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Stress Protein Modulation in HIV-1 Infected CD4-Expressing Cells

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ABSTRACT

Heat shock or stress proteins (HSPs), are a large family of phylogenetically conserved molecules that can be constitutively expressed at high levels under normal physiological conditions. These proteins are selectively synthesized following metabolic and environmental insult. This study was designed to determine whether cognate and/or inducible HSP production are altered in CD4-expressing lymphocytic cell lines concomitant with acute and chronic human immunodeficiency virus type 1 (HIV-1) infection. Our findings indicate that in CEM.NKR cells, HSP27 production is selectively altered at early stages of acute HIV-infection (6-24h post-infection), subsequent to virus internalization but prior to synthesis of viral progeny. Levels of HSP27 induction were viral dose-dependent and were not accompanied by any alterations in cellular proliferation. In contrast, acute HIV-infection was not associated with significant quantitative changes in the constitutive expression of HSP60, HSP70, or HSP90. Nevertheless, a transient, marked induction of select HSP70 subspecies was evident at early stages of infection, finally disappearing by 48-72h. Acute-infection of Jurkat cells resulted in similar patterns of *de novo* induction of HSP27 and HSP70 isoforms. Uninfected and chronically-infected CEM extracts showed little detectable constitutive HSP27. However, synthesis of select HSP27 and HSP70 homologues in chronically-infected cells was observed following exposure to a mild heat shock and doses of TNFα. Similar HSP70 homologues arose in chronically-infected cells treated with heat-shock and TNFα. These findings indicate that HSP pathways are uniquely modulated in CD4+ cells as a consequence of acute and chronic HIV-1 infection.

RÉSUMÉ

Les protéines de choc thermique ("heat shock proteins"; HSP) sont une grande famille de protéines phylogénétiquement conservées qui peuvent être exprimées à un haut niveau dans des conditions physiologiques normales. Ces protéines sont sélectivement synthétisées à la suite d'un changement métabolique et environmental. Cette étude a été conçue afin de déterminer quel type de production de HSP (inductible ou constitutive) est affecté dans les lymphocytes exprimant CD4 et infectés de façon chronique ou aigüe par le virus de l'immunodéficience humaine de type 1 (VIH-1). Nos découvertes indiquent que dans les cellules CEM.NKR, la production de HSP27 est affectée sélectivement aux stades précoces de l'infection par le VIH-1 (6-24h après l'infection), après l'internalisation du virus mais précédent la synthèse virale. Les niveaux d'induction de HSP27 étaient dépendant de la dose virale et n'avaient pas d'effet sur la prolifération cellulaire. Par contre, l'infection par le VIH n'était pas associée à des changements significatifs de l'expression constitutive de HSP60, 70, et 90. Toutefois, une induction différente, mais brève, de certaines sous-espèces de HSP70 était évidente aux stades précoces de l'infection, disparaissant après 48-72h. Des modèles similaires d'infection des isoformes HSP27 et HSP70 *de novo* ont été aussi observés pour les cellules Jurkat infectées. Des extraits de cellules CEM non-infectées et infectées chroniquement montraient peu de HSP27 détectable constitutivement. Cependant, la synthèse de certains homologues de HSP27 et HSP70 était observée dans les cellules chroniquement infectées à la suite de faible exposition à un choc thermique et au TNFα. Des isoformes semblables de HSP70 sont apparus dans les cellules chroniquement infectées traitées avec des chocs thermiques et TNFα. Ces découvertes indiquent que les voies d'expression de HSP sont uniquement modulées dans les cellules CD4+ en réponse à une infection aigüe ou chronique par le VIH-1.

Introduction

1. The Acquired Immunodeficiency Syndrome (AIDS)

1.1 Human Immunodeficiency Virus (HIV-1) and AIDS

1.1.1 History of HIV-1

The first recognition of AIDS as a clinical disease came in 1981 when the unusual co-occurrence of *Pneumocystis carinii* pneumonia and Kaposi's sarcoma was noted in previously healthy homosexual men (Centers for Disease Control, 1981, Gottlieb *et al*, 1981). The subsequent occurrence of this disease among hemophiliacs, intravenous drug users, blood transfusion recipients, and children born to mothers with AIDS, suggested that the unknown etiologic infectious agent was probably transmitted by blood and genital secretions (Levy, 1993).

