Murine Cytomegalovirus Ocular Infection in Immunocompetent and Cyclophosphamide-Treated Mice

Potentiation of Ocular Infection by Cyclophosphamide

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Conventional virologic and in situ nucleic acid hybridization methods were used to study immunocompetent and immunosuppressed 3-week old BALB/c mice inoculated intravitreally with 10⁴ plaque-forming units (pfu) of murine cytomegalovirus (MCMV). Immunocompetent mice experienced a self-limited ocular infection with peak virus titers of 10⁴⁵ pfu/ml in the retina-choroid fraction on day 4 of infection. Using biotinylated MCMV DNA probes, MCMV DNA was detected in cells of the iris, ciliary body, and rarely, the retina or choroid on days 4 and 7 of infection. With few exceptions, retinal architecture was preserved. By contrast, mice immunosuppressed with cyclophosphamide (200 mg/kg on day 0 and 100 mg/kg on days 5 and 11 after MCMV inoculation) had progressive ocular infection that culminated in a necrotizing retinitis. Virus titers in the retina-choroid fraction rose progressively (nearly 10⁸ pfu/ml in cyclophosphamide-treated mice on day 11 versus 10⁷ pfu/ml in immunocompetent mice). The MCMV DNA was detected in the iris and ciliary body of the immunosuppressed mice on days 4 and 7 and in the retina, on days 7, 11, and 14. On day 14 abundant MCMV DNA was found in most retinal layers, and extensive retinal necrosis was observed. These studies indicate that immunosuppression with cyclophosphamide potentiates MCMV ocular disease in mice, a finding analogous to CMV retinitis in immunosuppressed humans. Invest Ophthalmol Vis Sci 32:1749-1756, 1991

Human cytomegalovirus (HCMV) infections in immunocompromised patients frequently produce severe life- or sight-threatening illness.¹-⁷ Patients who are immunosuppressed by chemotherapy, for transplantation, or by human immunodeficiency virus type 1 (HIV-1) infection are at increased risk for severe pneumonitis, gastroenteritis, encephalitis, and retinitis. As many as 50% of the seronegative patients who receive renal, liver, cardiac, or marrow transplants develop primary HCMV infections, and most HCMV-seropositive patients show clinical or virologic evidence of HCMV reactivation or reinfection.³ In patients with acquired immune deficiency syndrome (AIDS), HCMV has emerged as the most frequent opportunistic viral infection and the most common cause of blindness.⁸-¹² Until recently these devastating complications of HCMV infection could not be treated effectively with antiviral drugs.¹³,¹⁴ Investigations regarding the pathogenesis and therapy of HCMV retinitis have been severely hindered by the absence of a suitable animal model. Although studies of murine cytomegalovirus (MCMV), including prior studies from this laboratory, indicate that MCMV produces anterior chamber disease when the virus is introduced intraocularly, the retina is usually spared in immunocompetent mice.¹⁵-¹⁷ Recently, Holland and colleagues¹⁸ observed histologic evidence of retinitis in MCMV-infected mice immunosuppressed concurrently with cyclophosphamide. In this report, we used virologic and molecular methods to characterize MCMV infection in immunocompetent mice and cyclophosphamide-treated mice. We observed that immunosuppressed mice had progressive retinal infection, documented by isolation of

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large quantities of infectious MCMV from ocular tissues and by detection of abundant amounts of MCMV DNA in retinal layers.

**Materials and Methods**

**Virus**

The MCMV, obtained originally from Dr. Earl R. Kern (University of Alabama, Birmingham, AL), was maintained in our laboratory by serial passage in 3-week-old female Swiss outbred mice. Virus stocks, prepared as 10% w/v homogenates of MCMV-infected salivary glands in Eagle’s minimum essential medium (MEM) with 10% fetal calf serum (FCS), regularly contained $5 \times 10^7$ plaque-forming units (pfu) of MCMV when titrated on monolayers of mouse embryo fibroblast (MEF) cells.

