Analysis of Hematopoietic Stem and Progenitor Cell Populations in Cytomegalovirus-Infected Mice

By Anne E. Gibbons, Patricia Price, and Geoffrey R. Shellam

We have studied the effects of murine cytomegalovirus (MCMV) infection on bone marrow stem and progenitor cell populations to find an explanation for the defects in hematopoiesis that accompany CMV infections in patients. Sublethal MCMV infection of BALB/c mice resulted in a 5- to 10-fold decrease in the numbers of myeloid (colony-forming unit–granulocyte-macrophage [CFU-GM]) and erythroid (burst-forming unit-erythroid [BFU-E]) progenitor cells in the marrow, but not in primitive myeloerythroid progenitor cell (colony-forming unit-spleen [CFU-S]) numbers. In contrast, we observed a 10- to 20-fold reduction in CFU-S as well as CFU-GM and BFU-E in lethally infected mice. Depletion of marrow CFU-GM was less severe in C57BL/10 and C3H/HeJ mice, which are more resistant to the effects of MCMV infection. Treatment of bone marrow cells with MCMV preparations in vitro did not reduce the numbers of CFU-GM, although up to 10% of the cells were productively infected. This finding suggests that CFU-GM were not susceptible to lytic MCMV infection in vitro and are probably not eliminated by lytic infection in vivo. Increases in the frequencies of Sca-1Lin- marrow cells, a population that includes cells with the characteristics of pluripotential stem cells, were observed in MCMV-infected BALB/c, C57BL/10, and DBA/2J mice. Increases in the frequencies of c-kitLin marrow cells were only seen in DBA/2J mice. MCMV infection did not impair the function of pluripotential stem cells because transplantation of marrow from MCMV-infected donors into irradiated recipient mice resulted in successful reconstitution of the T, B, and myeloid cell lineages.

© 1995 by The American Society of Hematology.

HUMAN CYTOMEGALOVIRUS (HCMV) infection is a serious complication in immunocompromised transplant and human immunodeficiency virus (HIV) patients and in neonates. Clinical manifestations of HCMV that involve the hematopoietic system include neutropenia, mononucleosis, thrombocytopenia, and hematolytic anemia. Furthermore, HCMV infection has been associated with delayed marrow engraftment and graft failure in bone marrow transplant patients. The presence of HCMV genomic material in peripheral blood cells indicates that the hematopoietic system may be an important target for infection by the virus.

It has been proposed that the detrimental effects of HCMV on hematopoiesis result from the infection of bone marrow stem or progenitor cells. Hence, several studies have assessed the ability of HCMV to infect cells of the bone marrow in vitro and then inhibit the development of hematopoietic colonies from precursor cells. These experiments have yielded mixed results. Rakusan et al11 found that HCMV inhibited myeloid and erythroid colony formation but could not show replication of the virus in bone marrow and cord blood mononuclear cells. Apperley et al12 reported that incubation of bone marrow mononuclear cells with HCMV did not affect hematopoietic colony formation or morphology, but stromal monolayers infected with HCMV were unable to support proliferation of myeloid progenitor cells. Simmons et al13 also showed myelosuppression in HCMV-infected long-term marrow cultures and associated this observation with infection of stromal elements and decreased production of granulocyte colony-stimulating factor (G-CSF). A study by Sing and Ruscetti14 showed that the formation of hematopoietic colonies in response to granulocyte-macrophage colony-stimulating factor (GM-CSF) and G-CSF was impaired by treatment of marrow cells with HCMV. Although there was no productive viral infection of marrow progenitor cells, HCMV-specific mRNA and immediate-early antigens were detected in some cells, suggesting that small numbers of bone marrow cells may be abortively infected and interfere with hematopoiesis in vitro. In a study by Maciejewska et al,15 HCMV-specific DNA was detected in erythroid and myeloid colonies after treatment of bone marrow cells with recombinant HCMV encoding the Escherichia coli Lac Z gene. LacZ+ activity was observed predominantly in cells of the myelomonocytic lineage, and a moderate inhibition of colony formation was observed when marrow cells were treated with high concentrations of virus. These investigators also showed that infectious virus could be recovered from cells derived from HCMV-infected progenitor cells, indicating that hematopoietic cells may harbor HCMV for several weeks and through several differentiation steps. Thus, the hematopoietic system may serve as a reservoir for latent HCMV and contribute to the dissemination of HCMV during active infections.

Because the CMVs are species specific, the infection of mice with murine CMV (MCMV) is often used as a model for in vivo studies of CMV disease pathogenesis. In addition, MCMV infection of mice can induce similar hematologic abnormalities to those described in patients with HCMV infections. MCMV infection also interferes with autoreconstitution of the bone marrow in sublethally γ-irradiated mice. We have shown previously that MCMV infection of BALB/c mice results in severe marrow atrophy, although there is little productive infection of marrow cells. Changes in the proportions of cells comprising the marrow were observed, as well as changes in peripheral blood cell populations. The experiments were performed using inbred mice of different strains because sensitivity to MCMV disease is genetically determined. The susceptibility of individual cells

From the Department of Microbiology, University of Western Australia, Queen Elizabeth II Medical Centre, Nedlands, WA, Australia. Submitted July 13, 1994; accepted February 21, 1995. Supported by the National Health and Medical Research Council of Australia.

