*Proc. Natl. Acad. Sci. USA*
Vol. 87, pp. 1386–1390, February 1990
Medical Sciences

# Mechanisms of cytomegalovirus-mediated myelosuppression: Perturbation of stromal cell function versus direct infection of myeloid cells

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*Communicated by E. Donnall Thomas, November 6, 1989*

ABSTRACT **Infection with cytomegalovirus (CMV) continues to be one of the most common complications following allogeneic bone marrow transplantation. To study the role of CMV in the suppression of hemopoiesis that frequently accompanies infection, we investigated the effect of CMV on the growth of isolated committed myeloid progenitors and on hemopoiesis in long-term bone marrow cultures. Laboratory strain AD169 had no effect on the growth and development of progenitor cells. In contrast, 40% of clinical isolates of CMV inhibited colony formation by up to 100%. In long-term bone marrow cultures all CMV isolates resulted in myelosuppression, which in the majority of cases was associated with the infection of stromal elements. Analysis of RNA from stromal cells infected with AD169 and one clinical isolate demonstrated a specific deficiency of granulocyte colony-stimulating factor transcripts. For a small proportion of the clinical isolates tested in long-term bone marrow cultures, suppression of hemopoiesis was correlated with infection of developing granulocytes. These studies suggest that CMV can impair hemopoiesis either through infection of stromal cells and consequent perturbation of growth factor production or by direct infection of myeloid cells.**

Human cytomegalovirus (CMV), a member of the herpesvirus family, infects the majority of the population by adulthood (1). The consequences of CMV infection ranges from subclinical asymptomatic illness to a rapidly progressive disseminated disease in immunocompromised individuals. In patients undergoing bone marrow transplants, CMV can be associated with delayed platelet engraftment (2), phenotypically abnormal peripheral blood leukocytes (3), and graft failure (4).

Several mechanisms acting alone or in combination could mediate the myelosuppression associated with CMV. Hemopoietic progenitor cells or accessory cells such as monocytes and T lymphocytes, hypothesized to provide growth factors, may be targets for infection (5–8). Alternatively, since the maintenance of primitive hemopoietic progenitor cells is crucially dependent on their interactions with stromal cells of the marrow microenvironment (9), the perturbation of stromal cell function by CMV could also result in myelosuppression.

We studied the myelosuppressive effects of CMV *in vitro* using a laboratory-adapted strain and low-passage clinical isolates to infect human long-term bone marrow cultures (LTBMC) and committed progenitor cells. These investigations have demonstrated that laboratory and clinical isolates of CMV caused a marked suppression of hemopoiesis in LTBMC that was correlated either with the infection of maturing myeloid cells or stromal and accessory cell components with perturbation of growth factor transcripts. In addition, certain clinical isolates of CMV markedly inhibited the growth of isolated progenitor cells. These results clearly demonstrate several mechanisms of CMV-mediated myelosuppression *in vitro* that involve distinct components of the hemopoietic system as targets for infection.

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## MATERIALS AND METHODS

**Virus.** Human CMV strain AD169 was obtained from Raleigh Bowden [Infectious Diseases at Fred Hutchinson Cancer Research Center (FHCRC)]. A single stock of AD169 virus at $2 \times 10^7$ plaque-forming units (pfu)/ml was used throughout the experiments described.

CMV isolates from 19 patients undergoing bone marrow transplants were also studied. Isolates were derived from throat swabs, urine, bronchial alveolar lavage, and biopsies of lung, stomach, and duodenal tissue. CMV from clinical samples was propagated in human foreskin fibroblasts (10) grown in minimal essential medium supplemented with 10% fetal calf serum (first-passage virus). All CMV strains were used at passage two or three. Virus was derived from sonicates of infected fibroblasts demonstrating 80–100% cytopathic effects. The quantity of virus was determined by plaque assay. Tests for mycoplasm contamination were negative.

