Involvement of cAMP and Protein Kinase C in Cytomegalovirus Enhancement of Human Immunodeficiency Virus Replication (43656)

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Abstract. **Cytomegalovirus (CMV) infection constitutes a serious threat to patients with acquired immune deficiency syndrome. Recently we reported that human immunodeficiency virus (HIV) infection of CD4$^+$ cells was associated with sustained elevation of cellular levels of cAMP. Moreover, cyclic nucleotide modulators enhanced HIV replication by increasing intracellular levels of cAMP. In this study, the effect of CMV on HIV replication in CMV/HIV mixed infection and its relationship to cAMP were examined. MT-4 cells, CMV strain AD169, and HIV strain IIIB were used. Optimal enhancement (4.4-fold increase) of HIV replication was observed when MT-4 cells were infected with CMV at Day 0 followed by HIV on Day 4 after infection, as determined by reverse transcriptase activity on Day 11 after infection. cAMP (measured by radioimmunoassay) levels in cells infected with CMV alone, HIV alone, or CMV/HIV together were 2-, 3-, and 5-fold above untreated cells, respectively. CMV also enhanced the replication of UV-irradiated HIV 4-fold and this was associated with a 2-fold increase in cAMP as well. Moreover, UV-irradiated CMV enhanced HIV replication 8.8-fold. The same dose of viable and UV-irradiated CMV used in the above experiments increased protein kinase C activity in these cells 3.0- and 8.0-fold, respectively. These findings might suggest that cAMP and protein kinase C are involved in CMV enhancement of HIV replication. These findings may have relevance to the identification of novel target sites for development of antiviral therapeutics.** [P.S.E.B.M. 1993, Vol 204]

Cytomegalovirus (CMV) infection is common in immunosuppressed individuals, such as organ transplant recipients (1–4) and patients with acquired immune deficiency syndrome (AIDS) (5–8). CMV infection in AIDS manifests as pneumonia (5), colitis (9–10), retinitis (11–13), and hepatitis (14). It has been reported previously that CMV enhanced the *in vitro* replication of human immunodeficiency virus (HIV) in mixed CMV/HIV infections. The precise mechanism of this enhancement has not been identified (15).

CMV infection with subsequent expression of the CMV immediate early genes in human fibroblasts (as well as CD4$^+$ and chronically infected lymphocytes) activate gene expression directed by HIV (Types I and II) long-terminal repeats. More than one nonoverlapping region of the HIV promotor has been shown to be capable of responding to CMV immediate early gene products (16–21).

Infection of T cells with HIV has been associated with both quantitative and qualitative deficiencies. The latter has resulted in a gradual depletion of CD4$^+$ target cells, and in physiological incompetence of the infected cells. This incompetence includes the inability of the T cells to respond properly to specific and nonspecific antigens, and the inability of the cells to make sufficient amounts of lymphokines required for regulation of the immune response. Uninfected cells may also be affected indirectly through the release or suppression of certain factors from HIV-infected cells. HIV infection was associated with altered intracellular levels of cAMP in CD4$^+$ cells, and it was suggested that this might have

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implications on the replication and pathogenesis of HIV (22–23).

In vitro, human cytomegalovirus infection has been shown to be associated with several physiological and biochemical responses. Among those reported were the induction of a Ca^{2+} influx that was followed by a sustained elevation of intracellular free [Ca^{2+}] as well as a transient inhibition and then activation of the oubain-sensitive Na^+/K^+-ATPase (24–26). A transient increase in cAMP early after infection has also been reported (27). Certain manipulations of these responses resulted in inhibition of CMV replication (24, 28).

Apparently cAMP-dependent protein kinase A appears to be involved in the cellular response to CMV and HIV infection. In this report, the cAMP responses of MT-4 cells after CMV-HIV mixed infections and their relation to enhancement of HIV replication were investigated.

Materials and Methods

Cells and Viruses. MT-4 cells ($CD4^+$ cell line) were used in this study. Cells were grown in RPMI 1640 medium and supplemented with 10% heat-inactivated fetal calf serum, 2 m*M* L-glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 0.075% $NaHCO_3$, and were maintained at 37°C.

Human CMV strain AD169 and HIV strain HTLV-IIIB were used in these experiments unless otherwise mentioned. HIV virus stocks were prepared from H9-HIV chronically infected cells and titrated as described previously (23).

