Ocular Reactivation of MCMV after Immunosuppression of Latently Infected BALB/c Mice

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PURPOSE. The purpose of this study was to identify the site(s) of MCMV latency and reactivation in the eye.

METHODS. Three months after supraciliary inoculation of 5×10^2 PFU of MCMV, BALB/c mice underwent immunosuppression with methylprednisolone and antibodies specific for CD4 T cells, CD8 T cells, and NK cells or with methylprednisolone alone. Control mice were infected but did not receive the immunosuppressants. After 2 or 3 weeks of immunosuppression, the mice were killed. Replicating virus and viral antigen were detected in the injected eyes, peripheral blood leukocytes (PBLs), and extraocular tissues by plaque assay and by staining for early antigen (EA) and β-galactosidase (β-gal), respectively.

RESULTS. In latently infected, nonimmunosuppressed control mice, replicating-virus–and viral-antigen–positive cells were not detected in the injected eyes or extraocular tissues. After immunosuppression with methylprednisolone and antibodies, EA and β-gal were detected, and replicating virus was recovered from the injected eye and from several extraocular sites, including liver, lungs, salivary glands, and kidneys. No virus was recovered from PBLs. β-Gal- or EA-positive cells were observed in the RPE of most mice, and a few virus-infected cells were also observed in the nuclear layers and ganglion cells. Microscopic changes, including retinal folding and detachment, photoreceptor atrophy, macrophage infiltration, and a few EA-positive cytomegalic cells, were observed in the injected eye of immunosuppressed mice.

CONCLUSIONS. After immunosuppression, MCMV reactivates in the injected eye and extraocular tissues, and RPE cells are the initial site of MCMV ocular reactivation in the eye. The timing of virus recovery from all sites suggests that MCMV observed in the injected eye is from in situ reactivation of virus and not from spread of virus from extraocular sites via infected PBLs. (*Invest Ophthalmol Vis Sci.* 2005;46:252–258) DOI:10.1167/iovs.04-0537

Human cytomegalovirus (HCMV) is a ubiquitous herpesvirus that generally causes asymptomatic infection, but it may also cause pneumonitis, hepatitis, gastroenteritis, myocarditis, and retinitis.[1] Persons who are immunologically immature (neonates) or undergo immunosuppression (patients with AIDS or recipients of organ allografts) are at risk of contracting CMV-related diseases. The host specificity of HCMV has prevented development of HCMV-infected animals as models to study the pathogenic processes whereby HCMV infects human cells, replicates within them, enters latency, and reactivates.[2] Murine cytomegalovirus (MCMV) is a mouse-specific CMV that causes pneumonitis, hepatitis, and retinitis in immunosuppressed mice.[1–4] Like HCMV, MCMV becomes latent after acute infection of immunocompetent mice.[2, 5–10] Because of similarities between HCMV and MCMV, MCMV-infected mice have been used to study the pathogenesis of cytomegalovirus infection and reactivation.

The eye is among the organs susceptible to CMV infection. Before the introduction of highly active antiretroviral therapy (HAART), CMV retinitis was the most common ocular opportunistic infection in patients with acquired immunodeficiency syndrome. In the pre-HAART era, although the percentage of patients with CMV retinitis varied depending on the study, up to 46% of patients with AIDS could be expected to have HCMV retinitis at some point during the course of the disease.[11–13] The eye is also a target of congenital or neonatal CMV infection because of an immature blood–retinal barrier.[14,15] In patients, HCMV retinitis may result from either a primary infection or from reactivation of latent infection.[12,14] A mouse model of acute MCMV retinitis that shares some features with HCMV retinitis has been used to study the pathogenesis of CMV retinitis in immunosuppressed mice.[3,4] Studies of latent and reactivated MCMV infection, in which viral DNA was detected in some inoculated eyes and extraocular tissues several months after clearance of infectious virus from the eye and from all extraocular sites, indicate that the virus becomes latent in the injected eye and in some extraocular sites (such as the salivary gland, kidney, spleen, liver, and lung) after initial ocular infection with MCMV.[16–20] It has also been shown that immunosuppression induces reactivation of MCMV in the injected eye and in some extraocular sites, as detected by recovery of replicating virus[20] and the presence of mRNA for gH, a late protein.[6] However, the site(s) of latency and reactivation of MCMV in the eye of the mouse have not been defined. It is also not known whether virus detected in the eye of immunosuppressed mice results from virus reactivation in situ in the eye or whether virus detected in the eye results from the spread of replicating virus from nonocular sites.

