

Differential Expression of Chemokines by Human Retinal Pigment Epithelial Cells Infected with Cytomegalovirus

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PURPOSE. To evaluate the effects of human cytomegalovirus (HCMV) infection on chemokine gene expression and secretion by human retinal pigment epithelial (HRPE) cell cultures.

METHODS. HRPE cells were infected with HCMV (strain AD169) at an MOI of 5. Culture supernatants, collected at various postinoculation days, were used for the analyses of chemokines by ELISA. The steady state levels of chemokine and chemokine receptor mRNA were analyzed by RT-PCR. Effects of interferon and MCP-1 on HCMV replication in HRPE cells were evaluated by plaque assays.

RESULTS. HRPE cells infected with HCMV exhibited characteristic cytopathic effects. The reduction in the levels of monocyte chemoattractant protein (MCP)-1 and -3 mRNA in HCMV-infected HRPE cells was observed in comparison to uninfected HRPE cells. In contrast, HCMV infection enhanced IL-8 mRNA levels, whereas regulated on activation normal T-cell expressed and secreted (RANTES) mRNA was not detectable in either control or infected HRPE cells. A significant decrease in MCP-1 ($P < 0.01$) and MCP-3 ($P < 0.05$), but a significant increase in IL-8 ($P < 0.05$), protein secretion was observed. Expression of the chemokine receptors CCR2, specific for MCP-1, and CXCR1 and CXCR2, specific for IL-8, were not altered by HCMV infection. Treatment of HRPE cultures with MCP-1 had no significant effect on HCMV replication in HRPE cells.

CONCLUSIONS. HCMV infection in HRPE cells resulted in the modulation of MCP-1, MCP-3, and IL-8. Because chemokines facilitate the activation of leukocytes and their migration to the sites of inflammation, the modulation of chemokine production by the virus suggests a role for chemokines in immune evasion and/or immunopathogenesis of CMV retinitis. (*Invest Ophthalmol Vis Sci.* 2003;44:2026–2033) DOI:10.1167/iovs.02-0980

Immunopathological processes during inflammation and infection are modulated by various cytokines, chemokines, and other factors produced by infiltrating leukocytes and the resident cells of the affected tissues.^{1–3} Chemokines, proteins

with conserved cysteines, act as chemoattractants to recruit the leukocytes.^{4–6} Most of the chemokines are classified as CC (α) or CXC (β) chemokines; in CC chemokines the first two cysteines are adjacent, whereas in CXC chemokines the first two cysteines are separated by one amino acid.^{2,4} Chemokines are soluble, diffusible small molecules that act on target cells by interacting with specific membrane receptors classified as CCR or CXCR.^{4–7} Most of the chemokine receptors recognize more than one chemokine of the same family (α or β), with various affinities. Chemokines may have specific as well as overlapping interactions with such inflammatory cells as neutrophils, monocytes/macrophages, and lymphocytes, depending on the presence and availability of the receptors.^{2–8} By regulating the leukocyte trafficking, chemokines play a critical role in the pathophysiological processes during wound healing, infection, and/or inflammation.^{2–8}

In immunologically mediated eye diseases, chemokines and their receptors have been shown to be essential in inflammatory cell recruitment to the cornea in onchocercal keratitis⁹ and to the iris/ciliary body in autoimmune anterior uveitis.¹⁰

In virus infections, chemokines and chemokine receptors have been identified as critical elements for the defense against viruses.^{11–13} The host uses chemokines and chemokine receptors to aid in viral clearance by attracting leukocytes to the foci of infection, by enhancing cytotoxic activity of infected cells, and by blocking entry of viruses that use chemokine receptors to gain entry into cells.^{14–16} Virus infections may also result in an alteration in transcription and translation of cytokines, chemokines, and other factors by the host cell.^{12,17} In response to the antiviral potential of chemokines, viruses have generated various strategies to block these responses as mechanisms for immune evasion.^{17–19} Selected viruses have been shown to express chemokine analogues, virus-encoded chemokine-binding proteins, and virus-encoded receptors.^{20–24}

Human cytomegalovirus (HCMV) infection causes life-threatening complications in immunocompromised hosts, such as patients with acquired immunodeficiency syndrome (AIDS) and recipients of organ and bone marrow transplants.^{25–29} The ocular diseases commonly observed in these patients manifest as cotton-wool spots, hemorrhages, and degeneration in the retina, and together these constitute the diagnosis of CMV retinitis.^{30–35} The precise mechanism of viral spread in the retina is not clear, but it has been suggested that HCMV leaks from the damaged blood vessels and subsequently infects cells in the various retinal layers.^{36,37} CMV antigens and/or viral inclusion bodies were detected in astrocytes, Müller cells, and retinal pigment epithelium (RPE).^{36,38} RPE appears to be disorganized and focally missing in some cases, resulting in the breakdown of the blood-ocular barrier followed by accumulation of the fluid in the subretinal space, causing retinal detachment.^{36–40}

RPE, a single layer of epithelium sandwiched between the neuroretina and choroid, plays a vital role in the normal functioning of the retina by engulfing the photoreceptor outer segments, transport of nutrients, and transport of waste mate-

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rial between retina and choroid.^{41,42} In addition, RPE, with its Bruch's membrane, acts as a blood-retinal barrier and keeps the neuroretina intact and attached to prevent retinal detachments. Because of its strategic location, RPE frequently encounters infectious agents and inflammatory molecules.^{43,44} In our previous studies, a human retinal pigment epithelial cell culture (HRPE) model was used to study the characteristics of HCMV replication and the use of antisense oligonucleotides to inhibit HCMV replication in these cells.^{45,46}

The demonstration of the presence of HCMV antigens and inclusion bodies, and damage to the RPE layer in CMV retinitis strongly suggests involvement of RPE in the pathogenesis of CMV retinitis.³⁶⁻³⁸ The presence of immunoglobulins in the retinal arterioles, and neutrophils and other immune cells in the retina of patients with CMV retinitis may be associated with intraocular inflammation.^{36,38,40} A variety of factors such as viral antigens, virus host-cell interactions, and immune complexes may be involved in the production of chemokines, which are agents involved in leukocyte trafficking. However, the cellular sources of these chemotactic factors are not known. Therefore, we investigated the secretion of chemokines by HCMV-infected HRPE cells and discussed its possible role in leukocyte trafficking in CMV retinitis and in immune-recovery uveitis.

