

# Pathogenesis of Mouse Hepatitis Virus Infection

## The Role of Nasal Epithelial Cells as a Primary Target of Low-Virulence Virus, MHV-S

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**Abstract** The pathogenesis of mouse hepatitis virus (MHV-S) infection in suckling and weanling mice was comparatively studied after intranasal inoculation. In sucklings, infectious virus as well as specific antigen was first detected in the nasal mucosa at 12 hr, then in the nerve cells of the olfactory bulbs. At this stage viral particles were demonstrated both in the supporting cells and olfactory cells of the nasal mucosa. In the posterior part of the brain and spinal cord, virus was detected on days 3 to 4 postinoculation when viral growth was clearly demonstrable in the liver, spleen and intestines. In weanlings too, infection was first established in the nasal mucosa, shedding infectious virus in the nasal washing until day 6 postinoculation, and later infection spread to the brain and spinal cord. In weanling mice, however, neither infectious virus nor viral antigen was detected in the liver or other visceral organs, while serum neutralizing antibody became detectable on day 5 postinoculation, increasing in titer thereafter. Histopathologically degenerative and necrotic changes were observed in the nasal mucosa and central nervous system of both age groups of animals coincidentally with the presence of viral specific antigen, while inflammatory response was much less prominent in sucklings. In the liver, spleen and intestines, however, some lesions were observed only in sucklings.

It has been known that there is a wide variance in virulence and organ tropism among many established strains or fresh isolates of mouse hepatitis virus (MHV) (18). Among these strains MHV-2 and MHV-3 are of high-virulence for mice inoculated by the intraperitoneal (i.p.) or intravenous route, multiplying not only in the liver but also in other organs including the nervous system (1, 23, 26) irrespective of age of the host (12), although some differences in susceptibility among mouse strains have been reported (2, 23). On the other hand, MHV-4 or JHM, a highly neurotropic strain, is not fully virulent when inoculated by the i.p. or other routes, but is capable of causing a prominent demyelination resulting in systemic paralysis when inoculated intracerebrally or intranasally (i.n.) (9).

In contrast to these extensive studies on virulent MHV, the pathogenesis of low-virulence MHV infection remains unclear. Such types of MHV are usually pathogenic only for suckling (21, 22) or athymic (13, 25) mice but not for weanling or adult mice. The natural infection of low-virulence MHV was suggested to occur

through nasal infection (22), but it is still unknown how the virus is transmitted from mouse to mouse after multiplying in the host.

In the previous paper (22) we reported that ICR mice gained an age-dependent resistance against low-virulence MHV-S irrespective of the route of inoculation and that the nasal cavity is the most probable route of natural infection (22). The present paper describes comparative studies on the response of susceptible suckling and resistant weanling mice to low-virulence MHV-S.

#### MATERIALS AND METHODS

*Mice.* Two- to 3-day-old suckling and 4-week-old weanling ICR mice purchased from a commercial breeder (Shizuoka Jikkendobutsu, Hamamatsu) were used throughout the experiments. The breeder colony had been routinely checked serologically to ascertain absence of MHV (7, 8). The sucklings were nursed by their dams before and after virus inoculation, and the weanlings were freely fed commercial pellets (NF, Oriental Yeast Co., Tokyo) and water.

*Virus and inoculation.* MHV-S kindly supplied by Dr. J.C. Parker, Microbiological Associate, Inc., Bethesda, U.S.A., was propagated on DBT cells as previously reported (10), and it was used at the 12th to 14th passage levels on DBT cells. The titer of the stocked virus materials showed 2 to  $8 \times 10^6$  PFU/0.2 ml by a plaque assay using DBT cells (11). The suckling and weanling mice were inoculated i.n. with 0.01 and 0.02 ml of virus materials, respectively.

*Sampling of organs and nasal washings.* After virus inoculation, 3 to 4 mice were sacrificed after every 12 hr, and tissues of brain, liver, spinal cord, intestine as well as blood were sampled from both age groups of animals. In suckling mice, the spinal cord with the vertebral column and associate muscles was sampled. In one experiment, the brains of suckling mice were cut at a level of the optic chiasm into the anterior and posterior parts (about 2:3), and the two parts were assayed separately for virus titers. After removing the brain and skin, the head of both aged mice was also examined for virus which will be referred to as "head without brain." In weanling mice, the lung, kidney, heart, thymus, spleen and salivary glands were also assayed for virus. The intestines of suckling mice were divided into two parts, small and large intestines with contents, and then assayed for virus titers.

For collection of nasal washings, about 1 ml of phosphate buffered-saline, pH 7.2 (PBS), was injected into one nasal cavity of mice immediately after killing by cervical dislocation. Then, washing containing desquamated epithelial cells of the cavities was collected from the other nostril, and it was subjected to freezing-thawing 3 times using dry ice and acetone.

