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The Effects of Cytomegalovirus on Human Immunodeficiency Virus Replication in Brain-Derived Cells Correlate with Permissiveness of the Cells for Each Virus

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Human cytomegalovirus (HCMV) is commonly found in the brains of patients with AIDS and in some cases can be detected in the same cells as can human immunodeficiency virus type 1 (HIV-1). In this study, we analyzed the patterns of replication of HIV-1 and HCMV in singly infected cells and the effects of dual infection in human brain-derived cell lines of three different origins: neuroblastoma cell lines SK-N-MC and SY5Y; astrocytoma/glioblastoma cell lines U373-MG and Hs 683; and undifferentiated glioblastoma cell lines A172 and T98G. To bypass the restriction at the adsorption/penetration step in these CD4-negative cells, we used HIV-1 (amphotropic retrovirus) pseudotypes. These HIV-1 pseudotypes infected the majority of the cells in the cultures and expressed high levels of HIV-1 gene products in all except the SY5Y cells. The cell lines differed in the ability to support HCMV infection, but coinfection with HIV-1 had no effect on HCMV replication. The A172 cells were completely nonpermissive for HCMV gene expression, while HCMV replication in the singly infected T98G and SK-N-MC cell lines was restricted at the level of some early gene products. This resulted in complete and partial inhibition, respectively, of viral DNA synthesis. Dual infection of the A172, T98G, and SK-N-MC cells had no effect on HIV-1 replication. The other three cell lines, U373-MG, Hs 683, and SY5Y, were fully permissive for HCMV replication. In the U373-MG and Hs 683 cells, HCMV markedly inhibited the synthesis of HIV-1 gene products. In contrast, a transient stimulation of HIV-1 production followed by a repression was observed in the dually infected SY5Y cells. We conclude from these results that under conditions in which both HIV-1 and HCMV can undergo fully permissive infection, HCMV can repress HIV-1 gene expression. In cells in which HCMV replication is limited but HIV-1 replicates well, there is no effect on HIV-1 gene expression. However, activation of HIV-1, at least transiently, may occur in cells in which HIV-1 gene expression is limited. These studies suggest that a threshold level of some HIV-1 gene product(s) may obscure activation or promote repression of HIV replication by HCMV.

Human immunodeficiency virus type 1 (HIV-1) infection of the brain can occur early in the course of systemic infection and is commonly associated with neuropsychological impairment in the late stages of AIDS. As the disease progresses, neurological disorders also increase in severity, leading to the syndrome referred to as the AIDS dementia complex (for reviews, see references 19, 42, 74, and 88). The relationship between HIV-1 infection and the clinical effects on the central nervous system (CNS), however, is poorly understood, and many fundamental questions relating to the progressive neurological dysfunction remain to be answered. For example, what are the cell types that are actually impaired during the progression of AIDS dementia complex? Are the affected cells directly infected by HIV-1, or is the damage due to some indirect effect of HIV-1 infection? What is the role of other viral cofactors also found in the brains of HIV-1-infected individuals? These are complex problems, and clearly both in vivo and in vitro studies are necessary to dissect the molecular mechanisms underlying the pathogenesis of the neurological disease.

The prevailing view is that macrophages and microglial cells represent the majority of detectable HIV-1-infected cells in the brain, and infection of these cells occurs in a CD4-dependent manner (42, 74). However, latent infection or low-level persistent infection with only limited viral gene expression may involve a much broader range of cell types, including capillary endothelial cells, glial cells, astrocytes, oligodendrocytes, and neuronal cells (11, 42, 74, 88). One difficulty in understanding the pathogenesis of HIV-related CNS dysfunction involves the apparent disparity between the degree of the neurological defects and the level of detectable HIV-1 replication in neurally derived cells. Because it seems unlikely that the neurological dysfunction observed in patients with AIDS results from a simple direct cytolytic effect induced by the HIV-1 infection in the brain, a number of indirect mechanisms of CNS injury have been proposed (42, 61, 74). Most hypotheses invoke some variation of the theme that either a virus-coded protein or induced cellular factor exerts a toxic effect on uninfected brain cells. The alternative argument is that persistent viral infection, with viral gene expression at a level that falls below the limits of most detection assays, is sufficient to cause cell dysfunction and death. In this regard, it has been proposed that low levels of HIV-1 infection of brain capillary endothelium and astrocytes could lead to disruption of the blood-brain barrier and the subsequent neuropathology.

Any consideration of the underlying mechanisms responsible for the AIDS-associated neurological disease must take into account the fact that other viral opportunistic infections frequently occur in the CNS of HIV-1-infected individuals (for reviews, see references 42, 44, and 86). Among these viruses, human cytomegalovirus (HCMV) has received increasing attention as a potential cofactor in the progression of HIV-1-

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associated pathology. This virus is a serious pathogen in patients with AIDS, and dual infection of cells with HIV-1 and HCMV has been documented in the brain, retina, and lungs of these individuals (5, 18, 55, 71). In the CNS, HCMV has been detected by in situ cytohybridization and immunostaining techniques in multiple cell types, including endothelial cells, glia, neurons, and monocytes-macrophages (51, 89). The ability of HCMV to replicate in brain-derived cells has also been confirmed by in vitro studies. Some cells, such as primary explant cultures of brain endothelial cells and cell lines of astrocytoma/glioblastoma and neuroblastoma origin, yield measurable titers of progeny virus, while other cell lines, notably those of undifferentiated glioblastoma origin, permit only limited HCMV immediate-early (IE) and early gene expression (14, 29, 60). In a human brain cell aggregate system, HCMV has also been shown to preferentially infect monocyte-derived macrophage/microglial cells (62), suggesting that HCMV and HIV-1 may be coinfecting these same cells in vivo.

One of the complications inherent in studying the molecular interactions between HIV-1 and other viruses is the difficulty of ensuring that the majority of the cells in the population are dually infected. This is a particular problem when the cells either are CD4 negative or express only low levels of CD4 such that the HIV-1 infection is restricted at the adsorption/penetration step. To bypass this obstacle, we devised a way to create HIV pseudotypes with expanded host range. This was accomplished by coinfecting CD4-positive cells with HIV-1 and an amphotropic retrovirus. As described previously (73), the resulting HIV-1 pseudotypes were able to infect and replicate efficiently in a broad range of CD4-negative cells. With the use of these HIV-1 pseudotypes, we then proceeded to study the molecular interactions between HIV-1 and HCMV in human foreskin fibroblast (FF) cells and U373-MG astrocytoma/glioblastoma cells, both of which are fully permissive for the growth of each virus (37). We found that although HCMV replication was unaffected by the presence of HIV-1, HIV-1 RNA and protein synthesis and virus production were significantly inhibited in the dually infected cells.

The aim of this work was to explore further the patterns of replication of HIV-1 and HCMV in several different types of brain-derived cells and the molecular interactions between these two viruses in dually infected cells. The particular cell lines used originated from undifferentiated glioblastomas (T98G and A172), astrocytoma/glioblastomas (Hs 683 and U373-MG), and neuroblastomas (SK-N-MC and SY5Y) and were chosen because preliminary data from our own laboratory as well as published reports (29, 60) indicated that they differed in the ability to support HCMV infection. In this study, we show that HIV-1 pseudotypes are able to infect and replicate to high titers in all of the cell types except the SY5Y neuroblastoma cell line. Through the use of a panel of antibodies to specific HCMV gene products, we also present a more detailed picture of HCMV replication in the various brain-derived cell lines than previously available. Our results document that in the dually infected cells, HIV-1 has no effect on the HCMV life cycle, whereas HIV-1 replication is affected in one of three ways. We find that HIV-1 replication is transiently stimulated by HCMV in SY5Y cells, which are fully permissive for HCMV but allow only limited HIV-1 gene expression in the absence of HCMV. In contrast, inhibition of HIV-1 is observed in cells fully permissive for both viruses, whereas no effect is detected in cells in which HCMV IE or early protein expression is restricted. Possible molecular mechanisms underlying these differences and their implications are discussed.

MATERIALS AND METHODS

Cells and viruses. Human FF, CEM, and CEM-1B (persistently infected with amphotropic retrovirus) cells were obtained and propagated as previously described (73). The human undifferentiated glioblastoma-derived cell lines T98G (CRL 1690) and A172 (CRL 1620) were obtained from the American Type Culture Collection (ATCC). The human astrocytoma/glioblastoma-derived cell lines U373-MG (ATCC HTB17) and Hs 683 (ATCC HTB 138) were obtained from Robert LaFemina and from the ATCC, respectively. The SK-N-MC human neuroblastoma-derived cell line was obtained from the ATCC (HTB 10), whereas the SY5Y human neuroblastoma-derived cells were kindly provided by Martin Muggenridge.

The T98G cells were maintained in Eagle's minimum essential medium containing nonessential amino acids, 1.0 mM sodium pyruvate, and 10% fetal bovine serum (FBS). The U373-MG, Hs 683, and A172 cell lines were maintained in Dulbecco's modified Eagle's medium with high glucose (4.5 g/liter) and 10% FBS. The SK-N-MC and SY5Y cell lines were propagated in OptiMEM (GIBCO BRL) containing nonessential amino acids, 1.0 mM sodium pyruvate, and 5% FBS. All media were supplemented with 0.26 mg of L-glutamine per ml and 180 U of penicillin-streptomycin per ml.

Stocks of HIV-1, of HIV-1 (amphotropic retrovirus) pseudotypes (designated HIV-1B), and of the AD169 strain of HCMV were propagated and titered as previously described (37, 73). The stocks of HIV-1 or HIV-1B pseudotypes typically contained 1,000 to 6,000 ng of HIV-1 antigen per ml as determined by enzyme immunoassay (Abbott Laboratories).

