Adoptive Transfer of Murine Cytomegalovirus-Immune Lymph Node Cells Prevents Retinitis in T-Cell-Depleted Mice

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Purpose. The purpose of this study was to determine whether adoptive transfer of murine cytomegalovirus (MCMV)-immune lymph node cells prevents retinitis in immunosuppressed mice.

Methods. Adult BALB/c mice were thymectomized and T-cell depleted using rat monoclonal antibodies specific for mouse CD4+ and CD8+ T-cells. The level of rat immunoglobulin G in the treated mice was monitored by enzyme-linked immunosorbent assay. Immune cells were labeled with PKH26-GH immediately before adoptive transfer, and flow cytometry was used to determine the percentage of adoptively transferred T-cells (PKH+, fluorescein isothiocyanate [FITC+]) in the spleens of the recipient mice 3 days after transfer. The ability of adoptively transferred cells to protect from retinitis was studied in T-cell-depleted mice injected with MCMV through the supraciliary route. Mice received 4 X 10⁷ in vitro-restimulated MCMV-immune cells, 4 X 10⁷ freshly isolated MCMV-immune cells, 4 X 10⁷ freshly isolated ovalbumin-immune cells, or no cells (control group).

Results. The best time to balance depletion of endogenous T-cells with persistence of transferred cells was 3 weeks after T-cell depletion. Both restimulated and freshly isolated MCMV-immune cells conferred protection from retinitis. Freshly isolated ovalbumin-immune lymph node cells did not prevent retinitis, indicating that protection was virus-specific and merely was not because of transfer of antigen-activated lymph node cells.

Conclusions. Adoptive immunotherapy has been used to prevent cytomegalovirus (CMV) infection in patients who have undergone transplantation, and, by extrapolation, the results of these studies suggest that adoptive immunotherapy with human CMV-specific immune cells might be used to prevent or ameliorate CMV retinitis in immunocompromised patients. Invest Ophthalmol Vis Sci. 1997;38:301-310.

Cytomegalovirus (CMV) infection is a common complication of patients who are immunosuppressed, especially after transplantation of bone marrow or other organs.¹,² Cytomegalovirus retinitis has emerged as a significant problem in patients with acquired immune deficiency syndrome, and, depending on the study, this potentially blinding ocular complication is observed in up to 46% of patients with acquired immune deficiency syndrome at some point during the course of their disease.³-⁶ Although CMV infection in an immunocompetent individual usually results in either minimal or no clinical disease⁷ and more than half of the adult population is CMV-seropositive,⁸-⁹ the consequences of a primary (or a reactivated) CMV infection in a patient who is immunosuppressed frequently are life or sight threatening or both.¹⁰-¹³ Whereas several components of the immune system, including T-cells, NK-cells, and macrophages, have been implicated in control of cytomegalovirus infections in humans and mice,¹⁴-¹⁷,¹⁸ most attempts to augment the immune system to control CMV or MCMV infections have involved adoptive transfer of components of the cellular immune system¹⁹.

Studies of specific immune reconstitution in human patients infected with CMV or at risk for CMV
A model of MCMV retinitis in immunosuppressed (either by depletion of T-cells or by treatment with methylprednisolone) BALB/c mice has been developed and studied in our laboratory. In this model, injection of \(5 \times 10^6\) plaque-forming units (pfu) of the Smith strain of MCMV into the supraciliary space of immunosuppressed BALB/c mice results in acute retinitis with histopathologic features resembling those of observed in eyes of human patients who are immunocompromised with CMV retinitis. Previously, we showed that adoptive transfer of MCMV-immune cells prevents retinitis in euthymic BALB/c mice infected with a high dose \(5 \times 10^6\) pfu of MCMV. However, adoptive immunotherapy has not yet been tried as a way to prevent or reduce the severity of retinitis in immunosuppressed mice. Accordingly, in the experiments presented herein, we determined whether adoptive transfer of nonrestimulated or in vitro-restimulated MCMV-immune lymph node cells or both could confer protection against retinitis in thymectomized T-cell-depleted BALB/c mice infected with MCMV through the supraciliary route. The results indicate that adoptive transfer of \(4 \times 10^7\) freshly isolated or in vitro-restimulated MCMV-immune lymph node cells prevented retinitis in \(>80\%\) of the mice. MCMV-immune cells were required for protection, and ovalbumin-immune cells did not protect. Protection from retinitis correlated microscopically with preservation of the retina and lack of histopathologic evidence of virus replication in the retinas of mice treated with MCMV-immune lymph node cells. The results of these experiments indicate that adoptive immunotherapy with MCMV-immune cells prevents retinitis in mice and suggest that adoptive immunotherapy with autologous or histocompatible CMV-immune cells also could be explored for use in treating human patients with CMV retinitis.

