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Resistance to Highly Virulent Mouse Hepatitis Virus Acquired by Mice after Low-Virulence Infection: Enhanced Antiviral Activity of Macrophages

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As early as 1 to 2 days after intranasal inoculation with a mouse hepatitis virus of low virulence, MHV-S, susceptible DDD mice became fully resistant to a normally lethal challenge with a highly virulent MHV-2. The resistance of MHV-S-pretreated mice was correlated with significantly decreased MHV-2 multiplication in the liver, spleen, and brain. Infection with MHV-S did not induce a high level of interferon in DDD mice, and no neutralizing antibody against MHV-2 was detected in the sera of mice until day 6 of MHV-S infection. The multiplication of MHV-2 was suppressed in peritoneal cells (PC) in vivo and peritoneal adherent cells (PAC) in vitro of MHV-S-pretreated mice as compared with those of normal mice. This suppression of virus multiplication was demonstrated in PAC collected during days 1 to 3 of infection but not in PAC collected from day 5 on. PC from MHV-S-pretreated mice were also suppressive to MHV-2 growth in DK cells as compared with PC from normal mice. By treatment of MHV-S-pretreated mice with silica, suppression of virus growth in the liver was partially diminished. These findings suggest that increased suppression of MHV-2 growth in PAC (mostly macrophages) of MHV-S-pretreated mice is responsible for resistance.

Coronaviruses are known for their wide range of natural hosts and of disease manifestation (11) and cause severe hygienic problems in experimental and domestic animals (J. A. Robb and C. W. Bond, Compr. Virol., in press). However, efforts to develop vaccines against virus infection have been limited (11). As a model of vaccination studies of coronavirus infections, we examined the effect of a low-virulence mouse hepatitis virus (MHV), MHV-S, on the infection of mice with the highly virulent virus MHV-2, using several strains of mice showing different susceptibility to MHV-2 (19). Mice of all strains acquired resistance against MHV-2 infection within several days after MHV-S inoculation, which was attributable to cross-reactive neutralizing antibodies elicited by MHV-S infection. However, as early as 1 to 2 days after MHV-S inoculation, some degree of resistance was observed in all strains examined. Among them, DDD mice, which were shown to be relatively resistant in terms of survival time as compared with other highly susceptible strains (19), tolerated the challenge infection of MHV-2, revealing a complete resistance.

On such a resistance appearing so early in virus infections, interferon (IF) seems to be an important factor. It was documented that IF is an antiviral substance by itself, but also suppresses virus growth by activating macrophages (12, 21).

In the present paper, we describe the resistance phenomenon and some factors involved in this resistance to virulent MHV, a resistance which appeared as early as 1 to 2 days after low-virulence MHV infection, focusing on the role of macrophages.

MATERIALS AND METHODS

Mice. Four-week-old DDD and CDF1 (BALB/c × DBA) mice, as well as ICR mice 25 to 30 weeks of age, were used. The first two strains were obtained from the animal supply section of this Institute, and ICR mice were purchased from a commercial breeder (Shizuoka Jikkendoubustu, Hamamatsu). Colonies of these mice were proven to be free from MHV infection by seromonitoring (3).

Cells and cell cultures. For the propagation and assay of viruses, DBT cells (5) were employed, which were grown in a growth medium consisting of 10% calf serum and 10% tryptose phosphate broth (Difco Laboratories, Detroit, Mich.) in Eagle minimal essential medium (EMEM; Nissui, Tokyo). Cells were maintained in a maintenance medium in which the proportion of calf serum was reduced to 5%, as described previously (5). DK cells derived from DDD mice were kindly supplied by K. Kai and cultured with the above medium.

