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Induction of Endogenous Human Cytomegalovirus Gene Expression after Differentiation of Monocytes from Healthy Carriers

JEAN TAYLOR-WIEDEMAN, PATRICK SISSONS, AND JOHN SINCLAIR*

Department of Medicine, University of Cambridge, Cambridge CB2 2QQ, United Kingdom

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Monocytes are one site of carriage of the human cytomegalovirus (HCMV) genome in healthy human carriers. However, as there are conflicting data detailing the level of HCMV gene expression during persistence in these cells, we have analyzed monocytes for evidence of viral immediate-early, early, and late transcription by using reverse transcription followed by PCR. We were unable to find evidence of HCMV lytic gene transcription in freshly isolated peripheral blood monocytes from HCMV-seropositive subjects. However, as differentiation of monocytes to monocyte-derived macrophages results in increased permissiveness to infection with HCMV in vitro, we examined whether such differentiation could result in reactivation of endogenous viral gene expression. Here we show that in vitro differentiation of monocytes does result in expression of endogenous HCMV immediate-early genes. Although this differentiation led to reactivation of endogenous viral immediate-early expression, we were unable to detect any early or late viral transcription. Cocultivation experiments correlated with this level of gene induction, as no productive infection was detected. These data strongly suggest a mechanism of persistence of HCMV in the peripheral blood that is independent of HCMV lytic gene expression and that initial phases of lytic gene expression in monocytes can be induced by differentiation of these cells to monocyte-derived macrophages.

Circumstantial evidence has long indicated that the peripheral blood of healthy human donors is able to transmit human cytomegalovirus (HCMV) infection to susceptible recipients (1, 33). Moreover, the incidence of transfusion-associated HCMV infection can be markedly reduced by using leukocyte-depleted blood products (5, 6, 10, 38). In a previous analysis of the peripheral blood of healthy human HCMV carriers, we identified the monocyte as the predominant site of carriage of HCMV genome in seropositive healthy subjects (31, 32). However, the well-established inability to coculture HCMV from peripheral blood (16) suggests that peripheral blood cells do not harbor infectious virus. Similarly, our knowledge of the extent of specific viral gene expression during persistence is still limited. One report (34) detected immediate-early 1 (IE1) but not late antigens in tissues from autopsy examinations. However, it is difficult to know what changes may have occurred in these tissues after death and, hence, rule out postmortem changes. Another report examining healthy subjects found that up to 2% of peripheral blood mononuclear cells (PBM) harbored HCMV IE RNA by in situ hybridization (24). However, Gnann and colleagues (11), using a similar detection system, found that the predominant probe-positive signal during reactivation in posttransplant kidneys was seen in infiltrating monocytes; no HCMV RNA was found in pretransplant kidneys in either lymphocytes or monocytes. These conflicting data suggest that the level of sensitivity of in situ hybridization may compromise detection of rare transcripts or possibly only a very few cells harbored most transcripts.

Consequently, we have examined the spectrum of HCMV gene expression in freshly isolated monocytes from asymptomatic healthy carriers to determine whether viral persistence occurs by low-level replication or by restricted gene expression. We have used reverse transcription (RT) followed by PCR (RT-PCR) to detect specific HCMV genes of all phases of the lytic cycle because this detection system is highly sensitive and specific. These genes included the differentially spliced major IE1 and IE2 transcripts located between map units 0.732 and 0.751 in the unique long region (2, 30), the major early gene located in the large repeat sequences between map units 0.011 and 0.032 (13), and the true late 28-kDa phosphoprotein (pp28) gene located between map units 0.63 and 0.65 in the unique long region (20).

Additionally, analyses of the ability of peripheral blood cells to express HCMV upon in vitro infection have clearly shown that freshly isolated monocytes are difficult to infect with HCMV and do not replicate virus (9, 23), although extended culture of monocytes with (15, 18) or without (28) specific differentiation signals can result in substantial numbers of cells permissive for HCMV infection. Similarly, it has been reported that in the T2 teratocarcinoma cell line (12) and the premonocyte THP-1 cell line (37), differentiation of these cells is essential for HCMV permissiveness. In these cases, the lack of permissiveness of these cells for HCMV is due to the presence of differentiation-specific negative regulators of IE expression which disappear upon differentiation of these cells to a permissive phenotype (17, 19, 21, 25–27). Consequently, we also examined whether in vitro differentiation had any effect on expression of endogenous HCMV in the monocytes of healthy carriers. Here we show that, consistent with in vitro infection data, freshly isolated monocytes from healthy seropositive HCMV carriers do not produce IE, early, or late HCMV RNA but that differentiation of these cells to monocyte-derived macrophages (MDM) results in induction of HCMV IE gene expression and that different methods of in vitro differentiation result in induction of different classes of IE transcripts.

* Corresponding author. Mailing address: University of Cambridge Department of Medicine, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QQ, United Kingdom. Phone: 223 336850. Fax: 223 336846.

MATERIALS AND METHODS

Subjects. Blood samples were acquired from the National Blood Transfusion Service and consisted of buffy coat samples from five healthy adult HCMV-seropositive donors; in four instances (subjects S1, S5, S6, and S9), blood was acquired from healthy laboratory volunteers known to be HCMV seropositive. The subjects (S1 to S9) were analyzed as follows: S1, monocytes only; S2, monocytes only; S3, monocytes only; S4, monocytes only; S5, phorbol 12-myristate 13-acetate (PMA)- and hydrocortisone (HC)-differentiated MDM only; S6, monocytes and PMA/HC-differentiated MDM; S7, monocytes, PMA/HC-differentiated MDM and granulocyte-monocyte colony-stimulating factor (GM-CSF)- and HC-differentiated MDM; S8, monocytes, PMA/HC-differentiated MDM, and GM-CSF/HC-differentiated MDM; and S9, GM-CSF/HC-differentiated MDM only.

