Human Cytomegalovirus Entry into Epithelial and Endothelial Cells Depends on Genes UL128 to UL150 and Occurs by Endocytosis and Low-pH Fusion

Brent J. Ryckman,1 Michael A. Jarvis,2 Derek D. Drummond,2 Jay A. Nelson,1,2 and David C. Johnson1*

Department of Molecular Microbiology and Immunology1 and Vaccine and Gene Therapy Institute,2 Oregon Health and Sciences University, Portland, Oregon 97239

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Human cytomegalovirus (HCMV) replication in epithelial and endothelial cells appears to be important in virus spread, disease, and persistence. It has been difficult to study infection of these cell types because HCMV laboratory strains (e.g., AD169 and Towne) have lost their ability to infect cultured epithelial and endothelial cells during extensive propagation in fibroblasts. Clinical strains of HCMV (e.g., TR and FIX) possess a cluster of genes (UL128 to UL150) that are largely mutated in laboratory strains, and recent studies have indicated that these genes facilitate replication in epithelial and endothelial cells. The mechanisms by which these genes promote infection of these two cell types are unclear. We derived an HCMV UL128-to-UL150 deletion mutant from strain TR, TRΔ4, and studied early events in HCMV infection of epithelial and endothelial cells, and the role of genes UL128 to UL150. Analysis of wild-type TR indicated that HCMV enters epithelial and endothelial cells by endocytosis followed by low-pH-dependent fusion, which is different from the pH-independent fusion with the plasma membrane observed with human fibroblasts. TRΔ4 displayed a number of defects in early infection processes. Adsorption and entry of TRΔ4 on epithelial cells were poor compared with those of TR, but these defects could be overcome with higher doses of virus and the use of polyethylene glycol (PEG) to promote fusion between virion and cellular membranes. High multiplicity and PEG treatment did not promote infection of endothelial cells by TRΔ4, yet virus particles were internalized. Together, these data indicate that genes UL128 to UL150 are required for HCMV adsorption and penetration of epithelial cells and to promote some early stage of virus replication, subsequent to virus entry, in endothelial cells.

Human cytomegalovirus (HCMV) is a ubiquitous herpesvirus that seldom causes clinical symptoms in healthy, immunocompetent adults. However, primary infection of neonates is a significant cause of life-threatening diseases and is associated with several neurological birth defects (2, 6). Persistent or recurrent infections in immunocompromised AIDS patients and transplant recipients result in significant morbidity and mortality with the retina, the lungs, and the gastrointestinal tract representing major sites of pathogenesis (6, 28). HCMV displays an extremely broad host cell range in immunocompromised hosts, infecting many different cell types, including epithelial cells, endothelial cells, smooth muscle cells, fibroblasts, neurons, monocytes, and macrophages (6, 28, 42). HCMV replication in retinal pigment epithelial cells appears to be largely responsible for the retinal damage that leads to blindness in HCMV retinitis, and infection of endothelial and smooth muscle cells may contribute to transplant vascular sclerosis, restenosis following angioplasty, and atherosclerosis (16, 28, 49, 58, 66). Epithelial and endothelial cells may also play important roles in viral persistence and dissemination of virus within the body and between hosts (16, 28, 55).

Given the significance of epithelial and endothelial cells to the pathology and epidemiology of HCMV disease, it is vital to understand how the virus replicates in these cells. Unfortunately, commonly studied laboratory strains, including AD169 and Towne, do not replicate well in cultured epithelial or endothelial cells. It has become generally accepted that the loss of broad host cell range exhibited by laboratory strains is the result of a number of genetic mutations and rearrangements that can be correlated with extensive serial propagation on fibroblasts. Strains that have not been extensively propagated (referred to as “clinical” or “wild-type” strains) generally harbor fewer mutations and retain the ability to infect a wider variety of cultured cell types (10, 13, 20, 35, 43).

Most of the genes located between UL128 and UL150 are either missing or mutated in laboratory strains AD169 and Towne, while HCMV Toledo and other so-called “clinical strains” such as TR, FIX, and Merlin retain these genes (10, 13, 20, 35). Several recent reports have implicated individual genes in the left end of this gene cluster (i.e., UL128, UL130, and UL131) in infection of epithelial and endothelial cells (20, 40, 60). Hahn et al. found that independent deletion of UL128, UL130, or UL131 from an endothelial tropic strain (FIX) resulted in a virus that could no longer infect endothelial cells (20). Additionally, they described a multicistronic mRNA encoding UL128, UL130, and UL131 and an additional transcript encoding just UL128 (20). Transcomplementation experiments suggested that UL130 and UL131 facilitate endothelial cell infection and replication, while the effect of deleting UL128 was probably due to reduced stability of the entire UL128-to-UL131 mRNA (20). Patrone et al. showed that the UL130 gene product is a virion envelope component and that the
UL130 protein encoded by Towne is less stable, and therefore less efficiently incorporated, than the UL130 protein of the wild-type FIX. When Towne was propagated on a cell line expressing the more stable FIX UL130 protein, progeny virions were able to infect endothelial cells for one replication cycle (40). Wang et al. reported that a recombinant AD169 containing a repaired UL131 gene (derived from TR) displayed enhanced infection of epithelial and endothelial cells (60). The mechanism or mechanisms by which UL131 enhances infection of epithelial cells remain to be determined. The AD169 UL131 repair virus was syncytial, suggesting that there are other mutations in AD169, which complicates interpretation of these results. Recently, the UL132 protein was shown to be a virion component of strain AD169 and deletion mutants were impaired for growth on fibroblasts, likely during late stages of maturation or egress (56). The UL132 gene is present in all HCMV strains; however, the amino acid sequence is highly variable (10, 13, 35). Genes to the right (i.e., UL133 to UL150) may also participate in infection of epithelial and endothelial cells, but their contribution has not been well characterized. Moreover, little is known about the mechanisms by which any of the UL128-to-UL150 gene products function to promote infection in epithelial and endothelial cells. Infection of epithelial or endothelial cells by laboratory strains of HCMV might potentially be blocked at any stage of virus replication, from entry and uncoating through assembly and egress of virions. However, in many cases early or immediate-early (IE) genes are not expressed in epithelial or endothelial cells, suggesting defects in entry, uncoating, or early gene expression.

