Intraocular Viral Replication After Systemic Murine Cytomegalovirus Infection Requires Immunosuppression

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Purpose. Human cytomegalovirus retinitis is the most common blinding complication of acquired immune deficiency syndrome. However, the pathogenesis of the disease is poorly understood. The authors sought to characterize intraocular viral replication after systemic murine cytomegalovirus (MCMV) infection in the normal and immunosuppressed Balb/c mouse.

Methods. Normal or immunosuppressed mice (400 rads radiation plus antilymphocyte serum) were infected intravenously with a recombinant MCMV (RM408) that carries an MCMV IE1 promoter—LacZ insert. In vivo MCMV replication and its tissue distribution were monitored by β-gal activity with x-gal staining on frozen tissue sections of multiple organs harvested from infected mice at different time points after inoculation.

Results. MCMV replication within the eye can be detected in the immunosuppressed Balb/c mouse but not in the normal host. Intraocular viral replication was noted first, and most frequently, in the ciliary body and was mainly restricted to the uveal tract. Intraocular viral replication coincided with the peak of systemic viral replication; however, the neurosensory retina was spared. In contrast, supraciliary inoculation of MCMV replication; however, the neurosensory retina was not caused by either a defect in the recombinant virus or the inability of the host tissue to support viral replication. Investig Ophthalmol Vis Sci. 1995; 36:2322-2327.

Human cytomegalovirus (CMV) retinitis is the major ocular complication of acquired immune deficiency syndrome (AIDS).1-3 Although more than 80% of the general population is infected with CMV, retinitis develops only in the late stages of AIDS or in other severely immunocompromised patients.4 Consequently, it is thought that the immune system is important in the protection of the eye from CMV, although the precise mechanism of the protection is unknown.
Murine cytomegalovirus has been used in the rodent to induce retinitis to provide insight into the pathogenesis of human CMV retinitis in AIDS. The latter complication presumably develops after either systemic viremia or in situ reactivation of latent virus within the retina. In contrast, experimental murine CMV (MCMV) has been induced in the rodent only by intraocular inoculation—either through the anterior chamber or the supraciliary space. Consequently, we have studied intraocular MCMV replication after systemic infection with a recombinant MCMV having an IE1 promoter–LacZ insert—RM408—to mimic the route of infection encountered in AIDS.

**METHODS. Mice.** Eight- to 12-week-old female Balb/c mice purchased from the Jackson Laboratories (Bar Harbor, ME) were used in all experiments. All mice were kept in the pathogen-free animal facility of the Division of Comparative Medicine, Washington University School of Medicine, under a 12-hour light–12-hour dark lighting cycle and were given unrestricted food and water. All animal procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

All experiments performed in this study were composed of 20 mice per experimental group. Furthermore, they were killed and examined histologically on days 3, 5, 7, 14, 21, and 28 after intravenous infection with MCMV virus.

**Virus.** Recombinant MCMV virus, RM408 (provided by Dr. Edward Mocarski of Stanford University), was used in all experiments. Virus stocks were obtained by growing RM408 on NIH3T3 cells (ATCC No. CRL 1658) in Dulbecco’s minimum essential medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum. All virus stocks were stored at —70°C. A fresh aliquot of the virus stock (10^6 plaque-forming units [PFU]/ml) was thawed and used in each experiment.

**Immunosuppression.** Adult Balb/c mice were exposed to 400 rads of irradiation from a ^137^Cs source (130 rads/minute) delivered by a Cammacell 40 irradiator (Atomic Energy of Canada, Ottawa, Canada) and intraperitoneally injected with rabbit anti-mouse lymphocyte serum (ALS, 0.2 ml/mouse; Accurate Chemical and Scientific, Westbury, NY) 1 day after irradiation.

**MCMV Infection.** Intraocular infection was accomplished by tail vein injection of freshly thawed virus stock. Two infectious dosages (1 or 5 X 10^6 PFU) were used. Supraciliary infection was accomplished as described by Atherton et al. Briefly, anesthetized mice were positioned under a surgical microscope. Through a limbal incision, a PE30 tube connected to a 10-µl Hamilton syringe was inserted into the supraciliary space incision; 1 X 10^6 PFU of RM408 in 2 µl volume was injected.