Isolation of monocytes by adherence. Adherent cells were isolated from PBM after Lymphoprep (Nycomed Pharma AS) gradient centrifugation, plated on 14-cm-diameter plastic petri dishes in phosphate-buffered saline (PBS), and incubated for 1 h at 37°C in 5% CO_2. Nonadherent cells were removed by three washes with PBS. Adherent cells were processed for DNA and RNA as detailed below.

DNA and RNA extraction. DNA was isolated by sodium perchlorate extraction (31), and total cellular RNA was isolated by using an RNA extraction kit (Promega) based on the method of Chomczynski and Sacchi (4). As the acid-phenol-based RNA isolation did not yield fully DNA-free RNA preparations, it was necessary to remove contaminating DNA from all isolated RNA by incubation with RQ1 RNase-free DNase. In this situation, RNA samples were processed as follows. After the 75% ethanol wash, RNA pellets were resuspended in 500 μl of RQ1 buffer (40 mM Tris-HCl [pH 8.3], 10 mM NaCl, 6 mM $MgCl_2$, 10 mM $CaCl_2$) with 10 U of rRNasin (Promega) and 15 U of RQ1). The mixture was incubated at 37°C for 15 min, and then 0.5 volume of stop solution (50 mM EDTA, 1.5 M sodium acetate, 1% sodium dodecyl sulfate [SDS]) was added. RNA was extracted with acid phenol-chloroform-isoamyl alcohol (25:24:1), precipitated with 1 volume of isopropanol, pelleted at 10,000 × *g* for 30 min at 4°C, and washed once with 75% ethanol. The ethanol was removed by aspiration, and the RNA pellet was resuspended in RNase-free water (Promega).

First-strand RT. First-strand RT was carried out with 10 μg of total RNA (approximately equivalent to 10^5 cells) in a 50-μl reaction in the presence of 10 μg of oligo(dT) primer per ml. Non-oligo(dT)-primed RNA samples served as a control to determine the presence of any contaminating DNA or cDNA. Initially, oligo(dT) or H_2O was added to separate aliquots of 10 μg of RNA and incubated at 68°C for 5 min, slowly cooled to 20°C over 10 min, and held for a further 5 min at 20°C. Samples were reversed transcribed in 50 μl containing 50 mM KCl, 50 mM Tris-HCl (pH 8.3 at 42°C), 6 mM $MgCl_2$, 5 mM spermidine, 1 mM each deoxynucleoside triphosphate (dNTP), 1 U of rRNasin (Promega), 4 mM dithiothreitol, and 4 U of reverse transcriptase (superRT; HT Biotechnology, Cambridge, United Kingdom) by incubation for 1 h at 42°C and then 8 min at 95°C. Samples were then cooled to 4°C and held at −70°C until PCR amplification. All incubations were performed in a Perkin-Elmer Cetus Thermocycler, using GeneAmp (Perkin-Elmer Cetus) 0.5-ml tubes.

PCRs. For nested intron-spanning IE1 PCR, the primers and probe were as follows: sense primer, 5′-GGTGCATTG GAACGCGGATT-3′; antisense primer, 5′-ATTCTATGCC GCACCATGTCCA-3′; nested sense primer, 5′-ATGGAGT CCTCTGCCAAGAG-3′; nested antisense primer, 5′-CAT AGTCTGCAGGAACGTCGT-3′; and probe, 5′-GACCCTG ATAATCCTGACGA-3′. cDNA from approximately 10^5 cells was amplified in a 40-μl reaction mix (50 mM KCl, 10 mM Tris-HCl [pH 8.5 at room temperature], 1 mM $MgCl_2$, 0.01% gelatin, 0.5% Tween 20, 0.1% Triton X-100, 1 mM each primer, 200 mM each dNTP, 1 U of Promega *Taq* polymerase). The nested PCR used 2 μl of the primary PCR mix as the template in a 20-μl reaction mix identical to that for the primary PCR except that it contained 1.5 mM $MgCl_2$.

For both the primary and nested PCRs, a hot start was used, which was followed by an initial denaturation of 94°C for 5 min and then 50 amplification cycles consisting of 94°C for 40 s, 55°C for 40 s, and 72°C for 90 s. The nested amplification cDNA product was 117 bp long, and the nested amplification DNA product was 231 bp long.

For nested intron-spanning IE2 PCR, the primers and probe were as follows: sense primer, 5′-GGTGCATTGGAACGCG GATT-3′; antisense primer, 5′-GCGCTGCTAACGCTGCAA GAG-3′; nested sense primer, 5′-GACCCTGATAATCCT GACGA-3′; nested antisense primer, 5′-ATACCGGCATGA TTGACAGCCT-3′; and probe, 5′-GACATCCTCGCCCAG GCT-3′. The reaction mix and cycling parameters were the same as described above except that the primary PCR required 0.25 mM spermidine and 0.5 mM $MgCl_2$. The lengths of the nested DNA, primary cDNA, and nested cDNA amplification products were 1,924, 406, and 266 bp, respectively.

Major early gene PCR amplified a 315-bp portion of the major early gene. The sequences of the primers and probe were as follows: sense primer, 5′-CGTTATCCGTTCCTCG TAGG-3′; antisense primer, 5′-GTTTCGTTGTTGTCGGT AGT-3′; and probe, 5′-CCTACCACGAATCGCAGATGA-3′. The reaction mix and cycling parameters were the same as described for the primary IE1 PCR except that the early PCR required 2.0 mM $MgCl_2$.

Nested late pp28 gene PCR amplified a portion of the major 28-kDa structural phosphoprotein gene. It was a nested but non-intron-spanning PCR, as this is an unspliced gene. The sequences of the primers and probe were as follows: sense primer, 5′-GAGGATGACGATAACGAGGA-3′; antisense primer, 5′-TCAAACAGCACATTAGACACACGG-3′; nested sense primer, 5′-GACAGTAGTAGCGGCAGCCA-3′; nested antisense primer, 5′-GACTCGCGAATCGTACGCGA-3′; and probe, 5′-GACCTAGACGAAGAGGACACCTCAAT-3′. The conditions were identical to those described above for the intron-spanning IE1 PCR except that 0.5 mM $MgCl_2$ was used for the primary PCR and 2 mM $MgCl_2$ was used for the nested PCR. The amplification products were 548 and 389 bp in length for the primary and nested PCRs, respectively.

