Reciprocal enhancement of gene expression and viral replication between human cytomegalovirus and human immunodeficiency virus type 1

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Biological interactions between human cytomegalovirus (HCMV) and the human immunodeficiency virus type 1 (HIV-1) were analysed in transfection and infection experiments, carried out in a human osteogenic sarcoma cell line (HOS) and in the same cell line chronically infected with HCMV (E155). When HOS and E155 cells were transfected with recombinant plasmids containing the HIV long terminal repeat (LTR) linked to the bacterial chloramphenicol acetyltransferase (CAT) gene, LTR-directed CAT expression was 20 times higher in E155 cells than in HOS cells. HOS cells co-infected with HCMV and HIV-1 showed enhanced production of the HIV-1 p24 antigen. In reciprocal experiments, an increase in HCMV immediate early gene expression was observed when HCMV-infected HOS cells and E155 cells were either transfected with a recombinant plasmid containing the HIV transactivator gene (pTAT), or when infected with HIV-1. DNA hybridization analysis of E155 and HCMV-infected HOS cells revealed higher levels of HCMV DNA in cells transfected with pTAT than in cells transfected with other non-specific recombinant plasmids. E155 cells transfected with pTAT also produced higher titres of infectious HCMV than control cultures of E155 cells transfected with other recombinant plasmids, including pMTAT carrying a mutant tat gene. The functional reciprocity in vitro between HCMV and HIV is discussed with respect to its possible implications for the clinical development of AIDS.

Introduction

AIDS and related immunological disorders have been aetiologically linked to human immunodeficiency virus type 1 (HIV-1) (Gallo et al., 1984; Levy et al., 1984; Curran et al., 1985). AIDS patients are often latently infected for months or years before overt symptoms appear, so much interest has centred on the factors that trigger or complement active HIV-1 infection. One possibility is co-infection with other viruses, especially herpesviruses. Several studies have demonstrated the ability of secondary infectious agents to trigger HIV growth in vitro (Gendelman et al., 1986; Zagury et al., 1986; Mosca et al., 1987; Rando et al., 1987; Horvat et al., 1989). Clinical findings have served to focus attention on human cytomegalovirus (HCMV), an opportunistic pathogen that has been detected in a number of different immunodeficiency syndromes, including AIDS (Macher et al., 1983; Mintz et al., 1983) and HCMV viraemia parallels the progression of AIDS symptoms (Fiala et al., 1986). An increased titre of HCMV antibody is observed as a significant indicator of the development of AIDS in HIV-seropositive homosexual men (Polk et al., 1987) and in some cases, multiple isolates of HCMV have been detected in HIV-infected patients (Drew et al., 1984; Spector et al., 1984). HIV and HCMV can co-infect brain cells of AIDS patients (Nelson et al., 1988) and AIDS victims, in their terminal state, have overwhelming disseminated HCMV infections (Macher et al., 1983).

HCMV, like HIV, can be transmitted both sexually and via blood transfusions and can infect both lymphocytes and monocytes (Rice et al., 1984), which are also the target cells for HIV-1 infection. HCMV infection alone can depress T lymphocyte helper/suppressor ratios (Drew et al., 1985) and, in allograft recipients, leads to a high incidence of opportunistic infections as well as graft rejection (Rubin et al., 1977; Hirsch & Felsenstein, 1984). This observation raises the possibility that immune suppression induced by one virus favours replication of another virus, with concomitant enhancement of the immunosuppressive capability of the second virus.

Molecular analyses have indicated that HCMV (or the
immediate early genes of HCMV) can increase HIV long terminal repeat (LTR)-directed transcription (Davis et al., 1987; Elfassi et al., 1987; Rando et al., 1987; Skolnik et al., 1988). In the present study, we have further analysed the molecular interactions of HCMV and HIV-1. We report that HCMV enhances HIV-1 transcription and the production of p24\textsuperscript{ag} protein in fibroblastoid cell lines, and that HIV-1 and its tat gene are capable of enhancing the expression of immediate early proteins and the full replication of HCMV in these cells. These results suggest a functional complementarity of these viruses that might be reflected \textit{in vivo} during the development of AIDS.