MATERIALS AND METHODS

Reagents

Human monocyte chemotactic protein (MCP)-1, MCP-3, IL-8, and regulated on activation normal T-cell expressed and secreted (RANTES) ELISA kits and human rIFN- β and MCP-1 were purchased from BioSource International (Camarillo, CA); human IL-1 β , macrophage-inflammatory protein (MIP)-1 α , and MIP-1 β ELISA kits from R&D Systems (Minneapolis, MN); fetal bovine serum (FBS) and cell culture medium from Life Technologies (Grand Island, NY); and PCR amplimer sets for human MCP-1, MCP-3, IL-8, and RANTES and multiplex cDNA amplification kits (CytoXpress) for human chemokine receptors (CCR 1-5) and (CXCR 1-4) from BioSource International. An RNA PCR reagent kit (GeneAmp) with DNA polymerase (Ampli Taq) was from Applied Biosystems, Foster City, CA.

HRPE Cultures

Primary cell lines of HRPE cells were prepared from human donor eyes as described previously.⁴⁷ The protocol regarding use of donor eyes adhered to the provisions of the Declaration of Helsinki for research involving human tissue. Briefly, RPE-choroid explants were placed in culture dishes with the RPE layer facing down in minimum essential medium (MEM) containing 20% FBS. Outgrowth of epithelial cells from the explants was monitored and the cells selected for further propagation in MEM containing 10% FBS, nonessential amino acids, and antibiotic-antimycotic mixture. The homogeneity of the cell population was confirmed by positive immunostaining with mAb to cytokeratin, an epithelial cell-specific cytoskeletal protein.⁴⁷ HRPE cultures at passages 7 to 12 were used for the experiments reported in this study.

HCMV Preparation

HCMV (strain AD169) was propagated in MRC-5 cells and prepared as described earlier.⁴⁵ HCMV stocks were prepared both in 2% FBS and in serum-free medium (SFM). Virus was propagated in MRC-5 cells and when most of the cells were infected, cells were lysed by freeze-thawing on dry ice. The culture extracts were clarified by centrifugation at 2000 rpm for 20 minutes, and the supernatants were frozen at -70°C . An aliquot of the viral stock was used for the determination of viral titers by plaque assays. Virus titers, measured on MRC-5 cells, were typically $1 \times 10^{6-8.7}$ plaque-forming units (PFU)/mL. Previous studies in our laboratory have shown that HCMV infection of HRPE

cells maintained in SFM results in slightly enhanced production of virus in comparison to medium containing 2% FBS.⁴⁵

SFM was used in HCMV infection studies with HRPE cells, because serum growth factors, cytokines, and chemokines would interfere with the production of chemokines by cells and determination of chemokines by ELISA. Once HRPE cultures reached confluence, SFM had no effect on the viability of cells or attachment of the cells to the dish during the course of the experiment. To compensate for the effects of absence of serum on HRPE cells, mock-infected HRPE cells from the same batch of cultures treated under similar conditions (except virus) were used for all the comparisons.

HCMV Viral Plaque Assays

HRPE cultures were grown to confluence in 24-well culture plates in MEM containing 10% FBS and other components, as described in the section on HRPE cultures. The cultures were washed with SFM and incubated in SFM (1 mL/well) or SFM containing indicated agent for 24 hours. Then, the cultures were washed twice with SFM and inoculated with 50 PFU of HCMV (strain AD169). The cultures were incubated for 2 hours at 37°C in a tissue culture incubator, with mild agitation every 15 minutes. After a 2-hour adsorption period, the inoculum was removed, and cells were washed twice with SFM and cultures overlaid with 1 mL of viral titration medium (MEM supplemented with 2% heat-inactivated FBS and 0.75% methylcellulose)⁴⁶ containing indicated concentrations of agents. Two days later, 1 mL of viral titration medium without methylcellulose but containing various agents was added to the wells. Media were replaced every other day. Ten days after inoculation, cells were washed, fixed with ethyl alcohol, and stained with Giemsa, and the plaques were counted.

Analysis of Chemokine mRNA Expression in HCMV-Infected HRPE Cells by RT-PCR

The PCR primers for human chemokines MCP-1, MCP-3, IL-8, and RANTES were obtained from BioSource International. The following primers were used: pp65, 5'-CAC CTG TCA CCG CTG CTA TAT TTG C-3' and 5'-CAC CAC GCA GCG GCC CTT GAT GTT T-3',⁴⁶ and GAPDH, 5'-CCA CCC ATG GCA AAT TCC ATG GCA-3' and 5'-TCT AGA CGG CAG GTC AGG TCC ACC-3'. HRPE cultures were inoculated with HCMV at an MOI of 5, as described earlier, and incubated in SFM without methylcellulose overlay. Total cellular RNA from the mock- and HCMV-infected HRPE cultures was prepared by an extraction process (RNASTAT-60; Tel-Test, Friendswood, TX). A RNA PCR kit was used for reverse transcription and PCR reactions in a single tube, as described.⁴⁸ Briefly, 1 μg of total RNA was reverse transcribed to cDNA by incubating with oligo d(T)₁₆, the reverse transcriptase primer, and MuL ν reverse transcriptase for 15 minutes at 42°C , 5 minutes at 99°C , and 5 minutes at 5°C , in a PCR system (GeneAmp 9600; Applied Biosystems). The synthesized cDNA was amplified by PCR with commercial DNA polymerase (Ampli Taq ; Applied Biosystems) in the presence of specific primers. Samples were heated for 105 seconds at 95°C and amplified for 25 cycles: 15 seconds at 95°C and 30 seconds at 60°C , followed by a final extension of 7 minutes at 72°C . PCR products were separated on an agarose gel containing ethidium bromide, photographed under UV light, and integrated with an image-acquisition system (Eagle Eye; Stratagene, San Diego, CA).

