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RESPONSES OF ANTIBODY CLASSES AND SUBCLASSES TO EQUINE HERPESVIRUS TYPE-1 (EHV-1) IN THE SERUM AND RESPIRATORY SECRETIONS OF MICE

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SUMMARY

A 30-50% mortality was observed in BALB/c mice inoculated with 5 x 10^6 p.f.u. EHV-1 intranasally. The absence of mortality in surviving mice following re-infection suggested the involvement of acquired protective immunity. This was indicated by a rapid decline of virus titre in the respiratory tissues which was correlated with a rapid increase of IgG antibody detected by means of ELISA. High IgG antibody titre was attributed to its subclass of IgG1. Generally, the titre of IgG1 in the serum was closely correlated to those in the upper and lower respiratory tracts. The antibody titre of IgG3 was very low at any time post-infection. The antibody titres of IgG2a and IgG2b were high but rapidly declined upon reaching a peak. IgA antibody titre detected was very low and only in the respiratory secretions. The titre of neutralising antibody detected was low both in the serum and the respiratory secretions. Based on these results, we suggested that IgG1 perhaps IgG2a and IgG2b are the most important antibody subclasses in rendering antibody-mediated protection against EHV-1 infection. In contrast, similar protection could not be mediated by neutralising antibody and IgA antibody.

Keywords: Equine herpesvirus type 1, class and subclass antibody responses, protection.

INTRODUCTION

Equine herpesvirus type-1 (EHV-1) is an important virus in horses. It causes various problems including respiratory problems, abortion and paralysis. The disease causes major economic losses worldwide. The virus was reported to replicate primarily in the respiratory mucosa, particularly in the ciliated epithelial cells of the bronchioles, in pneumocytes and also in the placenta and perhaps establish latency in trigeminal ganglia (Slater *et al.*, 1994). The mechanism of protection against EHV-1 infection, however, is not well defined. It has been reported that the antibody can mediate protection *in vitro* and *in vivo* (Burrows and Goodridge, 1978), and the antibody could be of serum or local origin. The protection rendered by local antibodies in the respiratory tract may affect the efficiency of the nasal mucosa as a first barrier to subsequent infection (Chen and Quinnan, 1989). Several different antibody classes and subclasses may be involved during antibody responses, and their distributions may reflect the type of desirable immune responses.

In this study, a mouse model was used to explore the characteristic of antibody response against EHV-1. This model has many advantages over previous established animal models. The mouse can be infected intranasally (i.n.) and the induced disease mimics that of in the natural host. The cell-associated viraemia produced last several days, and inoculation into pregnant mice causes premature parturition and birth of infected-offspring (Awan *et al.*, 1991). Based on these features, the model has been used to study acute infection (Awan *et al.*, 1990), abortion (Awan *et al.*, 1991), latency and re-activation (Field *et al.*, 1992), and immune responses (Azmi and Field, 1993) to EHV-1. The present communication presents an evidence of differences in antibody classes and subclasses in response to EHV-1 infection in mice. The findings may provide clues to the understanding of the mechanism of antibody-mediated protection in the natural host.

MATERIALS AND METHODS

Virus

The provenance of EHV-1 strain AB4 has been described previously (Awan *et al.*, 1990). The virus was grown in confluent rabbit kidney (RK-13) cell monolayers in Dulbecco minimal essential medium (DMEM) supplemented with 8% newborn calf serum. The virus was titrated by means of plaque-forming assay and used for animal inoculation and preparation of enzyme-linked immunosorbent assay (ELISA) antigen.

Inoculation of mice

Ten groups of 20 four-week-old specific-pathogen-free (SPF) BALB/c mice were used in the study. Mice were lightly anaesthetised with diethly ether and infected i.n. by instilling 40 µL virus inoculum containing 5 x 10^6 plaque forming unit (p.f.u.) EHV-1. A group of 20 control mice were inoculated with RK-13 cell lysate. Following inoculation, the mice were observed daily for development of clinical signs. All surviving mice were re-inoculated i.n. at 60 days following the primary inoculation.

Tissue samples

Following the primary and re-inoculations, four infected-mice were killed on days 3 and 5 post inoculation (p.i.), to determine virus titres in the lungs and nasal tissues. These tissues were minced and homogenised in DMEM without serum. The samples were assayed for the virus by means of plaque-forming assay.

