Novel Activation of γ-Interferon in Nonimmune Cells during Human Cytomegalovirus Replication (44114)

I. BOLDOGH,[1] T. K. BUI,[2] P. SZANISZLO, W. A. BRESNAHAN, T. ALBRECHT, AND T. K. HUGHES
Department of Microbiology and Immunology, University of Texas Medical Branch at Galveston, Galveston, Texas 77555-1019

Abstract. **This is the first study documenting the induction of γ-interferon (IFN-γ) in human embryonic fibroblasts during human cytomegalovirus (HCMV) replication. Infection of cells with HCMV resulted in the consistent production of IFN-γ RNA, as determined by RT-PCR and Northern blot analysis. Western blot analysis of cell lysates and immunoprecipitates from the cultural fluids of infected cells demonstrated the presence of IFN-γ at the protein level. Induction of IFN-γ required infectious HCMV, since high-dose ultraviolet inactivation of the virus stock eliminated IFN-γ production. Further, IFN-γ induction appears to be a late event in the virus replication cycle, since inhibition of HCMV DNA synthesis (e.g., phosphonoacetic acid) blocked the increase in IFN-γ. Soluble factor(s) released from HCMV-infected cells apparently did not contribute to the induction of IFN-γ, since virus stocks from which virus had been removed by sedimentation did not induce production of IFN-γ. The appearance of IFN-γ at late stages of HCMV infection and its elimination in the presence of an inhibitor (Actinomycin D) of RNA synthesis indicate a true transcriptional induction of this lymphokine at the RNA and protein levels. The significance of IFN-γ production with regard to the replication and pathogenesis of HCMV *in vitro* and *in vivo* will require further investigation.** [P.S.E.B.M. 1997, Vol 215]

A number of studies have shown that immunoregulatory cytokines may be produced and released from human cytomegalovirus (HCMV)–infected cells, supporting the possibility that cytokines may play a role in modulating the effects of HCMV infection. For example, *in vitro* infection of fibroblasts results in an increase in the expression of tumor necrosis factor–α (TNF-α), interleukin-6 (IL-6), IL-1, platelet-derived growth factor (PDGF), and transforming growth factor–β (TGFβ) (1, 2). Exposure of peripheral blood leukocytes (PBLs) to HCMV results in enhanced production of γ-interferon (IFN-γ), colony-stimulating factor (CSF), TNF-α, and IL-1β (3–6). Infection of bone marrow cells with HCMV results in increased production of IFN-α, IFN-γ, TNF-α (5), IL-6, and basic fibroblast growth factor (βFGF) (7), and transiently suppresses IL-1α and IL-1β production (8). Similar findings have been obtained in transplant recipients, where increased levels of IL-2, IL-6, and IFN-γ were noted in bronchoalveolar fluids (9, 10) and the levels of circulating IL-2 receptor in the sera were elevated (11). These observations made both *in vitro* and *in vivo* are consistent with the notion that HCMV infection is accompanied by modification of cytokine production.

While IFN-α and -β are produced by multiple cell types, it has been generally thought that IFN-γ is produced only by cells of immune lineage—that is, T cells ($CD8^+$, subsets of $CD4^+$), B cells, natural killer (NK) cells, and macrophages (12–15). Fibroblasts, in particular, are associated with the production of IFN-α and -β, and not with the production of IFN-γ. Recent data in the murine system, however, has indicated that cells of nonimmune origin may produce IFN-γ in response to conventional IFN-α and -β inducers (i.e., Newcastle disease virus and the synthetic polyribonucleotide poly rIrC) and, interestingly, in response to IFN-γ itself (16).

[1] To whom requests for reprints should be addressed at Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX 77555-1019.
[2] Present address: School of Osteopathy, Kirksville, MO 63501.
These studies were supported by grants (DE-11389 and DA-08354) from the National Institutes of Health. W. B. is a recipient of a James W. McLaughlin predoctoral fellowship.

Received July 29, 1996. [P.S.E.B.M. 1997, Vol 215]
Accepted December 5, 1996.

0037-9727/97/2151-0066$10.50/0

Considering the above effects of HCMV on the IFN system and on cytokines in general, we investigated the effect of HCMV infection on IFN-γ production in diploid human fibroblasts.

In this study, we demonstrate for the first time the induction of IFN-γ at the RNA and protein levels in human embryonic lung fibroblasts after HCMV infection. This induction occurs during the late stages of infection and requires viral DNA synthesis. These findings, when considered together with those describing the effect of HCMV infection on cytokine production by epithelial and endothelial cells, suggest that the possibility that IFN-γ may be involved in the replication and/or pathogenesis of HCMV infections.

Materials and Methods

Cells. Fibroblast cells of human origin (LU, WI-38) were maintained in Eagle's minimum essential medium (EMEM; Sigma Chemical Co., St. Louis, MO) containing 10% fetal bovine serum (FBS), supplemented with glutamine (292 mg/l), streptomycin (100 μg/ml), and penicillin (100 U/ml). WISH cells were propagated in EMEM supplemented with 5% FBS. The cell cultures were routinely examined for mycoplasma contamination using Hoechst 33258.

Virus Propagation. HCMV (strains AD-169, C-87, Davis and clinical isolates 83-1, 84-2 [17]) was propagated in LU cells, and the infectivity was determined by plaque assay as described previously (18). For selected experiments, culture fluids from infected cells were initially cleared of debris by low-speed sedimentation for 15 min in a Beckman JA-14 rotor at 10,000 rpm. HCMV was sedimented from the clarified fluids (Beckman SW-28 rotor; 27,000 rpm; 1 hr). This procedure allowed for a 100-fold concentration of the infectivity, as determined by plaque assay.

Virus Inactivation. Virus suspensions were inactivated by exposure to ultraviolet (UV) light. A Thomas UV lamp with a GE-G8T5 bulb was used to inactivate 1.5-ml aliquots of virus at 80 erg/sec/mm^2 (19, 20).

