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# PROTECTIVE IMMUNITY AGAINST EQUINE HERPESVIRUS TYPE-1 INFECTION FOLLOWING IMMUNISATION OF MICE WITH A NON-PATHOGENIC EQUINE HERPESVIRUS TYPE-4

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## SUMMARY

Protection against equine herpesvirus type-1 (EHV-1) infection was studied in mice following immunisation with its related heterologous virus EHV-4. The EHV was found to be non-pathogenic in mice. Inoculation of mice with either live or heat-inactivated EHV-4 stimulated IgG antibody response to both EHV-4 and EHV-1 antigens. Similar cross-reactive phenomenon was observed in mice inoculated with EHV-1. A significant level of virus-specific antibody was detected when the hyperimmune sera were tested by the virus neutralisation assay but not by ELISA. Antibody production following inoculation of mice either with live or heat-inactivated EHV were generally at similar levels. However, when mice were challenged with EHV-1, protection was only observed in mice immunised with live EHV-4. It is thus suggested that live EHV-4 can induce protective immunity against severe disease of EHV-1 and could possibly be used as an immunising agent to stimulate protective immunity against heterologous virus, EHV-1.

Keywords: EHV-1, EHV-4, cross-reaction, IgG antibody, immunity

## INTRODUCTION

Equine herpesvirus type-1 (EHV-1) and type-4 (EHV-4) are known to cause respiratory problems in horses world-wide. Beside respiratory problems, EHV-1 infection is also characterised by paralysis, abortion and delivery of weak foals. In contrast, EHV-4 causes problems, which are limited to the respiratory tract only and not directly associated with abortion and paralysis (Allen and Bryan, 1986). It has been reported that the two viruses are distinct but serologically related. Their glycoproteins gB and gD are antigenically related (Crabb and Studdert, 1990; Okazaki *et al.*, 1990). Such homology leads to production of cross-reactive antibodies.

Little is known about the correlation and degree of cross-reactions in relation to protection against both infections. In theory, immunisation of horses with one of these viruses should induce protection against another. It has been demonstrated that horses exposed to EHV-4 were protected against subsequent EHV-1 infection (Tewari *et al.*, 1993). Other studies, however, revealed a one-way protection against EHV-4 infection following immunisation with inactivated EHV-1 (Crandell *et al.*, 1980; Burrows *et al.*, 1984; Edington and Bridges, 1990). There was no study employing live EHV-4 as an immunising agent in horses.

One of the major setbacks of these trials was the use of animals of unknown immune status, which complicated the interpretation of results. Most horses in the field are infected by at least one of these viruses and remain latent life-long (Allen and Bryan, 1986). Therefore, there is a need to use animals of known immune status in such trials to explain the relevant mechanism of protection and immunity. Thus, the use of a mouse model is considered acceptable.

This study describes the serological cross-reaction between EHV-1 and EHV-4 and the protection against EHV-1 infection following immunisation of mice with EHV-4.

## MATERIALS AND METHODS

*Virus*

Equine herpesvirus-1 (EHV-1) strain AB4 was grown either in confluent rabbit kidney (RK-13) or Vero cells containing Earle's minimum essential medium (EMEM) supplemented with 8% new-born calf serum. Similarly, the equine herpesvirus-4 (EHV-4) strain 1778 was grown in equine embryonic lung (EEL) cells.

*Virus inoculation*

Four-week-old BALB/c mice were used in this study. Prior to virus inoculation, the mice were lightly anaesthetised with diethyl-ether before 40 µL of the virus inoculum containing $5x10^6$ plaque forming unit (pfu) of either EHV-1 or EHV-4 were administered intranasally (in) into each mouse. The control mice were similaly administered with RK-13 cell lysate. Following virus inoculation, the mice were observed daily for development of clinical signs including dyspnoea, tachypnoea, incoordination, abdominal breathing, ruffled hair and death.

