Transmission of equine herpesvirus 2 to the mouse: characterization of a new laboratory infection model

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Intranasal inoculation of BALB/c mice with a recent clinical isolate of equine herpesvirus 2 (EHV-2 strain MR) produced a productive infection characterized by clinical signs, including weight-loss and conjunctivitis, but no mortality. Infectious virus was isolated from the lung, trachea and nasal turbinates with the highest titres present in lung tissue; EHV-2 neutralizing antibody was detected in the serum on day 21 post-inoculation. No infectious virus was detected in neural tissue, blood or lymphoid tissues. An EHV-2-specific nested PCR confirmed the presence of EHV-2 DNA in the respiratory tissues. In addition, EHV-2 DNA was detected in the trigeminal ganglia and olfactory bulbs from the first day after inoculation and these tissues remained positive for EHV-2 DNA for at least 30 days, by which time EHV-2 DNA had been cleared from all other tissues.

Introduction

Existing isolates of equine herpesvirus 2 (EHV-2) are believed to comprise a genomically heterogeneous group of slowly cytopathic viruses (Browning & Studdert, 1988). Of the five equine herpesviruses recognized to date, EHV-2 and EHV-5 have been provisionally assigned to the subfamily Gamma-herpesvirinae on genomic criteria (Telford et al., 1993; Agius & Studdert, 1994). Previously, these viruses had been thought to be similar to other known cytomegaloviruses based on their biological properties (Roizman et al., 1992). However, the precise role of any of the current isolates of EHV-2 in relation to disease production in the horse is unknown and the viruses have been isolated from clinically normal horses as well as those showing clinical signs including upper respiratory tract disease, pneumonia, enlarged lymph nodes, conjunctivitis or general malaise (Studdert, 1971; Blakeslee et al., 1975; Sugiura et al., 1983; Jolly et al., 1986; Browning & Studdert, 1988).

Few experimental infections of horses with EHV-2 have been reported. Blakeslee et al. (1975) observed chronic follicular pharyngitis following intranasal inoculation of two 6-week-old foals. Wilks & Studdert (1976) inoculated three 7-month-old foals intranasally, genitally or intravenously, but no clinical signs were observed and no infectious virus was recovered from clinical specimens obtained 3 and 9 days post-inoculation (p.i.). Gleeson & Studdert (1977) inoculated EHV-2 by the intra-uterine route in a pregnant mare at mid-gestation but observed no abnormality during the pregnancy, although the virus was isolated from uterine fluid collected at 107 and 156 days p.i. The newborn foal was normal at birth but showed very mild signs of disease, between 4 and 11 days after birth. Thus, the pathogenesis of EHV-2 in the natural host has been difficult to study and its role as a primary cause of disease in the horse, or as a cofactor with other infections, remains equivocal.

The situation is further complicated by the fact that, based on serological tests (Bagust et al., 1972; Rose et al., 1974) and the detection of infectious virus or virus DNA (Edington et al., 1994; Rizvi et al., 1997) the infection is ubiquitous among equine stock and the precise infection status of naturally reared animals is very difficult to determine. Recently, we isolated a strain of EHV-2 from a pony which had acquired this infection naturally. DNA sequence data have been obtained to confirm the identity of the virus unambiguously as EHV-2. The virus grows relatively quickly in equine dermal (ED) cell cultures and has proved convenient to use in the laboratory. In this paper, we describe the successful transmission of this virus to mice. The pathogenesis of the infection is described, including unexpected data concerning the establishment of infection in neural tissues, and we propose that this is a potentially valuable model for the further study of this virus and its interactions with the host.

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Methods

**Virus.** The virus used for this study was isolated from a 10-month-old Welsh mountain pony which had acquired the infection naturally. The virus was co-cultivated from equine lymph nodes during an investigation of EHV-2 latency. From its growth characteristics it was identified as being a potentially useful isolate for laboratory study. The virus replicated relatively quickly in cultured ED cells, producing CPE within 24 h and discrete, well-delineated plaques. The virus was plaque purified three times in ED cells. Virus working stocks were prepared by inoculation of ED cells at low m.o.i. The infected cells were harvested after 3–4 days p.i. Virus working stocks typically contained 5 × 10⁷ p.f.u./ml and were stored in small aliquots at −70 °C until required for use.

**Characterization of virus**

(a) Restriction analysis. The virus was partially purified using a 10–50% sucrose gradient and viral DNA extracted using a standard protocol (Telford et al., 1995). Partially purified viral DNA was digested with different restriction enzymes (BamHI, HindIII, SalI and EcoRI), followed by electrophoresis through 0.5% agarose gels, stained with ethidium bromide and photographed. DNA from representative strains of EHV-1 (Ab4p; Telford et al., 1992), EHV-2 (86/87; Telford et al., 1995) and EHV-4 were similarly digested and included.