Infection of cells. Dual infection and HIV-1 infection of the brain-derived cell lines or of the FF cells (10^6 cells per T75 flask) were performed essentially as described previously (37, 73), with the following modifications. HIV-1B pseudotype or HIV-1 infections were performed with 10 ml of undiluted viral stocks or with stocks diluted 1:3 or 1:4 in fresh medium. Undiluted viral stocks were additionally supplemented with nonessential amino acids when used to infect T98G, SK-N-MC, and SY5Y cells. Mock-infected cells were exposed to an equivalent volume of a filtered supernatant from uninfected CEM or CEM-1B cells maintained in culture for 3 days. The cells were washed and refed with medium at 24 h postinfection (p.i.) and then mock infected or superinfected with HCMV (multiplicity of infection [MOI] of 3 to 5) at approximately 40 h p.i. After HCMV infection, the cells were refed with fresh medium each time aliquots were taken for the determination of HIV-1 antigen titers, typically at 1, 3, 5, and 7 days after HCMV infection.

For experiments in which the cells were infected with HCMV alone, the cells were plated at 10^6 cells per 100-mm-diameter dishes and infected 24 h later with approximately 5 PFU of HCMV per cell or mock infected with 1% dimethyl sulfoxide control supernatant from uninfected FF cells maintained in culture for 3 days. The virus was allowed to adsorb for 6 h, after which the cells were rinsed twice and refed with fresh medium. In some experiments, the medium contained 10 μg of 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG; ganciclovir) per ml to inhibit HCMV DNA replication.

Antibodies. A murine monoclonal antibody recognizing the HIV-1 $p24^{gag}$ protein obtained from American BioTechnologies, Inc. was used to detect HIV $p24^{gag}$ protein and its precursors by Western immunoblotting. The rabbit polyclonal antiserum to HIV-1 $p25/24^{gag}$ was provided through the AIDS Research and Reference Reagent Program, Division of AIDS Program, National Institute of Allergy and Infectious Diseases

(77), and was used for the detection of HIV antigens by immunostaining.

The mouse monoclonal antibodies CH16-0, recognizing the HCMV IE1 72-kDa and IE2 86-kDa proteins, and CH16-1, directed against the 52-kDa major DNA-binding protein of HCMV (open reading frame [ORF] UL44), were kindly provided by Lenore Pereira. The rabbit polyclonal antibody BSA-2.9 was used to detect the four early phosphoproteins of 84, 50, 43, and 34 kDa encoded by the 2.2-kb class of HCMV RNAs (ORF UL112-UL113) (76, 90, 91). The rabbit polyclonal antiserum, anti-C1, which recognizes the carboxy terminus of the HCMV assembly proteins (67, 87), was kindly provided by Wade Gibson. Mouse monoclonal antibody 8125, recognizing a late HCMV antigen, was purchased from Chemicon. Horseradish peroxidase (HRP)-linked sheep immunoglobulin G (IgG) directed against mouse IgG and HRP-linked donkey IgG directed against rabbit IgG were obtained from Amersham.

Immunostaining of cells. The cells were infected as described above, and mock-infected, singly infected, and dually infected samples were harvested at 3 days after HCMV superinfection (5 days after HIV-1 or HIV-1B infection) by trypsinization. Approximately 2×10^4 cells from each sample were seeded per well on multiwell slides (Bellco) and fixed in ice-cold 100% acetone prior to storage at −70°C. The cells were warmed to −20°C in a bath of acetone-methanol (1:1), then permeabilized in a bath of 100% methanol at −20°C, and washed three times in phosphate-buffered saline (PBS). Endogenous peroxidase activity was inhibited by a 30-min incubation in 0.3% hydrogen peroxide in PBS followed by additional washes in PBS. A 1:100 dilution of the rabbit polyclonal antiserum to HIV-1 $p25/24^{gag}$ was used to detect the cells expressing HIV *gag* antigens; the diluted antiserum was precleared by being treated twice with 1% mouse liver acetone powder (Sigma) for 30 min at 0°C. HCMV antigens were detected with the pool of mouse monoclonal antibodies including CH16-0, CH16-1, and 8125, which were diluted at 1:1,000, 1:1,000, and 1:30, respectively. After incubation of the cells with the antibodies for 2 h at room temperature, the antibodies were removed by five washes in PBS. The cells were then incubated for 1 h at room temperature either with HRP-linked sheep IgG anti-mouse IgG or with HRP-linked donkey IgG directed against rabbit IgG, both at a 1:100 dilution in PBS, and excess secondary antibody was removed with five washes of PBS. A positive reaction was identified on the basis of the brown peroxidase precipitate produced following incubation of the cells in 0.6 g of 3,3′-diaminobenzidine (DAB; Sigma) per liter plus 0.03% H_2O_2 in PBS. The cells were counterstained with Harris hematoxylin (Fisher) and mounted in glycerol buffered with PBS.

Western blot analysis. At 3 days after HCMV infection, cell lysates were obtained, and proteins from approximately 2.5×10^5 to 1×10^6 cells per lane were separated by electrophoresis on sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gels and transferred to nitrocellulose membranes as described previously (37, 73). The membranes were saturated for 2 to 16 h in Tris-buffered saline (TBS) containing 5% nonfat dry milk, 0.01% Antifoam A emulsion (Sigma), and 0.02% Tween 20 and incubated for 1 to 2 h with the primary antibody at a dilution of 1:1,000 in TBS containing 5% nonfat dry milk. After five washes of 20 min each in TBS containing 0.01% Antifoam A emulsion and 0.02% Tween 20, the membranes were incubated for 1 h with the HRP-linked anti-mouse or anti-rabbit IgG secondary antibody diluted 1:2,500 in TBS containing 5% nonfat dry milk, 0.01% Antifoam A emulsion, and 0.02% Tween 20. Incubations with the secondary antibodies were followed by five additional washes before incubation with the detection reagents. The detection of HIV or HCMV proteins was performed with the enhanced chemiluminescence (ECL) Western blotting detection kit (Amersham) according to the manufacturer's instructions.

In some cases, the bound antibodies were stripped from the blots by incubating the membranes in 50 ml of 100 mM 2-mercaptoethanol–2% SDS–62.5 mM Tris HCl (pH 6.7) at 50°C for 30 to 40 min, followed by two washes of 30 min each in TBS with 0.01% Antifoam A emulsion and 0.02% Tween 20 before the membranes were reprobed with another antibody as described above.

Analysis of HCMV DNA. At various times after HCMV infection, intracellular DNA was isolated (37) and aliquots (5, 1, and 0.2 μg) were slot blotted onto nitrocellulose filters with the Schleicher & Schuell Minifold II slot blot system according to the manufacturer's instructions. As a control, aliquots of the HCMV strain AD169 *Eco*RI fragment D (5, 0.5, and 0.05 ng) were also slot blotted onto the filters. The filters were then prehybridized and hybridized as described previously (37) with the purified HCMV *Eco*RI fragment D (79) labeled with [α-^{32}P]dCTP (3,000 Ci/mmol) by random priming with the Boehringer Mannheim Random Primed DNA labeling kit.

Biosafety. All experiments involving live HIV-1 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

Replication of HIV-1 and HIV-1B pseudotypes. Prior to using the brain-derived cell lines for dual infection, we compared the replication of HIV-1 in the various cells following infection with nonpseudotyped HIV-1 or HIV-1 (amphotropic retrovirus) pseudotypes (designated HIV-1B). As shown in Table 1, HIV-1B pseudotypes bypassed the restriction at the adsorption/penetration step in all of these CD4-negative cell lines, with HIV-1 synthesis increased up to 10,000-fold relative to the infection of these cells with nonpseudotyped HIV-1. The level of HIV-1 produced did vary in the different cell lines, further confirming the notion that cellular factors also influence the infection. HIV-1 replicated best in the A172, Hs 683, U373-MG, and SK-N-MC cells, with peak titers comparable to those produced in the CD4-positive CEM cells (73). Virus production was slightly lower in the T98G cells but was significantly reduced in the SY5Y cells. This lower level of HIV-1 production in the SY5Y cells was not cell type specific, as HIV-1 production in the other neuroblastoma cell line, SK-N-MC, was at least 100-fold higher than in the SY5Y cells.

Although the results of these experiments suggested that the HIV-1B pseudotypes could infect and replicate in all of these CD4-negative brain-derived cell lines, we could not eliminate the possibility that only a small percentage of the cells were infected. To address this question, we harvested the cells 5 days after infection with the HIV-1B pseudotypes and prepared them for immunostaining with an antibody specific for HIV-1 antigens. Figure 1 shows that the majority of the cells in all of the cultures, with the exception of the SY5Y cells, expressed HIV-1 *gag* gene products. In the case of the SY5Y cells, the level of HIV-1 gene products was below the limits of detection, and thus we could not determine the percentage of cells infected. However, there does not appear to be a high level of HIV-1 gene expression in a minor subpopulation of these cells.