MATERIALS AND METHODS

Virus and Cells

Two strains of MCMV (RM461 and K181) were used in these experiments. These viruses were kindly provided by Edward S. Mocarski and Cheryl Stoddart (Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA). RM461 was derived by insertion of a modified *Escherichia coli LacZ* gene (under the control of the HCMV major immediate-early promoter/enhancer) into the wild-type MCMV K181 genome at the *Hin*dIII L/J site, just downstream of immediate-early gene 2 (ie2). RM461 expresses the *E. coli lacZ* gene product β-galactosidase (β-gal) as an immediate-early viral gene product during the viral replication cycle.[21] Stocks of K181 were prepared from the salivary glands of MCMV-infected BALB/c mice. Stocks of RM461 were prepared in mouse embryo fibroblast (MEF) cells (BioWhittaker, Walkersville, MD) grown in tissue culture medium containing 5% fetal calf serum (HyClone, Logan, UT). The titer of

MCMV in virus stocks was determined by duplicate plaque assay on MEF cells.

Mice

Adult female BALB/c mice (Taconic Inc., Germantown, NY) were used in all experiments. All mice were housed in accordance with National Institutes of Health guidelines. Mice were maintained on a 12-hour light–dark cycle and were given unrestricted access to food and water. Mice were anesthetized with a rodent cocktail (0.5–0.7 mL/kg of a mixture of 42.9 mg/mL ketamine, 8.57 mg/mL xylazine, and 1.43 mg/mL acepromazine) before experimental manipulation. The treatment of animals in this study conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the Institutional Animal Care and Use Committee of the Medical College of Georgia.

Experimental Design

Immunocompetent adult female BALB/c mice were injected with 5×10^2 plaque-forming units (PFU) of RM461 via the supraciliary route. After 3 months (when replicating virus can no longer be recovered from any ocular or nonocular site[6]), latently infected mice were divided into three groups. Mice in group 1 were injected with methylprednisolone acetate (2 mg/mouse, intramuscularly, every 3 days) and with normal rat IgG (0.5 mg/mouse, intravenously, 1 day and 7 days after beginning treatment with methylprednisolone). Mice in group 2 were treated with methylprednisolone acetate (2 mg/mouse, intramuscularly, every 3 days) and with intravenously injected, T-cell-specific antibodies (0.45 mg anti-CD4 [GK1.5] and 0.1 mg anti-CD8 [2.43]; American Type Culture Collection, Manassas, VA) and anti-asialo GM_1 (10 μg/mouse; Wako Chemicals, Richmond, VA) 1 day and 7 days after beginning treatment with methylprednisolone. Mice in group 3 (control) were injected with PBS only. Animals were killed after 2 or 3 weeks of immunosuppression (i.e., a minimum of 14 weeks after inoculation of MCMV). Blood was collected by cardiac puncture and anticoagulated with heparin, and the mice were perfused with PBS. Spleens were harvested and single-cell suspensions were prepared for flow cytometry. Peripheral blood leukocytes (PBLs) were separated from blood (Histopaque-1119; Sigma-Aldrich, St. Louis, MO), according to the manufacturer's instructions. Eyes and extraocular tissues were removed, snap frozen, and sectioned on a cryostat. The slides were air dried and prepared for β-gal staining or for immunohistochemistry. For detection of reactivated replicating virus by plaque assay, the eyes, nonocular tissues, and PBLs were homogenized in serum-free tissue culture medium, with a hand-held tissue homogenizer (Biospec Products, Inc., Racine, WI). The homogenates were serially diluted and plated (in duplicate) on MEF cells.

For in vitro reactivation of latent MCMV, the eyes of latently MCMV-infected, nonimmunosuppressed mice were removed and separated into anterior and posterior segments with a dissecting microscope. The fragments of each segment of a single eye were placed in separate culture plate inserts (Millicell; Millipore, Bedford, MA) with a pore size of 3.0 μm, and each culture plate insert was placed in a well containing a monolayer of MEF cells. By this method, reactivated virus released from the tissue diffuses into the medium and infects the MEF cells. Because there is no direct contact of the minced tissue with the MEF cells, the cellular toxicity that frequently results from such contact is eliminated. After 1 day and then every 7 days thereafter, the culture plate inserts containing the minced tissue were removed to another well containing a fresh monolayer of MEF cells until the ocular fragments had been in culture for 3 weeks. MEF cultures were fixed and stained for virus plaques 3 weeks after the culture plate inserts had been removed.

Flow Cytometry

The non–cross-reactive antibodies FITC-anti-L3T4 (BD-PharMingen, San Diego, CA), PE-anti-ly-3.2 (BD-PharMingen), and FITC-anti-mouse pan-NK (BD-PharMingen), recognizing CD4, CD8, and DX5 (NK), re-

spectively, were used to determine the extent of lymphocyte depletion. Flow cytometry of stained spleen cell samples was performed (FACStar Flow Cytometer; BD Biosciences, San Jose, CA), and the flow cytometry results were analyzed using DP2 software (provided by the National Institutes of Health, Bethesda, MD).

