IgG subclass response against equine herpes virus types 2 and 5 in horses

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Contents

Abstract

Total-IgG and IgG-subclass response against the latent equine gamma herpes virus (EHV-2/5), was examined using ELISA and plaque purified EHV-2. Thirty two healthy Icelandic horses, 21 adults and 11 foals, were tested. All individuals were positive in total IgG ELISA except for one foal. They all showed a strong EHV-2/5 specific IgGb response but much lower IgGa and IgG(T), except for one adult which had an IgG(T) response as high as the IgGb response. The IgG values for all the adult horses were similar, indicating a stable response, whereas the values for the foals showed more variation which could reflect recent infections in some of them. The results demonstrate that IgGb is the dominating antibody in an EHV-2/5 response. This IgG subclass profile indicates a Th1 directed immune response as can be expected for a latent virus.

1. Introduction

1.1 Equine gamma herpes virus

The family of herpesviridae are double stranded DNA viruses with an envelope.[1] A significant aspect of the pathogenesis of herpesviridae in all animal species is their ability to cause latent infection, with viral persistence in neural and/or peripheral blood mononuclear cells.[2] The family of herpesviridae is classified into three subfamilies; the alpha, beta and gamma herpesvirinae. This classification is largely on the basis of biological properties.[3] Five different types of equine herpes viruses are known; equine herpes viruses 1, 3 and 4 are α-herpes viruses, equine herpes viruses 2 and 5 are γ-herpes viruses of the genus rhadinoviruses.[3,2] The horse is the only animal species known to have two distinct γ-herpes viruses.[25] The α-herpes viruses are characterized by a relatively short replication cycle and a rapid spread in cell culture. Some possess a broad host range. The γ-herpes viruses have a slow replication cycle and a restricted host range.[4,5] EHV-2 and EHV-5 share considerable cross-reactivity in enzyme linked immunosorbent assay (ELISA) and Western blot, suggesting EHV-2 and EHV-5 possess common epitopes.[6]

Horses become infected with EHV-2/5 as foals despite maternal antibodies, and may be infected successively with different virus variants.[7] EHV-2 is widely spread in horse populations, up to 89%[8] and it has been isolated from the leucocytes of healthy animals as well as from horses with different clinical signs. EHV-2 infection occurs mostly in young foals, they become infected between 2 and 4 months of age.[9] For EHV-5 the percentage of infected horses seems to be much lower, up to 24% for adult horses and 15% for foals.[9] Both EHV-2 and EHV-5 seem to be frequent in the Icelandic horse population.[10]

The clinical impact of equine γ-herpes virus infections is not fully known, but EHV-2 has been associated with pharyngitis, conjunctivitis, keratoconjunctivitis, upper respiratory tract disease, poor performance, chronic obstructive pulmonary disease, and severe pneumonia in some foals.[2] EHV-5 does not seem to be associated with respiratory disease.[9]

1.2 Immune response

The host response to antigen is mediated through a network of interacting immune cells and their products. Dendritic cells are the most important antigen presenting cells (APCs). Their function is the uptake and presentation of antigen. Most pathogens like bacteria, viruses and parasites are recognized by the immune system because they contain PAMP (pathogen-associated molecular pattern). The immunogens of pathogens that replicate in the cytoplasm of the host cell are presented at the cell surface by Major Histocompatibility Complex-I (MHC-I) to CD8$^+$ cells, which differentiate into cytotoxic T cells. Pathogens (taken) in intracellular vesicles are presented to naïve CD4$^+$ cells at the cell surface by MHC-II. When those CD4$^+$ cells are exposed to immunogenic peptides they may differentiate into either regulatory T cells or T-helper 1 or 2 effector cells. Those T-cells then stimulate B lymphocytes to produce immunoglobulins and/or CD8$^+$ cells to start killing.[11] Clear evidence suggests that the development of naïve Th cells to either Th1 or Th2 is influenced by both environmental and genetic factors acting at the level of antigen presentation.[12]

The APCs use cytokines to direct the immune response into different pathways, depending on the invasive pathogen. In general, Th1 responses are highly protective against infections mounted by intracellular pathogens, because of the ability of Th1-type cytokines to activate phagocytes and to promote the production by B lymphocytes of opsonizing and complement-fixing antibodies (IgG). Th1 responses may become dangerous for the host because of the strong and chronic inflammatory reaction evoked.[13] Extracellular pathogens mostly direct the immune response into a Th2 response. The Th2 cytokines activate antigen specific B lymphocytes for the production of IgM, IgA, IgE (fig.2) or neutralizing subtypes of IgG (IgG(T)).[11] Th1 cells are associated with cell-mediated inflammatory reactions and Th2 cells are associated with strong antibody and allergic responses.[14] IL-4 production stimulates differentiation into Th2 cells, whereas IFN-γ, IL-12, and IL-18 in the absence of IL-4 stimulates Th1 polarization[12,15] (fig. 1).
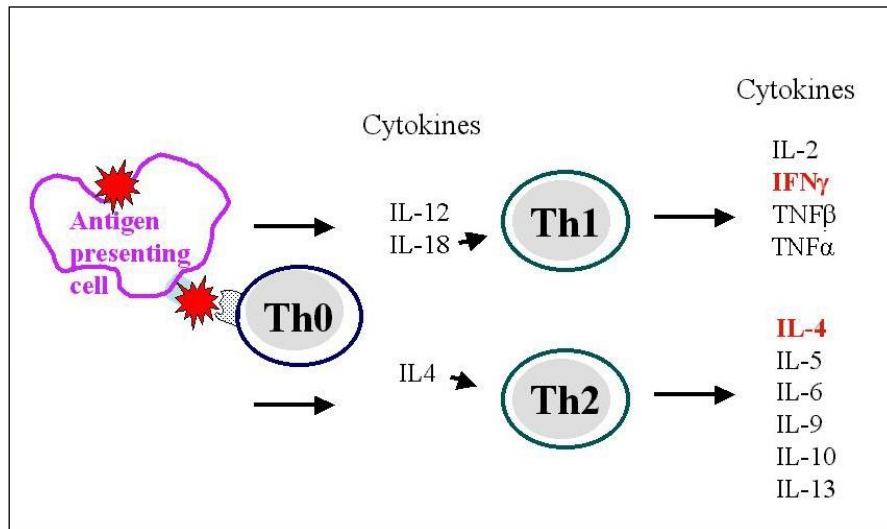
Fig. 1. Cytokines involved in Th1 and Th2 immune responses

Th2 cells are characterized by the production of IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13.[13,15,16] Th1 is characterized by the production of IL-2, IFN-γ and TNF-β[13,16] and TNF-α[17] (fig. 1).