Viruses and virus inoculation. A low-virulence strain of MHV-S (15, 17) and a highly virulent MHV-

2 (7), passaged through DBT cells 14 and 73 times, respectively, were employed as inocula. To produce inocula, DBT cells were inoculated with either MHV-S or MHV-2 and cultured with maintenance medium for 12 h. The supernatant was isolated, centrifuged at 2,000 rpm for 10 min to remove cell debris, divided into batches of small volume, and stored at −20°C. Stored virus titers of MHV-S and MHV-2 were 2×10^6 to 4×10^6 and 10^6 to 3×10^6 plaque-forming units (PFU) per 0.2 ml, respectively. A total of 10^5 PFU of MHV-S was inoculated into the mice intranasally (i.n.) 2 days before the challenge of 10^5 PFU of MHV-2 by the intraperitoneal (i.p.) route, unless otherwise stated. Mice inoculated with MHV-S i.n. were referred to as MHV-S mice, and mice not inoculated with MHV-S were referred to as Nor mice.

Virus assay. MHV-2 and MHV-S were plaque assayed using DBT cells as previously described (5, 17). Briefly, sampled tissues of the liver, spleen, and brain were homogenized in a chilled phosphate-buffered saline (PBS; pH 7.2) to make a 10% solution. They were then centrifuged at 2,000 rpm for 10 min, and the supernatant was serially 10-fold diluted. Each dilution was assayed for infectious viruses in triplicate on DBT cells. MHV-S and MHV-2 in the same sample were identified by their plaque morphology: MHV-S produces fusion-type and MHV-2 produces rounding-type plaques (5, 6).

IF assay. Sera from mice were assayed for IF. The assay of IF titers using vesicular stomatitis virus and L-929 cells was described previously (19).

Neutralizing antibody assay. Sera from infected mice were serially twofold diluted with EMEM containing 10% tryptose phosphate broth. A 0.5-ml sample of each dilution was mixed with the same volume of MHV-2 preparation containing approximately 200 PFU/0.2 ml in EMEM with 10% tryptose phosphate broth and incubated at 37°C for 45 min. Then the dilutions were plaque assayed on DBT cells. Neutralizing antibody titer was expressed by the reciprocal of the dilution producing a 50% reduction in plaque number as compared with the control, as described elsewhere (17).

Virus multiplication in PC in vivo. Mice pretreated with MHV-S (MHV-S mice) and untreated normal mice (Nor mice) were challenged i.p. with 10^5 PFU of MHV-2. At 3, 6, 12, and 24 h later, three mice in each group were sacrificed, and peritoneal cells (PC) were collected from the mice individually by washing the peritoneal cavity with 5 ml of chilled PBS. Cells were washed once by centrifuging at 1,000 rpm for 5 min and adjusted to a concentration of 5×10^5 cells per ml. This solution was then subjected to freeze-thawing three times, and virus titer in the supernatant was assayed.

Virus multiplication in cultured PAC. MHV-S mice and Nor mice were sacrificed, and PC were collected as described above. A sample of 10^6 PC in 1 ml of EMEM supplemented with 20% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.) were placed in a well of 15-mm diameter (Multiwell plate; Corning Glass Works, Corning, N.Y.) and incubated at 37°C for 60 to 90 min under a 5% CO_2 environment. Nonadherent cells were then removed by washing three times with EMEM. Remaining adherent cells (peritoneal adherent cells; PAC) were further cultured with the above medium for 2 or 24 h. The cells were washed with EMEM before the MHV-2 challenge at a multiplicity of infection of 1, and the supernatant was isolated at 4, 12, and 24 h after virus inoculation. Infectious viruses in the supernatant were plaque assayed on DBT cells. The adherent cells, after incubation for 2 and 24 h, consisted of at least 98% macrophages as determined by cellular morphology. By membrane immunofluorescence using anti-mouse brain θ and anti-mouse gamma globulin (gift from T. Kuhara), less than 4% and 1% of adherent cells cultured for 24 h were found to be positive for mouse brain θ and mouse gamma globulin, respectively.

