Direct Infection of CD34⁺ Progenitor Cells by Human Cytomegalovirus: Evidence for Inhibition of Hematopoiesis and Viral Replication

By Mojgan Movassagh, Joël Gozlan, Brigitte Senechal, Claude Baillou, Jean-Claude Petit, and François M. Lemoine

We successfully infected fluorescence-activated cell–sorted CD34⁺ cells from normal cord blood by the human cytomegalovirus (HCMV) laboratory strain Towne. An inhibitory effect of HCMV on clonogenic myeloid progenitors was observed in primary methylcellulose cultures. After an initial 7-day liquid culture of CD34⁺-infected cells, this inhibition was further amplified in secondary methylcellulose cultures, then involving both the myeloid and erythroid lineages. Under these conditions, viral DNA was detected both in erythroid and myeloid colonies using the polymerase chain reaction (PCR), but reverse transcription PCR (RT-PCR) failed to detect viral RNA. In contrast, when CD34⁺-infected cells were maintained in liquid suspension, both immediate, early, and late transcripts were detected as soon as day 3. In addition, viral production was demonstrated in the culture supernatants, thus confirming that a complete viral cycle occurred under liquid conditions. Furthermore, by resorting cells into CD34⁺ and CD34⁻ fractions, we showed by RT-PCR that viral replication took place in cells still expressing CD34 antigen, whereas no RNA was found in more differentiated cells that had subsequently lost their CD34 antigen. These findings suggest that HCMV replication can occur at the early steps of progenitor differentiation and may be involved in the viral-induced myelosuppression.

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sorted at 1,500 to 2,000 cells per second and collected in IMDM containing 50% FCS. In these conditions, the purity of sorted cells, assessed by analyzing an aliquot of cells immediately after sorting, attained more than 96% (range, 96% to 98.5%).

**Infection of CD34+ cells by HCMV.** In initial experiments, CD34+ cells were incubated with HCMV at different multiplicity of infection (MOI) values, ranging from 10 to 10-2 pfu/cell in IMDM + 10% FCS for 2 hours at 37°C. After determination of the optimal MOI, further experiments were performed at 1 pfu/cell. After infection, the cells were washed in phosphate-buffered saline (PBS) five times to remove unattached and passively adsorbed virus, counted, and resuspended in IMDM plus FCS. To control the efficiency of the washing procedure, viral detection was performed by polymerase chain reaction (PCR; see below) after the last washing. As negative controls, cells were mock-infected under the same conditions using either inactivated virus prepared by heating virus stocks at 56°C for 30 minutes or by using culture medium alone.

**Hematopoietic progenitor assay.** The number of clonogenic hematopoietic progenitors was assayed in methy1cellulose cultures as described elsewhere. Briefly, 1,500 to 3,000 infected and uninfected CD34+ cells were plated in 35-mm dishes (Greiner, Frickenhausen, Germany), in 1.1 mL of IMDM containing 0.8% methylcellulose (Fluka 4000, Buchs, Switzerland), 30% FCS, 1% bovine serum albumin, 100 μmol/L 2-mercaptoethanol (Sigma, St Louis, MO) 2 mmol/L glutamine, 100 μmol/L penicillin, 100 μg/mL streptomycin, 3 μmol/L recombinant human erythropoietin (rhEpo; Boehringer, Mannheim, Germany), 100 μg/mL rh interleukin-3 (rhIL-3; Genzyme, Boston, MA), 200 μg/mL rh granulocyte-macrophage colony-stimulating factor (rhGM-CSF; Genetics Institute, Cambridge, MA).

Duplicate cultures were incubated in a humidified atmosphere containing 5% CO2 in air at 37°C. BFU-E and CFU-GM were counted under an inverted microscope, after 16 to 18 days culture. Individual colonies were plucked and killed for PCR analysis (see below).