MATERIALS AND METHODS

Animals

Female euthymic BALB/c mice between 7 and 11 weeks of age were obtained from Taconic (Germantown, NY). Animals were maintained on a 12-hour-light cycle alternating with a 12-hour-dark cycle and were given unrestricted access to food and water. Animals were housed in accordance with National Institutes of Health Guidelines, and all procedures in this study conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Virus

The Smith strain of MCMV was used for all experiments. Virus stocks were prepared from the salivary glands of MCMV-infected BALB/c mice as described previously. Virus stocks and experimental samples were titered in duplicate by plaque assay on monolayers of mouse embryo fibroblasts. The titer of virus stocks was between \(10^6\) and \(10^7\) pfu/ml; a fresh aliquot of stock virus was thawed and diluted (if necessary) to the appropriate concentration for ocular and footpad injections. For virus recovery experiments, eyes, salivary glands, lungs, and spleens were homogenized in 1.0 ml of tissue culture medium without bovine serum, serially diluted and plated in duplicate on mouse embryo fibroblasts monolayers. After 5 days, plaques were counted and the titer of virus was expressed in plaque-forming units per milliliter. For plaque assays, the minimum level of detection was \(5.0 \times 10^6\) pfu/ml.

Ocular Inoculation

Mice were anesthetized with pentobarbital (0.65 mg/10 g body weight) and inoculated with \(5 \times 10^2\) pfu of MCMV in a volume of 2 µ l through the supraciliary route as described previously.

Preparation of Murine Cytomegalovirus-Immune Lymph Node Cells

The MCMV-immune lymph node cells were prepared by a modification of a method used to generate herpes simplex virus type-1 immune cells. Briefly, euthymic BALB/c mice were immunized through the footpad route with \(5 \times 10^4\) pfu of MCMV per mouse. Ten days later, the mice were killed, the draining lymph nodes were removed, and a single cell suspension was prepared by pressing the lymph nodes through sterile 70- 

\[\mu\text{m} \text{nylon mesh (Spectrum Medical Industries, Houston, TX). The cells were washed in Ca}^{2+}-, \text{Mg}^{2+}-\text{free Hank's balanced salt solution, and pelleted by centrifugation at } 200 \times g \text{ for 10 minutes. Erythrocytes were removed by treatment with ACK lysing buffer. The cells were resuspended in Hank's balanced salt solution, counted, and adjusted to the correct concentration for adoptive transfer. Some mice received these...} \]
Adoptive Transfer of Murine Cytomegalovirus-Immune Cells

After labeling, the cells were washed, counted, and cytokine-depleted. BALB/c mice were thymectomized after the injection of MCMV-immune lymph node cells at 37°C for 36 hours in RPMI-1640 with 10% fetal calf serum and 1000 units of recombinant interleukin-2 per milliliter. Recombinant interleukin-2 was prepared by culturing P815-IL2 cells (a gift from Dr. Bruce Ksander, Schepens Eye Research Institute, Boston, MA) in RPMI-1640 containing 10% fetal calf serum, 0.2% genetecin (Life Technologies, Gaithersburg, MD), and 1% Hepes buffer (Life Technologies). Flow cytometric analysis of samples of the MCMV-immune lymph node cells immediately after transfer showed that 37.9% of the cells were CD4+, 23.1% were CD8+, and 39.0% were CD4-, CD8- (data not shown).