Virus Irradiation. Virus inactivation was performed by exposure to a source of ultraviolet light. Briefly, virus stocks were irradiated in 35-mm dishes by 80 erg/sec/mm^2 of irradiation to various time intervals. The radiation source was a General Electric G8T5 "germicidal" bulb producing light at 254 nm. The dose rate was measured by a Black-ray UV-intensity meter (Ultraviolet Products Inc., San Gabriel, CA) (24). The infectivity of the UV-irradiated CMV and HIV was determined by plaque assay for the former and reverse transcriptase (RT) activity and HIV p24 Ag concentration for the latter in susceptible cells. The viral titer after UV irradiation of CMV was inhibited by 70% and >99.99% and of HIV was inhibited by 97.5% and >99.99% at 1 and 5 min, respectively.

Infection Experiments Using Cell-Free Viruses. MT-4 cells (5×10^6) were infected with CMV strain AD169 at a multiplicity of infection of 1–2 plaque-forming units (PFU)/cell for 1 hr at 37°C and with HIV at a maximum oxygen intake (MOI) of 0.02–0.05 $TCID_{50}$/cell for 90 min at the appropriate times of infection. The cells were then cultured at a density of 0.5×10^6/ml in RPMI 1640 medium in the presence or absence of the appropriate treatment.

Mock infection was carried out using cell-free supernatants of noninfected H9 cells and human fibroblast lysates (human foreskin fibroblasts used for the preparation of the CMV stocks) as controls for the HIV- and CMV-infected cells, respectively.

Measurement of Reverse Transcriptase Activity. Briefly, RT activity was measured in 2 ml of culture supernatant that was precipitated with polyethylene glycol overnight at 4°C. The solubilized samples were then assayed for Mg^{2+}-dependent RT activity in a buffer cocktail made of: 42 m*M* Tris-HCl (pH 7.8), 8.5 m*M* dithiothreitol, 10 m*M* $MgCl_2$, 3.4 m*M* NaCl, 25 μCi/ml of [^{3}H]thymidine triphosphate (10–20 Ci/mmol; New England Nuclear Corp., Boston, MA), and 0.5 unit/ml of oligo(dt)-poly(rA) template primer (Pharmacia/P-L Biochemicals, Piscataway, NJ) as described previously (29).

cAMP Determination. At selected time intervals of harvest, cells were clarified with low speed centrifugation (1500 rpm for 5 min). The pellet was then resuspended in 1 ml of acidified ethanol, vortexed, and left for 15 min at room temperature and then centrifuged for 10 min at 1000 rpm. This procedure was repeated once. The samples were dried, and the pellet was resuspended in 0.4 ml of distilled water then frozen at −20°C until assayed for cyclic nucleotide content. The cAMP was measured by radioimmunoassay using Amersham kits (Arlington Heights, IL) as described previously (30).

HIV P_{24} Antigen Capture Assay. The inactivated culture supernatants were assayed for HIV P_{24} antigen by EIA kits using the conditions described by the manufacturer (Coulter Immunology, Hialeah, FL).

PKC Determination. Protein kinase C (PKC) was extracted from the cells at the day of harvest. Briefly, 1×10^7 cells were spun down and washed twice with cold phosphate-buffered saline. The pellet was then reconstituted in 0.5 ml PKC sonication buffer (20 m*M* Tris-HCl, pH 7.5, 0.25 *M* sucrose solution, 1 m*M* EDTA, 1 m*M* EGTA, 2 m*M* dithiothreitol, 1 m*M* phenylmethylsulfonyl fluoride, and 50 μg/ml leupeptin) while the samples were kept on ice. The samples were sonicated for 30 sec, followed by ultracentrifugation at 100,000*g* for 1 hr at 4°C. The supernatants (cytosol) was withdrawn and the particulate matter was reconstituted in 0.25 ml buffer and sonicated as mentioned above. The PKC activity was then measured using Amersham kits under the same conditions as recommended by the manufacturer (Amersham International, PIC, UK).