Effect of HCMV infection on HIV-1 virus production. To

TABLE 1. Expanded tropism of HIV-1B pseudotypes in brain-derived cells[a]

Cells	Virus	HIV-1 virus production (ng of HIV-1 antigen per ml)		
		Day 2 p.i.	Day 5 p.i.	Day 12 p.i.
Undifferentiated glioblastoma				
A172	HIV-1	31	0.6	0.2
	HIV-1B	2,656	11,080	1,794
T98G	HIV-1	45	0.5	0.06
	HIV-1B	65	585	680
Astrocytoma/glioblastoma				
U373-MG	HIV-1	24	0.14	0.04
	HIV-1B	184	765	1,795
Hs 683	HIV-1	85	0.55	0.18
	HIV-1B	178	1,009	2,884
Neuroblastoma				
SK-N-MC	HIV-1	52	0.8	1
	HIV-1B	1,316	6,834	3,189
SY5Y	HIV-1	51	0.5	0.05
	HIV-1B	25	25	31

[a] Cells were plated at 10^6 cells per T75 flask and were infected the following day with 10 ml of cell-free supernatant containing either HIV-1B pseudotype or nonpseudotyped HIV-1. Antigen titers of the inocula used to infect the brain-derived cell lines ranged from 3,000 to 6,000 ng/ml for HIV-1B pseudotype and from 4,000 to 8,000 ng/ml for nonpseudotyped HIV-1. Cells were washed thoroughly at days 1 and 3 p.i. to remove the residual inoculum, and the cells were then refed every 2 days. The titer of HIV-1 antigen released in the supernatant of the cells from 2 to 12 days p.i. was determined with the Abbott HIV-1 p24 antigen assay.

study the effect of HCMV infection on HIV-1 replication in the different cell lines, the cells were mock infected or infected with HIV-1B pseudotypes and 40 h later were mock infected or superinfected with HCMV (MOI of 3 to 5). At various times p.i., the supernatants were obtained and the amount of HIV-1 was determined by the HIV-1 antigen test. Figure 2 shows HIV-1 production in the absence or presence of HCMV in the human brain-derived cell lines (Fig. 2A to F) and human FF cells (Fig. 2G). As a consequence of the HCMV infection, HIV-1 replication was affected in one of the following ways.

First, as we previously reported, HIV-1 production was inhibited by the HCMV infection in the FF cells (Fig. 2G) and in the astrocytoma/glioblastoma cells U373-MG (Fig. 2E). This marked repression was also observed in the other astrocytoma/glioblastoma cell line, Hs 683 (Fig. 2F). A 3- to 7-fold inhibition of HIV-1 production occurred by 3 days after HCMV infection, and the overall level of inhibition reached 20- to 50-fold as the HCMV infection progressed.

In contrast to these inhibitory effects, HIV-1 replication was unaffected by the presence of HCMV in the two undifferentiated glioblastoma cell lines, A172 (Fig. 2A) and T98G (Fig. 2B), or in the neuroblastoma cell line SK-N-MC (Fig. 2C). Although slight differences were detected in these three cell lines in the presence of HCMV, they never exceeded twofold. In A172 cells (Fig. 2A) and to a lower extent in SK-N-MC (Fig. 2C), U373-MG (Fig. 2E), and Hs 683 (Fig. 2F) cells, the level of HIV-1 released into the supernatant decreased with time both in the presence and in the absence of HCMV. This decrease was a consequence of the increase in cell density when the cultures became too confluent and was not observed when the cells were passaged more frequently (data not shown).

A different effect of HCMV on the HIV-1 infection was seen in the neuroblastoma cell line SY5Y (Fig. 2D). In the absence of HCMV, HIV-1 production was initially quite low (approximately 3 ng of HIV-1 antigen per ml of supernatant) but gradually began to increase as the cells approached confluence, reaching 50 ng of HIV-1 antigen per ml of supernatant at day

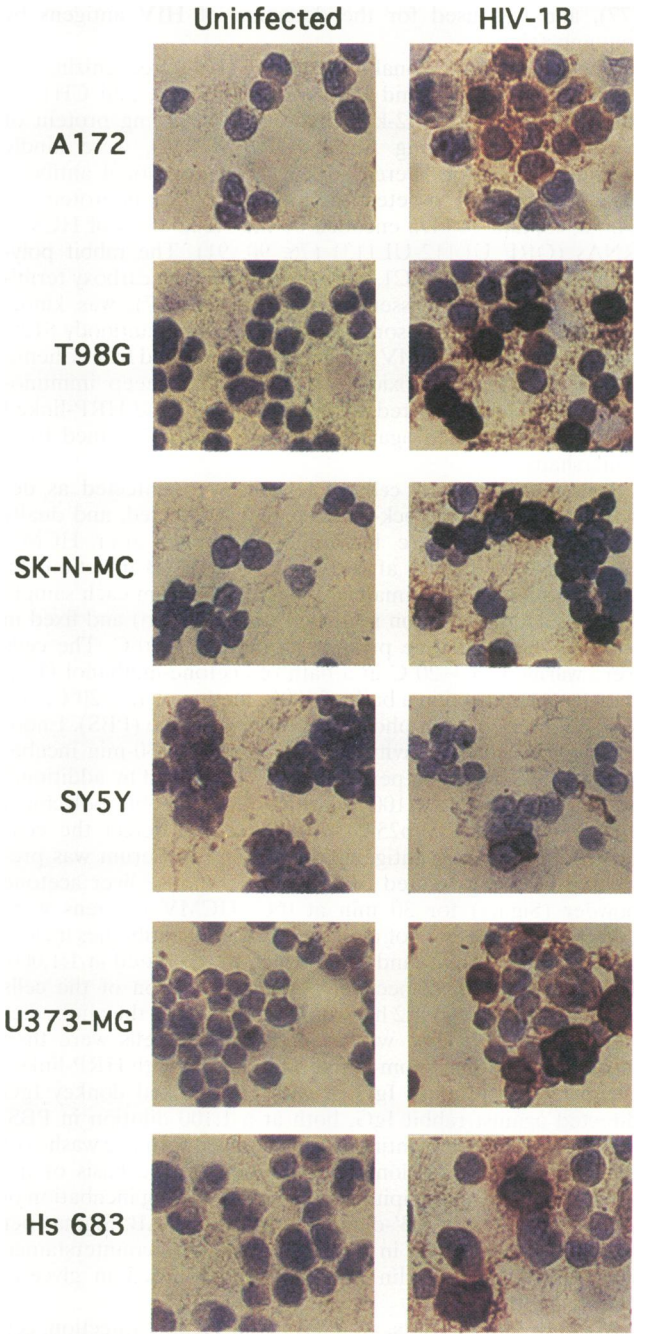

FIG. 1. Detection of HIV *gag* antigens in HIV-1B-infected cells by immunostaining. The different brain-derived cell lines were mock infected or infected with HIV-1B pseudotypes. The cells were harvested at 5 days after HIV-1B infection, deposited on multiwell slides, and analyzed by immunostaining with a precleared rabbit polyclonal serum to HIV $p25/24^{gag}$ antigens as described in Materials and Methods. The cells were counterstained with Harris hematoxylin, which stains the nuclei purple-blue, to provide a contrast with the brown color of the oxidized DAB.

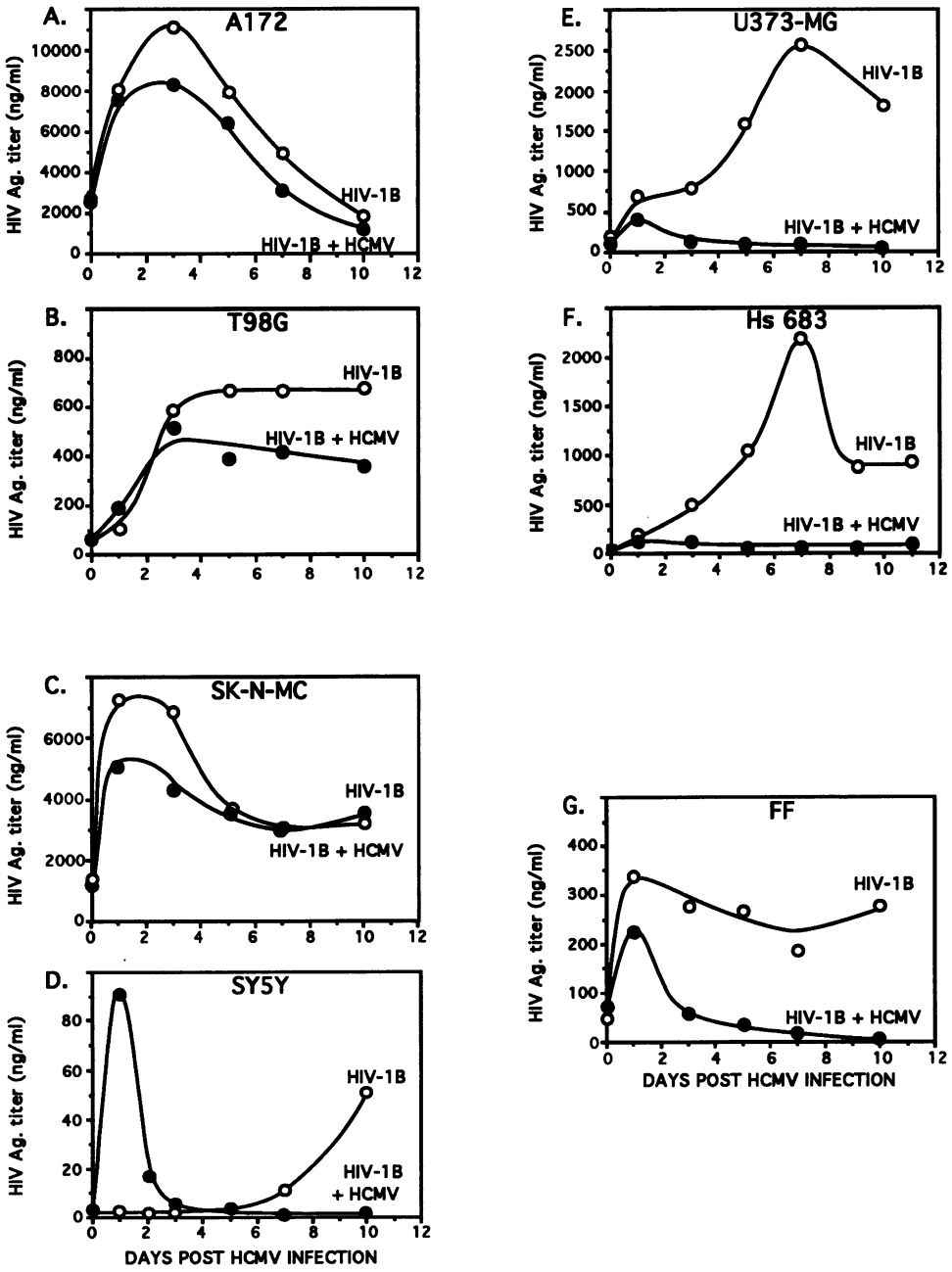

FIG. 2. Effect of HCMV infection on HIV-1 virus production. Undifferentiated glioblastoma (A172 [A] and T98G [B]), neuroblastoma (SK-N-MC [C] and SY5Y [D]), astrocytoma/glioblastoma (U373-MG [E] and Hs 683 [F]), and FF (G) cells were infected with HIV-1B pseudotypes for 40 h before HCMV infection. The cells were mock infected (○) or infected with HCMV at an MOI of 5 PFU/cell (●). At various times p.i., the supernatants were collected and the concentration of HIV-1 antigen released was determined by the Abbott HIV antigen enzyme immunoassay.

