Human Cytomegalovirus Strain Toledo Lacks a Virus-Encoded Tropism Factor Required for Infection of Aortic Endothelial Cells

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Human cytomegalovirus (HCMV) strains were investigated to identify those with altered tropism for endothelial cells. In viral replication kinetics analysis, HCMV strain Toledo replicated poorly in aortic endothelial cells (AECs), and the virus count was reduced by 2–3 log units, in comparison with strain AD169. Virus entry at the cell surface for each strain was equivalent. However, immunofluorescence studies revealed a lack of immediate early viral antigen 72 expression, and direct blot hybridization failed to detect viral genomes in the nucleus of Toledo-infected AECs. Complementation assays were done to determine whether endothelial cell infectivity was dependent on a virus-encoded tropism factor. Pseudotype virus in endothelial cell cultures indicated that AD169 could provide trans factors to rescue Toledo during infection of endothelial cells. Collectively, these results show that a viral function provides an endothelial cell tropism factor for HCMV and plays a role during postentry infection events.

Human cytomegalovirus (HCMV) is the leading cause of congenital infection in the Western Hemisphere and is a primary cause of morbidity in posttransplant and human immunodeficiency virus–infected persons. New results link HCMV infection to chronic vascular diseases, including transplant vasculopathy, restenosis, and atherosclerosis. Most recent studies have found a strong association between HCMV infection and posttransplant vasculopathy [1–8], an accelerated form of coronary artery disease seen in heart transplant patients, but others have not [9]. In one other report, the correlation was observed only when HCMV viremia was persistent [10]. Of interest, a prospective study involving patients treated with gancyclovir showed a lower incidence of posttransplant coronary artery disease in the absence of calcium blockers [11].

Even though seroepidemiologic studies linking HCMV infections to arterial restenosis after angioplasty remain controversial [12–16], a mechanistic study convincingly demonstrated an association between HCMV and restenosis [17]. That report correlated the presence of HCMV immediate early antigen (IEA) 2 in smooth muscle cells to high levels of p53 protein among biopsy specimens from restenotic lesions, leading to the hypothesis that neointimal lesions in restenosis are driven by blocking of p53 inhibition of cell cycle progression by HCMV IEA2. Additional evidence that mechanistically linked HCMV to neointimal hyperplasia was found in vitro studies, which showed that the viral US28 gene product (a chemokine receptor homologue) mediates smooth muscle cell migration in the presence of cysteine-cysteine chemokines [18].

Appreciation of atherosclerosis as an inflammatory disease has fostered interest in the role of infections in its etiology. A role in the atherosclerotic process has been proposed for various microbial pathogens, including HCMV [19–21]. However, the extent to which HCMV may be involved [22, 23] is not clear. It is conceivable that HCMV infection may elicit a subclinical inflammatory response in some seropositive persons who eventually become susceptible to the atherogenic effects of the virus [24]. This may also help to explain the disparate results achieved by groups working on the involvement of HCMV infection in atherogenesis.

Because the endothelium is located between the circulatory components and underlying tissues, recent work has focused on the role of endothelial cell dysfunction as an underlying mechanism in the development of vascular lesions. Endothelial cells are, in addition, natural sites of HCMV infection. Although the process of viral dissemination and spread in infection is still largely undescribed, endothelial cells are considered to play an important part [25–27]. Virus may be disseminated from the endothelial cell layer during acute disease [25, 26] or may serve as a potential reservoir for infection of smooth muscle cells in arterial walls. The viral factors necessary for endothelial cell tropism have yet to be identified. In our study, virus strains were screened for altered endothelial cell replication to examine the basis of this tropism. To study aspects relevant to pathogenesis of both acute HCMV infection and chronic vascular diseases, analyses focused on primary aortic endothelial cells (AECs).
Materials and Methods