Determination of Chemokine Levels in Culture Supernatant Fluids by ELISA

HRPE cultures were grown to confluence in 24-well plates in medium containing 10% FBS, washed with SFM, and incubated with SFM for 24 hours. Cultures were then inoculated with HCMV at an MOI of 5. After a 2-hour adsorption period, the virus inoculum was removed, the cells washed twice, and the cultures fed with fresh SFM. Culture supernatants were collected after 1 day of inoculation and replaced with fresh SFM. The culture supernatants were collected similarly after 3, 5, 7, and 9 days of inoculation, and each time replaced with fresh SFM, and stored at -70°C . The cultures were maintained in SFM throughout the

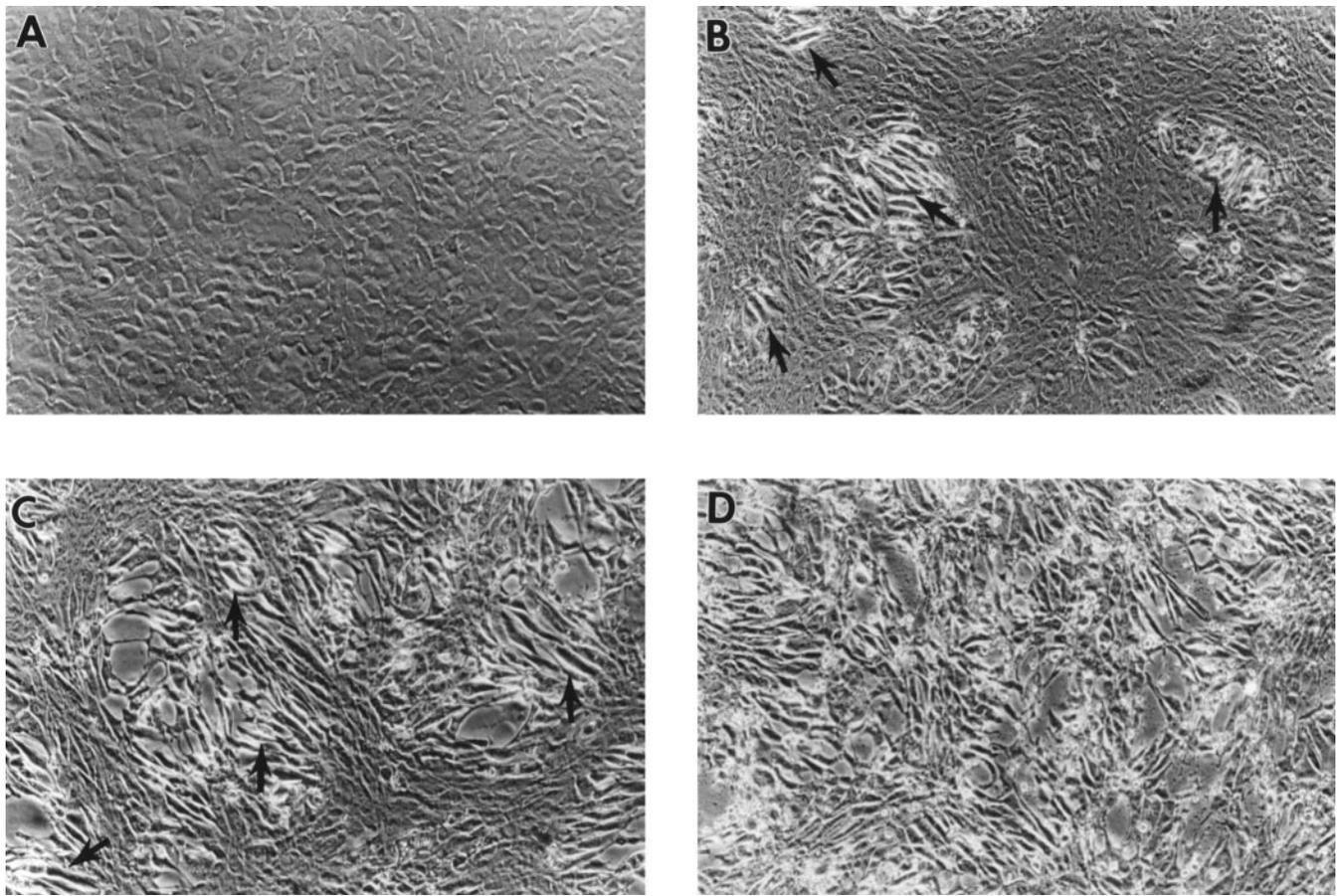


FIGURE 1. Representative phase-contrast photomicrographs of live HRPE cultures infected with HCMV. HRPE cultures were inoculated with HCMV at an MOI of 5. Cultures were observed under microscope and photographed on various postinoculation days. (A) Mock-infected cultures at PI day 9 and HCMV-infected cultures at PI days 3 (B), 5 (C), and 9 (D). Arrows: position of cells in the cultures exhibiting typical CPEs (i.e., swollen and shiny cells).

study period to avoid interactions and interference with serum growth factors, cytokines, chemokines, and other agents. Culture supernatant fluids from uninfected and HCMV-infected HRPE cells were clarified by centrifugation for 5 minutes at 14,000 rpm in a microfuge (Eppendorf, Fremont, CA). Levels of MCP-1, MCP-3, IL-8, RANTES, MIP-1, and IL-1 β were determined by ELISA, according to the manufacturers' instructions. Range of the standards for MCP-1, and MCP-3, IL-8, and RANTES were 30 to 2000 and 15 to 1000 pg/mL, respectively. Results obtained from the same batch of cultures grown under similar conditions and infected with HCMV were used for the statistical evaluation of the data for any given experiment.