The T-helper immune response in the horse can evoke the production of different immunoglobulin types and cytokines. The immunoglobulin classes of the horse are complex and it was believed they included four IgG subisotypes (IgGa, IgGb, IgGc and IgG(T)), IgA, IgM and IgE (fig. 2). There were no reports of the identification of equine IgD.[18]
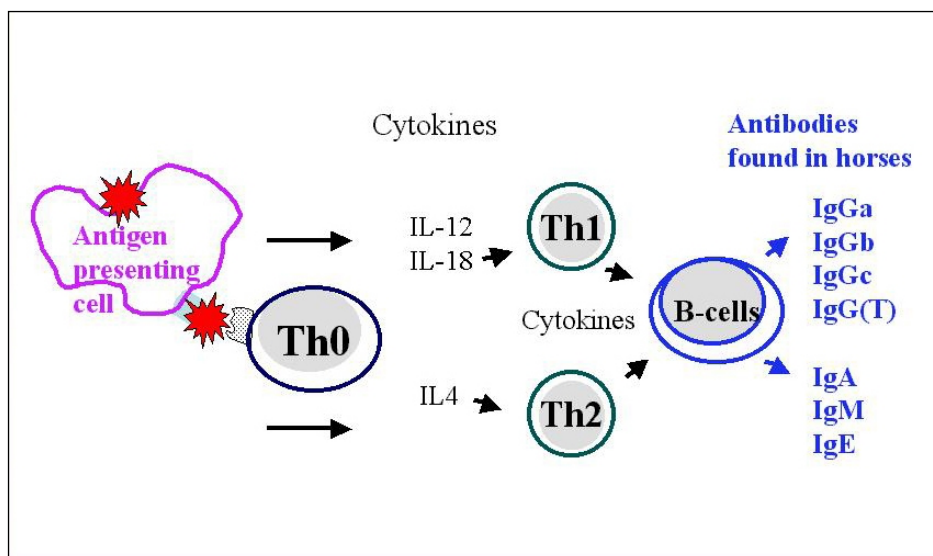

Fig. 2. Antibodies formed in horse Th1 and Th2 responses

However, recent studies on the IGHC genes of the horse, showed the existence of at least six, maybe seven IgG types in horses and has described an IGH-D gene for the first time.[18] IgGa, IgGb and IgGc are designated on the basis of their increasing anodal mobility in immuno-electrophoresis. The study of the IGHC genes showed the existence of two different types of IgG(T).[18] Additional features of IgG(T) include its failure to fix complement and its ability to inhibit complement fixation by IgGa and IgGb. It also has been shown that equine monocytes and neutrophils do not appear to have Fc receptor sites that will bind IgG(T), whereas they can bind other IgG subisotypes and IgM. These features suggest that IgG(T) is likely to be best adapted to toxin neutralization, and less efficient in complement fixation, opsonization and antibody-dependent cellular cytotoxicity.[17]

Several cytokines of the horse have been cloned, sequenced and expressed to use them for a better understanding of the development of the equine immune system. The equine cytokines characterized so far are IL-1, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IFN-α, IFN-β, IFN-ω, IFN-γ and TNF-α.[17,19]

1.3 Allergic response and insect hypersensitivity

Allergens are molecules which don't have PAMP. Therefore, they should not activate the immune response like pathogens do. Most individuals are tolerant to them, but they can evoke an allergic response in some individuals (fig. 3).
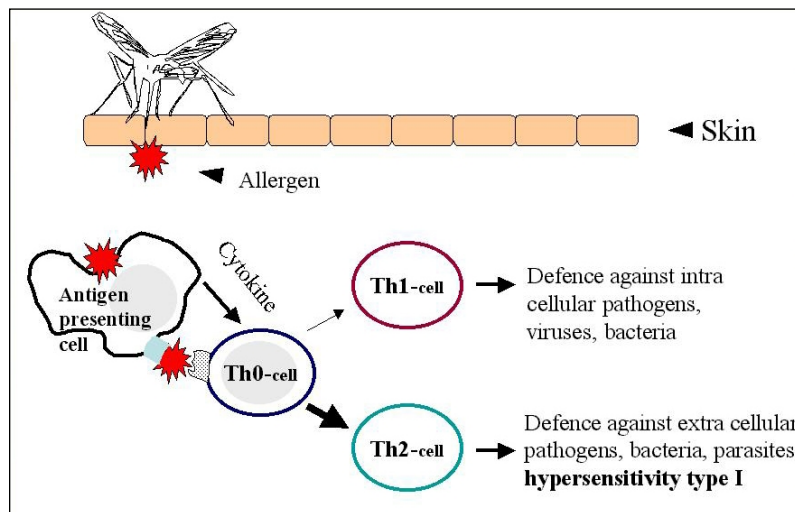

Fig. 3. Allergic response to insect bites

Those hypersensitivity reactions are classified into four types; I – IV. However, only the terms Type I and Type IV are used routinely. Type I or immediate hypersensitivity is characterized by the production of IgE antibodies against foreign proteins that are commonly present in the environment, and by the release of histamine, leukotrienes and other inflammatory mediators. Type IV are cell mediated reactions in which specific Th1 cells are the primary effector cells. IgE production is dependent on Th2 cells and any priming that generates a Th1 response will inhibit IgE production.[14]

Insect hypersensitivity is characterized by an immediate (type I) hypersensitivity reaction followed by an additional delayed (type IV) reaction.[20] It is a chronic, recurrent, seasonal dermatitis of horses, which can affect horses of all breeds. It is caused by the bites of midges of *Culicoides* spp. These are extremely small (1-3mm) flies, and they are most active during dawn and dusk, during warm weather when there is little or no breeze. They require standing water or decaying vegetation to breed.[21,22] The clinical signs of this insect hypersensitivity are a reflection of the initial pruritis (papules and skin sensitization), which causes intense irritation, scratching and rubbing, leading to serous effusion, excoriations, alopecia, lichenification, erythema, pigment disturbances, 'rat tail' and 'buzzed off' mane.[20,21]

One of the aims of research to insect hypersensitivity is to see whether it is possible to switch the immune response away from the Th2-pathway and obtain a strong Th1 focused immune response.