Address reprint requests to Patricia Price, PhD, Department of Microbiology, University of Western Australia, Queen Elizabeth II Medical Centre, Nedlands, WA, 6009, Australia.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1995 by The American Society of Hematology.

0006-4971/95/8602-0039$3.00/0

to MCMV infection is determined by H-2 genes. Cells from C3H/HeJ mice have the H-2^ phenotype and are 10-fold more resistant to infection with MCMV in vitro than cells from BALB/c mice that have the H-2^ phenotype. Replication of MCMV in the spleen is controlled by a non-H-2 gene designated Cmv-l, which restricts viral replication in the spleens of C57Bl/10 mice, but not BALB/c or C3H/HeJ mice. Other non-H-2 genes influence interferon, natural killer cell, and T-cell responses to the virus.

This study examines the effects of MCMV infection in vivo on hematopoietic stem and progenitor cell numbers and on the functional capability of marrow cells. The ability of bone marrow cells to support productive viral infection and form colonies of differentiated cells after treatment with MCMV in vitro is also assessed.

MATERIALS AND METHODS

Mice. Specific pathogen-free, inbred mice were obtained from the Animal Resources Centre (Murdoch, Western Australia) and housed under minimal disease conditions in the Department of Microbiology (University of Western Australia). Eight- to twelve-week-old female mice of the BALB/c, C57Bl/10ScSn, C57Bl/6J, and DBA/2J strains were used for most experiments. Female, ex-breeding BALB/c mice were used as recipients in some hematopoietic colony assays, and male BALB/c donor mice were infected at 8 weeks of age for radioprotection and reconstitution studies. Regular serologic screening established that mice in the holding center remained free from Sendai virus and Mycoplasma pulmonis.

Preparation of MCMV stocks. The strain of virus used in this study is designated as K181 (Perth). Restriction enzyme digest analysis of the viral DNA has shown that it has a pattern identical to the K181 strain. The stock was prepared by salivary gland passage in 3-week-old female BALB/c mice and its plaque forming unit (pfu) titer was determined. Tissue culture MCMV stocks were prepared by infection of confluent mouse embryo fibroblast (MEF) cultures with salivary gland-passaged virus under centrifugation at 800g for 30 minutes at room temperature. After incubation at 37°C for 90 minutes, the cells were suspended by pipetting with RPMI with 2% FCS, washed three times, and resuspended for counting by trypan blue exclusion.

Infectious center assays. Productively infected cells were quantified by coculture with embryonic fibroblasts. Briefly, serial twofold dilutions of marrow cells were prepared in 0.5 mL of RPMI containing 2% FCS, and 2 X 10^5 cells in 200 mL of phosphate-buffered saline (PBS) were transferred intravenously via the lateral tail vein into lethally irradiated recipient mice (750 rad) that were maintained in an isolator unit with tracycline drinking water (0.25 g/L; Pfizer, West Ryde, Australia). The mice were killed after 14 days and their spleens were removed to Bouin’s solution. Individual surface colonies, each comprising the progeny of a single stem cell, were counted and the numbers of CFU-S per donor were calculated. Irradiated mice receiving 200 mL of PBS intravenously were included in each experiment to assess endogenous colony formation in recipient mice.

Hematopoietic progenitor cell assays. Granulocyte/monocyte progenitor cells were enumerated in double layer semisolid agar cultures. Briefly, 5 X 10^5 bone marrow cells were plated in 0.5 mL of DMEM medium containing 10% FCS, penicillin, gentamycin, and L-glutamine. The cultures were incubated for 14 days at 37°C in a humidified incubator and the colonies were fixed and stained with crystal violet.

Collection of bone marrow cells. Suspensions of mouse bone marrow cells were obtained by flushing RPMI 1640 (RPMI; Gibco, Grand Island, NY) supplemented with 2% fetal calf serum (FCS; Gibco Laboratories, Auckland, New Zealand) through excised femurs and tibias. The cells were treated with NH4Cl (0.184 mol/L) to lyse erythrocytes, washed, and resuspended in RPMI containing 10% FCS for counting and assessment of viability by trypan blue exclusion.

Treatment of bone marrow cells with MCMV in vitro. For infection of cells in vitro, 0.25 mL of the virus preparation (containing 5 X 10^6 or 5 X 10^5 pfu MCMV) was added to an equal volume of cells adjusted to 2 X 10^7 cells/mL in RPMI containing 2% FCS in FCS-precoated 24-well tissue culture trays and centrifuged at 800g for 30 minutes at room temperature. After incubation at 37°C for 90 minutes, the cells were suspended by pipetting with RPMI with 2% FCS, washed three times, and resuspended for counting by trypan blue exclusion.

