The pathogenesis of equine herpesvirus type 1 in the mouse: a new model for studying host responses to the infection

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An infection was established in adult BALB/c mice by means of intranasal inoculation of the AB4 strain of equine herpesvirus type 1 (EHV-1). The acute infection was confined to the respiratory tract and blood. Virus was shown to replicate in the nasal mucosa, trachea and lung for several days producing clinical signs of disease. Viraemia was also detected and a small proportion of peripheral blood cells contained virus at the peak of the infection. Histological and electron microscopic evidence were obtained which proved that productive virus replication occurred in the ciliated epithelial cells lining the bronchi and in pneumocytes in the lung, resulting in the destruction of these cells. Both humoral and cell-mediated responses to the infection were detected and monitored. By means of immunoprophylaxis or chemotherapy it was possible to modify the course of the infection. This infection model has many striking features in common with that observed in the natural host and the observations suggest that the mouse is a convenient and relevant model in which to study both host responses to EHV-1 infection and modification of the pathogenesis by means of immunoprophylaxis or therapy.

Introduction

Equine herpesvirus type-1 (EHV-1) is transmitted as a respiratory infection among horses throughout the world (Bagust, 1971; Bagust et al., 1972). The infection alone, or in conjunction with other microbes, is responsible for respiratory disease. However, a particularly serious aspect of the infection is that abortion may result in pregnant mares and furthermore severe neurological signs, including hind limb paresis, have been associated with EHV-1 (Bitsch & Dam, 1971; Mumford & Edington, 1980; Carrol & Westbury, 1985; Edington et al., 1985). The epidemiology of EHV-1 is complicated by its coexistence in equines with EHV-4 (also known as EHV-1, subtype 2), which has serological cross-reactivity, yet is distinct on genomic and biological grounds from EHV-1 (Studdert et al., 1981; Studdert & Blackney, 1979; Fitzpatrick & Studdert, 1984). How the two viruses interact within horse populations or within a single host has yet to be defined. Viraemia appears to be a crucial feature of the pathogenesis and severe and prolonged viraemia may correlate with spread across the placenta to the foetus and the production of neurological signs associated with virus replication in the endothelial cells in the small blood vessels of the central nervous system (CNS) (Charlton et al., 1976). Natural immunity to infection by EHV-1 appears to be incomplete or short-lived and reinfection with the same or related strains with the production of clinical signs is documented (Bryans, 1969; von Steinhagen, 1988) and confirmed in this laboratory (Y.-C. Chong, H. J. Field & P. H. Duffus unpublished).

EHV-1 is an infection of major importance, especially in the thoroughbred industry, and there is much interest in the development of effective vaccines or chemotherapeutic agents to prevent infection or disease, or reduce virus shedding in infected animals. Research has been hampered by the lack of small laboratory animal models, although much progress has been made with the hamster (Doll et al., 1953, 1956), which has also been used to study
antiviral agents (Rollinson & White, 1983) and the immune responses to virus and virus proteins (Wilks & Coggins, 1977; Stokes et al., 1989). However, the pathogenesis of the infection in hamsters differs quite strikingly from that in the natural host; for example, the liver is a primary site of virus replication in the hamster and the histopathology does not resemble that observed in the natural respiratory disease.

Mice have previously been shown to be susceptible to EHV-1, but published work has employed only newborn mice inoculated intracerebrally and the infection did not resemble that in the natural host (Patel & Edington, 1983). Adult mice are generally considered refractory to infection. Evidence is presented in the present paper for a new murine model which suggests that mice can provide a relevant model system for several features of the infection apparent in the equine. Mice are not only very convenient for study, but a wealth of information exists already on their responses to herpesvirus infections. In this communication we confirm that the proposed new murine model for EHV-1 infection is one that can easily be manipulated in order to study many aspects of EHV-1 pathogenesis.

Methods

Mice strains. Six different strains of mice, AKR, BALB/c, C57, CBA, DBA and SWR/J were obtained from Bantin & Kingman. Mice were female and 3 to 4 weeks old on receipt. Animals were kept for 1 week prior to inoculation to minimize the effects of stress of transportation and acclimatization.

