Journal of Gastroenterology and Hepatology (1990) 5, 395–401

LIVER AND BILIARY

Protective effect of OK-432 (streptococcal preparation) on murine fulminant hepatitis following mouse hepatitis virus infection

MUTSUNORI SHIRAI, SEISHIRO WATANABE AND MIKIO NISHIOKA

Third Department of Internal Medicine, Kagawa Medical School, Kagawa, Japan

Abstract The effects of OK-432 (streptococcal preparation) on murine fulminant hepatitis were investigated. Hepatitis was induced by injection of mouse hepatitis virus type 2 (MHV-2) at a strength of either 1×10^3 or 1×10^4 plaque-forming units (PFU). Mice without OK-432 treatment died within 5 days, whereas mice pre-inoculated with OK-432 showed survival rates of 50% (1×10^3 PFU) or 10% (1×10^4 PFU) after 60 days. Survival time was not prolonged if OK-432 was injected after MHV-2. Examined histologically, mice not treated with OK-432 showed severe haemorrhagic necrosis of the liver, often panlobular. Treated mice showed less necrosis; the least necrosis was observed in those injected with OK-432 before MHV-2. In those mice injected first with OK-432 and then with 1×10^3 PFU of MHV-2 that survived 7 days, autopsy showed a very slight and focal hepatic necrosis, with follicular infiltration by lymphocytes and macrophages. Mitogenic reaction of spleen cells was remarkably less than normal in mice with MHV-2 injection. However, mice injected with OK-432 before MHV-2 (same treatment as mice showing high survival rates) showed relatively high reactivity in comparison with mice not treated with OK-432.

Key words: biological response modifier, fulminant hepatitis, immunotherapy, mouse hepatitis virus, OK-432.

INTRODUCTION

Mouse hepatitis virus (MHV) is a member of the coronavirus group, which belongs to the RNA viruses.[1–3] It is highly transmissible in mice:[4] MHV type 2 (MHV-2) fulminantly produces massive necrosis in the liver.[5,6] Therefore, hepatic injury caused by MHV-2 may be a good model to study the pathogenesis of human fulminant hepatitis.

Immunotherapy using biological response modifiers is now widely applied to various fields such as cancer treatment, and can also be applied to chronic hepatitis. As a biological response modifier, OK-432 (streptococcal preparation; low virulent SU-strain of type-3, group A *Streptococcus pyrogenes* of human origin, incubated with Penicillin G Potassium) is known to produce various immunological changes, including induction of interferon, activation of macrophages, and activation of natural killer lymphocytes.[7]

The effects of OK-432 on MHV-2 fulminant hepatitis in mice were investigated and the survival time and histological changes after inoculation with MHV-2 were evaluated.

METHODS

Balb/c female SPF mice (4 weeks old) were obtained from Charles River Japan Inc.

MHV-2 was maintained by serial passage cultures of mouse cell line DBT. The virus was purified by plaque cloning and propagated in DBT cells. Culture fluid harvested from infected cell cultures after incubation at 37°C for 24 h was

Correspondence: M. Shirai MD, Third Department of Internal Medicine, Kagawa Medical School, 1750-1, Ikenobe, Miki-cho, Kita-gun, Kagawa Prefecture, 761-07, Japan.

Accepted for publication 24 September 1989.

clarified by centrifugation and stored at −80°C. These materials were assayed for infectivity by the plaque count method in DBT cells, indicated as plaque forming units (PFU).[8-10]

OK-432 (Picibanil; Chugai Pharmaceuticals Co., Tokyo, Japan) is an immunopotentiator developed by Okamoto *et al.*[11] It is produced by incubating Penicillin G Potassium with the low virulent SU-strain of type-3, group A *Streptococcus pyrogenes* of human origin, and is known to produce various immunological changes in mice.[7,12,13] OK-432 was dissolved in 0.2 mL of physiological saline to use in all the mice as follows.

MHV-2 was injected intraperitoneally into mice of groups A, B, and C at 1×10^3 PFU/mouse, and groups D, E, and F at 1×10^4 PFU/mouse, dissolved in 0.2 mL of physiological saline (all groups, $n=10$) as shown in Fig. 1. OK-432 was injected intraperitoneally into groups A and D at 1.5 KE (Klinische Einheit)/head 4 days and 1 day before the injection of MHV-2, respectively. OK-432 (0.2 KE/mouse) was injected intraperitoneally into group A just after the injection of MHV-2, group B every day since the third day after virus injection, group D 2 days after virus injection and group E every day after virus injection.