Immunohistochemistry

Monoclonal antibody to an MCMV early gene product[22] was biotinylated (Sulfo-NHS-LC-Biotin; Pierce, Rockford, IL) according to the manufacturer's instructions. Frozen sections of ocular tissues were fixed with acetone for 5 minutes at room temperature. The slides were rinsed twice with PBS, and the sections were blocked with 3% normal goat serum (Vector Laboratories, Burlingame, CA) for 30 minutes. Biotin-labeled anti-EA was applied to the sections for 90 minutes at room temperature in a humidified chamber. The slides were then washed twice with PBS, treated with 0.5% H_2O_2 in absolute methanol for 30 minutes, and washed twice with PBS. Sufficient avidin-biotin complex (ABC) solution (ABC kit; Vector Laboratories) to cover the sections was applied for 45 minutes at room temperature in a humidified chamber. The slides were washed three times with PBS and incubated with 3,3'-diaminobenzidine (DAB; Sigma-Aldrich) at room temperature for 10 to 30 minutes. The reaction was stopped by washing slides in tap water. The sections were counterstained with methyl green, dehydrated, mounted, and examined microscopically for brown-stained cells, indicative of EA expression.

Monoclonal antibody against RPE65 (kindly provided by Michael Redmond, National Eye Institute, National Institutes of Health) was used to stain the cells of the retinal pigment epithelium (RPE).[23] After they were blocked, the sections were incubated overnight at 4°C in primary antibody to RPE65 (1:400) and then washed and incubated with Texas-red–labeled anti-rabbit (1:100; Vector Laboratories). Antibody to MCMV EA was labeled with FITC (Sigma-Aldrich) according to the manufacturer's instructions. To double stain MCMV EA and RPE65, the sections were stained first with anti-RPE65 and then reacted with FITC-labeled anti-MCMV EA. The slides were mounted with antifade mounting medium containing 4',6'-diamino-2-phenylindole (DAPI; Vectashield; Vector Laboratories) and examined with a confocal microscope for MCMV EA-positive green cells (FITC) and RPE65-positive red cells (Texas red). Biotin anti-mouse CD11b (Mac-1, 1:50; BD PharMingen) was used to stain macrophages/macroglia. Immunohistochemistry was conducted with Texas red–labeled avidin (Vector Laboratories). FITC-labeled anti-mouse CD3 (1:50; BD PharMingen) was used to stain T cells.

Staining for β-Gal in Frozen Sections

Frozen sections of ocular and nonocular tissues were fixed with 0.5% glutaraldehyde in PBS for 15 minutes and washed twice with PBS. The sections were reacted with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) a chromogenic substrate for β-gal. After incubation overnight at room temperature in substrate solution (PBS containing 2.4 mM X-gal, 12.5 mM K_3FeCN_6, and 12.5 mM $K_4FeCN_6 \cdot 3H_2O$), the sections were washed with PBS and counterstained with safranin-O. Tissue sections were dehydrated, mounted, and examined microscopically for blue-stained cells, indicative of immediate early (IE) gene expression.

RESULTS

In Vitro Reactivation of Ocular MCMV

To determine whether MCMV is latent in the eye, injected eyes were collected from nonimmunosuppressed mice that were latently infected with MCMV. After separation, the anterior and posterior segment of each eye was cocultivated separately with MEF cells using a culture plate insert (Millicell; Millipore), as described in the Materials and Methods section. The results showed that replicating virus was not detected in either the

TABLE 1. Lymphocyte Depletion in the Spleen of BALB/c Mice Latently Infected with MCMV

Group	Weeks of Treatment	Spleen Cells (n)/ %Depletion*	CD4$^+$ Cells (n)	%CD4$^+$/ %Depletion	CD8$^+$ Cells (n)	%CD8$^+$/ %Depletion	DX5$^+$ Cells (n)	%DX5$^+$/ %Depletion
1	2	5.4×10^5/99.19	2.1×10^5	39.55/98.28	8.3×10^4	15.35/98.26	1.1×10^4	1.94/99.53
	3	3.0×10^5/99.55	3.1×10^4	10.40/99.75	1.5×10^4	4.85/99.69	NT	NT
2	2	3.3×10^5/99.51	1.2×10^4	3.50/99.91	5.6×10^2	0.17/100.00	6.7×10^3	1.94/99.74
	3	2.8×10^5/99.58	9.2×10^2	0.33/100.00	1.1×10^2	0.0/100.00	NT	NT
3	—	6.7×10^7/—	1.2×10^7	18.50/—	4.8×10^6	7.10/—	2.7×10^6	4.05/—

Group 1, latently infected mice treated with methylprednisolone only; group 2, latently infected mice treated with methylprednisolone and T-cell— and NK-cell–specific antibodies; group 3, nonimmunosuppressed mice latently infected with MCMV. NT, not tested.
  * %Depletion compared with the controls (group 3).

anterior or posterior segment of any injected eye immediately after the mice were killed (1 day). After 1 week of culture, virus was detected in the anterior segment of 1 of 10 mice and in the posterior segment of 1 of 10 mice. After 2 weeks of culture, virus was detected in 1 of 10 anterior segments and 3 of 10 posterior segments. After 3 weeks in culture, replicating virus was recovered from 9 of 10 anterior and posterior segment samples.

