

EQUID HERPESVIRUS 1 IS NEUROTROPIC IN MICE, BUT LATENCY FROM WHICH INFECTIOUS VIRUS CAN BE REACTIVATED DOES NOT OCCUR

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Equid herpesvirus 1 (EHV-1) is the most common cause of virus-induced abortion in horses. After primary infection the virus becomes latent predominantly in the respiratory tract lymph nodes and the genome can also be detected in the peripheral nervous system. The role of mouse as a feasible model for the establishment of latency and reactivation of EHV-1 was investigated. Intracerebral and intranasal infections of 3- and 17-day-old mice were made and virus replication was confirmed by virus isolation and detected by indirect immunofluorescence (IIF) in brain. For reactivation studies, the mice were killed 8 weeks post infection and tissues were collected for cocultivation. In mice from both age groups, infectious virus was not detected by cocultivation. Following attempts to reactivate virus *in vivo* with corticosteroids, the viral antigen was detected at low levels by IIF and the expression of the gB gene by reverse transcription polymerase chain reaction (RT-PCR) in brain, trigeminal ganglia, olfactory lobe, lung and spleen. Virus was also detected by IIF following incubation of tissue explants in the growth medium containing pokeweed mitogen (PWM). These results show the limitations of the mouse model for investigating EHV-1 latency and highlights the issue of 'ineffective reactivation' of virus.

Key words: Equid herpesvirus 1 (EHV-1), mouse, latency, reactivation

Equid herpesvirus 1 (EHV-1) is the most common cause of virus-induced abortion in horses (Smith, 1997). The natural route of infection is via the upper respiratory tract, followed by cell associated viraemia, endothelial cell infection and infection of the fetus with subsequent abortion (Edington et al., 1991; Smith, 1997). After primary infection, the virus becomes latent predominantly in lymphoid tissues, particularly those draining the respiratory tract (Welch et al., 1992; Edington et al. 1994*a, b*) and the genome can be detected in the central nervous system (Slater et al., 1994*a*).

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Horses have been used as the natural host for studying EHV-1 infections for many years. These studies are limited by the fact that in outbred horses the α -herpesviruses EHV-1 and 4 are endemic along with EHV-2. Specific pathogen free (SPF) foals are expensive, and can only be produced in small numbers (Slater et al., 1994b).

Small laboratory animal models have been used for studying EHV-1 infection. Hamsters were first used by Doll et al. (1953) as an animal model. They have also been used to study antiviral agents (Rollinson and White, 1983) and immune responses to viral proteins (Wilks and Coggins, 1977; Stokes et al., 1989). However, the pathogenesis in the hamster model differs from the horse in that the liver is the primary site of viral replication in hamster.

Mice were originally shown to be susceptible to EHV-1 infection by Hatzios and Reagan (1960) and Plummer et al. (1973). Patel and Edington (1983) reported that when newborn mice were infected intracerebrally, the virus spread in neurones throughout the brain and that the infection did not resemble the neuropathology in the horse where a vascular thrombosis is the underlying pathology. However, infection of mice via the intranasal route (Awan et al., 1990; Inazu et al., 1992) appeared to mimic the natural EHV-1 infection. An acute infection was restricted to the nasal mucosa, respiratory tract and blood. Abortion was also produced in this model (Awan et al., 1991). In addition, Field et al. (1992) have used the murine model to show the recovery of EHV-1 from one of three infected mice using a wide variety of stimuli. A later study by Baxi et al. (1996) was characterised by the failure of recovery of infectious EHV-1 from latently infected mice. Thus, while the use of the mouse model may provide a useful method for investigation of acute infections, many aspects of EHV-1 infection and latency in mice still require clarification. The main aim of this study was to assess the suitability of the mouse as a useful model for latency of EHV-1 in horses.

Materials and methods

Virus

EHV-1 strain Ab4, passage 11 was propagated in RK13 cells in 10% fetal calf serum (FCS), 2 mM L-glutamine and non-essential amino acids (Patel et al., 1982). Penicillin (100 units/ml), streptomycin (100 μ g/ml) and fungizone (20 μ g/ml) were added to the medium. The inoculum used in the mice contained 10^4 pfu/ml.