**Isolation of Committed Progenitor Cells.** After informed consent as defined by the Internal Review Board of the Fred Hutchinson Cancer Research Center, bone marrow cells from normal CMV seronegative donors were obtained and mononuclear cells were isolated by density gradient centrifugation over Ficoll (Lymphoprep; Nyegaard, Oslo). Light density cells were treated with soybean agglutinin (11) (Vector Laboratories), and the nonagglutinating fraction was isolated by unit gravity sedimentation over 5% bovine serum albumin. After washing, cells bearing CD2, CD7, CD10, CD18, and CD19 antigens were depleted by two sequential incubations with monoclonal antibody-coupled immunomagnetic beads (Dynal). Cells coexpressing CD33 and CD34 were isolated from the remaining population by two-color flow microfluorimetry (FACS II; Becton Dickinson). These cells had a colony-forming unit (cfu) plating efficiency of between 40% and 75%.

**LTBMCs.** LTBMCs were established according to a modification of the technique of Gartner and Kaplan (12). LTBMCs were initiated in 60 × 15 mm dishes by plating $3 \times 10^7$ hemolyzed buffy coat cells together with $10^7$ mononuclear cells in 10 ml of Iscove's modified Dulbecco's medium

Abbreviations: CMV, cytomegalovirus; LTBMC, long-term bone marrow culture; pfu, plaque-forming unit(s); cfu, colony-forming unit(s); CFU-GM, granulocyte/macrophage progenitor cells; BFU-E, primitive erythroid colony-forming cells; CFU-Mix, multipotential colony-forming cells; moi, multiplicity of infection; M-CSF, macrophage colony-stimulating factor; G-CSF, granulocyte CSF; GM-CSF, granulocyte/macrophage CSF; IEA, immediate-early antigen; EA, early antigen; LA, late antigen; FITC, fluorescein isothiocyanate; DAPI, 4',6-diamidino-2-phenylindole; FHCRC, Fred Hutchinson Cancer Research Center.

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(IMDM) at 365 mosM supplemented with 100 units of penicillin G sodium per ml, 100 μg of streptomycin sulfate per ml, 0.4 mg of L-glutamine per ml (GIBCO), 0.01 mg of folic acid per ml, 0.04 mg of *myo*-inositol per ml, 0.01 mM 2-mercaptoethanol, 1 μM hydrocortisone sodium succinate (Sigma), and 12.5% each of lot-selected batches of horse serum (Flow Laboratories) and fetal calf serum (HyClone). Cultures were maintained at 37°C in an atmosphere of 5% $CO_2$ in air for 3–4 days and switched to 33°C thereafter. LTBMCs were fed on a weekly basis by demi-depopulation, as described (13).

**Clonogenic Assay of Progenitor Cells.** Committed granulocyte/macrophage progenitor cells (CFU-GM) were assayed in IMDM supplemented with 20% fetal calf serum, 10% human placenta-conditioned medium, 0.05 mM 2-mercaptoethanol, and 0.3% agar (Bacto agar; Difco). Primitive erythroid colony-forming cells (BFU-E) and multipotential colony-forming cells (CFU-Mix) were assayed in 1 ml of medium consisting of IMDM, 30% fetal calf serum, 0.9% bovine serum albumin, 5% phytohemagglutinin-stimulated leukocyte-conditioned medium, 1 unit of recombinant human erythropoietin (Amgen Biologicals), 0.05 mM 2-mercaptoethanol, and 1.2% methylcellulose.

**Incubation of Hemopoietic Cells with CMV.** Enriched progenitor cell populations were incubated at $10^4$ per ml in medium containing AD169 virus or sonicates of cells infected with clinical isolates for 2 hr at 37°C. As a control, cells were incubated with sonicates of uninfected fibroblasts under identical conditions. LTBMCs were grown for 2 weeks before exposure to CMV. Cultures fed 24 hr previously were incubated with 2 ml of medium containing virus for 2 hr at 37°C.

