The Pathogenesis of Murine Cytomegalovirus Ocular Infection

Anterior Chamber Inoculation

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To investigate the pathogenesis of ocular cytomegalovirus infections, 3-week-old BALB/c mice were inoculated with $10^4$ plaque-forming units of murine cytomegalovirus (MCMV) by the right anterior chamber and studied sequentially with the use of virus assays and in situ nucleic acid hybridization methods. During acute infection, MCMV was recovered from the right vitreous, lens, cornea, retina/choroid, and optic nerve. Titers of MCMV exceeded $10^3$ per ml of homogenate on days 4 and 7 after inoculation. With the use of biotinylated MCMV DNA probes, MCMV nucleic acids were detected in and adjacent to cells of the iris and ciliary body and occasionally within inflammatory lesions of the cornea. During chronic infection, MCMV was recovered, with the use of co-cultivation or explant methods, from ocular tissues of occasional mice inoculated with MCMV 1 yr earlier. Infectious MCMV was also recovered from the ocular homogenates of a group of mice immunosuppressed with antilymphocyte serum and cortisone. These studies indicate that cells of the uveal tract are permissive for MCMV and suggest that intrinsic persistence or latency of cytomegalovirus in ocular tissues could contribute to the pathogenesis of ocular infections in immunosuppressed hosts. Invest Ophthalmol Vis Sci 31:1575–1581, 1990

Human cytomegalovirus (HCMV), a ubiquitous lymphotropic herpesvirus, causes numerous clinical syndromes in humans.1-4 Approximately 1% of all newborns excrete HCMV at birth, and seroepidemiologic data indicate that most humans exhibit evidence of prior HCMV infection by late adulthood.3-6,8 Although most of these infections are asymptomatic, HCMV can cause severe disseminated disease in congenitally infected infants, immunosuppressed adults, or, rarely, in immunocompetent patients in whom the heterophile-negative infectious mononucleosis syndrome develops.1,2,7-9

In the congenitally infected infant or immunosuppressed adult, HCMV can infect and seriously damage the eye. Ocular abnormalities, including chorioretinitis or optic atrophy, affect 20–25% of symptomatic congenitally infected infants.6-10 Among adults, symptomatic ocular HCMV infections, particularly retinitis, occur almost universally during conditions that perturb cell-mediated immune responses, such as immunosuppressive therapy for organ or marrow transplantation, lymphoreticular malignancies, or the acquired immune deficiency syndrome (AIDS).11-14

Although the ophthalmologic features of HCMV infections have been well-characterized, the pathogenesis of ocular HCMV infections, particularly in adults with disorders such as AIDS, has not been fully determined.15,16 Observations during murine cytomegalovirus (MCMV) infection of mice, including those reported previously from this laboratory,17 suggest that MCMV can establish persistent or, possibly, latent infections of ocular tissues.18 However, the relationship between these animal studies and the pathogenesis of HCMV retinitis in humans with immune deficiencies such as AIDS requires additional investigation.

In the current experiments, we used intraocular MCMV infection of 3-week-old BALB/c mice to characterize more fully the pathogenesis of ocular MCMV infections. Our goals included the following: (1) to determine intraocular sites of MCMV replication, using in situ nucleic acid hybridization methods; and (2) to study ocular tissues during chronic infection for evidence of MCMV persistence.
Materials and Methods

Virus and Animals

The Smith strain of MCMV, obtained originally from Dr. Earl R. Kern, presently at the University of Alabama, Birmingham, was prepared as a 10% wt/vol homogenate of salivary glands harvested from MCMV-infected Swiss Webster mice. MCMV pools regularly contained $10^7$ plaque-forming units (pfu) of MCMV when titrated on monolayers of mouse embryo fibroblast (MEF) cells.