Mice

One hundred and twenty Balb/c mice were divided into two age groups (3- and 17-day-old) and used for intranasal ($n = 70$) and intracerebral ($n = 50$) inoculations (Table 1). A total of 10 control mice were mock infected with an inoculum of uninfected, sonicated (Rabbit Kidney) RK13 cells and sterile PBS.

Table 1

Outcome of intracerebral or intranasal inoculation of the Ab4 isolate of EHV-1 in 3-day-old and 17-day-old Balb/c mice

Route of inoculation	Age of mice	Total no. of mice	No. of mice killed at 3 and 6 d.p.i.	No. of mice for latency studies	No. of deaths recorded
Intracerebral	3-day-old	50	16	18	16
Intranasal	3-day-old	33	12	9	12
Intranasal	17-day-old	37	12	19	6

Experimental design

Intracerebral (i.c.) and intranasal (i.n.) inoculations were carried out as described by Patel and Edington (1983). For i.c. inoculation of 3-day-old Balb/c mice the inoculum (100 pfu/10 μ l) was introduced through a 25-gauge needle into the left cerebral hemisphere. For i.n. inoculation 300 pfu/30 μ l and 700 pfu/70 μ l volumes were used for 3- and 17-day-old mice, respectively. A total of 40 mice from all groups were sacrificed at 3 and 6 days post infection (d.p.i.) for acute studies (see Table 1), the remaining mice were used after a period of 10 weeks for direct detection of latent virus or for attempted reactivation. Euthanasia of mice was performed using CO₂ inhalation. Tissue samples from turbinates, lung, brain, spleen and bronchial lymph nodes were titrated for infectivity while frozen sections were examined using indirect immunofluorescence (IIF) as described previously (Patel et al., 1982). Similar tissues were taken from mice that died in the course of the acute infection.

Latency and reactivation

Different experimental procedures used for reactivation of viruses from various tissues (brain, olfactory lobe, trigeminal ganglia, lung, bronchial lymph node and spleen) were as follows (see Table 8):

In vivo:

1. Cyclophosphamide (250 mg) and dexamethasone (20 mg) (Shimeld et al., 1990) were given intraperitoneally [i.p.] on consecutive days and mice were killed 24 h later. The tissues listed above were subjected to cocultivation, or immediately examined by IIF and RT-PCR.

In vitro:

1. Cocultivation of tissues (Welch et al., 1992) with primary equine embryonic kidney (EEK) cells, RK13 or L1 murine cells.

2. Cocultivation was also carried out in the presence of IL-2 (20 U/ml) or pokeweed mitogen (PWM 100 µg/ml), which were known to reactivate EHV-1 from equine leukocytes (Smith et al., 1998).

3. Examination of frozen sections taken after explants had been incubated with IL-2 and PWM for 24 h.

Indirect immunofluorescence

EHV-1 antigen was detected by IIF using a rabbit polyclonal antiserum for screening (Thomson et al., 1976), or by a monoclonal antibody against EHV-1 gB (Edington et al., 1987).

RT-PCR

Total RNA from each tissue was prepared using the method of Chomczynski and Sacchi (1987). For reverse transcription, 1 µg of total RNA and 100 pmol of random hexamers pd(N)₆ (Amersham Pharmacia) in reverse transcriptase (RT) buffer (Gibco Life Sciences), with 200 nmol dTT and 50 mM of each deoxynucleoside triphosphate were heated to 65 °C for 5 min and chilled on ice for 5 min. After the addition of 5 units of RNasin (Gibco Life Sciences) and 100 units of MMLVRT (Gibco Life Sciences), the reaction mixture (total volume 20 µl) was incubated for 1 h at 37 °C, heated to 75 °C for 10 min, and chilled on ice. Primer sequences of the conserved gB gene of EHV-1 (294 bp product) were used and the sequences of the two primers are shown in 5'---3' orientation (Welch et al., 1992).