*Virus assay.* A 10% homogenate made from each organ with a glass grinder containing chilled PBS or nasal washings sampled and treated as mentioned above was centrifuged at 2,000 rpm for 10 min. The supernatant was diluted in 10-fold steps with Eagle's minimal essential medium (MEM) (Nissui, Tokyo), and each dilution was inoculated to monolayers of DBT cells in 60-mm petri dishes in triplicate for plaque assay as previously reported (11).

*Assay of neutralizing antibody.* Sera were collected from 3 infected weanling mice at an interval of 1 or 2 days after virus inoculation. They were diluted in 2-fold steps with MEM supplemented with 10% tryptose phosphate broth (TPB) (Difco, U.S.A.) after heating at 56 C for 30 min. To 0.5 ml of the serum dilutions were mixed equal volumes of a virus suspension containing approximately 200 PFU/0.2 ml. After incubation at 37 C for 45 min, 0.2 ml of the mixture was inoculated in triplicate on confluent DBT cells grown in 60-mm petri dishes. After 1 hr incubation for virus adsorption, the cells were overlaid with MEM supplemented with 10% TPB, 5% calf serum and 0.9% Bacto agar (Difco, U.S.A.). Two days later, cells were stained with neutral red (1: 10,000) and developed plaques were counted 8 hr after staining. Neutralizing titer of the serum was expressed as reciprocal of the serum dilution giving 50% plaque reduction as compared to the control. As for suckling mice, sera were collected on day 5 from 5 animals, pooled, heated at 56 C for 30 min and assayed in the same manner as in the cases of 4-week-old mice. No infectious virus was detectable in the sera after inactivation.

*Immunofluorescence.* Three liters of MHV-S propagated on DBT cells was subjected to concentration with ammonium sulfate followed by partial purification by discontinuous sucrose gradient centrifugation (24). A pool of 2 ml fractions showing a distinct virus band was diluted 1: 3 with PBS, and an equal volume of Freund's complete adjuvant (Difco, U.S.A.) was added. The mixture was injected in the amount of 4 ml into the footpads of a rabbit weighing 2 kg, and 2 weeks later, 4 ml of the virus material without adjuvant was injected intravenously as a booster. The rabbit was bled 1 week after the booster injection. The antiserum showed a neutralizing titer of 1: 16,000 by the 50% plaque reduction method using DBT cells. Labeling of the antiserum with fluorescein isothiocyanate was carried out according to the method of Kawamura (16).

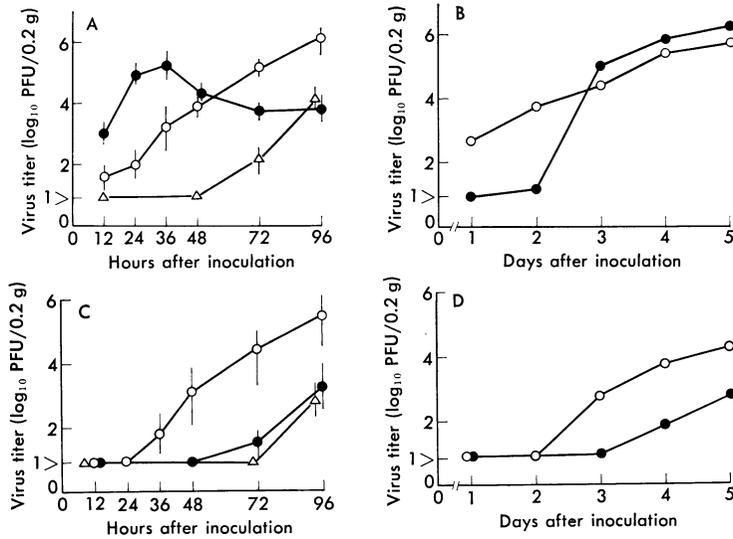
*Histopathology.* At 24 or 48 hr intervals, 3 to 4 weanling mice were sacrificed. Tissues from the main organs were sampled and fixed with 10% formalin, and sections were made and stained with hematoxylin and eosin. As for 2- to 3-day-old suckling mice, sampling was performed every 12 or 24 hr; some materials were fixed with Bouin's solution immediately after killing for microscopic examination.

*Electron microscopy.* Three to 4 suckling mice were sacrificed at 12, 24 and 36 hr after viral inoculation and tissues of the nasal mucosa were sampled and fixed in 0.1 M sodium cacodylate (pH 7.4) containing 2.5% glutaraldehyde. They were postfixed with 2% osmium tetroxide, dehydrated in ethanol, transferred to propylene oxide, and embedded in Epon 821. Sections 70 nm in thickness were made and stained with uranyl acetate and lead citrate, and then examined under electron microscopes, JEL 100S and Hitachi HU-12.