For tracing experiments, MCMV-immune lymph node cells were labeled with PKH26-GL (Sigma Chemical, St. Louis, MO) after the manufacturer’s directions. PKH26-GL is incorporated stably into the cell membrane and does not affect the biologic characteristics of the cells. The excitation wavelength of this compound is 551 nm, and the emission wavelength is 567 nm; the detection wavelength was 575 ± 26 nm. After labeling, the cells were washed, counted, and resuspended at an appropriate concentration for intravenous injection.

Preparation of Ovalbumin-Immune Lymph Node Cells

Ovalbumin (2.85 mg/mouse) (Sigma Chemical) in phosphate-buffered saline (PBS) was injected into euthymic BALB/c mice by footpad. Ten days later, the draining lymph nodes were removed, and a single cell suspension was prepared as described above.

T-Cell Depletion

Adult BALB/c mice were thymectomized after the procedure of Chin. Two weeks after thymectomy, 1.2 mg of rat antimouse CD4 monoclonal antibody (mAb) (GK1.5) and 150 μg of rat antimouse CD8 mAb (2.43) were injected intravenously. Two days later, the mice were injected with 1.5 mg of rat antimouse CD4 mAb only. The original stocks of both rat antimouse T-cell-specific hybridoma cell lines were obtained from the American Type Culture Collection (Rockville, MD), and mAb was prepared as described previously. The extent of T-cell depletion was monitored by flow cytometry of spleen cells. As shown previously, this method resulted in depletion of >95% of both CD4+ and CD8+ splenic T-cells.

Enzyme-Linked Immunosorbent Assay

The concentration of rat immunoglobulin G (IgG) was measured by enzyme-linked immunosorbent assay in 96-well plates by a modification of the method of Hornbeck. Mice were bled through the tail vein immediately after injection of rat mAbs specific for mouse CD4+ and CD8+ T-cells and on days 6, 13, 20, and 27 thereafter. The blood was allowed to clot overnight at 4°C, and the serum was separated from the clot by centrifugation and diluted. Rat IgG (Sigma Chemical) was used as the positive control and PBS as the negative control. The samples were diluted serially and incubated at 37°C for 1 hour to allow adsorption of protein to the plates. The plates were washed three times, blocking buffer (3% bovine serum albumin in PBS) was added to each well, and the plates were incubated for an additional hour. The fluid was discarded, and 50 μl of biotinylated goat antirat IgG (Hyclone, Logan, UT) was added to each well for 30 minutes. The wells were washed three times, and 50 μl of streptavidin-β-D-galactosidase (Life Technologies) was added to each well for 30 minutes. The wells were washed three times, and 100 μl of chlorophenol red-β-D-galactopyranoside (Boehringer Mannheim, Indianapolis, IN) was added to each well. The plates were read at 550 nm and 690 nm using a 400 ATC SLT microplate reader (TEGAN U.S., Research Triangle Park, NC). The standard curve and sample concentrations were determined using Delta Soft III (Biometallics, Princeton, NJ).

Histopathologic Evaluation of Eyes Injected With Murine Cytomegalovirus

Immediately after the mice were killed, the virus-injected eyes were removed and fixed in buffered formaldehyde solution. The eyes were embedded in paraffin, and representative sections were made at six levels throughout the eye to ensure that a focal area of retinitis would not be overlooked. The sections were stained with hematoxylin and eosin before microscopic examination. All sections of each injected eye then were examined for retinitis characterized by virus-infected cytomegalic cells in the retina and in the retinal pigment epithelium and focal or total retinal destruction as described previously. Animals were considered to be retinitis-positive if there was microscopic evidence of focal or total retinal infection.