Lymphocyte Depletion

The extent of splenic lymphocyte depletion was assessed after 2 or 3 weeks of immunosuppression of mice latently infected with MCMV and treated with methylprednisolone alone (group 1) or with methylprednisolone together with T-cell–and NK-cell–specific antibodies (group 2). As shown in Table 1, after treatment with methylprednisolone alone (group 1), >99% of spleen cells were depleted. Continued treatment did not significantly affect the extent of overall cell depletion, but the relative percentage of CD4$^+$ and CD8$^+$ cells decreased. Among the mice in group 2, treatment with T-cell–and NK-cell–specific antibodies in addition to methylprednisolone resulted in only slightly more depletion of total spleen cells. However, more CD4$^+$ and CD8$^+$ cells were depleted in the mice in group 2 than in the mice in group 1. For example, after 3 weeks of immunosuppression, the actual number of CD4$^+$ and CD8$^+$ cells in the mice in group 2 was reduced 33- and 136-fold, respectively, compared with the number of CD4$^+$ and CD8$^+$ cells in the mice in group 1.

Reactivation of MCMV in Immunosuppressed Mice

To determine whether MCMV reactivates in immunosuppressed mice, eyes, extraocular tissues, and PBLs were collected from immunosuppressed mice after 3 weeks of immunosuppression and from latently infected, nonimmunosuppressed control mice. Replicating, reactivated virus was recovered from the injected eye, the lungs, and the liver of the mice in group 2 (Table 2). Replicating virus was not recovered from these tissues in mice in group 1 or in mice in the nonimmunosuppressed control group. In addition, replicating virus was not recovered from the noninjected eye, salivary glands, kidneys, spleen, or PBLs of any mouse in any group.

Ocular Viral Antigen in Immunosuppressed Mice

Even though the virus-recovery studies show that MCMV reactivated in the injected eye of immunosuppressed mice, virus recovery provided no information about the sites in which virus had reactivated in the eye. To determine the location of reactivated MCMV, injected eyes from mice in each group were examined for β-gal (expressed as an IE gene) and/or for EA after 2 or 3 weeks of immunosuppression. As shown in Table 3, viral antigen was detected in the RPE of one of four mice in group 1 after 3 weeks of immunosuppression. In contrast, among mice in group 2, more animals and more sites were positive for viral antigen after 2 weeks of immunosuppression. Among eyes in which viral antigen was detected, the RPE layer appeared to be the initial and most frequent site of MCMV reactivation (Fig. 1B), although positive cells were observed occasionally in other sites, including the nuclear layers and the ganglion cells (Fig. 1C), the choroid (Fig. 1B), and the anterior segment (Fig. 1D). The pattern of EA staining was similar to that of β-gal staining, and β-gal-positive cells were usually observed in the same sites as EA-positive cells in adjacent sections (for example, Figs. 1E, 1F). As was observed with β-gal staining, the RPE layer of only one mouse in group 1 was EA positive, whereas most of the animals in group 2 expressed viral antigen in the RPE as well as in several other sites in the posterior and anterior segments (Table 4). None of the injected eyes of the mice in group 3 was positive for viral antigen (Fig. 1A).

To ensure that the results from experiments using immunosuppressed mice latently infected with RM461 were not influenced by insertion of the *LacZ* gene into the MCMV genome, mice latently infected with the parent virus K181 underwent immunosuppression with methylprednisolone and antibodies specific for CD4, CD8, and asialo-GM$_1$, and sites of EA expression were identified. After 3 weeks of immunosuppression, EA-positive cells were observed in the RPE layer of the injected eye of four of five of the mice and occasionally in other sites in both the posterior and the anterior segment. No

TABLE 2. Recovery of MCMV after Immunosuppression of BALB/c Mice Latently Infected with MCMV RM461

Group	Injected Eye	Uninjected Eye	Salivary Gland	Lung	Liver	Kidney	Spleen	PBL
1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
2	3/5	0/5	0/5	4/5	1/5	0/5	0/5	0/5
3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3

Groups are described in Table 1. The first number is the number affected, and the second number is the number in the group.

TABLE 3. β-Gal Expression in the Injected Eye after Immunosuppression of BALB/c Mice Latently Infected with MCMV RM461

| Group | Weeks of Treatment | Anterior Segment | | Posterior Segment | | | |
		Ciliary Body	Iris	RPE	Nuclear Layers	Ganglion Cell Layer	Choroid
1	2	0/4	0/4	0/4	0/4	0/4	0/4
	3	0/4	0/4	1/4	0/4	0/4	0/4
2	2	1/8	1/8	6/8	1/8	1/8	3/8
	3	2/8	2/8	7/8	3/8	3/8	3/8
3	—	0/5	0/5	0/5	0/5	0/5	0/5

Groups are described in Table 1. Data are as described in Table 2.

