THE ROUTE OF TRANSMISSION OF HEMAGGLUTINATING ENCEPHALOMYELITIS VIRUS (HEV) 67N STRAIN IN 4-WEEK-OLD RATS

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ABSTRACT

Four-week-old Wistar rats were inoculated with HEV by different routes. Animals died of encephalitis after intraperitoneal (i.p.), subcutaneous (s.c.) and intravenous (i.v.) as well as intracerebral (i.c.) and intranasal (i.n.) inoculation. However when inoculated subcutaneously, rats died a few days earlier than those inoculated i.p. and i.v., suggesting that the virus might be transmitted to the central nervous system (CNS) by the neuronal route rather than by blood stream.

Rats which were inoculated subcutaneously at the site of the neck (group A) began to die on day 4 p.i., a few days earlier than animals inoculated in the foot pad of the right leg (group B). On day 2 and 3 after inoculation, the virus titer in the brain was higher in group A, but group B animals showed higher virus titers in the lumber region of spinal cord than group A animals.

In order to follow the virus spread from the peripheral nerve to the brain, the virus was inoculated into the sciatic nerve of rats. The inoculated rats developed clinical signs on day 4 and began to die on day 6. On day 2, virus was detected in the posterior half of the spinal cord and migrated toward the anterior half and in the brain where it was present on day 3. The highest virus titers in the brain were recorded on day 4 to 6, meanwhile the virus titers in the spinal cord tend to decrease. By immunohistochemical study, antigen positive neurons were found in the spinal cord and brain on day 4. Viral specific antigen was detected in the neurons in the cerebral cortex and mid brain of some animals which had survived viral infection more than 24 days.

INTRODUCTION

The HEV 67N strain causes encephalomyelitis or vomiting and wasting syndrome in piglets [1,2]. In experimental infection of piglets, the virus spreads along the nerve pathways to the CNS, and virus replication is restricted to the neurons [3]. In our experimental studies of HEV 67N strain [4], the virus produced encephalomyelitis in mice when inoculated by several routes and propagated mainly in the nerve cells of the CNS. However, 20-day-old or older mice were resistant to the virus injected i.n., i.p. or s.c.

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Recently, the authors reported the successful propagation and plaque assay of HEV 67N strain in established cell line, SK-K cell culture [5].

The SK-K-passaged virus caused encephalitis in mice and in adult rats. Four-week-old rats inoculated i.p., i.v., s.c., i.c. died of encephalitis, but s.c. inoculated rats died a few days earlier than i.p. and i.v. ones. This suggests that the virus might spread to the CNS by the neuronal route rather than by blood stream. Rats were shown to be more sensitive than mice and thus might be useful for studying the pathogenesis of HEV infection.

In order to follow the virus spread from the peripheral nerve to the brain, the virus was inoculated into the sciatic nerve of the rats. The virus growth and its distribution in the spinal cord and the brain was studied by virus titration and immunohistochemistry in the different organs.

MATERIALS AND METHODS

Virus. Plaque-purified HEV 67N strain was propagated in SK-K cells and was assayed for infectivity in a plaque assay, as described [5].

Animal inoculation. Four-week-old male rats were obtained from a breeder colony, which was serologically negative for murine coronaviruses. Rats were inoculated i.c. and i.n with 0.02 ml of viral suspension and with 0.2 ml for i.p., s.c. and i.v inoculations. For inoculation into the sciatic nerve, animals were deeply anesthetized with pentobarbital and the right thigh was surgically operated to inoculate with 0.02 ml of the virus.

Infectivity of the brain and spinal cord. Ten percent tissue homogenates (w/v in Eagle's MEM) were prepared from the brain and spinal cord, and assayed for infectivity in SK-K cell system as described [5]. The spinal cord was divided into 2 pieces, anterior half and posterior one for infectivity assay.

Immunohistochemical study. Immunohistochemistry was performed on paraffin sections by ABC method using anti-67N mouse antibody (1:1000). For histopathological study, serial sections were stained with hematoxylin and eosin (HE).

RESULTS

Five rats were tested for susceptibility to the virus by each route of inoculation, and mortality and clinical signs were recorded over 14 days. Most rats died of encephalitis (Table 1) ; rats inoculated i.c. were most susceptible to the virus, whereas i.p. inoculated ones were most resistant. Among rats inoculated by other routes, s.c. inoculated rats began to die a few days earlier than i.n., i.p., and i.v. inoculated ones. This suggested the possibility that the virus might be transmitted to the CNS by neuronal route.

To test this hypothesis, 2 groups of rats (A and B) were inoculated s.c., at 2 different sites, with 10 PFU of virus. Rats in group A which had been inoculated at the site of neck, began to die on day 4, a few days earlier than animals in group B which had received the virus in the foot pad of right leg. The virus was also detected earlier in the brain and anterior half of the spinal cord of rats in group A, and the infectivity titers of these organs were higher. In contrast to this, the virus was isolated one day earlier from the posterior half of the spinal cord of rats in group B.

