Necrotizing Retinopathy After Intraocular Inoculation of Murine Cytomegalovirus in Immunosuppressed Adult Mice

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A light microscopic study was done to investigate retinal changes in healthy and immunosuppressed mice after intraocular inoculation of murine cytomegalovirus (MCMV). A 0.01-ml inoculum containing 10^5 plaque-forming units of MCMV was placed behind the lens in 138 4-week-old Swiss Webster mice. Ninety-eight mice were immunosuppressed with 0.2 mg/g of cyclophosphamide given intraperitoneally at the time of inoculation and 0.1 mg/g of cyclophosphamide every 5 days thereafter. Selected eyes were examined on postinoculation days 5, 10, 15, and 16-20. Evidence of viral infection was most prominent in uveal tissue. Uveal infection developed whether or not animals received cyclophosphamide, but retinal necrosis developed only in immunosuppressed mice. Focal retinal necrosis, primarily involving the outer retinal layers and retinal pigment epithelium, was first observed in an eye examined on day 10. Retinopathy from MCMV was present in three of five eyes (60%) examined on day 15, and in six of 16 eyes (37.5%) examined between days 16-20. Retinal disease was characterized by full-thickness retinal necrosis, scattered cytomegalic cells, intranuclear and intracytoplasmic viral inclusions, and acute and chronic inflammation. These results indicate that MCMV can produce a necrotizing retinopathy in mice and that immunosuppression facilitates infection. Although ocular MCMV infection in immunosuppressed adult mice is a potential model for study of human CMV retinopathy, many differences exist between human CMV and MCMV and between the ocular diseases they produce. Invest Ophthalmol Vis Sci 31:2326-2334, 1990

Human cytomegalovirus (CMV) retinopathy is a serious opportunistic infection of newborns and immunocompromised adults. Before 1982 it was a rare disease that occurred primarily in organ-transplant recipients who were receiving immunosuppressive drug therapy to prevent allograft rejection. It is now being seen with increasing frequency because of its association with the acquired immune deficiency syndrome (AIDS). Believed to occur in approximately 25% of patients with AIDS, CMV retinopathy is the most common AIDS-related intraocular infection.

Human CMV retinopathy is characterized by full-thickness necrosis of the retina. Without treatment, foci of infection enlarge relentlessly and eventually destroy the entire retina over several months. This opportunistic infection therefore adds greatly to the morbidity of the syndrome.

Human CMV retinopathy remains a poorly understood disease. Currently a good animal model of this infection does not exist. Human CMV is species specific and cannot be studied in animals. Murine CMV (MCMV) is a DNA virus that is morphologically similar to human CMV. It causes chronic salivary gland infection in mice and an acute, severe disseminated infection involving liver, spleen, lymph nodes, adrenal glands, and other visceral organs after intraperitoneal inoculation. MCMV infection in mice has been suggested as a possible model for study of various human CMV infections.

Although MCMV can infect ocular tissues of mice, necrotizing retinopathy has not been described in previous reports of ocular infection. This study was designed to determine whether MCMV can produce necrotizing infection of the retina, to assess the

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role of immunosuppression in the pathogenesis of MCMV ocular disease, and to compare the histologic features of MCMV-associated retinopathy to other necrotizing viral retinopathies in mice.

**Materials and Methods**

One hundred fifty 4-week-old female Swiss-Webster mice (Jackson Laboratories, Bar Harbor, ME) were studied. Mice were anesthetized before inoculations with a 0.03-ml intramuscular injection of a 1:1 mixture of ketamine (100 mg/ml) and xylazine (20 mg/ml). By means of a Hamilton syringe, 138 mice received a 0.01-ml inoculation of 10^5 plaque-forming units (PFU) of salivary gland-passed Smith strain MCMV (stored previously at -70°C) in the left eye. The inoculum was delivered through a 30-gauge needle inserted at the limbus and placed behind the lens. After inoculation, the needle was carefully retracted from the eye to minimize escape of fluid.

Ninety-eight MCMV-inoculated mice were given 0.2 mg/g of cyclophosphamide intraperitoneally at the time of viral inoculation and 0.1 mg/g of cyclophosphamide intraperitoneally every 5 days thereafter. The remaining 40 MCMV-inoculated mice were observed without immunosuppression as controls. Twelve mice were immunosuppressed according to the same regimen after receiving an intraocular injection of sterile phosphate-buffered media; they were studied as additional controls.

Mice were given filtered distilled water (acidified to pH 2.5 using hydrochloric acid) and sterilized food ad libitum. Bedding, food, and water were resterilized in an autoclave every 5 days.

Surviving MCMV-inoculated mice were chosen randomly and killed by cervical dislocation on days 5, 10, 15, and 20 after inoculation. Animals that were moribund on days 16, 17, 18, and 19 and not expected to survive to day 20 also were killed. All surviving immunosuppressed mice that did not receive viral inoculations were killed on day 20.

