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Human cytomegalovirus latent infection of granulocyte–macrophage progenitors

(herpesvirus/gene expression/myeloid/persistent infection)

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ABSTRACT **We have investigated the interaction of human cytomegalovirus (CMV) with cultured primary granulocyte–macrophage progenitors, a suspected natural site of viral latency, and have established conditions for latent infection and reactivation in this cell population. Progenitor cells from human fetal liver or bone marrow maintained a CD14$^+$, CD15$^+$, CD33$^+$ cell surface phenotype during propagation in suspension culture. Exposure to human CMV did not reduce growth or alter the phenotype of these cells during a 4-week culture period. Viral replication was not detectable in these cells, although viral DNA, as measured by PCR analysis, persisted in a high proportion of cultured cells in the absence of delayed early (β) gene expression. Viral gene expression was restricted such that only *ie*1 region transcripts were detected by PCR analysis of cDNA, and these transcripts were estimated to be present in no less than 2–5% of latently infected cells. Most of these transcripts remained unspliced, a result that strikingly contrasts with the splicing pattern normally seen during viral replication in permissive cells. Latent virus reactivated after prolonged, 16- to 21-day cocultivation of infected granulocyte–macrophage progenitors with permissive cells, results that support a role for the myelomonocytic cell population as a biological reservoir of latent human CMV and suggest that these cells may be the source of CMV DNA PCR-positive monocytes found in the peripheral blood of healthy carriers.**

Human cytomegalovirus (CMV), a ubiquitous species-specific herpesvirus and significant pathogen in immunocompromised individuals and neonates (1), is the best studied member of the betaherpesviruses (2). Although latent infection by CMV is widespread and reactivation of latent virus after either immunosuppression or progressive immunodeficiency is the single most important contributor to emergence of CMV disease, the site(s) of viral latency remain poorly characterized. Viral DNA has been detected in peripheral blood cells of healthy seropositive carriers (3, 4), and monocytes have been implicated as the most likely cell type harboring latent viral genomes (4). Although the CMV genome persists in monocytes, virus does not reactivate during cultivation under conditions that stimulate growth and differentiation (5). Thus, it has remained unclear whether monocytes, or other mononuclear cell types in peripheral blood, correspond to true sites of latency or reflect an occasional depository of viral DNA during sporadic reactivation (6–8).

A wide range of peripheral blood leukocytes has been shown to restrict CMV replication after infection (6, 7, 9–11). Monocytes and bone marrow-derived granulocyte–macrophage progenitors (GM-Ps) have been the focus of many experiments. These fail to support viral replication, but reports on viral gene expression have been inconsistent (10, 12–15). These investigations have suggested a role for GM-Ps as a reservoir of persistent or latent CMV infection.

MATERIALS AND METHODS

Cell and Virus Culture. Fetal liver cells (from 12- to 18-week abortuses) were cultured in Iscove's modified Dulbecco's medium (GIBCO/BRL) supplemented with 5% fetal bovine serum, 5% conditioned medium from the 5637 bladder carcinoma cell (ATCC HTB9), penicillin G (100 units/ml), and streptomycin (100 μg/ml) (16). Nonadherent cells were collected and transferred three times a week, depleting cultures of stromal cells and differentiated, adherent myelomonocytic lineage cells. Human foreskin fibroblast (HF) cells were grown in Dulbecco's modified Eagle's medium (GIBCO)/10% NuSerum (Collaborative Research). The *lacZ* derivative of human CMV strain Towne, RC256 (17) and low-passage isolate Toledo (18) were propagated on HF cells.

Detection of Viral Infectivity. Three freeze/thaw cycles (−80°C/37°C) were used in attempts to release virus from 10^5 GM-Ps. Plaque assays were conducted on HF cells and included centrifugal enhancement at inoculation (19). Infected GM-Ps were introduced into HF cell cultures to recover virus by cocultivation.

Nested DNA PCR and Cell Dilution. Cells or nuclei were counted and diluted to give an average of 100, 30, 10, 3, and 1 per tube, and DNA was extracted as described (20). Nuclei were prepared from cells lysed on ice in 20 mM Hepes·KOH, pH 7.9/5 mM KCl/0.5 mM $MgCl_2$/5 mM dithiothreitol/0.1% Triton X-100 and were monitored by phase-contrast microscopy. Initial PCR amplification was with *ie*1 primers IEP4BII and IEP2AII (Table 2) as described (21) using a Perkin–Elmer/Cetus thermocycler (94°C for 1 min, 62°C for 1 min, and 72°C for 2 min) for 30 cycles followed by nested primers IEP3B and IEP3A (94°C for 1 min, 52°C for 1 min, and 72°C for 2 min) for 30 cycles. The reaction contained 50 mM KCl, 10 mM Tris·HCl (pH 8.5), 2 mM $MgCl_2$, 1 μM of each primer, 200 μM of each dNTP, and 1.25 units of *Taq* polymerase (Perkin–Elmer/Cetus). After amplification, 10% of each reaction was separated by electrophoresis in 2.5% agarose gels. Gels were stained with ethidium bromide.

