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IMMUNISATION AGAINST EQUINE HERPESVIRUS TYPE-1 (EHV-1) INFECTION IN MICE: THE EFFECT OF DIFFERENT ANTIGEN PREPARATIONS AND INOCULATION ROUTES

M. Azmi

Department of Veterinary Pathology and Microbiology, Universiti Pertanian Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

SUMMARY

The effect of immunisation routes and the nature of EHV-1 antigens on immunity against EHV-1 infection was studied. Immunisation via the intravenous (i.v.), intramuscular (i.m.) and intraperitoneal (i.p.) routes with live EHV-1 induced higher IgG antibody response than via the intranasal (i.n.) route. However, the former immunisation routes partially or did not result in protection against lethal infection. Partial protection exhibited by reduction in virus titres was limited to the lower respiratory tract only. In contrast, despite very poor antibody responses following i.n. immunisation, the mice were protected against a similar infection. Thus, i.n. is suggested to be the most effective route of immunisation against EHV-1 infections. In general, i.n. immunisation using heat-, ultraviolet radiation- or formalin-inactivated antigens resulted in higher IgG antibody responses compared to the live virus. Heat-inactivated EHV-1 antigens induced the highest IgG antibody responses with a transient peak. None of these mice, however, was protected against subsequent lethal infections. The current findings could explain the failure of vaccination of horses using inactivated EHV-1 antigens which are normally given via the i.m. route. It is suggested that effective immunisation required live EHV-1 (of less pathogenic strains) as an immunising agent and i.n. as the route of immunisation.

Keywords: EHV-1, immunisation, route of immunisation, viral antigen, antibody response, protection.

INTRODUCTION

Equine herpesvirus type-1 (EHV-1) is an important virus causing respiratory problems, abortion and paralysis in horses. Immunisation with commercially available vaccines could reduce severe respiratory disease but not viraemia and abortions (Burki *et al.*, 1990). It is believed that the disease could only be successfully prevented if an appropriate vaccine could be developed for a suitable immunisation program. In general, immunity produced following vaccination or natural infection is of short duration. Horses could be infected despite the presence of high levels of neutralising antibody in the serum (Burrows and Goodridge, 1978). Several studies in horses have addressed the efficacy of various experimental vaccines. These studies, however, were limited mainly to the level of antibody responses against killed-virus antigens and no consideration was given to the effect of immunisation routes and the nature of immunising antigens. Problems faced in conducting such comprehensive studies were in part due to unavailability of suitable experimental horses and expensive maintenance cost. A similar study, however, could be carried out in depth in a suitable mouse model (Awan *et al.*, 1990; Azmi and Field, 1993). In the method of preparations were used; immunisation routes were varied and their effects on host antibody responses and protection against EHV-1 infection were determined.

MATERIALS AND METHODS

Virus

EHV-1 strain AB4 was grown in confluent rabbit kidney (RK-13) or Vero cell monolayers in Earle's minimum essential medium (EMEM) supplemented with 8% newborn calf serum. The virus was purified by ultracentrifugation in 20-60% potassium tartrate gradient and used for the preparation of immunising antigens.

Immunising antigens

Virus inocula containing 1.25×10^8 plaque forming unit (PFU) EHV-1/mL were inactivated by one of the following methods: i) heat-inactivation at 56°C for 30 min, ii) formalin-inactivation in 0.015% formaldehyde, and iii) ultraviolet radiation (UV) - inactivation with a dose of 1200 mJ/cm^2. All inactivated inocula were tested for surviving virus in RK-13 cell culture. Inocula containing 5×10^6

immunisation. In some cases, live virus was used at a dose of 2×10^6 PFU per mouse. For challenge inoculations, mice were normally given 40 µL inoculum containing 5×10^6 PFU live EHV-1.

Experiment 1

Five groups of eight female BALB/c mice (four-week-old) were inoculated intranasal (i.n.) with EHV-1, under diethylether anaesthesia. Each group of mice was given 40 µL virus inoculum containing 5×10^5, 8×10^5, 2×10^6, 5×10^6 or 1×10^7 PFU EHV-1. Following the inoculation, the mice were observed daily for development of clinical signs. At day 5 pos- inoculation (p.i.) surviving mice were killed and their respiratory tissues (nasal turbinates and lungs) collected for virus assay.