*Plaque forming assay*

This assay was used to determine the virus titres in infected cell cultures and respiratory tissues of mice inoculated with the virus. Lung and nasal turbinate samples were minced with a scissor and homogenised using an electric blender in 1 mL EMEM. Tissue homogenates were further disintegrated in a sonic water-bath before they were centrifuged at 3,000 rpm for 10 min. Ten-fold dilutions of the supernatants were prepared in EMEM before the samples were inoculated onto confluent RK-13 cell monolayers. The virus was allowed to adsorb for 45 min at 37°C. An overlay medium (EMEM) containing 1% carboxymethyl cellulose and 2% foetal calf serum was added and the cultures were incubated further at 37°C. The presence of cytopathic effects was examined after 2 or 3 days of incubation and plaques were stained with crystal violet solution prior to counting.

*Hyperimmune sera*

The EHV-1 and EHV-4 antigens were prepared and emulsified in complete Freund's adjuvant. The pre-immune serum was collected from each mouse before the hyperimmune serum was prepared by injecting two groups of 10 mice/group subcutaneously with the respective antigen. Subsequent booster injections were administered subcutaneously at days 14 and 21 using the same antigens that were emulsified in incomplete Freund's adjuvant. The pre-immune and hyperimmune sera were used in the virus neutralisation assay and ELISA as negative and positive controls respectively.

*Neutralising antibody assay*

Two-fold serial dilutions of heat-inactivated sera were mixed with 100 pfu of either EHV-1 or EHV-4. The mixtures were incubated at 37°C prior to the inoculation onto RK-13 cell monolayers for EHV-1 and onto the EEL cells for EHV-4. An overlay medium was added and the cell monolayers were incubated for 2-3 days before the number of plaques formed were determined. The neutralising antibody titres were determined based on the dilution of serum required to produce 50% reduction of plaques as compared to control (Bitsch, 1978).

*Enzyme-linked immunosorbent assay (ELISA)*

An indirect ELISA was developed based on the protocols of Voller *et al.* (1980), which has been described previously (Azmi, 1995). The virus antigens were purified by ultra-centrifugation at 24,000 rpm for 2 hr in 20-60% potassium tartrate gradient. The concentrations of viral antigens were determined using the standard BioRad protein assay kit before 0.5 ug of viral antigen was coated onto 96-well plates overnight at 4°C. The plates were then washed thrice with PBS-Tween20 before 2% BSA was added and incubated further at 37°C for 2 hrs. The plates were then washed thrice with PBS-Tween20 before the two-fold dilutions of serum samples were added and the plates were incubated again at 37°C for 2 hrs. After washing thrice with PBS-Tween20, the goat-anti mouse IgG peroxidase-labeled conjugate was added and incubated at 37°C for 2 hrs. The plates were then washed thrice in distilled water and allowed to react with the substrate, 2,2'-azino-bis (3-ethylbenzthioline-6-sulfonic acid), for 30 min at room temperature. Results were read at wavelength 492 nm before the end point titres were determined.

*Experimental design*

*Experiment 1*

A preliminary experiment was conducted to demonstrate the pathogenicity of EHV-1 and EHV-4 in mice. Two groups of four-week-old BALB/e mice, consisting of 20 mice per group were inoculated intranasally (in) with $5x10^6$ pfu of EHV-1 and $10^7$ pfu of EHV-4 respectively. The mice were observed daily for clinical signs of the disease. Four mice from each group were killed at days 3 and 5 post-inoculation. Nasal turbinate, lungs, liver, spleen and blood were obtained and assayed for virus by the plaque-forming assay. Blood samples were collected in microfuge tubes containing EDTA before the buffy coat layers were separated upon centrifugation at 12,000 rpm for 5 min. The buffy coat was treated with sterile distilled water for 10-15 seconds and the osmotic balance was reconstituted in EMEM. Cells were counted and cultivated in EEL cells to determine the presence of the virus.

*Experiment 2*

Two groups of 25 mice per group were inoculated intranasally with $10^6$ pfu of either live or equivalent amount of heat-inactivated EHV-1. A group of 25 control mice was inoculated with RK-13 cell lysate. All mice were challenged intranasally with $5x10^6$ pfu of live EHV-1 at day 28 p.i. before four mice from each group were killed at days 3 and 5 post-challenge. The nasal turbinate and lungs were collected and subjected to the plaque-forming assay.