The aim of this study was: 1) To evaluate the antibody response of Icelandic horses of different ages against equine gamma herpes virus. This was done by measuring total-IgG and IgM response. 2) To determine if the response was Th1 polarized like can be expected for a latent virus. This was done by measuring IgG subclasses. Cytokines are measured, but the results are not ready yet. The future aim is to be able to measure a Th1 profile that can be compared to the Th2 response observed in horses with type I hypersensitivity like insect hypersensitivity.

2. Materials and methods

2.1 Horses

Thirty two healthy Icelandic horses were divided into two different groups. The first group consisted of seventeen adult horses of 5-10 years old and four young horses of about 10 months of age at the time of sample collection. In the second group were eleven foals with the estimated age of 1 to 4 months. The age of the foals had to be estimated because the precise date of birth was not known; for this research it is assumed all the foals were born at the 15th of May. Table 1 shows the ages of the adults and the young horses, and the estimated ages of the foals.

Adult	Age (years)	Reg. nr.	Foal	Age (months)	Reg. nr.
1	6	Eq 705	1	1	647
2	8	Eq 706	2	2	880
3	7	Eq 572	3	3	881
4	7	Eq 573	4	3	882
5	7	Eq 808	5	3,5	888
6	6	Eq 809	6	4	891
7	10	F 3	7	4	894
8	8	Eq 849	8	2	1057
9	5	Eq 850	9	3	1103
10	6	Eq 851	10	3,5	1108
11	5	Eq 852	11	3,5	1111
12	6	Eq 853			
13	5	Eq 854	**Young**	**Age (months)**	**Reg. nr.**
14	5	Eq 855	1	10	556
15	5	Eq 846	2	10	557
16	6	Eq 847	3	10	558
17	6	Eq 848	4	10	559

Table 1. Ages at the time of sample collection and registration numbers of the adult horses, the young horses and the foals.

Six of the seventeen adult horses are owned by Keldur institute and immunized with Human Serum Albumin protein for the research of insect hypersensitivity. Pre-immunization blood from those horses was used. The rest of the adult horses could be grouped into horses of young age or ten months old, and older horses (age 5- 10). It was

made sure that those horses would be available again in the following years for collecting blood every time needed. Serum of the foals was collected for paternal testing.

The total IgG and the IgG subclass antibody titers were determined in all 32 horses, IgM antibody titers were only tested in the foals.

2.2 Sample collection

Blood samples were collected by jugular vein puncture into non-additive vacutainer (Greiner bio-one) tubes and allowed to clot successively for 1,5 hours at 37°C and 3 hours at 4°C. The tubes were centrifuged at 2000 xg for 30 minutes at a temperature of 4°C. Serum was removed and transferred to sterile tubes and stored at -20°C until further use.

2.3 EHV-2 ELISA antigen production

Equine primary kidney cells, 80-90% confluence, were washed 3 times in PBS and inoculated with plaque purified EHV-2 virus in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 2 mM glutamine, 100 IU penicillin and 100 IU streptomycin for 1 hour on a cradle at 37°C. Then DMEM supplemented with 2% Fetal Bovine Serum (FBS) (Invitrogen) was added and the cells were incubated for 7 days at 37°C or until 90-100% cytotoxic effect was observed. Cells for control antigen were treated the same without virus. After 7 days the cells were scraped off in the medium, washed in PBS, pelleted at 2000 xg, dissolved in lysis buffer to obtain 10 times concentration, frozen at -80°C, thawed in 50°C and sonicated twice for 15 seconds on ice. The cell lysate was then spun at 48.200 xg for 20 min and the supernatant stored at -80°C until used.

The virus antigen used is plaque purified EHV-2, but it is not possible to discriminate whether the horses are infected with EHV-2 or EHV-5 using ELISA, due to cross-reaction of those two closely related viruses.[6] Therefore we will use the term EHV-2/5 in this study.

2.4 ELISAs

To determine the total-IgG, the IgG-subclass and the IgM responses to equine gamma herpes virus infection, EHV-2-specific ELISA was used. Plates coated with the same batch of antigen were used for EHV total-IgG ELISA, EHV IgG subclass ELISA and EHV IgM ELISA. In the total-IgG and IgM ELISA the serum was tested on the control antigen at the same time as on the antigen. Only the first dilution of the sera of all the horses was tested on the control antigen for the IgG subclasses. In case there was some reaction on the control antigen, all the serum dilutions of that horse were incubated on the control antigen.

ELISA plates (MaxiSorb, Nunc) were coated with the EHV-2 antigen or control antigen preparation, diluted 1/1000 in ELISA 0.05 M carbonate-bicarbonate buffer, 100 µl/well. This was kept over night at 4°C and then frozen at -20°C for later use.

2.4.1 Total-IgG ELISA

After thawing the plates at 37°C, they were blocked with 200 µl 2% porcine skin gelatin in phosphate buffered saline (PBS-T) per well for 75 min. at 37°C. After every incubation a washing step was done with PBS-T washing buffer. Double dilutions in PBS-T were made of the sera, starting at a dilution of 1/100 (10 µl in 1 ml). Serum dilutions were incubated 90 min. at 37°C, 100 µl/well. Horseradish peroxidase-conjugated rabbit anti-horse IgG (Sigma) diluted in PBS-T (1/4000) was added, 100 µl/well, and incubated for 1 hour at 37°C. For color development 100 µl O-phenylenediamine dihydrochloride (OPD) solution was added to every well and incubated for 10 minutes at room temperature in dark. To stop the reaction 75 µl H_2SO_4 per well was used.

2.4.2 IgG subclass ELISA

To measure IgG subclass responses, the plates were incubated at 37°C for 1 hour with 100 µl/well mouse-anti-equine IgGa, IgGb, IgGc and IgG(T) (Serotec) diluted in PBS-T (1/1000) after the serum step. Then after washing, with horseradish peroxidase-conjugated goat-anti-mouse IgG (Dako) diluted in PBS-T (1/2000), 100 µl/well for 1 hour at 37°C. Blocking, serum dilutions, color development and stop of reaction were as described for the total-IgG assay.

2.4.3 IgM ELISA

To measure the IgM response, 100 µl mouse-anti-horse IgM (Serotec) diluted in PBS-T (1/1000) was added to every well after the serum step and incubated for 1 hour at 37°C. Then after washing 100 µl/well horseradish peroxidase-conjugated goat-anti-mouse IgG (Dako) diluted in PBS-T (1/2000) was incubated at 37°C for 1 hour. Blocking, serum dilutions, color development and stop of reaction were as described for the total-IgG assay.

