The Journal of Microbiology, March 2002, p.11-19
Vol. 40, No. 1

Protective Immune Reponses Induced by Non-infectious L-particles of Equine Herpesvirus Type-1: Implication of Cellular Immunity

Mohd Lila Mohd Azmi[1]*, Hugh John Field[1], Frazer Rixon[3], and John McLauchlan[3]

[1]Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400, Serdang, Selangor DE, Malaysia
[1]Department of Clinical Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 0ES, U.K.
[3]Institute of Virology, Hill Street Road, Glasgow, U.K.

(Received July 23, 2001 / Accepted February 1, 2002)

Mice immunized with equine herpesvirus type-1 (EHV-1) L-particles showed a significant increase ($p<0.05$) in serum antibody titers. Upon a booster dose four weeks later, antibody titers increased significantly. Interestingly, immunization via intravenous or intramuscular route induced significantly higher ($p<0.05$) antibody titers. However, mice iummunized with UV-treated L-particles, virions or immunization via intranasal route induced lower antibody titers. Upon challenge inoculation with wild-type EHV-1, our data showed there was a poor correlation between antibody titers and protection against virus replication. Therefore, the role of cell-mediated immunity towards protection was investigated. As predicted, the strongest cell-mediated immunity, as measured by delayed-hypersensitivity test, was detected in mice immunized with live virus particles. The magnitude of cell-mediated immune response correlated with the efficacy of L-particles as immunizing agent. The highest efficacy, as indicated in mice immunized via intranasal route, was highly correlated with cell-mediated immunity. A similar phenomenon was also demonstrated in mice immunized intranasally with UV-treated L-particles. However, the degree of protection was reduced when mice immunized intravenously or intramuscularly with UV-treated L-particles. In conclusion, protection conferred in these animals was highly implicated by immune cells and the least by antibodies. The route of immunization and the nature of the antigen also contributed to the efficacy of L-particles as immunizing agent. In contrast to that of herpes simplex virus type 1, our data showed EHV-1 non-infectious L-particles are highly suitable for immunization of the host against EHV-1 disease.

Key words: equine herpesvirus, L-particles, cell-mediated immunity, antibody response

Equine herpesvirus type 1 (EHV-1), a common pathogen in horses, is responsible for respiratory disease, abortion and central nervous system (CNS) disorders (Allen and Bryan, 1986). It is a member of the alphaherpesvirus subfamily (Allen and Bryan, 1986; Roizman *et al.*, 1992; Telford *et al.*, 1992). The immunity produced in horses from natural infection is short-lived and animals suffered from repeated attacks of the infection (Allen and Bryan, 1986). Several vaccines are available but not proven to be completely protective (Burrows *et al.*, 1984; Burki *et al.*, 1990).

Cells infected by herpesviruses release not only virions but also non-infectious virion-related particles termed L-particles. Herpesviruses that produce L-particles include herpes simplex virus type 1 (HSV-1), cytomegalovirus (CMV), pseudorabies virus (PrV), and equine herpesvirus type-1 (EHV-1) (Irmiere and Gibson, 1983; Szilagyi and Cunningham, 1991; McLauchlan and Rixon, 1992). L-particles comprise a tegument surrounded by an envelope, but lack the internal capsid and DNA. It was suggested that the process of L-particle component assembly is similar to that involved in normal virion maturation (Rixon *et al.*, 1992).

Some proteins are present only in L-particles (e.g. Vmw175 which is an IE-3 protein of HSV-1), and are not normally present in virions (McLauchlan and Rixon, 1992). Among the proteins contained in L-particles are all the necessary proteins for the initiation of virus replication e.g., for HSV-1, Vmw175 and α-TIF, although these two proteins appeared not to be the components of EHV-1 L-particles (Rixon *et al.*, 1992). Since L-particles possess envelope proteins, which resemble those of virions, L-particles would be expected to have properties similar to virions regarding the induction of immune responses (Paap-Vid and Derbyshire, 1978; Paap-Vid and Derbyshire, 1979). If non-infectious L-particles could be produced under

* To whom correspondence should be addressed.
(Tel) 60-3-8946-8305; (Fax) 60-3-8948-6317
(E-mail) azmi@vet.upm.edu.my

conditions to exclude the presence of infectious virions, such material has been suggested to have potential for a vaccine preparation. In the present study, EHV-1 L-particles were used to immunize mice via different routes of inoculation. The protection was tested by antibody production delayed-type hypersensitivity (DTH) responses and virus replication following the challenge of mice with live EHV-1.

Materials and Methods

EHV-1 L-particles and virions

Sucrose gradient purified L-particles and virions preparation of EHV-1 strain AB4p (AB4 plaque purified virus) were prepared as described previously (McLauchlan and Rixon, 1992). The pathogenicity of the parental strain of AB4 (AB4p) has been described previously (Gibson *et al.*, 1992). UV-treated L-particles and virions were prepared by means of UV-light irradiation (254 nm wavelength) at a dose 6×40,000 μJ/cm^2.

EHV-1 antigens for ELISA

EHV-1 strain AB4 was grown in Vero cells and purified by potassium tartrate gradient (20-60%) centrifugation at 24,000 rpm (SW40Ti, Beckman USA) for two hours. The purified antigen was re-suspended in PBS. The concentration of viral antigen was determined by means of standard BioRad protein assay and optimised for ELISA.