## RESULTS

### *Viral Growth*

Suckling mice 2- to 3 days old were inoculated i.n. with  $4 \times 10^5$  PFU of MHV-S and 3 or 4 mice were sacrificed after every 12 or 24 hr for titration for virus in organs.



Figs. 1A-D. Figures 1A to 1D show viral growth in 2- to 3-day-old suckling mice after i.n. inoculation with  $4 \times 10^5$  PFU of MHV-S.

Fig. 1A. Viral growth in the brain (○), head without brain (●) and spinal cord (△). Each point represents a mean value of 3 to 4 samples and the vertical bar the range of titers.

Fig. 1B. Viral growth in the anterior (○) and posterior (●) portions of the brain. Each point represents the virus titer of pooled materials.

Fig. 1C. Viral growth in the liver (○), intestine (●) and blood (△).

Fig. 1D. Viral growth in the small (○) and large (●) intestines.

The titers in the brain and spinal cord as well as the head without brain are shown in Fig. 1A. During an early stage of infection the head without brain was found to have titers 2 to 3 logs higher than that of the brain with a peak of about  $10^5$  PFU/0.2 g at 24 to 36 hr postinoculation, which decreased thereafter. But eventually about  $10^4$  PFU/0.2 g were recovered at 96 hr when most mice were moribund. On the other hand, the titer in the brain increased gradually and attained  $10^6$  PFU/0.2 g at the time of death. The pattern of viral growth in the spinal cord resembled that in the brain, although being 2 to 3 logs lower throughout the experiment.

In some sucklings which received the same inoculation as above, the anterior and posterior parts of the brain were examined separately for virus. As presented in Fig. 1B, until day 2 postinoculation, the virus titer was significantly higher in the anterior part than in the posterior one. On day 3, however, the titer in the latter exceeded slightly that in the former. And then, the titer of both parts attained  $10^6$  PFU/0.2 g on day 5 of infection.

Figure 1C shows the virus titers in the liver, blood and intestines of infected sucklings. In the liver, virus was first detected at 36 hr postinoculation and the titer increased with time attaining  $10^5$  PFU/0.2 g when moribund. Viral growth patterns in the blood and intestines were similar to that in the liver, though 2 to 3

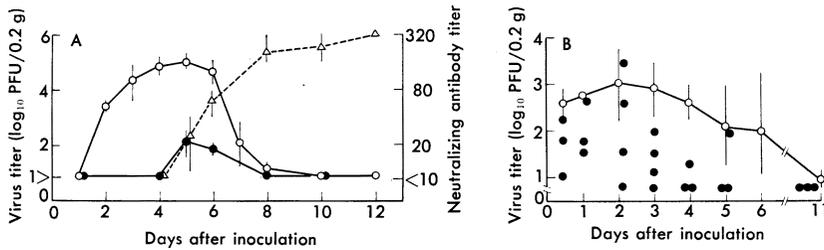


Fig. 2A, B. Figures 2A and 2B show viral growth and neutralizing antibody titer in 4-week-old mice after i.n. inoculation with  $4 \times 10^5$  PFU of MHV-S.

Fig. 2A. Growth of virus in the brain (○) and spinal cord (●), and neutralizing antibody titer (△). Each point shows the mean value of 3 samples with a vertical bar indicating the range.

Fig. 2B. Growth of virus in the head without brain (○) and nasal washing (●). Titers of the head without brain are shown by points expressing mean values and vertical bars expressing the range. Titers of nasal washings are shown individually.

logs lower. Viral growth in the small and large intestines is shown in Fig. 1D which indicates that the virus titer was 1 to 2 logs higher in the small intestine.

Virus assay was performed also in 4-week-old weanlings at 12, 24 or 48 hr intervals after inoculation of the same dose of virus. As shown in Figs. 2A and 2B, significant viral growth was observed in the brain, spinal cord (Fig. 2A) and head without brain (Fig. 2B), whereas no virus was demonstrated in the spleen or liver of the infected mice with a few exceptions (not included in the figures). Virus was first detectable from the head without brain and then 12 to 24 hr later in the brain. Peaks were attained on days 2 and 5 in the head without brain and brain, respectively. In the spinal cord, very low-titered virus was demonstrated with a delay by a few days. On day 10 postinoculation, virus was detectable in neither the brain nor spinal cord, but in one case it was detected from the head without brain. Infectious virus was detected in the nasal washings until day 5, although there were some variations in positivity of virus recovery and titer (Fig. 2B). None of the other visceral organs examined included infectious virus throughout the experimental period.