Quantitative Competitive PCR. Cells (8×10^4) or nuclei were suspended in 60 μl of 50 mM KCl/10 mM Tris·HCl, pH 8.5/2 mM $MgCl_2$/0.45% Nonidet P-40/0.45% Tween 20 and proteinase K (100 μg/ml), incubated 16 hr at 65°C, followed by 98°C for 10 min to inactivate proteinase K (22). Each sample was divided into six equal portions (1.3×10^4 cells) and analyzed in the presence of 3×10^3 to 1×10^6 copies of denatured human CMV *ie*1 cDNA (from pON2347, a construct containing the genomic *ie*1 region between −1021 and +947

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Abbreviations: CMV, cytomegalovirus; GM-P, granulocyte–macrophage progenitor; CFU-GM, colony-forming units of granulocyte–macrophage; β-Gal, β-galactosidase; RT-PCR, reverse transcription PCR; HF, human foreskin fibroblast.

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linked to a 1525-bp *Eco*RI/*Bam* I cDNA fragment representing exons 2, 3, and 4) as a competitive template. PCR amplification with primers IEP3C and IEP4BII (Table 2) was done for 30 cycles under the same conditions and analyzed by agarose gel electrophoresis as described for the nested PCR analysis.

Reverse Transcription (RT)-PCR. RNA was extracted from infected GM-Ps and control mixtures of CMV-infected HF cells and uninfected GM-P cells by standard methods (23). For all samples of 10^3 or fewer GM-Ps, 2 μg of RNase-free yeast tRNA (Sigma) was added before RNA extraction. RNA was treated with 5 units of RNase-free RQ1 DNase (Promega) in the presence of 100 units of RNasin (Promega) for 1 hr at 37°C, and cDNA was synthesized using 1 μg of random hexamer primers and SuperScript II murine leukemia virus reverse transcriptase (GIBCO/BRL) using the manufacturer's protocol. For the $\beta_{2.7}$ gene transcript, which is unspliced, 45 cycles of PCR were conducted with primers 2.7A and 2.7B using cycle parameters of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min. For the spliced UL112/113 transcript, an asymmetric nested amplification was done first with primers 112A and 113B for 30 cycles and then with primers 113D and 112A for 30 cycles using cycle parameters of 94°C for 1 min, 65°C for 1 min, and 72°C for 2 min. All primers and predicted PCR products are described in Table 2. For the *ie*1 transcript, 30 cycles of PCR was with primers IEP2AII and IEP3D (exon 2–3; Fig. 3*C*) or IEP4BII and IEP3C (exon 3–4; Fig. 3*D*) using parameters of 94°C for 1 min, 65°C for 1 min, and 72°C for 2 min or 94°C for 1 min, 62°C for 1 min, and 72°C for 2 min. After electrophoresis in 2.5% agarose gels, samples were visualized by using ethidium bromide. For the $\beta_{2.7}$ or UL112/113 transcript analysis, separated fragments were denatured and transferred to Hybridon-N$^+$ membrane (Amersham), hybridized with [^{32}P]dCTP-labeled (random primed, Amersham) DNA probes and subjected to autoradiography on Kodak X-Omat film.

RESULTS

We initiated studies using DNA PCR to track the viral genome in myeloid lineage cells of experimentally infected severe combined immunodeficiency (SCID)-hu mice that had been implanted with either human fetal thymus plus liver (24) or human bone plus bone marrow (25). Previous studies had shown that CMV replicates in human medullary thymic epithelium (26) and human bone marrow stromal cells (H.K., E.S.M., and M. Bonyhadi, unpublished work). Using bone marrow cells from infected implants, colony forming units of granulocyte–macrophage (CFU-GM) cells were identified by methylcellulose colony-forming assay (25). CMV DNA was detected in pools of ≈100 colonies, although virus was not recovered from cells after either freeze-thaw lysis or 12-day coculture with permissive HF cells. This work suggested that CMV may gain entry into GM-Ps and remain latent.

