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Human Cytomegalovirus Circumvents NF-κB Dependence in Retinal Pigment Epithelial Cells

Jindrich Cinatl, Jr.,* Stefan Marggraf,* Jens-Uwe Vogel,*† Martin Scholz, ‡ Jaroslav Cinatl, †§ and Hans Wilhelm Doerr*

The human CMV (HCMV) is a persistent virus that may cause severe inflammatory responses especially in immunocompromised hosts. In different cell types, HCMV infection leads to the activation of the pleiotropic transcription factor, NF-κB, which triggers virus replication but also propagates cell-mediated inflammatory mechanisms that largely depend on PG synthesis. We investigated the interactions of HCMV and the NF-κB-dependent PG synthesis pathway in cultures of retinal pigment epithelial (RPE) cells that are known to be infected in HCMV retinitis patients. Unlike in other cell types, HCMV increased neither NF-κB activity nor p65 and p105/50 mRNA levels in RPE cells. Both TNF-α and phorbol ester 12,13-tetradecanoylphorbol 13-acetate (TPA) enhanced NF-κB activity but only TPA increased HCMV replication. Cyclooxygenase-2 expression and PGE2 release was increased by TPA and TNF-α but not by HCMV infection. Stimulatory activity of TPA on HCMV replication was suppressed by protein kinase C inhibitors and inhibitors of p42/44 and p38 mitogen-activated protein kinases but not by NF-κB inhibitors. In conclusion, HCMV circumvents the NF-κB route in favor of the protein kinase C-dependent mitogen-activated protein kinase pathway in RPE cells. This virus/host cell interaction might be a mechanism that promotes HCMV persistence in immune-privileged organs such as the eye. The Journal of Immunology, 2001, 167: 1900–1908.

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tuman CMV (HCMV) is a major pathogen in immunocompromised individuals such as transplant recipients and AIDS patients. Numerous pathogenic changes in infected organs stem from virus replication in permissive cells resulting in their lysis. In contrast, HCMV may contribute to pathogenesis due to altered cellular gene expression that occurs independent of virus replication. For example, HCMV has been shown to alter the expression of cellular genes that code for proteins with proinflammatory activity, including adhesion molecules (1, 2), chemokines (3–7), and extracellular matrix proteins (8–10). HCMV may contribute to inflammatory mechanisms by modulation of multiple constituents of the PG synthesis pathway via arachidonic acid. In human fibroblasts and smooth muscle cells, HCMV induced cyclooxygenase (COX)-2 and cytosolic phospholipase A2 (cPLA2), whereas the expression of lipocortin-1, a negative regulator of cPLA2, was decreased (10, 11). In smooth muscle cells, NF-κB-dependent COX-2 contributes to the production of reactive oxygen intermediates that in turn stimulate NF-κB, a pathway that may be used by HCMV for its own replication (11). Moreover, immediately after infection HCMV activates NF-κB and other transcription factors (3, 12–15) that are required for viral DNA synthesis, thus allowing the productive infection of quiescent, differentiated cells that do not express these factors in sufficient amounts. The effects of HCMV on NF-κB are reminiscent to those of growth factors and hormones that result in transcription of numerous viral and cellular genes (for review see Ref. 16). More specifically, HCMV-mediated NF-κB activation may occur on the level of 1) binding of viral glycoproteins to cellular receptors (12, 15), 2) introduction of constituents of the virion (i.e., tegument protein pp71) with transactivation activity (17), or 3) transactivation of the NF-κB gene via HCMV immediate-early proteins 1 and 2 (IE1 and IE2), which are produced in infected cells before initiation of virus replication (11, 18–21). However, it has not been shown yet whether these HCMV-associated cellular and molecular changes may be relevant for the development of HCMV retinitis in the late stage of AIDS.