**Detection of β-galactosidase Activity.** The method of Sanes et al. was used to detect β-galactosidase (β-gal) activity. Briefly, freshly harvested organs were frozen in liquid nitrogen, and 5- to 6-µm sections were cut with a cryostat. The sections were air dried and fixed with 2% acetone and 0.2% glutaraldehyde in phosphate-buffered saline (PBS) for 5 minutes at room temperature. After they were washed twice with PBS, the sections were incubated with X-gal stain [1% x-gal [in 50% N,N-Diethylformamide to water], 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM MgCl2 in PBS] at 37°C overnight. After they were washed with PBS, the sections were counterstained with 1% safranin-O in 1% glacial acetic acid.

**RESULTS. Systemic Immunosuppression Is Required for Intraocular MCMV Replication After Intravenous Infection.** Viral replication is an essential step in the pathogenesis of MCMV retinitis. We first examined intraocular MCMV replication after intravenous viral infection in normal immunocompetent Balb/c mice. Two infectious dosages (1 X 10^6 and 5 X 10^6 PFU) were used, and 6 postinfection time points (days 3, 5, 7, 14, 21, and 28) were studied in experimental panels consisting of three or four mice at each dosage. We found no detectable β-gal activity in any ocular tissue of normal Balb/c mice with both dosages of RM408 infection during this period of observation. In contrast, intraocular MCMV replication demonstrated by β-gal activity was detected as early as the fifth day and lasted at least until day 21 in immunosuppressed Balb/c mice. Because all immunosuppressed mice with the 5 X 10^6 PFU viral inoculation were dead before postinfection day 14, the results beyond day 14 were collected only from low dose (1 X 10^5 PFU/mouse) viral infection.

**Intraocular Tissue Distribution of MCMV in Immunosuppressed Mice After Intravenous Infection.** At day 5 after intravenous infection, β-gal activity was first noted in the epithelium of the ciliary body (Fig. 1A). In the later stages of viral replication, β-gal activity was detected in the iris epithelium, choroid, and retinal pigment epithelium (RPE) (Fig. 1B). Viral replication (i.e., β-gal activity) in the iris appeared contiguous to that in the ciliary body, suggesting direct spread of the virus. In contrast, viral replication in the choroid and RPE was spatially separated from that of the ciliary body (Fig. 1C).

FIGURE 1. Systemic immunosuppression is required for intraocular MCMV replication after intravenous infection. Frozen section of an eye from an immunosuppressed Balb/c mouse at: (A) 5 days post-inoculation. Note the β-gal activity (blue staining) in the ciliary body, suggestive of intraocular MCMV replication. (B) 20 days post-inoculation. β-gal activity is noted within the iris, ciliary body and lens capsule. (C) 14 days post-inoculation. β-gal activity is noted in foci within the choroid, as well as the RPE.

β-gal activity also was detected within the anterior chamber angle and zonular fibers adjacent to the lens capsule (Fig. 1B) in the late stages of MCMV infection. However, no β-gal activity was detected in neuroretinal tissue at any stage of the experiment. Thus, the primary site of MCMV replication in this model appears to be the ciliary body, with subsequent spread to the iris and choroid.

The detection rate of β-gal activity by X-gal staining of the uveal tract from day 5 to day 21 after systemic infection is between 50% and 67% of eyes examined (3 of 6 eyes on day 5; 4 of 8 eyes on day 7; 5 of 8 eyes on day 14; and 4 of 5 eyes on day 21). No intraocular β-gal activity was detected on day 3 and day 28 after systemic infection.

Retinal Sparing of MCMV Replication Is Not Caused Either by a Defect in the Recombinant Virus or the Inability of the Neurosensory Retina to Support Viral Replication. The absence of MCMV replication in the retina could be caused by either the inability of the recombinant virus to replicate in neuroretinal tissue or the inability of neuroretinal tissue to support replication of the virus. We explored each of these possibilities by the inoculation of recombinant MCMV into the supraciliary space—a route known to cause necrotizing MCMV retinitis with the Smith strain.8 In 5 of 5 Balb/c mice immunosuppressed according to our previously described regimen, we found detectable β-gal activity in the ciliary body, iris, and retina 5 days after the inoculation of 1 × 10^9 PFU RM408 (Fig. 2A). On day 9, dense β-gal staining of the neuroretinal tissue reflected massive viral replication (Fig. 2B), which resulted in necrosis and destruction of the retina by day 21. Thus, the absence of MCMV in the neurosensory retina after systemic infection is not caused by either a defect in the recombinant virus or the inability of the neurosensory retina to support viral replication.

