Neurotropism of Mouse-adapted Haemagglutinating Encephalomyelitis Virus

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Summary

The propagation of a mouse-adapted strain (67N) of haemagglutinating encephalomyelitis virus in infected mice and murine cells was examined by viral re-isolation and immunostaining. Viral propagation was strictly limited to the neurons and to an established line of neuroblastoma cells in in-vivo and in-vitro experiments. These results provide adequate evidence that this virus is neurotropic.

Introduction

Members of the family Coronaviridae cause various diseases in man and animals, and show fastidious requirements for in-vitro propagation. Murine hepatitis virus (JHM strain), a member of the coronaviruses, is neurotropic in mice and rats, and the mechanisms of its viral persistency and demyelination in the central nervous system (CNS) have been well studied (Stohlman and Weiner, 1981). However, little information about the neurotropism of other coronaviruses has been reported.

Haemagglutinating encephalomyelitis virus (HEV), a swine coronavirus, causes a vomiting-wasting disease, encephalomyelitis and inapparent infection in pigs of various ages (Mitchell, 1963; Mengeling *et al.*, 1972). We previously reported that HEV had neuropathogenic properties for mice, and that mice were resistant age-dependently to HEV (Yagami *et al.*, 1986). The present study was aimed at elucidating the neurotropism of HEV by in-vivo and in-vitro experiments in mice and murine cultured cells, respectively.

Materials and Methods

Virus and Viral Assay

The 67N strain of HEV, passaged 15 times in the brains of suckling mice, was used. A virus stock was prepared and viral infectivity assays were carried out as described by Yagami *et al.* (1986). Briefly, 10-fold dilutions were prepared from a 10 per cent pooled suspension of three mouse brains, made in phosphate buffered saline (PBS) containing antibiotics and clarified by centrifugation at 2000 rpm for 10 min. Four 3-day-old suckling mice were inoculated intracerebrally (i.c.) with 5 µl of each dilution. The inoculated mice were observed daily for 10 days. The infectivity titre was calculated as the LD_{50} value.

Viral Inoculation of Mice

Outbred 3- and 42-day-old ICR mice (SLC Japan Inc., Japan) were infected with the 67N strain of HEV i.c. and intranasally (i.n.). Mice, lightly anaesthetized with ether, were inoculated with virus suspension (5 μl; ca 5 LD_{50}) i.c. or i.n., on the right side, with a micropipette and 27-gauge needle. The infected mice, in groups of six, were observed every 12 h and killed by bleeding under anaesthesia to collect several tissues at various stages of infection. Tissue specimens were used for viral re-isolation (three mice) and immunohistochemical observations (three mice). Animal experiments were performed humanely in accordance with the guidelines for animal experimentation of this University.

Cells and Cell Cultures

Several murine cell lines [L-929 (fibroblast), Ehrlich-Lettre Ascites (ELA; epithelial), DBT (neuroglia), Neuro-2a (neuroblastoma) and NB41A3 (neuroblastoma)], and also primary or secondary cultured cells, [mouse embryonic (ME) cells, cultured neurons and astrocytes] were used to examine the in-vitro growth of HEV. L-929, ELA, Neuro-2a and NB41A3 were obtained from the American Tissue Culture Collection (ATCC). Growth in Eagle's MEM (Nissui Co., Japan) was supplemented with 10 per cent fetal calf serum (FCS) and non-essential amino acids (for L-929, ELA, DBT and Neuro-2a). Growth in Ham's F-10 (Nissui) was supplemented with 20 per cent FCS (for NB41A3).

Neuronal cell cultures were established from the cerebral cortex of mouse fetuses, aged 18 days, as follows. Neuronal cells, dissociated with 0·2 per cent trypsin (Flow Laboratories, Inc.) and 0·01 per cent DNAse I (Sigma), were cultured on laminin (Sigma)-coated coverslips in SFM-101 medium (Nissui), supplemented with 1 per cent FCS. After 24 h of culture, the medium was replaced with SFM-101 containing 10^{-2} mM-cytosine arabinoside (Ara-C:Sigma) and maintained for a further 24 h, to inhibit the growth of fibroblasts. Subsequently, cultured neurons were used for viral inoculation and maintained with SFM-101 without Ara-C.

Astrocyte cultures were also established from the cerebral cortex of mouse fetuses aged 18 days, by a method described for rat astrocytes (Kato *et al.*, 1981). The cerebral cortex sections were carefully separated from the meninges, to avoid contamination with fibroblasts. Dissociated cells were cultured in petri dishes containing F-10 medium supplemented with 20 per cent FCS. After 7 days of culture, dissociated cells were subcultured in F-10 supplemented with 10 per cent FCS. The cultured neurons and astrocytes were identified by immunostaining with anti-neuron specific enorase rabbit serum (ZYMED Laboratories, U.S.A.) and with a monoclonal antibody [RC1; a specific immunohistochemical marker of radial glial cells (Edwards *et al.*, 1990)].

