Hydrocephalus in Suckling Rats Infected Intracerebrally with Mouse Hepatitis Virus, MHV-A59

Norio Hirano,* Naoaki Goto, Tetsuo Ogawa, Katsuhiko Ono, Toshiaki Murakami, and Kosaku Fujiwara

*Department of Veterinary Microbiology, Faculty of Agriculture, Iwate University, Morioka 020, and Department of Veterinary Pathology, Faculty of Agriculture, University of Tokyo, Bunkyo-ku, Tokyo 113

(Accepted for publication, June 6, 1980)

Abstract After intracerebral inoculation of mouse hepatitis virus, MHV-A59 strain, into 3- to 5-day-old Wistar rats, some survivors at 14 days postinoculation (p.i.) were found to lack the cerebral cortex and to have an accumulation of a considerable amount of cerebrospinal fluid. The virus titer in the brain increased exponentially after inoculation, reaching a maximum 4 to 6 days p.i. when immunofluorescence revealed virus-specific antigen within neurons in the cerebral cortex. A small amount of infectious virus was also detectable 14 days p.i. when the cerebral anomaly was evident. This brain malformation causing hydrocephalus was due to cerebral damage by viral infection.

Mouse hepatitis virus (MHV), which is a coronavirus (26, 35) prevalent in mouse colonies without apparent illness (10, 32), is known to cause fatal hepatitis (3, 12, 25, 29), encephalitis (1, 6, 12, 19, 25, 29), or enteritis (5) in mice. Recently, two coronaviruses, rat coronavirus (RCV) (31) and sialodacryoadenitis virus (SDAV) (4) from rats were shown to share a common antigen with MHV (4, 8), and Bhatt et al (3) demonstrated that SDAV is also capable of causing respiratory infection in mice. While Bhatt and Jacoby (2) observed some differences in pathogenicity between SDAV and RCV, the speculation has arisen that MHV, RCV, and SDAV might be only variations within the murine coronaviruses which are transmissible between mice and rats. Recently, our routine serological surveillance revealed a high incidence of antibody to MHV in rat sera in Japan (11).

The present study was carried out to determine the response of suckling rats to intracerebral inoculation with some MHV strains, and reveals that strain MHV-A59 has the ability to cause hydrocephalus at high frequency.

MATERIALS AND METHODS

Virus. Laboratory and wild strains of MHV, MHV-1 (12), MHV-2 (29), MHV-3 (8), JHM (6), MHV-S (34), MHV-A59 (25), NuA, NuU, and Nu66 (18),
were used. All these virus strains were propagated in DBT cells (14–16) and their infectivity was assayed on the same cells as described previously (14–16). Virus titer was expressed in plaque-forming units (PFU).

**Animals.** Wistar rats in a late stage of pregnancy were obtained from a commercial barriered breeding colony, which had been serologically checked for the absence of antibody to MHV (8). Suckling rats were nursed by their dams throughout the experiments.

**Inoculation.** Inoculation was by the intracerebral (i.c.), intranasal (i.n.), or intraperitoneal (i.p.) route, with 0.02 ml of virus for i.c. and i.n. and 0.2 ml for i.p. inoculation, respectively. In some cases, 2.5 mg of cortisone acetate (Cortone, Merck-Banyu, Tokyo) per rat was administered subcutaneously shortly after virus inoculation.

**Virus content of the organs.** Animals were killed at intervals and the brain, liver, and spleen were stored at −20 C. Blood was aseptically collected from the heart, diluted 10-fold in phosphate buffered saline (PBS, pH 7.2), and stored at −20 C until virus assay.

**Immunofluorescence and histopathology.** Samples of brain, liver, and spleen were frozen in n-hexane chilled with acetone and dry ice, and 6-μm sections were made by cryostat. After being fixed with cold acetone for 5 min, the sections were stained by the direct method with fluorescein isothiocyanate-conjugated rabbit IgG against MHV-2 at 37 C for 60 min, as described previously (13).