Virus strains and tissue cultures. EHV-1 strain 1939 was a gift from Dr J. Mumford, Animal Health Trust, Newmarket, U.K. and was originally isolated from an aborted foetus. The strain AB4 was a gift from Dr N. Edington, Royal Veterinary College, London, U.K. and was originally isolated from a case of equine herpes with neurological complications (paresis). The rabbit kidney fibroblast cell line (RK-13) was grown in Eagle’s minimal essential medium (EMEM) supplemented with 10% newborn calf serum. Equine embryonic lung cells and African green monkey kidney cells (Vero) were cultured in EMEM supplemented with 10% foetal calf serum (FCS). Cell culture was at 37°C in an humidified atmosphere containing 5% CO2. Viruses were routinely propagated in RK-13 cells in EMEM supplemented with 10% foetal calf serum (FCS). Cell culture was at 37°C in an humidified atmosphere containing 5% CO2. Viruses were routinely propagated in RK-13 cells in EMEM supplemented with 2% FCS, at a low m.o.i. and working stocks stored at -70°C in small volumes.

Preparation of hyperimmune serum. Virus was grown in RK-13 cells seeded in seven large (850 cm²) roller bottles. When the c.p.e. was 100% confluent the infected cells were harvested and, following centrifugation, were resuspended in 8 ml EMEM. The harvested material was then sonicated and the virus inactivated using 0.015% formalin. This preparation was inoculated intramuscularly into two rabbits with Freund’s complete adjuvant. At 2 week intervals thereafter further inoculations were performed with Freund’s incomplete adjuvant, giving a total of four injections. The second rabbit of the pair was given two additional booster injections with live virus in Freund’s incomplete adjuvant. The rabbits were bled before inoculation and after each antigen boost and their sera tested for antibody. The maximum titres obtained against EHV-1 in the post-immunization sera were 1/300, 1/512 (neutralizing) and 1/100000, 1/140000 (ELISA) for the first and second rabbit respectively.

Hyperimmune sera were also raised in seven mice using similar techniques, except that the antigen preparation was partially purified by means of potassium tartrate density gradient centrifugation and for the first injection virus was heat-inactivated. For two subsequent injections the antigen preparation was not heat-inactivated and contained live virus. Mice were given a total of three subcutaneous (s.c.) injections. The sera from pairs of mice were pooled and the maximum antibody titres to EHV-1 obtained in the hyperimmune pooled sera were 1/120 (neutralizing) and 1/100000 (ELISA).

Intranasal (i.n.) inoculation of mice with live virus. Mice were lightly anaesthetized with ether and 40 μl, or in some cases 50 μl, of EMEM containing a suspension of virus was placed on the nares until all the inoculum was inspired, which occurred within a few seconds. When all mice had been inoculated the surplus virus suspension was titrated again using RK-13 cells to confirm the dose.

Clinical assessment. Mice were examined daily and weighed individually. Obvious signs such as ruffled fur, crouching in corners, dyspnoea and abdominal respiration and dragging movements or deaths were recorded.

Virus isolation from murine tissues. Groups of mice were sacrificed by pentobarbitone sodium injection. The various organs were minced with scissors and homogenized in an electric blender in a small quantity of EMEM. The suspension was sonicated for 1 min at 0°C and centrifuged at 3000 r.p.m. for 10 min to remove cellular debris. Dilutions of the supernatant were performed in EMEM and samples were inoculated onto confluent RK-13 monolayers. After 45 min adsorption, EMEM containing 2% FCS and 1% carboxymethyl cellulose (CMC) was added and the cultures were incubated at 37°C. Cultures were examined after 2 or 3 days and plaques stained with crystal violet for counting. In some cases cultures that yielded no virus were homogenized and added to fresh RK-13 cells for further passage.

Infectious centre assays. Heparinized blood (2 mg/ml EDTA) was collected and the leukocytes were counted. The blood was centrifuged in microfuge tubes and theuffy coat was mixed in distilled water for 1 min to lyse the erythrocytes. The osmotic balance was restored with phosphate-buffered saline at 10-fold normal strength.

Cells were counted in a haemocytometer and a precise number of leukocytes was then added to preformed monolayers of RK-13 cells. After 30 min medium was added and incubation continued for a further 5 days; the development of plaques was determined as above. In some cases a sample of the same cell preparation was spun down onto a glass slide, acetone-fixed and prepared for immunofluorescence staining (Paul et al., 1979) (see below).

