

Hepatitogenicity of Three plaque Purified Mutants of Hepatotropic Mouse Hepatitis Virus, MHV-2

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ABSTRACT. Hepatitogenicity of three plaque purified mutant strains of mouse hepatitis virus, designated as MHV-2S, -2M and -2L, isolated from MHV-2 infected SR-CDF1-DBT cells was studied. After intraperitoneal inoculation with 2×10^5 PFU of parental MHV-2 and its mutants to 4-week-old female ICR mice, 40% of mice inoculated with MHV-2S and 20% of mice with -2M died in one week, whereas with -2L all mice survived. All mice inoculated with MHV-2 died in 3 days postinoculation (p.i.). Virus titer of the liver of mice inoculated with MHV-2, -2S and -2M reached peaks (MHV-2: 10^7 PFU/0.2 g, -2S: 10^5 PFU/0.2 g and -2M: 10^6 PFU/0.2 g) at 96 hr p.i., while with -2L a peak titer (10^3 PFU/0.2 g) was shown at 48 hr p.i. Immunofluorescence revealed MHV specific antigen in the liver of MHV-2S infected mice in and around necrotic areas though less extensive than that of parental MHV-2 infected mice. With MHV-2M specific fluorescence was restricted in degenerated hepatocytes in the small necrotic foci. In mice inoculated with MHV-2L only faint fluorescence was detected. Histopathologically, in the liver of MHV-2S infected mice zonal necrosis and cell infiltration were observed. There were spotty necrosis and focal cell infiltration in the liver of MHV-2M infected mice and only small inflammatory foci were seen in MHV-2L infected mice. Large number of extracellular virions were detectable in MHV-2S but not in -2M and -2L infected mice by electron microscopy.—**KEY WORDS:** hepatitis, hepatitogenicity, MHV, mouse, mutant.

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Fulminant hepatitis produced by intraperitoneal inoculation (i.p.) of hepatotropic mouse hepatitis virus, MHV-2, in mice has been expected to be a model of viral hepatitis of man and animals [8, 12]. In this experimental system, however, lesions developed so rapid by inoculation of even a small amount of virus and mice died in 3 or 4 days after infection [5]. Histopathologic lesions progressed in 1 to 4 days p.i., resulting in massive necrosis [5, 22]. Because of these rapid progress of the disease it is difficult to investigate the various phases of the disease and host reactions including immune processes.

To study the lesion of hepatitis in detail, hepatitis with slower progress has been developed using low virulence MHVs and athymic nude mice [2, 4, 20]. Though chronic active hepatitis, as seen in wasting disease of athymic mice [21], has been produced in this system [3, 19], various features of hepatic lesions observed were those of the immuno-deficient animal and may not represent the lesion of haired euthymic mice.

On the other hand, there were phenotypically various lesions such as destructive, degenerative or lytic necrosis in the liver of MHV-2 infected mice

[14], suggesting that there were several substrains with different pathogenicity in parental MHV-2.

To analyze the phenotypical lesions of hepatitis we isolated three altered plaque morphology mutants from MHV-2 infected SR-CDF1-DBT cells. This paper describes the hepatitogenicity of these three plaque mutant substrains of MHV-2 to susceptible mice, comparing with those of parental MHV-2.

MATERIALS AND METHODS

Mice: Mouse hepatitis virus free [1] 4-week-old ICR mice were purchased from a commercial breeder (Charles River Japan, Atsugi). They were kept in metal cage with filter cap and bred with autoclaved pellets for mice and rats (MF, Orient Yeast Co., Tokyo) and water.

Virus: Stocks of plaque purified mutant strains of mouse hepatitis virus, MHV-2S, -2M and -2L were used as inocula. These mutant strains were isolated from SR-CDF1-DBT (DBT) cells infected with wild type MHV-2 strain. After inoculation with MHV-2 (m.o.i.=1) culture fluid of DBT cell monolayer was harvested at 6 hr p.i. when the cytopathic effect

(CPE) not yet appeared. This procedure was repeated at least 30 times and culture fluid was diluted to make about 10 PFU on cultured DBT cells in 60 mm Petri dishes. In various sized plaques formed on the cell culture, 3 different sized, the smallest, intermediate and largest, plaque forming viruses were isolated. After plaques were purified 3 times, those substrains were designated as MHV-2S, -2M and -2L.