**Incubation of CD34+ in liquid culture.** After infection, purified CD34+ cells were cultured in suspension in IMDM, supplemented with 10% FCS, 100 μg/mL rhIL-3, 3 μg/mL rhEpo, 200 μg/mL rhGM-CSF, 50 ng/mL rh granulocyte-CSF (rhG-CSF; Rhou-Poulenc, Ivry, France) and stem cell factor (rhSCF; Amgen, Thousand Oaks, CA), and 100 μg/mL rhL-1 (Genzyme) and rhL-6 (a gift from Dr L. Aarden, Red Cross Hospital, Amsterdam, Netherlands). In this culture system, CD34+ cells can optimally proliferate, differentiate, and generate clonogenic progenitors in secondary methylcellulose assays. In a first set of experiments, PCR analysis of viral genes (see below) in cultured cells and detection of viral particles in the culture supernatant were carried out on days 3, 5, and 7. In a second set of experiments performed after 7 days of liquid culture, cells were counted, plated in methylcellulose to determine the effects of HCMV on the formation of clonogenic hematopoietic progenitors, and/or resorted into CD34+ and CD34− cell fractions, plated in replicate liquid cultures and maintained as above for an additional period of 7 days. In these secondary liquid cultures, viable cells from the different fractions were counted at days 3, 5, and 7 and compared with control experiments (noninfected cells). As described below, viral genes in these resorted cell fractions were detected by PCR analysis.

**PCR.** PCR was used to detect genomic viral DNA and reverse transcription-PCR (RT-PCR) for detection of immediate-early and late viral transcripts in infected cells and colonies.

Two sets of oligonucleotides were synthesized in the HCMV genome (Table 1): the first, located in the late gene encoding the major capsid protein (MCP), was used for viral DNA and late messenger RNA (mRNA) detection; and the second, located in the major immediate-early 1 (MIE1) antigen-encoding gene and flanking the exon 3-4 splice junction site was used for detection of viral immediate-early mRNA. PCR products from the MIE1 gene differed in length between DNA (395 bp) and complementary DNA (cDNA; 240 bp) samples. Because no introns have been reported in the late gene, expected lengths of DNA and cDNA PCR products are the same (263 bp). Two other sets of oligonucleotides were used, located in two cellular genes, coding for the β globin and the β2 microglobulin proteins, to check the quality of the DNA and RNA extractions.

PCR and RT-PCR were performed as described before, with slight modifications. Briefly, DNA was extracted from cells after a rapid lysis in presence of Nodinset P40 (NPw), Tween 20, and proteinase K and was amplified in the following reaction mix: 50 pmol of primers, 10 mmol/L Tris-HCl pH 8.3, 1.5 mmol/L MgCl2, 0.2 mmol/L of each dNTP, and 1.25 U of cloned Taq polymerase (Perkin Elmer Cetus, Norwalk, CT). Samples were covered with 100 μL of paraffin oil, denatured for 5 minutes at 94°C, and were amplified for 40 cycles in a thermal cycler as follows: 1 minute of denaturation at 94°C, 1 minute of annealing at 60°C, and 1 minute of polymerization at 72°C. This last step was extended to 10 minutes in the last cycle to ensure complete amplification.

Total RNA was extracted from cells with the RNAzol RNA extraction system (Bioprobe, Paris, France) and resuspended in 33 mL of diethyl-pyro-carbonate-treated water. Eight microliters was directly amplified in the MCP-encoding gene, without the RT step, to check that RNA extraction was free of contaminating DNA, whereas cDNA was synthesized from 25 μL of RNA with 100 U of murine Moloney reverse transcriptase (Life Technologies, Paisley, UK) and 100 pmol/L of random hexamers (pdN6; Pharmacia, Uppsala, Sweden), in a final volume of 45 μL. Thereafter, cDNA was divided into three parts and separately amplified for the two viral sequences tested and for the βg microglobulin control gene, by the same PCR procedure as described above.

