The Journal of Microbiology, September 2002, p.183-192 Vol. 40, No. 3

Protection of Specific-pathogen-free (Spf) Foals from Severe Equine Herpesvirus Type-1 (Ehv-1) Infection Following Immunization with Non-infectious L-particles

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(Received May 8, 2002 / Accepted June 15, 2002)

Cells infected with equine herpesvirus type-1 (EHV-1) produced both infectious and non-infectious virus-related particles. Compared to the whole virion, non-infectious particles termed L-particles were determined to lack 150 kDa protein, commonly known as nucleocapsid protein. The potential of L-particles to induce immune responses was studied in mice and foals. Intranasal immunization with L-particles or whole virions induced poor IgG antibody responses in mice. Interestingly, despite the poor antibody response, the conferred immunity protected the host from challenge infections. This was indicated by a significant reduction in virus titers in line with recovery towards normal body weight. Subsequently, the test on the usefulness of L-particles as immunizing agents was extended to foals. Immunization of specific-pathogen-free (SPF) foals resulted in similar results. As determined by a complement-fixing-antibody test (CFT), foals seroconverted when they were immunized either with inactivated L-particles or whole virions via intramuscular (i.m.) injections. The presence of the antibody correlated with the degree of protection. Beyond day 1 post challenge infection (p.i.), there was no virus shedding in the nasal mucus of foals immunized with whole EHV-1 virions. Virus shedding was observed in foals immunized with L-particles but limited to days 6 to 8 p.i. only. In contrast, extended virus shedding was observed in non-immunized foals and it was well beyond day 14 p.i. Viremia was not detected for more than four days except in non-immunized foals. Immunization in mice via intranasal (i.n.) conferred good protection. However, compared to the i.n. route, a greater degree of protection was obtained in foals following immunization via i.m. route. Despite variation in the degree of protection due to different routes of immunization in the two animal species, our results have established significant evidence that immunization with L-particles confers protection in the natural host. It is suggested that non-infectious L-particles should be used as immunizing agents for vaccination of horses against EHV-1 infection.

Key words: equine herpesvirus type-1, L-particles, SPF foal, immunity, antibody response

Equine herpesvirus type 1 (EHV-1) infections in horses result in respiratory disease, miscarriages and central nervous system (CNS) disorders (Mukaiya *et al.*, 2000). Symptoms of nervous involvement range from gait abnormalities, partial sensory impairment, paralysis and death. Miscarriages normally occur in mares following infection of the placenta, while *in utero* infection may cause severe pulmonary problems in neonates when they were born alive (Perkins *et al.*, 1999). The virus shed in nasal secretions and aerosol is believed to be a major source of EHV-1 that spreads among horses that share the same airspace. The virus is also transmitted transplacentally and via aborted fetuses. Following an infection, naïve susceptible animals will remain latent carriers. Stress and treatment with corticosteroids will reactivate the latent virus and produce virus replication and viral shedding. There are several vaccines available commercially, but existing immunizing agents employed in those vaccines have not proven to be effective. Infected animals which have recovered from natural infection may produce high antibody titers. However, such animals are not spared from repeated attacks from the virus. This generally indicates that harboured immunity is short-lived.

Virions of herpesviruses contain the genome at the core, which is housed in a nucleocapsid, and surrounded by an amorphous layer of tegument and envelope proteins form-

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ing the outer layer of the virion structure. The virus possesses a linear, double-stranded DNA genome of approximately 150 kbp, encoding some 76 distinct genes. There are about 29 viral polypeptides in the tegument and envelope; 15 to 18 polypeptides in the tegument and 11 glycoproteins in the envelope. At least ten membrane-anchored glycoproteins are encoded by EHV-1, the majority of which are present in the virion envelope and which mediate virus entry into cells. Many of these glycoproteins are immunologically important and serve to stimulate antiviral immunity in the host. Regulatory proteins of EHV-1, which are normally present in the tegument and other parts of virions other than the envelope, contribute minimally to cell-mediated antiviral immunity. In contrast, glycoproteins gC, gD, gB, gH and gL, that are present in the viral envelope, serve as important determinants to the virus pathogenicity and immunogenicity. These glycoproteins are important, without which the virus becomes unable to infect target cells. An infection of the target cells begins from primary attachment of glycoprotein C (gC) to the lipid-bilayer cell membrane. It is followed by more stable attachments of gB and gD. Subsequently gD interacts with the gH/gL complex, and along with gB this complex activates fusion events which enable the virus capsid to enter the cell (Neubauer *et al.*, 1997; Granzow *et al.*, 2001). Interference in these events will block virus entry and thus prevent virus replication. The importance of these glycoproteins in inducins the desired immune responses and protection to the host has been shown in many instances. Individual gC and gD are incorporated into individual solid matrix-antibody-antigen (SMAA) complexes or immuno-stimulating complexes (ISCOMs) and are injected into the host to induce antibodies that neutralise virus infectivity and mediate the lysis of EHV-1-infected target cells (Hannant *et al.*, 1993; Alber *et al.*, 2000). Immunization with baculovirus expressing EHV-1 gD also confers protective humoral and cellular immune responses (Tewari *et al.*, 1994). Mice immunized with a recombinant baculovirus, expressing the large and small subunits gB precursors, produce complement-dependent virus neutralising antibodies, and evoke protective immune responses against acute EHV-1 infection (Kukreja *et al.*, 1998; Munro *et al.*, 1999). The use of glycoproteins gB, gC and gD for immunization, employed either singly or in combination with at least two glycoproteins, also resulted in similar effects (Packiarajah *et al.*, 2000; Stokes *et al.*, 2000; Csellner *et al.*, 2000; Walker *et al.*, 2000; Ruitenberg *et al.*, 2000). Thus, protective immunity can be obtained by immunizing the host with glycoproteins which are expressed by recombinant expression systems or by the use of monoclonal antibodies to these glycoproteins for passive immunization.

