Entry of Human Cytomegalovirus into Retinal Pigment Epithelial and Endothelial Cells by Endocytosis

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Purpose. Human retinal pigment epithelial (RPE) cells and endothelial cells (HUVECs) are targets of human cytomegalovirus (HCMV) infection in vivo with significantly protracted replication in vitro compared with that in fibroblasts. This study analyzes the kinetics and mechanisms of HCMV entry into both cell types.

Methods. RPE cells were obtained from donor eyes. HUVECs were isolated from human umbilical cords. HCMV entrance was followed by electron microscopy and immunofluorescence in the presence of lysosomotropic agents and cytochalasin B.

Results. Human cytomegalovirus entered into RPE cells and HUVECs as early as 5 minutes after virus–cell contact. Entry was mediated by endocytosis, whereas HCMV enters fibroblasts through fusion. Most internalized viral particles and dense bodies appeared to be degraded within vacuoles. Viral entry, transport of viral proteins to the nucleus, and onset of viral transcription (immediate early [IE] protein expression) were significantly blocked by cytochalasin B. Lysosomotropic agents did not significantly reduce IE expression in RPE cells or HUVECs.

Conclusions. This study shows that HCMV penetrates these highly specialized relevant cells via endocytosis. The low level of infection and the delay in the onset of HCMV expression seen in these cells compared with fibroblasts may be related to the sequestration and degradation of incoming viral particles in endocytic vacuoles. (*Invest Ophthalmol Vis Sci.* 1999;40:2598–2607)

Human cytomegalovirus (HCMV), a species-specific member of the herpesvirus family, commonly infects immunocompetent individuals subclinically. However, CMV can become a serious pathogen associated with significant morbidity and mortality in a variety of clinical situations involving immunosuppression. Observations in humans and animals reveal that during acute infection under immunosuppressed conditions CMV infects almost all organs[1–5] and induces widespread pathology. Two targets of CMV infection and replication in vivo are endothelium[6–8] and retinal pigment epithelium.[9,10] There is increasing evidence for vascular involvement in such disseminated CMV infections, although the different cell types in the vascular wall seem to behave quite differently with respect to CMV infection.[11–14] Human umbilical vein endothelial cells (HUVECs) show low permissivity in vitro to the HCMV strain AD169, whereas smooth muscle cells and fibroblasts are productively infected.[15–17] Endothelial cells (HUVECs) and monocytes/macrophages appear to play a crucial role in the hematogenous spread of virus in patients suffering from acute HCMV infection.[5,6,12,18,19]

Human cytomegalovirus retinitis is an important etiology of blindness in AIDS patients. From 10% to 30% of AIDS patients have HCMV retinitis.[20–22] The pathologic features of this disease include transmission of virus from the retinal capillaries and necrosis of all 10 retinal layers, causing retinal detachment and blindness in untreated and sometimes in treated patients. The pathophysiology of retinal infection is still poorly understood. Human retinal pigment epithelial (RPE) cells constitute the external layer of the retina and are in close contact with photoreceptors. The outer segment tips of photoreceptors are renewed daily after specialized phagocytosis by RPE cells. This process is particularly essential to sight.[23]

Earlier reports showed that RPE cells are fully permissive to HCMV infection in vitro.[24] However, viral replication is significantly delayed in these cells compared with laboratory cell systems such as human diploid fibroblasts, which are widely used in HCMV research. A number of factors could influence HCMV cell tropism: efficiency of viral entry, transport of the virus within the cytoplasm, release of the viral genome and associated proteins into the nucleus, onset of

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genome transcription, posttranscriptional events, and synthesis of viral DNA. Recently, we determined that the endothelium may function as a barrier to HCMV infection and have shown in an in vitro system that impeded transport of HCMV genomes to the HUVEC nuclei might explain their low permissivity.[25]

Entry of enveloped viruses into animal cells involves virion binding to the cell surface receptor or receptors followed by either fusion of the viral envelope with the plasma membrane or by endocytosis, a process in which viruses are taken up into the cell within vesicles.[26] Attachment of the enveloped virus to the host cell membrane depends on proteoglycans,[27] glycoproteins, and cellular receptors.[28] Viral internalization most commonly occurs by receptor-mediated endocytosis into clathrin-coated vesicles, although endocytosis into smooth-walled vesicles and uptake into large smooth-walled vacuoles have also been documented.[28,29] For some enveloped viruses, this process is commonly followed by pH-dependent fusion between the viral envelope and the endosomal membrane. Alternately, direct fusion between the viral envelope and the host cell plasma membrane can occur and is a pH-independent process.

Various pathways are used by herpesviruses to penetrate host cells. Membrane fusion is the most common process, occurring either at the cell plasma membrane or within endocytic vesicles.[26] Herpes simplex virus enters human epidermal carcinoma and African green monkey kidney (Vero) cells by pH-independent fusion with the cell membrane.[30] Compton et al.[31] reported that HCMV enters fibroblasts by pH-independent fusion of the viral envelope with the plasma membrane, and we showed in the same cell system that HCMV virions, noninfectious enveloped particles, and dense bodies fuse with the cell membrane and that de-envelopment took place within 5 minutes after contact of inoculum with the cell culture.[32] However, among herpesviruses, differences in entry are observed depending not only on the type of virus but also on the cell types that are the targets of infection. For instance, Epstein–Barr virus (EBV) enters B lymphocytes by endocytosis[33,34] but enters epithelial cells by fusion with the cell membrane.[35,36]

In light of the inefficiency of in vitro infection of RPE cells and HUVECs,[16,24] we wished to investigate whether differences in viral entry and the onset of viral protein expression in these cells could explain their low permissivity compared with fibroblasts. Using electron microscopy and immunofluorescence, we found that HCMV entry into these cell types involves endocytosis. Endocytic uptake into vesicles and subsequent viral expression were pH independent. Immediate early protein expression was blocked in both cell types after cytochalasin B treatment. Incubation of RPE cells at a low temperature significantly blocked the detection of the viral tegument protein pp65 in the nucleus but not in fibroblasts. Thus, HCMV entry into RPE cells and HUVECs differs from that previously reported for fibroblasts. Despite virus entry by endocytosis being common to both cell types, other cellular factors apparently intervene in the ultimate level of virus expression in each cell type.

