Human Cytomegalovirus Inhibits Human Immunodeficiency Virus Replication in Cells Productively Infected by Both Viruses

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We have been studying the role of human cytomegalovirus (HCMV) as a potential cofactor in human immunodeficiency virus (HIV)-related disease. The clinical relevance of HCMV is highlighted by the fact that it is a principal viral pathogen in patients with AIDS and is known to infect the same cells as HIV. In this study, we focused on the molecular interactions between HIV and HCMV in human fibroblasts and in the human glioblastoma/astrocytoma-derived cell line U373 MG, cells which can be productively infected by both viruses. Because these cells are CD4$^-$, we used HIV pseudotyped with a murine amphotropic retrovirus as described previously (D. H. Spector, E. Wade, D. A. Wright, V. Koval, C. Clark, D. Jaquish, and S. A. Spector, J. Virol. 64:2298–2308, 1990). Initial studies showed that when cells were preinfected with HIV (Ampho-1B) for 5 days and then superinfected with HCMV, HIV antigen production dropped significantly in the coinfected cells but continued to rise in cells infected with HIV (Ampho-1B) alone. HCMV production, however, was unaffected by the presence of HIV. Further analysis showed that HIV steady-state RNA levels and *gag* and *env* protein production were also inhibited in the presence of HCMV. The transcriptional inhibition of HIV was particularly surprising in view of the previous results of several other laboratories as well as our own that HCMV infection stimulates HIV long terminal repeat-chloramphenicol acetyltransferase (LTR-CAT) expression in transient expression assays. To investigate this further, we transfected the HIV LTR-CAT construct into either uninfected cells or cells which had been preinfected with HIV. The cells were infected with HCMV 24 h posttransfection and assayed for CAT gene expression at 48 h after HCMV infection. Although there was some stimulation of the LTR-CAT in cells that were dually infected by HIV and HCMV, it was 16-fold less than that in the cells infected only with HCMV. This suggests that in the presence of the HIV infection, the stimulation of the HIV LTR-CAT gene by HCMV is significantly reduced. Experiments with UV-irradiated HCMV and the HCMV DNA polymerase inhibitor ganciclovir showed that HCMV transcription is necessary for the reduction in HIV production to occur; however, replication of the HCMV genome or any events which take place after DNA replication are not necessary. These results, coupled with the observation that inhibition is usually first seen between 8 and 24 h after HCMV infection, suggest that an HCMV early protein is involved in repression of HIV.

Although human immunodeficiency virus (HIV) has been implicated as the primary etiological agent of AIDS, a great deal of attention has focused on the potential role of other cofactors in the development or progression of the disease. Many questions relating to the complex clinical manifestations of HIV infection remain unanswered. What are the factors that determine which HIV-infected individuals will remain asymptomatic and which will develop AIDS? What governs HIV latency and the reactivation from latency? And what is the role of other viruses in the pathogenesis of the disease? Individuals infected with HIV are often infected with other viruses including human T-cell leukemia virus types I and II (HTLV-I and HTLV-II), human cytomegalovirus (HCMV), Epstein-Barr virus (EBV), varicella-zoster virus, herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), human herpesvirus 6 (HHV-6), papovaviruses, and hepatitis B virus, and the results of some studies suggest that the disease in those infected with a second virus progresses more rapidly than that in those infected with HIV alone (for a review, see reference 47).

There are a number of mechanisms by which other viruses may affect the clinical course of the HIV infection. One mechanism might involve additional immunosuppression of the host, in which infection with HIV and a second virus which induces immunoregulatory abnormalities might lead to a reduction in immune surveillance and enhanced replication of both viruses. In this regard, both HCMV and EBV are known to be immunosuppressive (6, 57, 67). Viral infection may also result in tissue inflammation and the recruitment of HIV-infected macrophages to the affected organ. It has also been documented that Fc receptors induced on the surface of HCMV-infected fibroblasts allow the uptake of antibody-coated HIV into these cells (41), and such a mechanism, if operating in vivo, might facilitate HIV infection of CD4-negative cells. Another mechanism might be through antigenic or mitogenic stimulation of the target cells for HIV. Relevant to this is the report that viral antigens from some viruses (HCMV, EBV, and HIV) but not others (HSV-1, HSV-2, varicella-zoster virus, HHV-6, adenovirus, hepatitis B virus, and vaccinia virus) can induce the production of monokines capable of activating HIV in chronically infected promonocyte and T-cell clones (12). Some viruses may also be able to directly stimulate HIV gene expression. For example, a number of studies have shown that HSV, EBV, varicella-zoster virus, HCMV, HHV-6, papovaviruses, hepatitis B virus, adenovirus, and HTLV can activate the HIV long terminal repeat (LTR) when linked

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to an indicator gene such as chloramphenicol acetyltransferase (CAT) (3, 17–19, 23, 28, 30, 38, 43, 44, 46, 49, 53–55, 61, 68–70). Additionally, there is evidence that under certain conditions, dual infection with HIV and with HSV, HHV-6, or HCMV may potentiate the HIV infection in tissue culture (1, 8, 27, 36, 62). However, some viruses may have the opposite effect and repress HIV gene expression. In support of this are the recent studies showing that coinfection of cells with HIV and with HHV-6 or EBV leads to a marked reduction of HIV expression (7, 25, 34). Finally, a striking feature of the retroviruses is their promiscuity with respect to phenotypic mixing as well as pseudotype formation (for reviews, see references 4 and 73). Thus, infection of a cell by both HIV and another enveloped virus might result in the formation of phenotypically mixed particles or pseudotypes of HIV with expanded host cell tropism. In fact, HIV can readily form phenotypically mixed particles and pseudotypes with the envelope glycoproteins from a large number of viruses including HTLV-I, HTLV-II, HSV-1, vesicular stomatitis virus, and amphotropic, ecotropic, xenotropic, and dualtropic murine leukemia viruses (5, 10, 11, 33, 35, 37, 50, 64, 74).