Western Blot Analysis and Immunoprecipitation. Cells were harvested by scraping and collected by sedimentation (800*g*, 5 min). The cells were lysed in lysis buffer (50 m*M* Tris-HCl, pH 7.4; 150 m*M* NaCl; 0.5% NP-40; 1 m*M* PMSF; 1 m*M* DTT; 25 μg/ml pepstatin A; 50 μg/ml leupeptin; and 0.2% aprotinin) and stored at −70°C until used. The protein content of cell lysates was quantified and equal amounts of protein were resolved by electrophoresis on 12.5% SDS polyacrylamide gels. For immunoprecipitation, 3 ml of serum-free fluids from HCMV-infected cultures were incubated with anti-human IFN-γ antibody (Biosource International Inc., Camarillo, CA) for 4 hr at 4°C. Antibody–IFN-γ complexes were recovered on Protein A-Sepharose beads (Sigma), washed four times with NP-40 lysis buffer, and dissolved in 2× sample buffer followed by separation on 12.5% SDS polyacrylamide gels. The proteins were transferred to nitrocellulose membranes (Amersham Corp., Arlington Heights, IL) and probed with antibody to IFN-γ or HCMV proteins. The primary and secondary (peroxidase-labeled anti-rabbit IgG) antibodies were applied sequentially and the membranes were washed in TBS-T containing 5% nonfat dried milk. Signal detection was accomplished using the ECL chemiluminescent detection system (Amersham).

Northern Blot Hybridization. Total RNA was isolated as described previously (21, 22), and its concentration was determined by spectrophotometry. The RNA was stored in the presence of ribonuclease inhibitor (RNasin; Promega, Inc., Madison, WI) at −80°C until used. Thirty micrograms of RNA per lane were fractionated on a 1.4% agarose gel after glyoxal denaturation (23) and alkaline transferred (24) onto a hybridization membrane (Hybond-N$^+$, Amersham). The blotted RNA was analyzed using oligonucleotide probes to IFN-γ (Clontech, Palo Alto, CA) or to a reference gene (G3PDH; Clontech). The oligonucleotides were labeled and the hybridizations were undertaken as described previously (25).

First-Strand cDNA Synthesis. The cDNA was synthesized from RNA by reverse-transcription (RT) using the SuperScript preamplification system from Gibco-BRL Inc. (Gaithersburg, MD). Briefly, total RNA (3 μg/reaction) was mixed with random hexamers and heated to 70°C for 10 min and quick-chilled on ice. The mixture was supplemented with 5× "synthesis buffer" (200 m*M* Tris-HCL, pH 7.5; 30 m*M* $MgCl_2$; 10 m*M* spermidine; 50 m*M* NaCl), dNTPs (1 m*M* of dATP, dGTP, dCTP, and dTTP), 30 m*M* DTT, and 20 U SuperScript reverse-transcriptase. The reverse-transcription took place at 42°C for 60 min, and the reaction was terminated by heating at 90°C for 5 min. The cDNA was stored at −80°C until used.

Polymerase Chain Reaction. Each polymerase chain reaction (PCR) reaction contained the following reagents from Perkin-Elmer Cetus (Norwalk, CT) in a total volume of 50 μl: 1× reaction buffer, 200 μ*M* dNTPs (dATP, dGTP, dGTP, dCTP; final concentration), primer 1 and 2 (0.5 μ*M* each), 30 m*M* $MgCl_2$, 2.5 U AmpliTaq DNA polymerase and experimental cDNA template. The oligonucleotide primers (sense: 5′-GCA TCG TTT TGG GTT CTC TTG GCT GTT ACT GC-3′; antisense: 5′-CTC CTT TTT CGC TTC CCT GTT TTA GCT GCT GG-3′) were purchased from Clontech. Denaturation (94°C for 1 min), primer annealing (60°C for 1 min), and extension (72°C for 1 min) were performed on a PTC-100 programmable thermal controller (MJ Research, Inc., Watertown, MA).

Characterization of PCR Product. The amplified products from PCR were separated by TBE- (0.089 *M* Tris-borate, pH 8.0; 0.002 *M* EDTA) agarose (3% NuSieve GTG and 1% SeaPlaque, FMC, Inc.) gel electro-

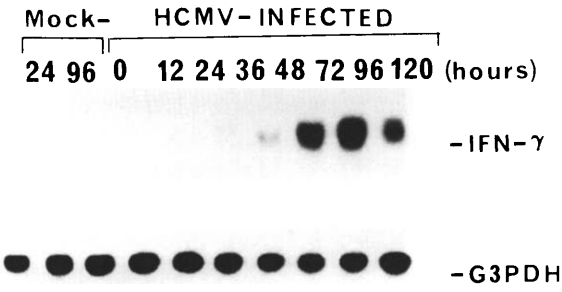

Figure 1. Kinetics of IFN-γ RNA induction in HCMV-infected cells as demonstrated by Northern blot analysis. Total RNA was isolated at selected times after infection as indicated in the figure and equal amounts (30 μg RNA/lane) were fractionated on agarose gels (22). The [^{32}P]-labeled oligonucleotide probe (Clontech) specific for human IFN-γ was hybridized to RNA from mock- or HCMV-infected cells. After removal of probe, the RNA containing filters were rehybridized with a [^{32}P]-labeled G3PDH oligonucleotide probe (Clontech).