**Methods**

Cell lines and viruses. The human osteosarcoma (HOS) clonal cells (TE-85, clone F-5) derived from a 13 year old Caucasian female (Rhim et al., 1975) were obtained from C. J. Chern (The Wistar Institute). The HOS and the HCMV chronically infected HOS cells (E155) (Furukawa et al., 1981) were grown in Eagle's modified minimal essential medium, supplemented with vitamins, glutamine and 7.5% foetal calf serum. The E155 cell line consistently yields HCMV titres of $10^9$ to $10^5$ p.f.u. per ml of culture medium (Furukawa et al., 1981). HOS and E155 cells were demonstrated, by karyotypic analysis, to contain only human chromosomes and to lack HeLa markers. Immunohistochemical tests (Osborn et al., 1984) confirmed the mesenchymal origin of the cell lines. HOS and E155 cells do not express the CD4 molecule, as assayed using a monoclonal antibody (MAb) directed against the OKT4 and OKT4a epitopes (Ortho Diagnostic Systems) and flow cytometric analysis (Ho et al., unpublished data; S. Bandopadhyay, The Wistar Institute, personal communication; Tateno et al., 1989).

HCMV (Towne strain) infectivity was assayed, as described by Wentworth & French (1970), using a human diploid fibroblast cell line (MRC-5) between passages 20 and 30. The virus stock contained $2 \times 10^6$ p.f.u, per ml. HIV-I-IIIb strain was propagated in SUPT-1 cells, as reported previously (Hoxie et al., 1986) and the virus stock contained $10^4$ to $10^6$ TCID\textsubscript{50} per ml.

\textbf{Recombinant plasmids.} The HIV recombinant plasmid pLTR-chloramphenicol acetyltransferase (CAT) contains portions of the HIV-LTR (−452 to +80 base pairs, relative to the mRNA start site) linked to the CAT gene (Rando et al., 1987). The HIV tat gene construct, p\textsc{tat}, contains the \textsc{tat} gene (Sanchez-Pescador et al., 1985) positioned between the simian virus 40 (SV40) early promoter and polyadenylation signals (Peterlin et al., 1986). Mutant tat-containing plasmid (p\textsc{mtat}) was constructed by cleaving the HIV proviral DNA, pZ6Neo (Srinivasan et al., 1987) with \textit{BamHI} restriction enzyme and subsequently cloning it into SV40 early promoter expression vector pKSV10. This construct, which is devoid of the tat initiation codon, lacked transactivation capability in cotransfection experiments with pLTR-CAT in HOS and HeLa cells (data not shown). The HIV \textit{art} gene construct, p\textsc{art} (or p\textsc{rev}) obtained from D. M. Knight (Centacor), has been described (Knight et al., 1987). The recombinant plasmid \textsc{prla}, which contains the HCMV immediate early (IE) genes 1 and 2, was obtained from G. Hayward (The Johns Hopkins University, School of Medicine, Baltimore, Maryland, U.S.A.) and has been described in detail (Pizzorno et al., 1988). The p\textsc{br}32 control plasmid was purchased from BRL.

Viral infection, viral antigen and infectivity assays. HOS cells were infected in 25 cm\textsuperscript{2} flasks with HCMV at an m.o.i. of 1 to 2, or with HIV-1 at an m.o.i. of 1. At 24 h post-infection (p.i.) cells were split into 24-well plates containing coverslips and at 4 days p.i. cells were co-infected with the reciprocal virus at the same m.o.i. Coverslips were fixed in cold acetone [80% acetone, 20% phosphate-buffered saline (PBS)] on day 2 after exposure to the second virus and assayed for the 68,000 M, HCMV IE protein by an immunofluorescence (IF) assay, using MAb E-13 (Chemicon International), which specifically detects this protein (Colimon et al., 1984) and a fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Cappel Laboratories) as described (Ho & Plotkin, 1987). Slides were viewed under a Leitz-Wetzlar Ortholux fluorescence microscope at 500-fold magnification, and 2000 cells were counted from each slide.

In a second set of experiments, HCMV-infected HOS, or uninfected HOS cells, grown in 25 cm\textsuperscript{2} flasks were incubated with HIV-1 overnight at 36 °C. Inoculum was removed and the cells were washed twice in PBS and trypsinized, cultures were then fed and maintained at 36 °C. Supernatants were collected at 2 h after trypsinization and at various time points thereafter (see Results) and assayed for residual inoculum using a commercial antigen-capture assay for p24\textsuperscript{ag} (DuPont). After 6 days, cells were again trypsinized and co-cultivated with $1 \times 10^4$ indicator SUPT-1 cells and monitored for c.p.e. The specificity of c.p.e. was confirmed by the antigen-capture assay.