10 after HCMV infection. In contrast, in the cells dually infected with HIV-1 and HCMV, we reproducibly observed a transient 26-fold stimulation of HIV-1 production between days 1 and 3 after HCMV infection. By day 3, however, the level of HIV-1 antigen released into the supernatant of the dually infected cells was comparable to that seen in the cells singly infected with HIV-1. The level of HIV-1 production in the dually infected cells further decreased as the cells continued to divide, suggesting that the initial stimulation observed in the presence of HCMV was then followed by inhibition of HIV-1 production.

Analysis of HIV-1 proteins. To investigate further the effect of HCMV infection on HIV-1 replication, we proceeded to analyze the production of HIV-1 proteins by Western blotting with a mouse monoclonal antibody to the structural *gag* proteins. In the experiment shown in Fig. 3, the different brain-derived cell lines were infected with HIV-1B pseudotypes for 40 h prior to infection with HCMV, cell extracts

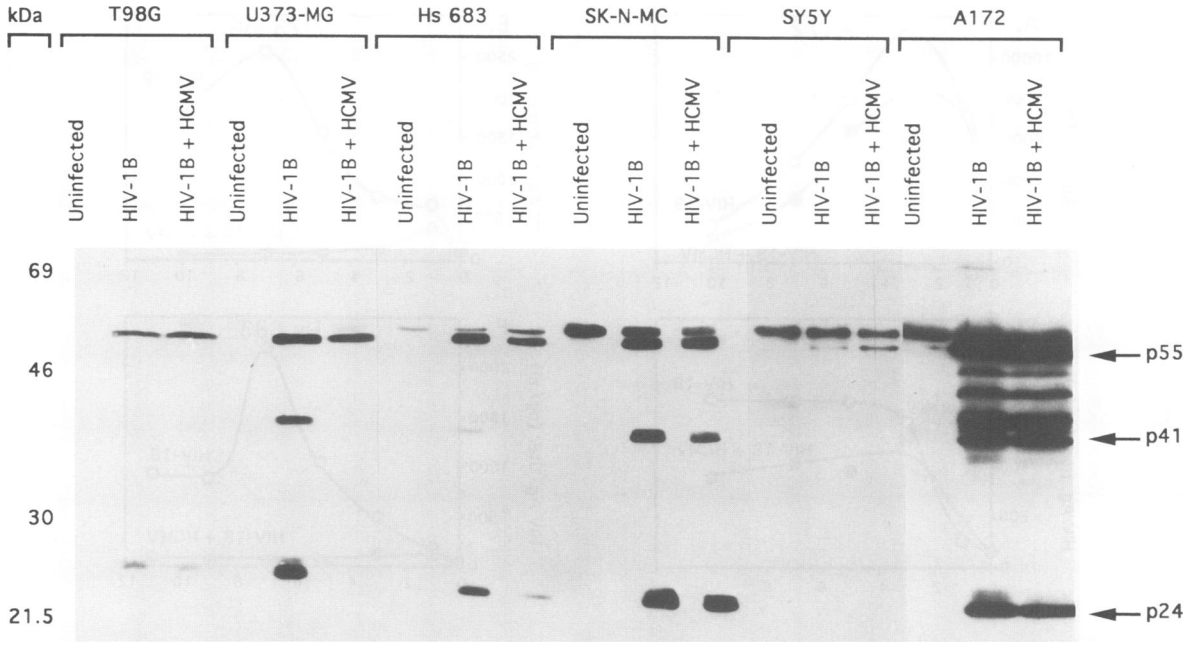

FIG. 3. Effect of HCMV infection on HIV-1 protein expression. The different brain-derived cell lines were mock infected or infected for 40 h with HIV-1B pseudotypes prior to infection or mock infection with HCMV. The cells were harvested at 3 days after HCMV infection, and cell lysates were analyzed by Western blotting with a mouse monoclonal antibody to the HIV-1 $p24^{gag}$ protein. Antigen-antibody complexes were detected with an HRP-linked sheep anti-mouse IgG and the ECL reagents (Amersham). The migration of molecular weight markers is indicated on the left, and the HIV-1 proteins detected are labeled on the right.

were prepared at 3 days after HCMV infection, and the intracellular steady-state concentrations of the HIV-1 proteins were determined by Western blotting. In the absence of HCMV, various amounts of the p55, p41, and p24 *gag* proteins were detected in the different cell lines, with the relative levels roughly corresponding to the amount of HIV-1 released into the supernatant. It should be noted that in some of the cell lines, this antiserum also detects a cellular protein that migrates slightly more slowly than the p55 protein.

Figure 3 shows that in both astrocytoma/glioblastoma cell lines, U373- MG and Hs 683, the accumulation of the HIV-1 *gag* proteins was inhibited by the HCMV infection, with the effect on the processed p41 and p24 proteins greater than that on the p55 precursor protein. In agreement with the data concerning viral release into the supernatant, HIV-1 *gag* protein synthesis was unaffected by the HCMV infection in the undifferentiated glioblastoma cells A172 and T98G and in the SK-N-MC neuroblastoma cells. In the SY5Y neuroblastoma cells, HIV-1 *gag* protein expression is at the limits of detection with this particular antibody, and only the $p55^{gag}$ precursor protein can be detected in samples infected by HIV-1B pseudotypes. Furthermore, as expected from the kinetics of viral release in the supernatant, at 3 days after HCMV infection, there was no difference in the concentration of the p55 precursor in SY5Y cells either singly infected with HIV-1B pseudotypes or dually infected with both HIV-1B and HCMV.

Extent of HCMV infection in singly and dually infected cultures as determined by immunostaining. Because interpretation of the effect of HCMV on the HIV-1 infection would be complicated if the majority of the cells in our experiments were not infected with HCMV, we proceeded to determine by immunostaining the percentage of cells in each population that were expressing HCMV gene products. The different cultures of brain-derived cell lines and FF cells were first infected with the HIV-1B pseudotypes and then superinfected with HCMV as described above. As controls, some cultures were left uninfected or were singly infected with the HIV-1B pseudotypes or HCMV. At the time point corresponding to 3 days after HCMV infection, the cells were harvested and immunostained with a pool of monoclonal antibodies directed against HCMV IE, early, and late antigens.

Figure 4 shows that in the cultures containing FF cells, the astrocytoma/glioblastoma cells U373-MG and Hs 683, and the SY5Y neuroblastoma cells, there was a high level of HCMV antigen expression in all of the cells whether they were dually infected with HIV-1B pseudotypes and HCMV or infected with HCMV alone. Although lower levels of HCMV proteins were observed in SK-N-MC and in T98G cell lines infected by HCMV alone or dually infected with HIV-1B pseudotypes and HCMV, HCMV antigens could be detected in almost all of the cells. However, in the A172 cells, any HCMV gene products present were below the limits of detection. As expected, no positive staining was observed in the FF cells and brain-derived cells infected by HIV-1B pseudotypes alone or mock infected.

As described above, analysis of the different cell lines by immunostaining for HIV-1 *gag* proteins indicated that 50 to 70% of each cell population infected by HIV-1B pseudotypes alone or dually infected (with the exception of the SY5Y cells, in which HIV-1 expression was below the limits of detection) expressed detectable levels of HIV-1 gene products. These results, coupled with the immunostaining data for HCMV antigens, suggest that except for the A172 cells, the majority of the cells in each population infected with HIV-1B were also infected with HCMV.

