Presence of human cytomegalovirus (HCMV) immediate early mRNA but not ppUL83 (lower matrix protein pp65) mRNA in polymorphonuclear and mononuclear leukocytes during active HCMV infection

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During an active human cytomegalovirus (HCMV) infection, leukocytes harbouring the HCMV lower matrix protein pp65 (ppUL83) are present in the peripheral blood and can be detected with the HCMV antigenaemia assay. In the present study, it was investigated whether the presence of pp65 in these cells was due to transcription of the virus genome or might be the result of uptake of this viral protein. Peripheral blood leukocytes of transplant recipients and AIDS patients with an active HCMV infection were investigated for the presence of HCMV immediate early (IE) antigen and pp65 using well characterized monoclonal antibodies, and for the presence of the corresponding mRNAs using non-radioactive in situ hybridization. Both mononuclear and polymorphonuclear cells were found to contain IE antigen and pp65. However, only mRNAs encoding IE antigen were found in these cells, whereas mRNAs encoding pp65 were not detected. In contrast, both IE antigen and pp65, as well as their corresponding mRNAs, were detected in the circulating late-stage HCMV-infected endothelial cells that were also present in the leukocyte fractions. These findings demonstrate that a restricted viral gene expression (transcription of IE genes) does occur in mononuclear and polymorphonuclear leukocytes. However, the abundant presence of the early antigen pp65 without detectable presence of the corresponding mRNA in these cells strongly indicates uptake of this protein by the phagocytic leukocytes, rather than de novo synthesis.

Introduction

During an active human cytomegalovirus (HCMV) infection, peripheral blood leukocytes have been shown to contain viral antigens (van der Bij et al., 1988b). Both immediate early (IE) antigens and the lower matrix protein pp65 (also called ppUL83; Landini & Spaete, 1993), which is a delayed early protein (Depto & Stenberg, 1989) and is the major constituent of dense bodies (Irmiere & Gibson, 1983), have been shown to be present in these cells. The lower matrix protein is the viral antigen that is most frequently and most abundantly present (Grefte et al., 1992). A sensitive, specific and rapid assay for early diagnosis and subsequent monitoring of an active HCMV infection has been based on these findings (HCMV antigenaemia assay; van der Bij et al., 1988a, b). The number of HCMV pp65-positive cells has been shown to correlate with disease activity (van den Berg et al., 1989, 1991; The et al., 1990) and the results obtained in several patient groups have since demonstrated the usefulness of this assay (Boland et al., 1990; Gerna et al., 1990; Boeckh et al., 1992; Erice et al., 1992; Ehrnst et al., 1993; Landini, 1993).

In addition, the presence of late-stage HCMV-infected cells of endothelial origin was recently demonstrated in the leukocyte fraction of the peripheral blood of patients with an active HCMV infection. These cells were shown to contain viral antigens from all three stages of the virus replication cycle, as well as numerous virus particles (Grefte et al., 1993a, b). It was concluded that they were productively infected with HCMV and probably involved in the dissemination of HCMV throughout the body.

The purpose of the present study was to investigate whether the presence of pp65 in peripheral blood leukocytes during an active HCMV infection, as detected with the HCMV antigenaemia assay, was due to transcription of the viral genome in these cells and de novo synthesis of this protein, or represented uptake/phagocytosis of pp65, probably by binding of virions and dense bodies followed by penetration and uncoating. The latter was hypothesized to be the case since the
number of leukocytes harbouring pp65 greatly exceeded that of IE antigen-positive leukocytes in patients with an active HCMV infection (Grefte et al., 1992). In addition, in experiments in vitro it had been shown that pp65 could be taken up by fetal lung fibroblasts with a subsequent nuclear localization of the protein in these cells (Grefte et al., 1992). A similar mechanism might account for the presence of pp65 in leukocytes. If this hypothesis were true, both IE and pp65 antigens, as well as IE mRNA, would be present in peripheral blood leukocytes during an active HCMV infection but pp65 mRNA would not. In contrast, both viral antigens and both viral mRNAs would be present in the circulating late-stage HCMV-infected endothelial cells that were expected to be present in the peripheral blood during an active HCMV infection.

