Age-Related Changes in Susceptibility of Mice to Low-Virulent Mouse Hepatitis Virus (MHV-2-CC) Infection

Hodaka SUZUKI1), Wijit KIATIPATTANASAKUL1), Satoru KAJIKAWA1), Shigeki TSUTSUI2), Hiroyuki NAKAYAMA1), Naoaki GOTO3), and Kunio DOI1)

1) Department of Veterinary Pathology, and 2) Department of Biomedical Science, Faculty of Agriculture, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113, and 3) Shin Nippon Biomedical Laboratories, Ltd., Tokyo Byouri Center, 2-7-7 Shinkawa, Chuo-ku, Tokyo 104, Japan

Abstract: This study was performed to examine mouse age-dependent changes in susceptibility to MHV-2-CC-infection and participation of macrophages in such changes in BALB/c mice. One-week-old mice were fully susceptible (mortality, 100%), 2-week-old semi-susceptible (36%), and 3- and 4-week-old fully resistant (0%) to MHV-2-CC, respectively. Such age-dependent differences corresponded well with the differences in the virus titers in the liver, spleen and blood and in the severity of liver lesions. In 1-week-old mice with peritoneal exudate cells (PEC) transferred from 4-week-old mice and infected with MHV-2-CC, a slight prolongation of survival time was recorded, although there was no difference in mortality. In 3-week-old mice infected with MHV-2-CC after silica-treatment to suppress macrophages, there was no significant change in susceptibility. In macrophages infected with MHV-2-CC in vitro, the virus replicated better in macrophages obtained from younger mice. These results suggest that macrophages may play a small role in the age-related development of resistance to MHV-2-CC infection in BALB/c mice.

Key words: age, BALB/c mice, macrophage, MHV-2-CC, susceptibility

Introduction

Mouse hepatitis virus (MHV) is a member of the coronavirus family and is known to produce various diseases in murine species, such as acute or chronic hepatitis [4, 14], encephalomyelitis [3] and enteritis [9]. Many MHV strains showing different organ tropism and pathogenicity have been isolated. MHV-2-CC is a low-virulent small-plaque mutant isolated from a cell line persistently infected with a highly virulent and hepatotropic strain, MHV-2 [8]. MHV-2-CC has been reported to produce chronic active hepatitis in athymic nude mice (nu/nu), but not in euthymic littermates (nu/+)[4, 13], suggesting the association of a defective immune system with the formation of liver lesions. MHV-2-CC-infected athymic nude mice are used as a model for human chronic active hepatitis [4, 13].

An age-related change in susceptibility [6, 17] has been reported in MHV-infection in mice. There have been many reports describing critical roles of macroph-
ages in the host mechanism of defense against MHV [18]. In MHV-2-infection, it was reported that the in vitro susceptibility of macrophages to the virus could reflect the in vivo susceptibility of mice [4, 16]. Moreover, there are reports that suppression [19] or transfer of macrophages [10, 12] could change susceptibility of the host to MHV-infection.

The present study was carried out to examine whether macrophages could participate in age-related change in susceptibility to MHV-2-CC in BALB/c mice.

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**Materials and Methods**

**Mice:** BALB/c mice purchased from Japan SLC Inc. (Hamamatsu) were used. The mice were kept under controlled conditions (temperature, 23 ± 2°C; relative humidity, 55 ± 5%; lighting, 12 hr (6:00–18:00); ventilation, 20 air changes an hour) in an isolator caging system (Niki Shoji Co. Ltd., Tokyo) and fed autoclaved pellets (MF, Orient Yeast Co., Tokyo) and water ad libitum.

**SR-CDF1-DBT (DBT) cells:** DBT cells which originated from astorocytoma were cultured in Eagle’s minimum essential medium (EMEM; Nissui Co., Tokyo) containing 10% calf serum (CS; Dainippon Seiyaku Co. Ltd., Osaka) and 10% trypsin phosphate broth (TPB; Difco Co. Ltd., Detroit, Michigan, U.S.A.) [7].

**Virus:** A low-virulent small-plaque mutant, MHV-2-CC, derived from a highly virulent hepatotropic strain, MHV-2, was used [8]. The virus was grown in DBT cells and the culture supernatant was diluted with EMEM to 1 × 10⁶ PFU/ml. 0.1 ml of the virus dilution was injected into mice intraperitoneally (i.p.).

**Thioglycollate medium (TGC):** TGC (BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD, U.S.A.) was dissolved in Millipore-filtered water. A 3% solution of TGC was autoclaved and injected i.p. at appropriate doses to induce peritoneal exudate cells (PEC) [17].

