Concurrent Murine Cytomegalovirus and *Klebsiella pneumoniae* Infections in Germfree Mice

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The effects of concurrent murine cytomegalovirus (MCMV) and *Klebsiella pneumoniae* infections were studied in germfree (GF) mice. The mice received sublethal doses, $5 \times 10^5$ pfu, of MCMV. *K. pneumoniae* was injected in doses of 40 to 100 cfu, which by itself killed 0-33% of GF mice. When *K. pneumoniae* was given to GF mice infected with MCMV, the mortality increased up to 100%, with distinct enhancement persisting until day 10 of the MCMV infection. The virus titer in various organs did not change after superinfection with *K. pneumoniae*, while the viable counts of *K. pneumoniae* in organs remained remarkably high until death, suggesting the cause of death to be severe generalized infection by the bacteria. When compared to specific pathogen-free (SPF) mice, GF mice were more susceptible to both MCMV and *K. pneumoniae* infection, had higher titers of the virus for longer periods in various organs, and showed extension in the duration of enhanced mortality by the bacteria. Histopathologically, the spleen and liver were found to be the most severely affected tissues, more so in GF than in SPF mice, with recovery from the changes being slower in the GF animals.

(Key words: germfree mice, murine cytomegalovirus, *Klebsiella pneumoniae*)

INTRODUCTION

Cytomegalovirus (CMV) has attracted attention because of its high prevalence in organ transplants, malignancies, and immunodeficiency disorders, and for its frequent association with concurrent bacterial infections (3, 10, 14, 15, 18). The latter phenomenon is well known clinically, but there have been few experimental studies examining dual infections.

The murine CMV (MCMV) has acquired popularity recently as a model for the study of CMV infection (9). A synergistic effect on mortality in mice has been reported in combined infections with MCMV and *Pseudomonas aeruginosa, Staphylococcus aureus*, and *Candida albicans* (5, 6). Bale et al. (1) have described marked enhancement of mortality of MCMV infected mice after challenge with *Escherichia coli*. In addition, it was demonstrated, in specific pathogen-free (SPF) mice, that mortality was greatly increased when primary MCMV infections were followed by superinfection with *Klebsiella pneumoniae* (11).

The germfree (GF) animal is a good model for host-parasite studies (4), in which the lack of normal flora may make possible the clarification of fundamental questions. The aim of the present study was to examine the effect of concurrent MCMV and *K. pneumoniae* infections employing GF mice.

MATERIALS AND METHODS

Mice: Four-week-old ICR, GF and SPF female mice were purchased from CLEA Japan Inc., Tokyo, Japan. GF mice, bred and maintained in plastic isolator systems, were free of detectable microflora. SPF mice were maintained in standard animal room quarters. All food (mouse chow CL2, CLEA) and water were sterilized in an autoclave. Animal experiments were carried out according to the Guideline for
Animal Experimentation of the Tokai University School of Medicine.

Murine cytomegalovirus: The preparation of the Smith strain of MCMV was as described previously (11). Briefly, MCMV was serially passaged in ICR mice. Salivary gland extract was prepared from mice infected 2 weeks previously with MCMV. The extract from the 11th serial passage, containing $5 \times 10^8$ plaque-forming units (pfu) per ml, was used as the virus stock for the infection experiments. Sublethal doses of MCMV, determined in GF mice as $5 \times 10^5$ pfu/mouse, were injected intraperitoneally (ip).

Klebsiella pneumoniae: The strain of K. pneumoniae, 155(01:kl), was injected ip in doses of 40–100 colony forming units (cfu). The stock bacterial suspension was kept at $-80^\circ$C. For use, a small portion of the frozen suspension was placed on trypticase soy agar (BBL Microbiology Systems, Cockeysville, MD, USA), spread and incubated overnight. A single colony was transferred into trypticase soy broth (BBL), incubated overnight and used in appropriate dilution in phosphate buffered saline.