To investigate this, we used non-radioactive in situ hybridization. Although this technique may be less sensitive than reverse transcriptase (RT)-PCR for mRNA detection, an in situ method was needed for this study in order to be able to differentiate between the mRNA content of the distinct leukocytes and that of the (remnants of) late-stage HCMV-infected endothelial cells that were also expected to be present. Non-radioactive in situ hybridization was preferred to radioactive in situ hybridization because of its better morphological resolution, higher probe stability and easier handling (Höltke & Kessler, 1990; Hukkanen et al., 1990; Dirks et al., 1993). Antisense RNA probes were judged superior to DNA probes because of their greater sensitivity (no probe reannealing and stability of RNA–RNA hybrids is greater than that of RNA–DNA hybrids allowing high-stringency washing and RNase treatment after in situ hybridization to remove unbound and non-specifically bound probe) and because of the possibility of using in vitro transcribed, digoxigenin-labelled sense RNA probes as a specificity control (Cox et al., 1984; Höltke & Kessler, 1990). Furthermore, to prevent non-specific hybridization as much as possible, we specifically investigated transcription from the HCMV IE and pp65 genes, instead of using large labelled (sub)genomic HCMV DNA fragments.

Methods

Patients. Six patients were selected on the basis of having an active HCMV infection as determined by the presence of HCMV pp65-positive leukocytes in peripheral blood samples (van der Bij et al., 1988b; Grefte et al., 1992). Blood was obtained from patients with various levels of HCMV antigenemia. Underlying disorders included AIDS (three patients), previous renal transplantation (two patients) and previous lung transplantation (one patient, evaluated at three timepoints). One AIDS patient (patient 4) received ganciclovir, another (patient 5) foscarnet.

Preparation of cells. Mononuclear and polymorphonuclear leukocytes were isolated separately from heparinized blood by density gradient centrifugation as described previously (van der Bij et al., 1988b). Suspensions of 1 × 10⁶ cells/ml were prepared and cytocentrifuged (100 µl/well) onto two-well epoxy-coated Cooke slides (Nutacon). Slides were air-dried, wrapped in aluminium foil and kept at ~80 °C until used. Cooke slides to be used for in situ hybridization experiments had been precoated with poly-t-lysine (Sigma). Control cells for in situ hybridization consisted of uninfected and HCMV-infected (laboratory strain Ad169) human embryonic lung fibroblasts (Middendorp et al., 1984, 1986) and mononuclear and polymorphonuclear leukocytes of a healthy HCMV seronegative volunteer.

HCMV antigen detection. Immunoperoxidase staining was carried out as described previously (Grefte et al., 1993a). Monoclonal antibodies used were a mixture of CMV-C10 and CMV-C11 (Clonab-CMV; Biotes), both directed against the lower matrix protein pp65 (Grefte et al., 1992) and E-13 (Seralab) which is directed against an epitope encoded by exon 2 of the major IE gene (Mazeron et al., 1992).

Probes and labelling. The pp65 open reading frame (UL83) was generated and amplified by PCR (primers 5′ GCGGGATCCTA-TGGAGTCCGCGGTGCCCG 3′ and 5′ GCGGGATCCACTCCGTGGTCTTTTGGGC 3′) on the genomic clone pp65-RSV (Grefte et al., 1992). This fragment was blunt-ended with the Klenow fragment of DNA polymerase, ligated into EcoRV-digested plBluescript II KS+ (Stratagene) and used to transform Escherichia coli DH5α. One clone (pPCR65E1) was used to detect pp65 mRNA. For the detection of IE gene 1 mRNA a cDNA clone (pHM124; kindly provided by Dr T. Stammlinger, University of Erlangen-Nürnberg, Germany) comprising exons 2, 3 and 4 of the major IE transcription region (UL123) inserted into pBluescribe was used. After linearization of clone pPCR65E1 with XbaI or XhoI (for pp65 mRNA detection) and of clone pHM124 with EcoRI or HindIII (for IE mRNA detection) both sense (negative control) and antisense (specific mRNA) labelled ssRNA probes were generated in the presence of digoxigenin-labelled UTP (Boehringer Mannheim) as run-off transcripts with T3 and T7 RNA polymerase (Boehringer Mannheim), respectively. This was performed as described by Höltke & Kessler (1990). Subsequently, the RNA probes were fractionated by limited alkaline hydrolysis according to Cox et al. (1984) to an average fragment length of 150 bases to improve probe penetration (Dirks et al., 1993). Some cross-hybridization of the IE1 probes with IE2 mRNA was anticipated, as these mRNAs share exons 2 and 3 (approximately 0.1 kb).

