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# Neurotropism of Swine Haemagglutinating Encephalomyelitis Virus (Coronavirus) in Mice Depending upon Host Age and Route of Infection

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## Summary

Mice aged 1, 4 or 8 weeks were inoculated with haemagglutinating encephalomyelitis virus (HEV), strain 67N, by the intracerebral (i.c.), intranasal (i.n.), intraperitoneal (i.p.), subcutaneous (s.c.), intravenous (i.v.) or oral route, with different doses. In 1-week-old mice, mortality and mean time to death were mostly the same regardless of the inoculation route, except for the oral route, which appeared to be the least effective. The virus killed 4-week-old mice readily by all routes of inoculation except the oral, and 8-week-old mice by i.c., i.n. or s.c. inoculation. In descending order of efficacy, the routes of HEV infection were: i.c., i.n., s.c., i.p., i.v. and oral. To follow the spread of HEV from peripheral nerves to the central nervous system (CNS), the virus was inoculated subcutaneously into the right hind leg of 4-week-old mice. The virus was first detected in the spinal cord on day 2, and in the brain on day 3. The brain titres became higher than those of the spinal cord, reaching a maximum of $10^7$PFU/0.2 g when the animals were showing CNS signs. Viral antigen was first detected immunohistochemically in the lumbar spinal cord and the dorsal root ganglion ipsilateral to the inoculated leg; it was detected later in the pyramidal cells of the hippocampus and cerebral cortex, and in the Purkinje cells of the cerebellum but not in the ependymal cells, choroid plexus cells or other glial cells. The infected neurons showed no cytopathological changes.

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## Introduction

Haemagglutinating encephalomyelitis virus (HEV), a coronavirus, causes vomiting and wasting disease, and encephalomyelitis in piglets (Andries and Pensaert, 1980a,b; Siddell *et al.*, 1983). Greig and Girard (1963) isolated the virus from the brains of sucking pigs with encephalomyelitis. The disease was produced experimentally in piglets by oronasal inoculation (Alexander, 1962). In England, Cartwright *et al.* (1969) reported the isolation of an antigenically similar virus from sucking pigs showing anorexia, depression and vomiting, without encephalomyelitis. In America, Mengeling and Cutlip (1976) demonstrated that both major clinical forms of the disease (vomiting and wasting,

and encephalitis) were reproduced by inoculation with the same field isolate from pigs.

Yagami *et al.* (1986) produced fatal encephalitis in mice aged 35 days by the intracerebral (i.c.) inoculation of mouse-passaged HEV (strain 67N), but mice aged 20 or more days were resistant, even to large doses, when the virus was administered by the intranasal (i.n.), intraperitoneal (i.p.) or subcutaneous (s.c.) route. Hirano *et al.* (1990) reported successful propagation and plaque assay of HEV 67N in cultures of the established cell line, SK-K. Rats aged 1, 2, 4 or 8 weeks were subsequently inoculated with SK-K-grown virus by Hirano *et al.* (2001a) in an experiment in which the routes of infection, in descending order of efficacy, were i.c., i.n., s.c., i.p., i.v. and oral. Rats aged 1 or 2 weeks

were generally similar in terms of mortality and mean time to death, regardless of inoculation route, except for the oral route. Eight-week-old rats inoculated by the i.c., i.n. or s.c. route died, but those inoculated by other routes survived. In rats inoculated by the i.c. route, HEV antigen was found in the pyramidal cells of the hippocampus and cerebral cortex, and later in the large-sized neurons of the pons and spinal cord and in the Purkinje cells of the cerebellum, but not in the ependymal cells, choroid plexus cells or other glial cells of the central nervous system (CNS).

In the field of neuroscience, neurotropic viruses such as pseudorabies virus (PRV), herpes simplex virus (HSV) and rabies virus have been used as trans-neuronal tracers for studying neuronal connections of rats and mice (Kuypers and Ugolini, 1990).