The focus of this report is on the interaction between HIV and HCMV, a member of the herpesvirus group. One difficulty in studying the full range of biological interactions between HIV and HCMV in tissue culture is that only human fibroblasts and the human glioblastoma/astrocytoma-derived cell line U373 MG are fully permissive for HCMV replication. However, since these cells are $CD4^-$, HIV replication is inhibited at the adsorption step. To bypass this difficulty, we devised a way to create HIV pseudotypes with expanded host range. As described previously (64), this was accomplished by coinfecting $CD4^+$ cells with HIV and a murine amphotropic retrovirus, which resulted in phenotypically mixed particles with the HIV genome and amphotropic retrovirus glycoproteins. These HIV pseudotypes, designated HIV (Ampho-1B), were able to infect and replicate to high titers in both $CD4^-$ human fibroblasts and the U373 MG cell line.

In the study reported here, we used these HIV (Ampho-1B) pseudotypes to study the molecular interactions between HIV and HCMV in the cells which allow both viruses to complete their replication cycle. Our results indicated that, in the coinfected human fibroblast or astroglial cell, HCMV replication proceeds normally. However, HCMV appeared to have a marked effect on the replication of HIV. The titers of HIV were substantially reduced, and both HIV RNA and protein synthesis were affected. This inhibition required HCMV viral gene expression but not viral DNA replication. We also showed that the inhibition of HIV gene expression is in direct contrast to the overall activation of an HIV LTR-CAT construct by the HCMV infection. Potential molecular mechanisms governing these interactions and their clinical implications are discussed.

MATERIALS AND METHODS

Cells. CEM cells, a $CD4^+$ T-helper lymphoma cell line, were propagated in RPMI 1640 medium containing 10% fetal bovine serum at 5×10^5 cells per ml. CEM-1B cells, a CEM cell line persistently infected with the mouse amphotropic retrovirus 4070A (9) and used to obtain HIV (Ampho-1B) pseudotypes as previously described (64), were propagated in the same manner, except that 400 μg of G418 per ml was added to the medium. Human foreskin fibroblasts (FF cells) were obtained and propagated in Dulbecco modified Eagle medium with 10% fetal bovine serum as previously described (64). The human glioblastoma/astrocytoma-derived cell line U373 MG (ATCC HTB17) was a gift from Robert LaFemina and was maintained in Dulbecco modified Eagle medium with high glucose (4.5 g/liter) containing 10% fetal bovine serum. All media were supplemented with 0.26 mg of L-glutamine per ml, 180 U of penicillin-streptomycin per ml, 1.4 μg of amphotericin B (Fungizone) per ml, and 45 μg of gentamicin sulfate per ml.

Virus stocks. The LAV strain of HIV, originally obtained from the Pasteur Institute, was propagated by low-multiplicity infection of CEM cells and stored as filtered cell supernatant at $-70°C$. The AD169 strain of HCMV was propagated by low-multiplicity infection of FF cells and stored in Dulbecco modified Eagle medium containing 10% fetal bovine serum and 1% dimethyl sulfoxide at $-70°C$.

Infection procedures. HIV (Ampho-1B) pseudotypes were generated by infecting CEM-1B cells at a concentration of 5 $\times 10^5$ cells per ml with a low multiplicity of HIV. During infection with HIV, the CEM-1B cells were maintained in medium without G418. In a standard low-multiplicity infection, a culture of CEM-1B cells was infected with a stock of HIV at a final concentration of 35 ng of HIV antigen per ml of cells. The amount of HIV antigen in the HIV stocks was determined by an HTLV-III antigen enzyme immunoassay as described by the manufacturer (Abbott Laboratories). At 24 h postinfection (p.i.), the cells were washed twice with phosphate-buffered saline (PBS) and resuspended in fresh medium. After approximately 75% of the cells displayed cytopathic effect (typically 3 to 4 days p.i.), the cells were resuspended in fresh medium for 48 h and the supernatant was then recovered and passed through a 0.45-μm-pore-size filter. These supernatants typically contained 1,000 to 3,000 ng of HIV antigen per ml. Each T75 or T25 flask of FF cells or U373 MG cells at approximately 50% confluence was mock infected or was infected either with undiluted supernatant containing the HIV (Ampho-1B) pseudotypes or with supernatant diluted 1:1 with fresh medium (total volume of 10 ml for the T75 flask or 5 ml for the T25 flask). After 24 h, the cells were washed twice with PBS and fed with fresh medium. Under these conditions, we have previously determined by in situ cytohybridization that 100% of the cells are infected with HIV (64). HCMV infections were performed at a high multiplicity of infection (MOI) of 3 to 5 in a volume of 5 to 8 ml of medium per T75 flask or 3 ml per T25 flask for 3 to 8 h. The volume was then brought up to 10 ml with medium (5 ml for a T25 flask), and the infection was allowed to proceed for a total of 8 or 24 h before washing the cells twice with PBS and adding fresh medium. In some experiments, at the time of infection with HCMV, the FF cells were maintained in medium containing 10 μg of ganciclovir (DHPG) per ml until harvested. The titer of HCMV was determined by plaque assay.

UV light treatment. Medium from FF cells infected with HCMV or uninfected FF cells was recovered after 3 days and exposed to UV light (Sylvania GTE G30T8 30-W lamp) at a distance of 46 cm for 2 h while being gently agitated. This supernatant was then used for the infection or mock infection.

Antibodies. Mouse monoclonal antibody to the HIV *gag* proteins p25, p40, and p55 was obtained from Genetic Systems Corp. Polyclonal antiserum to gp160B (HT3) was provided through the AIDS Research and Reference Reagent Program, AIDS Program, National Institutes of Health (40, 58), and rabbit anti-mouse and anti-goat immunoglobulin Gs (IgGs) were purchased from Jackson Immuno Research.

Western blot (immunoblot) analysis. Cells were trypsinized, washed once with PBS, resuspended in a minimal amount of PBS, lysed with an equal volume of 2× Western sample buffer (2% sodium dodecyl sulfate [SDS], 5 mM $NaPO_4$ [pH 7.0], 10% glycerol, 5% 2-mercaptoethanol, 0.1 M dithiothreitol, 0.01% bromophenol blue), and boiled for 10 min prior to storage at $-20°C$. Proteins from approximately 2.5×10^5 cells per lane were separated by electrophoresis on an SDS-12.5% polyacrylamide gel and transferred to nitrocellulose membranes. Filters were blocked overnight and probed the next day with antibodies and ^{125}I-labeled protein A as described previously (64).