EA-positive cells were observed in the injected eye of control mice. After immunosuppression, replicating virus was recovered from the injected eye of four of five mice, the salivary glands of four of five mice, and the lungs of three of five mice infected with the parent virus. No replicating virus was recovered from other sites, including the uninjected eye, kidneys, spleen, or PBLs.

To determine whether viral-antigen–positive, MCMV-infected cells in the area of the RPE were RPE cells, sections of eyes from latently infected, immunosuppressed mice were double stained with antibodies specific for MCMV EA and for RPE-65. As shown in Figure 2, most of the EA-positive cells in the area of the RPE were also RPE-65 positive.
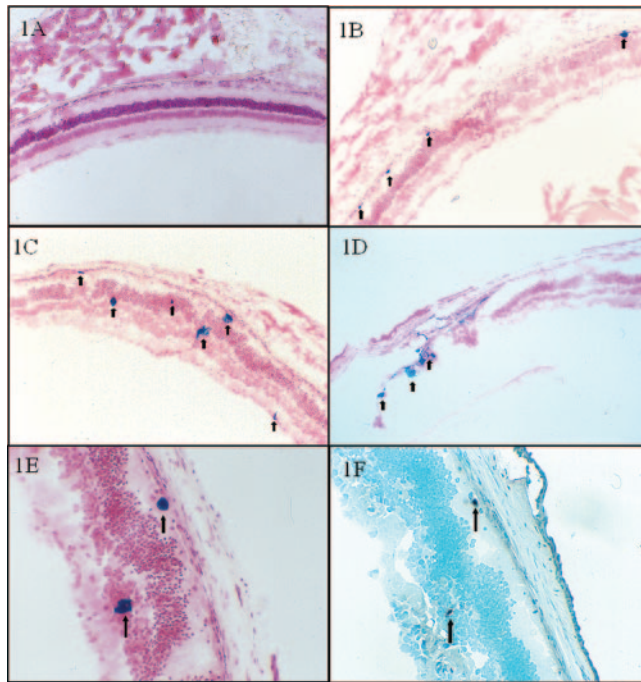

FIGURE 1. Photomicrographs of MCMV in the injected eye of latently infected BALB/c mice. No MCMV (β-gal)-positive cells were observed in the eyes of nonimmunosuppressed, latently infected mice (**A**). β-Gal-positive cells were observed in the eye after 3 weeks of immunosuppression with methylprednisolone and antibodies (**B-E**, *arrows*). The RPE was the initial and most frequent site of MCMV reactivation (**B**). β-Gal-positive cells were observed occasionally in other sites, including the nuclear layers and the ganglion cell layer (**C**), the anterior segment (**D**), and the choroid (**B**). The pattern of EA staining (**F**, *arrows*) was similar to that observed for β-gal staining (**E**, *arrows*), and β-gal-positive cells were usually observed in the same sites as EA-positive cells in adjacent sections (compare Figs. **E** and **F**).

Microscopic Changes Associated with Reactivation of MCMV in the Eye

Although typical MCMV retinitis and retinal destruction were not observed in immunosuppressed mice in either group 1 or 2, reactivation of virus was associated with several microscopic changes in the eye. The most common finding (9/15 mice) was retinal detachment and loss of the photoreceptors that appeared to be associated with the presence of virus in the RPE (Figs. 3A, 3B). EA-positive cytomegalic cells were also observed occasionally in injected eyes of immunosuppressed mice (Fig. 3C). Some viral-antigen–negative, macrophage-like cells were also present in the retina of these mice (Fig. 3D).

Immune Cells in the Retina of Mice Latently Infected with MCMV

To determine the identity of the macrophage-like cells, we stained sections of the eyes of normal mice and of mice latently infected with MCMV before and after immunosuppression with antibodies for Mac-1 and CD3. In normal mice, Mac-1–positive cells were occasionally found in the RPE layer (Fig. 4A), but no T cells were observed (Fig. 4B). Before immunosuppression of latently infected mice, some Mac-1- (Fig. 4C) and CD3- (Fig. 4D) positive T cells were observed in the injected eye, primarily in the choroid, the RPE, and photoreceptor layer. After 3 weeks of immunosuppression with methylprednisolone and antibodies, Mac-1-positive cells were observed in all layers of the retina (Fig. 4E), whereas only an occasional CD3-positive T cell was seen (Fig. 4F).

DISCUSSION

Similar to other members of the herpesvirus family, the CMVs remain with their host and establish lifelong latency after primary infection. Depending on the host and viral system, the question of whether the virus is truly latent or maintains a low-level, persistent viral infection has not been completely resolved. Operationally, latency has been defined as the inability to detect replicating virus despite the presence of virus DNA and perhaps some limited transcription. In the murine models of MCMV infection, latency is characterized by the ability to reactivate virus from cells of infected tissues after cocultivation with permissive cells in culture, even though infectious virus cannot be detected directly in disrupted tissues.[24,25] Immunosuppression[6,8,9,20] or immunologic modulation, such as allogeneic stimulation,[26,27] has been shown to induce reactivation of latent virus.

