Spread of Murine Cytomegalovirus to Inner Ocular Structures Following Disruption of the Blood–Retina Barrier in Immunosuppressed BALB/c Mice

Yanping Duan, Roy Hernandez, Ling Pang, and Sally S. Atherton

Purpose. The aims of this study were to determine whether disruption of the blood–retina barrier (BRB) increases spread of murine cytomegalovirus (MCMV) to the eye after intraperitoneal inoculation and whether systemic immunosuppression influences the location of MCMV in the ocular compartment.

Methods. The BRB of the left eye of normal and immunosuppressed mice was disrupted by supraciliary inoculation of tissue culture medium followed 2 hours later by intraperitoneal injection of MCMV. Plaque assay of homogenized ocular tissue was used to determine the frequency of virus-positive eyes and the titer of virus in the eyes. β-galactosidase staining of frozen sections was used to locate virus in the eyes.

Results. In nonimmunosuppressed mice, the frequency of virus isolation, as well as the titer of virus, were significantly higher in eyes in which the BRB had been disrupted. Although the frequency of virus isolation was the same in both eyes of immunosuppressed mice, the titer of virus was significantly higher in the eye in which the BRB had been disrupted. The most striking result was that the location of virus was different in the nondisrupted eyes of immunosuppressed mice than it was in the disrupted eyes of immunosuppressed mice. In the former, virus was seen only in the outer ocular structures (conjunctiva, sclera, lacrimal gland), whereas in the latter, virus was observed in the retina and anterior segment (iris, ciliary body) as well as the outer ocular structures.

Conclusions. The results of these studies suggest that ocular damage followed by increased spread of virus to and within the eye during systemic infection with CMV may be one mechanism by which development of CMV retinitis is facilitated in patients with acquired immune deficiency syndrome. Invest Ophthalmol Vis Sci. 1996;37:935–940.

Cytomegalovirus (CMV) is the most common cause of infectious retinitis and blindness in immunosuppressed patients, particularly those with acquired immune deficiency syndrome (AIDS). Spread of CMV into the eye during systemic CMV infection is one mechanism by which CMV retinitis is likely to occur in immunocompromised human patients. However, in the mouse, retinitis is not observed after intraperitoneal inoculation of MCMV, even though latent virus has been demonstrated in ocular tissue after systemic infection, suggesting that MCMV does reach the ocular compartment during systemic infection. The reason retinitis develops in immunosuppressed human patients with systemic CMV infection and but not in mice with systemic infection after intraperitoneal inoculation of MCMV is unclear but may be associated with breakdown of the blood–retina barrier (BRB) in the former. This idea is supported by results from studies in immunosuppressed mice in which disruption of the BRB (as a result of injection of MCMV by the supraciliary route) results in retinitis2,3 and from studies in human patients with AIDS in whom infection of the retina or of the retinal vasculature by human immunodeficiency virus type 1 (HIV-1) alone4 or in combination with other herpesviruses, such as CMV or HHV-6, has been suggested to damage the BRB.5,6 In addition, results of a recent study7 showed that the neurosensory retina was not infected after immunosuppressed mice were infected with MCMV by the intravenous route. Because the BRB of the mice injected with virus intravenously presumably was not disrupted, these results provide further support for the idea that disruption of the BRB may be required before MCMV can infect the inner retina in immunosuppressed mice.

Based on these observations, we hypothesized that disruption of the BRB (by supraciliary injection of tissue culture medium) would facilitate spread of MCMV to ocular tissue during systemic MCMV infection after intraperitoneal inoculation. To test this hypothesis, the BRB of immunosuppressed and nonimmunosuppressed mice was disrupted by supraciliary injection of tissue culture medium, and MCMV was inoculated by the intraperitoneal route. The number of virus-positive eyes, the titer of ocular virus, and the location of virus in the ocular compartment were determined. Results of these studies suggest that disruption of the BRB increases virus spread to the eye, irrespective of the immune status of the host, and that disruption of the BRB in immunosuppressed mice correlates with spread of virus to the inner ocular structures.
METHODS. Virus. Two viruses were used in these experiments. The original stock of MCMV was obtained from Dr. Robert Levy (Department of Microbiology and Immunology, University of Miami School of Medicine, Miami, FL), and an MCMV recombinant (RM461) was kindly provided by Drs. 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 MCMV genome at the HindIII L/J site just downstream of immediate-early gene 2 (ic2). RM461 expresses the E. coli lacZ gene product, β-galactosidase (β-gal), as an immediate-early viral gene product during the viral replication cycle. Stocks of both viruses were propagated from salivary gland of MCMV-infected BALB/c mice as described previously. Stocks of both viruses were propagated from salivary glands of MCMV-infected BALB/c mice as described previously and titered by plaque assay on monolayers of mouse embryo fibroblast cells (Bio Whittaker, Walkersville, MD) grown in Dulbecco’s minimal essential medium (DMEM) containing 10% fetal calf serum (HyClone, Logan, UT). Virus stocks were stored at −70°C. The average titer of the virus stocks was between 10^6 and 10^7 pfu/ml. A fresh aliquot of stock virus was thawed and diluted immediately before each experiment.