Preparation of nucleic acids. At various times p.i., cells were trypsinized, washed twice with ice-cold PBS, and stored as a pellet at $-70°C$. Pellets were resuspended in 1 ml of 4 M guanidinium thiocyanate–25 mM sodium citrate (pH 7.0)–0.5% sarcosine–0.1 M 2-mercaptoethanol (GT solution), and the solution was adjusted to 0.2 M sodium acetate (pH 5.0). This solution was then extracted once with a mixture of 1 ml of phenol and 0.2 ml of chloroform containing 2% isoamyl alcohol (2% CIA). Samples were placed on ice for 15 min and then centrifuged at $10,000 \times g$ for 20 min at 4°C. The aqueous layer containing total cell RNA was removed and extracted once more with an equal volume of phenol and 2% CIA and centrifuged as before. The aqueous layer was again removed, and the nucleic acids were precipitated with an equal volume of isopropanol at $-20°C$ for at least 1 h. Samples were centrifuged again as before, and the pellets were dried and resuspended in 0.2 ml of GT solution and transferred to a 1.5-ml Eppendorf tube. The centrifuge tubes were washed with 0.2 ml of GT solution, and this wash was also transferred to the Eppendorf tube. Samples were precipitated again with an equal volume (0.4 ml) of isopropanol at $-20°C$ for at least 1 h. Following centrifugation for 15 min at 4°C in an Eppendorf centrifuge, the pellets were rinsed with 70% ethanol, dried in vacuo, and resuspended in 10 mM Tris–0.1 mM EDTA (pH 8.0).

Northern (RNA) blot analysis. Total cell RNA was denatured and separated by formaldehyde agarose gel electrophoresis and blotted by the method of Fourney et al. (20), except that 1 μl of 1-mg/ml ethidium bromide was added to each sample prior to heating at 65°C. After transfer of RNA from the gel to a nitrocellulose filter, the filter was baked for 2 h at 80°C in a vacuum oven. It was then prehybridized at 45°C for 4 h in a solution containing 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50 mM $NaPO_4$ (pH 7.0), 10× Denhardt's reagent, 250 μg of salmon DNA per ml, 4 mM EDTA, and 0.5% SDS. Hybridization was performed at 45°C in a solution containing 50% formamide, 5× SSC, 40 mM $NaPO_4$ (pH 7.0), 1× Denhardt's reagent, 100 μg of salmon DNA per ml, 1 mM EDTA, 0.1% SDS, 10% dextran sulfate, and 2×10^6 cpm of the [32P]-labeled HIV LTR-specific probe per ml prepared as described below. The blot was washed in 1× SSC–0.1% SDS at room temperature twice for 20 min each wash, then washed in 0.1× SSC–0.1% SDS at 50°C twice for 20 min each wash, and rinsed in 0.1× SSC at room temperature. The blot was then subjected to autoradiography at $-70°C$ in the presence of intensifying screens.

The HIV LTR-specific probe was derived from an HIV LTR-CAT construct (provided by Paul Luciw) which contains sequences between -633 and $+185$ of the HIV LTR upstream from the CAT gene (52). This construct was cleaved with EcoRI and labeled with [α-32P]dCTP (3,000 Ci/mmol) by random priming with the Boehringer Mannheim

Random Primed DNA Labeling Kit and the manufacturer's instructions.

Analysis of HCMV DNA. Intracellular DNA was isolated as previously described (39). Aliquots of the DNA (200, 20, and 2 ng) were then slot blotted onto nitrocellulose filters with the Schleicher & Schuell Minifold II Slot Blot System according to the manufacturer's instructions. The filters were prehybridized and hybridized with [32P]-labeled HCMV EcoRI fragment D (66) as described above for the Northern blot hybridization. This fragment was labeled with [α-32P] dCTP (3,000 Ci/mmol) by random priming with the Boehringer Mannheim Random Primed DNA Labeling Kit.

Cell transfection. FF cell monolayers that had been infected with HIV (Ampho 1B) or with Ampho 1B for 24 h were transfected with the LTR CAT construct by using the DEAE-dextran technique previously described by Staprans et al. (65). Cells were then superinfected with HCMV at 24 h posttransfection and harvested for a transient expression assay 48 h later.

Transient expression assay. At 48 h after infection with HCMV, cells were harvested and washed once with 25 ml of cold PBS. The pellet from each flask was resuspended in 2 ml of cold PBS and split into two parts: one for the analysis of CAT activity and the other for the preparation of nuclear DNA and assessment of transfection efficiency. Soluble extracts were prepared and assayed for CAT activity essentially as described by Gorman et al. (24) except that the acetyl coenzyme A was increased to 4 mM for longer reaction times (10 h). Transfection efficiency was determined on the second half of the samples by isolation of transfected cell nuclear DNA and determination of relative amounts of plasmid DNA sequences present as described by Staprans et al. (65). Briefly, isolated nuclear DNA was digested with EcoRI, and equivalent amounts of DNA, determined by ethidium bromide staining intensities, were electrophoresed on agarose gels and then transferred to nitrocellulose filters. The filters were prehybridized and hybridized with the [32P]-labeled HIV LTR probe as described above for the Northern blot except the incubation temperature was 37°C. The relative hybridization signals detected by autoradiography were used to assess the relative copy number of plasmid DNAs in different samples.

Biosafety. All experiments involving live HIV were performed at the highest level of containment in a locked BL3 facility, and there was strict adherence to established National Institutes of Health procedures for research conducted at the BL3 level of containment.