Results

Effect of CMV Coinfection on HIV Replication. The effect of CMV on HIV replication was examined in MT-4 cells using the different infection mixtures of both viruses shown in Figure 1. The CMV strain AD169 and HTLV-IIIB were used to infect the cells. The replication of HIV was then determined on Days 4, 7, 11, and 14 after infection, by measuring the reverse

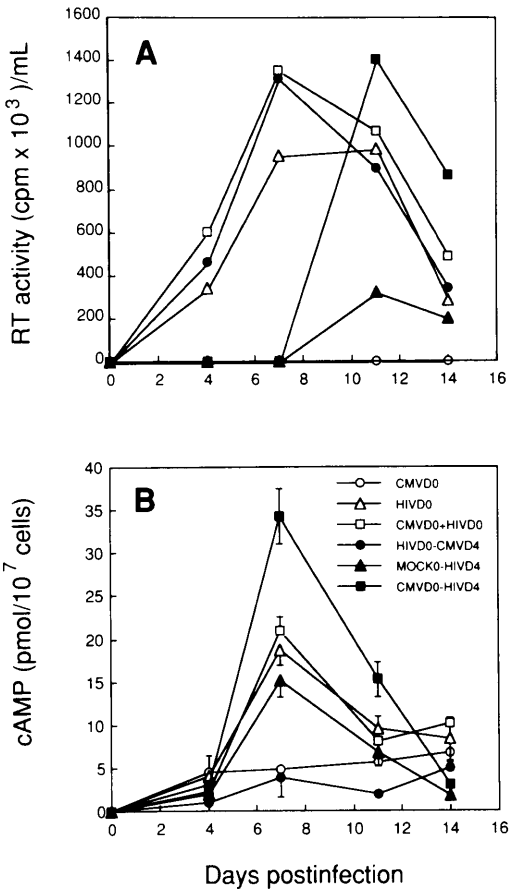

Figure 1. Effect of CMV coinfection on HIV replication and induction of cAMP in MT-4 cells, at selected time intervals after infection. HIV replication was determined by RT activity (A), expressed as cpm/ml (× 10^3). cAMP (B) was expressed as pmol/10^7 cells. Untreated cells had baseline cAMP levels. Data are representative of three independent experiments.

transcriptase activity and HIV p24 Ag content of the culture supernatants. The results as shown in Figure 1A revealed an increase in the RT activity of MT-4 culture supernatants, from the different CMV/HIV combinations utilized when compared with those from cultures infected with HIV alone. Significant enhancement was observed when the cells were infected with CMV at Day 0 and superinfected with HIV on Day 4. HIV replication was enhanced at Days 11 and 14 after infection 4.4-fold compared with the control cells. Levels of p24 Ag were similarly enhanced (data not shown). These data indicate that CMV/HIV coinfection of MT-4 cells was associated with enhancement of HIV replication.

Patterns of cAMP Response in CMV/HIV-Infected MT-4 Cells. Since it had been reported previously that HIV replication could be enhanced by increasing cellular levels of cAMP (31) and that CMV induced a transient increase of cellular cAMP in other cells (27), the patterns of cAMP responses in CMV/HIV mixed infection was examined next. Intracellular levels of cAMP were measured at various time points after CMV/HIV infections. The results as depicted in Figure 1B showed that most of the cAMP peak values were at Day 7 after infection. Cytomegalovirus induced a 2-fold increase in the cAMP levels when measured at Day 4 after infection, compared with the untreated cells. Also, a 2-fold increase in cAMP level was obtained when the cells infected with CMV at Day 0 were superinfected with HIV at Day 4. The peak levels of cAMP (Day 7) preceded the peak in RT activity (Day 11) in this experiment, suggesting the possibility of a relationship between cAMP and the enhancement of HIV replication.

Effect of CMV on the Replication of the UV-Irradiated HIV. To further elucidate the role of CMV on HIV replication, the CMV Day 0/HIV Day 4 experiment was chosen for the infection. The effect of CMV on UV-irradiated HIV was investigated next. MT-4 cells were infected with CMV at an MOI of 1 PFU/cell on Day 0 and were superinfected with infectious or UV-irradiated HIV at Day 4. The RT activity was then determined at Day 11 after infection. The results demonstrated that the CMV-infected cells produced a 4.4-fold increase in RT activity when viable or UV-irradiated HIV (1 min) were used for coinfection at Day 4. Moreover, CMV enhanced the replication of the UV-irradiated HIV, even in the samples that were irradiated for 5 min, when compared with the controls which contained no detectable RT activity in the absence of CMV (Fig. 2, A and B).

