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A hamster model of equine herpesvirus 9 induced encephalitis

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An acute and lethal infection of equine herpesvirus 9 (EHV-9), a new type of equine herpesvirus, was established in Syrian hamsters by intranasal inoculation. Clinical symptoms included the loss of body weight, nasal and ocular discharges and apparent neurological symptoms. Both LD_{50} and ID_{50} were equal at 33 plaque forming units. Histological and immunohistochemical examination demonstrated that the virus replicated in the olfactory mucosal cells and in the neurons of the olfactory bulbs, cerebrum and mesencephalon. The induction of encephalitis by intranasal but not by other routes of inoculation (i.v., i.p., i.m.) indicated that EHV-9 entered the brain via the olfactory nerve and then spread trans-synaptically to connecting neurons along the olfactory tract. This animal model should be useful for studying the pathogenesis and neurovirulence of this newly discovered neurotropic virus as well as other neurotropic herpesviruses. *Journal of NeuroVirology* (2000) **6**, 314–319.

Keywords: herpesvirus; neuropathogenesis; hamster

Introduction

We previously described the isolation and identification of a new equine herpesvirus from the epizootic encephalitis of Thomson's gazelle (*Gazella thomsoni*) (Fukushi *et al*, 1998). The outbreak occurred in a herd of gazelles from the end of September to the beginning of October 1993. Seven of ten gazelles without any symptoms or with apparent neurological symptoms died. All dead gazelles had nonsuppurative encephalitis, characterized by necrosis and degeneration of neurons, glial reaction, and perivascular infiltration of mononuclear cells in the cerebrum (Yanai *et al*, 1998). Intranuclear inclusion body was seen in the degenerated neurons in five animals. The herpesvirus was isolated from two animals at the late stage of the epidemic. These findings indicated that the infection was caused by the isolated herpesvirus. Generally, the herpesvirus is not considered to cause acute and lethal infection in the natural or reservoir host species. Because the gazelle was not considered a natural host of the isolated herpesvirus, the reservoir of the virus is still unknown. The isolated herpesvirus, gazelles herpesvirus 1 (GHV-1), was serologically closely related to EHV-1 and EHV-4 (Fukushi *et al*, 1998). However, DNA fingerprints of GHV-1 with *Bam*HI, *Bgl*II, and *Eco*RI were different from those of EHV-1 and other equine herpesviruses. DNA identity of GHV-1 was estimated to be about 95% to EHV-1 and EHV-8, and 60% to EHV-4, based on nucleotide sequences of the glycoprotein G gene and a conserved region of the glycoprotein B gene. Therefore GHV-1 has been established as a new equine herpesvirus, equine herpesvirus 9 (EHV-9).

Our previous studies have shown that the virulence of EHV-9 to the horse is different from EHV-1. EHV-9 caused pyrexia, interstitial pneumonia, and inapparent encephalitis accompanied with virus excretion and viremia by intranasal inoculation (Taniguchi *et al*, 2000). Histopathological findings of EHV-9 infection in the horse were characterized by perivascular infiltration of mononuclear cells and glial reaction in the olfactory and limbic systems without vascular lesions. These findings in the horse are different from EHV-1 myeloencephalitis which is characterized by vasculitis with secondary hypoxic degeneration in adjacent neural tissue (Allen and Bryans, 1986). Although these EHV-1 induced lesions have been

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considered to be due to immune-complex and vascular tropism of EHV-1, the pathogenesis is still not defined. On the other hand, EHV-9 has a relatively wide host range and specific pathogenesis characterized by tropism to respiratory and neural tissues. Therefore EHV-9 may become a potential pathogen for horses and other animals and induce neurological and respiratory disease in these animals.

