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Unrestricted Replication of Human Cytomegalovirus in Hydrocortisone-Treated Macrophages

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Monocytes differentiated in the presence of phytohemagglutinin P-stimulated T cells could be infected with human cytomegalovirus AD169 and produced low levels of infectious virus. Additional treatment with therapeutic levels of hydrocortisone resulted in a 10- to 100-fold increase in infectious virus production. Hydrocortisone-treated cells demonstrated immediate-early protein kinetics similar to that observed with human fibroblasts, whereas a delay of up to 24 h was observed with untreated cells. Late protein production was barely detectable by immunostaining without hydrocortisone treatment. In treated cells, however, late protein was detected and the levels correlated with the number of cells producing infectious virus. This system provides a model for human cytomegalovirus infection of macrophages in humans.

Human cytomegalovirus (HCMV) is a common cause of severe disease in immunocompromised patients, particularly those undergoing organ transplantation or with AIDS (1, 11). The virus can be either reactivated or transmitted via the cellular component of blood or inflammatory cells in a transplanted organ (1, 6, 14). Through in situ hybridization studies, viral nucleic acid has been detected in the monocytes and mononuclear cells of healthy seropositive individuals (14, 18). Viral RNA detected in these studies was of the immediate-early (IE) class (18). In addition, monocytes and mononuclear cells from viremic patients contain transcripts representing early and/or late (L) genes and produce infectious virus (2, 17, 23). Thus, the monocyte has been suggested as a possible reservoir for HCMV from which reactivation and dissemination can occur (14).

The life cycle of HCMV is controlled by the cascade regulation of the genome. IE protein synthesis precedes early polypeptide synthesis, and both are required for L protein synthesis and production of mature infectious virions (9). Several in vitro studies have reported that replication in monocyte-derived macrophages (macrophages) is restricted to IE protein expression (3, 16). Depending on the isolate of HCMV used, 0 to 15% (mean, 2 to 3%) of macrophages expressed IE but not L proteins. Clinical isolates produced 10- to 100-fold more IE-positive cells than AD169. However, in vitro studies have been unable to duplicate the switch from restricted replication as observed in asymptomatic individuals to active replication as seen in viremic patients.

The restriction on HCMV replication in vitro has been lifted in other cell systems by differentiation of the cell. Teratocarcinoma cells become permissive for HCMV after differentiation with retinoic acid (8). THP-1, a monocytic cell line, allowed a full cycle of viral replication following treatment with 12-*O*-tetradecanoylphorbol-13-acetate (25). In vivo, particularly following organ transplantation, a chemically induced immunosuppression is associated with HCMV replication and dissemination (24). Hydrocortisone (HC) is one of the components of this therapy associated with increased HCMV disease following organ transplantation (24). Additionally, in vitro human foreskin fibroblasts (HFF) and embryonic kidney cells treated with HC produced more infectious virus than did untreated cells (4, 12, 13, 22). Thus, for the development of an in vitro system for the replication of HCMV in monocytes, we hypothesized that both cellular differentiation and a chemical inducer of immunosuppression, like HC, would be most successful. We report here that T-cell-differentiated, HC-treated macrophages allow unrestricted HCMV replication resulting in the switch from IE to L viral protein production and the release of infectious virus.

Infection of macrophages with HCMV. In preliminary experiments we, like others (3, 16), were unable to detect the production of infectious HCMV from recently isolated monocyte-derived macrophages. Therefore, monocytes were differentiated in the presence of phytohemagglutinin P-stimulated autologous T cells. Peripheral blood mononuclear cells from HCMV-seronegative donors were suspended at a concentration of 2×10^6/ml in RPMI 1640 with glutamine, 20% fetal bovine serum, and penicillin (100 U/ml)–streptomycin (100 μg/ml) (medium). Peripheral blood mononuclear cells were stimulated for 3 days with phytohemagglutinin P (3 μg/ml of medium), nonadherent cells were removed, and adherent cells fed medium without phytohemagglutinin P. After 3 to 4 weeks of culture, cells were vigorously washed three times with phosphate-buffered saline, scraped from the flasks into cold medium, and readhered to tissue culture dishes. At this time the cells were 100% esterase positive (Fig. 1), >99% CD 11b (Mac-1) positive (CD 11b [MO-1; Coulter, Hialeah, Fla.] is a macrophage marker), and <0.1% CD 3 positive (CD3 [T3; Coulter] is a T-cell marker).