Materials and Methods

Human Research

The tenets of the Declaration of Helsinki were followed, and approval was obtained from the Office of Human Subjects Research.

Cell Cultures

Primary human RPE cell lines were established from donor eyes. Human eyes were obtained from the Bristol Eye Bank (a generous gift from M. Nash, Oxford, UK) after removal of corneas for transplantation. RPE cells were isolated with trypsin, resuspended in Ham's F-10 (GIBCO–BRL) medium with 10% heat-inactivated fetal calf serum (FCS), and transferred to a 25-cm^2 flask. RPE cells form monolayers like epithelial cells. At confluence, cells were typically hexagonal. Homogeneity was confirmed by positive immunostaining with monoclonal antibodies (Mab) to cytokeratins.[37] Cultures from three different primary cell lines were used at passages 3 through 9 for all further experiments.

HUVECs were isolated from umbilical cords as previously described.[16] Cells were grown in wells coated with fibronectin in a medium consisting of 40% (vol/vol) M-199 (GIBCO, Breda, The Netherlands), 40% (vol/vol) RPMI-1640 (GIBCO), 20% (vol/vol) newborn calf serum (PAN System, Germany), 20 U/ml heparin sodium salt (ICN, Zoetermeer, The Netherlands), 2 ml/L bovine brain extract, 200 mM L-glutamine (Serva, Heidelberg, Germany), and 50 μg/ml gentamicin (AUV, Cuyk, The Netherlands). Experiments used six different preparations of HUVECs, and all were performed with cells at passages 2 to 6.

Human foreskin fibroblasts (HFFs) were grown in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine and 10% FCS.

All cells were routinely tested and found negative for mycoplasma contamination.

Virus Culture

Human cytomegalovirus strain AD169 was propagated in human embryonic fibroblasts (HEFs) in Earle's modified Eagle's medium (ICN), supplemented with 2% (vol/vol) FCS (Integro, Zaandam, The Netherlands), or in HFFs in Dulbecco's modified Eagle's medium supplemented with 5% FCS (GIBCO–BRL). Cleared supernatants of human fibroblasts displaying 90% to 100% cytopathic effects were stored at −70°C. Virus titers were determined by a plaque titration assay in HFFs as previously described.[38] Titration was performed for all stocks using both passive particle adsorption and centrifugal enhancement[39]; centrifugation usually resulted in a 0.5 to 1 log enhancement of virus titers compared with passive adsorption. All virus stocks were negative for mycoplasma contamination.

Infection of Cells

HUVECs were cultured until 95% confluent, in 96-well culture plates (Costar, Badhoevedorp, The Netherlands) for biochemical experiments, in 12-well culture plates for electron microscopy and on glass slides for immunofluorescence. Cells were washed twice with phosphate-buffered saline (PBS) and preincubated with Iscove's modified Dulbecco's medium (IMDM) supplemented with 15% (vol/vol) newborn calf serum, 200 mM L-glutamine, and 50 μg/ml gentamicin (15% IMDM) for 1 hour at 37°C. Virus dilutions were made in IMDM supplemented with 2% (vol/vol) newborn calf serum, 200 mM L-glutamine, and 50 μg/ml gentamicin (2% IMDM). Cells were inoculated at different multiplicities of infection (MOI; 3 pfu/cell for HUVECs and 0.5 pfu/cell for HFFs) with HCMV diluted in 2% IMDM, by centrifugation for 45 minutes at 700*g* at 20°C, followed by a 75-minute incubation at 37°C. Subsequently, inoculum was removed, and cells were incubated with growth

medium for the indicated times. In addition, for some experiments cells were preincubated with medium containing biochemical reagents for 1 hour, infected with HCMV in the presence of these agents, and refed medium containing these agents for the indicated time.

RPE cells were seeded in 8-well LabTek glass slides (Nunc, Naperville, Il) at 60,000 cells/well for immunofluorescence and in 12-well culture plates for electron microscopy. At confluence, they were infected using MOIs of 10, 5, 1, 0.1, or 0.01 pfu/ml. High MOIs (MOI of 10 or 5) were used for analysis of viral entry, whereas high and low MOIs were used to study the effects of lysosomotropic agents.

Electron Microscopy

Human cytomegalovirus was absorbed onto HUVECs and RPE cells by centrifugation at room temperature for 5 minutes. The first sample was fixed immediately, whereas the others were incubated further at 37°C for an additional 5, 10, or 25 minutes or 1, 2, 4, 6, or 8 hours. At each time cells were prefixed with 1.6% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3), rinsed in the same buffer, post-fixed in 1% osmium tetroxide, dehydrated in methanol, and embedded in Epon in situ in the culture wells. After resin polymerization, cells were cut tangentially and vertically to the basal surface of the plastic blocks. Ultrathin sections were collected on uncoated grids and counterstained with uranyl acetate and lead citrate. Morphologic analysis was performed in a JEOL-JEM 100CX II electron microscope.

Indirect Immunofluorescence

To follow virus entry we performed indirect immunofluorescence using Mab F6a (Mab IgG2a), which detects the HCMV tegument and dense body protein pp65 (ppUL83).[40] Monoclonal antibody E13 (IgG1, a kind gift from M. C. Mazeron, Hopital Lariboisière, Paris, France) directed against exon 2 of immediate early IE proteins 1 and 2[41] was used to detect onset of viral genome expression.

RPE cells were seeded in 8-well LabTek glass slides (Nunc, Naperville, III) at 60,000 cells/well. HUVECs were seeded in gelatin-coated slides. At confluence, they were infected and sampled at various times as indicated below. Cells were air-dried, then fixed with 3% paraformaldehyde for 10 minutes, rinsed with PBS, and quenched with PBS–glycine 0.1 M for 10 minutes. Cells were then permeabilized with PBS–0.1% BSA–0.05% saponin for 20 minutes. After a 1-hour incubation with primary antibodies, slides were washed in PBS then incubated with a 1:100 dilution of fluorescein-conjugated anti-mouse IgG1 to detect immediate early (IE) proteins (Dako, Trappes, France), 1:100 dilution of Texas red–conjugated anti-mouse IgG2a to detect pp65 (Southern Biotechnology, Le Perray–en–Yvelines, France), or both. After final washing, slides were post-fixed with 10% formaldehyde in PBS, mounted in glycerol/PBS (70%/30%), and examined and photographed in a Leitz dialux microscope for counting of IE-positive nuclei.