Histology. Mice were killed with pentabarbitone sodium and small pieces of tissue were carefully removed and immediately fixed in 10% formal saline. Tissues were paraffin-embedded and sections prepared using standard methods. For immunohistochemistry, tissues were snap-frozen by immersion in liquid nitrogen. The tissues were later embedded frozen in CMC for cryostat sectioning. Sections were mounted on glass slides and fixed in cold acetone. The indirect immunofluorescence technique was employed using hyperimmune rabbit serum to EHV-1 (see above) and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin. Uninfected tissues were stained to provide control material and in all cases adjacent sections were stained using a preimmune serum from the same rabbit that was used to raise the EHV-1 antiserum.

Electron microscopy. Tissues were fixed in 4% glutaraldehyde and stained with osmium tetroxide using standard techniques. After embedding and sectioning, specimens were examined by transmission electron microscopy using a Hitachi H600 microscope. Some ultrathin
sections were stained with toluidine blue and examined by means of light microscopy. These sections were used to identify regions of histopathology in infected tissues for ultrastructural examination.

**Preparation of virus antigen and ELISA test for serum antibody.** Virus stocks were prepared in Vero cells (to avoid possible cross-reaction between rodent RK-13 cell antigens and murine sera). Several large roller bottles (850 cm$^2$) containing confluent monolayers of Vero cells were inoculated with EHV-1 (1939) at an m.o.i. of 0.1 p.f.u./cell and incubated at 37 °C. Compared with RK-13 cells c.p.e. developed very slowly in Vero cells, but a diffuse c.p.e. eventually became visible and the cultures were harvested after 10 days, when this became 100% confluent (infected cultures harvested at earlier times were not satisfactory). Cells were suspended in the infection medium and centrifuged at 3000 r.p.m. for 10 min. The supernatant was used as the source of virus antigen in the ELISA without further purification.

Dilutions of virus antigen were fixed to PVC microtitration plates (Flow Laboratories) by overnight incubation at 4 °C and titrated by the indirect ELISA using a commercial rabbit anti-mouse immunoglobulin conjugated to horseradish peroxidase (Dakopatts). The hyperimmune mouse anti-EHV-1 (see above) and preimmune sera were employed to determine the optimum dilution. A calibration curve was constructed and the cut-off value for adsorption was defined as 0.1 A$_{490}$ units (Clark & Barbara, 1987; Dutta et al., 1980, 1983).

Neutralization for serum antibody. Serial two-fold dilutions of the test sera (300 µl) were added to equal volumes of virus suspension containing 1000 to 2000 p.f.u./ml. The mixture was stirred for 1 h at 37 °C, then 0.1 ml samples were inoculated onto RK-13 cell monolayers and plaques counted after 2 to 3 days. The percentage of plaques compared with the preimmune serum control were plotted against test serum dilutions and the 50% plaque reduction titre was obtained (Thomson et al., 1976; Dutta & Campbell, 1977).

Skin test for cell-mediated immunity. Virus was grown as above in Vero cells to minimize cross-reaction. The virus was then inactivated by heating at 56 °C for 30 min and tested for surviving virus by plaque titration on RK-13 cell monolayers. An inoculum previously containing 10$^{5.9}$ p.f.u. was injected s.c. into the ear pinnae of anaesthetized mice. Control inocula contained medium alone. The ear thickness development was measured at daily intervals by means of a micrometer screw gauge. This technique has been shown to be a reliable indicator of the cell-mediated response in HSV-infected mice (Nash et al., 1980).

**Results**

**Susceptibility of different mouse strains and choice of virus strain**

Several different mouse strains were compared for their relative susceptibility to EHV-1. Groups of BALB/c, C57, CBA, AKR, SWR/J and DBA mice were inoculated i.n. with a standard dose of virus (strain 1939) and the target organs (turbinate bones, trachea and lungs) were removed at intervals after inoculation for virus titration. All mice strains showed virus replication in the target organs examined. BALB/c developed the highest titres of virus in the lung. C57, CBA and DBA were similar, but developed lung virus titres some 10-fold less than BALB/c mice. SWR/J and AKR mice showed much virus replication in nasal tissue, but relatively little in the lung (data not shown). Two different virus strains were then compared in BALB/c mice.