**Immunohistological Staining for CMV Antigens in LTBMCs.** LTBMC-adherent cell layers were stained *in situ* using anti-CMV antibodies (Syva); E3 (CMV major immediate-early antigen, IEA); H5 (early antigen, EA); C5 (late antigen, LA). Briefly, after rinsing with warm Hanks' balanced salt solution, cultures were fixed for 10 min with 3% formaldehyde in phosphate-buffered saline (PBS), washed with PBS, and then permeabilized with absolute methanol for 10 min at −20°C. Following rehydration in PBS, the layers were treated with 0.2 M glycine in PBS, washed with PBS, and incubated with the anti-CMV antibodies. Bound antibody was revealed either by immunoperoxidase staining or by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (γ-specific; Southern Biotechnology Associates, Birmingham, AL), followed by nuclear counterstaining using 4′,6-diamidino-2-phenylindole (DAPI). Where indicated, LTBMC layers were double immunolabeled using E3, H5, or C5 in combination with antibodies specific for different adherent layer components. Included among these are the following: rabbit anti-human factor VIII-related antigen (Dako; endothelial cells); E-3 (I. Bernstein, FHCRC; IgG3; antihuman umbilical vein endothelial cells); 6.19 (14) (C. Frantz, University of Rochester School of Medicine and Dentistry, Rochester, NY; IgG2a; stromal fibroblasts, adipocytes, endothelial cells); LeuM3 (CD14, Becton Dickinson, IgG2b; macrophages); goat antisera to collagen types I, III, IV, and V (Southern Biotechnology Associates); rabbit anti-human fibronectin (Calbiochem); rabbit antilaminin (courtesy of William Carter, Division of Basic Sciences, FHCRC). These antibodies were revealed using biotinylated goat anti-rabbit IgG, rabbit anti-goat IgG (Vector Laboratories), or FITC-conjugated isotype-specific anti-mouse Ig (Southern Biotechnology Associates), as appropriate, followed by streptavidin-Texas Red (Southern Biotechnology Associates) and nuclear counterstaining with DAPI. Histochemical staining for alkaline phosphatase on marrow stromal elements and nonspecific esterase activity in monocyte macrophages was performed according to standard procedures. Phagocytosis was assessed by a 30-min incubation at 37°C with a 1:200 dilution of India ink (Pelikan).

**RNase Protection Assays.** Total cellular RNA was prepared from LTBMCs 24 hr after feeding, using the method of Chirgwin *et al.* (15) followed by centrifugation through a 4.7 M $CsCl_2$ pad. Uniformly labeled antisense cRNA probes representing 445 nucleotides of the human macrophage colony-stimulating factor (M-CSF) coding sequence (16), 277 nucleotides of the human granulocyte CSF (G-CSF) coding sequence excluding the variably spliced region (17), or 497 nucleotides of the human granulocyte/macrophage CSF (GM-CSF) coding region (18) were prepared with the T7 promoter of the plasmid pGEM using [$^{32}$P]UTP and T7 RNA polymerase according to the manufacturer's recommendations (Promega). These probes were gel-purified and then mixed with 4-μg samples of whole cellular RNA in the presence of 3 M guanidium isothiocyanate in a reaction volume of 20 μl. The mix was denatured at 80°C for 15 min and then incubated overnight at 42°C. Following hybridization, the reaction mixture was diluted to 300 μl with TE buffer (10 mM Tris, pH 7.4/1 mM EDTA), mixed with 1–10 μl of RNase (1 mg of RNase A per ml/50,000 units of RNase T1 per ml), and incubated for 60 min at room temperature. The optimal concentration of RNase was determined for each probe using yeast RNA as a negative control, RNA from the human pancreatic carcinoma cell line MiaPaCa as a positive control for M-CSF (16), and the human bladder carcinoma cell line 5637 (19, 20) as a positive control for G-CSF and GM-CSF. Following digestion, the reaction products were digested with proteinase K (final concentration, 100 μg/ml) for 15 min at room temperature and extracted with phenol and then extracted twice with chloroform. Following ethanol precipitation in the presence of carrier RNA, the reaction products were size-fractionated on a 6% denaturing polyacrylamide gel and identified by autoradiography.

## RESULTS

**Effect of HCMV on the Growth of Isolated Progenitor Cells.** Highly enriched populations of progenitor cells, free of contaminating accessory cells, were incubated with AD169 or clinical isolates (Table 1). AD169 and 12 of 19 clinical isolates had no effect on the growth of colonies nor was viral IEA detected in cytospin preparations. However, 8 clinical isolates inhibited growth of CFU-GM, BFU-E, and CFU-Mix colonies, ranging from 17% to 100% of growth obtained in mock-infected control cultures.