Cells infected by herpesviruses, including herpes simplex virus (HSV-1), pseudorabies virus (PrV), and EHV-1, also produce virus-related light particles (L-particles) containing envelope and tegument proteins but lack the nucleocapsid and DNA (Szilagyi and Berriman, 1994). L-particles have been defined as non-infectious virion-related particles that lack the nucleocapsid but do contain teguments and envelopes. The tegument is a complex assemblage of virus proteins whose relative proportions within virions are essentially constant for a particular strain of virus. It has been demonstrated that the tegument and envelope can combine to assemble mature particles whose properties are indistinguishable from those of virions during the early events after infection (Szilagyi & Cunningham, 1991). The compositions of the tegument and envelope in virions and L-particles are also very similar, hence, interaction with the capsid is not a primary determinant for incorporation into either of these substructures of virions (Rixon *et al.*, 1992). Interactions between the tegument and envelope components might play a critical role in particle assembly and maturation. As the tegument and envelope contain all the necessary proteins, L-particles are as efficient as virions in supplying these proteins in the fully functional state. Thus, L-particles are biologically competent and have the potential to participate in the early stages of virus infection. L-particles at more than 1000 particles per cell may interfere with virion adsorption and penetration. They also induce a progressive increase in plaque numbers in cell culture. The enhancement obtained could be the role of L-particle tegument proteins including Vmw110 (ICP0) and Vmw65 (alpha-TIF) proteins, but not VP11/12, VP13/14, and *vhs* (McLauchlan *et al.*, 1994; Yang *et al.*, 1995; Sathananthan *et al.*, 1996). L-particles of each different virus, to a varying extent, may enhance the plaquing efficiency of their own individual viral DNA and were also effective in heterologous combinations (Dargan and Subak-Sharpe, 1997).

Interestingly, L-particles contain an intact envelope with all immunogenic membrane glycoproteins required to elicit a protective immune response. Therefore, L-particles are potentially superior immunogenic over the protein subunit or recombinant viruses that deliver each viral glycoprotein individually. It is suggested that L-particles could be used as an alternative immunogenic material of virus origin for preparation of vaccines against EHV-1 infection. In this report, we describe the host immune response following vaccination of mice and horses with EHV-1 L-particles. The antibody response and protective immunity were evaluated by serology and virus challenge tests.

Materials and Methods

EHV-1 L-particles and virions

The purified forms of L-particles and virion of EHV-1 strain AB4p were prepared as described previously (Szilagyi and Cunningham, 1991; McLauchlan and Rixon,

1992). Gibson *et al.* (1992) has described the pathogenicity of the parental strain of AB4 (AB4p) used in this study.

The morphology of EHV-1 virions and L-particles was visualized by means of electron microscopy technique according to the standard negative staining method that employs phototungstic acid. A procedure of discontinuous, denaturing gel electrophoresis SDS-PAGE was performed as described previously by McLauchlan and Rixon (1992). Briefly, viral proteins were separated on gels containing 12% acrylamide cross-linked with 2.5% (wt/wt) N,N' methylene bis-acrylamide. Polymerization was initiated by addition of 0.04% TEMED and 0.06% APS. Samples were heated to 100°C for 5 min in a boiling mix prior to loading on the gel. Electrophoresis was performed for approximately 1 hr at 120 V to 150 V or overnight at 40 V using the buffer system of Laemmli (1970). Proteins were detected by staining with Coomassie Brilliant blue for 20 min followed by destaining. This procedure was to reveal protein profiles of virions and L-particles.

Inoculation of mice

Three groups of 15 four-week-old female BALB/c mice were inoculated via the intranasal (i.n.) route as follows. The first group of mice was inoculated with cell-lysate and served as the control. The second group of mice was inoculated i.n. with 10^8 particles of purified live EHV-1 virions in 40 ml volume. The third group of mice was inoculated with 10^9 purified EHV-1 L-particles. Serum samples were collected from the tail vein and subjected to enzyme-linked immunosorbent assay (ELISA) to measure IgG antibody titers.