Increased replication of HCMV has previously been observed both in vivo and in vitro in the presence of HC (4, 12, 13, 22, 24). Because monocytes are sensitive to the effects of HC (5, 15, 19–21, 27), it was added to our culture system. The HC concentration most effective in increasing HCMV replication was determined by a dose response. Macrophages pretreated for 18 h and maintained with HC (Sigma, St. Louis, Mo.) (10^{-4} to 10^{-7} M) or no HC were infected at a multiplicity of infection (MOI) of 0.2 with supernatant-derived HCMV AD169, which had been passed through a 0.2-μm-pore-size filter and frozen in 1% dimethyl sulfoxide. At 7 days postinfection, supernatant was harvested for viral titration by plaque assay (26) and cells were harvested for

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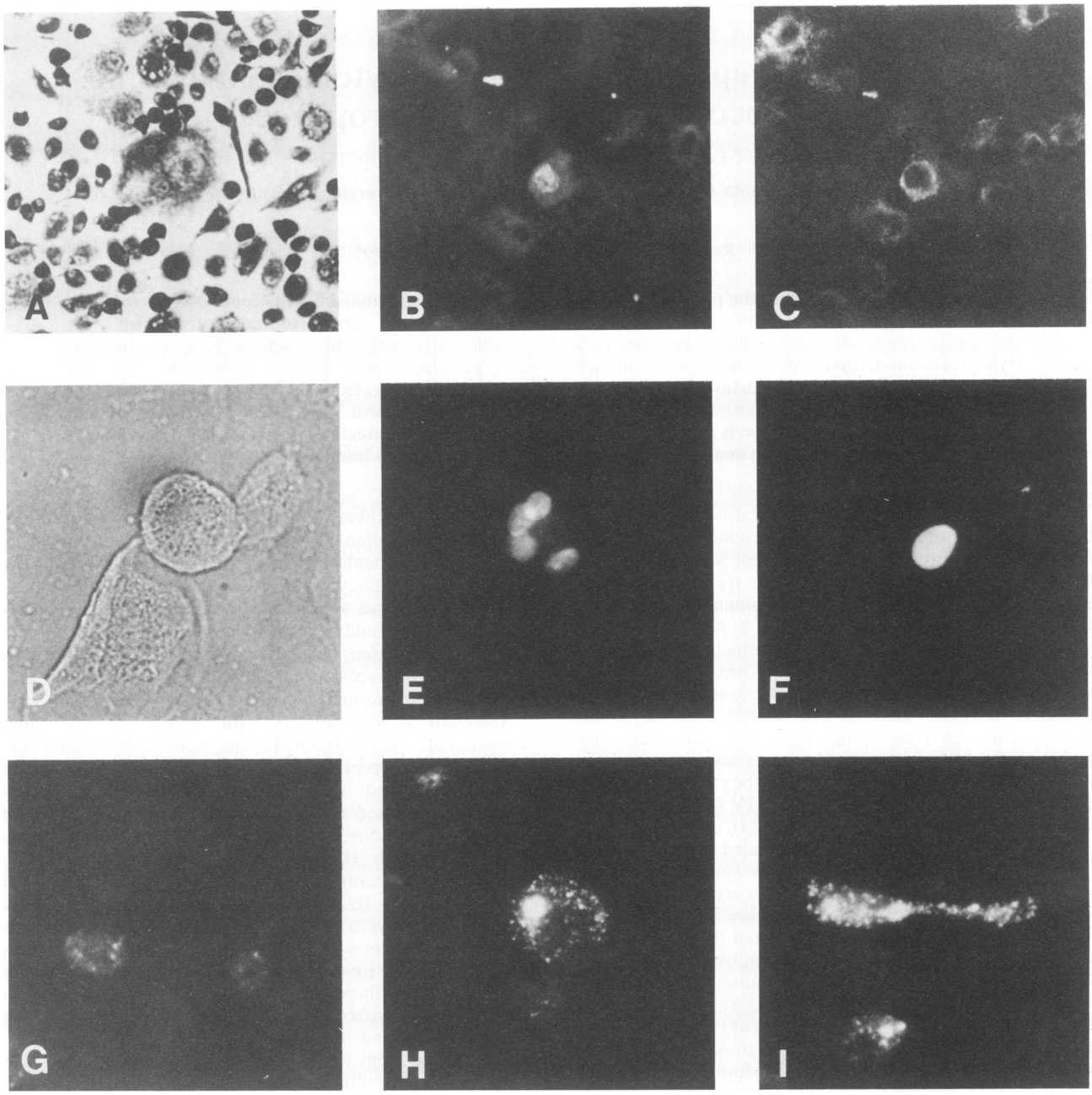