**Addition of CMV to LTBMCs: Effects on Hemopoiesis.** CMV has been demonstrated to infect and replicate in bone marrow-derived stromal cells of simian and human origin (21, 22). To investigate whether infection with CMV impaired the ability of marrow stromal cells to maintain hemopoietic development, LTBMCs established from normal seronegative donors were inoculated with AD169 virus, and cultures were monitored on a weekly basis by assessing nonadherent cell production and phenotype. The results of a representative experiment are shown in Fig. 1. Cultures inoculated with AD169 showed rapid and progressive decreases in weekly nonadherent cell production, including dramatic reductions in the numbers of nonadherent day 7 CFU-GM as well as day 14 BFU-E and CFU-Mix.

The effect of adding CMV clinical isolates to LTBMCs was similarly assessed (Fig. 2). All five CMV isolates tested resulted in variable but marked reductions in nonadherent cell production with time. Within 1 week of addition of virus, day 7 CFU-GM numbers fell by 130-fold, day 14 BFU-E fell by 80-fold, and CFU-Mix numbers were reduced to undetectable levels. Similar results have been obtained with an additional 10 clinical CMV isolates.

**Identification of CMV-Infected Cell Types in LTBMCs.** As summarized in Table 2 (Fig. 3 *A*, *B*, *E*, and *F*), adipocytes and nonphagocytic fibroblast-like cells were found to express

Table 1. Effect of AD169 and low-passage clinical isolates of CMV on colony formation by enriched bone marrow-derived progenitor cells

| Virus | Passage | No. of experiments | moi | % inhibition of colony formation* | | |
|---|---|---|---|---|---|---|
| | | | | CFU-GM | BFU-E | CFU-Mix |
| AD169 | ND | 6 | 0.1 | 0 | 0 | 0 |
| AD169 | ND | 6 | 1.0 | 0 | 0 | 0 |
| AD169 | ND | 6 | 10.0 | 0 | 0 | 0 |
| 2829 | 2 | 2 | 2.64 | 0 | 0 | 0 |
| 6855 | 2 | 2 | 0.58 | 0 | 0 | 0 |
| 5009 | 3 | 4 | 4.68 | 58 ± 8 | 31 ± 17 | 66 ± 13 |
| 5068 | 3 | 4 | 3.51 | 58 ± 16 | 38 ± 30 | 100 ± 0 |
| 5435 | 3 | 4 | 6.07 | 56 ± 9 | 33 ± 16 | 100 ± 0 |
| 5475 | 3 | 4 | 2.34 | 46 ± 18 | 32 ± 4 | 33 ± 20 |
| 5706 | 3 | 4 | 1.38 | 36 ± 8 | 44 ± 4 | 17 ± 8 |
| 8417 | 3 | 4 | 1.81 | 0 | 0 | 0 |
| 8592 | 3 | 4 | 3.74 | 0 | 0 | 0 |
| 8855 | 3 | 4 | 2.35 | 0 | 0 | 0 |
| 8962 | 3 | 4 | 1.48 | 0 | 0 | 0 |
| 9081 | 3 | 4 | 1.21 | 0 | 0 | 0 |
| 11206 | 2 | 2 | 1.04 | 0 | 0 | 0 |
| 11239 | 3 | 2 | 0.67 | 0 | 0 | 0 |
| 11386 | 2 | 3 | 0.91 | 0 | 0 | 0 |
| 11707 | 3 | 2 | 0.88 | 0 | 0 | 0 |
| 12509 | 2 | 2 | 0.93 | 66 ± 20 | 43 ± 13 | 50 ± 25 |
| 12623 | 2 | 2 | 1.91 | 56 ± 24 | 84 ± 8 | 100 ± 0 |
| UF179 | 2 | 2 | 0.52 | 89 ± 9 | 90 ± 8 | 100 ± 0 |

moi, Multiplicity of infection; ND, not determined. Values are presented as mean ± SEM.
*Enriched progenitor populations were plated at 250 cells per ml in triplicate. The actual number of colonies detected in "mock-infected" control cultures ranged from 50 to 87 per dish for CFU-GM and BFU-E and 1 to 3 per dish for CFU-Mix. Controls were mock infected by exposure to sonicates of uninfected foreskin fibroblasts.