The eye has an immune-privileged status and thus differs from other tissues in its way to eliminate pathogens or to prevent infections. The pathological features of HCMV retinitis include transmission of virus from retinal capillaries and necrosis of the retinal layers, causing retinal detachment and blindness in untreated patients (22–24). In the retina, HCMV has been detected in different cell types including retinal vascular endothelial cells, Müller cells/astrocytes, and retinal pigment epithelial (RPE) cells (22, 25, 26). The HCMV-directed cellular immune response is weak and occurs frequently in the absence of neutrophils (22, 27). The inefficiency of the leukocytes to eliminate HCMV may explain the smoldering character of the retinitis frequently observed in AIDS patients and the long persistence of the virus within the retina. Recently, it has been shown in vitro that HCMV-infected RPE cells prevent stimulated neutrophil adhesion and transepithelial migration due to augmented Fas ligand expression (28). Moreover, the extent of HCMV-induced modulation of cellular gene expression in RPE cells differed from that found in other cell types (4, 28).
Whether the distinct immunogenicity of the HCMV-infected retinal pigment epithelium is due to a reduced potential of HCMV to activate NF-κB and/or the PG synthesis pathway in RPE cells is not known. Therefore, we investigated the interdependency between HCMV infection, NF-κB activation, and expression of COX-2 in human RPE cell cultures.

**Materials and Methods**

**Cell culture**

Human RPE cells were isolated from three bulbs from different donors freshlyenucleated for corneal transplantation (tenets of the Declaration of Helsinki were followed). RPE isolation and culture were performed as described previously (28). RPE cells were grown in IMDM supplemented with 20% FBS. Homogeneity of cultured RPE cells was confirmed by positive immunostaining with mAbs to cytokeratins (pan) and to cellular retinaldehyde binding protein. mAbs were kindly donated by Dr. J. Saari (Department of Ophthalmology, University of Washington, School of Medicine, Seattle, WA; Ref. 29). The cell cultures used in this study were designated RPE-I, RPE-II, and RPE-III. Cells were routinely tested negative for mycoplasm and were not used in the experiments later than passage six. Human foreskin fibroblasts (HFF) were established and maintained in culture as described previously (30).

**Virus preparation**

The HCMV laboratory strain AD169 was obtained from American Type Culture Collection (Manassas, VA). The HCMV strain H91 was isolated from urine of an AIDS patient with HCMV retinitis (8). Virus stocks were prepared in HFF grown in MEM with 4% FBS. The respective titers were determined by plaque titration in HFF cells. Mock-infected inocula were prepared in an identical fashion, except that cell monolayers were not infected with HCMV.

**Virus infectivity assay**

Confluent cultures of RPE cells were incubated with HCMV at a multiplicity of infection (MOI) of 2. After incubation for 1 h required for virus adsorption, cells were washed with PBS and incubated in culture medium. As described in detail previously (30), cells producing HCMV-specific Ags were detected 24 and 72 h postinfection (p.i.) by immunoperoxidase staining using mAbs (DuPont, Bad Homburg, Germany) directed against 72 kDa IE Ag (IEA) and 67 kDa late Ag (LA), respectively.

**Stimulation of RPE cells**

Mock- or HCMV-infected RPE cells were incubated up to 24 h with TNF-α (200 U/ml; Roche, Mannheim, Germany) or 12,0-tetradecanoylphorbol 13-acetate (TPA; 300 ng/ml; Sigma, Deisenhofen, Germany). Cells were stimulated either before infection, during virus adsorption, or after virus adsorption.

To determine which signaling pathways were involved in the induction of HCMV Ag and COX-2 gene expression, infected cells (1 h adsorption period) were stimulated with TPA in the presence of specific inhibitors. Pretreatment of cells for 30 min) of various signal transduction pathway enzymes. Specific inhibitors of protein kinase C (PKC) used were bisindolylmaleimide I (5 μM) and Gö 6983 (1 μM). For the inhibition of mitogen-activated protein kinase (MAPK) PD 98059 (40 μM), a specific inhibitor of the extracellular signal-regulated kinase 1 (ERK1) kinase (MEK1) and SB 203580 (20 μM), which inhibits p38, were used. BAY 11-7082 (10 μM) was used as an inhibitor of NF-κB. All inhibitors were purchased from Calbiochem-Novabiochem (Bad Soden, Germany). No cytotoxic effect of any inhibitor on cellular viability was found as determined by trypsin blue staining.

**Extraction of cell nuclei**

To prepare nuclear extracts, the cells were harvested on wet ice with a cell scraper and washed twice in PBS (4 °C). The supernatant was removed, and the pellet was washed twice in 2 ml ice-cold buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, and 10 mM KCl) containing a protease inhibitor cocktail (230 μM aprotinin, 4 μM leupeptin, 3.3 μM antipain, 1.5 μM pepstatin A, 1 μM PMSF, and 1 mM DTT; Sigma) and was centrifuged. The supernatant was aspirated, and the cell pellet was resuspended in 80 μl buffer A, containing 0.1% Triton X-100. The crude nuclear pellet was resuspended in buffer C (20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT; 0.5 mM PMSF, and 25% glycerol (v/v)) supplemented with the protease inhibitory mixture as described above) and incubated on ice for 30 min. The nuclear fraction was again centrifuged at 20,800 × g for 12 min (4 °C) to collect the supernatant containing the nuclear protein extracts. Protein concentrations were determined by Bio-Rad DC protein assay (Bio-Rad, Hercules, CA) with BSA as a standard. Aliquots were stored at −80 °C until further use.