FIG. 1. Immunofluorescent analysis of HCMV-infected macrophages. Macrophages treated with HC were infected with supernatant-derived HCMV AD169. Slides were harvested at day 7 postinfection. IFA was performed with antibodies that reacted with CD 11b, with 72-kDa IE antigen, or with the 80-kDa L antigen. Secondary antibody was anti-mouse fluorescein isothiocyanate- or rhodamine β isothiocyanate-conjugated antibody (CD11b). (A) Esterase stain of uninfected cells; (B) HCMV IE antigen plus fluorescein isothiocyanate; (C) macrophage marker CD 11b plus rhodamine β isothiocyanate; (D) light microscopy of multinucleated cell from panel E; (E and F) IE antigen; (G) normal mouse immunoglobulin G plus fluorescein isothiocyanate; (H and I) L antigen. Magnification, ×200 (A) and ×400 (B through I).

immunofluoresent antibody assay (IFA) (Table 1). Viral staining was performed on acetone-fixed cells. The antibodies used included an anti-IE antibody (Dupont, Boston, Mass.) which binds the 72-kDa major IE protein and C5 antibody (7) (Syva, Palo Alto, Calif.), which binds an 80-kDa L protein. There were slight increases in protein expression and infectious virus production with 10^{-7} and 10^{-6} M HC. However, IE and L protein expression as well as infectious virus production were 5- to 10-fold above these levels when the concentration of HC used was increased to 10^{-5} M. A dose of 10^{-4} M was toxic to the macrophages.

Following the same procedures as the previous experiment, the optimum dose of 10^{-5} M HC which was used in all subsequent experiments, was compared with untreated macrophages. Cells and supernatant were harvested at 7 days postinfection, diluted, and plaque assayed on HFF (Table 1).

TABLE 1. Dose response of HCMV replication to HC enhancement

HC treatment (M)	Expt 1[a] results			Expt 2[b] results	
	% IFA-positive cells		Infectious virus (10^2/ml)	No. of infected cells/10^5 cells	Infectious virus (10^2/ml)
	IE	L			
10^{-5}	1.7	0.5	17.5	1,000	10.0
10^{-6}	0.14	0.10	2.50		
10^{-7}	0.06	0.01	1.00		
None	<0.01	<0.01	<0.5	1.00	0.10

[a] A total of 10^5 macrophages, treated with 10^{-4} to 10^{-7} M HC or untreated, were infected with supernatant-derived HCMV AD169 (MOI = 0.2). On day 7 postinfection, supernatant and cells were harvested for plaque assay and IFA, respectively. The 10^{-4} M HC was toxic for the macrophages.

[b] A total of 10^5 macrophages, treated with 10^{-5} M HC or untreated, were infected as described above. On day 7 postinfection, cells and supernatant were harvested for plaque assay.

In macrophages without HC, infected cells were detected at a frequency of 1 cell per 10^5 and supernatant virus equalled 10 infectious virions per ml. With the addition of HC, the frequency of infected cells increased to 1,000 cells per 10^5 total cells and 10^3 infectious virions per ml were released into the supernatant. Infected cells were also double stained for the macrophage marker CD 11b and IE protein of HCMV (Fig. 1), further demonstrating that replication was occurring in the macrophage.