The surviving mice treated in groups A and C were sacrificed 1, 2, 4 and 7 days after injection of MHV-2 (no mice treated in group C survived 7 days after the injection of MHV-2). The livers of all the mice were observed macroscopically, fixed in 10% formaldehyde solution, and embedded in paraffin. Various stainings, performed using paraffin sections, were microscopically observed. Serum glutamic oxaloacetic transaminase (SGOT) was examined using serum from mice treated as groups A, B and C on the first, 4th and 7th day after the injection of MHV-2.

Blastoid formation of the spleen cells in reaction to phytohaemagglutinin (PHA) concanavalin A (Con A) and PWM was investigated using normal mice ($n=5$) and mice treated the same as groups A ($n=5$) and C ($n=5$). Spleen cells obtained aseptically from sacrificed mice were washed 3 times by RPMI 1640, suspended in RPMI 1640 containing 10% fetal calf serum at

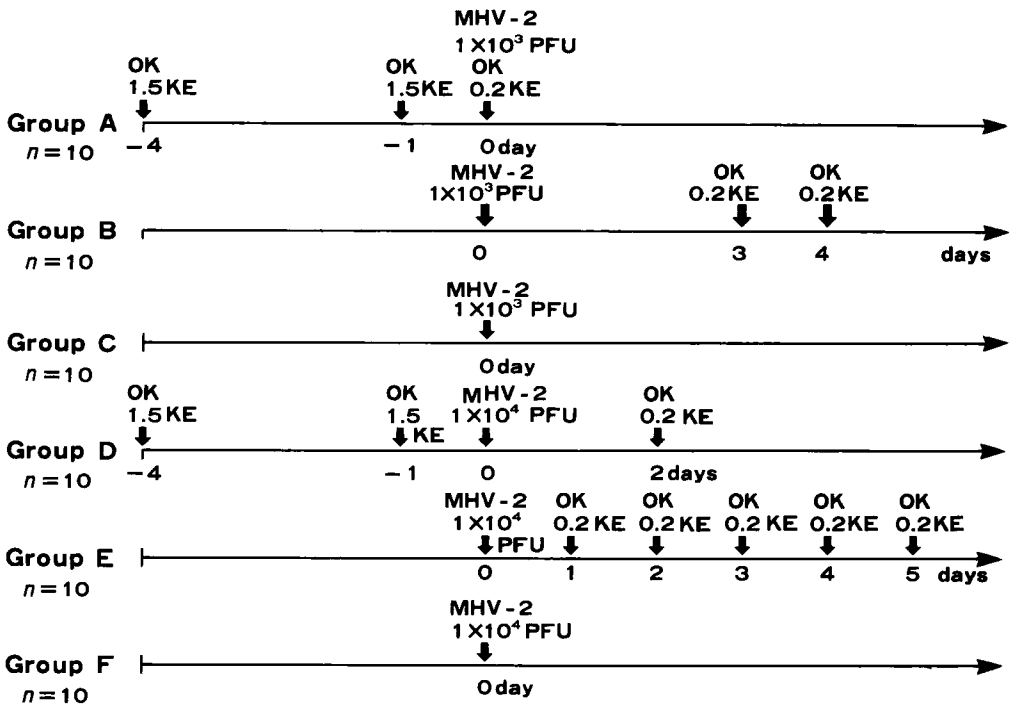

Figure 1 Experimental design. OK : OK-432.

1×10^6 cells/mL and incubated for 72 h at 37°, 5% CO_2 with 10 μg/mL of PHA, Con A and PWM (Difco Laboratory Co. Ltd, CA, USA). The proliferation assays were always performed in triplicate and 1 μCi/mL of [^{3}H]-thymidine (New England Nuclear Co. Ltd, Boston, USA) was added 18 h before harvesting with a Skatron (Lierbyen, Norway). Results are expressed as the mean (counts/min) of three replicate determinations ([^{3}H]-thymidine incorporation) after subtraction of background incorporation in medium culture.

The results were statistically examined by Student's *t*-test.