**Immunocytochemical staining of NF-κB**

Immunocytochemical analysis of NF-κB was conducted to assess subcellular NF-κB subunit localization. Cells grown in microchannel slides were fixed 2 h p.i. or after TNF-α or TPA pretreatment with aceton/ methanol solution and stained using a mouse mAb to the p65 subunit of NF-κB (aa 1–286; Santa Cruz Biotechnology, Heidelberg, Germany). Anti-rabbit PE-conjugated secondary Ab (Dianova, Hamburg, Germany) was used for the detection of primary Abs.

**RT-PCR**

Total RNA was isolated from RPE or HFF cells (mock- or AD169-infected) using TRIzol according to the manufacturer’s instructions (Life Technologies, Gaithersburg, MD). RNA was reverse transcribed using random hexamer priming. One microgram of total RNA was denatured at 70 °C for 10 min and chilled on ice. The denatured RNA was then co-incubated with 2.5 μM random hexamer oligonucleotides, 1 μM of each primer, 5 mM MgCl₂, 1.5 μM each dNTP, 1 μM RNase inhibitor (Roche, Mannheim, Germany), and 1 μl murine leukemia virus reverse transcriptase (Life Technologies) in 1× PCR buffer II (PerkinElmer, Norwalk, CT) for 1 h at 37 °C. The reverse transcriptase was inactivated for 5 min at 95 °C before amplification. The following sequences of primers were used: p50/p105 (NF-κB) sense, 5’-ATA GCA CTG GCA GCT TCA CA-3’; p50/p105 (NF-κB) antisense, 5’-AG CAG AAT TCC TCC GAA GC-3’ (position 195–1410) (31). The sequence of GAPDH primers used as control were as follows: 5’-TGG GAG AAG TGA TGG CTA CG-3’ (position 61–81) and 5’-GAA GGG GTC ATT GAT GGC AA-3’ (position 151–171) (8). Lipocortin I sense, 5’-TGG CCT TGC ATA AGG CTA AAA-3’ (position 222–242); lipocortin I antisense, 5’-CAG CAC GAA GTT CAT CAG CA-3’ (position 431–450) (32); cPLA₂ sense, 5’-CCA AAG TGA CAA AGG GGC CC-3’ (position 221–240); cPLA₂ antisense, 5’-GCT ACC ACA GGC ACA TCA CG-3’ (position 691–710); COX-2 sense, 5’-ATG AGA TTT GGG TAA AAT TCG T-3’ (position 580–601); COX-2 antisense, 5’-GAT CAT CTC TGC CTG AGT ATC-3’ (position 859–879); COX-1 sense, 5’-CAG CTC CTG GCC CGC CGT TT-3’ (position 525–544); COX-1 antisense, 5’-GTTG CAT CAA CAC AGG CGC CTC-3’ (position 804–824) (33); p65 sense, 5’-CTG ATG GAG TAC CCT GCT GC-3’ (position 606–626); p65 antisense, 5’-ATC TGG TGC GTC ATT TTA TGA AA-3’ (position 1184–1205). PCR amplification of the cDNA was conducted by adding 0.5 μg Taq DNA polymerase (Roche). PCR amplification of fragments was performed using 28 cycles in a DNA thermocycler using the following conditions: denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 1 min at 72°C. For quantification of the GAPDH fragment was as follows: denaturation for 1 min at 94°C, annealing for 1 min at 52°C. and extension for 1.5 min at 72°C in a PerkinElmer Thermocycler. PCR products were resolved alongside a DNA marker on a agarose gel, stained with ethidium bromide, and photographed. To ascertain that transcripts were specifically amplified, sequence analysis of PCR products was performed. Amplified sequences fully matched nucleotide sequences (results not shown).