The virus propagated in mouse brain was inoculated into each of the cells and serially passaged two or three times, to avoid retention of the unadsorbed virus in the medium. At the secondary or tertiary passage, the cultured cells were examined for cytopathic effect (CPE) and specific immunofluorescent antigen (IFA). In addition, the infectivity of the culture medium was assayed by the method described above.

Immunostaining

Viral antigen in paraffin-wax sections from infected mice was stained with anti-HEV rat serum, peroxidase-conjugated anti-rat IgG rabbit serum (CAPPEL), and 0·05 per cent 3,3-diaminobenzidine tetrahydrochloride. The sections were also counterstained with haematoxylin. Tissue sections from uninfected mice were used for control purposes. Similarly, HEV antigen in infected cultured cells was detected by the IFA method, with anti-HEV rat serum and fluorescein-conjugated anti-rat IgG rabbit serum (CAPPEL), after fixation with chilled acetone.

Results

HEV Propagation in Infected Mice

Viral distribution at intervals after inoculation was examined in i.c.- and i.n.-infected newborn and adult mice. The results are shown in Table 1. The infective viruses were limited to regions of the CNS, such as the olfactory bulb, the cerebrum and the cerebellum, with the exception of the lungs of i.n.-inoculated newborn mice at an early stage of infection. The infectivity titres were much higher in the CNS than in lung. Viral propagation in i.c.-infected mice was more rapid than in i.n.-infected ones, although little difference was demonstrated between the maximal viral titres.

Specific viral antigen was observed in the neurons of the olfactory bulb, cerebrum and cerebellum but not in liver, spleen or kidney. In the newborn mice infected i.c., the viral antigens were scattered diffusely throughout the olfactory bulb, cerebrum and cerebellum, and were most prominent in the neurons of the cortex (layers II to V), in the hippocampus and in the Purkinje cells of the cerebellum 2 to 3 days after infection (Figs 1a to 1c). They were rarely localized in the glial cells. Similar findings were observed at the terminal stage (5 days after infection) in i.n.-infected newborn mice. The antigen, however, was restricted to the olfactory bulb, trigeminal nerve and lower brainstem at an early stage (2 days after infection), suggesting neurotransmission through the tractus olfactorius and trigeminal nerve (Fig. 1d). In the adults, antigen-positive cells were reduced and located particularly in the neurons in the outer layer (II) of the cortex and hippocampus but not in the inner layers (III to V) or in the Purkinje cells (data not shown). A slight

Table 1
Viral distribution in i.c.- and i.n.-infected mice

Mouse age (days)	Organs	*Infectivity titres* at the stated number of days after									
		infection i.c.					infection i.n.				
		1	2	3	5	7	1	2	3	5	7
Newborn (3)	Olf. bulb	≦0.5	4.0	6.5	N	N	—	0.7	4.5	5.5	N
	Cerebrum	≦0.5	4.5	6.3	N	N	—	—	2.7	5.7	N
	Cerebellum	≦0.5	4.3	6.0	N	N	—	—	3.0	5.3	N
	Lung	—	—	—	N	N	0.7	—	≦0.5	—	N
	Liver	—	—	—	N	N	—	—	—	—	N
	Spleen	—	—	—	N	N	—	—	—	—	N
	Kidney	—	—	—	N	N	—	—	—	—	N
Adult (42)	Olf. bulb	—	≦0.5	≦0.5	2.7	N	—	≦0.5	≦0.5	1.0	2.0
	Cerebrum	≦0.5	≦0 5	≦0.5	3.0	N	—	—	≦0.5	1.0	2.7
	Cerebellum	≦0.5	≦0.5	≦0.5	1.3	N	—	—	≦0.5	≦0.5	1.0
	Lung	—	—	—	—	N	—	—	—	—	—
	Liver	—	—	—	—	N	—	—	—	—	—
	Spleen	—	—	—	—	N	—	—	—	—	—
	Kidney	—	—	—	—	N	—	—	—	—	—

Three mice each were examined for each point, and infectivity of 10 per cent pooled organ suspension was assayed. * Infectivity titre expressed as log10 $LD_{50}/5\,\mu l$ for 3-day-old mice. N = Not tested because of animals' death. — = Virus was not reisolated.

antigenic reaction was detected also in the tracheal epithelial cells of i.n.-infected newborn mice 1 to 3 days after infection.