RESULTS

Figure 2 shows survival rates for all the groups. High survival rate or prolongation of survival time was seen in groups A and D in which mice were treated with OK-432 before injection of MHV-2. Survival rates of 50% in group A and 10% in group D were recognized 60 days after injection of MHV-2. Mice in group C died within only 4 days and those in group F died within 5 days of injection of MHV-2. No prolongation of survival time was obtained in groups B and E in which mice were injected with OK-432 after MHV-2 injection.

Macroscopically, the livers of the dead mice demonstrated many spotty or confluent haemorrhagic necroses. Microscopically, they showed massive necroses (Fig. 3), often showing panlobular necrosis (Fig. 4). However, the grade of hepatic necrosis in dead mice in groups inoculated with OK-432, especially in group A, was generally lower than that in mice without inoculation of OK-432 (Table 1).

Microscopically, the livers of sacrificed mice treated in group A showed very slight pathological changes 1 day after injection of MHV-2, and scattered focal necroses accompanied by follicular lymphocytic infiltration with many macrophages 4 days after the injection (Figs 5, 6; Table 2). There was very slight or no hepatic necrosis 7 days after the injection in the surviving mice. There was slight hepatic necrosis with congestion 1 day after injection of MHV-2 and confluent hepatic necrosis up to a sublobular level 2 days after the inoculation in the mice treated in group C. In the same way as the dead group C mice, they

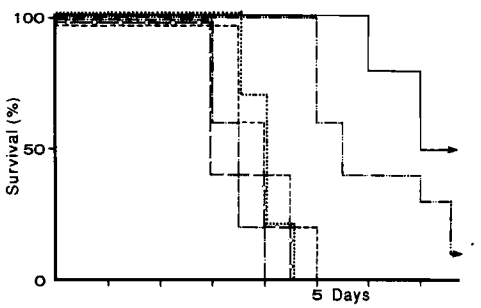

Figure 2 Survival after injection of MHV-2 in groups A(——), B(· · · · ·), C(—·—), D (—··—), E (—○—), and F (- - -). For all groups, $n = 10$.

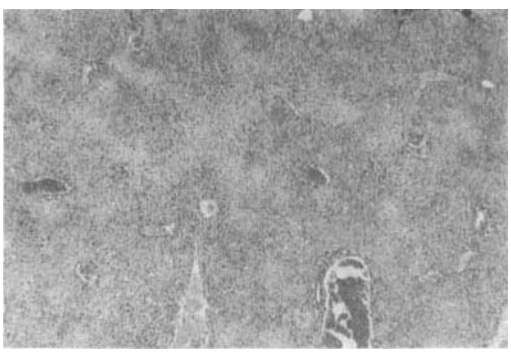

Figure 3 Diffuse haemorrhagic necrosis in the liver of a mouse without OK-432 treatment 3 days after infection (group C); (H&E × 11).

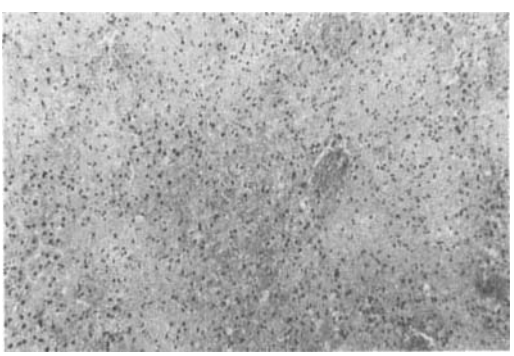

Figure 4 Massive, panlobular hepatic necrosis with poor inflammatory cell infiltration in a mouse of group C. The necrosis is mainly liquescent (group C); (H&E × 110).

Table 1 Severity of liver necrosis of dead mice

Group	Extent of liver necrosis at autopsy (dead mice)				
	I	II	III	IV	V
A*	0	0	0	3	2
B	0	0	0	5	5
C	0	0	0	2	8
D†	0	0	0	5	3
E	0	0	0	5	5
F	0	0	0	1	9

*Five mice survived. †Two mice survived.

I: none; II: very slight; III: slight; IV: moderate; V: severe. Values shown are numbers of mice.

