J. Comp. Path. 2011, Vol. 145, 271–281

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EXPERIMENTALLY-INDUCED DISEASE

Kinetics and Pathogenicity of Equine Herpesvirus-9 Infection following Intraperitoneal Inoculation in Hamsters

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Summary

The kinetics of infection and pathogenicity of equine herpesvirus-9 (EHV-9) was studied in a hamster model. Five-week-old Syrian hamsters and 5-day-old suckling hamsters were inoculated intraperitoneally with 10^5 and 4×10^4 plaque-forming units of EHV-9, respectively. EHV-9 antigens were detected by immunocytochemistry in the peritoneal macrophages, which may be the primary site of virus attachment and propagation at 6 h post inoculation (hpi). At 12 hpi, viral antigen was observed in the abdominal nerves and ganglia (mainly the coeliac ganglia). Virus antigen was detected in the dorsal root (spinal) ganglia, in parts of the spinal cord (particularly the mid-lumbar area) and in the myenteric plexuses at 36, 48 and 72 hpi, respectively. At 96 hpi, virus antigen was detected in the most caudal part of the brain. Polymerase chain reaction conducted on samples of the blood, spinal cord and brain revealed EHV-9 DNA in the spinal cord at 36 hpi and in the blood at 48 hpi and for 4 days after this initial detection. It is suggested that after initial propagation in the abdominal macrophages, EHV-9 infected the abdominal ganglia or myenteric plexuses and then travelled to the brain via the peripheral nerves and spinal cord. Examination of other organs also revealed the presence of EHV-9, suggesting that the virus might infect tissues other than those of the nervous system.

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Keywords: EHV-9; hamster model; neuropathogenesis

Introduction

Equine herpesvirus-9 (EHV-9), a varicellovirus in the subfamily Alphaherpesvirinae, is the newest member of the equine herpesviruses and is closely related to equine herpesvirus-1 (EHV-1). EHV-9 was first described in an outbreak of disease in Thomson's gazelles (*Gazella thomsoni*) in a Japanese zoo (Fukushi *et al*., 1997) and subsequently in a giraffe (*Giraffe camelopardalis reticulata*) with signs of encephalitis (Borchers *et al*., 2005; Kasem *et al*., 2008). Recently, EHV-9 was detected in a polar bear with progressive encephalitis (Donovan *et al*., 2009), raising fears of emerging infections in various wild and zoo animal species. Emerging EHV-9 infection is also a concern with respect to domestic animals, because the virus has exhibited a wide range of susceptible hosts and has been shown to be easily transmittable via the nasal route in goats (Taniguchi *et al*., 2000), pigs (Narita *et al*., 2000), dogs and cats (Yanai *et al*., 2003a, b) and common marmosets (Kodama *et al*., 2007).

The pathogenesis of EHV-9 following nasal infection has been studied in a suckling hamster model. EHV-9 propagated in the olfactory epithelium 12–24 h post inoculation (hpi) and then gained access to the brain through the olfactory and trigeminal nerves (El-Habashi *et al*., 2010a).

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0021-9975/$ - see front matter
doi:10.1016/j.jcpa.2011.01.009

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EHV-9 may also infect hamsters by the ocular, oral and intraperitoneal routes (El-Habashi *et al.*, 2010b). Animals infected via the oral and peritoneal routes exhibited milder lesions and fewer viral antigen-positive cells in the glomerular and mitral cell layers of the olfactory bulb than animals infected via the nasal route. This observation suggests that the virus most likely does not enter the brain through the olfactory nerve, but may enter through other nerves. Further investigations are needed to determine exactly how EHV-9 reaches the brain following infection by non-nasal routes and to identify the pathogenesis of EHV-9-induced encephalitis following these routes of administration. In particular, the primary site of propagation of the virus has not yet been identified, nor is it known how the virus gains access to the brain by these routes. Another unanswered question is the time required for the virus to travel to the brain. The pathogenesis of EHV-1 induced encephalitis and myelitis following intraperitoneal inoculation has been studied previously (Hasebe *et al.*, 2002), and other neurotropic viruses, such as Theiler's virus and poliovirus, have been studied in the CBA mouse model using different routes of infection in order to elucidate their detailed neuropathogenicity (Villarreal *et al.*, 2006).