Absorbances were read at 492 nm with an ELISA reader ('HTS 7000 Plus Bio Assay Reader' of Perking Elmer) immediately after stopping the reaction. The cut-off value for determining the titers was set at 0.300 since all the readings were lower than 0.300 on the control antigen, except for the serum of foal #9. In that specific case the control values were subtracted.

3. Results

3.1 Total-IgG responses

The total IgG responses to EHV-2/5 of the adult horses and the foals are shown in figs. 4a and 4b. The results show that total IgG antibody against EHV-2/5 could be detected in all adults and foals except for one foal (#9). None of the horses, except for foal #9 showed a response on the control antigen. Only for this foal the EHV-2/5 antigen absorbance values are subtracted with the control antigen absorbance values. For the rest of the foals and all the adults only the absorbance values on the EHV-2/5 antigen have been used.
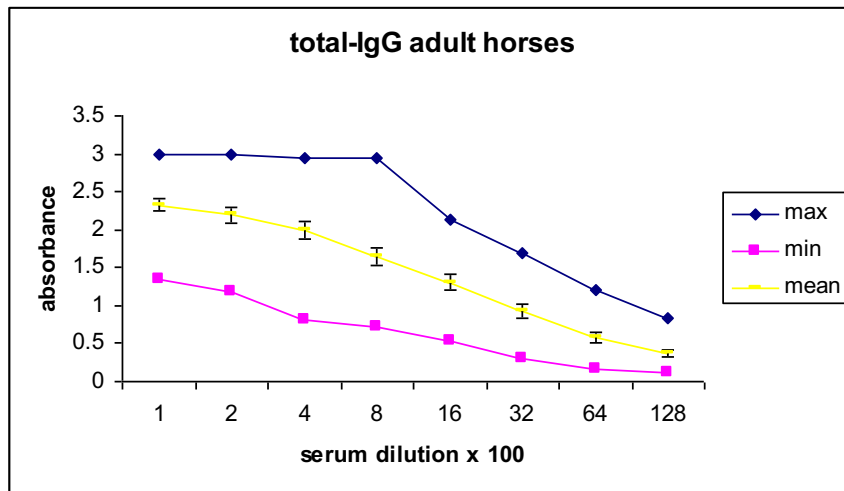

Fig. 4a. Serum total-IgG responses to equine gamma herpes virus (EHV-2/5) of 21 adults. The maximum, minimum and mean values and the standard error of the mean (sem) are shown for every serum dilution.
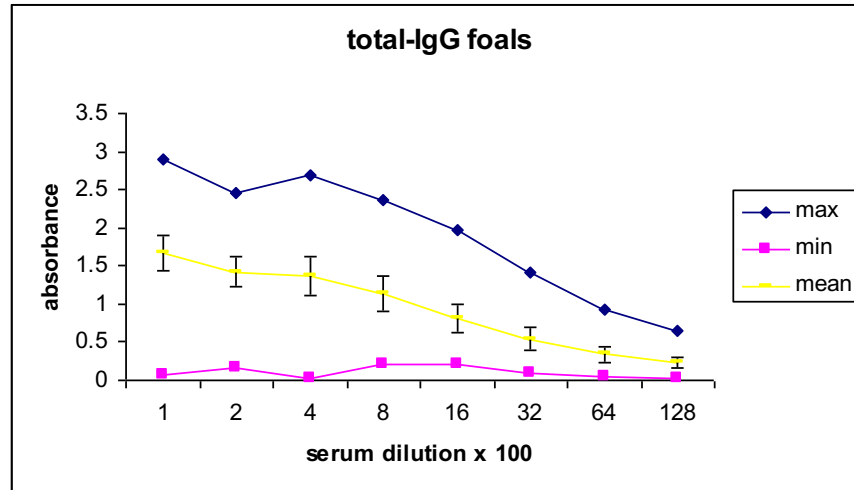
Fig. 4b. Serum total-IgG responses to equine gamma herpes virus (EHV-2/5) of 11 foals. Control antigen values are subtracted from EHV-antigen values only for foal #9. The maximum, minimum and mean values and the sem are shown for every serum dilution.

In fig. 4a and 4b it is shown that the distribution of absorbance values is more in the foals than in the adults. The max and min are closer to each other. There is also less variation in the standard error of the mean (sem) values of the adult horses compared to the foals (figs. 4a and 4b). No correlation was observed between age of foals and level of EHV-2/5 response. The foals with the lowest titer were 3 to 4 months old and the foals with the highest titer were 2 to 3,5 months old. (figs. 5 and 9)
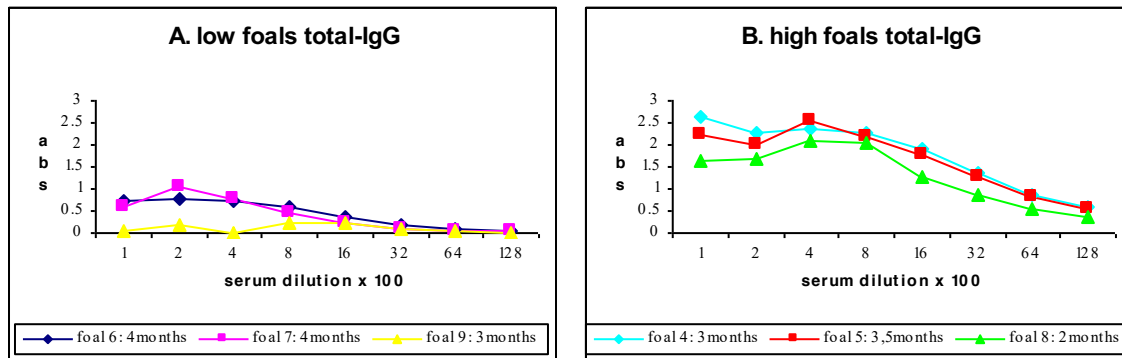

Fig. 5. Serum total-IgG responses to equine gamma herpes virus (type 2/5) of (A) the 3 foals with the lowest (foals #6, 7 and 9) compared to (B) the 3 foals with the highest (foals #4, 5 and 8) response. The y-axis is titled 'abs', which means 'absorbance'.

