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Murine cytomegalovirus (MCMV) spreads to and replicates in the retina after endotoxin-induced disruption of the blood-retinal barrier of immunosuppressed BALB/c mice

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The goal of this study was to determine whether disruption of the blood-retinal barrier (BRB) facilitates spread of MCMV to the retina in immunosuppressed (IS) BALB/c mice. IS mice were inoculated intravenously (i.v.) with murine cytomegalovirus (MCMV) or with macrophages infected with MCMV for 4 days *in vitro*. The BRB was disrupted by injection of sodium iodate (i.v.) or lipopolysaccharide (LPS, i.v. or anterior chamber). Frozen sections of ocular tissue were examined for MCMV antigens. The results showed that MCMV-infected cells were observed only in the choroid and ciliary body in IS mice with an intact BRB. After LPS injection, a few positive cells were observed in the retina of IS mice after i.v. injection of MCMV. In lipopolysaccharide (LPS)-treated IS mice, a few PKH-26–positive macrophages or MCMV-positive cells were observed in the retina at 1 or 2 days after injection of macrophages. No PKH-26–positive cells or virus-infected cells were noted in the retina of phosphate-buffered saline (PBS)-treated mice. Ten days after injection of virus-infected macrophages, MCMV-infected cells were observed in choroid and ciliary body of both LPS- and PBS-treated mice, but they were observed in the retina only in LPS-treated mice. The results support the idea that disruption of the BRB allows MCMV to spread to the retina of IS mice and that monocytes/macrophages disseminate MCMV to the retina in mice with a disrupted BRB. By extrapolation, damage to the BRB in immunosuppressed patients may facilitate spread of CMV-infected monocytes/macrophages to the retina. *Journal of NeuroVirology* (2005) **11,** 365–375.

Keywords: AIDS; animal model; blood-retinal barrier; cytomegalovirus; immunosuppression; retinitis

Introduction

Cytomegalovirus (CMV) infections are commonly observed in immunosuppressed patients such as organ transplant patients or patients with the acquired immunodeficiency syndrome (AIDS) (Hoover *et al*, 1993). Prior to the availability of highly active antiretroviral therapy (HAART), most of the CMV disease in AIDS patients was retinitis (Hoover *et al*, 1993, 1996; Gallant *et al*, 1992) and estimates of the percentage of patients with AIDS who developed CMV retinitis during the course of their HIV infection were as high as 40% (Jabs *et al*, 1992; Cohen, 1995).

Murine cytomegalovirus (MCMV) has been used to induce retinitis in the BALB/c mouse to provide insight into the pathogenesis of CMV retinitis in human patients. Although retinitis can be induced by intraocular inoculation of MCMV (Atherton *et al*, 1991, 1992; Bigger *et al*, 2000; Zhang and Atherton, 2002), during systemic infection, MCMV disseminated only to the ciliary body and/or uveal tract of the eye even in mice that had been deeply immunosuppressed by irradiation and treatment with antilymphocyte

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serum (Gao *et al*, 1996) or by retrovirus infection and systemic MCMV infection (Dix, 1998). Neither infection of the neurosensory retina nor clinical retinitis was observed. These results suggest that systemic immunosuppression is not the only determinant in development of CMV retinitis. This idea is supported by the observation that prior to the availability of HAART, a large percentage of AIDS patients developed retinitis whereas in immunosuppressed adult transplant recipients, the incidence of retinitis is low. For example, only 1% to 2% of renal transplant patients develop CMV retinitis (Espana-Gregori *et al*, 1994; Brown *et al*, 1988). In another study, only 10 cases of HCMV retinitis were identified during a 14-year follow-up of 5721 bone marrow transplant patients (Crippa *et al*, 2001). In addition to immunosuppression, identification of other factors that contribute to development of CMV retinitis could lead to new therapies to prevent or treat CMV retinitis in immunosuppressed patients.

Results

Lymphocyte depletion

The extent of splenic lymphocyte depletion was assessed 14 days after immunosuppression. As shown in Table 1, after MCMV infection of nonimmunosuppressed mice, the total number of spleen cells as well as the number of CD4+ and CD8+ lymphocytes was approximately twice that of the non–MCMV-infected control mice. After treatment with methylprednisolone alone (IS [immunosuppressed] group), more than 98% of spleen cells were depleted. Treatment with T cell specific antibodies in addition to methylprednisolone (deeply IS group) resulted in slightly more depletion of total spleen cells (2.1×10^5 versus 6.8×10^5 in the IS group). However, 26 times more CD4+ (1.4×10^4 versus 3.7×10^5) and 32 times more CD8+ cells (6.9×10^3 versus 2.2×10^5) were depleted in the deeply IS group than in the IS group.

Distribution of MCMV in the eyes of IS mice after intravenous infection

Fourteen days after intravenous (i.v.) inoculation of RM461 into deeply IS mice, early antigen (EA) positive cells were observed in the choroid of 8 of 10 eyes and in the ciliary body of 1 of 10 eyes; β-gal–positive cells were observed in the choroid of 8 of 10 eyes and in the ciliary body of 2 of 10 eyes (Figure 1A, B). No EA-positive or β-gal–positive cells were noted in the ganglion cell layer, the nuclear layers or the retinal pigment epithelium (RPE). No MCMV-positive cells were observed in the eyes of nonimmunosuppressed, MCMV-infected mice (not shown). To determine whether insertion of the *E.scherichia coli* LacZ gene affected the ability of RM461 to spread to the eye, 5×10^3 plaque-forming unit (PFU) of the parent virus, K181, was injected i.v. into deeply IS mice. Fourteen days later, the mice were sacrificed and ocular sections were stained for MCMV EA. The results were similar to those seen in RM461-infected immunosuppressed mice. EA-positive cells were observed only in the choroid (7 of 8 eyes) and the ciliary body (2 of 8 eyes); virus infected cells were not seen in the ganglion cell layer, the nuclear layers or the RPE (not shown).