Slide preparation. Preparation of slides for mRNA in situ hybridization was carried out according to Dirks et al. (1993). In short, cytocentrifuged preparations were thawed under a table ventilator. Slides were fixed in 0.9% sodium chloride solution containing 4% formaldehyde and 5% acetic acid for 20 min at room temperature (RT). After fixation, slides were rinsed in PBS, dehydrated in increasing concentrations of ethanol (50%, 70%, 96% and 100%) and washed for 10 min in xylene at RT. Slides were then washed twice in 100% ethanol, rehydrated in PBS and digested with 0.1% pepsin (P-7000; Sigma) in 0.01 M-HCl for 10 min at 37 °C to increase accessibility for macromolecular reagents. To inactivate pepsin and to preserve morphology, slides were post-fixed in 1% formaldehyde in PBS for 5 min. Subsequently, slides were rinsed in PBS, dehydrated in ethanol as described above and air-dried under a table ventilator for 15 min. A disadvantage of this fixation and protein digestion protocol was that it prevented the simultaneous detection of viral antigen. However, it proved to be crucial for the sensitive detection of the two viral mRNAs in these cells, and thus could not be circumvented.

In situ hybridization. The in situ hybridization procedure was based on that described by Knollema et al. (1992). Briefly, slides were hybridized overnight at 60 °C in a humidified chamber in hybridization
solution containing 42% deionized formamide, 0.25 M-sodium chloride, 3.3 mM-Tris-HCl pH 8.0, 0.4 mM-EDTA pH 8.0, 1 x Denhardt's solution (Sigma), 10% dextran sulphate, 0.5 mg/ml yeast tRNA (Clontech Laboratories) and 10% calf thymus DNA (Boehringer Mannheim). Probes were denatured at 65°C for 5 min before application. Final probe concentrations in hybridization solution were 1 to 5 ng/μl, as recommended as optimized by Dirks et al. (1993).

After hybridization, slides were washed three times for 10 min in 4 x SSC at RT. Next, to remove all unhybridized and non-specifically bound probe, slides were treated with a mixture of RNase A (20 μg/ml, Boehringer Mannheim) and RNase T1 (50 U/ml, Boehringer Mannheim) in 0.5 M-sodium chloride, 10 mM-Tris-HCl and 1 mM-EDTA pH 8.0, at 37°C for 30 min, followed by extensive washing in decreasing concentrations of SSC at increasing temperatures (starting in 2 x SSC at RT and ending with a final stringency of 0.1 x SSC at 65°C for 30 min). All solutions and glassware used before and during hybridization had been treated with diethylpyrocarbonate (Sigma) to inhibit RNase activity.

**Hybrid detection.** Following washes, cytocentrifuged preparations hybridized with digoxigenin-labelled RNA probes were incubated for 30 min at RT in 0.1 M-Tris-HCl pH 7.5 and 0.15 M-sodium chloride (buffer 1), containing 10% non-immune sheep serum (Dako), 2% BSA (Organon Teknika) and 2% HCMV-seronegative pooled human serum to prevent non-specific binding of the conjugate. Next, slides were incubated for 2 h at RT with alkaline phosphatase-conjugated sheep anti-digoxigenin antibody Fab fragments (Boehringer Mannheim), diluted 1:500 in buffer 1, containing 2% BSA and 2% HCMV-seronegative pooled human serum. This mixture was incubated with slides for 1 h at RT, rinsed twice in buffer 1 and once for 10 min in 0.1 M-Tris-HCl, 0.1 M-sodium chloride and 0.05 M-MgCl2, 6H2O pH 9.5 (buffer 2). Colour reaction was developed overnight in a freshly prepared solution of 0.34 mg/ml nitro blue tetrazolium (Boehringer Mannheim) and 0.17 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (Sigma) in buffer 2, and terminated in 0.1 M-Tris-HCl and 1 mM-EDTA pH 8.5. Slides were washed twice in PBS and mounted in Kaiser's glycerol gelatin (Merck). The dark-blue-purple precipitate, indicating the presence of hybridized mRNA, was revealed with bright-field microscopy.

To visualize nuclei, slides were counterstained with 1 μg/ml 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Boehringer Mannheim) for 15 min at RT in some experiments. These slides were mounted in Citifluor (Citifluor UKC Chemical Laboratories) followed by examination with combined fluorescence and bright-field microscopy.