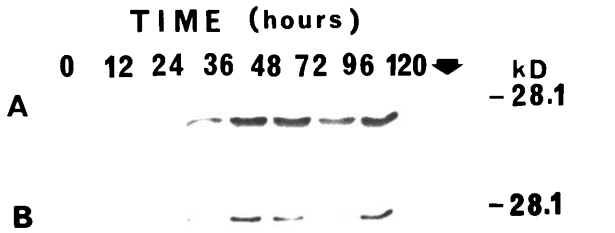

Figure 3. Detection of IFN-γ from cell lysates (A) and cultural fluids (B) of HCMV-infected cells. The cell lysates and cultural fluids were prepared at selected intervals after infection and processed for immunoprecipitation and/or Western blot analysis as described in Materials and Methods. The proteins on nitrocellulose membranes were probed by a monoclonal anti-human IFN-γ antibody purchased from Biosource International. The solid arrowhead marks the position of IFN-γ from a pool of cell lysates prepared at 72 hr of HCMV infection. The recombinant IFN-γ from Biosource International demonstrated an apparent molecular mass of approximately 17 kDa (data not shown).

phoresis. φX174/*Hae* III DNA was used as a size marker (Gibco-BRL). The DNA bands were visualized under UV light after ethidium bromide staining and photographed. PCR products were transferred onto hybridization membrane [Hybond-N$^+$ (Amersham)] by alkaline (0.4% NaOH) blotting as described by Li *et al.* (24). The abundance of PCR products bound to the membranes was analyzed by hybridization (25) to [^{32}P]-labeled oligonucleotide probes (specific activities were between 6.4 and 7.8 cpm/pmol) for target (IFN-γ) and reference (G3PDH) genes (Clontech). The levels of hybridization were visualized by autoradiography.

IFN-γ Assays. Interferon assays were performed by a slightly modified microplaque assay as described elsewhere on human WISH cells (26).

Results

Detection of IFN-γ RNA and Protein in HCMV-Infected Cells. Confluent or subconfluent LU cells were infected with HCMV at a multiplicity of infection (MOI) of five plaque-forming units (PFU) per cell. At various times (indicated in Fig. 1) after HCMV exposure, the infected cells were harvested, collected by sedimentation, and divided to process the cell lysates for determination of IFN-γ RNA (Northern blot and RT-PCR analysis) and protein (Western blot). The results presented in Figures 1 and 2 show that increasing amounts of IFN-γ RNA were produced in HCMV-infected cells. The molecular size of the RNA hybridized to the IFN-γ probe was 1.2 kb (27). The kinetics of IFN-γ RNA (or protein) appearence in HCMV-infected cells correlated with the intensity of infection. For example, in cells infected at 10 PFU/cell, IFN-γ RNA was detectable as early as 24 hr postinfection (p.i.). As the MOI was decreased to 3 or 1 PFU/cell, RNA appeared at progressively later times (60 hr p.i. or later), respectively (data not shown). Infection of cells at MOIs of 10, 5, 3, and 1 PFU/per cell resulted in *de novo* synthesis of HCMV specific antigens in >99%, >95%, >80%, and >60% of the cells observed at 24 hr p.i., respectively (data not shown). No IFN-γ RNA was detectable in RNA extracted from mock-infected cells using RT-PCR (data not shown).

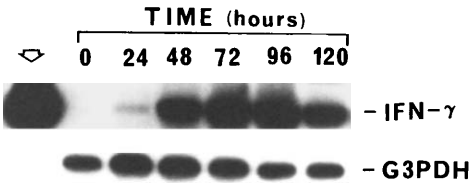

Figure 2. HCMV-mediated IFN-γ RNA induction as detected by RT-PCR analysis. Three micrograms of RNA from each RNA sample prepared at the times indicated were subjected to cDNA synthesis. The cDNAs were PCR amplified, fractionated on agarose gels, transferred onto nylon membranes, and hybridized with probes as described in the legend to Figure 1.

Since the results described above demonstrate that IFN-γ RNA was detected in HCMV-infected cells, we next examined if this RNA was translated into protein. Accordingly, protein lysates prepared from mock- or HCMV-infected cells (LU or WI-38) were subjected to Western blot analysis. The proteins resolved on SDS PAGE were transferred onto nitrocellulose membranes and probed with mouse anti-human IFN-γ antibody. The antibody demonstrated reactivity from 36 hr p.i., as illustrated in Figure 3A for LU cells. Similar results were obtained for WI-38 cells (data not shown). The results obtained using Western blot analysis were consistent with the results of Northern blot (or RT-PCR) analysis, indicating that the higher levels of IFN-γ RNA were reflected in higher protein levels for IFN-γ. Monoclonal antibody reacted with polypeptides of about 24 (Fig. 3) and 17 kDa (Fig. 4, Lanes 1, 2, and 4), which may correspond to glycosylated and non-glycosylated forms of IFN-γ (27). Recombinant human IFN-γ (rhIFN-γ, used as control) obtained from Biosource International demonstrated a molecular size of about 17 kDa (16.8 kDa; data not shown). At a MOI of 5 PFU/

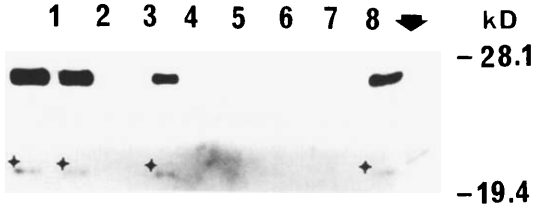

Figure 4. The effect of virus inactivation and selected inhibitors on HCMV-induced IFN-γ production. LU cells were infected with HCMV stock (Lane 1), HCMV sedimented from virus stock (Lane 2), or mock-infected with the supernatant fluids from which virus particles had been sedimented (Lane 3); HCMV stock inactivated by UV irradiation for 1, 10, or 20 min (Lanes 4, 5, and 6, respectively). Lanes 7 and 8 demonstrate the lack of IFN-γ production when PAA (700 μ*M*) was added from 1 to 72 hr p.i. (Lane 7) or actinomycin D (10 μg/ml) was added from 24 to 72 hr p.i. (Lane 8). The cells were harvested at 72 hr p.i. to prepare protein lysates. Equal amounts of protein were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with anti–IFN-γ antibody. The crossed dot (+) shows the non-glycosylated form of IFN-γ. The solid arrowhead marks the position of the same infected cell lysate pool described in the legend to Figure 3.

cell, the abundance of IFN-γ in the cell lysates increased until 96 hr p.i. and decreased thereafter. The kinetics of IFN-γ production apparent in this experiment seem to follow the progression of HCMV-mediated cytopathology and cell lysis in both 1LU and WI-38 cells (data not shown) (18). The cell lysates were also evaluated for HCMV IE (Fig. 5A) and a late (pp28) polypeptide (Fig. 5B). Figure 5 demonstrates that the appearences of IFN-γ and pp28 occur in parallel, indicating that both are produced in the late phase of HCMV replication. IFN-γ was not detectable at any time in lysates of mock-infected cells (data not shown).

