In Vivo Reactivation of Latent Murine Cytomegalovirus in the Eye by Immunosuppressive Treatment

Theodore Rabinovitch, Jang O. Oh, and Petros Minasi

Intravitreal inoculation of $10^3$ pfu of murine cytomegalovirus (MCMV) to 3-week-old BALB/c mice resulted in virus isolation from eye homogenates for 2 weeks and from co-cultured specimens of the same eye up to 5 weeks after inoculation, indicating that MCMV in the eye became latent 2 weeks after the virus inoculation. Immunosuppressive treatment with daily intramuscular injections of cyclosporine (40 μg/g) and cortisone acetate (125 μg/g) 9 weeks after intravitreal MCMV inoculation resulted in isolation of infectious virus from ten of 44 eye homogenates (10 of 22 mice) during a 3-week period, indicating in vivo reactivation of latent ocular MCMV. No virus was isolated from eye homogenates of similarly infected mice with sham immunosuppression (daily intramuscular saline injections), nor was any virus isolated from uninfected eyes with immunosuppressive treatment. Three weeks of daily cyclosporine and cortisone injections depleted L3T4+ cells to 6.0%, Lyt-2+ cells to 20% and anti-MCMV antibody to 10% of untreated mice. The results suggest that the eye can serve as a site of latent MCMV that can be reactivated by immunosuppressive means. Invest Ophthalmol Vis Sci 31:657-663, 1990

Cytomegalovirus (CMV) is an ubiquitous agent causing one of the most common human infections. CMV occurs in nearly 4% of live-born human infants, and by the age of 40 years the majority of adults exhibit serologic evidence of CMV infections. Most infections are subclinical. The virus remains in the body in a latent state, as borne out by several studies of humans infected with CMV and mice infected with murine CMV (MCMV). Disseminated infection due to this agent may occur throughout the life of an individual, primarily in the setting of abnormal host immunity, such as leukemia patients or organ graft recipients on immunosuppressive agents. Although infection in some of these patients may be due to newly acquired infection, it is generally thought that the disease occurs by reactivation of latent virus. The vital role of T cell defects in this presumed reactivation has been highlighted by the frequent association of CMV infection in patients with the acquired immunodeficiency syndrome (AIDS).

The eye is one of the susceptible organs to CMV infections. Ocular involvements of CMV such as uveitis and retinocchoroiditis have been reported in the literature as a manifestation of systemic CMV infection. Furthermore, recent studies indicate that CMV retinopathy is the most common ocular infection in AIDS patients. Whether these ocular infections are due to reactivation of latent virus within the eye or are from dissemination from peripheral organs to the eye is unknown. Although Hayashi et al and Bale et al reported the existence of latent MCMV in murine ocular tissues, there is still no convincing evidence that indicates the presence of latent CMV in the human eye. It is also unknown whether defects in the host's immunity can lead to the reactivation of latent virus in the eye. Our results indicate that the eye is a site of latency of MCMV and that latent virus in the eye can indeed be reactivated in vivo by immunosuppression.

Materials and Methods

Animals

Three-week-old BALB/c male mice obtained from a local vendor were used throughout the experiments. They were free from MCMV infection as shown by negative antibody against the virus. The investigation

From the Francis I. Proctor Foundation, University of California, San Francisco, California.

Supported in part by NIH research grants EY-01578 and EY-08037 to Jang O. Oh, by NIH core grant EY-02162, and by the E. A. Baker Foundation of Canada.

Submitted for publication: April 17, 1989; accepted October 11, 1989.

Reprint requests: Jang O. Oh, MD, PhD, Francis I. Proctor Foundation, Box 0412, University of California, San Francisco, CA 94143.
using animals, as described in this manuscript, conforms to the ARVO Resolution on the Use of Animals in Research.