Mice. Female BALB/c mice 4 to 6 weeks of age (Taconic, Germantown, NY) were used in all experiments. Mice were immunosuppressed by intramuscular injection of sterile methylprednisolone acetate suspension (1.0 mg/10 g body weight) (Upjohn, Kalamazoo, MI) every 4 days as previously described. Control mice were not immunosuppressed. For supraciliary injection (to disrupt the BRB), mice were anesthetized by sodium pentobarbital (0.65 mg/10 g body weight) (Upjohn, Kalamazoo, MI) every 4 days as previously described. Control mice were not immunosuppressed. For supraciliary injection (to disrupt the BRB), mice were anesthetized by sodium pentobarbital (0.65 mg/10 g body weight) (Upjohn, Kalamazoo, MI) every 4 days as previously described. Control mice were not immunosuppressed.

For virus recovery studies, the eyes were homogenized in 1.0 ml of serum-free tissue culture medium using a hand-held tissue homogenizer (Biospec Products, Racine, WI). The amount of virus in the tissues was determined by plaque assay and expressed as pfu/ml of tissue homogenate. For studies to determine the location of ocular virus, eyes were embedded in OCT, frozen at −70°C, and serial 10-μm frozen sections were prepared on a cryostat. The frozen sections were fixed with 0.5% glutaraldehyde in phosphate-buffered saline (PBS, 1 mM MgCl₂, 137 mM NaCl, 4 mM KCl, 8 mM Na₂HPO₄, and 1.5 mM KH₂PO₄) for 15 minutes and then washed with PBS twice for 10 minutes each time. The sections were reacted with X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Gibco BRL, Gaithersburg, MD), a chromogenic substrate for β-gal. After incubation at room temperature in substrate solution (PBS containing 2.4 mM X-gal, 12.5 mM K₃Fe(CN)₆, and 12.5 mM K₄Fe(CN)₆·3H₂O), the sections were washed with PBS and counterstained with safranin-O (Sigma, St. Louis, MO). Tissue sections were dehydrated, mounted, and examined microscopically for blue-stained cells, indicative of virus infection. Results from virus recovery studies were analyzed for significance (P < 0.05) using the Mann-Whitney test, and β-gal staining results were analyzed for significance by chi-square analysis.

RESULTS. Virus Recovery. To determine whether disruption of the BRB affects the titer of virus in the eye after intraperitoneal inoculation of MCMV in nonimmunosuppressed BALB/c mice, animals in groups 1 and 2 were killed 1 week after intraperitoneal injection of 5 × 10⁴ pfu. As shown in Table 1, among mice in group 1, significantly more eyes with a disrupted BRB were virus positive (11 of 14) than eyes with an intact BRB (5 of 14). Among mice in group 2, the number of virus-positive eyes was not significantly different from the number of virus-positive, nondisrupted right eyes of mice group 1 (3 of 10 versus 5 of 14). Although the amount of virus recovered from any eye was generally low, the mean titer of virus recovered from the left eyes of mice in group 1 was significantly higher than that recovered from the fellow right eye and also from both eyes of the mice in group 2.
TABLE 1. Recovery of MCMV From Eyes 1 Week After Intraperitoneal Inoculation

<table>
<thead>
<tr>
<th>Group 1*</th>
<th>Group 2†</th>
<th>Group 3‡</th>
<th>Group 4§</th>
</tr>
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<tbody>
<tr>
<td>Left Eye</td>
<td>Right Eye</td>
<td>Left Eye</td>
<td>Right Eye</td>
</tr>
<tr>
<td>Number positive/total</td>
<td>11/14</td>
<td>5/14</td>
<td>3/10</td>
</tr>
<tr>
<td>Mean titer PFU/ml</td>
<td>21§</td>
<td>9</td>
<td>8#</td>
</tr>
</tbody>
</table>

* Disruption of the BRB of the left eye 2 hours before intraperitoneal inoculation of 5 x 10⁴ PFU of MCMV.
† Intraperitoneal inoculation of MCMV only; no disruption of BRB.
‡ Frequency of virus recovery in the left eye significantly different from the frequency of virus recovery in the right eye and from both eyes of mice in Group 2 (P < 0.05, chi-square analysis).
§ Titer of virus in the left eye significantly different from the titer of virus in the right eye and from both eyes of mice in Group 2 (P < 0.05, Mann-Whitney test).
# Mean virus titer significantly different between comparable eyes from mice in Group 2 and Group 4 (P < 0.05, Mann-Whitney test).