The mean antibody titers of total-IgG of both the adult horses and the foals are shown in fig. 6. The graph shows that the titer of the total-IgG is much higher for the adults than for the foals.
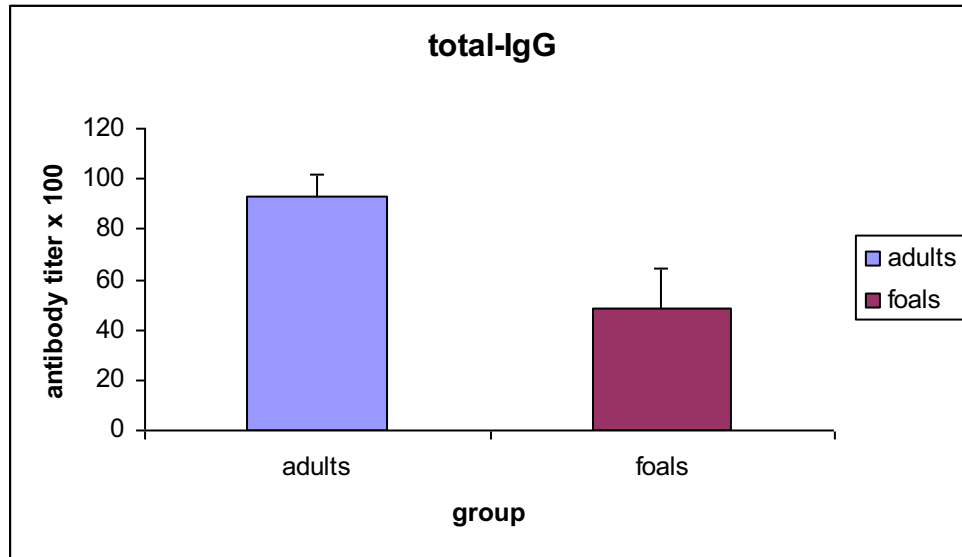

Fig. 6. Mean ELISA titers of total-IgG antibodies to equine gamma herpes virus (type 2/5) and the standard error of the mean (sem) of the adult horses and the foals.

3.2 IgG subclass responses

IgGa, IgGb and IgG(T) subclass responses to EHV-2/5 of the adult horses and the foals are shown in fig. 7. In all subclasses the mean responses of the adults are stronger than the mean responses of the foals, in agreement with the results of the total-IgG response. Mainly in the IgGb, but also in the IgGa response the distribution of absorbance values is more for the foals than for the adults, and again the sem is more variable. Both the adult horses and the foals respond strongest with IgGb, except for adult #10 and foal #9. Adult #10 shows an IgG(T) response which is equal to the IgGb response (fig. 8).
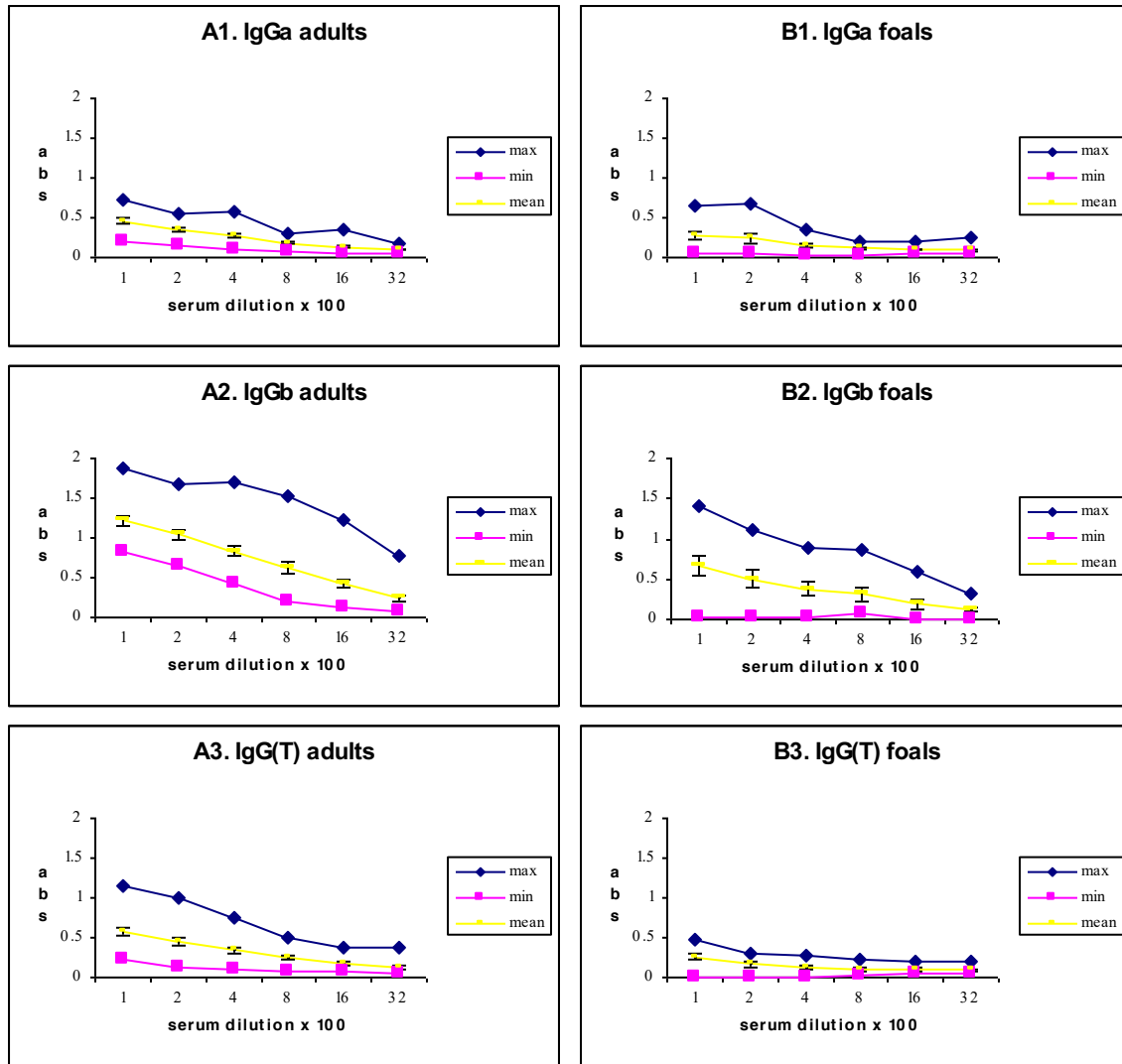
Fig. 7. Serum IgG-subclass responses to equine gamma herpes virus (EHV-2/5) of 21 adult horses (A1, A2, A3) and 11 foals (B1, B2, B3). Control antigen values are subtracted from EHV-antigen values for foal #9. The maximum, minimum and mean values and the sem are shown for every serum dilution. The y-axis is titled 'abs', which means 'absorbance'.
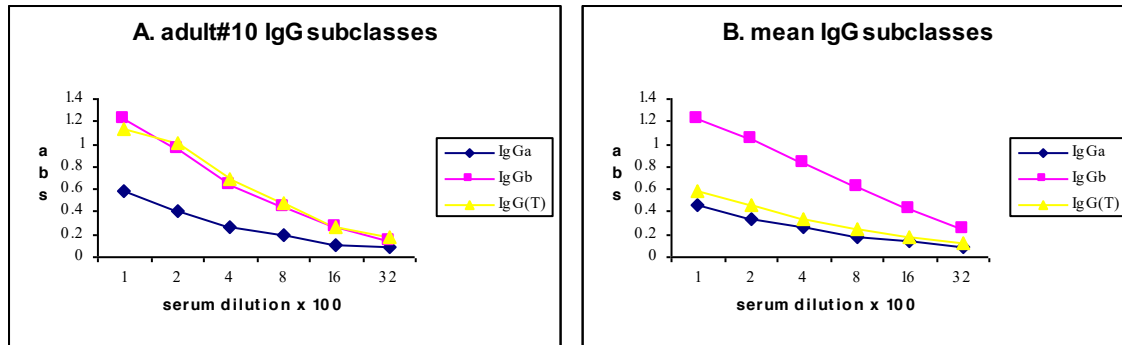
Fig. 8. IgG subclass response to equine gamma herpes virus (type 2/5) of (A) horse #10 compared to (B) the mean IgG subclass response of the group of 21 adult horses. The y-axis is titled 'abs', which means 'absorbance'.