Rodents, including Syrian hamster and mice, are known to be susceptible to EHV. The Syrian hamster has been used as an experimental animal for studying EHV-1 infection. Doll *et al* (1953) adapted EHV-1 to suckling hamsters. Adopted EHV-1 causes hepatitis and acute fatal infections (Doll *et al*, 1953) in addition to virus-induced abortion (Burek *et al*, 1975). The hamster has also been used to study antiviral agents (Rollinson and White, 1983) and immune responses to virus (Stokes *et al*, 1989; Wilks and Coggins, 1977). Recently mice have been used as experimental animals for studying respiratory infection and abortion (Awan and Field, 1993; Awan *et al*, 1991; Inazu *et al*, 1993). Intranasal inoculation of BALB/c mice with EHV-1 has been shown to induce acute respiratory infection and abortion. Our previous studies have shown that EHV-9 caused neurological disease in mice by intranasal inoculation (Fukushi *et al*, 1998). However, the use of hamsters for EHV-9 infection has not been investigated. Therefore, to develop a suitable animal model for studying the neuropathogenesis of EHV-9, we evaluated hamsters.

Results

Different routes of inoculation
We first evaluated the virulence of EHV-9 by different routes of inoculation. The inoculation routes studied were intranasal, intravenous, intraperitonial, intramuscular, intraocular and subcutaneous. Only intranasal inoculation of EHV-9 induced disease, including the loss of body weight and neurological symptoms, 3 days post inoculation. Neurological symptoms developed 3 days post inoculation. The symptoms included restless behavior, salivation, nasal and ocular discharges and crouching posture at the onset of disease followed by sensitive reaction to external stimulation, epileptic symptoms and incoordination of limbs at the climax, and then coma. Deaths ensued 1–3 days after the appearance of neurological symptoms.

The virus was recovered from the brain of hamsters inoculated intranasally with the virus, but not from other organs. Isolation of the virus from nasal swabs was not successful, however, EHV-9 DNA was detected by PCR in the same samples used for isolation. In hamsters inoculated with the virus by other routes, no symptoms and lesions were observed and EHV-9 was not recovered from any organs.

Virulence of EHV-9 in hamster
Dose dependency of pathogenesis and virulence was evaluated by intranasal inoculation of hamsters with EHV-9 (Figure 1). The incubation period was 3–5 days. Deaths were observed in animals which developed neurological symptoms. The animals died 1–3 days after the appearance of neurological symptoms. The virus was recovered from the brain of hamsters with neurological symptoms and not from animals with no symptoms. The titers of the virus recovered were 10^5 p.f.u./brain. LD_{50} and ID_{50} were both 33 p.f.u.

Pathological findings
Gross pathologic changes were not observed in all animals at necropsy. However, histopathological changes were observed in the brain and lungs of the animals inoculated intranasally with EHV-9 (see below).

Similar histopathological changes were observed in the central nervous system, and in lungs of both sick and dead animals. Histopathological changes of the central nervous system were observed in the olfactory bulbs, cerebrum and mesencephalon, but not in the cerebellum and medula oblongata (Figure 2). The histological changes of the central nervous system were degeneration and necrosis of neurons and rarefaction of surrounding tissues (Figure 3A). Acidophilic to amphophilic intranuclear inclusion body was frequently found in the nucleus of degenerated neurons. Mild to severe perivascular cuffing and focal and diffuse glial proliferation were also observed (Figure 3B,C). These neuropathologi-

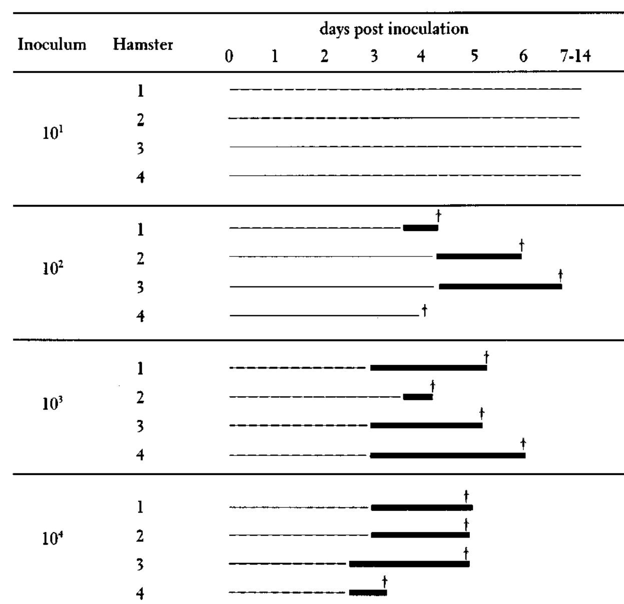

Figure 1 Dose dependency of pathogenicity and virulence of EHV-9 in hamsters. Critical period and death are shown by the bold line and a symbol (†), respectively.