Cell types and virus strains. Primary human AECs were purchased from Clonetics and cultured in medium purchased from the same manufacturer. Each cell lot was from a single donor. All experiments were repeated for ≥2 different donors. Low-passage AECs were used with <5 doublings. We used heparin-free media in the course of the experiments, beginning 2 doubling periods before time of infection, and throughout infection assays. Supernatants from HCMV-infected primary human embryonic lung fibroblasts (HELFs) were used as the inoculum source for infection studies. Virus titers were determined by plaque assays on primary HELFs [28]. HELFs were cultured in Iscove’s modified Dulbecco’s medium (IMDM) with 5% fetal calf serum (FCS). HCMV AD169, originally obtained from American Type Culture Collection stocks in 1986 (passage ≥200), and HCMV Toledo passages 8–12, originally from S. Plotkin (Aventis Pasteur, Doylestown, PA), were a gift of E. Mocarski (Stanford University, Stanford, CA). A recombinant green fluorescent protein (GFP)–expressing Toledo virus, derived from the same Toledo strain donated to this study by E. Mocarski, was provided by J. Vieira (Fred Hutchinson Cancer Research Center, Seattle). This recombinant virus, originally called “HV5.111,” contains a cassette insertion at an intergenic location between US9 and US10 [29]. The cassette contains the GFP expressed under control of the elongation factor 1–α promoter and selectable markers. The Powers (Po) strain, a low-passage HCMV clinical isolate, was provided by J. Nelson (Oregon Health Science Center, Portland) [30]. VH/E (endotheliotropic strain) and VH/Fb (non-endotheliotropic strain) were derived from a HCMV clinical isolate and were provided by J. Waldman (Ohio State University, Columbus) [31].

For preparation of monocyte-derived macrophages (MDMs), peripheral blood mononuclear cells were isolated from peripheral blood and adhered to plastic tissue culture dishes for 15 min in 1× PBS containing calcium and magnesium (Gibco BRL Life Technologies) [32]. Nonadherent cells were removed. The remaining adherent cell population was cultured in 1× IMDM with 10% FCS (Chemicon) just before infection.

Replication kinetics analyses. For single-step replication kinetics analysis, 5 × 10^4 primary HELFs or primary AECs were seeded into each well of 24-well culture plates (Corning). After 2 days, cells were infected with HCMV AD169 or HCMV Toledo. Input inoculum was based on plaque assay titrations and is shown as time point 0 in figures 1 and 2. For each sample, we collected the supernatant and monolayer fraction together from each cell culture well and stored them at −80°C. Samples were freeze-thawed 3 times and then subjected to plaque assay on primary HELFs [28]. Replicate experiments that examined triplicate samples were analyzed for each time point with each virus unless otherwise noted (figure 1). For multistep replication kinetics analysis, the same procedures were followed, except that cells were infected at an MOI of 0.05.

Indirect immunofluorescence (IF) detection. Cells were grown in chamber slides, infected with virus at an MOI of 3, and adsorbed for 3 h at 4°C. Inoculum was removed, samples were washed, and fresh media were placed on cells. Cells were fixed with acetone/methanol (1:1) for 10 min at −20°C. Fixed cells were rehydrated in IF buffer (25 mM HEPES, pH 8.2, with 0.9% NaCl) for 5 min at ambient temperature and blocked in IF buffer containing 2.5% rabbit serum, 2.5% newborn calf serum, 1% bovine serum albumin (fraction V; Sigma), and 50 μg/mL mouse IgG (Sigma). Primary antibodies were diluted in blocking solution without mouse IgG and consisted of HCMV IEA72 monoclonal antibody (Mab) 8131 (Chemicon International), used at 1:200 dilution; RGI#1204 (Rumbaugh-Goodwin Institute for Cancer Research) antibody directed against the UL57 early viral product, used at 1:2000 dilution; Mab 8128 (Chemicon) directed against pp65, used at 1:200 dilution; and Mab 8125 (Chemicon) directed against an HCMV late antigen (LA), used at 1:200 dilution. The secondary antibody used was rabbit anti–mouse IgG conjugated with fluorescein isothiocyanate (Dako) and used at a dilution of 1:30. Incubations were done for 45 min at ambient temperature, and washes used IF buffer with 0.1% Tween 20. We used Pathfinder mounting media (Kallestad) to enhance IF signal and inhibit IF quenching during visualization. Slides were visualized immediately with a Zeiss Axiovert 135 inverted microscope at a wavelength of 490 nm. To determine the percentage of viral antigen–positive cells in the cultures in the quantitative IF assays, the number of antigen-positive cells per microscopic field was divided by the total number of cells per field. Percentages from 5 fields were averaged, with a minimum of 200 cells per field.