**Virus**

Smith strain of salivary gland-passed MCMV (SG-MCMV) was obtained from the American Type Culture Collection and maintained in BALB/c mice. A working stock of MCMV was prepared by passing the SG-MCMV once in primary BALB/c mouse embryo fibroblast cells. The supernatant of the culture medium was mixed with 10% DMSO and stored at -70°C until use. The titer of the working stock of MCMV was 10^5 plaque-forming units/ml.

**Intraocular Inoculation**

Under general anesthesia with intramuscular injection of Ketamine HCl (10 µg/g) and Xylazine (5 µg/g), 0.01 ml of either virus suspension containing 10^3 pfu of MCMV or saline was injected intravitreally to both eyes through a 30-gauge hypodermic needle with the help of a dissecting microscope. Care was taken to avoid the lens during the intravitreal injection.

**Cells for Virus Isolation**

Two-week-old BALB/c mouse embryo were used to obtain primary cultures of embryo fibroblast cells. Monolayer cultures of secondary embryo fibroblast cells grown in wells of 24-well plastic cell culture plates (Costar, Cambridge, MA) were used for virus isolation from specimens. The growth medium for the cells consisted of Eagle's minimum essential medium (MEM) containing 5% heat-inactivated fetal calf serum and antibiotics. The cells in the plate were incubated at 37°C in a humid atmosphere of 5% CO₂ and 95% air.

**Virus Isolation from Blood and Tissues**

After incising the subclavian artery through a superficial skin incision, blood was obtained in a syringe and mixed with heparin (10 units/ml of blood). The blood was then separated into mononuclear cells and plasma by Ficoll-Hypaque gradientation. Mononuclear cells (10⁶ cells) or 0.1 ml of plasma were inoculated onto each of the duplicate wells of mouse embryo fibroblast cells. Ten percent tissue homogenate of each organ was prepared in MEM by grinding with mortar and pestle, centrifuging at 400 g for 10 min, and 1.0 ml of the supernatant was then inoculated into each of the duplicate wells of mouse embryo cells in a 24-well culture plate. Co-culture of tissue was carried out by placing a 1-2 mm³ fragment of tissue in each of two wells containing monolayer culture of mouse embryo fibroblast cells. The culture plates were then incubated at 37°C in a humid atmosphere of 5% CO₂ and 95% air, and the cell culture medium was completely replaced with fresh medium twice a week during the experimental period. The plates were examined for cytopathic effect (CPE) twice a week for 8 weeks, and any virus isolate from culture with positive CPE was confirmed as MCMV by immunofluorescence assay with fluorescein-conjugated specific anti-MCMV serum.

**Analysis of T Lymphocyte Subsets**

Mononuclear cells were fractionated from heparinized blood by Ficoll-Hypaque gradientation and were treated with both phycoerythrin-conjugated L3T4 monoclonal antibody and fluorescein-conjugated Lyt-2 monoclonal antibody (Becton Dickonson Monoclonal Center, Inc., Mountain View, CA). A minimum of 10⁶ cells were analyzed for L3T4⁺ (helper T cells) and Lyt-2⁺ cells (suppressor T cells) by the use of fluorescence-activated cell sorter, and the percent of each subset of cells compared to the total number of mononuclear cells in the mouse was obtained.

**Antibody Titration**

Serial two-fold dilutions of mouse plasma beginning at 1:5 were tested for antibody against MCMV by the standard indirect immunofluorescence technique with fluorescein-conjugated rabbit anti-mouse globulin (Beckton-Dickinson Monoclonal Center) using MCMV-infected mouse embryo fibroblast cells on coverslips as targets. The titer of antibody was the reciprocal of the highest dilution of plasma which showed positive fluorescence reaction.