ELISA

An indirect ELISA technique was developed based on well-established principles and protocols of Bidwell *et al.* (1976), Voller *et al.* (1980), Clark and Barbara (1987) and Azmi (1995) with some modifications. The test was carried out with a working volume of 50 μl of each reagent. The antigen was diluted in bicarbonate buffer to give a concentration of 10 μg per ml antigen protein. Each well of a 96-well plate (Dynateh. Immulon, USA) was coated with 50 μl of the antigen solution, and incubated at 4°C overnight. The plate was then washed three times with phosphate-buffered saline Tween 20 (PBST) using an automated microplate washer (Dynatech. MR 7000, USA). To block non-specific binding, 50 μl of 2% BSA- Fraction V (Sigma, UK) was added and the plate incubated at 45°C for two hours. The plate was washed three times as above. For the detection of serum antibodies, two-fold (or ten-fold) serial dilutions of test sera were added and the plate was incubated at 37°C for one hour. The plate was then washed three times. Fifty μl of pre-diluted goat anti-mouse peroxidase conjugated immunogolobulin (Sigma, UK) was added and allowed to react with antigen bound-mouse antibodies, by incubating the plate again at 37°C for one hour. Again the plate was washed three times. The 2,2-Azino-bis (3-ethylbenzthioline-6-sulfonic acid) (ABTS) substrate (Sigma, UK) diluted in citrate-phosphate buffer (CPB) and supplemented with 0.01% of 30% H_2O_2 was added and the plate incubated for 30-40 minutes at room temperature. Upon completion of the reaction, the plate was read immediately in a spectrophotometer (Dynatech. MR 7000, USA) at dual wavelength mode at absorbance 410-490 nm. Hyperimmune and pre-immune sera were included in the plate as positive and negative controls respectively.

Determination of ELISA Cut-off point

The end-point titration or cut-off point was determined by plotting ELISA data obtained, serum dilution against optical density (O.D.), based on two-fold or ten-fold serial dilutions of the individual test serum. This enabled the value of $\log_{10}$ dilution to be read from the curve, which corresponded to an optical density value of the mean of eight wells of preimmune sera plus three standard deviations. On the basis of the optical density value for an antigen with negative sera, the arbitrary optical density limit of 0.15 (mean +s.d.; mean =0.095; s.d.=0.017) was defined for negative sera diluted at 1: 100. Values above this were considered to be positive with 99.97% probability (Meulemans and Halen, 1992).

Delayed-type hypersensitivity

DTH test as a mean for the measurement of cell-mediated immune responses in mice was carried out by employing the skin test as described by Nash *et al.* (1980) and Azmi and Field (1993). The purified virus was heat-inactivated at 56°C for 30 min. The mice to be tested were lightly anaesthetized with diethyl ether. Using a 50 μl-Hamilton syringe fitted with a 27 gauge needle, 20 μl of the antigen (containing 10^6 pfu of heat-inactivated virus) was inoculated into the left ear pinna of the immunized mice. A similar quantity of an appropriate uninfected cell lysate suspension was inoculated into the right ear pinna as control. Inoculations were carried out in triplicate. Ear skin thickness was measured with the micrometer screw gauge at 48 h after antigen inoculation. Results were expressed as ear thickness increase (mmx 0.01). A control group of mice mock-infected with cell-lysate suspension was included in the experiment.

Virus isolation from tissues

Groups of four mice were killed at days 3 and 5 post-inoculation (p.i.). Nasal turbinates and lungs were collected individually in 1 ml ice-cold serum-free DMEM medium containing 8% antibiotic-antimycotic solution and 2% anti-PPLO agent in bijou bottles. The tissues were minced thoroughly with scissors, homogenized with an electric homogenizer (Thyristor Regler TR50, IKA-WERK, Germany). The suspensions were subjected to sonic vibration for 1-2 min in an ice-cold sonic water bath. The suspensions were then transferred to an eppen-

dorf tube and spun at 3,000 rpm for 10 min at 4°C in a refrigerated centrifuge (Hettich, Germany) to remove the cellular debris. The supernatants were kept at 70°C before being titrated by means of plaque assays. In some cases the whole undiluted supernatant was tested for the virus.

Virus titration by plaque forming assay

A confluent monolayer of Vero cells was grown on 24-well tissue culture plates (Tissue culture CLUSTER-Costar, USA). Ten-fold serial dilutions of the virus sample were prepared in serum- free DMEM medium. One hundred μl of each dilution was inoculated in duplicate onto the confluent monolayers (two wells were inoculated with DMEM medium only and acted as negative controls). The virus was allowed to adsorb at 37°C for 45 min. Five hundred μl of DMEM medium containing 2% FCS, 1.2% CMC, 1% antibiotic-antimycotic and 1% anti-PPLO solutions was added to each well, and the plate was incubated at 37°C. The plate was periodically monitored over 48-72 hours p.i. After 3-4 days incubation, the overlay medium was poured away and the monolayers were rinsed twice with PBS. Following this, the cell monolayer was fixed for 10 min with 70% methanol before being stained with 2.5% alcoholic crystal violet solution for 5-10 min. The plate was rinsed gently in tap water until plaques were clear for enumeration, dried at room temperature, and the plaques counted using an inverted-light microscope (Olympus CK2, Japan). The virus titer was calculated as the mean number of plaques in the last dilution that showed plaque formation multiplied by the reciprocal of that dilution and expressed as $\log_{10}$ pfu per sample. The limit of sensitivity was 10 pfu per sample.

Inoculation of mice

Several groups of 15 mice each were inoculated as shown in Table 1. The mice were primarily inoculated i.n. i.v. or i.m. with uninactivated or UV-treated materials. The number of particles per inoculum was 10^8 for virions and and 10^9 for L-particles. The inoculation was carried out by i.v. injection via the tail vein; and i.m. via the front or hind leg muscles. The mice, which were, inoculated i.n. with purified live virions, showed mild clinical signs e.g., ruffled hair and respiratory distress, but recovered by about one week p.i. No mice in the other groups showed clinical signs and none died. Four weeks later, all mice were given a second inoculation with the same dose and route as in the primary inoculation. Four weeks after the second inoculation, three mice from each group were skin-tested for DTH. The remaining mice were challenged i.n. with 6×10^6 p.f.u. of EHV-1 strain AB4 (N.B. not AB4p). Serum was collected from groups of four mice at various time points beginning from the day of primary inoculation until 14 days after the challenge inoculation, for subsequent analysis by means of ELISA. The clinical signs and reduction in body weight were observed and the weight of the spleen was measured individually.