Four weeks later, all mice were given a challenge inoculation i.n. with 6×10^6 p.f.u. of EHV-1. The body weight of individual mice was determined everyday until day 10 post-infection (p.i.). Groups of four mice were killed at days 3 and 5 p.i. Nasal turbinates and lungs were collected individually in 1 ml ice-cold serum-free Dulbeccios Minimum Essential Medium (DMEM) containing 8% antibiotic-antimycotic solution and 2% anti-PPLO agent. Tissues were minced thoroughly with scissors and homogenized with an electric homogenizer (Thyristor Regler TR50, IKA-WERK, Germany). The tissue suspension was subjected to sonic vibration for 2 minutes in an ice-cold sonic water bath. This was to release cell-associated viruses. The homogenate was transferred to Eppendorf tubes, and spun at 700 g for 10 min at 4°C in a refrigerated centrifuge (Hettich EBA 12R, Germany). This step was to remove cellular debris from the virus suspension. Supernatants were kept at 70°C and titrated by means of plaque forming assays. In some cases, the entire undiluted supernatants were tested for virus.

Inoculation SPF foals

A total of five specific-pathogen-free (SPF) Welsh pony foals were used in the study. Throughout the study, these animals were kept in individual isolation. The first and the second foals were inoculated i.n. and intramuscularly (i.m.) respectively with 10^9 L-particles; the third foal was injected i.m. with *uv*-inactivated virions at p.f.u. of 10^6. Since foals are very susceptible to live virus, purified virions were previously inactivated by means of *uv*-light irradiation (254 nm wavelength) at the dose of 6×40,000 $\mu J/cm^2$. Four weeks later, foals were given a second inoculation with the same materials via the same route. The two foals, serving as controls, were not inoculated. Following primary inoculation, foals were tested for seroconversion for complement-fixing antibodies by the use of complement-fixing antibody test (CFT). Four weeks after the second inoculation, each foal was given a challenge inoculation i.n. with 10^7 p.f.u. of virulent parental strain EHV-1, strain AB4.

Virus titration by plaque forming assay

A confluent monolayer of Vero cells was prepared in 24-well tissue culture plates (Costar, USA). A ten-fold serial dilution of the virus sample was prepared in serum-free DMEM medium. One hundred µl of each dilution was placed onto confluent monolayers in duplicate. The virus was allowed to adsorb into cells at 37°C for 45 min. Five hundred µl of overlay DMEM medium containing 2% fetal calf serum (FCS), 1.2% carboxylmethylcellulose (CMC), 1% antibiotic-antimycotic and 1% anti-PPLO solutions was added to each well, and the plate was incubated further at 37°C. The plate was periodically monitored over 48 to 72 hr p.i. After 3 or 4 days incubation, the overlay medium was removed and the plate was rinsed twice in phosphate buffered saline (PBS). The cell monolayer was fixed in 70% methanol for 10 min and stained in 2.5% alcoholic crystal violet solution for 5 to 10 min. The plate was rinsed gently in tap water and allowed to air-dry at room temperature. Plaques were counted in an inverted-light microscope (Olympus CK2, Japan). Virus titers were calculated as the mean number of plaques developed from the last sample dilution that showed plaques in monolayer multiplied by the reciprocal of that dilution and expressed as $\log_{10}$ p.f.u. per sample. The limit of sensitivity of the test was 10 p.f.u. per sample.

Detection of viremia by plaque forming assay

Blood samples of SPF foals were collected from the jugular vein into a tube containing EDTA. Blood samples were transferred into Eppendorf tubes and centrifuged in a microfuge at 5,000 g for 10 min. The buffy-coat layer was separated and treated with distilled water for 15 sec, thus causing lysis of erythrocytes. The osmotic balance was then quickly restored and cells were washed three times in DMEM. Cells were counted in a hemacytometer chamber by using an inverted light microscope. Cell-associated viruses were detected by co-cultivating peripheral

