Preferential Suppression of Myelopoiesis in Normal Human Bone Marrow Cells After In Vitro Challenge With Human Cytomegalovirus

By Garwin K. Sing and Francis W. Ruscetti

The pathogenic effects of human cytomegalovirus (CMV) infection in vitro on hematopoiesis were investigated. Normal human bone marrow cells from both seronegative and seropositive donors were challenged with CMV (Towne or wild-type strain) and tested for their responsiveness to the recombinant hematopoietic growth factors granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte-CSF (G-CSF), respectively. Regardless of the serostatus of the donor, infection with CMV resulted in a significant decrease in the proliferation and colony formation of hematopoietic progenitor cells in response to both growth factors, with more pronounced suppression in response to G-CSF being observed. Evaluation of the colony composition revealed a profound decrease in colonies of the granulocytic (CFU-G), or granulocyte-macrophage (CFU-GM) lineages, while suppression of multipotential (CFU-GEMM) and erythroid (BFU-E) colony-forming cells occurred after infection with wild-type but not the laboratory strain of CMV. Although no evidence of productive virus infection could be seen in colony-forming cells, in situ hybridization studies and immunohistochemical staining revealed the presence of CMV-specific mRNA and immediate-early antigens, demonstrating that a small proportion of cells were abortively infected. These studies demonstrate that CMV can infect bone marrow progenitor cells and interfere with normal hematopoiesis in vitro, which may help to explain the hematologic defects seen during acute infections with CMV in vivo.

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CLINICAL STUDIES have revealed that many viral infections in man are associated with hematopoietic depression, commonly manifested by leukopenia and thrombocytopenia, although there is little direct evidence for infection of bone marrow progenitor cells.

Acute infections with cytomegalovirus (CMV) may lead to severe hematologic disorders, such as mononucleosis, hepatosplenomegaly, lymphadenopathy, thrombocytopenia, and hemolytic anemia. CMV infection remains the most common infectious cause of morbidity after bone marrow transplantation (reviewed in reference 3). In the mouse model, infection with murine CMV (MCMV) has been shown to induce a rapid inhibition of megakaryopoiesis and granulocytopoiesis in vivo and in vitro. In addition, Mutter et al. recently demonstrated the inability of lethally irradiated mice to reconstitute their bone marrow after infection with MCMV, suggesting that the virus interferes at the level of the earliest progenitor cell.

Although abortive or productive virus infection has been demonstrated in normal human bone marrow, the effects of human CMV infection on hematopoiesis remain controversial. Using conditioned media as the source of colony-stimulating factors, Rakusen et al. demonstrated the suppression by CMV of granulocyte-macrophage (CFU-GM) and erythroid (BFU-E) colony formation, whereas Apperley et al. found no effects on colony formation. Therefore, to delineate more precisely the effects of CMV on the growth of specific hematopoietic subpopulations, we studied the effects of CMV challenge on the proliferation and colony formation by normal human bone marrow cells in response to purified recombinant granulocyte-macrophage and granulocyte colony-stimulating factors (GM-CSF and G-CSF, respectively).

MATERIALS AND METHODS

Cells and Virus

For the preparation of virus stocks, titration of virus, and assays for infectious centers, human embryonic fibroblast (HEF) Wi-38 cells were used. Cells were maintained in basal Eagle medium containing 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100 µg/mL streptomycin, and were subcultured weekly. Virus stocks of human CMV (HCMV) strain Towne (a gift from Dr M.F. Stinski, University of Iowa, Iowa City, IA) and a low-passage wild-type strain (a gift from Dr Wade Gibson, Johns Hopkins School of Medicine, Baltimore, MD) were propagated by serial passage at low multiplicities of infection (MOI) of 0.01 plaque-forming units per cell to avoid generation of defective particles. Virus titrations were performed on HEF cells with an overlay of 0.3% agarose (Bethesda Research Laboratories, Gaithersburg, MD) in 2x basal Eagle medium (GIBCO Laboratories, Chagrin Falls, OH) containing 10% FCS for 10 days at 37°C under a humidified atmosphere and 5% CO₂, as described previously. Virus titrations were performed in duplicate on clarified conditioned media from suspension cultures of hematopoietic cells after virus challenge.