IEA and LA ($IEA^+$, $LA^+$) in AD169-treated LTBMCs. CMV antigens could not be detected in macrophages or in granulocytic cells attached to the adherent layer (Fig. 3 *I* and *J*).

Although all 15 clinical isolates tested suppressed hemopoiesis in LTBMCs, the most acutely myelosuppressive isolates (5009, 5068, 5435, and 5475) showed antigen expression initially restricted to monocyte/macrophages (Fig. 3 *G* and *H*), developing granulocytes, and occasional blast cells (Fig. 3 *K* and *L*). With time there was a slow but progressive accumulation of $IEA^+$ and $EA^+$ stromal cells. Rare $LA^+$ cells were first observed 6 weeks after addition of virus, coinciding with the first appearance of cytopathic effects in stromal elements. None of the four isolates resulted in infection of >2% of stromal cells as compared with AD169.

By contrast, CMV isolates 8417, 8855, 8592, 8962, 9081, 11206, 11239, 11386, and 11707 gave a pattern of infection very similar to that of AD169 with $6.19^+$ fibroblast-like stromal cells $IEA^+$ and $LA^+$ and adipocytes $IEA^+$, whereas granulocytes remained negative. However, unlike AD169, these CMV isolates infected between 1% and 5% of macrophages and endothelial cells (Fig. 3 *C* and *D*).

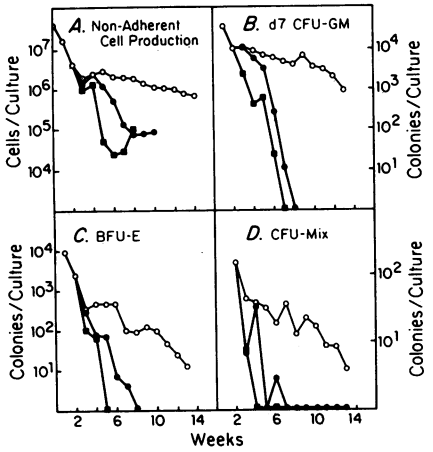


FIG. 1. Effect of CMV strain AD169 on hemopoiesis in LTBMCs. (*A*) Nucleated cell production. (*B*) Nonadherent day 7 CFU-GM. (*C*) BFU-E. (*D*) CFU-Mix. LTBMCs at week 2 were incubated with 2 ml of medium containing AD169 virus for 2 hr at 37°C at moi of 1.0 (■) and 0.01 (●). ○, Mock-infected control cultures. Each point represents the mean of four LTBMCs.

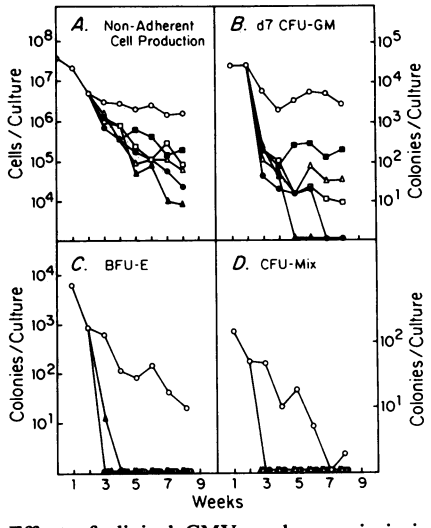


FIG. 2. Effect of clinical CMV on hemopoiesis in LTBMCs. LTBMCs were incubated with virus derived from sonicates of infected fibroblasts, as described in the legend to Fig. 1. ●, CMV 5009 (moi = 0.049); □, CMV 5068 (moi = 0.037); ▲, CMV 5435 (moi = 0.063); △, CMV 5475 (moi = 0.024); ■, CMV 5706 (moi = 0.014). ○, LTBMCs mock-infected with sonicates of uninfected fibroblasts. Each point represents the mean of three LTBMCs.