RESULTS

Effect of dual infection on HIV and HCMV virus production. We began our study of HIV-HCMV interactions by assessing the production of each virus during a coinfection. Human FF cells were mock infected or infected for 5 days with HIV (Ampho-1B) pseudotypes or with Ampho-1B alone. The cells were then mock infected or superinfected with HCMV (MOI 3 to 5). At various times p.i., the supernatants were obtained and the amount of HIV was determined by the HIV antigen test. Figure 1 shows that the production of HIV in the supernatants decreased markedly following infection with HCMV. FF cells infected with HIV (Ampho-1B) alone produced 150 ng of HIV antigen per ml at day 1 p.i., rising to 322 ng/ml by day 4. Production leveled out for 2 days and then rose to 755 ng/ml by day 14. However, when the HIV-infected cells were superinfected with HCMV on day 5, HIV production fell to 208 ng/ml 1 day
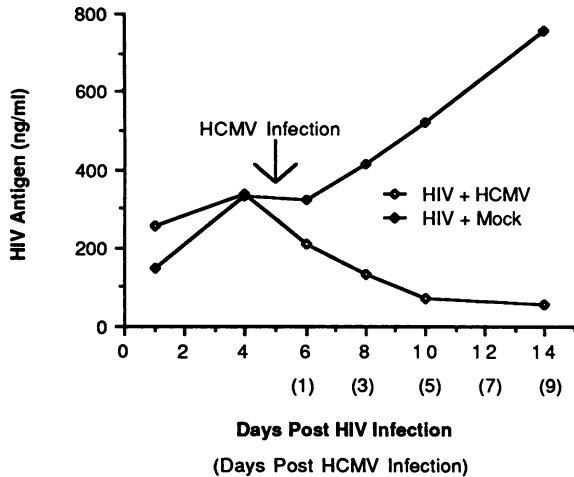
FIG. 1. Effect of HCMV infection on HIV virus production. Human foreskin fibroblasts were infected with HIV pseudotypes for 5 days to establish a persistent infection. The cells were then infected with HCMV (MOI 3 to 5) (◇) or mock infected (◆). At various times p.i., supernatants were obtained and the amount of HIV present was determined by using the Abbott HIV antigen enzyme immunoassay.

later and continued to fall to 72 ng/ml by day 10 (day 5 p.i. with HCMV).

In contrast to the inhibition of HIV production, HCMV synthesis was relatively unaltered. Figure 2 shows the production of HCMV virions in the supernatant as measured by plaque assay. The presence of the amphotropic virus alone (Ampho-1B) had no effect on the HCMV infection. The small (approximately threefold) drop in HCMV virus production in the cells dually infected with HIV and HCMV was due to a lower number of cells in these cultures.

Analysis of HIV proteins. To investigate further the inhibition of HIV synthesis by HCMV, we proceeded to analyze the production of HIV proteins by Western blot using a mouse monoclonal antibody to the structural *gag* proteins and a goat polyclonal antibody to the envelope glycopro-
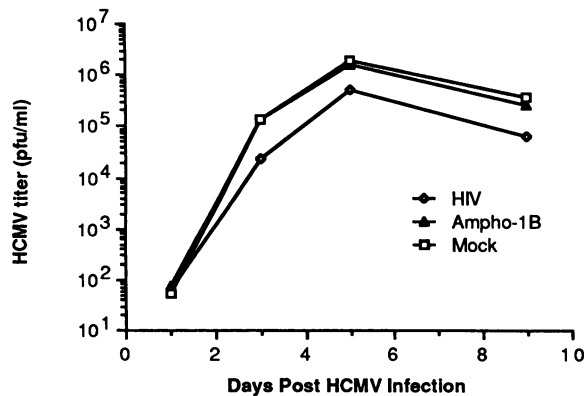

FIG. 2. Effect of HIV infection on HCMV virus production. Human foreskin fibroblasts were infected with HIV pseudotypes (◇) or amphotropic retrovirus (▲) for 5 days to establish a persistent infection or mock infected (□). The cells were then infected with HCMV (MOI 3 to 5). At various times p.i., supernatants were obtained and the amount of HCMV present was determined by plaque assay.
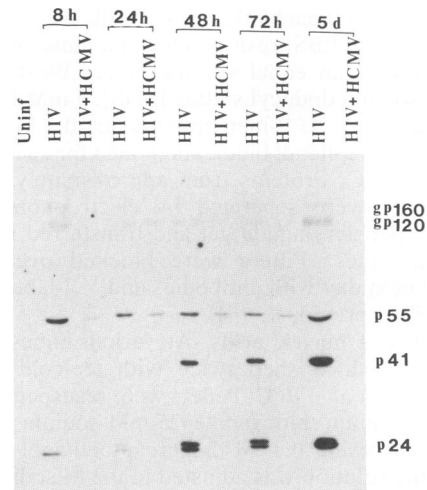

FIG. 3. Effect of HCMV infection on HIV protein synthesis. Human foreskin fibroblasts were infected with HIV pseudotypes for 40 h prior to infection with HCMV. At 8, 24, 48, and 72 h and 5 days after infection with HCMV, the cells were harvested and cell lysates were analyzed by Western blot with a mouse monoclonal antibody to the HIV *gag* proteins and a goat polyclonal antibody to gp160 HTLV-III. Following incubation with the specific antibodies and rabbit anti-mouse IgG plus rabbit anti-goat IgG, the blot was labeled with ^{125}I-protein A. Proteins are labeled on the right. Uninf, uninfected.

teins. In the experiment shown in Fig. 3, human FF cells were infected with the HIV (Ampho-1B) pseudotypes for 40 h prior to infection with HCMV. At 8, 24, 48, and 72 h and 5 days after infection with HCMV, cell extracts were prepared and the intracellular steady-state concentration of the HIV proteins was determined by Western blot. Figure 3 shows that the accumulation of the HIV proteins was inhibited as early as 8 h after HCMV infection. For the *gag* proteins, it also appears that the HCMV infection inhibited the processing of the precursor p55 protein to p41 and p24. Although HIV glycoprotein synthesis was inhibited, interpretation of the experiments was complicated by the presence of the gp120 cleavage product of gp160 in some lanes. Inhibition in this case may be partial, or residual gp120 from input virus may still be present. We noted that the precise time at which inhibition of HIV proteins may be seen does vary somewhat from experiment to experiment. However, in all cases, significant inhibition is seen by 24 h after infection with HCMV, thus suggesting that it is due to some event occurring early in HCMV's replicative cycle.