Pattern of HCMV IE and early protein expression. Since the foregoing experiments indicated that the differential effects of HCMV on the HIV-1 infection were not due to differences in the percentage of cells infected with HCMV, we proceeded to

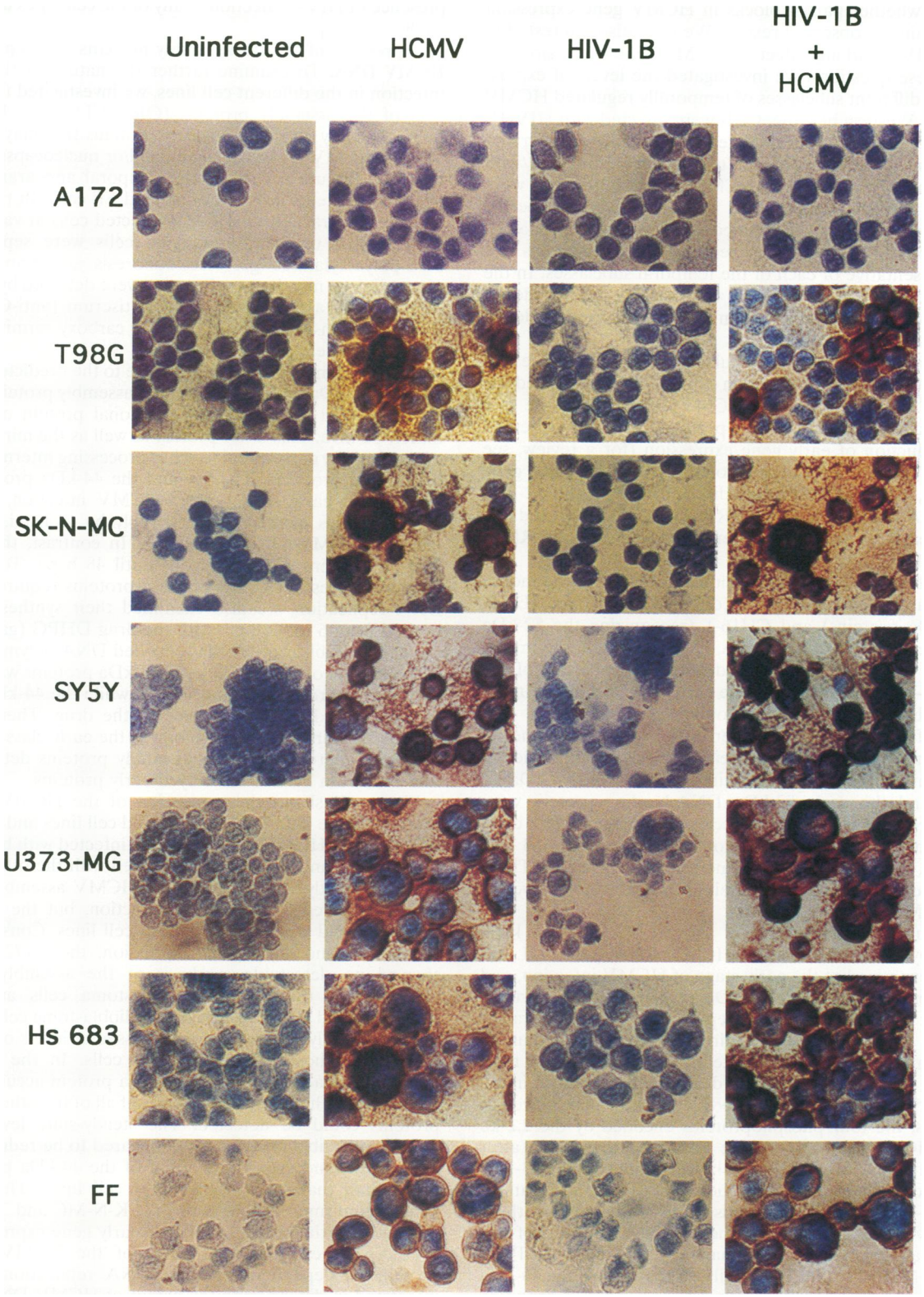

FIG. 4. Detection of HCMV antigens in singly and dually infected cells by immunostaining. The different brain-derived cell lines and the FF cells were mock infected or infected for 40 h with HIV-1B pseudotypes prior to infection or mock infection with HCMV. The cells were harvested at 3 days after HCMV infection, deposited on multiwell slides, and analyzed by immunostaining with a pool of monoclonal antibodies directed against HCMV IE, early, and late antigens as described in Materials and Methods. The cells were counterstained with Harris hematoxylin and stained with DAB as in Fig. 1.

determine whether partial blocks in HCMV gene expression might explain the observed results. We were also interested in whether HIV-1 had any effect on HCMV gene expression. To address these questions, we investigated the levels of expression of the different subclasses of temporally regulated HCMV proteins by Western blotting. Cells were infected with HIV-1B pseudotypes and then superinfected with HCMV as described above. Some cultures were left uninfected or infected with HCMV alone. At 3 days after HCMV infection, the cells were harvested and the steady-state concentrations of specific HCMV proteins were determined by Western blot analysis with antibodies directed against selected HCMV proteins that were representative of each of the temporal subclasses. In the following experiments, the same cell extracts used to determine HIV-1 protein expression in Fig. 3 were used to detect the various HCMV proteins (Fig. 5 and 6).

To determine whether the regulation of the viral cycle was modified at the level of IE protein expression, we analyzed the expression of the two major HCMV IE proteins, the IE1 72-kDa and the IE2 86-kDa gene products, which are essential for the induction of early gene expression (for a review, see reference 72). For the analysis of the extent of early gene expression, we used antibodies directed against the 52-kDa major DNA-binding protein (ORF UL44) (23, 58) and the family of early phosphoproteins encoded by the 2.2-kb RNAs (ORF UL112-113) (76, 90, 91).

The pattern of HCMV expression detected with the mouse monoclonal antibodies CH16-0 (directed against the IE 72- and 86-kDa proteins) and CH16-1 (recognizing the 52-kDa early protein) is shown in Fig. 5A. Consistent with the immunostaining results, there was no detectable expression of the 72- and 86-kDa IE proteins or the early 52-kDa protein in the A172 undifferentiated glioblastoma cells, suggesting that HCMV infection is blocked either at the adsorption/penetration step or at the level of IE protein synthesis in this particular cell line. In all of the other cell lines, the 72- and 86-kDa IE proteins and the early 52-kDa DNA-binding protein were detected. High levels of these proteins were present in FF cells, in the two astrocytoma/glioblastoma cell lines Hs 683 and U373-MG, and in the SY5Y neuroblastoma cells. These proteins were also expressed, albeit at a slightly lower level, in the SK-N-MC and T98G cells.

The four phosphoproteins of 84, 50, 43, and 34 kDa, encoded by the 2.2-kb class of HCMV RNA (ORF UL112-113), are expressed at the early stage of HCMV infection, and only the expression of the 50-kDa protein appears partially dependent on HCMV DNA replication (90). The rabbit polyclonal antiserum BSA-2.9, directed against the common amino terminus of these four proteins, was used to detect their expression in the different brain-derived cell lines and in FF cells (Fig. 5B). In U373-MG, Hs 683, and SY5Y cells as well as in FF cells, the four phosphoproteins encoded by the 2.2-kb class of HCMV transcripts were all detected by 72 h after HCMV infection. Although the relative amounts of the 43- and 34-kDa proteins were similar in these cells, the concentrations of the 50- and 84-kDa proteins were lower in the three brain-derived cell lines than in the FF cells. Synthesis of this family of early proteins was also markedly reduced in the T98G and SK-N-MC cells. The T98G cells expressed only the 43-kDa protein, and in the SK-N-MC cells, a faint band corresponding to the 43-kDa protein could be seen only after long exposures. As expected from the pattern of IE protein expression, the four proteins encoded by the 2.2-kb class of HCMV transcripts were not detected in the A172 cells. In addition, neither HCMV IE nor early gene expression was modified by the presence of HIV-1 infection in any of the cell types used in this study.

Expression of HCMV assembly proteins and replication of HCMV DNA. To examine further the nature of the HCMV infection in the different cell lines, we investigated the expression of the assembly proteins (ORF UL80 and UL80a), a family of early and delayed-early proteins that may be necessary for HCMV DNA packaging and/or nucleocapsid maturation (87). Figure 6A shows the temporal appearance of the assembly proteins in HCMV-infected FF cells. In this experiment, cell extracts from HCMV infected cells at various time intervals p.i. or from uninfected cells were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Proteins were detected by immunoblotting with a polyclonal rabbit antiserum (anti-C1) that is directed against a peptide from the carboxy terminus of the assembly protein precursor (67, 87).

Four proteins corresponding in size to the predicted 96-, 44-, 38-, and 33-kDa, carboxy-coterminal assembly proteins (67, 87) were detected along with an additional protein of approximately 62 kDa. This latter protein as well as the minor protein of 76 kDa may correspond to other processing intermediates or breakdown products. The 96- and the 44-kDa proteins were detected as early as 8 h after HCMV infection, and their concentrations increased considerably by 48 h p.i., after the onset of HCMV DNA replication. In contrast, the 38- and 33-kDa proteins did not appear until 48 h p.i. To ascertain whether expression of any of these proteins required HCMV DNA replication, we also examined their synthesis in cells treated from 6 to 72 h p.i. with the drug DHPG (ganciclovir), a specific inhibitor of HCMV-encoded DNA polymerase. The accumulation of the 96-, 38-, and 33-kDa proteins was partially inhibited in presence of ganciclovir, while the 44-kDa protein was unaffected by the presence of the drug. Therefore, the 44-kDa protein appears to belong to the early class of HCMV proteins, whereas the other assembly proteins detected with this antiserum behave as delayed-early proteins.

Figure 6B shows the expression of the HCMV assembly proteins in the different brain-derived cell lines and in FF cells infected with HCMV alone or dually infected with both HIV-1 pseudotypes and HCMV. As observed with the other HCMV proteins, steady-state levels of the HCMV assembly proteins were unaffected by the HIV-1 infection, but the pattern of expression did vary in the different cell lines. Consistent with lack of IE and early gene expression, the A172 cells also showed no detectable synthesis of the assembly proteins. However, in the SY5Y neuroblastoma cells and in the U373-MG and Hs 683 astrocytoma/glioblastoma cells, the four major assembly proteins were expressed at levels only slightly lower than those seen in the FF cells. In the SK-N-MC neuroblastoma cells, only the 44-kDa protein accumulated to high levels, although small amounts of all of the other assembly proteins could be detected. The steady-state levels of the HCMV assembly proteins also appeared to be reduced in the T98G cells, and even the amount of the 44-kDa protein was lower than that seen in the other cell lines. These results further confirmed that in both the SK-N-MC and T98G cells, there are partial blocks in HCMV early gene expression.