Since the genomic sequence (28) and biological characteristics (29, 30) of cell culture–adapted and non-adapted strains of HCMV differ in some respects, we also infected LU cells with other cell culture–adapted HCMV strains (Davis, C-87) or with non-adapted HCMV strains (17). When late cytopathic effects (18) were observed in 95% or more of the cells, the cells were harvested and cell lysates prepared. The cultural

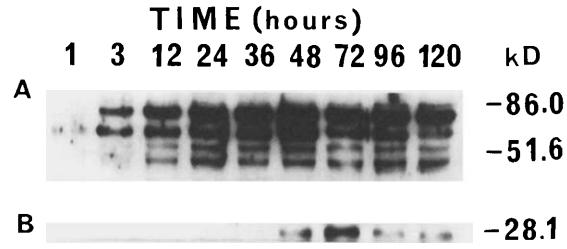

Figure 5. Progression of HCMV replication, demonstrated at the level of protein synthesis. HCMV-infected cells were harvested as indicated and the cell lysates were resolved by SDS-PAGE (with equal amounts of protein loaded in each lane), transferred to nitrocellulose filters, and probed with antibody to HCMV IE1 and IE2 proteins (A) (33) or HCMV late protein, pp28 (B) (34, 35).

fluids from infected cells were clarified and supernatant fluids subjected to immunoprecipitation. The cell lysates and immunoprecipitates of the clarified cultural fluids were investigated for the presence of IFN-γ by Western blot analysis. The results summarized in Figure 6 demonstrate induction of IFN-γ by all HCMV strains evaluated, with IFN-γ detectable in both the cell lysates and immunoprecipitated cultural fluids. The quantity of IFN-γ detected varied among the strains. The non-adapted strains, which provided for substantially lower multiplicities than did the adapted strains, demonstrated substantially lower levels of IFN-γ induction.

Induction of IFN-γ Requires Infectious HCMV. Since HCMV stocks contain infectious and defective virus particles, as well as soluble factors (1, 31, 32), that could affect cytokine production, the effect of several HCMV preparations on induction of IFN-γ was measured to determine the component in the virus stocks responsible for the increase in IFN-γ. The preparations evaluated included: HCMV stock, sedimented HCMV (purified from soluble factors that might induce IFN-γ production), and the supernatant fluids from which the virus had been removed (residual virus infectivity $<10^1$ PFU/ml). Cells were infected with equal MOIs (5 PFU/cell), except for the supernatant fluids, and harvested 72 hr later. As illustrated in Figure 4, HCMV stock (Lane 1) and purified HCMV (Lane 2) induced similar levels of IFN-γ. The virus-free supernatant fluids failed to induce detectable levels of IFN-γ (Fig. 4, Lane 3).

Cell cultures were also infected with HCMV (5 PFU/cell, measured prior to inactivation) that was inactivated by UV irradiation (1, 10, and 20 min at a dose rate of 80 ergs/sec/mm^2). The infected cells were harvested at 72 hr p.i., when maximum levels of IFN-γ induction were observed with non-irradiated virus. As demonstrated in Figure 4, Lane 4, 1 min of UV irradiation reduced, but did not eliminate, induction of IFN-γ. UV irradiation of virus stocks for 10 or 20 min (Lanes 5 and 6, respectively) did eliminate detectable IFN-γ production.

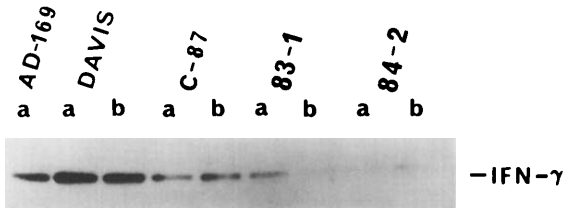

Figure 6. Induction of IFN-γ by tissue culture–adapted HCMV strains (AD-169, C87, Davis) or by non-adapted clinical isolates of HCMV (83-1, 84-2). Cell cultures were infected with adapted HCMV strains at a MOI of 5 PFU/cell or with non-adapted isolates at a multiplicity of approximately 1 PFU/cell. When late cytopathic effects were evident in more than 95% of cells, the cells and cultural fluids were harvested and processed for immunoprecipitation (cultural fluids) and/or Western blot analysis. a, cell lysate; b, immunoprecipitated cultural fluids. For AD169, the IFN-γ that was immunoprecipitated from cultural fluids is shown in Figure 3B.

Twenty-four hours prior to and at the time of harvesting cells for analysis of IFN-γ levels, cell cultures treated in parallel were fixed and evaluated for expression of HCMV-specific proteins by immunofluorescence using antibody to immediate early (33) and late (34, 35) HCMV proteins. The cells that were infected with non-irradiated HCMV stocks or HCMV exposed to UV light for 1 min induced both IE and late HCMV proteins. UV irradiation for 10 min resulted in delayed and reduced expression of HCMV IE proteins, with detectable fluorescence in about 60% and 80% of cells at 48 or 72 hr p.i., respectively, and no detectable expression of HCMV late proteins (20). After 20 min of irradiation, neither IE nor late HCMV-specific proteins were detected (20). Similar data were obtained when cell lysates from the LU cells infected with UV-irradiated HCMV were evaluated by Western blot analysis for IE and late HCMV-specific protein synthesis (data not shown). Since UV doses that eliminate expression of late HCMV synthetic events (HCMV DNA synthesis, expression of late viral genes) also result in a failure to detect IFN-γ, these data suggest that induction of IFN-γ requires virus capable of expressing late virus genes.