Measurement of cAMP revealed a 2-fold increase in the CMV-infected versus the mock-infected cells (Fig. 2C). The CMV-induced enhancement of HIV replication was consistently associated with an increase in cellular levels of cAMP above that of cells infected with either virus alone.

Effect of CMV-UV Irradiation on HIV Replication. To answer the question of whether CMV-induced enhancement of HIV replication required viable CMV or was dependent upon certain viral structural components, viable or UV-irradiated CMV were used to infect MT-4 cells at Day 0 with an MOI of 2 PFU/cell or its equivalent of the UV-irradiated virus before inactivation. A coinfection with viable HIV was performed on Day 4. Figure 3A shows that the intact and UV-irradiated CMV produced a 9- and 8.8-fold increase in RT activity, respectively, compared with the mock-infected cells. Furthermore, measurement of cAMP revealed a 2-fold increase in the CMV/HIV-infected cells compared with the HIV-infected controls. However, cAMP increase was not observed when coinfection was carried out with the UV-irradiated CMV (Fig. 3, B and C). These findings suggest that the CMV enhancement of HIV replication does not necessarily require viable virus and that a CMV structural component(s) independent of cAMP may also be a triggering factor.

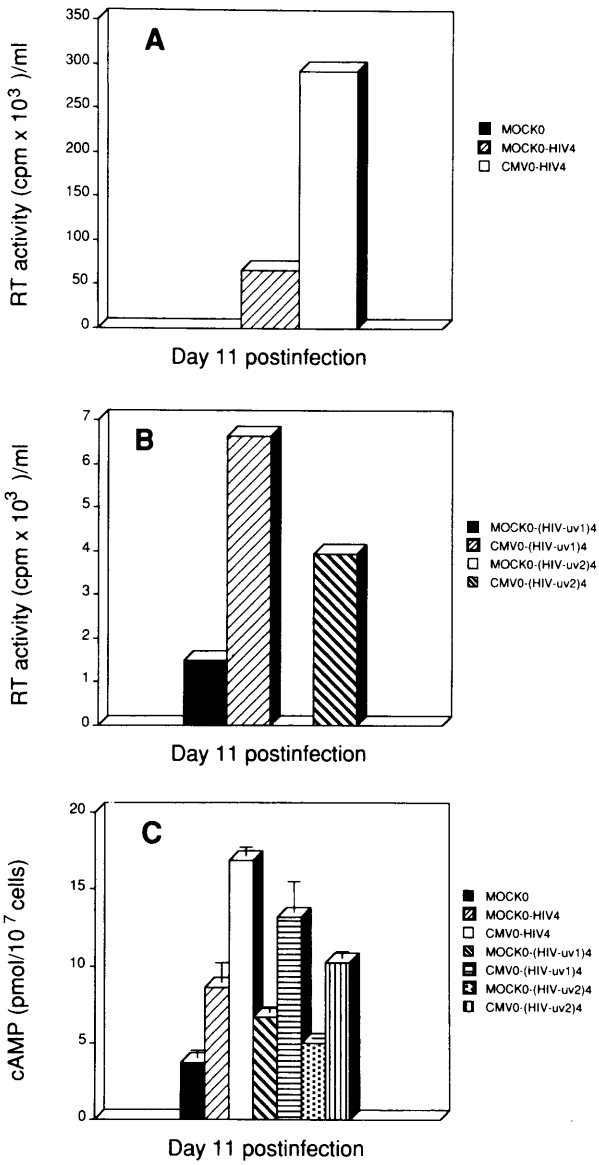

Figure 2. Effect of CMV coinfection on HIV replication and cAMP levels of the viable and UV-irradiated HIV in MT-4 cells at Day 11 after infection. UV_1 and UV_2 represent 1- and 5-min exposure of the virus to 80 erg/sec/mm^2. HIV replication (A & B) was determined by RT activity, expressed as cpm/ml (× 10^3). The variation of the triplicate samples was less than 10%. cAMP (C) was expressed as pmol/10^7 cells, and the experimental data points represent the mean of three samples ± SD representative of two independent experiments.