**Results**

**Detection of HCMV antigens and mRNA in mononuclear cells**

Isolated peripheral blood mononuclear and polymorphonuclear leukocytes from patients with an active HCMV infection were investigated for the presence of pp65 and IE antigens by immunoperoxidase staining, and for the presence of the corresponding messenger RNAs by non-radioactive in situ hybridization (Table 1).

Numbers of IE antigen-positive mononuclear cells (Fig. 1b) ranged from 0 to 35 per slide (i.e. per approximately 105 cytocentrifuged cells) and were lower than the numbers of pp65-positive mononuclear cells (Fig. 1a), which ranged from 0 to 166 per slide. Both antigens were located in the (peri)nuclear region. Besides mononuclear cells, several circulating late-stage HCMV-infected endothelial cells (up to 13 per slide) could be found. As demonstrated previously (Grefte et al., 1993a), these cells showed the simultaneous presence of IE antigen and pp65.

IE mRNA-positive mononuclear cells (Fig. 2a, c, d) were present in approximately the same numbers as the IE antigen-positive mononuclear cells of the same patient and ranged from 0 to 60 per slide. The IE mRNA signal was always strictly cytoplasmic. The endothelial cells present on these slides were also found to contain IE mRNA, although the signal was weak (Fig. 3a, e). However, late-stage HCMV-infected fibroblasts showed comparable IE mRNA levels (Fig. 4a). In contrast, no evidence was found for the presence of pp65 mRNA in mononuclear cells, although the endothelial cells that were present on the same slides showed an abundant expression of pp65 mRNA (Fig. 3b, f).

**Table 1. Numbers of HCMV antigen- and mRNA-positive mononuclear cells and circulating cytomegalic endothelial cells present per slide**

<table>
<thead>
<tr>
<th>Patient</th>
<th>HCMV antigen</th>
<th>HCMV mRNA</th>
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<tr>
<td></td>
<td>IE pp65</td>
<td>IE pp65</td>
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<tr>
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<tr>
<td>2 RTx</td>
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</tr>
<tr>
<td>3 AIDS</td>
<td>34 166 (+ 2 EC)</td>
<td>60 (+ 2 EC)</td>
</tr>
<tr>
<td>4 AIDS</td>
<td>25 118 (+ 1 EC)</td>
<td>35</td>
</tr>
<tr>
<td>5 AIDS</td>
<td>35 (+ 2 EC) 125 (+ 13 EC)</td>
<td>13 (+ 7 EC)</td>
</tr>
<tr>
<td>6 LTx-1†</td>
<td>10 113</td>
<td>13</td>
</tr>
<tr>
<td>LTx-2</td>
<td>12 110</td>
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</tr>
<tr>
<td>LTx-3</td>
<td>14 42</td>
<td>15</td>
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* RTx, Renal transplant recipient.
† EC, Cytomegalic endothelial cells.
‡ LTx, Lung transplant recipient investigated at three timepoints.

**Table 1. Numbers of HCMV antigen- and mRNA-positive mononuclear cells and circulating cytomegalic endothelial cells present per slide**
Immunoperoxidase staining of mononuclear (a and b) and polymorphonuclear (c and d) leukocytes of a patient with an active HCMV infection. Leukocytes were stained with a mixture of monoclonal antibodies CMV-C10 and CMV-C11, both directed against the lower matrix protein pp65 (ppUL83; a and c), and with monoclonal antibody E-13, directed against IE antigens (b and d). Counterstaining was carried out with haematoxylin. The bar represents 6 μm.

Detection of HCMV antigens and mRNA in polymorphonuclear cells

Numbers of IE antigen-positive polymorphonuclear cells (Fig. 1d) ranged from 0 to 346 per slide and, as in mononuclear cells, were lower than the numbers of pp65-positive polymorphonuclear cells (Fig. 1c), which ranged from 24 to 1829 per slide (Table 2). In polymorphonuclear cells these viral antigens were also located in the (peri)nuclear region. In contrast to mononuclear cells, polymorphonuclear cells seemed to contain less IE antigen than pp65 per cell, since the latter signal was usually considerably stronger. In addition, a few endothelial cells showing cytomegaly were seen in these polymorphonuclear leukocyte fractions. As expected, these endothelial cells contained both IE and pp65 antigen and mRNA.

Polymorphonuclear cells that contained IE mRNA were also present (Fig. 2e, g, h). However, the number of IE mRNA-positive polymorphonuclear cells was lower than the number of IE antigen-positive polymorphonuclear cells and circulating cytomegalic endothelial cells present per slide.

