Murine Cytomegalovirus Replication in the Lungs of Athymic BALB/c Nude Mice

John D. Shanley, Roger S. Thrall, and Stephen J. Forman

Murine cytomegalovirus (MCMV) infection in the lungs of T cell–deficient athymic BALB/c (Nu/Nu) mice and their immunocompetent heterozygous (Nu/+ ) littermates was examined. Following intranasal inoculation, MCMV replicated in the lungs of both Nu/Nu and Nu/+ mice, but virus titers were significantly higher in T cell–deficient mice. After subcutaneous inoculation, virus disseminated to lung tissue of athymic mice, leading to progressive MCMV replication in lungs that was not seen in the immunocompetent mice. Athymic mice failed to develop an antibody response to MCMV. Histologically, athymic mice uniformly developed focal interstitial cellular aggregates adjacent to blood vessels or airways, which progressively enlarged and coalesced. Pneumonitis was not seen in the lungs of any Nu/+ mice. Thus, MCMV can replicate in the lungs without pneumonitis in immunocompetent mice, but MCMV produces a progressive focal pneumonitis during deficiency of T cell–mediated immunity.

Human (H) cytomegalovirus (CMV) is an important pathogen in humans with immune abnormalities, especially in those with alterations of T cell immunity [1–3]. Although HCMV may affect many tissues, serious infections often involve the lungs, leading to respiratory compromise [4, 5]. In persons with advanced human immunodeficiency virus (HIV) infection, active HCMV replication is common in the lungs and other tissues [6, 7]; however, the role of HCMV as a source of pulmonary pathology in HIV infection is unclear. HCMV may be recovered from lung tissue in the absence of disease [7], and late in the course of HIV infection, HCMV has been associated with pneumonitis without other apparent microbial pathogens [7–9].

With a model in which to study the natural history of HCMV lung infections in the absence of T cell–mediated immunity, we examined the natural history of murine (M) CMV infection in the lungs of T cell–deficient athymic mice and in immunocompetent Nu/+ controls. The mice were infected subcutaneously (sc) and intranasally. The latter route of infection, which reliably produces early viral replication in lung tissue before dissemination of infection to other tissues, was used to study replication in lung tissues [10, 11]. By sc inoculation, the degree of viral replication in various tissues is related to virus dose [12]. Since the sc dose used induced minimal replication in the pulmonary tissue in immunocompetent mice, this route permitted examination of the role of deficiency in T cell immunity in dissemination of MCMV to the lungs.

Materials and Methods

Mice. For these experiments, 6- to 8-week-old female and male T cell–deficient, homozygous (Nu/Nu) BALB/c AnN nude mice and their immunocompetent heterozygous (Nu/+ ) littermates were obtained from Frederick Cancer Research Center (Frederick, MD). Pregnant CD-1 mice were purchased from Charles River Breeding Laboratories (Wilmington, MA). Animals were maintained in groups of 5 in isolator units in a laminar flow hood and given free access to food (HMR3000; Agway Prolab, Syracuse, NY) and water. The colony was monitored for infections due to MCMV and other pathogens. Sera from all heterozygous Nu/+ study animals were consistently negative for antibody to MCMV unless the animals had been experimentally infected.

Virus. MCMV Smith strain was originally obtained from M. C. Jordan (University of Minnesota, Minneapolis) as a 10% (wt/vol) homogenate of infected salivary gland tissue. The virus was passaged once in tissue culture using mouse embryo cells (MECs), and aliquots were prepared from clarified tissue culture supernatants [13]. The virus pool used for these experiments contained 2.0 × 10⁶/mL pfu. The inoculum for sham-infected controls was prepared from supernatants of uninfected MECs. Virus and control stocks were stored at −70°C. The virus stocks, screened by antibody generation assay (Microbiological Associates, Bethesda, MD), were free of other murine pathogens.

Cell culture methods. MECs were prepared from embryos of late-term pregnant CD-1 mice (Charles River Breeding Laboratories) by trypsin-EDTA desegregation and maintained in tissue culture using Eagle MEM (EMEM) containing 10% fetal calf serum (FCS), 100 U/mL penicillin, and 50 μg/mL gentamicin. Virus was quantified by plaque assay in MEC monolayers in 24-well tissue-culture clusters using an overlay of EMEM containing 20% FCS, 0.02 M HEPES (pH 7.5), 1% methylcellulose, and antibiotics [13].
Viral infection of mice. Intranasal infection was initiated by instilling 0.05 mL of stock virus (10⁶ pfu) into the nose under light anesthesia [9]. sc inoculation was done by administering 10³ pfu of virus in 0.2 mL of PBS [12]. Sham-infected control animals were inoculated in parallel using an equivalent volume of tissue-culture medium.