The results in nonimmunosuppressed BALB/c mice suggested that disruption of the BRB in an immunologically intact BALB/c mouse correlates with an increase in the number of virus-positive eyes. However, in humans, infections with CMV generally are observed in immunocompromised persons. To determine whether immunosuppression affects ocular spread of virus and/or the titer of virus in either eye, the left BRB was disrupted in immunosuppressed mice (group 3). Control mice for these experiments were immunosuppressed, but the supraciliary space of mice in this group was not injected with DMEM-0 (group 4). As shown in Table 1, virus was recovered from most of the eyes of immunosuppressed mice irrespective of the status of the BRB at the time of intraperitoneal inoculation of MCMV. However, as was observed in the studies with nonimmunosuppressed mice in groups 1 and 2, the titer of virus in left eyes of mice in group 3 was significantly higher than in the fellow right eye or in both eyes of mice in group 4 in which neither eye had been injected with DMEM-0 before intraperitoneal inoculation of virus. As shown in Table 1, the average virus titer was significantly higher in immunosuppressed mice when the results from the left eyes of immunosuppressed and nonimmunosuppressed mice were compared (group 1 versus group 3) and also when the results of comparable eyes of mice in group 2 and group 4 were compared.

TABLE 2. β-Gal Staining of MCMV in Ocular Tissue of Immunosuppressed Mice

<table>
<thead>
<tr>
<th>Time (PI) (weeks)</th>
<th>Group 3*</th>
<th>Group 4†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer Ocular Structures</td>
<td>Intraocular Structures</td>
<td>Outer Ocular Structures</td>
</tr>
<tr>
<td>Retina</td>
<td>AS</td>
<td>Retina</td>
</tr>
<tr>
<td>1</td>
<td>10/10</td>
<td>3/10</td>
</tr>
<tr>
<td>2</td>
<td>10/10</td>
<td>5/10</td>
</tr>
</tbody>
</table>

* Disruption of the BRB of the left eye 2 hours before intraperitoneal inoculation of 5 x 10⁴ PFU of MCMV.
† Intraperitoneal inoculation of MCMV only, no disruption of BRB.
‡ Conjunctiva, sclera, lacrimal gland.
§ Anterior segment (iris, ciliary body).
∥ Frequency of β-gal staining significantly different in this group than the same site in eyes of mice receiving only an intraperitoneal inoculation of virus (P < 0.05, chi-square analysis).
FIGURE 1. Photomicrographs illustrating the location of β-gal positive, virus-infected cells in the eye 2 weeks after intraperitoneal inoculation of $5 \times 10^4$ pfu of murine cytomegalovirus (RM461). In the immunosuppressed mouse with an intact blood–retina barrier at the time of virus inoculation, virus was observed in the sclera (A) but not in the choroid or retina. In the immunosuppressed mouse with a disrupted blood–retina barrier, virus was seen in the retinal pigment epithelium and sclera (B) and in the outer nuclear layer and photoreceptors (C). Magnification, ×670.
cells in the retina and retinal pigment epithelium with loss of the retinal architecture) was not observed in any eye at either time point (not shown).

**DISCUSSION.** The result of the studies presented herein demonstrate that disruption of the BRB correlated with increased spread of MCMV to the eye irrespective of the immune status of the host, mean virus titers were higher in eyes of immunosuppressed mice with or without a disrupted BRB, and virus was observed in the inner ocular structures only in eyes of immunosuppressed mice in which the BRB had been disrupted.

The first two results follow what might be predicted to occur based on previous studies in nonimmunosuppressed and immunosuppressed mice infected with MCMV by a variety of routes.\(^\text{10,11}\) Although the intraperitoneal route of infection in a mouse does not mimic a route by which human patients normally are infected with CMV, virus is observed in circulating leukocytes after intraperitoneal inoculation of MCMV.\(^\text{16}\) In CMV-infected human patients, peripheral blood leukocytes are frequently virus positive.\(^\text{13}\) The results of this study demonstrate that disruption of the BRB in nonimmunosuppressed BALB/c mice correlates with an increase in the number of virus-positive eyes and with recovery of a low, but significantly higher, titer of virus in the eyes with a disrupted BRB. The increase in virus titer may result from more virus-infected cells gaining access to the eye or from trapping of a few virus-infected cells in eyes with a disrupted BRB followed by limited virus replication in such eyes. The finding that a slightly higher titer of virus was recovered from eyes with a disrupted BRB supports the second idea. However, the result that virus replication was generally low is probably not surprising because high titers of virus are not recovered from the eye, and retinitis does not develop in nonimmunosuppressed mice after inoculation of \(5 \times 10^5\) pfu directly into the supraciliary space.\(^\text{9}\) In contrast to the findings in nonimmunosuppressed mice, most of the eyes of immunosuppressed mice were virus positive irrespective of the status of the BRB. This result likely reflects the observation that the frequency of virus-infected leukocytes in immunosuppressed mice is higher than in nonimmunosuppressed mice.\(^\text{14}\)