Table 2. Numbers of HCMV antigen- and mRNA-positive polymorphonuclear cells and circulating cytomegalic endothelial cells present per slide

<table>
<thead>
<tr>
<th>Patient</th>
<th>HCMV antigen</th>
<th>HCMV mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IE</td>
<td>pp65</td>
</tr>
<tr>
<td>RTx*</td>
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<td>24</td>
</tr>
<tr>
<td>2 RTx</td>
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<td>136</td>
<td>920 (+1 EC)</td>
</tr>
<tr>
<td>AIDS 5</td>
<td>346</td>
<td>1829</td>
</tr>
<tr>
<td>LTx-1†</td>
<td>93</td>
<td>496</td>
</tr>
<tr>
<td>LTx-2</td>
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<td>145</td>
</tr>
<tr>
<td>LTx-3</td>
<td>33</td>
<td>245</td>
</tr>
</tbody>
</table>

* RTx, Renal transplant recipient.
† EC, Cytomegalic endothelial cells.
‡ LTx, Lung transplant recipient investigated at three timepoints.

nuclear cells present in the same patient. As in mononuclear cells, no pp65 mRNA was detectable in polymorphonuclear cells and the strong hybridization signal in the few endothelial cells present in this cell fraction...
Fig. 2. Mononuclear (a to d) and polymorphonuclear (e to h) leukocytes of a patient with an active HCMV infection. Leukocytes were hybridized with digoxigenin-labelled antisense IE mRNA-specific probes, followed by hybrid detection and examination. Panels (a), (c), (d) and (e), (g), (h) show IE mRNA-containing leukocytes as demonstrated with bright-field microscopy; (b) and (f), fluoromicrographs of the leukocytes present in (a) and (e) respectively, after DAPI counterstaining to detect the IE mRNA-negative leukocytes. In (a), (b), (e) and (f), the bar represents 9 µm; in (c), (d), (g) and (h) (higher magnification), the bar represents 6 µm.

showed that pp65 mRNA, if present, would probably have been detected.

**Control experiments**

Several controls were included to ensure the sensitivity and specificity of the in situ hybridization procedure. In each experiment, HCMV-infected (laboratory strain Ad169) human embryonic lung fibroblasts were used as positive control cells. Both IE and pp65 mRNA were present in late-stage HCMV-infected fibroblasts, harvested 5 days post-infection (p.i.; Fig. 4a, b), as
Fig. 3. Circulating cytomegalic endothelial cells present in the mononuclear leukocyte fraction of a patient with an active HCMV infection. Cells were hybridized with digoxigenin-labelled antisense IE mRNA-specific probes (a, c and e) or with digoxigenin-labelled antisense pp65 (lower matrix protein ppUL83) mRNA-specific probes (b, d and f) followed by hybrid detection and examination. IE mRNA-containing (a and e) and pp65 mRNA-containing (b and f) circulating cytomegalic endothelial cells, demonstrated with bright-field microscopy; (c) and (d) are fluoromicrographs of the cells present in (a) and (b) respectively, after DAPI counterstaining (nucleus in d is partly obscured by the dark-blue precipitate, shown in b and indicating the presence of pp65 mRNA). In (a) to (d), the bar represents 9 μm; in (e) and (f), the bar represents 6 μm.
Fig. 4. Late-stage HCMV-infected human embryonic lung fibroblasts, hybridized with digoxigenin-labelled antisense IE mRNA-specific probe (a), with digoxigenin-labelled antisense pp65 (lower matrix protein ppUL83) mRNA-specific probe (b) and, as a negative control, with IE and pp65 digoxigenin-labelled sense probes (c) and (d) respectively. Hybridization was followed by hybrid detection and examination with a bright-field microscope. The bar represents 20 μm.

demonstrated by a distinct and reproducible cytoplasmic hybridization signal with IE and pp65 antisense RNA probes. In addition, it should be noted that IE mRNA and pp65 mRNA could be detected in HCMV-infected fibroblasts as early as 8 h p.i. (data not shown). Together, these results indicate an acceptable and comparable sensitivity. None of the probes ever hybridized to uninfected fibroblasts or to mononuclear or polymorphonuclear leukocytes of a healthy HCMV-seronegative volunteer, indicating the specificity for viral mRNAs.