Intron-spanning histidyl-tRNA synthetase gene PCR was used as a control PCR for the ability to amplify cDNA after RT. Histidyl-tRNA synthetase is a multicopy housekeeping gene (36). The sequences of the primers and the intraexonic probe were as follows: sense primer, 5′-GGAAGACTCCAA GCTTATCT-3′; antisense primer, 5′-GAACTTGCTATCAG GAACAC-3′; and probe, 5′-AGCCATGACCGGAGGCCGA TAT-3′. The reaction mix was the same as for the primary IE1 PCR with 2.5 mM $MgCl_2$ and required only 30 cycles of amplification. The primers amplified a 370-bp cDNA and spanned three introns.

RT-PCR. For all RT-PCRs, RNA from MDM or monocytes was reverse transcribed and amplified along with matched controls, which consisted of RNA and all other components of the RT mix except the oligo(dT) primer (designated RT-PCR sample controls in all figure legends). The RT H_2O control was an RT-PCR negative control and consisted of the complete RT

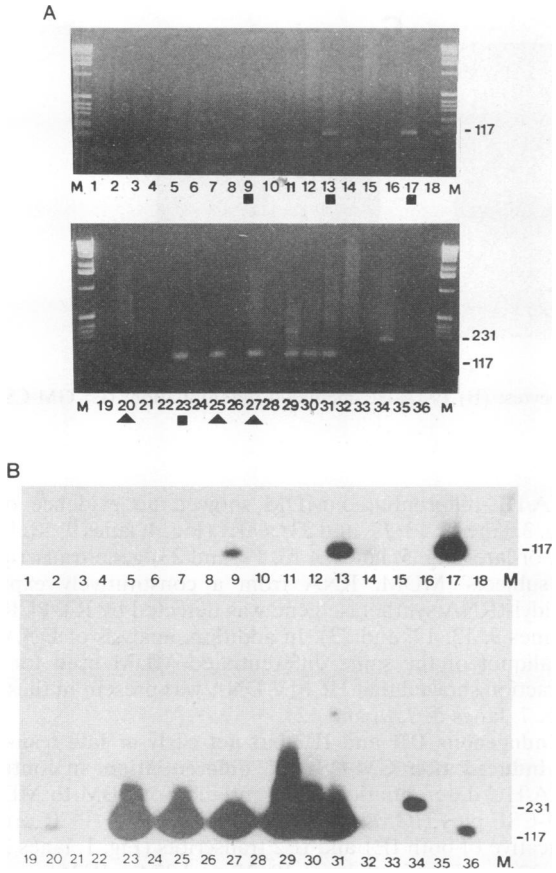

FIG. 1. IE1 expression. An ethidium bromide-agarose gel (A) and the Southern blot of that gel (B) are shown. A 1-kb marker ladder (GIBCO) is shown in lanes M. Sizes of nested products are indicated (DNA = 231 bp and cDNA = 117 bp). Lanes: 1, S1 RT-PCR; 2, S1 sample control; 3, S2 RT-PCR; 4, S2 sample control; 5, S3 RT-PCR; 6, S3 sample control; 7, S4 RT-PCR; 8, S4 sample control; 9, S5 PMA/HC MDM RT-PCR; 10, S5 PMA/HC MDM sample control; 11, S6 monocyte RT-PCR; 12, S6 monocyte sample control; 13, S6 PMA/HC MDM RT-PCR; 14, S6 PMA/HC MDM sample control; 15, S7 monocyte RT-PCR; 16, S7 monocyte sample control; 17, S7 PMA/HC MDM RT-PCR; 18, S7 PMA/HC MDM sample control; 19, S7 GM-CSF MDM sample control; 20, S7 GM-CSF MDM RT-PCR; 21, S8 monocyte RT-PCR; 22, S8 monocyte sample control; 23, S8 PMA/HC MDM RT-PCR; 24, S8 PMA/HC MDM sample control; 25, S8 GM-CSF MDM RT-PCR; 26, S8 GM-CSF MDM sample control; 27, S9 GM-CSF MDM RT-PCR; 28, S9 GM-CSF MDM sample control; 29 to 31, RT-PCR of RNA RNA from the equivalent of 10, 1, and 0.1 HCMV-infected fibroblast cells; 32 and 33, RT-PCR H_2O controls; 34, DNA PCR equivalent to 100 copies; 35, PCR H_2O control; 36, one copy of a pGEM-2 IE1 plasmid. MDM derived by PMA/HC and GM-CSF/HC are highlighted by boxes and triangles, respectively.

mix with H_2O instead of RNA, while RT-PCR positive controls were amplifications from a standard batch of RNA extracted from HCMV-infected fibroblast cells 96 h postinfection at a dilution equivalent to 10, 1, 0.1, or 0.01 infected cells without additional carrier RNA. Routinely, 10 μg of RNA from adherent monocytes (equivalent to 10^6 cells) was reverse transcribed and 5 μl was amplified in the various HCMV PCRs (the histidyl control PCR amplified only 2 μl of the RT mix). Finally, all reverse transcripts included water template controls, and all PCR amplifications included water template controls.

Southern blots. Agarose gels were blotted onto Zeta-ProbeR GT blotting membranes (Bio-Rad) by using an alkaline transfer. Samples from 1 to 2% agarose gels were transferred by using a 0.4 M NaOH solution over 2 to 3 h. Membranes were fixed by baking at 80°C for 30 min and then were prehybridized for 5 min at 42°C in 7% SDS and 0.25 M Na_2HPO_4. Hybridization was carried out in 7% SDS–0.25 M Na_2HPO_4–10% (wt/vol) polyethylene glycol 8000 for a minimum of 4 h at 42°C, using an end-labeled oligonucleotide probe. Blots were washed twice for 5 min at room temperature with 5% SDS–20 mM Na_2HPO_4 and then for 5 min at room temperature with 1% SDS–20 mM Na_2HPO_4. Autoradiography was carried out overnight at −70°C.