As a variation of the above experiment, we also investigated the effect of relative timing of the HCMV infection on the inhibition of HIV protein synthesis. For this experiment, human FF cells were infected under the following conditions: HIV (Ampho-1B) pseudotypes alone; HIV (Ampho-1B) pseudotypes followed by HCMV 24 h later; HIV (Ampho-1B) pseudotypes and HCMV together at time zero; or HCMV followed by HIV (Ampho-1B) pseudotypes 24 h later. At 72 h after infection with HIV (Ampho-1B) pseudotypes (corresponding to 48 to 96 h after infection with HCMV), the cells were harvested and cell lysates were analyzed by Western blot for the HIV-specific *gag* proteins. Figure 4 shows that in all cases the HCMV infection inhibited the accumulation of the HIV *gag* proteins. Inhibition was most pronounced, however, when the cells were prein-
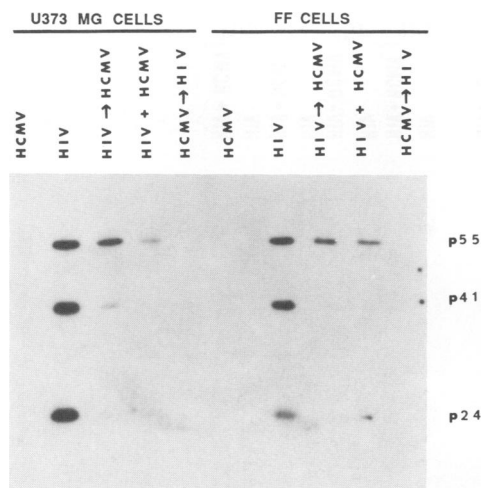
FIG. 4. Effect of timing of HCMV infection on HIV protein synthesis. U373 MG cells or human foreskin fibroblasts (FF cells) were infected with HCMV or HIV pseudotypes alone; HIV pseudotypes for 24 h and then HCMV (HIV → HCMV); HIV pseudotypes and HCMV simultaneously (HIV + HCMV); or HCMV for 24 h and then HIV pseudotypes (HCMV → HIV). Cells were harvested 72 h after HIV pseudotype infection (48 to 96 h after HCMV infection), and cell lysates were analyzed by Western blot with a mouse monoclonal antibody to the HIV gag proteins. Following incubation with the specific antibody and rabbit anti-mouse IgG, the blot was labeled with ^{125}I-protein A. Proteins are labeled on the right.

fected with HCMV or simultaneously infected with both HIV and HCMV. Although in this experiment the amount of time the cells were infected with HCMV varied from 48 to 96 h, we have also analyzed the samples at 72 h after HCMV infection (48 to 96 h after HIV infection) and have obtained the same pattern (data not shown).

The above experiments showed that dual infection of human FF cells with HIV and HCMV under conditions which would allow both viruses to undergo their complete replication cycle resulted in inhibition of HIV protein synthesis. To determine whether this was a general effect or specific to the FF cells, we performed the above experiment in U373 MG glial cells, which are fully permissive for both HCMV and HIV (Ampho-1B) pseudotypes. As shown in Fig. 4, the pattern of inhibition of HIV proteins in the U373 MG glial cells was identical to that seen in the FF cells.

HIV RNA synthesis in dually infected cells. During HIV infection, there are three major classes of RNAs: the multiply spliced species of approximately 2 kb which specify the rev, tat, and nef regulatory proteins; the partially spliced RNAs of approximately 4 kb which give rise to the env, vif, vpr, and vpu proteins; and the genomic transcripts of about 9 kb which specify the products of the gag and pol genes (for a review, see reference 15). These RNAs appear to be temporally regulated such that those of the 2-kb class are the first RNAs which appear in the cytoplasm. Synthesis of the rev protein then mediates the shift from early to late phase such that the partially spliced and genomic RNAs now accumulate in the cytoplasm (31). To examine the effect of the HCMV infection on the relative accumulation of the HIV RNAs, we infected human FF cells with the HIV (Ampho-1B) pseudotypes for 40 h prior to infection with HCMV. Total cellular RNA was then harvested at 8, 24, 48, and 72 h after infection with HCMV and subjected to Northern blot

analysis. Figure 5 shows that by 24 h after infection with HCMV, all three size classes of HIV RNA were inhibited in the coinfected cells. These results suggest that the HCMV infection is affecting either the synthesis of the primary transcript or the overall stability of the RNAs.

Effect of HCMV infection on transactivation of HIV LTR. The inhibition of HIV RNA synthesis by the HCMV infection was surprising in view of the reports by a number of laboratories that DNA viruses, including HCMV, can transactivate the HIV LTR when linked to a reporter gene. One possible explanation was that other cis-acting sequences in the HIV genome were affecting the response of the HIV LTR to the HCMV infection. Alternatively, the interaction of HIV and HCMV proteins in the dually infected cells might reduce any activating effects of HCMV-specified proteins. To investigate this further, human FF cells were mock infected or infected with HIV (Ampho-1B) pseudotypes or with Ampho-1B alone for 24 h. The cells were then transfected with the LTR-CAT construct and 24 h later either mock infected or infected with HCMV. Cell extracts were prepared at 48 h after infection with HCMV and analyzed for CAT activity (Table 1).

As expected, HCMV alone activated the LTR quite well, with CAT activity in the HCMV-infected cells 1,300-fold higher than basal activity and 185-fold higher than the activity in cells infected with HIV (Ampho-1B) alone. However, the level of transactivation of the HIV LTR-CAT in the cells dually infected with HIV and HCMV was approximately 16-fold lower than that in the cells infected with HCMV alone. This repression was not due to the presence of the Ampho-1B virus since HCMV infection of cells infected with Ampho-1B alone resulted in stimulation of CAT activity to a level 1,000-fold higher than the basal activity. These results suggest that there are interactions between the HCMV and HIV gene products (or possibly between HCMV gene products and the HIV proviral DNA) which affect the ability of HCMV to transactivate the HIV LTR even when linked to a reporter gene. However, the CAT activity was still 10 times higher in the dually infected cells than in the cells infected only with the HIV (Ampho-1B) pseudotypes. Since the net effect of the HCMV infection on HIV RNA and protein synthesis is one of repression, it appears that HCMV may interact differently with the HIV proviral genome generated during the infection than with an LTR-CAT construct introduced into the cell by transfection.

Inhibition of HIV replication requires HCMV gene expression but not HCMV DNA synthesis. The kinetics of inhibition of HIV gene expression by the HCMV infection suggested that it occurred early in the HCMV replication cycle. However, it was still possible that some component of the input HCMV virion particles was mediating this inhibition. An alternative explanation was that as the HCMV DNA genome replicated, it effectively competed for transcription factors needed by HIV. To address these possibilities, we performed two experiments.