In some experiments, PC were treated with antiserum to C3H thymocytes (anti-C3H thy1.2) prepared in AKR mice as described elsewhere (14) in the presence of complement to remove T lymphocytes from PC. A 0.5-ml sample of PC, adjusted at a concentration of 10^6 cells per 0.5 ml, was placed in a well with 0.1 m of anti-C3H thy1.2 serum (40-fold diluted in final) and incubated at 37°C for 30 min. Then, 0.1 ml of guinea pig serum was added as a complement source, and the PC were cultured for a further 30 min. Thereafter, cells were washed twice with EMEM to remove nonadherent and killed cells, and adherent cells were infected with MHV-2 as described above. Virus titers in the supernatants sampled at 12 h postinoculation were assayed. Under the above conditions, more than 90% of 10^6 DDD thymocytes were lysed, when examined by a trypan blue dye exclusion test.

Effect of PC on MHV-2 multiplication in DK cells. Subconfluent DK cells (1.5×10^5 cells per well) cultured in a 15-mm-diameter well (Multiwell plate) were infected with MHV-2 at a multiplicity of infection of 0.2 and incubated at 37°C for 1 h. The cells were washed with EMEM to remove free viruses, and 1.5×10^6 PC (DK to PC ratio, 1:10) from MHV-S mice or Nor mice were added. The mixture was cultured with EMEM containing 20% fetal calf serum, and virus titers in the supernatants were assayed on DBT cells. The collection of PC was the same as described above.

Silica treatment. Two kinds of silica were used. One was Dorentrup quartz, <5 μm, kindly supplied by Kenya Ôtaki, National Institute of Health, Japan, and the other was Min-U-Sil, <5 μm (10), kindly supplied by Philips Furmanski, Michigan Cancer Foundation. These materials were made up at 125 mg/ml in PBS and autoclaved. After ultrasonication, 0.4 ml was administered i.p. to each mouse 2 h before MHV-2 inoculation. The effect of the silica treatment was estimated by the virus titers in the liver of the mice and was statistically calculated by Student's *t* test.

RESULTS

Mortality and survival time of MHV-S mice and Nor mice after MHV-2 challenge. Twenty each of ICR, CDF1, and DDD mice were divided into two groups, each of which consisted of 10 mice. Mice in one group were inoculated i.n. with 10^5 PFU of low-virulence MHV-S, and those in the other group were inoculated with culture fluid. Two days later, all

mice were inoculated i.p. with 10^5 PFU of highly virulent MHV-2 and observed for 2 weeks. As shown in Fig. 1A, all Nor mice died within 8 days after the MHV-2 challenge due to MHV-2 multiplication, whereas all of the DDD, 90% of the ICR, and 40% of the CDF1 mice previously inoculated with MHV-S survived the otherwise lethal infection with MHV-2. Since the protective nature of the MHV-S inoculation was demonstrated most clearly in DDD mice, subsequent experiments were carried out on this strain.

To see when, after MHV-S inoculation, resistance appears to the MHV-2 challenge, 10 mice each were inoculated i.n. with 10^5 PFU of MHV-S at 12, 24, or 48 h before the MHV-2 challenge, leaving 10 mice as a control. As shown in Fig. 1B, none of the mice pretreated with MHV-S at 24 or 48 h before the MHV-2 challenge died. However, only limited protection was demonstrated in mice that received MHV-S at 12 h before the challenge.