Previous studies in rats showed that HEV infected neurons but not glial cells in the CNS via trans-synaptic pathways after inoculation by the s.c. route (Hirano *et al.*, 1998), i.c. route (Hirano *et al.*, 2001a) and i.n. route (Hirano *et al.*, 2001b); degenerative changes were not found in the HEV-infected neurons. Except for these studies in rats our experience of experimental HEV infection in laboratory animals is limited to 4-week-old mice, 2-month-old Syrian hamsters and 3-week-old Mongolian gerbils (Hirano *et al.*, 2001c).

The value of HEV as a trans-synaptic tracer for analysing the neuroanatomical connections of rats and mice would be enhanced by further information on infections in mice. The aim of the present study, therefore, was to define the neurotropism of HEV in mice of different ages, inoculated by various routes.

Table 1
Susceptibility of mice aged 1,4 or 8 weeks to inoculation with the virus by different routes

| Route | Dose (PFU) | Results* in mice aged (weeks) | | |
|---|---|---|---|---|
| | | 1 | 4 | 8 |
| Intracerebral | $10^5$ | ND | 5/4.2(4−5) | 5/4.8(4−6) |
| | $10^4$ | 5/3.0(3−4) | 5/4.2(3−4) | 5/5.6(5−6) |
| | $10^3$ | 5/3.6(3−4) | 5/5.3(5−6) | 5/6.6(6−7) |
| | $10^2$ | 5/3.6(3−4) | 5/6.5(5−8) | 4/7(7) |
| | 10 | 5/3.8(3−4) | 3/6.3(5−7) | 1/8(8) |
| Intranasal | $10^5$ | ND | 5/6.8(6−7) | 5/7.6(7−8) |
| | $10^4$ | 5/4.4(3−5) | 5/10.0(6−12) | 5/10.5(8−14) |
| | $10^3$ | 5/5.0(4−6) | 2/10.0(10) | 2/12.5(12−13) |
| | $10^2$ | 5/6.0(5−7)) | 0 | 0 |
| | 10 | 3/6.0(5−7) | 0 | 0 |
| Intraperitoneal | $10^6$ | ND | 5/4.4(4−5) | 3/6.3(6−7) |
| | $10^5$ | ND | 5/5.0(4−7) | 1/10(10) |
| | $10^4$ | 5/2.6(2−3) | 2/7(7) | 0 |
| | $10^3$ | 5/3.3(3−4) | 1/7(7) | 0 |
| | $10^2$ | 5/4.3(3−6) | 0 | 0 |
| | 10 | 4/4.3(4−5) | 0 | 0 |
| Subcutaneous | $10^6$ | ND | 5/5.4(4−6) | 5/6.5(6−7) |
| | $10^5$ | ND | 5/8.2(5−11) | 5/7.0(6−8) |
| | $10^4$ | 5/2.6(2−3) | 5/8.4(7−12) | 1/8(8) |
| | $10^3$ | 5/3.2(3−4) | 1/9(9) | 0 |
| | $10^2$ | 5/3.7(3−4) | 0 | 0 |
| | 10 | 1/7(7) | ND | ND |
| Intravenous | $10^6$ | ND | 5/5.6(5−6) | 0 |
| | $10^5$ | ND | 5/5.0(5−7) | 0 |
| | $10^4$ | ND | 2/6.0(5−7) | 0 |
| | $10^3$ | ND | 0 | 0 |
| Oral | $10^6$ | ND | 0 | 0 |
| | $10^5$ | 5/5.0(4−6) | 0 | 0 |
| | $10^4$ | 2/6.5(6−7) | 0 | 0 |