A group of 20 mice were inoculated intranasally with $10^6$ pfu of live EHV-1. Serum samples were collected from each mouse at days 0, 3, 5, 8, 14, 21, 28, 31, 36, 42 and 49 post-inoculation. On day 49 p.i., all mice were inoculated intranasally with $5x10^6$ pfu of live EHV-1. Serum samples were collected at days 3, 5, 8, 14 and 21 post-challenge. The IgG antibody titres to EHV-1 and EHV-4 were determined by ELISA.

*Experiment 3*

Two groups of 20 mice per group were primarily inoculated intranasally with $10^6$ pfu of live and equivalent amount of heat-inactivated EHV-4

respectively. All mice were challenged intranasally with $5x10^6$ pfu of live EHV-1 at day 28 post-inoculation. Tissue samples for virus assay were collected as described in the Experiment 2.

Another two groups of 20 mice were inoculated intranasally with $5x10^6$ pfu of live and equivalent amount of heat-inactivated EHV-4 respectively. Serum samples were collected at the intervals as described in Experiment 2. The mice were similarly challenged with $5x10^6$ pfu of live EHV-1 before sampling was carried out as described in Experiment 2.

Another group of 20 mice was inoculated intranasally with $5x10^6$ pfu of live EHV-1. Serum samples were collected at the intervals described earlier. The mice were then challenged with $5x10^6$ pfu of live EHV-4 at day 28 p.i.

*Experiment 4*

This experiment was conducted to determine the cross-reactivity of sera at various antibody titres by means of ELISA technique. One hundred and ninety-two serum samples were randomly selected from a collection of approximately 500 serum samples. The samples were obtained from mice that were immunised with either EHV-1 or EHV-4. The sera were obtained from sero-positive animals to either EHV-1 or EHV-4. The antibody titres ranged from very low following a single inoculation, moderate following secondary inoculation and high following multiple inoculations. The antibody titres to EHV-1 and EHV-4 antigens were determined by ELISA and results were plotted to demonstrate their cross-reactivity at different antibody levels.

## RESULTS

*Immunisation and protection against EHV-1 infection*

Following intranasal infection with EHV-1, the mice showed clinical signs, which included ruffled hair, respiratory distress, weight loss and occasional death. The virus was recovered from the nasal turbinate and lungs (Table 1). The infection was more severe when mice were inoculated at high dose of $10^7$ pfu per mouse with cumulative mortality approaching 100% by day 7 post-inoculation. The virus was also detected, but at lower levels in liver, spleen and blood. In contrast, inoculation of mice with EHV-4 failed to produce clinical signs and the virus was not detected in any of the target organs (Table 1).

In Experiment 2, the mice that were previously inoculated with live EHV-1 did not exhibit any sign of illness upon challenge with live EHV-1. The virus titres in nasal turbinate and lungs were reduced from maximum 2.91 $\log_{10}$ at day 3 post-inoculation to undetected level at day 5 post-inoculation (Table 2). The mice that were previously inoculated with heat-inactivated EHV-1 exhibited signs of illness while the EHV-1 titres detected in the nasal turbinate and lungs were significantly ($p<0.05$) similar to the control groups.

Similarly, the mice in Experiment 3 that were previously inoculated with live EHV-4 and challenged with live EHV-1 did not develop any sign of iillness (Table 2). However, clinical signs and virus replications were observed in mice inoculated with the heat-inactivated EHV-4 and challenged with live EHV-1. Virus titres in the nasal turbinate and lungs were significantly ($p<0.05$) similar to those of the control group.

*Antibody responses to EHV-1 and EHV-4 antigens*

Following primary inoculation of mice with EHV-1, the IgG titres against EHV-1 antigen remained low ($<1$ $\log_{10}$) but significantly ($p<0.05$) increased following second exposure to the homologous virus (Fig. 1). The same mice showed lower IgG titres to EHV-4 than to the EHV-1 antigen. A slight rise in the IgG antibody titres (approximately 1 $\log_{10}$) against EHV-4 antigens was observed following the second exposure to EHV-1.