Since full expression of some of the HCMV assembly proteins is dependent on viral DNA replication, we were interested in determining the extent of HCMV DNA replication, particularly in the SK-N-MC and T98G cells, which showed limited expression of not only the assembly proteins but also the UL112-113 family of proteins. For these experiments, total cell DNA was extracted from uninfected cells and from HCMV-infected cells at early (8 h) and late (72 h) points

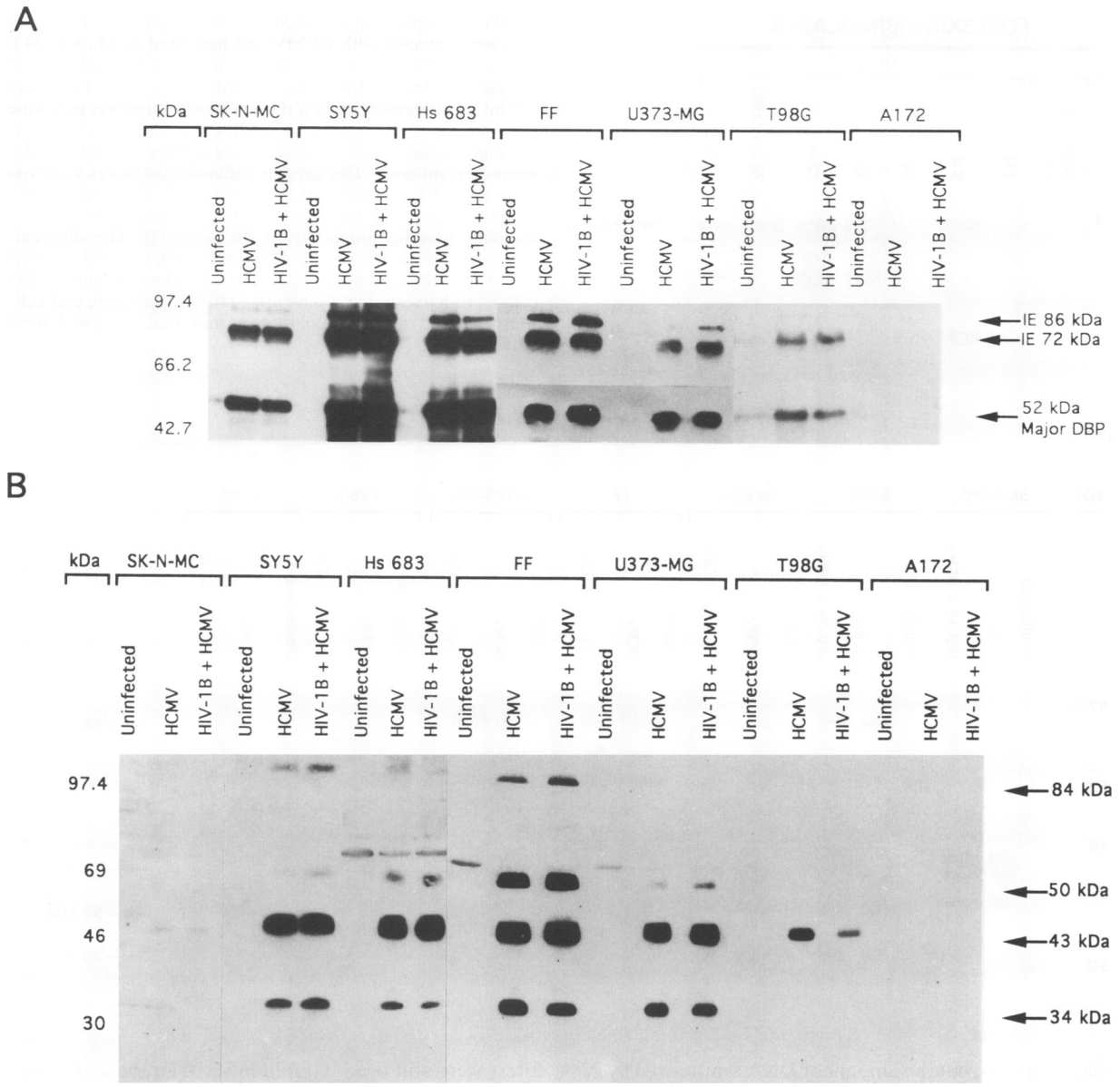

FIG. 5. Pattern of expression of HCMV IE and early proteins. The brain-derived cell lines and the FF cells were mock infected or infected for 40 h with HIV-1B pseudotypes prior to infection with HCMV. The cells were harvested at 3 days after HCMV infection, and cell lysates were analyzed by Western blotting. Following incubation of the blots with primary antibodies and HRP-linked secondary antibodies, the complexes were visualized with the ECL Western blotting detection kit (Amersham). The positions of molecular weight markers are indicated on the left, and the HCMV proteins are labeled on the right. (A) HCMV proteins were detected with a pool of two mouse monoclonal antibodies; one (CH16-0) directed against the HCMV IE1 86-kDa and IE2 72-kDa proteins and the other (CH16-1) directed against the HCMV early 52-kDa major DNA-binding protein (ORF UL44), followed by an HRP-linked sheep anti-mouse IgG. (B) The four HCMV phosphoproteins (ORF UL112-113), encoded at early times by the HCMV 2.2-kb family of RNAs, were detected with the rabbit polyclonal antibody BSA-2.9. An HRP-linked donkey anti-rabbit IgG was used as the secondary antibody.

p.i. DNA was also extracted at 72 h p.i. from HCMV-infected cells maintained in the presence of DHPG to inhibit viral DNA replication. The amount of HCMV DNA present was assayed by slot blot hybridization with HCMV *Eco*RI fragment D labeled with ^{32}P (Fig. 7). For comparative purposes, we also show hybridization to a slot blot of various quantities of purified plasmid DNA and total DNA extracted at 72 h p.i. from HCMV-infected FF cells maintained in the presence or absence of DHPG. At 8 h p.i., all of the brain-derived cell lines showed detectable amounts of HCMV DNA, which likely represents input virus. By 72 h p.i., the concentration of HCMV DNA increased at least 25- to 50-fold in the U373-MG, Hs 683, and SY5Y cells, and the levels were comparable to those seen in the fully permissive FF cells. In these three cell lines, as well as in FF cells, the accumulation of HCMV DNA was reduced in the presence of DHPG, indicating that the increase in viral DNA at 72 h p.i. was due to DNA replication. HCMV viral DNA replication also occurred in SK-N-MC

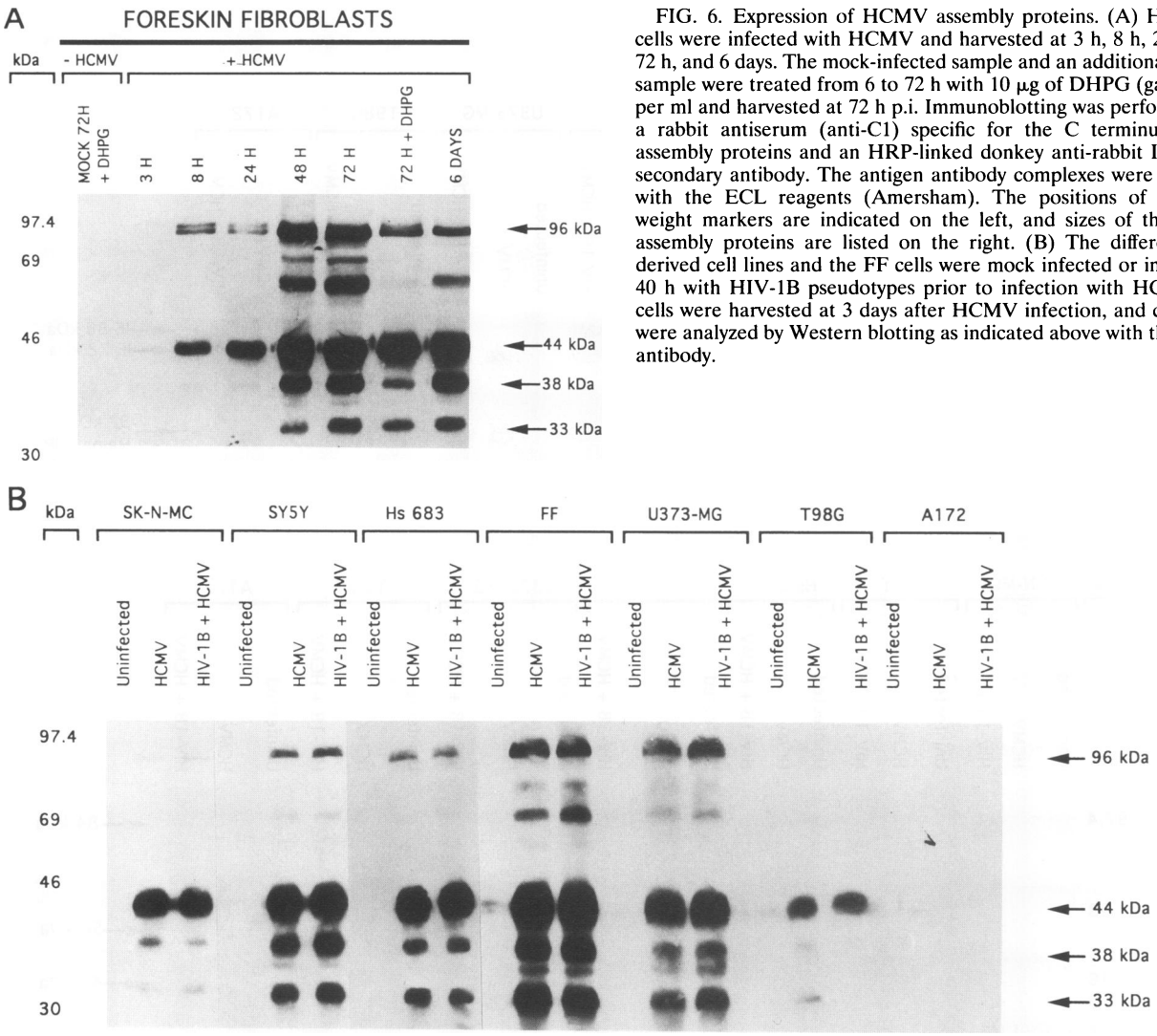

FIG. 6. Expression of HCMV assembly proteins. (A) Human FF cells were infected with HCMV and harvested at 3 h, 8 h, 24 h, 48 h, 72 h, and 6 days. The mock-infected sample and an additional infected sample were treated from 6 to 72 h with 10 μg of DHPG (ganciclovir) per ml and harvested at 72 h p.i. Immunoblotting was performed with a rabbit antiserum (anti-C1) specific for the C terminus of four assembly proteins and an HRP-linked donkey anti-rabbit IgG as the secondary antibody. The antigen antibody complexes were visualized with the ECL reagents (Amersham). The positions of molecular weight markers are indicated on the left, and sizes of the HCMV assembly proteins are listed on the right. (B) The different brain-derived cell lines and the FF cells were mock infected or infected for 40 h with HIV-1B pseudotypes prior to infection with HCMV. The cells were harvested at 3 days after HCMV infection, and cell lysates were analyzed by Western blotting as indicated above with the anti-C1 antibody.