\textbf{DNA transfection and transient gene expression assays.} HOS and E155 cells (5 x 10\textsuperscript{4} in a 60 mm dish) were transfected with caesium chloride-purified plasmid DNA (1 to 10 µg) using the calcium phosphate co-precipitation technique (Graham & van der Eb, 1973). The amount of transfected DNA in each experiment was adjusted to 30 µg using sonicated salmon testes DNA. At 4 to 6 h post-transfection, cells were treated with 15% glycerol for 2 min. Transfection efficiency in E155 and HOS cells was monitored by Southern blot hybridization analysis using a pLTR-CAT DNA probe. Cells were re-fed with fresh medium at 24 h post-transfection and assayed for CAT activity, as described (Gorman et al., 1982), for the indicated time periods before the addition of ethyleacetate. Reaction products were separated by ascending thin-layer chromatography and quantified by liquid scintillation counting.

To assay the expression of the HCMV IE proteins, cultured cells were harvested from individual dishes 2 days after transfection with different recombinant plasmids and resuspended at $2 \times 10^5$ cells/ml in PBS. Samples (100 µl) of this suspension were dispersed on glass slides using a cytospin centrifuge, and indirect IF was performed, as described earlier.

Analysis of \textit{viral DNA.} Total DNA was extracted as described (Rando et al., 1986) from E155 cells at 48 h post-transfection. Total DNA was also extracted from HOS cells infected with HCMV (at an m.o.i. of 1 to 2) 16 h before transfection with the same recombinant plasmids. Purified DNA was denatured in 0-4 M-NaOH for 10 min at room temperature, cooled on ice for 5 min and neutralized by the addition of an equal volume of 2 M-sodium acetate pH 5-5. Various dilutions of each denatured DNA sample were immobilized on nitrocellulose filter membranes using a 96-well filtration manifold (Schleicher & Schuell) and dried in vacuo at 80 °C for 2 h, before prehybridizing the samples at 68 °C in 6 × SSC, 0.5% SDS, 5 × Denhardt's buffer and salmon testes DNA (100 µg/ml). Samples were hybridized overnight at 68 °C with a \textit{32P}-labelled HCMV DNA probe (3.5 × 10\textsuperscript{6} c.p.m./ml), using the same buffer as in the prehybridization step. The HCMV probe was obtained by electrophoretic fractionation of \textit{BamHI}-restricted pRL43a fragments in a 0.8% low melting temperature agarose gel and phenol–chloroform extraction of the 5·1 kb fragment, which contains only DNA sequences corresponding to the HCMV IE gene region (Pizzorno et al., 1988). Nitrocellulose filters were washed in a solution containing 0.1% SSC and 0.5% SDS at 68 °C for 2 h and air-dried before autoradiography. Radioactivity was quantified by liquid scintillation counting.
Results

**HCMV increases HIV LTR-directed gene expression and HIV p24 antigen production**

Basal levels of CAT activity in E155 cells transfected with pLTR-CAT were 21.5-fold higher than in comparable transfected HOS cells (Fig. 1). The CAT assay, after cotransfection of both cell lines with pLTR-CAT and the control pBR322 plasmid, also revealed significantly higher enzyme activity in E155 cells (35-fold) than in HOS cells (Fig. 1). High CAT activity observed after cotransfection with pLTR-CAT and pTAT confirmed the ability of pLTR-CAT in both cell lines to be transactivated. Transfection efficiency in both cell lines was comparable as detected by a Southern blot assay.

Fig. 2 shows the results of experiments in which HOS cells were infected with HIV-1 alone or in combination with HCMV and the level of p24 antigen in the culture supernatant was monitored daily up to 6 days p.i. The level of detectable p24 antigen was increased in the co-infected cells, with peak levels at 4 to 5 days post-co-infection, as compared with cells infected with HIV alone. Expression of p24\textsubscript{pro} protein in 1 to 2% of acetone-fixed cells was confirmed by IF.

Production of infectious HIV-1 by the HOS cells was assessed by cocultivation with the SUPT-1 indicator cell line. Development of c.p.e. was monitored, recorded and then confirmed by p24\textsubscript{pro} antigen-capture assay. HOS cells infected with HIV-1 alone or co-infected with HIV-1 and HCMV were able to produce infectious virus.

**HIV-1 and its tat gene increase HCMV-IE DNA replication and its expression**

Hybridization of a molecular probe specific for the HCMV IE gene region with the total DNA isolated from HCMV-infected HOS cells and from E155 cells 48 h after transfection with 5 \( \mu \)g of pTAT, pART, pBR322 or salmon testes DNA revealed a measurable increase (two- to threefold) in the amount of HCMV IE DNA present in cells transfected with pTAT (Fig. 3a,b).