BALB/c mice, 24–27 days old at the time of the experiments, were purchased from Harlan Sprague Dawley, Indianapolis, Indiana. Mice were maintained in a controlled environment, 12-hr light/dark cycle, and provided food and water ad libitum. MCMV-infected and uninfected mice were housed separately. The animal experiments conformed to the guidelines of the ARVO Resolution on the Use of Animals in Research.

Virus Assay

Tissue homogenates and virus pools were assayed for MCMV with the use of confluent monolayers of MEF cells grown in 24-well plates and an agarose overlay as described previously. Culture results were expressed as the log pfu of MCMV per milliliter of homogenate or gram of tissue.

In Situ Hybridization Studies

Nucleic acid hybridization studies were performed with the use of modifications of techniques described previously. The probe consisted of MCMV DNA fragment HindIII A cloned into pACYC177 (courtesy of Dr. Ulrich Koszinowski, Institute for Microbiology, Ulm, Federal Republic of Germany). This fragment did not hybridize with DNA isolated from uninfected MEF cells.

The MCMV DNA HindIII A insert was gel purified and labeled with biotinylated deoxyuridine triphosphate (Enzo Biochem, New York, NY) by nick translation methods. Before hybridization, tissues were deparaffinized with xylene. Tissue sections were pretreated with 0.02 N HCl, 0.005 mg/ml proteinase K for 10–15 min, and RNase; postfixed in 4% paraformaldehyde; and treated with 3% hydrogen peroxide in methanol for 30 min to inactivate endogenous peroxidases. Tissues were reacted with 5 μl of a hybridization mixture containing 5 ng of biotinylated MCMV DNA probe/μl, 50% formamide, 10% dextran sulfate, 2× SSC (0.3 M NaCl and 0.3 M sodium citrate), and 250 μg/ml of salmon sperm DNA; denatured at 92°C for 3 min; and hybridized for 16 hr at room temperature (20 to 22°C).

Slides were developed with the use of the Detek 1-hrp kit (Enzo Biochem), with diaminobenzidine (DAB) as the chromogen, and lightly counterstained with Harris hematoxylin. The presence of MCMV nucleic acids was indicated by a brown precipitate. Controls in each run included (1) MCMV-infected MEF cells; (2) uninfected MEF cells; and (3) ocular tissues from sham-inoculated, control mice.

Experimental Protocol for Acute Infection

Mice were anesthetized with ether, and, with the use of a dissecting microscope to magnify the eye, $2 \times 10^4$ pfu of MCMV contained in 5 μl of minimum essential media (MEM) was inoculated into the right anterior chamber. Control mice received an equivalent dilution of normal salivary gland homogenate prepared from uninfected Swiss Webster mice. This homogenate was culture negative for MCMV.

On days 0, 1, 4, 7, 14, 21, and 28 after inoculation, mice were killed by ether inhalation, and specimens of eye, whole blood, spleen, and salivary gland were removed for virus assay or in situ hybridization studies. Spleen and salivary gland tissues from individual animals were removed and prepared as 10% wt/vol homogenates in MEM containing 10% fetal calf serum and antibiotics (penicillin 100 U/ml, streptomycin 50 mg/ml) with the use of ground glass homogenizers. Circulating leukocytes were obtained by separating heparinized whole blood on ficoll-hypaque (Histopaque, Sigma, St. Louis, MO) gradients using methods described previously. The right and left eyes were removed and, with the use of a dissecting microscope, were separated into ocular components, cornea, lens, retina/choroid, vitreous, and optic nerve. Ocular components were then pooled individually by using groups of three mice and homogenized in 1 ml of MEM. Tissues were stored at −70°C until assayed.

The eyes from five or six additional mice per day (one or two control and three or four MCMV-infected mice) were removed, fixed in fresh paraformaldehyde–lysine–periodate (PLP) for 24 hr, washed briefly with phosphate-buffered saline, and stored in 70% ethanol until embedded in paraffin. Five-micron-thick sections were then prepared from each eye, collected onto microscope slides pretreated with Denhardt’s medium, and stored at 4°C for nucleic acid hybridization studies.