#### *Neutralizing Antibody*

Sera collected from individual weanlings were assayed for neutralizing antibody titers. Since the circulating blood of the mice showed no infectious virus during the course of the experiment, the sera were subjected to antibody assay without any virus-killing treatment. As shown in Fig. 2A, neutralizing antibody appeared on day 5 postinoculation which increased gradually in titer until the end of the experimental period. No antibody was detected in pooled sera of infected sucklings.

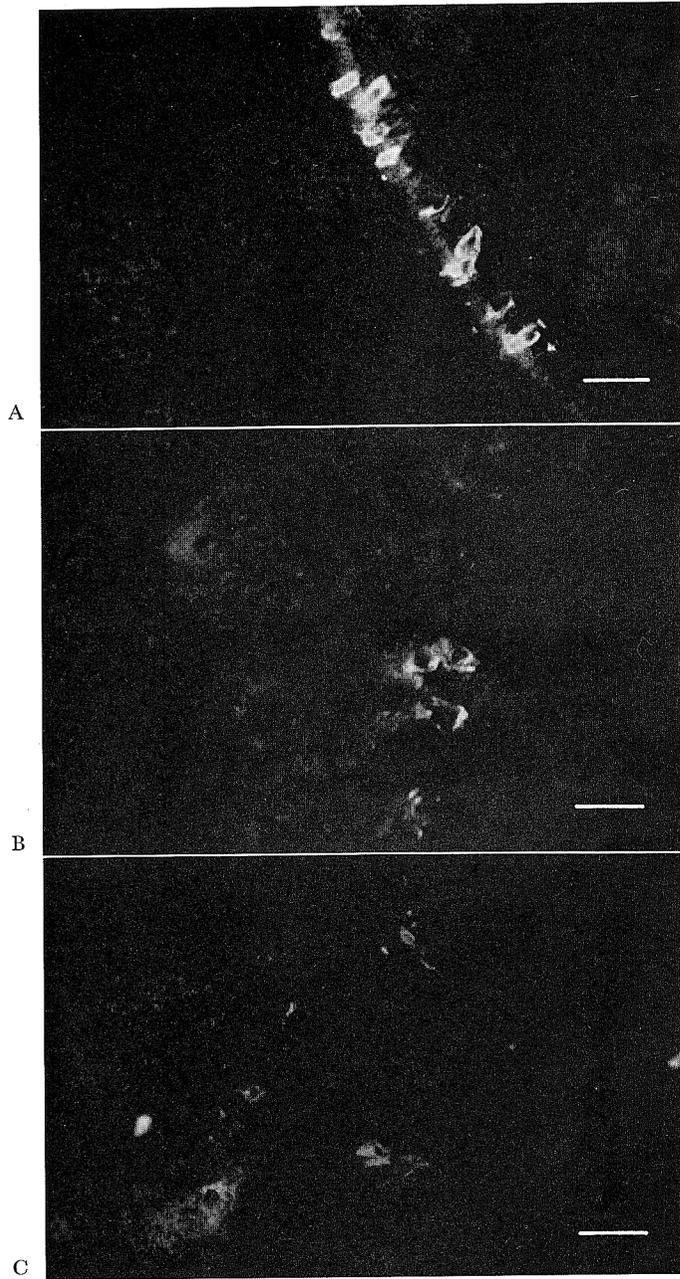


Fig. 3. Specific immunofluorescence observed in suckling mice; in nasal epithelial cells at 12 hr (3A), in mitral cells at the olfactory bulb at 48 hr (3B) and in epithelial cells of the large intestine at 96 hr (3C) after inoculation. Bar=50  $\mu$ m

### *Immunofluorescence*

Suckling and weanling mice having received i.n. inoculation with  $4 \times 10^5$  PFU of MHV-S were killed after every 12 or 24 hr and examined for presence of MHV-S antigen by immunofluorescence. As for suckling mice, specific antigen was first detected at 12 hr postinoculation in the epithelium of nasal mucosa (Fig. 3A). At 24 to 36 hr, viral antigen was distributed in the tunica propria of the mucosa as well as within the cytoplasm of a few neurons in the olfactory bulb (Fig. 3B). At 72 hr, some neurons in the cerebral white matter were found to have viral antigen. Specific fluorescence was demonstrated also in the liver and spleen at 72 hr and then in the intestines (Fig. 3C) at 96 hr postinoculation.

In 4-week-old weanling mice, the presence of viral antigen was restricted to the epithelium of the nasal mucosa on days 3 to 4 and some nerve cells on day 4 or later, while the antigen was not detected in any other organ throughout the course of the experiment.