The virus was never detected from the liver and spleen. Taken together, these results suggest that the virus might spread to the brain via peripheral nerves.

To follow the virus spread from the peripheral nerve to the brain, 1000 PFU of HEV were inoculated into the sciatic nerve of right leg. On day 4 p.i., the inoculated animals developed clinical signs consisting of ataxia, hypersensitivity and flapping ears, and they began to die on day 6. Some of severely diseased rats survived for more than 24 days.

The virus was first isolated only from the posterior half of the spinal cord on day 2 (Fig. 1). On day 3, the virus was also detectable in the anterior half of the spinal cord and the brain. The virus titers in the spinal cord increased to reach a maximum on day 4 and decreased thereafter. On day 5, the highest titers in the brain ranged from $10^{6.5}$ to 10^{7} PFU/0.2g. The virus could not be detected in the liver, spleen or blood.

Table 1. Susceptibility of 4-week-old rats to HEV 67N

Route of inoculation	Inoculum (PFU)	Dead/Tested	Mean time to death
i.c.	1×10^5	5/5*	4.8 (4-5)**
	1×10^3	5/5	5.0 (4-6)
	1×10	1/5	6.3 (5-7)
i n.	1×10^5	5/5	7 8 (6-12)
	1×10^3	5/5	9.0 (9-11)
	1×10	1/5	10.0 (10)
i.p.	1×10^6	4/5	6.8 (6-9)
	1×10^4	1/5	10.0 (10)
	1×10	1/5	10.0 (10)
s.c.	1×10^6	5/5	5.2 (4-6)
	1×10^4	5/5	5.4 (4-6)
	1×10	1/5	6.0 (6)
i.v.	1×10^6	5/5	6.0 (6)
	1×10^4	4/5	6.2 (6-7)
	1×10	1/5	7.0 (7)

* No. of animals dead/tested, 14 days postinoculation.
** Mean time to death in days (range).

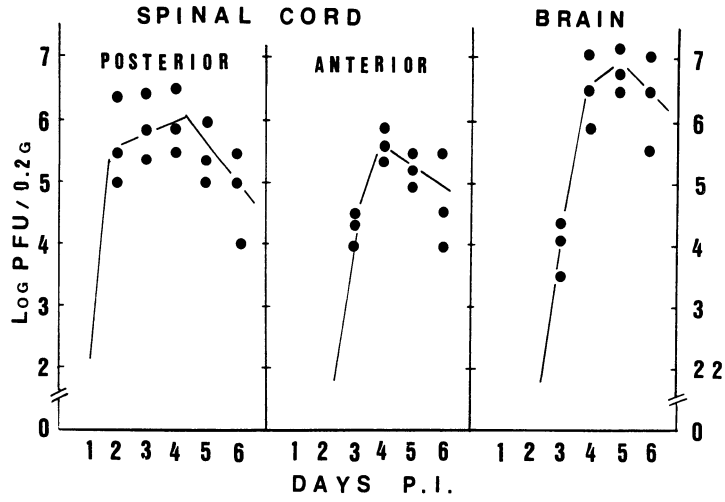

Fig. 1. Virus growth in the spinal cord (posterior half and anterior half) and in the brain of 4-week-old rats after inoculation with 1000 PFU of HEV 67N strain into the sciatic nerve.

Using the ABC method, viral specific antigens were detected on day 3 in the spinal cord of the infected rats (Fig. 2). On day 4, viral antigen positive cells were also found in the cerebral cortex of the brain (Fig. 3). Although the brain and spinal cord showed high infectivity titers, the virus antigen positive cells were not widely distributed in the CNS. Some neurons became necrotic in the spinal cord and brain, and a few mononuclear cells were seen in the perivascular and subarachnoidal space of the rats sacrificed on day 4 (HE staining). Antigen positive cells were identified as neurons by their distribution and shape.

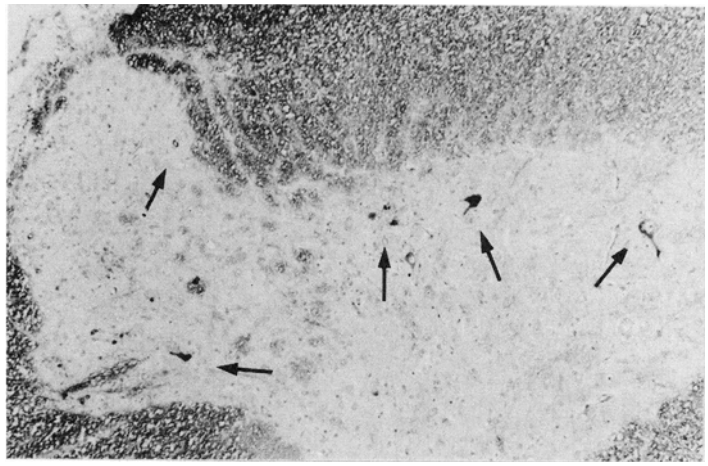

Fig. 2. Viral antigen in the neurons (arrow) in the spinal cord of rat sacrificed on day 4.