Experimental Protocol for Chronic Infection

Mice were inoculated with MCMV as described above and then housed for 12–13 months before study. At this time two groups of five MCMV-infected animals were killed and tissues harvested for
assay of latent MCMV. Eyes were separated with the use of a dissecting microscope into various components: cornea, lens, retina/choroid, vitreous, and optic nerve. For co-cultivation, the ocular components were minced finely and cultured individually with $10^5$ MEF cells in 24-well plates. For explant cultures, tissues were minced and added to confluent monolayers of MEF cells also grown in 24-well plates. Spleen and salivary gland tissues from individual animals were finely minced with scissors and co-cultivated or explanted with MEF cells.

An additional group of ten mice were treated with an immunosuppressive regimen consisting of daily injections of cortisone acetate (125 mg/kg) and biweekly injections of rabbit antimouse lymphocyte serum (ALS) (0.3 ml/mouse) (Accurate Chemical and Scientific Westbury, NY, serum lot #A1-A3940), a regimen known to reactivate latent MCMV. In normal mice this immunosuppressive regimen induced profound lymphopenia (mean of 246 ± 378 lymphocytes/mm$^3$ in ALS-cortisone-treated mice [n = 9] after 14 days of treatment versus 11,345 ± 2201 lymphocytes/mm$^3$ in control mice [n = 10] receiving normal rabbit serum and saline, $P < 0.000001$). By contrast, neutrophil counts were unaffected (mean of 4029 ± 5522/mm$^3$ in ALS-cortisone-treated mice versus 4097 ± 2050/mm$^3$ in controls, $P = 0.49$). After 18 or 21 days of immunosuppression, eye, spleen, and salivary gland tissues were collected, prepared as tissue homogenates, and assayed for infectious MCMV on confluent MEF cell monolayers.

**Results**

**Acute Infection**

Figure 1 summarizes the results of tissue culture assays for MCMV during acute infection (days 1 to 28). On day 1 after inoculation, small amounts of MCMV were recovered from components of the right eye, with the largest amount detected in the corneal fraction. The right optic nerve, right trigeminal nerve, as well as all components of the left eye had negative results for infectious MCMV.

By day 4 considerable quantities of MCMV were present in the right eye. With the exception of the optic nerve, which generally had negative results for MCMV, the amounts were approximately equal in the various ocular components. Again, all components of the left eye were culture negative for MCMV.

On day 7, infectious MCMV was also present in relatively large quantities in the right eye, and the greatest amounts were detected in the lens and retina/choroid. At this time, small amounts of MCMV (approximately 10 pfu/ml of homogenate each) were also detected in the vitreous and retina/choroid fractions of the left eye. Detection of MCMV in com-

![Fig. 1. MCMV titers in various ocular components harvested during acute MCMV infection, days 1-28. Each point represents the geometric mean data from three groups of tissue homogenates pooled from three mice per day (total of nine mice/day).](image-url)
ments of the left eye presumably reflected hematogenous dissemination because MCMV was detected in circulating leukocytes on days 4 and 7 (minimum of one of nine mice [11%] on day 4 and two of nine mice [22%] on day 7). By contrast, the left optic nerve uniformly had negative results for infectious MCMV, making neural dissemination unlikely. MCMV was also recovered from the spleen and salivary gland, an observation that confirms hematogenous dissemination of MCMV (Fig. 2).

By day 14 the quantities of MCMV in the various ocular components declined, such that MCMV was rarely detected in either eye by day 21. The decline in MCMV replication in the right eye corresponded to a decline of virus titer in spleen tissues. By contrast, MCMV persisted at high titers in the salivary gland.

**In Situ Hybridization Studies**

Eye tissues from control and MCMV-inoculated mice were studied by in situ nucleic acid hybridization on days 2, 4, 7, and 11. Tissues from animals inoculated with MCMV 2–7 days earlier gave positive results for the presence of MCMV nucleic acids (Fig. 3). The percentage of nucleic acid–positive eyes from MCMV-inoculated mice was 66% (two of three) on day 2, 100% (four of four) on day 4, 50% (two of four) on day 7, and 0% (zero of four) on day 11. Control mice uniformly had negative results for MCMV nucleic acids.