### *Histopathology*

At 12 to 24 hr postinoculation, degeneration and necrosis appeared in epithelial cells of the nasal mucosa and some necrotized cells were desquamated to fall into the nasal cavity (Fig. 4A) in sucklings. At 36 to 48 hr some proliferation of nasal epithelial cells was demonstrable, and thereafter a regenerating process was clear, resulting in structural recovery on days 3 to 4 postinoculation. At this stage of infection, the olfactory bulb underwent degeneration and necrosis of nerve cells with hyperemia in the arachnoid. On days 5 to 6, these necrotized cells increased in number with proliferation of glia cells. Later, the other parts of the brain and spinal cord were involved. In the liver, a few small necrotic foci were seen on day 3, which increased in size and number on days 4 to 5 (Fig. 4B). Inflammatory response at the necrotic sites was rather poor while a few monocytes were detected in some cases. At the same stage or later, some neutrophils were found in the red pulp of the spleen, and necrosis occurred in exocrine cells of the pancreas. Degeneration of the epithelium with accumulation of neutrophils was seen in the small and large intestines (Fig. 4C).

Also in infected weanling mice there were changes in the nasal mucosa and central nervous system but not in other organs such as the liver. On day 4 postinoculation, necrosis and desquamation of epithelial cells of the nasal mucosa as well as some meningitis and chorioependymitis were demonstrated in all cases examined. On days 7 to 8, however, the nasal mucosa turned to be recovered rapidly with replacement by proliferated epithelial cells. While necrosis appeared in neurons of the olfactory bulb on days 6 to 8 (Fig. 5A), recovery was rapid, leaving only a slight demyelination in the white matter of the midbrain on day 7 (Fig. 5B).

### *Electron Microscopy*

Electron microscopy of infected sucklings revealed necrotic changes with proliferation of epithelial cells of the nasal mucosa at 36 hr postinoculation. Viral particles were present in a space between the microvilli of supporting cells (Fig. 6A)

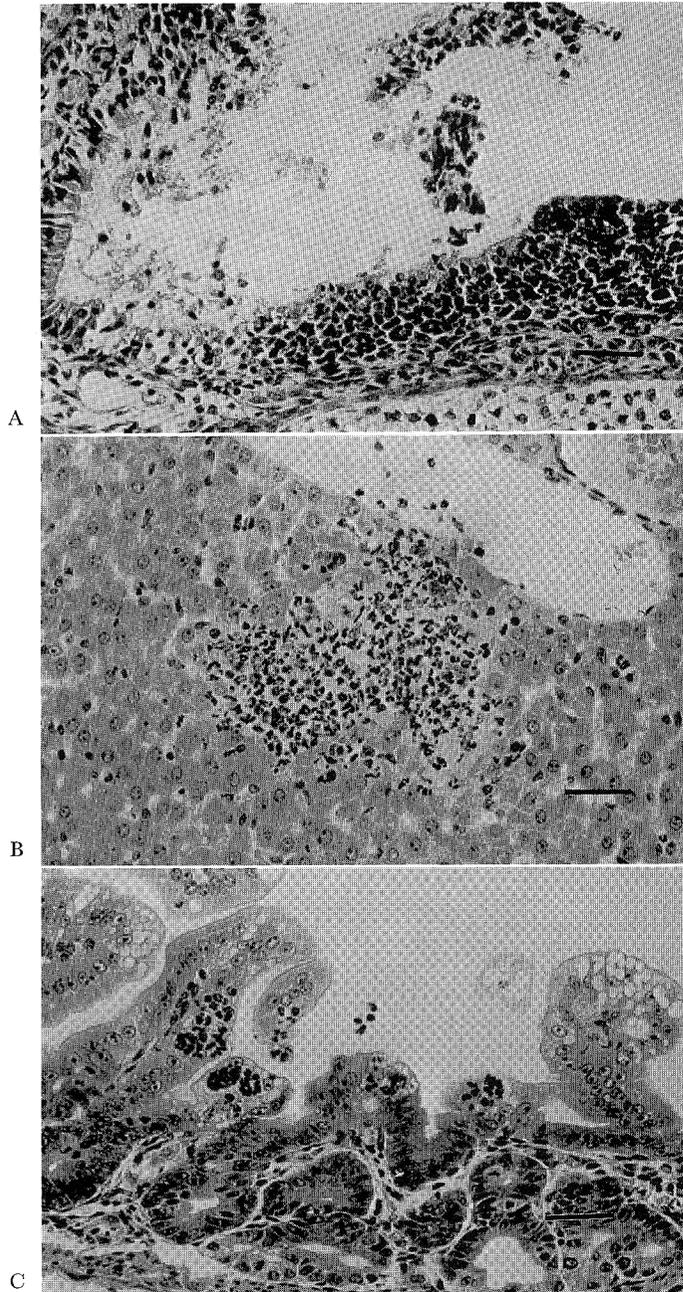


Fig. 4. Histopathological changes of suckling mice: degeneration and necrosis of the nasal epithelium at 12 hr (4A), necrotized lesion in the liver with accumulation of inflammatory cells on day 4 (4B) and degeneration and vacuolation of epithelium with neutrophils in the small intestine on day 5 (4C) after inoculation. Bar = 50  $\mu$ m