To establish cultures in which the direct interaction of CMV with GM-Ps could be investigated, fetal bone marrow or liver cells were cultivated under myelomonocytic cell culture conditions (16). These culture conditions supported the outgrowth of myelomonocytic lineage cells independent of stromal cells that are permissive for CMV (data not shown). Stromal cells from adult bone marrow have long been known to be permissive for CMV (12). The nonadherent cell population in these suspension cultures expressed CD14, CD15, and CD33 cell surface markers, as judged by multiparameter flow cytometry using a FACScan and murine monoclonal antibodies from Becton Dickinson (data not shown). Nonadherent cells in these cultures exhibited a myelomonocytic morphology (Fig. 1). Cultures initiated with fetal bone marrow or with fetal liver allowed the outgrowth of a similar nonadherent population of cells that continued to grow for ≈4 weeks. Nonadherent cells were transferred three times a week, initially to deplete stromal cells, but later to remove the large, adherent cells that developed from the small adherent cells. Methylcellulose colony-forming assay (24) detected between 1×10^3 and 5×10^3 CFU-GM per 10^6 starting fetal liver cells (Fig. 1). More than 50% of the cells used to initiate these cultures were CD33$^+$, and a small proportion were CD34$^+$ and capable of forming CFU-GM. After 2 weeks in culture, CFU-GM were no longer detectable by methylcellulose colony-forming assay, probably due to the loss, through differentiation, of the precursor population. Also, neither CD34$^+$ nor CD19$^+$ (B-cell lineage) cells were detectable when suspension cultures were evaluated 2 weeks after being established.

For infection with CMV, duplicate or triplicate samples of 10^6 fetal liver cells from 10 individual CMV-negative sources were cultured. Three days later, nonadherent cells were collected, counted, and exposed to CMV (strain RC256) at a multiplicity of infection of 3. We used RC256, a derivative of the Towne strain that carries the *lacZ* gene under control of the strong CMV β (delayed early) promoter ($\beta_{2.7}$), because this virus expresses abundant levels of β-galactosidase (β-Gal), which provided a simple sensitive indicator of viral replication in cells or tissues (17, 26). Infected cells were washed free of virus and then placed in culture. At weekly intervals, 10^5 GM-Ps were removed from duplicate or triplicate cultures from each independent fetal sample. Viral infection had no significant effect on growth or morphology of either nonadherent or adherent cells. Weekly samples were analyzed for infectious virus and for β-Gal expression as an indicator of viral β (delayed early) gene expression (Table 1). Neither plaque assay of freeze/thaw cell lysates nor infectious centers assay of intact cells yielded evidence of virus replication in the GM-P population, consistent with reports (12–15). Furthermore, cells did not stain with 5-bromo-3-chloro-indolyl β-D-galactoside. Thus, neither residual input infectious virus nor viral replication was detectable in these cultures.

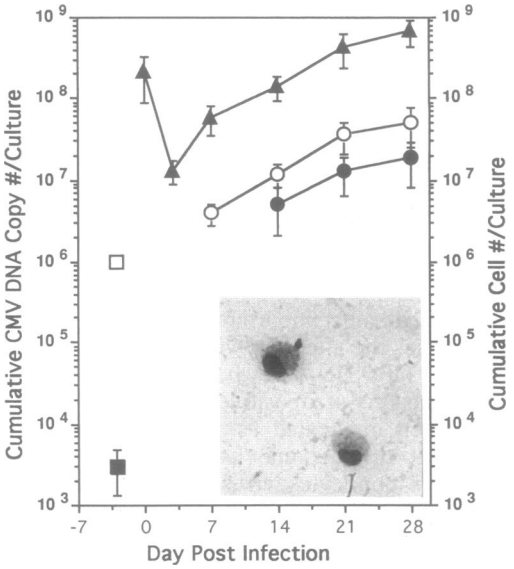

FIG. 1. Human CMV DNA maintenance in GM-P cultures. Six liver cell samples (two to three replicate cultures from each) from different sources were placed into culture (10^6 total cells per culture, containing $1–5 \times 10^3$ CFU-GM). Immediately after inoculation with 3 plaque-forming units of strain RC256 per cell and at the times indicated, CMV DNA copy number was determined using DNA from between 1×10^4 and 7×10^4 cells by quantitative competitive PCR (analysis of 1.3×10^4 cells is shown in Fig. 2*B*). The number of GM-Ps per culture was determined by direct counting, and the number of DNA-positive infected cells was determined by cell-dilution PCR studies (Table 1 and Fig. 2*A*). (*Inset*) Morphology of nonadherent GM-Ps. □, Starting cell number; ■, CFU-GM; ●, infected cell number; ○, total cell number; ▲, DNA copy number.