MCMV in the eyes of IS mice after systemic administration of sodium iodate

Because MCMV spread to the choroid but not to the nearby RPE during systemic infection of deeply IS mice, we hypothesized that the RPE plays a role in preventing virus spread from the choroid to the retina and that selective damage of RPE cells would facilitate spread of MCMV to the retina. To test this hypothesis we used sodium iodate, a chemical that selectively damages RPE cells and increases the permeability of blood-retinal barrier (BRB) (Noell, 1953; Reddy *et al*, 1977; Anstadt *et al*, 1982; Kitano *et al*, 1988; Taarnhoj and Alm, 1992). As described in Materials and Methods, horseradish peroxidase (HRP) was used to assess the integrity of the BRB. RPE cells of control mice appeared normal, and there was no uptake of HRP (Figure 2A). In mice injected with sodium iodate, some RPE cells exhibited rounding and uptake of peroxidase and there was occasional leakage of peroxidase across the RPE into the photoreceptor layer (Figure 2B). RPE-65 staining in the eye of sodium iodate treated mice showed that the normal tight intercellular junction disappeared in some areas (compare Figure 3A and B). Mice were then

Table 1 Lymphocyte depletion in the spleen of BALB/c mice

Group	*No. of spleen cells/% depletion**	*No. of CD4⁺ cells*	*%CD4⁺/% depletion*	*No. of CD8⁺ cells*	*%CD8⁺/% depletion*
Control	5.2×10^7/—	1.1×10^7	21.8/—	6.5×10^6	12.5/—
Non-IS	1.1×10^8/—	2.7×10^7	24.3/—	1.4×10^7	12.3/—
IS	6.8×10^5/98.69	3.7×10^5	53.9/96.77	2.2×10^5	32.7/96.58
Deeply IS	2.1×10^5/99.60	1.4×10^4	6.8/99.87	6.9×10^3	3.3/99.89

Control group: uninfected BALB/c mice.
Non-IS: BALB/c mice infected with MCMV, no immunosuppression.
IS: BALB/c mice infected with 5×10^5 PFU of MCMV, treated with methylprednisolone alone.
Deeply IS: BALB/c mice infected with 5×10^5 PFU of MCMV, treated with methylprednisolone and T cell specific antibodies.
*% depletion compared with controls.

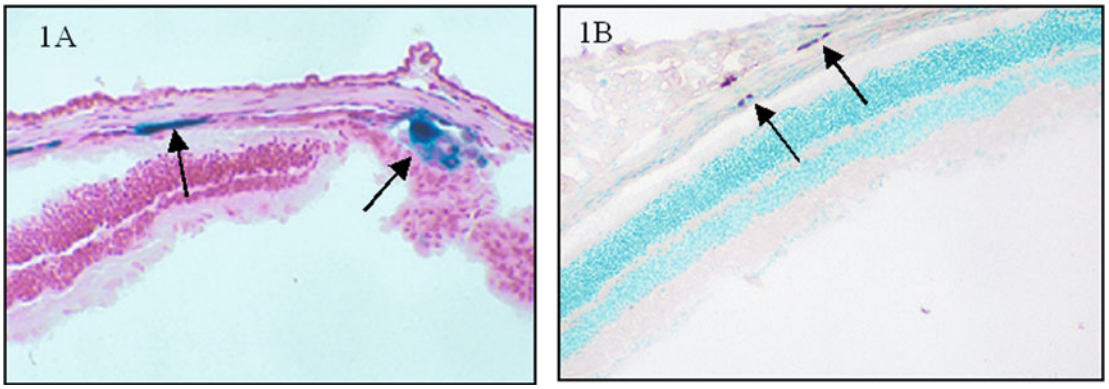

Figure 1 Photomicrographs of MCMV antigen in the eye of BALB/c mice immunosuppressed by treatment with methylprednisolone and T cell specific antibodies 14 days after intravenous inoculation of RM461. β-Gal–positive (**A**) and EA-positive (**B**) virus-infected cells (*arrows*) were observed in the choroid and ciliary body.

injected with sodium iodate (0.8 mg/mouse, i.v.), 1 day or 12 days after i.v. virus inoculation and sacrificed 14 days after virus inoculation. Examination of ocular sections stained for EA and IEA showed MCMV-positive cells in the choroid and/or ciliary body of most of the deeply IS mice. However, a few virus positive cells were also observed in the RPE of some sodium iodate treated mice (Figure 4A), and EA-positive cells were observed in the inner retina of one mouse (Figure 4B, Table 2). No virus-positive cells were observed in the RPE cells of PBS-treated deeply IS mice (Table 2). No virus-positive cells were seen in the RPE of non-IS mice or of mice treated with methylprednisolone alone after sodium iodate treatment (not shown).

Disruption of the BRB by lipopolysaccharide (LPS)

Systemic or intraocular injection of LPS induces uveitis characterized by disruption of the BRB and infiltration of leukocytes into the eyes of experimental animals (Taarnhoj *et al*, 2002; Rosenbaum *et al*, 1980; Li *et al*, 1995). Leukocytes were seen in the retina of IS mice after systemic injection of LPS (100 μg/mouse, i.v.) (Figure 5A) or injection of LPS into the anterior chamber (1 μg/mouse) (Figure 5B). In additional experiments, fluorescently labeled T cells and macrophages were observed in the retina of LPS-treated mice 1 to 2 days following intravenous inoculation. In contrast, PKH-26–labeled T cells or PKH-26–labeled macrophages were not observed in the retina of phosphate-buffered saline (PBS)-treated control mice. Together, these results indicate that LPS treatment disrupts the BRB.

Spread of MCMV to the retina after LPS-induced disruption of the BRB

To determine if disruption of the BRB allows MCMV to spread to the retina during systemic infection, MCMV was injected i.v. into deeply IS mice. Ten days after virus inoculation, LPS was injected systemically (100 μg/mouse, i.v.) or into the anterior chamber (1 μg/mouse). Four days after LPS treatment, MCMV-positive cells were observed in the choroid of all (16/16) of deeply IS mice. A few MCMV IEA- or