Nuclear entry assays. Duplicate sets of AEC and HELF cultures were infected as described in the subsection “Indirect IF detection.” For each set of infections, nuclei were collected from one group after the adsorption period at 4°C to use as a control for viral DNA contamination in the sample preparation process. For the other group, the infected cells were placed at 37°C after adsorption at 4°C. Nuclei were isolated from cells by use of a modification of a nontonic detergent lysis method [33] and then sepa-
Figure 2. Viral replication kinetics analysis of human cytomegalovirus (HCMV) AD169 and HCMV Toledo. For single-step kinetics analysis, aortic endothelial cells (AECs; A) and human embryonic lung fibroblasts (HELFs; B) were infected at an MOI of 3. Over the course of 10 days, samples were collected and examined for levels of virus produced, using a plaque assay on permissive fibroblast cultures. For multistep viral replication kinetics analysis, AECs (C) and HELFs (D) were infected at an MOI of 0.05. Over the course of 20 days, samples were collected and assayed as described for A and B. Detection limits for each assay were 3 pfu/mL. Error bars indicate SEM. HCMV AD169 (○; dashed line) and HCMV Toledo (■; solid line) are shown in all graphs.

rated from cytoplasmic proteins on 30% iodyxanol gradients (Gibco BRL Life Technologies). Total DNA was prepared from each sample of nuclei using an SDS lysis buffer (1% SDS in Tris-EDTA buffer), followed by digestion with proteinase K, extraction (using an organic phenol-chloroform), and ethanol precipitation. We used direct blot hybridization to assess the detectable viral DNA levels associated with each sample. Gels for Southern blot analysis were loaded with equivalent amounts of total DNA prepared from each sample after digestion with HindIII [34]. Probe was prepared by random prime labeling [34] of a cloned IEA1 sequence (clone pON 2347; sites HindIII to XbaI) [33].

Western blot analysis of infected monolayers. AECs and HELFs were infected with AD169 or Toledo at an MOI of 20 in 24-well plates. All infections were done with centrifugal enhancement (1800 g for 15 min) at 4°C and held for another hour at that temperature. Infections were made with and without 50 μg/mL of heparin. Plates were then brought to 37°C and maintained at that temperature for 6 h. Cell monolayers were washed 6 times with PBS, lifted with trypsin, washed, and pelleted. Pellets were resuspended in 50 μL of 1× loading buffer [35]. Protein concentrations for each infected cell sample were determined by the Bradford assay (BioRad), and equivalent amounts of total protein were added to wells and resolved on 12.5% SDS–discontinuous PAGE gels, as described elsewhere [35], with the following alterations: Samples were sonicated for 3–5 s to eliminate viscosity (not boiled), and blots were reacted with MAb directed against pp65 (RGI#1205-S; Rumbaugh-Goodwin Institute for Cancer Research) at a dilution of 1:1500. Secondary antibody (Vectastain ABC kit; Vector Laboratories) and colorimetric detection methods (3,3′-diaminobenzidine peroxidase substrate kit; Vector Laboratories) were used in accordance with manufacturer’s directions.

Confocal microscopy. Cells were grown in 24-well plates and infected as described in the subsection “Indirect IF detection” at an MOI of 3. They were trypsinized, washed twice, and spotted onto slides. After drying, cells were fixed and stained in a manner similar to that used for indirect IF detection, except that we substituted goat serum for rabbit serum in the blocking solution and the diluent. We used supernatant from hybridoma cell culture (1:2 dilution), containing mouse antibody directed against the HCMV small capsid protein (provided by W. Britt, University of Alabama, Birmingham) [36]. The secondary antibody was goat anti–mouse AlexaFluor 488 (Molecular Probes) used at 1:250 dilution. Images were collected by a BioRad MRC 1024 confocal microscope with T1 and T2 dichroics, an excitation laser line of 488 nm, and a 522DF32 emission filter.