Table 1. Detection of viral infectivity, β-Gal expression, and viral DNA in GM-P cell culture

PI weeks	Virus, % positive* F/T	Virus, % positive* IC	β-Gal, % positive* (X-gal stain)	Viral DNA, % positive* 10–30†	3–10†	1–3†
3	0	0	0	100	100	60
4	0	0	0	100	100	70

Ten samples of RC256-infected GM-P cells were harvested at 3 or 4 weeks postinfection (PI). Freeze/thaw (F/T)-released free virus was plaque-assayed on HF cells. GM-Ps (10^5) were cocultivated with HF cells for 12 days to detect infectious centers (C). β-Gal was detected by overlay of 10^5 cells with 5-bromo-3-chloro-indolyl β-D-galactoside (X-gal) as described (17). DNA isolated from the indicated numbers of cells was extracted and subjected to nested PCR assay using *ie*1 region primers and conditions described in Fig. 2*A*.
*Percentage positive of 10 culture samples (10^5 cells per sample).
†Number of cells assayed.

Despite the absence of infectious virus and β-Gal expression, a high proportion (between 10% and 100%, depending on the sample) of infected GM-Ps from 10 different tissue samples were positive for CMV DNA by cell-dilution PCR analysis (Table 1) with a nested set of primers for *ie*1 region (Table 2). The interaction of CMV with GM-Ps is summarized in Fig. 1. DNA copy number was determined immediately after infection and at 3, 7, 14, 21, and 28 days after infection by quantitative competitive PCR. The cumulative cell number was also determined by direct count of cells, and the percentage of cells that were genome positive was estimated by cell dilution followed by nested DNA PCR. As GM-Ps proliferated, cell numbers accumulating to 10^7–10^8 cells per culture, viral DNA accumulated in parallel with the number of cells. Cells and nuclei isolated from the same six cultures were compared by dilution analysis, and viral DNA was found mainly associated with the nuclei. An example in which viral DNA was detected in samples containing three or more cells or nuclei is shown in Fig. 2*A*. Results of this analysis for all samples showed that CMV DNA was present in between 10% and 100% of GM-Ps (Table 1). Quantitative competitive PCR (27) with an *ie*1 cDNA clone as competitor was used to estimate a viral genome copy number of 10–100 copies per genome-positive cell. This range was also observed when nuclear DNA was analyzed (an example is shown in Fig. 2*B*). Thus, CMV DNA was quantitatively associated with GM-P cells at genome copy numbers characteristic of latent infection by other herpesviruses.

The results suggested that the CMV genome was maintained in GM-Ps in the absence of productive viral replication. When cells were maintained in the presence of ganciclovir at concentrations of this drug known to inhibit productive viral replication (5 or 10 μM), GM-P cell number increased 3- to 10-fold over a period of 2 weeks, and viral genome copy

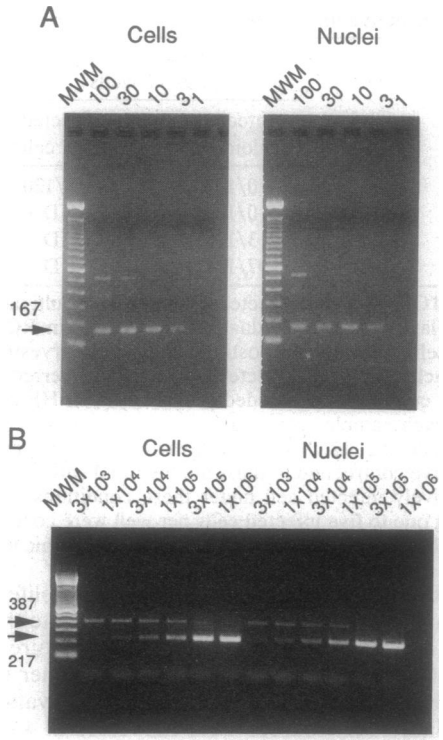

FIG. 2. (*A*) Retention of CMV DNA in cells and nuclei. Cells (*Left*) and nuclei (*Right*) were counted and diluted to give an average of 100, 30, 10, 3, and 1 per tube, as indicated above lanes, before DNA was prepared (20). Position of the 167-bp product generated by nested PCR with *ie*1-specific primers, first using IEP4BII and IEP2AII (Table 2) for 30 cycles and then using IEP3B and IEP3A for 30 cycles, is indicated next to the lanes of ethidium bromide-stained agarose gel. (*B*) CMV DNA copy number. DNA isolated from 1.3×10^4 cells (at left) or 1.3×10^4 nuclei (at right) was subjected to quantitative PCR with primers IPE3C and IEP4BII (Table 2) in the presence of 3×10^3 to 1×10^6 copies of a denatured human CMV ie1 cDNA plasmid as competitor; copy numbers are indicated above lanes. Arrows, position of 387-bp product from human CMV DNA and 217-bp product from the competitive template. Results reveal that $\approx 3 \times 10^4$ CMV genomes were present in these samples, equivalent to ≈20 genomes per genome-positive cell. MWM, 100-bp ladder (GIBCO/BRL).

increased proportionately (data not shown). No differences in morphology or cell growth characteristics were seen in infected cultures when compared with uninfected controls, and a low-passage strain of virus, Toledo strain (18), gave similar results to those described for the Towne strain derivative RC256. An additional 19 individual samples of fetal liver and three samples of fetal bone marrow cells were tested in this system using either strain of virus, and results consistent with those described here were obtained.