For confocal microscopy, slides were mounted in Vectashield (Vector Laboratories, Burlington, CA). Confocal laser scanning microscopy and double-fluorescence analysis were performed with a TCS4D confocal microscope (Leica, Nussloch, Germany) interfaced with argon/krypton lasers. Simultaneously, double-fluorescence acquisitions were performed using the 488- and the 568-nm laser lines to excite fluorescein isothiocyanate (FITC) and TRITC dyes using a 63× immersion Plan APO objective (NA = 1.4). Fluorescence was selected with the appropriate double-fluorescence dichroic mirror and band-pass filters and measured with blue-green and red side sensitive one photomultipliers.

Treatment of Cells to Inhibit Endocytosis

Chloroquine was used as a weak base to raise the pH of endosomes and lysosomes. Stock solutions of chloroquine (100 mM; Sigma, St. Quentin Fallavier, France) were prepared in PBS. Cells were washed twice and incubated in their respective media plus 0.05 or 0.1 mM chloroquine for 1 hour before and during virus adsorption. Cells were then washed twice and refed media containing their respective concentrations of chloroquine. Ammonium chloride (NH_4Cl) was also used to alkalinize the cytosol. Stock solutions of NH_4Cl for each experiment were freshly prepared in PBS. Cells were incubated in media containing 10 or 30 mM NH_4Cl for 1 hour before and during virus adsorption. All working dilutions were made in Ham's F-10 for RPE cells or IMDM for HUVECs. Lysosomotropic agents were present 1 hour before, during viral adsorption, and throughout the course of infection, unless indicated otherwise. Viability of cells was determined by trypan blue exclusion. For calculating the percentage of IE-positive nuclei, cells were subsequently stained with 0.5 ng/ml 4′,6-diamino-2-phenyl indole (DAPI) in PBS containing 2.3% (wt/vol) 1,4-diazobicyclo-(2,2,2)-octane. The number of FITC-positive and the total number of DAPI-stained nuclei were counted in three different wells using four microscopic fields per well.

RPE cells and HFFs were preincubated at 20°C for 1 hour and then infected at high MOI (10 pfu/cell) for 1 and 2 hours, respectively, for HFFs and RPE cells. Nuclear localization of pp65 was analyzed by immunofluorescence using a confocal microscope.

Statistical Analysis

Results are expressed as mean ± 1 SD and were analyzed statistically by using unpaired Student's *t*-test. Statistical analysis was performed with the Statview 4.5 software package (Abacus Concepts, Berkeley, CA). $P < 0.05$ was considered significant.

Results

HCMV Entry into RPE cells and HUVECs

Electron microscopic observations revealed that within 5 to 10 minutes of contact of RPE cells and HUVECs with virus at 37°C, virion and dense bodies close to the cell were taken up into invaginations of the cell membrane (Figs. 1A, 1B, 2A, and 2B) and internalized into vesicles. Initially, particles were captured in pits that were coated onto their cytoplasmic surface with a fuzzy layer (Figs. 1A, 1B; arrowheads). These saucer-shaped depressions had the morphologic appearance of clathrin-coated pits. However, single-membrane–bound vesicles and vacuoles containing engulfed particles seemed to coalesce with uncoated endosomes (Figs. 1C, 1E, 2A, 2B, and 2C). Sometimes viral particles enclosed in an extensive intracisternal system were observed (Fig. 1D). Bundles of microfilaments and microtubules were frequently present in proximity to vesicles (Figs. 1C, 1D, 1E, 2A, and 2C). As early as 15 minutes in RPE cells (Fig. 1F) and 2 hours in HUVECs (Fig. 2C), virions