RESULTS

Persistence of Rat T-Cell Specific Monoclonal Antibody

A potential confounding factor when transferring immune cells into thymectomized, T-cell-depleted mice
is eradication or inactivation of the adoptively transferred cells by residual rat mAb. Experiments were done using enzyme-linked immunosorbent assay to determine the level of rat mAb in thymectomized, T-cell-depleted mice. As shown in Figure 1, the level of rat IgG began to decline after day 6 and by day 27, the level of rat IgG was <100 μg/ml. This result suggests that there was a low potential for depletion or inactivation of the adoptively transferred cells by residual rat mAb when they were infused on day 21 as described in the following section.

Determination of the Best Time for Adoptive Transfer of Murine Cytomegalovirus-Immune Cells

Because the results of the above experiment suggested that rat IgG levels began to decline as early as 6 days after injection of T-cell-specific mAbs, to balance depletion of endogenous T-cells with survival of transferred cells, experiments were done to determine the time after depletion when survival of the transferred cells was the highest. For these studies, MCMV-immune cells were prepared as described in the Materials and Methods section, labeled with PKH26-GH, and injected intravenously into uninfected T-cell-depleted mice 7, 14, 21, and 28 days after injection of mAbs. Three days after cell transfer, the animals were killed and single cell suspensions were prepared from the spleens. The cells were stained with fluorescein isothiocyanate (FITC)-conjugated rat antismouse CD4 (Life Technologies) or FITC-conjugated rat anti-mouse Lyt2 (CD8) (Becton Dickinson, Bedford, MA), and the percent of PKH+, FITC+ CD4+ cells and the percent of PKH+, FITC+ CD8+ cells were determined by flow cytometric analysis of spleen cells.

Before adoptive transfer, the extent of T-cell depletion in the spleens of thymectomized, T-cell-depleted mice was determined by flow cytometry. As shown in Figure 2A (unstained) and Figure 2B (stained with FITC-conjugated rat antimouse CD8), the extent of CD8+ T-cell depletion in thymectomized T-cell-depleted mice was >95%. Depletion of CD4+ cells was equivalent (data not shown). As shown in Figure 2C (unstained) and Figure 2D (stained with FITC-conjugated rat antimouse CD8), 3 days after adoptive transfer, PKH+, FITC+ CD8+ adoptively transferred cells comprised a small, but consistent,

**FIGURE 1.** Detection of residual rat immunoglobulin G in thymectomized T-cell-depleted mice. The amount of rat IgG (μg/ml serum) remaining after T-cell depletion was measured by enzyme-linked immunosorbent assay 6, 13, 20, and 27 days after injection of rat antimouse T-cell monoclonal antibodies. Each value represents the mean ± standard error of the mean from five mice.

**FIGURE 2.** Flow cytometric results of recovery of adoptively transferred cells in the spleens of thymectomized T-cell-depleted mice. (A) Unstained control specimen and (B) specimen stained with fluorescein isothiocyanate-conjugated antimouse CD8 representative profiles from the spleens of thymectomized, T-cell-depleted mice 24 days after T-cell depletion. Depletion of CD4+ T-cells was equivalent (data not shown). (C) Unstained control specimen and (D) specimen stained with fluorescein isothiocyanate-conjugated antimouse CD8 representative profiles of adoptively transferred PKH-labeled cells harvested from the spleens of thymectomized T-cell-depleted mice 3 days after adoptive transfer of 4 × 10⁷ murine cytomegalovirus-immune lymph node cells (24 days after T-cell depletion). The percentage of adoptively transferred CD4+ (data not shown) or CD8+ cells among the total number of recovered spleen cells was determined by subtracting the background fluorescence from each group (see Fig. 3).
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FIGURE 3. Percent ± standard error of the mean of adoptively transferred (PKH+, fluorescein isothiocyanate-conjugated [FITC+]) CD4+ T-cells (A) and CD8+ T-cells (B) in the spleens of thymectomized T-cell-depleted mice 3 days after adoptive transfer of murine cytomegalovirus-specific immune cells. Maximum recovery of CD4+ PKH+ or CD8+ PKH+ cells was observed when cells were adoptively transferred 21 days after injection of rat antimouse T-cell monochlonal antibodies.