Serum samples

Pre-immune serum was first collected from individual mice. Hyperimmune serum was prepared by hyperimmunising 10 mice with purified-EHV-1 with complete and incomplete Freunds adjuvant. Pre-immune and hyperimmune sera were used as negative and positive controls, respectively. Serum samples from infected mice were collected following the primary and re-inoculations, on days 3, 5, 8, 14 and every week p.i. Nasal wash and broncho-alveolar lavage were collected from killed animals by infusing 1 mL sterile PBS into each nasal turbinate and trachea. The samples were spun at 3,000 rpm for 5 min to remove cell debris.

Detection of neutralising antibody

Two-fold serially diluted heat-inactivated serum, nasal wash or broncho-alveolar lavage containing antibody were mixed with 100 p.f.u. EHV-1. The mixture was incubated at 37°C prior to addition onto RK-13 cell monolayers. Overlay medium was added and the cell was incubated for 2-3 days. The number of plaques formed were counted and the neutralising antibody titre determined by calculating the dilution of serum or samples containing antibody required for 50% reduction of plaques of the control wells (Bitsch, 1978).

ELISA antigen

EHV-1 strain AB4 was grown in Vero cells, and purified by potassium tartrate gradient (20-60%) centrifugation at 24,000 rpm for 2 h. The purified antigens were resuspended in PBS. The concentration of viral antigen was determined by means of standard BioRad protein assay and optimised for ELISA.

ELISA technique

Serum samples, nasal wash or broncho-alveolar lavage were assayed for ELISA antibody against EHV-1 antigen. An ELISA plate was coated with 0.5 µg antigen overnight prior to the addition of antibody sample and conjugate. Samples containing antibody were serially diluted two-fold, beginning with 1:100 dilution. Rabbit anti-mouse immunoglobulin, i.e. anti-whole IgG, IgG1, IgG2a, IgG2b or IgG3, IgM or IgA was added prior to addition of goat anti-rabbit peroxidase conjugate. Substrate 2,2'-azino-bis (3-ethylbenzthioline- 6-sulfonic acid) (ABTS) was reacted with the conjugate and results read at a wavelength of 492 nm. The dilution factors of the samples were plotted against its optical density. The antibody titre was read (as $\log_{10}$ end-point dilution) when the optical density of ELISA samples reached 0.133 (mean O.D. of uninfected samples + 3 s.d.).

RESULTS

Virus isolation

Mice infected with EHV-1 showed clinical signs of respiratory distress, ruffled hair coat and inappetence by 2 to 3 days p.i. Mortality of 30% to 50% occurred by 3 to 5 days p.i. Following the primary i.n. inoculation, the virus titres in the lung and nasal turbinate tissues were approximately 5 x 10^6 p.f.u. on days 1 and 3, and gradually decreased by days 5, 7 and 9 p.i. (Figure 1a). Mice that survived for one week p.i.

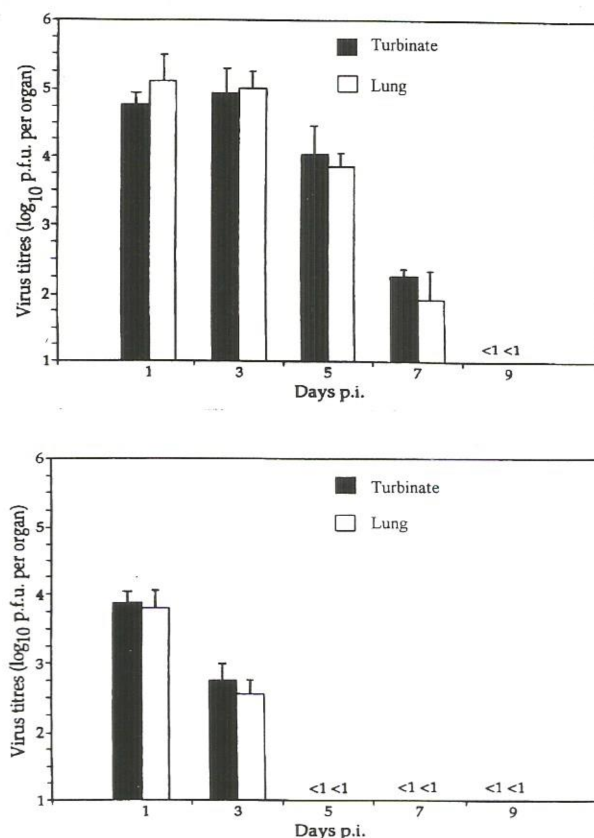

Figure 1. Virus titre (geometric mean; n=4) in the respiratory tissue; nasal turbinate and whole lung of mice following (a) primary inoculation and (b) re-inoculation with 5 x 10^6 p.f.u.