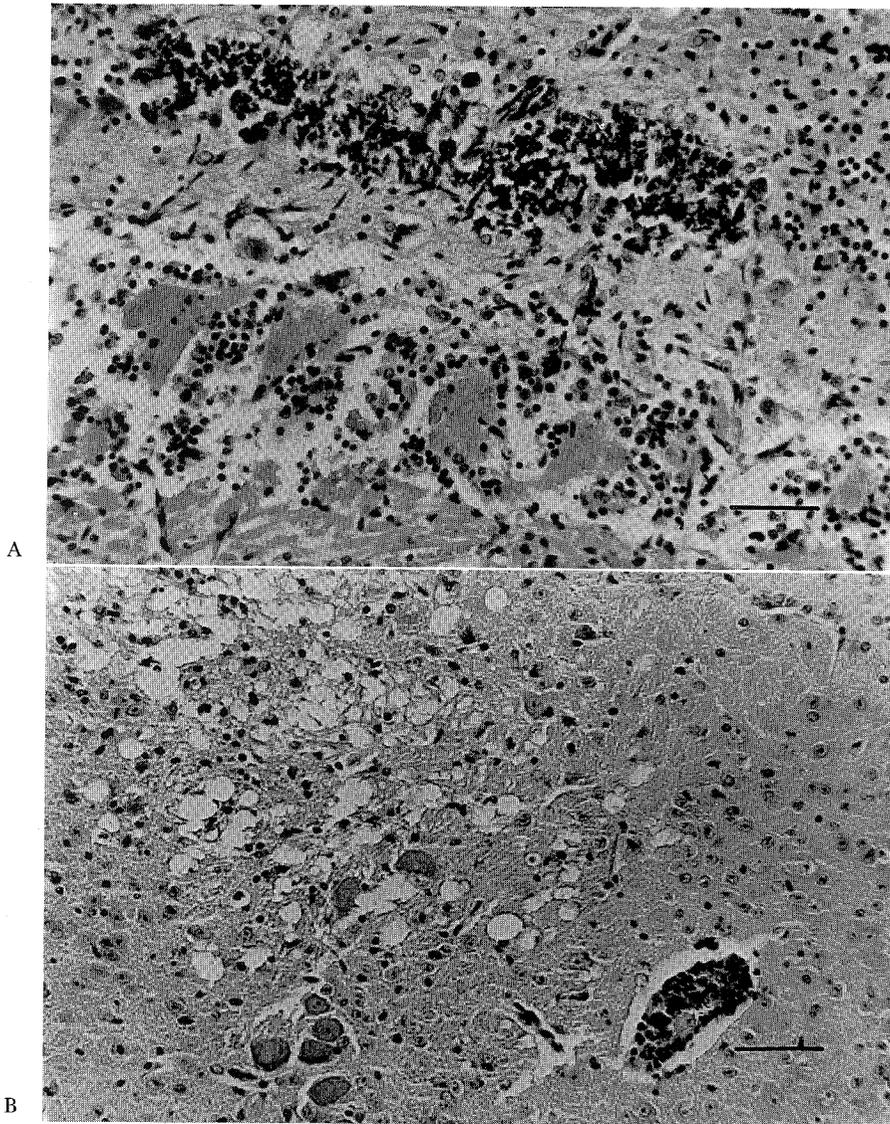


Fig. 5. Organization in the olfactory bulb (5A) and demyelinating changes in the midbrain (5B) of weanling mice on day 7 after MHV-S inoculation. Bar=100  $\mu$ m

and also in the cytoplasm of the same cells. The particles were also numerous in the olfactory cells (Fig. 6B). Most of the particles were seen in the cytoplasmic vesicles, being morphologically circular and 85 to 145 nm in diameter; they contained a donut-shaped nucleoid and some were covered with fuzzy surface materials.