Another five groups of eight female BALB/c mice (four-week-old) were each given similar inoculation dose as above, and its cumulative mortality was determined at day 10 p.i. Data obtained were used to determined the optimum dose of inoculations for subsequent experiments.

Experiment 2

Four groups of 25 four week old female BALB/c mice were each inoculated i.n. with 2×10^6 PFU EHV-1 per mouse, either by i.n. intravenous (i.v.), intra-peritoneal (i.p.) or intramuscular (i.m.) route. Inoculations were given in volumes of 40 µL for i.n. and 100 µL for i.v., i.m. and i.p. routes. Control mice were uninoculated. Four mice were killed for virus isolation at days 3 and 5 p.i. Serum samples were collected (at days 0, 3, 5, 8, 14 p.i. and weekly) and assayed for IgG antibody. Two months later, surviving mice were given challenge i.n. inoculation with 5×10^6 PFU EHV-1. Respiratory tissues were collected (at days 0, 3, 5, 8 and 14 post challenge inoculation) and virus titres determined in a plaque-forming assay. The most suitable route of immunisation determined in this experiment was used for subsequent experiments.

Experiment 3

Four groups of twenty female BALB/c mice (four-week-old) were inoculated i.n. either with heat-inactivated, formalin-inactivated, UV-inactivated or live EHV-1. The inocula contained 5×10^6 erstwhile PFU of inactivated or equivalent live EHV-1. A group of twenty control mice were mock-infected with RK-13 cell lysate. Serum samples were collected (at days 0, 3, 5, 8, 14 p.i. and weekly) and assayed for IgG antibody. Two months later, all mice were given challenge i.n. inoculation with 5×10^6 PFU EHV-1 and killed at days 3 and 5 post challenge inoculation. The virus was isolated from the respiratory tissues and titrated in a plaque-forming assay. Serum samples were collected from surviving mice (at days 0, 3, 5, 8 and 14 p.i.) and assayed for IgG antibody.

Virus isolation and titration

The lungs and nasal turbinates of mice were minced and homogenised in 1 mL phosphate-buffered saline (PBS). The suspension was sonicated and spun at 3,000 rpm for 10 min; the supernatant was assayed for virus in a plaque-forming assay in RK 13 cell culture.

Blood samples were collected in EDTA and centrifuged at 3,000 rpm for 15 min, and the buffy-coat layer separated. The buffy-coat cells were collected and washed thrice in PBS. The cell associated virus was assayed in RK-cell culture for plaque formation. The number of plaques produced was expressed as per 10^6 buffy-coat cells.

Enzyme linked immunosorbent assay (ELISA)

An indirect ELISA was developed based on well established principles and protocols (Voller *et al.*, 1980) and the details have been described previously (Azmi, 1995). Briefly, an ELISA plate was coated with purified EHV-1 antigens overnights and washed thrice. Following the washing step, diluted-serum samples (serially diluted two-fold) were then added, and the plate was then incubated at 37°C and washed. A goat-anti mouse IgG conjugate (Sigma) was added and reacted with 2,2'-azino-bis(3-ethylbenzthioline-6 sulfonic acid) (ABTS). Then, the plate was read in an ELISA reader and read at a wavelength of 490 nm. End point titres were determined by plotting the dilution factor of individual test sera against its optical density and when it reached 0.133 (mean O.D. of preimmune sera + 3 s.d.). Hyperimmune and preimmune sera were included as positive and negative controls respectively.

Statistical analysis

The statistical significance of differences between groups of data was determined using the two tailed Student's unpaired t test.

RESULTS

Dose-dependent EHV-1 infectivity

Mice inoculated i.n. with a dose of $>10^6$ PFU EHV-1 displayed clinical signs including ruffled hair, weight loss, dyspnoea and death in severe cases, from day 1 to 7 p.i. The intensity of clinical signs increased with increasing inoculum dose. When the inoculum dose was increased to 10^7 PFU EHV-1 per mouse, the mortality was 100% (Table 1). An intermediate dose of 5×10^6 PFU EHV-1 resulted in development of clinical signs with a mortality of approximately 50%. This dose was used in subsequent challenge infection experiments. In contrast, the infection was not reproducible when the inoculum doses of $< 10^6$ PFU EHV-1 per mouse mice were given. The mice exhibited inconsistent clinical signs; no mortality and no infectious virus recovered from the respiratory tissues beyond day 5 p.i.