**Results**

**Latency of MCMV in the Eye Following Primary Eye Infection**

Mice received bilateral intravitreal injections of MCMV (10³ pfu/eye). Histopathologic examinations in our preliminary studies showed that these mice developed only a mild chorioretinitis that lasted for 10 days. At weekly intervals, two to three mice were sacrificed, and organs including the eyes were collected for MCMV isolation from homogenate cultures and co-cultures of each organ. Both eyes were cut into two equal portions, and one portion of each eye was used for the homogenate culture and the other for the co-culture. As shown in Figure 1, infectious virus could be recovered from three of four eye homogenates in week 1, from one of four in week 2,
No. 4 REACTIVATION OF LATENT MCMV / Rabinovitch er ol 659
EYE
Trigeminal
Ganglion
Salivary
Gland
Blood
Mononuclear
Cells
Blood Plasma

- not
- done
- not
done

Fig. 1. Virus isolation from homogenates and co-cultures of various organs of mice after bilateral intravitreal inoculation of MCMV (10^3 PFU/eye).

but from none thereafter. In co-cultures, virus could be recovered from two of four eyes on week 4 and one of the four eyes on week 5, but no virus was detected thereafter, suggesting that virus in the eye becomes latent after week 4. No virus was recovered from trigeminal ganglia homogenates, but one of four co-cultures at week 2 and week 4 was positive for MCMV. Salivary gland samples were most consistently positive for virus isolation, with almost 100% of both homogenates and co-cultures positive up to week 5, and with 25% of co-cultures on week 6. Other than isolation of virus from mononuclear cells in one of four mice on week 6, no virus was recovered from any blood specimen at any other time.

Immunosuppressive Effect of Cyclosporine (CsA) and Cortisone

Daily injections of CsA (Sandoz Ltd., Basel, Switzerland) (40 μg/g IM), cortisone acetate (Merck, Sharp & Dohme, West Point, PA) (125 μg/g IP), or both were administered to each of three groups of ten mice that had been infected intravitreally with MCMV 9 weeks previously. Another group of uninfected mice served as a control and received daily saline injections instead. At weekly intervals, two to four mice from each group were sacrificed, and heparinized blood obtained for titration of antibody against MCMV, and for assaying L_3T_4^+ cells and Lyt-2^+ cells by flow cytometry. The percent of each cell subset in treated mice compared to untreated mice was calculated. The percent of each subset in the blood of untreated mice was 28.3 ± 5.7 for L_3T_4^+ cells and 4.2 ± 0.7 for Lyt-2^+ cells.

As shown in Figure 2, the combined injections of cortisone and CsA had a more rapid and sustained depleting effect on L_3T_4^+ cells than either agent alone. The amount of L_3T_4^+ cells remaining after 1 week of combined injections was only 21.9% of that in untreated mice. This dropped further to 11.5% by 2 weeks and to only 6.0% after 3 weeks of injections. Three weeks of cortisone injections alone significantly reduced the cells (5.6%), but cortisone alone was less effective than the combined injections at 1 and 2 weeks (65.2% and 27.2%, respectively). The depletion of the cells using CsA alone was not only ineffective in the first 2 weeks (92.5% at 1 week, 43.8% at 2 weeks) but the percent of L_3T_4^+ cells rose to 62.8% by 3 weeks.

Fig. 2. Effects of cyclosporine (CsA) and/or cortisone on L_3T_4^+ cells and Lyt-2^+ cells in the blood. Daily injections of CsA (40 μg/g IM), cortisone acetate (125 μg/g IP), or both were administered to three groups of ten mice that had been injected intravitreally to both eyes with MCMV (10^3 PFU/eye) 9 weeks earlier. Another group of un.injected mice served as a control and received daily saline injections intraperitoneally. At weekly intervals, two to four mice from each group were sacrificed, and blood from each mouse was used for assaying L_3T_4^+ cells and Lyt-2^+ cells by flow cytometry. The percent of L_3T_4^+ cells or Lyt-2^+ cells compared to that in the control (saline-injected) group was presented in this figure. Each vertical bar denotes one standard deviation.
Combined injections of CsA and cortisone also markedly reduced Lyt-2+ cells to 18.5% compared to untreated mice in the first week (Fig. 2), but no further significant reduction occurred by week 3. The ratio Lyt-2+ cells/Lyt-2- cells was 2.1:1.0 on week 3 following combined injections of CsA and cortisone, while it was 6.7:1.0 in untreated mice. Cortisone alone had little effect on Lyt-2+ cells until week 2 (50.0%) but by week 3 reduced the count to 11.4%. CsA alone never reduced Lyt-2+ cells lower than 42.8%.