These virus strains were prepared by MEM to 10^5 PFU/0.1 ml and inoculated to mice intraperitoneally. Some groups of mice were treated with 1 mg/mouse of corticosteroid (Prednisolon, Toshiba seiyaku, Japan) at the time viral inoculation.

Virus titration: The liver tissues of inoculated mice were taken at the interval of 24 hr, homogenated in MEM and centrifuged at $\times 4000$ rpm for 30 min. Supernatants of the homogenates were stored at -80°C until use. Virus titration was carried out on DBT cells as described by Hirano *et al.* [6].

Immunofluorescence: According to Sainte-Marie [15] sampled liver tissues were fixed in 95% ethanol and dehydrated at 4°C and embedded in paraffin at 58°C . Deparaffinized sections, 4–5 μm thick, were treated first with anti-MHV-2 rabbit serum, then with fluorescein-isothiocyanate conjugated anti-rabbit goat serum (Cappel, CA, U.S.A.) and examined under a fluorescence microscope.

Histopathology: The liver, spleen, pancreas, intestinal tracts and brain were collected and fixed in 10% neutral buffered formalin. For light microscopy, paraffin embedded tissues were cut in 4 μm thick and stained with hematoxylin and eosin (HE), Masson's trichrome stain and Watanabe's silver impregnation for reticulum fibert. Sections in 1 μm thick made from epoxy resin embedded tissues were also used for light microscopy, after staining with

toluidine blue.

Electron microscopy: Small blocks of the liver of mice inoculated with MHV-2 and its mutant strains were fixed in 5% glutaraldehyde in phosphate buffered saline pH 7.2 and postfixed in 1% osmium tetroxide. After dehydrated in ascending ethanol series, samples were immersed in propylen oxide and embedded in epoxy resin (Epok 812, Oken, Tokyo). The lesions were monitored by semi-thin sections stained with toluidine blue. Ultrathin sections were made, stained with uranyl acetate and lead citrate and observed under an electron microscope, JEM 100CXII, at 80 kv.

RESULTS

Mice inoculated with 2×10^5 PFU/0.1 ml of MHV-2S, -2M, -2L and parental MHV-2 were examined the mortality and surviving time of dead mice. At 7 day p.i., 40% of mice inoculated with MHV-2S and 20% with -2M died while mice inoculated with MHV-2L survived. All mice infected with MHV-2 died within 4 days. With cortico steroid treatment all mice inoculated with MHV-2S and -2M also died but 50% of mice died in those inoculated with MHV-2L. Mean surviving time of dead mice inoculated with MHV-2, -2S and -2M was 3.5, 5 and 4 days, respectively (Table 1).

Virus titers of the liver at 24 hr p.i. were 10^3 PFU/0.2 g in mice inoculated with MHV-2 and mutant strains, except with MHV-2L. At 72–96 hr p.i. in MHV-2 inoculated mice virus titer reached a peak of 10^7 PFU/0.2 g. Virus titer of mice inoculated with MHV-2S showed a peak of 10^5 PFU/0.2 g at 96 hr p.i. and decreased gradually thereafter, while some showed 10^4 PFU/0.2 g even at 144 p.i. The titer reached a peak of 10^6 PFU/0.2 g in mice inoculated

Table 1. Response of ICR mice infected with plaque mutant strains of MHV-2

Virus strain	Mortality	Mean time-to-death (days)	Virus antigen (72 hr p.i.)
MHV-2	10/10 ^{a)}	3.5 ^{b)}	+++
-2S	4/10	5	++
-2M	2/10	4	+
-2L	0/10	—	±
with corticosteroid			
-2S	6/6	3.5	+++
-2M	6/6	3.5	+++
-2L	3/6	4	++

a) No. of dead mice/No. of infected mice.

b) Mean time-to-death of dead mice.