**Silica suspension (Silica):** Silica (Sigma Chemical Co., St. Louis, MO, U.S.A.) was dissolved in phosphate-buffered saline (PBS), adjusted to 5 mg/ml, autoclaved and injected i.p. into mice in order to suppress macrophages [1].

**Virus titration:** Liver and spleen samples were homogenized in PBS containing kanamycin (kanamycin-PBS) and centrifuged. A mixture of one volume of blood and nine volumes of kanamycin-PBS was stored at −70°C. Serial 10-fold dilutions of the samples were made with EMEM. The virus titration on DBT cells was performed as described previously [7].

**Histopathology:** Samples of liver were fixed in Bouin’s solution and embedded in paraffin in a routine manner. Sections (4-μm) were stained with hematoxylin and eosin (HE). The severity of liver lesions was graded according to the site and area of inflammation (See the footnote to Table 2).

**Immunohistochemistry:** For the detection of viral antigens in the liver, immunohistochemical staining by the avidin-biotin-complex (ABC) method was performed as described previously [11]. A polyclonal anti-MHV-2 rabbit serum [11] was used as the primary antibody and biotinylated goat anti-rabbit IgG (Kirkegaard & Perry Lab., Gaithersburg, MD, U.S.A.) as the secondary antibody.

**Measurement of cell viability:** Viability of the macrophage culture was confirmed by measuring the activity of lactate dehydrogenase (LDH) released from cells into medium by using a cytotoxicity detection kit (Boehringer Mannheim, Germany). The cytotoxicity rate was calculated with the following formula: Cytotoxicity rate (%)=(LDH activity in sample supernatant-LDH activity in original medium)/(LDH activity in total cell lysate-LDH activity in original medium) × 100. The cytotoxicity rate induced by the virus (%) was expressed as (cytotoxicity rate of the test samples (%))-(average cytotoxicity rate of control samples (%)). This experiment was performed in triplicate.

**Statistical analysis:** Unpaired t-test was used to compare the data for the different groups and a P value <0.05 was considered significant.

**Experimental Designs:**

**Experiment 1. Age difference in the susceptibility to MHV-2-CC-infection**

BALB/c mice 1 to 4 weeks old (at least 10 mice of each age) were injected i.p. with MHV-2-CC and the mortality was checked every day for 2 weeks post infection (p.i.). The 1 and 2 week-old mice were kept with their nursing mothers. In addition, 1, 2 and 3-week-old mice were injected i.p. with MHV-2-CC. Liver, spleen and blood samples were taken at 1, 2, 3, 5, 7 and 10 days p.i. (from 3 mice each day) for virus titration. Liver samples were also examined histopathologically and immunohistochemically.
Experiment 2. Role of macrophages in MHV-2-CC-infection

*In vivo experiment*\#: Thirty 4-week-old BALB/c mice were injected i.p. with 1.5 ml of TGC. Four days later the mice were euthanized with ether and PEC were taken by washing the peritoneal cavity with PBS. The viable cells were counted and adjusted to $1 \times 10^7/0.1$ ml or $1 \times 10^7/0.1$ ml with EMEM. PEC (more than 90% of the cells were morphologically macrophages) were transferred to 1-week-old BALB/c mice (at least 10 mice in each group). MHV-2-CC was then injected i.p. and mortality was observed for 2 weeks. The liver, spleen and blood were collected at 1, 2, 3, 5 and 7 days p.i. (3 mice each day) from the mice treated in the same way for the virological and pathological examinations. Additionally, silica (100 mg/kg body weight) was injected i.p. into 10 1-week-old BALB/c mice 5 times for 2 weeks. When the mice were 3 weeks old, MHV-2-CC was injected i.p., and mortality was observed for 2 weeks. Then the mice were sacrificed at 1, 2, 3, 5, 7 and 10 days p.i. (3 mice each day) for the virological and pathological examinations.

*In vitro experiment*\#: PEC from 1- to 3-week-old BALB/c mice (10 mice of each age) were adjusted to $1 \times 10^8$ cells/1.0 ml with EMEM containing 10% fetal bovine serum (FBS; Immuno-Biological Laboratories, Fujioka) and then 1 ml of the suspension was placed into each well of a 24-well plate (Costar Co., Cambridge, MA, U.S.A.). After incubation at 37°C under 5% CO$_2$ for 10 to 15 hr, non-adherent cells were removed by washing three times with PBS. Adherent cells were then inoculated with MHV-2-CC at multiplicity of infection (m.o.i.) 1 and the cultures were incubated. Supernatants were taken at 6, 12, 24 and 48 hr p.i. The supernatants and cell lysate samples were stored at 4°C. The cytotoxicity rate and virus titers were determined as described above.