Infectious center assays. Productively infected cells were quantified by coculture with embryonic fibroblasts. Briefly, serial twofold dilutions of marrow cells were prepared in 0.5 mL of RPMI containing 2% FCS, and 2 X 10^5 cells in 200 mL of phosphate-buffered saline (PBS) were transferred intravenously via the lateral tail vein into lethally irradiated recipient mice (750 rad) that were maintained in an isolator unit with tracycline drinking water (0.25 g/L; Pfizer, West Ryde, Australia). The mice were killed after 14 days and their spleens were removed to Bouin’s solution. Individual surface colonies, each comprising the progeny of a single stem cell, were counted and the numbers of CFU-S per donor were calculated. Irradiated mice receiving 200 mL of PBS intravenously were included in each experiment to assess endogenous colony formation in recipient mice.

Hematopoietic progenitor cell assays. Granulocyte/monocyte progenitor cells were enumerated in double layer semisolid agar cultures. Briefly, 5 X 10^5 bone marrow cells were plated in 0.5 mL of DMEM medium containing 10% FCS, penicillin, gentamycin, and L-glutamine. The cultures were incubated for 14 days at 37°C in a humidified incubator and the colonies were fixed and stained with crystal violet.

Hybridomas. The monoclonal antibodies (MoAbs) used in this study included rat antibodies to Sca-1 (E13 161.7), c-kit (ACK2), CD2 (RM2.2), CD3 (KT3-1.1), CD5 (53.7.3), CD8 (53.6.72), B220 (RA3-6B2), Mac-1 (M1/70.15.11.5), Gr-1 (RB6-8C5), and erythrocytes (TER-119). All hybridomas were maintained in RPMI supplemented with 10% FCS, 2 mmol/L L-glutamine, 50 mmol/L 2-mercaptoethanol, and antibiotics. Antibody present in the culture supernatant of E13 161.7 and ACK2 were concentrated by precipitation with 50% ammonium sulfate, dialyzed against PBS (333 mM NaCl, pH 7.2), and then conjugated to biotin.

Fluorescence staining and flow cytometry. Bone marrow cell preparations were assessed for the presence of Sca-1"Lin^ cells using a modification of the method described by Spangrude et al. First, 2 X 10^6 bone marrow cells were incubated with a cocktail of lineage-specific antibodies for 45 minutes on ice. The lineage cocktail comprised rat antibodies to CD2, CD3, CD5, CD8, B220, Mac-1, Gr-1, and erythrocytes. The cells were then washed in PBS-azide and treated with fluorescein isothiocyanate (FITC-
bone marrow stem cells after MCMV infection

Untoward against Sca-1

numbers of CFU-S per donor calculated from two to four independent experiments.

Gates were set and fixed in PBS containing 4 mg/mL paraformaldehyde, Gates were set and fixed in PBS containing 4 mg/mL paraformaldehyde, 1 mg/mL sodium azide, and 0.5% wt/vol BSA. Fluorescence was detected with a FACScan flow cytometer and analyzed with the program LYSYS (Becton Dickinson, Mountain View, CA). Cells were washed with streptavidin phycoerythrin (Becton Dickinson Immunocytochemistry Systems, San Jose, CA). Finally, the cells were incubated with biotinylated MoAb preparations, and Lin+, or lineage-specific antibodies. Comparisons of antigen expression by marrow cells were performed using Student’s t-test. The level of significance was taken as P < 0.05.

Cell preparations were also assessed for the presence of Sca-1−, c-kit−, and Lin− cells using single-color staining methods; frequencies of these cells were similar when results were obtained from preparations stained by the single- or dual-color method.

Infection with sublethal doses of MCMV (4 × 10^4 pfu) or lethal (2.4 × 10^5 pfu) doses of MCMV resulted in acute myeloid and granulocyte-macrophage (CFU-GM) cell death (Fig 2B). The results presented are the mean numbers of CFU-GM per donor calculated from two to four independent experiments. Although there was little change in CFU-S numbers in sublethally infected mice, infection with a lethal dose of MCMV resulted in a fivefold decrease in numbers of CFU-S at day 2 postinfection (PI) and a 20-fold decrease at day 4 PI. Mice infected with lethal doses of MCMV died 5 to 10 days PI. These findings suggest that the profound loss of CFU-S may be a feature of lethal MCMV infection.