To investigate further if late viral synthetic events were essential for induction of IFN-γ, a well-characterized inhibitor (phosphonoacetic acid [PAA], 700 μ*M*) of HCMV DNA synthesis (36, 37) was added to the culture medium of HCMV-infected cells (MOI = 5) at 1 hr p.i., At 72 hr PI, the cells were harvested and evaluated for evidence of IFN-γ production by Western blot analysis. The failure to detect IFN-γ in lysates of those cells treated with PAA (Fig. 4, Lane 7) indicates that activities associated with late synthetic events in the HCMV replication cycle were related to the induction of IFN-γ.

Actinomycin D (Act-D) an inhibitor of transcription was also used in these studies. As demonstrated in Figure 4 (Lane 8), in the presence of Act-D (10 μg/ml) IFN-γ was not detected by Western blot analysis, suggesting that the IFN-γ gene was transcriptionally activated in HCMV-infected cells. These data largely exclude the possibility that IFN-γ RNA accumulated during HCMV infection and was transcribed into protein at late times in HCMV infection. The results of RT-PCR analysis (Fig. 2, Lane 2) and the lack of RNA detection at 3, 6, and 12 hr p.i. (data not shown) also support this assumption.

Effects of IFN-γ on Virus Replication. To determine if the levels of IFN-γ induced during HCMV infection affect virus replication, the following studies were undertaken. Parallel cultures of cells were treated with selected concentrations (0, 1, 10, 100, and 300 U/ml) of recombinant human IFN-γ (rhIFN-γ) prior to or following virus adsorption and continuing throughout the virus replication cycle. At 96 hr p.i., the infected cultures were harvested, and the infectivity determined by plaque assay. The results, summarized in Table I, show that a concentration of 1 or 10 U/ml of rhIFN-γ (in the range of the IFN-γ activities produced during HCMV replication) had no effect on HCMV replication. One unit of rhIFN-γ per milliliter appeared to increase slightly the virus yield, while 10 units/ml of rhIFN-γ resulted in an insignificant decrease. Higher concentrations of rhIFN-γ (100 or 300 units) demonstrated significant anti-HCMV activity, similar to the findings previously published by Yamamoto *et al.* (38).

Table I. Effect of IFN-γ on HCMV Replication

IFN-γ (IU/ml)	Virus yield (PFU/ml)	
	Pretreated[a](±SD)	Post-treated[b](±SD)
0	$5 \times 10^6 \pm 1.78$	$5 \times 10^6 \pm 0.75$
1	$6 \times 10^6 \pm 1.47$	$7 \times 10^6 \pm 1.86$
10	$5 \times 10^6 \pm 1.87$	$4 \times 10^6 \pm 2.16$
100	$1 \times 10^4 \pm 1.78$	$5 \times 10^4 \pm 0.63$
300	$3 \times 10^3 \pm 0.75$	$3 \times 10^3 \pm 1.51$

[a]Cells were treated with IFN-γ 12 hr prior to infection and throughout the infection.
[b]Cells were treated with IFN-γ after virus adsorption and throughout the infection.

To evaluate if the IFN-γ produced in HCMV-infected cells was biologically active, we collected media from the HCMV-infected and mock-infected cultures and tested for anti-VSV activity on WISH cells (26). The results showed that the cultural fluids from HCMV-infected cultures exhibited substantial antiviral activity from 48 hr p.i. The maximum level of antiviral activity was obtained at 72 hr p.i., when 8 ± 3 units/ml were measured (data not shown). As a control, rhIFN-γ was used.

In order to verify the type of IFN responsible for the anti-VSV activity in the cultural fluids of HCMV-infected cultures, we eliminated IFN-γ activity with neutralizing antibody from Biosource International. Following neutralization with the IFN-γ–specific antibody, no anti-VSV activity was detected. Further, the cultural medium was evaluated by immunoprecipitation using mouse anti-human IFN-γ. The immunoprecipitates were subjected to SDS PAGE and Western blotting. From 36 to 48 hr p.i., a band corresponding to 24 kDa was identified (Fig. 3B), which coincides with the molecular weight of glycosylated human IFN-γ (27).

Discussion

The present study appears to be the first evidence for induction of IFN-γ in human fibroblast cells and in particular by HCMV. The induction of IFN-γ in these cells by HCMV may be noteworthy, since several agents (Newcastle's disease virus, Poly rIrC, and IFN-γ itself) that were recently shown to induce IFN-γ RNA in mouse L929 cells or mouse embryo fibroblasts (16) inconsistently induced IFN-γ in the diploid human fibro-

blasts used in this study (data not shown). Thus, it would appear that, for human fibroblasts, HCMV is a consistent inducer of IFN-γ, while classical IFN inducers need to be evaluated further.

Traditionally, induction of IFN-α and -β has been related to virus infection, whereas induction of IFN-γ has been associated with exposure to mitogens or specific antigens (39). However, in this study we showed that the induction of IFN-γ was caused specifically by HCMV infection and not by soluble mediators present in the virus stocks. For example, neither the supernatant fluids from virus stocks nor inactivated virus stocks induced a detectable increase in IFN-γ. In fact, the latter series of experiments indicated that HCMV with the capacity to express late virus proteins was necessary for induction of IFN-γ at both the RNA and protein levels. Furthermore, the increase in IFN-γ after HCMV infection does not appear to be a consequence of lysis of the infected cells and passive release of existing IFN-γ. At the time of maximum IFN-γ levels, very little, if any, cell lysis was evident; and when cell lysis was evident beginning at 120 hr, IFN-γ RNA and protein levels and activities were decreasing.