ND, not done.
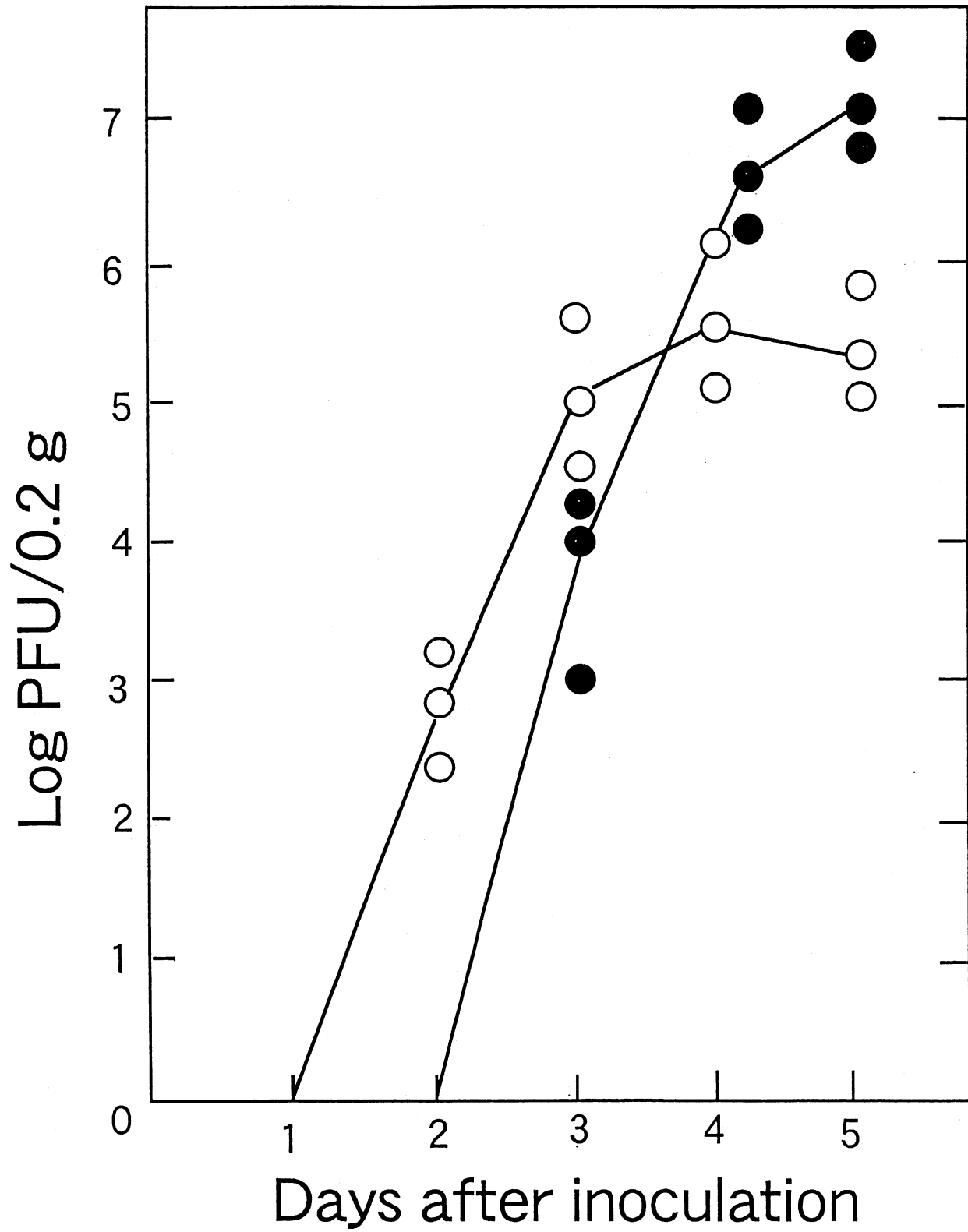*Deaths in groups of 5/mean number of days (and range) to death.

Fig. 1. HEV 67N growth in the spinal cord and brain of mice, killed in groups of three, after subcutaneous (s.c.) inoculation with $10^5$ PFU in the right hind leg. O- - -O, Spinal cord; ●- - -●, brain.

## Materials and Methods

### Mice

Specific pathogen-free ICR (outbred) mice were obtained from a commercial breeder (SLC, Hamamatsu, Japan) to supply offspring aged 1, 4 or 8 weeks. In most experiments, male mice were used and the animals were given free access to commercial pellets and water. Sucking mice were nursed by their dams. The mice were kept in lamina-flow units throughout the experiments, which were performed in accordance with the guidelines for animal experimentation of Iwate University.

### Virus and Assay

The plaque-purified 67N strain of HEV (Mengeling et al., 1972) was propagated and assayed for infectivity by the plaque method in SK-K cell culture, as described previously (Hirano et al., 1990), the infectivity titre being expressed in plaque-forming units (PFU). For viral assay of the brain and spinal cord, 10% (w/v) homogenates were prepared in Eagle's minimum essential medium, and the supernates were subjected to the infectivity assay after centrifugation at 2000 rpm (1000 $g$) at 4 °C for 10 min.