Table 1. Intranasal inoculation of mice with EHV-1 of different inoculum doses

Dose per mouse (PFU)	Virus titre		Mortality
	nasal turbinates	lungs	
5×10^5	1.4 ± 0.51*	-	0/8
8×10^5	2.64 ± 0.68	-	0/8
2×10^6	4.05 ± 0.42	1.83 ± 0.47	2/8
5×10^6	5.15 ± 0.25	3.74 ± 0.38	4/8
1×10^7	-	4.89 ± 0.54	8/8

* Geometric means ± s.d. ($\log_{10}$ PFU per organ) at day 5 p.i.; n=4.
- Virus titre below the level of detection (<10 PFU per organ).

Effects of route of inoculation on virus infectivity and antibody responses

The virus was consistently isolated on days 3 and 5 p.i. from nasal turbinates and lungs of mice inoculated intranasally (Table 2). In contrast, following i.v. inoculation, the virus was only detected in the lungs and at low titres (less than five plaques per lung were observed in 3 mice at day 3 p.i.). Low levels of virus were also detected either in the liver or the spleen following the i.n. or i.v. inoculation. The virus was recovered from the blood following the i.n. and i.v. but not i.m. and i.p. inoculations. Inoculations via the i.m. and i.p. routes did not cause disease in mice and the virus was not detected in either the respiratory tissues or the blood. In general, clinical signs were exhibited only in mice inoculated via the i.n. route.

In any case, IgG antibody was detected in the serum. Mice inoculated via the i.v., i.p. and i.m. routes produced high levels of IgG antibodies with peak titres ranging from 1.6 to 2.1 $\log_{10}$ by day 21 p.i. (Figure 1). The difference between the routes of inoculations was not significant (P>0.05). In contrast, mice inoculated intranasally produced very low serum IgG antibody levels with titres of < 0.8 $\log_{10}$ at any time p.i.

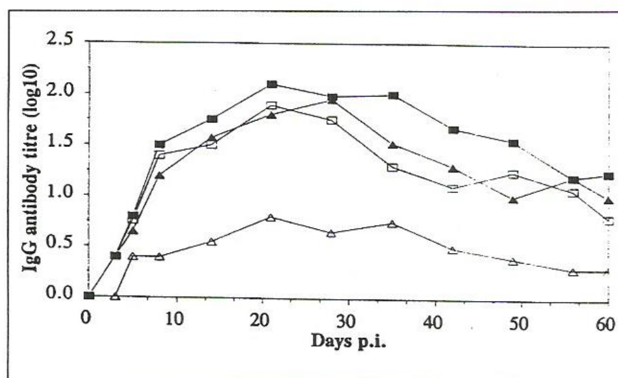

Figure 1. Serum IgG antibody titre (geometric mean; n=7) in mice following inoculation with live EHV-1 via the i.n. (Δ), i.v. (□), i.m. (■) or i.p. (▲) routes.

Effects of route of immunisation on protection against challenge infection

Upon challenge inoculation with 5×10^6 PFU EHV-1 at two months post-immunisation, clinical signs developed in the mice that were previously immunised via i.v., i.m. and i.p. routes. Approximately 30% of these mice died within 1 week p.i. In contrast, very mild clinical signs were displayed in the mice that were previously immunised intranasally and none died upon challenge inoculation.

The virus reduction in the nasal turbinate of the mice that were previously immunised via i.v., i.m. and i.p. routes, was not significant (Figure 2). However, significant virus reductions, ranging from 1 $\log_{10}$ to 1.2 $\log_{10}$ PFU were noted in the lungs but only at day

Table 2. Virus infectivity following different inoculation routes

	Days p.i.	Route of inoculation			
		i.n.	i.v.	i.m.	i.p.
Virus in nasal turbinates	3	4.10 ± 0.36*	-	-	-
	5	1.85 ± 0.25	-	-	-
Virus in lungs	3	4.25 ± 0.67	+	-	-
	5	2.18 ± 0.45	+	-	-
Virus in buffy coat cells	3	40†	30	-	-
	5	2	10	-	-
Cumulative mortality	1-10	1/8	0/8	0/8	0/8

* Virus titre, geometric mean ± s.d. ($\log_{10}$ PFU per organ) at day 5 p.i.; n=4.
† Number of infectious centre per 10^6 buffy coat cells.
+ Approximately 5 PFU per organ from 3/4 mice.