Reduction in lineage-committed precursor cells in MCMV-infected mice. Myeloid and erythroid progenitor cells were also enumerated in the marrow of BALB/c mice after infection with lethal or sublethal doses of MCMV as described above. A 5- to 10-fold decrease in colony-forming units–granulocyte-macrophage (CFU-GM) was observed 2 days after sublethal MCMV infection (Fig 2A). Similarly, BFU-E numbers in sublethally infected mice were reduced 10-fold at day 2 PI compared with uninfected mice (Fig 2B). There was a gradual increase in the numbers of both CFU-GM and BFU-E after this time. When mice were inoculated with a lethal dose of MCMV, the numbers of CFU-GM (Fig 2A) and BFU-E (Fig 2B) declined 2 and 4 days PI. Similar results were recorded in repeat experiments.

The effects of MCMV infection on the numbers of CFU-GM in different strains of mice were compared to assess whether host genetic composition could influence the development of hematopoietic abnormalities. BALB/c, C57BL/10, and C3H/HeJ mice were infected with a constant sublethal dose (4 × 10^4 pfu), or a strain-adjusted sublethal dose (see Materials and Methods) of MCMV. Two days after infection with the constant virus dose, CFU-GM numbers were reduced 5- to 10-fold in BALB/c mice and less than twofold in C57BL/10 mice. C3H/HeJ mice showed no significant change in CFU-GM numbers after infection with this virus dose (Fig 3A). The numbers of CFU-GM declined approximately twofold in C57BL/10 and C3H/HeJ mice inoculated with strain-adjusted doses of MCMV (Fig 3B). CFU-GM numbers increased to control levels after day 4 of MCMV infection in all strains examined. Again, repeat experiments gave similar results to those described.

Susceptibility of bone marrow cells to infection with MCMV in vitro: Effects on hematopoietic colony development. Bone marrow cells were treated with MCMV in vitro to determine whether they were capable of supporting productive viral infection and whether the virus could inhibit development of hematopoietic colonies from stem and progenitor cells. Treatment of cells was performed using centrifugal enhancement to achieve the maximum level of infection possible. Preliminary experiments indicated that infection of male or female mice with MCMV resulted in similar changes in marrow cellularity and composition.

RESULTS

CFU-S numbers are reduced by lethal but not sublethal infection of BALB/c mice. The numbers of CFU-S in the bone marrow of BALB/c mice were monitored after infection with sublethal (4 × 10^4 pfu) or lethal (2.4 × 10^5 pfu) doses of MCMV (Fig 1). The results presented are the mean numbers of CFU-S calculated from two to four independent experiments. Although there was little change in CFU-S numbers in sublethally infected mice, infection with a lethal dose of MCMV resulted in a fivefold decrease in numbers of CFU-S at day 2 postinfection (PI) and a 20-fold decrease at day 4 PI. Mice infected with lethal doses of MCMV died 5 to 10 days PI. These findings suggest that the profound loss of CFU-S may be a feature of lethal MCMV infection.

Reduction in lineage-committed precursor cells in MCMV-infected mice. Myeloid and erythroid progenitor cells were also enumerated in the marrow of BALB/c mice after infection with lethal or sublethal doses of MCMV as described above. A 5- to 10-fold decrease in colony-forming units–granulocyte-macrophage (CFU-GM) was observed 2 days after sublethal MCMV infection (Fig 2A). Similarly, BFU-E numbers in sublethally infected mice were reduced 10-fold at day 2 PI compared with uninfected mice (Fig 2B). There was a gradual increase in the numbers of both CFU-GM and BFU-E after this time. When mice were inoculated with a lethal dose of MCMV, the numbers of CFU-GM (Fig 2A) and BFU-E (Fig 2B) declined 2 and 4 days PI. Similar results were recorded in repeat experiments.

The effects of MCMV infection on the numbers of CFU-GM in different strains of mice were compared to assess whether host genetic composition could influence the development of hematopoietic abnormalities. BALB/c, C57BL/10, and C3H/HeJ mice were infected with a constant sublethal dose (4 × 10^4 pfu), or a strain-adjusted sublethal dose (see Materials and Methods) of MCMV. Two days after infection with the constant virus dose, CFU-GM numbers were reduced 5- to 10-fold in BALB/c mice and less than twofold in C57BL/10 mice. C3H/HeJ mice showed no significant change in CFU-GM numbers after infection with this virus dose (Fig 3A). The numbers of CFU-GM declined approximately twofold in C57BL/10 and C3H/HeJ mice inoculated with strain-adjusted doses of MCMV (Fig 3B). CFU-GM numbers increased to control levels after day 4 of MCMV infection in all strains examined. Again, repeat experiments gave similar results to those described.

Susceptibility of bone marrow cells to infection with MCMV in vitro: Effects on hematopoietic colony development. Bone marrow cells were treated with MCMV in vitro to determine whether they were capable of supporting productive viral infection and whether the virus could inhibit development of hematopoietic colonies from stem and progenitor cells. Treatment of cells was performed using centrifugal enhancement to achieve the maximum level of infection possible. Preliminary experiments indicated that infection of male or female mice with MCMV resulted in similar changes in marrow cellularity and composition.