The eye is among several organ targets of CMV infection. The results of the studies reported in this manuscript support the idea that after inoculation of MCMV via the supraciliary route, virus becomes latent in the eye and that latent virus may reactivate in the eye. Results from several studies suggest that

TABLE 4. Early Antigen Expression in the Injected Eye after Immunosuppression of BALB/c Mice Latently Infected with MCMV RM461

		Anterior Segment		Posterior Segment			
Group	Weeks of Treatment	Ciliary Body	Iris	RPE	Nuclear Layers	Ganglion Cell Layer	Choroid
1	2	0/4	0/4	0/4	0/4	0/4	0/4
	3	0/4	0/4	1/4	0/4	0/4	0/4
2	2	0/5	0/5	3/5	0/5	1/5	1/5
	3	1/5	1/5	3/5	2/5	2/5	0/5
3	—	0/3	0/3	0/3	0/3	0/3	0/3

Groups are described in Table 1. Data are as described in Table 2.

MCMV becomes latent in the eye after ocular (anterior chamber or intravitreal) or intraperitoneal inoculation. Bale et al.[18] reported that the eyes of 10% of mice infected with MCMV via the intraperitoneal route were positive for virus during latency. In later studies, use of in situ hybridization during acute infection after anterior chamber inoculation demonstrated that cells of the uveal tract are permissive of MCMV.[17] In the same studies, when latently infected mice underwent immunosuppression with cortisone acetate and antilymphocyte serum, infectious virus was recovered from 60% of the salivary glands and from 20% of the eyes, whereas none of the leukocytes was virus positive, confirming that MCMV becomes latent in the eye and was not spread to the eye via leukocytes harboring replicating virus. In another study, after inoculation of young (12- to 18-day-old) mice via the anterior chamber, latent MCMV was recovered from a small number of eyes by cocultivation several months after the acute infection and after clearance of infectious virus from all sites.[19]

In a study by Rabinovitch et al.,[20] intravitreal injection of 3-week-old BALB/c mice with MCMV resulted in mild chorioretinitis during the acute phase of the virus infection and that virus was cleared from all ocular structures within 2 weeks of virus inoculation. When the mice in this study were subjected to immunosuppression with cortisone acetate and cyclosporin A, replicating virus was recovered from the eye.[20] Previous results from our laboratory showed that after inoculation of 5×10^2 PFU of MCMV (RM461 or Smith strain) into the

supraciliary space of euthymic BALB/c mice, replicating virus was cleared from salivary gland by 5 weeks after infection (PI) and other sitesm including injected eyes, by 4 weeks PI. β-gal-positive, virus-infected cells were cleared from all sites by day 28 PI.[16,28] More recent studies by Kercher and Mitchell[29] using PCR and virus recovery demonstrated that MCMV becomes latent in the eye after inoculation via the supraciliary route. However, after immunosuppression of these mice with cyclophosphamide, replicating virus was recovered only after explantation of the ocular tissue and not directly from the eyes of the immunosuppressed mice. Taken together, the results of many studies of MCMV showing that replicating virus cannot be detected in the eyes of latently infected, nonimmunosuppressed mice after inoculation of virus via several ocular routes support the idea that a low level of replicating virus does not persist in the eye after acute ocular infection. Because replicating virus was recovered from both the anterior and posterior segments of the eye after cocultivation in vitro, these findings suggest that virus is latent in structures in both the anterior the posterior segments.

Early antigen (EA) and immediate EA were detected, and replicating virus was recovered from the injected eyes and some extraocular tissues of immunosuppressed mice, indicating that MCMV can reactivate in the injected eyes and extraocular tissues after immunosuppression. However, ocular reactivation of latent MCMV was observed only in mice that had undergone deep immunosuppression with a combination of methylprednisolone and antibodies. These results are at vari-
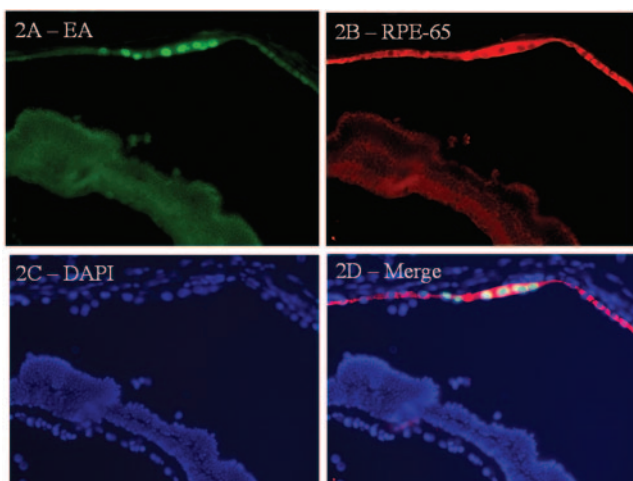