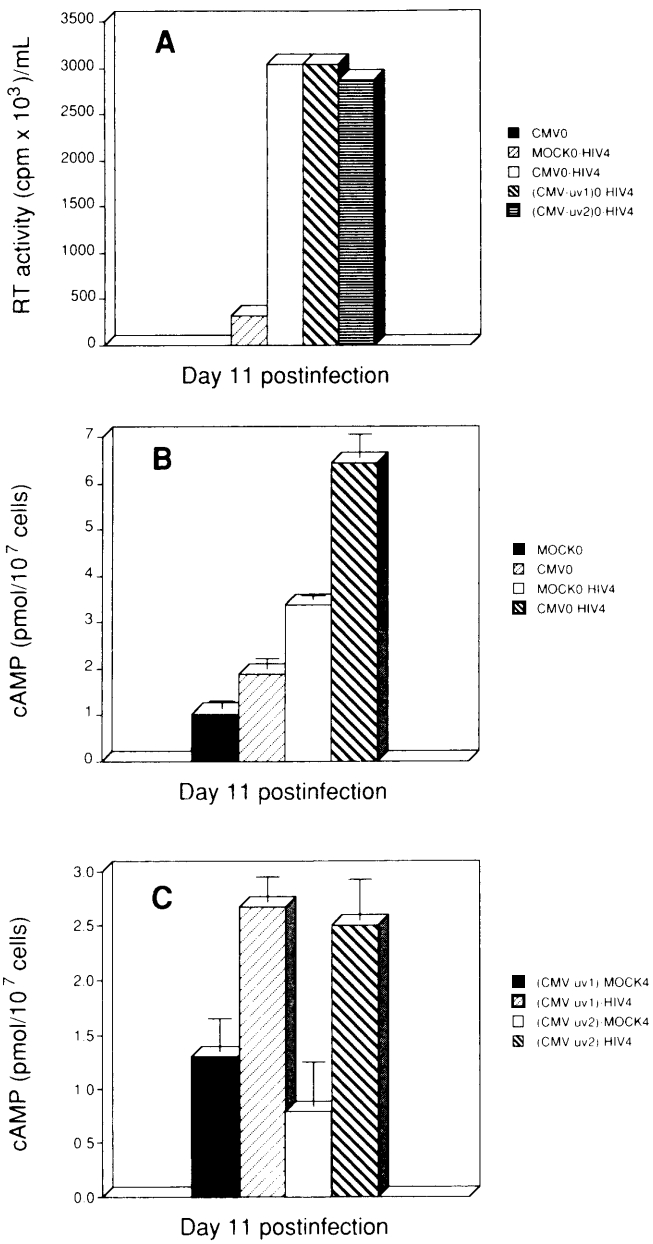

Figure 3. Effect of CMV UV-irradiation on HIV replication and cAMP concentrations in MT-4 cells at Day 11 after infection. UV_1 and UV_2 represent the same protocol mentioned in Figure 2. HIV replication as determined by RT activity (A) was expressed as cpm/ml (× 10^3). However, cAMP levels (B & C) were expressed as pmol/10^7 cells. The experimental data points represent the mean of three samples.

Effect of CMV Infection on Cellular Proliferation. The replication of HIV involves a double-stranded proviral DNA stage that is integrated in the cellular genome. Thus HIV replication and propagation has been shown to be dependent upon cellular activation and proliferation. To determine whether the CMV-mediated enhancement of HIV replication was secondary to CMV-induced cell activation, the proliferative response to CMV infection was evaluated. MT-4 cells were infected for 1 hr with CMV at an MOI of 1 PFU/cell or equivalent amounts of UV-irradiated CMV. The proliferative response was then measured at selected time intervals after infection (Fig. 4). CMV inhibited [^{3}H]thymidine uptake by the cells, indicating that its enhancing effect on HIV replication is not mediated through stimulating cell proliferation. Moreover, UV-irradiated CMV failed to enhance [^{3}H]thymidine uptake above uninfected control cells.

