Murine Cytomegalovirus Infection Model in Balb/c Mice-2. Pericarditis with Myocardial Involvement during Virus Infection

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Pericarditis with myocardial involvement was observed in mice infected with doses of murine cytomegalovirus (MCMV) designed to produce lethal, acute but non-lethal and asymptomatic infections. White spot was noted on the surface of the right ventricle from 4 to 6 days after MCMV infection. MCMV was isolated from the heart tissues as early as 2 days after infection. The virus titre reached a peak on day 6 and thereafter rapidly declined to undetectable level. Histopathological changes appeared on day 2 to 4 when necrosis of myocardial fibres occurred in the subpericardial region. This was followed by acute inflammatory cellular infiltration on day 6. After that organisation of fibrin took place and fibrotic lesion and cellular infiltration could still be observed after 18 days. The pathological changes could only be seen in the right ventricle. Peroxidase-labelled antibody technique was carried out to detect MCMV antigen in the cardiac tissue. MCMV antigen was found in the pericardium only at the early stage of infection.

(Key Words: murine cytomegalovirus infection, pericarditis, dose effect, MCMV antigen)

INTRODUCTION

In normal human subjects, cytomegalovirus (CMV) infection takes many forms of manifestations, from severe disseminated infection in congenitally or perinatally infected infants, to asymptomatic infection which is the most common form of CMV infection in adults. In the middle of the scale, an infectious mononucleosis syndrome, and a variety of illnesses such as hepatitis, splenomegaly, encephalitis and pneumonia were considered to be caused by CMV (3). Involvement of the heart during human CMV infection in previously healthy individuals, though comparatively rare, had been reported in a few cases (4, 12, 13, 14, 16). In these cases, pericarditis and myocarditis occurred with variable prognosis, ranging from complete recovery to death. However, the pathogenesis of CMV on the heart is not clear. There has not been any report on animal model developed for the study of CMV pericarditis and myocarditis. Wilson et al recently reported that murine cytomegalovirus (MCMV) was activated from hearts of latently infected mice but whether cardiac abnormality occurred during infection was not mentioned (15). In this study, we established the MCMV pericarditis model in Balb/c mice and examined the involvement of the heart in lethal, acute but non-lethal and asymptomatic MCMV infections.

MATERIALS AND METHODS

Virus:
The preparation of the Smith strain of MCMV was described previously (6, 7). Briefly, MCMV was serially passaged in ICR mice (CLEA, Tokyo). Salivary gland extract prepared from mice infected 2 weeks previously with MCMV was used for mouse inoculation. The salivary gland extract from the 11th serial passage contained $5 \times 10^{8}$ plaque-forming-units (pfu) per ml and was used as the virus stock for infection experiments (SGMCMV).

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Experimental design:

Three groups of 4-week-old male Balb/cA Jcl mice (CLEA, Tokyo) were infected intraperitoneally (ip) with an inoculum of 0.1 ml containing $2 \times 10^5$, $2 \times 10^5$ or $2 \times 10^4$ pfu of SGCMV. These three doses produced lethal, acute but non-lethal and asymptomatic infections respectively as reported previously (7). Control mice were injected with normal salivary gland extract. At stated time intervals, 3 mice from each group, including the control, were sacrificed. After examining for macroscopic change, the heart from each mouse was removed and washed gently in three changes of PBS to clear it of blood. A 10% homogenate was made, subjected to freezing-and-thawing 5 times. After centrifugation, the supernatant was assayed for MCMV on mouse embryo fibroblast (MEF) by a micro-plate plaque assay technique as described previously (6).

In a separate experiment, hearts from the MCMV-infected and control mice were preserved in 10% formalin. Paraffin sections were prepared. One set was stained by haematoxylin and eosin and another set was processed for peroxidase-labelled antibody staining for MCMV antigen.

As control experiments, the SGCMV was adjusted to $5 \times 10^7$ pfu/ml and inactivated by heating at 56°C for 30 min or by treatment with ether. The inactivated MCMV was checked for viability on MEF and then 0.1 ml was injected ip into mice. The inoculated mice were sampled and examined for MCMV and pathological changes in the heart as described above.

Preparation of antiserum to MCMV:

In order to ensure the purity of the MCMV used for the production of antiserum, the mouse-passaged SGCMV used for infection experiments was plaque-purified twice on cell culture of MEF. The cell-culture-MCMV thus obtained (CCMCMV) was used to immunize 7-week-old male ICR mice. An inoculum of $5 \times 10^7$ pfu was injected ip into mice. (Mice did not show any sign of infection, as the MCMV was attenuated by passage in cell culture). After 3 weeks, booster injection was given once weekly for two weeks. One week after the last injection, blood was collected and the sera were pooled. The pooled serum was absorbed by acetone-dried tissue powder prepared from normal ICR mice as follows: livers, spleens and hearts were pooled separately and made into homogenates in phosphate buffered saline (PBS). Ten volumes (vol/vol) of cold acetone was mixed with the homogenate and then centrifuged at 9000 rpm for 30 min at 4°C. The supernatant was discarded and the acetone treatment was repeated twice. The acetone-treated tissue was dried on 5 layers of filter paper overnight. The dried powder was collected. Twenty-five mg of liver, 12 mg each of spleen and heart powder were suspended in 1 ml of PBS. This was mixed with 1 ml of the antiserum and allowed to stand at room temperature for 2 hours with occasional shaking. After centrifugation the absorbed serum was diluted 1:5 before testing.