We were interested in the earliest events in HCMV infection of epithelial and endothelial cells, including entry. For other herpesviruses, entry into the cytoplasm of host cells frequently involves two stages that can be separated biochemically or genetically. The first step, adsorption or binding of herpesviruses onto cell surfaces, frequently involves very numerous, charged cell surface molecules such as heparan or chondroitin sulfate glycosaminoglycans (GAGs) (50, 57). For example, adsorption of herpes simplex virus (HSV) is difficult to saturate and does not require viral glycoproteins that are necessary for virus penetration (25). The second step, entry or penetration of herpesvirus into the cytoplasm, requires fusion between viral and cellular membranes. Adsorption onto GAGs may simply increase cell surface concentrations of virus, thus enhancing entry, or, alternatively, GAG binding may be required to trigger fusion (57).

Entry of herpesviruses appears to be a complex process involving several sequential stages in the activation of the viral fusion machinery and is best understood for HSV, which requires at least three glycoproteins: gD, which binds several different receptors, gB, and gH/gL (8, 15, 31, 47, 57). Moreover, it is now well established that some herpesviruses can enter different cell types through different mechanisms. For example, Epstein-Barr virus (EBV) can enter epithelial cells and transformed B cells by fusion at the plasma membrane, but enters normal B cells by endocytosis and pH-independent fusion with endosomes (33). Similarly, HSV enters some cells by fusion with the plasma membrane and others by endocytosis followed by low-pH-dependent fusion with endosomal membranes, and still others by pH-independent fusion with endosomes (34, 36–38, 63).

The early stages of HCMV replication, adsorption, and entry have largely been characterized using laboratory strains and human fibroblast cells. HCMV AD169 adsorbs or attaches onto cell surface heparan sulfate GAGs, as shown by competition experiments and digestion with heparan sulfate-specific enzymes (12). Entry of HCMV into fibroblasts involves fusion between the viral envelope and the plasma membrane and is not sensitive to compounds that raise endosomal pH (11). There is evidence that HCMV enters fibroblasts by utilizing cellular receptors, including epidermal growth factor receptor and certain integrins (14, 61, 62). Nucleocapsids are then transported to nuclear pores where the viral genome is released (39). Adsorption of AD169 onto endothelial cells was shown to be comparable to that of endotheliotropic HCMV, but there were apparently other defects in early virus replication preceding release of the genome into the nucleus (52). Given the diversity of entry mechanisms of other herpesviruses, it seems probable that HCMV, with its comparatively large coding capacity, can also utilize different entry pathways to infect different cell types.

In order to characterize early stages of HCMV replication in epithelial and endothelial cells, we utilized a clinical HCMV strain, TR, which infected these cells well, and derived a UL128-to-UL150 deletion mutant, denoted TRΔ4. The data presented suggest that one or more of the genes UL128 to UL150 facilitate adsorption and entry of wild-type HCMV on epithelial cells and that on both epithelial and endothelial cells, wild-type HCMV TR entry is through endocytosis followed by fusion with endosomal membranes in a pH-dependent fashion. In these experiments, both TRΔ4 and AD169 were internalized but failed to enter epithelial cells and were unable to induce IE gene expression. However, defects with both viruses could be overcome by chemically enhancing fusion of virus with cells using polyethylene glycol (PEG). In contrast, endothelial cells infected with TRΔ4 and AD169 did not express IE, even after PEG treatment, indicating that there is another block, subsequent to entry. These results describe aspects of HCMV entry into epithelial and endothelial cells and demonstrate that the HCMV UL128-to-UL150 gene cluster encodes proteins important in virus entry and other early stages of infection of these cells.