Expression of Chemokine Receptors in HCMV-Infected HRPE Cells

Expression of the chemokine receptors, CCR and CXCR, was analyzed by multiplex PCR kit (CytoXpress BioSource International). Total RNA from uninfected and HCMV-infected HRPE cells were prepared an extraction process (TRIzol; Life Technologies, Rockville, MD). The RNA was reverse transcribed to cDNA in the presence of oligo dT primers and MuLv reverse transcriptase. One microgram of cDNA was used for each multiplex PCR reaction, with CCR or CXCR primer pairs and primers for GAPDH. PCR reactions were performed as specified by the manufacturer to amplify six targets at the same time in a single-tube reaction. After the initial denaturing step at 96°C for 1 minute, 2 cycles of denaturation for 1 minute at 96°C, and annealing for 4 minutes at 58°C were performed. This was followed by 33 cycles of denaturation for 1 minute at 94°C and annealing at 58°C for 2.5 minutes. The final step consisted of extension for 10 minutes at 70°C followed by a soak

at 25°C for 2 minutes. PCR products were resolved by 4% agarose gel electrophoresis, stained with ethidium bromide, photographed under UV light, and integrated with the image-acquisition system (Eagle Eye; Stratagene).

RESULTS

HCMV Infection of HRPE Cultures

HRPE cultures inoculated with HCMV showed a typical morphologic appearance of HCMV infection, characterized by swollen cells that were shiny and easily identified by phase-contrast microscope (Fig. 1). Cytopathic effects (CPEs) of HCMV infection in HRPE cultures were graded by observing the cultures under phase-contrast microscope. Approximately 25% of the cells exhibited CPEs by 3 days, 50% by 5 days, 75% by 7 days, and almost all the cells by 9 days after inoculation (Fig. 1).

Analyses of mRNA Expression of Chemokines

We evaluated the steady state levels of chemokine mRNA by RT-PCR in HCMV-infected HRPE cultures to determine whether HCMV infection influences the levels. To confirm and evaluate the replication of HCMV in HRPE cells, the levels of HCMV pp65 mRNA were determined in mock- and virus-infected cells. As expected, pp65 mRNA was not detected in mock-infected cells, but in HCMV-infected cells, it was detected. The levels of pp65 mRNA were low in cultures at

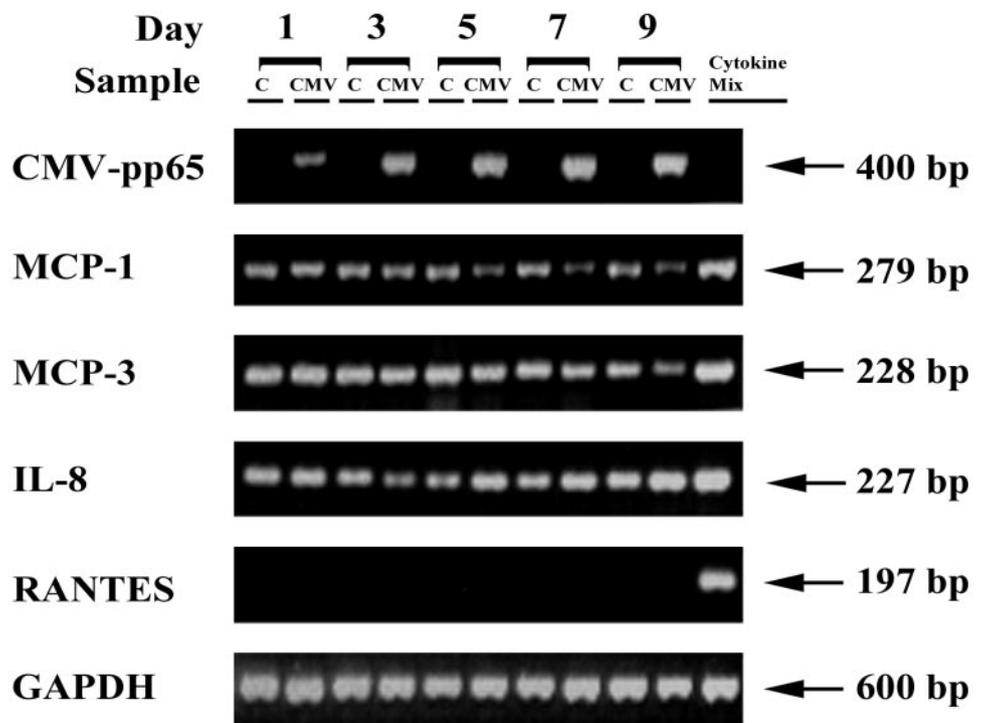


FIGURE 2. RT-PCR analyses of the steady state levels of chemokine mRNA in HCMV-infected HRPE cultures. HRPE cultures were either mock infected (C) or HCMV infected (CMV) at an MOI of 5 in SFM and total RNA prepared on various post-inoculation days (Day). One microgram of total RNA was used for RT-PCR analysis for each reaction for CMV-pp65, MCP-1, MCP-3, IL-8, RANTES, and GAPDH. Sizes of the PCR products are shown on the right side of the figure. PCR products in the right lane (Cyto mix, day 1) were derived from the total RNA prepared from HRPE cells treated with a mixture of cytokines (IFN- γ , TNF- α , and IL-1 β). All PCR reactions were subjected to 25 cycles. Results of one typical experiment are shown and are representative of those in three other individual experiments.