Table 2. Immunohistological staining for CMV antigens in LTBMCs infected with AD169 or low-passage clinical isolates of CMV

| Cell type | Phenotype | AD169 | | | Group I* | | | Group II† | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | IEA | EA | LA | IEA | EA | LA | IEA | EA | LA |
| Marrow fibroblast | Collagen types I, III, V$^+$; fibronectin$^+$; 6.19$^+$; Alk-Pase$^+$ | + | + | + | + | + | + | ± | ± | ± |
| Endothelial cell | FVIIIrAg$^+$; collagen type VI$^+$; laminin$^+$; 6.19$^+$; E-3$^+$ | − | − | − | + | + | + | − | ND | − |
| Adipocyte | Oil red 0$^+$; 6.19$^+$; FVIIIrAg$^-$; LeuM3$^-$ | + | ND | ND | + | ND | ND | − | ND | ND |
| Macrophage | LeuM3$^+$; phagocytic NSE$^+$; AC-Pase$^+$ | − | − | − | + | − | − | + | − | − |
| Granulocytic cell | | − | − | − | − | − | − | + | ND | ND |

Immunohistological staining was done using monoclonal antibodies E3, H5, and C5 to IEA, EA, and LA, respectively. ND, not determined. +, Presence of antigen in all cells of that phenotype; −, absence of antigen in all cells of that phenotype; ±, presence of antigen in a proportion of cells of that phenotype. Alk-Pase, alkaline phosphatase; AC-Pase, acid phosphatase; NSE, nonspecific esterase; FVIIIrAg, factor VIII-related antigen.

*LTBMCs infected with clinical isolates of CMV 8417, 8855, 8592, 8962, 9081, 11206, 11239, 11386, 11526, and 11707.

†LTBMCs infected with clinical isolates of CMV 5009, 5068, 5435, and 5475.

**Effect of CMV on Growth Factor Production by Adherent Cells.** Serum-free medium conditioned by the growth of AD169-infected and mock-infected LTBMC cultures was assayed for the presence of activities stimulating the growth of CFU-GM and BFU-E (Table 3). Conditioned medium from control cultures increased the growth of BFU-E relative to controls and supported moderate numbers of CFU-GM. Conditioned medium from CMV-infected cultures had moderate burst-promoting activity but lacked activities stimulating CFU-GM. Titration of the CMV-conditioned medium over the range of 1–20% against optimal concentrations of purified recombinant interleukin 3, GM-CSF, or G-CSF did not reveal the presence of any inhibitory activities (data not shown).

The same LTBMCs were used to evaluate the levels of RNA transcripts for G-CSF, M-CSF, and GM-CSF. RNA was extracted from the stromal adherent layers 24 hr after feeding and analyzed by means of an RNase protection assay. As shown in Fig. 4*A*, mock-infected control cultures demonstrated transcripts for all three CSFs. In contrast, LTBMCs infected with AD169 showed no detectable G-CSF mRNA.

Finally, CSF transcripts in normal LTBMCs infected with two different CMV clinical isolates were also studied. The results of the RNase protection assays (Fig. 4*B*) demonstrate no effect of either isolate on GM-CSF transcripts but varied effects on M-CSF transcripts. Infection with CMV I (8592) resulted in the disappearance of G-CSF transcripts as observed in LTBMCs infected with the AD169 strain of virus. Conversely, with CMV II (5009) levels of G-CSF, mRNA was increased with respect to control.