2L GGA AAG GAT ACA GCC ATA CGT C
2R CGT ACA CAA TAT CAC CGG TGG A

All amplifications were performed using a Perkin-Elmer DNA thermal cycler. A typical reaction consisted of 5 µl of cDNA, 59.6 µl of AnalR H₂O (BDH/Merck), 8 µl of (10×) NH₄ buffer (Bioline), 2.4 µl of 50 mM MgCl₂, 25 pmol of each primer, 50 mM of each deoxynucleoside phosphate and 5 units of BIOTAQTM polymerase (Bioline). Each reaction was overlaid with 80 µl of autoclaved mineral oil (Sigma). Negative control reactions (without the addition of reverse transcriptase and also using sterile H₂O) were also performed. The parameters of amplification were as follow: initial denaturation at 95 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 51 °C for 2 min, polymerisation at 72 °C for 3 min, with an additional final incubation for 7 min to complete all extensions. PCR products (294 bp) were resolved by electrophoresis in a 3% NuSieve agarose gel (FMC Bioproducts) and were visualised under UV light after ethidium bromide staining.

Results

Results from acute infections in 3-day-old and 17-day-old mice by i.c. and i.n. inoculations are summarised in Table 2.

Table 2

Isolation of Ab4 isolate of EHV-1 following intracerebral inoculation (100 pfu) of 3-day-old Balb/c mice

Days post infection	Detection method	Tissues		
		Brain	Lung	Spleen
3 d.p.i.	IIF	8/8	2/8	2/8
	VI*	>6(0.3)	0/8	0/8
6 d.p.i.	IIF	0/8	8/8	6/8
	VI*	0/8	4(0.3)	4(0.3)

*TCID₅₀/mg wet tissue (Log value ± Standard Deviation)

Three-day-old mice: acute infections

Intracerebral inoculation. Following i.c. inoculation of EHV-1 into 3-day-old mice (n=50), a total of 16 deaths were recorded (Table 1). Deaths occurred at 1, 2, 3, 5 and 7 days post infection (d.p.i.). On postmortem examination of each of these mice, virus was isolated from the brains of 13/16 and the lungs of 4/16 mice.

A total of 16 mice were killed on 3 or 6 d.p.i. (Table 2). At 3 d.p.i. cell culture yielded virus only from the brain. IIF showed that virus was restricted to the neuronal cells around the inoculation site. By 6 d.p.i. virus had cleared from the brain but was detected on cell cultures from lung and spleen and was confirmed by IIF in small bronchioles of the lung and more rarely, in the lymphoid cells of spleen.

Intranasal inoculation. Of 33 mice given EHV-1 i.n., 12 died at 2, 3, 4, 6, 8 and 10 d.p.i. (Table 1). Infectious virus was detected in the lung and brain of mice which died at 2, 3, 4, and 6 d.p.i.

In 12 mice killed on 3 and 6 d.p.i., virus was detected by IIF in the neuronal cells of the brain, small bronchioles of the lung and the lymphoid cells of the spleen. Virus was recovered from brain, lung, turbinate and spleen (at 6 d.p.i. only) on 3 and 6 d.p.i. (Table 3).

Seventeen-day-old mice: acute infections

From 37 seventeen-day-old mice given virus i.n. deaths were recorded at 5, 7 and 10 and 30 d.p.i. in four mice. Virus was detected by IIF in the lungs of mice that died at 5 d.p.i. and 7 d.p.i.

Table 3

Isolation of Ab4 isolate of EHV-1 following intranasal inoculation (500 pfu) of 3-day-old Balb/c mice

Days post infection	Detection method	Tissues			
		Brain	Lung	Spleen	Turbinate
3 d.p.i.	IIF	4/6	6/6	1/6	n/a
	VI*	4 (0.3)	6 (0)	4 (0.5)	6 (0.5)
6 d.p.i.	IIF	6/6	3/6	1/6	n/a
	VI*	4 (0.3)	5 (0.5)	0/6	6 (0.3)

n/a = Procedure not carried out; *TCID₅₀/mg wet tissue (Log value ± Standard Deviation)

A total of 12 mice were killed at 3 and 6 d.p.i. Virus was recovered from turbinate, lung and spleen at 3 d.p.i. and from brain at 6 d.p.i. By IIF virus was detectable in lung and bronchial lymph node at 3 d.p.i. and from brain and spleen at 6 d.p.i. (Table 4).