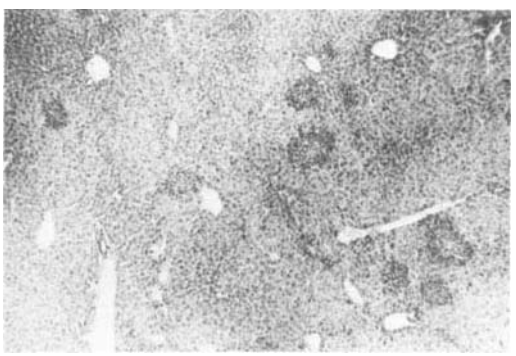

Figure 5 Focal necrosis scattering in the liver of a mouse that survived after injection with OK-432 before MHV-2, 4 days after infection; (H&E × 11).

Table 2 Time course of severity of liver necrosis and leucocyte infiltration

Extent of liver changes	*n*	Treatment* A	C
Necrosis			
Day 1	3	I–II	II–III
Day 2	3	II	III
Day 4	3	II–III†	IV–V
Day 7	3	I–I	—
Leucocyte infiltration of lesions			
Day 1	3	II	I–II
Day 2	3	II–III	I–II
Day 4	3	III–IV	I–II‡
Day 7	3	II–III	—

*Treated with the same treatments as groups A and C. †Grade IV in some areas. ‡Grade III in some areas.

I: none; II: very slight; III: slight; IV: moderate; V: Severe.

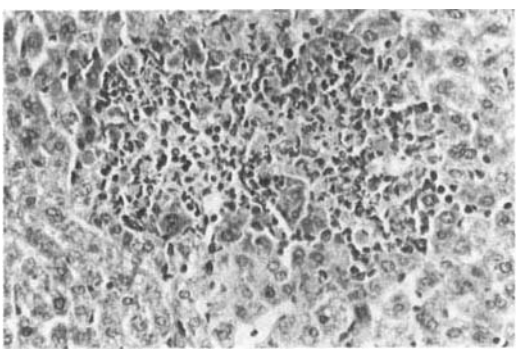

Figure 6 Focal hepatic necrosis accompanied by follicular lymphocytic infiltration with many macrophages in a mouse that survived after injection with OK-432 before MHV-2, 4 days after infection; (H&E × 220).

had severe massive necrosis of the liver 4 days after the injection.

The level of SGOT of a group A mouse that survived was significantly lower than that of mice treated in groups B and C on day 4 ($P < 0.01$; Fig. 7). This result was consistent with the histological findings.

The time course of the reaction of spleen cells to mitogen is shown in Fig. 8. Mice inoculated with MHV-2 reacted remarkably less than did normal mice ($P < 0.01$). However, the reactivity of mice injected with OK-432 before inoculation of MHV-2 (group A) was higher than that of the mice treated in group C on days 2 and 3 after MHV-2 inoculation ($P < 0.01$).

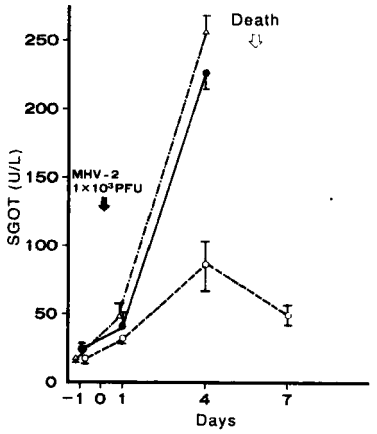

Figure 7 Changes in SGOT levels in mice after inoculation of MHV-2. The mice were treated with the same treatments as groups A(○——○), B(●——●) or C (Δ—.—Δ). For all groups, $n = 10$. After 7 days, six of the group A mice were still alive. The data are expressed as mean and s.e.m.

DISCUSSION

Intraperitoneal injection of 1×10^3 PFU of MHV-2 killed all the mice without OK-432 within 5 days of the injection. These mice had severe haemorrhagic liver necrosis. However, high survival rates or prolongation of survival time as well as histological remission were observed in mice with OK-432, especially in mice treated with OK-432 before injection of MHV-2. The protective mechanism of OK-432 is unknown in detail. A previous report has shown that OK-432 has inhibitory effects on fulminant hepatitis that was caused by galactosamine, and that the important mechanism is their activation of the reticuloendothelial system.[14] MHV-2 is an RNA virus similar to an enterovirus and also to the hepatitis A virus.[15,16] Generally, MHV-2 causes fulminant or acute hepatitis. The latter completely remits within a relatively short time. It is estimated that T cells play an important role in the immunological protection against MHV-2 infection, while the B cells are thought to produce antibodies to MHV-2 on the basis of information from the T cell.[17] However, humoral immunity may not be so important against MHV-2 infection because of the low efficacy of injection with neutralizing antibodies to MHV-2.[18] Recently, however, Nakanaga *et al.* reported a protective effect of monoclonal antibodies on lethal MHV-2 infection.[19] First MHV-2 accumulates in the spleen and then it proliferates in the Kupffer cells and macrophages of the liver. Therefore, it is necessary for the onset of fulminant hepatitis that MHV-2 is able to proliferate in the Kupffer cells or macrophages, before it can propagate in the liver.