**Table 1. Pathogenicity of EHV-1 and EHV-4 in mice**

| Virus inoculation | Cumulative mortality | | | Virus titre in turbinate ($\log_{10}$ p.f.u.) | | Virus titre in lungs ($\log_{10}$ p.f.u.) | |
|---|---|---|---|---|---|---|---|
| | day 3 | day 5 | day 7 | day 3 | day 5 | day 3 | day 5 |
| EHV-1 | 3/20 | 8/20 | 11/20 | 4.65±0.51 | 4.22±0.25 | 5.15±0.47 | 4.92±0.38 |
| EHV-4 | 0/20 | 0/20 | 0/20 | - | - | - | - |

**Table 2. Protection of mice against EHV-1 following an immunisation with EHV-1 or EHV-4 antigen**

| Immunising antigen | Cumulative mortality at day 7 | Virus titre in turbinate ($log_{10}$ p.f.u.) day 3 | day 5 | Virus titre in lung ($log_{10}$ p.f.u.) day 3 | day 5 |
|---|---|---|---|---|---|
| EHV-1$_L$ | 0/25 | 3.02±0.37 | <1 | 2.98±0.46 | <1 |
| EHV-1$_K$ | 13/25 | 4.85±0.41 | 3.25±0.47 | 5.18±0.26 | 3.62±0.35 |
| None | 13/25 | 5.12±0.55 | 3.46±0.31 | 4.90±0.41 | 2.85±0.53 |
| EHV-4$_L$ | 0/25 | 2.91±0.21 | <1 | 2.43±0.22 | <1 |
| EHV-4$_K$ | 15/25 | 5.15±0.57 | 4.35±0.44 | 4.81±0.36 | 3.56±0.34 |
| None | 10/25 | 5.06±0.48 | 4.16±0.22 | 5.11±0.26 | 3.61±0.51 |

$_L$ = live virus; $_K$ = inactivated virus

Cross-reactions between antibodies against EHV-1 and EHV-4 antigens were demonstrated in the sera of mice inoculated with EHV-4. Following exposure to live EHV-4, the antibody levels to EHV-4 started to increase by day 8. Similarly, serum IgG antibody to the heterologous virus EHV-1 was detected, but was generally lower than to the homologous virus (Figs. 2 and 3). A gradual increase in the IgG antibody to EHV-1 was noted following second exposure to EHV-1. In general, antibody titres at all points in time between the two groups of mice were not significantly ($p>0.01$) different.

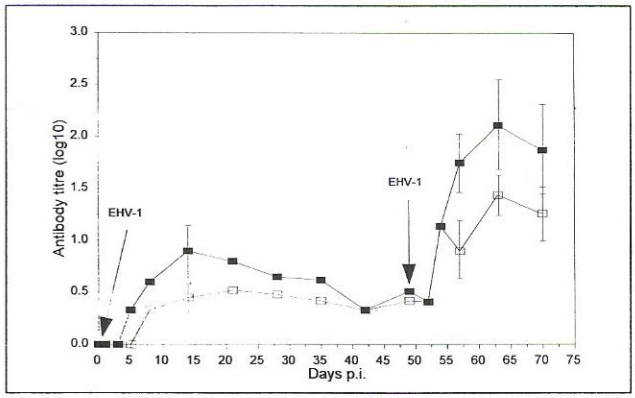


**Fig 1. EHV-1 and EHV-4 serum IgG antibody titres in mice primarily inoculated and re-inoculated with EHV-1. Mice were primarily inoculated intranasally with $10^6$ pfu of live and re-inoculated seven weeks later with the same dose of live EHV-1. Data points represent the mean of antibody titres (geometric mean±s.d.; n=5) to EHV-1 (▪) and EHV-4 (□) antigens.**

Mice that were primarily inoculated with EHV-1 showed poor IgG antibody responses to both EHV-1 and EHV-4 antigens (Fig. 4). However, there was a greater increase in the IgG antibody level to EHV-4 antigens following second inoculation with live EHV-4 compared to the antibody levels to EHV-1.