### Inoculation of Mice

The experimental design is shown in Table 1. Mice aged 1, 4 or 8 weeks ($n = 420$) were inoculated by the i.c., i.n., i.p., s.c., i.v. or oral route with various doses. The dose volume was 0.02 ml for i.c. and i.n. inoculation. For other routes of inoculation it was 0.2 ml, except that 1-week-old mice inoculated by the oral route were given 0.02 ml. Controls (two mice for each route of inoculation) were given fluid from uninfected SK-K cell culture. The animals were observed daily for 14 days after inoculation.

To examine the growth and spread of the virus in the CNS from peripheral tissue, 30 mice aged 4 weeks were inoculated subcutaneously over the right knee with $10^5$ PFU. Five mice were killed daily on each of days 1 to 5, three mice for viral assay, and two for immunohistochemistry (IHC).

### IHC

The infected mice were perfused with phosphate-buffered saline and a fixative containing 4% para formaldehyde under deep anaesthesia with pentobarbital. Sections of the brain and spinal cord, cut with a freezing microtome, were treated with anti-HEV 67N mouse antibody (1 in 1000) at 4 °C overnight, and then labelled with fluorescein isothiocyanate (FITC)-conjugated goat antiserum against mouse IgG at room temperature for 2 h. The sections were examined under a confocal laser scanning microscope (Olympus and Nikon, Tokyo, Japan).

## Results

### Inoculation of Mice

The results are shown in Table 1. Sucking mice aged 1 week given ≥100 PFU by all routes except for the oral route invariably died; i.v. inoculation in mice of this age was not tested. By the oral route, $10^4$ PFU killed only two of five mice; $10^5$ PFU, however, killed all animals. Sucking mice infected by the i.n., i.p. or s.c. route died in 2–7 days.

In mice aged 4 weeks, i.c. infection killed three of five mice given only 10 PFU, and all mice given ≥100 PFU, the time of death ranging from 3–8 days. Infection by the i.n., i.p., s.c. or i.v. route killed some or all animals, depending on dose. Mice of this age group were completely resistant to oral infection, even with the large dose of $10^6$ PFU.

In mice aged 8 weeks, only i.c. inoculation produced deaths (four of five mice) with a dose as small as 100 PFU. Inoculation by the i.n. and s.c. routes with doses of $10^4$ and $10^5$ PFU, respectively, was invariably lethal in 6–14 days. Intraperitoneal inoculation was comparatively ineffective, and inoculation by the i.v. and oral routes completely ineffective.

HEV was detected in the brains of mice that died, but not in animals that survived or in uninfected controls.

### Viral Growth in the Spinal Cord and Brain

Based on the results obtained in 4-week-old mice, mice of this age were inoculated by the s.c. route in the right hind leg with $10^5$ PFU. On day 4, the mice developed CNS signs. On day 6, five mice died. As shown in Fig. 1, the virus was first detected in the spinal cord on day 2 after inoculation and in the brain on day 3. On day 4, the infectivity titres of the spinal cord had increased, reaching a maximum of $10^6$ PFU/0.2 g; they declined later. The brain titres became higher than those of spinal cord, reaching on day 5 a maximum of $10^7$ PFU/0.2 g. Virus was not detected in the liver or spleen of any animal.