**Quantitative PCR**

HFF and RPE cells were grown in six-well plates and infected at confluence with HCMV at a MOI 2 for 4 h to enable virus adsorption and penetration. Where appropriate, virus was incubated with dextran sulfate (200 U/ml) or TPA (300 ng/ml) during the virus adsorption period. Cells were washed three times with PBS to remove unbound virus and were subsequently lysed by freezing and thawing. DNA was extracted and quantified with a quantitative HCMV DNA PCR kit according to the manufacturer’s instructions (Roche). Results are given as HCMV copies per 1 × 10⁵ cells.

**EMSA**

EMSA were conducted using a gel shift assay system kit (Promega, Mannheim, Germany) (34). Double-stranded NF-κB consensus oligonucleotide probes (5’-AGTTGAGGGGAGTTTTCCACGGC-3’) were end-labeled with [γ-³²P]ATP (3000 Ci/mmol at 10 μCi/ml; NEN, Mannheim, Germany). After T4 kinase end-labeling, oligonucleotide (35 fmol; 5,000–20,000 cpm) probe and nuclear protein (5 μg) were incubated for 20 min at room temperature in binding buffer containing 0.2% Nonidet P-40, 12
mM HEPES, 2.5 mM DTT, 4% glycerol (v/v), 140 mmol/L KCl, 1.6% Ficoll 400, 0.1 mM EDTA, 2 mg/ml BSA, and 2 μg/ml poly(dIdC) (Amersham Pharmacia Biotech, Freiburg, Germany). Where indicated, unlabeled competitive oligonucleotide (NF-κB) was added 10 min before the addition of radiolabeled probe in a 100-fold excess (34). Samples were run on a nondenaturing 4% polyacrylamide gel in 0.5 × TBE buffer at 2 W and 4°C for 2 h. Gels were vacuum-dried and visualized by exposure to a Kodak Biomax MS film (Kodak, Rochester, NY). For supershift assays, the reaction mixture was incubated with anti-p65 affinity-purified mouse mAb (F-6) to the p65 subunit (aa 1–286) of NF-κB (Santa Cruz Biotechnology) for 60 min at 4°C or with anti-p50 mAb. The oligonucleotide probe was added, and incubation was conducted as described above.

Electron microscopy

RPE cells infected with HCMV at MOI 2 were processed for ultrastructural analysis as described previously (35). Briefly, cells were pelleted and fixed with 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in ethanol, and embedded in Durcupan Fluka (Sigma). Thin sections were contrasted with uranyl acetate and lead citrate and viewed with a Jeol JEM, 2000 CX microscope (Arishima, Japan).

COX activity

The COX-2 activity was measured by determination of PGE₂ levels. Mock- or AD169-infected RPE cells (MOI 2) were incubated for 3 and 6 h in a culture medium supplemented with 2% FCS without or with TNF-α or TPA. The culture supernatants were removed at different time points and centrifuged at 3000 rpm for 10 min to remove cell debris. Immediately after centrifugation, the supernatants were shock-frozen in ethanol/dry ice and stored at −70°C until further use. Aliquots of collected samples were assayed for spontaneously released PGE₂ by an enzyme immunoassay according to the manufacturer’s instruction (Amersham Pharmacia Biotech).

Statistical analyses

Determination of statistical significance was conducted with the Student’s t test. Data groups were considered significantly different when p < 0.05.

Results

HCMV infection fails to stimulate NF-κB activity and transcription of p65 and p105/50 in RPE cells

In human fibroblasts and monocytes, HCMV infection induces translocation of NF-κB and DNA binding within minutes after infection. Moreover, increased levels of mRNA for NF-κB subunits including p65 and p105/50, which may be important for sustaining NF-κB activity, have been observed in infected cells (15, 36). Therefore, we studied the effects of HCMV infection on the DNA binding activity of NF-κB in RPE cells. As shown by EMSA, both mock- and HCMV-infected RPE cells expressed low levels of constitutive NF-κB activity, which did not increase during the 24-h observation period (Fig. 1A). The failure of HCMV to stimulate NF-κB activity was not due to a general dysfunction of NF-κB because TNF-α and TPA efficiently induced DNA binding activity after 30 min and sustained up to 24 h. Supershifts done with mAbs against the subunits p50 and p65 confirmed the specificity for NF-κB in the binding assay and showed the capability of