Table 2. Primers and predicted products for PCR analyses

Primer	Sequence	Pair	Predicted product, bp Unspliced	Spliced
IEP4BII	5′-CAATACACTTCATCTCCTCGAAAGG-3′	IEP2AII	721	
IEP2AII	5′-ATGGAGTCCTCTGCCAAGAGAAAGATGGAC-3′	IEP3D	263	151
IEP3B	5′-TCTGCCAGGACATCTTTCTC-3′	IEP3A	167	
IEP3A	5′-GTGACCAAGGCCACGACGTT-3′			
IEP3C	5′-CAACGAGAACCCCGAGAAAGATGTC-3′	IEP4BII	387	217
IEP3D	5′-CCAGACTCAGCTGACTGTTAACCTCCTTCC-3′			
2.7A	5′-CGGATTATCATTTCCCTCTCCTACC-3′	2.7B	238	
2.7B	5′-CCTTGCGGATTGACATTCTTGGTGGT-3′			
112A	5′-CCGGTTGATGAACCGGCAGAAGGAG-3′	113B	388	228
113B	5′-GAAAGGCCACCGCTTCAGACGTGTC-3′			
113D	5′-GGACTGCTGCTCCGTCTCGTTCTTG-3′	112A	307	150

Primers were either purchased commercially or prepared on an Applied Biosystems DNA synthesizer.

Table 3. Reactivation of latent virus

Days	Number of CPE-positive wells	
	Infected myeloid cells	Infected HFF cells
5	0/120	120/120
12	0/120	ND
16	3/120	ND
21	97/120	ND

Infected GM-Ps were subjected to long-term cocultivation with HF cell monolayers. Ten individual samples of strain RC256-infected myeloid cells, at 4 weeks postinfection were harvested, counted, and, for each sample, 300 infected cells were transferred into 48 wells of 96-well culture dishes seeded previously with HF cells. Twelve wells of each sample were stained with 5-bromo-3-chloro-indolyl β-galactoside at 5, 12, 16, and 21 days after initiating cocultivation. As a positive control and to illustrate the rapid spread of CMV from a productively infected cell under these conditions, 300 HF cells containing one to five infected cells per well were cocultivated with uninfected HF cells. ND, not done; CPE, cytopathic effect.

We noticed that the GM-Ps continued to proliferate during the 12 days of coculture with HF cells, despite the fact that they had already been in myelomonocytic culture for as long as a month. Therefore, we investigated whether virus might be recovered after a longer period of cocultivation. GM-Ps from 10 individual sources were harvested at 4 weeks after infection and seeded at 300 cells per well together with HF cells. These cultures were maintained for an additional 3-week period. Recovery of virus (plaque formation) was assessed at 5, 12, 16, and 21 days after cocultivation (Table 3). Although the GM-Ps remained virus-negative through the 12-day time point, by 21 days, 80% of samples yielded virus. For comparison, a few productively infected HF cells cultured in parallel yielded plaques within 5 days (Table 3), and an input of one cell-free infectious virus yielded a plaque within 7 days (data not shown). Thus, virus associated with GM-Ps is recovered with considerably delayed kinetics, consistent with reactivation from latency rather than persistence of small amounts of infectious virus. Reactivation occurred in all of the 10 samples studied when 300 GM-Ps were seeded, thereby showing reproducible recovery of infectious virus from the latently infected GM-Ps. When fewer than 300 GM-Ps were seeded, proportionately fewer wells showed reactivation, suggesting that every genome-positive cell in the culture was not capable of yielding virus under these conditions. Similar results were obtained with the low-passage Toledo strain of CMV (data not shown).

We surveyed 10 different latently infected GM-P cultures for evidence of viral β gene expression by direct RT-PCR. Fig. 3 *A* and *B* show examples of RT-PCR analyses from five different samples of RNA from 10^5 cells using PCR primers capable of detecting unspliced transcripts from the abundantly expressed $\beta_{2.7}$ gene (28) and from a family of spliced transcripts from the UL112-113 gene (29). Neither of these transcripts was detected, even though the conditions were sensitive enough to detect transcripts in a single infected HF cell (8 hr after infection) mixed in with 10^5 uninfected GM-Ps.