**Animals**

BALB/c mice, 24–27 days old at the time of inoculation, were purchased from Harlan-Sprague-Dawley (Indianapolis, IN). Control and MCMV-infected mice were housed separately in the University of Iowa Animal Care Facility and provided food and water ad libitum. Animal experiments conformed to the guidelines of the ARVO Resolution on the Use of Animals in Research.

**Virus Assay**

Virus pools and tissue homogenates were assayed for infectious virus using confluent monolayers of MEF cells grown in 24-well plates and an agarose overlay as described previously. The lower limit of detection for our method was 5 pfu/ml. Culture results were expressed as the log pfu of MCMV per ml of tissue homogenate or per ml of leukocyte suspension.

**In Situ Nucleic Acid Hybridization Studies**

Tissues from control and infected animals were studied for MCMV nucleic acids using modifications of procedures described previously. The probes were prepared from the MCMV DNA fragment Hind III A cloned into pACYC177 or fragment pAMB25 cloned into pACYC184 (both courtesy of Dr. Ulrich Koszinowski, Institute for Microbiology, Ulm, Federal Republic of Germany). The MCMV DNA fragments were cleaved from the plasmid, gel purified, and labeled with biotinylated deoxyuridine triphosphate (Enzo, New York, NY) using nick translation methods.

Before hybridization, tissue sections were deparaffinized with xylene and treated with 0.02 N HCl, 0.005 mg/ml proteinase K for 10–15 min, and RNases A (100 µg/ml), and T1 (10 units/ml). Tissue sections were then postfixed with 4% paraformaldehyde and treated with 3% hydrogen peroxide in methanol to inactivate endogenous peroxidases. The tissues were reacted with 5 µl of a hybridization mixture containing 5 ng of biotinylated MCMV DNA probe/µl, 50% formamide, 10% dextran sulfate, double concentrated SSC (0.3 M sodium chloride and 0.03 M sodium citrate), and 250 µg/ml salmon sperm DNA denatured for 3 min at 92°C, and hybridized for 16 hr at room temperature (20–27°C).

The slides were developed using the Detek I-hrp kit (Enzo), with diaminobenzidine as the chromogen, and lightly counterstained with Harris hematoxylin. They were coded and examined for MCMV DNA, indicated by the presence of a brown precipitate. Controls in each run included: (1) MCMV-infected MEF cells, (2) uninfected MEF cells, and (3) ocular tissues from mice that received control inocula.

**Protocol for Acute MCMV Infection**

The mice were anesthetized with ether or methoxyflurane, and using a dissecting microscope to magnify the eye, 3 × 10⁴ pfu of MCMV in 5 µl of MEM was introduced into the right eye behind the lens. Control mice received an equivalent dilution of a normal salivary gland homogenate prepared from uninfected Swiss-Webster mice. This homogenate was culture-negative for MCMV.

On days 1, 4, 7, 11, 14, and 21 after inoculation, the mice were killed in groups of three to five control or MCMV-infected mice each by ether or methoxyflurane inhalation, and specimens of eye, spleen, salivary gland, and whole blood were collected for virus assay or in situ hybridization studies. Spleens and salivary glands from individual animals were removed, prepared separately as 10% w/v homogenates in MEM containing 10% FCS and antibiotics (penicillin 50 units/ml, streptomycin 50 µg/ml) and frozen at −70°C until assayed. Leukocytes from individual animals were collected by centrifugation of whole blood on Ficoll-Hypaque gradients (Sigma Diagnostics, St. Louis, MO) using procedures described previously. The right eyes were removed and separated by microdissection into ocular components: cornea, lens, retina–choroid, vitreous, and optic nerve. Individual ocular components from two or three mice were pooled, homogenized in 1 ml of MEM, and stored at −70°C until assayed.