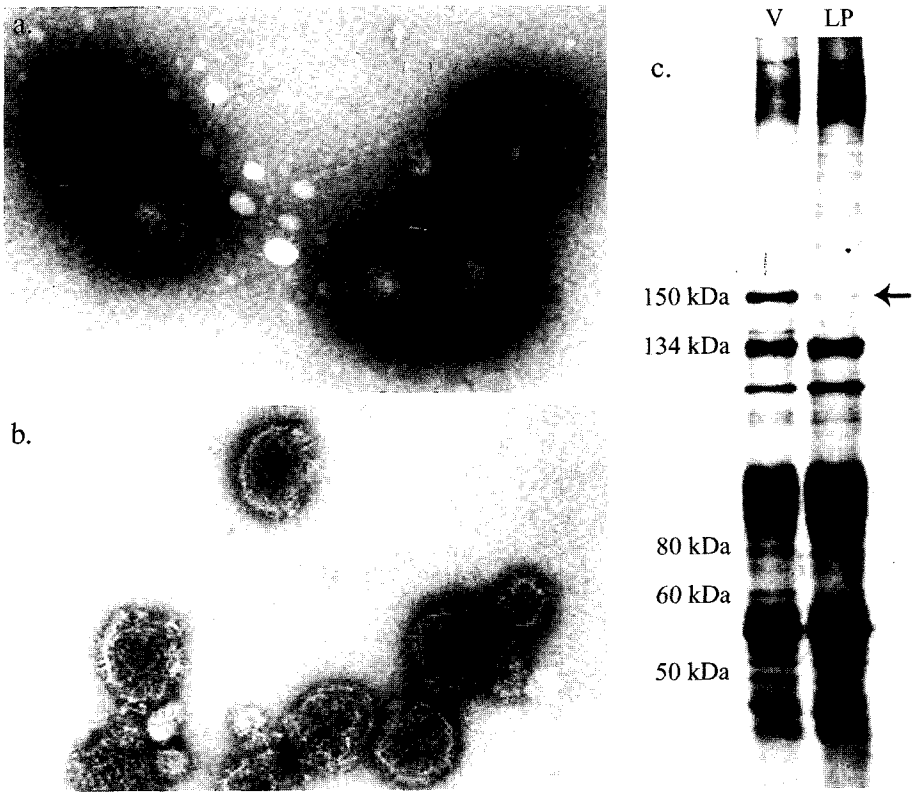

Fig. 1. The analysis of L-particles: (a) photomicrograph of whole virions (magnification 80,000×); (b) photomicrograph of L-particles (magnification 80,0000×) showed the absence of nucleocapsids in the core, and (c) protein profile of L-particles to indicate the disappearance of 150 kDa capsid protein from L-particles (L) as compared to the whole virion (V).

blood leucocytes (PBLs) directly into 24-well plates containing confluent RK-13 cell monolayers and incubated at 37°C for 8 to 12 days. Crystal violet solution was used to stain cell monolayers in the plate and the number of plaques appearing per 10^6 PBLs was determined. Whenever no virus plaque was observed prior to cell staining, the cell monolayer was harvested and used for a second passage of virus cultivation. The result of the second co-cultivation, however, was not directly related with the number of infected PBL cells. Results of this test were scored either "negative" or "positive" with regard to the appearance of virus plaques regardless of their amount.

Complement-fixing antibody test (CFT)

The complement-fixing antibody test was conducted in accordance with well-established protocols (Jenny *et al.*, 1958)). Pre-immune and positive sera were used as controls. In brief, horse serum samples were heat-inactivated at 56°C for 30 min to inactivate the indigenous complement. Serum samples were two-fold serially diluted in veronal buffered saline (VBS). Purified viral antigens (100 μg per ml) were mixed with an equal amount of diluted sera (25 μl each) in a round-bottom 96-well microtiter plate (Dynatech, USA). Guinea pig complement (Nordic Immunology, Sweden) was prepared in VBS and 25 μl of complement solution containing 4 CHU_{50} (50% complement hemolytic unit) was added to each well. The reagents were mixed by shaking and the plate was incubated at 37°C for 1 hr in a humidified chamber. A suspension of 2% sheep red blood cells (SRBC) was prepared in VBS and sensitized with 10 HU_{50} (50% hemolytic unit per ml) of rabbit anti-SRBC antisera (Nordic Immunology, Sweden). Fifty ml of sensitized-SRBC suspension was added to each well. The plate was then incubated at 37°C for 30 min. Subsequently, the plate was centrifuged (Hettich EBA 22R, Germany) at 250 g for 1 min and the absorbance was read spectrophotometrically at 545 nm. The serum sample dilution was determined to be positive when the hemolytic system had an absorbance of 0.66 or less (equivalent to <20% hemolysis as in the negative controls). All serum samples were tested in duplicate and an average complement-fixing antibody titer determined.

ELISA

The indirect enzyme-linked immunosorbent assay (ELISA) was based on well-established principles and protocols with some modifications (Azmi, 1995). The test was carried out with a working volume of 50 μl of each reagent. The purified viral antigen was diluted in bicarbonate

buffer to give a concentration of 10 μg per ml antigen protein. Each well of a 96-well plate (Dynatech Immulon, USA) was coated with 50 μl of the antigen solution, and incubated at 4°C overnight. The plate was washed three times with phosphate-buffered saline Tween 20 (PBST) using an automated microplate washer (Dynatech. MR 7000, USA). Fifty μl of 2% BSA- Fraction V (Sigma, UK) was added and the plate incubated at 45°C for two hr. The plate was washed three times as aforementioned. Ten-fold serially diluted test sera were added and the plate was incubated at 37°C for one hr. The plate was washed three times. Fifty μl of pre-diluted goat anti-mouse peroxidase conjugated anti-IgG immunoglobulin (Sigma, UK) was added and allowed to react with antigen-bound mouse IgG antibodies, at 37°C for one hr. The plate was washed three times. The 2,2-azino-bis (3-ethylbenzthioline-6-sulfonic acid) (ABTS) substrate (Sigma, UK) prepared in citrate-phosphate buffer (CPB) contained 0.01% of 30% H_2O_2 was added and the plate was incubated at room temperature in the dark for 30 to 40 min. The plate was read in a spectrophotometer (Dynatech. MR 7000, USA) at dual-wave length mode with absorbance of 410 and 490 nm respectively. Hyperimmune and pre-immune sera were included in the plate as positive and negative controls. 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 that 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 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).