Inoculated eyes were enucleated immediately after death. A total of 76 eyes (40 from MCMV-inoculated, immunosuppressed animals; 28 from MCMV-inoculated, nonimmunosuppressed animals; and 8 from media-inoculated, immunosuppressed animals) were chosen randomly, fixed in formalin, and embedded in paraffin. The remaining eyes, which had been designated for other investigations, were not used for calculation of disease incidence. Four-micron thick sections were stained with hematoxylin and eosin. At least two step levels were examined for each eye by light microscopy for the presence of retinal necrosis, cytomegalic cells, viral inclusions, and inflammatory cells. Contralateral eyes of MCMV-inoculated animals were not examined.

This study conformed to the ARVO Resolution on the Use of Animals in Research.

**Results**

Within 1 week of MCMV inoculation, all immunosuppressed animals had clinical signs consistent with systemic viral infection, including ruffled fur, weight loss, and lethargy. Many were moribund at the time they were killed. None of the control animals had clinical signs of systemic MCMV infection by day 20. Culture or histopathologic evidence of nonocular MCMV infection was not sought.

In the MCMV-inoculated, immunosuppressed group, mortality increased throughout the course of the study (Table 1). Mortality remained low during the course of the study in MCMV-inoculated mice that did not receive immunosuppression and in mice that were not inoculated with virus, despite immunosuppression.

In immunosuppressed mice the earliest sign of intraocular MCMV infection was observed on day 5; numerous enlarged histiocytes with prominent intranuclear inclusions in uveal tissue were present at the site of injection (Fig. 1). Retinal necrosis was not observed in any eyes enucleated on day 5.

On day 10 necrosis, inflammation, and cytomegalic cells with viral inclusions were seen in the iris (Fig. 2), ciliary body, choroid, and extrascleral tissues. De-
Fig. 1. Inoculated eye, day 5. Light micrograph shows presence of numerous enlarged histiocytes with prominent intranuclear inclusions (arrows) in uveal tissue at the site of injection (hematoxylin and eosin, original magnification ×50, inset ×780).

spite infection of uveal tissue, the retinas were not involved in most eyes. Only one of 12 eyes examined on day 10 had retinal necrosis; a discrete focus of destruction, more severe in the outer retinal layers, was seen (Fig. 3).

On day 15, more severe disease of the iris and choroid was noted, and three of five eyes examined had retinal disease. Retinal pigment epithelial infection was occasionally seen without involvement of the overlying retina. Retinal disease was limited to isolated foci of full-thickness necrosis (Fig. 4). In one eye there was also a discrete retinal lesion characterized by cytoplasmic swelling of cells in the outer layers, which may represent early MCMV retinopathy. The choroid and retinal pigment epithelium underlying this lesion showed acute and chronic inflammation, necrosis, and occasional cytomegalic cells with intranuclear inclusions.

Between days 16 and 20, six of 16 eyes had retinal necrosis involving all layers of the retina. In one eye the entire retina was necrotic (Fig. 5). In the other eyes there were multiple foci of necrosis throughout the retina. Retinal disease was characterized by full-thickness retinal necrosis, scattered cytomegalic cells,
intranuclear and intracytoplasmic viral inclusions, and acute and chronic inflammation. All animals had evidence of uveal tract infection, and acute and chronic inflammatory cells were present in all ocular tissues. No relationship was found between the severity of systemic disease and the presence of MCMV retinopathy.

In mice that were given MCMV inoculations but were not immunosuppressed, there was acute and chronic inflammation at the injection site and in the vitreous, iris, ciliary body, and rarely the inner retinal layers. Hemorrhage was also noted in some eyes. Retinal necrosis was not observed and no cytomegalic cells were seen in any eyes.

Eyes from immunosuppressed mice that were given intraocular injections of sterile media showed focal inflammation and hemorrhage at the limbal injection site only. No retinal necrosis was observed.

The percentage of mice that developed necrotizing MCMV retinopathy at various times after inoculation is summarized in Table 2. For animals surviving to days 16–20, the difference in prevalence of necrotizing MCMV retinopathy between immunosuppressed animals (6 of 16, 37.5%) and nonimmunosuppressed animals (0 of 19) was statistically significant \( P = 0.00493 \), Fisher's exact two-tailed test.

Discussion

A distinct, species-specific DNA virus, MCMV is similar in ultrastructural and biologic features to
human CMV. It infects many organs, including liver, spleen, thymus, bone marrow, lung, kidney, salivary glands, and cells of the central nervous system. The clinical manifestations of disseminated infection include lethargy, hunching, weight loss, and ruffled fur. Infections can be fatal.