The macrophage culture consisted of three morphologically distinct cell types: multinucleated giant, single round, and single elongated (Fig. 1). In order to determine whether one of the cell types was preferentially infected by HCMV, macrophages were scraped from their original flask and readhered to chambered culture slides. HC-treated cells were then infected with AD169 (MOI = 10) and harvested after 7 days. All three cell types, multinucleated giant, single round, and single elongated cells, were positive for HCMV proteins (Fig. 1). However, even in the presence of viral replication there was no cytopathic effect.

Kinetics of infectious virus and protein production. Growth curves were measured with macrophages, both with and without HC treatment. To ensure that all susceptible cells became infected and to facilitate the quantitation of IFA-positive cells, these and subsequent cultures for IFA were infected with an MOI of 10, 50-fold more virus than was used in the initial experiments (Table 1). Results are reported per 10^5 cells, and data representative of two to three experiments are shown. Macrophage cultures were infected with HCMV. Then supernatant was obtained for plaque assay, and cells were harvested for IFA every 2 to 3 days for 14 days. HFF were infected concurrently for IFA controls. Macrophages treated with HC obtained peak virus titers of 4×10^3 at day 7 postinfection, followed by a reduction to 2×10^2 at days 9 to 11. In contrast, macrophages without HC consistently produced 10 infectious virus particles throughout the 14-day period (Fig. 2). Thus, a peak of acute HCMV replication was observed in HC-treated but not untreated cells.