Results

The whole virions and L-particles mixture can be separated by sucrose gradient purification with regard to a sharp lower band containing almost exclusively virions and a more diffuse upper band consisting of L-particles. As visualised in an electron microscope, a dense core structure, normally present in EHV-1 virions (Fig. 1a) disappeared from the L-particles (Fig. 1b). The L-particles appeared to be spherical entities with significant variation in size, on average smaller in diameter (140 nm) than that of virions (180 nm). Many polypeptides of the viral envelope and the tegument were found to be common in both types of particles (Fig. 1c). Results showed L-particles did not contain 150 kDa polypeptides, which represents the capsid protein of complete virions. L-particles are composed of an outer envelope, i.e. a bilaminar membrane with protruding glycoprotein spikes, and a uniformly granular tegument, but lack any nucleocapsid. L-particles may also contain unique inclusion vesicles as revealed in the form of one or more spherical objects embedded in the tegument of a large proportion of L particles but not observed in virions (Rixon *et al.*, 1992).

Mice primarily inoculated with purified live virions showed mild clinical signs of the disease e.g., ruffled hair and respiratory distress, but recovered by about one week p.i. No mice in other groups showed clinical signs or mortality. However, no clinical signs were observed in any group of mice upon second inoculation. Upon challenge inoculation at four weeks after the second dose of immunization, control mice that had received a placebo were highly susceptible to EHV-1 infection. Accordingly, mice contracted a severe disease as characterized by respiratory distress, significant reduction in body weight and mortality in some cases. At day 8 p.i., significant virus titers were observed in lungs and turbinates. In contrast, mice primed with whole virions were protected fully from the development of the severe disease of EHV-1. Virus titer in the lungs and nasal turbinate was reduced significantly as observed on day 3 p.i. and declined to an undetectable level by day 5 p.i. (Fig. 2). Similarly, virus titers in mice previously immunized with L-particles declined significantly as compared to those of the control mice but the virus titers were somewhat higher than those of the mice previously immunized with whole virion. Viruses were detected in respiratory tissues on day 5 p.i.; nevertheless, virus titers were declined to undetectable levels by day 8 p.i. No signs of EHV-1 disease were developed and no mortality was observed in mice previously immunized with whole virions or L-particles. These results indicated that immunization with L-particles conferred protection from the development of respiratory disease. However, a classical protection from virus replication in respiratory tissues was afforded when the whole virion was used in

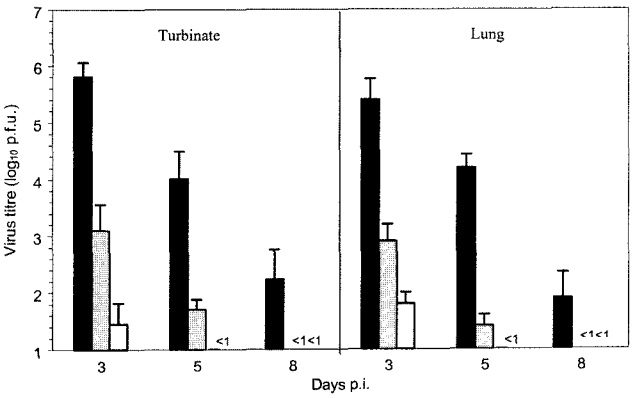

Fig. 2. The efficacy of L-particles as an immunizing agent in mice. Virus titers in nasal turbinate and lungs were significantly reduced upon immunization with L-particles (▩) and whole virions (□), as compared to non-immunized (■) mice. Immunization with whole virions resulted in a significantly rapid virus clearance from the respiratory tissues.

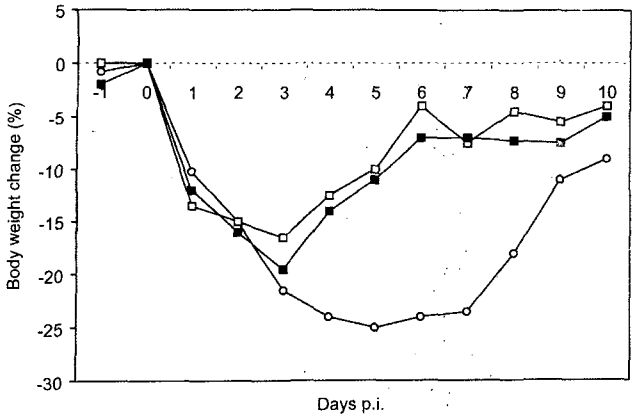

Fig. 3. The rate of recovery from infection based on body weight. Mice immunized with L-particles (□) recovered at a similar rate as mice immunized with whole virions (■) towards the normal body weight. However, the body weight performance of non-immunized mice (○) was very poor.