HEV Propagation in Cultured Cells

As shown in Table 2, the 67N strain of HEV was able to propagate in established line NB41A3 cells, and primary cultured neurons but not in L-929, DBT, ELA, Neuro-2a or mouse embryonic cells. A high titre of virus was detected, particularly in NB41A3 cells, which showed a mild CPE and detachment. The CPE was not clear in infected neurons because complete monolayers of primary neuron cultures were not obtained, but virus titres similar to those in NB41A3 cells were detected.

As for IFA in the infected cells, bright fluorescence was observed in NB41A3 cells and in primary cultured neurons. The antigen was widely distributed, not only in the perikaryon but also in the dendrites of cultured neurons. On the contrary, no antigen was demonstrated in L-929, DBT, ELA, mouse embryonic cells, or astrocytes (Figs 2a–d).

Infective virus was recovered from the culture medium of NB41A3 cells and neurons but not from other cell cultures. A high virus titre was shown in the growth medium of the infected NB41A3 cells.

Discussion

The pathogenic mechanism of HEV infection in pigs appears to be quite different from that of other coronaviruses. The disease is initiated as an inapparent infection of the respiratory tract, and the virus spreads via peripheral nerves to the CNS. Subsequently, the infection of neurons which regulate peristaltic functions of the digestive tract results in a vomiting disease

Table 2
Viral propagation in cultured murine cells

Cells	No of preliminary passages of virus	Cytopathic effects	Infectivity titres*	Immunofluorescent antigen (IFA)
Cell lines				
L929 (fibroblast)	3	—	—	—
ELA (epithelial)	3	—	—	—
DBT (neuroglia)	3	—	—	—
NB41A (neuroblastoma)	3	+	2.7	+
Neuro2A (neuroblastoma)	3	—	—	—
Primary or secondary cells				
Embryonic fibroblast	2	—	—	—
Neuron	2	—	2.5	+
Astrocyte	2	—	$\gtrsim 0.3$	—

* Expressed as log10 $LD_{50}/5$ µl for 3-day-old mice.
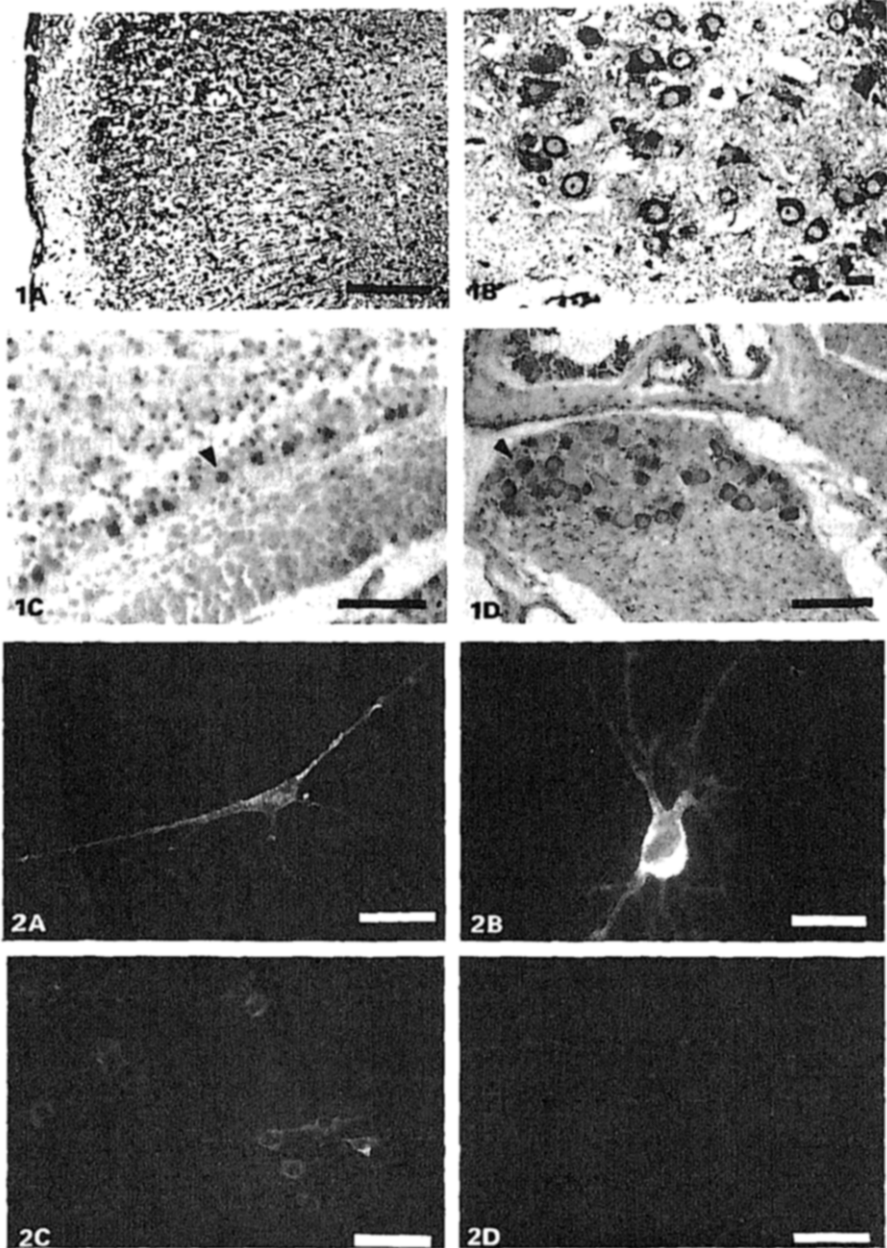
Fig 1. HEV antigen was present in neurons in layers II to V of cerebral cortex (A and B), and in cerebellar Purkinje cells (C) in newborn mice 2 days after inoculation i.c. The antigen was also present in neurons of the trigeminal nerve (D) 3 days after inoculation of newborn mice i.n. Immunoperoxydase-haematoxylin staining was used. Scale bars = 100 μm in A, 10 μm in B and 50 μm in C and D.

