Murine Cytomegalovirus Infection Model in Balb/c Mice-1. Virological and Pathological Profiles in Mice Inoculated with Various Virus Doses

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By intraperitoneal injection of murine cytomegalovirus (MCMV) in Balb/c mice at doses of $2 \times 10^6$, $2 \times 10^5$ and $2 \times 10^4$ pfu per mouse, lethal, acute but non-lethal and asymptomatic infection were produced respectively. 100% mortality was found in mice infected with the lethal dose, as well as high MCMV titres in liver and spleen, severe necrosis of the spleen and severe degeneration of the liver. In these mice leukocyte counts in blood was not raised and inflammatory responses in the peritoneum, spleen and liver were not observed. Mice with acute but non-lethal infection showed less severe pathological changes, moderate spleen necrosis was followed by spleen enlargement. In this group of mice, severe mononucleosis was observed, and moderate inflammatory reactions were noted in the peritoneum, liver and spleen. Mice asymptptomatically infected exhibited slight mononucleosis in the blood and splenomegaly.

(Key Words: Murine cytomegalovirus infection, dose effect, mononucleosis, spleen necrosis, inflammatory responses)

INTRODUCTION

The murine cytomegalovirus (MCMV) model resembles the human CMV infection in many aspects. For example, congenital infection, viral immunosuppression and persistent infection have been demonstrated in both CMV infections. Therefore, MCMV has been considered a valid and practical model for studying various aspects of CMV infection including pathogenesis, interactions between the virus and immune system, and control of persistent infection (6). Recently, we have employed the murine model to investigate the mechanism by which MCMV enhances mortality during concurrent MCMV and bacterial infection (9). In the study, it was demonstrated that doses of MCMV administered to mice had a decisive effect on the phenomenon of enhanced mortality, which might be due to differing degrees of inflammatory reactions brought about by various doses. Therefore, the pathological state of the MCMV-infected host might have an important effect on whatever phenomenon we might be observing in the animal model. Most studies on MCMV infection have been conducted on a single dose of MCMV and detailed studies comparing different doses in a given strain of mice are lacking. The present study gives a general virological and pathological profile of mice infected with graded doses of MCMV, designed to produce lethal, acute and asymptomatic infections. The data may provide some perspectives in the design and interpretation of results in future studies using this model.

MATERIALS AND METHODS

Mice:
Four-week-old male Balb/c A Jcl mice were purchased from CLEA, Tokyo.

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Virus:
The preparation of the Smith strain of MCMV was as described previously (9). Briefly, MCMV was serially passaged in mice by injecting intraperitoneally (ip) into 4-week-old ICR mice. Fourteen days after injection, the salivary glands from the infected mice were made into 10% (wt/vol) homogenate which was subjected to freezing-and-thawing and then centrifugation. The salivary gland extract from the 10th serial passage titred $2 \times 10^8$ plaque-forming-units (pfu) of MCMV per ml, and was used as the virus stock. Salivary gland extract from MCMV-infected mice was used as the source of MCMV for two reasons: firstly, the virus is rapidly attenuated by passage in cell cultures, losing its virulence to mice by even one passage; and secondly, MCMV multiplies to constant high titres in salivary glands.

Virus assay:
MCMV was assayed on confluent monolayers of mouse embryo fibroblast (MEF) by a micro-plate plaque assay technique as described previously (9).

Experimental design:
Three groups of mice were injected with $2 \times 10^6$, $2 \times 10^5$, and $2 \times 10^4$ pfu of MCMV respectively, by the ip route. As controls, mice were injected with 10% homogenate of normal salivary gland. At given time intervals, 3 mice from each group were sacrificed. Heart blood was collected in heparinized tubes. The peritoneum was washed with 2 ml of Hanks' balanced salt solution (HBSS) with heparin and the washing was collected. Organs were removed aseptically. Half of the organ was preserved in 10% formalin for histological examination. The other half was washed in sterile HBSS three times to eliminate contamination by body fluids. A 10% homogenate was made, subjected to freezing-and-thawing 5 times to release the intracellular viruses. After centrifugation at 2000xg for 25 min, the supernatant was assayed for MCMV. Total white cell counts and differential white cell counts were performed on the blood and peritoneal washing by standard haematological techniques. Virus assay was done on these samples after lysis by freezing-and-thawing. Histological examination was carried out by the standard techniques of paraffin sectioning followed by staining with haematoxylin and eosin. Control mice were sampled at the same time and treated in the same manner.