Table 1. Inoculation of mice with virions or L-particles of EHV-1 strain AB4p

Inoculum*	Route of inoculation			
	i.n.	i.v.	i.m.	Control†
UV-exposed L-particles	^	^	^	ND
Normal L-particles	^	^	^	ND
Live virions	^	^	^	ND
Inactivated virions	^	^	^	ND
Uninoculated (control)	ND	ND	ND	^

*inocula contained purified material.
†control mice were uninoculated.
i.n.= intranasal, i.v.= intravenous, i.m.=intramuscular
‡n=15.
^Inoculation done, ND not done.

Statistical analysis

The statistical significance of differences between groups

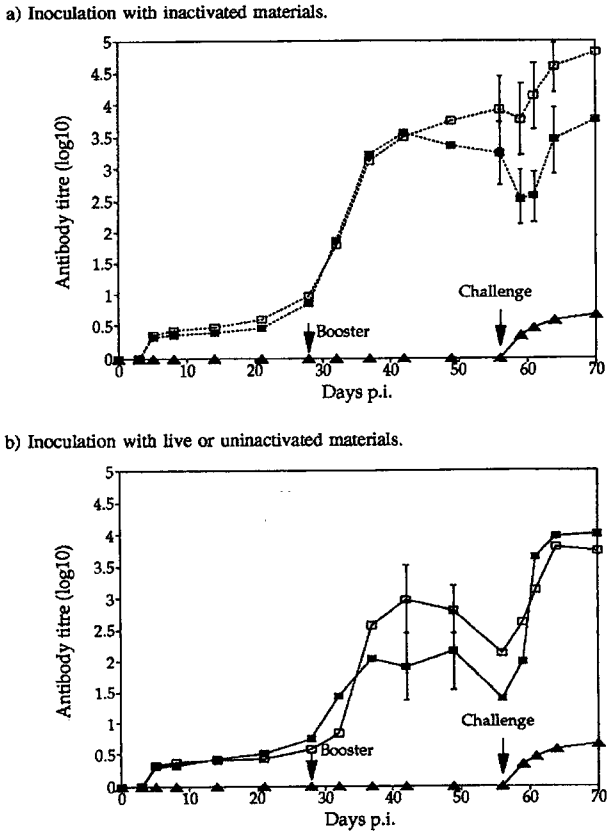


Fig. 1. Serum IgG antibody response following i.m. inoculation with EHV-1 virions or L-particles. Mice were inoculated twice at four week intervals as follows: *(a)* UV-inactivated virions (■) UV-exposed L-particles (□); *(b)* live virions (■) or normal L-particles (□). Control mice were uninoculated (▲). Four weeks after the second inoculation, mice were given a challenge inoculation with 6×10^6 p.f.u. of EHV-1. Data points represent the antibody titre (geometric mean± s.d.; n=4).

of data was determined using the two-tailed Students unpaired t-test.

Results

Antibody responses following intramuscular inoculation

Following primary i.m. inoculation with any antigens prepared, IgG antibody titers were low and remained at about 0.5 $\log_{10}$ (Fig. 1). There were no significant differences detected among the groups. When mice were given the second i.m. inoculation, the antibody response rapidly increased and peaked at about 14 days p.i. The highest antibody titer detected in mice inoculated with UV-inactivated virions or UV-treated L-particles (approximately 3.5 $\log_{10}$ at day 42 p.i.) while the lowest titer (approximately 1.8 to 2.0 $\log_{10}$) was noted in mice inoculated with live virions. Inoculation with normal L-particles gave rise to higher antibody titers (approximately 1 $\log_{10}$ higher at day 42 p.i.). Generally, inoculation with antigens that had been treated with UV light resulted higher antibody response as compared to those inoculations with antigens that had not been treated with UV light.

Following the peak, antibody titers declined except in those mice inoculated with UV-treated L-particles. When these mice were challenged with EHV-1at day 56 p.i., antibody titers rapidly increased and peaked by day 8 post challenge. The highest antibody titer was again produced in mice inoculated with L-particles that previously treated with the *uv* light.

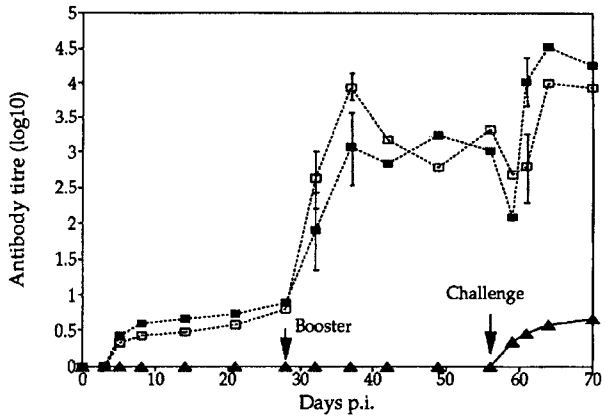
Antibody responses following intravenous inoculation

Following a primary i.v. inoculation with any immunizing antigens, IgG titers remained low (between 0.4 to 0.8 $\log_{10}$; Fig. 2). After the second i.v. inoculation, antibody response was rapidly increased and peaked at about 8 days later. L-particles treated with the UV *light* again induced higher antibody titers (approximately 3.8 $\log_{10}$). The lowest antibody titers was observed in mice inoculated with normal L-particles (approximately 2.5 $\log_{10}$). Following i.n. challenge inoculation at day 56 p.i., 0.5 $\log_{10}$ reduction in antibody titers was noted at day 3 post challenge and the titers rapidly increased from less than 3 $\log_{10}$ at day 59 p.i. to >3.8 $\log_{10}$ at day 64 p.i. (8 days after the challenge inoculation). The highest antibody titers were noted at day 64 p.i. (with the titer of approximately 4 $\log_{10}$) in mice which had been previously inoculated with live or UV-inactivated virions. Antibody titers remained high until day 77 p.i. which was the last day of observation.