Serologic methods. Titers of serum antibody to MCMV were determined by ELISA as previously described using glycine-extracted MCMV and control antigens [14].

Evaluation of viral infection. At various intervals after virus inoculation, mice were killed and sera and tissues were obtained. To quantify virus, tissues were prepared as 10% (wt/vol) homogenates with EMEM containing 10% newborn calf serum and 10% DMSO and stored at −70°C until assay. We used a minimum of 4 mice in each study group.

Histologic evaluation. For qualitative histologic evaluation, tissues were fixed with 10% buffered formalin or Bouin’s fixative. Paraffin-embedded sections were then stained with hematoxylin-eosin and examined by light microscopy. For transmission electron microscopy, samples were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer. After being embedded in epoxy resin, sections were stained with osmium tetroxide and uranyl acetate.

Statistical analysis. Where appropriate, treatment groups were compared by Student’s t test or by χ² test.

Results

We compared the course of acute MCMV infection in athymic BALB/c Nu/Nu mice and immunocompetent Nu/+ controls after intranasal viral challenge (figure 1, table 1). MCMV replication was initially detected in the lungs of both Nu/+ and Nu/Nu mice on day 3. These mice had comparable virus titers throughout the study. In immunocompetent mice, viral replication was primarily confined to the lung and salivary glands. In contrast, virus was isolated from athymic mice in all organs tested, and the level of viral replication progressively increased during the study.

We examined antibody production to MCMV during the test period (table 1). In immunocompetent mice inoculated intranasally, serum antibody to MCMV was detected after day 7 and progressively increased. Athymic mice infected with MCMV failed to generate serum antibody to MCMV.

We also examined the virologic events of acute MCMV infection after sc viral challenge (figure 2, table 2). In the Nu/+ mice, viral replication was restricted to the salivary gland and was not detected in lung tissue. In contrast, in athymic mice, there was viral replication in multiple organs after day 7 and virus titers progressively increased over 18 days. Antibody production to MCMV was noted only in the Nu/+ mice and was detected after day 7. No serum antibody was produced by the athymic Nu/Nu mice.

Previous studies have suggested that intranasal and sc inoculation of MCMV rarely lead to lethal infection in immunocompetent mice [10-12]. We compared the long-term outcome of MCMV infection in Nu/Nu and Nu/+ mice after intranasal and sc challenge (figure 3). None of the MCMV-infected Nu/+ or sham-infected Nu/Nu mice died, regardless of challenge route. In contrast, all of the Nu/Nu mice infected with MCMV intranasally or sc died within 4 weeks of inoculation.

We also examined histologic changes in the lungs of athymic Nu/Nu mice and immunocompetent controls during acute MCMV infection after intranasal and sc challenge. The lungs of all immunocompetent mice remained histologically normal during infection by either route, despite impressive virus recovery after intranasal challenge. Histologic changes in the lungs of athymic mice infected by either route were similar (they were first noted 7 days after inoculation and were present in all mice examined by day 14). Initial changes were isolated foci of cellular aggregates in the lung interstitium. These foci contained large mononuclear cells that were occasionally associated with inclusion-bearing cells and were most often proximate to blood vessels or airways (figure 4). At days 14 and
Table 1. MCMV titers (log_{10} pfu/mL) in tissues of BALB/c athymic (Nu/Nu) and immunocompetent (Nu/+ ) mice at various times after intranasal inoculation with 10^5 pfu of MCMV.