Combined injections of CsA and cortisone also reduced antibody against MCMV in plasma; 1 week after the treatment antibody was reduced by 66.7% compared to untreated mice and at 3 weeks by more than 90% (Table 1).

Because of their most effective suppression on LYT-2+ and Lyt-2+ cells as well as antibody in the blood, the combined injections of CsA and cortisone were chosen as the immunosuppressive means to study in vivo reactivation of latent MCMV in the eye of immunosuppressed mice.

In Vivo Reactivation of Latent MCMV in Eyes by Cyclosporine and Cortisone

Two groups of mice received bilateral intravitreal injections of MCMV (10^3 pfu/eye). Mice in the third group served as a control and received bilateral intravitreal injections of saline instead of the virus. MCMV in the eye became latent 4 weeks after the intravitreal inoculation of the virus (Fig. 1). Therefore, 9 weeks later, well after latency of the virus was established in eyes of mice in virus-injected groups, mice in one of the virus-injected groups and mice in the saline-injected group were subjected to an immunosuppressive treatment with daily injections of CsA (40 μg/g IM) and cortisone (125 μg/g IP), while mice in the remaining virus-injected group received sham immunosuppressive treatment with daily saline injections. At three weekly intervals, two to three mice from each group were sacrificed, and eyes and other organs were obtained for MCMV isolation from tissue homogenates. This experiment was repeated three times and the results are shown in Table 2.

In the virus-injected mice with immunosuppression, ocular homogenates were positive for virus isolation in ten of 44 eyes in ten of 22 mice. Most of the virus isolates (9/10) were obtained during the first 2 weeks of immunosuppression. Virus was also recovered from four of 22 salivary gland homogenates. This low rate of virus isolation from the gland was unexpected and the cause is unknown at present. Virus isolation was positive in two of 22 mononuclear cell cultures from immunosuppressed mice but homogenates of trigeminal ganglia and blood plasma remained negative. In the virus-injected mice with sham immunosuppression (saline injection), no virus could be isolated from homogenates of any tissue except mononuclear cells; mononuclear cells from one of 18 previously infected mice yielded virus without immunosuppressive treatment. The saline-injected mice with immunosuppression failed to yield virus from homogenates of any organs. All the virus isolates were confirmed as MCMV by indirect immunofluorescence assay with specific anti-MCMV serum.

**Discussion**

The establishment of latent MCMV in ocular tissues was first reported by Hayashi et al. They inoculated ICR/Sic mice intracameraly with Smith strain MCMV, and detected virus in homogenates of eyes as early as 2 days and as late as 21 days post-inoculation. Then, using BALB/c mice, they recovered virus from co-cultivated eye specimens at 5, 6 and 7 months post-inoculation. From the results, they concluded that the eyes were latently infected with the virus. Later, Bale et al also observed that ocular homogenates were positive on days 3, 5 and 7 after intraperitoneal injection of MCMV to Swiss Webster mice. In another series of experiments in different mice, they found that explants of eyes were positive on days 60, 90 and 120. These results suggested to them that the eye harbored latent MCMV. Since MCMV is known to produce chronic persistent infections in mice, and since both co-cultures and homogenate cultures were not carried out with the same eye concurrently in these studies, it is uncertain whether the viruses recovered from the co-cultured