Groups of mice were inoculated with AB4 at 10$^{6.0}$ p.f.u./mouse or with strain 1939 10$^{6.5}$ p.f.u./mouse. It was notable that the clinical signs produced by AB4 were subjectively more severe, despite the slightly lower dose received, but the virus titres observed in the lungs and turbinates and the number of infectious centres detected in blood did not show a significant difference between the two virus strains.

Because of the advantage of reproducible clinical signs the combination of BALB/c mice and strain AB4 at a dose of approximately 10$^7$ p.f.u./mouse were routinely used for all subsequent experiments described below.

**Intranasal inoculation of BALB/c mice with EHV-1 strain AB4: clinical signs**

From 48 h after inoculation the mice began to show abnormal signs and by day 3 all were hunched with ruffled fur and infected animals appeared smaller, which was confirmed by measuring the weights of individual mice. The infected animals showed a conspicuous weight reduction for 4 to 5 days, then gradually recovered the normal rate of gain, delayed by about 1 week. Some mice showed signs suggestive of mild neurological involvement, for example the slight dragging of hind limbs. From the third day irregularities in breathing were noted. Mice died from the fourth to the seventh day after inoculation. Approximately 50% of animals recovered from the infection and became clinically normal by day 10, but it was several weeks before the weights of these mice regained that of the controls. Mice were also mock-infected i.n. with an inoculum of a preparation of sonicated, uninfected RK-13 cells, or a preparation of heat-inactivated virus. None of these mice developed significant weight loss or any other clinical signs of disease.

**Virus isolation from solid tissues**

BALB/c mice were inoculated with EHV-1 strain AB4 and the nasal turbinate bones, trachea, lung, liver, spleen, brain, eyeball, adrenal gland, cervical lymph nodes and uterus were homogenized and tested for the presence of infectious virus at intervals during the clinical course of the disease. Virus was consistently isolated from the nasal tissues, trachea and lungs from all mice tested during the first week after inoculation. Virus was occasionally isolated from the CNS, eye and liver, particularly from moribund mice. Infectious virus could not be detected in the other tissues tested, including lymphoid tissue, adrenal glands, spleen and uterus. The pattern of virus isolation with time from lungs and
turbinates, the organs which appeared to be the most important sites of virus replication, is shown in Fig. 1. Virus titres reached a peak at 3 to 5 days after inoculation, coincident with the time of maximum clinical signs. The presence of infectious virus could be detected in lungs and turbinate bones for at least 8 days, but virus was cleared from these tissues by day 12 after inoculation.

**Histological findings in the lungs of infected mice**

Histological evidence of virus replication was observed in the lung. By indirect immunofluorescence small foci of EHV-1 antigen-containing cells were clearly visible at 24 h post-infection (p.i.). At later times large foci of antigen-positive cells were observed, the majority of which appeared to be associated with small bronchioles (Fig. 2a). By the fifth day p.i. all the antigen staining appeared to be localized to the mucosal and submucosal tissues associated with the bronchioles (Fig. 2b). Haematoxylin-eosin-stained sections revealed heavy infiltration of the lungs by inflammatory cells and the complete loss of the normal alveolar architecture in large areas of the infected lungs (Fig. 3b). The ciliated epithelial cells lining the bronchioles were abnormal and desquamated and in some cases, together with inflammatory cells they obliterated the lumen of the bronchiole. The pathological changes in the mucosae were even more apparent in ultrathin sections prepared for electron microscopy and observed under the light microscope (Fig. 4a).

**Electron microscopy of infected lungs**

Thin sections of lung tissue were examined by means of light microscopy and areas of lung tissue showing typical herpes histopathology (Fig. 4a) were selected for ultrastructural study. In both alveolar and bronchiolar tissues many cells were observed to contain signs of herpesvirus replication, including disrupted chromatin, changes to the nuclear membrane, the presence of dense cored virions in the nucleus and enveloped particles both in the cytoplasm and external to cells. Several infected cells were positively identified as ciliated epithelial cells (Fig. 4b). In addition type I and type II pneumocytes were observed containing virions in all stages of replication (Fig. 5a).

Of particular interest was the fortuitous observation of a leukocyte containing virus particles (Fig. 5b), which
Host response to EHV-1 infection

Fig. 3. Histopathology of EHV-1-infected mouse lung. Mouse lung tissue stained by means of haematoxylin and eosin; (a) normal mouse prior to inoculation, (b) consolidated lung 3 days p.i. showing heavy infiltration by inflammatory cells in a region of the lung in which almost complete destruction of alveolar architecture (small arrows) and desquamation of bronchiolar respiratory mucosa (large arrow) was observed. Mice were from the same experiment as for Fig. 1. Bar marker represents 100 µm for (a) and (b).