![FIGURE 1.](http://www.jimmunol.org/) Activation of NF-κB in mock-infected, HCMV-infected, and TNF-α- or TPA-stimulated cultured RPE cells (A) and in mock- or AD169-infected HFF (B). Nuclear extracts were prepared 0.5, 1, 2, and 24 h after treatment. EMSA was conducted with 5 μg nuclear extract and oligonucleotides containing NF-κB sites as described in Materials and Methods. Infection of HFF with HCMV AD169 induced gel shift after 0.5 h (B). Supershifts with anti-p65 or with anti-p50 Abs are shown for both cell types. NS, Nonspecific.
either subunit to bind to DNA (Fig. 1A). HFF infected with HCMV (positive control) stimulated NF-κB already after 0.5 h (Fig. 1B). Immunocytochemical analysis of cytoplasmic to nuclear translocation of NF-κB p65 is depicted in Fig. 2. Consistent with the results obtained by EMSA, p65 is detectable primarily in the cytoplasm of mock- and AD169-infected RPE cells. In HFF but not in RPE cells, infection with HCMV resulted in translocation of NF-κB p65. Both TNF-α and TPA induced a marked increase in the amount of nuclear staining and a corresponding decrease in cytoplasmic staining in HFF and RPE cells.

In addition, HCMV infection failed to increase levels of mRNA for both p65 and p105/50 NF-κB subunits 4 and 24 h after virus inoculation, whereas in infected HFF, mRNA of both NF-κB subunits was found to be increased (Fig. 3).

**TPA but not TNF-α increases HCMV replication in RPE cells**

We now studied whether stimulated NF-κB activity leads to enhanced sensitivity to HCMV infection and enhanced virus replication in HCMV-infected RPE cells (as it has been shown for other cell types). Cellular sensitivity to HCMV infection was evaluated by quantitating the intracellular (RPE and HFF) content of HCMV DNA by quantitative PCR immediately after the virus adsorption/penetration period (4 h; Fig. 4) and by serial immunostaining against HCMV IEA (24 h p.i.) and LA (72 h p.i.) in three different HCMV-infected RPE cell lines (Fig. 5). Quantitative PCR revealed similar intracellular amounts of HCMV DNA in infected HFF and RPE cells, indicating that adsorption/penetration of HCMV virions is comparable between cell types even after stimulation with TNF-α or TPA (Fig. 4). Despite similar amounts of intracellular HCMV DNA, dramatic differences in virus replication (IEA and LA expression) were observed. In RPE cell cultures infected at MOI 2 with AD169 strain, only 0.5–0.9 and 0.3–0.7% of cells expressed IEA and LA, respectively (Fig. 5), whereas >99% of infected HFF exhibited IEA and LA expression (data not shown). RPE cells were incubated with TNF-α at different times before, during, or 1 h after the virus adsorption/penetration period. No effects on the number of HCMV IEA- and LA-expressing cells were observed, regardless of the stimulation strategy. In contrast,

**FIGURE 4.** HCMV DNA in nonstimulated, TPA-treated, and TNF-α-treated HFF and RPE cells. DNA was extracted 4 h p.i. and quantified by quantitative PCR. As a negative control, virus adhesion to the cell surface was inhibited by dextran sulfate (DS).

**FIGURE 2.** Translocation of NF-κB from cytoplasm to nucleus in RPE cells (A, C, E, and G) and HFF (B, D, F, and H). Immunostaining of NF-κB p65 in mock-infected (A and B), HCMV AD169-infected (C and D), TNF-α-treated (E and F), and TPA-treated (G and H) cells.

**FIGURE 3.** p65 and p50/105 gene transcription in mock-infected and HCMV-infected cultured RPE cells (A) and HFF cells (B). Cells were harvested 4 and 24 h p.i. and processed to determine p65, p50/105, and GAPDH mRNAs by RT-PCR.
TPA (300 ng/ml) added 1 h after virus adsorption/penetration increased the number of cells expressing HCMV IEA and LA 19- to 42- and 9- to 22-fold, respectively (Fig. 5). TPA added 1 h before infection, at the time of virus inoculation, or after virus adsorption enhanced HCMV replication to a similar extent (data not shown). When TPA was added 12 or 24 h before infection, no effects were observed. Increased permissivity of TPA-treated RPE cells was confirmed by ultrastructural observations (Fig. 6). In TNF-α-treated RPE cell cultures infected with the laboratory strain HCMV AD169 and the clinical strain HCMV Hi91 (data not shown), low numbers of cells expressing only viral nucleocapsids in the nucleus were observed (Fig. 6, A and B). In cultures treated with TPA, 5-15% of cells were found to be productively infected. Higher numbers of viral nucleocapsids in TPA-treated cells were found when compared with TNF-α-treated cells (Fig. 6C). Moreover, in TPA-treated RPE cells, HCMV morphogenesis, an indicator of productive infection, was found. In Fig. 6D, enveloped virions as well as numerous dense bodies in the cytoplasm of infected, TPA-treated RPE cells are depicted. These results suggest that HCMV can infect RPE and HFF in a similar efficacy. However, the virus replication is limited in RPE cells due to intracellular regulatory mechanisms downstream of HCMV adhesion/penetration.