Twenty-five microliters of the amplified products was analyzed by electrophoresis on 2% agarose gel, Southern blotting and probing with the 3P kinase-labeled oligoprobe (CMCP3 or IES3). To determine whether specimens were suitable for nucleic acid amplification, all the samples were controlled by amplification of a cellular gene, using primers flanking the β globin gene for DNA detection, and primers located in the β2 mi.-

**Detection of HCMV p65 antigen.** Immunocytochemistry was performed as previously described to detect the p65 lower matrix protein HCMV antigen in the nucleus of infected cells. Infected or mock-infected cells (50,000 to 100,000 per slide) were collected after infection and centrifugated onto glass slides. The fixation was performed in a solution of 5% formaldehyde in PBS for 10 minutes, followed by an additional step of cell permeabilization, using a solution of 0.5% NP40 in PBS, for 10 minutes. Slides were then incubated for 30 minutes at 37°C with a MoAb (clone IC3: Argène), washed twice in PBS−2% FCS, and incubated with a secondary antibody, an FITC-conjugated F(ab)2 fragment of sheep antimouse IgG. Cells were counterstained with 0.1% Evans Blue.

**Virus recovery.** To determine the presence of infectious particles in culture supernatants, specimens were inoculated into 24-well plates containing confluent monolayers of HEF, at various times postinfection. Plates were then centrifuged at 2,300g for 45 minutes and incubated at 37°C for 48 hours. An immediate-early HCMV antigen was then detected by an immunoperoxidase reaction using E13 MoAb as described elsewhere.

**Statistical analysis.** Results are expressed as means ± SEM. Comparisons between CMV-infected cells and uninfected cells were

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HUMAN CMV INFECTION OF CD34\(^+\) PROGENITOR CELLS

RESULTS

**HCMV infection of CD34\(^+\) cells.** The initial experiments were designed to determine optimal conditions for HCMV infection of CD34\(^+\) cells. Thus, cells were incubated with the Towne strain for 2 hours at an MOI value of 10 to 10\(^{-3}\) infectious particles per cell or with medium alone (negative control). Thereafter, amplification of HCMV DNA was performed after intensive washing. The results of one representative experiment out of five (Fig 1) showed that HCMV DNA could be detected by PCR in infected cells at an MOI value of 10 to 10\(^{-2}\) infectious particles per cell. Under these conditions, no viral DNA was detected in the supernatant of the last washing (data not shown).

The HCMV matrix phosphoprotein pp65 is known for its rapid uptake and migration into the nucleus of infected HEF cells. Using immunocytochemistry, the pp65 could be detected as expected in the nuclei of fibroblasts 2 hours after infection, but we were unable to detect its nuclear expression in CD34\(^-\)infected cells, even at high virus-to-cell ratio (up to 10 pfu per cell). Longer exposure (12 hours) of CD34\(^+\) cells to HCMV did not change the results (data not shown).

**Effects of HCMV on the growth of CD34\(^-\) progenitor cells in semisolid cultures.** To evaluate whether HCMV might disturb the clonogenic potential of hematopoietic progenitors, highly enriched CD34\(^+\) cells were incubated with HCMV at various MOI values (10 to 10\(^{-2}\) infectious particles per cell) as described above and then were plated in methylcellulose cultures. The results from six separate experiments, presented in Table 2, show that HCMV at high MOI values (1 to 10) significantly inhibited (P < .05) the formation of myeloid, but not of erythroid colonies. No noticeable change in the morphological aspect of the colonies was observed.

Molecular analysis of HCMV genome after gene amplification of DNA from pooled BFU-E and CFU-GM (Fig 2) consistently shows the presence of viral DNA at MOI values of 10, 1, and 10\(^{-3}\) infectious particles per cell, whereas at an MOI value of 10\(^{-2}\) infectious particles per cell, the results were negative in most of the experiments. When colonies from CD34\(^+\) cells infected at an MOI value of 1 infectious particle per cell were individually analyzed, the presence of viral HCMV DNA was detected in 66% and 88% of 24 BFU-E and 26 CFU-GM colonies, respectively. However, RT-PCR using cDNA synthesized from RNA preparations failed to detect immediate-early or late viral transcripts (data not shown). This pattern of detection of viral nucleic acids suggest that HCMV remains latent within these colonies.