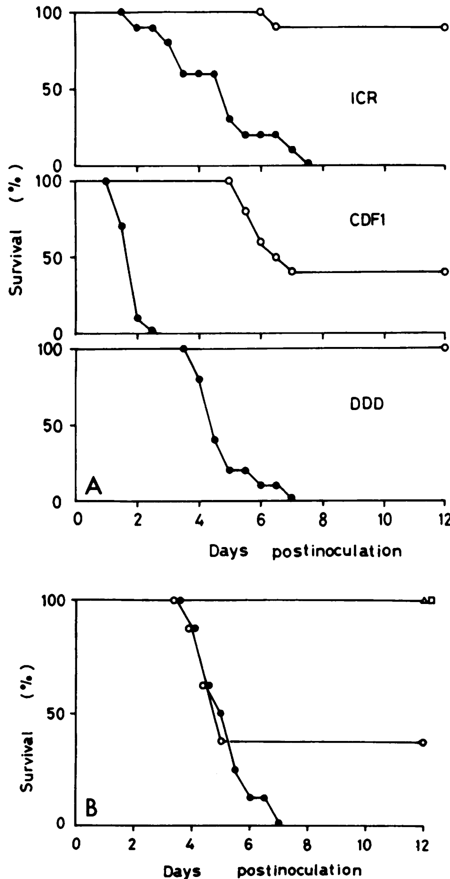

FIG. 1. *(A) Survival times of ICR, CDF1, and DDD mice after 10^5 PFU of MHV-2 were inoculated i.p. Mice were inoculated (○) or not inoculated (●) with 10^5 PFU of MHV-S i.n. 2 days before MHV-2 challenge. (B) Survival time of DDD mice after MHV-2 inoculation. DDD mice were inoculated with MHV-S at 12 (○), 24 (△), or 48 (□) h before the MHV-2 challenge, or not inoculated (●). Each group consisted of 10 mice.*

Multiplication of MHV-2 in MHV-S mice and Nor mice. MHV-S mice and Nor mice were challenged i.p. with 10^5 PFU of MHV-2, and virus titers in the brain, liver, and spleen were assayed for each individual mouse. A prominent difference was observed in MHV-2 titers of the liver (Fig. 2B): more than 10^5 PFU of MHV-2 was demonstrated in Nor mice, whereas less than 10^2 PFU of virus was detected at the peak in MHV-S mice. Such differences were also found in the brain and spleen between the two groups of mice (Fig. 2A and 2C). The virus, which produced fusion-type plaques, was isolated from the brains of MHV-S mice. This virus was considered to be MHV-S and not MHV-2 by the morphology of the plaques, as shown in our previous work (5, 6). Such fusion-type plaques were not observed in the liver or spleen throughout the experimental periods.

Production of IF and neutralizing anti-

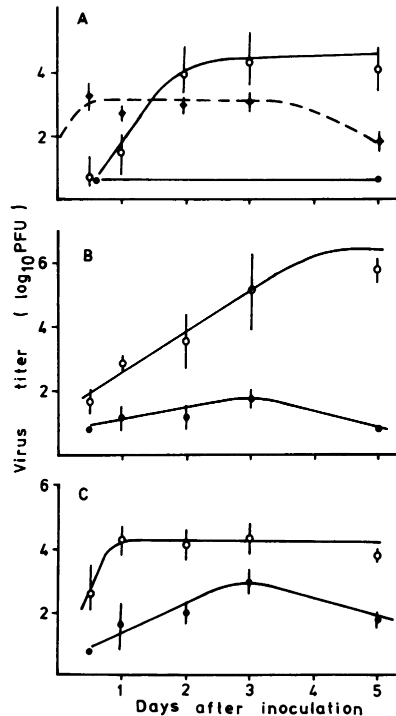

FIG. 2. *Virus titers in the brain (A), liver (B), and spleen (C) after 10^5 PFU of MHV-2 were inoculated. Mice preinoculated (●) or not inoculated (○) with MHV-S were challenged with 10^5 PFU of MHV-2, and titers of MHV-2 (○, ●) and MHV-S (◆——◆) were examined. Each point represents the mean value, and the vertical bars indicate the range of three to four samples.*

bodies. Batches of mice were inoculated with MHV-S. Sera from three mice were sampled at 24 and 48 h, and the rest of the mice were inoculated with MHV-2 i.p. at 48 h after MHV-S inoculation. They were bled at 24-h intervals thereafter. Another group of mice was inoculated only with MHV-2 i.p., and sera were sampled at 24-h intervals. A portion of each serum was used for the assay of IF. Inoculation of MHV-S i.n. did not elicit high-titered IF, whereas the MHV-2 challenge i.p. produced high titers in Nor mice, but not in MHV-S mice (Fig. 3).