HOS cells infected with either HIV-1 or HCMV and then co-infected with the opposite virus 4 days later showed a 5-9- and 7-5-fold increase, respectively, in the frequency of cells positive for the HCMV-IE 68000 \( M_r \) protein, as compared with HCMV singly infected cells when assayed by IF 2 days after the second viral infection (Table 1, experimental conditions 1 and 2). Similar analysis after dual infections allowed to proceed for 11 days after initial HCMV infection indicated a two- to threefold higher frequency than in the control cultures (data not shown).

HOS cells were also infected with HCMV, transfected 16 h later with 10 \( \mu \)g of pTAT, pART, pBR322 or
Fig. 3. Detection of HCMV DNA in HOS and E155 cells by hybridization analysis. HCMV-infected (1 to 2 p.f.u./cell) HOS cells (a) and E155 cells (b) were transfected with the plasmids indicated. HOS cells were transfected 16 h.p.i. Total DNA was isolated from HOS cells 0 (2 h after glycerol treatment), 1, 2 and 3 days post-transfection and DNA was isolated from E155 cells 2 days post-transfection. The extracted DNA was applied to a nitrocellulose filter membrane and analysed for the presence of HCMV DNA using a 32P-labelled 5.1 kb BamHI fragment of pRL43a. After autoradiography the amount of 32P-labelled HCMV DNA present on the nitrocellulose filters was quantified by liquid scintillation counting, and the results are presented below the photographs as c.p.m. per sample. Control DNAs include 1.25 µg of uninfected HOS cellular DNA (UN) and 0.25 µg of pTAT, pBR322 or pRL43a. The concentration of diluted HCMV-infected HOS cellular DNA is indicated.

Table 1. Effects of HIV on the number of HCMV IE protein-positive HOS cells

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>pTAT</th>
<th>pART</th>
<th>pBR322</th>
<th>Salmon testes DNA</th>
<th>HCMV and HIV</th>
<th>HCMV alone</th>
<th>Enhancement (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. HIV infection then HCMV infection*</td>
<td>624</td>
<td>105</td>
<td>5.9</td>
<td>314</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. HCMV infection then HIV infection†</td>
<td>839</td>
<td>112</td>
<td>7.5</td>
<td>260</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. HCMV infection then transfection‡</td>
<td>600</td>
<td>412</td>
<td>2.3</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. pHCMV-IE cotransfection§</td>
<td>50</td>
<td>24</td>
<td>5.5</td>
<td>18</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Infection with HIV followed by HCMV infection 4 days later.
† Infection with HCMV followed by HIV infection 4 days later.
‡ HOS cells were infected with HCMV 16 h before transfection with pTAT, pART, pBR322 or salmon testes DNA.
§ Ten µg of pRL43a was cotransfected into HOS cells along with 10 µg of pTAT, pART, pBR322 or salmon testes DNA.

HIV-tat gene increases infectious HCMV production

Culture supernatant from E155 cells was used in infectivity studies to determine whether HIV-1 gene products affected the yield of infectious HCMV from E155 cells. Cell-free supernatants, obtained from E155 cells 2 days after transfection with 5 µg of pTAT, pMTAT, LTR-CAT, pART, pBR322 or sonicated salmon testes DNA, were incubated with MRC-5 cells under agar overlay and HCMV-induced plaques were counted 10 days later. About 6 to 10 times more infectious HCMV was obtained using supernatant from pTAT-transfected E155 cells than with supernatants...
Table 2. Enhancement of HCMV yield in E155 cells transfected with HIV genes

<table>
<thead>
<tr>
<th>Transfector</th>
<th>Titre of infectious virus yield* (p.f.u. × 10^{-3}/ml)</th>
</tr>
</thead>
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<tr>
<td>pTAT</td>
<td>13.0 ± 1.8</td>
</tr>
<tr>
<td>pMTAT</td>
<td>1.8 ± 0.7</td>
</tr>
<tr>
<td>pART</td>
<td>2.0 ± 0.8</td>
</tr>
<tr>
<td>pLTR-CAT</td>
<td>1.2 ± 0.9</td>
</tr>
<tr>
<td>pBR322</td>
<td>1.2 ± 0.9</td>
</tr>
<tr>
<td>Salmon sperm</td>
<td>1.2 ± 0.5</td>
</tr>
</tbody>
</table>

* Mean values from four titration plates of MRC-5 cells.

derived from the other transfections, including the pMTAT (Table 2). The experiment was repeated four times under identical conditions, with similar results.