**Effects of HCMV on the growth of CD34\(^+\) cells in liquid culture.** To evaluate the effects of HCMV on the proliferation and differentiation of CD34\(^+\) cells, the latter were incubated with HCMV at 1 MOI and seeded in liquid culture in the presence of a combination of seven growth factors for 7 days, as described in Materials and Methods. The results from five separate experiments after 7 days of culture showed that, compared with noninfected cultures, the number of viable cells and the absolute number of CD34\(^+\) cells was sig-

**Table 1. Primers and Probes for HCMV DNA and mRNA Detection**

<table>
<thead>
<tr>
<th>Primers or Probes</th>
<th>Location*</th>
<th>Sequence (5' to 3')</th>
<th>Product Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMCP1</td>
<td>1198-1217</td>
<td>GTGATCGTCGCTGCGAAAA</td>
<td>263 (mRNA or DNA)</td>
</tr>
<tr>
<td>CMCP2</td>
<td>1461-1442</td>
<td>GAGGCCTGCGAAGTCTA</td>
<td></td>
</tr>
<tr>
<td>CMCP3 (probe)</td>
<td>1326-1364</td>
<td>GCCGGAGATCGCGTTACAC</td>
<td></td>
</tr>
<tr>
<td>IES 1</td>
<td>338-357</td>
<td>AACGAGTGACCGAGGATGC</td>
<td>240 (mRNA)</td>
</tr>
<tr>
<td>IES 2</td>
<td>571-552</td>
<td>ACCTTACCTCTCGAAAGGC</td>
<td>395 (DNA)</td>
</tr>
<tr>
<td>IES 3 (probe)</td>
<td>424-453</td>
<td>GTGCGGATAGAATCAAGG</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: bp, base pair; CMCP, major capsid protein encoding gene; IES, immediate-early 1 encoding gene.

* Numbered from the presumed start site of the mRNA; sequence data from Chea et al.\(^{16}\) and Stenberg et al.\(^{17}\)

![Fig 1. Detection of viral DNA in HCMV-infected CD34\(^+\) cells by PCR. Amplified products (263 bp) were blotted onto a nylon membrane and probed with the \(^{32}\)P-labeled oligonucleotide CMCP3. One of five experiments is shown. HCMV-infected HEF fibroblasts were used as a positive control; noninfected HEF fibroblasts were used as a negative control. Results are shown for CD34\(^+\) cells infected at MOI values varying from 10 to 10\(^{-2}\) infectious particles per cell.](https://www.bloodjournal.org)

![Table 2. Inhibition of the Colony Formation After Infection of CD34\(^+\) Cells by HCMV](https://www.bloodjournal.org)

<table>
<thead>
<tr>
<th>MOI</th>
<th>Percentage of Inhibition</th>
</tr>
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<tbody>
<tr>
<td>10</td>
<td>24 ± 3*</td>
</tr>
<tr>
<td>1</td>
<td>29 ± 4*</td>
</tr>
<tr>
<td>10(^{-1})</td>
<td>13 ± 8</td>
</tr>
<tr>
<td>10(^{-2})</td>
<td>4 ± 3</td>
</tr>
</tbody>
</table>

Values are the mean ± SEM of 6 separate experiments seeded in duplicate. The absolute number of CFU-GM and BFU-E are 52 ± 12 and 145 ± 37 for 3,000 plated cells, respectively. Abbreviation: MOI, multiplicity of infection, each value represents the ratio of viable viral particles per cell.