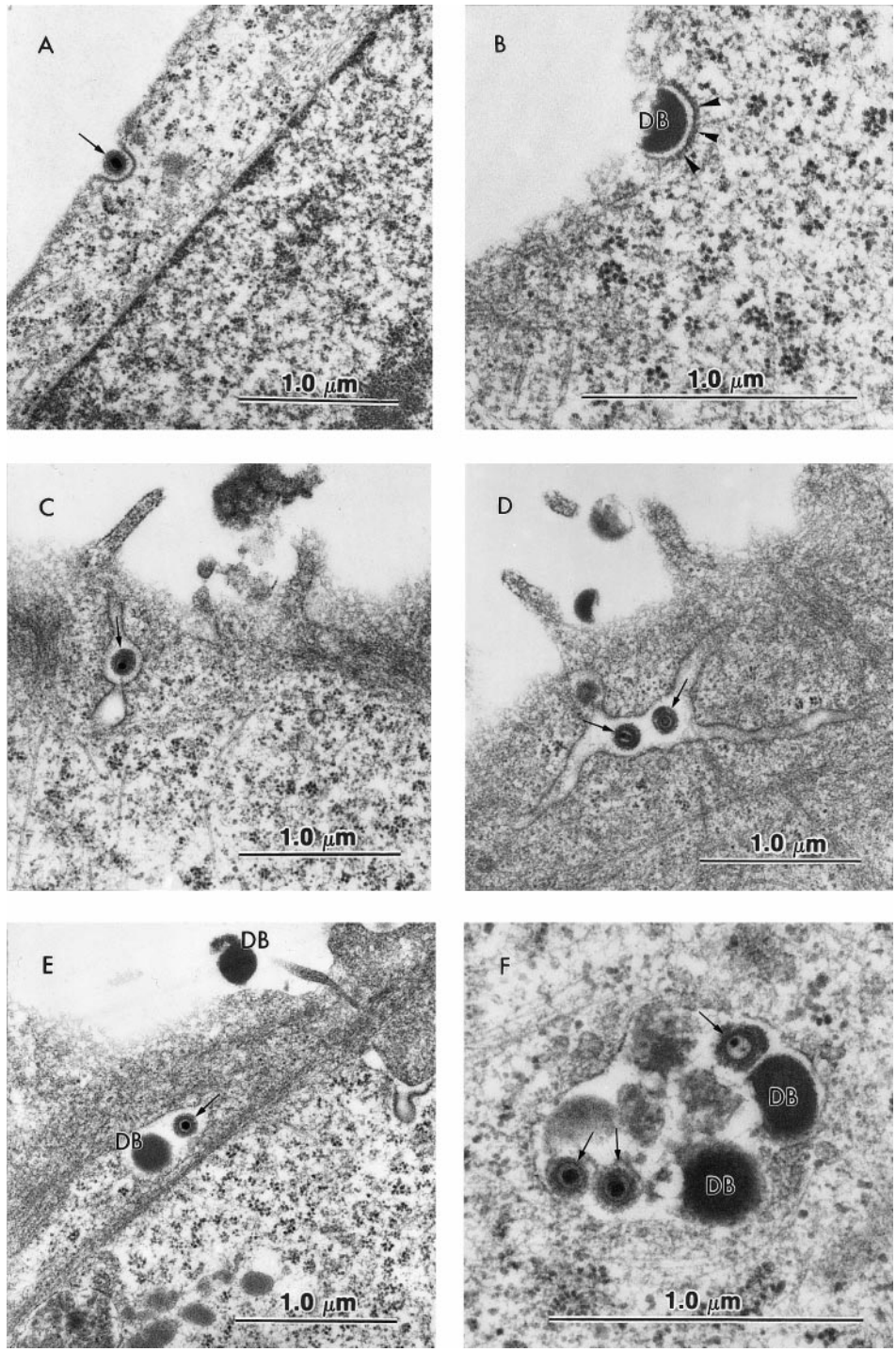

FIGURE 1. Human cytomegalovirus virions (*arrows*) and dense bodies (DB) in the initial phases (5–15 minutes of virus-cell contact) of entry into RPE cells. (**A**) Engulfment of the virus particle into an invagination of the cell membrane. (**B**, **C**, **D**, **E**) The vacuoles form, containing virus particle and sometimes dense bodies, then move toward the cell center. (**F**) At 15 minutes, virions and dense bodies enclosed in phagocytic vacuoles underwent degradation.

and dense bodies were enclosed in large vacuoles. Virus particles were detected inside all RPE cells and HUVECs. Viral structures appeared to be in the process of degradation. Viral envelopes were disrupted and dense debris accompanied viral structures within vesicles (Figs. 1F, 2C, and 2D). Sometimes, naked capsids were present within vesicles. Fusion of the viral envelope with endosomal membranes was never observed, neither were free nucleocapsids seen in the cytosol. Caveolae resembling micropinocytotic vesicles were seen at the vacuolar margin (Fig. 2C, arrowheads). These vacuoles contained morphologically unidentifiable dense material and damaged nucleocapsids.

Thus, the mechanism of HCMV entry into RPE cells and HUVECs morphologically involves endocytosis, which differs from entry by fusion observed in fibroblasts.[31,32]

Effect of Different Procedures Inhibiting Endocytosis

In light of the apparent entry by endocytosis, several substances that interfere with this process were used. Some lyso-

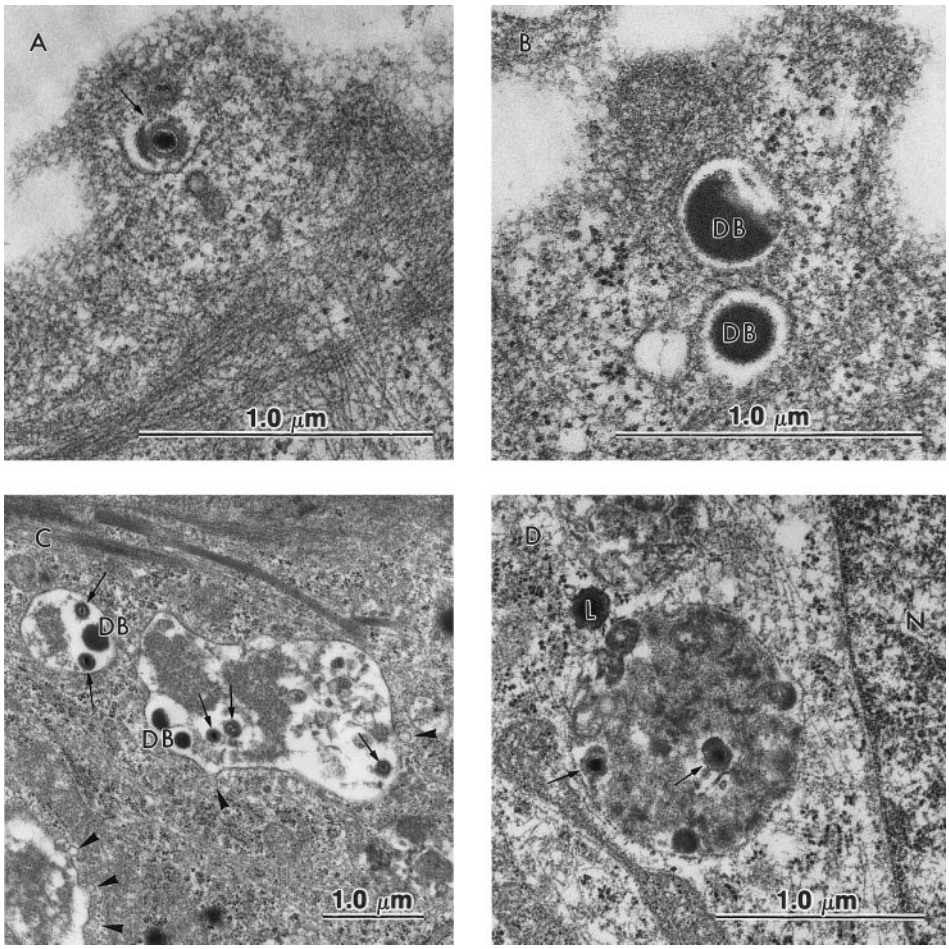

FIGURE 2. Entry of HCMV virions (*arrows*) and dense bodies (DB) into HUVECs. (**A**, **B**) During the first 15 minutes of viral adsorption, virion and dense bodies are observed in invaginations and vacuoles at the cell periphery. (**C**) After 2 hours, numerous virions, DB, naked capsids, and debris are present in large vacuoles. A caveolae (*arrowheads*) can be seen at the vacuole margin. (**D**) At 8 hours, vacuoles containing dense material and damaged nucleocapsids (*arrows*) were observed near the nucleus (N). L, lipid droplet.

somotropic agents, such as ammonium chloride and chloroquine, act mainly to prevent acidification of endosomes.[42] Cytochalasin B acts primarily by inhibiting F-actin polymerization, which plays a role in endocytosis and cytoplasmic transport processes.