Virus titers and viable counts of bacteria in organs: Samples of peritoneal washings, blood, spleen, liver, kidney, and lung were collected from infected animals at various times during the course of infection. Usually three mice were used in each experimental group. MCMV was assayed on confluent monolayers of mouse embryo fibroblast by a microplate plaque assay technique (11, 16). Before virus assay, the homogenates were subjected to freezing and thawing five times and centrifugation at 2,000×g for 25 minutes. Viable counts of bacteria were determined by standard methods (11).

Histological examination: Standard techniques of paraffin embedding, sectioning, and staining with haematoxylin and eosin were used.

RESULTS

Mortality following challenge with K. pneumoniae 4 days after MCMV infection

Figure 1 shows the mortality of GF mice challenged with K. pneumoniae 4 days after

![Graph](image-url)

**Fig. 1** Cumulative mortality rates in GF and SPF mice superinfected with $1 \times 10^7$ cfu of K. pneumoniae (KP) 4 days after infection with $5 \times 10^7$ pfu of MCMV. Nine to ten mice were used in each group. • • •, MCMV alone; --, KP alone; ---, MCMV and KP combined.
MCMV infection. GF mice, infected only with $5 \times 10^5$ pfu of MCMV, showed 0% mortality, although the infected mice showed clinical signs of infection such as diminished activity and ruffled fur from about 3 days post infection. *K. pneumoniae* alone, when given ip at a dose of 100 cfu, usually resulted in 33% of the mice dying, usually on day 4. When the same dose of *K. pneumoniae* was given to GF mice infected previously with $5 \times 10^5$ pfu of MCMV, the mortality rate increased markedly.

SPF mice were less susceptible than GF mice to the MCMV and *K. pneumoniae* infections. **Mortality rate following challenge with *K. pneumoniae* at various time intervals after MCMV infection of GF mice**

The degree of increase in mortality clearly was related to the interval between the MCMV and *K. pneumoniae* infections (Figure 2). Significant increases were noted at intervals of 7 and 10 days, but was less pronounced when the interval was extended to 14 days.

**Virus titers in various organs after infection with MCMV**

The virus titers in various organs of mice infected with MCMV alone, or superinfected with *K. pneumoniae* on day 4, are shown in Figure 3. In the group of GF mice infected with both pathogens, all mice died after 2 days, making further sampling impossible. After a single infection with $5 \times 10^5$ pfu of MCMV, the virus titers in organs of GF mice were approximately 100-fold higher than in SPF mice, with the spleen being the most affected target organ. The virus titers in GF mice were maintained even after 10 days of MCMV infection. The virus titers in the organs of the groups superinfected with $4 \times 10^5$ cfu *K. pneumoniae* were not significantly higher than those of the groups infected with MCMV alone.

**Fig. 2** Mortality of GF mice superinfected with *K. pneumoniae* (KP) at various times after MCMV infection. Mice infected with $5 \times 10^5$ pfu of MCMV, then challenged with $4 \times 10^5$ cfu of KP. Six to eight mice were used in each group. - - - , MCMV alone; ---, KP alone; ______, MCMV and KP combined.
Fig. 3 Levels of MCMV in various organs of GF and SPF mice infected with MCMV alone or superinfected with *K. pneumoniae* (KP) on the 4th day. -- MCMV alone; •--•, MCMV and KP combined; --, lowest detectable level of MCMV. Each point represents the mean value from 3 to 5 mice.

**Viable counts of bacteria in various organs of MCMV-infected mice superinfected with *K. pneumoniae***

Figure 4 graphically depicts the viable counts of bacteria in the organs of the superinfected mice and shows that the counts of *K. pneumoniae* remained remarkably high until death at around 2 days post *K. pneumoniae* infection.

**Histopathological changes resulting from MCMV infection**

Table 1 summarizes the histopathological changes observed during the course of MCMV infection in the GF and SPF mice. In GF mice, the spleen and liver were the organs most severely affected during the acute phase of the virus infection.