neuroblastoma cells, but the amount of DNA synthesized by 72 h p.i. was at least fivefold lower than in the above cell lines. In the A172 and T98G undifferentiated glioblastoma cells, although DNA from input virus could be seen at 8 h p.i., there is no evidence for replication of the DNA. In fact, the amount of DNA present at 72 h p.i. was considerably lower than that seen at 8 h p.i., probably as a result of degradation or loss during cell division.

Release of infectious HCMV into the supernatant. To determine whether the various levels of HCMV proteins detected in these cells correlated with virus production, we assayed the amount of infectious HCMV released into the supernatant of the cells at 3, 5, and 7 days p.i. (Table 2). As expected, no infectious HCMV particles were detected in the supernatant of the A172 cells, and only very low levels of HCMV were released into the supernatant of the T98G cells, with titers ranging from 200 PFU/ml at 3 days p.i. to 10 PFU/ml at 7 days p.i. The release of HCMV from the SK-N-MC cells was slightly higher, ranging from 300 PFU/ml at 3 days p.i. to 900 PFU/ml at 5 days p.i. Peak HCMV titers in the supernatants from the SY5Y, Hs 683, and U373-MG cells were 7- to 20-fold higher than those from the SK-N-MC cells, but these titers were still over 100-fold lower than those obtained from the infected FF cells. Thus, even in the case of the Hs 683, U373-MG, and SY5Y cells, in which HCMV viral DNA replication and protein synthesis are comparable to that in the fully permissive FF cells, the release of infectious virus into the supernatant is greatly diminished. Preliminary experiments, however, suggest that most of the infectious virus in these brain-derived cell lines remains cell associated (data not shown).

DISCUSSION

Infection of brain-derived cell lines by HIV-1. The many reports that only low levels of HIV-1 are produced following infection of brain-derived cells have led to the hypothesis that in these cells, the virus may persist in an indolent or latent form rather than being engaged in a fully productive infection. However, it is not yet clear whether the restriction observed in brain-derived cells is caused by inefficient entry due to the lack of the CD4 receptor or by limiting cellular factors required to support a fully productive infection. Even in experiments in which clones of persistently infected cells have been isolated, it

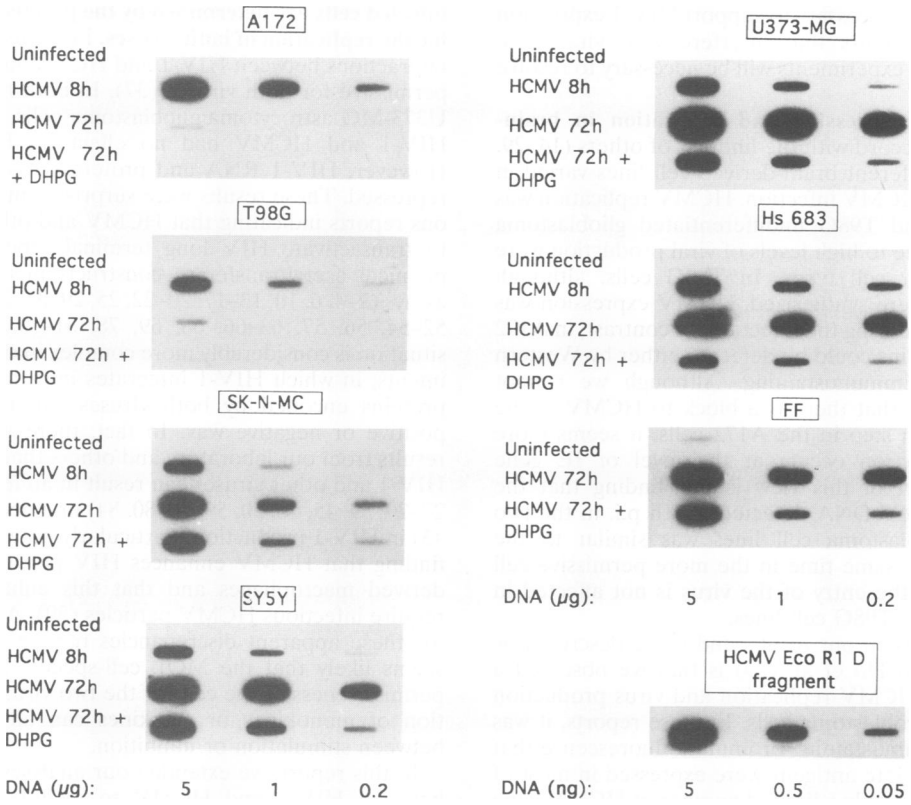

FIG. 7. HCMV DNA replication. Total DNA was harvested from uninfected cells and from HCMV-infected cells at 8 h (early times) or 72 h (late times) after HCMV infection. DNA was also extracted at 72 h from HCMV-infected cells maintained in the presence of 10 μg of DHPG (ganciclovir) per ml. The samples were slot blotted onto nitrocellulose filters and hybridized with ^{32}P-labeled HCMV strain AD169 *Eco*RI fragment D. The amount of DNA loaded in each slot is indicated at the bottom. As a control, indicated amounts of HCMV *Eco*RI fragment D were also analyzed in a similar manner.

remains possible that selection against cells producing high levels of virus may have occurred because of their reduced proliferative ability. Relevant to this question is the report that infection of glioma cells by HIV-1 appears to have an adverse effect on cell growth (7).

Previously, we showed that HIV-1 (amphotropic retrovirus) pseudotypes could bypass the restriction of HIV-1 infection at the adsorption/penetration step in a number of CD4-negative cell lines (73), and thus with these pseudotypes, we could begin to address questions relating to the intracellular events modulating the course of HIV-1 infection. In this study, we extended our analysis to include additional CD4-negative brain-derived cell lines and demonstrated that all were permissive for HIV-1B pseudotype entry, whereas HIV-1 infection was partially or totally blocked at the adsorption/penetration step. Only in the SK-N-MC cells was there any evidence of sustained virus production following infection with the nonpseudotyped HIV-1. In contrast, a high level of productive infection was maintained in five of the six brain-derived cell lines infected with the HIV-1B pseudotypes. Therefore, it seems likely that the major block to HIV-1 infection in these CD4-negative brain-derived cells results from a restriction of HIV entry and not from an indolent, nonproductive infection.

The SY5Y neuroblastoma cell line, however, did present a strong restriction for HIV-1 production and gene expression. In these cells, no virus was detected after infection with nonpseudotyped HIV-1, whereas HIV-1B pseudotype infection was characterized by a low level of viral production. Virus could be detected in the supernatants of the HIV-1B-infected cells, although the level of viral replication was below the limits of detection by immunostaining. However, because we did not observe a minor population of cells expressing a high level of HIV-1 antigen after infection with HIV-1B pseudotypes, it is likely that the majority of the cells are infected but HIV-1 expression is restricted. It is possible that the SY5Y cells lack

TABLE 2. Release of HCMV from infected cells[a]

Cells	HCMV virus production (PFU/ml)		
	Day 3 p.i.	Day 5 p.i.	Day 7 p.i.
Undifferentiated glioblastoma			
A172	0	0	0
T98G	2.0×10^2	1.0×10^2	10
Neuroblastoma			
SK-N-MC	3.0×10^2	9.0×10^2	7.0×10^2
SY5Y	$<1.0 \times 10^2$	3.0×10^3	6.5×10^3
Astrocytoma/glioblastoma			
U373-MG	3.0×10^2	1.9×10^4	1.7×10^4
Hs 683	4.0×10^2	6.0×10^3	1.3×10^4
FF	$>2.5 \times 10^2$	6.5×10^5	2.3×10^6

[a] The different brain-derived cell lines and the FF cells were infected with HCMV at an MOI of 5 PFU per cell. At various times p.i., the supernatants were collected, and the titer of infectious HCMV present in the medium was determined by plaque assay on FF cells.

the intracellular factors necessary to support HIV-1 expression or contain negative factors that interfere with viral gene expression. Additional experiments will be necessary to resolve this question.