5 p.i. In contrast, significant virus reductions were noted both in the nasal turbinates and the lungs of the mice immunised intranasally. Approximately 2 to 2.5 $\log_{10}$ PFU virus reductions were noted at day 3 p.i. and no virus was detected in either tissues by day 5 p.i.

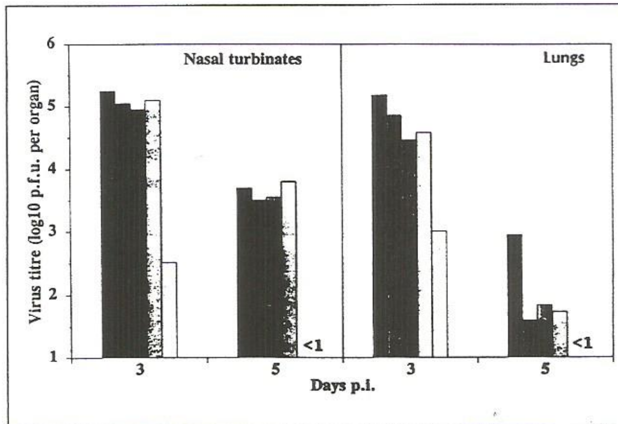

Figure 2. Virus titres (geometric mean; n=4) in nasal turbinates and lungs of mice following challenge inoculation with EHV-1. The mice were previously immunised for 60 days with live virus via i.n. (❒), i.v. (■), i.m. (■) and i.p. (■) routes, or unimmunised mice served as controls (■).

Assay of serum samples by means of ELISA indicated mice that were previously immunised via any of the four routes seroconverted (Figure 3). A rapid rise in IgG antibody titres was observed in mice previously immunised via i.v., i.m. and i.p. routes. Antibody titres increased maximum levels by day 5 p.i. with titres from the different routes of immunisation ranging from 3.1 to 3.8 $\log_{10}$. In contrast, in a similar challenge inoculation, the mice previously immunised intranasally produced IgG antibody of lower titres. The rise in antibody titres was slow, reaching approximately 1.5 $\log_{10}$ at day 5 p.i. and increasing to 2.8 $\log_{10}$ by day 14 p.i.

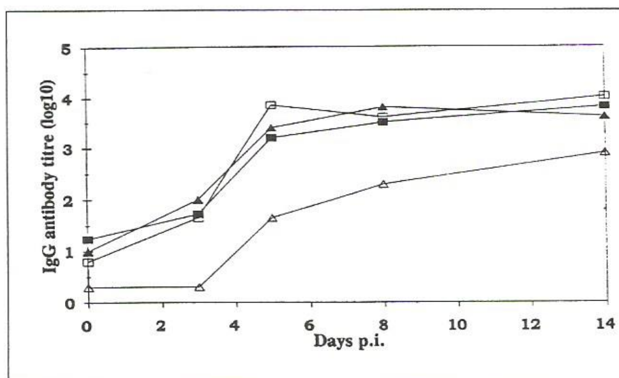

Figure 3. Serum IgG antibody titres (geometric mean; n=4) following challenge inoculation with EHV-1. The mice were previously immunised for 60 days with live virus via the i.n. (Δ), i.v. (❒), i.m. (■) or i.p.

Immunisation with EHV-1 antigen of different preparations

Effects on antibody responses

Following i.n. inoculation with EHV-1 antigens (heat-inactivated, formalin-inactivate, UV-inactivated and live virus), IgG antibodies were detected in the serum (Figure 4). Mice inoculated with live virus were found to produce IgG antibody consistently of low titres (<1 $\log_{10}$). In contrast, inoculation with any of the three inactivated antigens resulted in production of IgG antibody of high titres. The highest antibody titre was noted in mice inoculated with heat-inactivated virus, i.e., 1.8 $\log_{10}$ at day 14 p.i. The mice that were inoculated with formalin-inactivated or UV-inactivated virus antigen also produced high antibody level with peak titres of approximately 1.4 $\log_{10}$ at day 14 p.i. However, from thereafter the antibody titres gradually declined reaching lowest levels by 60 days p.i.