For infectious center assays, 10⁶ virus-challenged cells were mixed with 3 x 10⁵ Wi-38 indicator cells, seeded in Petri dishes, and allowed to attach overnight, after which they were overlaid with a 0.3% agarose suspension as described above.

Cells and virus stocks were routinely tested for mycoplasma contamination and found to be negative. Mycoplasma testing was performed by culturing samples of virus and cell suspensions for 7 to 10 days in PPLO broth (Difco, Detroit, MI) containing 10% yeast lysate and 20% horse serum. Subsequently, portions of these cultures were grown on PPLO agar (Difco) supplemented with 20% horse serum and 10% yeast lysate. Petri dishes were microscopically examined for mycoplasma growth after 14 and 28 days.

Inoculation of Bone Marrow Cells With CMV

Unless specified, all experiments were performed with the Towne strain of CMV. Infection of bone marrow cells was performed by

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incubation with supernatant HCMV at a MOI of 0.1 for 3 hours at 37°C.

Bone marrow cells were obtained from healthy donors who had given informed consent. Detection of antibodies to CMV in donor blood was performed by the enzyme-linked immunosorbent assay (ELISA) (Whittaker M.A. Bioproducts, Walkersville, MD). The mononuclear cells were isolated by centrifugation on Ficoll-Hypaque gradients, washed twice with phosphate-buffered saline (PBS), and suspended in Iscove’s modified Dulbecco’s medium (IMDM) containing 10% FCS. Adherent cells were adsorbed out by pre-incubation in tissue culture flasks for 2 hours before use.

Reagents

Recombinant human GM-CSF (rHuGM-CSF) was obtained from Immunex Corporation (Seattle, WA), and recombinant human G-CSF (rHuG-CSF) was from AMGEN (Thousand Oaks, CA). Stock solutions (10 μg/mL) were stored at 4°C and diluted in tissue culture medium containing 10% FCS for use. Human recombinant erythropoietin (Epo) was purchased from AMGEN and stored at −20°C before use. The 5637 conditioned medium was prepared by harvesting culture fluids from 5637 bladder carcinoma cells (ATCC, Rockville, MD) 10 days after seeding. The medium was then dialyzed against PBS overnight and stored at 4°C.

Colony Assays

*Multipotential and erythroid colony-forming cells.* The assay for human multipotential (CFU-GEMM) and erythroid colony-forming cells (BFU-E) was performed as described previously.12 Bone marrow cells (10⁶) were plated in 35 mm Lux standard tissue culture dishes containing a 1 mL mixture of IMDM, 0.35% Seaplaque agarose (FMC Bioproducts, Rockland, ME), 30% FCS, 1% detoxified bovine serum albumin (BSA; Sigma Chemical Co, St Louis, MO), 2 U/mL Epo, 2 × 10⁶ mol/L hemin, and a predetermined optimal concentration of GM-CSF (10 ng/mL). In some experiments, 10% 5637 conditioned medium was used as the source of GM-CSF. Dishes were incubated at 37°C in a humidified atmosphere flushed with 5% CO₂ in air.

Colonies were scored with an inverted microscope after 14 days of incubation, and the colony- or cluster-bearing agarose layers were occasionally fixed with 3% glutaraldehyde in methanol and stained with benzidine and/or Jenner-Giemsa for morphologic analysis.13

*CFU-GM.* Colony (greater than 50 cells per aggregate) and cluster (3 to 50 cells per aggregate) formation of bone marrow cells was stimulated by varying dilutions of GM-CSF or G-CSF. Cells (10⁶) were plated in 1 mL 0.35% agarose culture medium as described above, except that Epo and hemin were omitted. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air and scored for colonies and/or clusters after 7 and 14 days of incubation.