RESULTS

CFU-S numbers are reduced by lethal but not sublethal infection of BALB/c mice. The numbers of CFU-S in the bone marrow of BALB/c mice were monitored after infection with sublethal (4 × 10^4 pfu) or lethal (2.4 × 10^5 pfu) doses of MCMV (Fig 1). The results presented are the mean numbers of CFU-S calculated from two to four independent experiments. Although there was little change in CFU-S numbers in sublethally infected mice, infection with a lethal dose of MCMV resulted in a fivefold decrease in numbers of CFU-S at day 2 postinfection (PI) and a 20-fold decrease at day 4 PI. Mice infected with lethal doses of MCMV died 5 to 10 days PI. These findings suggest that the profound loss of CFU-S may be a feature of lethal MCMV infection.

Reduction in lineage-committed precursor cells in MCMV-infected mice. Myeloid and erythroid progenitor cells were also enumerated in the marrow of BALB/c mice after infection with lethal or sublethal doses of MCMV as described above. A 5- to 10-fold decrease in colony-forming units–granulocyte-macrophage (CFU-GM) was observed 2 days after sublethal MCMV infection (Fig 2A). Similarly, BFU-E numbers in sublethally infected mice were reduced 10-fold at day 2 PI compared with uninfected mice (Fig 2B). There was a gradual increase in the numbers of both CFU-GM and BFU-E after this time. When mice were inoculated with a lethal dose of MCMV, the numbers of CFU-GM (Fig 2A) and BFU-E (Fig 2B) declined 2 and 4 days PI. Similar results were recorded in repeat experiments.

The effects of MCMV infection on the numbers of CFU-GM in different strains of mice were compared to assess whether host genetic composition could influence the development of hematopoietic abnormalities. BALB/c, C57BL/10, and C3H/HeJ mice were infected with a constant sublethal dose (4 × 10^4 pfu), or a strain-adjusted sublethal dose (see Materials and Methods) of MCMV. Two days after infection with the constant virus dose, CFU-GM numbers were reduced 5- to 10-fold in BALB/c mice and less than twofold in C57BL/10 mice. C3H/HeJ mice showed no significant change in CFU-GM numbers after infection with this virus dose (Fig 3A). The numbers of CFU-GM declined approximately twofold in C57BL/10 and C3H/HeJ mice inoculated with strain-adjusted doses of MCMV (Fig 3B). CFU-GM numbers increased to control levels after day 4 of MCMV infection in all strains examined. Again, repeat experiments gave similar results to those described.

Susceptibility of bone marrow cells to infection with MCMV in vitro: Effects on hematopoietic colony development. Bone marrow cells were treated with MCMV in vitro to determine whether they were capable of supporting productive viral infection and whether the virus could inhibit development of hematopoietic colonies from stem and progenitor cells. Treatment of cells was performed using centrifugal enhancement to achieve the maximum level of infection possible. Preliminary experiments indicated that infection of male or female mice with MCMV resulted in similar changes in marrow cellularity and composition.
to 10% of bone marrow cells were productively infected with the virus (Table 2). Similarly, the development of erythroid colonies from BFU-E was not affected by treatment of bone marrow cell cultures from BALB/c mice with tissue culture-derived MCMV preparations (data not shown).

Attempts to enumerate CFU-S in bone marrow cell preparations treated with MCMV in vitro were unsuccessful because the high level of productive virus infection of bone marrow cells resulted in MCMV infection and disease in irradiated recipient mice.

**Enumeration of hematopoietic stem cells by flow cytometry.** The frequencies of marrow cells expressing Sca-1 or c-kit antigens in the absence of blood cell lineage-specific antigens (ie, Sca-1<sup>-Lin<sup>-</sup> or c-kit<sup>-Lin<sup>-</sup></sup>) were assessed in MCMV-infected and uninfected mice (Table 3). Previous studies have shown that cells with these surface phenotypes are highly enriched for both pluripotential stem cell and CFU-S activity. These experiments were performed in BALB/c, C57BL/6, and DBA/2J mice because BALB/c and DBA/2J mice are highly susceptible to MCMV disease, whereas C57BL/6 mice are more resistant. The Ly-6E.1 molecule expressed by BALB/c mice and the Ly-6A.2 molecule expressed by C57BL/6 and DBA/2J mice are allelic variants of the same molecule, and both are recognized by the anti-Sca-1 MoAb. We found significant increases in the frequencies of Sca-1<sup>-Lin<sup>-</sup> cells at days 2 and 4 PI compared with uninfected mice for each of the strains examined. Although marrow cell yields were decreased in the infected mice, we calculated that the actual numbers of Sca-1<sup>-Lin<sup>-</sup> cells had increased 20-fold at day 4 PI in BALB/c mice (from 0.2 × 10<sup>5</sup> to 3.9 × 10<sup>5</sup> cells per donor) and 10-fold in the other strains (from...
Table 1. The Effects of Exposure of Bone Marrow Cells From BALB/c Mice to MCMV In Vitro on Development of Granulocyte/Monocyte Colonies From CFU-GM