Prevention of PCR contamination. All preamplification PCR (pre-PCR) work was carried out in an area that was separate from any postamplification PCR work by the use of a laminar flow hood dedicated to the preparation of PCR-safe solutions and of DNA and RNA from uninfected tissues. In addition, all equipment used for pre-PCR work was dedicated to that purpose. Finally, to prevent micropipette contamination, sterile aerosol-resistant tips (Continental Laboratory Products) were used for pre-PCR work. For the nested PCR, all reaction mixes were initially prepared in the PCR-safe hood. Then, target DNA from the primary PCR was added outside the hood on a PCR bench dedicated to nesting, again using aerosol-resistant tips. To prevent handling contamination, sterile disposable gowns were routinely used and gloves were changed whenever the PCR-safe hood was entered. All equipment and chemicals for PCR were dedicated to that purpose. Where possible, all chemicals were purchased as solutions. If this was not possible, chemicals were purchased solely for PCR and solutions were prepared only under PCR-safe conditions in the laminar flow hood. Chemicals were purchased in small quantities and made to solution by adding double-processed sterile water (Sigma) directly to the bottle contents. Only disposable plastics were used. In addition, all PCR racks were rinsed in 0.25 M HCl prior to use in the PCR-safe hood.

Differentiation of freshly isolated monocytes. Freshly isolated adherent human monocytes were differentiated in the presence of 5×10^{-5} M preservative-free hydrocortisone sodium succinate (18, 22) over a period of 6 days in Iscove's modified Dulbecco's medium (Gibco, BRL) containing 15% donor horse serum (Sera-lab), 15% fetal calf serum (Advanced Protein Products, Ltd.), 2 mM L-glutamine (Flow), penicillin (100 IU/ml)-streptomycin (100 μg/ml) (Gibco, BRL), and 3 g of sodium bicarbonate (Flow) per liter and then incubated overnight with 2×10^{-8} M PMA (Sigma) (27, 35). PMA was solubilized in dimethyl sulfoxide at a concentration of 1 mg/ml then diluted to 10 μg/ml in PBS and stored at −20°C until use. Alternatively, primary human monocytes were differentiated in the presence of 5×10^{-5} M HC (18, 22) for 12 h followed by the addition of 5 ng of recombinant GM-CSF (Genzyme) per ml (14, 20a) for a further period of 9 days in Iscove's modified Dulbecco's medium (GM-CSF MDM) described above.

Cocultivation of MDM with fibroblasts. One T-75 flask of MDM ($>10^6$ cells) was placed on ice for 10 min prior to gentle scraping of cells into the medium. Cells and medium were then passaged onto an 80 to 90% confluent flask of MRC5 cells, and a control flask of MRC5 cells of equal confluency and passage number was set up in parallel. After 24 h, medium on both flasks was changed to minimal essential medium containing 10% fetal calf serum. If there was no evidence of cytopathic effect, monolayers were passaged 1:2 after 14 days and observed for a second 14-day period. Cells were refed 10 ml of

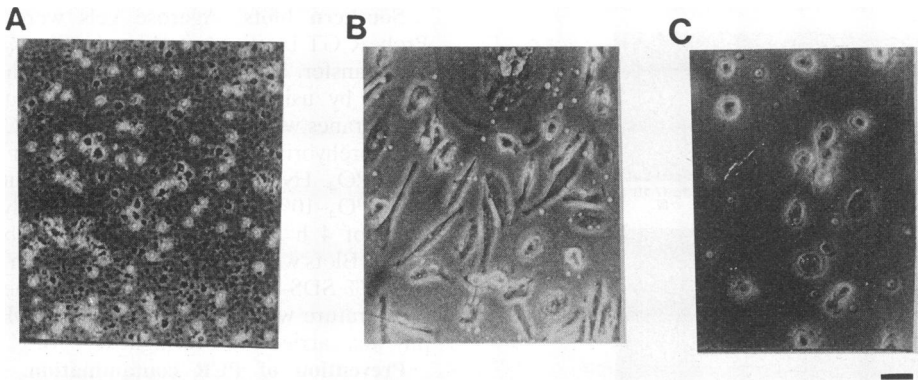

FIG. 2. Phenotypic changes in MDM. (A) Undifferentiated primary monocytes; (B) PMA/HC-mediated differentiation; (C) GM-CSF/HC-mediated differentiation. In all photographs, the bar represents 100 μm.

minimal essential medium containing 10% fetal calf serum every 5 to 7 days.

RESULTS

Transcriptional analysis of endogenous HCMV expression in freshly isolated monocytes. Freshly isolated monocytes from seven seropositive subjects were analyzed for evidence of IE, early, and late HCMV gene expression. In no case were transcripts observed (Fig. 1, lanes 1, 3, 5, 7, 11, 15, and 21 for IE1; Fig. 3, lanes 1, 3, 5, 7, 11, 15, and 21 for IE2; Fig. 4, lanes 1, 3, 5, 7, 11, 15, and 21 for early; Fig. 5, lanes 1, 3, 5, 7, 11, 15, and 21 for late) even though all control RT-PCRs detecting the constitutively expressed gene, expressing histidyl tRNA-synthetase, confirmed that the RTs had been completed successfully (Fig. 6, lanes 1, 3, 5, 7, 11, 15, and 21). In addition, DNA extracted from aliquots of cells which had been harvested at the same time as those for RNA extraction showed that the HCMV genome was present in these cells (Fig. 7, lanes 1 to 4, 6, 8, and 11).