The eyes from an additional five or six mice/day (one or two control and four MCMV-infected mice) were removed, fixed in fresh periodate-lysine-paraformaldehyde for 24 hr, washed briefly in phosphate-buffered saline, and stored in 70% ethanol at 4°C un-
til embedded with paraffin. Paraffin-embedded eyes were then sectioned at 5 μm. Ocular sections were collected onto microscope slides treated with aminoolkylsilane and stored at 4°C for nucleic acid hybridization studies. Adjacent sections from each eye were stained with hematoxylin and eosin.

Protocol for Cyclophosphamide-Induced Immunosuppression of MCMV-Infected Mice

Mice (Group 1) were infected intravitreally with MCMV as described and treated with cyclophosphamide (Elkins-Sinn, Cherry Hill, NJ) intraperitoneally at a dose of 200 mg/kg on day 0 and 100 mg/kg on days 5 and 11. This regimen induced 60% to 100% mortality in MCMV-infected mice by day 14. In one experiment cyclophosphamide was given on day 4 instead of day 5. Controls consisted of: (1) MCMV-infected mice that did not receive cyclophosphamide (Group 2) and (2) cyclophosphamide-treated mice that received normal salivary gland intravitreally instead of MCMV (Group 3). Tissues from the three groups of mice (typically three or four mice from Group 1, three mice from Group 2, and two mice from Group 3 per day per experiment) were collected on days 1, 4, 7, 11, and 14 and processed for virus assay and in situ nucleic acid hybridization studies as described.

Results

Virus Assays of Ocular Tissues From Immunocompetent Mice

The results of virus assay for days 1–14 after intravitreal MCMV inoculation of immunocompetent BALB/c mice are summarized in Figure 1 (broken lines). On day 1 small quantities (approximately 10^1 pfu of MCMV/ml of homogenate) were detected in several ocular fractions, including the cornea and retina–choroid. Subsequently, the amounts of MCMV detected in various ocular components rose, indicating replication of MCMV in ocular tissues. On day 4, all assayed ocular fractions contained between 10^1 and 10^3 pfu of MCMV/ml. Greatest quantities, approximately 10^3 pfu/ml, were present in the retina–choroid fraction. By day 7, the amounts of MCMV in the ocular components began to decline, and by day 14, MCMV was detected only in the optic nerve fraction. By day 28, all ocular components from immunocompetent mice were culture negative for MCMV.

We also detected MCMV in spleens and salivary glands harvested concurrently, a feature that undoubtedly reflected hematogenous dissemination of MCMV after intravitreal inoculation. Titers of MCMV were highest on day 4 in the spleen (Fig. 2) and on day 14 in the salivary gland (approximately 10^9 pfu/ml).

In Situ Nucleic Acid Hybridization Studies of Tissues From Immunocompetent Mice

Using in situ hybridization methods, we detected MCMV DNA in ocular tissues harvested on days 4, 7, and 14 (Table 1). Eighty-three percent (10 of 12) of

<table>
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<th>Day</th>
<th>Immunocompetent [no. positive (%)]</th>
<th>CP-treated [no. positive (%)]</th>
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<tbody>
<tr>
<td>4</td>
<td>10/12 (83)</td>
<td>4/4 (100)</td>
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<tr>
<td>7</td>
<td>8/14 (57)</td>
<td>7/7 (100)</td>
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<tr>
<td>11</td>
<td>0/6 (0)</td>
<td>4/5 (80)</td>
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<tr>
<td>14</td>
<td>1/6 (17)</td>
<td>4/4 (100)</td>
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CP = cyclophosphamide.

* Number of animals with MCMV DNA-positive ocular tissues versus number of animals studied.

Fig. 1. Summary of MCMV titers in various ocular fractions harvested during MCMV infection of immunocompetent mice (broken lines) and during MCMV infection of mice treated with cyclophosphamide (CP) (solid lines). The lower limit of detection was 5 pfu of MCMV/ml.

