Immune response against equine gammaherpesvirus in Icelandic horses

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1. Introduction

Five different herpesviruses are known to infect horses among those are the two closely related gammaherpesviruses EHV-2 and EHV-5. EHV-2 has been detected in around 90% of horses worldwide, but EHV-5 is much less investigated and probably less frequent (Borchers et al., 1997; Craig et al., 2005; Nordengrahn et al., 2002; Reubel et al., 1995). We have shown that both viruses are present in horses in Iceland (Torfason et al., 2008). Horses are usually infected at the age of 1–6 months via the upper respiratory tract and develop a latent infection (Bell et al., 2006; Fu et al., 1986; Murray et al., 1996). The EHV-2 is thought to reside in the B-lymphocytes (Drummer et al., 1996). Horses frequently harbour both viruses and can get re-infected with different strains of EHV-2 (Browning and Studdert, 1987). The clinical impact of the gammaherpesviruses is not clear but EHV-2 has been associated with respiratory disease, keratoconjunctivitis, fever and inappetence (Borchers et al., 2006; Browning and Studdert, 1988; Kershaw et al., 2001). As EHV-2 and EHV-5 share many epitopes the specific immune response against the two viruses cannot be distinguished in broad spectrum antibody tests such as whole virus ELISA (Agius et al., 1994).
and biochemical properties (Lunn et al., 1998; Montgomery, 1972; Sheoran et al., 1998). However, molecular characterization of the equine Ig heavy chain genes has recently shown that IgGa corresponds to IgG1, IgGb to IgG4 and IgG7, IgGc to IgG6 and IgG(T) to IgG3 and IgG5 (Lewis et al., 2008; Wagner, 2006; Wagner et al., 2004). The most abundant IgG subclass in serum of adult horses and in colorectum is IgG4/7 (Lopez et al., 2002; Sheoran et al., 2000). It is critical in defence against bacterial and viral infections (Lopez et al., 2002; McGuire et al., 1997; Nelson et al., 1998; Sheoran et al., 1997).

No publications could be found on the cytokine or IgG subclass response in horses infected with γ-EHV. In the α-herpesvirus infection caused by EHV-4, IgG1 and IgG4/7 are induced at a high level while IgG6 and IgG3/5 are low or undetectable. The EHV-4 specific IgG1 antibody response disappeared and could not be detected in healthy EHV-4 positive horses. By contrast the IgG4/7 levels were sustained in recovered horses and were therefore considered to be the long lasting antibody response against the virus (Mizukoshi et al., 2002). Similarly in natural equine influenza infection protective immunity has been associated with the humoral IgG1 and IgG4/7 together with mucosal IgA (Nelson et al., 1998).

As in most viral infections, a Th1 directed cellular immune response plays the major role in protection against the α-herpesvirus EHV-1. In vivo EHV-1 infection induces IFN-γ production in lymphocyte subsets upon in vitro EHV-1 stimulation (Breathnach et al., 2005; Paillot et al., 2005) and protective immunity to EHV-1 is characterized by an IFN-γ polarized immune response (Coombs et al., 2006).

The aim of this study was to provide data on cytokine and the IgG subclass response in natural γ-EHV infection.

2. Materials and methods

2.1. Horses

A total of 41 healthy Icelandic horses were used for the study, 20 adults age 3–10 years, 10 foals age 10 months and 11 foals age 1–4 months.

2.2. Plaque purification

The forth passage of EHV-2 isolated from peripheral blood mononuclear cells (PBMCs) from an Icelandic horse (Torfason et al., 2008) was plaque purified as follows: foetal equine primary kidney cells (EqFKCs) were grown in six-well plates (NUNC) until 80-90% confluent in Dulbecco’s minimal essential medium (DMEM) (Invitrogen) supplemented with 2 mM glutamine, 100 IU penicillin, 100 IU streptomycin and 10% foetal bovine serum (FBS) (Invitrogen). The cells were washed three times in phosphate-buffered saline (PBS) and inoculated with EHV-2 virus diluted in growth medium without FBS for 1 h at 37 °C. The cells were washed again in PBS before an overlay of 1% SeaPlaque GTG agarose (FMC BioProducts) in growth medium with 10% FBS was added. The plates were then incubated in humidified atmosphere containing 5% CO₂ at 37 °C and observed for cytopathic effect. After approximately 7 days, single plaques were picked and plaque purified again.