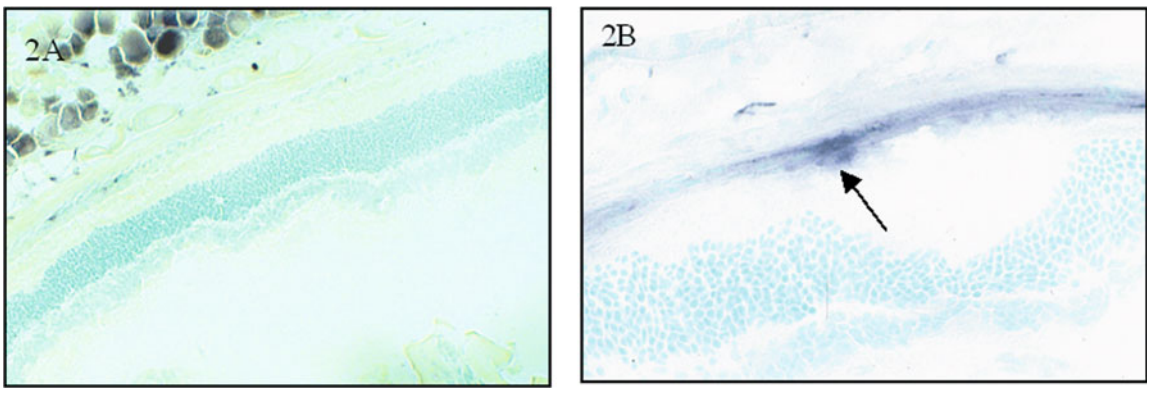

Figure 2 Photomicrographs of peroxidase staining in the eyes of sodium iodate-treated mice and PBS-treated control mice 30 min after intravenous injection of horseradish peroxidase. There was no uptake of horseradish peroxidase by the RPE cells of control mice (**A**). In the mice injected with sodium iodate, some RPE cells exhibited rounding and uptake of peroxidase. Leakage of peroxidase across the RPE into the photoreceptor layer was observed occasionally (**B**, *arrow*).

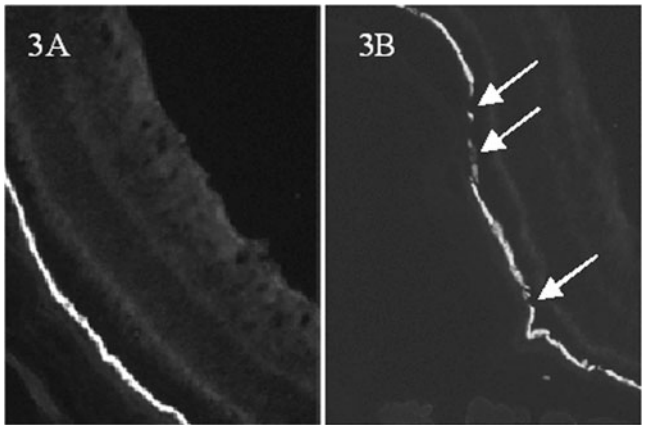

Figure 3 Photomicrographs (shown in grey scale) of RPE-65 staining in the eyes of PBS-treated control mice (**A**) and sodium iodate–treated mice (**B**). In sodium iodate–treated mice, the normal tight intercellular junction in the RPE layer was interrupted in some areas as indicated by the arrows (**B**).

EA-positive cells were observed in the retina of 4 of 8 mice after i.v. injection of LPS (Figure 6A) and in the retina of the injected eye of 7 of 8 mice after injection of LPS into the anterior chamber (Figure 6B). No virus-infected cells were observed in the retina of deeply IS mice treated with PBS (Table 3). And no viral antigen positive cells were observed in the retina of mice treated with LPS and immunosuppressed with methylprednisolone alone (not shown).

MCMV infection of macrophages

Because LPS treatment was associated with infiltration of leukocytes in the eyes, we hypothesized that MCMV-infected leukocytes could spread virus to retina. To test this hypothesis, T cells and macrophages were separately isolated and incubated

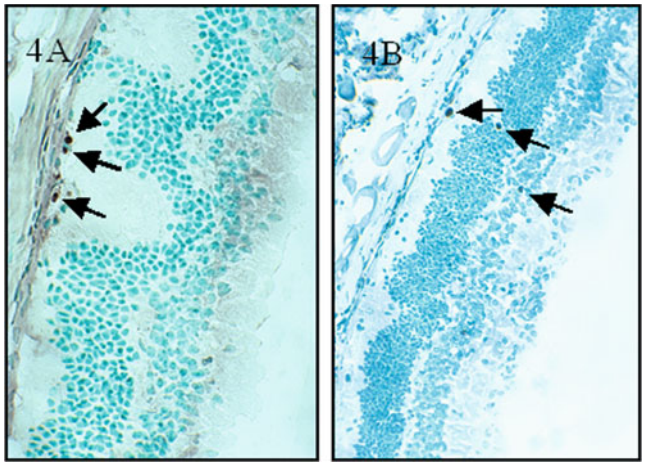

Figure 4 Photomicrographs of MCMV EA staining in the eyes of IS mice 14 days after intravenous inoculation of RM461 and systemic administration of sodium iodate. A few EA positive cells were observed in the RPE (**A, B**). EA-positive cells were also observed in the inner retina of one mouse (**B**).

Table 2 Detection of MCMV antigen in the eye following i.v. injection of MCMV into deeply IS-mice treated with sodium iodate

Group	*Anterior segment*	*RPE*	*Inner retina*	*Choroid*
Sodium iodate A	2/8	1/8	0/8	7/8
Sodium iodate B	2/8	3/8	1/8	8/8
Control A	2/8	0/8	0/8	8/8
Control B	2/8	0/8	0/8	7/8

Sodium iodate A: sodium iodate, intravenous injection 1 day after virus inoculation.
Sodium iodate B: sodium iodate, intravenous injection 12 days after virus inoculation.

with MCMV *in vitro*. After 4 days of *in vitro* incubation with MCMV, approximately 5% of macrophages were IEA or EA positive (Figure 7), whereas no T cells were MCMV positive (not shown). Plaque assay of freeze-thawed macrophages showed an average of 2.2 PFU of MCMV/macrophage. Experiments were then performed to determine if MCMV-infected macrophages release infectious virus. First, the titer of MCMV in the culture medium of macrophages 4 days after inoculation with MCMV was assayed and determined to be an average of 7.4×10^4 PFU/ml. Using Millicell culture plate inserts (Millipore Corporation), cocultivation of MCMV-infected macrophages with MEF demonstrated typical MCMV cytopathic changes beginning after 2 days of cocultivation. MEF cells were MCMV EA positive after 3 days of cocultivation. Together, the results of the plaque assay and cocultivation studies support the idea that MCMV is able to infect and replicate in macrophages.