IgGc responses were negative for all the adult horses and the foals in serum dilution 1/100 (table 2). It can not be excluded that the monoclonal antibody (mAb) against IgGc doesn't work properly.

Adult	Absorbance	Foal	Absorbance
1	0.1973	1	0.1362
2	0.1979	2	0.0992
3	0.1502	3	0.1367
4	0.1624	4	0.1835
5	0.1709	5	0.1815
6	0.1047	6	0.0602
7	0.1451	7	0.0907
8	0.1439	8	0.1274
9	0.1574	9	0.0553
10	0.1058	10	0.1122
14	0.1798	11	0.0958

Table 2. IgGc absorbance values for equine gamma herpes virus (type 2/5) ELISA of some adult horses and the foals at serum dilution 1/100
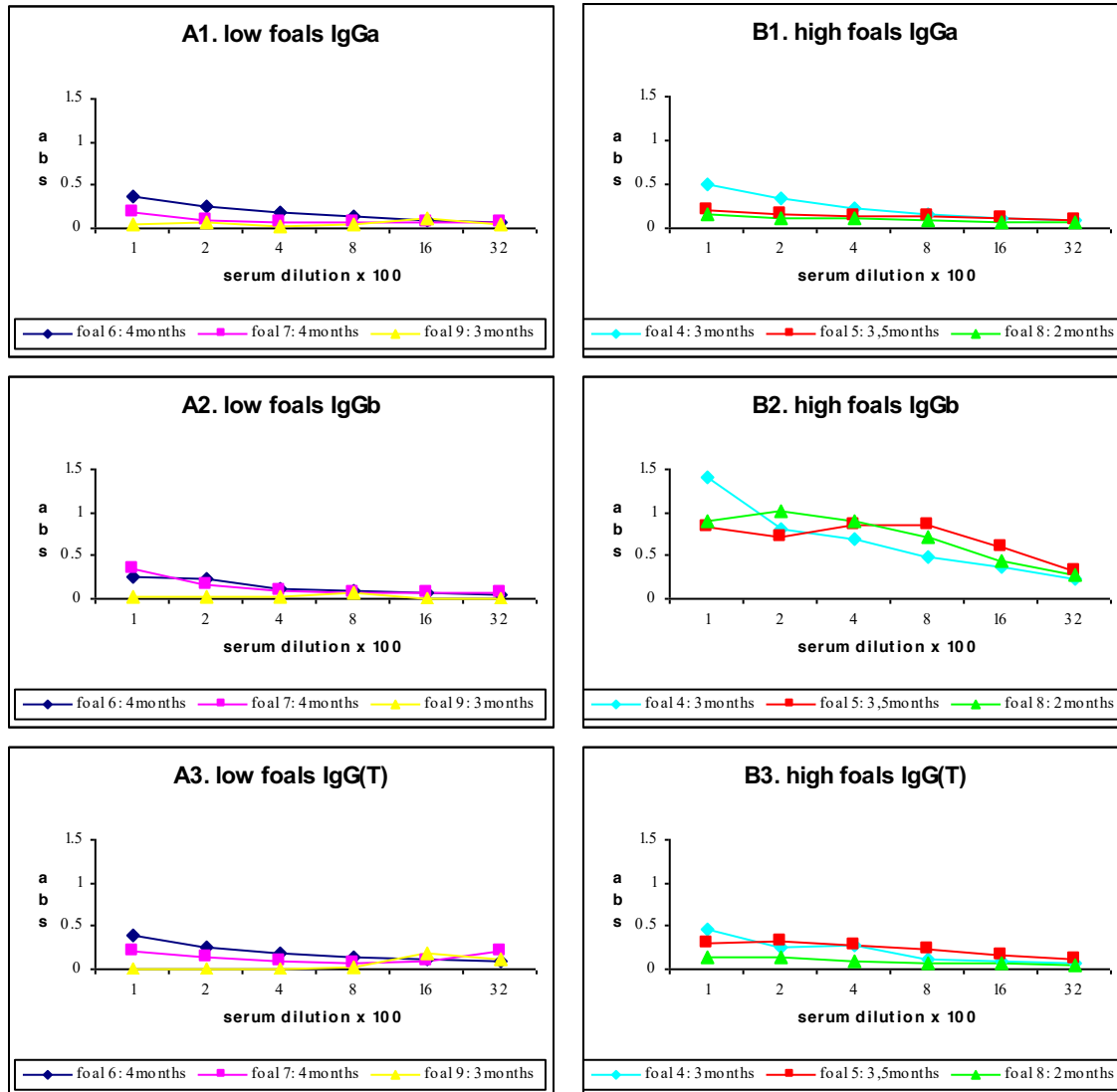
Fig. 9. Serum IgG subclass responses to equine gamma herpes virus (type 2/5) of (A1, A2, A3) the 3 foals with the lowest (foals #6, 7 and 9) response compared to (B1, B2, B3) the 3 foals with the highest (foals #4, 5 and 8) response. The y-axis is titled 'abs', which means 'absorbance'.

