Immunosuppression Reactivates and Disseminates Latent murine Cytomegalovirus

(Accepted 17 June 1977)

SUMMARY

When searched for by standard virological methods, murine cytomegalovirus (MCMV) becomes undetectable by 4 months after inoculation of mice. However, a 2-week regimen of immunosuppression with anti-lymphocyte serum and corticosteroid results in reactivation and dissemination of the virus in virtually all animals. This system should be useful in defining the pathogenesis of generalized cytomegalic disease resulting from reactivation of latent virus in immunocompromised individuals.

In this laboratory, a latent infection is defined as one in which the virus (or the virus genome) is harboured in the animal in a non-replicating state. In contrast, a chronic or persistent infection is one in which infectious virus can be recovered at any time from tissues by the application of standard methods.

As with other herpesviruses (Stevens, 1977), there is evidence that cytomegalovirus (CMV) is able to persist within the human host in a latent state for many years after initial infection (Weller, 1971; Lang, 1972; Lang et al. 1976). Under certain circumstances the virus may subsequently reactivate, sometimes producing disease. In this regard, disseminated CMV infection in patients whose defence mechanisms are impaired either by underlying disease or therapeutic measures is a major problem thought to result from reactivation of the latent virus (Lang et al. 1976). Specifically, recipients of organ transplants and patients with various malignancies, particularly leukaemias, often develop life-threatening CMV infections (Rifkind, Goodman & Hill, 1967; Meyers et al. 1975; Abdallah, Mark & Mergan, 1976). In addition, transfusion of blood is frequently followed by CMV infection in the recipient (Prince et al. 1971). Here, latent virus is either transferred within cellular blood components, possibly lymphocytes, or reactivated from recipient tissues as a result of immunological or other stimuli (Lang, 1972).

Experimental infections in mice with murine CMV (MCMV) have been studied as a model for human disease (Brodsky & Rowe, 1958; Mannini & Medearis, 1961; Henson, Smith & Gehrke, 1966; Selgrade & Osborn, 1974). In all aspects investigated thus far, the pathogenesis of virus infection in this model closely parallels current concepts of CMV infection in man. Thus the course of the acute infection has been characterized, and the subsequent development of chronic or persistent MCMV infection, in which large amounts of infectious virus continue to be produced in the salivary glands and kidneys, has been defined (Medearis, 1964). However, until recently (Olding, Jensen & Oldstone, 1975), little attention has been focused on the development of murine models in which virus infection persists in the animal in a latent state. In this report, we describe a MCMV infection in which virus no longer detectable in mouse tissues can be reactivated and disseminated following immunosuppressive measures.

The virus used was the Smith strain of MCMV, prepared as a 10% (w/v) homogenate of salivary gland tissue after passage in mice (Osborn & Walker, 1970). Plaque assays
were accomplished in secondary BALB/c mouse embryo cells (MEC) under a tragacanth overlay (Selgrade & Osborn, 1974). In experiments, the initial infection was established by subcutaneous inoculation of 4-week-old C3H/St mice (Strong Laboratories, La Jolla, California) with 1000 plaque-forming units (p.f.u.) of MCMV. This route of inoculation and dosage of virus was employed since several other methods in common use resulted in indefinite shedding of virus from salivary glands and kidneys. Here, the virus replicated to a peak titre of approx. $5 \times 10^4$ p.f.u./g of salivary gland tissue by 4 weeks after inoculation, and no virus was detected in the other tissues (kidney, lung, liver, spleen, thymus, pancreas) examined at 2-week intervals. In these assays, organs were homogenized as a 10% (w/v) suspension in a Ten-Broeck grinder. After centrifugation for 10 min at 200 g, 0.2 ml of undiluted homogenate was inoculated on to mouse embryo cell monolayers, allowed to adsorb for 30 min, overlaid with 5 ml of tissue culture medium, and observed for cytopathic effects over a 10-day period. Between 4 and 8 months after inoculation, salivary gland homogenates were negative for virus in 47 of 52 (90%) mice tested; in the remaining 5 mice (10%), however, MCMV was present at low titre ($10^2$ p.f.u./0.2 ml homogenate or less). Because of this, all mice underwent surgical salivary gland biopsy to detect those with persistent active infection before ‘reactivation’ experiments were initiated. Here, approximately half of the tissue in each lobe of the submandibular gland was resected from anaesthetized mice, and a 10% tissue homogenate was prepared for direct virus assay on MEC monolayers. Mice found to have active MCMV infection by this procedure were excluded from subsequent experiments. To verify further that mice with negative salivary gland biopsies were free of infectious MCMV, pertinent tissues (salivary glands, thymus, lung, liver, pancreas, kidney and spleen and bone marrow cell suspensions) from 10 animals were co-cultivated on allogeneic mouse embryo fibroblasts for 8 weeks. No virus was detected by this technique. Finally, salivary gland homogenates from 29 mice with previously negative biopsies were inoculated on to MEC both by the standard adsorption technique described above and by use of centrifugal force (1900 g for 30 min), which has been reported to increase the sensitivity of MCMV assay by 10- to 100-fold in two different laboratories. (Osborn & Walker, 1968; Hudson, Misra & Mosmann, 1976). No virus was detected by direct adsorption while 1 of 29 homogenates was positive by centrifugal inoculation, a statistically insignificant difference.