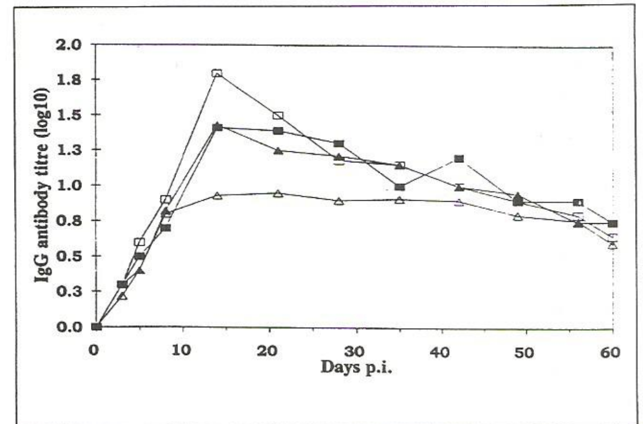

Figure 4. Serum IgG antibody titres (geometric mean; n=7) in mice following i.n. inoculation with live virus (Δ), heat (❒)-, UV (■)- and formalin (▲)-inactivated antigens.

Protection against challenge infection

The degree of protection following i.n. immunisation with different antigen preparations was assessed by challenge infection with 5×10^6 PFU EHV-1 two months later. Following challenge inoculation, all mice excluding those previously inoculated with live virus exhibited severe clinical disease. In general, cumulative mortality approaching 50% was noted in the disease animals. In contrast, the mice that were previously immunised with live virus exhibited very mild clinical signs which disappeared within 3 days p.i. These mice were then fully recovered from the clinical disease.

Upon virus assay on mouse respiratory tissues collected at days 3 and 5 after challenge inoculation, there was no significant virus reduction noted in the nasal turbinates or lungs of the mice previously immunised with any of the inactivated EHV-1 antigens

those in unimmunised mice. In contrast, significant virus reductions (approximately 2-2.5 log_{10} PFU at day 3 p.i.) either in the nasal turbinates or lungs were noted and only in mice previously inoculated with live virus. No virus was detected in these mice by day 5 p.i.

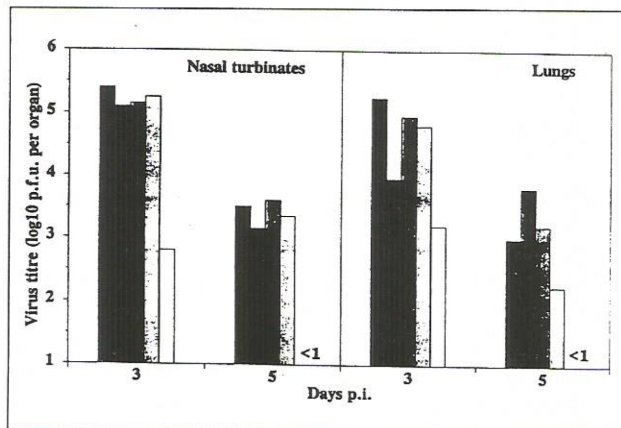

Figure 5. Virus titres (geometric mean; n=4) in nasal turbinates and lungs of mice following challenge inoculation with EHV-1. The mice were previously immunised intranasally for 60 days with live virus (❒), heat (■)-, UV (■)- and formalin (■)- inactivated antigens. Mice inoculated with RK-13 cell lysates (■) served as controls.

In general, rapid rises in IgG antibody titres were noted in mice previously immunised with inactivated virus antigens (Figure 6). The mean antibody titres were approximately 2.0 and 4.5 log_{10} at days 5 and 8 p.i. respectively. In contrast, a slower rate of rise in IgG antibody titre occurred in mice previously immunised intranasally with live virus, with titres of 1.4 and 2.2 log_{10} at days 5 and 8 p.i., respectively. The difference of approximately 1.4-2.4 log_{10} between the two antibody titres at day 8 p.i. was significant (P<0.01).

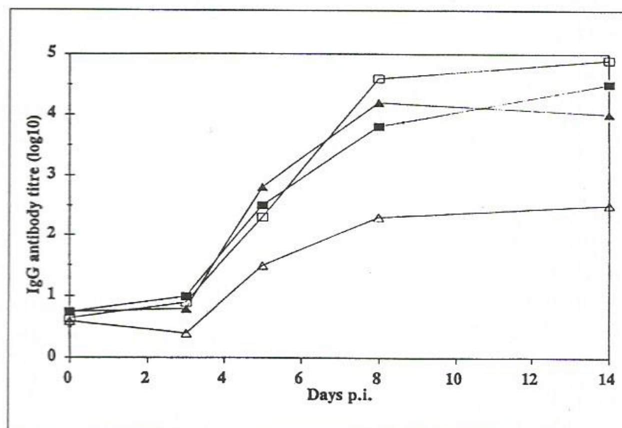

Figure 6. Serum IgG antibody titres (geometric mean; n=4) following challenge inoculation with EHV-1. The mice were previously immunised intranasally for 60 days with live virus (Δ), heat (❒)-, UV (■)- and