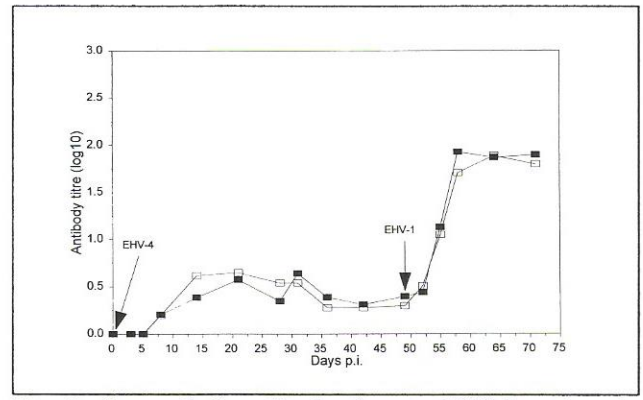


**Fig. 2. Serum IgG antibody titre to EHV-1 antigen in mice inoculated with EHV-4 and challenged with EHV-1. Mice were primarily inoculated intranasally with $5x10^6$ pfu of live (▪) or with a similar amount of heat-inactivated (□) EHV-4. Seven weeks later, all mice were re-inoculated with $5 \times 10^6$ pfu of live EHV-1. Data points represent the mean of antibody titres (geometric mean; n=5) to EHV-1 antigen.**

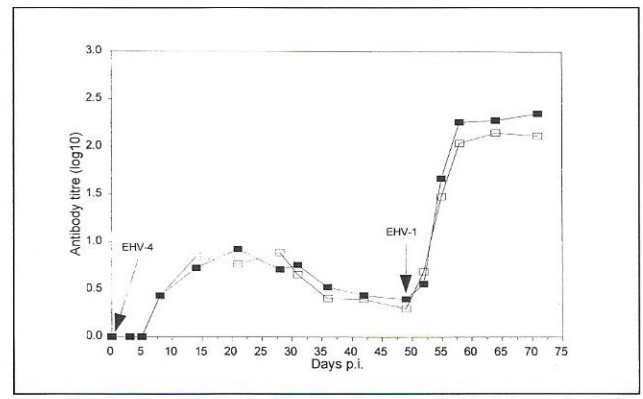


**Fig. 3. Serum IgG antibody titre to EHV-4 antigen in mice inoculated with EHV-4 and challenged with EHV-1. Mice were primarily inoculated intranasally with $5 \times 10^6$ pfu of live (▪) or with a similar amount of heat inactivated (□) EHV-4. Seven weeks later, all mice were re-inoculated with $5 \times 10^6$ pfu of live EHV-1. Data points represent the mean of antibody titres (geometric mean; n=5) to EHV-4 antigen.**

*Antibody cross-reaction and specificity*

The cross-reaction and specificity of heterologous and homologous antibody were analysed based on the data obtained by ELISA and virus neutralisation test. Antibodies to EHV-1 and EHV-4 antigens were found to be highly cross-reactive. The virus-specific antibody was not detected when the antibody titres to the homologous antigens were below 1 $\log_{10}$ (Fig. 5). However, some degrees of specificity to the homologous antigens were observed when antibody titres to the homologous antigen were increased to 5 $\log_{10}$. When both virus neutralisation and ELISA were used to test the hyperimmune sera, a two-way antibody cross-reaction was observed (Table 3). The degree of cross-reaction detected by ELISA was considerably high.

**Table 3. Serologic cross-reaction between EHV-1 and EHV-4 following ELISA and virus neutralising antibody assay**

| | neutralising ab titre | | ELISA IgG titre | |
|---|---|---|---|---|
| Viral ag | EHV-1 | EHV-4 | EHV-1 | EHV-4 |
| EHV-1 | 1:80[a] | 1:16 | 1:100000[b] | 1:50000 |
| EHV-4 | 1:20 | 1:60 | 1:80000 | 1:100000 |

[a]maximum serum dilution to neutralise 50% of plaque formed by EHV-1

[b]end-point dilution of sera for determination of ELISA antibody titre

Generally, the neutralising antibody titres against heterologous virus were lower. This indicated the presence of a one way antibody cross-reaction to EHV-1 and EHV-4 antigens.