Peroxidase-labelled antibody staining:

Peroxidase-labelled antibody staining was carried out according to Nakane and Pierce (10). Paraffin sections were dewaxed and treated with hydrogen peroxide and normal sheep serum to eliminate non-specific staining. The treated sections were stained with the absorbed mouse anti-MCMV serum for 1 hour at room temperature. Sheep anti-mouse Ig, peroxidase-linked F(ab')2 fragment (Amersham International plc, England) was used as the second antibody. Coloration was developed with 20mg diaminobenzidine and 17μl hydrogen peroxide in 0.05M Tris buffer adjusted to pH 7.6. The anti-MCMV serum was titrated by staining paraffin sections of spleen and liver that were shown to contain cytomegalic inclusion bodies. A final dilution of 1:20 of the anti-serum was considered appropriate for use.

RESULTS

Macroscopic Involvement of the Heart

As described previously, mice infected with $2 \times 10^6$ pfu of MCMV resulted in lethal infections, with no mice surviving for more than 6 days (7). The heart was visibly involved, showing at first small white spots on the surface of the right ventricle, rapidly becoming relatively enlarged white patches near the time of death.

Mice acutely infected with $2 \times 10^5$ pfu showed signs of heart involvement on day 4 as a small whitish spot appeared on the right ventricle. From day 8 the white spots increased in size and number and were still present after 18
Fig. 1  Titres of MCMV and macroscopic pathological changes in hearts of mice infected with graded doses of MCMV. Each point of the MCMV titre (O—O) represents the average of 3 mice ± standard deviation. Macroscopic lesions (———) were graded as follows: 1 = a single small spot present; 2 = multiple small spots present; 3 = appearance of confluent white patch. *: no mice in this group survived beyond this day.
days. All mice examined after day 4 showed macroscopic pathological change.

Mice infected with $2 \times 10^4$ pfu showed barely visible whitish specks on the right ventricle on day 8. From day 8 to day 18, small white spots were observed only in 6 out of the 12 mice examined.

**Virological findings:**

MCMV was detected in mice infected with all three doses (Fig. 1). Relatively high titres of MCMV was detected in mice infected with $2 \times 10^6$ pfu of MCMV as early as 2 days post infection and continued to increase until the time of death.

With the dose of $2 \times 10^5$, the virus titres were much lower. MCMV titre reached a peak on day 6 and rapidly declined to an undetectable level on day 8. Macroscopic changes were not remarkable at the time of MCMV multiplication and became marked after the decline of virus titre.

Mice infected with the $2 \times 10^4$ pfu dose showed a low titre only on day 6.

**Histopathological changes:**

In mice infected with the lethal dose, mild necrosis was seen in the myocardial fibers in the subpericardial region. On day 4, the necrosis was intensified but inflammatory cell infiltra-
tion was not marked. On day 6, the dying mice showed extensive fibrosis in the pericardium and the underlying tissues.

Several stages of histopathological changes were seen in mice with acute but non-lethal infection (Figs. 2, 3, 4, 5). On day 4, a small focus of myocardial fibers in the subpericardial region appeared eosinophilic indicating mild necrosis (Fig. 2). On days 6 and 8, there was acute inflammatory cell infiltration including many polymorphonuclear cells in the pericardium and the underlying interstitium. From day 11, the cellular infiltration decreased and organization of fibrin could be seen. The organi-

Fig. 4

Fig. 5

Haematoxylin and eosin stained sections of the heart of mice infected at various times with $2 \times 10^5$ pfu of MCMV. On day 4 of infection (Fig. 2) eosinophilic stained myocardial fibres could be seen. On day 6 (Fig. 3), acute inflammatory cell infiltration including polymorphonuclear cells was observed. On day 11 (Fig. 4), cellular infiltration decreased and consisted of mainly mononuclear cells while fibrin organization was started. On day 18 (Fig. 5), fibrin organization was extensive and cellular infiltration could still be seen.
zation process continued in subsequent days and cellular infiltration was still visible 18 days after infection.

In all the sections examined, histopathological changes occurred only in the pericardial region of the right ventricle and was not present in the left ventricle (Fig. 6) or atria.

In the asymptotically infected mice, mild cellular infiltration was observed in the mice from day 6 to day 8 of infection followed by organization of fibrin which continued into day 18. The degree of involvement was slight, usually limited to a single focus. However, pathological changes were observed in 10 out of 12 mice examined from day 8 to day 18 after infection i.e. some mice which were negative on gross examination were found to have slight histopathological changes.

**Control experiments:**

Control mice injected with normal salivary gland extract did not show any macroscopic or histopathological changes in the heart all through the 18 day experimental period, also virus was not isolated from the tissues.