DISCUSSION

The major findings of the present study are as follows: i) virus infectivity was not reproducible when the i.n. inoculum dose was <10^6 per mouse, ii) i.n. inoculation with 10^7 PFU EHV-1 resulted in 100% mortality, iii) inoculations via i.v., i.m. and i.p. routes induced high antibody responses but it did not confer full protection but may be only partially reduced the severity of disease manifestation in the lungs only, i.v.) i.n. inoculation with any of the three inactivated antigens induced higher antibody responses but it did not confer protection against subsequent lethal infection, and v) i.n. inoculation with low inoculum doses of live viruses induced very low antibody responses but the mice were protected from subsequent lethal EHV-1 infection.

Inoculum doses and inoculation routes appeared to be important determining factors for the development of acute diseases in mice. Inoculations with >10^6 PFU EHV-1 will result in the development of the disease only when it was given via the i.n. route. Serum antibody responses were found to be very low at most points in time post infection. In contrast, when the live virus was introduced via other routes, i.e. i.v., i.m. or i.p., the disease was not produced. However, compared to those following i.n. route, the induced antibody responses were found to attain a high level when immunisation was given via the other three routes. Therefore, it is suggested that the infection and virus replication in the respiratory tissues following i.n. inoculation could be immunosuppressive and as a result the antibody responses would be very poor. The current data seems to further support the evidence of immunosuppression in horses in the early stages of EHV-1 infection (Hannant *et al.*, 1991). Despite low antibody responses, mice previously inoculated via i.n. route were protected from subsequent infections. In contrast, there was no complete protection in those mice inoculated via other routes, despite having higher antibody responses. These data indicated that, although it may lead to immunosuppression of antibody responses, introduction of immunising antigens into the respiratory tract is required to obtain the desired immunity. Higher antibody responses could be obtained via other routes of immunisation, but they were not protective. Immunisations via i.v., i.m. or i.p. route did not result in the deposition of immunising antigens at the natural site of infection (in the lungs) and the immune responses would be poor. It is suggested that i.n. could be the best route of immunisation of introduction to stimulate local immune responses for maximum protection. Since high antibody responses seems not to mediate complete protection, the present data further support the evidence that protection against EHV-1 infection is mediated by other mechanisms, perhaps cell-mediated immunity (Azmi and Field, 1993). However, this did not completely rule out the importance of antibody-mediated protection in EHV-1

A low inoculum dose of live EHV-1 that was given i.n. caused very mild and transient clinical signs in mice. In theory, live EHV-1 could be used for immunisation to obtain protective immunity. In the present study, when the same amount of different inactivated antigens were given i.n., the antibody responses were greater than those observed with the live virus, but no protection was conferred. No protection conferred following immunisation with inactivated antigens was probably in part due to the damaging effects of inactivation process; UV-light damaged viral genome, heat-inactivation and formalin-inactivation destroyed viral proteins. A similar study showed that when a dose of live influenza viruses was injected i.v. into mice; the virus undergoes defective growth in host cells and did not generate infective progeny (Effros *et al.*, 1977). This implies that protective immunity can only be developed when there is virus replication at the site of natural infection.

In conclusion, the current findings may explain why vaccination of horses using inactivated EHV-1 antigens via i.m. routes failed to confer long-lasting immunity. It is suggested that such immunity could be only obtained with an active virus replication at the site of natural infection and this is not possible with inactivated viruses. Less pathogenic strains of viruses or genetically modified viruses such as thymidine kinase-deficient mutant could be used for the preparation of effective immunising agents (Corrochano *et al.*, 1993).

REFERENCES

Awan, A.R., Chong, Y.C. and Field, H.J. (1990). The pathogenesis of equine herpesvirus-1 in the mouse: a new model for studying host responses to the infection. *J. Gen. Virol.*, **71:** 1131-1140.