postinoculation (PI) day 1 and increased significantly with time after inoculation (Fig. 2). MCP-1 mRNA was detected in both mock- and HCMV-infected HRPE cultures. There was a decrease in the intensity of the bands in virus-infected cultures compared with that in time-matched, mock-infected cultures at PI days 5, 7, and 9 (Fig. 2). Similarly, MCP-3 mRNA was detected in both uninfected and HCMV-infected HRPE cells. The decrease in the MCP-3 mRNA levels in HCMV-infected HRPE cells was less pronounced than that observed in MCP-1. In contrast to MCP-1 and -3, IL-8 mRNA levels increased in HCMV-infected HRPE cells. This was seen clearly in cultures at PI day 5, 7, and 9. RANTES mRNA was not detected in either uninfected or HCMV-infected cultures. HRPE cultures treated with a mixture of cytokines (IFN- γ , IL-1 β , and TNF- α) elevated mRNA levels of MCP-1, MCP-3, and IL-8, and induced RANTES mRNA (Fig. 2, Cyto mix). The bottom panel shows the bands of PCR products amplified with GAPDH primers with the same amount of total RNA preparations as indicated in the top panels. These results demonstrate that chemokine gene expression was differentially regulated by HCMV infection of HRPE cells.

Chemokine Secretion

Next, we examined the secretion of chemokine proteins by HRPE cells infected with HCMV to verify whether altered levels of chemokine mRNA results in alteration in chemokine secretion. HRPE cultures were inoculated with HCMV at an MOI of 5, similar to the conditions described in the mRNA studies. The levels of chemokines secreted at various postinoculation days by mock- and HCMV-infected HRPE cells were determined. Mock-infected cultures secreted MCP-1 in the range of 1500 to 2200 pg/mL. HCMV infection resulted in a gradual decrease in MCP-1 secretion compared with mock-infected cultures (Fig. 3A) and was significantly lower at PI days 3, 5, 7, and 9 ($P < 0.005$). MCP-1 secretion was almost completely abolished at PI days 7 and 9. MCP-3 secretion by mock-infected HRPE cultures was in the range of 200 to 350 pg/mL, much lower than MCP-1. A significant decrease ($P < 0.05$) in MCP-3 secretion was

observed in HCMV-infected HRPE cells at PI days 5, 7, and 9 (Fig. 3B).

In contrast to MCP-1 and MCP-3, IL-8 secretion was significantly enhanced by HCMV infection of HRPE cells (Fig. 3C). Uninfected HRPE cells secreted IL-8 in the range of 28 to 120 pg/mL and this secretion was elevated significantly ($P < 0.05$) at PI days 3, 5, 7, and 9. Very low levels of RANTES (12–18 pg/mL) were detected in mock-infected HRPE cultures. HCMV infection had no effect on RANTES secretion (Fig. 3D) corresponding to RT-PCR results in which no mRNA was detected. Treatment of HRPE cultures with a mixture of cytokines (IFN- γ , IL- β , TNF- α) resulted in a severalfold increase in the secretion of MCP-1, MCP-3, IL-8, and RANTES (data not shown), suggesting that HRPE cells have the potential to produce these chemokines with appropriate stimulation. Mock- and HCMV-infected cells did not secrete either MIP-1 α or MIP-1 β (data not shown). Because IL-1 β is known to induce MCP, IL-8, and RANTES, we examined the culture supernatants for the presence of IL-1 β . At all time points, IL-1 β was not detectable in either mock- or HCMV-infected HRPE cells.

Chemokine Receptor Expression

ELISA data indicated that the levels of MCP-1 and -3 in the culture supernatants decreased and were almost undetectable, whereas IL-8 secretion increased significantly as a result of HCMV infection. To investigate whether changes in the levels of the secreted chemokines are due to up- or downregulation of their membrane receptors, we examined the expression of CC and CXC receptor messages in mock- and HCMV-infected HRPE cells by RT-PCR. Results of the CC chemokine receptors for MCP-1, MCP-3, and RANTES, are shown in Fig. 4A. CCR2, the specific receptor for MCP-1, -2, and -3 was not expressed in either uninfected or HCMV-infected HRPE cells. CCR1, the specific receptor for RANTES, MIP-1 α , and MCP-3 was prominently expressed in HRPE cells and downregulated in HCMV-infected cells at PI days 7 and 9. The other CC chemokine receptors were expressed at very low levels, and there appeared to be no significant change. Because the specific recep-

