Tropism of human cytomegalovirus for endothelial cells is determined by a post-entry step dependent on efficient translocation to the nucleus

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Marked interstrain differences in the endothelial cell (EC) tropism of human cytomegalovirus (HCMV) isolates have been described. This study aimed to define the step during the replicative cycle of HCMV that determines this phenotype. The infection efficiency of various HCMV strains in EC versus fibroblasts was quantified by immunodetection of immediate early (IE), early and late viral antigens. Adsorption and penetration were analysed by radiolabelled virus binding assays and competitive HCMV-DNA-PCR. The translocation of penetrated viral DNA to the nucleus of infected cells was quantified by competitive HCMV-DNA-PCR in pure nuclear fractions. The intracytoplasmic translocation of capsids that had penetrated was followed by immunostaining of virus particles on a single particle level; this was correlated with the initiation of viral gene expression by simultaneous immunostaining of viral IE antigens. The infectivity of non-endotheliotropic HCMV strains in EC was found to be 100–1000-fold lower when compared to endotheliotropic strains. The manifestation of this phenotype at the level of IE gene expression indicated the importance of initial replication events. Surprisingly, no interstrain differences were detected during virus entry. However, dramatic interstrain differences were found regarding the nuclear translocation of penetrated viral DNA. With nonendotheliotropic strains, the content of viral DNA in the cell nucleus was 100–1000-fold lower in EC when compared to endotheliotropic strains, thereby reflecting the strain differences in IE gene expression. Simultaneous staining of viral particles and viral IE antigen revealed that interstrain differences in the transport of penetrated capsids towards the nucleus of endothelial cells determine the EC tropism of HCMV.

Introduction

A hallmark of human cytomegalovirus (HCMV) is its broad host-cell range during acute infection in vivo, including epithelial cells, fibroblasts, smooth muscle cells, endothelial cells (EC) and macrophages (Ng Bautista & Sedmak, 1995; Roberts et al., 1988, 1989; Sinzger et al., 1993, 1995, 1996; Wiley & Nelson, 1988). EC and macrophages were suggested to play a major role in the haematogeneous dissemination of the virus (Grefte et al., 1995; Ibanez et al., 1991; Lathey & Spector, 1991; Sinzger & Jahn, 1996). In cell culture significant interstrain differences have been described regarding the infectivity of HCMV variants in EC and macrophages (Minton et al., 1994; Sinzger et al., 1999b; Waldman et al., 1989). Interestingly, the non-pathogenic fibroblast-adapted strains like AD169 or Towne were less efficient in these cell types as compared to low passage isolates. Therefore it can be hypothesized that interstrain differences in HCMV infection of these cell types reflect mechanisms that determine virulence in vivo.

To date, little is known about the underlying mechanisms of HCMV interstrain cell tropism variations. It has been suggested that the infectivity of HCMV strains in macrophages might depend on the efficiency of virus entry (Minton et al., 1994). Unfortunately, difficulties in propagating cultured macrophages in vitro limit the availability of these cells for...
more detailed analyses. In contrast, EC can be subpassaged after primary isolation and grown to higher cell numbers, thus making them a more accessible model for investigating HCMV cell tropism. The phenotype of endotheliotropic versus non-endotheliotropic HCMV strains has been well defined (MacCormac & Grundy, 1999; Sinzger et al., 1999b; Waldman et al., 1991). However, there are no reports of interstrain comparative analyses to address the issue of which step of the virus life-cycle is critical for expression of this phenotype. We thus chose to investigate the EC culture model in order to assess the step during virus replication that limits HCMV infection in these cells. Evidence is presented demonstrating that nuclear translocation of virus particles that have penetrated the cell is a critical event that determines EC tropism of HCMV.

**Methods**

**Cells.** Human foreskin fibroblasts (HFF) were cultured in MEM containing 2.4 mmol/l glucose, 100 µg/ml gentamicin and 5% foetal calf serum (MEM5). Fibroblasts were used for experiments between passages 10 and 25. Human umbilical vein endothelial cells (HUVEC) were kindly provided by J. Waldman (Waldman et al., 1991). Strains TB40/E and TB40/F were derived in our laboratory from a bone marrow transplant recipient on EC and fibroblasts, respectively, and were kindly provided by J. Waldman (Waldman et al., 1991). Strain AD169 is a highly passaged fibroblast-adapted laboratory strain of HCMV. Strain KSA16/3 was isolated following co-infection of EC by strain AD169 and a clinical isolate (Sinzger et al., 1999b). For preparation of virus stocks, HFF were infected at an m.o.i. of 0.1. Supernatants of infected cultures were harvested 6 days post-infection (p.i.) and stored at 80°C after removal of cell debris by centrifugation for 10 min at 2800 g. For preparation of purified virions, viral particles were pelleted for 70 min at 80000 g and purified on glycerol–tartrate gradients (Irmiere & Gibson, 1983). The infectious titre in HCMV preparations was determined by TCID<sub>50</sub> assays (Mahy & Kangro, 1996) in fibroblasts on 96-well-plates.