Azmi, M. and Field, H.J. (1993). Cell-mediated antiviral response to equine herpesvirus type-1 demonstrated in a murine infection model by means of adoptive transfer of immune cells. *J. Gen. Virol.*, **74:** 275-280.

M. Azmi (1995). Response of antibody classes and subclasses to equine herpesvirus type-1 (EHV-1) in the serum and respiratory secretions of infected-mice. *J. Vet. Malaysia*, **7**: 15-20.

Burki, F., Rossmanith, W., Nowotny, N., Pallan, C., Mostl, K. and Lussy, H. (1990). Viraemia and abortions are not prevented by 2 commercial equine herpesvirus-1 vaccines after experimental challenge of horses. *Vet. Q.*, **12:** 80-86.

Burrows, R. and Goodridge, D. (1978). Equid herpesvirus-1 (EHV-1): some observations on epizootiology of infection and on the innocuity testing of live virus vaccine. *In*: Proc. 24th. Ann. Conf. Am. Assoc. Eq. Pract., St Louis. pp. 17-29.

Corrochano, L.M., Madueno, F., Field, H.J. and De La Fuente, R. (1993). Sequence analysis of thymidine kinase-defective mutants of equine herpesvirus type-1 (EHV-1). *Gene*, 126: 257-260.

Effros, R.B., Doherty, P.C., Gerhard, W. and Bennink, J. (1977). Generation of both cross-reactive and virus-specific T cell population after immunization with serologically distinct influenza A viruses. *J. Exp. Med.*, **145:** 557-568.

Hannant, D., O'Neill, T., Jessett, D.M. and Mumford, J.A. (1991). Evidence of non-specific immunosuppression during development of immune response to equid herpesvirus-1. *Equine Immunol.* **12**(Suppl): 41-45.

Stokes, A., Murray, P.K., Allen, G.P. and Pullen, L.A. (1989). A hamster model of equine herpesvirus type-1 (EHV-1) infection - passive protection by monoclonal antibodies to EHV-1 glycoprotein 13, glycoprotein 14 and glycoprotein 17/18. *J. Gen. Virol.*, **70:** 1173-1183.

Voller, A., Bidwell, D. and Barlett, A. (1980). Enzyme-linked immunosorbent assay in research. *In*: Manual of Clinical Immunology. H. Friedman (Ed.). American Society of Microbiology. pp. 359-371.

RINGKASAN

PENGIMUNAN TERHADAP JANGKITAN HERPESVIRUS EKUIN TIP-1 (EHV-1) DALAM MENCIT : KESAN PERSEDIAAN ANTIGEN DAN JALAN PENGINOKULATAN YANG BERBEZA

Kesan jalan pengimunan dan sifat antigen EHV-1 terhadap keimunan menentang jangkitan EHV-1 telah dikaji. Pengimunan secara intravena (i.v.), intraotot (i.m.) dan intraperitoneum (i.p.) menggunakan EHV-1 hidup mengaruh gerakbalas antibodi IgG yang lebih tinggi daripada secara intranasum (i.n.). Bagaimanapun, tiga jalan pengimunan yang disebut terdahulu ini hanya separa atau tidak langsung menghasilkan perlindungan terhadap jangkitan maut. Perlindungan separa yang ditunjukkan oleh pengurangan dalam titer virus telah terhad kepada trakus pernafasan bawah sahaja. Disebaliknya, walaupun gerakbalas antibodi itu buruk berikutan pengimunan i.n., mencit masih terlindung daripada jangkitan yang sama. Oleh demikian, i.n. disarankan sebagai jalan pengimunan paling berkesan terhadap jangkitan EHV-1. Umumnya, pengimunan i.n. mengguna antigen, tertakrif haba, sinaran ultra-ungu atau formalin menghasilkan gerakbalas antibodi IgG lebih tinggi daripada virus hidup. Antigen EHV-1 tertakrif haba mengaruh gerakbalas antibodi IgG paling tinggi dengan kemuncak sementara. Bagaimanapun, tiada satu daripada mencit ini terlindung daripada jangkitan maut susulan. Penemuan kini mungkin menerangkan mengapa berlakunya kegagalan pemvaksinan pada kuda mengguna antigen EHV-1 tertakrif yang biasa diberi melalui jalan i.m.. Apa yang disarankan ialah pengimunan berkesan memerlu EHV-1 hidup (daripada strain kurang patogenik) sebagai antigen pengimun dan i.n. sebagai jalan pengimunan.