The aims of the present study were (1) to determine the primary site of virus propagation following intraperitoneal inoculation of hamsters with EHV-9, (2) to characterize the pathway by which EHV-9 accesses the central nervous system (CNS), (3) to define the kinetics of development of encephalitis induced by EHV-9 and (4) to determine whether EHV-9 has any effect on the internal organs of infected animals.

Materials and Methods

Virus Culture

Madin-Darby bovine kidney (MDBK) cells were used for the propagation of EHV-9. The inocula were prepared by culturing the virus from the original seed stocks of EHV-9 (P19, 5th passage in MDBK cells). The virus was titrated by plaque-forming assay on MDBK cells.

Animals

Four 5-week-old male and twelve 10-week-old female Syrian hamsters that were 10 days pregnant were purchased from a breeder (SLC Inc, Hamamatsu, Japan). The animals were housed in plastic cages and kept in an isolated biohazard cabinet for approximately 1 week of acclimatization. The animals were provided with a basal pellet diet (Oriental MF, Oriental Yeast Co., Tokyo, Japan) and bottled water *ad libitum*. The experiment was conducted in accordance with laws and Standard Operating Procedures related to the use of laboratory animals. The experimental protocol was approved by the Animal Experiment Committee of the Faculty of Applied Biological Science at Gifu University.

Virus Inoculation

Four 5-week-old hamsters were injected intraperitoneally with a single dose of 10^5 plaque-forming units (PFUs) of EHV-9 virus and were maintained until the end of the experiment (15 days post inoculation [dpi]) or the appearance of neurological signs. The 12 pregnant dams were left to give birth, after which fifty 5-day-old suckling hamsters were inoculated intraperitoneally with 4×10^4 PFU of EHV-9 virus. Of the five suckling hamsters that were killed at intermediate intervals of 6, 12, 24, 36, 48, 72, 96, 120, 144 and 168 hpi, four were subjected to histopathological and immunohistochemical analysis and one provided samples for polymerase chain reaction (PCR) studies. Five uninfected animals were kept as a control group. The animals were checked for clinical signs at least three times daily.

Collection of Peritoneal Cavity Cells

Cells were collected from the peritoneal cavity as described by Goafa *et al.* (1996). After death, the abdominal skin was treated with 70% ethanol and then the outer skin layer was opened and 10 ml of cold phosphate buffered saline (PBS; pH 7.2, 0.01 M) containing 3% fetal calf serum was injected into the abdominal cavity using a 27-gauge needle attached to a 5 ml syringe. The abdomen was gently massaged and then the PBS solution was carefully aspirated using a 25-gauge needle attached to a 5 ml syringe. The cell suspension obtained was centrifuged at 400g for 8 min. The supernatant was discarded and the cells were resuspended in PBS in order to prepare smears for immunocytochemistry (ICC).

Pathological Investigations

The brain, different levels of spinal cord, lungs, liver, spleen and small and large intestines were taken from the juvenile hamsters and fixed in 10% neutral buffered formalin. The suckling hamsters were bisected in the sagittal plane and were fixed similarly. Samples were processed routinely and embedded in paraffin wax. Sections (5 μm) were stained with haematoxylin and eosin (HE). The suckling hamsters were not decalcified.

Fixed tissue sections were also subjected to immunohistochemistry (IHC) by the avidin–biotin complex (ABC) method as described previously (Yanai *et al.*, 1998). The primary antibody was a rabbit antibody specific for EHV-9 (1 in 800 dilution; Veterinary Microbiology Laboratory, Gifu

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