<table>
<thead>
<tr>
<th>Days after inoculation</th>
<th>Salivary gland</th>
<th>Lung</th>
<th>Spleen</th>
<th>Liver</th>
<th>Antibody titer*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nu/+ mice</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>&lt;0.5</td>
<td>3.1 ± 0.01</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;10</td>
</tr>
<tr>
<td>7</td>
<td>3.3 ± 0.08</td>
<td>4.8 ± 0.08</td>
<td>&lt;0.5</td>
<td>1.1 ± 0.3</td>
<td>&gt;10</td>
</tr>
<tr>
<td>14</td>
<td>6.2 ± 0.1</td>
<td>4.9 ± 0.08</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&gt;10</td>
</tr>
<tr>
<td><strong>Nu/Nu mice</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>3</td>
<td>&lt;0.5</td>
<td>3.9 ± 0.08</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;10</td>
</tr>
<tr>
<td>7</td>
<td>4.7 ± 0.2</td>
<td>4.6 ± 0.02</td>
<td>2.2 ± 0.04(^1)</td>
<td>2.7 ± 0.08(^1)</td>
<td>&lt;10</td>
</tr>
<tr>
<td>14</td>
<td>7.4 ± 0.06(^1)</td>
<td>4.8 ± 0.06</td>
<td>4.3 ± 0.05</td>
<td>5.5 ± 0.06(^1)</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

* Log_{10} pfu/g in tissue homogenates pooled from ≥5 mice determined by plaque assay.
* Reciprocal of ELISA antibody titer.
\(^1\) P < .01 (Student’s t test) from titers of matched Nu/+ mice (same day).

21, as infection progressed, these foci expanded and often coalesced but continued to be surrounded by normal lung tissue (figure 5). Inclusion-bearing cells were occasionally found in the endothelium of small blood vessels. Late in the course of infection (day 21), some mice had accumulation of acidophilic fluid in the alveoli, although the airways were consistently normal throughout the course of infection. There was no difference in the wet weight of the lungs of MCMV-infected or uninfected athymic and immunocompetent mice infected by either route (data not shown).

The lungs of MCMV-infected athymic mice were examined by electron microscopy 14 days after inoculation. The focal areas of cellular aggregates contained nondescript interstitial mononuclear cells. On occasion, interstitial mononuclear cells containing viral nucleocapsids were found in the nucleus and cytoplasm. Other lung structures were mostly normal.

**Discussion**

In both humans and animals, cellular immunity plays a critical role in the course and outcome of CMV infections [1, 2, 15]. In situations of unrelenting viral replication, such as seen with AIDS, disease may result from direct viral damage to tissues [7]. In other situations, host cell immunity to CMV infection may play a role in the pathogenesis of disease [15]. The studies described here reiterate the importance of host cellular immunity in limiting the progression of viral replication and development of disease in the lungs due to direct viral effects in the absence of T cell–mediated immunity.

The administration of MCMV to T cell–deficient nude mice resulted in progressive viral replication with dissemination to multiple organs, including the lung [13, 16]. Infection damaged lung and other tissues, ultimately leading to death. In contrast,
**Table 2.** MCMV titers (log_{10} pfu/mL) in tissues of BALB/c athymic (Nu/Nu) and immunocompetent (Nu/+), mice at various times after subcutaneous inoculation with 10^3 pfu of MCMV.

<table>
<thead>
<tr>
<th>Days after inoculation</th>
<th>Salivary gland</th>
<th>Lung</th>
<th>Spleen</th>
<th>Liver</th>
<th>Antibody titer</th>
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<td></td>
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<tr>
<td><strong>Nu/+ mice</strong></td>
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<tr>
<td>3</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;10</td>
</tr>
<tr>
<td>7</td>
<td>1.5 ± 0.03</td>
<td>1.1 ± 0.03</td>
<td>&lt;0.5</td>
<td>1.0 ± 0.8</td>
<td>NA</td>
</tr>
<tr>
<td>14</td>
<td>5.8 ± 0.4</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>≥10</td>
</tr>
<tr>
<td>18</td>
<td>5.7 ± 0.03</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>≥10</td>
</tr>
<tr>
<td><strong>Nu/Nu mice</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>3</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;10</td>
</tr>
<tr>
<td>7</td>
<td>3.9 ± 0.3</td>
<td>1.6 ± 0.6</td>
<td>1.6 ± 0.08</td>
<td>&lt;0.5</td>
<td>NA</td>
</tr>
<tr>
<td>14</td>
<td>6.6 ± 0.6^1</td>
<td>3.7 ± 0.2^2</td>
<td>3.1 ± 0.4^2</td>
<td>&lt;0.5</td>
<td>&lt;10</td>
</tr>
<tr>
<td>18</td>
<td>7.0 ± 0.2^2</td>
<td>4.5 ± 0.01</td>
<td>4.3 ± 0.08^2</td>
<td>4.0 ± 0.3^2</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

**NOTE.** NA, not available.

* Log_{10} pfu/g in tissue homogenates pooled from ≥5 mice determined by plaque assay.