(b) Cloning and sequencing. BamHI digests of partially purified viral DNA were cloned into the pGEM-3Z vector (Promega). Seventeen clones were obtained ranging in size from 21 kb to 500 bp. Two clones, BamHIn and q, were sequenced by cycle sequencing using the Thermo Sequenase core sequencing kit (Amersham). The sequence of these clones was analysed using programs from the Sequence Analysis Software Package of the Genetics Computer Group (1991). The nucleotide sequences were compared with virus nucleotide sequences using the Sequenase core sequencing kit (Amersham). The sequence of these DNA were cloned into the pGEM-3Z vector (Promega). Seventeen clones was analysed using programs from the Sequence Analysis Software Package of the Genetics Computer Group (1991). The nucleotide clones was analysed using programs from the Sequence Analysis Software Package of the Genetics Computer Group (1991). The nucleotide sequences were compared with virus nucleotide sequences using the

**Mouse inoculation.** Female, BALB/c mice were obtained at age 3 weeks from Bantin & Kingman (Hull, UK). After 1 week’s acclimatization, a group of 50 mice was lightly anaesthetized using a mixture of oxygen and isoflurane (Rhone-Poulenc) and a 50 µl drop of medium which contained 1 × 10⁶ p.f.u. of virus was placed on the nares until inspired.

**Assessment of clinical signs.** Mice were examined daily for their general condition. Each mouse was weighed daily and abnormal signs — ruffled fur, crouching in corners, hunched back, dyspnoea and conjunctivitis — were assessed subjectively and noted. A group of mice mock-infected with ECV cell lysate was used for comparison.

**Detection of infectious virus.** Groups of three mice were euthanased on days 0, 1, 3, 5, 8 and 30 p.i. Tissue samples were collected into 1 ml EMEM. Tissues were minced with scissors, and homogenized using an electric blender. Following sonication in an ice-cold water bath for 1 min and centrifugation at 3000 r.p.m. for 10 min, 10-fold dilutions of the supernatant were inoculated onto confluent monolayers of ED cells. After 1 h adsorption, the cells were overlaid with EMEM containing 2% foetal calf serum (FCS) and 1% carboxymethyl cellulose (CMC) and incubated at 37 °C in a humidified incubator in an atmosphere of 5% CO₂. Cultures were examined daily for 7–10 days at which time the cells in wells not showing CPE were repassaged. To confirm the identity of virus recovered from murine tissue, virus from representative samples was grown up from wells of ED cells showing CPE and was partially purified and subjected to restriction analysis as described above. The restriction patterns obtained from the viruses isolated from mice were compared with the inoculum virus.

**Detection of viraemia.** Peripheral blood mononuclear cells (PBMC) were harvested from blood samples; 1 × 10⁶ PBMC per well were inoculated onto confluent monolayers of ED cells and 2 ml of maintenance medium containing 1% CMC was added to each well. The plates were then centrifuged at 200 g for 5 min to layer the PBMCs onto the ED cell monolayer. Cultures were then incubated at 37 °C in 5% CO₂ for 7–10 days at which time the cultures were repassaged onto fresh monolayers of ED cells.

**Detection of latent virus.** The methods employed have previously proved successful for the isolation of EHVIs from naturally infected ponies (Welch et al., 1992; our unpublished results). Briefly, the tissues were dissected into approximately 1 mm³ fragments and added to 24-well plastic tissue culture plates. Medium (EMEM plus 10% FCS) containing 1 × 10⁶ ED cells in suspension was added immediately to each well. For lymphoid tissue, a cell suspension was obtained from the tissue and this was mixed with a suspension of ED cells as above. The cultures were observed daily and further ED cells were added as required. After 14 days the cells were harvested by trypsinization and the cells seeded onto new plates for further incubation. The process was repeated once.

**Detection of EHV-2 DNA by nested PCR.** An EHV-2-specific nested PCR (nPCR) was used to detect the presence of EHV-2 DNA. PCR primers complementary to a region within ORF64 (template protein) were designed and synthesized. Outer pair sequences were 126089n 5’TATGAAAGTAACTACAGA 3’ and 126371c 5’TCCCGCGCAAGCTCCTGCT 3’. Inner pair sequences were 126146n 5’GAGCACATGGCTTCGGAG 3’ and 126292c 5’TTGATCGAGGGTAAACA 3’. Tissue was minced with a scalpel and high molecular mass DNA was extracted by standard proteolytic digestion (Kitchin et al., 1990; Slater et al., 1994). DNA extractions were carried out in an EHV-free flow cabinet to prevent accidental contamination of samples and negative control samples were carried through all stages of preparation. The PCR reaction mixes (50 µl), which were prepared in a dedicated EHV-free area, contained 0.5 µM of each primer, 0.2 mM dNTPs, 2.5 mM MgCl₂, 1.5 units Tag polymerase, 1× reaction buffer and 1 µg template DNA. The cycling profile employed was denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, anneal at 58 °C (inner pair) or 61 °C (outer pair) for 30 s, extend at 72 °C for 1 min. A 1 µl volume of the first round reaction was used as template for the second round. Using purified viral DNA as template, first and second round products of the expected 282 bp and 140 bp sizes were made. The minimum sensitivity of the nested reaction was found to be approximately 0.1 fg of purified EHV-2 DNA, which equates to less than 10 copies of EHV-2 DNA.