To determine if MCMV-infected macrophages spread to the retina after LPS-induced disruption of the BRB, macrophages were infected with MCMV *in vitro* as described above. After 4 days, the macrophages were labeled with PKH-26 (Figure 8) and 1×10^5 cells were injected into the tail vein of deeply IS mice. Thirty minutes after injection of labeled cells, LPS was injected systemically (100 μg/mouse, i.v.) or into the anterior chamber (1 μg/mouse) and animals were sacrificed 1 or 2 days later. A few labeled macrophages were seen in the retina of mice injected with LPS via either route. Labeled cells were observed in the inner retina (Figure 9), whereas some were seen in the photoreceptor layer (Figure 10). Labeled cells were not observed in the retina of PBS-treated control mice (not shown). Staining for MCMV antigen demonstrated few MCMV-infected cells in the photoreceptor layer and in the inner retina of LPS-treated mice (i.v. or anterior chamber injection; Figure 11A and B, respectively). No MCMV antigen–positive cells were observed in the retina of PBS-treated mice (not shown).

To determine if virus that had been carried to the retina by macrophages infected retinal cells, LPS- and PBS-treated deeply IS mice (prepared as described above) were sacrificed 10 days after injection of virus-infected macrophages. MCMV-infected

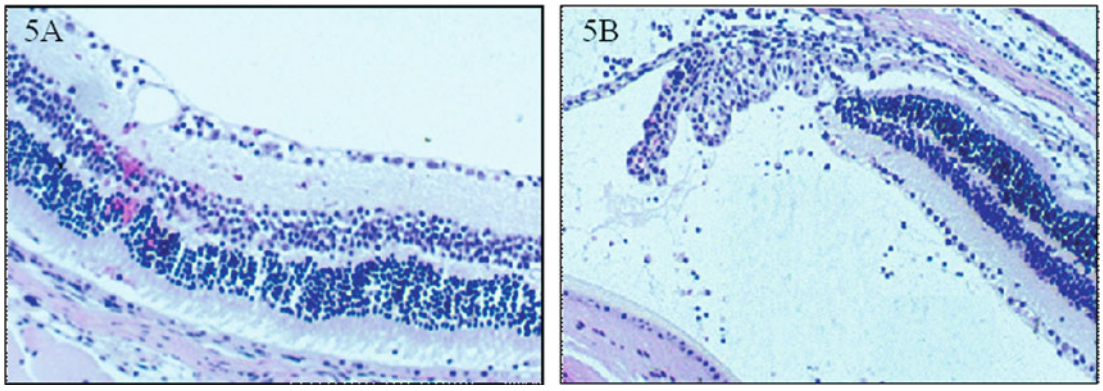

Figure 5 Photomicrographs of hematoxylin and eosin staining in the eye of IS mice treated with LPS. A few leukocytes were seen in the retina of IS mice injected intravenously with LPS (**A**). More leukocytes were observed in retina of IS mice injected with LPS via the anterior chamber route (**B**).

cells were observed in the choroid and ciliary body of both LPS- and PBS-treated mice. However, MCMV-infected cells were observed in the retina only in LPS-treated mice (Table 4). Virus-infected cells were observed in the retina of 7 of 8 mice treated with LPS i.v. and in the retina of 8 of 8 mice injected in the anterior chamber with LPS (Table 4). More MCMV-infected cells were observed in the retina of mice in the latter group than in the former (Table 5, also compare Figure 12A and B). Most of the MCMV-infected cells in the LPS-treated mice were observed in the inner nuclear layer in proximity to the retinal vasculature. Only a few RPE cells were MCMV positive. Although viral antigen–positive cells were noted in the inner retina, neither typical necrotic MCMV retinitis nor retinal disruption was observed in deeply IS, LPS-treated mice after administration of macrophages infected with either RM461 or K181.

Discussion

The results of the studies presented herein show that after intravenous inoculation of lacZ-containing recombinant MCMV RM461 or parent virus K181 into mice immunosuppressed by treatment with methylprednisolone and antibodies, immediate early antigen– or early antigen–positive virus-infected cells were observed in choroid and ciliary body. No positive cells and pathological changes were noted in posterior segment. Because immunosuppressed mice inoculated with MCMV via the supraciliary route develop retinitis (Atherton *et al*, 1991, 1992; Bigger *et al*, 2000; Zhang and Atherton, 2002) whereas immunosuppressed mice systemically infected with MCMV do not (Gao *et al*, 1996; Dix, 1998), the results of these studies support the idea that the BRB, which is present at the retinal pigment epithelium (outer BRB) and the retinal vascular endothelium (inner BRB), plays important role in the prevention of virus spread to retina. By extrapolation, abnormalities or disruptions of the BRB may help to explain why human CMV (HCMV) retinitis occurs in only a subset of human immunodeficiency (HIV)-infected patients and why most CMV infections of the eye develop coincident with systemic CMV infection.

There are several ways by which abnormalities of the BRB have been postulated to occur in

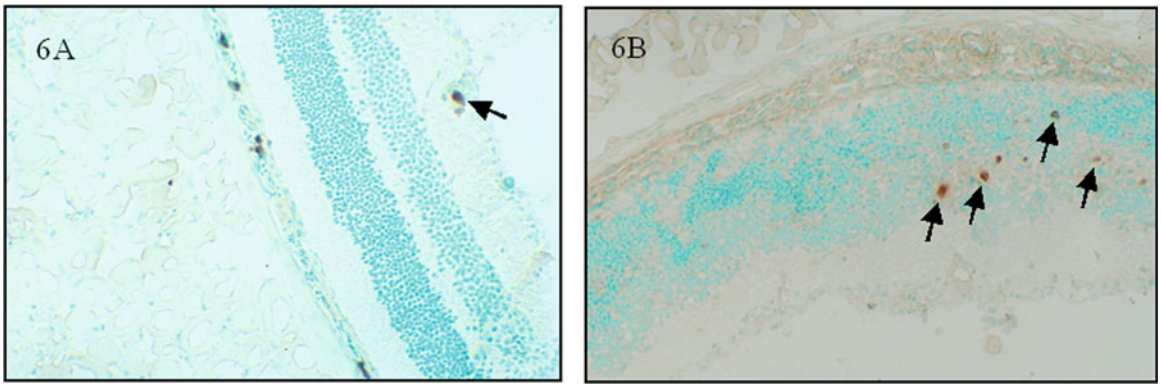

Figure 6 Photomicrographs of MCMV EA staining in the eye of IS mice 14 days after intravenous inoculation of MCMV and LPS treatment. A few MCMV EA-positive cells were observed in the retina of IS mice injected intravenously with LPS (**A**). Virus-infected cells were also observed in the inner retina following injection of LPS into the anterior chamber of the same eye (**B**).