Transcription from a region of the α gene, *ie*1, was readily detected in all six latently infected GM-P cultures, even when

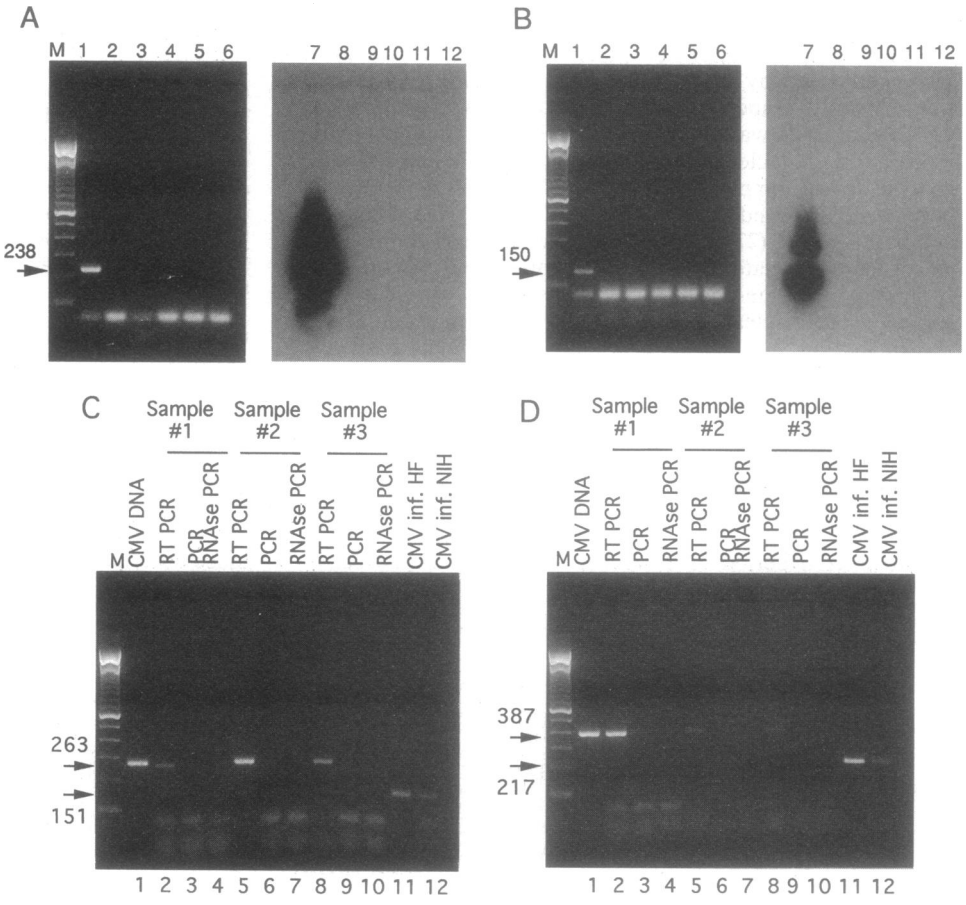

FIG. 3. Electrophoretic separation (2.5% agarose gels) of RT-PCR products of α and β gene transcripts in GM-Ps at 4 weeks after infection. (*A*) $\beta_{2.7}$ gene expression. (*B*) UL112/113 gene expression. (*C*) Expression between *ie*1 exons 2 and 3. (*D*) Expression between *ie*1 exons 3 and 4. The left set (lanes 1–6) of *A* and *B* show ethidium bromide-stained bands, and the right set (lanes 7–12) show autoradiographic results of hybridization with [^{32}P]dCTP-labeled $\beta_{2.7}$ or UL112/113 DNA probes. (*C* and *D*) Ethidium bromide-stained gels. *A* and *B* (lanes 2–6 and 8–12) use RNA from five different samples of 10^5 infected GM-Ps, and *C* and *D* (lanes 2–10) use RNA from three different samples (nos. 1, 2, and 3) of 10^3 infected (inf.) GM-Ps. Controls included RNA extracted from one infected HF cell (8 hr) mixed with 10^5 uninfected GM-P cells (*A* and *B*; lanes 1 and 7), from 10 infected HF cells (4 hr) mixed with 10^3 uninfected GM-P cells (*C* and *D*; lane 11), or from 10 infected NIH 3T3 cells (4 hr) mixed with 10^3 uninfected GM-P cells (*C* and *D*; lane 12). In *C* and *D*, three analyses were done for each sample, one in which reverse transcriptase was included in the reactions (RT PCR), and, as controls, one in which reverse transcriptase was left out of the reaction (PCR), and one where RNA was treated with 20 ng of RNase A (Sigma) for 1 hr at 37°C before RT-PCR (RNase PCR). All RNA samples were also treated with RNase-free RQ1 DNase. PCR of CMV DNA from a sample of 10^3 infected GM-Ps is also shown for comparison in lane 1. The $\beta_{2.7}$ transcript RT-PCR used 45 cycles of PCR with primers 2.7A and 2.7B, and the UL112/113 transcript RT PCR used a partially nested amplification first with primers 112A and 113B for 30 cycles and then with primers 112A and 113D for 30 cycles. For the *ie*1 transcript analysis, 30 cycles of PCR with either primers IEP2AII and IEP3D (exon 2–3; *C*) or IEP4BII and IEP3C (exon 3–4; *D*) were used. Lane M, 100-bp ladder (GIBCO/BRL). Primers and predicted PCR products are listed in Table 2. PCR products are denoted by an arrow next to the panels.