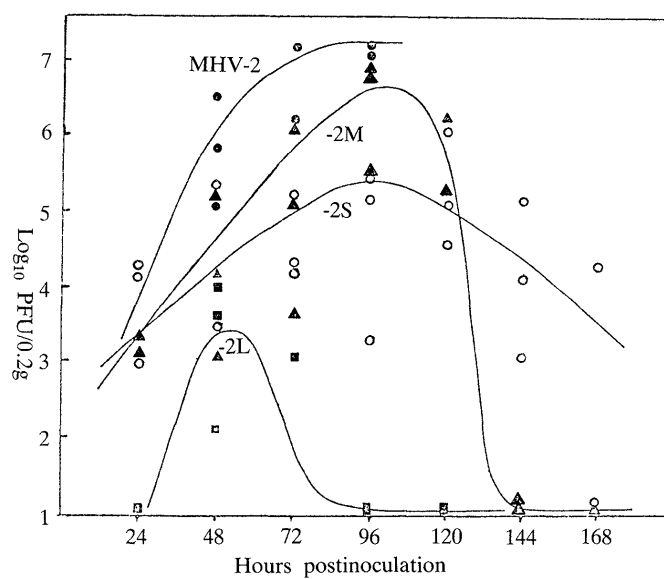


Fig. 1. Virus titers of MHV-2 (●), -2S (○), -2M (▲) and -2L (■) after intraperitoneal inoculation with 2×10^5 PFU.

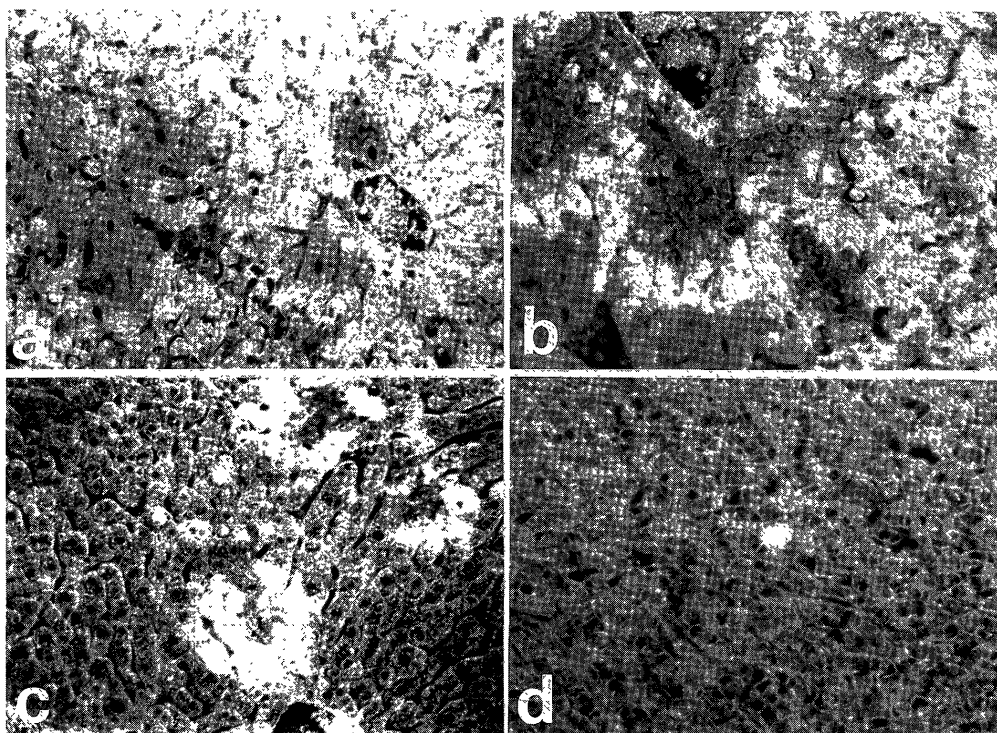


Fig. 2. Immunofluorescence of the liver of mice infected with MHV-2 and its mutant strains.
 a. Diffuse specific fluorescence of the liver of a mouse infected with MHV-2. 96 hr p. i. $\times 200$
 b. MHV specific fluorescence of necrotic areas of a mouse infected with MHV-2S. 96 hr p. i. $\times 200$
 c. Viral antigen in necrotic foci of the liver of a mouse infected with MHV-2M. 96 hr p. i. $\times 200$
 d. A small specific fluorescence in the liver of a mouse infected with MHV-2L. 48 hr p. i. $\times 200$

with MHV-2M but declined rapidly and less than 1 PFU/0.2 g at 144 hr p.i. MHV-2L inoculated mice showed a peak titer of 10^3 FPU/0.2 g at 48 hr p.i. (Fig. 1).

