

Human Cytomegalovirus Suppression of and Latency in Early Hematopoietic Progenitor Cells

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Bone marrow cells (BMC) are involved in the pathogenesis of human cytomegalovirus (HCMV) infections, and the hematopoietic cells are probable sites of HCMV latency in healthy donors. In vitro studies have indicated both a direct inhibitory effect of HCMV on proliferation and differentiation of myeloid bone marrow progenitors and an impairment of bone marrow stroma cell function by HCMV. The purpose of the present study was to establish whether the suppressing effect could be limited to subsets of immature CD34⁺ BMC and to investigate the role of immature cell populations as possible sites of HCMV latency. CD34⁺ cells from healthy HCMV-seropositive and -seronegative donors were sorted according to the expression of HLA-DR (CD34⁺HLA-DR⁺ and CD34⁺HLA-DR⁻ cells). The progenitor growth of hematopoietic progenitor cells from seronegative donors was examined by colony and single-cell assays after in vitro infection with HCMV. To determine the susceptibility of the CD34⁺ cells to HCMV infection in vitro and in vivo, cells of both subsets from seronegative and seropositive donors were analyzed for the presence of HCMV DNA by polymerase chain reaction. HCMV infection in vitro inhibited the interleukin-1 α (IL-1 α)-, IL-3-, granulocyte colony-stimulating factor-,

granulocyte-macrophage colony-stimulating factor-, and stem cell factor-induced proliferation in single-cell assays of CD34⁺ HLA-DR⁻ cells by 34%. In contrast, the colony growth of the CD34⁺HLA-DR⁺ subset was suppressed in cells from only 3 of the 8 donors. However, in vitro HCMV infection of the CD34⁺HLA-DR⁺ progenitor cells inhibited the proliferation of all donors tested when hematopoietic growth factors were used individually to promote progenitor growth. In addition, the formation of burst-forming units-erythroid and colony-forming units-granulocyte, erythrocyte, monocyte, megakaryocyte was reduced 40% to 60% by HCMV in vitro. In contrast, the growth of high proliferative potential colony-forming cells was not inhibited after in vitro HCMV infection. Furthermore, HCMV DNA was detected in both CD34⁺HLA-DR⁻ and CD34⁺HLA-DR⁺ progenitors from in vitro-infected HCMV-seronegative donors and cells from HCMV-seropositive donors. Taken together, the early progenitors defined as CD34⁺HLA-DR⁻ and CD34⁺HLA-DR⁺ are directly suppressed in their proliferation by HCMV in vitro, and hematopoietic stem cells are also sites of HCMV latency in healthy HCMV-seropositive donors.

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HUMAN CYTOMEGALOVIRUS (HCMV) infection may cause severe disease in immunocompromised patients.^{1,2} Blood transfusions and organ transplantations from HCMV-seropositive donors to HCMV-seronegative patients may lead to fatal HCMV infections in the recipients.³⁻⁸ Especially in patients receiving bone marrow transplants, HCMV infection has been related to delayed engraftment and even graft failure.⁹⁻¹³ Furthermore, bone marrow suppression with leukopenia is a common feature of HCMV infectious disease, particularly in the immunocompromised patients and newborn infants.¹⁴⁻¹⁷

The majority of the adult population is HCMV-seropositive (60% to 80%) and carriers of latent HCMV.¹⁸ Therefore, the pool of HCMV-seronegative donors for blood transfusions, bone marrow, and organ transplantations is limited. In the case of bone marrow transplants, the demand for an HLA-match sometimes makes the transplantation of cells

from a seropositive to a seronegative donor necessary, causing a risk for transfer of HCMV.

HCMV has been shown to be able of productive infection in endothelial-like and fibroblastoid stroma cells of the bone marrow.¹⁹ Given that HCMV may be transferred by blood transfusions and bone marrow grafts, the suspicion emerged that cells of the hematopoietic system could possibly be sites of HCMV latency in healthy, HCMV-seropositive persons.²⁰⁻²² It has been shown that by leukocyte depletion one may prevent the transfer of HCMV during blood transfusions,^{23,24} and screenings by polymerase chain reaction (PCR) have detected HCMV DNA in mononuclear cells from peripheral blood.^{25,26} However, latent virus has only been successfully reactivated and isolated from monocytes stimulated to differentiate.²⁷

HCMV has been shown to inhibit the proliferation and differentiation of hematopoietic precursors in vitro.²⁸⁻³⁰ We and others have shown that CD34⁺ bone marrow cells may be infected in vitro^{20,31} and that this infection correlated with a growth inhibition of hematopoietic progenitors by approximately 40%. CD34⁺ bone marrow cells may be further separated into immature and mature hematopoietic subsets according to the expression of other surface markers such as major histocompatibility complex (MHC) class II (HLA-DR),³²⁻³⁵ CD38,³⁶ CD33,³⁷ and Thy-1.^{38,39} To examine the presence of latent HCMV in immature progenitor cells and to study the susceptibility of hematopoietic progenitors to HCMV in vitro, we examined the latency of HCMV in and the growth inhibitory effect of HCMV on CD34⁺ bone marrow cells separated according to the expression of HLA-DR. In addition, we examined the in vitro effect of HCMV infection on the colony formation of CD34⁺ burst-forming units-erythroid (BFU-E), colony-forming units-granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM), and

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high proliferative potential colony-forming cells (HPP-CFC).