Heat or ether treatment of the SGMCMV resulted in complete inactivation of the virus as judged by titration on MEF. Mice injected with these suspensions remained healthy and the hearts were all negative for MCMV isolation and were normal on pathological examination.

**Demonstration of MCMV Antigen:**

Peroxidase-labelled antibody staining was performed on all the paraffin sections. Only occasional cells in the pericardium stained positively in the early stage of infection, from day 4 to day 6. However, the cells were not well defined. Figure 7A showed the staining pattern in the heart section of a mouse 4 days after infection with $2 \times 10^6$ pfu of MCMV. The ability of the absorbed mouse anti-MCMV serum to detect MCMV antigens in paraffin section was tested by staining sections of liver and spleen of MCMV-infected mice. Positive staining was demonstrated in cells present in the red pulp of the spleen and parenchyma of the liver (Fig. 7B and C).

**Fig. 6** Pericarditis lesions (arrow) were found only in the right ventricle.
DISCUSSION

It has been increasingly recognised that virus is a common causative agent of pericarditis and myocarditis in human. Coxsackie B virus is implicated in about 40% of cases in some studies while the etiology of the remaining cases were undecided (2, 5). Human CMV has been reported to cause pericarditis and myocarditis in previously healthy individuals although the number of studies was small (4, 12, 13, 14, 16). Due to the high prevalence of CMV infection in the general population, the actual incidence of myocarditis associated with CMV may be higher than has been reported. Development of an animal model of CMV myocarditis is es-
sential to the understanding of the pathogenesis and natural history of the disease.

We have reported the establishment of lethal, acute but non-lethal and asymptomatic MCMV infection models by infecting Balb/c mice with $2 \times 10^6$, $2 \times 10^5$ and $2 \times 10^4$ pfu of MCMV, respectively (7). In this study, we found that pericarditis coupled with myocarditis could be produced in Balb/c mice infected with the three doses of MCMV. In general, the MCMV titre and degree of pathological changes correlate with the dose used. Our preliminary observation showed that the degree of heart involvement probably depends on the number of passages in mice of the MCMV, less marked involvement being noted with MCMV of lower passages.

In non-lethal infections, MCMV could only be isolated from the heart tissue in the early stage of infection and the virus was no longer detectable when the gross and histological pathology became remarkable. This may be a general feature of viral myocarditis and may explain the finding of others that, in human myocarditis cases, it was not possible to isolate virus from myocardial tissues obtained from endomyocardial biopsy even when histological study confirmed the presence of myocarditis (2).

Histological studies revealed that the lesions caused by MCMV were limited to the pericardial region of the right ventricle. It was reported that pericarditis occurred spontaneously in aged DBA mice and these lesions were also limited to the right ventricle (1). In our experiment, young Balb/c mice were used, and control mice injected with normal salivary gland were sampled at the same time as infected mice. None of the control mice showed any pathological changes in the hearts, nor was MCMV isolated from them. However, the fact that both MCMV-induced and natural-occurring pericardial lesions in different strains of mice were limited to the right ventricle may suggest that this region might be by some reason more susceptible to pericardial abnormality.

The haematoxylin and eosin stained sections revealed the involvement of the pericardium and the underlying myocardium during MCMV infection. However, by the peroxidase-labelled antibody staining technique, MCMV antigen was detected exclusively in the pericardium and only at the early stage of infection. This may show the primary involvement of the pericardium in MCMV infection.

In order to maintain the virulence of the MCMV used for infection experiments, MCMV was serially passaged in mice and the salivary gland extract was used for mouse inoculation. In doing so, there is always a slight chance that the virus stock might become contaminated by any other virus carried in the mice although SPF mice were used and maintained in a SPF environment. To rule out this possibility, the MCMV stock was inactivated by heat or ether before it was injected into mice. MCMV is a heat sensitive virus possessing an envelope and hence is also ether sensitive. Viruses that have been shown to cause myocarditis in mice include encephalomyocarditis virus and Coxsackievirus (8, 9), both belonging to the picornavirus group which is ether resistant. Treatment by ether and heat of the MCMV virus stock completely abolished the infectivity of the virus in mice and cell culture. This insured that the virus stock was not contaminated. Furthermore, a purified strain of MCMV was used to raise anti-MCMV serum in mice. Using this serum MCMV antigen was detected in the pericardial lesion. This gives further proof that the pericarditis observed was due to MCMV and was not caused by any other agent.

A significant finding was that hearts from mice asymptotically infected with MCMV were positive for virus isolation and pathological examination. Especially, histopathological change was detected in most of the mice suggesting that the incidence of CMV infection of the heart was high in asymptomatic CMV infection. This finding coupled with the fact that latent infection is a common feature in CMV infection become more significant when cardiac transplantation in human is considered. The incidence of primary and secondary CMV infection in recipients after transplantation has been shown to be high (11). The heart from an apparently healthy donor may carry CMV in a latent state and could be a source of CMV infection in the recipient. Indeed, one recent study has shown that MCMV could be reactivated from cardiac explants of mice latently infected with MCMV (15). The pericarditis model described here may be used to further study the long term effect of MCMV infection of the heart and latency of CMV in cardiac tissues.
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