For cell-free infection of cell cultures, medium was removed and replaced by fresh MEM5 60 min prior to infection. Virus preparations were then added for 90 min at 37°C. Subsequently, cells were washed with fresh medium and maintained at 37°C. For virus adsorption, cells were incubated with virus preparations on ice to prevent virus entry. To allow for virus penetration and replication, cells were shifted to 37°C after a 90 min adsorption period. After infection, cells were washed and maintained at 37°C in the appropriate medium. For cell-associated propagation of virus, infected cultures were subpassaged when they had grown to confluence.

For single-step growth curves, HUVEC grown to subconfluence in 75 cm² culture flasks were infected with HCMV preparations as described before at a virus concentration of 10⁶ TCID<sub>50</sub>/ml (m.o.i. = 1). After 90 min of incubation, cultures were washed six times with medium to remove residual virus and were then cultured for 10 days at 37°C in RPMI 1640 without heparin or ECGS. Starting at day 1 p.i., 2 ml supernatant was removed daily from infected cell cultures and replaced by 2 ml of fresh medium; the supernatant samples were stored at −80°C prior to determination of the infectious titre.

**Transfection of UL122/123 plasmid prR47.** Plasmid prR47 is a pUC18-based plasmid containing the complete UL122/123 gene region of AD169 in a 6.7 kb EcoRI–SalI insert (Stamminger et al., 1991). HFF or HUVEC were seeded into six-well culture plates at a density of 200000 cells per well 24 h prior to transfection. For transfection 1 µg of plasmid DNA was introduced into the respective cell culture using Superfect reagent (Qiagen). At 48 h after transfection, transfected cells were trypsinized, cytocentrifuged onto glass slides, and fixed with acetone at room temperature for 5 min. Viral IE antigens were detected by indirect immunoperoxidase staining as described below.

**Radiolabelling of HCMV.** For use in binding assays HCMV was radioactively labelled in culture by incorporation of [³⁵S]methionine. Infected HFF grown in 175 cm² plastic flasks at 30% CPE were incubated with 15 ml medium containing 6.25 MBq [³⁵S]methionine (Amersham) for 2 days. Cell debris in the supernatant was removed by centrifugation at 2800g for 10 min and [³⁵S]methionine-labelled virus was collected from the medium by centrifugation at 80000 g for 70 min in a Beckman ultracentrifuge. The radioactively labelled virus preparations were washed three times with ultracentrifugation to minimize the amount of unincorporated [³⁵S]methionine and resuspended in 1 ml MEM.

**Monoclonal antibodies, immunoblotting and immunoperoxidase staining.** To analyse viral gene expression, monoclonal antibodies (MAbs) against viral proteins from different phases of the HCMV replicative cycle were used. In detail, MAbs were reactive against the immediate early (IE) proteins IE72 and IE86 (pUL122/123, MAb E13; Biosoft, Paris, France), the early protein p52 (pUL44, MAb BSS10; Biotest, Dreieich, Germany), the early late protein pp65 (pUL83, MAb 28-77; kindly provided by W. Britt, Birmingham, AL, USA), the late major capsid protein (pUL86, MAb 28-4; kindly provided by W. Britt), and the late tegument protein pp150 (pUL32, MAb XP1; Behringwerke, Marburg, Germany) (Jahn et al., 1990). MAbs against vimentin (Dako) and lamin B (Calbiochem) were used to detect cytoskeleton components and nuclear components, respectively.

For immunoblotting, protein samples were prepared by lysis of cells in sample buffer containing 2% SDS and 15% dithiothreitol, separated by SDS–PAGE, blotted on nitrocellulose, and detected with the ECL Western blotting detection system (Amersham).