Table 3 Detection of MCMV antigen in the eye following i.v. injection of MCMV into deeply IS-mice treated with LPS

Group	*Anterior segment*	*RPE*	*Inner retina*	*Choroid*
LPS IV	4/8	3/8	4/8*	8/8
LPS AS	5/8	3/8	7/8#	8/8
PBS IV control	2/8	0/8	0/8	8/8
PBS AS control	4/8	0/8	0/8	8/8

*LPS IV compared to PBS IV control: $\chi^2 = 5.50$, $P < .05$.
#LPS AS compared to PBS AS control: $\chi^2 = 12.44$, $P < .01$.

HIV-infected individuals. HIV infection of the retinal vasculature has been postulated to occur in HIV-infected individuals (Pomerantz *et al*, 1987; Cantrill *et al*, 1988). It has also been suggested that HIV infection of the endothelial cells might facilitate CMV infection of the retina by allowing virus-infected leukocytes to enter the retina through a compromised inner BRB. In support of this idea, dual infection of retinal cells by HIV and CMV has been documented (Skolnik *et al*, 1989). However, another study found no evidence of HIV infection of the retina, which led the investigators to speculate that seeding of the retina by CMV resulted from a local process such as deposition of immune complexes or local release of cytokines, which in turn might facilitate entry of virus-infected leukocytes into the retina from the systemic circulation (Faber *et al*, 1992). CMV has been observed in the cells of RPE, which are permissive for the virus (Miceli *et al*, 1989), and changes in the function of the inner retinal cells and of the RPE have been observed in HIV-infected patients with CMV retinitis (Harrison and van Heuven, 1999).

Cotton-wool spots which are areas of microinfarction in the nerve fiber layer are the most common fundus lesions in HIV, occurring in up to 50% of patients (Skiest, 1999). These lesions, which resolve and recur regularly in AIDS patients, are caused either by deposition of circulating immune complexes in the microvasculature, resulting in vessel occlusion, or active infection of vascular endothelial cells, causing ischemia and necrosis (Skiest, 1999). It has also been suggested that cotton wool spots may represent areas of HIV infection of endothelial cells and that such infection may play a role in CMV retinitis by providing a site by which virus enter the retina (Skiest, 1999; Gonzalez *et al*, 1996). However, because not all patients with CMV retinitis have cotton wool spots (Skiest, 1999), there is likely to be more than one route by which virus enters the eye.

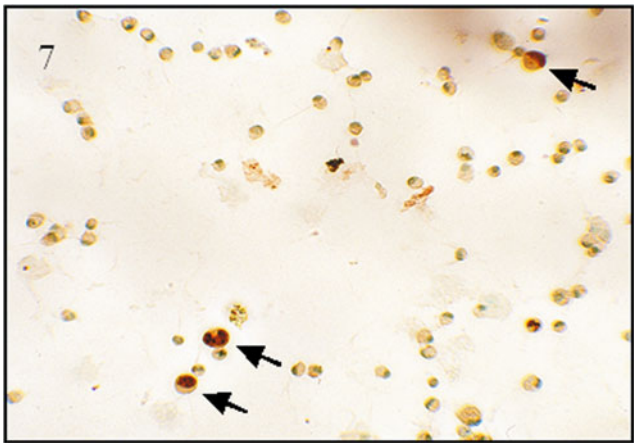

Figure 7 Photomicrograph of MCMV EA staining of macrophages infected with MCMV for 4 days *in vitro*. About 5% of the macrophages were virus antigen (EA) positive (*arrows*).

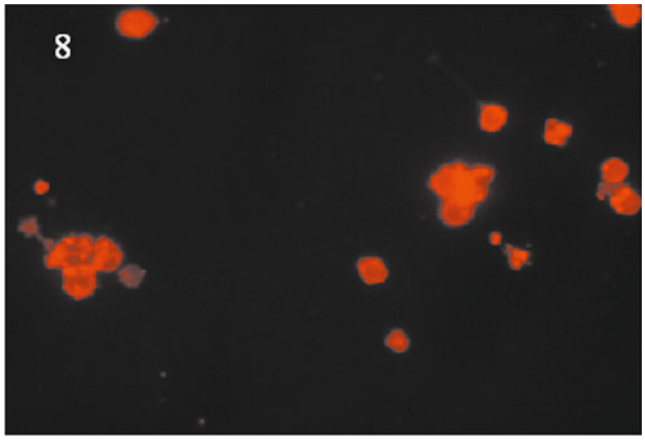

Figure 8 Photomicrograph of macrophages labeled with PKH-26.

Our results provide evidence that after breakdown of the BRB, MCMV is able to spread to and replicate in the retina during systemic infection. After treatment of lS mice with LPS, the BRB was disrupted and leukocytes, including a few virus-infected macrophages, migrated to photoreceptor layer through outer BRB and inner nuclear layer through inner BRB. Several days later, more virus-infected cells were noted in the retina and most of these cells were located in the inner nuclear layer and were in proximity to the retinal vasculature. This finding is similar to the observation in HCMV retinitis reported by Rao *et al* (1998) who reported that HCMV was present primarily in Müller cells and perivascular glial cells and suggested that the inner BRB is disrupted.