To determine whether inhibition of the HIV infection was due to some factor associated with the input HCMV virus inoculum, we treated the HCMV virions with UV light. Under these conditions, HCMV should be able to enter the cell, but HCMV gene expression should be inhibited (26). In the experiment shown in Fig. 6, FF cells were infected with HIV (Ampho-1B) pseudotypes for 40 h and then incubated with UV-treated conditioned medium from uninfected cells or with UV-treated medium containing HCMV. Cell extracts were prepared 8, 24, and 72 h later and analyzed by Western blot for HIV gag and envelope proteins. Under these con-
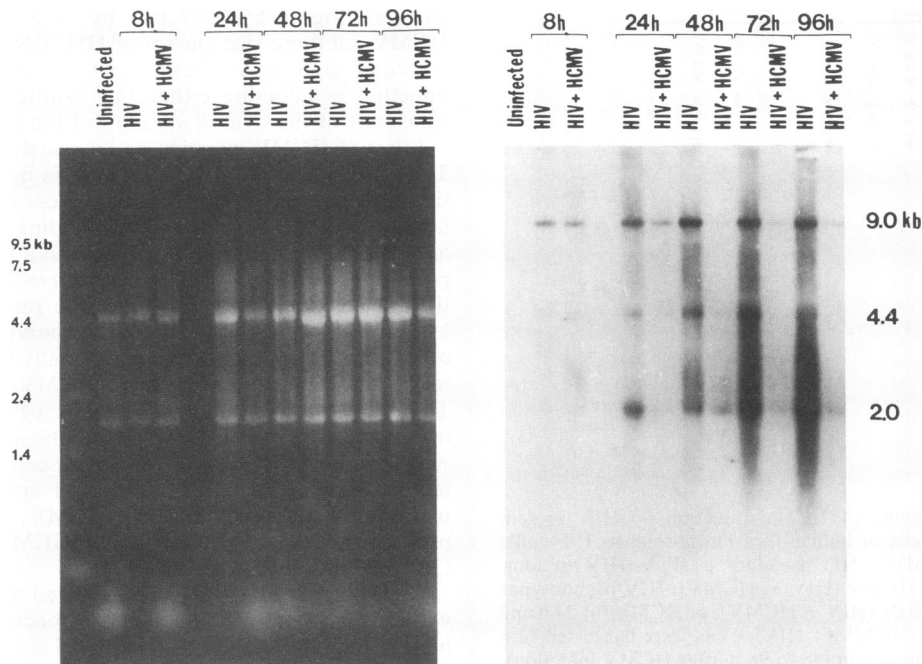
FIG. 5. Effect of HCMV infection on HIV RNA synthesis. Human FF were infected with HIV pseudotypes for 40 h prior to infection with HCMV. Total RNA was isolated at 8, 24, 48, 72, and 96 h after HCMV infection and was analyzed by Northern blot. On the left is a photograph of the ethidium bromide-stained gel, and on the right is the autoradiogram of the Northern blot following hybridization with a probe to the HIV LTR. The positions of the size markers are shown on the left of the stained gel. The sizes of the three HIV mRNA species are shown on the right of the autoradiogram.

ditions, HCMV was unable to inhibit the HIV infection, thus suggesting that at least some HCMV gene expression is required for the inhibitory effect.

To assess whether HCMV viral DNA replication was required for inhibition of the HIV infection, we used the drug DHPG, which is a specific inhibitor of the HCMV-encoded DNA polymerase. In this experiment, FF cells were infected with HIV (Ampho-1B) pseudotypes for 40 h and then mock infected or superinfected with HCMV in either the presence or absence of 10 µg of DHPG per ml. At days 1, 3, 5, and 7 after HCMV infection, supernatants were obtained and the amount of HIV present was determined by the HIV antigen

assay (Fig. 7B). To confirm that the drug inhibited HCMV DNA synthesis, DNA was isolated from the infected cells at 7 days after HCMV infection and the amount of HCMV DNA present was assayed by slot-blot hybridization with an HCMV-specific probe (Fig. 7A). As shown, HCMV DNA synthesis was inhibited greater than 10-fold by the presence of DHPG, and DHPG had no effect on the replication of HIV

TABLE 1. Effect of HIV-HCMV coinfection on transactivation of HIV LTR-CAT

FF cells[a]	Relative CAT activity[b]
Uninfected...	1
HCMV infected.......................................	1,332
HIV pseudotype infected	7.2
HIV pseudotype plus HCMV infected	84
Ampho-1B infected	1.2
Ampho-1B plus HCMV infected...................	1,036

[a] Human FF monolayers were mock infected or infected with HIV pseudotypes or Ampho-1B alone for 24 h. The cells were then transfected with the HIV LTR-CAT plasmid by the DEAE-dextran technique. At 24 h posttransfection, the cells were either mock superinfected or superinfected with HCMV, and all monolayers were harvested at 48 h after superinfection.

[b] CAT assays were performed with 0.5 to 10.0 µg of extract and incubated at 37°C for 10 h. The relative CAT activity was determined by comparing the corrected percent conversion of substrate chloramphenicol for cells transfected with LTR-CAT alone and cells transfected with LTR-CAT and infected with one or more of the viruses used. Numbers are corrected for micrograms of extract assayed and the transfection efficiency as determined by an analysis of nuclear plasmid DNA (see Materials and Methods).
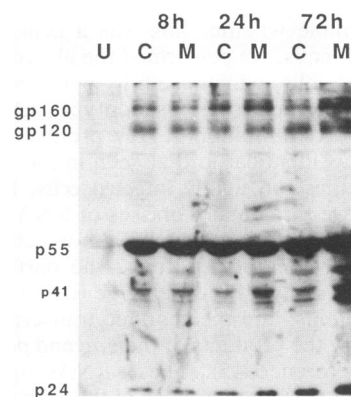