MATERIALS AND METHODS

Cell preparation. Preservative-free heparinized bone marrow samples from healthy adults that were previously screened for HCMV serostatus in an IgG enzyme immunoassay (EIA) CMV-G assay (Abbott, Chicago, IL), were obtained by aspiration using protocols approved by the Institutional Review Board. CD34⁺ cells were highly enriched by immunomagnetic separation, as described in detail elsewhere.⁴⁰ Briefly, low-density mononuclear cells (1,077 g/mL; Lymphoprep; Nycomed Pharma, Oslo, Norway) were incubated with Dynabeads M-450 (Dynal, Oslo, Norway) coupled with a monoclonal antibody (MoAb 561) against CD34.⁴⁰ Rosetting cells were collected with a magnet and cells were detached from the beads by incubation with a polyclonal antiserum against murine Fab fragments (DETACHaBEAD; Dynal). Immunomagnetically purified CD34⁺ cells (purity >90%) were labeled with fluoresceinated anti-CD34 and anti-HLA-DR MoAbs. Fluorescence-activated cell sorting was performed on a Coulter Epics Elite flow cytometer (Coulter Electronics, Hialeah, FL), as previously described.⁴¹ The purity of the sorted cell fractions was greater than 98%.

MoAbs. Fluorescein isothiocyanate (FITC)-labeled anti-HLA-DR, phycoerythrin (PE)-labeled anti-HPCA-2 (CD34), and Simultest Control (IgG1 FITC + IgG1 PE) were used for fluorescence-activated cell sorting. The MoAbs were purchased from Becton Dickinson Immunocytometry Systems (San Jose, CA). FITC-labeled anti-HCMV antibody E-13 against IE1 and IE2 gene products was purchased from Seralab (Sussex, UK).

Virus. Highly purified virus stocks from low passage human embryonic fibroblast (HE) cells were used. Low-passage HCMV from a clinical isolate was propagated at low virus to cell ratios to minimize generation of defective particles. The supernatant was harvested when extensive (>90%) cytopathogenic effect was evident and was centrifuged briefly at 1,500 rpm to remove cell debris. The supernatant was centrifuged at 20,000 rpm for 90 minutes. The pellet was placed on a gradient of potassium tartrate-glycerol prepared in 0.05 mol/L Tris-HCl, pH 7.4, 0.10 mol/L NaCl (TN buffer); centrifuged in a ultracentrifuge for 60 minutes; and harvested as previously described.^{42,43} The virus was resuspended in medium, titrated in low-passage HE cells, and frozen in aliquots at -70°C. Mock preparations were made simultaneously. Virus stocks were tested for endotoxin contamination (QCL-1000 chromogenic endpoint LAL-kit; Bio Whittaker, Walkersville, MD), and the HE cells were screened for mycoplasma. Mycoplasma testing was performed by DNA staining with bisbenzimidides (Hoechst 33258; Behring Werke AG, Marburg, Germany).

Inoculation of hematopoietic cells with HCMV. CD34⁺, CD34⁺HLA-DR⁺, and CD34⁺HLA-DR⁻ bone marrow cells from HCMV-seronegative donors were washed once in medium, resuspended, and infected with mock or HCMV at 1.0 pfu per cell for 16 hours at 37°C by continuous movement. The cells were washed twice. The viability was ensured by trypan-blue exclusion.

Cytokines. Purified recombinant human interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) were obtained from Sandoz Pharma Ltd (Basel, Switzerland). Purified recombinant human IL-1 α (IL-1 α) was provided by Dr S. Lyman (Immunex, Seattle, WA). Purified recombinant human stem cell factor (SCF) and granulocyte colony-stimulating factor (G-CSF) were provided by Dr I. McNiece (Amgen Corp, Thousand Oaks, CA).

Colony growth. Separated bone marrow cells were resuspended in Iscove's modified Dulbecco's medium (IMDM) supplemented with 15% fetal calf serum (GIBCO, Life Technologies Ltd, Paisley, UK), 1% L-glutamine, and 1% detoxified bovine serum albumin

(Sigma Chemical Co, St Louis, MO) and antibiotics (complete IMDM). Cells (1×10^3) were plated in 35-mm Lux petri dishes (Nunc, Inc, Naperville, IL) in 1 mL of complete IMDM, with 0.3% Seaplaque agarose (FMC Bioproducts, Rockland, ME) and predetermined optimal concentrations of G-CSF (30 ng/mL), GM-CSF (30 ng/mL), IL-1 α (10 ng/mL), IL-3 (30 ng/mL), and SCF (100 ng/mL) as single factors or in combinations as specified. Dishes were incubated in a fully humidified atmosphere at 37°C in 5% CO₂ and scored for growth of colony-forming unit-culture (CFU-C) after 14 days of incubation. The growth of HPP-CFCs was assessed after 28 days of incubation. Single-cell proliferation assays were performed in Terasaki plates (Nunc, Roskilde, Denmark) at a concentration of 1 cell per well in 20 μ L of complete IMDM containing the same combination of cytokines as mentioned above. The single-cell deposition was controlled by microscopy of the wells shortly afterwards. An average of 87% of the wells contained 1 cell, with the remaining wells containing 0 or 2 cells. The frequency of responding progenitors (>10 cells/well) was determined after 10 days of incubation with 300 wells scored per group.

