Hyperthermia enhances cytomegalovirus regulation of HIV-1 and TNF-α gene expression

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Iwamoto, Gary K., Audrey M. Ainsworth, and Pope L. Moseley. Hyperthermia enhances cytomegalovirus regulation of HIV-1 and TNF-α gene expression. *Am. J. Physiol.* 277 (*Lung Cell. Mol. Physiol.* 21): L1051–L1056, 1999.—The immediate-early (IE) genes of human cytomegalovirus (CMV) can be expressed in monocytic cells and are known to regulate viral and cellular genes. Reactivation of human immunodeficiency virus (HIV-1) may be stimulated by a variety of factors including other viruses and inflammatory cytokines. These studies examine the role of hyperthermia and CMV in the regulation of HIV-1 and tumor necrosis factor (TNF)-α. THP-1 cells were transfected with the CMV IE genes. HIV-1 and TNF-α transcription were assessed with chloramphenicol acetyltransferase promoter constructs. Hyperthermia sufficient to stimulate production of heat shock proteins was used to stimulate the cells. Hyperthermia significantly enhances the effect of CMV IE gene products on the expression of HIV-1 and TNF-α. The increases in HIV-1 transcription appear to be in part due to increases in TNF-α. Heat shock proteins induced by hyperthermia may play an important role in the viral regulation of monocytic function by CMV.

THP-1 cells; endotoxin; tumor necrosis factor-α; heat shock proteins; human immunodeficiency virus

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HUMAN IMMUNODEFICIENCY VIRUS (HIV-1) infection is characterized by a prolonged period of clinical latency. The development of acquired immune deficiency syndrome (AIDS) occurs through the reactivation of viral expression (3, 15, 23, 25). A variety of factors appear to upregulate the viral expression in HIV-1-infected cells (3, 15, 23, 25, 31–33). Cytomegalovirus (CMV) has been implicated as an important cofactor in the disease progression of AIDS. Experimental and clinical studies (1, 4–6, 11, 21, 22, 34) demonstrated that the interaction between CMV and HIV-1 furthers disease progression and leads to increased HIV-1 replication. CMV immediate-early (IE) proteins can affect cellular function and enhance inflammatory cytokine production (9, 12, 13). Heat shock proteins (HSPs) from virally infected cells and cancer cells can stimulate an immune response and also stimulate tumor necrosis factor (TNF)-α production (2, 24, 28, 29). Viral proteins complexed with HSPs may play an important role in the generation of the immune and inflammatory responses seen in many viral infections. We hypothesized that hyperthermia sufficient to induce HSP accumulation acts in concert with CMV IE proteins to increase HIV-1 transcription and inflammatory cytokine transcription.

We further examined the effect of hyperthermia as a stimulus of HIV-1 transcription and TNF-α transcription alone and in combination with CMV IE proteins.

METHODS

Reagents. Lipopolysaccharide (LPS; *Escherichia coli* 026: B6), silica TLC plates, and ethyl acetate were obtained from Sigma (St. Louis, MO). Acetyl-CoA and DEAE were obtained from Amersham (Arlington Heights, IL). *FAST* CAT was obtained from Molecular Probes (Eugene, OR). Merifluor CMV-immunofluorescent CMV identification reagent was obtained from Meridian Diagnostics (Cincinnati, OH).

Tissue culture. THP-1 cells, a monocytic leukemia cell line, was obtained from American Type Culture Collection (Manassas, VA) (30). The cells were maintained in RPMI 1640 medium (ICN, Gaithersburg, MD) with 4 mM L-glutamine and 10% fetal bovine serum (HyClone, Logan, UT).

Plasmids. Plasmid pJN201 contains the CMV IE promoter upstream from the human CMV IE1, -2, and -3 genes (8). The plasmids were purified twice over cesium chloride gradients. The *Limulus* amebocyte lysate assay QCL-1000 was obtained from BioWhittaker (Walkersville, MD). Endotoxin assays were performed as suggested by the manufacturer. TNF-α-chloramphenicol acetyltransferase (CAT) constructs were kindly provided by Dr. Lois Geist (University of Iowa, Iowa City) (9). The TNF-α constructs contained from −650 to +1 bp of the TNF-α promoter. Endotoxin levels were <0.1 endotoxin unit/ml (1 pg/ml) in all solutions and plasmid preparations used during transfection. HIV-1-CAT contains the HIV-1 long-terminal repeat upstream from the CAT gene (18).