<table>
<thead>
<tr>
<th>MCMV Treatment</th>
<th>GM Colonies/10⁶ Bone Marrow Cells</th>
<th>Productive Infection/10⁶ Bone Marrow Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGV</td>
<td>1,490 ± 80</td>
<td>&lt;20</td>
</tr>
<tr>
<td>UV-SGV</td>
<td>1,560 ± 40</td>
<td>&lt;20</td>
</tr>
<tr>
<td>NSG</td>
<td>1,440 ± 80</td>
<td>&lt;20</td>
</tr>
<tr>
<td>TCV</td>
<td>1,500 ± 80</td>
<td>&lt;20</td>
</tr>
<tr>
<td>UV-TCV</td>
<td>1,300 ± 40</td>
<td>&lt;20</td>
</tr>
<tr>
<td>RPMI/2% FCS</td>
<td>1,560 ± 80</td>
<td>&lt;20</td>
</tr>
</tbody>
</table>

*Aliquots of 5 x 10⁶ marrow cells were exposed to 5 x 10⁶ pfu or equivalent concentrations of salivary gland-derived virus (SGV), SGV inactivated by exposure to UV light (UV-SGV), normal salivary gland homogenate (NSG), 5 x 10⁶ pfu of tissue culture-derived virus (TCV), TCV inactivated by exposure to UV light (UV-TCV), or RPMI supplemented with 2% FCS, which was used as a diluent for the cell and virus preparations.
† Mean numbers of granulocyte/monocyte colonies ± SEM for triplicate cultures.
‡ Productively infected cells enumerated by infectious center assay.

Table 2. The Effects of Exposure of Bone Marrow Cells From Different Strains of Mice to MCMV In Vitro on Development of Granulocyte/Monocyte Colonies From CFU-GM

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>MCMV Infection ( moi)*</th>
<th>GM Colonies/10⁶ Cell</th>
<th>Productive Infection/10⁶ Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>0</td>
<td>1,400 ± 140</td>
<td>&lt;20</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>1,300 ± 80</td>
<td>2,030</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>1,480 ± 60</td>
<td>9,160</td>
</tr>
<tr>
<td>C57BL/10</td>
<td>0</td>
<td>1,660 ± 100</td>
<td>&lt;20</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>1,580 ± 120</td>
<td>4,120</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>1,900 ± 180</td>
<td>27,420</td>
</tr>
<tr>
<td>C3H/HeJ</td>
<td>0</td>
<td>1,460 ± 80</td>
<td>&lt;20</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>1,600 ± 80</td>
<td>35,240</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>1,420 ± 60</td>
<td>126,970</td>
</tr>
</tbody>
</table>

* A total of 5 x 10⁶ marrow cells were exposed to 5 x 10⁶ or 5 x 10⁶ pfu MCMV or RPMI supplemented with 2% FCS to give the multiplicities of infection ( moi) indicated.
† Mean numbers of granulocyte/monocyte colonies ± SEM for triplicate cultures.
‡ Productively infected cells enumerated by infectious center assay.

BALT/c mice were assessed in radioprotection and reconstitution studies. Preliminary experiments indicated that infection of male or female mice with MCMV resulted in similar changes in marrow cellularity and composition. Hence, we used male mice infected with 4 x 10⁶ pfu of MCMV as marrow donors and female mice as recipients, allowing us to assess the origin of reconstituting peripheral blood cells in some recipients by in situ hybridization of blood cell smears with a DNA probe which recognizes mouse Y-chromosome—specific DNA sequences.

The numbers of marrow recipients that survived for 12 weeks after transplantation were similar with infected and uninfected donors (Table 4), suggesting that marrow cells from both MCMV-infected and uninfected mice were capable of mediating radioprotection. However, this result should be interpreted with caution because 2 of 8 mice survived irradiation without transplantation. We could not conclude whether marrow cells from MCMV-infected mice differed in their ability to protect mice from the effects of lethal irradiation compared with marrow cells from uninfected mice. The results do indicate that marrow cells from all groups of donors were capable of reconstituting the T-, B-, and myeloid cell lineages in irradiated recipients. There were no significant differences in the proportions of cells of each of these lineages in mice transplanted with marrow from MCMV-infected or uninfected donor mice (Table 4). The mice that survived irradiation without marrow transplantation had a higher frequency of cells expressing Gr-1 antigens in their spleens, but the significance of this result is unclear because there were only 2 mice. Cells that reacted with the Y-chromosome—specific probe were identified in blood cell smears prepared from mice that received marrow from donors infected with MCMV 2 days previously, but not in blood smears from mice that did not receive male bone marrow.