Phenotypic appearance of MDM. In all cases, differentiation of monocytes to MDM resulted in significantly enlarged cells compared with primary adherent monocytes (Fig. 2); PMA/HC treatment resulted in MDM with the most varied phenotypes, which included rounded as well as flattened cells with pseudopods (Fig. 2B). Most cells were mononuclear, but some cells were binuclear; no multinucleated giant cells were observed. GM-CSF/HC differentiation (Fig. 2C) resulted in mainly rounded cells, but only single nuclei were observed. With both methods, cells were actively phagocytic, engulfing most platelets that had attached to the plastic flask after the initial adherence.

Endogenous HCMV IE1 expression is induced in MDM after differentiation with PMA plus HC. MDM from four healthy seropositive individuals were examined for evidence of HCMV gene transcription after differentiation with PMA plus HC. In all healthy seropositive carrier MDM, RT-PCR products indicative of IE1 transcripts were observed (Fig. 1, lanes 9, 13, 17, and 23), while the respective RT-PCR sample controls were negative (lanes 10, 14, 18, and 24). To preclude that this IE expression was a result of viremia rather than induction of IE expression by monocyte differentiation, freshly isolated monocytes were obtained from three of these four subjects at the same time as cells isolated for differentiation and analyzed for evidence of IE expression by RT-PCR. There was no evidence of IE1 transcripts or other transcripts in any of these monocyte controls (lanes 11, 17, and 23). Further analysis of PMA/HC-differentiated MDM showed no evidence of IE2 (Fig. 3, lanes 9, 13, 17, and 23), early (Fig. 4, lanes 9, 13, 17, and 23), or late (Fig. 5, lanes 9, 13, 17, and 23) gene transcripts. In all subjects' MDM, RNA from a constitutively expressed histidyl-tRNA synthetase gene was detected by RT-PCR (Fig. 6, lanes 9, 13, 17, and 23). In addition, analysis of DNA from an aliquot of the same differentiated MDM used for RNA extraction showed that HCMV DNA was present in these cells (Fig. 7, lanes 5, 7, 9, and 12).

Endogenous IE1 and IE2 but not early or late transcripts are induced after GM-CSF/HC differentiation. In contrast to PMA/HC differentiation, differentiation of PBM to MDM by GM-CSF plus HC resulted in detection of RT-PCR products indicative of both IE1 and IE2 transcripts (Fig. 1, lanes 20, 25, and 27 for IE1; Fig. 3, lanes 19, 25, and 27 for IE2) from three healthy individuals. Major early and late pp28 transcripts, however, were not detectable (Fig. 4, lanes 19, 25, and 27 for early; Fig. 5, lanes 19, 25, and 27 for late) in these cells. In these subjects' MDM, RNA from a constitutively expressed histidyl-tRNA synthetase gene was again detected by RT-PCR (Fig. 6, lanes 19, 25, and 27), and HCMV DNA was detectable in monocytes before (Fig. 7, lanes 8 and 11) and after (Fig. 6, lanes 10, 13, and 14) differentiation. An analysis of all MDM showed no productive virus infection even after extensive coculture with permissive fibroblasts.

DISCUSSION

We have previously reported that monocytes are the predominant site of carriage of HCMV genome in the peripheral blood of healthy human subjects. Here, we present an analysis of these cells for evidence of HCMV gene expression. A panel of sensitive RT-PCRs was developed to analyze IE (IE1 and IE2), early (major early), and late (pp28) viral gene expression. The level of sensitivity of these analyses was equivalent to at least one HCMV-infected cell. In addition, all analyses included RT-PCRs which detected a constitutively expressed gene to ensure successful RT and also included RT controls that contained all components of an RT mix but without oligo(dT) primer. These extensive controls allowed an unequivocal analysis of endogenous HCMV gene expression. In addition, rigorous measures were taken to prevent PCR contamination. Using these RT-PCR analyses, we were unable to detect evidence of polyadenylated IE, early, or late transcripts in freshly isolated monocyte RNA of healthy carriers from the equivalent of 10^5 cells, despite the fact that HCMV DNA was detectable in these cells and that a control RT-PCR to detect