Fig. 4. Selection of infected tissue for ultrastructural study. (a) Ultrathin section taken from the lung of a mouse 4 days p.i. stained with toluidine blue showing typical herpesvirus pathological changes in the mucosal cells of a bronchiole. A similar field was selected for ultrastructural study. Bar marker represents 100 µm. (b) Electron micrograph showing ciliated epithelial cells containing herpesvirus particles. Several degenerating cells with cilia evident (c) have become detached from the mucosal surface. Bar marker represents 5 µm.

provided unequivocal evidence of virus replication within leukocytes (see below). Observation by scanning electron microscopy revealed the desquamation of the epithelial surfaces within 2 days of the infection such that large areas were completely devoid of ciliated cells. These observations by means of scanning and transmission microscopy will be described in greater detail in a further communication (A. R. Awan et al., unpublished). Control, uninfected mice examined concurrently showed none of these pathological changes.

Detection of viraemia

An important feature of equine herpes in the natural host is the viraemic spread of virus during the acute disease. Accordingly, blood samples were examined from infected mice for the presence of virus. This was accomplished by plating out buffy coat cells together with a monolayer of uninfected RK-13 cells to detect infectious centres. Plaques appeared during the acute phase of the infection, which suggested that approximately 1 in 5 × 10⁴ cells behaved as infectious centres (Table 1). Similar preparations of cells were spun down onto glass slides using a cytocentrifuge and acetone-fixed for immunofluorescence staining. A small proportion of cells were observed to contain EHV-1 antigen (Fig. 6) and this correlated closely with the number of virus-producing cells previously determined by means of the infectious centre assay (Table 1). The particular cell type that contained virus antigen has yet to be defined; but one likely possibility is considered to be the monocyte-macrophage.

Development of immune responses in infected mice

Mouse sera were analysed for the presence of antibodies specific to EHV-1 by means of an ELISA and a virus
Fig. 5. Electron microscopic evidence for the productive infection on pneumocytes and leukocytes. (a) Type II pneumocytes identified by means of the lamellar body (l) showing clumped chromatin (cr) and an accumulation of herpesvirus particles with dense cores present in the nucleus (v). (b) Leukocyte contained within a small capillary adjacent to a clump of virus-infected cells. Several virus capsids (see inset) are visible within the nucleus of the leukocyte. Capillary wall (w) containing the leukocyte (l) and a red blood cell (r) adjacent to a type II pneumocyte (p) within the alveolar septum. Bar markers represent 2 μm (a and b) and 0.5 μm (inset).

Fig. 6. Detection of viraemia by immunofluorescence staining. Cytospin preparation of buffy coat cells from a mouse 5 days p.i. Staining by means of indirect immunofluorescence reveals two EHV-1 antigen-containing cells (arrow). Bar marker represents 100 μm.

Fig. 7. Development of specific antibody in EHV-1-infected mice. Mice were inoculated i.n. with EHV-1 strain AB4 at dose of 10⁶.9 p.f.u. live (●) or heat-inactivated (○) virus or a mock-infected RK-13 cell extract (□). Antibody titres to EHV-1 were determined by means of ELISA and the values are the geometric means with standard deviation obtained from five mice tested individually at each time point.

Table 1. Viraemia in mice infected with EHV-1 strain AB4, detected by means of infectious centre assay using cells obtained from the buffy coat

<table>
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<th>Individual mouse no.</th>
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* Infectious centres calculated/10⁶ buffy coat cells plated on RK-13 monolayers.
† ND, Not determined.