RNA from as few as 10^3 cells was evaluated by RT-PCR (three examples are shown for each of two different *ie*1 splice junctions in Fig. 3 *C* and *D*). Analyses using RNA from uninfected GM-Ps were uniformly negative. Surprisingly, the signal we observed was largely derived from unspliced RNA in the infected GM-Ps, rather than from spliced RNA typical of either productively infected HF or abortively infected mouse NIH 3T3 fibroblasts. An overwhelming majority of *ie*1 region RNA remained unspliced in all six independent GM-P cultures examined and was readily detected in RNA from 10^3 cells by RT-PCR. The presence of unspliced RNA was demonstrated between exons 2 and 3 (Fig. 3*C*) or between exons 3 and 4 (Fig. 3*D*) of the *ie*1 gene. Using semiquantitative PCR conditions, we estimated that each RNA sample contained at least 10^3 copies of cDNA before amplification. Between 70 and 99% of these transcripts remained unspliced, as estimated by probing PCR reaction products with internal oligonucleotide probes (data not shown). This high relative abundance of unspliced transcript is unusual and indicates that *ie*1 region expression during latency may be different than occurs during productive infection (2). RNA isolated from a cell dilution series was evaluated by RT-PCR analysis, and we estimated that no less than 2–5% of CMV genome-positive cells contained this unspliced transcript. Using monoclonal antibodies, we failed to detect any evidence of the 72-kDa *ie*1 gene product in these cells (data not shown), which was consistent with the relative absence of appropriately spliced transcript. Further analysis has shown that ≈10% of this unspliced RNA appeared to be polyadenylylated (data not shown).

DISCUSSION

The importance of latency to pathogenesis of human CMV has been evident for >30 yr (1), yet little concrete information currently exists on the nature or expression of the latent CMV genome. Unlike the neuronal site that characterizes latency of alphaherpesviruses, such as herpes simplex virus, or the B lymphocyte that is clearly important for latency of the gammaherpesvirus Epstein–Barr virus, the site of human CMV latency has been elusive. Despite the fact that sensitive PCR methods have reproducibly shown association of the viral genome with mononuclear leukocytes (3, 4), the absence of a manipulable system to reactivate virus has limited any understanding of these cells as sites of latent infection. Our work establishes a system to study CMV persistence in human bone marrow-derived primary myelomonocytic cells, a potential reservoir of latent CMV in healthy carriers (4). The system described here opens the way to investigate the establishment, maintenance, and reactivation phases of latent infection.

We have found that CMV infection of GM-Ps is noncytocidal and that viral gene expression is restricted. Previous studies have suggested that the GM-P population restricts CMV replication, although stromal cells support CMV replication (12–15). Interestingly, CMV gene expression was similarly restricted in the large adherent cells that develop in these cultures. We identified an atypical, unspliced *ie*1 region transcript in infected GM-Ps, and the expression of this transcript can now be evaluated directly as a marker of latency in healthy carriers.

The ability to activate virus after extended cocultivation with permissive cells indicates that CMV replication in GM-Ps depends upon growth or differentiation that occurs during cocultivation. The dependence of CMV replication on the differentiation state of cells—in particular, monocytes (5–7)—has been the topic of study in several laboratories. We can now investigate the importance of viral and host cell functions in latent infection using the GM-P cell culture system.