Immunofluorescence revealed that viral antigens were seen diffusely in the liver of parental MHV-2 infected mice at 72–96 hr p.i. when virus titer reached a peak (Fig. 2a). In the liver of MHV-2S infected mice specific fluorescence was seen in accordance with the zonal degeneration of hepatocytes and cell debris in the necrotic areas (Fig. 2b). Focal degeneration appeared in the liver of MHV-2M infected mice and specific fluorescence was observed in and around the foci (Fig. 2c). Small fluorescence spots were seen in the liver of MHV-2L inoculated mice at 48 hr p.i. (Fig. 2d) and MHV antigen could not be detected thereafter.

Histopathologically, there was massive necrosis of hepatocytes leaving a small number of seemingly intact cells around the central vein in the liver of mice inoculated with MHV-2 (Fig. 3a). MHV-2S infected mice showed degenerative and hyalinized hepatocytes in the liver at 96 hr p.i., then those

lesions confluent and made zonal necrosis in whole of the liver (Fig. 3b).

MHV-2M infected mice showed spotty foci consisting of dark stained, round and hyalinized hepatocytes scattered at the early stage of infection.

Those lesions proceeded to focal necrotic and necrobiotic hepatocytes with various inflammatory cells. The focal necrosis became the largest in size at 96 hr p.i. (Fig. 3c) and eosinophilic hyalinized cells increased. At 144 hr p.i. the necrotic foci diminished in size and degenerated hepatocytes decreased in number, leaving reticular network. Only small inflammatory foci were left and regeneration of hepatocytes with a few mitosis were seen around the foci at 168 hr p.i. Viral antigen could not be detected at that time.

The liver of mice infected with MHV-2L showed some small foci containing several degenerated hepatocytes and few inflammatory cells mainly in the periphery of portal area or hepatic vein at 24 to 48 hr p.i. (Fig. 3d). At 72 hr p.i. or later there was no lesion in the liver.

Corticosteroid treated mice inoculated with

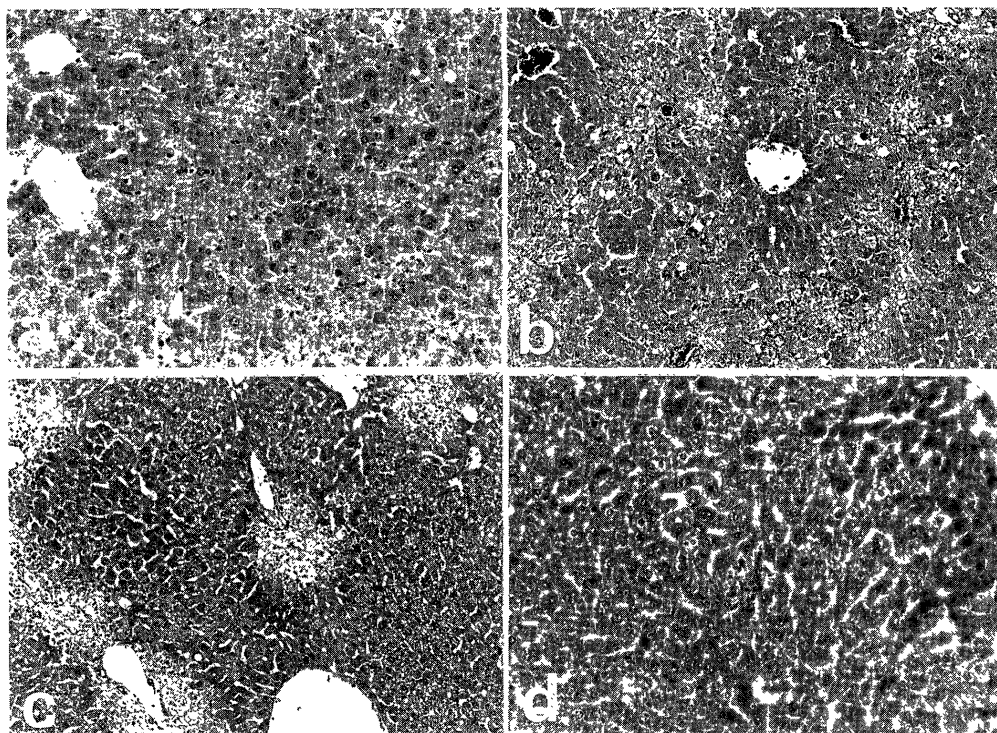


Fig. 3. Necrotic lesions of the liver of mice infected with MHV-2 and its mutant strains.