The remaining portions of sera collected as described above were also subjected to the assay of neutralizing antibodies against MHV-2. No antibodies were detected in the sera of mice inoculated with MHV-2 only (Fig. 4). However, antibodies appeared in the sera of MHV-S mice 6 days after MHV-S inoculation, namely, 4 days after MHV-2 inoculation.

Virus multiplication in PC in vivo and in cultured PAC. The multiplication of MHV-2 in PC in vivo was compared between MHV-S mice and Nor mice as described in Materials and Methods. Multiplication of MHV-2 was prominently suppressed in the PC of MHV-S mice as compared with those of Nor mice (Fig. 5).

Virus multiplication was measured after in vitro challenge with MHV-2 on cultured PAC from MHV-S mice and Nor mice. The PAC were incubated at 37°C for 2 h before in vitro infection with MHV-2. PAC from the MHV-S mice showed a significant suppressive effect on MHV-2 multiplication as compared with PAC from Nor mice (Fig. 6). The same results were obtained when PAC were incubated at 37°C for 24 h. When PAC were treated with anti-C3H thy1.2 serum to remove T lymphocytes in PAC, there was no change in virus multiplication (Table 1). No infectious MHV-S was detected in the PC

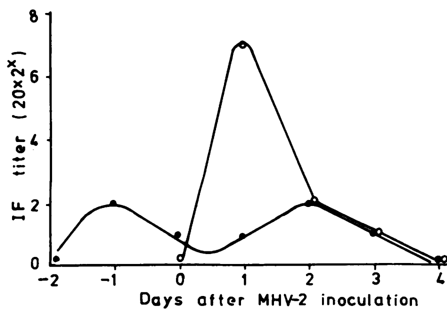

FIG. 3. *IF titers in the sera of mice inoculated with MHV-S on day −2 and with MHV-2 on day 0 (●), and of mice inoculated with MHV-2 only (○). Each point is the value of pooled sera from three to four mice.*

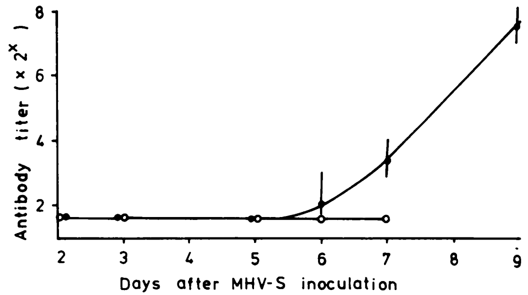

FIG. 4. *Neutralizing antibody titers against MHV-2 in the sera after MHV-S inoculation. Mice were inoculated (●) or not inoculated (○) with MHV-S on day 0, and they were challenged with MHV-2 on day 2. Each point represents the mean value, and vertical bars indicate the range of three to four samples.*

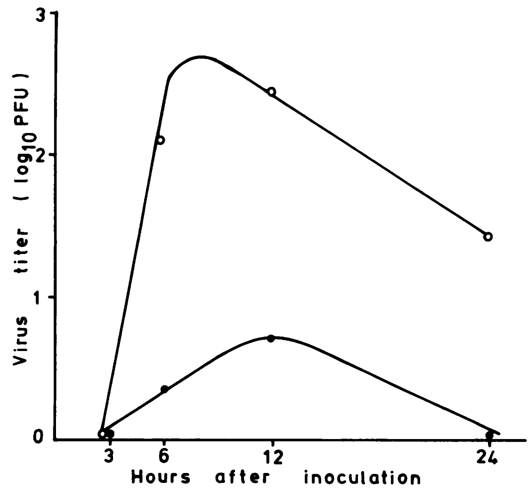

FIG. 5. *Multiplication of MHV-2 in vivo in PC of MHV-S-inoculated (●) or non-inoculated (○) mice. Each point represents the mean value of three samples. For details, see the text.*

and in the cultured PAC from MHV-S mice throughout the experimental period.