Therefore, cultures were treated with various concentrations of the compounds described above. Assessment of their effects was evaluated by immunofluorescent detection of IE proteins. IE proteins are the first viral proteins to be expressed in infected cells and appear in cell nuclei.[43] Their detection is therefore indicative of efficient transport into and expression of an active viral genome in the nucleus. Expression of IE proteins is not synonymous with full viral replication[44] and could be induced by noninfectious enveloped particles shown to contain varying amounts of uncondensed DNA.[45] Irrespective of the multiplicity of infection used (range, 0.01–10 pfu/cell), nuclear IE proteins were seen in only a fraction of RPE cells 24 hours after infection (Fig. 3). However, by electron microscopy the vast majority of cells contained virus-filled vesicles 15 minutes after cell-virus contact (data not shown). At low MOIs, there was a linear relationship between the number of IE-positive cells and the multiplicity of infection (Fig. 3). At high MOIs (10, 5, and 1), the number of cells expressing IE proteins 24 hours after infection did not vary markedly and reached a plateau of 13% in RPE cells and 5% in HUVECs. However, this maximal level was significantly less than that attained in fibroblasts (100%). Below a MOI of 0.5, the number of IE-positive cells decreased linearly with the MOI in all cells.

At high MOIs, ammonium chloride and chloroquine did not seem to influence HCMV entry and subsequent nuclear detection of IE antigens in either RPE cells or HUVECs (data not shown). However, at low MOI in RPE cells, the number of IE-positive nuclei was somewhat reduced, from 6500 ± 325 cells/cm^3 to 5300 ± 250 and 5200 ± 285 cells/cm^3, respectively, after chloroquine (50 μM) and ammonium chloride (30 mM) treatment. At these concentrations, we did not observe any cytotoxicity.

Cytochalasin B decreased the number of HCMV IE-positive nuclei in HUVECs in a dose-dependent manner irrespective of the MOI. At a MOI of 0.5 and a dose of 1 μM cytochalasin B, the level of IE-positive nuclei was decreased by 80% compared with untreated cells, which showed 5% IE-positive nuclei (Fig. 4). In RPE cells infected only at a low MOI (0.06) the number of IE-positive nuclei were reduced (70%) compared with untreated cells, which showed 6% IE-positive nuclei (Fig. 4). Cytochalasin B did not influence the expression of IE protein in HFFs, even when they were infected at very low MOI (0.003). Cytochalasin B concentrations above 5 μM were cytotoxic, because at 10 μM the number of viable cells was decreased by 20% compared with control culture viability.

Endocytosis can be modulated at different levels by lowering the cell temperature between 17°C and 20°C depending on the experimental model. It has been shown in rat RPE cells that maximum rod outer segment binding to the cell surface with minimum ingestion occurs at 17°C.[46] In Hela cells, incubation at 19.5°C induces accumulation of a toxin in endosomes

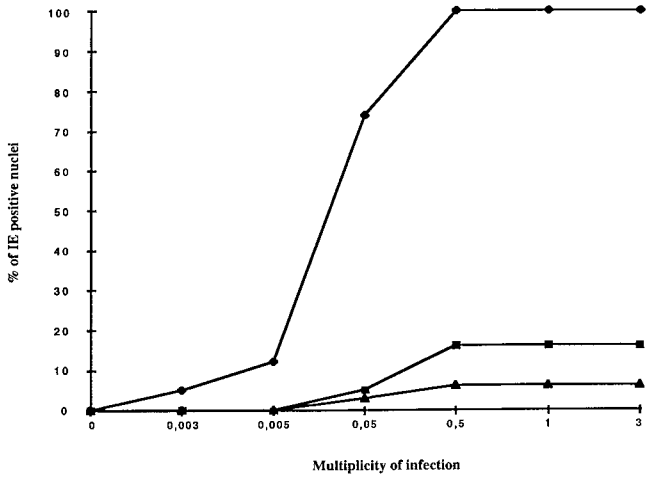

FIGURE 3. Relationship of MOI to percentage of cells showing IE fluorescence in RPE cells (■), HUVECs (▲), and fibroblasts (●). Cells were infected at different MOI, and the numbers of cells expressing IE proteins in nuclei were counted 24 hours after infection. A plateau in the number of cells was attained at the highest MOIs (> 0.5 pfu/ml), indicating that only a subpopulation of RPE cells and HUVECs is capable of undergoing the initial steps of productive infection (IE protein synthesis). Note that all fibroblasts show IE positivity, whereas only 13% of RPE cells and 5% of HUVECs were positive at high MOIs.

and blocks its transport to the Golgi apparatus.[47] We therefore compared HCMV entry into RPE cells and human fibroblasts at low temperature, using the appearance of pp65 in the nucleus as a measure of viral protein entry. Figure 5 shows that no pp65 was detected in the nuclei of RPE cells maintained at 20°C, whereas fibroblast nuclei were positive for pp65 under the same conditions. When RPE cells maintained during virus adsorption at 20°C were washed and returned to 37°C, pp65 appeared in nuclei within 1 hour, which shows that the virus was indeed adsorbed to cells at 20°C.

Immunofluorescence of Incoming Viral Proteins and the Onset of Viral IE Protein Synthesis

Electron microscopic observations were complemented by immunofluorescence studies. We used antibodies directed against pp65, a tegument protein predominantly present in enveloped virus particles (15% of protein mass) and in dense bodies (95% of the protein mass)[48] to follow the progression of pp65 from the cell surface to the nucleus of RPE cells and HUVECs. At the earliest times (1–2 hours, respectively, for RPE cells and HUVECs; Figs. 6A and 7A) after virus-cell contact, both cell types showed granular cytoplasmic pp65 fluorescence. pp65-positive nuclei were observed 1 to 2 hours after viral adsorption (Figs. 6A and 7B). The number of positive nuclei increased regularly with time and was greater in RPE cells than in HUVECs. There was a progressive reduction of pp65 in the cytoplasm of both cell types that was concomitant to the appearance of pp65 in the nucleus. However, in both cell types, many nuclei were pp65 negative after 12 hours, when the cytoplasmic signal had almost disappeared.