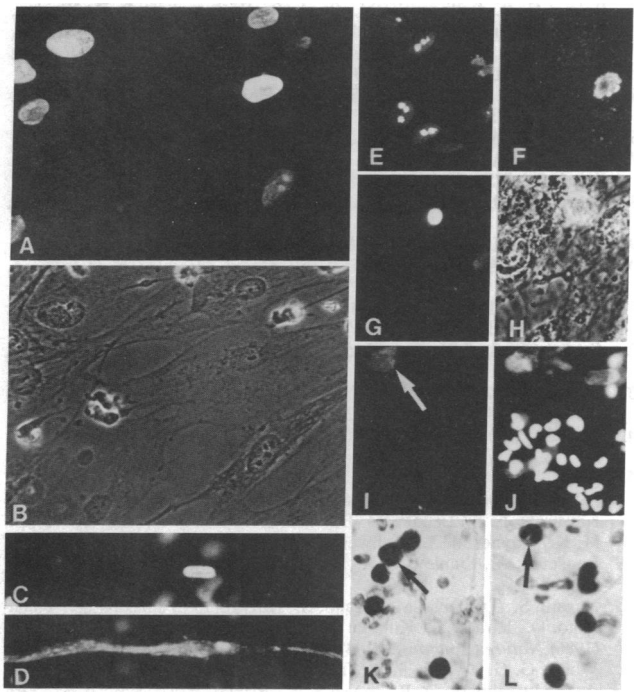


FIG. 3. Immunohistological staining for CMV antigens in the adherent layer of LTBMCs. (*A*) Immunofluorescence staining for IEA in the nuclei of stromal cells infected with AD169. (×270.) (*B*) Phase-contrast image of the field depicted in *A*. (×270.) (*C* and *D*) Double immunofluorescence staining for intranuclear IEA (FITC, *C*) and factor VIII rAg (Texas Red, *D*) in a single endothelial cell in a culture infected with CMV 8592. (×270.) (*E*) Staining with EA in the nucleus of stromal cells infected with AD169. (×170.) (*F*) LA in the cytoplasm of a stromal cell infected with AD169. Note prominent inclusion body. (×170.) (*G*) IEA in the nucleus of a macrophage in a culture infected with CMV 5009. (*H*) Phase-contrast of the field in *G*. (×270.) (*I*) IEA in the nucleus of a single stromal cell (arrow) in a culture infected with AD169 virus. (*J*) Same field showing nuclear counterstain with DAPI. Note absence of IEA in developing granulocytic cells. (×270.) (*K* and *L*) Immunoperoxidase staining for IEA in a culture infected with CMV 5068. Note IEA in cells with blast-like morphology (arrow, *K*) and a granulocytic cell (arrow, *L*). (×440.)

## DISCUSSION

Although many reports document the presence of CMV IEA in leukocytes (6–8, 23–27), evidence of viral replication with a potential role in myelosuppression has not been demonstrated (6, 7, 25), with one exception (27). Our current report demonstrates growth inhibition of myeloid, erythroid, and multipotential progenitor cells by 8 of 20 clinical isolates of CMV. These results are at variance with those of Apperley *et al.* (22), who failed to show inhibition of growth with 4 of 4 low-passage clinical isolates.

We were unable to find any effect of the laboratory-adapted strain of CMV AD169 on the growth of isolated progenitors. Such differences between AD169 and low-passage clinical isolates of CMV have been reported by

Table 3. Bioassay of serum-free conditioned medium (CM) from AD169-infected and control LTBMCs

| CM added | BFU-E | CFU-GM |
|---|---|---|
| HPCM + Epo | 132 ± 8 | 121 ± 5 |
| Control CM + Epo | 162 ± 4* | 78 ± 4 (+ 34 ± 7 clusters) |
| AD169 CM + Epo | 107 ± 17† | 1 ± 0 (+ 18 ± 3 clusters) |

Data represent the mean ± SEM of the number of colonies obtained from $10^4$ accessory cell-depleted bone marrow cells. CM was 10% and erythropoeitin was used at 1.5 units. HPCM, human placenta-CM.

*Large multicentric, ≥6 clusters per BFU-E.

†Very small monocentric colonies.