<table>
<thead>
<tr>
<th>Duration of daily injections</th>
<th>Antibody titer in plasma</th>
<th>Reduction of antibody titer in CsA and cortisone group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CsA and cortisone†</td>
<td>Saline§</td>
</tr>
<tr>
<td>------------------------------</td>
<td>--------------------------</td>
<td>------------------------------------------------------</td>
</tr>
<tr>
<td>1 week</td>
<td>42.7 ± 10.5</td>
<td>128.0 ± 5.7</td>
</tr>
<tr>
<td>2 weeks</td>
<td>48.0 ± 7.3</td>
<td>192.0 ± 12.0</td>
</tr>
<tr>
<td>3 weeks</td>
<td>16.0 ± 1.8</td>
<td>170.7 ± 9.6</td>
</tr>
</tbody>
</table>

* Mean titer ± 1 standard deviation.
† ((100 - (Titer of CsA + cortisone group / Titer of saline group) × 100) %
‡ Daily injections of CsA (40 μg/g IM) and cortisone acetate (125 μg/g IP) for 1, 2 and 3 weeks.
§ Daily injections of saline for 1, 2 and 3 weeks.
Table 2. Isolation of MCMV from homogenates of eyes with latent MCMV and from other organs following immunosuppressive treatment

<table>
<thead>
<tr>
<th>Organ</th>
<th>With immunosuppression†</th>
<th>Without immunosuppression‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 week</td>
<td>2 weeks</td>
</tr>
<tr>
<td>Exp 1</td>
<td>3/6 (3/3)</td>
<td>0/4 (0/2)</td>
</tr>
<tr>
<td>Eye Exp 2</td>
<td>2/6 (2/3)</td>
<td>2/6 (2/3)</td>
</tr>
<tr>
<td>Exp 3</td>
<td>1/6 (1/3)</td>
<td>1/6 (1/3)</td>
</tr>
<tr>
<td>Total</td>
<td>6/18 (6/9)</td>
<td>3/16 (3/8)</td>
</tr>
<tr>
<td>Grand total</td>
<td>10/44§ (10/22)</td>
<td>ND</td>
</tr>
<tr>
<td>Salivary gland</td>
<td>1/9</td>
<td>2/8</td>
</tr>
<tr>
<td>Total</td>
<td>4/22</td>
<td>ND</td>
</tr>
<tr>
<td>Trigeminal ganglia</td>
<td>0/9</td>
<td>0/7</td>
</tr>
<tr>
<td>Total</td>
<td>0/19</td>
<td>ND</td>
</tr>
<tr>
<td>Blood MNC1</td>
<td>1/9</td>
<td>0/8</td>
</tr>
<tr>
<td>Total</td>
<td>2/22</td>
<td>ND</td>
</tr>
<tr>
<td>Blood plasma</td>
<td>0/9</td>
<td>0/8</td>
</tr>
<tr>
<td>Total</td>
<td>0/22</td>
<td>ND</td>
</tr>
</tbody>
</table>

* No. of eyes or organs with positive MCMV isolation/No. of specimens tested. Numbers in parentheses indicate number of mice with positive MCMV isolation from eyes/No. of mice tested. The isolation rates from organs other than the eye represent the sum of the rates in three separate experiments.
† Daily injections of cyclosporine (40 μg/g IM) and cortisone acetate (125 μg/g IP) for 1, 2 and 3 weeks.
‡ Daily injections of saline for 1, 2 and 3 weeks.
§ P < 0.001.
P < 0.001.
MNC = Mononuclear cell.

eyes actually represented reactivated latent viruses or merely viruses from chronic persistent infection of the eyes. In our experiments, therefore, co-cultures of the eyes were performed simultaneously with cultures of homogenates of the same eyes. Infectious virus could be recovered from eye homogenates up to post-infection week 2 but from none of the homogenates thereafter. The results suggest that no persistent virus infection was present in the eye following intraocular injection of MCMV. Furthermore, in all cases when the co-cultured eye specimens became positive for MCMV post-infection week 4 and thereafter, the homogenates of the same eyes were negative for the virus. Therefore, the virus isolates from the co-cultures after post-infection week 4 were indeed from in vitro reactivation of latent virus in the eye rather than from persistently infected eye tissue with MCMV. The results clearly indicate that the mouse, the eye is a site of MCMV latency. However, the recovery rate from the co-cultured eye was low; at best, 50% of co-cultured eyes revealed virus. This may in part be due to the fact that the co-cultured method is a rather insensitive method of detecting latent virus. In some cases, co-cultures of latently infected tissues only become virus-positive after a long period of time, and in still other instances, viruses can never be activated by this method despite additional evidence indicating that latent virus is present.22