The finding that the location of virus was different in eyes with a disrupted BRB than in nondisrupted eyes was unexpected. Among immunosuppressed mice, only eyes with a disrupted BRB contained virus in the inner ocular structures (retina, retinal pigment epithelium, iris, ciliary body), whereas in intact eyes of immunosuppressed mice, virus was observed only in the outer ocular structures (conjunctiva, sclera). The difference in the ocular location of virus between the eyes of immunosuppressed mice with an intact BRB (outer ocular structures) and that of immunosuppressed mice with a disrupted BRB (inner ocular structures) suggests that disruption of the BRB may be necessary before the inner ocular structures become accessible to virus. By extrapolation, this result suggests that even if a high percentage of circulating leukocytes is virus positive, infection of the inner ocular structures may not be possible without disruption of the BRB. Although these studies do not address the mechanism by which the BRB in an immunocompromised patient may be disrupted and allow ingress of virus, results from other studies suggest that such disruption could occur. Both HIV-1 and HHV-6 have been observed in the retina, retinal vasculature, or both,\(^\text{5,6}\) and the presence of either or both of these viruses in the retinal vasculature might affect the integrity of the BRB.

Finally, in spite of the location of virus in the inner ocular structures and the significantly higher titer of virus in eyes with a disrupted BRB, none of the immunosuppressed mice had histopathologic evidence of retinitis. From the current study and from results of previous studies, it appears to be difficult to induce clinical disease even if eyes are MCMV-positive. The failure of ocular virus to induce disease may result from several factors. First, virus may be located at a site or sites in which it cannot replicate. Second, the amount of virus may be too low to cause disease even in an immunosuppressed mouse. Third, the 2-week time span of these experiments may not have been long enough to allow an initial small inoculum of virus to replicate and cause disease. Fourth, BRB was disrupted 2 hours before virus inoculation, and the BRB at the site of injection may have been reestablished and prevented continued seeding of the eye by circulating virus-infected leukocytes. Studies are in progress to identify factors besides immunosuppression and disruption of the BRB that might contribute to the ability of MCMV to gain access to the eye during systemic infection after intraperitoneal injection or to influence its ability to replicate and cause retinitis once it has entered the ocular compartment.

**Key Words**
blood–retina barrier, cytomegalovirus, immunosuppression, BALB/c mouse, virus infection

**References**

Comparison of Three Tonometers for Measuring Intraocular Pressure in Rabbits
Lisa S. Abrams, Susan Vitale, and Henry D. Jampel

Purpose. Rabbits are used commonly for the evaluation of drugs and surgery to lower intraocular pressure (IOP). The authors compared the accuracy and variability of three tonometers for measuring IOP in rabbits.

Methods. The anterior chamber of adult rabbits was cannulated with a 25-gauge needle connected to an elevated bottle of balanced salt solution. The bottle was raised and lowered to control IOP. A fluid-filled pressure transducer also was placed in the anterior chamber. Intraocular pressure was decreased in increments of 5 to 10 mm Hg from 50 to 0 mm Hg and was recorded with each of these instruments: the hand-held applanation tonometer, the Tono-pen XL, and the pneumatonometer.

Results. The Tono-pen XL underestimated the IOP, whereas the pneumatonometer slightly overestimated IOP. Under closed stopcock conditions, at IOPs between 3 and 30 mm Hg, the Tono-pen XL was as accurate as the pneumatonometer but had a smaller variance; the hand-held applanation tonometer had lower accuracy and higher variability.

Conclusions. The Tono-pen XL is the tonometer of choice for measuring IOP in rabbits within the range of IOP 3 to 30 mm Hg. All tonometers were less accurate when the IOP was elevated markedly. Invest Ophthalmol Vis Sci. 1996;37:940–944.

Rabbits often are used in the evaluation of new medications and surgical procedures for glaucoma. Although the measurement of intraocular pressure (IOP) in rabbits has been extensively studied, the optimal method has not been established. The pneumatonometer is frequently used, but it has the theoretical disadvantage of being a high displacement tonometer. The hand-held applanation tonometer has only occasionally been used. The Tono-pen XL (Mentor, Norwell, MA), a miniaturized Mackay-Marg tonometer, has recently come into use as a convenient, portable, accurate instrument for the measurement of IOP in humans, and it might be a good choice for measuring IOP in rabbits. In the current study, we sought to compare the usefulness of the pneumatonometer,