DISCUSSION

We have established that there is a significant reduction in the numbers of progenitor cells committed to the myeloid...
and erythroid lineages in the bone marrow of BALB/c mice infected with lethal or sublethal doses of MCMV. Because depletion of these cells occurs at the same time as mice experience leukopenia and atrophy of the spleen, the hematologic changes could result from changes in blood cell production in the marrow. In contrast, we found a significant decrease in myeloid progenitor cells only in lethally infected mice, indicating that the loss of more primitive hematopoietic cell populations or marrow exhaustion is a feature of lethal MCMV disease.

The reduction in numbers of CFU-GM in MCMV-infected BALB/c, C57BL/10, and C3H/HeJ mice correlated with previously reported differences in their sensitivity to MCMV disease; CFU-GM depletion was most pronounced in susceptible BALB/c mice and less pronounced in moderately resistant C57BL/10 and resistant C3H/HeJ mice, even when these mice received doses of virus adjusted for differences in their sensitivity to the effects of MCMV. We suggest that there may also be differences in the way individual patients respond to infection with HCMV, and the effects of the virus on marrow function may be influenced by the patient’s immunocompetence, the presence of other infections or illnesses, and genetic factors. Comparisons of the pathogenesis of MCMV disease in mice that are sensitive or resistant to the deleterious effects of the virus could also suggest some possible therapeutic strategies for investigation. For example, examination of cytokine profiles may indicate factors that are critical for eradication of the virus or for the maintenance of marrow function during infection.

Bone marrow cells were treated with MCMV in vitro to determine whether hematopoietic progenitor cells could serve as targets for MCMV infection. Virus treatment was performed using centrifugal enhancement to achieve the maximum level of infection possible. However, we found no significant differences in the ability of MCMV-infected or mock-infected preparations of marrow cells to form colonies of differentiated cells from CFU-GM, although up to 10% of the cells were productively infected by the virus. The experiments were performed with both tissue culture- and salivary gland-derived MCMV preparations, as well as UV-inactivated virus preparations, normal salivary gland homogenates, and culture medium as control treatments. These results differ from those of Busch et al., who reported that salivary gland-derivied (but not tissue culture-derivied) MCMV preparations could inhibit myeloid colony formation from CFU-GM, even after inactivation of infectious MCMV by UV irradiation. The differences in results may be related to differences in the length of time for which marrow cells were exposed to virus or control preparations in each study. Apparently, it is possible that the isolates of MCMV used for the studies differed in their ability to induce a myelosuppressive property in salivary gland tissue. Because we used infectious center assays to identify productively infected marrow cells, we could not eliminate the possibility that CFU-GM were abortively or latently infected with MCMV. However, CFU-GM counts were unaffected by treatment of marrow cells with virus in vitro, despite the fact that nonproductive infection of cells was likely to be greater in these cultures than in virus-infected mice. Indeed, Maciejewski et al. have shown that human hematopoietic progenitor cells can be infected with HCMV (detectable by in situ hybridization) and continue to differentiate and proliferate, giving rise to colonies of myeloid cells.

Variations in the ability of laboratory and clinical isolates of HCMV to infect myeloid cells and inhibit hematopoietic colony formation have been reported previously, leading to suggestions that isolates of HCMV differ in their tropism for cells of the hematopoietic system. We also found variations in the ability of isolates of MCMV derived from wild mice to decrease the numbers of marrow CFU-GM in BALB/c mice inoculated with the same number of pfu of each virus (data not shown). Hence, it appears that isolates of MCMV may also differ in their tropism for hematopoietic cells and their ability to suppress hematopoiesis.

We enumerated cells in the bone marrow of sublethally infected mice that expressed Sca-1 or c-kit antigens in the absence of blood cell lineage-specific antigens to indicate whether MCMV infection could alter the frequency of the most primitive hematopoietic stem cells in the bone marrow. The frequency and numbers of Sca-1”Lin” cells increased.
after MCMV infection in BALB/c, C57BL/6, and DBA/2J mice, suggesting that there may be an enrichment for pluripotent stem cells in the marrow of MCMV-infected mice. However, we did not observe an increase in c-kit+Lin- cells in MCMV-infected BALB/c or C57BL/6 mice. In addition, we did not find a decrease in the number of marrow cells required to prevent recipient mice from the effects of lethal irradiation. Hence, it is possible that MCMV infection induces a population of Sca-1+Lin- cells that are not detectable in the CFU-S or radioprotection assays. In view of these results, it would be interesting to determine whether MCMV infection affects marrow cells and to investigate whether infection targets early progenitor cells or an immature precursor stem cell. This may involve decreased production of colony-stimulating factors including stem cell factor or interleukin-12 (IL-12), both potent stimulators of hematopoietic cells of several lineages, particularly in synergy with other cytokines. Alternatively, production of factors that suppress the growth and development of hematopoietic cells may increase after MCMV infection. Candidate factors include macrophage inflammatory protein, transforming growth factor, tumor necrosis factor (TNF), interferon, and prostaglandins. To date, inhibitors of hematopoietic stem cell proliferation have not been found in HCMV-infected marrow cultures. However, the hypothesis is attractive because it is unlikely that large numbers of stromal cells are infected in MCMV-infected mice, but the release of inhibitory factors by a few infected marrow stromal cells could profoundly affect hematopoiesis. In addition, we propose that the depletion of CFU-GM observed in MCMV-infected mice may result from maturation and migration of cells to the marrow sites of viral replication and inflammation in the host. The finding that serum colony-stimulating activity increases after MCMV infection, as does the frequency of marrow cells expressing phenotypic markers of mature blood cells, supports this hypothesis. Migration of cells from the marrow may occur in response to inflammatory and chemotactic cytokines produced after MCMV infection; we are currently investigating the role of TNF as a factor that may initiate this process.