Our previous results showed that RPE cells are the major and early targets of MCMV infections following MCMV inoculation into the superciliary space of BALB/c mice (Zhang and Atherton, 2002). In contrast, the results of the studies presented herein indicate that during systemic MCMV infection of IS mice, the underlying intact RPE layer is spared from infection even though virus is able to spread to the choroid. However, a few RPE cells were infected with MCMV after they were selectively damaged and the outer BRB was disrupted by treatment with sodium iodate. These results, together with those of other investigators (Miceli *et al*, 1989; Rao *et al*, 1998),

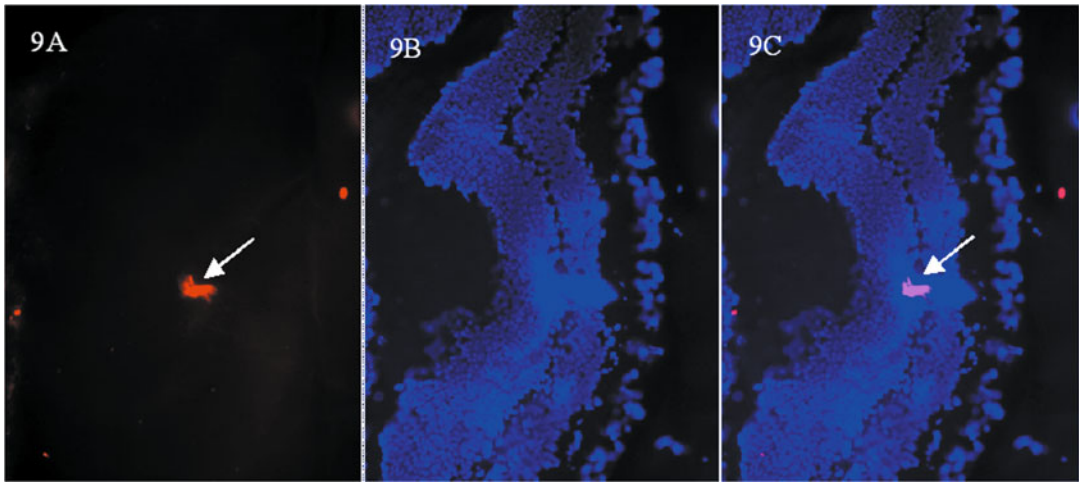

Figure 9 Photomicrographs showing PKH-26–labeled macrophages in the inner retina of IS mice 1 to 2 days after LPS treatment and injection of PKH-26 labeled macrophages (**A**, PKH-26–labeled cells; **B**, DAPI staining; **C**, merge).

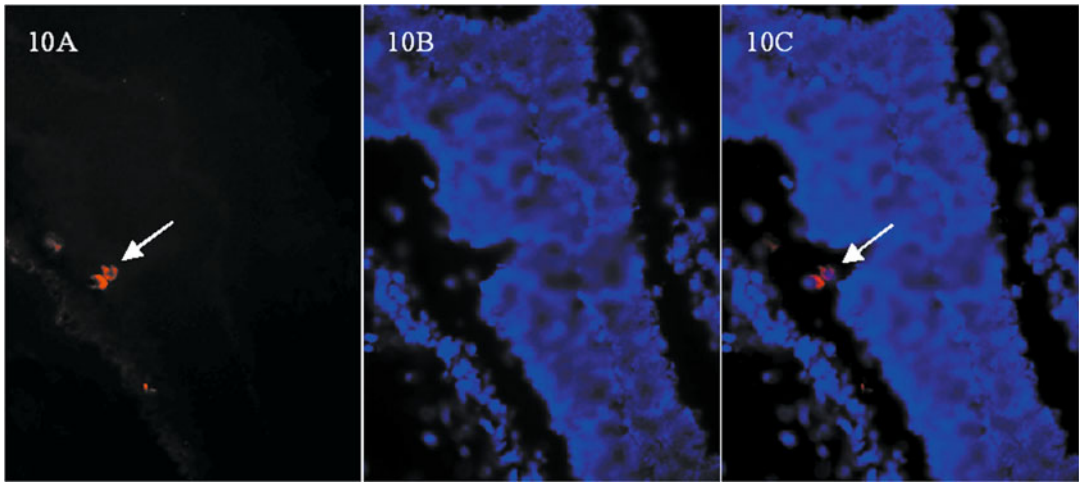

Figure 10 Photomicrographs showing PKH-26–labeled macrophages in the photoreceptor layer of IS mice 1 to 2 days after LPS treatment and injection of PKH-26 labeled macrophages (**A**, PKH-26–labeled cells; **B**, DAPI staining; **C**, merge).

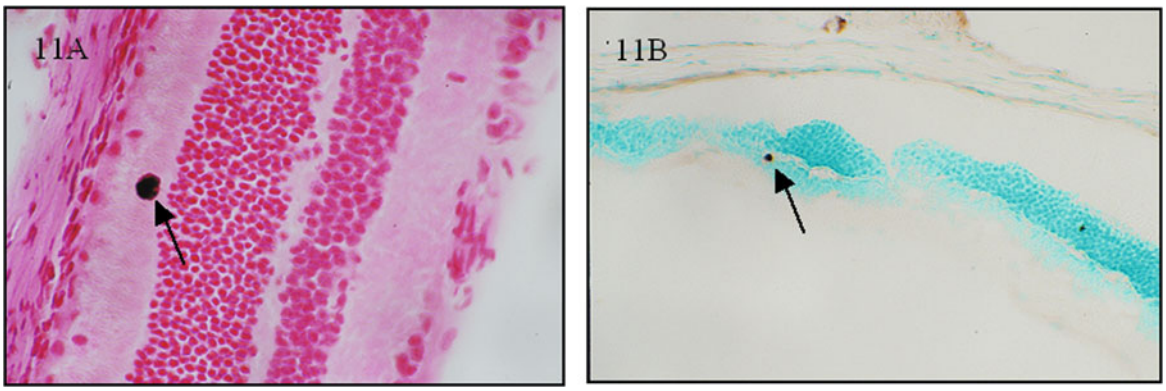

Figure 11 Photomicrographs of MCMV antigen staining in the retina of IS mice 1 to 2 days after LPS treatment and intravenous injection of MCMV-infected macrophages. MCMV-infected cells were observed in the photoreceptor layer (**A**) and in the inner retina (**B**).

Table 4 Detection of MCMV antigen in the eye following i.v. injection of MCMV infected macrophages into deeply IS-mice treated with LPS

Group	*Anterior segment*	*RPE*	*Inner retina*	*Choroid*
LPS IV	4/8	3/8	7/8*	8/8
LPS AS	5/8	3/8	8/8#	8/8
PBS IV	2/8	0/8	0/8	8/8
PBS AS	4/8	0/8	0/8	8/8

*LPS IV compared to PBS IV Control: $\chi^2 = 12.44$, $P < .01$.
#LPS AS compared to PBS AS Control: $\chi^2 = 16.00$, $P < .01$.

suggest that RPE cells are another major target of CMV infection.