### Viral Spread from the Peripheral Nerves to the CNS

After s.c. inoculation, HEV antigen was first detected immunohistochemically on day 2 in
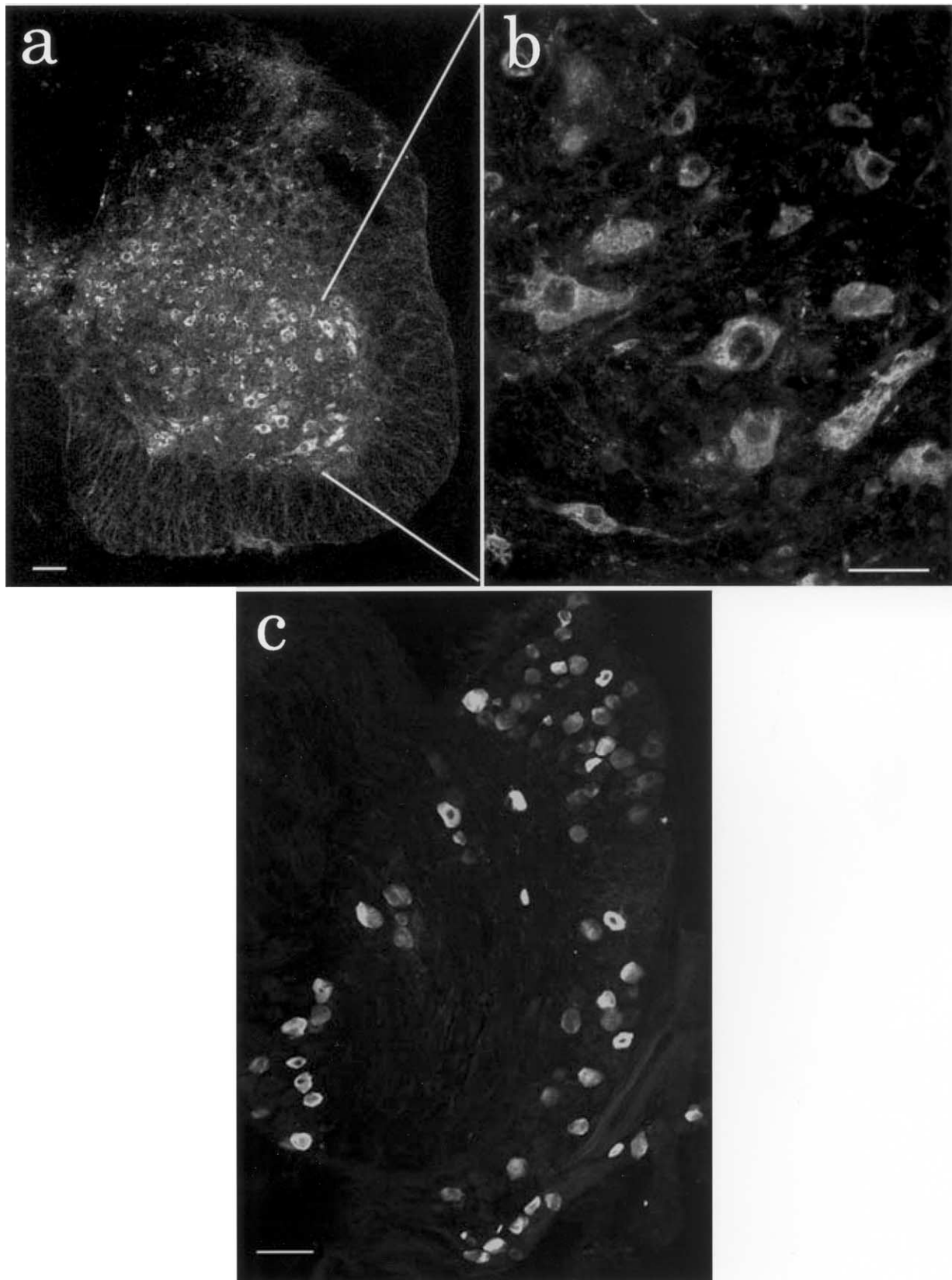
Fig. 2a–c. (a and b) Antigen-positive neurons in the spinal cord on day 3 after s.c. inoculation. (c) Antigen-positive cells in the dorsal root ganglion ipsilateral to the inoculated leg on day 3. IHC. Bars, 100 μm (a and c); 50 μm (b).
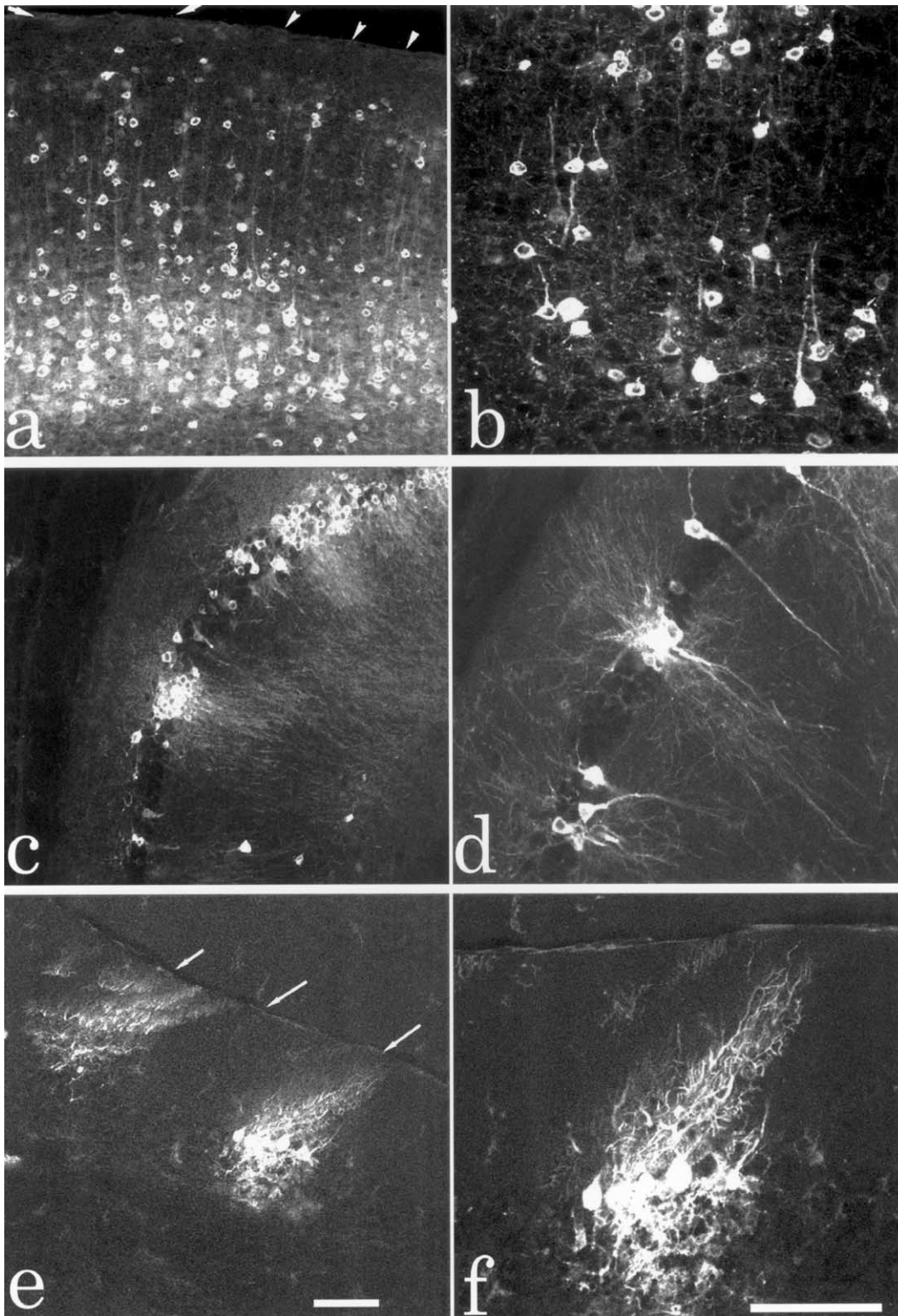
Fig. 3a–f. (a and b) Antigen-positive pyramidal cells in the cerebral cortex on day 4. (Arrowheads: pial surface). (c and d) Antigen-positive pyramidal cells in the hippocampus on day 4. (e and f) Antigen positive Purkinje cells of the cerebellum on day 4. (Arrows: pial surface). IHC. Short bar, 100 μm (e). Long bar, 100 μm (f). Figs 3a and c are at the same magnification as 3e. Figs 3b and d are at the same magnification as 3f.