Antibody Responses Following Intranasal Inoculation

Following primary i.n. inoculation with any prepared materials, IgG antibody titers remained very low at between 0.4 to 0.7 $\log_{10}$ (Fig. 3). When these mice were given a second i.n. inoculation, the antibody response increased slightly, but was significantly lower (<1.5 $\log_{10}$) compared with the other two inoculation routes (≥ 2 $\log_{10}$; as described following i.v. inoculation). When challenged at day 56 p.i., antibody titers did not significantly ($P<0.05$) increase. In short, in any of the three cases, i.n. inoculation raised very low antibody responses.

a) Inoculation with inactivated materials.



b) Inoculation with live or uninactivated materials.

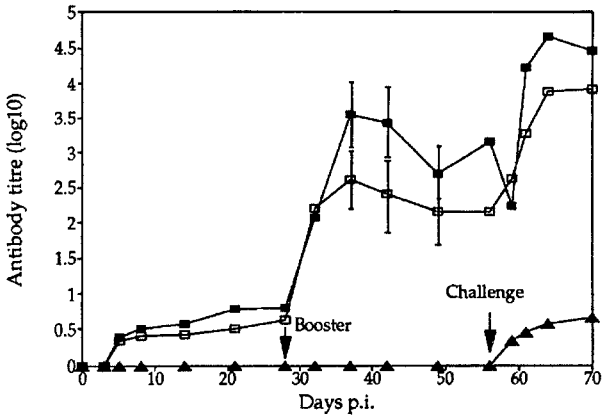


Fig. 2. Serum IgG antibody response following i.v. inoculation with EHV-1 virions or L-particles. Mice were inoculated twice at four week intervals as follows: *(a)* UV-inactivated virions (■) UV-exposed L-particles (□); *(b)* live virions (■) or normal L-particles (□). Control mice were uninoculated (▲). Four weeks after the second inoculation, mice were given a challenge inoculation with 6×10^6 pfu of EHV-1. Data points represent the antibody titre (geometric mean± s.d.; n=4).

DTH responses to EHV-1 L-particles and virions preparations

Three mice from each experimental group were tested for DTH response. Purified EHV-1 antigen, mentioned above, was employed in the skin test. The result obtained are shown in Table 2. In all cases the maximum responses were obtained in mice that had been immunized with live virions. Mice inoculated with normal L-particles showed

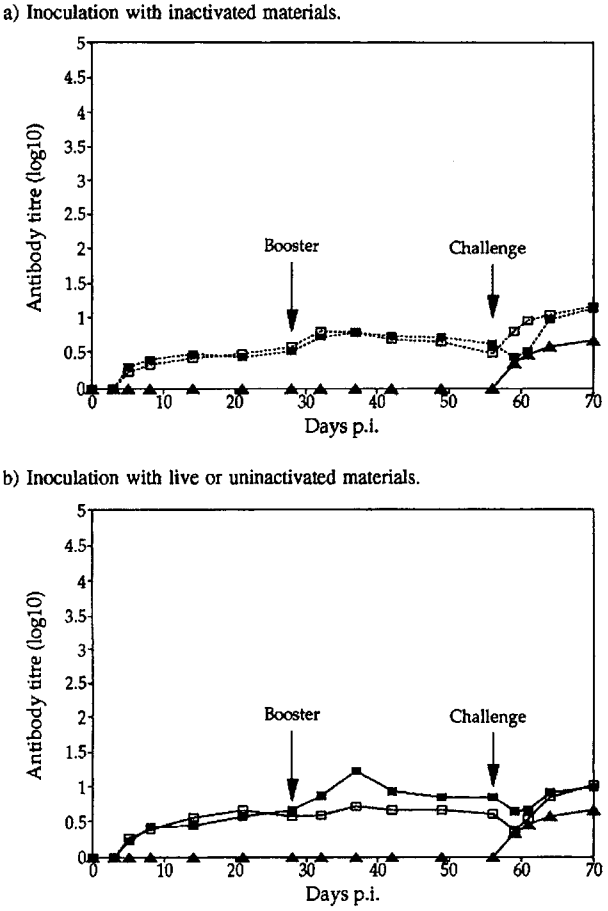


Fig. 3. Serum IgG antibody response following i.n. inoculation with EHV-1 virions or L-particles. Mice were inoculated twice at four week intervals as follows: *(a)* UV-inactivated virions (■) or UV-exposed L-particles (□); *(b)* live virions (■) or normal L-particles ( ). Control mice were uninoculated (▲). Four weeks after the second inoculation, mice were given a challenge inoculation with 6×10^6 pfu of EHV-1. Data points represent the antibody titre (geometric mean± s.d.; n=4).

a higher magnitude of responses than inoculation with UV-treated L-particles, and with the maximum in mice inoculated i.n.

Clinical signs and virus replication following virus challenge

Following challenge inoculation of mice, clinical signs observed were different among the groups. Body weight was reduced from 1 day post challenge inoculation. In severe cases, mice lost 20-25% of their normal body weight. Less weight reduction was noted in mice inoculated with live virions i.v or i.n, or with normal L-particles i.n. One week after the challenge inoculation, surviving mice regained their body weight to 5-10% less than the uninoculated mice.