Inactivation of Viral Infectivity

One milliliter samples of CMV stock were inactivated with periodic agitation in 35 mm plastic plates with a UV transilluminator (Fotodyne Inc, New Berlin, WI) at a distance producing 24 ergs/s/mm² for 15 minutes. Virus inactivation was determined by comparison of the titres of irradiated and nonirradiated virus.

Virus neutralization was performed with a mycoplasma-free polyclonal hyperimmune rabbit antiserum raised against crude supernatants of the AD169 strain of CMV (Lee Biomolecular Research Inc, San Diego, CA). Serum dilutions of 1:100 to 1:1,000 were sufficient to neutralize 5 × 10⁶ plaque-forming units (pfu)/mL CMV (Towne strain). In addition, the serum was photochemically disinfected and treated with 4′-aminomethyltrioxsalen to inactivate possible contaminants.

**DNA Synthesis**

Mock-infected or CMV-infected bone marrow cells were incubated with varying dilutions of CSF in multiwell tissue culture plates in a humidified atmosphere with 5% CO₂. Cells were cultured at 10⁶ cells per well in triplicate for a period of 5 days (when maximum proliferation in the presence of CSF alone was obtained), and 1 μCi methyl-thymidine (New England Nuclear, Boston, MA; 67 Ci/mmol) was added to each well for the final 16 hours of culture. Cells were harvested with a Skatron cell harvester, and isotope uptake was determined by liquid scintillation counting.

**Immunocytochemical Staining for Viral Proteins**

Control or virus-infected cells were air-dried on Titertek multiwell slides (Flow Laboratories, McLean, VA) and fixed in ice-cold acetone for 20 minutes. Cells were incubated with monoclonal antibody E-3 (Genetic Systems, Seattle, WA), which recognizes the major 72 Kd immediate-early protein.14 Antibody labeling was detected with a biotin-conjugated sheep anti-mouse IgG (Vector Laboratory, Inc, Burlingame, CA) followed by glucose oxidase-conjugated streptavidin (Vector Laboratory).

**Measurement of Cytokine Activity in Supernatant Fluids**

Ten milliliter aliquots of supernatant fluid were harvested from G-CSF– and GM-CSF–treated bone marrow cultures of 10⁶ cells/mL at 1, 3, 5, and 10 days postinfection and stored at −70°C. These fluids were assayed for human interferon γ by radioimmunoassay (Centocor, Malvern, PA) and for interferon α using an antiviral assay for reduction of the cytopathic effect of vesicular stomatitis virus on WISH cells.15 Human tumor necrosis factor α was assayed using a specific ELISA kit (Endogen, Boston, MA). Transforming growth factor β (TGF-β) was measured using a growth inhibition of mink lung fibroblasts in the presence and absence of a neutralizing antibody to TGF-β.16

**Identification of CMV mRNA Transcripts by In Situ Hybridization**

The method for in situ hybridization was based on that described by Lawrence and Singer.17 Briefly, air-dried cells were fixed in 4% paraformaldehyde in PBS for 10 minutes at room temperature. They were prehybridized for 60 minutes in a solution containing 80 μg/mL salmon sperm DNA, 250 μg/mL Escherichia coli tRNA, 50% deionized formamide, 5× saline sodium citrate (SSC), and 5× Denhardt’s solution. Twenty microliters of this solution, together with the addition of a [³²P]-labeled cDNA probe for the CMV mRNA encoding the immediate-early antigens (specific activity of 1 × 10⁶ cpm/μg DNA; a gift from Dr Gary Hayward, Johns Hopkins School of Medicine), was added to each of the spots. As a control, a probe consisting of the human immunodeficiency virus (HIV) long terminal repeat (LTR) was also used. The cells were covered with a glass cover slip and incubated at 37°C overnight. The following day, the coverslips were removed, and slides were washed in solutions of varying concentrations of SSC and formamide. They were then counterstained in acid hematoxylin (Sigma), and dipped in Kodak NTB2 emulsion. After 2 to 5 days exposure, the slides were developed and mounted for viewing. Potential nonspecific binding of CMV probe was determined by ribonuclease A pretreatment (20 mg/mL in PBS at 37°C for 1 hour) with subsequent reduction of signal, showing specificity of hybridization to RNA (data not shown).
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RESULTS