\(* P < .05\).
Positive control
negative control

DNA extracted from 10 pooled BFU-E or CFU-GM was amplified by PCR using CMCP1 and CMCP1 primers. Then, amplified products (263 bp) were blotted onto a nylon membrane and probed with the "P-labeled oligonucleotide CMCP3. HCMV-infected HEF fibroblasts were used as positive control; noninfected pooled BFU-E were used as negative control. Similar results were obtained with noninfected pooled BFU-E. Viral DNA was detected in BFU-E and CFU-GM colonies generated from CD34' progenitor cells infected with HCMV at MOI values of 10, 1, and 10⁻¹ infectious particles per cell. At an MOI value of 10⁻² infectious particles per cell, no HCMV DNA was detected (not shown).

Table 3. Inhibition by HCMV of the Proliferation and Differentiation of CD34' Cells After 7-Day Liquid Culture

<table>
<thead>
<tr>
<th>No. of Experiments</th>
<th>Percentage of Inhibition*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD34' Cells</td>
</tr>
<tr>
<td>1</td>
<td>37</td>
</tr>
<tr>
<td>2</td>
<td>44</td>
</tr>
<tr>
<td>3</td>
<td>57</td>
</tr>
<tr>
<td>4</td>
<td>21</td>
</tr>
<tr>
<td>5</td>
<td>20.5</td>
</tr>
</tbody>
</table>

Noninfected or infected CD34' cells were cultured for 7 days. Then, cells were counted, stained with CD34-PE MoAb, and analyzed by flow cytometry. The absolute number of CD34' cells at day 7 was calculated as follows: % of CD34' cells x total cell number. Percentage of inhibition was calculated by dividing the results in infected cultures by the results in noninfected cultures x 100.

* P < .05.

Table 4. Inhibition by HCMV of CFU-GM and BFU-E Colonies Generated by CD34' Infected Cells After 7-day Liquid Culture

<table>
<thead>
<tr>
<th>No. of Experiments</th>
<th>CFU-GM</th>
<th>BFU-E</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>45*</td>
<td>43.5*</td>
</tr>
<tr>
<td>2</td>
<td>78*</td>
<td>54*</td>
</tr>
<tr>
<td>3</td>
<td>65*</td>
<td>80*</td>
</tr>
<tr>
<td>4</td>
<td>31*</td>
<td>55*</td>
</tr>
<tr>
<td>5</td>
<td>26*</td>
<td>28*</td>
</tr>
</tbody>
</table>

Absolute number of CFU-GM and BFU-E are 117 ± 14 and 43 ± 2 for 50,000 plated cells, respectively.

* P < .05.
† This experiment was performed with resorted CD34' cells.

DISCUSSION

HCMV-induced inhibition of hematopoiesis, suspected on the basis of clinical data, has been demonstrated in vitro by several previous studies. However, the mechanisms involved in this narrow suppression are complex and poorly understood. In particular, direct infection of hematopoietic progenitor cells remains controversial. The present study indicates that HCMV can directly infect hematopoietic progenitor cells from normal cord blood, leading to a significant inhibition of both GM and erythroid lineages; can persist in these cells throughout their proliferation and differentiation; and under some conditions, can replicate in infected progenitors.
The evidence obtained here supporting direct infection of CD34+ cells by HCMV were as follows: (1) the consistent detection of viral DNA after incubation of highly purified CD34+ cells with HCMV; (2) the persistence of viral DNA after clonogenic differentiation of infected cells in methylcellulose cultures, demonstrating that progenitors were indeed infected with HCMV in spite of the fact that the ~4% of CD34- cells contaminating the CD34+ sorted fraction could also have been infected; and (3) the extent of the viral cycle observed after proliferation and differentiation of infected CD34+ cells in liquid cultures. These arguments are indirect because we could not demonstrate the presence of
the lower matrix pp65 antigen in infected cells. However, its rapid uptake and migration in the nucleus of infected cells has been documented in human fibroblast but not in progenitor cells, except in one report in which a clinical isolate was used, without any details on the infection procedures. In addition, the immunocytochemistry technique used for detection of pp65 antigen is probably less sensitive than the other techniques used in this study, such as PCR amplification and viral recovery in the supernatant.