MATERIALS AND METHODS

Cells and viruses. Neonatal, normal human dermal fibroblasts (NHDF) were obtained from Cascade Biologies (Portland, OR) and grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 12% fetal bovine serum (FBS) (HyClone). The retinal pigment epithelial cell line ARPE-19 was obtained from the American Type Culture Collection (ATCC) (RCCL-2302) and was grown in a 1:1 mix of DMEM and Ham's F-12 medium (Invitrogen) supplemented with 10% FBS. Transformed human umbilical vein endothelial cells (hHUVECs) were provided by Ashlee Moses (Oregon Health and Sciences University, Portland, OR) and were grown in EBM-2 plus the supplied EGM-2 supplement kit (Cambrex). HCMV strain AD169 was obtained from ATCC. The wild-type strain TR was derived from the vitreous fluid of a patient with AIDS-related HCMV retinitis and was cloned into a bacterial artificial chromosome (BAC), and infectious virus was produced by transfecting NHDF cells (35, 54). All strains of HCMV were propagated on NHDF cells by infection at a multiplicity of less than 0.1 in DMEM plus 5% FBS for 10 to 16 days. Cell-associated virus was harvested by sonication of cell pellets, and the cellular debris was removed by centrifugation at 8,000 × g for 20 min. Virus particles were concentrated by centrifugation at 50,000 × g for 1 h through a 20% sorbitol cushion. Virus titers were determined by plaque assay on NHDF cells. HSV-1 gD-null mutant vRR1097 (44) was provided by Richard Roller (Univer-
recently described (31). Briefly, cells were grown to near confluence on glass coverslips and infected with HCMV and then fixed with 2% formaldehyde in PBS for 15 min followed by three postfixation washes in PBS. Fixed cells were permeabilized and blocked for 1 h at 1 h in IF buffer and then stained with primary antibodies for 1 h, washed with IF buffer, and stained with secondary antibodies for 1 h. For experiments quantifying the percentage of cells infected, cells were subsequently stained with 1.4 μM 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) in PBS for 10 min. Coverslips were mounted with Fluoromount-G (Southern Biotech, Birmingham, AL) and analyzed on a Nikon Microphot-FX immunofluorescence microscope.

**Lysoosmotropic agents.** A 1.5 M stock of NH₄Cl, pH 7.3, in PBS, was made fresh for each experiment. Chloroquine was prepared as a 25 mM stock in PBS, and bafilomycin A1 (Sigma) was prepared as a 10 μM stock in dimethyl sulfoxide. Cells were preincubated for 1 h in either normal medium or medium containing drug and then infected with 5 PFU per well of HCMV strain TR using centrifugal enhancement. Drugs were either present throughout the experiment or added at 4 h after inoculation. Cells were fixed at 20 h (fibroblasts and epithelial) or 8 h (endothelial) and analyzed by immunofluorescence for IE86.

**Centrifugal and PEG enhancement of infection.** Cells growing on glass coverslips were inoculated with 10 PFU per well of HCMV. Culture dishes were centrifuged at 800 × g for 2 h at room temperature and then moved to 37°C for 1 h. The cells were washed twice with PBS and were subsequently treated with polyethylene glycol or returned to 37°C until immunofluorescence analysis was performed. PEG 6000 (Fluka) was prepared as a 60% (wt/wt) solution in PBS and diluted with warm PBS to 40% or 44%. Cells were treated with diluted PEG for 30 s and washed immediately five times with warm PBS. To control for effects of PEG on endothelial cells, t-HUVECs were incubated with HSV-1 (R19) or TR (derived from Vero cells that had been treated with anti-gD monoclonal antibody) and then infected with HCMV AD169 for 1 h. The cells were washed twice with PBS and then treated with 4% paraformaldehyde for 30 min. After washing the cells with Dulbecco's PBS, they were permeabilized with 0.5% Triton X-100 and then incubated with the appropriate antibodies. After washing, the cells were mounted with Fluoromount-G (Southern Biotech, Birmingham, AL) and analyzed on a Nikon Microphot-FX immunofluorescence microscope. Briefly, cells were grown to near confluence in 2-cm² multwell culture dishes and cooled to 4°C on ice and radiolabeled particles were added in a total volume of 150 μl. Cultures were centrifuged at 800 × g for 2 h at 4°C, and then unbound virus was removed by washing with cold PBS. Cultures were then shifted to 37°C for various times and then cooled back to 4°C on ice. To remove cell surface virions, cells were then treated for 2 h at 4°C with proteinase K (PK: 2 mg/ml; Fisher Scientific) in PBS or with 0.2% bovine serum albumin in PBS. The cells were then scraped from wels, pelleted at 1,000 × g for 5 min, and washed with cold PBS, and radioactivity was quantified by liquid scintillation.

**HCMV cell-to-cell spread assay.** NHDF and ARPE-19 cells were grown to near confluence on glass coverslips and infected with 20 PFU per well of HCMV. Infected ARPE-19 cells were centrifuged and treated with PEG. Cells were maintained in appropriate media containing 0.3% pooled human immunoglobulin for 21 days and then fixed and analyzed by immunofluorescence for IE86 and HCMV gB.

**RESULTS**

**Construction of a recombinant HCMV lacking epithelial and endothelial cell tropism.** HCMV strain TR was derived from an ocular specimen (54) and, after a very limited number of passages on human fibroblasts, was cloned into a BAC (35). HCMV TR derived from both the original clinical specimen and virus derived from the BAC clone can infect epithelial and endothelial cells much more efficiently than HCMV AD169 or Towne (60; I. Smith, unpublished results; M. Jarvis, unpublished results). TR contains all of the genes UL128 to UL150 (35); however, little is known about the mechanisms by which these genes promote HCMV infection of epithelial or endothelial cells. The goal of our experiments was to determine which stages of HCMV replication were affected in epithelial and endothelial cells when the UL128 to UL150 genes were deleted. AD169 contains deletions and mutations in this region but also likely harbors mutations elsewhere in the virus genome that may complicate analyses. Therefore, to specifically address the role of the UL128 to UL150 genes in infection of epithelial and endothelial cells, we produced an HCMV strain TR lacking these genes in order to mediate recombination between the ends of a linear DNA species and homologous sequences within the BAC-cloned HCMV genome. An expression cassette containing the LacZ/Amp' genes flanked by 60 nucleotides of sequences upstream of the UL128 start codon and downstream of the UL150 stop codon was generated by PCR and used to transform recombination-competent bacteria that harbor the HCMV TR-BAC. Transformants were selected for growth on ampicillin, and the resulting clones were screened by restriction digest and Southern blot analysis for correct recombination. The UL128 to UL150 region of HCMV strain TR spans two EcoRI fragments of 22.3 kb and 10 kb (Fig. 1A, line 3). Replacement of UL128 to UL150 was predicted to remove one EcoRI site and thereby collapse the two fragments into a single 15-kb fragment (Fig. 1A, line 4). Southern blot analysis using a probe specific for the Amp' sequences confirmed the presence of a single insertion of the LacZ/Amp' cassette in the intended location within the genome (on a 15-kb EcoRI fragment) in