Suppression of GM-CSF or G-CSF Responsiveness After Infection With CMV

In order to test the effects of CMV exposure on the proliferation of bone marrow cells in response to GM-CSF or G-CSF, cells were challenged with CMV (Towne) at a MOI of 5 pfu per cell and cultured for 72 hours in the presence of varying concentrations of G-CSF or GM-CSF. After pulsing for the final 6 hours of culture with [3H]-thymidine, the cells were harvested and counted. Cells infected with CMV were totally insensitive to the stimulatory effects of both GM-CSF and G-CSF (Fig 1). Addition of the highest concentration of either growth factor failed to enhance proliferation above the control levels seen with mock-infected cells cultured in the absence of growth factor. Furthermore, the background proliferation of cells cultured in the absence of CSF was also suppressed when compared with mock-infected controls. From trypan-blue exclusion, the percentage of viable virus-challenged cells did not differ significantly from those of controls during the 3-day course of the assay (Table 1). However, at later time periods (6 to 14 days), a 20% to 40% reduction in cell viability was observed, which was more pronounced when cells were either cultured in the absence of CSF or in G-CSF, compared with cells grown with GM-CSF. Similar results were obtained irrespective of the donor’s serostatus (three seronegatives and four seropositives).

Suppression of growth was also obtained when bone marrow cells were precultured in the presence of GM-CSF for 8 days before challenge with CMV (Fig 2); again, the level of proliferation of infected cells at optimal concentrations of GM-CSF was lower than that of mock-infected cells grown in the absence of GM-CSF. Thus, exposure to CMV not only suppressed the ability of resting bone marrow cells to respond to CSF, but also suppressed the proliferation of actively growing cells that had been stimulated with CSF previously.

The specificity of these observed effects was demonstrated in three ways. First, it could be shown that the suppressive effects of CMV challenge could be diluted out with decreasing multiplicities of infection (Table 2). Second, the addition of specific antibodies to CMV, but not control antisera, effectively neutralized the suppressive effect (Fig 3). Third,
cells of the myeloid-monocytic lineage (CFU-GM) was GEMM or BFU-E were seen, whereas colony-formation by and virus-infected nonadherent bone marrow cells contained colonies, no significant suppression of the earliest CFU-
01, were scored. With regard to day colony formation was inhibited by greater than 75% inhibited by approximately 60% (Fig
suspensions of CMV for 90 minutes, washed, and plated in conditioned medium from suspension cultures of control UV-inactivated virus had no effect on GM-CSF induced proliferation of bone marrow cells, while viable CMV markedly inhibited the bone marrow cell growth response (Table 3). Conditioned medium from suspension cultures of control and virus-infected nonadherent bone marrow cells contained no detectable levels of tumor necrosis factor, interferon γ or α, or TGF-β (data not shown). These regulatory cytokines are known to inhibit the growth of progenitor cells in response to CSFs, and, hence, it appears unlikely that CMV-induced suppression was mediated by these factors.