Assay for effect on CFU-GEMMs and BFU-Es. Separated bone marrow cells were resuspended in Iscove's methylcellulose medium (Methocult GF H4435), which contained optimized levels of growth factors supplied with cytokines, including erythropoietin (3 U/mL), SCF (50 ng/mL), GM-CSF (20 ng/mL), IL-3 (20 ng/mL), IL-6 (20 ng/mL), and G-CSF (20 ng/mL; Stem Cell Technologies Inc, Vancouver, British Columbia, Canada). CD34⁺ cells (1×10^3) were plated in 35-mm Lux petri dishes. Scoring for growth of CFU-GM, BFU-E, and CFU-GEMM was performed after 14 to 18 days of incubation.

Detection of HCMV-DNA. Cells were harvested from an average of 20 to 30 colonies in soft agar after 28 days of incubation from both HCMV-seronegative donors infected with mock or HCMV and HCMV-seropositive donors. The cells were washed in phosphate-buffered saline (PBS) and pelleted, and the supernatant was removed. They were then stored at -70°C until PCR was performed. The pellet was prepared for PCR by the addition of 100 μ L lysis buffer containing 10 mmol/L Tris-HCl, pH 8.3, 1 mmol/L EDTA, and 300 μ g/mL proteinase K per 10^6 cells. The cell suspension was incubated for 60 minutes at 55°C. The proteinase K was heat-inactivated at 94°C for 15 minutes. The cell mixture was then cooled on ice for 5 to 10 minutes and centrifuged for 1 minute at 6,000g. Ten microliters of lysed supernatant was amplified in a total volume of 50 μ L reaction mixture (20 mmol/L KCl; 10 mmol/L Tris-HCl [pH 8.3]; 3 mmol/L MgCl₂; 200 μ mol/L of dATP, dGTP, dCTP, and dTTP each; 1 U AmpliTaq DNA Polymerase [Perkin Elmer PCR reagents; Roche Molecular Systems, Inc, Branchburg, NJ]; dH₂O; and 0.25 μ mol/L of each primer) in the PCR I reaction by incubation at 94°C for 1.5 minutes, 65°C for 1.5 minutes, and 72°C for 1.0 minutes for 20 cycles. PCR I mix (2.5 μ L) was added to the PCR II mix, with a total volume of 50 μ L and incubation at 94°C for 2 minutes, 55°C for 1.5 minutes, and 72°C for 2 minutes. The PCR reaction was a nested CMV PCR, which amplifies a region of the major immediate early (MIE) gene spanning from exon 4 to exon 5. The outer sense primer for PCR I was MIE4 5' CCAAGCGCCTCTGATAACCAAGCC 3', and the antisense primer was MIE5 5' CAGCACCATCCTCCTCCTCTTCTCTGG 3'.⁴⁴ The inner sense primer for PCR II was MIE4 5' CCACCCGTGGTGCCAGCTCC 3', and antisense primer was MIE5 5' CCCGCTCCTCTGAGCACCC 3'.⁴⁵ The primers were supplied by Genosys Products Medprobe A.S. (Oslo, Norway).

Detection of HCMV-RNA. Cells were harvested as for the detection of HCMV-DNA, but with the use of RNase-free buffers, tips, and tubes. Control cells from HCMV- or mock-infected permissive human embryonal fibroblasts (HE) and a semipermissive human lung adenocarcinoma cell line (A549) was prepared by the same