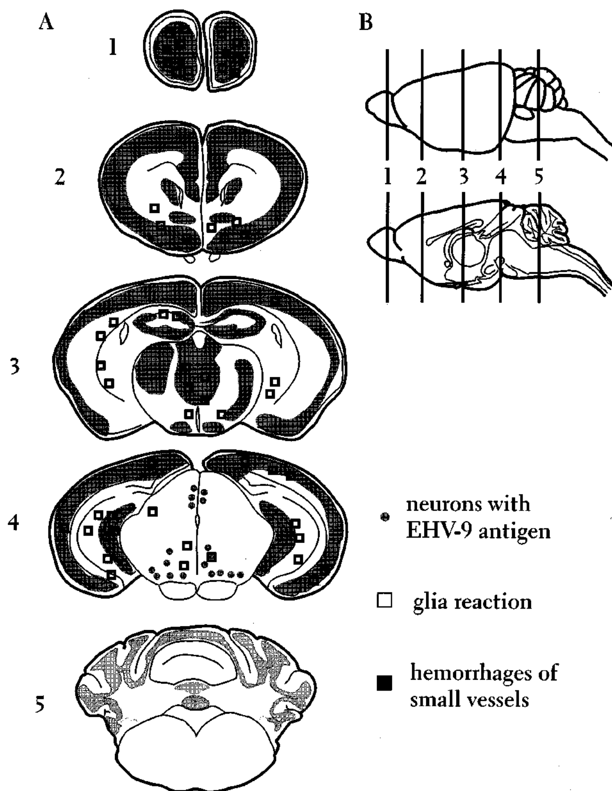

Figure 2 Distribution of lesions in the brain of hamsters inoculated with EHV-9 intranasally. (**A**) Distribution of lesions in each section. (**B**) Levels of brain sections.