- Massive necrosis of the liver of a mouse infected with MHV-2. 96 hr p. i. HE $\times 160$.
- Zonal necrosis of the liver of a MHV-2S infected mouse. 96 hr p. i. HE $\times 160$.
- Focal necrosis scattering in the liver of a mouse infected with MHV-2M. 96 hr p. i. HE $\times 160$.
- A small necrotic lesion in the liver of a mouse infected with MHV-2L. 48 hr p. i. HE $\times 160$.

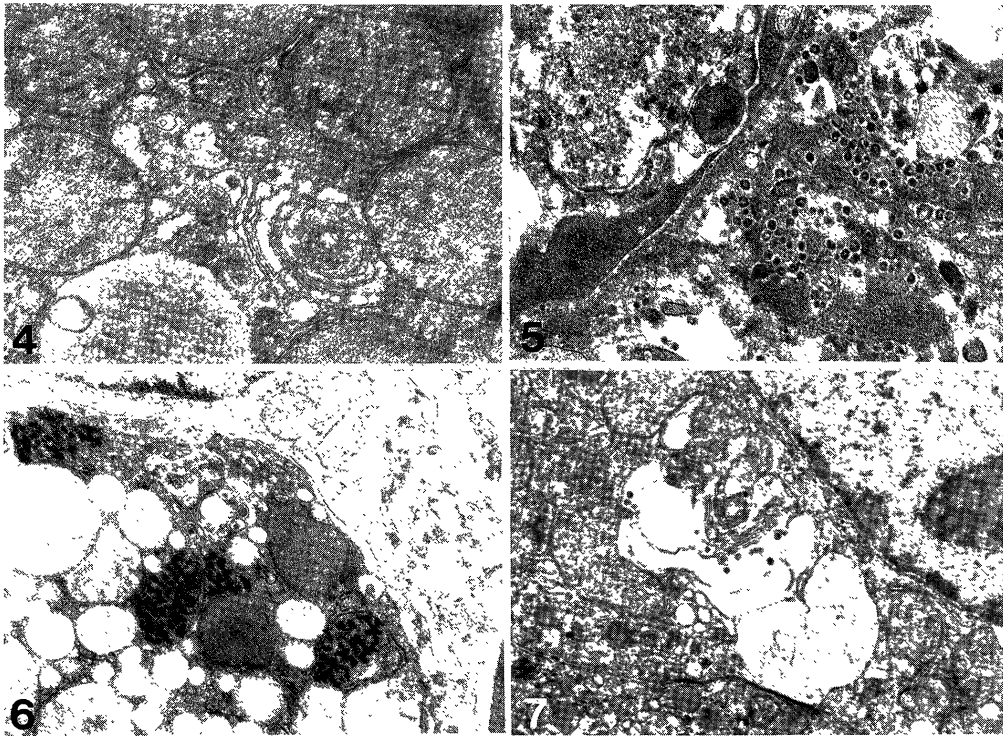


Fig. 4. Virions in the cisternae of Golgi complex of a hepatocyte of a mouse infected with MHV-2S. $\times 19,000$.

Fig. 5. Many virions in degenerated area and the space of Disse of a mouse infected with MHV-2S. $\times 16,000$.

Fig. 6. Granular aggregations of ribosome-like substance of a degenerated hepatocyte infected with MHV-2M. $\times 7,600$.

Fig. 7. Virions in dilated cisternae of Golgi complex of a hepatocyte of MHV-2L infected and corticosteroid treated mouse. $\times 9,000$.

Table 2. Types of lesion of the liver of ICR mice infected with plaque mutant strains of MHV-2

Virus strain	Lesion			Inflammatory reaction ^{a)}	Regeneration
	Type	Growth	Frequency		
MHV-2	Necrotic	Diffuse	+++	—	—
-2S	Necrotic	Zonal	++	++	—
-2M	Degenerative	Focal	++	++	—~++
-2L	Degenerative	Spotty	+	+	++
mutant MHV + Corticosteroid	Necrotic	Diffuse	+++	—	—

a) Inflammatory reaction and regeneration were examined 72 hr p. i.

MHV-2S -2M and dead mice inoculated with -2L showed hepatic massive necrosis just like those seen in mice inoculated with parental MHV-2. Viral antigens were seen diffusely in the necrotic areas. In survived mice inoculated with MHV-2L the pathologic

features were the same as those without corticosteroid treatment (Table 2).