Table 4

Isolation of Ab4 isolate of EHV-1 following intranasal inoculation (1000 pfu) of 17-day-old Balb/c mice

Days post infection	Detection method	Tissues				
		Brain	Lung	Spleen	Bronchial lymph node	Turbinate
3 d.p.i.	IIF	0/6	3/6	1/6	4/6	n/a
	VI*	0/6	4 (0.5)	3 (0.3)	n/a	> 6 (0)
6 d.p.i.	IIF	2/6	0/6	1/6	0/6	n/a
	VI*	4 (0.3)	0/6	0/6	n/a	6 (0.7)

n/a = Procedure not carried out; *TCID₅₀/mg wet tissue (Log value ± Standard Deviation)

Latency

Forty-six mice from these three groups were kept for a further two months to carry out latency and reactivation studies (Table 1). Four different experimental protocols were used for the reactivation of latent virus as described above.

For *in vivo* reactivation, cyclophosphamide and dexamethasone were given i.p. to 14 mice. No infectious virus was recovered when animals were killed after 24 h. By RT-PCR, EHV-1 gB was amplified and IIF staining was positive but only in a low number of animals (Table 8).

In *in vitro* experiments when cocultivation, with or without IL-2 or PWM, was used infectious virus could not be detected following cocultivation of tissues from 32 mice in any of these groups with any of the three monolayers (EEK, RK

13, or murine L1 cells). However, viral antigen was detected by IIF at low levels in different tissues (Table 8).

Three-day-old mice: intracerebral inoculations

Infectious virus was not recovered from any tissue (Table 5). Virus antigen was detected by IIF only in a few nerve cells of the trigeminal ganglia and the lymphoid cells (in the range of 10–15 cells per section) of the spleen of two mice.

Table 5

Reactivation of virus from mice infected intracerebrally at 3 days of age

Reactivation protocol	Detection method	Tissues					
		Cerebrum	Olfactory lobe	Trigeminal ganglia	Lung	Bronchial lymph node	Spleen
<i>In vitro</i> * 20 U/ml IL-2	IIF	0/13 ^x	0/13	0/13	0/13	0/13	0/13
<i>In vitro</i> * 100 µg/ml PWM	IIF	0/13	0/13	2/13	0/13	0/13	2/13
<i>In vivo</i> [†] cyclophosphamide and dexamethasone	IIF	0/5	0/5	2/5	2/5	0/5	3/5
	RT-PCR	1/5	1/5	1/5	0/5	0/5	0/5

*Virus isolation: *In vitro* cocultivation was performed and all the tissues were negative after 3 passages; [†]Virus isolation from homogenised tissues was performed and all the tissues were negative after 3 passages; ^xNumber positive/Total number examined

The remaining 5 mice were injected with cyclophosphamide and dexamethasone and were necropsied on day 3 as described above. After necropsy, no infectious virus was recovered by cocultivation of tissues. Virus was detected by IIF in trigeminal ganglia neurones (2–5 cells per section) and lung macrophages in two mice and the spleens of three mice. The frequency of positive cells in lung and spleen was from 15–20 cells per section. The gB gene was amplified by RT-PCR in brain, olfactory lobe and trigeminal ganglia from one mouse.

Three-day-old mice: intranasal inoculations

Five mice were killed after two months. After 24 h of explantation, virus was detected by IIF from trigeminal ganglia, lung, bronchial lymph node and from spleen tissues incubated in PWM-containing medium in one mouse; but infectious virus was not recovered from any of the mice. The frequency of positive cells was lower in trigeminal ganglia (an average of 5 cells per section) as compared to the positive cells in other tissues (15–20 cells/section).

The remaining four mice were injected with cyclophosphamide and dexamethasone and were killed after three days. Virus was detectable by IIF in the lung macrophages of one mouse and in the lymphoid cells of the spleen of two mice, but not by virus isolation (Table 6).