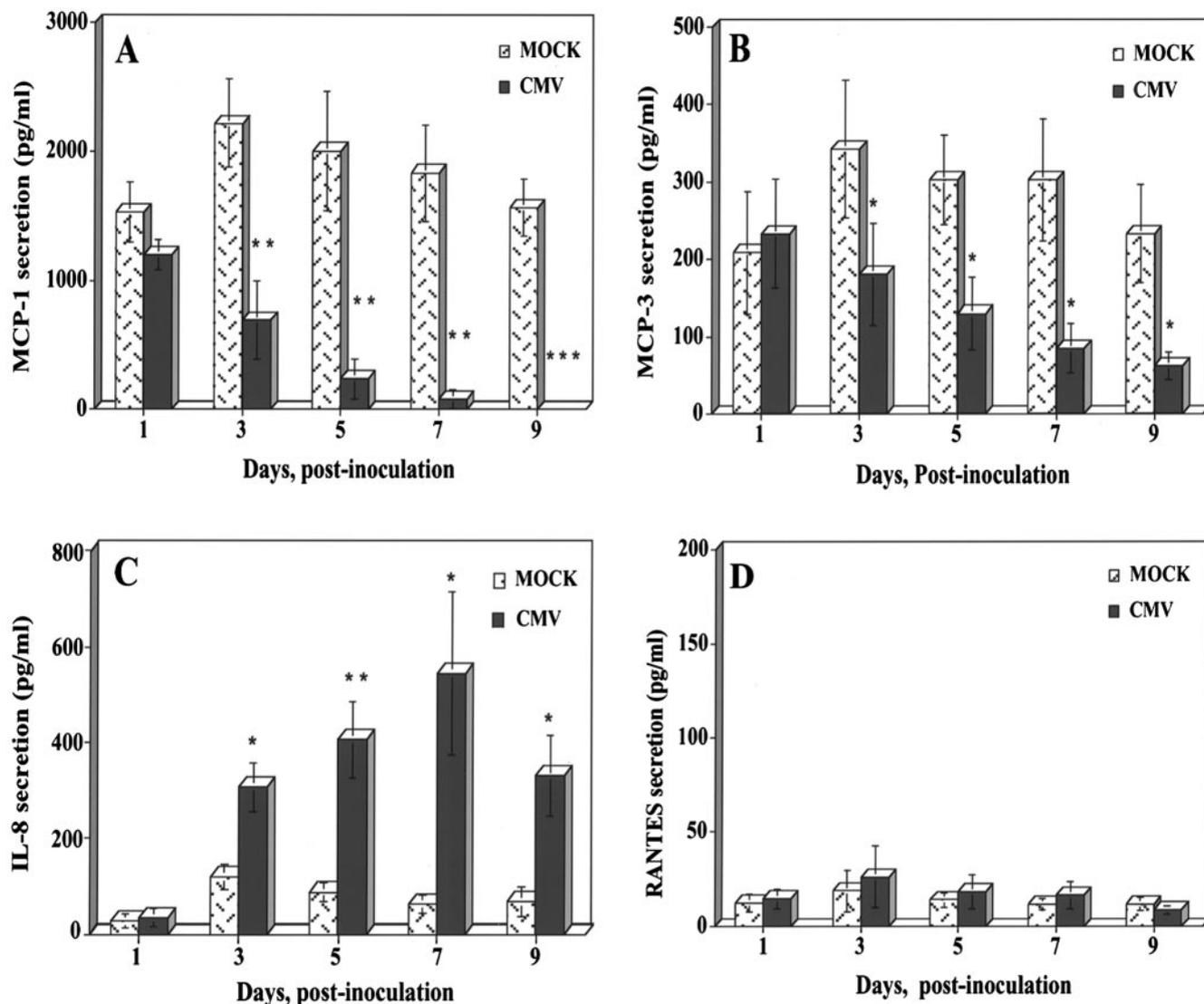


FIGURE 3. Chemokine secretion by HCMV-infected HRPE cultures. HRPE cells were infected with HCMV (CMV) at an MOI of 5 or mock infected (MOCK) and incubated in SFM (SFM). Culture supernatants were collected at the indicated postinoculation days and replaced with fresh SFM. The levels of chemokines present in the culture supernatants were determined by ELISA: (A) MCP-1, (B) MCP-3, (C) IL-8, and (D) RANTES. The range of the standards for MCP-1, MCP-3, IL-8, and RANTES were 30 to 2000 and 15 to 1000 pg/mL, respectively. Data are the mean \pm SEM of results in four individual experiments, each with duplicate samples. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

tor for MCP-1 (CCR2) was unaffected by HCMV infection, it is unlikely that the very low levels of MCP-1 found in culture supernatants were due to receptor binding and/or receptor-mediated internalization of this chemokine. The receptors for IL-8, CXCR1 and CXCR2, were expressed at very low levels and were not affected by HCMV infection at all the time points studied (Fig. 4B). In contrast, CXCR4, a specific receptor for stromal cell-derived factor (SDF)-1, was prominently expressed both in mock- and HCMV-infected HRPE cells. These results indicate that elevated IL-8 levels in the supernatants of HCMV-infected HRPE cells were not due to downregulated IL-8 chemokine membrane receptors.

Effect of Chemokine Treatment on CMV Replication

Because MCP-1 secretion by HRPE cells was significantly altered by HCMV infection, we evaluated the direct effects of this chemokine on HCMV replication in HRPE cells. HRPE cultures were grown to confluence in 24-well plates, pre-

treated with the chemokine for 24 hours, and overlaid with methylcellulose for plaque assays after inoculation with 50 PFU of HCMV. The agents were included in the culture medium for the entire incubation period during plaque assays, and plaques were counted on day 10. IFN- β , a known inhibitor of viral replication, was used as a positive control. Treatment of HRPE cells with MCP-1 had no effect on the replication of HCMV, whereas, as expected, IFN- β completely inhibited HCMV plaque formation (Fig. 5).

DISCUSSION

HCMV-induced retinitis is characterized by vasculitis, inflammation, and degeneration of the retina and retinal detachment, leading to sight-threatening complications, especially in immunocompromised individuals.³⁰⁻³⁵ Immunopathologic changes observed in the retina may be due to the replication of the virus and damage to the retinal cells, and secondary effects may be due to inflammatory reactions associated with the infiltrat-