We have also analyzed the kinetics of IE protein expression in both cell types. This event is indicative of the onset of viral genome transcription. Immediate early–positive nuclei were first detected in RPE cells and HUVECs at 4 hours (Fig. 6C) and 6 hours, respectively, and were more numerous at 8 hours (Fig. 7D) after viral adsorption. When observed from 4 to 8 hours after viral adsorption, some IE-positive nuclei coexpressed pp65 in both cell types.

DISCUSSION

RPE cells and HUVECs are two of the most relevant targets of HCMV infection in vivo.[6,9,10,12] However, the kinetics of viral replication are significantly slower in these cells than in human diploid fibroblasts, the standard laboratory substrate used for HCMV propagation. In particular, Detrick et al.[24] reported previously that HCMV replication in primary human RPE cells progresses very slowly. The kinetics of replication in RPE cells are similar to those observed in endothelial[16] and astrocytoma (Bodaghi B. and Michelson S., unpublished observations) cells. In addition, at high MOIs the proportion of cells showing IE protein expression plateaus at different levels depending on the cell type: 100% for HFFs, 13% for RPE cells, and 5% for HUVECs. Hyper-IE events occurring between the time of CMV contact with the host cell and the onset of any viral genome expression could play an important role in determining the outcome of viral replication in a given cell type. A better understanding of these events might help to explain the apparent diversity in viral cycle kinetics in different cell types. To gain insight into the reason for low infection levels and delayed viral genome expression in RPE cells[24] and HUVECs,[25] we addressed the question of viral entry into these cells.

In general, herpesviruses enter cells by fusion.[26] This mechanism is also used by HCMV to enter fibroblasts by pH-independent fusion of the viral envelope with the cell surface.[31] Our results reported here, which are based on electron microscopic observations, show that the mechanism of HCMV entry into RPE cells and HUVECs is different from that reported for fibroblasts.[31,32] We clearly show that HCMV is taken up by

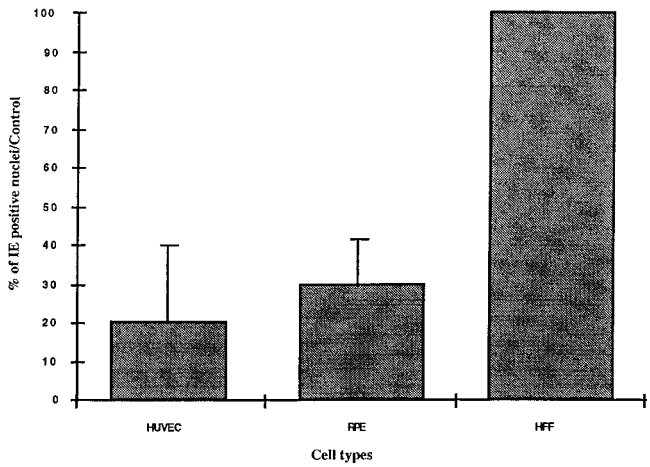

FIGURE 4. Effect of cytochalasin B on the number of cells expressing IE proteins. RPE cells and HUVECs were infected at MOIs corresponding to those showing a linear relationship between pfu/cell of virus and number of IE-positive cells (see Fig. 3). Cells were treated (or not) with cytochalasin B before infection and during virus adsorption (2 hours). The number of nuclei expressing IE proteins was counted and expressed relative to the number of nuclei in untreated cells. Cytochalasin B inhibited virus transport into the nuclei in RPE cells and HUVECs as indicated by a decrease in the number of IE-positive nuclei compared with HFFs.

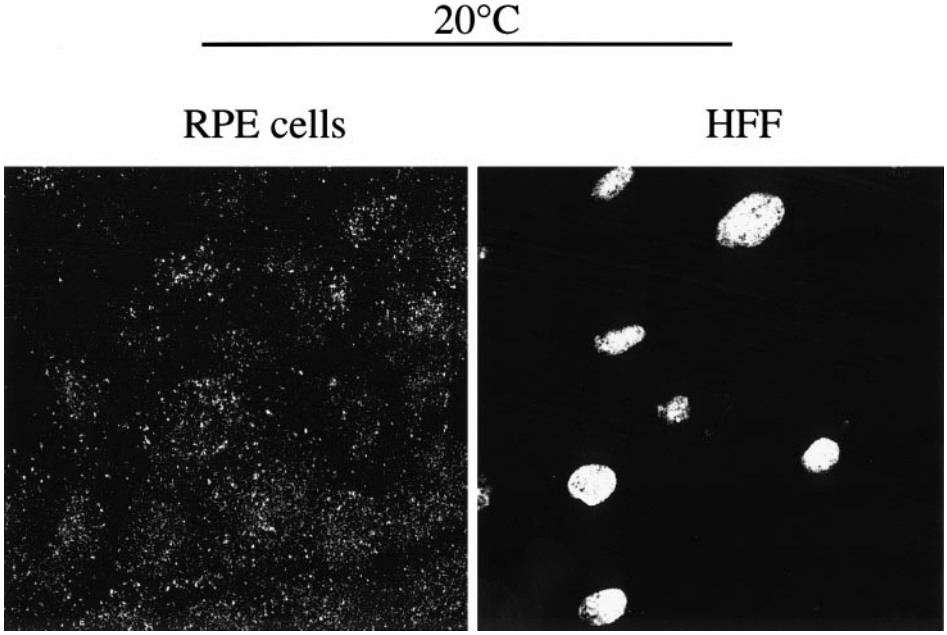

FIGURE 5. Effect of low temperature on CMV entry into RPE cells. RPE cells and HFFs were preincubated at 20°C for 1 hour, then were infected at high multiplicity for 1 hour at the same temperature. Detection of pp65 in nuclei showed that RPE cells had neither nuclear nor cytoplasmic pp65, whereas many HFF nuclei were pp65 positive. A temperature block of endocytosis thus prevented CMV entry into RPE cells but not fibroblasts.