sired previously (38). Briefly, cells were grown to near confluence in 2-cm² multwell culture dishes and cooled to 4°C on ice and radiolabeled particles were added in a total volume of 150 μl. Cultures were centrifuged at 800 × g for 2 h at 4°C, and then unbound virus was removed by washing with cold PBS. Cultures were then shifted to 37°C for various times and then cooled back to 4°C on ice. To remove cell surface virions, cells were then treated for 2 h at 4°C with proteinase K (PK: 2 mg/ml; Fisher Scientific) in PBS or with 0.2% bovine serum albumin in PBS. The cells were then scraped from well, pelleted at 1,000 × g for 5 min, and washed with cold PBS, and radioactivity was quantified by liquid scintillation.

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UL128-to-UL150 genes in TR-4 replaced with LacZ/Ampr sequences (10 kb and 22.3 kb) are indicated. (Line 4) Schematic diagram of EcoRI restriction sites (Ec) and the predicted fragment sizes of homologous recombination with TR-BAC sequences. The positions of EcoRI restriction sites (Ec) and the predicted fragment sizes (10 kb and 22.3 kb) are indicated. (Line 4) Schematic diagram of UL128-to-UL150 genes. AD169 lacks most of the UL128-to-UL150 genes and has multiple compensatory mutations that enhance virus adsorption onto epithelial cells by 5- to 10-fold compared with that onto cells that were not centrifuged (data not shown). For this reason, most other studies utilized centrifugal enhancement.

To compare the adsorption of TR, TRΔ4, and AD169 onto ARPE-19 cells, [3H]thymidine-labeled HCMV virions were partially purified and characterized by immunoblotting for levels of pp65. The specific radioactivities of TRΔ4 and TR were very similar, and AD169 was approximately half that of TR. Radiolabeled particles were centrifuged onto cells at 4°C, the cells were washed extensively, and radioactivity associated with cells was determined. TRΔ4 reproducibly bound at approximately 10% the levels observed for TR, suggesting that at least one of the UL128-to-UL150 genes promotes adsorption to epithelial cells (Table 1, experiment 1). In this experiment, AD169 bound well to epithelial cells (Table 1). In a second experiment, cells were incubated with TRΔ4 that was three times more concentrated (compared with TR) and adsorption was increased to approximately 50% the level observed for TR (Table 1, experiment 2). This indicates that at higher virus doses, or when TRΔ4 particles are more concentrated, adsorption can be enhanced. The observation that AD169 bound better than TR may reflect the fact that AD169 has been extensively passaged in culture and may have acquired compensatory mutations that enhance virus adsorption onto cultured cells. A similar defect in TRΔ4 binding was observed on fibroblasts (not shown). These results suggest that the UL128-to-UL150 gene cluster encodes one or more proteins that increase adsorption of wild-type HCMV onto epithelial cells and fibroblasts.

**TABLE 1. Binding of radiolabeled HCMV strains onto ARPE-19 epithelial cells**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Relative binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>TR</td>
<td>100</td>
</tr>
<tr>
<td>TRΔ4</td>
<td>11 ± 5</td>
</tr>
<tr>
<td>AD169</td>
<td>144 ± 20</td>
</tr>
</tbody>
</table>

Relative specific radioactivity (cpm/units of pp65): TR = 1, TRΔ4 = 1.1, and AD169 = 0.4.

The amount of radiolabeled TR that bound onto cells was arbitrarily set at 100.

Similar quantities of virus, as judged by pp65 content, were incubated with cells.

Three times more TRΔ4 (as judged by pp65) was incubated with cells compared with TR.

ND, not done.
FIG. 2. Effects of polyethylene glycol and centrifugation on infection of ARPE-19 epithelial cells by HCMV. Replicate cultures of ARPE-19 cells were inoculated with 10 PFU/cell of HCMV strain TR, TRΔ4, or AD169 and were either incubated at room temperature for 2 h or centrifuged at 800 × g at room temperature for 2 h. All cultures were then shifted to 37°C for 2 h and subsequently treated with either PBS or 44% PEG for 30 s. After 48 h, cells were fixed and analyzed by immunofluorescence to detect HCMV IE86. Representative fields are shown.
HCMV TRΔ4 and AD169 are defective for entry into epithelial cells. To study entry of HCMV into epithelial cells, 10 PFU/cell of TR, TRΔ4, or AD169 was applied to cells and cultures were either centrifuged or not centrifuged. The percentage of cells infected was then determined by indirect immunofluorescence detection of the HCMV IE86 protein. Without centrifugation, strain TR caused IE expression in 76% of ARPE-19 cells, while TRΔ4 and AD169 infected only 0.5% and 0.8% (Fig. 2 and Table 2). Centrifugation increased infection by TR to 90% and resulted in a 3.6-fold enhancement of infection by TRΔ4 and AD169 so that 1.8 and 2.6% of cells were infected. Therefore, while adsorption was enhanced by centrifugation, the number of cells that were infected by AD169 or TRΔ4, as judged by IE expression, was still dramatically lower than that observed with TR. Given that relatively high input doses of virus were applied to the cells in these experiments, the failure of TRΔ4 and AD169 to induce IE expression was probably not due solely to defects in virus binding or adsorption.