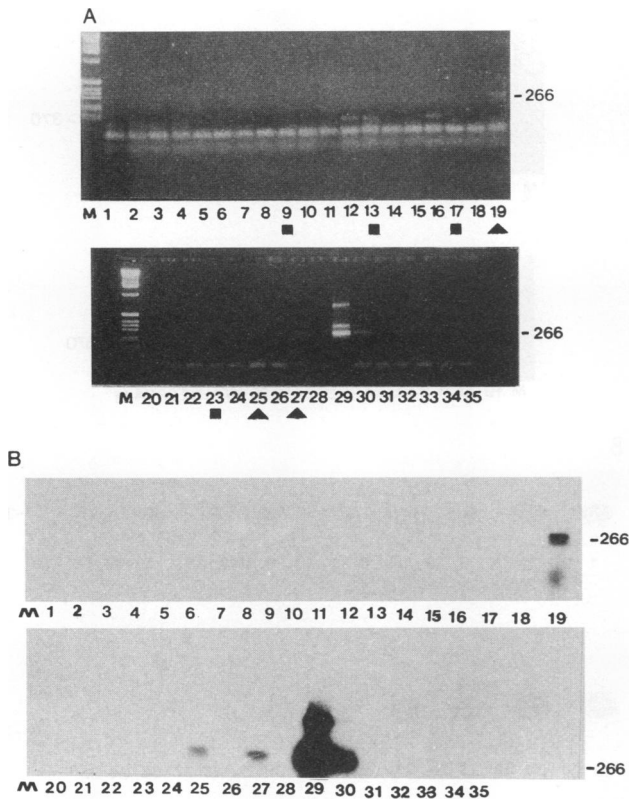

FIG. 3. IE2 expression. An ethidium bromide-agarose gel (A) and the Southern blot of that gel (B) are shown. A 1-kb marker ladder (Gibco) is shown in lanes M. The size of the nested product is indicated (cDNA = 266 bp). Lanes: 1, S1 RT-PCR; 2, S1 sample control; 3, S2 RT-PCR; 4, S2 sample control; 5, S3 RT-PCR; 6, S3 sample control; 7, S4 RT-PCR; 8, S4 sample control; 9, S5 PMA/HC MDM RT-PCR; 10, S5 sample control; 11, S6 monocyte RT-PCR; 12, S6 monocyte sample control; 13, S6 PMA/HC MDM RT-PCR; 14, S6 PMA/HC MDM sample control; 15, S7 monocyte RT-PCR; 16, S7 monocyte sample control; 17, S7 PMA/HC MDM RT-PCR; 18, PMA/HC MDM sample control; 19, S7 GM-CSF MDM RT-PCR; 20, S7 GM-CSF MDM sample control; 21, S8 monocyte RT-PCR; 22, S8 monocyte sample control; 23, S8 PMA/HC MDM RT-PCR; 24, S8 PMA/HC MDM sample control; 25, S8 GM-CSF MDM RT-PCR; 26, S8 GM-CSF MDM sample control; 27, S9 GM-CSF MDM RT-PCR; 28, S9 GM-CSF MDM sample control; 29 to 31, RT-PCR of RNA from the equivalent of 10, 1, and 0.1 HCMV-infected fibroblast cells; 32 and 33, RT-PCR H_2O controls; 34 and 35, PCR H_2O controls. MDM derived from PMA/HC and GM-CSF/HC are highlighted by boxes and triangles, respectively.

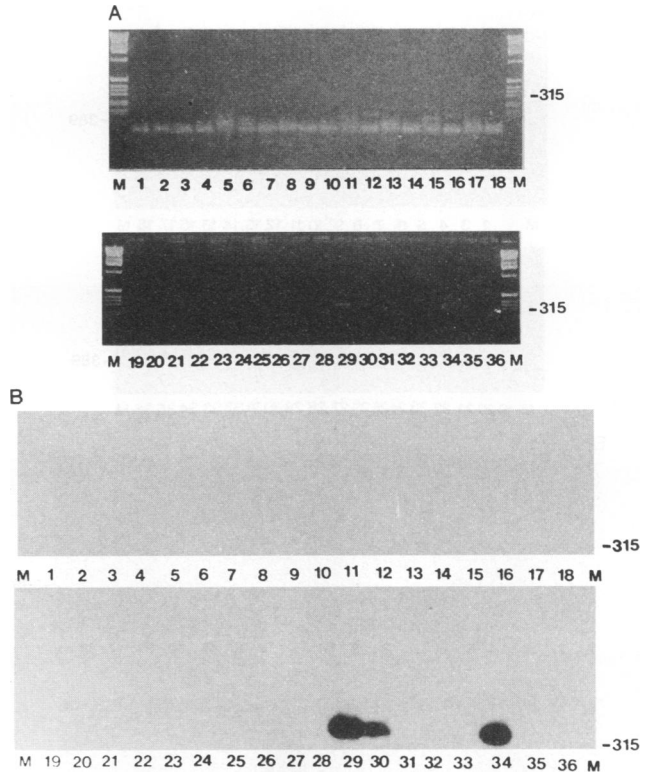

FIG. 4. Early expression. An ethidium bromide-agarose gel (A) and the Southern blot of that gel (B) are shown. A 1-kb marker ladder (GIBCO) is shown in lanes M. Sizes of nested products are indicated (DNA and cDNA = 315 bp). Lanes: 1, S1 RT-PCR; 2, S1 sample control; 3, S2 RT-PCR; 4, S2 sample control; 5, S3 RT-PCR; 6, S3 sample control; 7, S4 RT-PCR; 8, S4 sample control; 9, S5 PMA/HC MDM RT-PCR; 10, S5 PMA/HC MDM sample control; 11, S6 monocyte RT-PCR; 12, S6 monocyte sample control; 13, S6 PMA/HC MDM RT-PCR; 14, S6 PMA/HC MDM sample control; 15, S7 monocyte RT-PCR; 16, S7 monocyte sample control; 17, S7 PMA/HC MDM RT-PCR; 18, S7 PMA/HC MDM sample control; 19, S7 GM-CSF MDM RT-PCR; 20, S7 GM-CSF MDM sample control; 21, S8 monocyte RT-PCR; 22, S8 monocyte sample control; 23, S8 PMA/HC MDM RT-PCR; 24, S8 PMA/HC MDM sample control; 25, S8 GM-CSF MDM RT-PCR; 26, S8 GM-CSF MDM sample control; 27, S9 GM-CSF MDM RT-PCR; 28, S9 GM-CSF MDM sample control; 29 to 31, RT-PCR of RNA from the equivalent of 1, 0.1, and 0.01 HCMV-infected fibroblast cells; 32 and 33, RT-PCR H_2O controls; 34, PCR control of infected cell DNA equivalent to 10 copies; 35 and 36, PCR H_2O controls.

a constitutively expressed housekeeping gene showed that all RTs had been completed successfully. While we accept that our analysis would not have detected any nonpolyadenylated transcripts from these regions of the HCMV genome, these data suggest that within our limits of detection, HCMV persistence in monocytes of healthy carriers is independent of viral lytic gene expression.

There are many conflicting reports with respect to endogenous HCMV gene expression during latency. With the exception of one study (7), HCMV has never been isolated from the buffy coat of healthy donors even though a cumulative total of over 1,500 buffy coats from blood donors have been analyzed in four different studies (16). This clearly suggests that peripheral blood of healthy carriers does not contain infectious virus. Reports of HCMV IE expression in healthy carriers (24) conflict with others (11) and may reflect either limitations in specificity of the detection method or limitations in sensitivity. Similarly, reports of HCMV IE1 expression autopsy samples (34) are difficult to interpret because of the unknown effect of postmortem changes with respect to viral gene expression. For instance, it is well known that environmental stress may induce a heat shock response (3), and heat shock has been shown to partially activate HCMV in semipermissive cells (40, 41). It is possible, therefore, that any IE1 expression seen in autopsy samples is a reflection of this enhanced viral activity in response to postmortem changes and not a true representation of gene expression seen in healthy carriers.