To the contrary, the data obtained in this study, when considered together, strongly suggest that the increase in IFN-γ levels is a true induction resulting from cellular transcriptional activation during the late phase of HCMV replication. Several observations support this view. One, IFN-γ RNA and protein are not detected at early times in the virus replication cycle; in addition, at later times the increase in IFN-γ RNA is sensitive to the action of actinomycin D. Two, an inhibitor of HCMV DNA synthesis, PAA, blocks the increase in IFN-γ. Three, high doses of UV irradiation that block viral DNA synthesis also prevent the increase in IFN-γ. As noted previously, HCMV infection has been associated with the induction of a number of other cytokines (31) that appear to be mediated by HCMV IE proteins (2). The timing and quite possibly the mechanism for induction of IFN-γ by HCMV in human fibroblasts seem to be distinct from those observed for these other cytokines. Whether the IFN-γ promoter is directly influenced by regulatory proteins specified by HCMV during later phases of replication (40) is a subject of further investigation.

Natural human IFN-γ displays molecular masses of about 17, 20, or 25 kDa, depending upon the level of glycosylation sites occupied (27, 41, 42). The IFN-γ detected in HCMV-infected fibroblasts demonstrated molecular masses of about 24 kDa (about 90% of the total IFN-γ detected based on densitometric analysis of the autoradiographic bands) and 16–17 kDa, which may correspond to the glycosylated and nonglycosylated forms of IFN-γ, respectively (27, 41, 42). It is not known presently if the lack of partially glycosylated (~20 kDa) IFN-γ in human fibroblasts infected with HCMV is a characteristic of the cells or the virus infection. In either case, at the level of glycosylation, the HCMV/human fibroblast system appears to be different from other cells producing natural IFN-γ (39, 41).

The role of IFN-γ in HCMV replication and in the cellular response to virus infection needs to be investigated. It is known that binding of IFN-γ to its receptor results in the rapid phosphorylation of receptor-dependent (STAT-1a) and -independent (Jak1, Jak2) tyrosine kinases followed by a number of events, such as activation of protein kinase (PK)–C, Na^+/H^+ antiporters, and Ca^{2+}/calmodulin-dependent PKs (43, 44). These events result in changes in intracellular pH (pH_i), cell volume, and activation of primary response genes, such as MHC class I and II, and ICAM-1, etc. (45). Similarly, HCMV-mediated cellular responses also result in activation of PKCs, PKAs, amiloride-sensitive Na^+/H^+ antiporters, changes in intracellular ionic levels (e.g., Na^+, Ca^{2+}), pH_i, $[Ca^{2+}]_i$, and upregulation of MHC class I and II (46). Since both IFN-γ production and development and progression of cytomegaly (46) are late events in virus replication, it is possible that HCMV-induced IFN-γ is involved in the development and progression of the cellular changes associated with efficient HCMV replication.

Cytokine induction during viral infection has been demonstrated for a number of viruses (31, 32, 47). The cytokines can play a major role in several pathogenic processes during the course of virus disease and may strongly influence the outcome of the virus-host interaction. The IFN-γ levels observed in HCMV-infected cultures are unlikely to adversely affect HCMV yields (Table I) and are more likely to be related to cellular pathogeneis of virus infection. High levels of circulating IFN-γ have been reported in HCMV-infected bone marrow transplant recipients (48) and in infants with congenital HCMV disease (49). Whether IFN-γ released from HCMV-infected fibroblasts contributes significantly to these circulating levels of IFN-γ is not known. Furthermore, the effect of high circulating levels of IFN-γ appears to be controversial based on studies of rat CMV (RCMV). For example, in immunocompetent animals, neutralization of endogeneous IFN-γ activity significantly reduced the number of RCMV antigen-expressing cells in the spleen, the predominant site of RCMV replication (50). High does of IFN-γ, however, protected immunosuppressed rats against a lethal RCMV challenge (50).

IFN-γ produced by HCMV-infected cells *in vivo* may be important, particularly at the local sites of infection. For example, HCMV infection is often associated with ulcerative lesions of the digestive system in HIV-infected individuals (51, 53). Fibroblasts are among the cell types infected in these lesions (54). In this local environment, IFN-γ, which is a key cytokine in the inflammatory response, could have a profound effect, ini-

tiating the proinflammatory cascade (27, 55), modifying the T helper cell population toward Th_1 (27), activating macrophages, and increasing HLA class I and II and ICAM-1 (56, 57). Since low levels of cytokines have profound effects through their ability to synergize with other cytokines (such as TNF-α), the IFN-γ levels observed in this study could substantially affect the pathogenesis of HCMV infection.