procedure. mRNA was isolated directly by the Dynabead mRNA Direct Kit (DynaL A.S., Oslo, Norway), with some modifications. The mRNA was eluted with 15 μ L of elution solution containing 0.5 U RNAGuard (Pharmacia-Biotech, Lilleström, Norway) per microliter and kept at 65°C for 2 minutes. The tube was then placed in the Dynal MPC (DynaL A.S.), and the supernatant containing the mRNA was transferred to a new tube and stored at -70°C until reverse transcription and PCR amplification of cDNA was performed. The reverse transcription (RT) of mRNA and PCR amplification of cDNA were both performed in the same tube and by using a thermostable DNA polymerase, rTth DNA polymerase, for both reactions. The reaction mixture, a total volume of 50 μ L, contained 25 mmol/L Bicine; 115 mmol/L potassium acetate; 8% (wt/vol) glycerin (pH 8.2); 300 μ mol/L of dGTP, dATP, dTTP, and dCTP; 2.5 mmol/L Mn(OAc)₂ solution; 5 U rTth DNA polymerase (GeneAmp EZTth RNA PCR kit; Perkin Elmer, Roche Molecular Systems Inc); dH₂O; and 0.45 μ mol/L of each primer. The RT and the cDNA amplification was performed on a GeneAmp PCR System 9600 (Perkin Elmer) for 30 minutes at 60°C, followed by a denaturation at 94°C for 5 minutes and then 50 cycles with 94°C for 40 seconds, 55°C for 15 seconds, and 72°C for 90 seconds. A nested PCR strategy was performed with 1 μ L of the primary RT-PCR product in a total volume of 50 μ L reaction mixture containing 20 mmol/L KCl; 10 mmol/L Tris-HCl (pH 8.3); 3 mmol/L MgCl₂; 200 μ mol/L dATP, dGTP, dCTP, and dTTP; 1 U AmpliTaq DNA polymerase (Perkin Elmer); dH₂O; and 0.25 μ mol/L of each primer. The PCR products were visualized by electrophoresis and staining with ethidium bromide, with the nested amplified cDNA product being 275 bp, easily distinguishable from the size of the corresponding nested HCMV-DNA product at 579 bp. The RT-PCR was a nested PCR strategy, which amplifies a region of the MIE gene spanning from exon 2 to exon 4. The outer antisense primer used for the reverse transcripts was MIE exon 4, 5'-CCGTCTGGGTATATTTTTTC-3'. This primer was used together with the outer sense primer MIE exon 2, 5'-GACACGATGGAGTCCTCTGC-3', in the primary amplification of cDNA. The inner sense primer was MIE exon 2, 5'-CAAGAGAAA-GATGGACCCTG-3', and antisense primer MIE exon 4, 5'-GTGCTCCTTGATTCTATGCC-3'.⁴⁶ The primers were supplied by Genosys Products, Medprobe A.S. (Oslo, Norway).

RESULTS

The direct effect of HCMV on the growth of CD34⁺HLA-DR⁺ and CD34⁺HLA-DR⁻ progenitor cells. HCMV infection has been correlated with delayed engraftment in bone marrow transplanted patients.⁹⁻¹³ In addition, HCMV infection in vitro has been shown to inhibit the growth of hematopoietic progenitors in colony assays.^{19,28,29,47} Because we and others recently have shown that HCMV has the ability to infect and directly inhibit the growth of CD34⁺ cells in vitro,^{20,31} it was of interest to examine whether this effect was limited to a subset of CD34⁺ cells. The most immature hematopoietic progenitor cells have been shown to lack the MHC class II antigen HLA-DR³²⁻³⁵; therefore, CD34⁺ BM cells were separated according to the expression of HLA-DR. The progenitor growth of CD34⁺, CD34⁺DR⁻, and CD34⁺DR⁺ cells was assessed in a single-cell assay. The proliferation was induced by the combination of G-CSF (30 ng/mL), GM-CSF (30 ng/mL), IL-1 α (10 ng/mL), IL-3 (30 ng/mL), and SCF (100 ng/mL), and the number of responding mock- and HCMV-infected progenitors was scored at 10 days postinfection. In accordance with previous results, HCMV inhibited the growth of CD34⁺ progenitor cells by

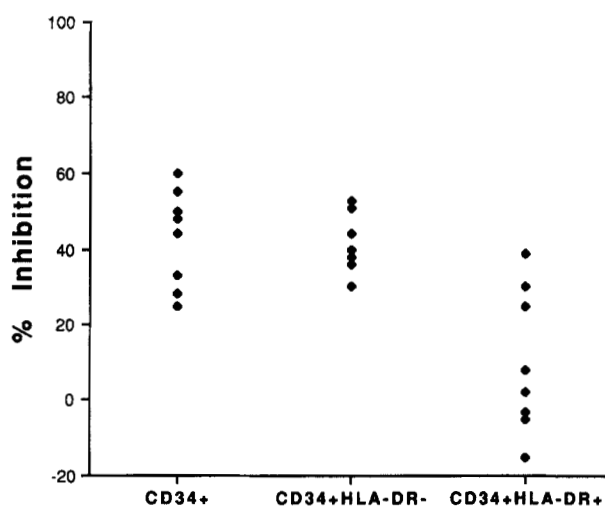


Fig 1. HCMV directly inhibits the growth of CD34⁺ bone marrow progenitors. CD34⁺, CD34⁺DR⁻, and CD34⁺DR⁺ bone marrow cells were purified as described in the Materials and Methods and infected with 1 pfu HCMV or mock per cell for 16 hours. The cells were seeded in single-cell proliferation assay as described in the Materials and Methods, and colony growth was stimulated with predetermined optimal concentrations of IL-1 α , IL-3, SCF, G-CSF, and GM-CSF. The wells were scored for proliferation after 10 days of incubation. The results are presented as the percentage of inhibition of proliferation compared with mock control of 8 experiments with at least 2,400 wells scored in each subset and an average of 40 wells with proliferation in the control in each experiment.