fraction of the total spleen cells. Figure 3A summarizes the percent of adoptively transferred CD4+ T-cells in the spleen at days 10, 17, 24, and 31, and Figure 3B summarizes the percent of adoptively transferred CD8+ T-cells on the same days. The best recovery of both CD4+ and CD8+ cells was observed when cells were adoptively transferred 21 days after the second injection of rat antimouse T-cell-specific mAb.

Infectivity of Adoptively Transferred Murine Cytomegalovirus-Immune Cells

Because immune cells are produced by footpad injection of live virus, there is the possibility that a low titer of virus carried in the adoptively transferred lymph node cells might cause disease in a thymectomized, T-cell-depleted host. Accordingly, before performing adoptive transfer experiments to test the ability of adoptively transferred cells to protect from retinitis, it was necessary to determine if the adoptively transferred cells caused ocular disease in immunosuppressed recipient mice. Immune cells were transferred to 10 thymectomized, T-cell-depleted uninfected mice, and within 24 hours of cell transfer, 2 µl of serum-free tissue culture medium was injected into the supraciliary space of each mouse. This injection violates the blood–ocular barrier and in the absence of virally induced inflammation, provides a route of access into the ocular compartment. One week after supraciliary injection, the mice were killed, and the salivary glands, lungs, and spleen of each animal were homogenized and titered. One half (five) of the injected eyes were examined microscopically for histopathologic evidence of retinitis (cytomegalic cells, inflammation, and/or retinal destruction) as described previously. The other five injected eyes were homogenized and titered for infectious virus. The eyes (5), lungs (10), and spleens (10) were negative for virus (<5 pfu/ml). The salivary glands (10) contained only a low titer of virus (mean titer, 2.04 × 10^1 ± 0.30 pfu/ml).

Adoptively Transferred Murine Cytomegalovirus-Immune Cells Protect Against Retinitis

Based on the above experiments that showed that (1) adoptively transferred immune cells could be shown in the spleen of T-cell-depleted recipient mice and (2) by themselves, immune cells did not cause ocular disease in a thymectomized, T-cell-depleted host, experiments were done to determine whether adoptive transfer of MCMV-immune cells could prevent retinitis in thymectomized T-cell-depleted mice. For these experiments, thymectomized, T-cell-depleted BALB/c mice were divided into four groups. Twenty-one days after injection of rat antimouse T-cell mAbs, lymph node cells were adoptively transferred through tail vein as follows: group 1, 4 × 10^7 in vitro-restimulated MCMV-immune lymph node cells; group 2, 4 × 10^7 freshly isolated (nonrestimulated) MCMV-immune lymph node cells; group 3, 4 × 10^7 freshly isolated ovalbumin-immune lymph node cells; group 4, PBS only (0.2 ml). All mice were injected with 5 × 10^2 pfu of MCMV through the supraciliary route. One week later, all mice were killed, and the titer of virus in each of the salivary glands and in one half of the injected eyes was determined by plaque assay. The remaining eyes were fixed, embedded in paraffin, sectioned at six levels throughout the eye, and examined microscopically for retinitis as described in the Materials and Methods section.
Although the titer of virus in the eyes of mice in group from that of the PBS-treated control mice (group 4).