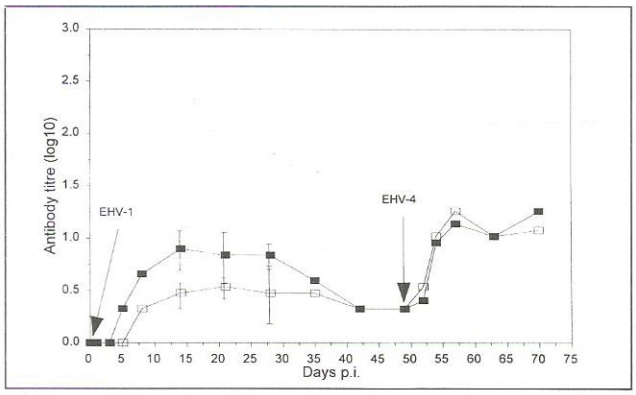


**Fig. 4. EHV-1-specific and EHV-4-specific serum IgG antibody response in mice primarily inoculated with live EHV-1 and re-inoculated with EHV-4. Mice were primarily inoculated intranasally with 5 x $10^6$ pfu of live and re-inoculated seven weeks later with 5 x $10^6$ pfu of live EHV-4. Data points represent antibody titres (geometric mean±s.d.; n=5) to EHV-1 (▪) and EHV-4 (□) antigens.**

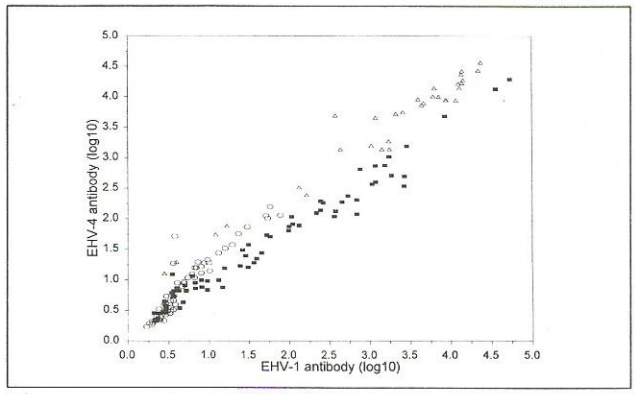


**Fig. 5. Determination of cross-reactive IgG antibodies to EHV-1 and EHV-4 antigens and their correlation with heterologous antigens. Serum samples of different antibody titres were obtained from mice inoculated with EHV-1 (▪) only, EHV-4 (□) only, and EHV-4 as primary inoculation followed by EHV-1 as second inoculation (⌓).**

## DISCUSSION

The pathogenesis of EHV-1 infection in mice following intranasal inoculation with doses ranging from $10^6$ to $10^7$ pfu has been described previously (Awan *et al.*, 1990; Inazu *et al.*, 1993; Field *et al.*, 1994). This study has demonstrated that although EHV-4 is serologically related to EHV-1, the former is non-pathogenic and not replicating in mice. In contrast EHV-1 caused a disease which was characterised by clinical signs mimic those in horses (Awan *et al.*, 1990). The results of this study support previous evidences that EHV-4 can only be grown in cells of equine origin. The EHV-1 and EHV-4 belong to the same subfamily *alphaherpesvirinae* of herpesviruses but replication of EHV-1 is not restricted to equine cells only (Allen and Bryan, 1986).