Effects of CMV Challenge on Colony-Forming Cells

The colony-forming ability of progenitor cells was tested after exposure to CMV. Cells were incubated with cell-free suspensions of CMV for 90 minutes, washed, and plated in the presence of CSF. Both 7 and 14 day colonies and clusters were scored. With regard to day 14 GM-CSF-induced colonies, no significant suppression of the earliest CFU-GEMM or BFU-E were seen, whereas colony-formation by cells of the myeloid-monocytic lineage (CFU-GM) was inhibited by approximately 60% (Fig 4). A more striking suppression of colony growth was seen in response to G-CSF, where colony formation was inhibited by greater than 75% (Fig 5). Since CFU-GEMM and BFU-E are regarded as belonging to the less mature stages of hematopoietic differentiation, this would suggest that the suppressive effects of CMV are exerted on cells of the more mature stages of differentiation, such as CFU-GM or CFU-G. This is supported by the data presented in Table 4. CMV virtually abolished the formation of the more mature day 7 colony-forming cells irrespective of the CSF present, whereas approximately 20% of the less mature day 14 CFU-GM were still able to be formed in these cultures.

However, it is well established that fresh isolates of CMV have a higher degree of infectivity than laboratory strains. Similarly, we found that the wild-type strain was more potent in suppressing colony formation than the Towne strain, although this was dependent on the CSF present. In the presence of GM-CSF, both strains of virus suppressed CFU-GM by approximately 80% (Table 3), while in the presence of G-CSF or 5637 conditioned medium, the degree of suppression of CFU-GM colonies by wild-type CMV was higher than that effected by Towne CMV. The wild-type strain also suppressed the formation of CFU-GEMM and BFU-E, whereas the Towne strain had no effect. Thus, it would appear that different strains of CMV have differential effects on bone marrow cells. To test whether nonadherent cells were capable of supporting virus replication, nonadherent cells were removed from adherent cells after 5 days postinfection and recultured in fresh medium with CSF.

### Table 2. Suppressive Effects of Varying Concentrations of CMV on Bone Marrow Cell Responsiveness to CSF

<table>
<thead>
<tr>
<th>CSF</th>
<th>Virus Concentration (pfu/cell)</th>
<th>[3H]-Thymidine Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5</td>
<td>337 ± 14</td>
</tr>
<tr>
<td>None</td>
<td>0.5</td>
<td>5,678 ± 761</td>
</tr>
<tr>
<td>None</td>
<td>0.05</td>
<td>5,159 ± 276</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>5,227 ± 959</td>
</tr>
<tr>
<td>GM</td>
<td>5</td>
<td>870 ± 244</td>
</tr>
<tr>
<td>GM</td>
<td>0.5</td>
<td>15,504 ± 1,934</td>
</tr>
<tr>
<td>GM</td>
<td>0.05</td>
<td>32,571 ± 2,402</td>
</tr>
<tr>
<td>GM</td>
<td>0</td>
<td>55,607 ± 3,653</td>
</tr>
<tr>
<td>G</td>
<td>5</td>
<td>991 ± 801</td>
</tr>
<tr>
<td>G</td>
<td>0.5</td>
<td>38,985 ± 6,400</td>
</tr>
<tr>
<td>G</td>
<td>0.06</td>
<td>72,461 ± 1,735</td>
</tr>
<tr>
<td>G</td>
<td>0</td>
<td>132,928 ± 12,312</td>
</tr>
</tbody>
</table>

Bone marrow cells (10⁵/mL) were challenged with the appropriate concentration of CMV for 2 hours, after which cells were washed three times and resuspended in medium containing 10 ng/mL CSF. Cells were cultured for 72 hours and pulsed with [3H]-thymidine for the final 16 hours of culture. Results are expressed as cpm ± SEM of three experiments performed in triplicate.

### Fig 3. Effect of anti-CMV-positive sera on the suppression of GM-CSF responsiveness by hematopoietic cell progenitors. Virus was incubated with specific or control antisera for 30 minutes at 37°C before inoculation of bone marrow cells. Virus-challenged cells were then cultured with 5 ng/mL GM-CSF as described in Fig 1. Results represent the mean ± SEM of three experiments performed in triplicate.