were a long term survival. When these mice were given a challenge inoculation at day 60 p.i., they were protected from developing a severe disease. None of these mice died compared to the control group which displayed 50% mortality (data not shown). In addition, there was rapid clearance of infectious virus from the respiratory tissues between 3 to 5 days p.i. (Figure 1b).

Neutralising antibody

Neutralising antibody was undetectable in all samples following the primary inoculation. Nevertheless, low neutralising antibody was noted following the challenge inoculation (Table 1). Generally, the highest neutralising antibody titre was detected in the nasal wash (1:8) and broncho-alveolar lavage (1:10) 14 days after the challenge inoculation, and the least in the serum (<1:8).

IgA antibody response

Following the primary inoculation, there was no IgA detected in either of the three samples, i.e. serum, nasal wash or broncho-alveolar lavage. However, starting from day 3 post challenge inoculation, a low IgA antibody titre (approximately 0.3 to 0.5 $\log_{10}$) was detected in nasal wash and broncho-alveolar lavage (Figure 2a). The serum IgA, however, remained below the level of sensitivity of the assay throughout the period of observation.

IgM antibody response

IgM antibody was detected following the primary or challenge inoculation. In all cases, IgM antibody titres reach peaks at 8 days after virus inoculation (Figure 2b) The IgM antibody titre in broncho-alveolar lavage was always lower than in the serum and nasal wash, however, the difference among the three samples was not significant ($p>0.05$). The pattern of IgM antibody response as detected in all three sample types following the challenge inoculation resembled to that of primary inoculation.

IgG antibody response

Following the primary inoculation, the level of IgG in nasal wash and broncho-alveolar lavage increased to a peak at day 21 p.i. with titres of 1.7 to 2 $\log_{10}$ which were higher than those detected in the serum, i.e. 1.2 $\log_{10}$ (Figure 2c). Beyond day 21 p.i., IgG antibody declined, however the titre in the serum remained lower than in the nasal wash and broncho-alveolar lavage. A sudden boost of IgG antibody levels was observed in all samples following the challenge inoculation. The rise was significantly greater ($p<0.05$) in nasal washing and broncho-alveolar lavage than in the serum. The rise in IgG antibody titre was rapid with a peak titre of 5 $\log_{10}$ at day 5 post challenge inoculation for both nasal wash and broncho-alveolar lavage, and 3.5 $\log_{10}$ at day 14 post challenge inoculation for the serum.

Table 1. Neutralising antibody titre in the serum and respiratory secretions.

Virus inoculation (5×10^6 p.f.u. EHV-1)	Days p.i.	Sample containing antibody		
		Serum	Nasal wash	Broncho-alveolar lavage
Primary inoculation	0	<1:2*	<1:2	<1:2
	5	<1:2	<1:2	<1:2
	14	<1:2	<1:2	<1:2
	21	<1:2	<1:2	1:2
	28	<1:2	<1:2	<1:2
Challenge inoculation	0	<1:2	<1:2	<1:2
	5	1:2	1:4	1:4
	14	1:4	1:8	1:10
	21	1:8	1:8	1:8
	28	1:4	1:8	1:8

* All values are expressed as dilution of antibody to reduced 50% of plaque formation
Pooled sera were collected from five individual mice.