**RESULTS**

**Neurovirulence of Nine Strains of MHV**

Ten 3- to 5-day-old rats were inoculated i.c. with $10^5$ to $10^6$ PFU of nine strains of MHV. As shown in Table 1, typical central nervous system (CNS) symptoms were observed in the JHM- and MHV-A59-inoculated groups, and

Table 1. Pathogenicity of different strains of MHV for 3- to 5-day-old rats after i.c. inoculation

| Strain   | Virus Dose (PFU) | CNS symptoms | Mortality | Virus isolation |
|----------|-----------------|--------------|-----------|----------------|
| MHV-1    | $1 \times 10^6$ | 0/10^a)      | 0/10b)    | 0/10c)         |
| MHV-2    | $3 \times 10^6$ | 0/10         | 0/10      | 5/10           |
| MHV-3    | $1 \times 10^6$ | 0/10         | 0/10      | 5/10           |
| JHM      | $1 \times 10^6$ | 10/10        | 0/10      | 8/10           |
| MHV-A59  | $1 \times 10^6$ | 10/10        | 3/10      | 6/6            |
| MHV-S    | $3 \times 10^6$ | 0/10         | 0/10      | 0/10           |
| NuA      | $6 \times 10^5$ | 0/10         | 3/10      | 6/7            |
| NuU      | $1 \times 10^5$ | 0/10         | 0/10      | 3/10           |
| Nu66     | $1 \times 10^5$ | 0/10         | 0/10      | 0/10           |

*a) No. cases showing CNS symptoms/No. tested.

*b) No. dead/No. tested, 14 days p.i.

*c) No. positive (more than $10^3$ PFU/0.2 g)/No. tested, 14 days p.i.
death occurred in the MHV-A59 and NuA groups. In the MHV-A59 group, CNS symptoms consisting of trembling, spasms, ataxia, and convulsions appeared in all rats 3 to 6 days p.i., and 3 of the 10 infected animals died 5 to 8 days p.i. while 7 survived for 14 days. Although all JHM-inoculated animals developed CNS symptoms, they survived for 14 days. In the NuA group 3 of the 10 rats died within 7 days without showing any CNS symptoms. None of the animals inoculated with the other six strains showed CNS symptoms, and all remained healthy for the 14 days of observation. On the 14th day p.i. all survivors were sacrificed for virus assay. As presented in Table 1, more than $10^8$ PFU of virus per 0.2 g was recovered from the brains of those infected with all strains except MHV-1, MHV-S, and Nu66. Marked accumulation of cerebrospinal fluid and loss of cerebral cortex were found in all seven animals inoculated with MHV-A59, whereas these changes were not produced by the other eight strains.

**Neurovirulence of MHV-A59**

As the brain lesion was found in only MHV-A59-inoculated animals, the neurovirulence of MHV-A59 was examined in detail. Thirty 5-day-old rats from three litters were inoculated i.c. with $1 \times 10^6$ PFU of strain MHV-A59. As shown in Fig. 1, 20 died within 14 days; the remaining 10, showing brain lesions with hydrocephalus, survived for 14 days. Neither death nor cerebral anomaly was observed in suckling rats of the same age after inoculation with uninfected DBT cell culture fluid.

The response of 5-day-old rats to different doses ($1 \times 10^3$ to $10^6$ PFU) of MHV-A59 was determined. As shown in Table 2, the development of cerebral lesions was most marked in the $10^6$ PFU group. In animals inoculated i.c. with $10^5$ and

![Fig. 1. Survival time of 5-day-old rats from three litters of 10 inoculated i.c. with $1 \times 10^6$ PFU. B: with brain anomaly, V: virus positive (more than $10^3$ PFU/0.2 g). ■, died; □, survived.](image-url)
10^6 PFU, the cortical lesions were bilateral while inoculation with 10^4 PFU produced unilateral lesions at the injection site in three of the five animals. Infectious virus was detected in the brains of animals inoculated with 10^4 to 10^6 PFU. Neither cerebral lesions nor virus were detected in those inoculated with 10^3 PFU.

To observe virus growth in the brain, 1 × 10^6 PFU of MHV-A59 was inoculated i.c. into six litters of 5-day-old rats, and at intervals brain virus titers and

### Table 2. Response of 5-day-old rats to different doses of MHV-A59

| Virus dose (PFU) | Brain anomaly | Virus isolation (PFU/0.2 g) |
|------------------|---------------|-----------------------------|
| 1 × 10^6         | + (B)^a       | 2 × 10^3                    |
|                  | + (B)         | 6 × 10^3                    |
|                  | + (B)         | 2 × 10^5                    |
|                  | + (B)         | 1 × 10^4                    |
|                  | + (B)         | 2 × 10^6                    |
| 1 × 10^5         | + (B)         | 8 × 10^3                    |
|                  | + (B)         | 3 × 10^3                    |
|                  | + (B)         | 2 × 10^5                    |
|                  | + (U)         | 4 × 10^2                    |
|                  | + (B)         | 5 × 10^4                    |
| 1 × 10^4         | + (U)         | 1 × 10^5                    |
|                  | + (U)         | 3 × 10^3                    |
|                  | + (U)         | 2 × 10^4                    |
|                  | -             | 2 × 10                      |
|                  | -             | 2 × 10^2                    |
| 1 × 10^3         | -             | -                           |
|                  | -             | -                           |
|                  | -             | -                           |
|                  | -             | -                           |

^a^ Bilaterally (B) or unilaterally (U).