The first indication that AIDS was caused by a retrovirus came in 1983 when Luc Montagnier's group at the Pasteur Institute in France isolated a reverse transcriptase-containing virus from the lymph node of a man infected with lymphadenopathy-associated virus (LAV) (Barré-Sinoussi *et al*, 1983). At around the same time, Gallo and colleagues reported on the isolation of human T cell leukemia virus (HTLV) from individuals with AIDS, and soon after, he identified another human retrovirus distinct from HTLV that he called HTLV-III, which was found to exist in the peripheral blood mononuclear cells (PBMC's) of several AIDS patients (Gallo *et al*, 1983, 1984). In 1984, the search for the AIDS virus was further developed when Levy *et al* reported the identification of another possible agent for the disease, AIDS-associated retrovirus (ARV) (Levy *et al*, 1984). The three viruses (LAV, HTLV-III, and ARV) were quickly found to have similar characteristics and in 1986, the AIDS virus was renamed the human immunodeficiency virus, HIV (Coffin *et al*, 1986).

1.1.2 HIV-1 Classification

In 1911, the first retrovirus was discovered, when a filterable agent known as Rous Sarcoma Virus (RSV) was found to cause disease in chickens (reviewed in Varmus, 1988). With the dramatic discovery almost sixty years later of the enzyme reverse transcriptase, the understanding of how a retrovirus functions became new fertile ground for research in the field of virology (Baltimore, 1970). Within one year, the first human retrovirus, the human foamy virus (HFV), was isolated and since then, the understanding of how these viruses function has been greatly developed (Achong *et al*, 1971). Retroviruses can be divided into three subsets: *Oncovirinae*, which includes all oncogenic retroviruses, *Lentivirinae*, which includes HIV-1, and *Spumavirinae*, the only group not associated with human disease, which includes the foamy viruses, like HFV (Weiss, 1996).

HIV-1 is a member of the lentivirus family, a unique genus of the *Retroviridae*. Despite the fact that one of the first viruses to be found by man was actually part of the lentivirus family (equine infectious anemia virus in 1911), it was only identified as such in recent years (Levy, 1993). Other identified lentiviruses include Visna virus of sheep, caprine arthritis-encephalitis virus (CAEV) of goats, and the bovine (BIV), feline (FIV), and simian immunodeficiency viruses (SIV) (*Ibid*). Lentiviruses are a unique subset of retroviruses in that they all induce slow, progressive, fatal disease. Common clinical signs of a lentivirus include a persistent viremia, a weak humoral immune response, as well as autoimmune diseases, pneumonitis, and central nervous system disorders (Fauci, 1988).

Of interest, a separate virus with similar properties to HIV-i was identified in Western Africa in 1986 (Clavel *et al*, 1986). Due to the remarkable sequence homology and serologic reactivity with HIV-1, this virus was named HIV-2 (Clavel *et al*, 1987). This virus was also found to cause AIDS, although the pathogenic course of HIV-2 appears to be much longer (Brun-Vezinet *et al*, 1987).

In recent years, HIV-1 has been further reclassified into two major sets. Group M is the major group and it contains nine clades.

The second is group O, which was isolated more recently in Cameroon and which also possesses the same degree of variability as group M (reviewed in Montagnier, 1996). Certain clades are found in certain regions, with subtype B predominating in North America and Europe. The main difference between the clades seem to be in the amino acid compositions of the different strains. The amino acid composition of each clade differs from that of the others by at least 20% in the envelope region and 15% in the gag region. Furthermore, within each clade, the differences in the envelope region can be up to 10% and those in gag up to 8% (reviewed in Essex, 1996, Louwagie *et al*, 1992). It has also been found that the clades differ by way of transmission; while certain clades are transmitted through heterosexual contact, others are associated with homosexual and intravenous drug encounters (Essex, 1996). However, the main reasons for the distribution of virus subtypes relates to the genetic structure of the envelope region, which appears to be the most important area for vaccine development (*Ibid*).

1.1.3 Epidemiology and Clinical Picture

The initial infection of HIV-1 is usually either unnoticed or is accompanied by a flu-like illness associated with fever, sore throat, muscle pain, and diarrhea. During this period, the virus is actively replicating and can be detected within the blood and cerebrospinal fluid (Weiss, 1993). The disease then enters a long period of latency, which usually lasts between 2-10 years, during which the immune system is functional. It is during this time that the virus causes a slow and relentless depletion of the helper (CD4+) T cell subset. This results in the progressive compromised ability to fight certain opportunistic infections (Fauci, 1988, Weiss, 1993). The last stage of HIV-1 infection, full blown AIDS, then sets in with the appearance of various infections including those by protozoa (*Pneumocystis carinii*), mycobacteria (*M. Avium*), fungi (candida albicans), viruses (herpes simplex virus, cytomegalovirus), and rare malignancies (Burkitt's lymphoma, Kaposi's sarcoma) (reviewed in Weiss, 1993). While

certain long-term survivors have been found to live with the virus for over 10 years, HIV-1 infection usually results in a fatal outcome.