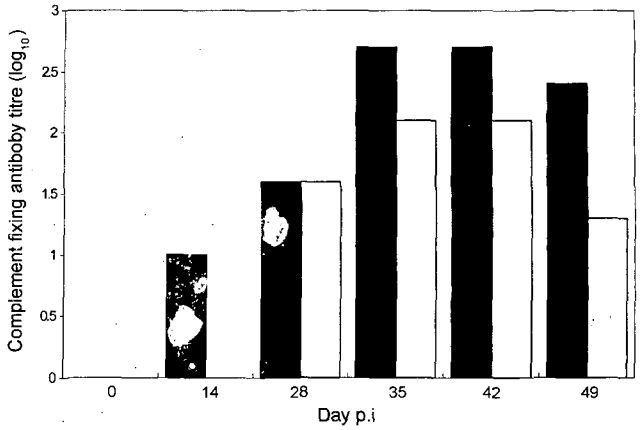

Fig. 5. Complement fixing antibody titers in SPF foals. Foals immunized via i.m. with whole virions (■) or L-particles (□) were seroconverted. No antibodies to EHV-1 antigen were detected in foals immunized with L-particles via the i.n. route.

immunization, and this is congruent with previous observations (Mohd-Azmi *et al.*, 2002). These results were substantiated by the physical performance of animals as measured accordingly with reference to their body weight (Fig. 3). Mice immunized with whole EHV-1 virions or L-particles showed early recovery to their normal body weight beginning from day 4 p.i. In contrast, poor body weight performance and recovery was observed in non-immunized control mice.

Generally, IgG antibody titers were relatively low following primary inoculation with whole virions or L-particles. Although the second inoculation was given four weeks later, it failed to induce a significantly higher antibody response (Fig. 4). This is not surprising since this phenomenon has been well documented in other studies (Azmi & Field, 1993; Mohd-Azmi *et al.*, 2002). Furthermore, antibody titers do not correlate well with the degree of immunity against EHV-1 infection. Despite low antibody response, mice immunized i.n. with whole virions or L-particles were protected from severe EHV-1 disease. These results were used as a basis for subsequent experimental immunization in SPF foals.

Foals inoculated with whole virions or L-particles i.m. were seroconverted i.e., antibody titers of 1 $\log_{10}$ at day 14 p.i., as determined by complement-fixing antibody test (CFT). Antibody titers in these foals increased further to at least 2 $\log_{10}$ after second inoculation at day 28 p.i. (Fig. 5). However, three weeks later, after the second inoculation, the foal inoculated with L-particles had at least 1 $\log_{10}$ lower antibody titer as compared to that of the foal inoculated i.m. with the whole virion. Surprisingly, despite a booster dose four weeks after the primary inoculation, at no time were antibodies detected in the foal

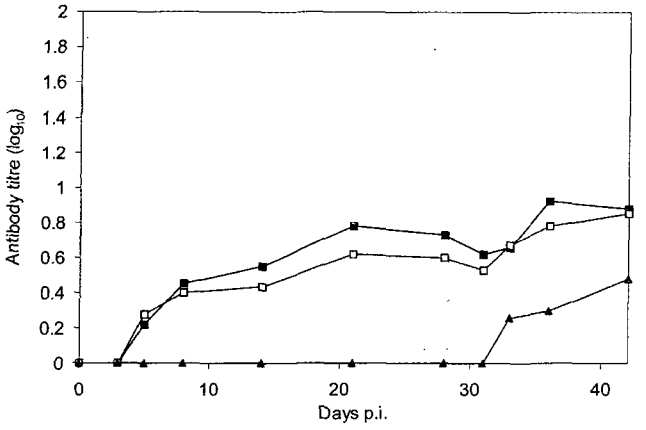

Fig. 4. Antibody response to EHV-1 as measured by means of ELISA. Immunization of mice with with L-particles (■) or whole virions (■) resulted in poor serum IgG antibody response. Poor antibody titers were noted in non-immunized mice (▲) upon virus challenge inoculation at day 28 p.i.