**Location of Ocular Virus**

As listed in Table 1, both in vitro-restimulated and nonrestimulated MCMV-immune lymph node cells protected the mice from retinitis. There was no significant difference between the incidence of retinitis in mice in group 1 and in group 2. Adoptive transfer of freshly isolated ovalbumin-immune lymph node cells did not protect against retinitis, and the incidence of retinitis in group 3 mice was not different from that of the PBS-treated control mice (group 4). The titer of ocular virus was reduced significantly by adoptive transfer of either in vitro-restimulated or freshly isolated MCMV-immune cells and was 3.5 times and 5.4 times less in the eyes of groups 1 and 2 mice, respectively, than it was in the eyes of the mice in group 4.

**DISCUSSION**

Previous studies have shown that inoculation of 5 × 10^6 pfu of the Smith strain of MCMV in T-cell-depleted or methylprednisolone-immunosuppressed BALB/c mice results in acute retinitis with histopathologic features similar to those observed in patients with acquired immune deficiency syndrome. Cellular immunity has a major role in controlling cytomegalovirus infections, and augmentation of the cellular immune system has been used to prevent or ameliorate CMV infections in patients who are immunosuppressed. Immune cell therapy with virus-specific cells has not yet been used as a treatment for CMV retinitis. To begin to explore this issue, experiments were performed to test whether MCMV-immune lymph node cells could protect against MCMV retinitis in thymectomized T-cell-depleted BALB/c mice and to determine whether protection from retinitis was virus specific.

Several factors could complicate protection studies in immunosuppressed mice. For example, residual rat antimouse mAb used to deplete endogenous host

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Incidence of Retinitis (%)</th>
<th>Virus Recovery (PFU/ml)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Eye</td>
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<tr>
<td>1</td>
<td>4 × 10^7 in vitro restimulated murine cytomegalovirus-specific lymph node cells</td>
<td>2/11 (18)*</td>
<td>2.76 × 10^3 †</td>
</tr>
<tr>
<td>2</td>
<td>4 × 10^7 nonrestimulated murine cytomegalovirus-specific lymph node cells</td>
<td>1/7 (14)*</td>
<td>1.79 × 10^3 †</td>
</tr>
<tr>
<td>3</td>
<td>4 × 10^7 nonrestimulated ovalbumin-specific lymph node cells</td>
<td>6/8 (75)</td>
<td>4.97 × 10^3</td>
</tr>
<tr>
<td>4</td>
<td>Phosphate-buffered saline (no cells)</td>
<td>8/10 (80)</td>
<td>9.58 × 10^3</td>
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* P < 0.01 versus group 4 (chi-square).
† P < 0.05 versus group 4 (Mann-Whitney test).
‡ P < 0.01 versus group 4 (Mann-Whitney test).

As listed in Table 1, both in vitro-restimulated and nonrestimulated MCMV-immune lymph node cells protected the mice from retinitis. There was no significant difference between the incidence of retinitis in mice in group 1 and in group 2. Adoptive transfer of freshly isolated ovalbumin-immune lymph node cells did not protect against retinitis, and the incidence of retinitis in group 3 mice was not different from that of the PBS-treated control mice (group 4). The titer of ocular virus was reduced significantly by adoptive transfer of either in vitro-restimulated or freshly isolated MCMV-immune cells and was 3.5 times and 5.4 times less in the eyes of groups 1 and 2 mice, respectively, than it was in the eyes of the mice in group 4.