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**Fig. 2.** Virus titer in the brains of suckling rats after i.c. inoculation of MHV-A59 (1 × 10^6 PFU at 5 days of age).
Fig. 3. Brain anomalies in suckling rats after i.c. inoculation at 5 days of age. 14 days p.i.
A: Affected brain having a large amount of cerebrospinal fluid and loss of cerebral cortex.
Left, uninfected control. B: Brain sections showing cerebral lesions. Left series, uninfected control.

Fig. 4. Virus-specific antigen in neurons of the cortex from an infected rat inoculated at 5 days of age. A: Specific fluorescence in pyramidal cell layer of cerebral cortex. B: Specific antigen in the dendrites and cytoplasm of pyramidal cells.

Fig. 5. Extensive necrosis of the cerebral cortex in a rat inoculated at 5 days of age. 8 days p.i.
virus-specific immunofluorescence were determined. As shown in Fig. 2, one day p.i. a small amount of virus was detected in the brain. The virus titer in the brain increased exponentially, reaching a peak of $10^7$ to $10^8$ PFU/0.2 g 4–6 days p.i. when typical CNS symptoms had developed. At this stage of infection the brain cortex appeared cloudy and hyperemic. By 8–10 days p.i. hydrocephalus was observed and there was a gradual decrease in virus titer. At autopsy 14 days p.i. or later, all the inoculated animals were found to have a large amount of cerebrospinal fluid and loss of the cerebral cortex, as shown in Fig. 3.

Virus-specific antigen was first detected in neuronal cells of the cerebral cortex 2 days p.i. By 4–6 days specific fluorescence had become prominent in the dendrites and cytoplasm of many neurons in the cortex, as presented in Fig. 4. At 8–10 days p.i., however, no intense fluorescence was found in the severely affected cortex. Histopathologically some neurons in the cortex were pyknotic 2 days p.i. By 4–6 days neuronal necrosis was frequently seen in the cortex, and marked perivascular infiltration of neutrophils was found in the meninges, cerebral cortex, and midbrain. By 8–10 days necrosis in the cortex had become more extensive, with infiltration of numerous macrophages (Fig. 5). Surrounding these necrotic areas there was marked proliferation of microglial cells. The ependymal cells were not affected during the infection.

Response of Rats to Different Routes of Infection and Age

Three groups of 5-day-old rats were inoculated i.c., i.n., or i.p. with $1 \times 10^6$ PFU of strain MHV-A59. As shown in Table 3, CNS symptoms and anomaly were seen at autopsy 14 days after i.c. inoculation, and the virus was recovered from the brain. The virus was also isolated from five survivors without brain anomaly after i.n. inoculation but not from the i.p. group. As age dependency of the response to i.c. inoculation has been reported for MHV-2 infection in mice (17), strain MHV-A59 ($1 \times 10^6$ PFU) was inoculated i.c. into 7-, 14-, and 30-day-old rats. Cortisone was given subcutaneously shortly after inoculation to half of each group except for the 7-day-old rats. CNS disorders appeared in the 7-day-old rats 3–8 days p.i. and 3 of the 10 died within 10 days p.i., as shown in Table 4. Virus was recovered from seven 7-day-old rats, with brain anomaly, which survived for 14 days. However, 14- and 30-day-old rats remained healthy for 14 days even

| Route | Mortality | Brain anomaly | Virus isolation |
|-------|-----------|---------------|-----------------|
| i.c.  | 8/10<sup>a</sup> | 2/2<sup>b</sup> | 2/2<sup>c</sup> |
| i.n.  | 0/10      | 0/10          | 5/10            |
| i.p.  | 0/10      | 0/10          | 0/10            |

<sup>a</sup> No. dead/No. tested, 14 days p.i.
<sup>b</sup> No. affected/No. surviving, 14 days p.i.
<sup>c</sup> No. positive (more than $10^8$ PFU/0.2 g)/No. tested, 14 days p.i.
HYDROCEPHALUS IN MHV-INFECTED SUCKLING RATS

The propagation of MHV in the brain of rats and hamsters has been reported for JHM (1, 3, 6, 28) and MHV-A59 (25) strains. The present study demonstrated the propagation of MHV-A59 in suckling rats, and cerebral lesions were established, with brain virus titers of $10^7$–$10^8$ PFU/0.2 g at maximum levels. The infected rats developed CNS symptoms such as trembling, spasms, ataxia, and convulsions. Virus-specific immunofluorescence was detected in neurons of the cerebral cortex. Anomalies in the brain were produced only with strain MHV-A59 of the nine strains tested. After i.e. inoculation of suckling rats with MHV-2, MHV-3, JHM, NuA, and NuU, $10^3$ (or more) PFU of virus per 0.2 g were recovered from the brain but no brain lesions were produced. No virus was detectable after i.e. inoculation with MHV-1, MHV-S, and Nu66.