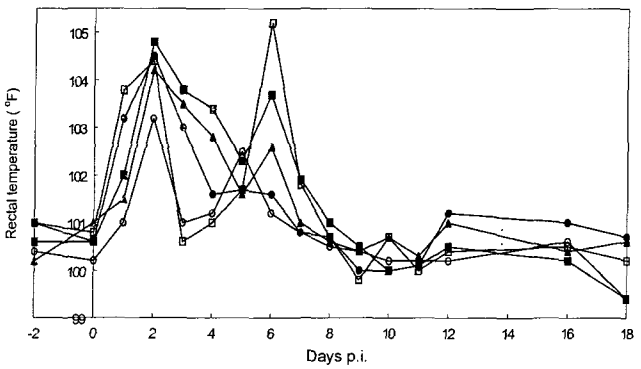

Fig. 6. Rectal temperature of foals following challenge inoculation with EHV-1. Pyrexia was noted in all foals. Biphasic pyrexia noted in non-immunized foals (▩ and ▲) and the foal immunized with L-particles via i.n. (□). To some extent, a smaller degree of biphasic pyrexia was noted in foals immunized previously with L-particles (●) or whole virions (○) via the i.m. route.

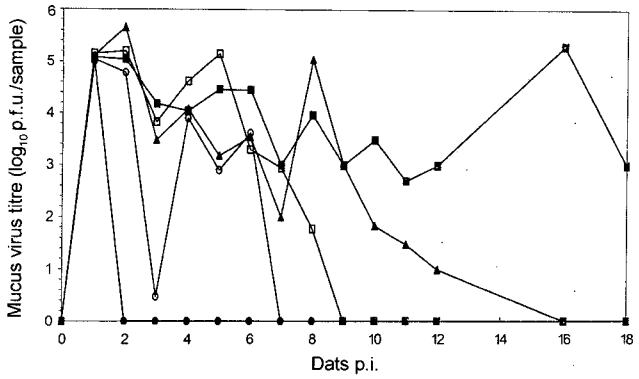

Fig. 7. Virus shedding in the nasal mucus of foals upon challenge inoculation. Protracted period of virus shedding noted in non-immunized foals (■ and ▲). The period of virus shedding declined in foals immunized with L-particles via i.n. (□) or i.m. (○) route. The shortest period of virus shedding was noted in the foal immunized with whole virion via the i.m. route (●).

immunized i.n. with L-particles.

Immunization with whole virions or L-particles did not prevent foals from developing pyrexia upon subsequent EHV-1 infection (Fig. 6). Upon challenge inoculation, biphasic pyrexia was noted in all five foals with rectal temperatures ranging from 102-105°F; peaked at days 2 and 6 p.i. Enlargement of palpable lymph nodes was noted in all three immunized foals. The infected non-immunized control foals were subjectively more depressed and weaker. However, clinical signs developed in immunized foals were somewhat milder than that of the two control foals. Nasal mucus samples were tested for virus in RK-13 monolayers. Infectious virus was detectable in all mucus samples of foals beginning from day 1 following challenge inoculation (Fig. 7). No virus was detected in the mucus after day 1 p.i. in the foal which had been previously immunized i.m. with inactivated virions. Viruses were detected in the mucus of foals immunized with L-particles either via the i.m. or i.n. route, with titers between 3 to 5 log_{10} p.f.u. per sample. The virus was undetectable in the mucus of these foals beyond day 8 p.i. In contrast, the virus was detected in the mucus of two non-immunized control foals until as long as 18 days p.i.

Following challenge inoculation, no foals escaped from viremia. Viremia was detected as confirmed by means of infectious center assay but with varying duration. It was noted a two-day viremia in the foal immunized i.m. with inactivated virions and four-day viremia in foals immunized with L-particles i.m. or i.n. route (Table 1). A longer period of viremia, which was extended up to 9 days p.i., was observed only in the non-immunized control foals. No viremia was observed in foals beyond day 9 p.i.

Discussion

L-particles are distinct from defective interference particles (DIP) where the latter is related with mutation and the maintenance of virus propagation in infected cells (Harty *et al.*, 1993). In contrast, the former is related with abnormal formation of enveloped particles without nucleocapsids under unfavorable conditions as produced by temperature sensitive mutants. The availability of EHV-1 L-particles that lacked nucleocapsid proteins and genomic materials has enabled the analysis on the use of non-replicating virus-related particles as immunizing agents. Results clearly indicated that L-particles contained all necessary proteins, which are essential for virus entry. This study showed L-particles potentially induced immune responses in the host and conferred protection against EHV-1 infection.

Immunization of mice via the i.n. route with live virus consistently resulted in better immunity than that via the i.m. route (Azmi and Field, 1993). The replicating viruses may strongly induce the functionally active lymphocytes, which are always available in the mouse respiratory system, especially in the lungs, that involve T or B cells (Abraham *et al.*, 1990). Other immune factors including natural killer (NK) cells may mediate lysis of virally infected cells by means of antibody-dependent cell-mediated cytotoxicity. Localised production of cytokines to include interferon and tumour necrosis factor will also inhibit virus replication directly (Vacheron *et al.*, 1990). However, there was no evidence available to describe such mechanisms in the respiratory tract of horses. Our results showed that immunization of foals with inactivated antigens via i.m. conferred better protection than that of the i.n. route. It is very likely that the differences in animal species and cell mass may contribute to the differ-