The impact of HCMV on hematopoietic colony formation was first investigated using a primary methylcellulose assay and then after initial culture of CD34+ cells in liquid suspension. Primary cultures demonstrated significant growth inhibition of myeloid progenitors cells at high virus-to-cell ratios (1 and 10 infectious particles per cells), whereas a slight, but nonsignificant inhibition was observed on erythroid progenitors. The use of highly purified CD34+ cells in these experiments indicates that the inhibitory effect of HCMV on clonogenic progenitors is due to a direct cell–virus interaction instead of an indirect mechanism involving stromal or accessory cells. When infected cells were cultured in suspension for 7 days before plating in methylcellulose, a more pronounced decrease in the number of myeloid colonies was observed, associated at that time with a significant inhibition of the erythroid lineage. To investigate whether this amplified effect of HCMV in liquid culture could be due to viral replication and subsequent reinfections of other cells, we tried to detect late viral transcripts and virus production in culture supernatants. Both tests were positive, and furthermore late RNA was detected within cells still expressing CD34 antigen. Nevertheless, we cannot exclude the possibility that HCMV might also induce some inhibitors of hematopoiesis only active in liquid culture, as suggested by previous reports showing the synthesis of an IL-1 inhibitor by HCMV-infected monocytes. Furthermore, a higher sensitivity to HCMV of the earliest progenitors, only clonogenic after few days in liquid culture, could also explain the increase of the myelosuppression; recent data from Sindre et al showing that HCMV-induced inhibition is more pronounced after infection of bone marrow CD34+ HLA-DR+ cells support this hypothesis.

A large percentage of the colonies generated from infected progenitors exhibited viral DNA, but failed to transcribe immediate-early or late CMV genes. These findings are in accordance with a recent report from Minton et al but contrasts with others reporting that an early HCMV promoter is active in infected colonies, as demonstrated by the detection of virus-transduced LacZ activity. This discrepancy may be explained by modifications of the enhancer sequences in the recombinant strain used, different sensitivities of the methods used for the detection of HCMV transcripts, or residual β-galactosidase activity in the virus preparations. The origin of CD34+ cells used in our study (ie, cord blood instead of bone marrow) does not explain the absence of immediate-early or late CMV transcripts because similar results were obtained using bone marrow CD34+ cells (data not shown). However, our results indicate that the virus genome can persist within these cells during their differentiation in methylcellulose culture, as previously reported.

Because analysis of colonies only investigates the subset of CD34+ cells that are able to differentiate in methylcellulose cultures, we also studied the extent of the HCMV cycle in CD34+ cells grown in liquid culture. In these conditions, we observed effective viral replication, demonstrated by detection of late mRNA, and virus production. Transcription of the late HCMV mRNA was only found in cells still expressing the CD34 antigen, whereas cells entering the differentiation process, which subsequently lose the CD34 antigen, failed to transcribe viral genes. Furthermore, the virus-induced inhibition of the cellularity was restricted to CD34+ cells (see Fig 5), indicating a specific sensitivity of this population to HCMV. These findings confirm that HCMV expression is dependent on the state of cell differentiation, but contrasts with the results observed in the monocytic cell line THP-1 and in peripheral blood monocytes, in which HCMV sustains its replication until cell differentiation in macrophage phenotype. However, the extent of viral replication at the very early steps of hematopoietic progenitor differentiation explored in our study has not been investigated before.

The pattern of viral latency in infected progenitor cells that give rise to mature colonies in methylcellulose culture or to CD34+ cells in liquid cultures, together with the effective viral replication observed in cells still expressing CD34 antigen, suggest an inhibitory role of viral replication on the differentiation processes and, in turn, on hematopoiesis. Factors that permit viral replication in some cells, and not in others, remain to be determined. Nevertheless, our data may have implications for the treatment of HCMV-induced myelosuppression, given recent reports of hematologic improvement and successful bone marrow engraftment during ganciclovir therapy of symptomatic HCMV infection.

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