Transfection. Transient transfections of THP-1 cells were performed with the DEAE-dextran transfection method (26). Cells (10×10^6) were exposed to plasmid DNA in 10 ml of a solution containing DEAE-dextran (7.5 µg/ml) for 40 min, washed once in RPMI 1640 medium containing 1.5 U/ml of heparin, and then washed in the same medium without heparin. Transfected cells were cultured in 100-mm tissue culture dishes at a concentration of 1×10^6 cells/ml in RPMI 1640 medium with 10% fetal bovine serum. The cells were harvested for CAT assays. Transfection efficiency was monitored with a fluorescently labeled antibody to CMV IE1 protein.

Immunofluorescent detection of CMV. The cells were monitored for transfection efficiency by fluorescent staining with an antibody to CMV IE1 protein 24 h after stimulation. The cells were cytospun onto slides, fixed in methanol for 20 min, stained with Merifluor CMV identification reagent (Meridian Diagnostics) for 20 min, rinsed in PBS, and mounted with fluorescent antibody mounting medium. Cells were counted under ultraviolet (UV) spectroscopy.

CAT assay. CAT assays were performed 24 h after stimulation as described by Gorman et al. (10). The acetylated derivatives were separated from nonacetylated chloramphenicol by ascending chromatography with chloroform-methanol (85:15). *FAST* CAT is a modified chloramphenicol with only one acetylation site attached to fluorochrome and provides a linear range of acetylation over a three-magnitude range. All

experiments were run within the linear range of this assay. TLC plates were read and quantified with a Molecular Dynamics (Sunnyvale, CA) scanner.

Statistics. CAT assays were calculated as the multiple of increase over the control value and compared by *t*-tests. A *P* value < 0.05 was used as the cutoff for significance.

RESULTS

Hyperthermia augments HIV-1 transcription. The ability to stimulate HIV-1 transcription and replication in the absence of HIV-1 tat protein may play an important role in stimulating HIV-1 replication in latently infected cells. HIV-1 replication is induced by a wide range of stimuli including cytokines, viruses, UV light, heat, and endotoxin. We first examined the ability of hyperthermia and CMV IE genes to modulate HIV-1 transcription in monocytic cells. In these studies, HIV-1 transcription was measured with an HIV-1-CAT promoter construct. THP-1 cells were either transfected with HIV-1-CAT (0.05 µg/ml) or cotransfected with HIV-1-CAT (0.05 µg/ml) and the CMV IE gene (pJN201; 0.05 µg/ml). The cells were either left unstimulated or stimulated with LPS (1.0 µg/ml) 4 h after heat for 2 h at 42°C. The cells were harvested 24 h after LPS stimulation, and CAT assays were performed (Fig. 1). Compared with that in control transfected unstimulated cells, HIV-1-CAT activity increased 4.1 ± 0.3-fold in control transfected LPS-stimulated cells, 0.9 ± 0.2-fold in CMV IE transfected unstimulated cells, and 30.2 ± 13.0-fold in CMV IE transfected LPS-stimulated cells. After heat exposure, HIV-1-CAT activity increased 1.1 ± 0.3-fold in unstimulated cells, 8.5 ± 1.5-fold in LPS-stimulated cells, 51.3 ± 20.5-fold in CMV IE transfected unstimulated cells, and 129.7± 43.7-fold in CMV IE transfected LPS-stimulated cells.