PEG can chemically induce fusion by dehydrating the surfaces of juxtaposed membranes (30). In this way, PEG can force the entry of otherwise entry-defective herpesviruses when adsorbed onto the cell surface (31, 46, 48). To test the hypothesis that AD169 and TRΔ4 are defective for entry on epithelial cells, cells were inoculated with TR, AD169, or TRΔ4 with or without centrifugation, and then subjected to a brief treatment with 44% PEG. As shown in Fig. 2 and Table 2, PEG treatment dramatically increased the number of cells infected with TRΔ4 and AD169 (88- and 47.5-fold, respectively). Centrifugation followed by PEG treatment increased infection by TRΔ4 and AD169 so that 62% and 70% of cells were infected, respectively. These results indicate that there was sufficient TRΔ4 and AD169 adsorbed onto the cells to initiate infection when entry was forced with PEG. Furthermore, the dramatic increase in infection by TRΔ4 and AD169 with PEG treatment suggests that these UL128-to-UL150 mutants are defective for entry into epithelial cells.

Infection of endothelial cells by HCMV lacking UL128 to UL150 is blocked at postentry stages. As with epithelial cells, AD169 also poorly infects endothelial cells (17, 20, 60). To characterize entry into endothelial cells and the requirement for the UL128-to-UL150 genes, t-HUVECsin were infected with TR, TRΔ4, or AD169 and treated with PEG. Without centrifugal enhancement or PEG treatment, HCMV strain TR infected 85% of endothelial cells while TRΔ4 and AD169 infected less than 1% and 6%, respectively (Fig. 3 and Table 3). Centrifugation alone had no effect on the number of cells infected by either TRΔ4 or AD169 (Fig. 3 and Table 3). PEG treatment, with or without prior centrifugation, had no effect on TRΔ4 infection and only marginally enhanced infection by AD169 (Fig. 3 and Table 3).

Despite the effectiveness of PEG on epithelial cells, it was possible that the PEG treatment failed to cause fusion between HCMV and endothelial cells. To determine if endothelial cells are somehow less sensitive to PEG, an entry-defective HSV mutant was used. The gD gene of HSV vRR1097 was replaced by a gene coding for GFP (44). Glycoprotein D is essential for entry of HSV into all cells characterized to date (57), and PEG has been shown to enhance the infectivity of HSV virions that lack gD (31). Therefore, HUVECsin were incubated with vRR1097 (grown on noncomplementing Vero cells) and then centrifuged and treated with 40% PEG. No infected cells (i.e., GFP-expressing cells) were detected in cultures that were not treated with PEG, whereas levels of over 50 infected cells per culture were observed after PEG treatment (data not shown). This indicated that PEG treatment is capable of promoting entry of another herpesvirus into these endothelial cells and, therefore, likely had a similar effect on TRΔ4 entry. From these experiments, the failure of TRΔ4 to enter endothelial cells cannot be excluded. However, since PEG promotes entry and yet did not result in additional IE86 expression by TRΔ4, it appears that the UL128-to-UL150 genes are required for a stage of infection subsequent to entry that could include disassembly of capsids, transport to nuclear pores, release of viral DNA into the nucleus, or IE gene expression.

Internalization of HCMV into epithelial and endothelial cells. Enveloped viruses reach the cytoplasm by numerous mechanisms, including fusion with the plasma membrane and endocytosis followed by fusion with endosomal membranes (33, 37). PEG essentially bypasses normal entry processes by inducing fusion with the plasma membrane. To more directly follow the internalization of HCMV, [3H]thymidine-labeled virions were purified from fibroblast culture supernatants and adsorbed onto fibroblast, epithelial, or endothelial cells at 4°C to synchronize uptake. Cells were then shifted to 37°C, and after various times, uptake of virus into cells was measured by treating cells with PK to release any cell surface virus. The internalization at any time point was quantified by subtracting the fraction of HCMV that was PK resistant at 0 min (5 to 15%) from the fraction that was PK-resistant virus at that time point. This assay measures only internalization: either fusion with the plasma membrane, endocytosis, or other uptake processes that remove the virus from the cell surface. First, the internalization kinetics of radiolabeled TR particles was compared between fibroblasts and epithelial cells. Fibroblasts internalized more TR than epithelial cells, and in both cases, the internalization began to plateau by 120 min (Fig. 4A). Subsequent experiments compared the fraction of each strain that was internalized after 180 min at 37°C. Approximately 25 to 45% of the cell-bound TR, TRΔ4, and AD169 were internalized into fibroblasts in the 3-h period (Fig. 4B). AD169 was more efficiently internalized into fibroblasts than other viruses. This was not surprising given that AD169 has been propagated extensively on fibroblasts. On epithelial cells, TRΔ4 and AD169 were internalized at least as efficiently as TR, although, for all three viruses, internalization was lower than on fibroblasts that express the UL128-to-UL150 genes. Therefore, it appears that the UL128-to-UL150 genes are required for a stage of infection subsequent to entry that could include disassembly of capsids, transport to nuclear pores, release of viral DNA into the nucleus, or IE gene expression.
FIG. 3. Effects of polyethylene glycol and centrifugation on infection of t-HUVECs by HCMV. Replicate cultures of t-HUVECs were inoculated with 10 PFU/cell of HCMV strain TR, TRΔ4, or AD169 and subsequently centrifuged as in Fig. 2 and treated with 40% PEG. After 48 h, IE86 was detected by immunofluorescence. Representative fields are shown.
This is consistent with the previous report that HCMV infects fibroblast cells by pH-independent fusion at the plasma membrane (11). Furthermore, these results indicate that NH₄Cl, chloroquine, and bafilomycin do not directly damage HCMV virions and that effects observed on other cell types are likely to result from modification of cellular processes. When added 1 h before and during virus infection, NH₄Cl and bafilomycin dramatically reduced the number of IE86-expressing epithelial and endothelial cells (Fig. 5A and B). These drugs had no effect when added 4 h after virus. Treatment with chloroquine produced a similar but less dramatic effect on both epithelial and endothelial cells (Fig. 5C). These results indicate that