RPE cells and HUVECs via endocytosis. Within minutes after cell-virus contact, virions and dense bodies were localized to endosomes and afterward began undergoing morphologic degradation. We never observed fusion of viral envelopes with cell surface or endosomal membranes, nor did we see free particles or nucleocapsids in the cytosol. This contrasts markedly with the early presence of nucleocapsids in the cytosol of fibroblasts.[32] It is well established that incubation of cells at low temperatures (17°C–20°C) blocks endocytosed material at the level of early endosomes.[46,47] Under these conditions, pp65 was no longer transported to the nucleus in RPE cells, whereas it was detected in the nuclei of fibroblasts. Electron microscopic observation of cells maintained at 20°C and infected confirmed that virus remains at the cell membrane (Topilko A., unpublished observations). How viral DNA manages to pass from these compartments to the nucleus thus remains to be determined.

Mechanisms of entry are closely related to the type of host cell undergoing infection.[3,26,49,50] Entry of EBV into B lymphocytes is pH-dependent and occurs after internalization via the endosomal membrane, whereas EBV entry into epithelial cells is pH-independent and results from direct fusion of the viral envelope with the cell membrane.[35,36] Another, original mechanism is used by the duck hepatitis B virus, which enters hepatocytes after endocytosis but without passage through acidic intracellular compartments.[51] In this case, virus is actively taken up by the host cell. Many enveloped animal viruses, such as Semliki Forest[52,53] and avian influenza virus,[54] penetrate into cells by an endocytic process involving clathrin-coated pits. Virus also enters cells by non–clathrin-coated endocytosis (for review see Ref. 55). Studies are currently under way to define the compartments through which CMV passes on its way to the nucleus in both cell types.

When the timing of events linked to viral entry and onset of viral genome expression and the response of cells to different chemical agents affecting endocytosis are compared in RPE cells, HUVECs, and fibroblasts, it becomes apparent that CMV interacts differently with each cell type. Human cytomegalovirus nucleocapsids are released directly into the cytosol of fibroblasts, whereas virus enters endocytotic vesicles in RPE cells and HUVECs. However, the nuclear localization of the incoming virion tegument protein pp65[56,57] in fibroblasts follows different kinetics than in RPE cells and HUVECs. In RPE cells and HUVECs, pp65 appears in the nucleus 1 to 2 hours after virus-cell contact, whereas in fibroblasts pp65 can already be detected in some nuclei within 10 minutes and most nuclei within 30 minutes (Michelson S., unpublished observations). Nuclear transport of a functional viral genome is evidenced by the onset of IE protein expression. In RPE cells and HUVECs, IE protein expression was not detected earlier than 4 to 6 hours postinfection, whereas in fibroblasts it appeared within the first hour after virus-cell contact.[43] In addition, the number of cells that eventually become IE positive after 24 hours is 100% for fibroblasts but only 13% for RPE cells and even less (6%) for HUVECs.

Although viral entry into RPE cells and HUVECs is clearly dependent on endocytosis, differences between these cell types rapidly become apparent at a post-entry level. By electron microscopy and immunofluorescence based on pp65 detection, the number of cells that contain virus particles and dense bodies is essentially 100% for both cell types. However, pp65 transport is more efficient in RPE cells than in HUVECs, because more RPE cell nuclei show pp65 fluorescence than do HUVECs. Irrespective of the MOI, lysosomotropic agents did not affect IE protein expression in HUVECs. IE protein level was also unaffected in RPE cells at high MOI. However, at low MOI, these agents decreased IE protein expression in RPE cells incompletely. These results seem to support the notion that pH-dependent fusion of the virus with the endosomal membrane is unlikely to be the major mechanism for the entry of the virus into these cell types. In addition, treatment with cytochalasin B significantly reduced the number of IE-positive nuclei in RPE cells but only at low MOI, whereas it consistently did so in HUVECs while not affecting IE protein expression in HFFs.

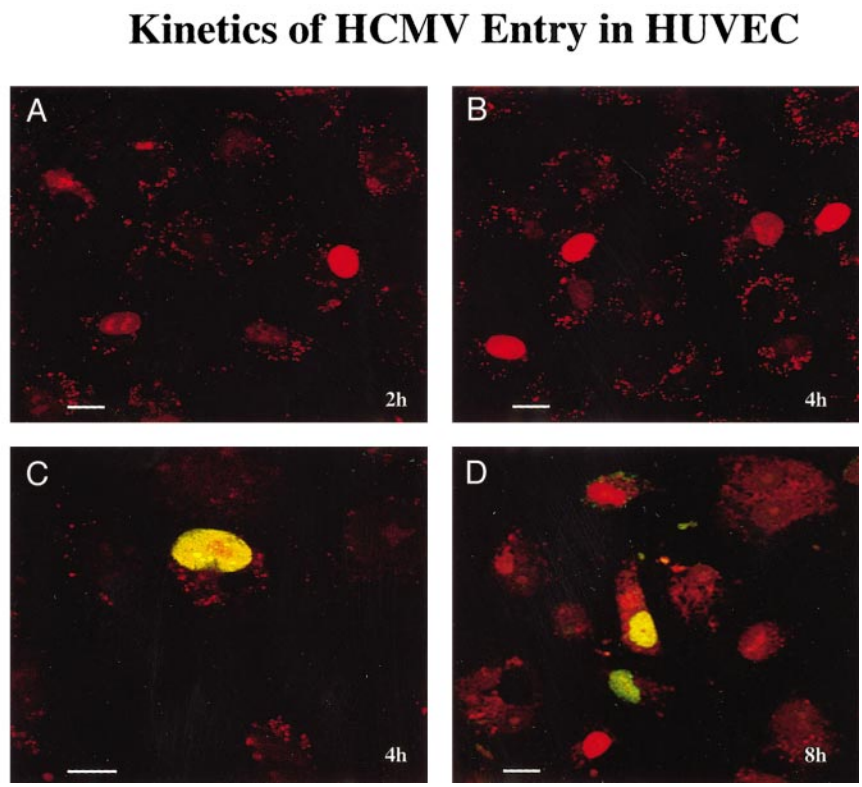

FIGURE 6. Kinetics of entry into HUVECs and onset of CMV IE protein expression. Immunofluorescence detection of the viral tegument protein pp65 (*red*) showed granules in the cytoplasm and in some nuclei 2 hours after contact with the virus (**A**). The number of positive nuclei increased at 4 hours (**B**), and IE-positive nuclei (**C**, *green*) were detected. (**D**) At 8 hours after infection, pp65 was less prominent in the cytoplasm (*red*), and some IE-positive nuclei showed colocalization of pp65 (*yellow*). Scale bar, 10 μm.