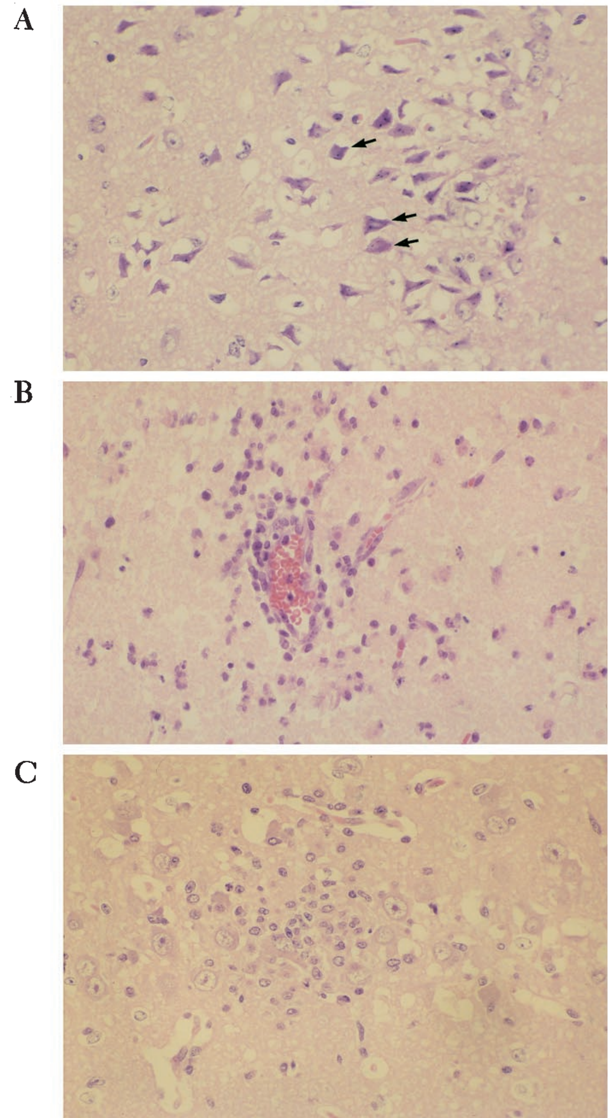

Figure 3 Histopathology of the cerebral cortex layers II to III (**A**), III (**B**) and IV (**C**). Note degenerated neurons (arrows) in (**A**), perivascular cuffing in (**B**) and focal glia reaction in (**C**). H-E stain, ×100.

cal changes were localized in the mitral cell layer, glomerular layer and granular layer of the olfactory bulbs. Severe neuropathological changes were also observed in layers II to IV, a part of layer V of cerebral cortex, hippocampus and dentate gyrus. Cellular reactions including perivascular cuffing and glial reactions were observed in the cerebral medulla and thalamus but rarely in the cerebral cortex.

Pathological findings of the nasal mucosal membrane were localized hypertrophy of mucosal epithelial cells and infiltration of the submucosa with lymphocytes.

Lung histopathology included hypertrophy of alveolus septum and infiltration of the interstitium with lymphocytes, neutrophils, and macrophages (data now shown). Moderate activation of lymphocytes was found in the bronchial lymphoid tissue.

No pathological changes were observed in other organs, including liver, spleen, kidney and thymus.

Immunohistochemical findings
EHV-9 antigen was detected by immunohistochemical staining in neurons containing intranuclear inclusion, degenerated neurons and apparently normal neurons (Figure 2). The antigens were localized in the cytoplasm and neuronal fibers (strong stain) and the nucleus (intermediate to weak stain). Staining of inclusion bodies were not consistent.

Immunohistochemical stain correlates with inflammatory reaction. The neurons containing EHV-9 antigens were frequently found in the glomerular, mitral cell, and granular cell layers of the olfactory bulbs, layers II to IV, less frequently in layers V and VI of the cerebral cortex, stratum pyramidale and stratum radiatum of hippocampus, granular layer of dentate gyrus, lateral olfactory stria, pyriform lobe, cortical amygdaloid body, and entorhinal cortex (Figure 4A,B), and were less frequently found in the

callosal body, caudate nucleus, anterior commissure, medial olfactory stria and thalamus. A few EHV-9 antigen possessing cells were also found in the cerebral peduncle of mesencephalon. No positive cells were seen in the cerebellum and medula oblongata.

EHV-9 antigens were also found in supporting cells and olfactory cells in the nasal mucosa (Figure 4C).