Changes in the frequencies of hematopoietic progenitor cell populations are not unique to MCMV infections. Decreases in the progenitor cell numbers in the marrow are also seen in mice infected with lymphocytic choriomeningitis virus (LCMV). This virus interferes with generation of myeloid, erythroid, and lymphoid cells and is associated with prolonged suppression of immune responsiveness. Thomsen et al. reported that hematopoietic dysfunction in LCMV-infected mice was partly attributable to the activation of NK cells in the bone marrow and hypothesized that activated NK cells caused rejection of progenitor cells. Other mechanisms, such as production of soluble factors that influence progenitor cell proliferation, may also be involved. Decreases in hematopoietic progenitor cell numbers can also follow the addition of B19 parvovirus, hepatitis A or B, and Dengue viruses to marrow cells in culture. These effects have been attributed to replication of the viruses in subsets of progenitor cells resulting in cytotoxicity or alterations the proliferative capacity of progenitor cells. In addition, in vivo colony formation by marrow progenitor cells can be suppressed by antibodies in the serum of patients with HIV or T lymphocytes from patients with Epstein-Barr virus infection.

The interference of CMV infection with normal hematopoietic function is now well documented in humans as well as in mice infected with MCMV. The clinical consequences include prevention of graft reconstitution in bone marrow transplant patients and increases in the susceptibility of patients to secondary infections as a result of their altered hematologic profile. The hematopoietic system may also provide an important target for infection, thereby acting as a vehicle for dissemination and persistence of the virus. We have shown that there are alterations in the frequencies of hematopoietic progenitor cells in the bone marrow of MCMV-infected mice. These changes are unlikely to be the result of a single mechanism acting on the hematopoietic system, but may be caused by a combination of events. First, the reductions in progenitor cell numbers may result from an increase in the rate of maturation and migration of cells from the marrow to sites of viral replication and inflammation in the host. Production of factors that regulate hematopoietic stem and progenitor cell proliferation may be altered as a consequence of MCMV infection of stromal cells. Finally, MCMV infection may alter the responsiveness of hematopoietic stem cells to proliferative signals by downregulating receptors for essential growth factors. The infection of mice with MCMV has proven to be a useful model for studying the effects of CMV infection on hematopoietic cell populations, and future work may suggest immunotherapeutic strategies for the control of HCMV-induced hematopoietic disorders.

ACKNOWLEDGMENT

The authors thank Dr Peter Klinken for the gift of erythropoietin and advice on performing BFU-E assays; Dr Gerald Spangrude for advice on staining of bone marrow cell preparations for flow cytometry; Dr Ken Shortman for providing the hybridomas E13 161.7, RM2.2, RB6-SC5, and TCR 119; Dr Suzanne Cory for the gift of ACK2 antibody; and Ying Fan for performing in situ hybridizations for Y-chromosome DNA.

REFERENCES


40. Palfree RG, Dumont FI, Hammerling U: Ly-6A.2 and Ly-6E.1 molecules are antispherical and identical to MALA-1. Immunogenetics 32:197, 1986


42. Williams DE, Eisenman J, Baird A, Rauch C, Van Ness K, March CJ, Park LS, Martin U, Machikuzu DY, Boswell HS, Burgess...

43. Zsebo KM, Williams DA, Geissler EN, Broudy VC, Martin FH, Atkins HL, Hsu RY, Birkett NC, Okino KH, Murdock DC, Jacobsen FW, Langley KE, Smith KA, Takeishi T, Cattanach BM, Galli SJ, Suggs SV: Stem cell factor is encoded at the Sl locus of the mouse and is the ligand for the c-kit tyrosine kinase receptor. Cell 63:213, 1990

44. Huang E, Nocka K, Beier DR, Chua TY, Buck J, Lahm HW, Wellner D, Leder P, Besmer P: The hematopoietic growth factor KL is encoded by the Sl locus and is the ligand for the c-kit receptor, the product of the W locus. Cell 63:225, 1990


Analysis of hematopoietic stem and progenitor cell populations in cytomegalovirus-infected mice

AE Gibbons, P Price and GR Shellam