**Histology.** Mice were killed by pentobarbitone sodium injection and a variety of respiratory, lymphoid and neural tissues collected. The tissues were cut into small pieces and fixed in 10% buffered formal saline. Tissues were paraffin-embedded, sections prepared using standard methods and stained with haematoxylin and eosin.

**Electron microscopy.** Tissues were collected from mice on days 2 and 3 p.i. cut into small pieces and fixed in 4% glutaraldehyde. The tissues were embedded, sectioned and stained with osmium tetroxide using standard techniques. The sections were examined by transmission electron (Hitachi H-600) microscopy.

**Virus neutralization test.** Blood was collected from a group of
four mice on days 0 and 21 p.i. The blood was pooled and serum separated for use in a virus neutralization test. Serum samples were serially diluted twofold in EMEM down to 1/512 and 100 p.f.u. virus in 100 µl was added to an equal volume of each dilution. The mixture was incubated for 1 h at 37 °C and then the whole was inoculated onto ED cells as above. The neutralizing titre of the serum was defined as the maximum serum dilution giving 50% reduction in plaques.

Results

Characterization of virus isolate by restriction enzyme and DNA sequence analysis

The virus was identified as EHV-2 on the basis of similarity of restriction pattern and sequence homology to the published sequenced strain of EHV-2 (86/87). The BamHI, HindIII, EcoRI and SalI restriction patterns of the isolate were different from EHV-1 and EHV-4 but similar to EHV-2 (strain 86/87). Furthermore, the nucleotide sequence of the BamHI n and q fragments showed close to 100% identity with the sequenced strain of EHV-2 (Telford et al., 1995) and the homologous genes were identified as ORF17 and ORF64. No sequence homology to any other herpesvirus was seen.

Inoculation of BALB/c mice

Clinical signs. Mice inoculated intranasally with EHV-2 developed clinical signs which became apparent on the first day after infection and remained for up to 4 days (Table 1). The effects were most severe on days 2 and 3 p.i. and in addition to the general clinical signs, mice developed conjunctivitis which was most severe on days 2 and 3 p.i. All mice were clinically normal by 1 week p.i. and no further changes were observed. No abnormal signs were recorded in a group of mock-infected mice. The mice showed a significant loss of body weight (Fig. 1). This was about 25% between days 2–4 p.i. and by day 8 the infected mice remained underweight in comparison with uninfected controls.

Virus isolation from tissues. Infectious virus was detected in the nasal turbinates, trachea and lung on day 1. The mean titres were 3.6, 4.0 and 4.8 log10 p.f.u. per organ respectively. Only lung tissue was positive for infectious virus, with a mean titre of 3.6 log10 p.f.u. per lung, on day 3 p.i. DNA from virus obtained from these respiratory tissues was subjected to restriction analysis and the restriction patterns, following digestion with BamHI, EcoRI and HindIII, were identical to those obtained from the inoculum virus. All non-respiratory tissues were negative for infectious virus on all occasions tested and no infectious centres were obtained from the PBMC. At 30 days p.i. no infectious virus was detectable in any tissue using the conventional virus isolation procedures described above. In an attempt to detect the presence of latent virus, tissue samples were assessed by co-cultivation with susceptible ED cells but no virus was isolated by this method (Table 2).

Histological and EM evidence for infection. Histological evidence of virus replication was observed in the lung on day 2 and 3 p.i. Lung sections (stained with haematoxylin and eosin) showed infiltration of inflammatory cells into the alveoli and there was complete loss of the normal alveolar architecture in several areas of the lung corresponding to 25–50% of the lung tissue examined. The architecture of the bronchioles was disrupted and the ciliated epithelial cells lining the bronchioles were abnormal: some sections of the epithelial cells appeared to have desquamated. Signs of virus replication were observed in the nuclei of infected lung cells on electron microscopy.
Table 2. EHV-2 detection in various tissues of mice during acute and latent phases following intranasal inoculation of BALB/c mice with EHV-2 strain MR

V, infectious virus isolated by plaque assay; V*, infectious virus detected by co-cultivation; PCR, EHV-2 DNA detected by nested PCR.