For *in situ*-detection of viral antigens in infected cells, indirect immunoperoxidase staining was done as follows. At various time-points after infection, cells grown in 24-well dishes were fixed with 80% acetone for 5 min at room temperature. Fixed cells were reacted with antibodies against viral antigens for 60 min at 37°C followed by incubation with peroxidase-conjugated goat anti-mouse Ig—Fab′₃ polyclonal sera (De Beer Medicals, Hilvarenbeek, Netherlands). Finally, antigens were detected by staining with diaminobenzidine (DAB; Sigma) and observation with a light microscope.
Fig. 1. For legend see opposite.
**Analysis of virus adsorption and penetration.** For adsorption assays, HFF and HUVEC grown in 24-well dishes were incubated with $[^{35}S]$methionine labelled virus at 4 °C for various times in triplicate. Negative controls contained heparin (100 IU/ml) to prevent virus adsorption. After incubation with virus the cells were washed three times with medium and lysed in 200 µl 1 M NaOH prior to scintillation counting of radioactivity in a β-counter (Wallac 1409) to determine the extent of virus binding. For penetration assays, HFF and HUVEC grown in 75 cm² plastic flasks were incubated with $^{35}$S-labelled virus for various times at 37 °C. Subsequently, cells were washed twice with ice-cold trypsin–EDTA and were incubated with trypsin–EDTA for 1 h on ice to eliminate adsorbed virus. Cells were pelleted by centrifugation and washed with MEM. Cells were then lysed in 1 M NaOH and radioactivity determined by scintillation in a β-counter. For analyses of penetration at low levels of infection, cells were treated as described above at m.o.i. = 0.1. Trypsin-treated cells were harvested by centrifugation and after lysis
Fig. 2. Course of HCMV infection in EC. (A) Kinetics of viral antigen expression by HCMV strains AD169 and TB40/E in EC after infection at an m.o.i. of 10. IE antigen (MAb E13, UL122/123, 4 h p.i.), early antigen p52 (MAb BS510, UL44, 24 h p.i.) and late antigen MCP (MAb 28-4, UL86, 72 h p.i.) were detected in EC by indirect immunoperoxidase staining. The time-course of antigen expression in infected EC is indicated by bars. (B) Single-step growth curve of HCMV strain AD169 after infection of EC monolayers at an m.o.i. of 1. Infectivity in supernatants from infected EC culture was determined by TCID<sub>50</sub> assays. (C) Electron micrograph of an EC 5 days p.i. with HCMV strain AD169. Numerous viral capsids are visible in the nucleus of the infected cell.

of cells with buffer containing 0.5% SDS and 0.1 mg/ml proteinase K, DNA was extracted with phenol–chloroform (Ausubel et al., 1989) and analysed by quantitative HCMV-DNA-PCR.

Electron microscopy. Cells were rinsed in PBS and fixed in 1.5% glutaraldehyde in 0.2 mol/l PBS for 30 min at 4 °C. After three washes for 10 min each in PBS containing 7.5% sucrose at room temperature cells were post-fixed in 1% osmium tetroxide in PBS for 60 min, dehydrated in a graded series of ethanol and embedded in Araldite (Merck). Ultrathin sections (70 nm) were picked up on 300 mesh nickel grids and stained with 1% uranyl acetate and 1.5% lead citrate. Sections were examined with a Zeiss EM 902 transmission electron microscope.

Analysis of nuclear localization of viral DNA. For quantification
µpHM 471 carries a 100 bp deletion within the amplified gene region. The plasmid pHM 471 (kindly provided by T. Stamminger) was added to exon 4 of the HCMV IE gene UL123. An aliquot of 1000 copies of nuclear DNA probes were quantified by competitive PCR using primers Quantitative PCR assays.

Viral nucleic acids within cellular or nuclear DNA probes were quantified by competitive PCR using primers P1 (5’ GGT CAC TAG CCG TTG TAT GAT GAC CA 3’) and P2 (5’ TTC TCA GCC ACA ATT ACT GAG GAC AGA GGG A 3’) within exon 4 of the HCMV IE gene UL123. An aliquot of 1000 copies of plasmid pHM 471 (kindly provided by T. Stamminger) was added to each sample of a logarithmic dilution series of the DNA preparations. pHM 471 carries a 100 bp deletion within the amplified gene region. The reactions were done in a total volume of 50 µl consisting of 2.0 mM MgCl₂, 0.25 mM each dNTP, 10 pmol each primer, 1 × PCR buffer (Boehringer Mannheim) and 1 U Taq polymerase (Boehringer Mannheim). Thermal cycling was performed as follows: 35 cycles of 94 °C for 1 min, 57 °C for 1 min, and 72 °C for 1 min. Amplification products were visualized by electrophoresis in agarose gels, ethidium bromide staining and UV light illumination. Equivalence of the natural HCMV IE amplification product and the pHM471 plasmid amplification product indicated the presence of 1000 HCMV-DNA copies in the analysed sample.

Immunostaining of viral particles after penetration. Cells were grown to subconfluence in 25 cm² culture flasks in appropriate culture medium. Prior to infection, cells were preincubated for 30 min with MEM5. Cells were then incubated with cell-free virus preparations for 0.5–4 h at 37 °C. To remove adsorbed virus particles that had not penetrated, cells were treated with trypsin–EDTA (Gibco) for 20 min. Tryptase was inactivated by washing with MEM5 and, after resuspension in PBS, cells were cytocentrifuged on glass slides. Cells were fixed with acetone for 5 min at room temperature and immunostained. For detection of viral particles, MAb XP1, directed against the viral tegument protein pp150, was used as a primary antibody. Subsequently, rabbit anti-mouse Ig polyclonal antiserum, peroxidase-antiperoxidase complexes (PAP; Dako) from mouse, biotinylated swine anti-mouse Ig antibodies (Dako) and peroxidase-conjugated streptavidin–biotin complex (Dako) were added. Signals were detected using DAB or metal-enhanced (Co-)DAB visualization of viral particles. If simultaneous detection of viral IE antigen in the same cell preparations was desired, an indirect immunoalkaline phosphatase staining of ppUL122/123 was performed subsequent to Co-DAB staining of the pp150 antigen. Incubation with MAb E13 was followed by incubation with peroxidase-conjugated goat anti-mouse Ig–Fab’2 polyclonal sera (De Beer Medicals). Signals were then detected using DAB as chromogen, resulting in gold-brown nuclear staining. Slides were mounted with glycerol–gelatin and staining observed with a Polyvar microscope (Cambridge Instruments) using interference contrast (DIC).