To test when the PAC of DDD mice become able to suppress MHV-2 multiplication in vivo after MHV-S inoculation, PC were collected from DDD mice which had been inoculated with MHV-S at 0 (non-inoculated), 1, 2, 3, 5, or 10 days before, and cultured for 2 h in vitro. Then, they were infected with MHV-2, and virus titers in the supernatant were examined at 12 h postinoculation. Virus multiplication was most suppressed in PAC collected from mice inoculated with MHV-S at 2 days before, and some degree of suppressive effect was demonstrable in cells from mice inoculated at 1 or 3 days before (Fig. 7). However, PAC collected from mice inoculated with MHV-S at 5 or 10 days before did not show this capacity.

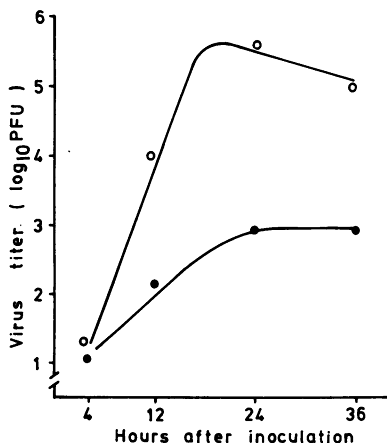

FIG. 6. *Multiplication of MHV-2 in cultured PAC from MHV-S inoculated (●) or non-inoculated (○) mice. Each point represents the mean value of three samples. For details, see the text.*

TABLE 1. *Effect of anti-C3H thy1.2 serum on MHV-2 multiplication in PAC*[a]

Treatment	PFU[b] in PAC from	
	MHV-S mice	Nor mice
Anti-θ + complement	3.5	5.4
Anti-θ + PBS	3.2	5.5
PBS + complement	3.5	5.4
PBS + PBS	3.2	5.3

[a] Virus titers in the supernatant of PAC at 12 h postinoculation were plaque assayed on DBT cells.
[b] Log_{10} PFU per 0.2 ml.

Effect of PC on MHV-2 multiplication in DK cells. To test whether PC from MHV-S mice are capable of suppressing MHV-2 growth in other cells, MHV-2 multiplication in DK cells was compared with respect to the presence or absence of PC from MHV-S mice or Nor mice. Multiplication of MHV-2 in DK cells was suppressed to a level of less than 1/100 when cultured with PC from MHV-S mice, as compared with the PC from Nor mice, or no PC (Fig. 8).

Effect of silica treatment. To study further the role of phagocytic cells in the resistance conferred by the inoculation of MHV-S, MHV-S mice were injected with silica (50 mg per mouse) by the i.p. route 2 h before MHV-2 challenge. Since silica treatment under the experimental conditions did not cause the MHV-S mice to die from the MHV-2 challenge, the titers of MHV-2 in the liver were compared 4 days after the MHV-2 challenge. Silica treatment caused MHV-S mice to exhibit MHV-2 multiplication in the liver (Fig. 9), and statistically significant differences ($P < 0.01$) were observed in the titers between MHV-S mice and silica-treated MHV-S mice. However, silica treatment did not completely abolish the resistance of mice, as shown by the significant difference ($P < 0.01$) in virus titers between MHV-S mice treated with silica and Nor mice. There was no difference in the effect between Dorentrup quartz and Min-U-Sil.

DISCUSSION

Resistance to highly virulent MHV-2 was established in susceptible DDD mice (19) as early as 1 to 2 days after i.n. inoculation of low-viru-