neutralization test. The results obtained from the ELISA are shown (Fig. 7). Antibody was first detected on the 12th day after inoculation and peaked at day 25, followed by a marked fall by day 30. The inoculation of a similar dose of heat-inactivated virus showed similar antibody kinetics, except that the sharp fall in ELISA titre at day 30 was not apparent. Sera were also tested for virus neutralization, but it was notable that no neutralizing activity could be detected at any time after inoculation with either live or inactivated virus, in contrast to the hyperimmune sera previously raised in mice by repeated s.c. injection of virus antigen together with adjuvant.
Fig. 8. Delayed-type hypersensitivity to virus antigen in EHV-1-infected mice determined by means of a skin test. Twenty μl heat-inactivated EHV-1 strain AB4 grown in Vero cells was inoculated into the skin of the left ear of groups of five mice; the right ears were inoculated with EMEM. Skin thickness was measured daily. (●) Originally inoculated with live virus (EHV-1 strain AB4 grown in RK-13 cells); (○) originally inoculated with heat-inactivated virus; ([ ]) originally inoculated with mock-infected RK-13 cells. Solid lines refer to antigen-inoculated left ear, broken lines are control right ears inoculated with medium.

The injection of heat-inactivated antigen into the infected mouse skin produced a powerful skin reaction of the classical delayed-type response (Fig. 8), which suggested that cell-mediated responses are also activated during the primary infection. More specific tests for T cell immunity are currently under investigation.

The effects of active or passive immunization on the acute phase of the infection

The prior injection of a single dose of heat-inactivated EHV-1 (10^6-9 p.f.u./mouse) had little or no effect on the subsequent course of infection upon challenge with live virus. The virus titres in turbinates and lungs in control animals did not differ significantly from those obtained from mice previously given the killed virus, moreover the clinical signs in mice previously exposed to killed virus appeared subjectively more severe than in the unimmunized infected controls. However, when hyperimmune, rabbit polyclonal anti-EHV-1 neutralizing antibody (1/300 neutralizing, 1/100000 ELISA) was inoculated s.c. 24 h prior to challenge with live virus this did ameliorate the course of the infection, as determined by virus isolation from the tissues. The sera of passively immunized mice were titrated for the presence of rabbit antibody on the third and fifth days p.i. (fourth and sixth days after antibody transfer) and ELISA titres were obtained that ranged from 1/39000 to 1/63000. Mice inoculated with preimmune sera also yielded low positive titres (approx. 1/1000), suggesting the presence of cross-reactive material in the murine sera interfering with the ELISA. The mice sera were also tested for neutralizing antibody and values of the order of 1/16 to 1/32 were obtained at both time points.

Although little difference in tissue virus titre could be detected on day 3 after virus inoculation, the tissues of the infected mice were found to contain significantly less virus on day 5 in passively immunized animals compared with the controls given preimmune sera. The geometric mean titres of infectious virus obtained from the turbinates of immunized mice was 0.25 log₁₀ and 1 log₁₀ lower than the controls on days 3 and 5, respectively, whereas the lung virus titres were reduced by 0.25 and 4 log₁₀, respectively, thus showing a protective effect. Furthermore a significant difference was observed between passively immunized and non-immunized mice in respect of viraemia as judged by infectious centre-forming cells (Table 2).

Table 2. Reduction in viraemia, as determined by infectious centre assay of buffy coat cells obtained from mice given prophylactic passive neutralizing antibody prior to infection with EHV-1 strain AB4

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<th>Individual mouse no.</th>
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* Infectious centres calculated/10^6 buffy coat cells plated on RK monolayers.
† ND, Not determined.

Chemical modulation of virus growth in the mouse model

The administration of the corticosteroid flumethasone in the drinking water (approx. 1 mg/kg/day from 2 days prior to virus inoculation for 1 week) had the effect of heightening virus titres approximately 10-fold and lengthening the period of virus growth in the lung such that virus was detected for 10 days after inoculation, but even in the corticosteroid-treated mice, virus was still cleared from the tissues by 12 days after inoculation (data not shown). Conversely, the administration of an inhibitor of EHV-1 replication (hydroxyphosphonylmethoxypropyl adenosine) (De Clercq et al., 1989) given from 1 day before virus inoculation led to a rapid
clearance of virus from the lungs and circulation, as judged by virus titres and infectious centre assay, respectively, and by histological examination of lung tissue. Furthermore no deaths occurred and the clinical signs of disease were minimal (Field & Awan, 1990).

Discussion

This paper describes a novel murine model for the laboratory study of EHV. The model has several convenient features, including the production of clinical signs and, importantly, offers a quantitative method for assessing virus growth in several target tissues including the circulation, over a period of approximately 1 week after inoculation.