In the past, the murine model of MCMV infection has been widely used to study in vivo reactivation of latent MCMV. Cytotoxic agents such as cyclophosphamide,6,8,23 or antithymocyte serum with or without corticosteroids6 have been used to reactivate latent MCMV from various organs. Immunologic stimulation by whole blood24 or spleen cell transfer7 also has led to reactivation of latent virus. However, as far as we know, no such in vivo reactivation of latent CMV in the eye has been reported previously. In our attempts to reactivate latent MCMV in the eye, we recovered virus from eye homogenates in ten of 22 animals following the immunosuppressive treatment with CsA and cortisone, while no virus was recovered from animals receiving saline (P < 0.001). The virus recovered from the eye homogenates could not be of extraocular origin, such as from the trigeminal ganglia, since no virus could be recovered from homogenates of this tissue. Similarly, it was unlikely that the salivary gland was disseminating the virus to the eye because the recovery rate of the virus from homogenates of the gland (18.2%) was far lower than...
that from the eye (45.5%). Furthermore, the possibility of hematogenous spread of the virus to the eye from other organs, such as salivary glands, is remote because the virus was infrequently present in the blood (9.0%) at any time. Therefore, the immunosuppression by daily injections of CsA and cortisone must have induced reactivation of latent MCMV within the eye in vivo. Our present study is the first to our knowledge to report such in vivo reactivation of latent MCMV in the eye.

The mechanisms by which the immunosuppressive treatment reactivates latent MCMV are poorly understood at present. The activity of MCMV has been known to be affected by the host’s cellular immune system. MCMV-infected mice whose T cells are reduced by antilymphocyte injections, gamma irradiation or frankly absent in congenitally athymic conditions suffer from disseminated, often lethal infection. Conversely, reconstitution of T cell function by intravenous injection of immune spleen cells to athymic mice can impede the progress of MCMV infection. As to the specific T cell subset that predominates in the control of MCMV, Shanley et al. found that adoptive transfer of splenic T lymphocytes with L3T4 markers prevented virus multiplication in the adrenal glands of athymic nude mice. On the other hand, Reddounced and his associates concluded that control of virus multiplication in lungs of mice immunodepleted by total-body gamma irradiation was a function of the Lyt-2 subset of T lymphocytes. These seemingly complex immunologic mechanisms for the MCMV infection are further complicated by the fact that MCMV itself can cause alterations in T lymphocytes, with reductions of L1T4 and Lyt-2 cells, and the inversion of the Td/Tt ratio as seen in AIDS.

The combined immunosuppressive treatment with CsA and cortisone in our experiment resulted in a marked reduction of blood L1T4 cells, Lyt-2 cells as well as humoral antibody with the change of Td/Tt ratio. These reductions were accompanied by in vivo reactivation of MCMV in the eye. It is unclear whether T cells and/or antibody also help to keep the virus in a latent state. Recently, the transfer of Lyt-2 memory T lymphocytes from latently infected donors to infected immunodeficient recipients was shown to exert a specific antiviral effect. Presumably, even while the virus is latent, these T cells function to hold the virus in check. Extrapolating these data to the results of our experiments suggests the possibility that the reduction in cytotoxic suppressor T lymphocytes and/or helper T lymphocytes remove the natural control mechanisms that may keep the virus in its latent state, and may allow it to become reactivated. Further studies are in progress to evaluate the roles these lymphocytes and antibody play in the regulatory mechanisms of latent MCMV infection in the eye.

Key words: murine cytomegalovirus, reactivation of latent virus, immunosuppression, helper T lymphocyte, suppressor T lymphocyte

References