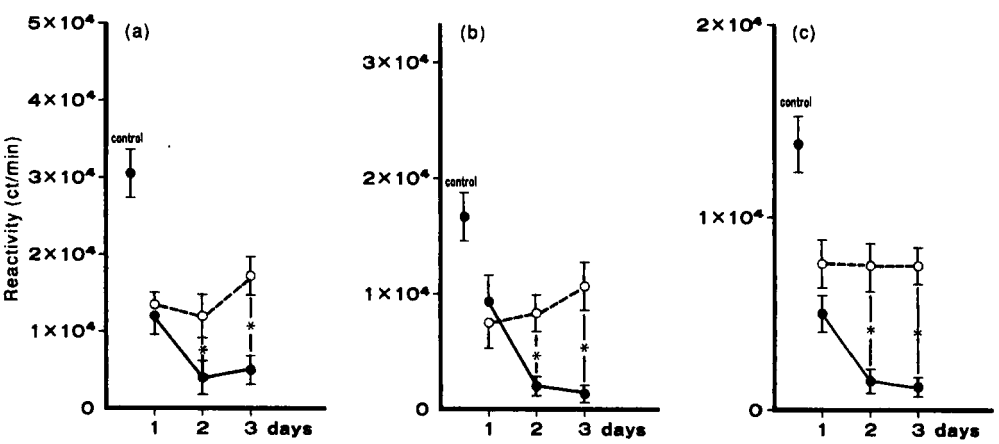

Figure 8 Time course of reactivity of the spleen cells to mitogens: (a) PHA, (b) Con A and (c) PWM. The mice injected with OK-432 before inoculation of MHV-2 (group A, ○——○) showed significantly higher reactivity than that of the mice not treated with OK-432 before MHV-2 (group C, ●——●) on days 2 and 3. Mice were sacrificed on days 1, 2, and 3 after MHV-2 injection. Values are expressed after subtraction of reactivity in medium culture. For groups A and C, and also controls, $n = 5$. The data are expressed as mean and s.e.m. *$P < 0.01$ using Student's *t*-test.

In this study, mice treated with OK-432 prior to injection of MHV-2 showed augmentation of T and B cell proliferation in response to mitogens. This resulted in protection against fulminant MHV-2 infection which has an immunosuppressive effect on T and B Cells.

Given the survival of the mice of group A, it may be also thought that OK-432, injected previously, augmented the activities of the intraperitoneal macrophages, lymphocytes and the reticuloendothelial system, including Kupffer cells, and that this was followed by the release of interferon, an anti-viral agent (data not shown; OK-432 is known to be a strong interferon inducer[7]). Activated macrophages might inhibit viral proliferation in the peritoneal cavity, and rapidly mediate T cell activation and T cell-dependent MHV-2-specific antibody production by B cells.[19] Large amounts of the MHV-2 are thought to be removed by this protective mechanism, and alive viruses invaded systemic circulation and the liver, where they may be inactivated. On the other hand, it is suggested that in groups B and E injected with OK-432 after MHV-2 inoculation, the injection of OK-432 was not successful in suppressing the proliferation of the viruses, which had already propagated diffusely in the liver, then fulminant hepatitis developed.

The protective effects of recombinant interleukin 1 and interleukin 2 on MHV-2 fulminant hepatitis were also investigated. However, successful results were not obtained (unpubl. data).

It is clear that pre-injection of OK-432 protects mice from fulminant hepatitis, although the mechanisms remain unclear. This fact suggests that immunotherapy might be introduced for fulminant hepatitis in humans. The mechanisms of the protective effect of OK-432 are being studied in greater detail.