Effect of Viable and UV-Irradiated CMV on PKC Activity in MT-4 Cells and Peripheral Blood Mononuclear Cells. To investigate the possibility that the CMV structural component(s) has an alternative mech-

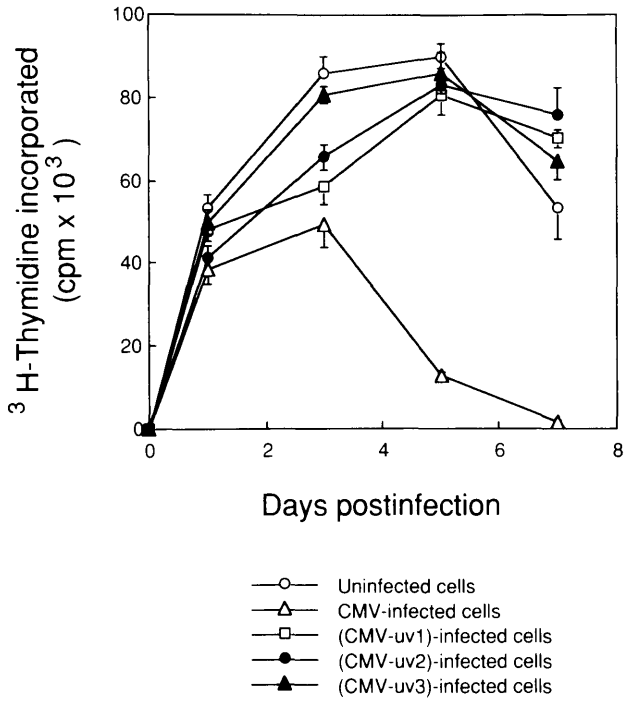

Figure 4. Effect of viable and UV-irradiated CMV on the proliferation of MT-4 cells at selected time intervals after infection. UV_1, UV_2, and UV_3 represent 1-, 5-, and 15-min exposure of the virus to 80 erg/sec/mm^2, respectively. Cellular proliferation was determined by the rate of [^{3}H]thymidine uptake by the cells and was expressed as (cpm × 10^3). There was no significant differences in cell number between CMV-infected and uninfected cells at Day 7 as determined by trypan blue exclusion. The experimental data points represent the mean ± SD of three samples representative of three independent experiments.

Table I. Effect of Viable and UV-Irradiated CMV on PKC Activity in MT-4 Cells and PBMC

Sample	PKC activity[a] (pmol/min)	Fold activation
Expt 1		
MT-4	2.4 ± 0.4	—
MT4 + CMV	6.4 ± 0.9	3.0
MT4 + CMV-UV	19.0 ± 1.4	8.0
Expt 2		
PBMC	2.5 ± 0.5	—
PBMC + CMV	2.7 ± 0.7	1.1
PBMC + CMV-UV	13.1 ± 2.1	5.2

[a] PKC activity was determined on Day 11 after infection. Samples were run in duplicates ± SD. Representative of three independent experiments.

anism (if any) to induce HIV replication that is independent of cAMP, MT-4 cells and peripheral blood mononuclear cells (PBMCs) were infected at Day 0 with viable and UV-irradiated (10 min) CMV at an MOI of 1 PFU/cell or its equivalent of the UV-irradiated virus before inactivation. The cells were harvested on Day 11 after infection for PKC determination. The results as shown (Table I) revealed that the UV-irradiated CMV induced a 8.0- and 5.2-fold increase in PKC activity in MT-4 cells and PBMC, respectively, compared with the control cells. These findings might indicate that a CMV structural component(s) enhanced HIV replication via a PKC-dependent pathway.

Effect of Protein Kinase Inhibitors on CMV-Induced Enhancement of HIV Replication. To answer the question of whether CMV enhancement of HIV replication was mediated by protein kinases A and C, MT-4 cells (5×10^6/limb) were treated with mixed CMV/HIV infection in the presence of 10 μM H7 or H8 (specific inhibitors of PKC and PKA, respectively) and the RT activity was measured on Day 11 after infection. The results as depicted in Table II revealed that CMV enhancement of HIV replication was abrogated by H8. HIV replication in the presence of CMV was enhanced by 1.7-fold compared with a 0.5-fold increase in the presence of the PKA inhibitor. On the other hand, UV-irradiated CMV enhanced HIV replication 4.8-fold. However, in the presence of H7 and H8 the fold increase was only 1.6 and 3.8, respectively. These findings indicate that PKA and PKC are directly involved in CMV enhancement of HIV replication.

Effect of CMV on HIV Patients' Isolates. The effect of CMV on the replication of HIV in PBMC obtained from HIV-infected individuals was examined next. The phytohemagglutinin-stimulated normal PBMC were infected with CMV, while the control cells were mock infected. Both cells were then cocultivated with PBMC from HIV-infected patients at a ratio of 1:2 as described previously (31). The culture supernatants were obtained at selected time intervals of cocultivation and their HIV P24 Ag was measured. The results using cells from four different patients showed an enhancement and/or earlier expression of P24 Ag when compared with cocultures without CMV infection (Fig. 5). These findings might help demonstrate that CMV plays a crucial role in reactivation of HIV in patients with AIDS.