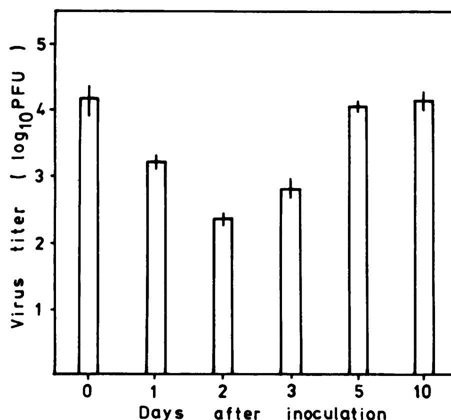

FIG. 7. *Multiplication of MHV-2 in cultured PAC from mice inoculated with MHV-S at various days before. At indicated days after MHV-S inoculation, PC were collected, and PAC were cultured and infected with MHV-2. The titer of MHV-2 in the supernatant of culture at 12 h postinoculation was plaque assayed.*

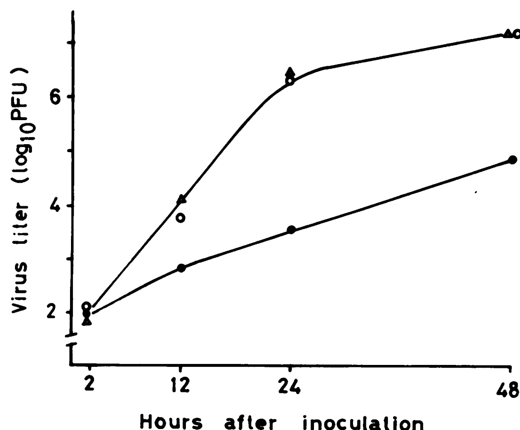

FIG. 8. *Effect of PC on MHV-2 growth in DK cells. PC were collected from mice inoculated (●) or not inoculated (○) with MHV-S and were cocultured with DK cells infected with MHV-2, and virus titers in the supernatant were examined. Control (△) shows MHV-2 growth in DK cells in the absence of PC.*

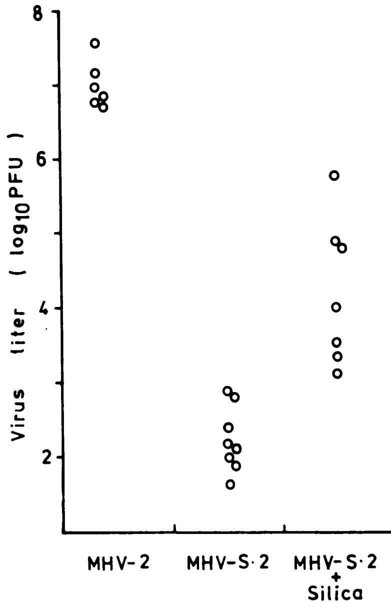

FIG. 9. *Effect of silica treatment on MHV-2 growth in the liver. The titers of MHV-2 in the liver were examined 4 days after MHV-2 challenge. Mice were inoculated with only MHV-2 (MHV-2), with MHV-S and MHV-2 (MHV-S·2), or with MHV-S and MHV-2 together with silica treatment (MHV-S·2 + silica).*

lence MHV-S. This phenomenon was also observed in other highly susceptible ICR and CDF1 mice, though the resistance was less complete. The resistance observed in DDD mice was shown to be due to the suppressed growth of virulent MHV-2 in target organs. As for the development of such resistance in an early stage of MHV-S infection, there seemed little possibility that neutralizing antibodies played any important role, as no neutralizing activity was detected in the sera of mice taken at that early stage.

As for the innate resistance of mice to infections with MHV-3 (9, 20) and MHV-S (19a). The role of macrophages has been emphasized so far. Even in infections with highly virulent MHV-2, the importance of macrophages in the resistance of mice has been reported (2, 8, 16). Our previous work has also shown that the macrophages of DDD mice were responsible for the lesser susceptibility of these mice to MHV-2 infection relative to other highly susceptible CDF1 mice (19). These facts led us to examine the multiplication of MHV-2 in macrophages of MHV-S-pretreated resistant mice. MHV-2 multiplication was suppressed at least 100 times more in the PC of MHV-S mice than in the PC of Nor mice. The restricted multiplication in PC of MHV-S mice in vivo correlated very well with the impaired virus multiplication in cultured PAC obtained through the first 3 days of MHV-S infection. This suppression of MHV-2 growth did not result from interference by MHV-S, since no infectious MHV-S was recovered from PAC of MHV-S mice. The multiplication of MHV-S in mice inoculated i.n. was restricted at nasal epithelial cells and in the brain, and no antigen was detected at the other parts of the body by immunofluorescence (18). From these facts, it seems unlikely that noninfectious virus or some viral components of MHV-S interfere with the multiplication of MHV-2 in PAC.