Complementation assays with pseudotype viruses. Coinfection of fibroblast cultures was done using GFP-Toledo and AD169 viruses, each at an MOI of 3. On postinfection (pi) day 5, progeny virus in supernatants from the first round of replication was used to infect AECs. Progeny viruses were a mixed population of individual parental viruses and complemented viruses in which either parental viral genome was packaged with a mixed range of structural proteins derived from GFP-Toledo or AD169 viruses. It is well established that genetic recombinants occur less frequently than structurally complemented pseudotype viruses after dual-virus infection of permissive cells. Pseudotype virus was used at an MOI of 3, based on GFP-positive plaques, to infect AECs. In parallel,
AECs were infected with GFP-Toledo or AD169 alone at an MOI of 3. Cultures were examined on pi day 5 for evidence of Toledo replication by titration of supernatants from AECs on HELFs (ratio of GFP-positive plaques to clear plaques) and for visible plaque formation on AECs.

Analysis of Toledo virus infection efficiency after pseudotype infection of AECs was undertaken using DNA from purified nuclei, Southern blot, and probing. We used a Toledo-specific probe directed against the UL141 open-reading frame [37]. AECs were infected in 6-well plates with pseudotype virus, AD169, or GFP-Toledo alone, with an MOI of 5. After 7 days of infection, AEC nuclei were harvested from cell monolayers (as described in the subsection “Nuclear entry assays”) for nuclear entry assays. Gels for Southern blot analysis were loaded with equal amounts of total DNA after digestion with HindIII. A Toledo-specific probe was prepared by random prime labeling [34] of an HCMV UL141 [37] PCR product. UL141 is present in Toledo but absent in AD169 viruses. The PCR used the forward and reverse primers ATCCTAGGACTAGTTCTTAAACATCCTGGGAGATAGA and GAAGATCTATTATATTTAATCTTTGCTTGAACAGGGGTA and Platinum Pfx DNA polymerase (Invitrogen Life Technologies), according to the manufacturer’s instructions, using buffers supplied with the enzyme. Template was 100 ng of DNA from Toledo-infected HELFs.

Limiting dilutions were done on supernatants from AECs after pseudotype virus infection, so that individual plaques could be isolated and genotyped. AEC cultures were infected at an MOI of 5 with pseudotype virus. On pi day 7, the supernatants from AEC infections were collected and titered. Limiting dilutions were set up on permissive fibroblast monolayers. Infections from culture wells containing only single plaques were selected in 13 separate samples. DNA from plaque-purified viruses was digested with HindIII, loaded onto an agarose gel, Southern blotted, and probed with Toledo-specific UL141 probe.

Results

**Significant restriction of HCMV Toledo in AECs.** HCMV strains were screened for altered endothelial cell tropism by comparison of replication efficiencies in primary AEC cultures at an MOI of 0.1 (figure 1). The replication kinetics profiles revealed 2 patterns of viral growth late after infection (~18 days), without significant loss of cells in the monolayers. These were represented by viruses that were replication competent in AECs (Po, AD169, or VH/E) and by those that were not (Toledo, GFP-Toledo, and VH/Fb), as shown in figure 1. We selected a virus strain from each group (AD169 and Toledo) for further study.

Single-step viral replication kinetics analysis showed that HCMV AD169 could replicate to significant levels in AEC cultures (figure 2A). However, HCMV Toledo replication was reduced by as much as 1000-fold, compared with that of AD169, by pi day 10. Although an initial peak of viral replication was detected for Toledo at pi day 5, these levels had decreased 100-fold by pi day 10. Toledo strain did not replicate to any detectable levels under low-multiplicity conditions (MOI, 0.05) in the AEC cultures, unlike AD169, for which levels of 1000 pfu/mL were attained consistently by pi day 10 (figure 2C). Overall, these results show there was significant replication of HCMV AD169 in AECs, whereas replication of the Toledo strain was dramatically restricted. During replication kinetics analysis, no plaques or significant cell loss was observed in AEC culture, even under low MOI conditions maintained for extended periods of time. Both viruses replicated equivalently in primary fibroblast cultures under the same MOI conditions (figure 2B and 2D).