<table>
<thead>
<tr>
<th>Tissue/organ</th>
<th>Days p.i.:</th>
<th>Acute infection</th>
<th>Latent infection</th>
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<tr>
<td></td>
<td>1 V/PCR</td>
<td>3 V/PCR</td>
<td>5 V/PCR</td>
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<tr>
<td>Lung</td>
<td>+ / +</td>
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Herpesviruses were observed both in the nucleus and in the cytoplasm of the infected lung cells (Fig. 2).

Detection of EHV-2 DNA. All the samples which tested positive for infectious virus were also shown to be positive for EHV-2 DNA by means of nPCR (Table 2). However, in addition the trigeminal ganglia and olfactory bulbs were uniformly positive by nPCR from day 1. The lung samples remained positive on days 5 and 8 p.i. although infectious virus was cleared at these times. Samples tested on day 30 p.i. yielded positive results for both neural tissues examined: trigeminal ganglia and olfactory bulbs. Spleen and PBMC were negative for EHV-2 DNA in all cases both early and late after inoculation.

Serological response. Serum was collected from the mice immediately before virus inoculation and again on day 21 p.i. EHV-2 neutralization antibodies were detected on day 21 with a titre of 1:32 compared with 1:4 prior to inoculation.

Discussion

The important findings to emerge from this study were: a fast-growing clinical isolate of equine herpesvirus was confirmed to be EHV-2 by sequence analysis; intranasal inoculation of BALB/c mice resulted in a productive infection in the respiratory tract with translocation of virus to neural tissues as evidenced by nPCR; finally, murine neural tissues showed a prolonged presence of EHV-2 DNA.

EHV-2 is a member of the *Gammaherpesvirinae* (Telford et al., 1995); members of this subfamily generally have a restricted host range. The previously reported attempts to adapt EHV-2 to laboratory species have not used the natural route of inoculation but no productive infection was reported following intraperitoneal and intracerebral routes of inoculation in young and suckling mice, hamsters, young guinea-pigs and rabbits (Plummer & Waterson, 1963; Karpas, 1966; Erasmus, 1970; Browning & Studdert, 1988). To our knowledge, the only...
productive EHV-2 infection in a laboratory animal reported to date is the study of Plummer et al. (1972) in which young rabbits were inoculated in the lumbar spinal cord; this was reported to result in an acute focal meningoencephalitis with the involvement of both white and grey matter lasting for 7–16 weeks with a persistent infection of the spinal cord for over 20 months.

In the present study an isolate of EHV-2 was obtained that productively infected cultures of equine dermal cells but not rabbit kidney cells in the first or second passage. It was therefore unexpected that a successful infection was produced in BALB/c mice. The evidence that a productive infection was achieved includes the following: (i) clinical signs developed including marked weight-loss, (ii) virus was isolated from the lung up to day 3 p.i. (iii) histopathological changes were evident in the respiratory tract consistent with infection, (iv) thin sections of lung examined by EM provided morphological evidence of herpesvirus-infected cells, (v) EHV-2 DNA was detected in ganglia and CNS tissues for at least 30 days p.i. Similar observations were not obtained when mice were inoculated intranasally with EHV-4 (Azmi & Field, 1993) or heat-killed EHV-1 in which cases no clinical signs or virus replication were observed. This supports our contention that the inoculum alone could not be responsible for the observed changes in mice following EHV-2 inoculation.

While the murine infection model has some features that resemble EHV-2 infections that have been reported in the natural host there were also important differences. For example, virus isolation has been reported from conjunctiva (Collinson et al., 1994) of naturally infected horses and EHV-2 has frequently been isolated from cultures of equine lymphocytes (Gleeson & Coggins, 1985; Roeder & Scott, 1975) However, similar results were not obtained in the present infection model although conjunctivitis was observed in mice inoculated in the present study. Virus isolation from the eye was, however, not attempted in this study.

Interestingly, we were able to detect viral DNA in the trigeminal ganglia and olfactory bulbs of mice during the acute infection and later for several weeks. The biological significance of this DNA needs further investigation; however, EHV-2 DNA has recently been detected by nPCR in the trigeminal ganglia of half of 12 ponies tested recently in our laboratory (accompanying paper: Rizvi et al., 1997). This suggests that EHV-2 does have definite neurotropic properties.

EHV-2 is reported to comprise a heterogeneous group of viruses and the characteristics of different strains of EHV-2 are likely to vary (Browning & Studdert, 1987, 1988). The present strain appears to be unusually cytopathic in cultured ED cells producing rapid CPE and yielding relatively high titres of infectious virus. The new EHV-2 infection model described in this paper should be valuable for the study of EHV-2 pathogenesis and latency and it may also be useful to investigate interactions between EHV-2 and other equine herpesviruses, e.g. EHV-1 where EHV-2 may act as a transactivator (Edington et al., 1994) and hence a co-factor in latency and reactivation of EHV-1.

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References


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