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### Table 3. Effects of polyethylene glycol and centrifugation on HCMV infection of t-HUVECs

<table>
<thead>
<tr>
<th>Virus</th>
<th>% IE86 positive (fold increase)</th>
<th>No treatment</th>
<th>Spin</th>
<th>PEG</th>
<th>Spin + PEG</th>
</tr>
</thead>
<tbody>
<tr>
<td>TR</td>
<td>85.8</td>
<td>94.5 (1.1)</td>
<td>89.5 (1.04)</td>
<td>95.2 (1.11)</td>
<td></td>
</tr>
<tr>
<td>TRΔ4</td>
<td>&lt;1</td>
<td>&lt;1 (1)</td>
<td>&lt;1 (1)</td>
<td>&lt;1 (1)</td>
<td></td>
</tr>
<tr>
<td>AD169</td>
<td>5.9</td>
<td>5.8 (1)</td>
<td>7.9 (1.34)</td>
<td>12.7 (2.15)</td>
<td></td>
</tr>
</tbody>
</table>

*Viruses were incubated with cells at 10 PFU/cell.

b Ratio of IE86-positive nuclei to DAPI-stained nuclei (n > 450 cells). Values in parentheses represent fold increase compared to no treatment. “Spin” represents centrifugation of cells and virus at 800 × g for 2 h at room temperature. “PEG” represents treated with 40% PEG for 30 s.
efficient HCMV infection of epithelial and endothelial cells requires acidification of endosomes.

Mutant HCMVs lacking UL128 to UL150 fail to spread on epithelial cells. Many herpesviruses are capable of spreading from cell to cell without being released to the extracellular space. Often, these viruses remain attached to one cell while spreading across narrow cell junctions to an adjacent cell (reviewed in reference 24). Spread across cell junctions in epithelial tissues, as well as in cultured epithelial cells, involves processes that mimic entry of extracellular virus, but there can be important differences between these processes. For example, HSV gE/gI functions selectively in cell-to-cell spread and not in entry of extracellular virus (24, 26). In contrast, pseudorabies virus gD is essential for entry of extracellular virus but is not required for cell-to-cell spread, either in cultured cells or in tissues (41, 45).

To characterize the role of the UL128-to-UL150 genes in epithelial cell-to-cell spread, ARPE-19 cells were infected with TR, TR/H9004, or AD169 by using centrifugal enhancement and PEG to promote entry. Cultures were then incubated at 37°C for 21 days in the presence of neutralizing antibodies and subsequently analyzed by immunofluorescence for the HCMV proteins IE86 and gB. Expression of gB would suggest that viruses are expressing late-gene products and presumably producing infectious progeny. HCMV TR, TR/H9004, and AD169 spread similarly on fibroblasts, producing robust plaques consisting of more than 300 cells (Fig. 6). Cells at the periphery of each plaque contained only IE86 protein, indicating that they represented an earlier stage of infection than cells nearer the center of the plaques that also expressed gB. When approximately 20 PFU/dish of TR, TR/H9004, and AD169 was applied to ARPE-19 cell monolayers and cells were treated with PEG, there were approximately 60 to 80% the numbers of plaques produced by TR/H9004 and AD169 compared with wild-type TR, similar to the entry observed in high-multiplicity-of-infection experiments. However, spread of TR/H9004 to adjacent epithelial cells was never observed; only single infected cells that expressed both IE86 and gB were detected (Fig. 6). By comparison, TR spread to approximately 5 to 10 cells during 21 days. As on fibroblasts, gB was detected primarily in ARPE-19 cells toward the center of the plaques, indicating outward virus spread. AD169 also largely failed to spread to adjacent epithelial cells in these experiments. This was somewhat different from earlier results in which single infected cells and small foci were observed with AD169 on ARPE-19 cells (22). In these previous experiments, the cells were pretreated with neuraminidase, which may have promoted plaque formation and spread. Alternatively, AD169 may be intermediate between TR and TR/H9004. However, comparison of TR and TR/H9004 suggests that wild-type HCMV relies on one or more of the UL128-to-UL150 genes for cell-to-cell spread between cultured epithelial cells.