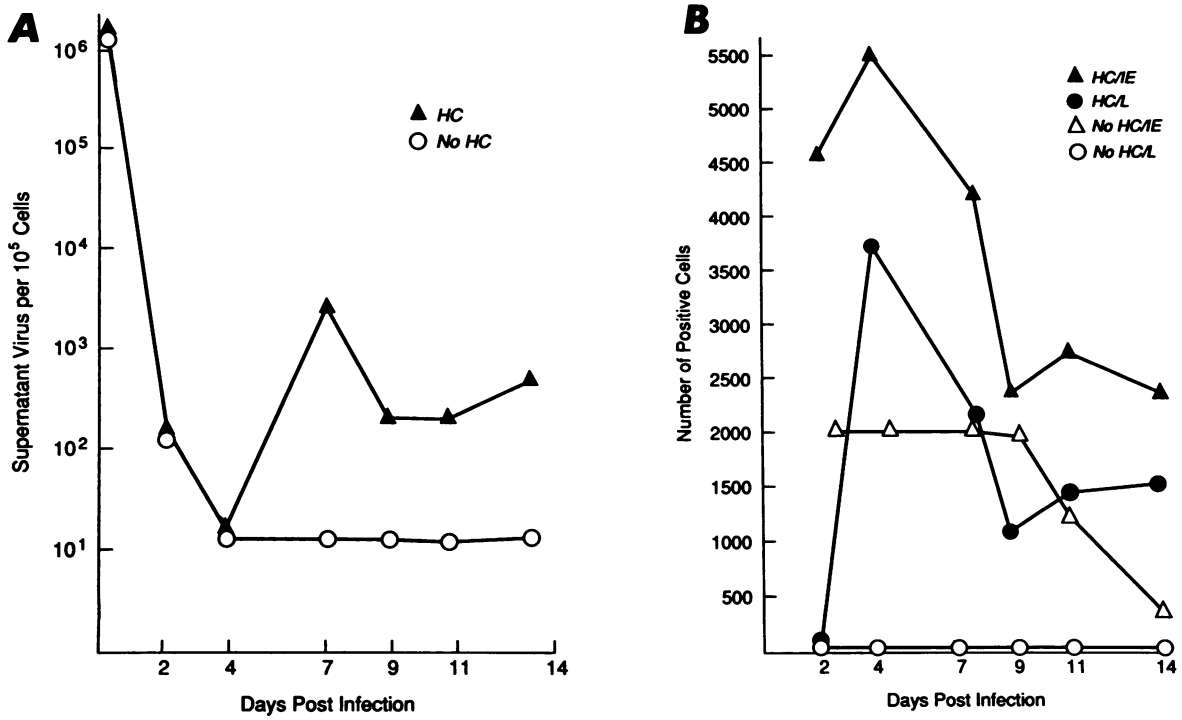

FIG. 2. Kinetics of HCMV replication in macrophages grown with and without HC. A total of 10^5 cells were infected with supernatant-derived HCMV AD169 (MOI = 10). Supernatant and cells were harvested on days 2, 4, 7, 9, 11, and 14 postinfection. Supernatant was assayed by plaque assay on HFF cells (A), and cells were assayed by IFA for IE and L proteins (B). The total number of positive cells was calculated as percent positive cells × total cells per well. HFF were positive for both IE and L proteins by 2 days postinfection. (B) HC-treated macrophages positive for IE (HC/IE) and L (HC/L) proteins and untreated macrophages positive for IE (No HC/IE) and L (No HC/L) proteins.

All HCMV-infected cells in HFF cultures were positive by IFA for both IE and L proteins on day 2 and remained positive throughout the culture period until cells were destroyed by the cytopathic effect of replicating virus (data not shown). Macrophages treated with HC were positive for IE proteins at day 2 postinfection, reaching a peak between days 4 and 7 postinfection. L protein was not observed in HC-treated macrophages until 4 days postinfection. The total number of positive cells was also highest on that day (Fig. 2). Although L-protein production was delayed compared with that in HFF, it did occur, providing additional evidence for HCMV replication in HC-treated macrophages during a 14-day culture period. In macrophages without HC, there was no evidence of acute HCMV replication. A constant level of IE expression was maintained from day 2 through day 9 postinfection, followed by a decline. Only minimal L-protein expression was observed from 4 days postinfection through the 14-day period. The total number of L-protein-positive cells ranged from 1 to 7 cells per 10^5 cells (Fig. 2). Without HC treatment, macrophages initially expressed IE proteins which decayed with time, and only a few cells were fully permissive, allowing for L-antigen expression. Therefore, the protein kinetics of IE- and L-protein expression support the growth curve. In HC-treated macrophages the peak observed for both IE and L protein production occurred just before the increase in infectious virus release. The low level of chronic viral release observed for untreated macrophages can be explained by the very low numbers of cells producing L protein and the decay of IE protein. In an additional experiment, when cultures were assayed over a 28-day period, no subsequent peaks of viral replication or protein expression were observed for either culture system, but titers never dropped below 10 (data not shown). Thus, HC treatment appears to make a subpopulation of macrophages permissive to HCMV replication, permitting both primary replication and a chronic productive infection. This is analogous with the in vivo situation, in which even during a disseminated infection only a subpopulation of macrophages appears to be infected with HCMV (2, 17, 23).

Kinetics of IE-protein expression. In order to further investigate differences in the early kinetics of IE-protein production, cells were infected as described above and assayed for IE-protein expression at 8, 24, 48, 72, and 96 h postinfection. All HFF were positive for IE protein by 8 h postinfection and remained positive through the 96-h period (data not shown). Macrophages treated with HC were also positive for IE at 8 h at a low level and remained positive through the period (Fig. 3). The highest percentage of positive cells was much lower (8.6% macrophages versus 100% HFF), but the kinetics of expression was similar for HC-treated macrophages and fibroblasts. However, in macrophages without HC, delayed detection of IE proteins was observed. IE proteins were not observed in macrophages without HC until 24 h postinfection, at which time 0.6% (increasing to a maximum of 2.3%) of the cells were positive (Fig. 3). Therefore, HC treatment and not T-cell differentiation alone was able to accelerate IE-protein expression in macrophages to the point observed in a permissive cell like the HFF. In contrast, HC was unable to increase the number of macrophages expressing IE protein to that observed in fibroblasts.