Hyperthermia significantly increased HIV-1 transcription in CMV IE transfected cells, with an increase from 0.9- to 51.3-fold over the control value in unstimulated cells (*P* = 0.03) and from 30.2- to 129.7-fold over the control value in LPS-stimulated cells (*P* = 0.047). Heat alone increased HIV-1 transcription in LPS-stimulated cells twofold. Hyperthermia significantly enhanced HIV-1 transcription in CMV IE transfected cells. In previous studies (9, 12, 13) with the CMV IE genes under the control of the CMV IE promoter, stimuli such as LPS increased expression of the CMV IE proteins in THP-1 cells. To evaluate whether hyperthermia increased CMV IE protein expression, we used a fluorescent-labeled antibody against CMV IE1 protein and counted cells by fluorescent microscopy. THP-1 cells transfected with the CMV IE genes demonstrated 3–5% of the cells expressing CMV IE1 protein; with the addition of LPS, 17–20% of the cells expressed CMV IE1 protein. Hyperthermia did not change CMV IE1 protein expression in either the CMV IE transfected cells or the CMV IE transfected cells stimulated with LPS. These experiments examined the percentage of cells expressing CMV IE1 protein but did not quantitate the level of expression of CMV IE1 protein per cell. The increases in HIV-1 transcription may be due to
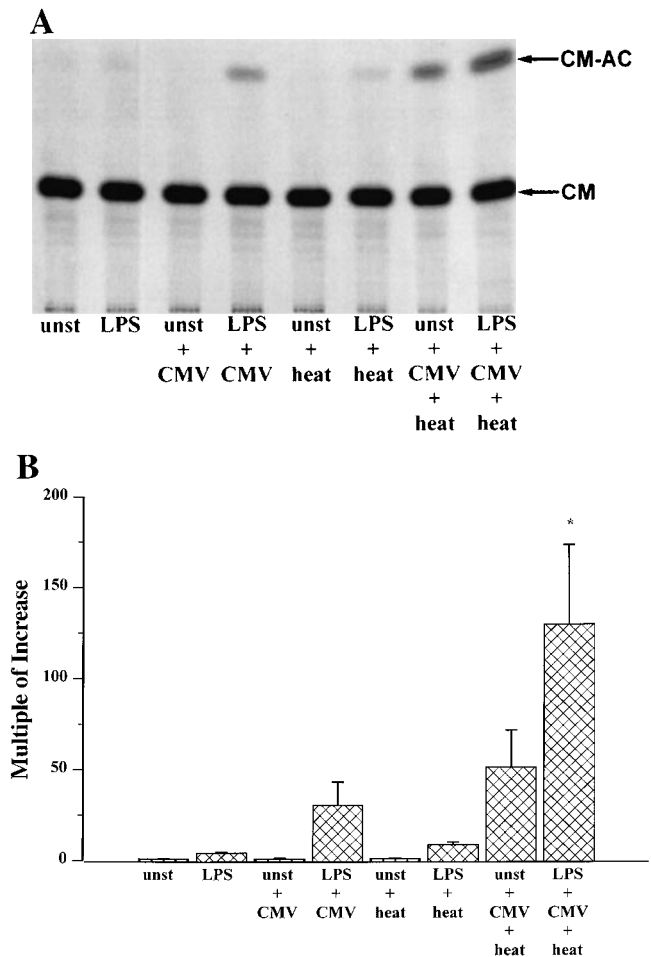
Fig. 1. Effect of cytomegalovirus (CMV) intermediate-early (IE) proteins and hyperthermia on human immunodeficiency (HIV)-1 chloramphenicol (CM) acetyltransferase (CAT; CM-AC) activity. *A*: representative CAT assay with HIV-1-CAT. LPS, lipopolysaccharide; unst, unstimulated. *B*: results of 3 experiments. HIV-1-CAT activity increased 4.1 ± 0.3-fold in control transfected LPS-stimulated cells, 0.9 ± 0.2-fold in CMV IE transfected unstimulated cells, and 30.2± 13.0-fold in CMV IE transfected LPS-stimulated cells compared with that in control transfected unstimulated cells. In heat-exposed cells, HIV-1-CAT activity increased 1.1 ± 0.3-fold in unstimulated cells, 8.5 ± 1.5-fold in LPS-stimulated cells, 51.3 ± 20.5-fold in CMV IE transfected unstimulated cells, and 129.7± 43.7-fold in CMV IE transfected LPS-stimulated cells. Hyperthermia-treated CMV IE transfected cells with (*P* = 0.047) or without (*P* = 0.03) LPS stimulation were significantly increased (*) over CMV IE transfected cells that were not exposed to hyperthermia.