40% (Fig 1). Furthermore, a distinct inhibitory effect of HCMV on the progenitor growth of CD34⁺DR⁻ cells was observed (30% to 40%; Fig 1). However, the ability of HCMV to affect the growth of the CD34⁺DR⁺ progenitors varied. The growth of CD34⁺DR⁺ progenitors obtained from 3 of 8 donors was inhibited (30% to 40%) by HCMV in vitro (Fig 1), whereas the growth of CD34⁺DR⁺ progenitors from 5 donors was not affected (Fig 1). Because the CD34⁺DR⁺ cell population has been shown to contain lineage committed progenitors responsive to single hematopoietic growth factors, we examined the effect of HCMV on progenitor growth of CD34⁺DR⁺ cells in the presence of single growth factors. HCMV- and mock-infected CD34⁺ and CD34⁺DR⁺ cells were stimulated with IL-3 or G-CSF alone; with the combination of IL-3 and G-CSF; or with the combination of IL-3, G-CSF, and SCF in single-cell progenitor assays. In contrast to our observations with G-CSF-, GM-CSF-, IL-1 α -, IL-3-, and SCF-promoted progenitor growth, in the presence of single cytokines or combinations of two and three cytokines, both the progenitor growth of CD34⁺ and CD34⁺DR⁺ cells from all donors examined were inhibited by HCMV (Fig 2). Thus, HCMV can directly inhibit the proliferation of both CD34⁺DR⁻ and CD34⁺DR⁺ progenitor subsets in vitro; however, the inhibitory effect on DR⁺ progenitors depended on the growth factors used to promote progenitor growth.

The effect of HCMV on BFU-Es, CFU-GEMMs, and CFU-GMs. The hematopoietic progenitor cell population defined as CD34⁺ beside the myeloid progenitors (CFU-GM) also

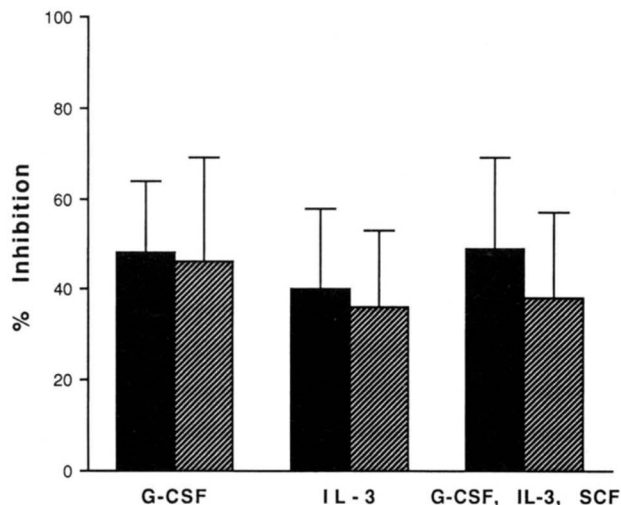


Fig 2. The direct effect of HCMV on HGF-induced CD34⁺DR⁺ hematopoietic proliferation. Progenitor cells were infected with mock or 1 pfu HCMV per cell for 16 hours, and cells were seeded in single-cell assay and scored for proliferation after 10 days of incubation. The results are presented as the percentage of inhibition compared with mock control and represent the mean (\pm SD) of four experiments with at least 1,200 cells scored in each group and an average of 40 wells with proliferation in the control in each experiment. (■) CD34⁺; (▨) CD34⁺HLA-DR⁺.

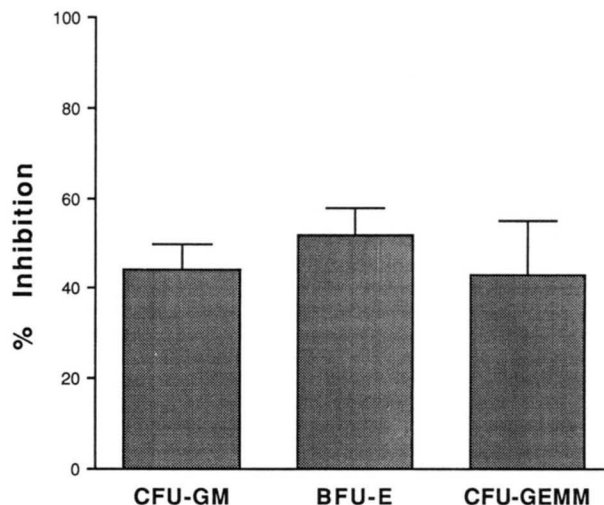


Fig 3. The effect of HCMV on erythroid (BFU-E), multilineage (CFU-GEMM), and myeloid (CFU-GM) colony formation. The cells were infected with mock or 1 pfu HCMV, and 1×10^3 cells were plated in a methylcellulose colony assay and incubated for 14 to 18 days before scoring of colony growth. The results are represented as the mean \pm SD percentage of inhibition compared with mock of three experiments. Each experiment was performed in triplicate, and the average of colonies in the mock control was 50.