The data that we present here, showing a lack of HCMV lytic gene expression in freshly isolated PBM of healthy carriers, suggests that HCMV latency in monocytes is not

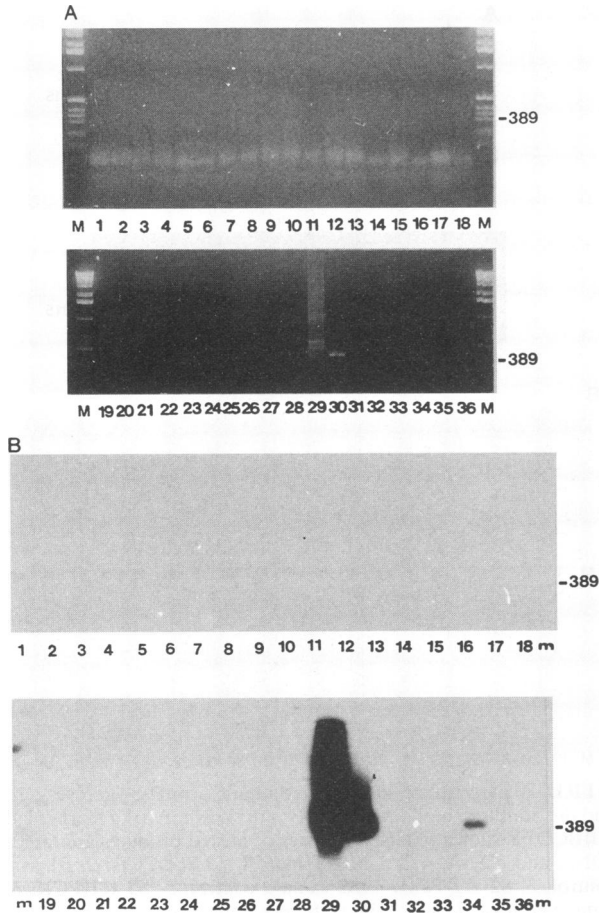

FIG. 5. Late expression. An ethidium bromide-agarose gel (A) and the Southern blot of that gel (B) are shown. A 1-kb marker ladder (Gibco) is shown in lanes M. Sizes of nested products are indicated (DNA and cDNA = 389 bp). Lanes: 1, S1 RT-PCR; 2, S1 sample control; 3, S2 RT-PCR; 4, S2 sample control; 5, S3 RT-PCR; 6, S3 sample control; 7, S4 RT-PCR; 8, S4 sample control; 9, S5 PMA/HC MDM RT-PCR; 10, S5 PMA/HC MDM sample control; 11, S6 monocyte RT-PCR; 12, S6 monocyte sample control; 13, S6 PMA/HC MDM RT-PCR; 14, S6 PMA/HC MDM sample control; 15, S7 monocyte RT-PCR; 16, S7 monocyte sample control; 17, S7 PMA/HC MDM RT-PCR; 18, S7 PMA/HC MDM sample control; 19, S7 GM-CSF MDM RT-PCR; 20, S7 GM-CSF MDM sample control; 21, S8 monocyte RT-PCR; 22, S8 monocyte sample control; 23, S8 PMA/HC MDM RT-PCR; 24, S8 PMA/HC MDM sample control; 25, S8 GM-CSF MDM RT-PCR; 26, S8 GM-CSF MDM sample control; 27, S9 GM-CSF MDM RT-PCR; 28, S9 GM-CSF MDM sample control; 29 to 31, RT-PCR of RNA from the equivalent of 1, 0.1, and 0.01 HCMV-infected fibroblast cells; 32 and 33, RT-PCR H_2O controls; 34, PCR control of infected cell DNA equivalent to 10 copies; 35 and 36, PCR H_2O controls.