ACKNOWLEDGEMENTS

The authors thank Drs K. Kyuwa and M. Hayami, members of the Department of Animal Pathology, Institute of Medical Science, University of Tokyo.

REFERENCES

1. Robb J.A & Bond C.W. Coronaviridae. In: Conrat H.F., Wagner R.R., eds, *Comprehensive Virology, Vol. 14.* Plenum Publishing Corporation, New York. 1979; 193–247.
2. Siddell S., Anderson R., Cavanagh D. *et al.* Coronaviridae. *Intervirology* 1983; **20**; 181–9.
3. Wege H., Siddell S. & Meulen V. The biology and pathogenesis of coronaviruses. *Curr. Top. Microbiol. Immunol.* 1982; **99**: 165–200.
4. Bang F.B. & Warwich A. Mouse macrophages as host cells for the mouse hepatitis virus and the genetic basis of their susceptibility. *Proc. Natl Acad. Sci. USA* 1960; **46**: 1065–75.
5. Hirano N., Murakami T., Taguchi F., Fujiwara K. & Matumoto M. Comparison of mouse hepatitis virus strains for pathogenicity in weanling mice infected by various routes. *Arch. Virol.* 1981; **70**: 69–73.
6. Neison J.R. Acute hepatitis associated with mouse leukemia. I. Pathological features and transmission of the disease. *J. Exp. Med.* 1952; **96**: 293–302.
7. Ishida N. & Hoshino T. *OK-432* Excerpta Medica, Tokyo. 1985.
8. Hirano N., Fujiwara K., Hino S. & Matumoto M. Replication and plaque formation of mouse hepatitis virus (MHV-2) in mouse cell line DBT culture. *Arch. ges. Virusforsch.* 1974; **44**: 298–302.
9. Hirano N., Fujiwara K. & Matsumoto M. Mouse hepatitis virus (MHV-2). Plaque assay and propagation in mouse cell line DBT cells. *Jpn. J. Microbiol.* 1976; **20**: 219–25.
10. Hirano N., Murakami T., Fujiwara K. & Matumoto M. Utility of mouse cell line DBT for propagation of mouse hepatitis virus. *Jpn. J. Exp. Med.* 1978; **48**: 71–5.
11. Okamoto H., Shoin S., Koshimura S. & Shimizu R. Studies on the anticancer and streptolysin-S forming abilities of hemolytic streptococci. *Jpn. J. Microbiol.* 1976; **11**: 323–36.
12. Oshimi K., Kano S., Takaku F. & Okumura K. Augmentation of mouse natural killer cell activity by a streptococcal preparation, OK-432. *J. Natl Cancer Inst.* 1980; **65**: 1265–9.
13. Ichimura O., Suzuki S., Sugawara Y. & Osawa T. Characterization of mouse natural killer cell activating factor (NKAF) induced by OK-432: Evidence for interferon- and interleukin 2-inde pendent NK cell activation. *Brit. J. Cancer* 1984; **50**: 97–108.
14. Matsumata T., Kanematsu T., Sonoda T. *et al.* Acute liver failure in rats inhibited by intrasplenic administration of OK-432. *J. Surg. Res.* 1986; **40**: 43–8.
15. Hirano N., Takenaka S. & Fujiwara K. Pathogenicity of mouse hepatitis virus for mice depending upon host age and route of infection. *Jpn. J. Exp. Med.* 1975; **45**: 285–92.
16. Rowe W.P., Hartley J.W. & Capps W.I., Mouse hepatitis virus infection as a highly contagious,

prevalent, enteric infection of mice. *Proc. Soc. Exp. Med.* 1963; 112: 161–5.

17. FUJIWARA K., TAMURA T., TAGUCHI F., MACHII K. & SUZUKI K. Immunisation de la souris 'nude' contre le virus de l'hépatite murine par transfert de lymphocytes sensibilisés. *C. R. Soc. Biol.* 1976; 170: 509–13.
18. TAGUCHI F., HIRANO N., KIUCHI Y. & FUJIWARA K. Difference in response to mouse hepatitis virus among susceptible mouse strains. *Jpn. J. Microbiol.* 1976; 20: 293–302.
19. NAKANAGA K., YAMANOUCHI K. & FUJIWARA K. Protective effect of monoclonal antibodies on lethal mouse hepatitis virus infection in mice. *J. Virol.* 1986; 59: 168–71.