contains erythroid (BFU-E) and multilineage (CFU-GEMM) progenitors. Therefore, we investigated the effect of HCMV infection on the colony formation of CD34⁺BFU-Es and CFU-GEMMs in a methylcellulose assay. The number of responding progenitors defined as CFU-GM, BFU-E, and CFU-GEMM was scored 14 to 18 days after HCMV or mock infection. Comparable to the inhibitory effect of HCMV on myeloid progenitor growth in single-cell assays, in the methylcellulose assay, HCMV inhibited the colony formation of the CFU-GMs by 40% to 60% (Fig 3). In addition, HCMV also inhibited the growth of the committed erythroid progenitors (BFU-E) and the growth of the multipotential progenitors (CFU-GEMM) by 40% to 60% (Fig 3).

The effect of HCMV on HPP-CFCs from CD34⁺ cells. HCMV highly inhibited the progenitor growth of CFU-GM, BFU-E, and CFU-GEMM in vitro and also immature progenitors characterized as CD34⁺DR⁻. Therefore, we further examined the ability of HCMV to affect the proliferation of the progenitors with high proliferative potential, defined as HPP-CFCs.^{48,49} Colonies greater than 0.5 mm in diameter from unseparated CD34⁺ and the CD34⁺DR⁺ cells were scored after 28 days of incubation. In contrast to the inhibitory effect of HCMV observed on CFU-GMs, BFU-Es, and CFU-GEMMs, HCMV infection did not affect the formation of HPP-CFCs compared with mock-infected cells (Table 1).

HCMV DNA and RNA detection in hematopoietic progenitors after in vitro HCMV infection. The inhibitory effect of HCMV on the hematopoietic progenitors may be related to binding of virus to receptors on the cell surface, uptake of virus, and/or expression of viral proteins. We investigated whether the inhibitory effect of HCMV on bone marrow cells from HCMV-seronegative donors correlated with an

uptake of virus. Therefore, both HCMV-infected and mock-infected cells harvested from colonies in soft agar derived from CD34⁺, CD34⁺DR⁻, and CD34⁺DR⁺ progenitors were screened by a nested PCR method for the presence of HCMV DNA. In all populations examined, HCMV DNA could only be detected in the HCMV-infected cells, whereas the mock-infected cells were negative (Table 2). Thus, in correlation with the ability of HCMV to inhibit progenitor growth, there was a successful uptake of HCMV in the infected cells. However, no HCMV immediate early proteins (IE1 and IE2) essential for later HCMV protein production could be detected in these cells; in addition, RT-PCR performed on the harvested colonies derived from the infected cells were negative for the presence of HCMV IE1 RNA (data not shown).

The latency of HCMV in hematopoietic progenitor cells. Because we have shown that both CD34⁺DR⁻ and

Table 1. The Effect of HCMV on HPP-CFC Formation

Donor	CD34 ⁺		CD34 ⁺ DR ⁺	
	Mock	HCMV	Mock	HCMV
1	13 \pm 1	13 \pm 2	15 \pm 1	12 \pm 1
2	14 \pm 1	15 \pm 2	18 \pm 2	19 \pm 3
3	15 \pm 0	17 \pm 3	10 \pm 1	10 \pm 0

CD34⁺ and CD34⁺DR⁺ progenitor cells were infected with 1 pfu HCMV or Mock for 16 hours and then seeded in a soft agar colony assay. Colony growth was stimulated with the combination of optimal concentrations of IL-1 α , IL-3, G-CSF, GM-CSF, and SCF, and colony growth (\geq 0.5 cm) was scored after 28 days of incubation. The results represent colony counts from 3 donors, and the colony assay was performed in duplicate.

Table 2. Detection of HCMV DNA in Cells From Harvested Colonies of In Vitro HCMV and Mock-Infected CD34⁺ Progenitors From HCMV-Seronegative Donors

Donors	CD34 ⁺ Mock	CD34 ⁺ HCMV	CD34 ⁺ HLA-DR ⁻ Mock	CD34 ⁺ HLA-DR ⁻ HCMV	CD34 ⁺ HLA-DR ⁺ Mock	CD34 ⁺ HLA-DR ⁺ HCMV
1	-	+	-	+	-	+
2	-	+	NA	NA	NA	NA
3	-	+	NA	NA	NA	NA
4	-	-	-	-	-	-
5	-	+	-	+	-	+
6	-	+	NA	NA	-	+
7	-	+	-	+	-	+
8	-	+	-	+	-	+

CD34⁺, CD34⁺HLA-DR⁻, CD34⁺HLA-DR⁺ cells were infected with 1 pfu of a clinical isolate of HCMV or Mock. After 16 hours of infection, the cells were washed twice and then seeded in soft agar containing IMDM and hematopoietic growth factors. Cells from an average of 20 to 30 colonies derived from hematopoietic progenitors were harvested after 28 days, washed twice in PBS, and lysed, and a nested HCMV PCR was performed as described in the Materials and Methods.

Abbreviation: NA, not available due to small amount of cells/test material.