Virus titers in the respiratory tissues were determined at days 3 and 5 post challenge inoculation. At days 3 and 5 post challenge, virus titers in turbinates and lungs of

Table 2. DTH response in mice immunized with L-particles or virions of EHV-1

Route	Inoculum*	Time after antigen inoculation (hour)	
		24	48
Intravenous	UV-exposed L-particles	26.3± 2.1†	26.0± 2.0
	Normal L-particles	26.7± 2.9	31.0± 1.7
	Inactivated virions	32.0± 3.6	27.0± 4.6
	Live virions	38.0± 2.7	37.0± 1.7
Intramuscular	UV-exposed L-particles	21.7± 3.2	26.0± 1.7
	Normal L-particles	27.3± 1.5	29.0± 1.0
	Inactivated virions	25.0± 2.0	28.0± 3.0
	Live virions	31.7± 3.1	30.7± 5.7
Intranasal	UV-exposed L-particles	31.7± 1.2	26.0± 3.6
	Normal L-particles	41.0± 3.0	35.0± 4.4
	Inactivated virions	27.0± 3.6	26.0± 3.0
	Live virions	39.3± 6.7	41.7± 1.2
Control	None	9.3± 3.1	7.7± 0.6

*mice were immunized with either one of four prepared antigens.
†ear skin thickness increases; mean± s.d.; n=3.
§uninoculated mice were tested for non-specific skin reaction.

unimmunized control mice reached approximately 10^6 pfu and 10^4 pfu respectively (Figs 4, 5 and 6). The maximum virus reduction (>2 $\log_{10}$ in turbinates and lungs) was observed in mice inoculated i.n. with live virions or normal L-particles; the least virus reduction was demonstrated in mice inoculated i.n. with UV-inactivated virions. A partial virus reduction (≥1.5 $\log_{10}$ in the lungs only, but not in the turbinates) was noted in all cases in which mice were immunized with L-particles. Mice inoculated with inactivated virions demonstrated less reduction in virus titer.

Following challenge inoculation, the weight of the spleen was measured individually. The weight of spleen of uninfected mice was approximately 90-105 mg. The maximum increase in spleen weight was observed in mice inoculated with live virions i.n. (Fig. 7). The increase in weight was also observed in mice inoculated i.n. with normal L-particles.

Discussion

Results obtained from the experimental immunization of mice with EHV-1 L-particles or virions are summarized as follow: *(i)* i.m. and i.v. inoculations gave higher antibody response, in contrast to i.n. inoculation which produced a very low response. *(ii)* Higher DTH response were detected in mice inoculated with live virions or normal L-particles. *(iii)* Significant virus reductions were observed in mice which had been inoculated with normal L-particles or live virions.

In any case, serum antibody response was found to be

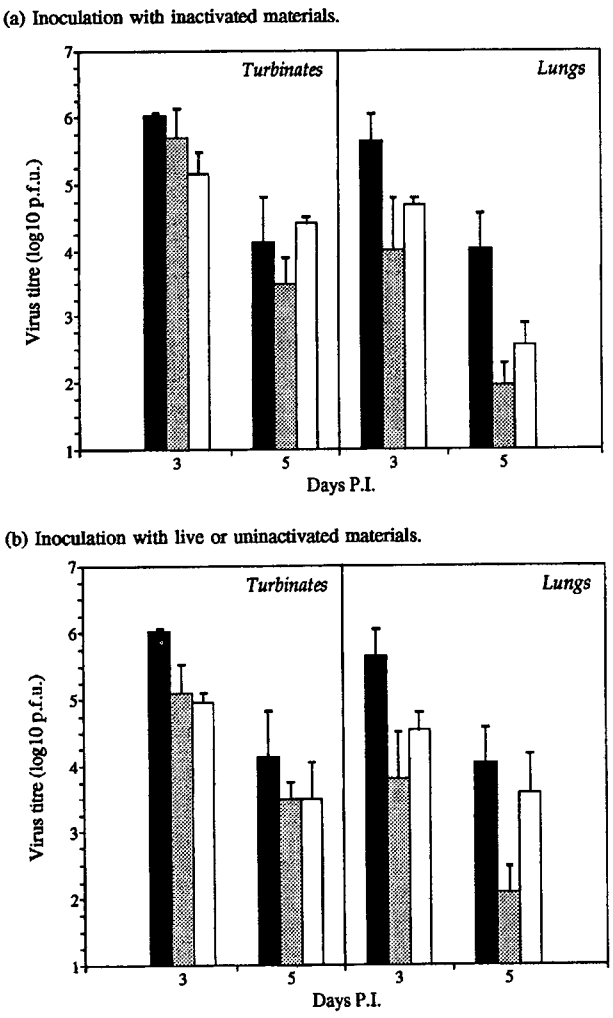


Fig. 4. Virus replication following i.m. challenge inoculation with EHV-1. Mice had been immunized twice (4 week interval) i.m. as follows: *(a)* inactivated L-particles (□) or inactivated virions (□), and *(b)* uninactivated L-particles (□) or live virions (□). Control mice were unimmunized (■). Four weeks after the second inoculation, mice were challenged with 6×10^6 pfu of EHV-1. Turbinates and lungs virus titres were measured at days 3 and 5 p.i. Bars represent the virus titre (geometric mean± s.d.; n=3).