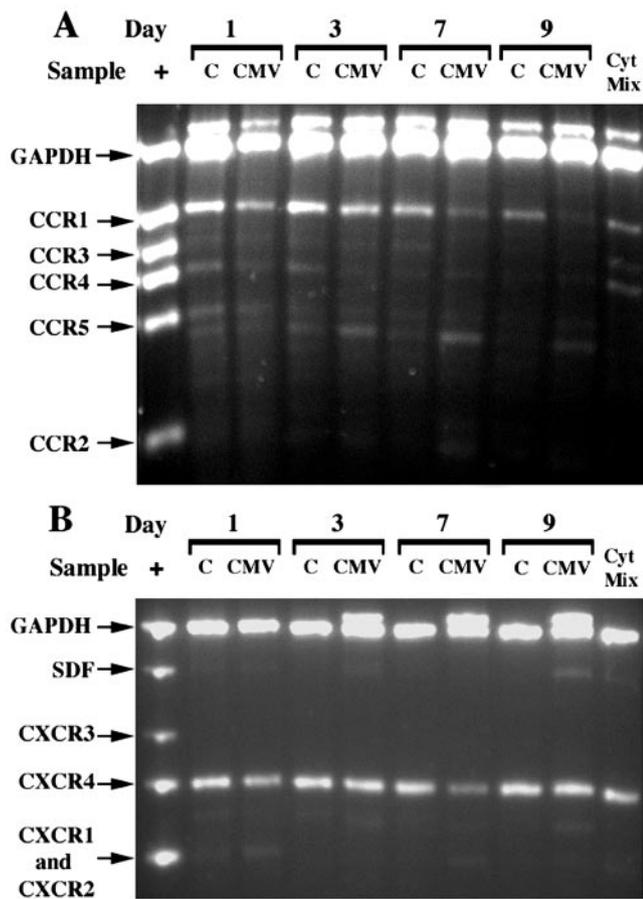


FIGURE 4. Expression of the chemokine receptors CCR and CXCR by HCMV-infected HRPE cultures. Total RNA was prepared from mock-infected (C) and HCMV-infected (CMV) HRPE cells at indicated post-inoculation days (Day). One microgram of synthesized cDNA was used for each multiplex PCR reaction in the presence of CCR or CXCR primer pairs to amplify six targets in a single-tube reaction at the same time. PCR products in the left lane (Cyt Mix) were derived from the total RNA prepared from HRPE cells treated with a mixture of cytokines (IFN- γ , TNF- α , and IL-1 β) for 1 day. The sizes of the PCR products: (A) CCR1, -2, -3, -4, and -5 and GAPDH were 363, 163, 318, 287, 246, and 500 bp. (B) CXCR1 and -2, -3, and -4; SDF-1; and GAPDH were 200, 301, 248, 400, and 500 bp. +, PCR positive controls of CCR and CXCR chemokine receptors. The results of one typical experiment are representative of two other individual experiments.

ing leukocytes.^{36,38,40} The presence of HCMV antigens in the Müller cells, astrocytes, and pigment epithelial cells has been demonstrated in the retinas of patients with CMV retinitis.^{36,37} We used cultured HRPE cells as a model to investigate the primary cellular responses of retinal resident cells in the expression of chemokines and chemokine receptors in response to HCMV infection. The present study demonstrated that HCMV infection resulted in downregulation of mRNA levels of MCP-1 and -3 and upregulation of mRNA levels of IL-8. The changes in the secretion of these chemokines reflected the alteration in the steady state levels of mRNA, suggesting the regulation of chemokine transcription in HRPE cells by HCMV.

The altered secretion of IL-8, MCP-1, and MCP-3 by retinal cells may regulate the recruitment and trafficking of leukocytes to the sites of infection within the retina. Through this process, chemokines may be involved in the initiation and perpetuation of immunologic and immunopathologic processes in the retina. IL-8, produced by macrophages, lymphocytes, fibroblasts, endothelial cells, and epithelial cells, is a chemoattractant primarily for neutrophils and to a lesser extent the lympho-

cytes.²⁻⁵ Activated neutrophils release granules, induce respiratory burst, and produce superoxide radicals as a part of the antimicrobial defense mechanism.²⁻⁵ Earlier studies have shown increased secretion of IL-8 by an HCMV-infected human monocyte cell line (THP-1), human astrocyte, fibroblast, and retinal pigment epithelial cultures.⁴⁹⁻⁵² In contrast, HCMV infection of human primary endothelial cells did not alter the production of IL-8.⁵³ In this study, we showed enhanced secretion of IL-8 by HRPE cells infected with HCMV. It is also possible that an increase in IL-8 levels in HCMV-infected HRPE cultures is due to viral CXC chemokine-1 (vCXCL 1),⁵⁴ a viral late gene product secreted into the medium. This viral chemokine competes with IL-8 for cellular CXCR 2 receptor, thereby releasing IL-8 for enhanced production. It is of interest to note that elevated levels of IL-8 present in the culture supernatants of RPE and other cells were shown to activate neutrophil migration.^{51,52}

Production of RANTES was not observed in HCMV-infected HRPE cells, suggesting that this chemokine is not associated with the viral pathogenesis involving HRPE cells. It is important to note that in HRPE cultures, RANTES was not constitutively expressed, but can be induced by the inflammatory cytokines IL-1, IFN- γ , and TNF- α . Failure to detect RANTES in culture supernatant was not due to sequestration by viral or cellular chemokine receptors, because we did not detect induction of RANTES mRNA. In contrast to the infection in HRPE cells, HCMV infection stimulates RANTES transcription in human foreskin fibroblasts⁵⁵ and primary endothelial cells.^{24,55} However, RANTES production decreases in these cultures because of sequestration by US28, a viral chemokine receptor that is involved in binding and/or internalizing extracellular RANTES.^{24,55}

Hirsch and Shenk⁵⁶ studied MCP-1 gene expression in HCMV-infected human foreskin fibroblasts and suggested that