HC requirement. To determine whether HC was required before viral infection for enhancement of HCMV replication, HC was added to macrophages at days −1, 0, and +3 postinfection and maintained through the culture period. At 14 days postinfection, all cells were assayed for expression

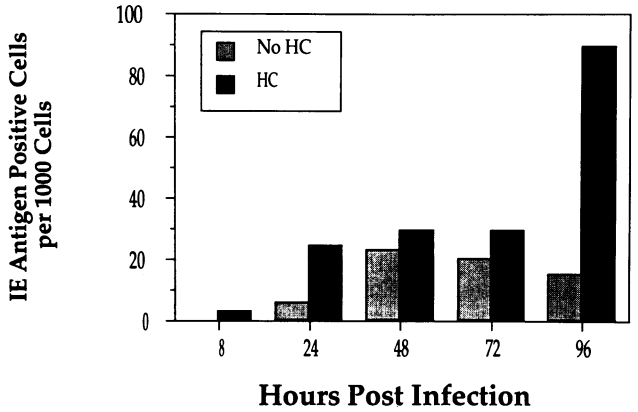

FIG. 3. Kinetics of IE antigen expression. A total of 10^5 macrophages were cultured with or without HC on individual wells of tissue culture slides and then infected with HCMV AD169 (MOI = 10). At 8, 24, 48, 72, and 96 h postinfection, cells were harvested, fixed in cold acetone, and then assayed by IFA for IE proteins. HFF were positive at 8 h.

of IE and L proteins by IFA. All HC-treatmented cells demonstrated an enhancement in HCMV expression compared with expression in untreated cells. Macrophages without HC contained 1.5% IE-protein-positive cells and no detectable L protein. The percentages of macrophages expressing IE and L proteins in cultures either pretreated for 1 day or treated after a 3-h adsorption period were equivalent: 9 and 4%, respectively. In macrophages treated with HC 3 days postinfection, a median increase was observed with 4% IE-protein-positive and 2% L-protein-positive cells. Thus, it was not necessary for cells to be pretreated with HC to allow unrestricted HCMV replication. HC added at up to 3 days postinfection could enhance HCMV antigen expression.

The mechanism by which HC enhances HCMV replication in macrophages is unclear. Glucocorticoid hormones like HC have been reported to have wide-ranging effects on macrophages in vitro. The production and/or release of inflammatory molecules, including interleukin-1, interleukin-6, tumor necrosis factor α, superoxide anions, and CFU-granulocyte macrophage colony-inhibitory molecules, is inhibited (15, 19, 21, 27). Cell surface expression of HLA-DR and the receptor for gamma interferon is enhanced (5, 20). Differentiation of mouse myeloid leukemia cells following HC treatment has also been reported (10). Thus, it is possible that HC treatment indirectly enhances HCMV replication in macrophages by altering cellular function and changing the viral-cellular interactions to favor viral replication. Alternatively, HC or physiological effects in response to HC may have direct effects on HCMV replication. Increased titers of HCMV during HC treatment of permissive cells have been reported (4, 12, 22). The addition of HC after virus infection can enhance HCMV replication (12, 22). Also, we have observed elevated levels of IE and early/late mRNAs (hybridized to *Eco*RI fragments J and D, respectively) between 1 and 3 days postinfection in HC-treated permissive HFF compared with levels in untreated HFF (unpublished observations). HC-receptor complexes may bind directly to HCMV DNA, or second messengers produced as the result of HC stimulation may interact with viral nucleic acid (DNA or RNA). Experiments are in progress to examine HC-macrophage-HCMV interactions.

Our in vitro data support the in vivo observation that

HCMV replication and disease are increased following immunosuppressive therapies using glucocorticoids during transplantation (1, 24). Macrophages, which under normal conditions are semipermissive for HCMV, are capable of acute replication during HC treatment. Thus, the cellular host range of HCMV is expanded and the total number of cells producing virus is increased. Because HCMV does not appear to be lytic for macrophages, HCMV-infected macrophages may also participate in the dissemination of HCMV which can occur during immunosuppressive therapy following organ transplantation (1).

In summary, our experiments demonstrate that HCMV can replicate in macrophages in vitro and that this replication is enhanced by HC treatment of cells. HC increases the number of cells expressing L protein and infectious virus. Acceleration of IE-protein production, to the time point observed in HFF cells, also occurred as the result of HC treatment. L protein expression, however, was still delayed compared with that in HFF cells. The overall replication observed, therefore, has a different pattern than that previously reported for HFF. These differences observed in viral replication may have important implications regarding HCMV pathogenesis and suggest that generalizations regarding HCMV replication in fibroblasts may not always be applicable to in vivo situations. The system described here will permit these questions to be investigated in macrophages.

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