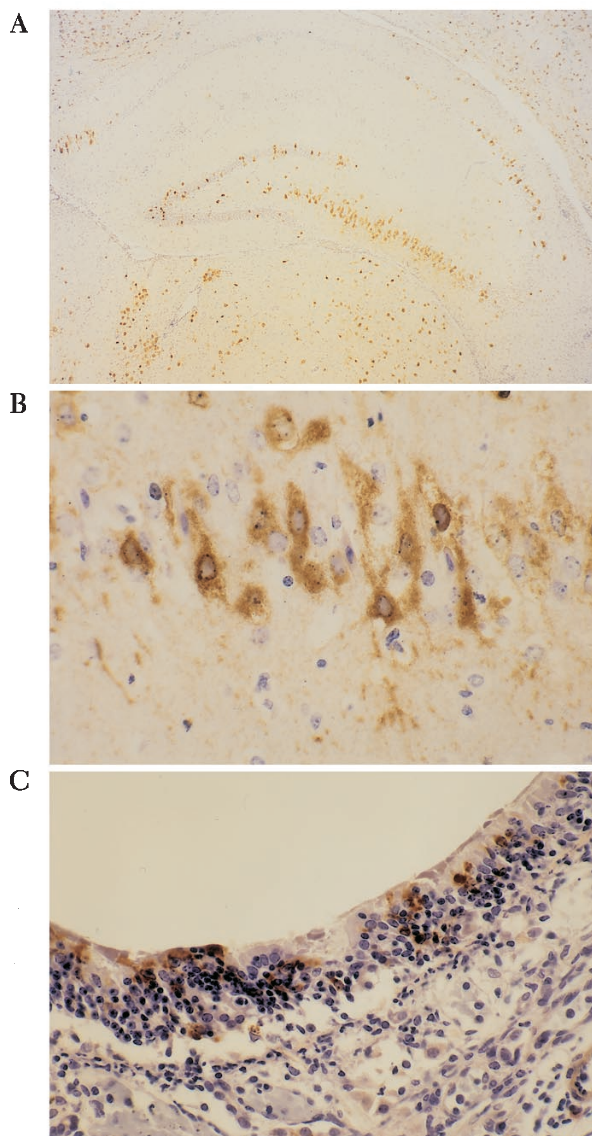

Figure 4 EHV-9 antigen positive cells in the limbic system and in the olfactory mucosa. (**A**) Frontal section at the thalamus. EHV-9 antigen positive cells in the cerebral cortex, hippocampus, dentate gyrus, entorhinal area, medial and lateral nuclei of thalamus were stained. H-E stain, ×25. (**B**) A higher magnification of (**A**) EHV-9 antigen positive cells in the pyramidal cell layer of the hippocampus were stained. Note the cell body and axon of neurons were stained. ABC stain, ×100. (**C**) Demonstration of EHV-9 antigen possessing cells in the olfactory mucosa by ABC stain, ×100.

Discussion

EHV-9 caused lethal encephalitis in hamster, characterized by degeneration and necrosis of neurons which accompanied intranuclear inclusion body, perivascular infiltration of mononuclear cells, and focal glial reaction in the olfactory bulbs and cerebrum, and mild interstitial pneumonitis, but no obvious histopathological changes in other organs. EHV-9 antigens were detected in the degenerated neurons, apparently normal neurons, and neural fibers but not in glial cells. These neuropathological findings of EHV-9 in hamsters were similar to EHV-9 induced encephalitis in Thomson's gazelles. The pathological findings in gazelles is non-suppurative encephalitis, characterized by necrosis and degeneration of neurons, glial reactions and perivascular cuffing with no vascular lesions in the cerebrum (Yanai *et al*, 1998). The histopathological changes were mainly located in the cerebrum, especially the frontal lobe. These findings indicated that EHV-9 possesses strong neurotropism. Therefore EHV-9 may induce similar neuropathology in the brain of animals.

The LD_{50} and ID_{50} titers of EHV-9 in hamsters were equal 33 p.f.u. All affected hamsters died indicating that EHV-9 is a virulent neuropathogen. In comparison, the LD_{50} titers of EHV-9 infection in mice were about 400 p.f.u. Some of the affected mice recovered from the EHV-9 infection, suggesting that mice are less susceptible to EHV-9 than hamsters (Fukushi *et al*, 1998). These findings indicated that the hamster is the most susceptible host for EHV-9 and that the hamster model should serve as a suitable model for studying the pathogenesis of EHV-9.

We found EHV-9 antigen in neurons and neural fibers but not in glial cells, indicating that susceptible cells of EHV-9 in the central nervous system of hamster are neurons (neuronotropism). However, other neurotropic herpesviruses such as herpes simplex virus 1 (HSV-1) and pseudorabies virus (PRV) are known to infect glial cells as well as neurons (Johnson, 1998). Therefore, the neuronotropism might be the most distinct characteristic property of EHV-9, differentiating it from other neurotropic herpesviruses.