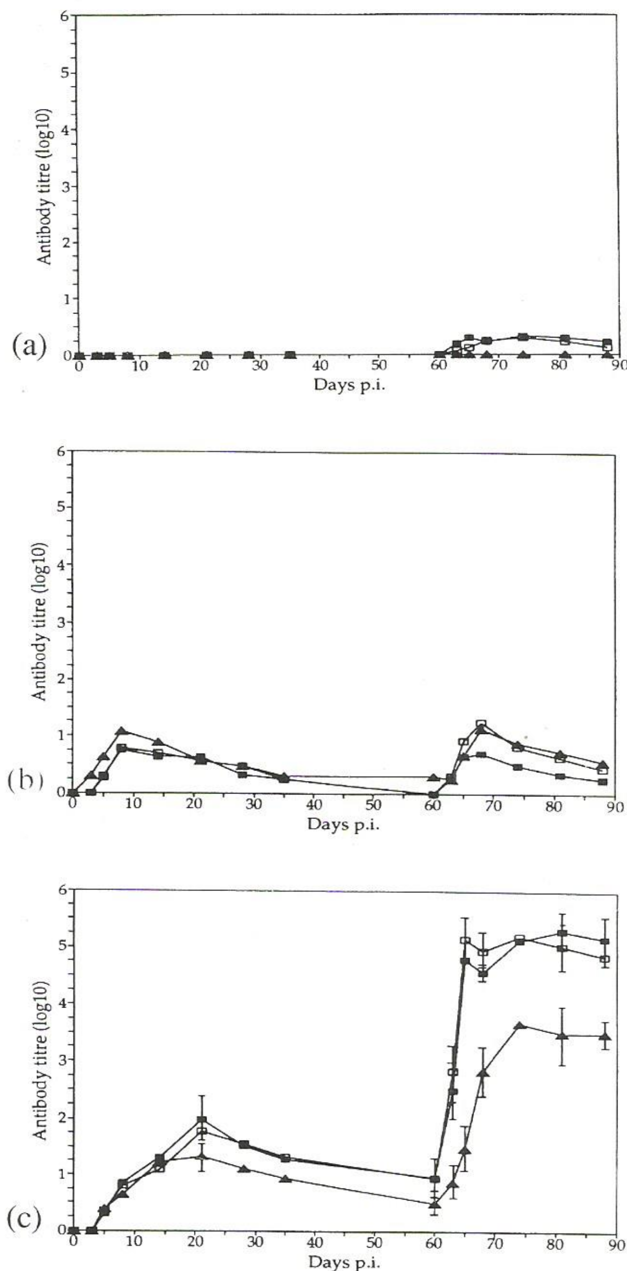

Figure 2. Antibody titres (geometric mean; n=4) for (a) IgA, (b) IgM and (c) whole IgG in the serum (▲), nasal wash (❑) and broncho-alveolar lavage (■) following primary and re-inoculation with 5 x 10^6 p.f.u. EHV-1. Some s.d. bars were omitted for clarity.