CMV IE and hyperthermia stimulation of inflammatory cytokines.

Hyperthermia augments CMV IE gene-associated TNF-α promoter activity. We next examined the ability of hyperthermia and CMV IE gene products to increase cytokine transcription. We evaluated TNF-α because of its ability to stimulate HIV-1 transcription. To evaluate the effect of the CMV IE genes on the TNF-α promoter, cotransfection experiments were performed with a TNF-α construct containing the region from −650 to +1 bp of the TNF-α promoter upstream from the CAT gene. THP-1 cells were either transfected with TNF-α-CAT (0.5 µg/ml) or cotransfected with TNF-α-CAT (0.5 µg/ml) plus the CMV IE gene (pJN201; 0.05 µg/ml). The

A

← CM-AC

← CM

unst   LPS   unst   LPS   unst   LPS   unst   LPS
              +   +   +   +   +   +
             CMV   CMV   heat   heat   CMV   CMV
                                          +   +
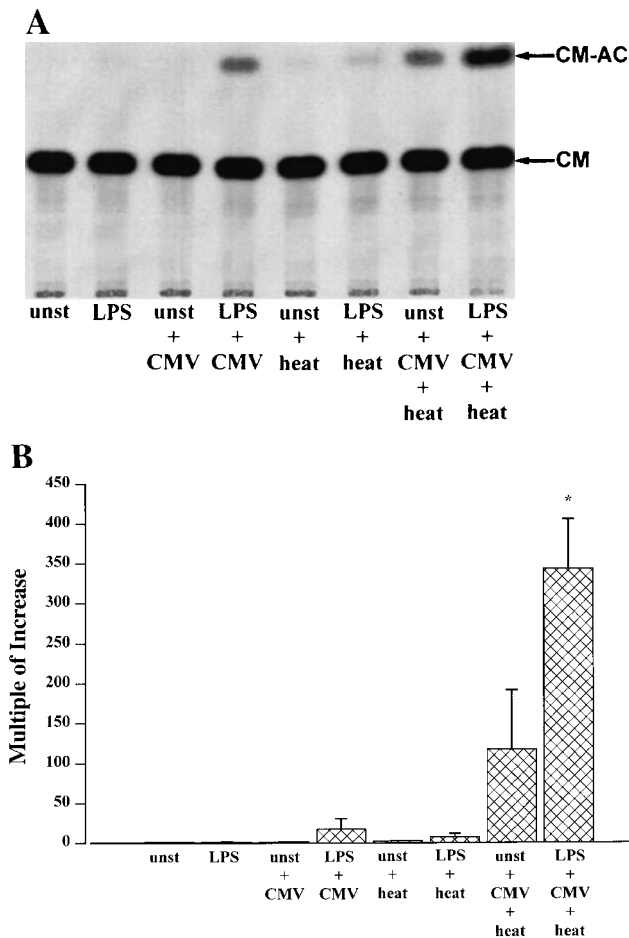   heat   heat

B

Fig. 2. Effect of CMV IE proteins and hyperthermia on tumor necrosis factor (TNF)-α-CAT activity. *A*: representative CAT assay with TNF-α-CAT. *B*: results of 3 experiments. TNF-α-CAT activity increased 1.4 ± 0.5-fold in control transfected LPS-stimulated cells, 1.5 ± 0.2-fold in CMV IE transfected unstimulated cells, and 17.3 ± 13.1-fold in CMV IE transfected LPS-stimulated cells compared with that in control transfected unstimulated cells. In heat-exposed cells, TNF-α-CAT activity increased 2.4 ± 0.3-fold in unstimulated cells, 7.2 ± 4.4-fold in LPS-stimulated cells, 116.8 ± 74.8-fold in CMV IE transfected unstimulated cells, and 343.2 ± 62.3-fold in CMV IE transfected LPS-stimulated cells. Hyperthermia-treated CMV IE transfected cells with LPS stimulation were significantly increased compared with CMV IE transfected LPS-stimulated cells that were not exposed to hyperthermia (* $P = 0.003$).