**DISCUSSION**

The ability of HCMV to replicate and spread in diverse cell types and tissues is important in the pathology and epidemiology of HCMV diseases. However, knowledge of the mechanisms by which HCMV infects and replicates in several relevant cell types is lacking. This is largely due to the fact that commonly studied laboratory strains of HCMV (e.g., AD169 and Towne) do not efficiently infect epithelial cells, endothelial cells, or monocytes/macrophages. Furthermore, many of the HCMV mutants available were derived from laboratory strains, adding to the difficulty of studying infection of these relevant cell...
types. The limited host cell range of AD169 and other labora-
tory strains is apparently due to a subset of the genetic muta-
tions that have accumulated during extensive propagation on
fibroblasts. Recently, certain clinical strains (including TR and
FIX) have been frozen as BAC clones, and these viruses retain
the capacity to infect endothelial and epithelial cells, as well as
monocytes/macrophages (19, 35; D. Streblow and J. Nelson,
unpublished results). There is obviously considerable interest
in identifying the gene or genes that differ between laboratory
and clinical strains and which mediate infection of these cell
types. The genes in the UL128-to-UL150 region have been the
focus of several studies of HCMV infection of endothelial and

FIG. 6. Plaque formation by HCMV on fibroblasts and epithelial cells. Fibroblasts or ARPE-19 epithelial cells were infected with HCMV strain
TR, AD169, or TRΔ4 at approximately 20 PFU/well, and the cells and virus were centrifuged at 800 × g for 2 h at room temperature. The epithelial
cells were then treated briefly with 44% PEG. All cultures were incubated at 37°C in the presence of neutralizing antibody for 21 days, fixed, and
analyzed by immunofluorescence for HCMV IE86 protein (red) and gB (green). Representative fields are shown. The magnification of the panels
showing epithelial cells was three times greater than that for fibroblasts.
epithelial function cells, but molecular mechanisms of how these viral
genome function have, by and large, not yet been reported.

Here we focused on early stages in HCMV replication in
epithelial and endothelial cells and investigated the role of the
UL128-to-UL150 genes in adsorption and entry into these
cells. Our experiments showed that the UL128-to-UL150 mu-
tant, TRΔ4, adsorbed onto epithelial cells poorly compared to
wild-type TR. Furthermore, at relatively high multiplicities of
infection, TRΔ4 infected less than 2% of epithelial cells (as
determined by IE protein expression), whereas wild-type TR
infected over 90% of these cells. This is consistent with our
previous observations that AD169 infected epithelial cells
poorly and that infection was somewhat enhanced by centrifu-
gation and neuraminidase treatment (22). Here we showed that
when virus entry into epithelial cells was “forced” with the
chemical fusogen PEG, TRΔ4 and AD169 infected up to 70%
of cells, leading to IE and gB expression. These observations
demonstrated that there was sufficient TRΔ4 adsorbed onto
cells that most cells expressed the IE gene after PEG treat-
ment. Moreover, the results showed that the UL128- to-UL150
genes are required for entry of HCMV into epithelial cells at
a stage subsequent to adsorption. Once entry occurred through
PEG-induced fusion, the HCMV UL128-to-UL150 mutant
had the capacity to undergo postentry processes: i.e., capsid
disassembly, translocation to the nucleus, and gene expression.
However, in what could be misinterpreted as confounding ob-
servations, radiolabeled TRΔ4 and AD169 were internalized
into epithelial cells as efficiently as TR. The simplest explana-
tion for this is that HCMV is taken up by endocytosis and that
productive entry occurs by fusion of the virion envelope with
endosomal membranes. Thus, in the case of TRΔ4 and AD169,
substantial amounts of virus were taken up by cells, as mea-
sured by the internalization assay, yet there was a failure of
virus to enter cells presumably by fusion with endosomes. Data
shown in Fig. 4 indicate that endocytosis is not complete since
substantial amounts of radiolabeled virus remain on the cell
surface even after 3 h. Therefore, treatment with PEG would
be expected to overcome defects in endocytosis or fusion with
endosomal membranes by forcing entry of those HCMV par-
ticles that remain on the cell surface.

We also observed that drugs that raise endosomal pH
(NH4Cl, chloroquine, or bafilomycin) inhibited infection of
epithelial cells by wild-type HCMV. Together, these results
strongly support the hypothesis that HCMV enters epithelial
cells by endocytosis and low-pH-dependent fusion with endo-
somal membranes. It is possible, although unlikely, that
HCMV entry into epithelial cells can also involve fusion with
the plasma membrane. In this case, the internalization assay
might follow uptake of a subpopulation of virus particles into
a nonproductive pathway. However, this is unlikely given the
dependence of HCMV entry on low pH. These results argue
that one or more UL128-to-UL150 genes promote entry at
dendosomes. One likely candidate is UL131, which was recently
shown to increase infectivity of AD169 in ARPE-19 epithelial
cells (60).