In an attempt to reactivate a possible latent MCMV infection in such virus-free mice immunosuppression with rabbit antiserum prepared against murine lymphocytes (ALS; Microbiological Associates) was initiated. This reagent was shown in our laboratory to be cytocidal in vitro for 97% of C3H/St splenic lymphocytes in a 2 h assay. Control groups included unmanipulated animals and mice given normal rabbit serum (NRS) by the same regimen (0.3 ml intraperitoneally twice weekly for 21 days). After 21 days the animals were killed and salivary gland homogenates assayed directly for infectious MCMV. As an initial screening procedure to detect systemic MCMV infection, spleens were also removed from the mice, chopped into 1 x 1 mm fragments, and maintained on MEC in tissue culture flasks for 4 weeks. The results of these experiments are presented in Table 1 (experiments 1 to 3). None of the unmanipulated mice yielded virus, and only 1 of 23 (4.4%) mice given NRS had MCMV in the salivary gland homogenate. However, 11 of 24 (45.8%) mice treated with ALS developed active MCMV infection of the salivary glands. As is also shown in Table 1, MCMV was recovered within 2 weeks from splenic tissue of 3 of 12 (25%) mice given ALS but from none of the unmanipulated or NRS-treated mice.

From these initial experiments it was concluded that the mice harboured MCMV in a state that could not be detected by direct virus assay of tissues, and that immunosuppression...
Table 1. Recovery of MCMV from salivary gland and spleen tissue of unmanipulated mice, mice given normal rabbit serum, mice given anti-lymphocyte serum, and mice given anti-lymphocyte serum and cortisone acetate*

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Unmanipulated</th>
<th>NRS†</th>
<th>ALS‡</th>
<th>ALS/cortisone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Salivary gland</td>
<td>Spleen tissue</td>
<td>Salivary gland</td>
<td>Spleen tissue</td>
</tr>
<tr>
<td>1</td>
<td>0/5</td>
<td>—</td>
<td>0/7</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>0/5</td>
<td>0/4</td>
<td>0/7</td>
<td>0/4</td>
</tr>
<tr>
<td>3</td>
<td>0/6</td>
<td>0/4</td>
<td>1/9</td>
<td>0/5</td>
</tr>
<tr>
<td>Total</td>
<td>0/16</td>
<td>0/8</td>
<td>1/23</td>
<td>0/9</td>
</tr>
<tr>
<td>4</td>
<td>0/4</td>
<td>0/4</td>
<td>1/5</td>
<td>1/5</td>
</tr>
<tr>
<td>5</td>
<td>2/5</td>
<td>0/5</td>
<td>2/5</td>
<td>0/5</td>
</tr>
<tr>
<td>6</td>
<td>0/5</td>
<td>0/5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Total</td>
<td>2/30</td>
<td>0/22</td>
<td>4/33</td>
<td>1/19</td>
</tr>
</tbody>
</table>

* Dosage regimens and assay procedures are given in the text. Results are expressed as number of mice positive for MCMV/number of mice tested. Selected viruses isolated from immunosuppressed mice were grown in murine 3T3 cell monolayers and shown to be MCMV by a specific indirect immunofluoresence test using murine anti-MCMV antibody and fluorescein-conjugated rabbit anti-mouse gamma globulin.
† NRS – normal rabbit serum.
‡ ALS – anti-lymphocyte serum.