2.3. Production of ELISA antigen

Twice plaque purified EHV-2 was diluted in growth medium containing 2% FBS and grown in EqFKC cells for 7 days or until 90–100% cytopathic effect was observed. Cells for control antigen were treated the same without virus. After 7 days the cells were scraped off, washed in PBS, pelleted at 2000 × g, dissolved in lysis buffer (0.01 M Tris, 0.002 M EDTA, 0.2 M sucrose, 2% (v/v) Triton-X100, 200 Kiu/ml aprotinin, 40 μl/ml Sigma protease inhibitor cocktail, pH 8.4) to obtain 10-fold concentration (Blixenkrone-Moller et al., 1991), then frozen at −80 °C, thawed in 50 °C and sonicated twice for 15 s on ice. The cell lysate was spun at 48,200 × g for 20 min and the supernatant stored at −80 °C.

2.4. ELISA

ELISA plates (MaxiSorb, Nunc) were coated with EHV-2 or control antigen preparation diluted 1/1000 in ELISA coating buffer (sodium carbonate–bicarbonate buffer pH 9.6) overnight at 4 °C (100 μl/well) and stored frozen at −20 °C until use. Washes and dilutions were done in PBS–T (PBS with 0.05% Tween 20). The coated plates were washed and blocked with 2% porcine skin gelatine (Sigma) for 90 min at 37 °C. Plates were washed and incubated with serum samples in twofold dilutions starting at 1/100 for 90 min at 37 °C. Subsequently, plates were washed and incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-horse IgG (Sigma, A9617) for 1 h at 37 °C. Bound antibody was detected with H₂O₂/3,3′-diaminobenzidine dihydrochloride (Dako) incubated at room temperature (RT) for 10 min. After 30 min, 75 μl of 4 M H₂SO₄ per well were added and the optical density OD₄₉₂ was read. For measuring γ-EHV specific IgG isotypes monoclonal antibodies against horse IgGa, IgGb and IgG(T) (Serotec, MCA 1902, MCA 1901, MCA 1900) were added in dilution 1/1000 after the serum incubation step and incubated at 37 °C for 1 h. Subsequently, the plates were washed and incubated with HRP-conjugated goat anti-mouse (Dako, P0447) for 1 h at 37 °C and then proceeded as described above. Based on the absorbance values for the horse sera on the control antigen an arbitrary cut-off of 0.3 OD was used to determine positive and negative reactions.