cells were either left unstimulated or stimulated with LPS (1 mg/ml) 4 h after heat for 2 h at 42°C. The cells were harvested 24 h after LPS stimulation, and CAT assays were performed (Fig. 2). Compared with that in control transfected unstimulated cells, TNF-α-CAT activity increased 1.4 ± 0.5-fold in control transfected LPS-stimulated cells, 1.5 ± 0.2-fold in CMV IE transfected unstimulated cells, and 17.3 ± 13.1-fold in CMV IE transfected LPS-stimulated cells. After heat exposure, TNF-α-CAT activity increased 2.4 ± 0.4-fold in unstimulated cells, 7.2 ± 4.4-fold in LPS-stimulated cells, 116.8 ± 74.8-fold in CMV IE transfected unstimulated cells, and 343.2 ± 62.3-fold in CMV IE transfected LPS-stimulated cells.

These studies demonstrate a significant increase in TNF-α with CMV IE transfected cells, increasing from 17.3-fold in LPS-stimulated cells to 343.2-fold in heated LPS-stimulated cells ($P = 0.03$). The increases in TNF-α transcription were not due to changes in CMV IE gene expression induced by hyperthermia. These studies suggest that hyperthermia induces changes that either enhance the ability of CMV IE gene products to act as transcription factors or induce other factors that work in concert with CMV IE gene products to enhance TNF-α transcription.

Hyperthermia induces 70-kDa HSP production. To evaluate the effect of hyperthermia on the cells, we examined changes in HSP production by measuring HSP70 production by Western blot analysis. We evaluated 70-kDa HSP (HSP70) production in THP-1 cells exposed to heat at 42°C for 2 h. HSP70 increased over time, with a peak in HSP70 protein production as measured by Western blot 8 h after heat exposure (Fig. 3). HSP70 protein production persisted up to 48 h. We also examined HSP70 production in our system. No HSP70 was seen in transfected cells, LPS-stimulated cells, or cells transfected with the CMV IE gene unless they were also exposed to heat (data not shown). The increases in HSP70 production parallel the increases in TNF-α transcription.

Hyperthermia augments TNF-α promoter activity at later time points. Hyperthermia induces a translational block with no protein synthesis for several hours. HSPs such as HSP70 are the first proteins produced after resolution of the translational block. We examined whether there were continued increases in TNF-α transcription at later time points. Experiments were repeated with a delay of 24 h between heat shock and LPS stimulation. THP-1 cells were either transfected with TNF-α-CAT (0.5 µg/ml) or cotransfected with TNF-α-CAT (0.5 µg/ml) plus the CMV IE gene (pJN201; 0.05 µg/ml). Cells were either left unstimulated or stimulated with LPS (1 µg/ml) 24 h after heat for 2 h at 42°C. The cells were harvested 24 h after LPS stimulation, and CAT assays were performed (Fig. 4). Compared with that in control transfected unstimulated cells, TNF-α-CAT activity increased 2.0 ± 0.6-fold in control transfected LPS-stimulated cells, 2.7 ± 0.7-fold in CMV IE transfected unstimulated cells, and 5.3 ± 1.5-fold in CMV IE transfected LPS-stimulated cells. After heat exposure, TNF-α-CAT activity increased 5.9 ± 2.4-fold in unstimulated cells, 12.8 ± 5.2-fold in LPS-stimulated cells, 139.8 ± 53.5-fold in CMV IE transfected unstimulated cells, and 103.1 ± 44.7-fold in CMV IE transfected LPS-stimulated cells. Hyperthermia-exposed CMV IE transfected cells with ($P = 0.03$) or without ($P = 0.02$) LPS stimulation were significantly increased over the cells that were not exposed to
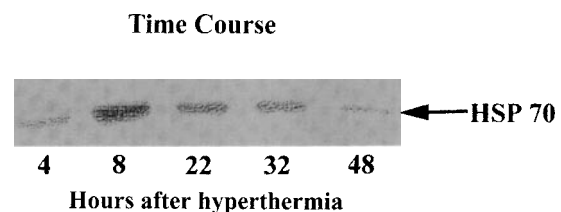
Time Course