with ALS resulted in ‘reactivation’ of the infection in nearly half of the animals. To determine whether a greater frequency of reactivation could be achieved with more potent immunosuppression, cortisone acetate (125 mg/kg/day i.p.) was added to the regimen. Because a significant number of deaths occurred among mice given the ALS/cortisone combination, the experiments were terminated after 16 days. As shown in Table 1 (experiments 4 to 6), the ALS/cortisone regimen caused reactivation of the MCMV infection in 23 of 24 (97 %) mice as evidenced by the development of active salivary gland infection. Immunosuppression with ALS alone was associated with active salivary gland infection in 6 of 9 (67 %) mice while 3 of 10 (30 %) mice given NRS developed active infection. Surprisingly, MCMV was recovered from the salivary glands of 2 of 5 (40 %) unmanipulated mice in experiment no. 5 (the only occasion on which this occurred among a total of 65 mice used in these and other experiments). This most probably represents spontaneous reactivation. Data presented in Table 1 also indicate that virus was recovered from spleen tissue in 19 of 25 (76 %) mice given the ALS/cortisone regimen but infrequently or not at all from mice given ALS, NRS or left unmanipulated.

To define the extent of the MCMV infection in mice given the ALS/cortisone regimen, homogenates of several organs were assayed for virus 16 days after treatment was started (Table 2). As can be seen, no virus was recovered from organ homogenates of unmanipulated control animals. Among mice given ALS and cortisone, however, MCMV infection was found to be widespread, involving the liver, lungs, kidneys, spleen, and salivary glands. In addition, recent experiments (Jordan et al. unpublished data) indicate that such immunosuppressed mice are viraemic.

These results show that mice harbouring undetectable MCMV develop disseminated infection following application of immunosuppressive measures. Whether the virus reactivates in a single organ system and then disseminates, or reappears simultaneously in multiple organs cannot be established from these experiments. Our inability to detect MCMV by either direct assay or co-cultivation of organs supports the conclusion that the virus is
Table 2. Isolation of MCMV from mouse organ homogenates of control and ALS/cortisone-treated animals*

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Salivary gland</th>
<th>Kidney</th>
<th>Lung</th>
<th>Liver</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>0/9</td>
<td>0/9</td>
<td>0/9</td>
<td>0/9</td>
<td>0/9</td>
</tr>
<tr>
<td>ALS/cortisone</td>
<td>10/10</td>
<td>8/9</td>
<td>8/8</td>
<td>9/9</td>
<td>9/10</td>
</tr>
</tbody>
</table>

* Results expressed as number of mice positive for virus/number tested.

maintained in a non-replicating and, therefore, latent state in these animals. Obviously, this interpretation is subject to the usual qualifications which accompany negative results dependent on sensitivity of assays, but it should be noted that more sensitive methods for detection of infectious virus are not now available.

Most previous investigations in the mouse have been concerned with the problem of a readily detectable persistent MCMV infection. Thus, Gardner et al. (1974) have shown that dissemination of MCMV occurs after treatment of chronically infected mice with anti-thymocyte sera, and Wu et al. (1975) demonstrated an increase in MCMV titres in the spleen and kidneys during skin graft rejection. It should be stressed again, however, that these studies show dissemination or augmentation of a pre-existing productive or replicating virus infection rather than activation of a latent one.

Finally, in an important series of experiments, Olding et al. (1975), showed that MCMV establishes latent infections in splenic B-lymphocytes following inoculation of mice either in utero or as newborns. In this system, latent MCMV can be reactivated by in vitro co-cultivation with allogeneic but not syngeneic fibroblasts. A more recent report by the same investigators indicates that 75% of these mice do not have detectable MCMV in other organs suggesting that the virus is in fact latent in the animal (Olding, Kingsbury & Oldstone, 1976). The MCMV strain used in these experiments was attenuated by continuous passage in tissue culture, resulting in diminished virus replication in the liver and spleen during the acute infection (Osborn & Walker, 1970). We have also been able to recover latent MCMV from murine splenic lymphocytes using virus supplied by Olding but not with the virulent strain used in the experiments reported here (Jordan et al., unpublished data). This suggests that the virulent virus strain either does not establish latent infections in splenic lymphocytes or is more difficult to reactivate in vitro. Further studies are in progress to determine the site and mechanism by which virulent MCMV is maintained in latently infected animals.

Technical assistance provided by J. Balay is acknowledged. This work was supported by PHS grant A1-06246 and American Cancer Society, California Division, Grant No. 842.

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(Received 27 April 1977)