In the spleen, the red pulp was necrotic and the white pulp much reduced at 4 days post infection. Numbers of characteristic, inclusion-bearing large mononuclear cells, were seen in the necrotic areas. Seven days after infections, the spleen showed further deterioration: the whole organ was nearly depleted of lymphocytes and many inclusion-bearing cells were still present.

In the liver, foci of cellular infiltration consisting of polymorphonuclear and mononuclear cells were seen 4 days after MCMV infection. Again, inclusion-bearing cells were present. Seven days after infection, the foci of infiltration in the liver had enlarged considerably.

In SPF mice, the pathological changes in these two organs had started to recover by the seventh day postinfection, whereas in GF mice, the severe lesions were maintained throughout the 14 days of observation.

**DISCUSSION**

Previous studies with SPF mice demonstrated that MCMV-infected mice exhibit a markedly enhanced susceptibility to infection by *K. pneumoniae* (11). The present study employing GF mice was conducted for comparison with the above phenomena observed in SPF mice.

We found that an acute infection with MCMV markedly enhanced the susceptibility of GF mice to ip challenge with *K. pneumoniae*. The degree of increase in mortality was dependent upon the interval between the MCMV and *K. pneumoniae* infections. With
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Fig. 4 Viable counts of *K. pneumoniae* (KP) in organs of control and MCMV-infected mice (interval of 4 days between MCMV and KP infections). ○ ○ ○, KP only; ● ● ●, MCMV and KP combined; − − −, lowest detectable level of KP. Each point represents the mean value from 3 to 5 mice.

<table>
<thead>
<tr>
<th>Days after MCMV infection</th>
<th>Spleen</th>
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<th>Lung</th>
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<td>14</td>
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Note: Mice were infected with $5 \times 10^5$ pfu of MCMV. − = normal; + = severe changes; ++ = more severe changes; +++ = most severe changes; NT = not tested.

SPF mice, the phenomenon of enhanced mortality was not observed when *K. pneumoniae* was given 10 days after MCMV infection (11). In GF mice, however, high mortality still resulted when the *K. pneumoniae* superinfection was administered 10 days after MCMV infection, although this was less marked when *K. pneumoniae* was introduced on day 14, corresponding to the prolonged histopathological changes and virus growth during the primary infection with MCMV. Recovery from the acute phase of MCMV infection clearly is delayed in GF mice, and likely underlies the phenomenon of continued enhanced mortality after superinfection by *K. pneumoniae*.

In GF mice challenged with *K. pneumoniae* 4 days after MCMV infection, the viable counts of bacteria in various organs remained remarkably high until death. However, the virus titers in these organs were similar to those in GF mice infected with MCMV alone. These results indicate that the increased mortality observed in combined MCMV and *K. pneumoniae* infections is due to severe generalized infection by the bacteria. This finding supports our hypothesis based on previous experiments using SPF mice (11).

Splenec necrosis in mice infected with MCMV has been touched upon briefly in previous reports (12, 13). In the present study, it was
shown that GF mice infected with MCMV developed marked pathological changes in the spleen and liver during the acute phase of infection, with the changes being maintained throughout the experimental period. On the contrary, in SPF mice, pathological changes in the spleen and liver showed progressive signs of recovery from the acute phase. These data confirm the association between the prolonged pathological changes and the prolonged clinical signs and virus growth in GF mice infected with MCMV.

Infection with MCMV has been shown to suppress both humoral and cell-mediated immune responses (2, 7, 8, 9). Additionally, it was demonstrated that immune responses in GF mice are more markedly depressed than in their SPF counterparts during MCMV infection and that recovery from the depression is much delayed (17). The relationship observed between depression of the immune response and peak virus titer in the organs, especially in the spleens, of infected GF and SPF mice raises the likelihood of MCMV-induced depression being a reflection of viral destruction or functional impairment of immunocompetent cells. In extension of these previous findings, the present study provides evidence that MCMV infection markedly enhances susceptibility to infection by K. pneumoniae in mice, probably through induction of multiple alterations in host defense mechanisms.

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REFERENCES