The cells that carry CMV into the eye have been studied extensively. Dissemination of CMV in the blood occurs mainly via leukocytes and these cells are the major source of HCMV spread (Taylor-Wiedeman *et al*, 1991). Monocyte-derived macrophages generated by allogeneic stimulation of peripheral blood mononuclear cells exhibit HCMV reactivation, replication, lytic and productive infection, and are able to transmit HCMV to endothelial cells (Soderberg-Naucler *et al*, 1997, 2001). CMV infection of endothelial cells in various sites has been documented, and circulating CMV-infected endothelial cells may be able to infect endothelial cells at distant sites (Percivalle *et al*, 1993; Grefte *et al*, 1993). Recently, it has been shown that dendritic cells play an important role in HCMV binding and dissemination (Halary *et al*, 2002). In these studies, macrophages infected with MCMV were able to migrate through a disrupted inner and outer BRB to the retina during systemic infection resulting in MCMV infection of the retina. Not surprisingly, immunosuppression facilitated the replication of MCMV in the retina. Because spread and replication of MCMV in the inner retina were only noted in deeply IS mice that had been treated with methylprednisolone and antibodies against CD4 and CD8, the results of the studies presented in this article suggest that immunosuppression, especially extensive depletion of CD4 and CD8 T cells, is needed for replication of MCMV in the retina. This mouse model of CMV infection of the retina mimics aspects of virus spread during systemic HCMV infection of immunocompromised patients and may be an additional tool in future studies using animal models to unravel the pathogenesis of HCMV ocular infection.

Table 5 Comparison of the number of MCMV antigen positive cells in the inner retina following i.v. injection of MCMV infected macrophages into deeply IS-mice treated with LPS

Group	*No. of positive cells*
LPS-IV-MCMV-Macrophages	4, 8, 13, 9, 0, 3, 22, 12
LPS-AS-MCMV-Macrophages	6, 19, 7, 32, 18, 10, 23, 75

LPS-AS group compared with LPS-IV group: $t = 1.95$, $P < .05$.

Materials and methods

Cells and virus

Mouse embryo fibroblast (MEF) cells (Whittaker M.A. Bioproducts, Walkersville, MD) grown in tissue culture medium containing 10% fetal calf serum (HyClone, Logan, Utah) were used for virus infection. MCMV strains used in these experiments were mutant MCMV RM461 strain and the parental strain, K181. The original stocks of these viruses were 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 *E. coli* LacZ gene (under the control of the HCMV major immediate early promoter/enhancer) into the MCMV K181 genome at the HindIII L/J site just downstream of immediate early gene 2 (ie2) (Stoddart *et al*, 1994). RM461 expresses the *E. coli* lacZ gene product, β-galactosidase (β-gal), as an immediate early viral gene product during the viral replication cycle. Because RM461 contains the LacZ gene inserted close to the sgg1 gene and it replicates less efficiently in salivary glands than K181, RM461 was propagated in tissue culture (Stoddart *et al*, 1994). K181 was prepared from the salivary glands of MCMV-infected BALB/c mice.

Mice

Six- to eight-week-old female BALB/c mice (Taconic, 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-h light cycle alternating with a 12-h dark cycle and were given unrestricted access to food and water. All ocular injections were done after the mice had been anesthetized with a mixture of 42.9 mg/ml ketamine, 8.57 mg/ml xylazine, and 1.43 mg/ml acepromazine at a dose of 0.5 to 0.7 ml/kg body weight. The treatment of animals in this study conformed to the Association for Research in Vision and Ophthalmology Statement on 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.

Immunosuppression

Two types of immunosuppression were used in these experiments. Mice were injected intramuscularly with methylprednisolone (2 mg/mouse) every 3 days (IS) or mice were injected with monoclonal antibodies (Ab) against CD4 (GK1.5, 0.45 mg/mouse, i.v.) and CD8 (2.43, 0.l mg/mouse, i.v.) (American Type Culture Collection, Manassas, VA) 1 day after the first injection of methylprednisolone (deeply IS).

Sodium iodate damage to the RPE

Immunosuppressed mice were injected with sodium iodate (30 mg/kg, i.v.; Sigma, St. Louis, MO), 1 day

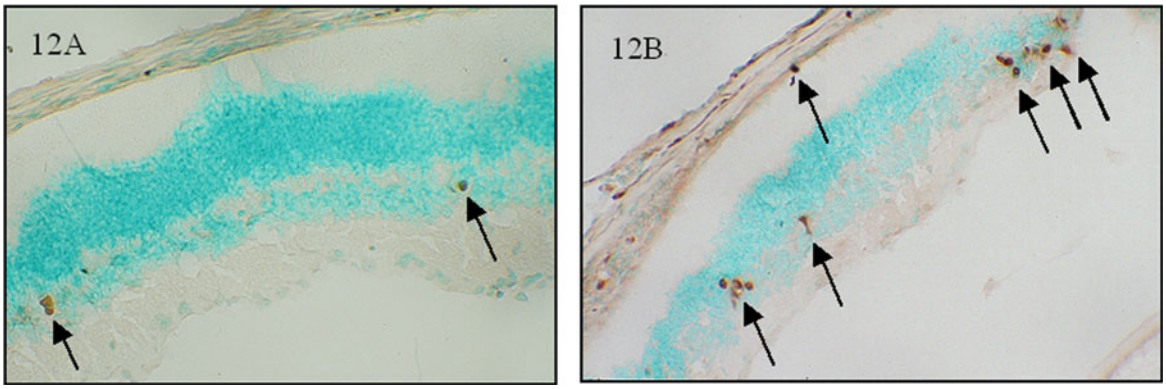

Figure 12 Photomicrographs of MCMV antigen staining in the retina of IS mice 8 to 10 days after LPS treatment and intravenous injection of MCMV-infected macrophages. A few MCMV-infected cells were observed in the inner retina of the mice injected intravenously with LPS (**A**). In contrast, more MCMV-infected cytomegalic cells were noted in the retina of eyes of IS mice in which LPS had been injected into the anterior chamber (**B**).