Interestingly, intranasal inoculation of mice with live EHV-4 at the same dose as the infective dose of EHV-1 conferred protection against subsequent challenge with live EHV-1. In contrast, no protection was conferred when the heat-inactivated EHV-4 was used to immunise the mice. This is similar to the previous findings when mice were inoculated with heat-inactivated EHV-1 (Azmi, 1995). The mechanism of protection following a single dose of immunisation with live EHV-4 is not yet fully understood. It was believed that defective replication of EHV-4 might have occurred in the mouse respiratory tissues and was strong enough to stimulate protective immune responses against EHV-1 (Azmi and Field, 1993). No protection was conferred following immunisation of mice with heat-inactivated EHV-1 indicated that the process of inactivation has destroyed protective immunogenic property of the live virus.

Cross-reactions between EHV-1 and EHV-4 have been shown by using both ELISA and virus neutralising assay. This is consistent with the previous findings that suggested that many highly homologous glycoproteins of both types of viruses are immunodominant (Nicholson *et al.*, 1990; Riggio and Onions, 1993). The important glycoproteins of EHV-1 and EHV-4 are the gB, gC and gD (Turtinen and Allen, 1982; Crabb and Studdert, 1990; Crabb *et al.*, 1991; Ahmed *et al.*, 1993). The cross-reactive phenomenon has also been shown in ELISA for IgG antibody both at high and low antibody titres to EHV-1 and EHV-4. In contrast, the virus neutralisation assay revealed that hyperimmune sera prepared against one virus contained significantly high neutralising antibody titres to its homologous than to the heterologous antigens. This is due to the fact that neutralising antibody is more virus-specific than the antibody demonstrated by ELISA. Interestingly, the patterns of antibody response observed in relation with cross-protection were found to be similar to a different study using specific pathogen-free foals (Tewari *et al.*, 1993).

The ELISA IgG antibody titre was found not to directly correlate with protection against virus infection. Despite significantly high antibody titres produced in mice following immunisation with heat-inactivated EHV-4, there was no cross-protection against subsequent challenge with EHV-1. Furthermore, low serum neutralising antibody titres were unlikely to mediate a significant protection to EHV-1 infection. The live virus in infected cells may replicate at low level and effectively stimulate the cell-mediated immune responses. Thus, protective cell-mediated immunity can only be conferred by immunising mice with live, not inactivated EHV-4. This study has demonstrated that antibody did not seem to be the most important factor for protective immune mechanisms against EHV-1 infection. However, antibodies may play an important role in other situations especially in the neutralisation of extracellular viruses (McKendall *et al.*, 1979; Morse and Morahan, 1981; Horimoto *et al.*, 1989) and in the antibody-mediated killing of virus-infected cells (Horimoto *et al.*, 1989; Stokes *et al.*, 1991; Chong *et al.*, 1992).

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## RINGKASAN

*KEIMUNAN PERLINDUNG TERHADAP JANGKITAN HERPESVIRUS TIP-1 EKUIN BERIKUTAN PENGIMUNAN MENCIT DENGAN SATU HERPESVIRUS TIP-4 EKUIN BUKAN PATOGEN*

*Perlindungan terhadap jangkitan herpesvirus tip-1 ekuin (EHV-1) telah dikaji dalam mencit berikutan pengimunan dengan virus EHV-4 heterologus kaitan. Herpesvirus ekuin ini di dapati bukan patogen dalam mencit. Penginokulatan mencit sama ada dengan EHV-4 hidup atau teraktif haba merangsang gerakbalas antibodi IgG terhadap kedua-dua antigen EHV-4 dan EHV-1. Fenomena tindakbalas silang dicerap dalam mencit terinokulat EHV-1. Suatu aras antibodi khusus virus tererti dikesan apabila serum hiperimun diuji dengan asai peneutralan virus tetapi bukan dengan ELISA. Penghasilan antibodi berikutan penginokulatan mencit sama ada dengan EHV hidup atau teraktif umumnya pada aras yang sama. Bagaimanapun, apabila mencit dicabar dengan EHV-1, perlindungan dicerap dalam mencit terimun dengan EHV-4 hidup. Dengan demikian, adalah disarankan yang EHV-4 hidup boleh mengarauh keimunan pelindung terhadap penyakit EHV-1 teruk, dan mungkin boleh diguna sebagai agen pengimunan untuk merangsang keimunan pelindung terhadap virus EHV-1 heterologus.*