2.5. Cytokine mRNA measurement

Peripheral blood mononuclear cells were obtained by centrifugation of anticoagulated horse blood on Ficoll-Paque™ PLUS (Amersham Biosciences) and washed several times in PBS. The PBMCs were then resuspended at 5 × 10⁶ cells in 750 μl of plaque purified EHV-2 virus, m.o.i. 1 or alternatively, culture media supplemented with 10% normal horse serum as negative control, and incubated on a rocking platform for 2 h at 37 °C. Cells were then seeded in a 24-well plate (Nunc) at 5 × 10⁶ cells/well. Culture media supplemented with 50 μM 2-mercaptoethanol and 10% normal horse serum, was added to obtain 1.5 ml/well and additionally a well with 1 μg/ml phytohemagglutinin (PHA, Sigma) as a positive control.
The PCR block was preheated to 95°C and the plate was inserted. The starting copy number of IFN-γ and IL-4 was 20 copies per well. For each reaction, the PCR master mix was prepared as follows: 1 μl 20 μM (1 μM) forward primer, 1 μl 20 μM (1 μM) reverse primer, 0.5 μl 10 μM (0.25 μM) probe, 2 μl 2 mM dNTP (0.2 mM), 2 μl 10× Thermopol Reaction Buffer, 0.1 μl Taq DNA polymerase (5000 U/ml) and 11.4 μl dH2O. The DNA was placed in triplicate wells of a 96-well polystyrene microplate (Low Profile Multiplate, MJ Research Inc.). Specific TaqMan primers and fluorescent probes were designed for each template and manufactured by TAG Copenhagen. The primers and probe sequences used are listed in Table 1. Separate plasmids encoding β-actin (pBluescript II SK-β-actin), IFN-γ and IL-4 (pUC18-IFN-γ and pUC18-IL-4 kindly provided by Dr. David Horohov, University of Kentucky) were used to establish a standard curve of known template concentration in each reaction. The DH5α E. coli harbouring the plasmids were propagated in LB medium, purified with QIAprep (QIAGEN) and the concentration of DNA was measured in a UV spectrophotometer. Serial dilutions were made for IFN-γ and IL-4 vectors from 6 × 10² to 6 × 10⁴ copies per μl ((0.2–2) × 10⁻⁷ ng/μl) and for β-actin 6 × 10⁴ to 6 × 10³ copies per μl. PCR master mix was prepared for each reaction as follows: 1 μl 20 μM (1 μM) forward primer, 1 μl 20 μM (1 μM) reverse primer, 0.5 μl 10 μM (0.25 μM) probe, 2 μl 2 mM dNTP (0.2 mM), 2 μl 10× Thermopol Reaction Buffer, 0.1 μl Taq DNA polymerase (5000 U/ml) and 11.4 μl dH2O. The DNA was placed in triplicate wells of a 96-well polystyrene microplate (Low Profile Multiplate, MJ Research Inc.), 2 μl of either the reverse transcribed cDNA or the diluted vectors or 2 μl dH2O that served as a negative control. The final volume of the PCR mix in each well was 20 μl. The thermocycling profile was as follows: 95°C for 2 min, 40 cycles of 95°C for 15 s, 60°C for 1 min. The PCR block was preheated to 95°C before the sample plate was inserted. The starting copy number of IFN-γ, IL-4 and β-actin in each sample was quantified by using the Opticon Monitor Software to set the cycle threshold ct line of the standard curve. The copy number of β-actin was used as an internal standard to normalize the data. Stimulation index (SI) was calculated according to the formula (Coombs et al., 2006).

Cytokine copy number in EHV-2/

β-actin copy number in EHV-2

Cytokine copy number in medium/

β-actin copy number in medium

### Table 1

<table>
<thead>
<tr>
<th>Primers and probes</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Probe</th>
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<tbody>
<tr>
<td>β-Actin</td>
<td>5'-GGCATCTCTGACCCTCAAGTA-3'</td>
<td>5'-CCTTCTCATGTCGCCGACT-3'</td>
<td>5'-FAM-CATCGACACGGACATGCTA-BHQ-3'</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5'-GGCAACCACACGAGGCAG-3'</td>
<td>5'-GGACCTCTACACATATTACC-3'</td>
<td>5'-FAM-ACTGTTTAAACACGGACGACAG-3'</td>
</tr>
<tr>
<td>IL-4</td>
<td>5'-CTGGAAGTGCTCTCAACAG-3'</td>
<td>5'-AGTACAGGCGCTCCCTTTG-3'</td>
<td>5'-FAM-CAGTCGCTCAAGGACATGCTA-BHQ-3'</td>
</tr>
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3. Results

3.1. IgG response to γ-EHV

As is shown in Fig. 1 the 20 adult horses and the ten 10-month-old foals had high IgG response to γ-EHV. The young foals were also clearly positive except the orphan foal that was without any detectable antibody response and was not included in Fig. 1. Three of the foals had titer equal to the adult horses, four somewhat lower and three very low.