The mean antibody titers of the IgG subclasses of both the adult horses and the foals are shown in fig. 10. This graph shows that the titers of all IgG subclasses are higher for the adult horses than for the foals.
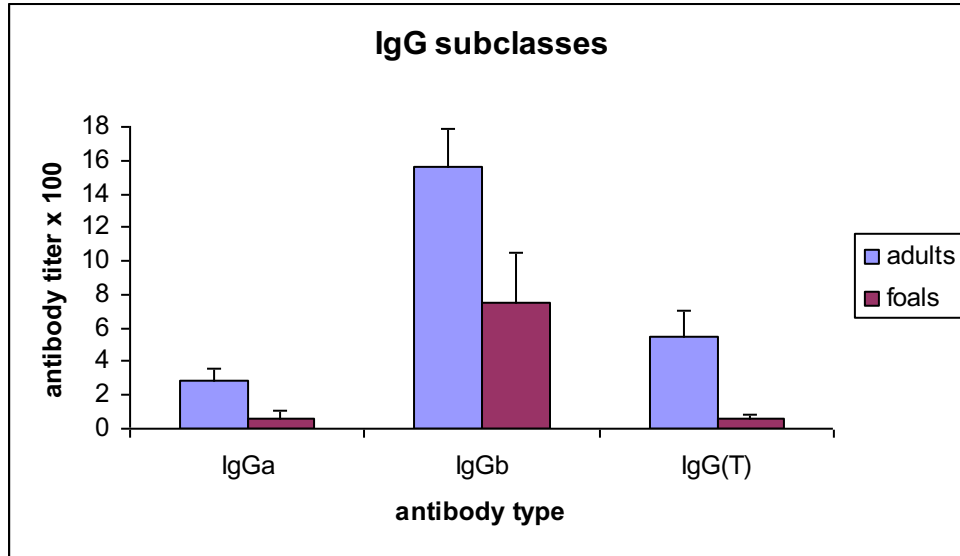
Fig. 10. Mean ELISA titers of IgG subtype antibodies to equine gamma herpes virus (EHV-2/5) and sem of the adult horses and foals.

3.3 IgM responses

All the foals were tested for IgM antibody responses. The absorbance values were very low (table 3). Just two of the foals had a very low titer, the others had no titer, if cut-off value was set at 0.300.

Foal	Absorbance
1	0.2488
2	0.4220
3	0.2839
4	0.1691
5	0.2415
6	0.2590
7	0.1425
8	0.2230
9	0.1344
10	0.3478
11	0.1632

Table 3. IgM absorbance values for equine gamma herpes virus (type 2/5) ELISA of the foals at serum dilution 1/100

4. Discussion

In order to investigate the antibody response and Th1, Th2 polarization of the immune response against EHV-2/5, total-IgG and IgGa, IgGb, IgGc and IgG(T) of 32 Icelandic horses was measured in ELISA. The horses were divided into 17 adult horses age 5 – 10 years plus 4 young horses (10 months old) and 11 foals with the estimated ages of 2 – 4 months (table 1). The virus antigen used is plaque purified EHV-2, but it is not possible to discriminate whether the horses are infected with EHV-2 or EHV-5 using ELISA, due to cross-reaction of those two closely related viruses.[6] Therefore we will use the term EHV-2/5 in this study.

4.1 Total-IgG response

EHV-2 is widely spread in horse populations, up to 89%.[8] In this study similar results were found. All horses showed an IgG response (figs. 4a,b) to equine gamma herpes virus (EHV-2/5), except for foal #9. For this foal the absorbance values on the control antigen are subtracted from the absorbance values on the EHV-2/5 antigen. The values are only subtracted for foal #9 and not for the others, because there seemed to be some nonspecific binding in the serum of this foal since the values on the control antigen were more or less the same as the values on the EHV-2/5 antigen. This could be due to natural antibodies.

4.1.1 Maternal antibodies

Foal #9 was known to be without a mother from right after its birth and didn't get any colostrum or milk, and therefore no maternal antibodies. It is likely that the other foals have maternal antibodies. It has been reported for the influenza virus that specific antibodies of the IgGa, IgGb and IgG(T) subtypes are most likely passively transferred to foals via colostrum.[23] The IgGa and IgGb antibodies to influenza virus in postsucking foals were detectable in all tested foals until 26 weeks.[23] Furthermore, foals become infected with EHV-2 at a young age, between 2 and 4 months[9], despite maternal antibodies.[7] All of the foals in this study, except for #1 (table 1), were between 2 and 4 months of age at the time of blood collection. Therefore, the antibody titers found in the

foals (figs. 4b and 6) could be maternal as well as antibodies produced in response to an infection with EHV-2/5. The stage of the response can differ in between foals. In one foal there can be a new infection while in another there could be just maternal antibodies present at the time of sample collection. For example the foals #4, 5 and 8, with the high titers (figs. 5 and 9) might be recently infected. This could be part of the explanation why there is more distribution in the antibody response of the foals compared to the adults (figs. 4a,b). To see whether this spread could be in correlation with age, the 3 foals with the lowest (numbers 6, 7 and 9) and the 3 foals with the highest (numbers 4, 5 and 8) immunoglobulin response and their age (table 1) are compared in figs. 5 and 9. The other five foals showed more or less the same intermediate responses. It doesn't seem there is a correlation between difference in antibody titers and age. Two of the foals with the lowest response are the oldest foals in the group, but in the group of the highest foals is one foal of approximately 3,5 months old. Furthermore, in the group with the highest titers there is a foal of approx. 2 months old while the foal of approx. 1 month old is in the group with intermediate titers. The bigger distribution between the foals can also be due to a difference in antibodies in the colostrum or because of a difference in uptake of colostrum between the foals. However, to be able to say more about the presence of maternal antibodies in relation to the time of infection and the level of antibody titers, foals should be followed for a longer period of time, taking regular blood samples, where the first should be a pre-sucking sample.