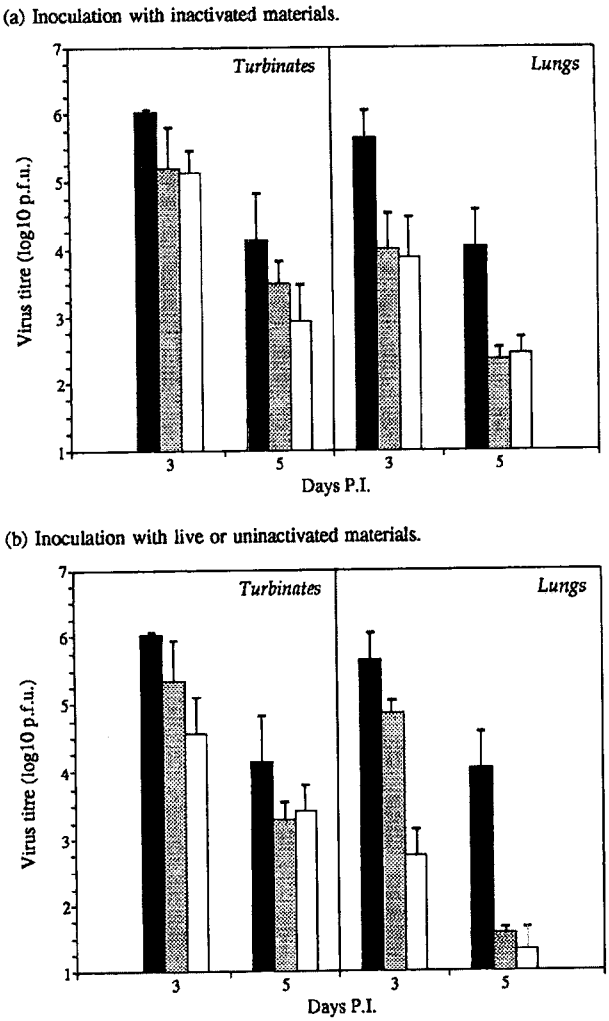


Fig. 5. Virus replication following i.v. challenge inoculation with EHV-1. Mice had been immunized twice (4 week interval) i.v. as follows: *(a)* UV-exposed L-particles (□) or inactivated virions (□), and *(b)* normal L-particles (□) or live virions (□). Control mice were unimmunized (■). Four weeks after the second inoculation, mice were challenged with 6×10^6 pfu of EHV-1. Turbinates and lungs virus titres were measured at days 3 and 5 p.i. Bars represent the virus titre (geometric mean ± s.d.; n=3).

very low following primary immunization. Following secondary inoculation, i.v.- and i.m.-inoculated mice showed a significant boost in antibody response, however, titers remained very low in i.n.-inoculated mice. This indicated the antibody response was well suppressed upon active EHV-1 replication in mice. The degree of suppression in antibody response in mice inoculated with live virus (or normal L-particles) was higher than in mice inoculated with UV-treated virus (or UV-treated L-particles). In all cases, when mice were challenged, antibody responses increased significantly except in mice that were previously immunized via i.n. route. Generally, immunization via i.n. route resulted very poor serum antibody responses as compared to other routes of immunization. The pattern of antibody response is consistent with the previous findings as reported by Azmi (1995).

In contrast, despite low antibody responses, the highest DTH response was shown in mice inoculated with live virus. Mice immunized via i.n. showed higher responses than i.v. or i.m. route. Upon challenge infection, mice that had been immunized i.n. with live virus (or with normal L-particles) showed no clinical signs of infection. It was also associated with a significant reduction in virus titers from respiratory tissues. These results are consistent with the process of recovery as indicated by an improvement in body weight. Protection was also correlated with an increase in the weight of the spleen. Heavier spleen weights are probably related to active cell-mediated immune

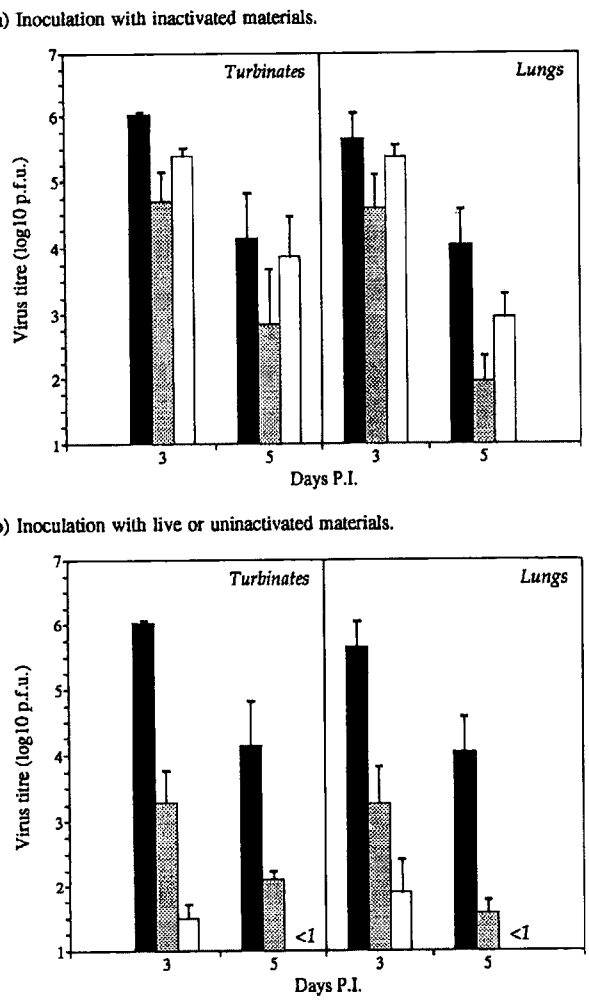


Fig. 6. Virus replication following i.n. challenge inoculation with EHV-1. Mice had been immunized twice (4 week interval) i.n. as follows: *(a)* UV-exposed L-particles (▨) or inactivated virions (□), and *(b)* normal L-particles (▨) or live virions (□). Control mice were unimmunized (■). Four weeks after the second inoculation, mice were challenged with 6×10^6 pfu of EHV-1. Turbinates and lungs virus titres were measured at days 3 and 5 p.i. Bars represent the virus titre (geometric mean ± s.d.; n=3).