Results

The endotheliotropic phenotype of HCMV variants is determined by the initial events of virus replication

Cytomegalovirus strains included in our analysis displayed marked interstrain differences in the ability to grow in EC. Both with a cell-associated and with a cell-free mode of virus propagation, the attempt to grow a particular strain continuously in EC sharply distinguished two groups of viruses. Non-endotheliotropic viruses like AD169, TB40/F and VHL/F failed to increase the fraction of initially infected cells in the EC culture. Thus they were unable to disseminate, and continuous passaging of inoculated cultures eventually resulted in complete loss of infectious virus. In contrast, endotheliotropic strains like KSA16/3, TB40/E and VHL/E increased the number of infected cells by focal expansion (Sinzger et al., 1999b). These viruses were able to disseminate in endothelial monolayers, yielding progressively higher titres of infectious virus and subsequently reaching 100% CPE.

However, for analysis of the mechanism underlying this phenomenon, focal expansion of HCMV in cell monolayers was unsuitable, and synchronized supernatant-associated infections were desired for dissection of distinct steps of the virus replication cycle. Therefore, the endotheliotropic phenotype of the various HCMV strains was quantitatively determined after infection of cell monolayers with cell-free virus preparations (Fig. 1, Table 1). Fibroblast cultures and EC cultures were infected in parallel with each virus strain at an m.o.i. of 0.5 and the fraction of infected cells was determined at 24 h p.i. by immunodetection of viral IE antigen. Again, HCMV strains KSA16/3, TB40/E and VHL/E were found to be endotheliotropic, whereas infectivity in EC was dramatically reduced with HCMV strains AD169, TB40/F and VHL/F (Fig. 1). Taken together, the infectivity of nonendotheliotropic strains was about 100–1000-fold reduced in HUVEC as compared to HFF (Table 1). In contrast, endotheliotropic

**Table 1. Comparison of supernatant-associated infection efficiency of various HCMV strains in fibroblasts (HFF) and umbilical vein EC (HUVEC) at an m.o.i. of 0.5**

<table>
<thead>
<tr>
<th>Virus preparation</th>
<th>Percentage of infected cells</th>
<th>HFF</th>
<th>HUVEC</th>
<th>Ratio HFF/HUVEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB40/E</td>
<td>60</td>
<td>40</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>TB40/F</td>
<td>50</td>
<td>0.5</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>VHL/E</td>
<td>60</td>
<td>40</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>VHL/F</td>
<td>70</td>
<td>0.5</td>
<td>140</td>
<td></td>
</tr>
<tr>
<td>KSA16/3</td>
<td>60</td>
<td>30</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>AD169</td>
<td>60</td>
<td>0.1</td>
<td>600</td>
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strains displayed only a slight reduction of infectivity in HUVEC as compared to HFF. This dramatic phenotypic difference of HCMV variants in easy-to-standardize supernatant-associated infectivity assays could now be subjected to detailed analysis of dissected events during the virus life-cycle.

The finding that this phenotype is manifest at the IE stage of the replicative cycle (Fig. 1) suggested that initial events are critical for infectivity in this cell type. However, this did not exclude an additional block later in the virus replication cycle. Therefore we analysed the kinetics of viral gene expression to follow the course of virus replication in those cells that had started viral gene expression (Fig. 2A). EC were infected by HCMV strains AD169 and TB40/E at an m.o.i. of 10. Subsequently, IE, early and late viral proteins were detected at various times p.i. After infection with nonendotheliotropic strain AD169, IE antigen became detectable at 4 h p.i. in a few cells and was detectable in 1% of cells at 24 h p.i. IE antigen remained detectable throughout the whole replicative cycle. Early antigen became detectable at 24 h p.i. and late antigen became detectable at 48 h p.i. (Fig. 2A) in 1% of cells. After infection with endotheliotropic strain TB40/E, antigens were expressed with the same kinetics but in up to 100% of cells (Fig. 2A). This indicated that (i) those cells that started viral gene expression also proceeded through the early and late stages of virus replication and (ii) that the kinetics of viral gene expression in EC resembled the kinetics as known from fibroblast cultures. Finally, the permissive and productive nature of HCMV infection in EC was demonstrated by single-step growth curves together with electron microscopical detection of capsids in late stage infected EC, even for non-endotheliotropic strains like AD169 (Fig. 2B, C). Endotheliotropic and nonendotheliotropic HCMV variants were only distinguished by the efficiency to initiate infection in EC. Once infection was initiated it was permissive on a single cell level irrespective of the virus strain. Together, these experiments emphasized the critical role of initial replication events for expression of the endotheliotropic phenotype of HCMV variants.