**Restriction of HCMV Toledo infection in AECs before IEA expression.** We used quantitative indirect IF assay (QIFA) to examine the frequency of infected cells and the distribution of viral IEA, early antigen (EA), and LA in infected AECs. By ≥3 days after infection, 80% of the AD169-infected endothelial cells expressed IEA, compared with only 2%–6% of endothelial cells infected with HCMV Toledo (figure 3A). By pi day 7, ~80% of AD169-infected AECs expressed EA, and, by pi day 10, ~80% showed LA (figure 3B, 3C, and 3G). Again, <3%–7% of Toledo-infected AECs expressed EA or LA (figure 3B, 3C, and 3G). Levels of viral antigen were equivalent for AD169 and Toledo within infected fibroblasts and were expressed in 80%–90% of the cells for IEA, EA, and LA by pi days 1, 2–3, and 3, respectively (figure 3D–3F).

QIFA results were consistent with those of replication kinetics analysis. The majority of AD169-infected endothelial cells were positive for each viral antigen class (figure 3), and expression of viral antigens in all temporal classes was restricted in HCMV Toledo–infected cells. Both strains had equivalent levels of expression of viral antigens in all temporal classes in fibroblast cultures. In addition, AD169 and Toledo infection in another permissive cell type, MDMs, also demonstrated equivalent numbers of IEA-positive cells 24 h after infection (data not shown). No plaques were observed for either AD169 or Toledo infections on endothelial cells. In the Toledo- and AD169-infected endothelial cell cultures, the cell monolayers were still intact, with no visible cell loss, and were clearly demonstrated to be viable by trypan blue exclusion assays (data not shown).

**Failure of direct blot hybridization to detect HCMV Toledo genome in AEC nuclei.** Because the QIFA studies revealed a lack of IEA expression in Toledo-infected AECs, experiments were done to examine the efficiency of viral genome entry into the host cell nucleus. By Southern blot hybridization, viral DNA prepared from nuclei of AECs infected with AD169 and collected 20 h after infection showed positive signal (figure 4A, lane 1). DNA prepared from nuclei of a parallel AD169 infection done at 4°C lacked signal after Southern blot hybridization (figure 4A, lane 3). This confirmed that the detection in lane 1 resulted from viral DNA reaching the nucleus and not from contamination of viral DNA associated with plasma membranes during isolation of the nuclei. Toledo-infected AECs showed absence of viral DNA signal in the cell nuclei at 37°C and 4°C (figure 4A, lanes 2 and 4, respectively). These results were consistent with those of a second blot, in which the same conditions were used, except that infection was done in the
Figure 3. Quantitative immunofluorescence comparison of expression of human cytomegalovirus antigens over time during infection of aortic endothelial cell (A–C and G) and human embryonic lung fibroblast cultures (D–F). Infections were done at an MOI of 3. Immunofluorescence demonstrated AD169 (○; dashed line) and Toledo (■; solid line) infection, examined using antibody to viral IEA72 (A and D), antibody to UL57 early antigen (B and E) or pp65 early antigen (G), and antibody to late antigen (C and F). Y-axis shows percentage of positive fluorescence. IEA, immediate early antigen; LA, late antigen.

presence of phosphonoacetic acid (300 μg/mL media) to block viral DNA replication, and samples of nuclei were collected 36 h after infection (data not shown). Fibroblast control samples harvested after infection at 37°C showed equivalent levels of viral DNA signal for both viruses (figure 4B, lanes 1–4). Fibroblasts infected at 4°C with either virus (figure 4B, lanes 5 and 6) showed no detectable viral genome in the host cell nuclei [38]. Equal amounts of total DNA were loaded in all cases.

Equivalence of virus entry at the host cell surface for Toledo and AD169. To determine whether virus entry events at the cell surface in AECs were involved in the restriction of Toledo replication, we compared AD169 and Toledo strains, using
Comparison of human cytomegalovirus (HCMV) strains Toledo and AD169 host cell nuclear entry. Aortic endothelial cells (AECs) and human embryonic lung fibroblasts (HELFs) were infected with either HCMV Toledo or HCMV AD169 at an equivalent MOI of 3. DNA was prepared from nuclei and digested with HindIII, and gels were loaded with equivalent amounts of total DNA for Southern blot analysis. A. Samples of nuclei from infected AECs. Lane 1, AD169-infected AECs; lane 2, Toledo-infected AECs; lane 3, AECs infected with AD169 and held at 4°C; lane 4, AECs infected with Toledo and held at 4°C. B. Samples of nuclei from infected HELFs. Lane M, marker (cross-hybridization with 2-kbp marker band routinely seen); lanes 1 and 2, AD169-infected HELFs; lanes 3 and 4, Toledo-infected HELFs; lane 5, HELFs infected with AD169 and held at 4°C; lane 6, HELFs infected with Toledo and held at 4°C.