Separate groups of mice, 10 per group, were injected with the three doses of MCMV and used for mortality rate and signs of infection, and measurement of body weight.

RESULTS

Gross observations
The mortality, changes in body weight and relative spleen size of mice injected with the three doses of MCMV are shown in Table 1. The dose of $2 \times 10^6$ pfu of MCMV produced 100% mortality. Mice exhibited ruffled fur, total inactivity and greatly reduced body weight. The dose of $2 \times 10^5$ pfu produced acute but non-lethal infection. Mice showed diminished activities and lower body weight than the control. However, all recovered towards the end of the observation period. The dose of $2 \times 10^4$ pfu produced asymptomatic infection.

Gross examination of the internal organs revealed that the spleen was most visibly affected. At the highest dose, blackened spleens of reduced size were observed. With the intermediate dose, spleen size was reduced at first, showing white patches on day 4. This was followed by spleen enlargement before returning to normal size on day 14. Mice infected with the lowest dose showed splenomegaly on day 6 to 8.

Other changes were observed in the lung. Mice dying from doses of $2 \times 10^6$ pfu had haemorrhage in the lung. Pleural effusion was seen in some mice infected with $2 \times 10^5$ pfu on day 8 and was also present in the group infected with $2 \times 10^4$ pfu although to a slight extent.

With the group of mice infected with $2 \times 10^5$ pfu, a white spot was observed in the heart from day 8 after infection. A detailed study of the involvement of the heart with MCMV is being published (10).

Virus titres in tissues
The virus titres in various tissues are shown in Fig. 1. At the peritoneum which was the site of inoculation, the high initial titre was rapidly reduced, followed by a peak rise at day 6. A low grade viremia was demonstrated with the lower doses of $2 \times 10^5$ and $2 \times 10^4$ but not with the high $2 \times 10^6$ pfu dose. This was in
Table 1 Gross observation of mice infected with various doses of MCMV.

<table>
<thead>
<tr>
<th>Inoculation dose (pfu)</th>
<th>Days after infection</th>
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<tr>
<td></td>
<td>2</td>
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<tr>
<td>Mortality (%)</td>
<td></td>
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<tr>
<td>$2 \times 10^6$</td>
<td>0</td>
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<tr>
<td>$2 \times 10^5$</td>
<td>0</td>
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<tr>
<td>$2 \times 10^4$</td>
<td>0</td>
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<tr>
<td>Average body weight</td>
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<tr>
<td>$2 \times 10^6$</td>
<td>16.0</td>
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<tr>
<td>$2 \times 10^5$</td>
<td>18.1</td>
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<tr>
<td>$2 \times 10^4$</td>
<td>19.3</td>
</tr>
<tr>
<td>control</td>
<td>19.5</td>
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<tr>
<td>Relative spleen size*</td>
<td></td>
</tr>
<tr>
<td>$2 \times 10^6$</td>
<td>↓</td>
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<tr>
<td>$2 \times 10^5$</td>
<td>→</td>
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<tr>
<td>$2 \times 10^4$</td>
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</tbody>
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*Relatively spleen size was graded by comparing with the spleen of the normal control. ↓, decreased; ↑, increased; →, no difference.

contrast to the other tissues where the virus titres correlate with the inoculation doses. During the early stage of infection, the spleen was the organ most severely infected, with the highest titres occurring on day 4. The peak titres in the kidney and lung of the groups infected with $2 \times 10^6$ and $2 \times 10^5$ pfu were similar, but the group with lethal infection had much higher titres in the liver and spleen. This indicates that the infected liver and spleen may be related to fatality in lethal infection by MCMV. All three groups of mice showed moderate high titres in the lung, and MCMV was still isolated from the lungs 14 days after infection. Titres in the salivary glands did not rise until day 6 and reached a peak around day 14 when infection in almost other organs had subsided. The same peak titre was reached regardless of the inoculation dose.