1. St. Jeor S, Admiraud J, Bruening E, Riolo J. Induction of cytokines by human cytomegalovirus. In: Michelson S, Plotkin S, Eds. Multidisciplinary Approach to Understanding Cytomegalovirus Disease. Amsterdam: Elsevier Science, pp 123–126, 1993.
2. Michelson S, Alcami J, Kim S-J, Danielpour D, Bachelerie F, Picard L, Bessia C, Paya C, Virelizier J-L. Human cytomegalovirus infection induces transcription and secretion of transforming growth factor beta–1. J Virol **68:**5730–5737, 1994.
3. Einhorn L, Einhorn S, Wahren B. Interferon induction in human leukocytes after in vitro exposure to cytomegalovirus and Epstein-Barr virus. Intervirology **23:**140–149, 1985.
4. Lolli F, Sundqvist VA, Castanga A, Ljungman P, Linde A, Anderson G, Olsson T, Wahren B. T and B cell responses to cytomegalovirus antigens in healthy blood donors and bone marrow transplant recipients. FEMS Immunol Med Microbiol **7:**55–62, 1993.
5. Dudding L, Haskill S, Clark BD, Auron PE, Sporn S, Huang ES. Cytomegalovirus infection stimulates expression of monocyte-associated mediator genes. J Immunol **143:**3343–3352, 1989.
6. Woodroffe SB, Garnett HM, Danis A, Layton JE. Alteration in IL-1 alpha and IL-1 beta, G-CSF, and GM-CSF secretion by endothelial cells infected with a clinical isolate of CMV. In: Michelson S, Plotkin S, Eds. Multidisciplinary Approach to Understanding Cytomegalovirus Disease. Amsterdam: Elsevier Science, pp 127–134, 1993.
7. Lagneaux L, Delforge A, Snoeck R, DeClercq E, Stryckmans P, Brown D. Perturbation of cytokine production by bone marrow stromal cells after cytomegalovirus infection. *In:* Michelson S, Plotkin SA, Eds. Multidisciplinary Approach to Understanding Cytomegalovirus Disease. Amsterdam: Elsevier Science, pp 111–116, 1993.
8. Westergren-Thorsson G, Sarnstrand B, Fransson LA, Malmstrom A. TGF-beta enhances the production of hyaluronan in human lung but not skin fibroblasts. Exp Cell Res **186:**192–195, 1990.
9. Humbert M, Delattre LM, Fattal S, Cerrina J, Simonneau G, Rain B, Dartevelle P, Duroux P, Galanaud P, Emilie D. In situ production of IL-6 within human lung allografts displaying rejection and cytomegalovirus pneumonitis. Tranplantation **56:**623–627, 1993.
10. Humbert M, Cerrina J, Rain B, Simonneau G, Dartevelle P, Duroux P, Galanaud P, Emilie D. Activation of macrophages and cytotoxic cells during cytomegalovirus pneumonia complicating lung transplantations. Am Rev Respir Dis **145:**1178–1184, 1992.
11. Humbert M, Emilie D, Cerrina J, Simonneau G, Rain B, Leroy-Adurie F, Fattal S, Dartevelle P, Duroux P, Galanaud P. Soluble interleukin-2 receptor and neupterin serum levels after lung/heart-lung transplantations: Absence of predictive value for late allograft rejection. Transplantation **146:**1092–1094, 1991.
12. Epstein LB, Kreth HW, Herzenberg LA. Fluorescence activated cell sorting of human T and B lymphocytes. II. Identification of the cell type responsible for interferon production and cell proliferation in response to mitogens. Cell Immunol **12:**407–421, 1974.
13. Vankelencom H, Billiau A. Inteferon-γ in neuronimmunology and endocrinology. In: Hughes TK, Ed. Advances in Neuroimmunology. Vol **2:** pp 139–161, 1992.
14. Dayton MA, Knobloch TJ, Benjamin D. Human B-cell lines express the interferon gamma gene. Cytokine **4:**454–460, 1992.
15. Fultz MJ, Dieffenbach CW, Vogel SN. Induction of IFN-γ in macrophages by lipopolysaccharide. Int Immunol **5:**1383–1392, 1993.
16. Rady PL, Cadet P, Bui TK, Tyring SK, Baron S, Stanton GJ, Hughes TK. Production of interferon-γ messenger RNA by cells of non-immune origin. Cytokine **7:**793–798, 1995.
17. Fons MP, Graves K, Cavallo R, Pollard R, Albrecht T. Human cytomegalovirus: Development and progression of nuclear inclusions by primary clinical isolates and laboratory-adapted strains. Proc Soc Exp Biol Med **181:**416–422, 1986.
18. Albrecht T, Cavallo T, Cole NL, Graves K. Cytomegalovirus: Development and progression of cytopathic effects in human cell culture. Lab Invest **42:**1–7, 1980.
19. Albrecht T, St. Jeor SC, Funk FD, Rapp F. Multiplicity reactivation of human cytomegalovirus inactivated by ultra-violet light. Int J Radiat Biol **26:**445–454, 1974.
20. Boldogh I, AbuBakar S, Deng CZ, Albrecht T. Transcriptional activation of cellular oncogenes *fos, jun,* and *myc* by human cytomegalovirus. J Virol **65:**1568–1571, 1991.
21. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem. **162:**156–159, 1987.
22. Boldogh I, AbuBakar S, Albrecht T. Activation of proto-oncogenes: An immediate early event in human cytomegalovirus infection. Scence **247:**561–564, 1990.
23. Maniatis T, Fritsch EF, Sambrook J. Molecular Cloning: A Laboratory Manual. NY: Cold Spring Harbor, Cold Spring Harbor Laboratory, 1982.
24. Li JKK, Dasker B, Kowalik L. Rapid alkaline blot-transfer of viral dsRNA. Anal Biochem **163:**214–216, 1987.
25. Boldogh I, Huang ES, Rady P, Arany I, Tyring S, Albrecht T. Alteration in the coding potential and expression of H-*ras* in human cytomegalovirus-transformed cells. Intervirology **37:**321–329, 1994.
26. Campbell JB, Grumberger T, Kochum MA, White SL. A microplaque reduction assay for human and mouse interferon. Can J Microbiol **21:**1247–1253, 1975.
27. Farrar MA, Scheiber RD. The molecular biology of interferon-γ and its receptor. Ann Rev Immunol **11:**571–611, 1993.
28. Cha T-A, Tom E, Kemble GW, Duke GM, Mocarski ES, Spaete RR. Human cytomegalovirus clinical isolates carry at least 19 genes not found in laboratory strains. J Virol **70:**78–83, 1996.
29. Brown JM, Kaneshima H, Mocarski ES. Dramatic interstrain differences in the replication of human cytomegalovirus in SCID-hu mice. J Infect Dis **171:**1599–1603, 1995.
30. Quinnan GV, Delery M, Rook AH, Frederick WR, Epstein JS, Manischewitz JF, Jackson L, Ramsey KM, Mittal K, Plotkin SA. Comparative virulence and immunogenicity of the Towne strain and a non-attenuated strain of cytomegalovirus. Ann Intern Med **101:**478–483, 1984.
31. Gonczol E, Plotkin SA. Cells infected with human cytomegalovirus release a factor(s) that stimulates cell DNA synthesis. J Gen Virol **65:**1833–1837, 1984.
32. Geist LJ, Hunninghake GW. Cytomegalovirus as a *trans*-activator of cellular genes. Sem Virol **5:**415–420, 1994.
33. Mazeron MC, Jahn G, Plachter B. Monoclonal antibody E-13 (M-810) to human cytomegalovirus recognizes an epitope encoded by exon 2 of the major immediate early gene. J Gen Virol **73:**2699–2703, 1992.
34. Ree MC, Landini MP, Coppolecchia P, La Placa M. A 28,000 molecular weight human cytomegalovirus structural polypeptide studied by means of a specific monoclonal antibody. J Gen Virol **66:**2507–2511, 1985.
35. Meyer H, Bankier AT, Landini MP, Brown CM, Barrell BG, Ruger B, Mach M. Identification and prokaryotic expression of the