**Location of Ocular Virus**

Although the titer of virus in the eyes of mice in group 1 and group 2 was significantly less than in the control mice, virus was recovered from the injected eyes of these mice. Because mice in group 1 and group 2 were protected from retinitis, the recovery of virus suggested that the location of ocular virus might be different in mice that received MCMV-immune cells than it was in mice in either of the control groups (groups 3 and 4). To address this question, MCMV-injected eyes from mice in all four groups were removed 7 days after infection, fixed, embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically for virus infection as indicated by cytomegalic cells in the iris and ciliary body and cytomegalic cells and focal or total retinitis in the posterior segment. As shown in Figure 4, cytomegalic cells were seen only in the anterior segment of mice in group 1 (in vitro-restimulated MCMV-immune lymph node cells) (Figs. 4A, 4B) and group 2 (nonrestimulated MCMV-immune lymph node cells) (data not shown). In contrast, cytomegalic, virus-infected cells were observed in both the anterior and posterior segment of mice in group 3 (ovalbumin-immune lymph node cells) (Figs. 4C, 4D) and of mice in group 4 (PBS-treated control) (Figs. 4E, 4F). Taken together, comparison of the microscopic examination results of the injected eyes of mice in all of the groups suggests that in mice treated with restimulated or freshly isolated MCMV-immune lymph node cells, virus infection was confined to the anterior segment. Animals treated with ovalbumin-immune lymph node cells or with PBS-developed retinitis and virus-infected, cytomegalic cells were observed in both the anterior segment and the posterior segment, suggesting that replicating virus recovered from these eyes was from both the anterior segment (the site of injection) and the retina.

![Image](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933422/)
FIGURE 4. Photomicrograph illustrating virus infection in the anterior segment and posterior segment of injected eyes of thymectomized T-cell-depleted mice (15 to 20 mice/group) injected with 5 × 10⁵ plaque-forming units of murine cytomegalovirus through the supraciliary route. The retinas of mice that received restimulated murine cytomegalovirus-immune lymph node cells mice were not infected (A), whereas evidence of virus replication (cytomegaly) of iris epithelial cells was observed (B). Retinitis was observed in injected eyes of mice that received ovalbumin-immune cells (C), and virus infection also was observed in the iris (D) of these mice. Focal destruction of the retina (E) and infection of the iris (F) also were observed in control mice injected with phosphate-buffered saline alone. Cytomegalic cells and areas of virus infection are shown (arrowheads) (original magnification, X343).

T-cells also could deplete adoptively transferred cells. Accordingly, the aim of the initial experiments of this study was to find a time after T-cell depletion when the amount of residual rat antimouse T-cell-specific IgG did not affect the ability of the transferred cells to protect. Together, the results from enzyme-linked immunosorbent assay studies and from flow cytometric analyses of adoptively transferred cells in the spleen showed that the best time for cell transfer was approximately 21 days after mAb injection.

An additional potential difficulty with adoptive transfer of MCMV-immune cells in immunosuppressed mice is inclusion of cells in the transfer inoculum that contain replicating virus or latent virus or both. After adoptive transfer into immunosuppressed mice, replication of infectious virus or reactivation of latent virus would be expected to increase the amount of total virus and depending on the distribution of cells, might exacerbate disease in one or more tissue sites, such as the eyes or lungs. The results of the studies reported herein show that adoptive transfer of MCMV-immune cells did not cause retinitis in uninfected immunosuppressed mice with a disrupted blood-retinal barrier. However, recovery of a low titer of virus in the salivary glands of these mice suggests that although the adoptively transferred inoculation
was virus-negative by plaque assay (data not shown), a few cells in the adoptively transferred inoculum were virus-positive. Other investigators have used PCR to show that MCMV-immune cells are negative for MCMV DNA sequences. There are several differences between the previous study and the one presented in this article. In the previous study, donor mice were immunized through the intraperitoneal route, treated with thymic humoral factor (THFγ2), and virus-specific immune cells were transferred approximately 4 weeks after the first injection of virus. In our studies, donor mice were inoculated through the footpad route and without further manipulation or additional injections of virus, draining lymph nodes were transferred 10 days after infection (fresher isolated) or 12 days after infection (in vitro-restimulated cells).

Our virus recovery studies were done 7 days after transfer of MCMV-immune cells into uninfected, T-cell-depleted mice, and it is possible that with prolonged immunosuppression and a longer experimen-tal timetable, the small amount of virus recovered in the salivary glands (mean titer, 2.04 × 10^1 pfu) might be able to replicate to higher titers and cause systemic or local infection. However, because the duration of the protection studies in infected mice also was 7 days, even a small amount of virus in the adoptive transfer inoculum should not have contributed significantly to the titer of virus nor to the outcome of these experiments.