Table 6

Reactivation of virus from mice infected intranasally at 3 days of age

Reactivation protocol	Detection method	Tissues					
		Cerebrum	Olfactory lobe	Trigeminal ganglia	Lung	Bronchial lymph node	Spleen
<i>In vitro</i> [*] 20 U/ml IL-2	IIF	0/5 ^x	0/5	0/5	0/5	0/5	0/5
<i>In vitro</i> [*] 10 µg/ml PWM	IIF	0/5	0/5	1/5	1/5	1/5	1/5
<i>In vivo</i> ⁺ cyclophosphamide and dexamethasone	IIF	0/4	0/4	0/4	1/4	0/4	2/4
	RT-PCR	0/4	0/4	0/4	0/4	0/4	0/4

^{*}Virus isolation: *In vitro* co-cultivation was performed and all the tissues were negative after 3 passages; ⁺Virus isolation from homogenised tissues was performed and all the tissues were negative after 3 passages; ^xNumber positive/Total number examined

Seventeen-day-old mice: intranasal inoculations

After two months of infection no virus was recovered by cocultivation and/or from explant tissues of 14 mice. However, virus was detected by IIF in the lung and spleen of four mice and in the trigeminal ganglia and bronchial lymph node of three mice incubated in a medium containing 100 µg/ml PWM. Of the five mice, which were injected with cyclophosphamide and dexamethasone on consecutive days, virus was detected by IIF in alveolar cells (possible macrophages) in the lungs of four mice, lymphoid cells of bronchial lymph node in three mice, the spleen of four mice, and neurones of trigeminal ganglia from two mice. By RT-PCR amplifications, virus was detected in the brain of two mice and olfactory lobe and trigeminal ganglia of one mouse. EHV-1 was shown to be present in relatively higher number of cells by IIF, especially in lung and spleen compared to the two groups of mice infected when three days old, but the difference was not statistically significant. A correlation between the detection of EHV-1 gB antigen by PCR or IIF was found only in trigeminal ganglia (Table 7).

Table 7

Reactivation of virus from mice infected intranasally at 17 days of age

Reactivation protocol	Detection method	Tissues					
		Cerebrum	Olfactory lobe	Trigeminal ganglia	Lung	Bronchial lymph node	Spleen
<i>In vitro</i> [*] 20 U/ml IL-2	IIF	0/14 ^x	0/14	0/14	0/14	0/14	0/14
<i>In vitro</i> [*] 10 µg/ml PWM	IIF	0/14	0/14	3/14	4/14	3/14	4/14
<i>In vivo</i> ⁺ cyclophosphamide and dexamethasone	IIF	0/5	0/5	2/5	4/5	3/5	4/5
	RT-PCR	2/5	1/5	1/5	0/5	0/5	0/5

^{*}Virus isolation: *In vitro* cocultivation was performed and all the tissues were negative after 3 passages; ⁺Virus isolation from homogenised tissues was performed and all the tissues were negative after 3 passages; ^xNumber positive/Total number examined

Table 8

Summary of reactivation experiments in mice

Reactivation	No. of mice	Tissues					
		Cerebrum	Olfactory lobe	Trigeminal ganglia	Lung	Bronchial lymph node	Spleen
<i>In vivo</i> cyclophosphamide + dexamethasone (IIF on tissues)	14	0/14 ^x	0/14	4/14 (28%)	7/14 (50%)	3/14 (21%)	8/14 (64%)
<i>In vivo</i> cyclophosphamide + dexamethasone (RT-PCR on tissues)	14	2/14 (21%)	2/14 (14%)	2/14 (14%)	0/14	0/14	0/14
<i>In vitro</i> pokeweed mitogen (IIF on tissues)	14	0/32	0/32	6/32 (19%)	5/32 (15%)	4/32 (12%)	7/32 (19%)

Discussion

The use of the horse to investigate latency of EHV-1 is compromised by the coexistence of EHV-4 in the population. The search for a more convenient laboratory animal model has focussed on hamsters and mice. The hamster model had major drawbacks due to the liver being the site of viral replication.

After the preliminary efforts of different researchers (Hatzios and Reagan, 1960; Plummer et al., 1973; Patel and Edington, 1983), Awan et al. (1990) developed a murine model using the intranasal route of infection, in which abortion was also produced. The present work was primarily designed to look at the latency and reactivation kinetics of EHV-1 in the mouse, while the acute studies identified the sites in which primary replication was occurring.