the lumbar spinal cord and lumbar dorsal root ganglion (DRG) ipsilateral to the inoculated leg. On day 3, the viral antigen was detected in many neurons in the ventral and dorsal horn of the lumbar spinal cord (Fig. 2a, b) and large-sized neurons in the DRG (Fig. 2c) but not in the contralateral DRG. In the brain, antigen was first detected in pyramidal cells of the cerebral cortex and hippocampus. On day 4, it was detected in the contralateral half of the spinal cord and DRG. In the brain, antigen-positive neurons had increased in number and were distributed widely in the cerebral cortex (Fig.3a and b), hippocampus (Fig. 3c and d), pons and midbrain. As shown in Fig. 3a, b, the antigen-positive neurons in the cortex were distributed in layers III and V but not II and IV. In the cerebellum, viral specific antigen was detected in the Purkinje cells (Fig. 3e, f) but in only a few granular cells. HEV antigen tended to be found in the large-sized neurons but not in the ependymal cells, choroid plexus cells or other glial cells. Infected neurons appeared to be free from cytopathological changes.

## Discussion

Yagami *et al.* (1986) reported that mice aged ≥ 20 days were resistant to i.n., i.p. or s.c. inoculation with mouse-passaged HEV in the large dose of $10^6$ $LD_{50}$. However, in the present study, the virus readily killed 4-week-old mice by i.c., i.n., i.p., i.v. or s.c. inoculation, and 8-week-old mice by routes of inoculation other than i.p., i.v. or oral. These differences in results between the two studies may have been due to the use of different mouse strains. The present study showed that the neurotropism of HEV 67N in mice was dependent on the age of the host at the time of infection and on the route of inoculation. The results were largely similar to those obtained in rats of different ages by various routes of inoculation (Hirano *et al.*, 2001a).

Following s.c. inoculation into the hind leg, the virus was first recovered from the spinal cord and later from the brain. HEV antigen was first detected immunohistochemically in the lumbar spinal cord and lumbar DRG ipsilateral to the inoculated leg and later in pyramidal cells of the cerebral cortex and hippocampus, and in Purkinje cells of the cerebellum. In the cortex, antigen-positive neurons were distributed in the cell layers III and V. HEV antigen was detected mainly in neurons, but not in ependymal cells, choroid plexus cells or other glial cells. These findings were similar to those in previous studies of rats inoculated by the s.c. route (Hirano *et al.*, 1998), suggesting that the

virus spreads trans-synaptically along the neuronal pathways from the peripheral nerves to the CNS. Recent immunohistochemical studies (unpublished) in which CNS sections from HEV-infected rats and mice were double- or triple-labelled with antibodies to HEV, glial fibrillary acidic protein (for astrocytes) and TB4 antibody (for microglial cells) confirmed that HEV does not infect glial cells.

Among neurotropic viruses, rabies virus, PRV, HSV and Borna disease virus (BDV) are known to spread from peripheral nerves to the CNS. There are, however, differences in the neurotropic properties of these viruses. Rabies virus infects predominantly neurons, such as pyramidal cells in the hippocampus and cerebral cortex and Purkinje cells in the cerebellum, but not glial cells; cytolytic and inflammatory reactions in the CNS do not occur in mice inoculated in the hind leg (Johnson, 1965). PRV (Rinaman *et al.*, 1993), and BDV (Carbone *et al.*, 1989), however, infect not only neurons but also glial cells. PRV causes cytolytic damage in the CNS (Rinaman *et al.*, 1993; Sur *et al.*, 1995). BDV produces severe inflammation in the CNS (Hirano *et al.*, 1983; Carbone *et al.*, 1989). In contrast, the brains of rats infected with HEV by the s.c. (Hirano *et al.*, 1998), i.c. (Hirano *et al.*, 2001a), or i.n. route (Hirano *et al.*, 2001b) showed no distinct neuronal changes of inflammatory reactions. It was recently found (unpublished observation) that HEV spread in the regenerated axons but not in Schwann cells of the peripheral nervous system after transection and suturing of the sciatic nerve of rats. The present study showed that HEV was strictly neurotropic but did not cause distinct pathological changes in the CNS, thus resembling rabies virus. HEV, being harmless for human beings, is handled more easily than rabies virus or HSV in the laboratory. It may prove valuable as a new trans-synaptic tracer for analysing neuroanatomical connections in the CNS of mice and rats.

## Acknowledgments

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