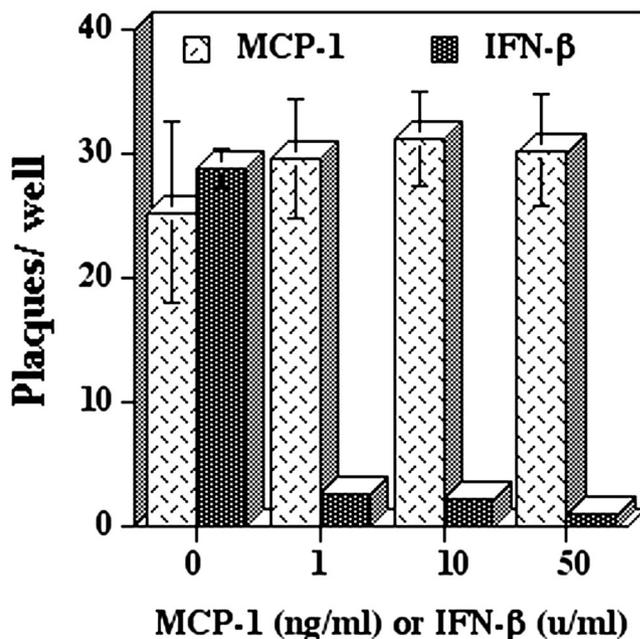


FIGURE 5. Viral plaque assays for the determination of the effects of MCP-1 and IFN- β on HCMV replication in HRPE cultures. HRPE cells grown to confluence in 24-well plates were inoculated with 50 PFU of HCMV and overlaid with medium containing methylcellulose. MCP-1 or IFN- β were present in the medium 24 hours before inoculation and thereafter until the cultures were fixed. After 8 to 10 days, cultures were fixed and stained and the plaques counted. Data are the mean \pm SEM results, with triplicate samples for each value and are representative of two such experiments.

HCMV infection negatively regulates MCP-1 transcription in these cells. In another study with human foreskin fibroblasts, Bodaghi et al.²³ observed significant reduction in MCP-1 production, whereas MCP-1 mRNA levels were not altered. They suggest chemokine sequestration by viral US28 protein to be a major cause of the reduced production of MCP-1. In primary endothelial cells, expression of MCP-1 was unaffected by infection with the clinical isolate of HCMV.⁵³ Mock-infected HRPE cells continued to produce MCP-1, whereas MCP-1 production was progressively decreased in HCMV-infected HRPE cells, with a complete shutoff by PI days 7 and 9. The steady state levels of MCP-1 mRNA also decreased significantly in HCMV-infected HRPE cells, suggesting that HCMV infection inhibits transcription of the MCP-1 gene. The presence of MCP-1 (500–1200 pg/mL) in the vitreous of normal humans has been reported,^{57,58} suggesting that under healthy conditions, some of the retinal cells produce MCP-1. In pathologic conditions, such as proliferative diabetic retinopathy and proliferative vitreoretinopathy, MCP-1 levels are elevated significantly, indicating the possible role of MCP-1 in retinal pathophysiology.^{57–59} However, there are no reports regarding the levels of MCP-1 in the vitreous in retinal infectious diseases.

Approximately 50% of the population is infected with HCMV, and the virus exists as a latent infection in immunocompetent individuals, who for the most part are asymptomatic.⁶⁰ This dampening of viral replication and pathogenesis is due to the anti-viral activity of chemokines in the host immune system. However, HCMV has developed ways to subvert the host immune system,^{17–19} by the alteration of transcription and secretion of immune molecules such as cytokines and chemokines, and/or by sequestration of the secreted chemokines.^{12,22,23} HCMV expresses four genes—US27, US28, UL33 and UL78—that encode proteins with homology to the CC chemokine receptors.^{20,22,61} These receptors may act as a part of the sequestration mechanism to prevent secreted chemokines from accessing the target immune cells or to reduce the chances of chemokine binding to the membrane receptors of the infected cell.^{21–24,61} Because we have studied HCMV infection in HRPE cells with demonstrated cytopathic effects, expression of all the viral proteins including viral chemokine receptors, may be expressed throughout the infection period. We examined the possibility of involvement of the host cell chemokine receptors, in addition to viral chemokine receptor analogues, in the regulation of MCP-1, MCP-3, and IL-8 production by HCMV-infected HRPE cells. Expression of CXCR1 and CXCR2 (the receptors for IL-8) and of CCR-2 (the receptor for MCP-1)^{4–8} were unchanged, suggesting that altered levels of these chemokines in the extracellular environment is mainly due to the effect on transcriptional and/or translational systems. However, we have not examined the role of US28, a viral chemokine receptor analogue that can bind MCP-1 and vCXCR-1, a viral chemokine that can compete with IL-8 for CXCR2, in our system. These factors may have an additional role in the alteration of MCP-1 and IL-8 levels, by sequestration in the extracellular medium.

CMV retinitis is a leading cause of visual loss in immunocompromised patients. However, very little is known about the nature of immune mechanisms involved in retinal pathogenesis. In this study, HRPE cells responded to HCMV infection and regulated the gene expression and secretion of the chemokines, MCP-1, MCP-3, and IL-8. The presence of neutrophils and other immune cells observed in the retinal tissue in patients with CMV retinitis,^{35,38} further supports the role of RPE-secreted IL-8 in viral pathogenesis. The decrease in MCP-1 and -3 in the extracellular environment may have the effect of preventing the migration and activation of leukocytes to the sites of infection, thus aiding the virus in evading the host immune system and establishing a latent infection in the retina.

The devastation of the immune system during AIDS and the incident CMV retinitis has been partially reversed with the advent of active antiretroviral therapy. However, because of the restoration of some immune cells, these patients may now experience immune-recovery uveitis.⁶² The severity of this disease is enhanced in patients with CMV retinitis and is characterized by vitritis and optic disc and macular edema. The data presented, focusing on host cell–virus interactions, suggest that chemokine modulation by CMV-infected RPE may be one of the contributing factors in immune-recovery uveitis.

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