Fig. 2. Titers of MCMV in spleen in immunocompetent mice (broken lines) and mice treated with cyclophosphamide (CP) (solid lines). The lower limit of detection was 5 pfu of MCMV/ml. Differences were significant at P < 0.00001 for days 11 and 14.
the mice (one eye studied/mouse) harvested on day 4 were positive for MCMV DNA, and 57% (8 of 14) were positive on day 7. By contrast, only one of the eyes harvested on days 11 or 14 (n = 12) contained MCMV DNA. These results corroborate the virologic studies, which indicated a brief, self-limited ocular MCMV infection.

On days 4 and 7, MCMV DNA was detected in the ciliary body and iris, and on day 4, we also observed MCMV DNA in occasional cells of the retina and choroid in a small number of animals (two of eight, 25%). The eyes of immunocompetent mice contained numerous inflammatory cells in and adjacent to the ciliary body and iris. Although we detected MCMV DNA in occasional retinal cells, the retinal architecture was normal in nearly all immunocompetent animals.

Viral Assays of Tissues From Cyclophosphamide-Treated Mice

In contrast to the transient ocular infection experienced by immunocompetent MCMV-infected mice, cyclophosphamide-treated mice had progressive ocular infections. Beginning on day 7 of MCMV infection, nearly all ocular fractions from immunosuppressed mice contained quantities of MCMV that greatly exceeded those of immunocompetent animals (Fig. 1). For example, the titer of MCMV in the retina–choroid fraction of immunosuppressed mice were nearly $10^3$ pfu/ml of homogenate on days 7 versus approximately $10^2$ pfu/ml in immunocompetent mice. On days 11 and 14, titers of MCMV remained high in the ocular tissues of cyclophosphamide-treated mice, whereas MCMV declined to low or undetectable levels in immunocompetent mice.

Cyclophosphamide-treated, MCMV-infected mice also had sustained, higher titers of MCMV in their spleens, and MCMV was detected in their circulating leukocytes. As summarized in Figures 2 and 3, the results for spleen and leukocyte cultures paralleled those of ocular tissues, providing further evidence for cyclophosphamide enhancement of MCMV infection. By contrast, salivary glands titers were approximately the same in immunosuppressed and immunocompetent animals.

In Situ Hybridization Studies of Tissues From Cyclophosphamide-Treated Mice

The MCMV DNA hybridization studies confirmed that cyclophosphamide-treated MCMV-infected mice had a progressive ocular infection that culminated in an extensive necrotizing retinitis. On day 4 of infection, all eyes (n = 4) from cyclophosphamide-treated mice contained MCMV DNA in cells of the ciliary body and/or iris, and appeared similar to immunocompetent, MCMV-infected mice.

By day 7, however, quantitative and qualitative differences were observed. All of the eyes from cyclophosphamide-treated, MCMV-infected mice (n = 7) contained MCMV DNA versus 57% (8 of 14) of the immunocompetent mice (Table 1), and MCMV DNA-containing cells were more abundant in cyclophosphamide-treated mice, a feature illustrated in Figure 4. In addition, the anterior chambers of immunosuppressed mice contained fewer inflammatory cells than did comparable tissues from immunocompetent animals. Retinal involvement was also first evident on day 7 when MCMV DNA was detected in cells of the outer nuclear layer of an occasional cyclophosphamide-treated mouse (Fig. 4C).

By days 11 and 14, progressive ocular infections were observed in the inoculated eyes of cyclophosphamide-treated mice. Ocular tissues from nearly all immunosuppressed mice contained MCMV DNA on these days, whereas tissues from all but one immunocompetent mouse were negative (Table 1). The MCMV DNA was present in abundant quantities on days 11 and 14 and involved all layers of the retina and also the ciliary body, iris, and choroid (Figs. 5, 6).