← HSP 70

4   8   22   32   48

Hours after hyperthermia

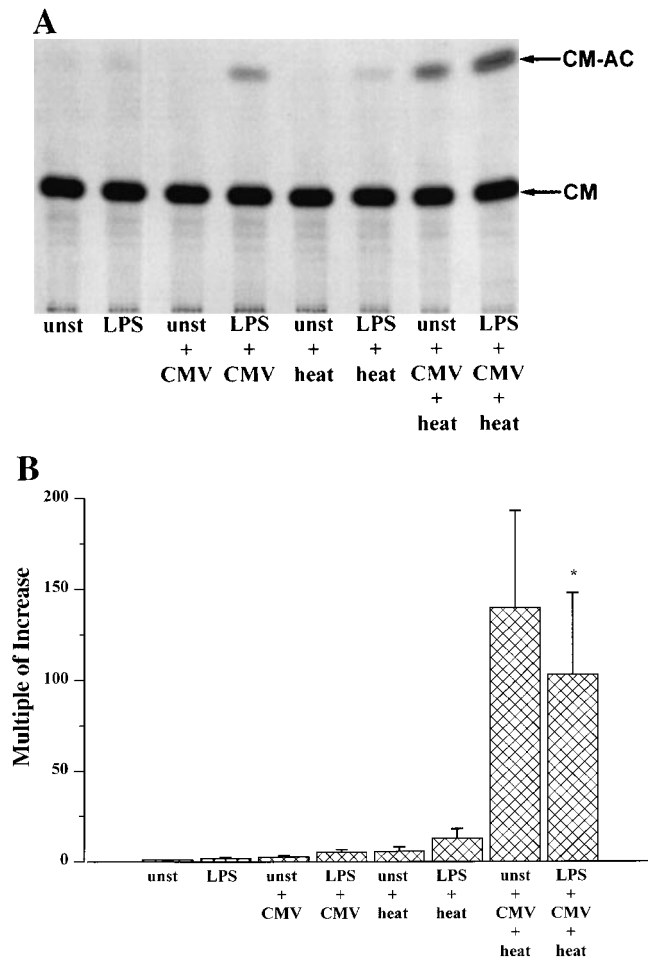Fig. 3. Western blot stained for 70-kDa heat shock protein (HSP70).

A

B

Fig. 4. Effect of CMV IE proteins and hyperthermia on TNF-α-CAT activity 48 h after heat exposure. *A*: representative CAT assay with TNF-α-CAT. *B*: results of 5 experiments. TNF-α-CAT activity increased 2.0 $\pm$ 0.6-fold in control transfected LPS-stimulated cells, 2.7 $\pm$ 0.7-fold in CMV IE transfected unstimulated cells, and 5.3 $\pm$ 1.5-fold in CMV IE transfected LPS-stimulated cells compared with that in control transfected unstimulated cells. In heat-exposed cells, TNF-α-CAT activity increased 5.9 $\pm$ 2.4-fold in unstimulated cells, 12.8 $\pm$ 5.2-fold in LPS-stimulated cells, 139.8 $\pm$ 53.5-fold in CMV IE transfected unstimulated cells, and 103.1 $\pm$ 44.7-fold in CMV IE transfected LPS-stimulated cells. Hyperthermia-treated CMV IE transfected cells with ($P = 0.03$) or without ($P = 0.02$) LPS stimulation were significantly increased (*) over cells that were not exposed to hyperthermia.

hyperthermia. Hyperthermia continued to stimulate TNF-α transcription at later time points when there is also production of HSP70.