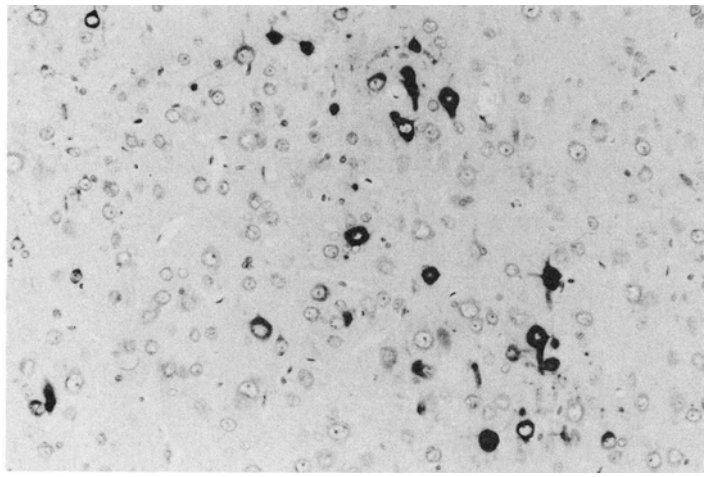

Fig. 3. Viral antigen in the neurons of the cerebral cortex of the brain on day 4 after inoculation.

As mentioned above, some diseased rats survived for more than 24 days after developed severe CNS symptoms. Viral antigens were detected in neurons of the cerebral cortex in the brain of rats sacrificed on day 24 (Fig. 4), and also in the neurons of the midbrain and area around the 3rd ventricle. In the 3rd ventricle, ependymal cells were antigen negative, suggesting that the virus spread was not established via the central canal. In the cerebral cortex, antigen positive neurons apparently increased in number and were more widely

distributed than in the early stage of infection. These findings suggest strongly that a persistent infection of HEV was established, and that the virus spread widely in the CNS with time in the survived rats. The direct inoculation with a small dose of the virus into the nerve might lead to a persistent infection.

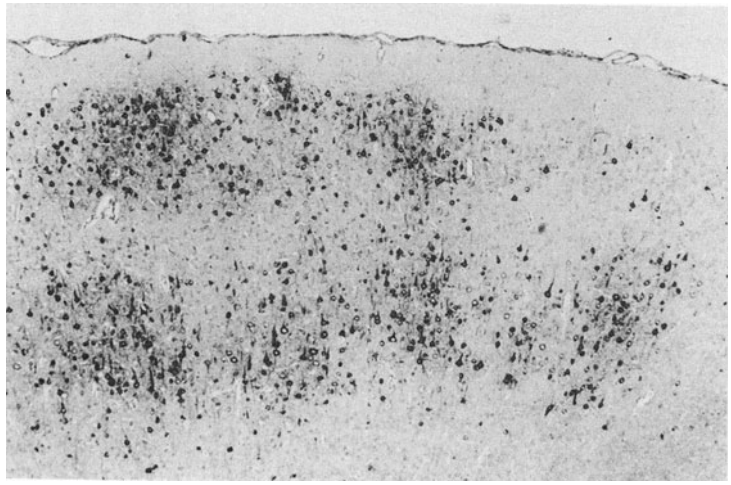

Fig. 4. Virus antigen in the numerous neurons in the cerebral cortex of the survived rats after developed severe clinical signs on day 24

DISCUSSION

HEV is a neurotropic coronaviruses causing a vomiting and wasting disease in piglets. In the diseased animals, the virus reaches the CNS through the nerve pathways and then spreads within brainstem and spinal cord. Virus replication was shown to be restricted to neurons [3].

In our previous studies on HEV infection in mice [4], all mice inoculated i.c. died of encephalitis regardless of age but 20-day-old or older mice did not die following i.n., i.p. or s.c. inoculation. In the diseased mice, the virus propagated mainly in the CNS and neuronal cells were the main targets of virus replication.

In the present study, we demonstrate that rats inoculated s.c. with the virus began to die a few days earlier than those inoculated i.n., i.p. and i.v. and that the virus spreads from peripheral nerve to the CNS.

Our experiments showed that the virus spread from the foot to the brain in 3 days. The results of virus isolation from the liver and spleen and the identification of virus growth in the brain strongly suggest that the virus might spread by neuronal route.

After inoculation with a small dose into the sciatic nerve, animals developed clinical signs on day 4, when viral titers were at their peak value in the spinal cord, and began to die on day 6 when the brain infectivity reached a maximum of 10^7 PFU/0.2g. The virus growth in the brain was concomittant with the development of clinical signs and this virus growth was restricted to the neurons which served as a main target of the virus replication.

The present study demonstrates that HEV spread via neuronal route from peripheral nerve to the brain, and that persistent infection of HEV was established in rats after direct inoculation in the sciatic nerve. The virus spread in the CNS and persistent infection in the rat would be a useful model for studying the pathogenesis of chronic and persistent infection with human and animal coronaviruses.

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