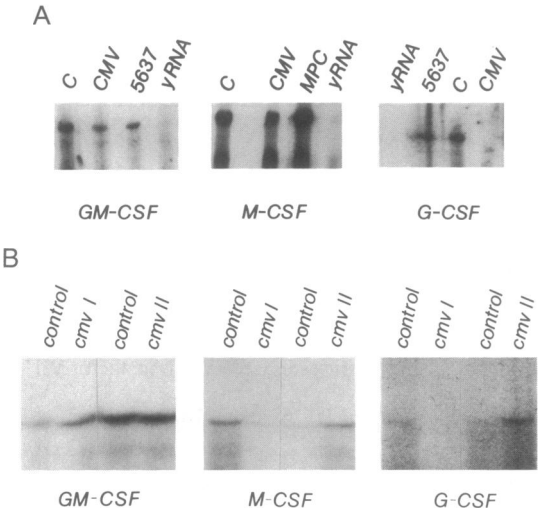


FIG. 4. RNase protection analysis of G-CSF, GM-CSF, and M-CSF transcripts in control and CMV-infected LTBMCs. (*A*) A group of cultures established from one donor was infected with AD169 virus at moi = 0.1 at week 2. Total RNA was extracted from infected and control cultures at week 4, and equal quantities were hybridized with uniformly labeled cRNA probes for G-CSF, GM-CSF, and M-CSF. Yeast RNA (yRNA) or RNA from MiaPaCa (MPC) or 5637 was used as negative and positive controls for M-CSF, G-CSF, and GM-CSF, respectively. (*B*) Results of a similar analysis of RNA from LTBMCs infected with two clinical isolates of CMV: CMV I, 8592 (moi = 0.012); CMV II, 5009 (moi = 0.027). RNA was isolated from infected and control LTBMCs 4 weeks after addition of virus and probed as above.

others (6, 22–24), suggesting that AD169 may have undergone a shift in cell tropism as a result of extended *in vitro* propagation on fibroblasts. However, approximately two-thirds of the clinical isolates also failed to inhibit progenitor growth and in this respect resembled AD169.

Of the eight clinical isolates shown to inhibit progenitor growth, four showed little tendency to infect adherent stromal cells of LTBMCs and were unique in their ability to induce IEA in developing granulocytic cells. This suggests that the suppression of hemopoiesis seen with these isolates is by means of their ability to infect primitive hemopoietic cells and inhibit their development.

The addition of AD169 and each of the remaining clinical isolates to LTBMCs resulted in a marked suppression of hemopoiesis in association with infection of stromal cells. Infection of stromal cells with CMV *in vitro* has been demonstrated in human, simian, and murine systems (21, 22, 28). We therefore investigated what effect CMV had on stromal cell function by assaying conditioned medium from CMV-infected LTBMCs for its ability to stimulate colony formation by progenitor cells and by analyzing the expression of CSF genes in infected stromal cells. The former experiments demonstrated that conditioned medium from CMV-infected cultures specifically lacked activities capable of stimulating the growth of granulocytic colonies but did have burst-promoting activities. Assays for inhibiting activities were negative, suggesting that the lack of CFU-GM growth was due to the absence of colony-stimulating activities. By using a sensitive nuclease protection assay we could demonstrate that AD169-infected stromal cells lack G-CSF transcripts but have unaltered levels of GM-CSF, a result in keeping with the bioassay results and the data of Migliaccio *et al.*, which showed that CFU-GM-stimulating activity is accounted for by G-CSF whereas the burst-promoting activity is attributable to GM-CSF (29).

Analysis of CSF transcripts in stromal cells infected with clinical isolates of CMV presented a more complex picture. Of two isolates tested, one showed evidence of a decrease in G-CSF transcripts, and one had no effect on growth factor mRNA levels. The latter isolate was one of the few found to infect granulocytic cells rather than stromal elements, further supporting the hypothesis that the myelosuppressive effects of this isolate may be by means of infection of myeloid cells. However, it is conceivable that this and the other CMV strains may have effects on the levels of other growth factors produced in LTBMCs. For example, others have shown that infection of monocytes with CMV abrogates their production of interleukin 1 (23).

In conclusion, we have demonstrated that CMV-mediated myelosuppression can be produced *in vitro*, by means of infection of myeloid cells or infection of stromal cells. Comparable mechanisms may be operant *in vivo*. Based on these data, it seems reasonable to suggest that in some patients with CMV-associated graft failure, treatment with specific growth factors may improve marrow function.

This work was supported in part by Grants HL36444, CA18221, and DK34431, awarded by the National Institutes of Health, Department of Health and Human Services.

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