or 12 days after virus inoculation. To determine permeability of the BRB after injection of sodium iodate, normal adult female BALB/c mice received a single injection of sodium iodate (30 mg/kg, i.v.) or the same volume of PBS. One day later, the mice were injected with HRP (200 mg/kg, i.v.; Sigma) as a tracer. Thirty minutes after injection of HRP, the mice were sacrificed and perfused with PBS. Both eyes of each animal were removed, snap-frozen, and sectioned on a cryostat. The slides were fixed with acetone for 5 min, washed with PBS 3 times for 1 h each time, and incubated with diaminobenzidine (DAB) solution for 5 m. The reaction was stopped by washing the slides in tap water for a minimum of 5 min. Tissue sections were counterstained with methyl green, dehydrated, mounted, and examined microscopically. RPE cells were visualized using a monoclonal antibody specific for RPE65 (kindly provided by Dr. Michael Redmond, National Eye Institute, National Institutes of Health, Bethesda, MD) as described below.

Disruption of the BRB by injection of LPS
LPS (Sigma) was injected into IS mice systemically (100 μg/mouse, i.v.) or into the anterior chamber (1 μg/mouse). Disruption of the BRB was determined 1 or 2 days after LPS injection. After sacrifice, both eyes were removed, fixed in buffered formalin, embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically for the presence of leukocytes in the anterior and posterior segments.

Leukocyte labeling and injection in LPS-treated mice
T cells were separated from the spleens of normal mice. Immediately after sacrifice, the spleens were collected and a single cell suspension was made. Dead cells were removed by centrifugation in Histopaque-1017 (Sigma) following the manufacturer's directions and the cell preparation was enriched for T cells by passage through a nylon wool column. To obtain macrophages, normal mice were injected with 3% Brewer thioglycollate medium (Sigma). Four days later, the mice were sacrificed and the macrophages were collected from the peritoneal cavity by gentle washing with Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS). After collection, the cells were washed, labeled with PKH-26, a red fluorescent dye (Sigma), concentrated to 1×10^5 cells/0.2 ml and injected into the tail vein of normal or IS mice. Ten minutes after injection of the PKH-26–labeled cells, LPS was injected systemically (100 μg/mouse, i.v.) or into the anterior chamber (1 μg/mouse). Control mice received an equivalent volume of PBS. One or two days after injection of labeled cells, the mice were sacrificed; both eyes were removed, snap-frozen, sectioned on a cryostat, and examined microscopically for red-stained fluorescent leukocytes.

MCMV infection of leukocytes in vitro
T cells or macrophages isolated from the spleen or peritoneal cavity as described above were adjusted to a concentration of 5×10^5 cells/ml in RPMI medium containing 5% FBS. Five million PFU of MCMV was added and the cells were incubated at 37°C for 2 h. After washing in Hank's balanced salt solution (HBSS), the cells were incubated at 37°C in RPMI medium containing 10% FBS. After 4 days, the medium was collected and the titer of virus in the medium was determined by plaque assay. The cells were harvested and washed three times in HBSS. MCMV antigen was detected by dropping an aliquot of cells on a slide and staining for MCMV. For virus recovery, 1×10^5 macrophages in 1 ml of medium were subjected to four cycles of freezing and thawing and centrifuged at 2500× g for 5 min. The supernatant was collected and the titer of virus was determined by plaque assay. A total of 1×10^5 MCMV-infected or uninfected (control) macrophages were placed in 0.4 μm Millicell culture plate inserts (Millipore, Billerica, MA) and cocultivated with MEF. After 3 days of cocultivtion, the MEF cells were harvested and stained for MCMV EA.

Systemic infection with MCMV
Two days after injection of methylprednisolone or methylprednisolone and antibodies, mice were inoculated i.v. with 5×10^5 PFU of RM461, 5×10^3 PFU of Kl81, or 1×10^5 macrophages infected with MCMV for 4 days *in vitro*. Ten to 14 days later, animals were deeply anesthetized, killed, and perfused with PBS. The spleen were removed for flow cytometry. Eyes were enucleated and were immersed in optimal cutting temperature (OCT) compound (Tissue-Tek; VWR Scientific, Houston, TX), snap frozen on dry ice, and sectioned on a semiautomatic cryostat (Microm HM505E; Zeiss, Houston, TX) at 12 levels, 100 μm apart.

Flow cytometry
Spleens were harvested and single cell suspensions were prepared for flow cytometry. The non–cross-reactive antibodies, florescein isothiocyanate (FITC)–anti-L3T4 (PharMingen, San Diego, CA) and phycoerythrin (PE)–anti-ly-3.2 (PharMingen), recognizing CD4 and CD8, respectively, were used to determine the efficiency of lymphocyte depletion. Flow cytometry of stained spleen cell samples was performed using a Becton Dickinson FAC Star Flow Analyzer (Becton Dickinson, San Jose, CA), and the flow cytometry data were analyzed using DP2 software.

Immunohistochemistry
Monoclonal antibody to an MCMV early gene product, pp56 (Pande *et al*, 1991), was separated from the supernatant of hybridoma 25G11 by ammonium sulfate precipitation and biotinylated with Sulfo-NHS-LC-Biotin (Pierce Chemical Company, Rockford, IL) according to the manufacturer's instructions. Immunohistochemistry was done using the ABC streptavidin–horseradish peroxidase kit (Vector Laboratories, Burlingame, CA) and developed using DAB (Sigma) as described previously (Bigger *et al*, 2000; Zhang and Atherton, 2002). Monoclonal antibody against RPE65 was used to stain the cells of the RPE. Following blocking, the sections were incubated overnight at 4°C in primary antibody to RPE65 (1:400). After washing, the sections were reacted with Texas red labeled anti rabbit (1:100, Vector Laboratories). The slides were mounted with Vectorshield mounting medium with DAPI (Vector Laboratories) and examined microscopically for red stained RPE65-positive cells.

Staining for β-gal in frozen sections
Frozen sections of ocular tissue were fixed with 0.5 glutaraldehyde in PBS for 15 min and then washed with PBS twice for 10 min each time. 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 $K_3Fe(CN)_6$, and 12.5 mM $K_4Fe(CN)_6 \cdot 3H_2O$), 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 gene expression.

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