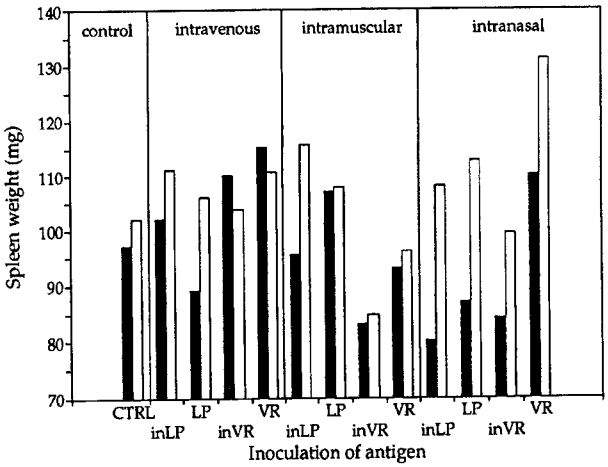


Fig. 7. The weight of spleens following challenge inoculation of mice previously immunized with EHV-1 virions or L-particles. The immunization was carried out as follows: UV-exposed L-particles (in LP); normal L-particles (LP); inactivated virions (inVR); or live virions (VR) via i.v., i.m. or i.n. The control mice (CTRL) were uninoculated. The mean weight of spleens (n=3) was determined at days 3 (■) and 5 (□) p.i.

responses which are likely to be related with recruitment of many immune cells against the infectious virus. In conclusion, immunization with L-particles or virions resulted in a protective immunity which was mediated by the immune cells. Immunization via i.n. route also resulted in the strongest cell-mediated immune response as compared with protection as induced by other means of immunization. These results are highly consistent with previous findings (Alber *et al.*, 1995; Allen *et al.*, 1995; Edens *et al.*, 1996; Siedek *et al.*, 1999).

Protection studies with L-particles for immunization of mice have been reported for HSV-1. However, in that case the HSV-1 L-particle preparations failed to give evidence of protection. This was probably due to the existence of certain virus transactivation factors (i.e. α-TIF and *vhs*) in HSV-1 L-particles (McLauchlan *et al.*, 1992) that may enhance virus infection. However, this phenomenon could not occur in EHV-1 since its L-particles did not appear to contain these factors, and therefore EHV-1 L-particles are highly suitable for a vaccine preparation. Protective immunity as observed following inoculation of mice with live virus suggested that virus replication is probably required to enhance cell-mediated immune responses ($CD4^+$ and $CD8^+$ T cells). However, this may suppress the antibody response during EHV-1 infection (Azmi and Field, 1993). The mechanism of antibody suppression, however, has yet to be defined. Consistent with previous observations, immunizations via i.n. route with inactivated preparations gave no protection to the mice. However, with the same material preparation, immunizations via i.v. and i.m. routes conferred partial protection, as indicated by a significant virus reduction in the lung but not in the nasal turbinate. Such protection is likely to be associated with high serum antibody responses, as demonstrated in this study. The antibody is, however, poorly neutralizing, therefore protection may have been mediated by another mechanism, e.g. ADCC.

In general, envelope glycoproteins of most viruses are important in inducing a desired immune response. Fractions of EHV-1-envelope containing glycoproteins, when used to immunize hamsters, gave protection from EHV-1 challenge infection (Papp-Vid and Derbyshire, 1978). The non-infectious L-particles of EHV-1 are completed with major glycoproteins that are normally present in mature virions. Therefore, immunization with L-particles confers a similar type of protection as described for individual glycoproteins. Inasmuch, hamsters immunized with particular individual envelope glycoproteins (i.e. gB, gC and

gD) were protected from EHV-1 infection (Guo, 1990, Tewari *et al.*, 1994; Tewari *et al.*, 1995; Osterrieder *et al.*, 1995; Stokes *et al.*, 1996). Particular envelope molecules had been used for vaccine preparation e.g. immune-stimulating complex (ISCOM) vaccine (Cook *et al.*, 1990; Ertuk *et al.*, 1992). In many cases, the acquired protection was mainly correlated with neutralizing antibody (Stokes *et al.*, 1989; Shimizu *et al.*, 1989). Immunization with EHV-1 gD was reported to confer protection in mice. In that experiment a neutralizing antibody response was observed, however, this was not thought to account for protection (Tewari *et al.*, 1994). Experiments with EHV-1 L-particles gave further indication that antibodies are not the main factor in protective immunity against EHV-1 infection. Envelope glycoproteins presented on EHV-1 L-particles probably elicit strong cell-mediated immune responses. In many cases, non-replicative materials (including EHV-1 L-particles or synthetic peptides) may stimulate cell-mediated response of a particular type only. Many may stimulate $CD4^+$ T cells rather than $CD8^+$ T cells. Therefore, it is desirable to delineate the protective immunodominant epitopes for the particular type of immune response desired. Our study has shown that the non-infectious L-particle of EHV-1 capable of inducing protective immunity as mediated mainly by cell-mediated immune components as well as antibody response, to a certain degree. L-particles, therefore, are highly recommended as a starting material for a suitable EHV-1 vaccine.

References

Alber, D.G., J. Greensil, R.A. Killington, and A. Stokes. 1995. Role of T cells, virus neutralizing antibodies and complement-mediated antibody lysis in the murine response against equine herpesvirus type-1 (EHV-1) infection of C3H ($H\text{-}2^k$) and BALB/c ($H\text{-}2^d$) mice. *Res. Vet. Sci.* 59, 205-213.

Allen, G. P. and J.T. Bryans. 1986. Molecular epizootiology, pathogenesis and prophylaxis of equine herpesvirus-1 infection. *Prog Vet. Microb. Immun.* 2, 78-144.

Allen, G., M. Yeargan, L.R. Costa, and R. Cross. 1995. Major histocompatibility complex class 1-restricted cytotoxic T-lymphocyte responses in horses infected with equine herpesvirus 1. *J. Virol.* 69, 606-612.

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

Azmi, M. and H.J. Field, 1993. Cell-mediated antiviral response to EHV-1 in a murine model by means of adoptive transfer of immune spleen cells. *Journal of General Virology* 74, 275-280.