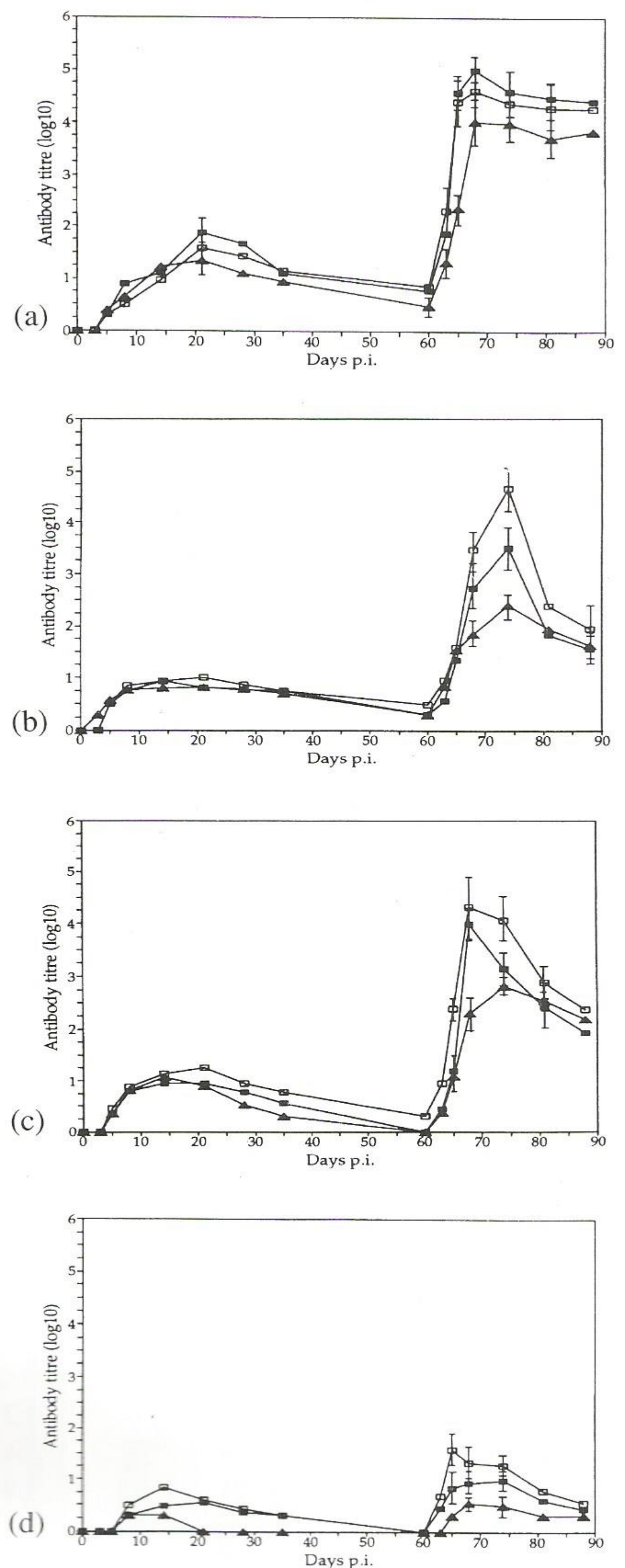

Figure 3. IgG antibody titres (geometric mean; n=4) for subclasses (a) IgG1, (b) IgG2a, (c) IgG2b and (d) IgG3 in the serum (▲), nasal wash (❑) and broncho-alveolar lavage (■) following primary and re-inoculation with 5 x 10^6 p.f.u. EHV-1. Some s.d. bars were omitted for clarity.

IgG subclass antibody response

All subclasses of IgG, i.e. IgG1, IgG2a, IgG2b and IgG3 were detected in the serum, nasal wash and broncho-alveolar lavage. The level of IgG1 closely corresponded to the level of whole IgG antibody (Figure 3a).

The level IgG2a and IgG2b antibodies always remained less than 1 $\log_{10}$ at all time following the primary inoculation (Figures 3b and 3c). No significant difference was noted between the levels of IgG2a and IgG2b. IgG2b was not detected in the serum and

brochial-alveolar lavage by day 60 p.i. However, a rapid increase in IgG2a and Ig2b was noted following the challenge inoculation and with the highest titre in nasal wash (4 to 4.5 $\log_{10}$), broncho-alveolar lavage (3.4 to 3.9 $\log_{10}$) and the least in the serum (2.2 to 2.7 $\log_{10}$). Following the challenge inoculation, IgG2a antibody increased to a peak after 14 days (day 74 post primary inoculation) with a titre of 2.2 $\log_{10}$ in the serum, 3.4 $\log_{10}$ in broncho-alveolar lavage and 4.6 $\log_{10}$ in nasal wash. The IgG2b antibody reached a peak after 8 days (day 68 p.i.) with a titre of 3.8 to 4 $\log_{10}$ both in the nasal wash and broncho-alveolar lavage, and after 14 days (day 74 p.i.) in the serum (titre of 2.8 $\log_{10}$). Surprisingly, IgG2a and IgG2b antibody titres rapidly declined to approximately 2 $\log_{10}$ by after day 28 following the challenge inoculation (day 88 p.i.).

In general, among all IgG antibody subclasses, IgG3 antibody detected was the least (Figure 3d). Following the primary inoculation, IgG3 was detected only by 8 p.i. The peak IgG3 antibody titre for nasal wash and broncho-alveolar lavage was approximately 0.8 $\log_{10}$ and occurred by day 14 post primary inoculation. The lowest antibody titre detected was in the serum of which gradually declined toward an undetectable level by 60 days. A higher IgG3 antibody level was detected following the challenge inoculation with titres reaching maximum values of 1.4 $\log_{10}$ in nasal wash after 5 days, 1 $\log_{10}$ in broncho-alveolar lavage after 14 days, and the least, 0.5 $\log_{10}$ in the serum after 8 days.