### Table 3. Effects of UV-Inactivated CMV on GM-CSF-Induced Cell Proliferation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GM-CSF Concentration (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Mock-infected</td>
<td>15,106 ± 3,218</td>
</tr>
<tr>
<td>UV/CMV</td>
<td>16,154 ± 2,108</td>
</tr>
<tr>
<td>CMV</td>
<td>536 ± 28</td>
</tr>
</tbody>
</table>

Proliferation assays were performed as described in Materials and Methods. Results represent the mean ± SEM of two donors (one seronegative and one seropositive) assayed in duplicate.
HCMV INHIBITS MYELOID PROGENITOR GROWTH

8-7-6-5-4-3-2-1-0

Fig 4. Effect of CMV challenge on the growth of CFU-GEMM-(A), BFU-E- (B), or CFU-GM- (C) derived colonies in response to GM-CSF. Results represent the mean ± SEM of seven experiments performed in duplicate.

After a further 10 days' incubation, supernatants were removed to test for CMV infectivity, and nonadherent cells were cocultured with HEF cells to test for infectious centers. No plaques were obtained, either from the supernatants or from the infectious center assay. Other workers have demonstrated that productive infection of certain immature cells, such as teratocarcinoma cells or THP-1 monocytes, can only occur after treatment with differentiating agents. To test whether hematopoietic progenitor cells could also support virus replication after differentiation, we cultured fresh bone marrow cells in the presence of GM-CSF or G-CSF for 3 days before challenge with CMV, and cells were tested for virus production at 5 and 10 days postinfection. Again, no infectious virus production could be demonstrated, either in the plaque or the infectious center assay.

However, when slide preparations of cells picked from 14-day colonies grown in CSF were probed for CMV-specific mRNA using a [32P]-labeled EcoRI J fragment of the CMV genome, a small proportion of cells (less than 10%) within each colony hybridized to the probe (Fig 6). The specificity of the reaction could be demonstrated by the fact that no hybridization was seen in mock-infected cells, nor was any hybridization seen when an irrelevant probe such as the HIV LTR was used.

 Immunohistochemical staining of cells grown in suspension cultures with antibodies to the major immediate-early protein of CMV revealed an equally small proportion (less than 10%) of cells expressing viral protein (Fig 7). Similar numbers of cells were found to express viral mRNA or protein, irrespective of whether the Towne or the wild-type strain was used as the inoculum. Thus, although nonadherent progenitor cells are unable to support viral replication, there is a small population of cells that can be abortively infected with CMV.

DISCUSSION

Although the high incidence of CMV infections in bone marrow and various organ transplants has been well documented, the pathology of acute infections in these patients is predominantly associated with interstitial pneumonitis, and very few studies have been performed to assess the effects of CMV infection on hematopoiesis in humans. Verdonck et al[10] reported a delay in platelet recovery in leukemic patients infected with CMV, as opposed to CMV-negative patients, after autologous bone marrow transplantation, whereas no differences in the recovery of neutrophils
Table 4. Effects of Towne or Wild-Type CMV on Colony Formation