Brain lesions were found in all survivors inoculated i.e. with MHV-A59 at ages less than 7 days, and their development and severity were closely associated with the inoculum dose. Such lesions were produced only after i.e. inoculation. Furthermore, 14- and 30-day-old rats were resistant to i.e. inoculation even with the administration of cortisone which is known to enhance MHV infection in mice (17, 18). These results indicate that the malformation is produced only when rats less than 7 days old are inoculated with MHV-A59 by the i.e. route and that the age of the host and the route of infection are important for the development of hydrocephalus in rats, as reported for MHV-2 infection in mice (17). Virus growth in the brain was parallel with the development of clinical signs, and virus-specific antigen was demonstrated in numerous neurons of the cerebral cortex by direct immunofluorescence. In addition, histopathological changes in the cortex were correlated with the presence of virus antigen detected by immunofluorescence.

As for the pathogenicity of MHV-A59 for rats, our observation was different with cortisone treatment, and neither brain lesions nor virus were detected at autopsy.

DISCUSSION

Table 4. Response of rats to infection with MHV-A59
(1 x $10^6$ PFU) at different ages

| Age in days | Cortisone acetate (2.5 mg/rat) | Mortality | Brain anomaly | Virus isolation |
|-------------|--------------------------------|-----------|---------------|----------------|
| 7           | -                              | 3/100a)   | 7/7b)         | 7/7c)          |
| 14          | +                              | 0/4       | 0/4           | 0/4            |
|             | -                              | 0/4       | 0/4           | 0/4            |
| 30          | +                              | 0/4       | 0/4           | 0/4            |
|             | -                              | 0/4       | 0/4           | 0/4            |

a) Cortisone acetate (2.5 mg/rat) was given subcutaneously shortly after inoculation.
b) No. dead/No. tested within 14 days p.i.
c) No. positive (more than $10^2$ PFU/0.2 g)/No. tested, 14 days p.i.
from that of Manaker et al (25), who reported that i.c.-inoculated newborn Fisher rats showed no evidence of disease. However, brain lesions could also be induced in suckling Fisher rats as in Wistar rats (unpublished data), suggesting that the virus could produce such a lesion in other strains of rat.

Concerning MHV infection of the CNS, i.c. inoculation of the JHM strain into mice caused initial damage in oligodendroglia cells followed by demyelination, as reported by Lampart et al (23), Weiner (36), and Powell and Lampart (33). Goto et al (13) demonstrated that mitral cells of olfactory bulbs were initially affected in weanling mice by i.n. infection. Recently, Nagashima et al (28) observed the development of demyelinating encephalitis in weanling rats after i.c. inoculation with the JHM strain and detected virus antigen in oligodendroglia cells by immunofluorescence. In the present study virus-specific fluorescence and maximum virus titers were observed in numerous neurons of the cortex during the early stage of infection; however, hydrocephalus was produced in the late stages, when virus was not detected. These observations indicate a difference in target cells and pathogenicity between the JHM and MHV-A59 strains.

The first observation of experimental hydrocephalus as a sequence of viral encephalitis in hamsters infected with non-neuroadapted mumps virus was reported by Johnson et al (21). Influenza A in rhesus monkeys (24), parainfluenza type 2 in hamsters (20), reovirus type 1 in hamsters, ferrets, mice, and rats (22), Ross River virus (27) and Newcastle disease vaccine virus (7) in mice have also been shown to induce hydrocephalus. These experiments show that nonfatal infections with those viruses result in stenosis of the aqueduct leading to hydrocephalus. Recently, Norrby and Kristensson (30) reported that subacute encephalitis and hydrocephalus in hamsters were caused by measles virus released from persistently infected cell cultures, and that no definite lesion of the aqueduct was found in animals with hydrocephalus. In the present study aqueductal stenosis was not found in the affected animals, and it was thought possible that hydrocephalus was produced as a sequence to the destruction of immature developing nervous tissues by the virus.

The present study shows that hydrocephalus was produced at high frequency in suckling rats after i.c. inoculation with the MHV-A59 strain of MHV. Such an experimentally produced disease may provide a useful model for studying brain anomalies due to intrauterine or neonatal viral infection of animals and man.

This study was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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(Received for publication, January 14, 1980)