IE promoter function is independent of cell type

The inefficiency of IE antigen expression by HCMV strain AD169 in EC might be caused by inefficient functioning of the respective promoter in this cell type. To test this possibility, we transfected a plasmid containing the complete UL122/123 gene of AD169 into fibroblasts and EC (Fig. 3). The fraction of
Fig. 4. (A) Quantitative analysis of HCMV adsorption to EC (HUVEC, ○) versus fibroblasts (HFF, ●). Subconfluent monolayers of HUVEC or HFF were incubated with $^{35}$S-labelled virus at 4 °C for various intervals. Virus inocula were normalized to result in an m.o.i. of 50, with the total radioactivity of the inocula varying with labelling efficiency. After several washings, cells were lysed and adsorbed virus was determined by counting of bound radioactivity in a β-counter. (B) Quantitative analysis of HCMV penetration into EC (HUVEC, ○) versus fibroblasts (HFF, ●). Subconfluent monolayers of HUVEC or HFF were incubated with labelled virus at 37 °C for various intervals. Virus inocula were normalized to result in an m.o.i. of 50, with the total radioactivity of the inocula varying with labelling efficiency. After extensive washings, unpenetrated virus was removed by trypsin treatment. Cells were lysed, and penetrated virus was analysed by counting of bound radioactivity in a β-counter. (C) Quantitative comparison of HCMV penetration into EC (HUVEC) vs. fibroblasts (HFF) at low m.o.i. Subconfluent monolayers of HUVEC or HFF were infected for 90 min with various virus strains at an m.o.i. of 0-1. Unpenetrated virus was removed by trypsin treatment. The intracellular content of viral DNA was quantified by competitive PCR using primers within exon 4 of the HCMV IE.
cells expressing IE antigens was detected by immuno-
peroxidase staining at 48 h p.i. Detection of IE antigen in nuclei
of transfected cells would indicate activity of the IE promoter
in the respective cells. Both EC and fibroblast cultures
expressed IE antigen at an efficiency of 10% of cells. This
demonstrated that the IE promoter is functional in EC and
indicated that the block in AD169 replication in EC is located
prior to IE gene expression.

No interstrain differences occur during HCMV entry
into EC

The cell tropism of many viruses is regulated at the level of
adsorption to and penetration into target cells. Therefore, we
analysed entry of HCMV variants into EC and fibroblasts in
order to determine whether interstrain differences in EC
tropism are associated with interstrain differences in
adsorption/penetration into EC. For quantitative analysis of
entry events, we performed binding studies with 35S-labelled
virus preparations (Fig. 4A, B). Analysis of virus adsorption
was done at 4 °C to prevent virus entry. For analysis of virus
penetration, cells were incubated at 37 °C for various times.
Unpenetrated virus was removed by trypsin treatment.
Adsorption of HCMV to fibroblasts was 2–6-fold more
efficient as compared with EC. Interstrain differences, if present
at all, were scant (Fig. 4A). Penetration into fibroblasts was
6–10-fold more efficient as compared to EC. Again, no dramatic
interstrain difference was found between endotheliotropic and
nonendotheliotropic HCMV variants (Fig. 4B). For analysis of
virus entry at low m.o.i., we repeated the penetration
experiments at infection multiplicities of 0–1, employing
quantitative DNA PCR assays as a read-out system. Under
these conditions penetration into fibroblasts was again slightly
more efficient as compared to EC (factor 2–10) but no
interstrain differences were found (Fig. 4C).

These findings might explain that even with endo-
theliotropic HCMV strains infection of fibroblasts is slightly
more efficient as compared to EC (Table 1). However, the
dramatic inefficiency of strains AD169, TB40/E and VHL/F in
infecting EC could not be explained at the level of virus entry.

Efficiency of nuclear translocation of viral DNA is
correlated with the endotheliotropic phenotype of
HCMV variants