3.2. IgG subclass response to γ-EHV

In both the adult horses and the 10-month-old foals the IgG4/7 was the chief response (Fig. 1). The IgG1 titer was constantly much lower. The IgG3/5 titer was similar to the IgG1 titer except in two adult horses and one 10-month-old foal where it was higher. In this foal and in one of these two adults, the titer of IgG3/5 exceeded also the IgG4/7 titer. The young foals also responded mostly with IgG4/7 but with lower and more variable titer (Fig. 1). Nine out of 10 young foals were positive for IgG4/7, using 1/100 dilution as a cut off point, whereas only 3 out of 10 were positive for IgG1 and IgG3/5 and these were the same three. The three youngest foals which were 1 and 2 months of age had only IgG4/7 response, probably of maternal origin whereas the three foals that showed some IgG1 and IgG3/5 response were 3–4 months old.

3.3. Cytokine response

We looked at the IFN-γ and IL-4 expression in ten of the adult horses after in vitro stimulation of PBMCs with EHV-2. All the horses produced IFN-γ but hardly any IL-4 (Fig. 2).

4. Discussion

The adult horses and the 10-month-old foals all responded strongly to γ-EHV, whereas there was more variation among the younger foals. Fu et al. (1986) looked at EHV-2 titer in 16 mares and their foals with ELISA. Soon after colostrum consumption the foals developed similar antibody levels as their dams. Thereafter the maternally derived antibody titer declined to a low level at 2 months of age and was then replaced by the actively produced antibody to natural EHV-2 infection (Fu et al., 1986). We cannot state whether the antibodies of the foals in our study are of maternal origin or actively produced. The antibody titer did not correlate to the age of the foals. This is not surprising since they are from random flocks of horses that could differ with regard to level of active virus and re-infections.
To the best of our knowledge, there are no reports on IgG subclass response against γ-EHV. In horses latently infected with EHV-4 only IgG4/7 but no IgG1 EHV-4-specific antibodies were detected in ELISA in serum dilution 1/800 (Mizukoshi et al., 2002). Likewise using this dilution only one of our adults and no 10 months old foal would pass as positive for IgG1 whereas in dilution 1/800 only one adult and no 10 months old foal would be IgG4/7 negative. Mizukoshi et al. did not look at IgG3/5 titer in the naturally EHV-4 infected horses but only one out of four experimentally infected foals showed an IgG3/5 in very low titer. In our study, the IgG3/5 was somewhat higher than IgG1 titer and seven adults and two 10-month-old foals would have passed as positive for IgG3/5 in the dilution 1/800. The young foals also responded mostly with IgG4/7 but with lower and more variable titer (Fig. 1).
out of 10 young foals were positive for IgG4/7 if a 1/100 dilution was used, whereas only 3 out of 10 were positive for IgG1 and IgG3/5. The early immunoglobulin repertoire of neonatal foals is comprised of IgG1, IgG4/7 and IgG3/5 and IgG4/7 is the most abundant subclass in equine colostrum (Sheoran et al., 2000). Endogenous produced IgG1 and IgG3/5 are detected within the first 2 months of life but IgG4/7 not until after 63 days (Holznagel et al., 2003; Sheoran et al., 2000). Accordingly the three foals that were 1 and 2 months of age only showed IgG4/7 response in our study, probably of maternal origin, whereas the three foals that showed some IgG1 and IgG3/5 response are 3–4 months old.

IFN-γ and cytotoxic T lymphocytes (CTLs) are essential in protection against most viral diseases. IFN-γ production has been shown to be important in EHV-1 infection (Breathnach et al., 2005; Paillot et al., 2005) and its production increases with age, suggesting that EHV-1-specific memory cells increase after repeated exposure to EHV-1 antigens (Paillot et al., 2005). In accordance to this the 10 adult horses we tested all expressed IFN-γ but hardly any IL-4 (Fig. 2). In conclusion our results demonstrate that IgG1, IgG4/7 and IgG3/5 are induced in γ-EHV infection, IgG4/7 is the dominant subclass and the production of IFN-γ exceeds that of IL-4.

Acknowledgement

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References