Fig. 2. HEV antigen was present in cultured neurons (A and B) and the established neuroblastoma line NB41A3 (C), but not in Neuro-2a (D) or other murine cells (data not shown), 72 h after inoculation. Immunofluorescent staining was used. Scale bars = 50 μm in A, C and D, and 25 μm in B.

(Andries and Pensaert, 1980a, b). In oronasally infected newborn pigs, the viral antigens have been demonstrated in epithelial cells of the tonsils at an early stage of infection and in neurons of the cortex, medulla oblongata, pons and midbrain at a later stage (Narita *et al.*, 1989a, b). In-vitro propagation of HEV has been reported only in primary and secondary cell cultures of swine kidney (Mengeling *et al.*, 1972) and thyroid (Sato *et al.*, 1983), and in lined ESK and SK-K cells (Mengeling, 1973; Hirano *et al.*, 1990) derived from swine kidney.

In the present study, propagation of HEV was strictly limited to neurons in infected mice, and to cultures of neurons and an established line of neuroblastoma cells (NB41A3). This is the first report of HEV proliferation in cultured neurons. These results provide sufficient evidence that HEV is a neurotropic virus. The lower titre of the virus in the lung of i.n.-inoculated newborn mice than in the CNS may have been due to the uptake of virus by pulmonary macrophages or epithelial cells. It is probable that the HEV strain used had become more neurotropic for mice as a result of mouse brain passage. It would be interesting to ascertain whether its powers of propagation in pig brain had increased.

In the present study, HEV was capable of propagating in NB41A3 but not in Neuro-2a cells. Both NB41A3 and Neuro-2a have been independently established by cloning from a neuroblastoma (C-1300) in a strain A albino mouse, and both have retained the characteristics of neuroblastoma cells (Hay *et al.*, 1988). However, there are substantial differences between them. For instance, NB41A3 cells secrete acetylcholine esterase together with the enzymes for the synthesis of the neurotransmitters, choline acetylase and tyrosine hydroxylase, as adrenergic neuron (Hay *et al.*, 1988), whereas Neuro-2a has not been shown to secrete these enzymes. Recently, Schultze *et al.* (1990) reported that HEV attached to an N-acetyl-9-O-acetylneuraminic acid-containing receptor which was destroyed by acetylesterase. These enzymes of NB41A3 might be related to the difference in HEV propagation between the two lines of neuroblastoma cells.

Previously, we demonstrated the age-dependency of mouse susceptibility to fatal HEV infection (Yagami *et al.*, 1986). The present study shows additional differences between infected newborn and adult mice, particularly in the distribution of HEV antigen in the layers of the cerebral cortex. These findings resemble those of Ogata *et al.* (1991) in rats infected with Japanese encephalitis virus (JEV). These authors suggested that the neurotropism of JEV was dependent on the degree of neuronal maturation in the cortex, which paralleled a particular pattern of disappearance of antigen from the inner layers to the outer layers of the cortex. Further work is needed to elucidate the relation between age-dependency of HEV infection and neuronal maturation, and localization of receptors on susceptible cells.

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