CD34⁺DR⁺ cells could be infected by HCMV in vitro, we also examined the possible site of latency in the human bone marrow in vivo. CD34⁺ progenitors from healthy HCMV IgG-seropositive donors were examined for the presence of HCMV DNA. CD34⁺DR⁻ and CD34⁺DR⁺ bone marrow cells were plated in colony assays in the presence of GM-CSF, G-CSF, SCF, IL-1 α , and IL-3, and colonies were harvested after 28 days of incubation and screened in a nested PCR for the detection of HCMV DNA. We found that HCMV DNA was present in CD34⁺DR⁻ and CD34⁺DR⁺ cells in all seropositive donors examined (Fig 4 and Table 3). In contrast, cells obtained from colonies derived from HCMV-seronegative donor cells were all negative for HCMV DNA (Fig 4). In addition, both immunofluorescence and RT-PCR screening of the cells from the seropositive subsets turned out to be negative for the presence of immediate early proteins or RNA (data not shown). Thus, in accordance with the ability of HCMV to infect CD34⁺DR⁻ and CD34⁺DR⁺ cells in vitro, HCMV can also successfully infect and establish latent infection in immature hematopoietic precursors in vivo, but with no detectable HCMV IE protein production.

DISCUSSION

Hematopoietic progenitor cells, bone marrow stroma cells, and endothelial cells are possible sites of HCMV latency in the human body.⁵⁰ Proliferation and differentiation of CD34⁺ bone marrow cells have been shown to be inhibited after HCMV infections in vitro.^{20,31} The mechanism of this inhibitory effect and the characteristics of the hematopoietic precursors affected by HCMV are still unclear. Previous findings have indicated that clonogenic bone marrow progenitors, including CD34⁺ cells, could be infected with HCMV in vitro.^{31,51} Based on this knowledge, we examined whether the immature bone marrow cells characterized by the lack of expression of the HLA-DR antigen were suscepti-

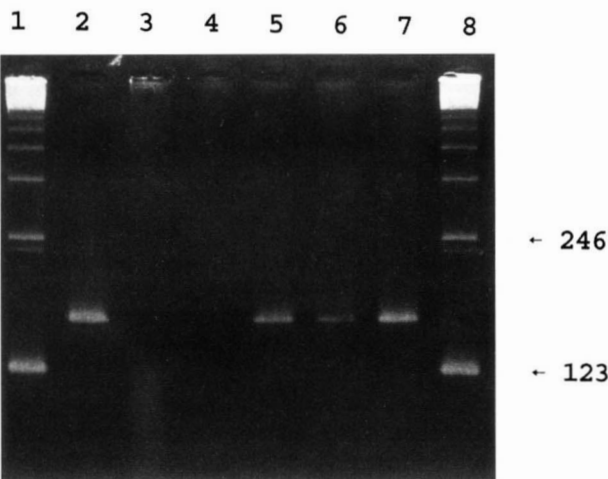


Fig 4. HCMV DNA detection in human hematopoietic progenitor cells from HCMV-seropositive donors. Cellular DNA was isolated from bone marrow CD34⁺ cells, CD34⁺HLA-DR⁻ cells, and CD34⁺HLA-DR⁺ cells responsive to IL-1 α , IL-3, SCF, G-CSF, and GM-CSF. The cells were harvested from an average of 20 to 30 colonies in soft agar. A nested PCR was performed and the PCR products were visualized by ethidium bromide staining after agarose gel electrophoresis. Lanes 2 and 3 show HCMV- and mock-infected control HE fibroblasts, respectively. Lane 4 shows harvested colonies derived from CD34⁺ progenitors of an HCMV-seronegative donor as a negative control. Lanes 5, 6, and 7 show harvested colonies derived from CD34⁺, CD34⁺HLA-DR⁻, and CD34⁺HLA-DR⁺ progenitors, respectively. Lanes 1 and 8 show size markers.

ble to an HCMV infection in vitro. We also examined the effect of HCMV on the growth of CD34⁺ erythroid, myeloid, multilineage, and high proliferative potential bone marrow progenitor cells. The purpose was to establish whether the inhibitory effect of HCMV on hematopoiesis was limited to a particular subset of CD34⁺ cells characterized by phenotype and/or functional ability. In addition, we examined if a possible inhibitory effect of HCMV was related to uptake of HCMV virus and whether immature hematopoietic progenitors were a possible site of HCMV latency in vivo.

Our results indicate that the direct growth inhibitory effect

Table 3. Detection of HCMV DNA in Cells From Harvested Colonies of CD34⁺ Progenitors From HCMV-Seropositive Donors

Donors	CD34 ⁺ Cells	CD34 ⁺ HLA-DR ⁻ Cells	CD34 ⁺ HLA-DR ⁺ Cells
1	-	+	+
2	+	NA	NA
3	+	NA	NA
4	+	+	+
5	+	+	+
6	+	+	+

CD34⁺, CD34⁺HLA-DR⁻, CD34⁺HLA-DR⁺ cells were seeded in soft agar plates containing hematopoietic growth factors. Cells from 20 to 30 colonies derived from the progenitors were harvested after 28 days of incubation, washed twice in PBS, and lysed, and a nested HCMV PCR was performed as described in the Materials and Methods.