Time Course of Systemic and Intraocular MCMV Replication. Intraocular and systemic MCMV replication postsystemic viral infection were studied in a time dependent manner by histologic examination of various organs. Frozen tissue sections of the spleen, liver, salivary gland, and eye were monitored for β-gal staining after inoculation. Three or four mice were examined at each time point.

No viral replication can be detected in normal Balb/c mice by x-gal stain in both viral dose groups. In contrast, in immunosuppressed mice MCMV replication was first detected in the spleen at day 3 (Fig. 3A). Massive viral replication was restricted to the spleen and liver, and peaked at days 5 to 7 (Fig. 3B). By day 21 viral replication in these organs was eliminated (Fig. 3C). Interestingly, MCMV replication in the salivary gland was detectable by β-gal staining during the first week of the infection. However, the intensity of β-gal staining within the salivary gland was less than that in the spleen and liver, suggesting that recombinant MCMV replication in the salivary gland is poor.

In contrast to the systemic organs, intraocular MCMV replication started later, on day 5, the same time as massive systemic viral replication. The peak of intraocular viral replication was not reached until day 21, a time when systemic MCMV replication was almost eliminated. By day 28, intraocular MCMV replication was not detectable. Thus, it appears that MCMV first replicates within the systemic organs of the host before hematogenous spread to the eye. Within the eye, the
FIGURE 2. MCMV replication after supraciliary inoculation. (A) Five days after inoculation of \(1 \times 10^3\) PFU RM408 in the supraciliary space \(\beta\)-gal activity is detected in the ciliary body, iris and retina. (B) By day 9 dense \(\beta\)-gal staining of the neuroretinal tissue represents probable viral replication within the neurosensory retina, as well as the uvea.

replication of the virus is independent of the time course observed for viral replication within extraocular organs.

DISCUSSION. Cytomegalovirus retinitis is the major cause of blindness in patients with AIDS. Murine CMV retinitis has been used as an experimental model for the study of the pathogenesis of this disease. However, the production of disease in the mouse has required the direct intraocular inoculation of the Smith strain of the virus to induce a necrotizing retinitis. We studied intraocular MCMV replication after systemic infection because this is presumably the route by which the virus spreads to the eye in patients with AIDS.

The ocular manifestations of systemic MCMV infection have not been studied thoroughly. Bale and colleagues have reported that MCMV can be recovered from an ocular homogenate during the acute phase of systemic MCMV infection. However, the primary intraocular tissue in which the virus resides was not identified. The MCMV recovered from ocular homogenates, and explants may be derived from either

resident ocular cells or contaminating blood–borne cells.

Intraocular viral replication is the first step in the pathogenesis of MCMV retinitis. Important information in understanding the pathogenesis of CMV retinitis should occur by defining the systemic conditions necessary for intraocular viral replication and identifying the primary intraocular target cell(s). In this report, viral replication is inferred by examining for the translation product of the LacZ gene–β-gal. Because the LacZ gene is controlled by the IE 1 promoter, the expression of LacZ does not necessarily indicate viral replication. However, the sequential spread of β-gal staining with time, both in adjacent tissue and discontinuously, suggests that we were observing viral replication.

The examination of frozen tissue sections harvested from intravenously infected Balb/c mice for β-gal staining revealed the following:

1. Intraocular MCMV replication after systemic infection requires systemic immunosuppression.
2. The ciliary body is the main portal of ocular entry for the virus after systemic infection.
3. MCMV can replicate in the epithelium of the ciliary body and iris, an unidentified cell in the choroid, and in the RPE.
4. MCMV replication cannot be detected in the neurosensory retina after intravenous infection even in the immunosuppressed host.
5. The absence of viral replication in the neurosensory retina is not caused by either a defect in the recombinant virus or in the inability of the tissue to support viral replication.
6. The time course of systemic and intraocular MCMV replication is different, with massive systemic viremia preceding the development of intraocular MCMV replication.

Our animal model resembles the clinical situation in humans in that MCMV replication occurred only in an immunocompromised host. A study of the time course of MCMV replication after intravenous infection suggested that massive viremia is required to establish foci for subsequent intraocular MCMV replication. The generalized immunosuppression used in our model, sublethal irradiation plus ALS treatment, affects all components of the immune system but is relatively transient. More precise modes of immunosuppression (i.e., elimination of specific subsets of immune cells) are under study to dissect further the precise protective mechanisms of the immune system against intraocular MCMV replication.