It is possible that AD169 and TRΔ4 possess mutations that
reduce virus replication in epithelial cells at stages subsequent
to IE and gB expression. It might be possible to address this by
measuring infectious virus yield after forcing HCMV into ep-
ithelial cells with PEG. However, this approach has a signifi-
cant drawback. The percentage of cells infected by TRΔ4 after
PEG treatment was 60% to 70%, compared with 98% for TR.
Therefore, cells would not be synchronously infected and com-
parison of virus yield would be difficult. It is also possible that
the construction of TRΔ4 produced secondary mutations out-
side of the UL128-to-UL150 region, and one could imagine that
these secondary mutations could affect entry. However, AD169 (which also lacks the UL128-to-UL150 genes) behaved
similarly or identically in entering epithelial cells; PEG in-
creased IE expression by a similar amount. It is difficult to
imagine that TRΔ4, which was constructed by using the high-
fidelity BAC system, could have acquired identical secondary
mutations (outside the UL128-to-UL150 region) as are present
in AD169. Our principal conclusion involving TRΔ4 was re-
stricted to the notion that the UL128-to-UL150 genes play a
role in entry. There may be other differences between AD169
and TRΔ4, but this conclusion is well substantiated without a
rescued version of the UL128-to-UL150 mutant by the similar
phenotype of AD169 and reversal by PEG experiments.

Endothelial cells were also efficiently infected by HCMV TR
but not by AD169 or TRΔ4. In contrast to the results with
epithelial cells, PEG treatment did not substantially increase
the number of endothelial cells infected by AD169 or TRΔ4.
Here again, radiolabeled particles of all three strains were
internalized with similar efficiencies. These results, coupled
with the observations that infection of endothelial cells by TR
was inhibited by NH4Cl, bafilomycin, and chloroquine, support
the hypothesis that HCMV enters endothelial cells through
endocytosis followed by fusion with endosomal membranes.
Furthermore, since PEG did not substantially increase infec-
tion by TRΔ4 or AD169, it is likely that HCMV UL128-to-
UL150 genes are necessary for some stage of endothelial cell
infection subsequent to entry but before gene expression.
These postentry steps may include translocation of the nucleo-
capsid to the nucleus, capsid disassembly, release of the ge-
nome across the nuclear pore, or immediate-early transcrip-
tion and translation. Our results are consistent with previous
reports that AD169 and other nonendotheliotropic HCMV
strains accumulate in the cytoplasm of endothelial cells and
that viral DNA is poorly translocated into the nucleus (52, 53).
The observed accumulation of virion components in the cyto-
plasm (52) could relate to a lack of fusion with endosomal
membranes, so that enveloped virions build up in endosomal
compartments, rather than in the cytoplasm. Our observations
that PEG did not promote infection of endothelial cells by
TRΔ4 or AD169 suggest that some step subsequent to entry is
also blocked, although it is likely that there are also defects in
entry into these cells. UL128-to-UL150 mutants may have mul-
tiple defects in initiating infection in endothelial cells. The
UL130 and UL132 proteins are virion components (40, 56, 59)
and, along with UL131, are good candidates for proteins that
might promote entry or an early replication step in endothelial
cells. Experiments are in progress to identify the specific
UL128-to-UL150 proteins that facilitate infection of endothe-
リアル cells.

Endocytosis of HCMV particles by endothelial and epithe-
lial cells was previously observed by electron microscopy (3).
However, as with many electron microscopy experiments, the
biologic relevance of these observations was not clear, espe-
sically since the particle/PFU ratio of AD169 has been esti-
mated to be as high as 490 (1). Moreover, these authors reported that treating the cells with NH4Cl or chloroquine had only marginal effects on the number of cells infected (3). However, since they also observed inefficient infection of endothelial and epithelial cells by AD169, even in the absence of drug, the observed entry may not reflect the major pathway of HCMV entry.

Our conclusions that HCMV enters epithelial and endothelial cells by endocytosis followed by low-pH-dependent fusion, combined with earlier observations that HCMV enters fibroblasts by plasma membrane fusion (11), fit well with observations involving gamma- and alphaherpesviruses. Miller and Hutt-Fletcher showed that EBV enters normal B cells by endocytosis and pH-independent fusion, but infects Raji-transformed B cells and epithelial cells by direct fusion with the plasma membrane (33). Similarly, HSV can enter different cells by (i) direct fusion with the plasma membrane, (ii) endocytosis and low-pH-dependent fusion, or (iii) endocytosis and pH-independent fusion (34, 36, 37, 63). Thus, herpesviruses have adapted to different cells through a variety of different entry pathways. For EBV, there is good evidence that different envelope glycoproteins can function in entry into different cell types (4). This may also be the case for HCMV, a virus that infects many different cell types and that has more coding capacity to dedicate to different host cells. However, our knowledge of HCMV entry is still relatively rudimentary when compared with that of alpha- or gammaherpesviruses because there are few viral mutants.

Efficient cell-to-cell transmission of HCMV is likely important to allow virus spread in solid tissues, dissemination to other tissues, and avoidance of the host immune system. Herpesviruses that fail to enter also fail to spread. This is appreciated to be as high as 490 (1). Moreover, these authors reported that treating the cells with NH4Cl or chloroquine had only marginal effects on the number of cells infected (3). However, since they also observed inefficient infection of endothelial and epithelial cells by AD169, even in the absence of drug, the observed entry may not reflect the major pathway of HCMV entry.

In summary, we have shown that HCMV enters epithelial and endothelial cells by endocytosis followed by low-pH-dependent fusion with endosomes. HCMV UL128-to-UL150 mutants infected epithelial cells poorly, although virions were internalized, suggesting that one or more of the UL128-to-UL150 genes are required for fusion with endosomal membranes. With endothelial cells, PEG did not overcome the deletion of UL128-to-UL150 genes, supporting the hypothesis of other defects at postentry stages of early replication.

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