**HCMV infection fails to increase COX-2 mRNA transcription and PGE₂ secretion in RPE cells**

HCMV increases expression of NF-κB-dependent COX-2 in HFF and smooth muscle cells due to activity of both NF-κB and HCMV IE proteins. This mechanism is known to be involved in PGE₂ synthesis and thus discussed to be strongly associated with HCMV pathogenesis. RT-PCR was done to study whether HCMV may differentially trigger the transcription of COX-2, cPLA₂, lipocortin-1, and COX-1 genes (major enzymes involved in the PG synthesis pathway) in fibroblasts and RPE cells. Total RNA was obtained 1, 2, or 4 h.p.i. from mock-infected and AD169-infected RPE and HFF cells. Cells were either nontreated or stimulated with TPA or TNF-α according to the experiments described above. At all time points analyzed, HCMV induced COX-2 transcription in HFF but not in RPE cells as shown representatively for 4 h in Fig. 7. Both TNF-α and TPA treatment increased COX-2 mRNA levels in both mock- and AD169-infected RPE and HFF cells. Cells were either nontreated or stimulated with TPA or TNF-α according to the experiments described above. At all time points analyzed, HCMV induced COX-2 transcription in HFF but not in RPE cells as shown representatively for 4 h in Fig. 7. Both TNF-α and TPA treatment increased COX-2 mRNA levels in both mock- and AD169-infected RPE and HFF cells. mRNA levels of cPLA₂ were increased in HCMV-infected HFF, whereas mRNA levels of lipocortin-1 were decreased (Fig. 7B).

These results were confirmed by measuring PGE₂ as an end product of the COX-2-associated PG synthesis pathway. ELISA with supernatants harvested at 3 and 6 h p.i. revealed that TNF-α...
and TPA treatment increased PGE\textsubscript{2} release in HFF and RPE cells (Fig. 8). In mock- and AD169-infected RPE cells, TNF-\textalpha/H9251 and TPA treatment increased PGE\textsubscript{2} release up to 7- and 60-fold (31 pmol/ml for mock-infected cells; 280 \pm 32 pmol/ml for TNF-\textalpha-treated cells; 2510 \pm 292 pmol/ml for TPA-treated cells). However, no HCMV-induced secretion of PGE\textsubscript{2} was found in RPE cells, whereas in infected HFF PGE\textsubscript{2} levels were increased up to 40-fold (3 h p.i.; Fig. 8).

TPA stimulates HCMV replication through PKC and MAPK independently of NF-\kappaB

TPA is a potent inducer of PKC leading to a variety of cellular responses including stimulation of NF-\kappaB activity. To define the proposed yet unknown PKC-dependent/NF-\kappaB-independent pathway that may control the HCMV replication in RPE cells, we evaluated the effects of Gö 6983 and bisindoylmaleimide, specific inhibitors of PKC. As shown in Fig. 9, Gö 6983 and bisindoyl-

**FIGURE 7.** Gene transcription of COX-2, cPLA\textsubscript{2}, lipocortin-1, and COX-1 in RPE cells (A) or HFF cells (B). Mock-infected (MOCK) or HCMV-infected (AD169) cells were either nonstimulated or treated with TNF-\textalpha or TPA. Cells were harvested 4 h after infection/stimulation and processed to determine mRNAs of COX-2, cPLA\textsubscript{2}, lipocortin-1, COX-1, and GAPDH by RT-PCR.

**FIGURE 8.** Cellular PGE\textsubscript{2} release in cultured RPE cells (A) or HFF cells (B). Mock-infected (MOCK) and HCMV-infected (AD169) cells were either nonstimulated or treated with TNF-\textalpha or TPA. Cell culture supernatants were harvested after 3 and 6 h p.i., and the content of PGE\textsubscript{2} was measured by ELISA. *p < 0.05 compared with mock-infected cells.