Western blot detection for pp65, a virion particle-associated protein. The results (figure 5A) showed equivalent detection of pp65 in AECs for Toledo (lanes 2) and AD169 (lane 4) infections. Heparin-treated control samples [38] for each virus (figure 5A, lanes 1 and 3) had no detectable signal. Fibroblasts infected with Toledo in the absence of heparin (figure 5B, lane 1) or with AD169 in the absence of heparin (figure 5B, lane 3) also showed equal amounts of pp65 for both viruses. Heparin-treated control samples for each virus in fibroblasts (figure 5B, lanes 2 and 4, respectively) showed no pp65. Overall, these results suggested that virus entry at the host cell surface in AECs was equivalent for the 2 strains.

To further support these findings, we used confocal microscopy to monitor internalization of virus, 6 h after infection at 37°C, with an MAb directed against the small virion capsid protein. As shown in figure 6, there was internalized signal for both AD169 and Toledo virus strains in AECs and in fibroblasts incubated at 37°C. To assess background levels of surface-bound material, AECs were infected and incubated at 4°C for 6 h. IF signals were rare in the infected samples held at 4°C, and uninfected negative control samples held at 4°C showed low detectable signal, primarily in AECs (figure 6).

AD169 supports replication of Toledo with trans factors during infection of AECs. We used complementation approaches with pseudotype virus infections of AECs to test whether endothelial cell infectivity was dependent on the presence of a virus-encoded tropism factor. Pseudotype viruses from this first round of replication on permissive fibroblast cells were used to infect AECs. As seen in figure 7, pseudotype virus infections demonstrated the presence of plaques (30–60 cells/plaque) on the endothelial cell monolayers; individual parental virus infections showed no plaques. Because GFP signal in AECs was too low to be detected, supernatants from infected AECs were examined by titration on HELFs, where GFP expression was easily detected. The lack of detectable GFP signal in AECs may be due to variations in the strength or efficiency of the reporter gene’s promoter expression in AECs. On average, 64% of total plaques (range, 60%–68% in 3 separate experiments, with a total of 4 replicates) from these culture supernatants were GFP positive. In addition, titers of GFP-Toledo virus recovered from pseudotype infections were ~2 logs higher than those seen for GFP-Toledo infection alone in AECs in 2 separate experiments. These results support improved infection efficiency in AECs for the complemented Toledo.

AD169 spreads very slowly in AEC cultures. Using the antibodies listed in the subsection “Indirect IF detection” in Materials and Methods to detect IEA72 and LA, AD169-infected AECs showed 2–3 positive cells per focus by pi day 7 and 12–20 positive cells per focus by pi day 21 (data not shown). Despite the finding of IF foci, plaques were never observed by phase-contrast microscopy, even 21 days after AD169 infection (data not shown). Toledo infection never showed plaques by phase contrast or foci by IF detection. Overall, the dramatic plaque phenotype observed by phase contrast and the predominant GFP-expressing virus seen in supernatants demonstrated that AD169 provided trans factors to rescue the infection efficiency of Toledo in AECs.

To further assess these findings, we used pseudotype virus,
Figure 6. Confocal microscopic detection of human cytomegalovirus (HCMV) small capsid protein. Human embryonic lung fibroblasts (HELFs) and aortic endothelial cells (AECs) were infected, at an MOI of 3, with HCMV Toledo and HCMV AD169 strains. Representative cells were imaged at oil magnification ×60 with 4.0 zoom; a 5-μM bar is shown in Toledo-infected samples. AlexaFluor 488 (Molecular Probes) signal clearly detected the presence of small capsid virion protein in cells infected at 37°C with both virus strains; signal was rarely seen in infected cells held at 4°C. Low background signal was seen in uninfected control samples.