Histopathological findings:

The liver and spleen had the most marked histopathological changes. With the dose of $2 \times 10^6$ pfu, necrotic foci and cytopsial inclusion bodies were seen in the spleen as early as day 2. Most of the spleen was degenerated on day 4 (Fig. 2). The red pulp was entirely necrotised and contained many inclusion-bearing cells. Only small areas of the white pulp remained. On day 6, the whole spleen became almost acellular except for many inclusion bearing cells. No remarkable inflammatory reactions could be observed. In the liver, acidophilic bodies were seen in the parenchymal on day 2, signifying early hepatic cell degeneration. On day 4, inclusion bodies were present in many parenchymal cells and degeneration was severe (Fig. 3). On day 6, 60-70% of cells bore inclusion bodies and the architecture of the liver was completely destroyed. Necrotic foci were also present. Inflammatory changes in the liver were also minimal.

In mice infected with $2 \times 10^5$ pfu, necrosis could be seen in the spleen from day 2 to day 6, but to a much milder extent. On day 2, there was an infiltration of polymorphonuclear cells in the red pulp where small necrotic foci could be seen. In the white pulp, many tingible-body-macrophages were present. On day 4, much of the red pulp was necrotised and inclusion-bearing cells could be seen (Fig. 4). The white pulp was also reduced and contained histiocytic cells. The same picture was also observed on day 6. On day 8, a large number of cells resembling mature and immature plasma cells were present in the spleen. These cells gradually decreased in number on subsequent days. On day 14, the spleen regained its normal struc-
Fig. 1  Titres of MCMV in various tissues of mice injected intraperitoneally with various doses of MCMV: (○—○), 2 × 10^6 pfu per mouse; (△—△), 2 × 10^5 pfu; (□—□), 2 × 10^4 pfu. Each point represents the average titre of three mice ± standard deviation. *: no mice in this group survived beyond this day. ......lowest detectable level.
Fig. 2  Haematoxylin-eosin (HE) stained section of the spleen of mouse infected 4 days previously with $2 \times 10^6$ pfu of MCMV. Many inclusion-bearing cells (arrow) can be seen in the severely necrotised red pulp. The white pulp (WP) was almost all destroyed.

Fig. 3  HF stained section of the liver of mouse infected 4 days previously with $2 \times 10^6$ pfu of MCMV. The liver was losing its normal architecture. Many inclusion bodies were seen in the parenchymal cells (arrow).
Fig. 4  HE stained of the spleen of mouse infected 4 days previously with $2 \times 10^5$ pfu of MCMV. Some inclusion-bearing cells can be seen (arrow) in the necrotised red pulp and the white pulp was affected to a lesser extent (WP).

Fig. 5  HE stained section of the liver of mouse infected 4 days previously with $2 \times 10^5$ pfu of MCMV. Infiltration of inflammatory cells could be seen surrounding the infected cell.
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In the liver, small foci of leukocyte infiltration could be seen on day 2. On day 4, inclusion-bearing cells were seen frequently surrounded by inflammatory cells (Fig. 5). Kupffer cells seemed mildly activated. From day 8, regenerative processes had started. The liver had a normal structure on day 14.

With the dose of $2 \times 10^4$, there was minimal degenerative changes in the liver and spleen (data not shown). In the liver, an occasional focus of leukocyte infiltration could be seen on day 2 and 4. Otherwise, the liver retained the normal structure all through the experimental period. In the spleen, very slight necrotic changes was observed in the red pulp on day 4 but the white pulp was intact. On day 6, the spleen was filled with a large number of cells resembling plasma cells. The number of these cells decreased on day 8. By day 14, normal structure of the spleen was recovered.

There were no remarkable changes in other tissues examined, namely the kidney, pancreas, thymus and regional lymph nodes. In the lung of mice infected with the highest dose, haemorrhage was observed together with some inclusion bearing cells. In the lung and kidney of other mice, an occasional inclusion bearing cell was observed.

**White cell count:**

Mice infected with $2 \times 10^5$ pfu showed marked leukocytosis, and a large number of white blood cells were found in the peritoneum (Fig. 6). The peritoneal infiltrate consisted

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Fig. 6  Total white blood cell count in heart blood and peritoneal washing of three groups of mice infected with various doses of MCMV. ($\bullet$ $\rightarrow$), $2 \times 10^6$ pfu per mouse; (■ $\blacktriangle$), $2 \times 10^5$ pfu; (□ $\uparrow$ $\downarrow$) $2 \times 10^4$ pfu: (+), control mice. Each point represents the average count of three mice ± standard deviation *No mice in this group survived beyond this day.
of nearly 100% mononuclear cells, some were large and had atypical morphology. Although absolute neutrophil count in the blood was also increased (Fig. 7), the leukocytosis was mainly due to increase in mononuclear cells, many of which were large and atypical. Therefore severe mononucleosis was demonstrated in this group of mice.