Table 1. Viremia detected in SPF foals following challenge inoculation with EHV-1

Inoculum (route)	Days after challenge inoculation											
	-2	0	1	2	3	4	5	6	7	8	9	10-28
Virions (i.m.)	-	-	-	-	-	-	+	-	+	-	-	-
L-particles (i.n.)	-	-	-	-	-	+	+	+	-	-	-	-
L-particles (i.m.)	-	-	-	-	+	+	+	+	-	-	-	-
control 1	-	-	+	-	+	+	+	+	+	-	+	-
control 2	-	-	-	-	+	+	+	+	+	-	-	-

+ viraemia.
– no viraemia

ences in types of immune cells and defense mechanisms available in the lungs. Differences in these results might also be subjected to the capability of the virus to replicate in the host and the effective route of immunization. The L-particle is neither infectious nor replicating. Since the present immunogen does not replicate and is unable to spread infectiously between cells beyond the site of inoculation, it is safe to predict that the efficacy of L-particles for immunization would be at least similar or superior to that of killed viruses. The preparation of L-particles does not involve the chemical treatment of potential antigens where such process may alter the antigenic conformation of the viral proteins. Therefore, the L-particle, in theory, is a naturally better immunogen than killed viruses. However, results showed the non-replicating L-particle, as an immunogen, is less effective than the live virus to confer protection to the host. Therefore, the amount of L-particles required for immunization should be increased to compensate for the lower bioavailability of viral antigen due to the inability of the L-particles to replicate as compared to the live virus.

Our results indicated that an active virus replication is required to induce an optimum immune response. When live viruses infect a permissive cell, the cell will actively synthesize and process viral proteins. Viral antigens synthesized will form a complex with the class I major histocompatibility complex. This complex is displayed on the cell membrane to stimulate $CD8^+$ cytotoxic T cells resulting in the specific killing of virally infected cells. In contrast, cells exposed to non-replicating virus will process viral antigens and form a complex with the class II major histocompatibility complex. In contrast, the antigen-class II MHC complex is displayed on the cell membrane. This induces the $CD4^+$ T cell-mediated immune response which is responsible for the production of specific antibodies against the virus. Antibodies may neutralize extracellular viruses but they are not effective against those which remain in infected cells. The replication cycle of intracellular viruses will only be interrupted when infected cells are destroyed by $CD8^+$ cytotoxic T cells.

L-particles were tested in mice for immunization against herpes simple virus type-1 infection. However, the experiment did not produce evidence of protection conferred in mice. In contrast to EHV-1, L-particles of HSV-1 contained certain virus transactivation factors to include alpha-TIF and *vhs* (McLauchlan *et al.*, 1992). Both factors enhance virus infection to the permissive cells. However, EHV-1 L-particles do not appear to contain these factors. Despite poor antibody responses, both in IgG and IgA antibody isotypes (Azmi, 1995), immunization with L-particles or whole virions conferred immunity against subsequent EHV- challenge infection in mice. This study also supported further the protective immunity conferred following immunization of mice with L-particles is associated with the role of cell-mediated immune responses (Mohd-Azmi *et al.*, 2002).

Horses inoculated with EHV-1 may produce high antibody titers. However, virus replication is associated with marked suppression of T and B cells (Charan *et al.*, 1997). The suppression is likely to be associated with the condition where T cells of $CD4^+$ and $CD8^+$ phenotypes harbour the virus during the viremic phase (Baxi *et al.*, 1996). This shows that the presence of live virus interferes with the normal function of the immune cells. In addition, the use of live virus for the development of vaccines against EHV-1 infection is complicated with the establishment of latent virus in trigeminal ganglia. For instance, defective mutants of viruses that are known to be less pathogenic i.e. thymidine-kinase defective mutant (Carvalho *et al.*, 2000; Ferrari *et al.*, 2000), a vaccine candidate, can also establish latent infection in the ganglia. Since horses are very sensitive to virus replication in the nervous system, immunizing agents that are neither infectious nor replicating are desirable. However, it is difficult to prepare such immunizing agents without prior disruption of virus particles or modification of immunogenic properties of the original viral glycoproteins. Therefore, the availability of non-infectious L-particles has created an advantage over conventional viral particles. There is no need for further inactivation treatment that normally modifies the tertiary structure of epitopes and the immunogenicity of antigens. In conclusion, L-particles of EHV-1, which are naturally non-infectious, containing all necessary glycoproteins similar to that of complete virions and can be produced in a significant amount, are suitable materials for preparation of reliable vaccines against EHV-1 infection. A certain degree of discrepancies e.g., differences in antibody responses and the pattern of virus replication, is expected. It occurs due to unavoidable factors including species differences and the nature of virus replication in different host species. Compared to other animal models, this study showed that the mouse EHV-1 infection model is the most relevant animal model for the study of the efficacy and the usefulness of L-particles as immunizing agents prior to laborious and expensive trials in SPF foals.

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