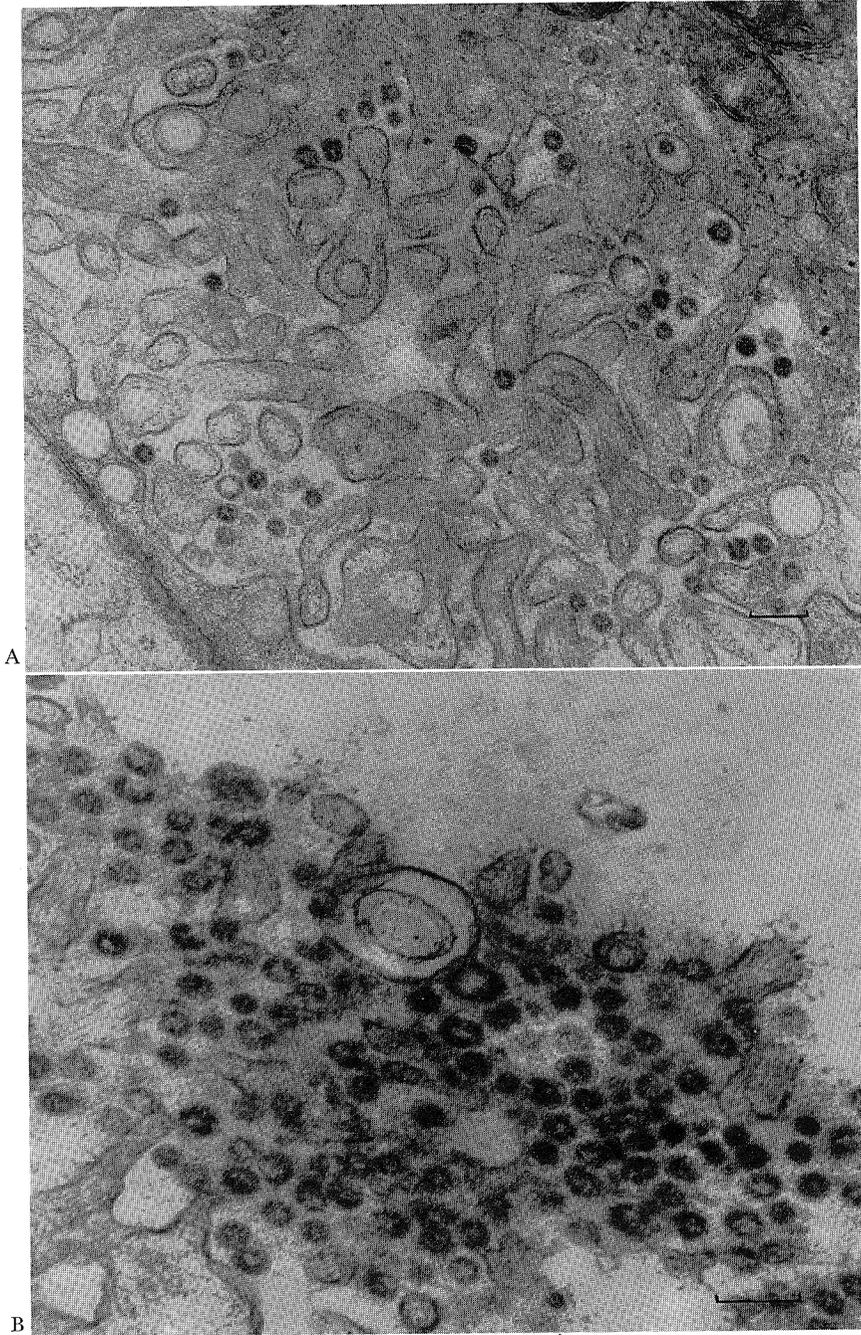


Fig. 6. Viral particles in a space between microvilli of supporting cells (6A) and in the olfactory cells (6B) of suckling mice at 36 hr after inoculation. Bar=200 nm

## DISCUSSION

It was shown in a previous paper (22) that MHV-S was highly virulent for suckling mice aged 1 week or less while being practically non-virulent for 4-week-old mice. The age-dependent resistance may be due to the ability of mice to suppress viral growth in the liver after i.n. infection despite the lack of such a suppression in the brain. It was also suggested that natural infection of MHV in mice might presumably occur via the i.n. route (22). Then, the present investigation was designed to see the comparative pathogenesis of MHV-S infection in suckling and weanling mice.