Ocular MCMV infections are well known, but in previous reports, disease was found to involve primarily the uveal tract, thereby limiting its utility as a model of human disease. Schwartz and associates injected 10^4 PFU of Smith strain MCMV behind the lens of nonimmunosuppressed, 3- to 4-week-old Swiss CD-1 mice. From postinoculation days 2–6 necrosis, inflammation, and cytomegalic cells were found in the uvea. Morphologic alterations without necrosis or inflammation were noted in the outer retin...
Table 2. Incidence of murine cytomegalovirus retinopathy

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<th>Group</th>
<th>Days after inoculation</th>
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<tr>
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<td>+MCMV, +CP</td>
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Data are expressed as number of mice with retinal necrosis divided by the number of mice examined (percentage).

ina that the investigators attributed to infection of the underlying retinal pigment epithelium.

Bale and associates injected 2 × 10⁴ PFU of Smith strain MCMV into the peritoneal cavity of 3-week-old nonimmunosuppressed Swiss-Webster mice; they were later able to isolate virus from ocular tissue and fluids. They did not observe pathologic changes in retinal tissue, however. Virus could also be recovered from explant cultures of the ocular tissue as long as 120 days after inoculation, which suggests that MCMV may establish latent infections in the eye.

Hayashi and associates injected Smith strain MCMV (0.012–0.025 ml of 10⁶/ml median tissue culture infective dose) into the anterior chamber of 12- to 18-day-old nonimmunosuppressed ICR/Sic mice. They were able to identify viral antigen in the iris, choroid, retina, and sclera by immunofluorescence techniques up to 2 weeks after inoculation. Although they observed transient retinitis, uveitis, and scleritis, they saw no retinal necrosis. When virus was injected into older mice (8–10 weeks), virus could be identified in the iris only.

Necrotizing CMV retinopathy in humans appears to occur only in severely immunosuppressed patients. In those receiving immunosuppressive drug therapy, CMV retinopathy resolves when it is discontinued. Rare reports of CMV retinopathy in immunocompetent adults have not been confirmed. This study suggests that immunosuppression is also necessary for development of necrotizing MCMV retinopathy in adult mice; retinal necrosis occurred only in animals treated with cyclophosphamide. Mortality was also higher in immunosuppressed mice, and only immunosuppressed mice developed clinical signs of disseminated infection.

Cyclophosphamide was chosen for immunosuppression because of its known effects on acute and latent MCMV infections in mice. Shanley and associates found increased viral titers in lung tissue when mice were given cyclophosphamide every 5 days after viral inoculation. Selgrade and associates found increased mortality and increased viral titers in target tissues when mice were injected with cyclophosphamide 1–3 days after viral inoculation. These effects were thought to be related to altered natural killer cell function. Mayo and associates found that single or serial injections of cyclophosphamide resulted in reactivation of presumably latent MCMV infections.

Herpes simplex virus type 1 (HSV-1) infection in mice has been used previously as a model of necrotizing viral retinopathy. When HSV-1 is injected into the anterior chamber of one eye of immunocompetent BALB/c mice, a necrotizing retinopathy develops in the contralateral, un inoculated eye after spread of virus through the central nervous system. The retina of the inoculated eye does not develop retinal necrosis, which has been attributed to protective immune mechanisms. Immunosuppressed BALB/c mice develop bilateral retinal necrosis. If the virus is inoculated translimbally or into the vitreous of immunocompetent BALB/c mice, retinal necrosis also occurs in the inoculated eye. Bilateral retinal necrosis occurs in outbred ICR mice inoculated with HSV-1 via a limbal injection into the anterior chamber. Similarly, it is likely that ocular MCMV disease varies with host susceptibility factors related to different animal strains, with immune status, and with the route of viral inoculation.

These variables may account for differences in the patterns of MCMV ocular disease observed between this study and previously published reports describing ocular MCMV infection.

There are several differences between MCMV- and HSV-1-associated retinal necrosis. HSV-1 causes more rapid and more severe retinal disease than MCMV. Susceptible animals develop retinal disease in HSV-1-inoculated eyes within 4–7 days, and the retinas are totally destroyed by postinoculation day 10. In contrast, necrotizing MCMV retinopathy occurred in only 25% of virus-inoculated eyes within 4–7 days, and the retinas are totally destroyed by postinoculation day 10. In this study, the earliest case of MCMV retinopathy was seen at day 10. The incidence of MCMV retinopathy was greatest among animals examined at day 15 (60%), although the numbers are too small to draw reliable conclusions about incidence.
The distribution of virus and the course of infection differ between MCMV and HSV-1 retinopathies. We found extensive uveal disease in all MCMV-inoculated eyes, whether or not they were treated with cyclophosphamide. Uveal tissue does not appear to be a major site of HSV-1 infection. The virus can be identified in iris and ciliary body after anterior chamber inoculation, but it diminishes over time in normal mice. A severe inflammatory response can be seen in the choroid adjacent to areas of retinal necrosis, but neither viral particles nor antigens are seen on histopathologic examination.