FIGURE 2. Photomicrographs of double staining for MCMV EA and RPE-65 in the injected eyes after immunosuppression of mice latently infected with MCMV. MCMV EA-positive cells in the RPE layer (**A**), RPE-65-positive cells (**B**), DAPI nuclear-stained cells (**C**), and a merged image indicating that the EA-positive cells in the RPE were also RPE-65 positive (**D**).
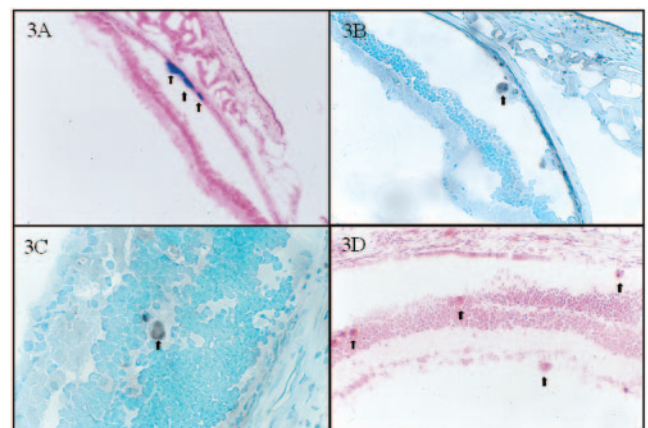

FIGURE 3. Photomicrographs of tissue changes associated with MCMV reactivation in the injected eyes after immunosuppression of mice latently infected with MCMV. MCMV-positive cells were observed in the RPE, and retinal detachment was observed (**A**, **B**, *arrows*). MCMV-positive cytomegalic cells (*arrows*) were seen in the retina (**C**) and RPE (**B**). Infiltration of β-gal (virus) negative macrophage-like cells was noted in the retina (**D**, *arrows*).
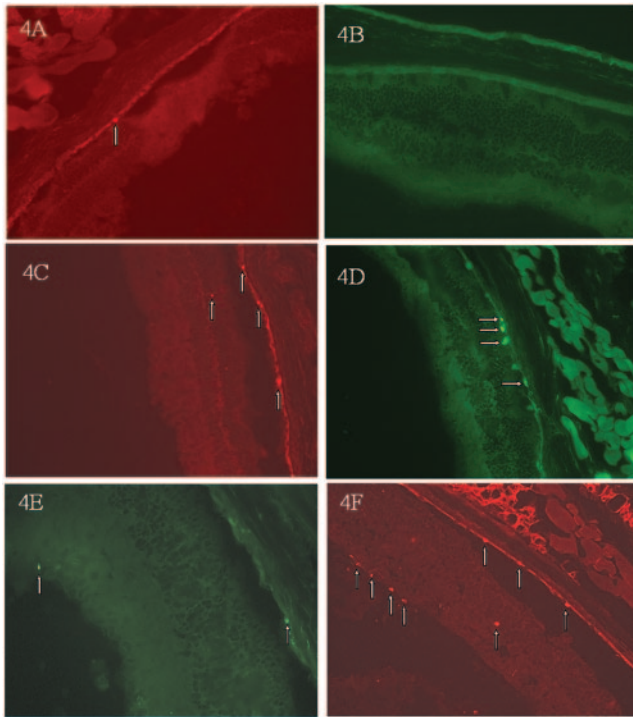
Figure 4. Photomicrographs of immune cells (*arrows*) in the eyes. A few Mac-1-positive cells (**A**) but no T cells were observed in the RPE of normal mice (**B**). Mac-1-positive cells (**C**) and CD3-positive T cells (**D**) were observed in the choroid, RPE, or photoreceptor layer in the injected eyes of nonimmunosuppressed latently infected mice. After 3 weeks of immunosuppression, few CD3-positive T cells were observed in the injected eye (**E**). Mac-1-positive cells were present in all layers of the retina (**F**).

ance with those reported in latently infected mice that had cyclophosphamide immunosuppression alone[29] and suggest that both the type of and the extent of cell deletion are involved in determining whether replicating virus is recovered from ocular tissues. When mice were treated for 3 weeks with methylprednisolone alone, only a few EA- or IEA-positive RPE cells were observed in the eye of a single mouse, and replicating virus was not recovered from any ocular or extraocular site. In contrast, in deeply immunosuppressed mice (treated with methylprednisolone and antibodies), the injected eyes of most of the mice were EA- or β-gal-positive as early as 2 weeks after the initiation of immunosuppression, and replicating virus was recovered from injected eyes and extraocular sites, including liver, lung, and salivary gland after 3 weeks of treatment. Although after 3 weeks of treatment the total number of spleen cells was similar in the mice treated with methylprednisolone plus antibodies and the mice treated with methylprednisolone alone, flow cytometric analysis revealed that more $CD4^+$ and $CD8^+$ cells were depleted in the mice treated with methylprednisolone and antibodies than in the mice treated with methylprednisolone alone. Because depletion of $CD4^+$ and $CD8^+$ T cells was nearly 100% after 3 weeks of deep immunosuppression, this finding suggests that extensive depletion of T cells is needed before MCMV can reactivate and replicate in the eye of latently infected mice.