We also studied the contralateral (uninoculated) eyes harvested on days 7 (n = 8), 11 (n = 10), and 14 (n = 3) from cyclophosphamide-treated mice. One mouse had MCMV DNA in the ciliary body and iris, but none had MCMV DNA in the retina or choroid. In addition, the retinal architecture was normal in all of the contralateral eyes.

Pooled serum from a group of mice (n = 10) inoculated with the MCMV pool were screened for antibodies to several murine viral pathogens (assay done by Whittaker M. A. Bioproducts, Walkersville, MD). This serum contained antibodies to MCMV and min-
ute virus of mice (MVM). To exclude MVM as a potential confounding variable, we did several additional experiments. We prepared a biotinylated MVM probe (MVM DNA courtesy of Dr. John Bodnar, Northeastern University, Boston, MA) and probed (1) MEF cells infected with the MCMV pool and (2) ocular tissues from an MCMV DNA-positive mouse. All were negative for MVM DNA. We also inoculated a group of immunocompetent and cyclophosphamide-treated mice with a different MCMV pool (Courtesy of Dr. John Shanley, University of Connecticut, Newington, CT) that did not induce specific MVM antibodies and studied tissues harvested on days 7 (n = 8) and 10 (n = 9). We observed abundant MCMV DNA in the ciliary body, iris, or retina of cyclophosphamide-treated animals, a finding that further confirms ocular infection with MCMV.

Histopathologic Observations

Hematoxylin and eosin-stained sections were reviewed from ocular tissues harvested on days 11 and 14, the peak period of infection in immunosuppressed mice. Cyclophosphamide-treated MCMV-infected mice had extensive pathologic abnormalities in their inoculated eyes (Figs. 5B, 6A–B). The iris, ciliary body, and cornea were necrotic, and large eosinophilic cells were present in the iris and ciliary body. The retinas of cyclophosphamide-treated, MCMV-infected mice displayed focal or diffuse areas of necrosis, often hemorrhagic, most evident in animals harvested on day 14 (Figs. 6A–B). Retinal necrosis was evident in all nine of the cyclophosphamide-treated animals studied on days 11 and 14. In general, extensive retinal necrosis correlated with the presence of abundant MCMV DNA.

Immunocompetent, MCMV-infected mice had inflammation of the ciliary body and iris on both days 11 and 14. On day 11, two animals had areas of retinal necrosis (Fig. 5A), but corresponding tissues were negative for MCMV DNA. On day 14, one animal had focal retinal atrophy; the retinas appeared normal in the other five animals. Tissues from mice that received cyclophosphamide and normal salivary gland homogenates (n = 8) had normal retinal histology.

Discussion

We investigated the pathogenesis of intravitreal MCMV infection in immunocompetent mice and in
mice treated concurrently with the immunosuppressive drug, cyclophosphamide. Immunocompetent mice had transient, self-limited ocular infections characterized by replication of MCMV in the ciliary body and iris. Although we occasionally detected MCMV DNA in retinal cells, MCMV was apparently cleared from the retina and other ocular components with minimal or no disruption of the retinal architecture. These results thus paralleled those seen previously in immunocompetent mice inoculated with MCMV via the anterior chamber.17

By contrast, cyclophosphamide-treated, MCMV-infected mice had progressive ocular infections that culminated in extensive necrotizing retinitis. On days 7, 11, and 14 of infection, titers of MCMV in ocular tissues of immunosuppressed mice greatly exceeded the viral titers in immunocompetent animals. Moreover, we detected abundant MCMV DNA in most retinal layers and observed marked disruption of retinal architecture in most immunosuppressed, MCMV-infected mice. Because such changes rarely occurred in mice receiving MCMV alone or in mice treated only with cyclophosphamide, we conclude that concurrent immunosuppression with cyclophosphamide potentiates ocular MCMV infection and facilitates MCMV retinitis.