PD 98059 or SB 203580 inhibited TPA-induced NF-\kappaB nuclear activity (Fig. 10). These findings further confirm the proposed NF-\kappaB-independent, MAPK-dependent regulation of HCMV replication in RPE cells. Alternatively, BAY 11-7082, a specific inhibitor of NF-\kappaB, completely suppressed TPA-induced NF-\kappaB nuclear activity (Fig. 10) but had no significant effects on TPA-induced increase in numbers of HCMV Ag-positive cells (Fig. 9).

**Discussion**

The results presented here show that HCMV replication in RPE cells is unexpectedly regulated via MAPKs but not via the NF-\kappaB route. Moreover, in contrast to other cell types, the NF-\kappaB-dependent production of COX-2, an important constituent of the PG synthesis pathway, was not found to be induced by HCMV. It has been suggested that the RPE contributes to the immune-privileged status of the retina as a part of the blood-retina barrier, e.g., by the secretion of immunosuppressive factors (39, 40). We speculate that our findings may partly contribute to explain the special inflammatory pathology in HCMV retinitis patients, characterized by low viral replication and weak leukocyte infiltration of the immune-privileged retina.

The hallmark of HCMV infection in different human cell types such as fibroblasts, smooth muscle cells, and monocytes is a rapid induction of NF-\kappaB DNA binding activity (11, 12, 15, 36, 41). This initial and very rapid increase in binding activity appears to be due to the release of preformed cytosolic NF-\kappaB heterodimers resulting from the binding of the major HCMV envelope glycoproteins, gB and gH, to their cognate cellular receptors (12, 36). However, HCMV infection was also shown to transactivate the
COX-2 gene expression is involved in the production of PGE2 and HCMV DNA replication.

p65 and p105/p50 NF-κB activity nor augmented transcription of genes encoding both BAY 11-7082.

NF-κB activity is relevant for vi-

FIGURE 9. Expression of HCMV IEA (A) and LA (B) in infected cultured RPE cells without stimulation or after treatment with TPA. HCMV Ag-positive cells were immunostained and counted microscopically. The relative TPA-induced increase in numbers of cells expressing HCMV IEA and LA is given. HCMV-infected RPE cells were cultured in the presence of the PKC inhibitors Gö 6983 and bisindoylmaleimide I, the MEK1 inhibitor PD 98059, the p38 inhibitor SB 203580, and the inhibitor of NF-κB, BAY 11-7082. * p < 0.05 compared with cells without inhibitor.

promoters for the two NF-κB subunits, p105/p50 and p65, which may be important for the sustained increase in NF-κB activity during the course of the infection. The transactivation of NF-κB genes occurs through modulation of cellular factors (independent of viral gene expression) and/or through transactivation activity of the major HCMV IE gene products (12, 36). It has been widely accepted that HCMV involves the NF-κB pathway to support HCMV DNA replication.

In contrast, HCMV-induced NF-κB activity is relevant for virus-associated immunopathological mechanisms that involve NF-κB-dependent gene expression (7). In HCMV-infected smooth muscle cells, the COX-2 promoter was up-regulated by both NF-κB and HCMV IE proteins (11). The NF-κB-dependent COX-2 gene expression is involved in the production of PGE2 and other prostanoids pathologically important as proinflammatory mediators. COX-2 represents the inducible isoform of COX that is constitutively expressed in different cell types of the CNS including RPE (42, 43). PGE2 is the major PG in the retina and may play a role in the initiation and modulation of ocular inflammation and angiogenesis (44–47).

The goal of this study was to determine the effects of HCMV on NF-κB and the associated PG synthesis pathway in RPE cells derived from immune-privileged human retina. We were surprised to find that in RPE cells HCMV infection neither induced NF-κB activity nor augmented transcription of genes encoding for both p65 and p105/p50 NF-κB subunits in RPE cells. HCMV infection also failed to modify NF-κB-dependent COX-2 expression and other enzymes involved in PG synthesis including cPLA2 and lipocortin-1, which were previously shown to be deregulated in infected fibroblasts (10). It should be mentioned that constituents of the PG pathway such as cPLA2 are regulated by phosphorylation at the posttranslational level and may be induced by HCMV in smooth muscle cells (13). Thus, the level of cPLA2 mRNA, and even protein expression, may not reflect the activation status of the gene product.