The suppressed virus multiplication in the cultured PAC of MHV-S-mice was due neither to T cells nor to B cells contained in PC, since few T or B cells were detected in PAC, and, in the case of T cells, anti-C3H thy1.2 serum had no influence on the suppression. Other cell populations in PAC, such as the nonphagocytic adherent cells reported by Nathan et al. (13), are unlikely to figure as factors influencing MHV-2 growth in PAC, since they were reported as immunoglobulin M positive on their surface (13), and we could not detect such cells in PAC. Thus, the restriction of MHV-2 multiplication in the PC and the PAC (mostly macrophages) of MHV-S mice, observed in vivo and in vitro experiments, respectively, seems to be an important factor in explaining the protective phenomenon manifested during the early stages of MHV-S infection. We are now investigating whether the PAC from MHV-S mice are also suppressive in the growth of other viruses unrelated to MHV.

To examine whether macrophages are really involved in the resistance of mice in vivo, we first examined whether the transfer of PC from MHV-S mice to Nor mice confers resistance against MHV-2 infection in the recipient Nor mice (data not shown). Though more than 10^7 PC were transferred, no significant protection was observed. The failure might be because the number of transferred PC was too few for complete protection. Peritoneal macrophages are generally believed not to proliferate in the recipient animals after transfer, contrary to lymphocytes or bone marrow cells, so that great numbers of macrophages may be needed for the protection. Since it seems very difficult to transfer enough macrophages, resistant MHV-S mice were treated with a silica, which was known to decrease macrophage activity (1). Administration of silica before MHV-2 inoculation permitted the multiplication of MHV-2 in the liver of MHV-S mice. This finding seems compatible with the idea that macrophages are actually responsible for the suppression of MHV-2

growth in MHV-S mice. Some other additional factors, however, might also be involved in the phenomenon, since the resistance was not completely abolished by silica treatment.

As for other possible factors involved in the resistance that appeared so early in MHV infection, natural killer cells can be suspected, since these cells were reported to be generated in an early stage of MHV infection (4). However, we could not detect activity in the spleen cells to suppress virus growth in DK cells (data not shown), suggesting that natural killer cells are less important in the establishment of early resistance.

IF has been reported to be an important host defense factor in MHV-3 infection in mice (21, 22), and this is suggested in MHV-S infection (19a). The low titer of IF found in MHV-S mice before the MHV-2 challenge might participate in the resistance, along with the increased resistance of macrophages. It might be postulated that IF is predominantly involved in the resistance through other mechanisms, such as a mediator for macrophage activation (21). Such a situation was discussed by Weiser and Bang when they reported (23) that susceptible mice became resistant to MHV-2 after injection of concanavalin A, and this resistance was also expressed in macrophages.

Thus, the resistance acquired by DDD mice against MHV-2 infection as early as 2 days after MHV-S inoculation was shown to be due to the antivirally activated macrophages, though other protective mechanisms could not be thoroughly ruled out. When mice were inoculated with MHV-S at 5 to 10 days before MHV-2 challenge, macrophages did not show any resistance to MHV-2 infection (shown in Fig. 7), but mice showed complete resistance against MHV-2 infection. At this stage of MHV-S infection, another mechanism(s) is suggested to be operating in the resistance of animals. Antibody produced as a result of MHV-S infection can be important as an anti-MHV-2 substance at 5 to 10 days after MHV-S inoculation, because anti-MHV-S antibody is known to effectively neutralize highly virulent MHV-2 (data not shown).

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