In the case of EHV-1, the study of latency in mice is still in infancy, although Field et al. (1992) reported the reactivation of EHV-1 from mice following the application of different stimuli. A total of 17/58 (29%) stimulated mice yielded EHV-1 from either blood or turbinate samples. In the present study we attempted to reactivate EHV-1 (Ab4) from Balb/c mice 10 weeks after primary infection. However, infectious virus could not be isolated on cell culture whatever *in vivo* or *in vitro* strategy was used. Nevertheless virus was detected at low frequency by IIF in the lung, spleen, bronchial lymph nodes and semilunar ganglia when these tissues were incubated in the presence of medium containing PWM. By IIF, virus was detected in macrophages of the lung, lymphoid cells of spleen and bronchial lymph nodes, and the neuronal cells of trigeminal ganglia.

In vivo reactivation of HSV-1 with cyclophosphamide and dexamethasone followed by cocultivation and RNA-PCR for the detection of different HSV-1 genes has also been used by Shimeld et al. (1990) and Minigawa et al. (1994). A similar procedure was used for the detection of virus in the present study. Again IIF and RT-PCR only detected virus in similar tissues. PCR amplification of gB RNA transcripts was positive in the cerebrum, olfactory lobe and semilunar ganglia only. Surprisingly IIF and PCR results did not correlate with each other except in trigeminal ganglia. Lung, spleen and bronchial lymph nodes showed positive IIF staining by using a monoclonal against EHV-1 gB, whereas gB transcripts could not be amplified in these tissues by PCR. One possible explanation for this may be that the section of tissues examined did not contain detectable amount of viral transcripts.

In all these reactivation experiments infectious virus could not be recovered from mice. This is in spite of the fact that in the acute phase of infection EHV-1 is neurotropic following either *i.c.* or *i.n.* inoculation.

Incomplete reactivation of HSV-1 in Balb/c mice following cadmium reactivation has been reported by Fawl et al. (1996). *In vitro* establishment of non-productive infection by HSV-1 in keratinocyte culture has also been reported (Syrijanen et al., 1996; Hukkanen et al., 1999). Non-productive infection

of HSV-1 was characterised by the lack of detection of infectious virus in culture supernatant and in the direct homogenate of the culture. PCR for HSV-1 DNA showed the presence of HSV-1 DNA in this culture. The establishment of a quiescent HSV-1 infection in neurally differentiated PC12 cells have also been reported by Danaher et al. (1999). Spontaneous shift of HSV-1 productive, persistently infected macrophage-like culture to a non-productive culture has also been documented by Tirado et al. (1998). Although viral proteins were detectable by immunoblots, attempts to recover infectious virus were unsuccessful.

The failure to reactivate the infectious virus in the present study is also substantiated by the findings of Baxi et al. (1996). Following the administration of dexamethasone and/or cyclophosphamide, there was no shedding of infectious virus from any of the murine tissues. However, they were able to detect the viral DNA in the olfactory bulb, trigeminal ganglia and peripheral blood mononuclear cells by nested PCR but not by *in situ* hybridisation.

In the present study EHV-1 was shown to establish virus that persisted at a low level in mice following acute infection, but it was not possible to reactivate infectious virus from these mice. This differs from HSV-1, which establishes latent infection in the mice, and these latent infections are easily reactivable to produce infectious virus (Stevens and Cook, 1971).

Latent EHV-1 and EHV-4 infections in horse have been shown to reactivate from lymphoid tissues following the administration of corticosteroids (Edington et al., 1985; Edington et al., 1994a, b; Browning et al., 1988; Welch et al., 1992) and also by IL-2 or chorionic gonadotrophin stimulation of leukocytes (Smith et al., 1998). Borchers et al. (1999) also have been able to detect latency associated transcripts of EHV-1 and EHV-4 by PCR from trigeminal ganglia of the latently infected horses. But as in our present study, so far the recovery of infectious virus from the trigeminal ganglia of horses has not been recorded. The results of the present study indicate that the mouse model has considerable limitations as a model for latency and reactivation studies of EHV-1 in relation to the natural host but support the concept that α -herpes virus infections may produce a persisting genome hindering effective reactivation.

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