**Discussion**

In this study, we found that HIV-1 or its tat gene product augmented expression of the HCMV IE gene and that the HIV-1 tat gene product increased titres of infectious HCMV in E155 cells and levels of HCMV DNA in E155 cells and in HCMV-infected HOS cells. A slight increase in HCMV replication in a T lymphoblastoid cell line persistently infected with HIV was also demonstrated recently (Casareale et al., 1989). By contrast, Skolnik et al. (1988) reported that a Jurkat cell line constitutively expressing the HIV-1 tat gene did not increase detectable levels of HCMV DNA, nor did the HIV-1 tat gene in a vaccinia virus recombinant construct increase detectable levels of particular HCMV early proteins in human embryonic lung cells. Possibly, the tat-induced increase observed in HCMV IE gene expression and in the HCMV viral titres is mediated by a cellular intermediate, induced by the tat gene product in HOS and E155 cells, rather than the result of a direct interaction between HIV-1 gene products and HCMV. However, in the study by Skolnik et al. (1988), expression of HCMV IE proteins was not described and the magnitude of expression of the HCMV late proteins with and without HIV-1 infection was six to 12 cells and 10 to 20 cells (0.03 to 0.06% and 0.05 to 0.1% of total cell number), respectively; cell numbers that are difficult to interpret.

Consistent with previous reports (Rando et al., 1987; Elfassi et al., 1987), we also find that HCMV can enhance HIV-1 LTR-directed transcription. The HCMV IE gene region encodes several different proteins (Stinski et al., 1983; Stenberg et al., 1984) and recent evidence has implicated the IE 2 gene in a number of trans-activating and autoregulating events (Hermiston et al., 1987; Pizzorno et al., 1988), including activation of HIV-1 LTR-directed transcription (Davis et al., 1987; Rando et al., unpublished results). The isolation of HIV from peripheral blood leukocytes usually requires an exogenous signal associated with T-cell activation (Zagury et al., 1986) and the enhancer region of the HIV-LTR contains a recognition sequence for the transcriptional factor NF-kB, which is associated with T cell activation (Nabel & Baltimore, 1987). Gimble et al. (1988) have recently described the induction of a nuclear factor that binds to the NF-kB recognition sequence when HeLa cells are infected with herpes simplex virus type 1. It is possible that HCMV enhances expression of NF-kB or other transcriptional factors, thereby enhancing HIV-LTR expression. We also observed increased levels of HIV-1 p24 antigen in the culture medium of HOS cells co-infected with HIV-1 and HCMV, although there was no real change in the time course of detection of p24 antigen during the 6 days p.i.

HIV infection of HOS and E155 cells, neither of which express the CD4 antigen, strongly suggests an alternative pathway for infection of human fibroblastoid cells. These cells then join the growing list of CD4-negative, HIV-infectible cell types, including colorectal (Adachi et al., 1987), glial (Cheng-Mayer et al., 1987), fibroblastoid (Tateno et al., 1989) and neuronal cells (Harouse et al., 1989). The E155 cell line was developed by serial passages of HCMV-infected HOS cells over 2 years (Furukawa et al., 1981) and maintained in our laboratory for more than 5 years. E155 cells constantly produce HCMV proteins in 5 to 80% of cells (higher expression if the cells are split more than once in 7 to 10 days) and a low level of infectious virus. The site of HCMV latency in vivo is not clear, but peripheral mononuclear cells (Rice et al., 1984), kidney mesangial cells (Heieren et al., 1988) and liver cells (Singh et al., 1988) have all been implicated. Infection of E155 cells with HIV-1 might mimic the situation in vivo; some cells harbour the HCMV genome in inactive or replicating form and superinfection of these cells with HIV-1 leads to more productive replication of both viruses.

About 80 to 90% of acutely HCMV-infected HOS cells expressed IE proteins, using an m.o.i. of 10 to 15, but only 1 to 2% of the cells expressed late proteins, as detected by the IF assay, using a MAb to the major matrix late proteins. No infectious virus production was detectable in the cells (data not shown). It will be of interest to determine whether HIV-1 infection can turn the non-productive HCMV infection into a productive one.

The present studies in HOS and E155 cells not only indicate a broadened host-range of HIV-1 infection, but also provide a cell system in which at least some of the HIV-1 and HCMV genes are expressed well, enabling...
detailed analysis of the molecular and biological interactions between these viruses.

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References


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