental virus
OH-03	Equine herpesvirus type 1 (EHV-1) – parental virus
pOH03	Bacterial artificial chromosome of the EHV-1 strain OH-03
pOH-ΔgG1	pOH-03 with EHV-1 gG1 (ORF70) deleted
vOH-ΔgG1	pOH-03 with EHV-1 gG1 deleted, gp2 (ORF71) restored
pOH-gG4	pOH-03 with gG of EHV-4 inserted
vOH-gG4	pOH-3 with EHV-4 gG inserted, gp2 restored
pOH-gG4hyp1	pOH-03 with the hypervariable region of EHV-1 gG inserted in EHV-4 gG
vOH-gG4hyp1	pOH-03 with hypervariable region of EHV-1 gG inserted in EHV-4 gG, gp2 restored
pOH-gG1hyp4	pOH-03 with the hypervariable region of EHV-4 gG inserted in EHV-1 gG
vOH-gG1hyp4	pOH-03 with the hypervariable region of EHV-4 gG inserted in EHV-1 gG, gp2 restored

2.3. Mutagenesis of pOH-03

In order to replace the EHV-1 gG gene by the corresponding EHV-4 sequences, a complete deletion mutant was generated first using two-step Red-mediated recombination, exactly as previously described (Lee et al., 2001; Tischer et al., 2010). To this end, we used primers NTa and NTb that harbored 40 bp homologous flanks at either side of the deletion (Table 2B). Next, the gG sequence of interest, along with the positive selection marker, was amplified from the previously constructed transfer plasmids (see above) using the following primer sets: (i) primers NT5 and NT6 to generate pOH-gG4, (ii) primers NT31 and NT32 to generate pOH-gG4hyp1 and (iii) primers NT41 and NT42 to generate pOH-gG1hyp4 (Tables 1 and 2B).

For reconstitution of recombinant viruses from BACs, 1–5 μg BAC DNA was transfected into RK13 cells using the calcium phosphate precipitation method (Rudolph et al., 2002a). This method was also applied for restoration of gp2, where 1 μg of a plasmid containing ORF71 was co-transfected with 5 μg BAC DNA into RK13 cells and non-fluorescing plaques were picked and purified to homogeneity by three rounds of plaque purification exactly as described earlier (Rudolph and Osterrieder, 2002b).

2.4. Restriction enzyme fragment length polymorphism (RFLP) analyses and sequencing

To confirm the genotypes of the generated mutants, RFLP was performed. To this end, BAC DNA was prepared by alkaline lysis (Sambrook et al., 1989), digested with the restriction enzymes *Bam*HI, *Eco*RI or *Hind*III, and separated on 0.8% agarose gels for 20 h at 60 V. In addition, BAC and viral DNA were analyzed by PCR using the forward primer NT11 and reverse primer NT12 (Table 2), which target gG. The amplified fragments were sequenced and sequence data were verified by the Vector NTI 9.1 software (Invitrogen) contig function.

2.5. Western blotting

To evaluate expression of gG of the different recombinant viruses, NBL-6 cells were infected at a multiplicity of infection