Abbreviation: NA, not available due to small amount of test material.

of HCMV on the CD34⁺ progenitors previously described is not limited to the more differentiated progenitors.³¹ In the single cell proliferation assays, we observed a significant direct suppression of HCMV on the growth of the early hematopoietic progenitors defined as CD34⁺HLA-DR⁻.

The growth of the more mature hematopoietic progenitors, CD34⁺HLA-DR⁺ cells, was also suppressed by HCMV. However, there was an apparent donor variation in the HCMV-related growth inhibition when these progenitors were stimulated with the combination of IL-3, IL-1 α , G-CSF, GM-CSF, and SCF. Progenitor growth of CD34⁺DR⁺ cells from some donors was significantly suppressed by HCMV, whereas the growth of these progenitors from others was not inhibited. In addition, HCMV infection after stimulation with single cytokines gave inhibition in all donors tested. This finding indicates that the HCMV-inhibitory effect on progenitor growth in vitro in specific subsets of CD34⁺ cells is dependent on the combination of growth factors used to promote progenitor growth, in which the stimulation with multiple cytokines may lead the CD34⁺DR⁺ cells into lineages less perceptible to the inhibitory effects of HCMV infection.

The growth-inhibitory effect of HCMV on CD34⁺ progenitors appears to be connected to the binding, uptake, and processing of HCMV in the progenitor cells. In accordance with the inhibitory effect of HCMV on the progenitor growth, we were able to detect HCMV DNA from colonies derived both from early and committed hematopoietic progenitors, CD34⁺DR⁻ and CD34⁺DR⁺, respectively. However, in agreement with previous results obtained by our group and others,^{19,31,51} we were unable to identify HCMV IE proteins and IE1 RNA, which is essential for later HCMV protein production, by immunofluorescence and RT-PCR in these colonies (data not shown), which may indicate that no immediate early HCMV proteins are produced in these progenitors after HCMV infection.

Previous studies have shown that HCMV DNA can be detected in both erythroid and myeloid colonies after an HCMV infection in vitro.⁵⁰ In contrast to previous observations¹⁹ but in agreement with the results of Rakusan,²⁸ we found not only that the growth inhibitory effect of HCMV was restricted to the myeloid progenitors but also that the erythroid progenitors, defined as BFU-E, and multipotential progenitors (CFU-GEMM) were inhibited. The reported differences in the ability of HCMV to inhibit erythroid progenitor growth may be due to the different virus strain used. Accordingly, Sing and Ruscetti²⁹ have reported that the inhibitory effect of HCMV on erythroid progenitors was dependent on virus strain.

In contrast to the growth-inhibitory effect of HCMV on myeloid, erythroid, and multilineage progenitors, the CD34⁺HPP-CFC population was not affected by HCMV. This result is in contrast to the inhibitory effect on the CFU-C formation of CD34⁺DR⁻ in a single-cell assay. CD34⁺DR⁻ progenitors have been shown to be enriched of immature progenitors including HPP-CFCs compared with CD34⁺DR⁺ progenitor populations.⁵¹ However, this population also contains more committed progenitors than HPP-CFCs, showing that the CD34⁺DR⁻ cells can be further separated according to func-

tional capacities of the progenitors with CD38 as an alternative marker for selecting a more pure progenitor population.⁵² Therefore, our results indicate that there is a CD34⁺ progenitor population, defined functionally, not suppressed by HCMV in vitro. Furthermore, we were able to detect HCMV DNA in colonies derived from the HPP-CFCs. According to our findings, the difference in the effect of HCMV on the CFU-GM, BFU-E, and CFU-GEMM populations and on the HPP-CFCs cannot be solely explained by a difference in HCMV uptake in these populations. In addition, no immediate early HCMV proteins have been detected in these cells and RT-PCR detection for HCMV immediate early RNA was negative (data not shown). Taken together, these findings indicate that the inhibitory effect of HCMV observed on the bone marrow progenitor growth may be related to events taking place after HCMV uptake and before HCMV protein production and may be connected to the uptake and processing of HCMV tegument proteins such as pp65, pp71, and pp150.^{53,54} These events may differ between subpopulations of CD34⁺ progenitors.

We were able to detect HCMV DNA in both CD34⁺DR⁻ and CD34⁺DR⁺ progenitor cells from HCMV-seropositive donors, whereas the CD34⁺ progenitor cells from HCMV-seronegative donors were negative for HCMV DNA in the same PCR assays. This strongly supports the importance of hematopoietic progenitors as host cells for latent HCMV.

In conclusion, these results support our in vitro findings, indicating that phenotypic immature progenitors are inhibited by HCMV, and these cells may be a site of latency in healthy donors. With this new knowledge it seems likely that the bone marrow may be an important reservoir for HCMV in seropositive donors.

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