DISCUSSION

The combination of CMV IE proteins and hyperthermia significantly enhances HIV-1 (Fig. 1) and TNF-α (Fig. 2) transcription. CMV is an important cofactor in the pathogenesis of HIV-1 infection. One of the most common coinfections found in HIV-1-infected subjects is CMV. Coinfection with CMV and HIV-1 has been demonstrated in brain, lung, and retinal tissues (7, 19, 20, 27). Experimental and clinical studies (1, 4–6, 11, 21, 22, 34) suggested that the interaction between CMV and HIV-1 leads to increased HIV-1 replication and

disease progression. CMV may affect HIV-1 infection by stimulating production of inflammatory cytokines known to upregulate HIV-1 replication (9, 12, 13). CMV IE gene products may stimulate HIV-1 replication by direct interaction with the HIV-1 promoter or through enhanced cytokine production, which then stimulates HIV-1 replication. In these studies, the increase in TNF-α transcription suggests that increased inflammatory cytokine production could contribute to the increased HIV-1 transcription. These studies did not demonstrate increased TNF-α protein production.

CMV IE proteins have been shown to increase interleukin-1, interleukin-6, and TNF-α production in LPS-stimulated cells. In previous studies (9, 12, 13), LPS has been needed as a cofactor with CMV IE proteins to increase inflammatory cytokine production. One mechanism by which LPS enhanced the effect of CMV IE gene products on inflammatory cytokine production in monocytic cells was by increasing expression of the CMV IE gene products. Hyperthermia, however, did not increase CMV IE gene expression in our experiments. The present studies demonstrate an increase in TNF-α transcription by hyperthermia and CMV IE proteins in the absence of LPS. A possible cofactor in enhancing the effect of CMV IE gene products on TNF-α and HIV-1 transcription may be HSPs.

Hyperthermia alone can enhance HIV-1 expression approximately twofold as seen in a study by Kretz-Remy and Arrigo (14) and in our experiments. The increases seen with the addition of the CMV IE gene products are significantly higher than the changes due to hyperthermia or CMV IE gene products alone. Our studies demonstrated a significant increase in TNF-α transcription with the presence of CMV IE proteins and hyperthermia. The increased transcription due to the addition of CMV IE gene products occurs at time periods of 24–48 h after exposure to heat when there was also an increase in HSP70.

Hyperthermia induces mitochondrial uncoupling, generation of oxygen radicals, a translational block, and generation of HSP accumulation. Increased inflammatory cytokine production has been demonstrated when cells are exposed to bacterial HSPs, and HSPs from virally infected cells and cancer cells can induce an immune response and stimulate TNF-α production (2, 24, 28, 29). Although hyperthermia sufficient to cause cellular HSP accumulation was used as a stimulus, it is tempting to consider that the HSPs are responsible, in part, for our findings. HSPs are a family of proteins central to the heat shock response (16, 17, 35). They are an acidic group of proteins (isoelectric point 5.0–6.5) and vary in size from 27 to 110 kDa. Accumulation of HSPs is associated with a tolerance to a variety of stresses including heat, UV irradiation, ischemia, and cytotoxic cytokines such as TNF-α. HSPs are ubiquitous intracellular transports or chaperones. Management of proteins and protein fragments is a common function of all HSPs. Mechanisms by which HSPs may enhance the effect of CMV IE proteins include protection of the CMV IE proteins from degradation, which increases the length of time the proteins are present in the

cell, and facilitation of transport of the CMV IE proteins to other cells, or they may act as chaperones enhancing CMV IE protein transport into the nucleus.

The data taken as a whole support the model that CMV coinfection drives an inflammatory response that stimulates HIV-1 replication. The inflammatory response induced by hyperthermia and CMV IE proteins increases both HIV-1 and TNF-α transcription. Hyperthermia alone did not significantly increase HIV-1 or TNF-α transcription. HSPs may enhance the ability of viral proteins to regulate cellular functions. An emerging role for HSPs is in the induction of immune responses. HSPs from virally infected cells and cancer cells can induce an immune response and stimulate TNF-α production (2, 24, 28, 29). The ability of HSPs to enhance viral regulation of cellular function may play an important role in many immune responses. Heat as well as other stresses may enhance HIV-1 replication by stimulating HSP production and leading to further disease progression.

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