**Results**

**Experiment 1. Age difference in the susceptibility to MHV-2-CC-infection**

The mortality rates for mice infected with MHV-2-CC are summarized in Table 1. One-week-old mice were completely susceptible, but 2-week-old mice were semi-susceptible. All the 3- and 4-week-old mice survived. Therefore, 1- to 3-week-old mice were used for further investigations on age-dependent changes in susceptibility. One-week-old mice showed the maximum liver virus titer at 2 days p.i. and all mice died by 10 days p.i. The titer of 2-week-old mice was one tenth that of 1-week-old mice, and viral replication was no longer detected at 10 days p.i. The titer of 3-week-old mice was one hundredth that of 1-week-old mice, and became undetectable at 7 days p.i. (Fig. 1a). The spleen virus titer reached its maximum level at 1 day p.i. in all age groups, and thereafter decreased gradually (in 1-week-old mice) or rapidly (in 2- and 3-week-old mice) (Fig. 1b). The virus titers of 2- and 3-week-old mice became undetectable at 10 and 7 days p.i., respectively. Viremia was severe in 1-week-old mice, but it was mild in 2- and 3-week-old mice (Fig. 1c).

As shown in Table 2, liver lesions developed most rapidly and most severely in 1-week-old mice. Immunohistochemically, a few viral antigen-positive cells were found around inflammatory foci. Similar but less severe lesions were also found in 2-week-old mice. The lesions in 3-week-old mice were mildest.

**Experiment 2. The role of macrophages in MHV-2-CC-infection**

*In vivo experiment*\#: Although no significant differences between PEC-transferred and non-transferred (control) groups were found in cumulative mortality, the survival times of the control, 10$^6$ PEC-transferred and 10$^7$ PEC-transferred groups were 6.92, 8.39 and 8.75 days, respectively, suggesting a slight prolongation of survival time due to PEC-transfer (Fig. 2). The virus titer in the liver changed similarly in both groups, although the transferred group had a slightly higher titer than the controls (Fig. 3a). The spleen virus titer of the PEC-transferred group was higher than that of the control one throughout the experiments (Fig. 3b). In the blood (Fig. 3c), control mice had higher virus titers than PEC-transferred ones, contrary to the results

<table>
<thead>
<tr>
<th>Age (Weeks)</th>
<th>No. dead/No. tested*</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13/13</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>5/14</td>
<td>36</td>
</tr>
<tr>
<td>3</td>
<td>0/10</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0/10</td>
<td>0</td>
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</table>

*14 days post infection
in the liver and spleen. As shown in Table 3, liver lesions tended to develop more rapidly and to be more severe in the control group than in the transferred one, but final lesions were similar.

None in the silica-treated or untreated (control) groups died during the experiments. Both groups had similar changes in the liver virus titer, though the titer was 10-fold higher in the silica-treated group than in control one at 5 days p.i. (Fig. 4a). In the spleen, the titers of both groups were similar (Fig. 4b). Slight viremia was found in the control group, but no virus replication was detected in the blood from the silica-treated group (Fig. 4c). As shown in Table 4, liver

Fig. 1. Virus titers in 1- to 3-week-old mice injected i.p. with 10⁵ PFU of MHV-2-CC (Mean ± S.E.M.). ○: 1W, ●: 2W, ▲: 3W. *: Significant differences are found between 1- and 2-week-old mice. #: Significant differences are found between 1- and 3-week-old mice. +: Significant differences are found between 2- and 3-week-old mice.

Table 2. Age difference in liver lesions

<table>
<thead>
<tr>
<th>Age (Weeks)</th>
<th>Day(s) p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1  2  3  5  7  10</td>
</tr>
<tr>
<td>1W</td>
<td>+  +  +  +++  ++++ N.T.**</td>
</tr>
<tr>
<td>2W</td>
<td>+  +  +  +++  ++++  +</td>
</tr>
<tr>
<td>3W</td>
<td>±  +  +  +++  +++  +</td>
</tr>
</tbody>
</table>

*Grade of lesions: –: No lesions, ±: Slight inflammation in perivascular area, +: Moderate inflammation in perivascular area, ++: Small inflammatory foci in liver parenchyma, +++: Middle or large inflammatory foci in liver parenchyma, ++++: Confluent inflammatory foci, **N.T.: Not Tested.

Fig. 2. Cumulative mortality rate of PEC-transferred and control 1-week-old mice injected i.p. with 10⁵ PFU of MHV-2-CC. PEC were obtained from 4-week-old mice and 10⁵ or 10⁶ PEC were transferred to each mouse. ○: Non-transferred, ●: 10⁵ PEC-transferred, ▲: 10⁶ PEC-transferred, ▲: 10⁷ PEC-transferred. Significant differences are found between Non-transferred and 10⁵ or 10⁶ PEC-transferred mice in the survival time.
lesions in the control group were more severe than those in silica-treated mice throughout the experiments.