gene coding for the highly immunogenic 28 kilo Dalton structural phosphoprotein (pp28) of human cytomegalovirus. J Virol **62:**2243–2250, 1988.
36. Huang ES. Human cytomegalovirus IV. Specific inhibition of virus-induced DNA polymerase activity and viral DNA replication by phosphonoacetic acid. J Virol **16:**1560–1565, 1975.
37. Bresnahan WA, Boldogh I, Thompson EA, Albrecht T. Human cytomegalovirus inhibits cellular DNA synthesis and arrests productively infected cells in late G1. Virology **224:**150–160, 1996.
38. Yamamoto N, Shimokata K, Maeno K, Nishiyama Y. Effect of Recombinant human interferon-γ against human cytomegalovirus. Arch Virol **94:**323–329, 1987.
39. Gunther G, Otto B. Interferon. An overview of the state of basic research with special regard to interferon-γ. Arznemittel-Forschung **43:**182–188, 1993.
40. Puchtler E. Stamminger T. An inducible promoter mediates abundant expression from the immediate-early 2 gene region of human cytomegalovirus at late times after infection. J Virol **65:**6301–6306, 1991.
41. Kelker HC, Le L, Rubin BY, Yip YK, Nagler C, Vilcek J. Three molecular weight forms of natural human interferon-γ revealed by immunoprecipitation with monoclonal antibody. J Biol Chem **259:**4301–4304, 1984.
42. Arakawa T, Hsu Y-R, Chang D, Stebbing N, Altrock B. Structure and activity of glycosylated human interferon-γ. J Interferon Res **6:**687–695, 1986.
43. Darnell JE, Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science **264:**1415–1421, 1994.
44. David M. Transcription factors in interferon signaling. Pharmacol Ther **65:**149–161, 1995.
45. Benveniste EN, Benos DJ. TNF-α–and IFN-γ–mediated signal transduction pathways: Effects on glial cell gene expression and function. FASEB J **9:**1577–1584, 1995.
46. Albrecht T, Boldogh I, Fons MP, Lee CH, AbuBakar S, Russell JM, Au WW. Cell-activation responses to cytomegalovirus infection: Relationship to the phasing of CMV replication and to the induction of cellular damage. Subcell Biochem **15:**157–202, 1989.
47. Shearer GM, Clerici M, Lucey DR. Cytokines and HIV infection. Sem Virol **5:**449–455, 1994.
48. Winstone DJ, Ho WG, Champlin RE. Cytomegalovirus infections after allogeneic bone marrow transplantation. Rev Infect Dis **12:**S776–S791, 1990.
49. Rhodes-Feuillette A, Canivet M, Champsaur H, Gluckman E, Mazeron MC, Peries J. Circulating interferon in cytomegalovirus infected bone-morrow-transplant recipients and infants with congenital cytomegalovirus disease. J Interferon Res **Special Number:** 91–98, 1992.
50. Haagmans BL, van der Meide PH, Stals FS, van en Eertweigh AJ, Claassen E, Bruggeman CA, Horzinek MC, Schijns MC. Suppression of rat cytomegalovirus replication by antibodies against gamma interferon. J Virol **68:**2305–2312, 1994.
51. Kamel PL. Cytomegalovirus-associated perianal ulcerations in AIDS. J Clin Gastroenterol **14:**105–108, 1992.
52. Kyriazis AP, Mitra SK. Multiple cytomegalovirus-related intestinal perforations in patients with acquired immunodeficiency syndrome. Report of two cases and review of the literature. Arch Pathol Lab Med **116:**495–499, 1992.
53. Murray RN, Parker A, Kadakia SC, Ayala E, Martinez EM. Cytomegalovirus in upper gastrointestinal ulcers. J Clin Gastroenterol **19:**198–201, 1994.
54. Flaitz CM, Nichols CM, Hicks MJ. Herpesviridae-associated persistent mucocutaneous ulcer in acquired immunodeficiency syndrome. Oral Surg Oral Med Oral Pathol **81:**433–441, 1996.
55. Reiter Z. Interferon: A major regulator of natural killer cell-mediated cytotoxicity. J Interferon Res **13:**247–257, 1993.
56. Ibrahim L, Dominguez M, Yacoub M. Primary human lung epithelial cells in vitro: Response to interferon-γ and cytomegalovirus. Immunology **79:**119–124, 1993.
57. Khoury EL, Pereira L, Greenspan FS. Induction of HLA-DR expression on thyroid follicular cells by cytomegalovirus infection in vitro. Evidence for a dual mechanism of induction. Am J Pathol **138:**1209–1223, 1991.