^1 Reciprocal of ELISA antibody titer.

^2 P < .01 (Student's t test) from titers of matched Nu/+ mice same day.

Inoculation of immunocompetent mice under these conditions produced limited nonlethal infections. After sc inoculation, viral replication was limited to the salivary gland. Intranasal inoculation produced viral replication in the lungs as well. No immunocompetent mice died.

In the absence of T cell immunity, the lung appears to be very susceptible to MCMV replication. After sc inoculation, virus disseminated to the lungs of athymic mice and resulted in progressive viral replication. In contrast, intranasal inoculation of MCMV produced comparable levels of viral replication in the lungs of both immunocompetent and athymic mice. None of the immunocompetent mice developed pneumonitis, indicating that viral replication can occur in the absence of histologic evidence of tissue damage. In contrast, viral infection in the lungs of athymic mice was uniformly associated with progressive focal pneumonitis.

Studies that have investigated the role of MCMV in producing interstitial pneumonitis have yielded confusing and conflicting results [17]. The majority have indicated that MCMV can replicate in the lungs of immunocompetent mice with minimal or no histologic evidence of disease. Induction of pneumonitis during MCMV infection generally requires significant alteration of host immunity by such means as antilymphocyte serum, cyclophosphamide, graft-versus-host disease (GVH), or irradiation [10, 11, 17–20]. With cyclophosphamide or GVH, MCMV infection induces a diffuse pattern of pneumonitis distinct from that seen in the present studies [10, 11, 19]. Pneumonitis in this setting is associated with an influx of T cells to the lungs.

![Figure 3](https://academic.oup.com/jid/article-abstract/175/2/309/816562/106521)

**Figure 3.** Survival of sham- and MCMV-infected athymic nude (Nu/Nu) mice and MCMV-infected immunocompetent BALB/c mice after (A) intranasal inoculation with 10^5 pfu or (B) subcutaneous inoculation with 10^3 pfu of MCMV.
the lungs and is not correlated with virus titers in pulmonary tissue [10, 21–23]. Inhibition of cellular immunity, but not inhibition of viral replication, prevents the development of pneumonitis, indicating the pneumonitis is mediated by immunopathologic mechanisms. In T cell–deficient mice, pneumonitis occurs during MCMV infection as a progressive focal nodular interstitial pattern distinct from the diffuse pneumonitis seen in other murine models of CMV lung infection. As infection progresses, the focal areas coalesce, suggesting damage is mediated by direct viral effects on lung tissue.

The observations in this animal model have direct relevance to HCMV disease in persons with AIDS. In patients with HIV-induced immunodeficiency and serologic evidence of prior HCMV infection, active virus shedding in the absence of tissue damage is common [3, 6]. As the immune deficiency progresses, HCMV replication may damage retinas, adrenal glands,
and gastrointestinal structures [3, 6, 7]. In this situation, the disease process may be arrested with antiviral agents such as ganciclovir or foscarnet, suggesting that damage is the result of direct viral replication [24, 25].

The role of CMV as an agent of pneumonitis in AIDS has been clouded because of its frequent association with other pathogens [7]. Evidence of interstitial pneumonia due solely to HCMV is relatively uncommon in HIV infection and generally occurs late in the course of AIDS [7–9]. As seen in this model, virus can be recovered from bronchoalveolar lavage and lung tissue of HIV patients in the absence of disease [7, 26]. Late in the course of disease, HCMV replication may progress sufficiently to induce pneumonitis, presumably by direct virus disruption of lung structure [8, 9, 27]. It is rarely diagnosed antemortem and is most frequently recognized at autopsy [9, 27]. Two histologic patterns of CMV pneumonia in AIDS have been described [9]: a focal interstitial pneumonitis characterized by focal mononuclear cell infiltration and diffuse alveolar damage characterized by interstitial edema and hyaline membranes [9, 28]. In the latter, HCMV is found throughout lung tissue. There is generally extensive disruption of pulmonary structures, and cellular infiltrates are primarily composed of scavenger leukocytes. These two patterns are similar to those seen in our animal model. Thus, this model of MCMV infection has relevance to the events occurring with CMV infection in severe immunodeficiency and may be useful in evaluating strategies for antiviral intervention.

Acknowledgments

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References