EHV-9 seemed to enter the brain by the olfactory route along the neuronal tract via trans-synaptic transmission. This assumption was further supported by our histopathological findings showing that EHV-9 antigen containing cells were localized to the olfactory mucosal cells and neurons of the olfactory tract, limbic system, and cerebral cortex. Several routes of entry of viruses into the central nervous system has been postulated. There are neural, olfactory, and hematogenous routes (Johnson, 1998). Infection by EHV-9 in hamster was established by intranasal inoculation only, indicating that EHV-9 entered the central nervous system

via the olfactory route only, but not the neural nor hematogenous route. On the other hand, other neurotropic herpesviruses including HSV-1 and PRV may enter the central nervous system via other routes by intravenous, intramuscular, and intraperitoneal inoculations (Johnson, 1998), suggesting that EHV-9, unlike other neurotropic herpesvirus, has a strict neurotropism. Based on these findings we propose that EHV-9 enters from the nasal mucosa along the olfactory pathway and spread trans-synaptically via its connections to the hippocampus, amygdala and cerebral cortex.

In summary, we propose that hamster should be the most susceptible and useful model for studying the pathogenesis and neurovirulence of EHV-9, a newly discovered neurotropic virus infection as well as other neurotropic herpesviruses.

Materials and methods

Hamster
Three weeks old male specific pathogen free Syrian hamsters (SLC, Shizuoka, Japan) were used. The average body weight at the time of inoculation was approximately 50 g. Food and water were given *ad libitum* through experiments.

Viral culture
Madine-Derby bovine kidney (MDBK) cells were used for propagation of viruses. Seed stocks of EHV-9 P19, 5th passage in MDBK cells, were used for inoculation. The titer of virus suspension was 1×10^5 p.f.u./ml.

Inoculation of animals
Intranasal inoculation was done by delivering 25 μl of viral suspension (10^3 p.f.u.) into each nasal cavity while animals were under anesthesia. Intravenous, intraperitoneal, intramuscular, and intraocular inoculations were done by injecting 50 μl of virus suspension. Control animals were inoculated with Eagle's MEM. Animals were observed for 7 days post inoculation. Animals were sacrificed by giving an overdose of anesthetic when symptoms appeared. Animals were dissected according to the necropsy procedure. Tissues were fixed in 4% buffered paraformaldehyde.

Determination of ID_{50} and LD_{50}
Serial 10-fold dilution of viral suspensions were inoculated intranasally to four animals per dilution. Animals were observed for 2 weeks post inoculation. Dead animals were necropsied immediately. Surviving animals were euthanized and necropsied to obtain brains for viral isolation. ID_{50} and LD_{50} were calculated by Reed and Muench method (Reed and Muench, 1938).

Histopathological examination
Brain and other major organs were examined. Organs were fixed in 4% paraformaldehyde in phosphate buffer, embedded in paraffin, sectioned and stained with hematoxylin and eosin (HE). Brains were sectioned coronally at several levels.

Immunohistochemistry
Immunohistochemistry was performed with paraffin sections. Tissue sections were treated with proteinase K (100 μg/ml) for 60 s at room temperature, then incubated with anti-EHV-9 serum obtained from infected horse (Taniguchi *et al*, 2000) at 1:400 for 1 h at 37°C or overnight at 4°C. Reactions were detected by biotinylated anti-horse IgG in the avidin-biotinylated enzyme-complex (ABC immunoperoxidase) method (Vectastain ABC, Funakoshi, Tokyo, Japan).

Virological examination
Viral isolation was attempted with samples collected at necropsy and autopsy including the olfactory bulbs, cerebrum, cerebellum, spinal cord, nasal concha, lungs, liver, kidney, and spleen. The tissues were homogenized and resuspended in MEM. Heparinized peripheral blood was centrifuged at 5000 r.p.m. for 5 min to obtain buffy coat. Contaminated erythrocytes were lysed by treating with distilled water for 1 min (Awan *et al*, 1990). Leukocytes were suspended in MEM. Nasal swabs were suspended in MEM. Suspensions were filtrated through 0.45 μm pore-size membrane filter. Isolation was done by inoculation of MDBK cell monolayer. Inoculated cells were incubated at 37°C for 2–4 days and observed for cytopathic effect (CPE). The recovered viruses were identified by DNA fingerprinting (Fukushi *et al*, 1998).

PCR detection of virals DNA in tissues
PCR detection of EHV-9 DNA was done using tissue suspension and paraffin-embedded tissue sections as described elsewhere (Kirisawa *et al*, 1993; Fukushi *et al*, a manuscript in preparation).

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