HCMV viral gene expression and replication in brain-derived cell lines. In accord with the findings of others (14, 29, 60), we found that different brain-derived cell lines varied in the ability to support HCMV infection. HCMV replication was restricted in A172 and T98G undifferentiated glioblastoma cells, whereas moderate to high levels of viral production were detected in the other cell types. In T98G cells, although HCMV IE proteins were synthesized, HCMV expression was blocked at early times during the infection. In contrast, in A172 cells, no HCMV proteins could be detected either by Western blot analysis or by immunostaining. Although we cannot exclude the possibility that there is a block to HCMV at the adsorption/penetration step in the A172 cells, it seems more likely that the restriction occurs at the level of IE gene expression. In support of this view is our finding that the amount of input HCMV DNA detected at 8 h p.i. in the two undifferentiated glioblastoma cell lines was similar to the amount present at the same time in the more permissive cell lines, suggesting that the entry of the virus is not affected in either the A172 or the T98G cell lines.

One difference between our results and those described by Poland et al. (60) and Ho et al. (29) is that we observed a partial restriction of HCMV replication and virus production in the SK-N-MC neuroblastoma cells. In these reports, it was demonstrated by immunostaining or immunofluorescence that HCMV IE, early, and late antigens were expressed in most of the SK-N-MC cells, but only a limited number of HCMV gene products were assayed. With an expanded set of immunological reagents, however, we found that there was a major block in the synthesis of the family of early proteins encoded by the 2.2-kb transcripts. Although the function of these proteins is still unknown, we have proposed previously that they might play a role in the synthesis of delayed-early or late RNA transcripts or in the replication of viral DNA on the basis of their nuclear localization, the timing of their synthesis, and their regulation by transcriptional and posttranscriptional mechanisms (72, 75, 76, 90, 91). In support of this view, we find that in the SK-N-MC cells, both DNA replication and the synthesis of the HCMV late gene products appeared to be less efficient than in the more permissive cell lines. It is tempting to speculate that the lack of the early proteins encoded by the 2.2-kb transcripts is of some importance in the restriction observed in these later events, but at present we do not have direct evidence for this hypothesis.

In the more permissive brain-derived cell lines U373-MG, Hs 683, and SY5Y, no major block in the HCMV viral cycle was detected, but the infectious titers were approximately 100- to 1,000-fold lower than those obtained following infection of FF cells. This lower level of viral release does not appear to be due to a block in HCMV protein synthesis, as Western blot analysis with serum from an HCMV-seropositive individual showed that the pattern of early and late proteins was similar to that in FF cells (data not shown). Alternatively, the reduced efficiency of viral release may result from differences in the processing and the egress of HCMV late antigens through the Golgi apparatus in these cells as previously described for the U373-MG cell line (33). We have also noted that most of the virus produced in the brain-derived cell lines remains cell associated, whereas approximately 50% of the infectious viral particles are released in the supernatant of the HCMV-infected FF cells (data not shown).

The interactions between HCMV and HIV-1 in dually infected cells are determined by the permissiveness of the cells for the replication of both viruses. Previously, we described the interactions between HIV-1 and HCMV in two cell types fully permissive for both viruses (37). In both human FF cells and U373-MG astrocytoma/glioblastoma cells, dual infection with HIV-1 and HCMV had no effect on HCMV replication. However, HIV-1 RNA and protein synthesis were markedly repressed. These results were surprising in view of the numerous reports indicating that HCMV and other viruses are able to transactivate HIV long terminal repeat (LTR)-chloramphenicol acetyltransferase constructs in transient expression assays (2–4, 6, 10, 13–17, 20–22, 25, 29–31, 34–37, 40, 41, 47–49, 52–54, 56, 57, 63–66, 68, 69, 78, 81–83, 85). However, the situation is considerably more complex in dual-infection experiments, in which HIV-1 integrates into the host genome and proteins encoded by both viruses can interact in either a positive or negative way. In fact, there are even conflicting results from our laboratory and others that dual infection with HIV-1 and other viruses can result in an increase (1, 9, 12, 24, 27–29, 39, 45, 46, 50, 59, 70, 80, 84) or a decrease (8, 26, 32, 37, 43) in HIV-1 production. Particularly noteworthy is our recent finding that HCMV enhances HIV production in monocyte-derived macrophages and that this enhancement does not require infectious HCMV particles (39). Although the reasons for these apparent discrepancies remain to be elucidated, it seems likely that the MOI, cell-specific factors, the relative permissiveness of the cell for the two viruses, and the production of monokines or cytokines can all affect the balance between stimulation or inhibition.

In this report, we extended our analyses of the interactions between HIV-1 and HCMV to include a range of brain-derived cells that differ in the ability to support HCMV infection. Our studies show that following dual infection of these cells with HCMV and HIV-1B pseudotypes, HCMV gene expression and virus production proceed normally, but HIV-1 replication shows one of the following patterns.

First, in cell types that were fully permissive for replication of the HIV-1B pseudotypes and HCMV, including the Hs 683 and U373-MG astrocytoma/glioblastoma cell lines and human FF cells, we found that both HIV-1 protein expression and viral production were inhibited in the dually infected cells. Previously, we showed that inhibition of HIV-1 production in FF cells requires HCMV gene expression but not DNA replication, suggesting that the subsequent events were not implicated in the inhibition process (37). This notion is further supported by our observations that the two astrocytoma/glioblastoma cell lines appear to be as permissive as the FF cells with respect to HCMV IE and early gene expression and viral DNA replication but do release greatly reduced levels of infectious particles into the supernatant.

The second pattern was observed in cells that were permissive for the HIV-1B pseudotypes but were restricted for the synthesis of HCMV IE or early gene products. In the A172, T98G, and SK-N-MC cells, the presence of HCMV had no effect on HIV-1 gene expression or virus production. Our observations differ somewhat from those of Ho et al. (29), who reported a two- to threefold increase of HIV-1 production in SK-N-MC cells infected for 2 days with HCMV prior to superinfection with HIV-1. We also have noted a small stimulation (less than twofold) of HIV-1 production when these cells are dually infected with HCMV and nonpseudotyped HIV-1. However, even a low level of stimulation has not been seen in multiple dual-infection experiments involving HIV-1B pseudotypes in these cells (data not shown). We do not believe that this difference is related to the presence of the amphotropic retrovirus in the supernatant of HIV-1B pseudotypes or

to the strain of HIV-1 used in our experiments. In preliminary experiments with SK-N-MC neuroblastoma cells and Hs 683 and U373-MG astrocytoma/glioblastoma cells containing a stably integrated HxB2D HIV-1 genome, HCMV superinfection inhibits HIV-1 production in both astrocytoma/glioblastoma cell lines but has no effect on HIV-1 gene expression in the SK-N-MC cells (32). It seems more likely that the differences observed during dual infection involving HIV-1 or HIV-1B pseudotypes are a consequence of the relative number of cells infected. Only a small fraction of the cell population becomes infected by nonpseudotyped HIV-1, whereas each cell in the population contains the HIV-1 genome when they are infected by HIV-1B pseudotypes or when the cells are established with an integrated HxB2D genome. It is possible that HCMV infection leads to induction of some plasma membrane modifications or an increase in the availability of an alternative receptor for nonpseudotyped HIV-1, thus facilitating HIV-1 entry. Such effects would result in an increase in the number of cells infected by HIV-1 but not in the level of HIV-1 protein expression in a given cell.

Of particular interest was the third type of effect, which was observed in the SY5Y neuroblastoma cell line. Cells infected with the HIV-1B pseudotypes alone expressed only low levels of HIV-1 antigens. However, in cells dually infected with the HIV-1B pseudotypes and HCMV, HIV-1 virus production was stimulated between 1 and 3 days after HCMV infection and then was inhibited as the HCMV infection progressed.

Although the molecular mechanisms responsible for these effects remain to be elucidated, we propose the following hypothesis. At early times in the HCMV infection, the IE gene products can stimulate HIV-1 gene expression, including the production of the regulatory proteins Tat and Rev. Alternatively, it may even be possible that the simple interaction of an HCMV particle (infectious or noninfectious) with a cell results in the enhancement of HIV infection as we have observed in monocyte-derived macrophage (39). As the concentrations of these HIV-1 regulatory proteins rise, the overall level of HIV-1 gene expression also increases. If the cells are permissive for HCMV infection, then once the HCMV early gene products reach a critical level, inhibition of HIV-1 gene expression ensues. Given the results of many transient expression assays from both our laboratory and others (2–4, 6, 13, 22, 38, 49, 52, 57, 63, 64, 85), it seems likely that the HCMV IE gene products do have the potential to stimulate HIV-1 gene expression. If, however, virus-cell interactions or the IE proteins are implicated in the activation of HIV-1 expression in SY5Y cells, then one might ask why a similar stimulation is not observed in the other brain-derived cell lines. Since our experiments address only the net effect of the dual infection on HIV-1 expression in these cells, it may be that there is a transitory stimulation, but its duration is so short that it is not easily detected. Alternatively, it is possible that in cells fully permissive for HIV-1 gene expression, the transactivation mediated by Tat obscures any stimulatory activity of the HCMV on the integrated HIV-1 genome.

In support of this latter possibility, preliminary data from our laboratory indicate that the transactivation of an integrated HIV-1 LTR by HCMV infection is significantly less than that mediated by HIV-1 (38). The opposite effect is observed, however, when the HIV-1 genome or an HIV-1 LTR-chloramphenicol acetyltransferase construct is introduced into a cell by transient transfection; i.e., the stimulatory activity of HCMV is greater than that of HIV-1 (37, 38). Although these results are preliminary, they may explain the seemingly contradictory conclusions drawn from transient expression assays and dual-infection experiments. At this point, we do not know whether HCMV interferes with Tat-mediated activation of the integrated HIV-1 genome or whether some other HIV-1 gene product interacts with an HCMV gene product to mediate the inhibitory effect. Further studies addressing the role played by HIV-1 regulatory proteins in conjunction with the early events of HCMV infection will be required to decipher the complex mechanisms of stimulation and inhibition observed in these cells.

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