AD169, and Toledo to infect AECs, and nuclei were harvested. Equal amounts of DNA were blotted and probed with a Toledo-specific UL141 probe (figure 8). The greatest level of Toledo-specific signal was observed in the pseudotype virus–infected cells (figure 8A, lane 2). After prolonged exposure of the same blot, faint positive signal was also seen in the Toledo-infected cells (figure 8B). Uninfected samples and AD169-infected samples (figure 8B, lanes 1 and 3, respectively) showed no signal, in contrast to what would be expected for the Toledo-specific UL141 probe [37].

Finally, to confirm that the GFP-Toledo derived from the pseudotype infections on AECs was actually complemented GFP-Toledo and not a recombinant virus, we analyzed 13 plaque-purified viruses from pseudotype infection of AECs. The Toledo-specific UL141 probe showed that 8 of 13 viruses hybridized specifically to the UL/b′ region probe with a band of similar mobility to that of a Toledo control sample. The same isolates also expressed GFP during infection of fibroblasts.

Discussion

A dramatic difference in viral replication efficiency was observed between HCMV strains AD169 and Toledo during infection of primary AEC cultures. The plaque-purified early passage Toledo strain replicated poorly in AECs and was restricted at the very earliest stages of replication. Generalized defects in viral replication can be ruled out as an explanation for the differences in infection efficiency of HCMV Toledo and AD169, because both strains replicated equivalently in HELFs. These virus strains also showed similar infectivity rates, measured by IEA72 expression, during infection of MDM cultures. Although an initial low level of viral replication was detected for Toledo, with a peak 5 days after infection, these levels had decreased 100-fold by pi day 10. This replication pattern is consistent with the infection and subsequent loss of a minor percentage of permissive cells in the cultures. The failure to detect viral replication in low MOI multistep kinetics analysis also supports this conclusion. The AEC supplier (Clonetics) confirmed that the cultures contained ≤1% nonendothelial cells (e.g., smooth muscle cells), which are permissive for HCMV replication [39].

Another report described an endothelial cell–adapted Toledo strain virus (Toledo E) that was capable of replicating in human venous endothelial cells derived from umbilical cord (HUVEC). However, the Toledo strain that we worked with clearly dem-
Figure 7. Complementation assay using pseudotype virus. Permissive fibroblast cultures were coinfectected with green fluorescent protein (GFP)-expressing Toledo and AD169 viruses, each at an MOI of 3. On day 5 after infection, supernatant viruses (pseudotype virus) from human embryonic lung fibroblasts were used to infect aortic endothelial cells (AECs) (MOI of 3, based on GFP-Toledo). In parallel, sets of AECs were infected with GFP-Toledo alone or AD169 alone (MOI of 3). By day 5, plaques were clearly visible on pseudotype virus–infected AECs (Pseudotype panel). Magnification ×50.

Figure 8. Southern blot with Toledo-specific probe to detect signal from aortic endothelial cell (AEC) nuclei. A, Eight-hour exposure. B, Seventy-two–hour exposure of blot shown in A. Lane 1, Nuclei from uninfected AECs; lane 2, Nuclei from pseudotype virus–infected AECs; lane 3, nuclei from AD169-infected AECs; lane 4, nuclei from Toledo-infected AECs. Lane 2 shows the predominant signal. A small portion of this signal is seen in B on the far left. B demonstrates that lane 4 shows faint signal after 72 h that was not seen after 8 h of exposure for Toledo-infected AECs. As expected, uninfected and AD169-infected lanes show no signal.

...onstrated a restricted infection phenotype in AECs. Replication ability of Toledo E occurred only after several serial passages in HUVEC culture, a process that may have selected for genetic changes in the virus strain [40]. An alternative explanation for these differences may be that the virus behaves differently in endothelial cells derived from different vascular beds. AECs are adult-derived arterial macrovascular cells. We have not tested our strain of Toledo in HUVEC umbilical venous endothelia.

QIFA results were consistent with productive infection of the majority of the AECs with HCMV AD169. In contrast, a low percentage of cells infected with HCMV Toledo showed detectable IEA72, UL57, pp65 (UL83), or LA when examined by IF only. Virus entry at the host cell surface in AECs was equivalent for the 2 strains, as shown by Western blot and IF microscopy for detection of structural antigens of the virus. This was also reported for endothelial cell–adapted strains of HCMV, in which radiolabeled virus was able to attach and efficiently enter HUVEC [41]. These findings are consistent with other studies, in which HCMV entered a variety of cells of human and nonhuman origin [42].