Electron microscopically, in hepatocytes inoculated with MHV-2S, virions were often observed in the cisternae of smooth endoplasmic reticulum and

Golgi complex with budding processes (Fig. 4). A number of virions were also seen in degenerated areas and the space of Disse (Fig. 5). In the cytoplasm of affected hepatocytes there were electron-dense granular aggregates, degenerated endoplasmic reticula and various amorphous substances. Morphologic alteration of hepatocytes of MHV-2M inoculated mice were swelling of mitochondria and proliferation of smooth endoplasmic reticula. Virions were seen in enlarged cisternae of endoplasmic reticulum and Golgi complex, but less frequent than those seen in MHV-2S infected hepatocytes. Extracellular virions were few in number. In the cytoplasm of degenerated hepatocytes granular aggregation of ribosome-like substance was often seen (Fig. 6).

In the liver of MHV-2L infected mice degeneration of hepatocytes consisted of mitochondrial swelling and chromatolysis of nuclei was a common feature. Virions could not be detected, but in corticosteroid treated and dead mice large number of virions were observed in the cytoplasm (Fig. 7).

DISCUSSION

Several mutant strains of MHV, both neurotropic and hepatotropic, have been isolated from original MHV strains. These mutants were shown to have various pathogenicity which was different from parental viruses to susceptible animals and used for analyzing the pathogenic features such as persistence of the infection or demyelination [7, 9, 11, 16].

From hepatotropic MHV-2 a low virulent strain, MHV-2-CC, which caused active hepatitis in athymic nude mice but was not virulent to haired euthymic mice after intraperitoneal inoculation had been isolated [3, 7]. These findings suggest that MHV-2 contains different substrains with different pathogenicity and those may relate to develop the various types of the disease in inoculated mice.

In the present experiment three plaque purified mutant substrains were isolated from early releasing virus of MHV-2 infected DBT cells. When inoculated these mutant viruses in ICR mice, the most susceptible mice to MHV infection [18], the mortality differed by substrains used. This indicated that hepatitis was caused by parental MHV-2 consists of various phenotypical lesions, and that some of which might be separable by employing different substrains from the parental virus.

Virus titers of the liver of mice inoculated with

hepatotropic strains of MHV reached a peak at 2 to 5 days after inoculation and then mice died, when mice escaped from death virus titer decreased after reached the peak in accord with the increase of neutralizing antibody titer [5]. In immuno-deficient mice virus titer gradually increased until mice died [3]. And, there was no case in MHV inoculated mice showing the rapid decrease of virus titer with recovering processes of the lesion.

In MHV-2M infection the peak titer of the liver of inoculated mice was nearly as high as that of MHV-2 infected mice, however, the virus titer decreased rapidly thereafter and no virus could be detected later. The rapid reduction of viral antigen was thought to mean that the antigen was sensitive to some host resistance factors. Interestingly, the specific immune process to the viral antigen seems not to be involved in this viral reduction occurred with very short term to develop the immune response and in case of MHV-2S infection virus titer was still detected at that time, seen in Fig. 1.

Histopathologic findings of the liver of mice inoculated with hepatotropic MHVs revealed many coagulative necrotic foci in which there were acidophilic and globular bodies with or without remnant of the nuclei [11]. In the present examination, different types of necrosis, such as diffuse destructive necrosis in MHV-2S infection, focal coagulative changes in -2M and small degenerative changes in -2L were observed. Acidophilic bodies were prominent in the degenerative type of lesions. These findings might show that there were several phenotypical different necrosis in the lesion of parental MHV-2 infection and each necrosis was caused by a different substrain with different pathogenicity.

Electron microscopically, in the liver of mice infected with hepatotropic MHVs virions were clearly seen in the cytoplasmic cisternae of hepatocytes and virions were seen much more numerous in the perisinusoidal or intercellular space than in hepatocytes [17, 23]. Numerous extracellular virions were seen in the liver of MHV-2S as well as MHV-2 infected mice, while in MHV-2M infected mice virions could not be seen in the perisinusoidal area, except for the case of mice died. In low virulent MHV-2L infected and corticosteroid treated mice extracellular virions were seen, though there were small in number.

These suggest that releasing of virions from the host cell is an important factor to determine the mortality of infected mice and MHV-2M and -2L are

thought to have some defects in the process of viral releasing.

Although further studies of hepatitogenesis of newly isolated mutant viruses are required, these mutant strains may be useful tools for studying the pathogenesis of experimental viral hepatitis.

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