Mice infected with $2 \times 10^4$ pfu showed slight leukocytosis in the blood and an early leukocyte infiltration in the peritoneum (Fig. 6). Some atypical mononuclear cells could be seen in the blood, and, as in the former group, the leukocytes in the peritoneum were almost all mononuclear cells.

The group infected with $2 \times 10^6$ pfu did not show any increase in leukocyte counts both in the blood and peritoneum. Over 50% of the cells in the blood appeared degenerate or atypical.

![Graph](image)

**Fig. 7** Absolute neutrophil count in heart blood of mice infected with various doses of MCMV: (○○○), $2 \times 10^6$ pfu per mouse: (▲▲▲), $2 \times 10^5$ pfu: (■■■), $2 \times 10^4$ pfu: (+), control mice.

**DISCUSSION**

Using inoculation doses of $2 \times 10^6$, $2 \times 10^5$, and $2 \times 10^4$ pfu of MCMV per mouse, it was possible to produce in Balb/c mice, lethal, acute but non-lethal, and asymptomatic infections, respectively. The lethally infected mice exhibited high virus titres and severe pathological changes in the spleen and liver. These suggest that the infected spleen and liver might be the main cause of fatality.

In the group of mice with acute but non-lethal infection, marked inflammatory responses were demonstrated in the peritoneum, liver and spleen, accompanying severe leukocytosis in the blood. The group of asymptotically infected mice also showed a slight inflammatory response. It was of interest to note that leukocytosis and inflammatory reactions were not demonstrated in mice infected with the lethal dose. One possible reason for this observation is that, at this dose, severe MCMV infection of the stem cells in the bone marrow and spleen occurred, resulting in an inability to produce leukocytes. Another possibility is that most leukocytes were rapidly infected with MCMV, became degenerate and were cleared in lymphoid organs. And, in the spleen and liver, the necrotic and degenerate processes were so rapid that inflammatory reactions could not readily be observed in histological sections. It had been shown in animal models and in humans that CMV in blood was associated with the leukocyte fraction, predominantly with the mononuclear cells (1, 2, 4, 5). In the present study, low grade viremia was demonstrated in mice infected with non-lethal doses of MCMV but not in the group infected with the lethal dose. This may be due to the small number of circulating leukocytes in the latter group, so
that MCMV was below detectable level.

Both spleen necrosis and enlargement had been reported during MCMV infection (7, 8, 11). These somewhat contrasting findings had been thought to be due to the difference in strains of mice used (11). In our present study it was shown that spleen necrosis occurred at the early stage of infection, corresponding to the period of virus growth in the spleen. The severity was proportional to the virus titre attained, and reached the highest level at the time of peak virus titre. Slight necrosis was also observed in the group asymptptomatically infected with MCMV during the early stage of infection though the spleen appeared normal by gross examination. Spleen enlargement corresponded with the decline of virus titre in the spleen. This was also associated with the appearance of large number of cells resembling mature and immature plasma cells. The exact nature of these cells is unknown but may play a role in the recovery from MCMV infection.

One interesting finding is that the lung was uniformly susceptible to MCMV infection regardless of the inoculation dose, judging from the moderate titres attained by all three groups. Yet, apart from pleural effusion in some mice, there was little other histopathological changes. Human CMV has been known to be a significant problem in bone marrow transplant patients, causing pneumonitis which has a high attendant mortality rate (3, 12). Typical pneumonitis changes were not demonstrated in our mouse model despite relatively high virus titres in the lung. One possible explanation is that the intraperitoneal route of infection is not suitable for the establishment of pulmonary infection. The intranasal route or droplet infection may be a better alternative. Secondly, bone marrow transplant patients have been invariably exposed to irradiation and immunosuppressive drugs. Therefore the condition of the host is different to our model which makes use of a normal host. The present model can be modified to study the effect of exposure to irradiation and immunosuppressive drugs on low dose MCMV infection.

In summary, we have presented some basic virological, haematological and histopathological data on infection of Balb/c mice by graded doses of MCMV, which may provide some useful background information for future studies.

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REFERENCES