In human CMV retinitis, the primary target cell for the virus appears to be within the neurosensory retina. In contrast, the primary intraocular target in MCMV replication appears to be the epithelium of the uvea and RPE. The neurosensory retinal tissue is spared from viral replication during the entire 4-week observation period, even when intraocular MCMV replication has occurred in adjacent tissues.

It could be assumed that recombinant MCMV cannot replicate within the neurosensory retina because the recombinant virus is defective for neuroretinal tissue. The original report concerning the recombinant virus suggests the MCMV IE 2 gene, which is disrupted in the virus, is not important for viral growth. However, the minimal replication we observed in the salivary gland of our immunosuppressed mice does indicate that RM408 is distinctly different from the Smith strain of MCMV. Mocarski and colleagues reported that RM408 replication within the salivary gland is greatly reduced during the acute phase of systemic viral infection. However, when we infected immunosuppressed Balb/c mice through the supraciliary route, virus replicated in the neuroretinal tissue, causing necrotizing retinitis within 3 weeks. This observation established that the recombinant virus can replicate within the neurosensory retina and that the neuroretinal tissue can support viral growth.

Therefore, undefined host defense mechanisms must protect the neuroretinal tissue from MCMV replication after intravenous spread.

The precise mechanisms by which the neurosensory retina is spared from MCMV replication after systemic infection is under study. Sublethal irradiation and ALS treatment only provides temporary immunosuppression, with recovery of the host immune response within 3 weeks. The reduced systemic viral replication observed during the second week after intravenous infection reflects the recovery of the host immune system. It is possible that the neurosensory retina is less permissible than the epithelium of the uvea and the RPE for viral replication. Therefore, although MCMV replication continues within the anterior chamber, the reduced viremia associated with a recovering host immune response may be responsible for the absence of viral replication within the neuroretinal tissue. Experiments to analyze the precise mechanism of neurosensory retinal resistance to viral replication after systemic infection are under investigation.

**Key Words**

AIDS, cytomegalovirus (CMV) retinopathy, immunosuppression, murine cytomegalovirus (MCMV), mouse

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In Vivo Measurement of Lipofuscin in Stargardt’s Disease—Fundus flavimaculatus

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Purpose. Several histopathologic studies have concluded that Stargardt’s disease (Fundus flavimaculatus) is associated with abnormally high levels of lipofuscin-like material in the retinal pigment epithelium. The purpose of this study was to determine whether this material has the same fluorescence characteristics as lipofuscin in vivo and whether noninvasive measurements identify a significant elevation in this material.

Methods. Five patients with autosomal recessive Stargardt’s disease were included in this study, as were 45 healthy controls. All patients had the angiographic dark choroid sign. The intensity and emission spectra of lipofuscin fluorescence were measured by noninvasive fundus spectrophotometry at 7° temporal to the fovea.

Results. The fluorescence intensities in the five patients with Stargardt’s disease were significantly higher (P < 0.0001) than those observed in normal subjects of the same age. The emission spectra in the patients are similar in shape to those measured in normals, but flecks appear to shift the spectra toward shorter wavelengths.

Conclusions. The spectral characteristics of the fluorophore observed in patients with Stargardt’s disease are consistent with those of retinal pigment epithelial lipofuscin. These patients have abnormally high levels of lipofuscin, confirming previous histopathologic observations. Noninvasive retinal pigment epithelial lipofuscin measurements may be a useful adjunct in the diagnosis of Stargardt’s disease—F. flavimaculatus. Invest Ophthalmol Vis Sci. 1995;36:2327–2331.

Stargardt’s disease (juvenile macular degeneration) is a hereditary macular degeneration characterized by diminished central visual acuity in the first several decades of life, the appearance of small yellowish lesions or flecks at the level of the retinal pigment epithelium (RPE) at the posterior pole, and atrophic changes in the macula. Fluorescein angiography often shows pronounced hypofluorescence of the choroid (dark choroid). Early in the disease, electrophysiologic, night vision, and color vision are relatively normal. Fundus flavimaculatus is similar to Stargardt’s disease except that the disease has a more progressive course and is diagnosed later in life (the macula is initially spared).