Examination of host cell nuclear entry showed that the AD169 viral genome could reach the host cell nucleus in AECs but that Toledo was restricted at or before this stage. Although other studies have reported that AD169 virus is unable to enter HUVEC nuclei [43], differences may exist in the AD169 strains examined, because there has been some passage divergence between AD169 in the United States and in Europe [44]. We are in the process of examining AD169 (Cambridge strain; gift of E. Mocarski) for levels of infectivity in the AEC cultures. However, the AD169 used in this report efficiently entered and produced infectious progeny, as also has been observed elsewhere [30].

The characteristic of AD169 that was common to our study and to other studies is its inability to efficiently spread (poor foci formation) in endothelial cell cultures [45, 46]. Although we and others found that strain AD169 does not demonstrate foci formation or significant cell-to-cell spread in endothelial cells in the short term (5–7 days), this strain clearly can undergo the complete viral replication cycle [30]. Multiple viral factors may be required for endothelial cell tropism and may operate in different ways, including infectivity and cell-to-cell spread. None of the AD169-infected AEC cultures showed signs of plaques or detectable cell loss, even by the latest time points in replication kinetics analysis. Significant virus levels were produced from AD169-infected AECs, which was consistent with other studies [30]. A pronounced plateau effect in virus levels produced by AD169-infected AEC cultures after pi day 10 also was found by multistep replication kinetics analysis, suggesting...
that either the virus is unable to spread to new cells after the first round of infection or the overall rate of cell-to-cell spread is very low.

Pseudotype progeny virus derived from coinfection of permissive fibroblasts with both parental virus strains contained a population of complemented Toledo that could replicate aggressively on the endothelial cells, forming IF foci and plaques (as seen by phase-contrast microscopy). Furthermore, infection efficiency was increased for complemented Toledo. Specifically, supernatants from pseudotype virus infections showed a titer for GFP-Toledo that was \( \sim 2 \) log greater than that in supernatants from AECs with GFP-Toledo infection alone. Southern blot analysis of DNA from nuclei showed that pseudotype virus infection resulted in a complemented GFP-Toledo virus that could reach the nucleus in AECs. Finally, genotypic analysis of plaque-purified viruses isolated after pseudotype virus infection of AECs showed that 62% of viruses (8 of 13) had patterns consistent with Toledo genome. Overall, these results show that AD169 can provide virus-encoded trans factors to rescue the replication defect for Toledo in endothelial cells.

Although AD169 can complement Toledo infection in AECs, there are no known genetic regions present in AD169 that are missing in Toledo. In fact, Toledo, which has the UL/b region, has more genetic material than AD169 [37]. Differences may be due to subtle sequence variations that affect gene expression or regulation. An alternative possibility is that a functional viral endothelial tropism factor is expressed by AD169 that may induce specific cellular proteins. These specific host cell proteins themselves may directly complement Toledo. Finally, it is evident that the viral factor we describe is not the ribonucleotide reductase homolog (M45) recently shown to be an endothelial cell tropism factor in murine cytomegalovirus [47]. We observed no difference in cell loss between the competent and defective virus strains in AEC cultures. Furthermore, the prenuclear restriction observed with the Toledo strain is not involved in the phenotype for the mutant M45 gene. In murine endothelial cells, the M45 mutant virus enters the host cell nucleus and expresses IEA and EA genes [47]. Collectively, these observations indicate that a postentry viral function such as cytosol transport, capsid docking at the nuclear pore complex, or translocation of the viral DNA across the nuclear membrane is required for HCMV infection in AECs. Our study establishes a system that can be used to examine the genetic determinants specifically required for HCMV replication efficiency in primary AECs. Genetic variations between HCMV strains have been considered to be the basis for cell or tissue differences in viral tropism and have led to observations of virus strain–specific differences in replication that provide valuable opportunities to map specific viral tropism determinants [48, 49]. Studies are in progress to map the regions of the virus that encode these endothelial cell tropism factors by using cosmid rescue.

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References