As early as 12 hr after virus inoculation in 2- to 3-day-old mice, a high virus titer in the head without brain as well as viral antigen in the nasal mucosa was demonstrated, suggesting that the inoculated virus grew first within the epithelial cells of the nasal mucosa. The virus titer increased logarithmically in this site, reaching a maximum at 36 hr postinoculation when viral antigen was densely distributed within the nasal epithelial cells as well as in the tunica propria. Numerous viral particles were observed in the epithelial cells of the nasal mucosa as well as in a space between microvilli of the epithelium, suggesting that MHV-S grown in the nasal mucosa and shed into the nasal cavity might be a source of transmission to other individuals. Concomitantly with viral growth, there were degeneration and necrosis in the nasal mucosa followed by a regenerating process. At this stage of nasal infection there was no significant viral growth in any other part of the body. Also, in 4-week-old mice, an immunofluorescent study and virus titration revealed that virus multiplied first in the nasal epithelial cells. Thus, the present study revealed that MHV did multiply and produce some lesions in the nasal mucosa in both susceptible suckling mice and resistant weanling ones, as suggested in our previous studies using MHV-JHM (9), although it had not been considered as yet that MHV can multiply and cause some histopathological changes in the nasal mucosa (1).

It is really of importance that the nasal epithelial cells serve as an initial target in natural infection of MHV. In both suckling and weanling mice, neither infectious virus nor viral antigen was detected in the bronchi or lungs. Also in rat coronavirus (RCV) and sialodacryoadenitis virus (SDAV) infections, the epithelium of the nasal mucosa of the rat seems to serve as the initial target (3, 14), as has been observed at the early stage of MHV-S infection in the mouse. However, RCV has a tropism to the respiratory system producing histopathological changes and SDAV to the salivary and lacrimal glands rather than the respiratory system. The 3 murine coronaviruses, MHV-S, RCV and SDAV, can be said to have a similarity in the tropism at the early stage of infection (3). The phenomenon observed in MHV-S infection is also compatible with human coronavirus infection which involves only the upper respiratory tract (19).

The viral growth in the brain seems to follow that occurring in the nasal mucosa of both sucklings and weanlings, and virus was first detected at the anterior portion of the brain, mainly in nerve cells of the olfactory bulb. The mitral cells of the bulb were shown to have viral antigen at an early stage of infection, indicating that the virus grown in olfactory epithelial cells gains access to mitral cells through or along

the olfactory fiber, as mentioned by Johnson (15) in herpes simplex virus infection and by Goto et al (9) in JHM infection. This concept is also supported by the electron microscopic observation that viral particles were found in the olfactory cells of the nasal epithelium as early as 36 hr postinoculation.

In suckling mice, viral antigens became to be distributed widely in the posterior part of the brain 3 to 5 days postinoculation and thereafter also in the spinal cord, mainly in nerve cells and sometimes in the ependyma or meninges. This suggests that infection in the anterior part of the brain is transmitted to the posterior part through nerve cells, cerebrospinal fluid and/or blood stream, while there appeared no or little tissue damage in the meninges. Since infectious virus appeared in the liver on day 2, it is likely that the infection of the liver and other visceral organs is due to virus disseminated after multiplication in the nasal mucosa, although viremia failed to be detected.

The virus grown in the liver may be liberated into bile and then into the intestinal lumen, where it may have a chance to infect the epithelial cells of the mucosa. At the last stage of infection, infectious virus was found also in the large intestine with some virus specific antigen or histopathological changes, although the titer was much lower than in the small intestine. From these findings, virus excreted in feces is supposed to be mostly derived from the liver or the small intestine. Transmission of MHV-S from the liver to the lumen of intestine has a similarity to that of human hepatitis A virus infection (4, 6), although no viral multiplication in the intestines has been evidenced in the latter (5).

The excretion of MHV in feces of mice was reported by Rowe et al (21) and we also experienced that naturally infected but apparently healthy adult mice were shedding infectious virus in the feces (unpublished observation). But we could not detect MHV-S in the feces or intestines of weanling mice. In suckling mice, however, infectious virus and viral antigens were demonstrated in the intestines and it can be considered that the transmission may occur also through feces. Thus, the mode of MHV transmission resembles that of other members of coronavirus, human coronavirus (19) and swine transmissible gastroenteritis virus (17).

It can be concluded that in MHV-S infection in suckling mice, virus first grows in the nasal mucosa of mice and the infection spreads later in two directions, either to the olfactory bulb probably through nerve fibers and then to the posterior parts of the brain and spinal cord, or to the liver and spleen via blood stream resulting in excretion of the virus in feces. In 4-week-old mice, however, no or little infectious virus was detected in the liver or other visceral organs, although high titered virus demonstrable in the brain was probably disseminated from the nasal mucosa as was observed in suckling mice.

MHV-S infection was reported to be characterized by demyelinating changes in the central nervous system in 3- to 4-week-old mice as shown in JHM (20). In the present experiments, demyelination was frequently encountered in 4-week-old mice on days 7 to 8, being severe on day 14 postinoculation. In 2- to 3-day-old suckling mice, however, no demyelinating changes were observed, probably because of too acute course of the infection. Pathogenesis on such demyelinating process

in MHV-S infection is now being studied and the results will be published later.

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