The disease in mice has several features that very closely resemble those in the natural host. These include the early involvement of respiratory mucosae, the restriction of primary virus replication to the respiratory tract and the subsequent cell-associated viraemia. These are all features of the natural disease in the horse (Coggins, 1979; Patel et al., 1982; Scott et al., 1983). In the mouse virus replication was proven to occur within ciliated epithelial cells lining the bronchi and types I and II pneumocytes. The particular blood cells that harbour the virus have yet to be identified, but some preliminary evidence suggests that cells of the monocyte-macrophage series are the most likely candidates.

Following the inoculation of mice with EHV-1 an active immune response was detected using tests for both humoral and T cell immunity. It was noticeable that the ELISA antibody levels waned markedly on the last occasion tested. This is in contrast to the inoculation of BALB/c mice with HSV, where antibody levels remain high for many months after inoculation (Nash et al., 1980; Wildy et al., 1982; Simmonds et al., 1987) and furthermore neutralizing antibody was readily detected from 8 days after inoculation of mice with HSV. In the present study neutralizing antibody to EHV-1 was not detectable in the infected mice. In mice given repeated injections of EHV-1 together with adjuvant the neutralizing and ELISA antibody titres reached approximately 1/120 and 1/100000, respectively. These sera were obtained 6 weeks after primary inoculation; no data being available for earlier times. However, mechanisms other than neutralizing antibody appear to be responsible for the rapid and complete clearance of virus, which occurred by day 12 in the experimentally infected mice and it is likely that cell-mediated responses have an important role, as shown previously for HSV in the mouse (Nash et al., 1985).

A relatively short-lived humoral response to EHV-1 in the horse is documented (Bryans, 1969; Allen & Bryans, 1986) and neutralizing antibodies are not readily detected at early times after true primary infection of specific pathogen (EHV-1)-free foals (Y.-C. Chong et al., unpublished). In the murine system on the basis of a single time point (30 days after inoculation) it was notable that antibody levels detected by ELISA fell more quickly in the group of mice given live rather than inactivated virus, suggesting the possibility of a specific immunosuppressive effect of live virus. This suggestion will be pursued in future long-term experiments on antibody production. No comparable information was obtained on the kinetics of the T cell immune response to infection in the mice. The skin test for delayed-type hypersensitivity could not be carried out repeatedly, therefore no data were obtained as to the intensity of the reaction with time after the original exposure to virus. However, at 30 days after virus infection a vigorous reaction was demonstrated and in one group of mice tested 78 days after virus inoculation the delayed-type hypersensitivity response was found to be apparently undiminished.

A very important feature of the biology of the natural equine infection is the establishment of a latent infection in animals that have recovered from the acute disease. This is evidenced by the fact that stimuli such as stress or administration of corticosteroids result in recrudescence and virus shedding (Edington et al., 1985; Browning et al., 1988). So far it has not been possible to prove the establishment of a latent infection with EHV-1 in the mice. However, following a course of flumethasone administration to mice several weeks after the primary virus inoculation, occasionally virus-containing cells have been detected in the blood and antibody titres, as measured in the ELISA show a significant rise. These preliminary observations suggest that virus may persist in the mice in some form and these findings will now be followed more exhaustively.

We believe this i.n. murine infection model can now be used to study the effects of specific immunologically active proteins in tests for humoral and T cell responses or their suppression. The epidemiology of EHV-1 is complicated by the coexistence among horses of a second antigenically related virus infection, EHV-4 (Browning et al., 1988), but infections in the horse with strains of this virus are thought to lead only rarely to the serious complications described for EHV-1. However, the viruses EHV-1 and EHV-4 have cross-reactive antigens and their inter-relationship within infected animals is still unclear (Fitzpatrick & Studdert, 1984; Browning et al., 1988). It is hoped that in the future such complex interactions can be better explored using the murine model.

Our work with antiviral agents already shows that the
murine model can also be used to demonstrate effects of an effective inhibitor on virus growth in vivo. The ability, for example, to clear the viraemia would be of great clinical significance if it is reflected in the equine host. This is because viraemia and the associated replication of virus in the endothelial cells of the vessels is thought to predispose to the development of neurological signs (Charlton et al., 1976) and to the infection of the foetus, leading to abortion (Allen & Bryans, 1986). These findings concerning the efficacy of inhibitors of EHV-1 in the murine model will be the subject of a further publication.

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References


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