Our in situ hybridization studies provide additional insights regarding the pathogenesis of MCMV ocular infections. Our results confirm that cells of the ciliary body, iris, and cornea are permissive sites for MCMV in immunocompetent mice. By contrast, retinal cells were usually spared. The in situ hybridization studies also indicate that retinal infection in immunosuppressed mice did not result from direct contact of retinal cells with the intravitreal virus inoculum. Rather, MCMV first replicated in the ciliary body and iris and then progressed to involve the choroid. With regard to the retina, MCMV DNA was detected first in cells of the outer nuclear layer. This suggests that MCMV reached the retina from the choroid, possibly through contiguous spread of virus through the retinal pigment epithelium, which also contained MCMV DNA.

When the contralateral, uninoculated eyes from cyclophosphamide-treated mice from days 7, 11, and 14 were studied by in situ hybridization, we detected MCMV DNA in the ciliary body of one of 21 mice. This animal was found dead on day 7, presumably from severe, systemic MCMV infection. This finding, although a single observation, confirms that ocular tissues can be seeded during MCMV viremia and that the ciliary body represents the initial permissive site for MCMV.

Potentiation of ocular MCMV infection by immunosuppression resembles certain pathogenetic features of acquired HCMV infection. However, compared with HCMV-induced ocular disease, MCMV produced more extensive disease of the ciliary body and iris. Although these structures can be affected by HCMV in immunosuppressed hosts, such as those with AIDS,21 extensive necrosis of the iris or ciliary body are not commonly recognized features of HCMV ocular infections.

Cell-mediated immune responses, particularly cytotoxic T-cell responses and natural killer cell activity, have dominant roles in recovery from HCMV infections.22-24 Deficiencies in these immune functions, whether congenital, iatrogenic, or acquired (eg, AIDS or immunosuppression for organ or marrow transplantation) place the host at substantial risk for invasive HCMV disease, including retinitis.8 The HCMV infection induces leukocyte-associated viremia that disseminates the virus to many target tissues including the eye. Deficiencies in immune responses, in-
Fig. 6. Eyes harvested on day 14 and probed for MCMV DNA. (A) An eye harvested from a cyclophosphamide-treated, MCMV-infected mouse reveals abundant MCMV DNA-containing cells and marked retinal disorganization (original magnification ×41). (B) A higher magnification from another cyclophosphamide-treated, MCMV-infected mouse confirms intense staining for MCMV DNA within the retina (original magnification ×82). (C) Retina from an immunocompetent MCMV-infected mouse shows normal histology and only background staining (original magnification ×82) (all DAB and Harris hematoxylin).

including those induced by cyclophosphamide in this animal system, allow cytomegaloviruses to replicate unabated and induce retinitis.

Although defects in cell-mediated immune responses undoubtedly play the dominant role in the pathogenesis of HCMV retinitis, other factors may be important. In patients with AIDS, additional variables, including bidirectional interactions between HCMV and HIV-1,25 direct HIV infection of the retina26-27 or microvascular abnormalities in the eye that may be associated with a disturbed blood-retinal barrier (BRB)28 could contribute to the pathogenesis of HCMV retinitis. Our experiments provide some observations relevant to this issue. Although we observed substantial viremia and immunosuppression in cyclophosphamide-treated mice, we did not observe retinitis in the contralateral eye, at least through day 14. This suggests that additional factors (eg, a disrupted BRB) may be required to produce cytomegalovirus retinitis. This hypothesis requires further investigation.

In summary, these studies indicate that cyclophosphamide treatment potentiates ocular infection in mice inoculated intravitreally with MCMV. Ocular infection in immunosuppressed mice progressed unabated and induced extensive necrotizing retinitis documented by histologic and nucleic acid hybridization studies. Our results suggest that MCMV infection of cyclophosphamide-treated mice can be used to determine the critical variables that contribute to the pathogenesis of cytomegalovirus retinitis.

Key words: murine cytomegalovirus, retinitis, AIDS, immunosuppression

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