Our study establishes that both low-passage as well as high-passage laboratory strains of CMV retain the biological potential for latent infection of GM-Ps. During latent infection viral DNA is stably associated with growing cells, and a ganciclovir-resistant parallel increase in viral DNA along with cell number occurs during culture. Our results suggest a mechanism for maintaining the viral genome in latently infected GM-Ps that is distinct from that used during productive replication. The detection of atypical ie1 region transcripts suggests that the products may play some role in latent infection, possibly in genome maintenance. In addition to yielding information on the nature of the viral genome and viral gene expression, this system will enable the direct investigation of the role of atypical transcripts from the *ie*1 region in establishment or maintenance of latent infection.

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1. Ho, M. (1991) *Cytomegalovirus: Biology and Infection* (Plenum, New York), 2nd Ed., pp. 205–300.
2. Mocarski, E. S. (1993) in *The Human Herpesvirus*, eds. Roizman, B., Whitley, R. & Lopez, C. (Raven, New York), pp. 173–226.
3. Bevan, I. S., Daw, R. A., Day, P. J., Ala, F. A. & Walker, M. R. (1991) *Br. J. Haematol.* **78**, 94–99.
4. Taylor-Wiedeman, J., Sissons, J. G., Borysiewicz, L. K. & Sinclair, J. H. (1991) *J. Gen. Virol.* **72**, 2059–2064.
5. Taylor-Wiedeman, J., Sissons, P. G. P. & Sinclair, J. H. (1994) *J. Virol.* **68**, 1597–1604.
6. Ibanez, C. E., Schrier, R., Ghazal, P., Wiley, C. & Nelson, J. A. (1991) *J. Virol.* **65**, 6581–6588.
7. Lathey, J. L. & Spector, S. A. (1991) *J. Virol.* **65**, 6371–6375.
8. Schrier, R. D., Nelson, J. A. & Oldstone, M. B. (1985) *Science* **230**, 1048–1051.
9. Scott, D. M., Rodgers, B. C., Freeke, C., Buiter, J. & Sissons, J. G. (1989) *J. Gen. Virol.* **70**, 685–694.
10. Rice, G. P., Schrier, R. D. & Oldstone, M. B. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 6134–6138.
11. Braun, R. W. & Reiser, H. C. (1986) *J. Virol.* **60**, 29–36.
12. Reiser, H., Kuhn, J., Doerr, H. W., Kirchner, H., Munk, K. & Braun, R. (1986) *J. Gen. Virol.* **67**, 2595–2604.
13. Apperley, J. F., Dowding, C., Hibbin, J., Buiter, J., Matutes, E., Sissons, P. J., Gordon, M. & Goldman, J. M. (1989) *Exp. Hematol.* **17**, 38–45.
14. Simmons, P., Kaushansky, K. & Torok-Storb, B. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 1386–1390.
15. Sing, G. K. & Ruscetti, F. W. (1990) *Blood* **75**, 1965–1973.
16. Baines, P., Masters, G., Booth, M. & Jacobs, A. (1987) *Exp. Hematol.* **15**, 809–813.
17. Spaete, R. R. & Mocarski, E. S. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 7213–7217.
18. Plotkin, S. A., Starr, S. E., Friedman, H. M., Gonczol, E. & Wiebel, R. E. (1989) *J. Infect. Dis.* **159**, 860–865.
19. Woods, G. L., Young, A., Johnson, A. & Thiele, G. M. (1987) *J. Virol. Methods* **18**, 207–213.
20. Kondo, K., Kondo, T., Okuno, T., Takahashi, M. & Yamanishi, K. (1991) *J. Gen. Virol.* **72**, 1401–1408.
21. Porter-Jordan, K., Rosenberg, E. I., Keiser, J. F., Gross, J. D., Ross, A. M., Nasim, S. & Garrett, C. T. (1990) *J. Med. Virol.* **30**, 85–91.
22. Higchi, R. (1989) in *PCR Technology: Principles and Applications for DNA Amplification*, ed. Erlich, H. A. (Stockton, New York), pp. 31–38.
23. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159.
24. Namikawa, R., Weilbaecher, K. N., Kaneshima, H., Yee, E. J. & McCune, J. M. (1990) *J. Exp. Med.* **172**, 1055–1063.
25. Kyoizumi, S., Baum, C. M., Kaneshima, H., McCune, J. M., Yee, E. J. & Namikawa, R. (1992) *Blood* **79**, 1704–1711.
26. Mocarski, E. S., Bonyhadi, M., Salimi, S., McCune, J. M. & Kaneshima, H. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 104–108.
27. Gilliland, G., Perrin, S., Blanchard, K. & Bunn, H. F. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 2725–2729.
28. Greenaway, P. J. & Wilkinson, G. W. (1987) *Virus Res.* **7**, 17–31.
29. Staprans, S. I. & Spector, D. H. (1986) *J. Virol.* **57**, 591–602.