Adoptive transfer experiments with MCMV-immune cells freshly isolated from the draining lymph nodes showed that these cells prevented retinitis as effectively as in vitro-restimulated MCMV-immune lymph node cells. This result indicates that in vitro restimulation is not required to prevent retinitis. However, we do not know whether cell type or types are responsible for protection. Previous studies in CD4-depleted mice, CD8-depleted mice, and in CD4 and CD8-depleted mice showed that depletion of CD8+ T-cells correlates with development of MCMV retinitis after supraciliary inoculation. Based on these results as well as on those of other investigators showing that T-cells rather than B-cells or other types of nonspecific effector cells are important in protection from CMV and MCMV infection, it is likely that T-cells also are involved in protection from retinitis mediated by adoptively transferred MCMV-immune cells. Additional studies, in which separated populations of MCMV-immune CD4+ cells, CD8+ cells alone, or in combination with other cell types, are necessary to answer this question. The finding that transfer of freshly isolated ovalbumin-immune cells did not protect suggests that in this system, antigen-specific cells are required for protection and that protection cannot be effected by transfer and reconstitution using activated but nonspecific lymph node cells.

Protection in these studies correlated with adoptive transfer of 4 × 10^7 MCMV-immune cells. Preliminary studies were done to determine the ability of a lower number of adoptively transferred immune cells to protect from retinitis, and the results of these studies indicated that the highest level of protection was achieved when 4 × 10^7 cells were transferred (not shown). In other systems of MCMV infection, the number of adoptively transferred cells needed to protect has varied. Rager-Zisman and colleagues reported the highest level of protection from systemic disease in mice receiving 1 × 10^7 MCMV immune cells, and Shanley demonstrated reduction of viral titer in the adrenal glands of nude mice that received 2 × 10^7 immune cells. Reddehase and co-workers showed that the titer of MCMV in the lungs was reduced by adoptive transfer of 2 × 10^6 immune lymphocytes. Together, the results from these investigators suggest that the number of immune cells required for protection varies with the system and with what end point is being examined. Perhaps a large number of immune cells is required for protection from MCMV retinitis because of limited access of transferred cells to the ocular compartment.

In view of the lack of microscopic evidence of retinitis, recovery of virus from the injected eyes of retinitis-protected mice was somewhat surprising. Although the eyes of mice receiving an adoptive transfer of MCMV-immune lymph node cells were protected from retinitis and the titer of virus in the eyes of these mice was significantly reduced, the eyes were virus-positive. Histopathologic examination results showed that most of the virus replication (as judged by the presence of cytomegalic cells) was confined to the anterior segment. Taken together, the virus recovery studies and the microscopic examination results suggest that in some tissues, such as the eye, where all components may not be infected with virus, results from virus titrations from the whole organ by themselves may not provide an accurate picture of what is occurring in that tissue.

Further studies to identify the protective cells and the immune specificities of the cells that protect are needed to explore the mechanism by which virus-specific adoptively transferred cells prevent MCMV retinitis. Nevertheless, much remains to be learned about adoptive transfer of immune cells to protect from MCMV retinitis. Therefore, the findings that (1) the incidence of retinitis in T-cell-depleted BALB/c mice was reduced significantly by adoptive transfer of syngeneic MCMV-immune lymph node cells and (2) in the absence of virus infection in the host, adoptively transferred cells could not infect the eye (even when the blood–ocular barrier was damaged) suggest that...
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adoptive transfer of autologous, in vitro-expanded CMV-specific cells eventually might be attempted for treatment of CMV retinitis in human patients.

Key Words
adoptive immunotherapy, BALB/c mouse, immunosuppression, murine cytomegalovirus, retinitis

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References
30. Reddahese MJ, Weiland F, Münch K, Jonjić S, Láska A,