Molecular analysis of CMV entry into retinal cells can help us to understand possible CMV and HIV-1 interactions in these cells. HIV-1 antigens have been identified in the retinal, endothelial, and neuroretinal cells isolated from AIDS patients.[58,59] Retinal glial cells are fully permissive for CMV replication.[60] Like endothelial cells, they express CD4 molecules at their surface. It has been shown that US28, a CC-chemokine receptor homologue encoded by CMV during the IE phase of viral

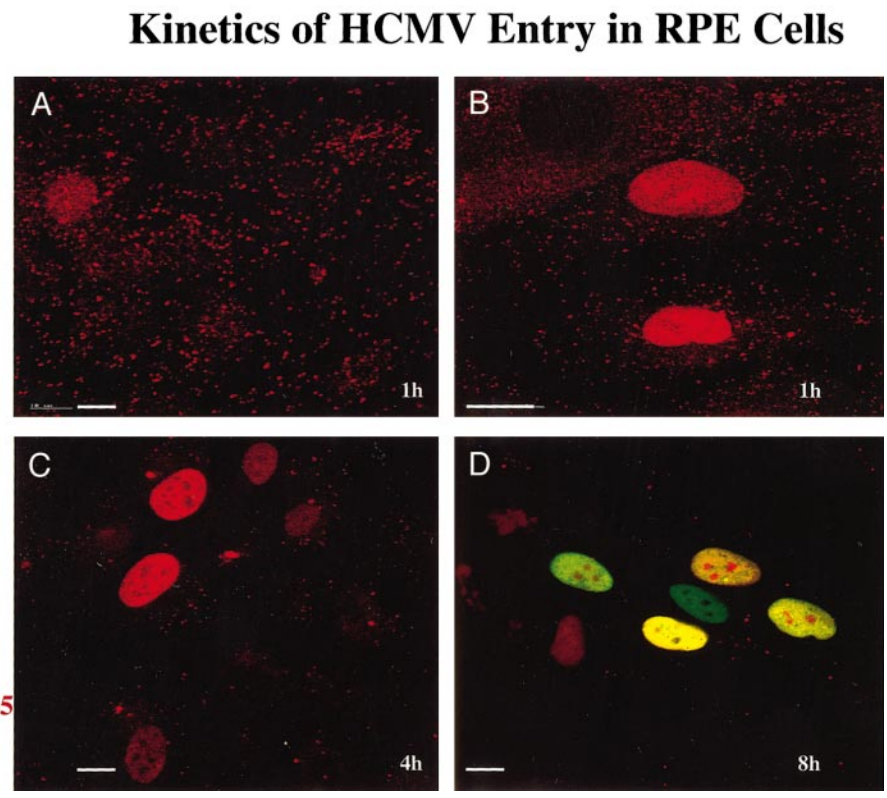

FIGURE 7. Kinetics of entry into RPE cells and onset of CMV IE protein expression. Immunofluorescence detection of the viral tegument protein pp65 (*red*) shows granules in the cytoplasm 1 hour after contact with the virus (**A**). (**B**) pp65 is transported to the nucleus within 1 hour where it accumulates (**C**). IE protein expression (**D**, *green*) was detected 8 hours after infection, and in some nuclei was seen colocalized with pp65 (*yellow* nuclei). Cytoplasmic pp65 fluorescence was no longer observed at late times. Scale bar, 10 μm.

replication, could be used by HIV as a coreceptor.[61,62] Interactions between these two viruses may influence retinal disease in patients with AIDS.

Our observations on the kinetics of CMV entry and onset of viral genome expression in RPE cells and HUVECs support the following interrelated conclusions. First, the penetration of CMV into these cells is not the main factor that determines permissivity of cells for viral replication. Second, the mode of viral entry is probably an initial controlling factor in viral replication; the efficiency of CMV expression seems to be greater when the nucleocapsid is liberated directly into the cytosol (fibroblasts) than when most of the virus is delivered to vesicles (RPE cells and HUVECs).

Finally, expression of CMV infection in a given cell type may be regulated differently at several levels. The first would be mode of entry. Within 15 minutes of virus-cell contact, CMV is detected, by both electron microscopy and immunofluorescence detection, in the vast majority of fibroblasts, RPE cells, and HUVECs. However, as we have shown here in RPE cells and HUVECs, CMV enters by endocytosis, as opposed to fusion in fibroblasts.[31,32] This means that in RPE cells and HUVECs, the viral nucleocapsid does not seem to gain direct access to the cytosol as it does in fibroblasts. Within the limits imposed by electron microscopy, we have never observed fusion of virion envelopes with plasma membranes or nucleocapsids in the cytosol. Hence, if fusion occurs in either RPE cells or HUVECs, it is an extremely rare event. Many particles and dense bodies are directed to large vesicles of RPE cells and HUVECs. This could serve to diminish the efficacy of infection in two ways: by imprisoning the virus in a compartment from which it cannot egress easily, thereby slowing its transport into the nucleus, and by exposing the virus to attack by nucleases and proteases within the vesicles. The observed decomposition in vesicles by electron microscopy and the late onset of reduced IE protein expression in RPE cells and HUVECs compared with fibroblasts suggest that both factors may be operative in the former cell types. Furthermore, incoming viral DNA remains predominantly perinuclear in the majority of HUVECs, and only a small portion of nuclei eventually become CMV DNA positive by in situ hybridization.[25] Hence, the outcome of CMV infection of a given cell may well be a function of the cell type, as well as of the virus itself.

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