Murine cytomegalovirus nucleic acids were identified in cells of the iris (Fig. 3B) and ciliary body (Fig. 3C) and within inflammatory reactions in the cornea adjacent to the site of inoculation. On day 4, MCMV-positive eyes also contained numerous inflammatory cells in the anterior chamber (Fig. 3A), affecting iris, ciliary body, and occasionally the cornea. Despite recovery of infectious MCMV from the retina/choroid fraction, we found no evidence of MCMV replication in the retinal cells using in situ nucleic acid hybridization methods. Histologically, the retina appeared similar to that of control animals.

### Chronic Infection

In initial studies of chronically infected animals, we attempted to determine whether MCMV could be recovered from ocular tissues using conventional methods for detecting latent or persistent MCMV. In co-cultivation and explant experiments, MCMV was recovered from the ocular fractions of two of ten (20%) animals (Table 1), a finding confirmed by passage of the MCMV isolates and in situ nucleic acid hybridization studies of infected fibroblast monolayers.

We also assayed tissue homogenates from mice undergoing systemic immunosuppression with cortisone acetate and antilymphocyte serum. In the first experiment, no infectious MCMV was recovered from the homogenates of salivary gland or eye tissue harvested from mice after 18 days of immunosuppression. In a second group of mice immunosuppressed for 21 days, infectious MCMV was recovered from salivary gland homogenate of three of five mice (60%) and from the pooled homogenates of retina/choroid, indicating the presence of infectious virus in at least one eye (Table 1). The amount of virus recovered from retina/choroid was small, approximately 5 pfu/ml of homogenate. MCMV was not isolated from circulating leukocytes harvested concurrently. Detection of MCMV was again confirmed by hybridization of the infected MEF cell monolayer with the MCMV DNA probe.

### Discussion

In these studies, MCMV replication in structures of the anterior segment of the eyes was confirmed during acute infection by conventional virus assay and by in situ nucleic acid hybridization techniques.
fectious virus was largely restricted to the inoculated eye, although small amounts of MCMV were recovered from the contralateral eye during viremia. We found no convincing evidence for neural transmission of MCMV. Using the in situ nucleic acid hybridization methods, we detected evidence of MCMV replication in cells of the iris, ciliary body, and, occasionally, within inflammatory reactions in the cornea near the site of inoculation. These molecular studies thus extend previous animals studies by identifying ocular sites that are permissive for MCMV in immunocompetent hosts.

Despite recovery of infectious MCMV from several ocular fractions, including the retina/choroid, we did not detect MCMV nucleic acids in the retinal layers or observe major pathologic evidence for MCMV infection. Although we cannot completely exclude the possibility that small foci of retinal infection were missed or that MCMV replication was below the limits of our hybridization methods, these results suggest MCMV replication was restricted to the anterior segment of the eye. Recovery of infectious MCMV from ocular fractions that were negative by in situ hybridization likely reflected contamination from infected ocular fluids or incomplete separation from infected components of the anterior chamber.

Animal age, immune status, route of MCMV inoculation, and virulence of MCMV are important determinants of the location and severity of acute murine ocular infection. Hayashi and co-workers observed that inoculation of salivary gland-derived MCMV into the anterior chamber of 12–18-day-old mice resulted in disseminated infection of the retina, uveal tract, and sclera. When inoculated intravitreally, MCMV also caused widespread infection, although the uveal tract was the most severely affected site. By contrast, anterior chamber inoculation produced disease limited to the uveal tract in adult mice.