Following virus penetration, nuclear transport of viral
particles and release of viral DNA are prerequisites for the
initiation of virus replication. As the phenotype of non-
endotheliotropic HCMV strains could neither be explained by
inefficient penetration nor by inefficiency of the IE gene in EC,
we hypothesized that inefficient nuclear translocation of viral
DNA might determine this phenotype. A major prerequisite
for the investigation of the nuclear translocation of viral DNA
was the preparation of pure nuclear fractions of infected cells.
In particular, the cytoskeleton had to be separated from nuclei,
as herpesviral particles are known to bind to the cytoskeleton
after penetration (Sodeik et al., 1997). To this end, nuclei were
liberated from infected cells with a Dounce homogenizer after
swelling in hypotonic buffer and subsequently separated from
cytoskeleton contaminants by density gradients. Western blot
assays of the resulting subcellular fractions were done to prove
the purity of the preparations. Absence of vimentin immuno-
reactivity in the presence of nuclear marker lamin B indicated
the purity of the nuclear fractions. While standard protocols
like sucrose-gradient centrifugation of Dounce-homogenized
cell preparations failed to fulfil this criterion, iodixanol
gradients did (Fig. 5A). For all subsequent experiments,
iodixanol-gradient centrifugation of Dounce-homogenized cell
preparations was used. The viral DNA content in nuclei of
infected cells was determined by quantitative competitive
DNA PCR (Sinzger et al., 1999a). This sensitive approach was
preferred because other methods like Southern blotting or in
situ-hybridization would entail infections at abnormally high
multiplicities, which in turn might favour abnormal routes of
virus trafficking. Three nonendotheliotropic strains (AD169,
TB40/F and VHL/F) and two endotheliotropic strains (TB40/E
and VHL/E) were included in this analysis. The nuclear
HCMV-DNA content of all endotheliotropic variants was
about equal in fibroblasts and EC (Fig. 5B). In contrast, all
nonendotheliotropic strains displayed 100–1000-fold de-
creased HCMV-DNA titres in the nuclei of infected EC as
compared to fibroblasts (Fig. 5 B).

Efficiency of nuclear transport of penetrated viral
particles determines the endotheliotropism of HCMV
variants

The inefficient nuclear translocation of nonendotheliotropic
HCMV genomes in EC could be explained by impairment of
transport of viral particles from the cell periphery towards the
nucleus. Alternatively, uncoating of viral DNA from properly
translocated particles could be disturbed leading to a stalling of
DNA-containing capsids at the nuclear membrane. To test the
‘transport’ hypothesis versus the ‘uncoating’ hypothesis, we
sought to visualize single penetrated virus particles by
immunostaining. When reagents against various structural
proteins were tested we found that antibodies against
tegment protein pp150 were most suitable for this purpose.
While antibodies against tegument protein pp65 resulted in
gene UL123. An aliquot of 1000 copies of plasmid pHM 471 was added to each sample of a logarithmic dilution series of the
DNA preparations. pHM 471 carries a 100 bp deletion within the amplified gene region. Equivalence (white arrowheads) of the
natural HCMV IE amplification product and the plasmid amplification product indicated the presence of 1000 HCMV-DNA
copies in the analysed sample dilution. Lanes in each gel image represent (from left to right) marker DNA and 10⁻¹–10⁻²-fold
fractions of total DNA samples from infected cells.
Fig. 5. (A) Flow chart of the nuclear localization assay. Nuclear fractions of infected cells were prepared as indicated in the flow chart. In the left-hand part of the figure, phase contrast micrographs of swollen cells, fractionated cells and purified nuclei are presented. In the right-hand part of the figure Western blot analyses of the nuclear fraction from sucrose gradients and iodixanol gradients are compared. Reactivity of the vimentin antibody indicates remnants of the cytoskeleton in the sucrose fraction, while the iodixanol fraction is free of cytoskeleton signals. Reactivity of the lamin B antibody indicates the presence of the nuclear membrane. (B) Quantitative analysis of nuclear translocation of viral DNA after virus entry. Nuclear fractions of
diffuse cytoplasmic staining at initial times after penetration and no signals were obtained with antibodies against the major capsid protein MCP, pp150 staining resulted in well-defined punctate signals. Three lines of control experiment indicated that pp150 immunostaining actually detected capsid particles. (i) When cells were incubated with high titre virus preparations for 90 min under adsorption conditions, signals were located at the cell surface. These signals were removed when cells were trypsinized subsequent to virus adsorption but were found within the cytoplasm when the temperature was shifted to 37 °C for 20 min subsequent to virus adsorption, thus allowing for virus penetration (Fig. 6 A). (ii) The number of these pp150 signals correlated well with the m.o.i., whereas the intensity of single signals was independent of the m.o.i. (Fig. 6 C). This pattern can be explained by particle-associated protein rather than by solubilized protein. (iii) Moreover, numerous signals were obtained with gradient-purified virions (Fig. 6 B, C) whereas only very few signals were detected with dense body fractions containing only remnants of infectious virus (data not shown). Together, this indicated that pp150 staining signals represented penetrated virus capsids. This is in accordance with previous reports about a tight association of pp150 with capsids and dissociation of pp65 from the capsid after detergent treatment (Benko et al., 1988; Chen et al., 1999; Gibson, 1996). Failure to immunostain MCP in penetrated virus might indicate that the respective epitope is not accessible at the surface of intact capsids. As a result of these pretesting pp150 immunostaining was employed for detection of penetrated capsids. As we observed that infectivity/particle efficiency was superior with unmanipulated infectious supernatants as compared with ultracentrifuged and gradient-purified preparations, we preferred unmanipulated preparations for subsequent assays.