FIG. 6. Effect of UV light on inhibition of HIV protein synthesis by HCMV. Human FF were infected with HIV pseudotypes for 40 h and then infected with HCMV which had been UV irradiated for 2 h (lanes C) or mock infected (lanes M). Cells were harvested at 8, 24, and 72 h after HCMV infection, and cell lysates were analyzed by Western blot with a mouse monoclonal antibody to the HIV gag proteins and a goat polyclonal antibody to gp160 HTLV-III. Following incubation with the specific antibodies and rabbit anti-mouse IgG plus rabbit anti-goat IgG, the blot was labeled with ^{125}I-protein A. Proteins are labeled on the left.
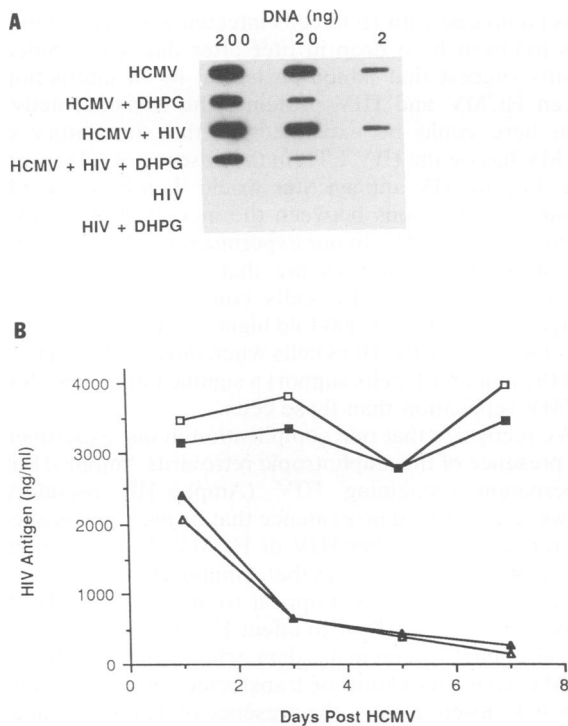
FIG. 7. Effect of DHPG on inhibition of HIV virus production by HCMV. Human FF were infected with HIV pseudotypes for 40 h and then mock infected or superinfected with HCMV in the presence or absence of 10 μg of DHPG per ml. (A) At 7 days after HCMV infection, total DNA was isolated and analyzed by slot-blot hybridization with a probe specific for HCMV. The conditions of infection are shown on the left and the amount of DNA loaded is shown above. (B) At various times after infection with HCMV, supernatants were obtained and the amount of HIV present was determined by using the Abbott HIV antigen enzyme immunoassay. Days after HCMV infection are shown on the horizontal axis. HIV antigen is shown on the vertical axis. □, HIV pseudotype alone; ■, HIV pseudotype plus DHPG; △, HIV pseudotype plus HCMV; ▲, HIV pseudotype plus HCMV plus DHPG.

in cells infected with the HIV (Ampho-1B) pseudotypes alone. However, the kinetics of inhibition of HIV by the HCMV infection in the dually infected cells was the same in the presence and absence of DHPG, thus suggesting that HCMV viral DNA replication is not necessary for inhibition to occur.

DISCUSSION

The clinical relevance of HCMV as a potential cofactor in the development of AIDS is demonstrated by the fact that this virus is a principal viral pathogen in patients with AIDS and is a significant cause of morbidity and mortality in these individuals (for a review, see reference 59). Multiple organ systems may be involved, with such clinical manifestations as pneumonia, adrenalitis, colitis, hepatitis, retinitis, encephalitis, and epididymitis. HCMV is also known to infect in vivo the same cell types as HIV including T cells, monocytes, endothelial cells, and glial cells (2, 16, 21, 22, 29, 32, 42, 45, 56, 60, 71, 72). Of special note are the studies which have shown that dual infection of a cell by both HIV and HCMV occurs in vivo in the brain tissue and retinas of patients with AIDS (48, 63).

Our premise when we began our studies was that HCMV would potentiate the HIV infection. This premise was based on the many studies which have shown that a number of viruses including HCMV can activate the HIV LTR when it is linked to an indicator gene such as CAT (3, 17–19, 23, 28, 30, 38, 43, 44, 46, 49, 53–55, 61, 68–70). Furthermore, there had been several reports indicating that under certain conditions cell cultures infected with HIV and either HSV, HHV-6, or HCMV produced higher levels of HIV (1, 8, 27, 36, 62). In fact, we showed in this study that coinfection of cells which are fully permissive for the replication of HIV and HCMV results not in activation but in an inhibition of HIV RNA, protein, and virus production. This repression appeared to be enhanced the earlier HCMV enters the cell relative to HIV. In experiments in which HCMV entered the cell 24 to 40 h after HIV, the earliest time at which inhibition was detected varied from 8 to 24 h after HCMV infection; however, the extent usually reached 80% by 3 days after HCMV infection. The important determinant in the timing of this repression may be the precise multiplicity of the HCMV infection, with a higher MOI resulting in a more rapid shutoff of HIV synthesis. In any case, we saw that inhibition of proteins appears at the same time as, or slightly after, RNA inhibition, indicating that the shutoff is primarily transcriptional. However, inhibition of translation or protein degradation cannot be excluded at this time.

Although the mechanism of the HCMV-mediated inhibition of HIV replication remains unknown, our experiments demonstrated that HCMV DNA replication is not necessary for repression, nor are any other events which follow replication, such as production of late proteins. Furthermore, the fact that UV treatment of HCMV prior to infection abrogated the inhibition indicates that HCMV gene expression is a prerequisite, although we cannot exclude the possibility that there is some other UV-sensitive factor in the initial HCMV inoculum which is essential for the repression. These results, coupled with the observation that inhibition occurs as early as 8 h after HCMV infection, suggest that an HCMV early protein is central to this process. Our findings are consistent with those of Cockley et al. (13), who found that HCMV can inhibit the replication of HSV-1 and HSV-2 but not adenovirus or vesicular stomatitis virus in human embryonic lung cells. That communication also reported that UV irradiation of the HCMV particles prevented the inhibition of HSV replication from occurring and that viral DNA synthesis was not necessary for this shutoff. The lack of HCMV-mediated inhibition of adenovirus replication supports the notion that the repression of HIV by HCMV is not simply a general interference with host cell metabolism. In addition, although it has been shown that some cellular transcripts, such as fibronectin, decrease in abundance in response to HCMV infection of fibroblasts, the steady-state concentration of other transcripts either remains the same (e.g., actin) or increases (examples are ornithine decarboxylase, thymidine kinase, heat shock protein 70, and brain creatine kinase mRNAs [14, 51]). Finally, the lack of an effect on vesicular stomatitis virus synthesis argues that the generation of interferon does not account for the inhibition observed.