Table II. Effect of H7 and H8 on CMV-Induced Enhancement of HIV Replication in MT-4 Cells

Treatment	RT activity[a] (cpm × 10^3/ml)	Fold increase
Mock 0-HIV4	289 ± 92	
Mock 0-HIV4 + H7[b]	706 ± 284	2.4
Mock 0-HIV4 + H8[b]	96 ± 24	0.3
CMV 0-HIV4	497 ± 107	1.7
CMV 0-HIV4 + H7[b]	1035 ± 169	3.6
CMV 0-HIV4 + H8[b]	144 ± 44	0.5
(CMV-UV)0-HIV4	1398 ± 246	4.8
(CMV-UV)0-HIV4 + H7[b]	477 ± 78	1.6
(CMV-UV)0-HIV4 + H8[b]	1088 ± 345	3.8

[a] RT activity was assayed in duplicates (means ± SD).
[b] Final concentration of H7 and H8 was 10 μM. Representative of three independent experiments.

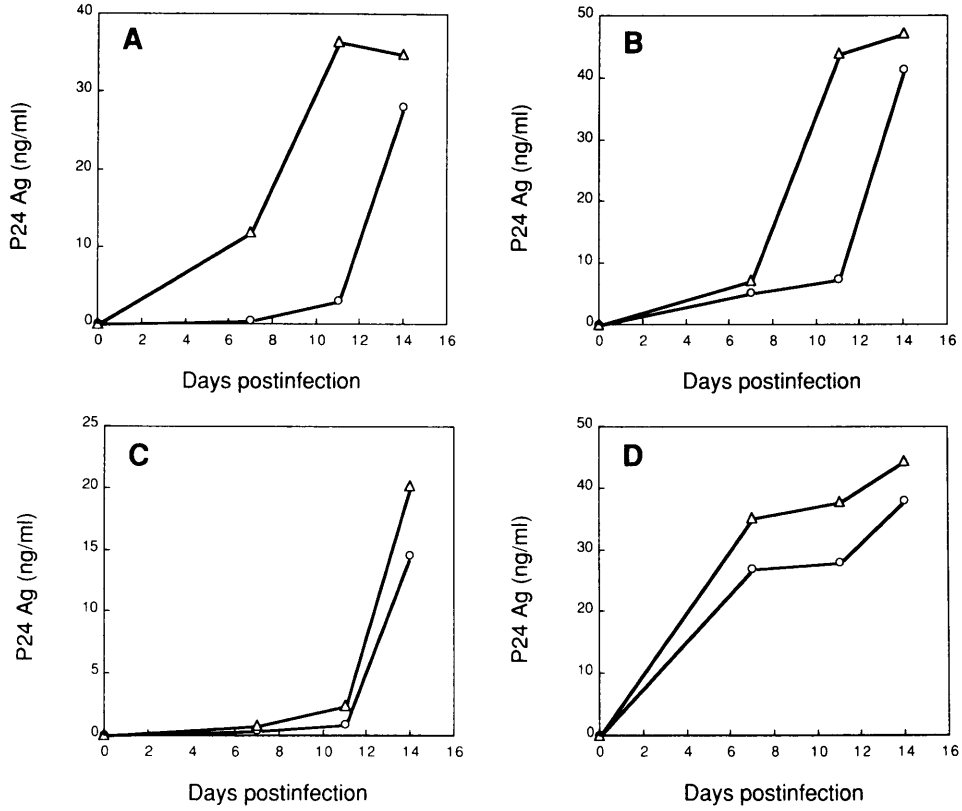

Figure 5. Effect of CMV on the isolation of HIV from PBMC obtained from HIV-infected patients. CMV-infected, phytohemagglutinin (PHA)-stimulated normal donor PBMC were cocultivated with the patients' cells (△). Meanwhile, cocultivated PHA-stimulated donor cells without CMV represent the control group (○). At selected time intervals, HIV replication was monitored by determining HIV P24 Ag content (ng/ml) in the coculture supernatants. Panels A, B, C, and D represent data points from four different patients.