Using this approach we analysed the localization of viral particles from various HCMV strains in HFF versus HUVEC after virus penetration. Cells were incubated with virus preparations at 37 °C for 4 h to allow for penetration and for IE gene expression as a marker for initiation of infection. HFF were additionally incubated with 10-fold dilutions of the respective virus to normalize the different degree of virus penetration (see Fig. 4). After the incubation period, cells were trypsinized to remove residual adsorbed but unpenetrated virus and then cytocentrifuged onto glass slides. Double immunodetection of structural protein pp150 (pUL32) and nonstructural newly synthesized IE protein (pUL122/123) was performed to follow the route of virus particles after penetration and to correlate virus particle staining patterns with the initiation of viral gene expression. Confirming the aforementioned penetration data (Fig. 4), the level of virus penetration was normalized between HUVEC and HFF when 10-fold diluted virus preparations were used for HFF cultures (Fig. 6 D). Under conditions of normalized penetration, endotheliotropic strains VHL/E and TB40/E displayed efficient nuclear translocation of virus capsids and initiation of viral gene expression as evidenced by IE antigen detection in both cell types. In sharp contrast, nonendotheliotropic HCMV variants AD169, VHL/F and TB40/F failed to translocate virus particles towards the nucleus in HUVEC cells, and no viral IE gene expression was detectable in these cells. Nuclear localization of particles and viral gene expression occurred in only very few cells with nonendotheliotropic strains. Despite their inefficiency in HUVEC, these strains were efficient in nuclear localization and gene expression in HFF. Data are shown for TB40/E versus TB40/F (Fig. 6 D) but were identical for VHL/E versus VHL/F and AD169.

In summary, the EC tropism of HCMV variants appeared to be determined by the efficiency of nuclear transport of virus particles after successful penetration.

Discussion

The cell tropism of viruses is commonly determined during the initial stage of the replicative cycle (Tyler & Fields, 1996). For example, the coreceptors CCR5 and CXCR4 determine the tropism of HIV variants for lymphocytes and macrophages, respectively (Dittmar et al., 1997; Grivel & Margolis, 1999; Penn et al., 1999). Another critical step seems to be the initiation of viral gene transcription, mediated by cell type-specific transcription factors (Cripe et al., 1987; Gloss et al., 1987; Kyo et al., 1995; Ori & Shaul, 1995). In this study we show that the EC tropism of HCMV variants is also determined prior to IE gene expression, but surprisingly neither adsorption/penetration nor the IE promoter function contribute significantly to cell tropism differences. Transfection of the isolated IE gene of nonendotheliotropic variant AD169 into fibroblasts and EC yielded efficient IE gene expression in both cell types, despite 1000-fold reduced infectivity in EC after infection with the complete virus particle. Regarding the efficiency of adsorption/penetration, virus entry appeared to be slightly reduced in EC as compared to fibroblasts but no infected cells were prepared as indicated in (A). The nuclear content of viral DNA 90 min p.i. was quantified by competitive PCR using primers within exon 4 of the HCMV IE gene UL123. An aliquot of 1000 copies of plasmid pH471 was added to each sample of a logarithmic dilution series of the DNA preparations. pH471 carries a 100 bp deletion within the amplified gene region. Equivalence (white arrowheads) of the natural HCMV IE amplification product and the plasmid amplification product indicated the presence of 1000 HCMV-DNA copies in the analysed sample dilution. Lanes in each gel image represent (from left to right) 123 bp marker and 10⁴–10⁷-fold fractions of total nuclear DNA samples from infected cells. With nonendotheliotropic strains AD169 and TB40/F, nuclear transport of viral DNA appears to be about 100–1000-fold reduced in HUVEC as compared to HFF. With endotheliotropic strains VHL/E and TB40/E, nuclear transport of viral DNA is about equal in the two cell types.
interstrain differences occurred at that level. The slightly reduced virus entry into HUVEC might explain why even endotheliotropic HCMV strains are slightly more efficient in fibroblasts. However, the dramatic interstrain differences in EC tropism could not be explained by different adsorption or penetration efficiencies of these viruses. The question was, why fibroblast-adapted HCMV strains like AD169 fail to start gene expression in EC despite their ability to penetrate and to activate their IE promoter upon transfection. This could only be explained by the inefficient release of viral DNA into the cell nucleus after penetration into the cells. Actually, we found dramatic interstrain differences in the efficiency of nuclear translocation of viral DNA in EC as compared to fibroblasts. While all strains efficiently released viral DNA to the nuclei in fibroblasts, only EC-propagated strains were effective in EC. Fibroblast-adapted strains were about 100–1000-fold less