<table>
<thead>
<tr>
<th>Virus</th>
<th>CSF</th>
<th>CFU-GM</th>
<th>Clusters</th>
<th>CFU-GEMM</th>
<th>BFU-E</th>
<th>CFU-GM</th>
<th>Clusters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>None</td>
<td>5 ± 3</td>
<td>105 ± 27</td>
<td>0</td>
<td>0</td>
<td>10 ± 3</td>
<td>154 ± 38</td>
</tr>
<tr>
<td>Towne</td>
<td>None</td>
<td>0</td>
<td>5 ± 3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>72 ± 3</td>
</tr>
<tr>
<td>WT</td>
<td>None</td>
<td>ND</td>
<td>ND</td>
<td>0</td>
<td>0</td>
<td>6 ± 2</td>
<td>31 ± 5</td>
</tr>
<tr>
<td>Control</td>
<td>GM</td>
<td>199 ± 51</td>
<td>146 ± 86</td>
<td>16 ± 8</td>
<td>49 ± 4</td>
<td>142 ± 65</td>
<td>38 ± 10</td>
</tr>
<tr>
<td>Towne</td>
<td>GM</td>
<td>0</td>
<td>18 ± 4</td>
<td>14 ± 2</td>
<td>44 ± 6</td>
<td>30 ± 8</td>
<td>46 ± 7</td>
</tr>
<tr>
<td>WT</td>
<td>GM</td>
<td>ND</td>
<td>ND</td>
<td>7 ± 5</td>
<td>34 ± 6</td>
<td>32 ± 4</td>
<td>49 ± 19</td>
</tr>
<tr>
<td>Control</td>
<td>G</td>
<td>150 ± 14</td>
<td>153 ± 58</td>
<td>0</td>
<td>3 ± 1</td>
<td>169 ± 11</td>
<td>109 ± 25</td>
</tr>
<tr>
<td>Towne</td>
<td>G</td>
<td>6 ± 2</td>
<td>29 ± 8</td>
<td>0</td>
<td>3 ± 1</td>
<td>33 ± 17</td>
<td>67 ± 21</td>
</tr>
<tr>
<td>WT</td>
<td>G</td>
<td>ND</td>
<td>ND</td>
<td>0</td>
<td>2 ± 1</td>
<td>19 ± 4</td>
<td>49 ± 19</td>
</tr>
<tr>
<td>Control</td>
<td>5637 CM</td>
<td>265 ± 23</td>
<td>51 ± 22</td>
<td>10 ± 4</td>
<td>45 ± 13</td>
<td>212 ± 21</td>
<td>20 ± 8</td>
</tr>
<tr>
<td>Towne</td>
<td>5637 CM</td>
<td>12 ± 4</td>
<td>14 ± 7</td>
<td>8 ± 2</td>
<td>39 ± 6</td>
<td>50 ± 9</td>
<td>32 ± 11</td>
</tr>
<tr>
<td>WT</td>
<td>5637 CM</td>
<td>ND</td>
<td>ND</td>
<td>0</td>
<td>2 ± 1</td>
<td>15 ± 2</td>
<td>22 ± 6</td>
</tr>
</tbody>
</table>

Results represent the mean ± SEM of three donors (two seronegative and one seropositive) assayed in duplicate.

Abbreviations: WT, wild-type; CM, conditioned medium; ND, not done.

was observed. In contrast, Petursson et al. observed a rapid suppression of megakaryocytopoiesis and granulocytopoiesis in mice after intravenous inoculation with MCMV, which was also observed in vitro. Furthermore, granulocytopenia is frequently observed in patients with acute CMV infections while receiving treatment for acute myelogenous leukemia.

In this work, we demonstrate that human cytomegalovirus suppresses hematopoiesis of normal human progenitor cells.

Fig 6. (A) Nonadherent progenitor cells infected with Towne strain CMV hybridized with the [32P]-labeled probe pUC-EcoRI J at an original magnification of 40×. (B) Nonadherent mock-infected progenitor cells hybridized with the [32P]-labeled probe pUC-EcoRI J.
Bone marrow cellular responsiveness to both GM-CSF and G-CSF was totally abrogated after exposure to CMV (Fig 1). The fact that even at maximal concentrations of CSF, [³H]-thymidine uptake of virus-challenged cultures was suppressed in relation to the background levels attained by mock-infected cultures suggests that the virus suppresses cycle-active cells present at the time of infection. This is supported by the data shown in Fig 2, where actively replicating cells prestimulated with GM-CSF for 8 days were again suppressed from further replication when assayed at 3 days postinfection. The specificity of the viral-induced suppression is demonstrated by the fact that suppression was dependent on the concentration of the viral inoculum and was neutralized by CMV-specific antibody (Table 2, Fig 3). Since no correlation was seen between lack of cellular proliferation and cell viability (Table 1), it would suggest that extensive cytopathology was not the cause of this reduction in cell growth. These experiments were performed with cells from three seronegative and four seropositive donors, with similar results obtained for each donor, demonstrating that this suppressive effect occurred irrespective of donor serostatus.