Discussion

The present data indicate that CMV enhanced the replication of HIV in MT-4 cells, confirming and extending observations reported by others using different cell systems. The enhancement of HIV replication was associated with increased intracellular cAMP levels. The enhancement of HIV replication may be mediated by the cAMP-dependent protein kinase A. Additionally, the isolation of HIV from PBMC of HIV-infected patients was enhanced after cocultivation with normal PBMC infected previously with CMV.

In T cells, multiple events have been shown to be activated via a PKA-dependent pathway (32). Infection of MT-4 cells with either CMV or HIV in the present study increased intracellular cAMP, while coinfection of the same cells with both viruses enhanced the cellular cAMP levels, and the latter paralleled an increase in RT activity. The latter enhancement was also observed when CMV and inactivated HIV were combined, where the increased HIV infectivity was associated with increased cellular levels of cAMP when compared with the mock-infected control. Furthermore, the PKA inhibitor H8 abrogated the CMV-induced enhancement of HIV replication (Table II). Increasing intracellular levels of cAMP by phosphodiesterase inhibitors, by activation of adenylate cyclase, or by directly adding dibuteryl cAMP also has been shown to enhance HIV replication (33). Also, exposure of cells to x-irradiation was shown to increase cellular levels of cAMP (34). The x-irradiation of HIV-infected MT-4 cells has been associated with a dose-dependent increase of both cellular cAMP and RT activity (35). cAMP serves as a multifunctional relay for mitogenic stimuli acting as an intracellular signal for the proliferation of eukaryotic cells. Its action is mediated via activation of PKA through phosphorylation of specific protein substrates that trigger DNA synthesis and oncogene expression. Thus, secondary to PKA activation, cAMP induces several of the well-known pleotypic biochemical markers of cell progression toward replication (36).

HIV replication is dependent upon cellular activation and proliferation. Though it is possible that CMV enhanced HIV replication by the antigenic stimulation of cells by CMV structural components, it is unlikely that this pathway is a major contributor, since viable CMV inhibited MT-4 cellular proliferation as determined by [^{3}H]thymidine uptake. The possibility that CMV upregulates the HIV receptor on MT-4 cells was also examined. However, the rate of adsorption of

radiolabeled HIV to CMV-infected cells was not different from its rate of adsorption to control uninfected cells (data not shown). Moreover, the expression of CD4 molecules on the surface of CMV-infected MT-4 cells was not enhanced when measured by flow cytometric analysis (data not shown).

Supporting the hypothesis for the role of cAMP in the CMV-mediated enhancement of HIV, a recent report showed that PKA pathway activators (forskolin, 8-bromo cAMP, and prostaglandin E2) synergized with CMV immediate early proteins in the transactivation of HIV long-terminal repeats in a $CD4^+$ human lymphoblastoid cell line derived from JURKAT cells (37). Furthermore, CMV has been shown to encode for three G-protein-coupled receptor homologs. In other systems, the G-protein-coupled receptor homologs trigger increases or decreases of cellular cAMP (38).

The fact that UV-inactivation of CMV also enhanced the replication of HIV indicates that a structural component(s) of CMV might also be involved in mediating this effect. The enhancement, however, was not secondary to stimulation of cellular proliferation by those structural components as the rate of proliferation with UV-irradiated CMV-infected cells was not higher than in mock-infected cells as determined by [^{3}H]thymidine uptake and apparently was independent of changes in cAMP.

A role for PKC in the CMV-mediated events cannot be ruled out, since T cell activation by phytohemagglutinin in the absence of infection has been shown to be preceded by enhanced PKC activity. Furthermore, CMV stimulated PKC activity (Table I) and PKC inhibitor H7 resulted in marked inhibition of the UV-irradiated CMV-induced enhancement of HIV replication (Table II). Moreover, PKC has been reported to influence some cellular responses, including the hydrolysis of phosphatidylinositol to inositol phosphates and diacylglycerol (39, 40). The latter in the presence of Ca^{2+} ultimately lead to the activation of PKC. Moreover, the replication of HIV has been shown to be sensitive to PKC inhibitors (41).

The present data suggest that CMV enhance HIV replication by several mechanisms: one dependent upon infectious virus that is cAMP-dependent, and another triggered by structural components of CMV, which is probably receptor mediated, independent of cAMP, and perhaps PKC mediated.

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