DISCUSSION

The ELISA technique employed in this study had successfully detected antibody responses against EHV-1 in mice infected i.n. The classes of antibody detected are of IgG (whole), IgM and IgA classes; IgG subclasses, i.e. IgG1, IgG2a, IgG2b and IgG3. All mice that survived from EHV-1 infection had exhibited these antibodies in the serum, nasal and broncho-alveolar lavage but at different titres over the period of infection.

Generally, IgM antibody titre in the serum, nasal wash and broncho-alveolar lavage following primary inoculation resembled those observed following challenge inoculation. IgM to IgG class switching was observed following primary inoculation of which whole IgG was detected at 1-2 weeks after the IgM level had reached a peak at 1 week p.i. Despite poor IgG antibody response in primary infection, an amnestic response of IgG was exhibited with a rapid boost to high antibody titre within 1 week following challenge inoculation. A similar pattern of antibody response was noted in the nasal wash and broncho-alveolar lavage. Thus, it is suggested that a strong IgG antibody response is responsible for complete protection against re-infection. The protective role of this antibody has been shown in another study in which passive immunisation with the monoclonal antibody protected hamsters from EHV-1 infection (Stokes *et al.*, 1989).

When IgG antibody subclasses was examined, it was noted that IgG1 is a major antibody contributing to the IgG antibody titre with IgG3 the poorest contributor. IgG2a and IgG2b antibodies were elevated to high titres, however, the titre of these antibodies appeared to decline more rapidly than the other IgG subclasses after reaching a peak. This observation may suggest that IgG1 antibody played a protective role against EHV-1 infection.

Based on previous observations on other virus infections (Ogra *et al.*, 1971; Murphy *et al.*, 1982; York *et al.*, 1989), it has been suggested that IgA may mediate protection in the respiratory tracts. In this study, however, IgA antibody titre detected was low in the respiratory secretions and only detected following challenge inoculation. At no time was IgA detected in the serum following primary or challenge inoculation. In the nasal secretion, IgA was detected only after challenge inoculation. IgG was the major antibody detected in the respiratory secretions. The presence of IgA antibody may not be significant, however, together with IgG1, IgG2a and IgG2b and some neutralising antibodies may contribute protective local immune responses against re-infection in the respiratory tract. This was evidenced by 100% survival and rapid decline of infectious EHV-1 in the tissues of the upper and the lower parts of the respiratory tract. However, neither neutralising antibody nor IgA alone could provide complete protection. This evidence may support the finding in horses where viraemia still occurred in the presence of neutralising antibody (Bryans, 1969).

This study has provided a useful evidence of antibody-mediated immune response against EHV-1 which may facilitate a better understanding of the immunity to EHV-1 infection in horses.

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RINGKASAN

GERAK BALAS KELAS DAN SUBKELAS ANTIBODI TERHADAP HERPES VIRUS EKUIN TIP-1 (EHV-1) DALAM SERUM DAN REMBESAN PERNAFASAN MENCIT

Kematian sekadar 30-50% telah dicerapkan dalam mencit BALB/c yang diinokulat secara intranasum dengan $5x10^6$ p.f.u. EHV-1. Tiadanya kematian dalam mencit berikutan jangkitan semula menyaran penglibatan keimunan pelindung teroleh. Ini telah ditunjuk oleh penurunan cepat dalam titer virus dalam tisu pernafasan yang terkorelasi dengan peningkatan cepat antibodi IgG dikesan melalui ELISA. Titer antibodi IgG tinggi disabitkan dengan subkelasnya, IgG1. Umumnya, titer IgG1 terkorelasi rapat dengan apa yang didapati dalam trakus pernafasan atas dan bawah. Titer antibodi IgG3 sentiasa sangat rendah pada tempoh pascajangkitan. Titer antibodi IgG2a dan IgG2b tinggi tetapi cepat turun apabila mencapai kemuncak. Titer antibodi IgA dikesan paling rendah dan hanya dalam rembesan pernafasan. Titer antibodi peneutralan yang dikesan adalah rendah kedua-duanya dalam serum dan rembesan pernafasan. Berasaskan penemuan ini, kami menyarankan IgG1 dan mungkin juga IgG2a dan IgG2b merupakan subkelas antibodi paling penting dalam memberi perlindungan berantarakan antibodi terhadap jangkitan EHV-1. Disebaliknya, perlindungan serupa tidak dapat diantarakan oleh antibodi peneutralan dan antibodi IgA.