Furthermore, in each experiment a hybridization with sense RNA probes was included as a negative control. These probes were complementary to the antisense (IE and pp65 mRNA-specific) probes, and had been transcribed and fractionated under the same conditions. None of these sense RNA probes was ever found to hybridize with any of the cell fractions or fibroblasts (Fig. 4c, d).

Finally, RNase treatment of slides prior to in situ hybridization abolished every signal observed in untreated slides, indicating the specific detection of viral messenger RNAs.

Discussion

The aim of this study was to investigate whether the presence of pp65 in peripheral blood leukocytes during an active HCMV infection, as detected with the HCMV antigenaemia assay, was due to transcription of the viral genome in these cells and de novo synthesis of this protein, or might represent passively or actively acquired pp65.

Leukocytes of immunocompromised patients with an active HCMV infection were studied for HCMV pp65 and IE antigen expression, followed by pp65 and IE mRNA detection. The use of a non-radioactive in situ hybridization procedure, with a relatively mild fixation and hybridization protocol and without a long exposure time, proved to be crucial in this study. It ensured the maintenance of a good morphology of the investigated leukocytes throughout the procedure and allowed the
proper identification of polymorphonuclear, mononuclear and circulating late-stage HCMV-infected endothelial cells. The latter, which were shown to contain IE and early HCMV transcripts and most probably also contain late HCMV transcripts, were present in both the mononuclear and polymorphonuclear leukocyte fractions from patients with an active HCMV infection.

Results showed that both viral antigens were present in mononuclear and polymorphonuclear leukocytes during an active HCMV infection. As demonstrated before, the pp65-positive leukocytes outnumbered the IE antigen-positive leukocytes (Grefte et al., 1992). However, although IE mRNA was readily detected in both mononuclear and polymorphonuclear leukocytes, no pp65 mRNA was detected in either cell type. In contrast, pp65 mRNA could readily be detected in the circulating late-stage HCMV-infected endothelial cells that were present in the same cytocentrifuged preparations. Also, pp65 mRNA could be detected in HCMV Ad 169-infected fibroblasts as early as 8 h p.i., when this early late gene was expressed at a low level, indicating that the sensitivity of the in situ hybridization procedure would be high enough to detect even low levels of pp65 transcription in peripheral blood leukocytes.

In addition, the results obtained with the circulating late-stage HCMV-infected endothelial cells, and with the in vitro HCMV-infected fibroblasts, demonstrate the tight coupling of transcription and translation of these HCMV genes in these cells. High levels of pp65 were accompanied by large amounts of pp65 messenger. Alternatively, low levels of IE antigen were reflected by the presence of only small amounts of IE messenger. The absence of detectable amounts of pp65 mRNA in leukocytes during an active HCMV infection, when much pp65 protein is detectable, also suggests that the presence of HCMV pp65 in leukocytes does not result from pp65 gene transcription and de novo protein synthesis in these cells but is more likely to be the result of uptake of this protein. Also, the observation that IE mRNA could easily be detected in leukocytes, although IE antigen was present in much lower amounts than pp65 antigen in these cells, gives additional support to this argument.

Finally, the previously reported finding that two other early late HCMV antigens, the DNA-binding protein p52 and the glycoprotein H homologue, were not present in peripheral blood leukocytes during an active HCMV infection is further evidence that viral gene expression is limited in these cells (Grefte et al., 1992).

Nevertheless, the present findings demonstrate that a restricted level of viral gene expression does occur in mononuclear and polymorphonuclear leukocytes. Monocyte-like cells (Fig. 2d), small lymphocyte-like cells (Fig. 2c) as well as terminally differentiated polymorphonuclear leukocytes (Fig. 2e, g, h) were shown to contain HCMV IE mRNA during an active HCMV infection. However, the numbers of IE mRNA-positive polymorphonuclear leukocytes during an active HCMV infection were lower than the numbers of IE antigen-positive polymorphonuclear leukocytes (Table 2). This is in contrast to the situation in mononuclear leukocytes (Table 1). Since polymorphonuclear cells contain lower amounts of IE antigen than mononuclear cells, less mRNA is probably present per cell. This may have been below the detection limit of the in situ hybridization procedure in some of the polymorphonuclear cells.