The Towne strain of CMV exerted a differential effect on hematopoiesis in that colony formation by progenitor cells of the more committed stages, such as the CFU-GM or CFU-G, were inhibited by as much as 75%, whereas colony formation by cells of the earlier multipotential or erythroid colonies was not affected (Figs 4 and 5). Similarly, virus-induced suppression of the more mature 7-day CFU-GM was significantly higher than that of 14-day CFU-GM (Table 3), supporting our contention that CMV preferentially interferes with the growth of more mature cells.

While both the Towne and wild-type strains of CMV exerted similar levels of suppression of CFU-GM in the presence of GM-CSF, the wild-type strain was more potent in suppressing colony formation (as well as suppressing the formation of CFU-GEMM and BFU-E colonies) when GM-CSF or 5637 conditioned medium was used. Thus, different strains of CMV have differential tropisms for specific progenitor cells.

These results are in direct contrast to the recent observations of Apperley et al, who found no evidence of suppression of hematopoiesis by CMV in bone marrow cells of either seropositive or seronegative donors. The reasons for this discrepancy are unclear but may be attributed to a number of factors. Differences in the strains of CMV used might play a role: Apperley et al used the AD169 strain of CMV, as well as a number of fresh clinical isolates. As we have demon-
strated in this work, the Towne strain and wild-type strain of CMV suppressed colony formation to different degrees, depending on the type of CSF present. Furthermore, Raku san et al observed large differences in 7-day GM colony inhibition between individual clinical isolates of CMV, and the degree of suppression exerted by the various strains ranged from 0 to 100%. Differences might also be seen with varying culture conditions. Our observations were made with a purified preparation of recombinant CSFs, as opposed to lymphocyte conditioned medium.

In agreement with previous results, there was no evidence of virus production in nonadherent cells from suspension cultures even when cells were first differentiated with GM-CSF or G-CSF before infection. However, nonadherent progenitor cells could be abortively infected with CMV, as demonstrated by in situ hybridization and immunohistochemical staining for the immediate-early antigen of CMV. The fact that these were nonadherent cells and not detached cells from the adherent population can be verified by the fact that they were picked from 14-day colonies grown in soft agar. Nevertheless, within each population of colony-forming cells, the percentage of cells abortively infected with CMV was consistently less than 10%. This is similar to CMV infection of peripheral blood mononuclear cells, where a low frequency of incomplete virus replication is also observed.

Previous studies have shown that CMV-infected monocytes can play a role in the suppression of lymphocyte proliferation, while others have shown that CMV can stimulate the production of interferon γ, a potent inhibitor of hematopoiesis. Thus, there is the possibility that the observed suppression of myelopoiesis was mediated through an indirect effect involving accessory cells. This appeared unlikely, however, since tests for cytokines known to have regulatory effects on hematopoiesis, such as interferons γ, α, and β, tumor necrosis factor α, and TGF-β, all proved to be negative. In addition, nonadherent cells were depleted from the bone marrow before the initiation of the cultures, making it unlikely that mature monocytes had any significant influence on the growth properties of progenitor cells.

We have demonstrated that infection of bone marrow progenitor cells with CMV in vitro can severely inhibit the ability of these cells to respond to purified recombinant colony stimulating factors. However, with the exception of congenital CMV infection, CMV is rarely the cause of severe disease in immunologically healthy people. Indeed, in normal persons with acute CMV infections, transient neutropenia is only rarely seen. Our results indicate that in the immunocompromised host, CMV can have direct damaging effects on hematopoiesis. This may have serious consequences in the light of current attempts to use recombinant CSFs for the treatment of various hematologic disorders, particularly when considering the high rates of seroconversion among certain high risk groups of patients.

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Preferential suppression of myelopoiesis in normal human bone marrow cells after in vitro challenge with human cytomegalovirus

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