4.1.2 Re-infection and re-activation

More variation in responses of the foals compared to responses of the adults could also be explained by the superinfection of the adult horses and/or the re-activation of the virus present in the adult horses. We know all the adults have been infected with EHV-2/5 at least once in their life since they all had an IgG response, and they are too old to have maternal antibodies. It is likely they have been infected more than once since it has been reported horses may be infected successively with different virus variants.[7] It has also been reported that the latent equine gamma herpes viruses are reactivated throughout the lifespan of the horse.[5] According to this the adult horses have probably reached more or less the same antibody level because of the regular challenge of the immune system by EHV-2/5.

4.2 IgG subclass response

Reports on EHV-4 and influenza infections show that it is very likely that IgGa antibody responses are transient, while IgGb antibody responses are sustained.[23,24] These studies of Wilson et al. and Mizukoshi et al. indicated that IgGa as well as IgGb antibodies are present at a high level recently after infection, but that IgGa antibodies decline much faster than IgGb antibodies. IgGa antibodies seemed to rapidly decrease after recovery from EHV-4 infection and only IgGb antibody seemed to be sustained in recovered horses.[24] My study is in agreement with this, since IgG subclass response to EHV-2/5 is mainly subtype IgGb and a little IgGa and IgG(T) (figs. 7 and 10). However, it is suggested that horses possessing IgGa antibody must be infected just before sample collection, since total-IgG and IgGb antibodies were detected in all horses which already had been infected with EHV-4, but IgGa antibody was not detected in any of those horses.[24] In contrast to influenza, EHV-2/5 is a latent virus. It has been shown that there is regular re-activation and also some superinfection of the EHV-2/5. The restimulation of the immune system by viral re-activation and especially by superinfection could be the reason for the IgGa titer.[5,7]

EHV-2/5 IgG(T) levels are a little higher than IgGa levels, but much lower than IgGb levels in all the horses, except for adult #10. This horse responded with an IgG(T) level which was equal to the IgGb (fig. 8). Unlike IgGa and IgGb, IgG(T) is known for its failure to fix complement and its ability to inhibit complement fixation by IgGa and IgGb, and it has been suggested that IgG(T) is likely to be best adapted to toxin neutralization.[17,24] Furthermore, IgG(T) appears to be highest IgG subtype found in allergic horses, as shown in an experiment on insect hypersensitivity (Svansson et al. *Unpublished*). Why adult #10 shows a high IgG(T) response to EHV-2/5 is unclear.

4.3 IgGc

When IgGc responses were not detected in any of the horses after the first IgG-subclass ELISAs, they were just only measured in the first serum dilution (1/100) for some of the adults and all the foals (table 2) to see if there was any need of determining the IgGc subclass on all the dilutions of some horses. IgGc titers could not be detected in any of those adult horses or foals. This doesn't prove there is no IgGc response, but since

we have not been able to show an IgGc response in Icelandic horses with this mAb, it might be not working properly enough.

4.4 IgM

In order to see whether the foals were newly infected or not, IgM responses were measured. Only two foals had a low response (table 3). Both those foals showed an intermediate response for the IgG (subclasses). We couldn't be sure with those low titers whether the detected responses were due to anti-IgM antibody reaction or due to some non-specific reaction. To test if the anti-IgM antibodies worked properly, it was decided to test them on serum of the HSA-immunized horses, because IgM is the antibody present the first, shortly after infection.[17] The serum used was collected before immunization, and 1 and 2 weeks after immunization. It was incubated on a plate coated with HSA-protein. Since the horses haven't naturally been in contact with HSA-protein, this should be the first contact to which they should respond with the production of IgM. Therefore we expected to see no titers in the sera of week 0, but much higher titers in the sera of week 1 and/or 2. However, the results showed that the titers for week 1 or 2 were not higher than for week 0. Since there was no horse clearly IgM positive it is possible that the IgM specific mAb does not work. We can therefore not conclude anything on IgM responses.

The EHV-2/5 specific IgG subclass response detected in this study is likely to be a typical Th1 pattern. Similar results have been shown for EHV-4 infection of horses.[24] In order to be able to compare a typical Th1 response to viruses with a (Th2) response in allergic horses, the cytokine profile against EHV-2/5 infection should also be measured. This work is already in progress.

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Appendix

I. Abbreviations

APC – antigen presenting cell
DMEM – Dulbeco's minimal essential medium
EHV – equine herpes virus
ELISA – enzyme-linked immunosorbent assay
FBS – fetal bovine serum
HSA – human serum albumin
IFN – interferon
Ig – immunoglobulin
IGHC – Ig H chain constant
IL – interleukin
IU – international units
mAb – monoclonal antibody
MHC – major histocompatibility complex
OPD – O-phenylenediamine dihydrochloride
PAMP – pathogen-associated molecular pattern
PBS-T – phosphate buffered saline with Tween20
sem – standard error of the mean
TGF – transforming growth factor
Th – T helper
TNF – tumor necrosis factor

II. Solutions and buffers

Carbonate-bicarbonate buffer:
　　　　1 liter distilled water
　　　　1,59 g Na_2CO_3
　　　　2,93 g $NaHCO_3$
　　　　pH 9,6

Lysis buffer:
　　　　0.01 M Tris
　　　　0.002 M EDTA
　　　　0.2 M Sucrose
　　　　2% (V/V) Triton-X100
　　　　200 KIU/ml aprotinin
　　　　pH 8,4

OPD solution:
　　　　4 OPD tablets
　　　　12 ml distilled water
　　　　5 μl H_2O_2

PBS-T dilution buffer:
　　　　5 PBS (Phosphate buffered saline) tablets, Sigma P-4417
　　　　1 liter distilled water
　　　　500 μl Tween 20 (0.05%), Sigma-Aldrich P-1379

PBS-T washing buffer:
　　　　400 ml 5xPBS, 1600 ml distilled water and 1.0 ml Tween 20 (sigma-aldrich P-1379)
　　　　5xPBS: - 250 g NaCl
　　　　　　　　- 6.25 g KH_2PO_4
　　　　　　　　- 89.5 g Na_2NPO_4
　　　　　　　　- 6.25 g KCl
　　　　　　　　- 5 liters of distilled water