In vitro experiment: Virus titers of macrophage cultures from 1- to 3-week-old mice are shown in Fig. 5a. No distinct age-dependent changes in susceptibility to MHV-2-CC replication were observed, although the macrophage cultures obtained from younger mice tended to have higher titers. Similarly, the macrophage cultures from younger mice were likely to show higher LDH leakage (Fig. 5b).

**Discussion**

Age-related changes in susceptibility have been reported in MHV- infection [6, 17]. In the present study, mouse resistance to MHV-2-CC- infection also developed between 2 and 3 weeks of age. As the virus titers in the liver, spleen and blood of 1-week-old mice were higher than those of 2- and 3-week-old ones, the susceptibility to MHV-2-CC is thought to be related to the degree of viral replication. The difference between the virus titers of 2- and 3-week-old mice was prominent in the liver but not so clear in the spleen and blood. This suggests that the difference between 2- and 3-week-old mice in susceptibility is due to both viral replication in the liver and by an age-related increase in elimination of the virus by the immune system.

PEC-transferred mice had slightly higher liver and spleen virus titers but a lower blood virus titer and

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**Table 3. Differences between liver lesions in PEC-transferred and control mice**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day (s) p.i.</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>1  2  3  5  7</td>
</tr>
<tr>
<td>PEC-transferred</td>
<td>-* ++ ++ +++ ++++</td>
</tr>
<tr>
<td>Control</td>
<td>+ ++ ++ +++ ++++</td>
</tr>
</tbody>
</table>

*Grade of lesions: See the footnote to Table 2.

**Table 4. Differences between liver lesions in silica-treated and control mice**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day (s) p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1  2  3  5  7  10</td>
</tr>
<tr>
<td>Silica-treated</td>
<td>-* - + ++ ± ±</td>
</tr>
<tr>
<td>Control</td>
<td>± ++ ++ +++ ++ +</td>
</tr>
</tbody>
</table>

*Grade of lesions: See the footnote to Table 2.
**Fig. 4.** Virus titers in silica-treated and non-treated 3-week-old mice injected i.p. with 10⁵ PFU of MHV-2-CC. Silica was inoculated i.p. every 3 days from 2 weeks before MHV-2-CC infection (Mean ± S.E.M.). — — — : Control, — — — — : Silica-treated. No significant differences are found between Silica-treated and Control mice.

**Fig. 5.** Virus titers (5a) and LDH leakage (5b) in macrophage cultures (1 x 10⁶ cells each well) from 1-to 3-week-old mice inoculated with MHV-2-CC at multiplicity of infection (m.o.i.) 1 (Mean ± S.E.M.). — — : 1W, — — — — : 2W, — — — — — : 3W. *: Significant differences are found between 1- and 2-week-old mice. #: Significant differences are found between 1- and 3-week-old mice. *: Significant differences are found between 2- and 3-week-old mice.
longer survival time compared to control mice. These data suggest that viremia may be important as a cause of the death of mice following MHV-2-CC-infection, and macrophages may play two contrary roles in supporting viral replication in the liver and spleen and in the depression of viremia. Although the liver virus titer was lower in the control group, liver lesions developed more rapidly and were more severe in the control group than in the PEC-transferred one. These findings suggest that macrophages might inhibit liver lesions without suppressing virus replication in the liver. The silica-treatment brought about no significant suppression of virus replication in the liver and spleen.

There have been many reports indicating that macrophages play an important role in MHV-infection [18]. There have also been some reports stating that such treatments as macrophage-transfer [10, 12] or macrophage-suppression [19] changed the susceptibility of mice to MHV, and that the susceptibility of mice to MHV in vivo was well correlated with the susceptibility of macrophages to MHV in vitro [2], but the susceptibility to MHV-2-CC was not clearly changed by either macrophage-transfer or macrophage-suppression in this series of experiments. On the other hand, close correlation between viral replication in both mice (in vivo) and mouse macrophage cultures (in vitro) and mouse age was found, and this suggests that macrophages, though probably not the sole factor, may be a little involved in the age-dependent development of resistance.

It is reported that T-lymphocytes [12], natural killer cells [20], interferons [21] and interleukins [5] could be involved in the protection of mice from MHV, and it is said that macrophages produce cytokines such as interferon [15], but the non-specific defence system may be critical in the early stage of infection because many mice died before 7 days p.i. in the present study. In conclusion, the results of the present study suggest that macrophages might play a small role in the age-related development of resistance to MHV-2-CC-infection in BALB/c mice.

References