These findings confirm and extend those of Dankner et al. (1990), who demonstrated IE mRNA to be present predominantly in monocytes and polymorphonuclear leukocytes during an active HCMV infection by using radioactive in situ hybridization. However, in contrast to our findings, these authors also claimed to have found early late viral transcripts in these leukocytes. Unfortunately, they did not show this in the accompanying figures. In our view, it may have been that the early late mRNA signal they found was in fact present in the circulating late-stage HCMV-infected endothelial cells which must have been present in these leukocyte fractions, but were not recognized as such at the time, and therefore did not provide an unequivocally clear picture. In addition, Turtinen et al. (1987), by using the same technique, also found IE mRNA to be present in monocytes, but they could not detect IE mRNA in polymorphonuclear leukocytes. Since our findings demonstrated less IE mRNA in polymorphonuclear leukocytes than in mononuclear leukocytes, their negative results observed with these cells may have been caused by a slightly less sensitive assay. Together, the described findings demonstrate that the viral genome is present and transcriptionally active in both polymorphonuclear and mononuclear cells, suggesting that these cells, in addition to the circulating cytomegalic endothelial cells (Grefte et al., 1993a, b), may play a role in the dissemination of the virus. Although no pp65 mRNA was found in mononuclear cells, it should be noted that this does not exclude the possibility that monocytes, upon differentiation into macrophages (which normally occurs outside the blood vessels), may become able to support a complete viral replicative cycle, and produce infectious virus. Evidence from in vitro experiments in support of this hypothesis is accumulating (Taylor-Wiedeman et al., 1991; Lathey & Spector, 1991; Ibanez et al., 1991; Sinclair et al., 1992; Maciejewski et al., 1993).

The presence of IE mRNA in polymorphonuclear cells (Gerna et al., 1992; Bitsch et al., 1993) and mononuclear cells (Bitsch et al., 1993), and of mRNA encoding late HCMV proteins (Bitsch et al., 1993; Gozlan et al., 1993)
HCMV mRNA detection in leukocytes

in these leukocyte fractions from patients with an active HCMV infection has also been demonstrated by RT–PCR. However, the leukocytes in these RT–PCR studies were isolated via density gradient centrifugation or starch or dextran sedimentation prior to RNA extraction, and our results demonstrate that circulating late-stage HCMV-infected endothelial cells may well have been present in these cell fractions. Therefore, the cellular origin of the viral mRNAs detected with this technique cannot be stated with certainty. In our view, the detection of late viral transcripts in these RT–PCR studies might well have been caused by the presence of (remnants of) these late-stage HCMV-infected endothelial cells.

Taken together, the described observations support the hypothesis that the presence of pp65 in leukocytes during an active HCMV infection, in contrast to the presence of IE antigen in these cells, does not result from gene transcription and subsequent de novo protein synthesis. Instead, the phagocytic leukocytes have probably acquired this abundant viral protein from their surroundings in an active or passive way. The site where pp65 may be acquired by leukocytes during an active HCMV infection (i.e. where pp65 is produced in large amounts and where it is accessible to leukocytes) could be the productively HCMV-infected endothelial cells (either circulating or still attached to the vascular wall)). These cells are present during an active HCMV infection and have been shown to contain high amounts of pp65 and pp65-containing dense bodies (Greffe et al., 1993a, b). However, at present, it cannot be excluded with certainty that minor levels of pp65 mRNA are present in peripheral blood leukocytes. To clarify this, future experiments will involve the use of in situ RT–PCR with pp65-specific primers on leukocytes from patients with an active HCMV infection.

In conclusion, HCMV IE mRNA, IE antigen and the lower matrix protein pp65 were demonstrated to be present during an active HCMV infection in peripheral blood mononuclear and polymorphonuclear leukocytes, whereas pp65 mRNA could not be detected in these cells. In contrast, both mRNAs and antigens were found to be present in circulating late-stage HCMV-infected endothelial cells. Thus, a restricted level of viral gene expression does occur in mononuclear cells and polymorphonuclear cells. The presence of the lower matrix protein pp65 (ppUL83) in these cells, as detected with the HCMV antigenemia assay, is however most probably the result of uptake of pre-existing protein by the phagocytic leukocytes, rather than pp65 gene transcription and de novo protein synthesis.

In our view, at least three kinds of cells that may be involved in HCMV dissemination can be distinguished in the blood during an active infection, late-stage HCMV-infected endothelial cells, mononuclear and polymorphonuclear leukocytes displaying restricted viral gene expression, and mononuclear and polymorphonuclear leukocytes that have acquired the viral matrix protein pp65 from an as yet unknown source.

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