Retinal pigment epithelial infections with MCMV occur in the absence of obvious retinal disease. Retinal pigment epithelial infection with HSV-1 can be seen in the absence of retinal infection, but it is usually seen in eyes with extensive retinal necrosis.

Retinal necrosis results from productive viral infection, rather than immune-mediated mechanisms, in both HSV-1 and MCMV disease. We identified MCMV infection of retinal tissue by the presence of cytomegalic cells and viral inclusions, shown to be reliable indicators of infection.

Disruption of the outer nuclear and photoreceptor layers is the first manifestation of HSV-1 retinopathy. Histopathologic studies, however, indicate that Mueller cells and ganglion cells in the inner retinal layers are the primary sites of infection. Mueller cell processes form the inner limiting membrane, which would be the first structure to be encountered by infectious particles in the vitreous. Disruption of the Mueller cell processes, which also provide support for other cells of the retina, is hypothesized to be the cause for extensive disruption of the normal retinal architecture. In contrast, the distribution of cytomegalic cells in early foci of necrosis indicates that MCMV infection first involves the outer layers of the retina. The early destruction of outer retina may reflect the direct extension of infection from uveal tissue to the retina. It may, however, also reflect different susceptibilities of various retinal cell types to infection, or it may be an artifact of the inoculation technique. It is possible that the trauma associated with injection allowed inoculum to dissect between the neural retina and underlying tissues. (The histopathologic studies of HSV-1 infection in mice we discussed followed anterior chamber inoculation, thereby avoiding the possibility of mechanical effects on the retina.)

Further studies will be required to determine the natural history of this MCMV retinopathy.

Use of MCMV retinopathy as a model of human disease may be limited by several factors. The incidence of necrotizing retinal infection is fairly low, and mortality is high at the time necrosis is seen. Several histopathologic features of MCMV ophthalmic infections differ from human CMV infections. In this and previous studies, severe and extensive choroidal infections are common and may be the initial site of infection. In human disease the retina is the primary tissue infected by CMV. Choroidal inflammation is seen only in association with extensive overlying retinal and retinal pigment epithelial infection. Spread of productive viral infection to the choroid is minimal and occurs infrequently. Infection of the ciliary body has been reported but is rare.

The extent to which MCMV infections in mice can be used as a model for the study of antiviral drug therapy remains uncertain, because MCMV and human CMV appear to have different sensitivities to various antiviral drugs. Human CMV in vitro shows little inhibitory effect from acyclovir, but MCMV is sensitive. A marked difference in sensitivity of human CMV and MCMV using plaque-reduction experiments. The median effective dose (ED50) was 0.4–0.6 μM for MCMV, but it was 10.0–77.6 μM for human CMV. Because of species specificity, the embryonic fibroblasts used in the assay system were different, a factor that can affect ED50. Nevertheless, the difference appeared to be real; using data about the effect of host cells on ED50 for HSV, which grows in both human and mouse embryonic fibroblasts, the investigators concluded that human CMV is sevenfold less sensitive to acyclovir than MCMV.

Acyclovir is more active than ganciclovir against MCMV in vitro, another acyclic nucleoside analogue of 2'-deoxyguanosine, which has recently been approved by the Food and Drug Administration for treatment of human CMV retinopathy. Conversely, ganciclovir is a much more potent inhibitor of human CMV than acyclovir in vitro.

It may be difficult to extrapolate in vitro results to clinical situations, however. Shanley and associates, for example, showed that although acyclovir was more active against MCMV than ganciclovir in vitro, ganciclovir was more effective in reducing tissue titers of MCMV in vivo. Nevertheless, limited clinical data suggest that the response to drugs will differ between MCMV and human CMV. Acyclovir has been shown to be effective for the treatment of disseminated MCMV infections in mice, the drug reduces viral titers in target organs other than salivary glands and reduces mortality. Acyclovir is not effective for the treatment of CMV retinopathy and other tissue-invasive infections in humans, although prophylactic
use may increase survival and prevent development of CMV infections in bone marrow and kidney allograft recipients. Whether drug therapy of MCMV infections will provide useful information about the treatment of human disease requires further study.

Additional investigations using techniques such as electron microscopy, immunohistochemical staining, and in situ nucleic acid hybridization will be needed to confirm retinal infection and viral distribution in this model. Our results, however, indicate that ocular MCMV infection in immunosuppressed adult mice can cause a necrotizing retinopathy, and therefore it may be useful for studying some aspects of human infection. Nevertheless the many differences between MCMV and human CMV, and between various features of the ocular infections they produce, require that further comparative studies be done to determine the extent to which MCMV retinopathy can be used as a reliable model of human disease.

Key words: immunosuppression, murine cytomegalovirus, opportunistic infection, retinitis, uveitis

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