The dramatic differences between RPE cells derived from immune-privileged retina and human fibroblasts lead us to speculate that HCMV has evolved a specific immune escape mechanism in RPE cells by circumventing the NF-κB route. This would be in accordance with previously published data on reduced neutrophil adhesion to HCMV-infected RPE cells (28). Overall, these HCMV-associated effects may prevent inflammatory reactions and thus may contribute to the maintenance of the retinal immune-privilege in vivo.

We studied whether HCMV replication in infected RPE cells, in turn, can be induced by augmented NF-κB activity. It is well known that the major HCMV IE promoter (MIEP) contains four NF-κB consensus binding sites (41) that are important for efficient transcription (48, 49). Although the effect of the NF-κB binding site was strongly dependent on the cell type used (50), several in vitro and in vivo studies demonstrate the importance of NF-κB activity for HCMV (re)activation. This is supported by findings that host factors such as TNF-α may be important for HCMV (re)activation possibly through up-regulation of the MIEP by NF-κB. (Re)activation of latent HCMV infection in transplant recipients correlated with TNF-α levels (51), and TNF-α-dependent activation of the HCMV promoter in the monocytic cell line HL-60 was mediated by NF-κB (49). Moreover, TNF-α induced HCMV (re)activation in latently infected monocyte/granulocyte progenitor cells (52). In smooth muscle cells, reactive oxygen intermediates were shown to stimulate transcription of HCMV IE genes through NF-κB (53).

Surprisingly, although both TPA and TNF-α potently stimulated NF-κB activity and NF-κB-dependent COX-2 expression in RPE...
cells, virus replication was stimulated by TPA but not by TNF-α. These findings suggest that NF-kB does not activate HCMV MIEP in RPE cells. TPA can activate both classical and novel members of the PKC isoenzyme family due to interaction with a phorbol ester binding site in their amino-terminal domain (54, 55). These effects of TPA mimic PKC activation by diacylglycerol and result in phosphorylation and activation of downstream targets including constituents of several signaling pathways such as p42/44 (ERKs) and p38 MAPK cascades (37, 38, 56, 57). Here, we demonstrate that PKC stimulates HCMV gene expression in RPE cells through activation of MAPKs and that inhibition of MEK1 and p38 MAPKs significantly suppressed HCMV gene expression in TPA-treated RPE cultures. It is probable that the MAPK-dependent activity of serum response factor and ELK-1 proteins (58) may play a role in HCMV MIEP stimulation in RPE cells as it was shown before in human monocytes and T cells (59). In contrast, MAPK activity alone may not be sufficient for stimulation of HCMV by TPA because PKC activation modifies various other cellular pathways that might influence HCMV MIEP relevant mechanisms, downstream of MAPK.

In previous studies with HCMV-infected human fibroblasts HCMV IE gene products were involved in stimulation of ERK and p38 kinase activity that appeared to be necessary for efficient viral replication (60, 61). Inhibition of ERK did not influence IE expression but decreased expression from an HCMV early gene promoter (60). Virus-induced MAPK p38 activity did not affect virus replication during IE or early times after infection but was essential for the initiation of HCMV DNA replication in infected human fibroblasts (62). In contrast, we now demonstrate that TPA-induced MAPK activity augments the expression of HCMV IE proteins in RPE cells, suggesting that this mechanism allows the compensation of the circumvented NF-kB route. Breening et al. (63), found that transcription from the HCMV MIEP can be up-regulated by a variety of environmental stresses in a p38 MAPK-dependent manner. Therefore, it is possible that stimuli leading to activation of MAPK via PKC may contribute to HCMV (re)activation in RPE cells within the retina without using NF-kB.

In conclusion, unlike in other cell types, HCMV infection circumvents the NF-kB and RANTES production by human cytomegalovirus infection of human fibroblasts and endothelial cells by acting concurrently on AP-1 and ERK pathways activated by cytomegalovirus infection of smooth muscle cells. J. Clin. Invest. 100:2054.


CORRECTIONS


In Figure 1A the first label at the top of the third picture is incorrect. The corrected figure, which indicates the super shifts of TNF-α (second picture)- and TPA (third picture)-stimulated cultured RPE cells, is shown below.


Due to publisher error, the title in the article was misprinted. The corrected title is shown below.

Sensitized Mast Cells Migrate Toward the Antigen: A Response Regulated by p38 Mitogen-Activated Protein Kinase and Rho-Associated Coiled-Coil-Forming Protein Kinase.