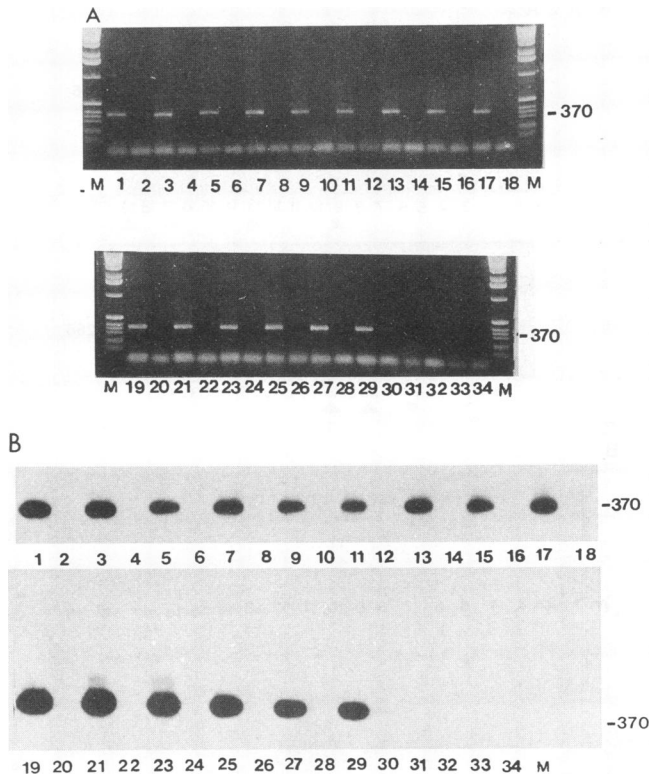

FIG. 6. Histidyl-tRNA synthetase expression. An ethidium bromide-agarose gel (A) and the southern blot of that gel (B) are shown. A 1-kb marker ladder (Gibco) is shown in lanes M. The size of the nested product is indicated (cDNA = 370 bp). Lanes: 1, S1 RT-PCR; 2, S1 sample control; 3, S2 RT-PCR; 4, S2 sample control; 5, S3 RT-PCR; 6, S3 sample control; 7, S4 RT-PCR; 8, S4 sample control; 9, S5 PMA/HC MDM RT-PCR; 10, S5 PMA/HC MDM sample control; 11, S6 monocyte RT-PCR; 12, S6 monocyte sample control; 13, S6 PMA/HC MDM RT-PCR; 14, S6 PMA/HC MDM sample control; 15, S7 monocyte RT-PCR; 16, S7 monocyte sample control; 17, S7 PMA/HC MDM RT-PCR; 18, S7 PMA/HC MDM sample control; 19, S7 GM-CSF MDM RT-PCR; 20, S7 GM-CSF MDM sample control; 21, S8 monocyte RT-PCR; 22, S8 monocyte sample control; 23, S8 PMA/HC MDM RT-PCR; 24, S8 PMA/HC MDM sample control; 25, S8 GM-CSF MDM RT-PCR; 26, S8 GM-CSF MDM sample control; 27, S9 GM-CSF MDM RT-PCR; 28, S9 GM-CSF MDM sample control; 29 and 30, RT-PCR of RNA from the equivalent of 0.1 and 0.001 HCMV-infected fibroblast cells; 31 and 32, RT-PCR H_2O controls; 33 and 34, PCR H_2O controls.

dependent on low-level replication of virus and is consistent with the observation that leukocytes from healthy subjects are coculture negative for HCMV. Either HCMV persists in monocytes without any gene expression at all or some as yet undefined latent HCMV transcripts are expressed in these cells. As in vitro-differentiated MDM have been shown to have increased HCMV permissiveness (15, 18) and in vivo terminally differentiated alveolar macrophages are completely permissive for HCMV infection (8), it is clear that some degree of differentiation is necessary for permissive infection of freshly isolated human monocytes with HCMV. We therefore attempted to induce endogenous HCMV gene expression by using various methods of monocyte differentiation and activation. Using GM-CSF plus HC to differentiate monocytes from healthy HCMV carriers, we have been able to induce IE1 and IE2 transcripts but not major early or late pp28 gene expression from endogenous virus. Consistent with this lack of full lytic expression, cocultivation of PMA/HC- and GM-CSF/HC-differentiated MDM showed no evidence of full productive infection even after 28 days of culture. Interestingly, differentiation of monocytes induced by PMA plus HC resulted in induction of IE1 but not IE2 poly(A)$^+$ RNA. We do not know why these different conditions result in differential induction of IE1 and IE2 transcripts. Although both RNAs are differential spliced products of the same transcription unit, and these differentiated cells would have contained primary transcripts

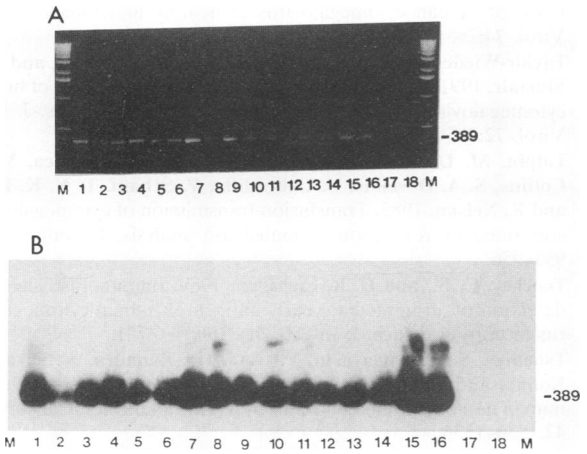

FIG. 7. HCMV pp28 DNA PCR. An ethidium bromide-agarose gel (A) and the Southern blot of that gel (B) are shown. A 1-kb marker ladder (Gibco) is shown in lanes M. The size of the nested product is indicated (DNA = 389 bp). All subjects' DNA amplifications were carried out on 1 μg of DNA. Lanes: 1, S1; 2, S2; 3, S3; 4, S4; 5, S5; 6, S6 monocytes; 7, S6 PMA/HC MDM; 8, S7 monocytes; 9, S7 PMA/HC MDM; 10, S7 GM-CSF MDM; 11, S8 monocytes; 12, S8 PMA/HC MDM; 13, S8 GM-CSF MDM; 14, S9 GM-CSF MDM; 15 and 16, HCVM-infected cell DNA amplifications from the equivalent of 500 and 50 copies of HCMV; 17 and 18, PCR H_2O controls.

containing both IE1 and IE2 sequences, it is possible that no mature spliced polyadenylated RNA containing IE2 sequences was present in these cells, as splice site selection and splicing can occur prior to polyadenylation (39). Consequently, our RT-PCR analysis, which was based on polyadenylated RNA, would have been unable to detect these primary transcripts containing IE2 sequences. Our results, therefore, suggest that the control of major IE RNA splicing and/or polyadenylation site selection is, at least in part, controlled by cellular factors which are differentiation dependent. Such discordant expression of IE1 and IE2, resulting from posttranscriptional control, probably at the level of splice site and/or polyadenylation site selection, has been shown during HCMV infection (29). However, we also accept that it is possible that this discrepancy reflects differences in the relative sensitivities of the IE1 and IE2 RT-PCRs. Finally, attempts to use more extensive methods of differentiation with and without macrophage activation have, as yet, not lead to full virus production as detected by cocultivation. The data presented here represent the first report of the induction of endogenous HCMV gene expression from the monocytes of healthy carriers as a result of in vitro differentiation. While we were not able to reactivate full virus production, our differentiation conditions were able to reactivate endogenous HCMV IE expression in monocytes from a number of healthy carriers. We think it likely that specific cellular interactions as well as other cytokines are necessary for full HCMV reactivation, but clearly, differentiation of monocytic cells to MDM plays as essential role in the induction of the first phase of lytic gene expression during reactivation of HCMV.

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