At this point, it appears that there are at least two distinct mechanisms of inhibition which are operating. At one level, there is some inhibitory function which can affect gene expression directed by both the HIV proviral genome introduced via infection and by the LTR linked to an indicator

gene placed inside the cell by DEAE-dextran-mediated transfection. The activity of the HIV LTR-CAT construct is 16-fold lower in cells infected with HIV and HCMV than it is in cells infected with HCMV alone. This inhibition is likely due in some way to the presence of HIV proteins or cis-acting genomic sequences, which together with HCMV gene products affect either the initiation or elongation of HIV-LTR-directed transcription or the stability of the transcripts generated. Nuclear runoff assays should help resolve at what point this inhibition is occurring, and these experiments are in progress.

Although, as discussed above, there may be an inhibitory mechanism which acts on both the HIV LTR-CAT construct and the HIV proviral genome, we still must deal with the observation that, in the dually infected cells, the net effect on the HIV LTR-CAT is one of stimulation while HIV proviral gene expression is inhibited. What accounts for two such different, yet profound effects? One possibility is that there is differential stability of the HIV and CAT mRNAs. Arguing against this, however, is our recent observation that in transient assays HCMV infection can activate HIV gene expression and virus production from an infectious clone of HIV introduced into the cell by DEAE-dextran-mediated transfection. This observation may actually be providing us with an important clue to the differential effect. There are at least two ways in which the HIV DNA genome introduced by infection differs from that introduced by transfection. First, HIV DNA transfected into the cell remains primarily unintegrated, at least in short-term assays, while HIV DNA resulting from infection and reverse transcription of the HIV RNA integrates into the host chromosome. HIV DNA that is integrated may assume a conformation rendering it inaccessible to the stimulatory factors or more accessible to the inhibitory factor. Alternatively, DNA introduced by transfection may be in a nuclear compartment which has a high concentration of the activating factors. A second possibility relates to the copy number of the HIV genome in any given cell. When introduced by infection, this copy number will be relatively low, particularly in our experiments in which HIV is introduced into the CD4-negative cells by means of HIV pseudotypes. Under these conditions, there is likely little or no reinfection of the cells occurring. In contrast, when the DNA is introduced by DEAE-dextran-mediated transfection, although only a few cells will take up the DNA, the copy number in any given cell will be high. If there are both activating and inhibitory products present which have specificity for the HIV LTR, and if only the inhibitory factor is present in limiting amounts, then the presence of excess copies of the HIV LTR could act as an effective competitor.

The results of our studies do differ from those of Skolnik et al. (62) and Ho et al. (27), which suggest that enhancement of HIV replication rather than inhibition occurs when cells are coinfected with HCMV. There are a number of reasons for this apparent contradiction of results. Skolnik et al. (62), in their experiments, used lymphoblastoid and monocytic cell lines, which permit little if any HCMV gene expression. Furthermore, in their study they did not document that infectious HCMV or even HCMV particles were required for the stimulation. It remains possible that some stimulation could have been provided by the conditioned medium containing the HCMV inoculum.

In the study by Ho et al. (27), the cells used were human osteogenic sarcoma (HOS) cells, a fibroblastoid line. These cells were persistently infected with HCMV for a long time and then superinfected with HIV at an MOI of 1 to 2. A transient increase in HIV antigen was seen in the coinfected cells compared with HOS cells infected with HIV alone; this was followed by a drop in titer after day 4 p.i. Since our results suggest that inhibition is due to an interaction between HCMV and HIV proteins, the transient activation seen here could be explained by the stimulatory effect HCMV has on the HIV LTR in the absence of HIV proteins. The drop in HIV antigen titer would then be the expected result of interactions between the increased levels of HIV proteins and HCMV. In our experiments, cells were infected at multiplicities which ensure that all cells replicate both viruses. Our human FF cells consistently produce HIV antigen titers 100- to 1,000-fold higher than those reported by Ho et al. (27) for the HOS cells when infected by HIV alone, and the human FF cells support a significantly higher level of HCMV replication than these cells.

We recognize that one complication in our experiments is the presence of the amphotropic retrovirus Ampho-1B in the supernatant containing HIV (Ampho-1B) pseudotypes. However, we found no evidence that its presence is affecting the replication of either HIV or HCMV. We showed in the transient expression assays that Ampho-1B does not activate the HIV LTR nor does it appear to interfere with HCMV's activation of the LTR or to affect HCMV virus production. We also have preliminary data which indicate that while HCMV gives activation of transfected infectious clones of HIV in transient assays, the presence of Ampho-1B does not affect this activation, thus ruling out a cooperative role in the inhibition (unpublished data).

The idea that the presence of herpesvirus proteins and DNA sequences during a coinfection can result in a net inhibition of HIV rather than activation is further supported by the work of Carrigan et al. (7) and Levy et al. (34), who found that coinfection of peripheral blood mononuclear cells with HHV-6 and HIV resulted in a suppression of HIV production. This contrasts with reports that HHV-6 transactivates the HIV LTR and enhances HIV replication in dually infected peripheral blood mononuclear cells (19, 36). The reasons for the discrepancy in these results remain unknown.

Another herpesvirus, EBV, also has been shown to downregulate HIV reverse transcriptase activity in B cells but not T cells (25). This is in contrast to the transient assays which show that EBV immediate-early proteins can activate the HIV LTR (30, 38). These studies, along with the results presented in this report, demonstrate that interactions between HIV and other viruses may be very complicated and emphasize the importance of combining plasmid and wholevirus experiments.

If HCMV can both stimulate and inhibit HIV in vitro, how might this affect the course of disease in vivo? In cells in which HIV is unintegrated, present in high copy number, or expressing few of its gene products, HCMV infection of the cell may be able to stimulate HIV gene expression and viral production. Activation of HIV may also occur in dually infected cells in which HCMV gene expression is limited. Alternatively, in cells in which both HIV and HCMV are undergoing active viral replication, HCMV may be able to inhibit HIV gene expression and force HIV back into latency. We hypothesize that the complex molecular interactions between HIV and HCMV can influence the fine balance of HIV productive infection, latency, and reactivation from latency, thereby contributing to the pathogenesis of HIV-related disease.

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