Fig. 6. For legend see facing page.
Fig. 6. (A) Immunostaining of viral particles with antibody XP1 against the viral tegument protein pp150 after incubation of HUVEC with HCMV strain TB40/E at an m.o.i. of 10 under conditions allowing for adsorption of virus (90 min, 4 °C), adsorption and removal of adsorbed virus (90 min, 4 °C; 20 min trypsin) or adsorption and penetration of virus (90 min, 4 °C; 20 min, 37 °C). Cell spots were prepared by cytocentrifugation. Signals were amplified by a five-step indirect immunoperoxidase technique and visualized with DAB as chromogen (brown punctate staining). Counterstaining of nuclei was done with haematoxylin. (B) Immunostaining of viral particles with antibody XP1 against the viral tegument protein pp150 after incubation of HFF with gradient-purified virions of HCMV strain TB40/F at an m.o.i. of 1 for various time intervals at 37 °C. Remnants of adsorbed virus were removed by trypsin treatment for 20 min at 37 °C and cells were cytocentrifuged onto glass slides. Signals were amplified by a five-step indirect immunoperoxidase technique and visualized with Co-DAB as chromogen (black punctate staining). A slight counterstaining of nuclei was done with haematoxylin. Examples of stained virus particles are indicated by the arrowheads. (C) Double immunodetection of viral particles and viral antigen expression using antibody XP1 against the viral tegument protein pp150 and antibody E13 against IE antigens of HCMV after incubation of HFF with gradient-purified virions of HCMV strain TB40/F at an m.o.i. of 1 for 4 h at 37 °C. Remnants of adsorbed virus were removed by trypsin treatment for 20 min at 37 °C and cells were cytocentrifuged onto glass slides. Signals were amplified by a five-step indirect immunoperoxidase technique and visualized with Co-DAB as chromogen (black punctate staining). Viral IE antigens were visualized by a two-step immunoperoxidase staining using antibody E13 and DAB as chromogen (brown nuclei). No counterstaining was done. Examples of stained virus particles are indicated by the arrowheads. (D) Double immunodetection of viral particles and viral antigen expression using antibody XP1 against the viral tegument protein pp150 and antibody E13 against IE antigens of HCMV after incubation of EC (HUVEC) and fibroblasts (HFF) for 4 h at 37 °C. Remnants of adsorbed virus were removed by trypsin treatment for 20 min at 37 °C and cells were cytocentrifuged onto glass slides. Viral particles were visualized by a five-step indirect immunoperoxidase technique using antibody XP1 and Co-DAB as chromogen (black punctate staining). Viral IE antigens were visualized by a two-step immunoperoxidase staining using antibody E13 and DAB as chromogen (brown nuclei). Examples of stained virus particles are indicated by the arrowheads.
efficient in EC and this was accompanied by a 100–1000-fold reduced infectivity. Inefficient nuclear import of AD169 DNA in EC had been demonstrated by in situ-hybridization (Slobbe van Drunen et al., 1998). However, as these data were not quantified and no comparisons with other HCMV strains were made, this observation could not be directly linked to interstrain differences in the EC tropism of HCMV variants. Our quantitative comparisons of initial replication events now provide direct evidence that HCMV interstrain differences in EC tropism are actually due to inefficient translocation of the viral genome into the nucleus of the infected cells.

Regarding the mechanism underlying the inefficiency of nuclear DNA import, our results differ from the observations reported by Slobbe van Drunen et al. (1998). While these authors described accumulation of signals around the nucleus, we found that in contrast viral particles of nonendotheliotropic HCMV strains accumulate in the cytoplasm of EC and do not reach the nucleus (Fig. 6). Thus, while the former report would imply that release of viral DNA at the nucleus is blocked our data strongly suggest that the transport of virus particles towards the nucleus is impaired. This discrepancy might be partially explained by the use of different assays for virus detection. Condensed genomes within penetrated but still intact capsids might be undetectable by in situ hybridization and the application of very high m.o.i.s might favour aberrant entry pathways.

In our approach, combining immunostaining of particles with the detection of viral IE antigen, we could directly correlate the nuclear transport of penetrated virus with the successful initiation of viral gene expression even at low to moderate m.o.i.s. Using this approach an unusual mechanism for the determination of virus cell tropism was found. Nonendotheliotropic HCMV variants are distinguished from endotheliotropic HCMV variants by their dramatic inefficiency in nuclear translocation of penetrated virus particles in EC, although all HCMV variants are efficient in fibroblasts. Thus there appears to be both an interstrain difference between HCMV variants regarding initial events in EC and a cell type-specific difference between EC and fibroblasts regarding transport processes.

It is tempting to speculate about the possible interactions between viral and cellular structures that mediate the efficiency of nuclear transport of virus particles. During the transport of capsids from the cellular membrane to the nucleus, interactions of the viral tegument or capsid with components of the microtubule system might be important, as has been shown previously for herpes simplex virus (Sodeik et al., 1997). The use of different motor proteins in HUVEC and HFF would provide an explanation for the cell type differences observed. At present, these considerations are still speculative. However, a virus–cell system is now available for detailed analyses of the nuclear transport processes involved in initiation of HCMV infection. Our finding that the transport of penetrated HCMV particles is a critical event in the virus life cycle might furthermore indicate that this step is a potential target for future antiviral strategies.

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References


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