After inoculation of 5×10^2 PFU of RM461, virus spread to the anterior and the posterior segments of the injected eye, as well as to some extraocular sites.[14,25] Although a few β-gal-positive cells were also observed in the nuclear layers, the RPE cells appeared to be the initial and major targets of acute MCMV infection after inoculation of virus via the supraciliary route.[16,28] These studies showed that when latently infected

mice had 3 months of immunosuppression after virus inoculation, MCMV reactivated in the eye, and the sites of MCMV reactivation in the injected eye were similar to the sites during acute infection. RPE was also the initial and major site of MCMV reactivation. Many β-gal- or EA-positive cells were found in the RPE of most mice 2 or 3 weeks after immunosuppression, although occasional virus-positive cells were observed in the anterior segment, nuclear layers, ganglion cells, and choroid. Our present results, as well as previous studies in our laboratory[6] and in that of other investigators,[18] indicate that MCMV cannot be recovered from PBLs after immunosuppression. Our previous studies of latency also showed that although PBLs contain viral genomes, late antigen (gH) RNA could not be detected in immunosuppressed mice.[6,16] These results, together with other observations that noninjected eyes were negative for virus; that the choroid and ciliary body, but not the RPE, became infected during systemic infection of immunosuppressed mice[28,30]; and that latent virus was reactivated in vitro from injected eyes by cocultivation suggest that some or perhaps all of the virus recovered from the eye results from in situ reactivation of latent virus and subsequent replication and not from spread of replicating virus from nonocular sites via MCMV-infected PBLs.

Our results also showed that the sites of EA expression in the injected eyes of IS mice latently infected with parent MCMV K181 were similar to the mice infected with the mutant virus RM461. Replicating virus was also recovered from injected eye and lung in both groups. The results confirmed previous observations from our laboratory[31] and from others[21] that these two strains of MCMV replicate similarly in the eye, as well as in several other organs, such as the lung and liver. RM461 replication in these tissues was not influenced by insertion of the *LacZ* gene into the MCMV genome. In the studies described herein, replicating virus was recovered from the salivary glands of four of five mice infected with K181, but no virus was recovered from salivary glands of mice latently infected with RM461. Replication of RM461 in the salivary gland is lower than that of the parent virus, K181, because RM461 contains the *lacZ* gene inserted close to the *sgg1* gene, which regulates MCMV replication in salivary glands.[21]

After immunosuppression, microscopic changes such as retinal detachment and loss of photoreceptors and occasional cytomegalic cells were observed in the retina of the injected eye of MCMV latently infected mice. These changes appeared to be due to reactivation of MCMV in the eye and not to an effect of the immunosuppression, since comparable changes were not observed in the noninjected eyes of immunosuppressed mice. In addition, because the microscopic appearance of the retinas of the injected eyes of nonimmunosuppressed mice latently infected with MCMV remained normal, it is unlikely that the retinal changes were due to the virus injection or to the limited amount of viral replication that occurs after supraciliary inoculation of MCMV.

Resting microglia moderately express CD11b, which is rapidly upregulated after activation.[32] Our results showed that more Mac-1-positive cells were observed in the retina of the injected eye after immunosuppression. The role these activated microglia play in retinal damage during replication of replicated virus is not clear. Although viral antigen-positive cells were observed in the retina of the injected eye after immunosuppression, fulminant retinitis was not observed, and the titer of replicating virus in the eye was low (average <40 PFU/eye). Immune cells in situ such as activated microglia may play a role in the restriction of viral replication and spread in the retina. Alternatively, because activated retinal microglia are associated with degenerative retinal diseases and photoreceptor loss,[33–37] the microscopic changes observed in the retina of

immunosuppressed, latently infected mice may be attributable to the effects of these cells.

The result that virus reactivated in situ in the mouse eye during immunosuppression suggests that spread of virus from nonocular sites or from systemic infection may not always be necessary for induction of CMV retinitis. Although most CMV infections of the retina are coincident with systemic CMV infection, some patients who are congenitally infected with HCMV and in whom HCMV retinitis develops do not have evidence of concurrent systemic CMV infection.[14]

Although it has been suggested that reactivation of MCMV is differentially regulated in the eye,[29] the results of the studies presented herein suggest that the extent and timing of immunosuppression are also critical elements during in vivo viral reactivation. Although the preponderance of the evidence suggests that virus can reactivate in the eye of the mouse, additional studies are needed to determine why reactivation of ocular virus does not lead to fulminant retinitis, even in deeply immunosuppressed mice.

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