Fig. 3. (A, Top left) An eye harvested 4 days after MCMV inoculation via the anterior chamber and probed for MCMV nucleic acids. Inflammatory cells are present at the angle of the anterior chamber (X62). (B, Top right) A high-power view of the iris in (A) (L, lens). Several cells contain MCMV nucleic acids as indicated by the intranuclear precipitates. Numerous inflammatory cells are also present (X165). (C, Bottom left) The ciliary body from the eye of another mouse harvested on day 4 and probed for MCMV nucleic acids. A large cell in the center of the field (arrow) shows intranuclear staining for MCMV nucleic acids (X165). (D, Bottom right) Tissues from a sham-inoculated control mouse demonstrate only background staining (L, lens; X82) (all Harris hematoxylin and DAB).
Table I. Recovery of MCMV from chronically infected* mice

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* Inoculated with MCMV 1 yr earlier. Data expressed as number of animals positive for MCMV per number studied.
† Vitreous fraction.
‡ Retina/choroid fraction. Result in Experiment 2 is data from pooled tissue indicating at least one positive animal.
ND, not done.

(8 to 10 weeks old) receiving virulent salivary gland-derived virus or in young mice receiving attenuated tissue culture-derived MCMV. The older age of the mice used in the current experiments could thus account for the observation that MCMV replication was identified only in the anterior segment.

Replication of MCMV in cells of the anterior segment could be relevant to certain ocular findings of congenital HCMV infections. Hittner and co-workers, for example, observed abnormalities of the anterior vascular capsule of the lens in two infants with congenital HCMV infection. Among patients with HCMV-related ocular abnormalities studied by Frenkel and colleagues, one had Peter’s anomaly, a lesion that has been attributed to anoxia or focal infection. The current observations and those of Hayashi et al suggest that HCMV replication in the anterior ocular segment could contribute to these unusual ophthalmologic features of HCMV infections.

Uveal permissiveness for MCMV may also be relevant to HCMV ocular disease that occurs in immunosuppressed adults. HCMV has been detected in cells of the iris and ciliary body of patients with AIDS and HCMV retinitis. The ciliary body, through its cellular continuities with the choroid and neurosensory retina, could potentially serve as a reservoir or conduit for HCMV infection of the latter structures.

Two prior studies provided data that suggest that MCMV persists or establishes latency in murine ocular tissues. We observed that infectious MCMV could be isolated from the intraocular fluids of mice inoculated ip with MCMV 90 days earlier, a finding that suggested persistence of MCMV. Furthermore, we detected evidence of latent or low-level persistent MCMV infection by recovering virus from explants of ocular tissues harvested 4 months after animal inoculation. Hayashi and co-workers corroborated the latter observation by isolating MCMV from ocular tissues assayed by co-cultivation methods 2–10 months after intraperitoneal or anterior chamber inoculation. The current experiments provide additional evidence that latent or persistent MCMV infections develop in murine ocular tissues.

The cellular site or sites of MCMV persistence in ocular tissues remain to be characterized. Thus far, latent virus has been recovered from retina, vitreous, iris, cornea, sclera, and optic nerve. A unifying explanation for these observations would be persistent infection of intraocular inflammatory cells, monocytes, or tissue macrophages, a hypothesis also proposed by Hayashi and colleagues. Lymphocytes and/or monocytes have an important role in latent or persistent systemic cytomegalovirus infections of humans and other animals.

Human cytomegalovirus retinitis in patients with immune deficiency disorders such as AIDS could reflect several modes of infection, including primary HCMV infection, reinfection with new HCMV strains, or reactivation of latent or persistent HCMV infection. At present, ocular HCMV disease is presumed to be the result of disseminated, systemic HCMV disease. However, the ability to detect MCMV in ocular tissues during chronic infection of mice and to recover infectious virus during immunosuppression, albeit in small numbers of animals, suggests that intrinsic latency or persistence of virus within ocular cells could conceivably contribute to the pathogenesis of HCMV ocular disease in immunosuppressed hosts.

Key words: cytomegalovirus, retinitis, latency, uveitis, AIDS

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References