Bidwell D.E., A.A. Buck, H.J. Deisfeld, B. Enders, J. Haworth, G. Hudldt, N.H. Kent, C. Kirsten, P. Mattern, E.J. Ruitenberg, and A. Voller. (1976). The enzyme linked immunosorbent assay (ELISA). *Bull. WHO* 54, 129-139.

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

Burrows, R., D. Goodridge, and M.S. Denyer. 1984. Trials of an inactivated equid herpesvirus-1 vaccine: challenge with subtype-2 virus. *Veterinary Record* 114, 369-374.

Clark, M.F. and D.J. Barbara. 1987. A method for the quantitative analysis of ELISA Data. *J. Virol. Meth.* 15, 213-222.

Cook, R.F., T. Oneill, E. Strachan, B. Sundquist, and J.A. Mumford. 1990. Protection against lethal equine herpesvirus type-1 (subtype-1) infection in hamsters by immune stimulating complexes (ISCOMS) containing the major viral glycoproteins. *Vaccine* 8, 491-496.

Gibson, J.S., J.D. Slater, A.R. Awan, and H.J. Field. 1992. Pathogenesis of equine herpesvirus-1 in specific pathogen-free foals: primary and secondary infections and reactivation. *Archives of Virology* 123, 351-366.

Guo, P. 1990. Characterization of the gene and an antigenic determinant of equine herpesvirus type-1 glycoprotein-14 with homology to gB-equivalent glycoproteins of other herpesviruses. *Gene* 87, 249-255.

Irmiere, A. and W. Gibson. 1983. Isolation and characterization of a non-infectious virion-like particle released from cells infected with human strains of cytomegalovirus. *Virology* 130, 118-133.

Mclauchlan, J. and F.J. Rixon. 1992. Characterization of enveloped tegument structures (L-particles) produced by alphaherpesviruses: integrity of the tegument does not depend on the presence of capsid or envelope. *J. Gen. Virol.* 72, 269-276.

Mclauchlan, J., C. Addison, M.C. Craigie, and F.J. Rixon. 1992. Non-infectious L-particles supply functions which can facilitate infection by HSV-1. *Virology* 190, 682-688.

Meulemans, G. and P. Halen. 1992. Enzyme-linked immunosorbent assay (ELISA) for detecting infectious laryngotracheitis viral antibodies in chicken serum. *Avi. Path.* 11, 361-368.

Osterrieder, N., R. Wagner, C. Brandmuller, P. Schmidt, H. Wolf, and O.R. Kaaden. 1995. Protection against EHV-1 challenge infection in the murine model after vaccination with various formulations of recombinant glycoprotein gp 14 (gB). *Virology* 208, 500-510.

Paap-Vid, G. and J.B. Derbyshire. 1978. The protective antigen of equine herpesvirus type-1. *Can. J. Comp. Med.* 42, 219-226.

Paap-Vid, G. and J.B. Derbyshire. 1979. The virus neutralizing activity of antibodies specific to the envelope and nucleocapsid of equine herpesvirus type-1. *Canadian Journal of Comparative Med.* 43, 231-233.

Rixon, F.J., C. Addison, and J. Mclauchlan. 1992. Assembly of enveloped tegument structures (L-particles) can occur independently of virion maturation in herpes-simplex virus type 1-infected cells. *J. Gen. Virol.* 73, 277-284.

Roizman, B., R.C. Desrosiers, B. Fleckenstein, C. Lopez, A.C. Minson, and M.J. Studdert. 1992. The family *Herpesviridae:* an update. *Arch. Virol.* 123, 425-449.

Siedek, E.M., M. Whelan, N. Edington, and A. Hamblin. 1999. Equine herpesvirus type 1 infects dendritic cells in vitro: stimulation of T lymphocytes proliferation and cytotoxicity by infected dendritic cells. *Vet. Immun. and Immunopath.* 67, 17-32.

Shimizu, M., K. Satou, and N. Nishioka, 1989. Monoclonal antibodies with neutralizing activity to equine herpesvirus 1. *Arch. Virol.* 104, 169-174.

Stokes, A., P.K. Murray, G.P. Allen, and L.A. Pullen. 1989. A Hamster model of equine herpesvirus type-1 (EHV-1) infec-

tion-passive protection by monoclonal-antibodies to EHV-1 glycoprotein-13, glycoprotein-14 and Glycoprotein-17/18. *J. Gen. Virol.* 70, 1173-1183.

Stokes, A., D.G. Alber, R.C. Cameron, R.N. Marshal, G.P. Allen, and R.A. Killington. 1996. The production of a truncated form of baculovirus expressed EHV-1 glycoprotein C and its role in protection of C3H (H-2K^k) mice against virus challenge. *Virus Research* 44, 97-109.

Szilagyi, J.F. and C. Cunningham. 1991. Identification and characterization of a novel non-infectious herpes-simplex virus-released particle. *J. Gen. Virol.* 72, 661-668.

Telford, E.A.R., M.S. Watson, K. McBride, and A.J. Davison. 1992. The DNA sequence of equine herpesvirus-1 *Virology* 189. 304-316.

Tewari, D., J.M. Whalley, D.N. Love, and H.J. Field. 1994. Characterization of immune responses to baculovirus expressed equine herpesvirus 1 glycoprotein D and H in a murine model. *J. Gen. Virol.* 75, 1735-1741.

Tewari, D., S.V. Nair, M. De Ungria, G.L. Lawrence, M. Hayden, D.N. Love, H.J. Field, and J.M. Whalley. 1995. Immunization with glycoprotein C of equine herpesvirus-1 is associated with accelerated virus clearance in a murine model. *Arch. Virol.* 140, 789-797.

Voller, A., D. Bidwell, and A. Barlett. 1980. Enzyme linked immunosorbent assay, in research. In: Manual of clinical immunology. Edited by H. Friedman. *American Society of Microbiology* pp. 359-371.