Despite the fact that HIV is probably the most investigated infectious agent in history, the worldwide level of infection is spreading at an alarming rate. It is estimated that about 20 million people worldwide are infected with HIV-1, with that number likely to increase to 40 million by the year 2010 (Haynes, 1996). More than one half of the reported AIDS cases in North America are associated with HIV transmission by homosexual and bisexual males, but cases involving intravenous drug users have continued to increase (Levy, 1993). Furthermore, AIDS has become the leading cause of death among persons 25-44 years of age in the United States (*Ibid*).

Today, most evidence suggests that all but 1-2 million of the worldwide cases of HIV-infection are in developing countries. Sub-Saharan Africa and southeast Asia now appear to be the regions with the most incidence and prevalence of HIV-1 infection. In these areas, the spread of HIV shows an entirely different pattern from North America and Europe where HIV-infection is mainly spread through heterosexual contact. Due to poor public health conditions, and lack of adequate birth control, the spread of this virus continues to be alarming (reviewed in Essex, 1996, Montagnier, 1996).

Most researchers cite the incomplete understanding of the mechanism by which HIV kills the immune system to be a major obstacle in the design of effective therapeutic strategies. We still do not know why AIDS takes so long to develop following HIV-infection. Finally, the lack of an adequate animal model and the recent discovery of the variability of the virus have hindered vaccine and cure strategies. Nonetheless, HIV research has grown profoundly over the last decade, and work on HIV has led to numerous important discoveries. A better understanding of the virus's relationship with the immune system remains an important goal (Cohen, 1993).

1.1.4 HIV-1 Structure and Genome

Electron microscopy has revealed that the HIV-virion is an enveloped spherical structure that is approximately 100-120 nm in diameter (Figure 1). The outer coat consists of a phospholipid bilayer envelope derived from the host cell membrane. The surface of the virus is made up of 72 knobs that contain the envelope glycoproteins. Each knob is a glycoprotein made up of 2 components: gp120, the external surface (SU) envelope protein that is believed to play a role in binding HIV-1 to target cells, and gp41, the transmembrane (TM) protein that has shown to be involved in viral fusion (reviewed in Greene, 1993, Morrow *et al*, 1994). Underneath the outer coat is a matrix (MA) protein (p17) that associates with the inner surface of the phospholipid bilayer and may play a role in the stabilization of the interior and exterior parts of the virion. The structural capsid (CA) protein (p24) contains two identical RNA strands with which the viral RNA-dependent DNA polymerase (reverse transcriptase or RT), and the nucleocapsid (NC) proteins (p6, p9) closely associate. The HIV virion also contains tRNA molecules ($tRNA^{LYS3}$) that are used for the initiation of reverse transcription, and viral protease (PR) and integrase (IN) enzymes (reviewed in Greene, 1993, Morrow *et al*, 1994).

The genomic size of HIV-1 is 9.8 kb in length with open reading frames coding for several viral proteins (Figure 2). The HIV genome shares the basic structure of all known retroviruses including: *gag*, *pol*, and *env*. The *gag* gene codes for a p55 precursor polyprotein that can be proteolytically cleaved into the viral core proteins, p24, p17, p9, and p6. The *pol* gene codes for a protease (PR) that cleaves the *gag* and *pol* precursor proteins, RT p66/p51 that makes proviral DNA from viral RNA, and an integrase (IN) that is required for proviral incorporation into the host DNA. The *env* gene codes for the precursor polyprotein gp160, that is processed into gp120 and gp41 (reviewed in Haseltine, 1991).

The HIV-1 genome has 5' and 3' ends flanked by identical sequences of DNA known as the long terminal repeats (LTR). LTR's contain regulatory elements such as the TATA promoter, SP-1

Figure 1: The HIV-1 Virion

Schematic representation of the mature HIV-1 virion including: the external glycoproteins, gp120 (SU) and gp41 (TM), the matrix p17 (MA), capsid p24 (CA) and nucleocapsid p6/p9 (NC) proteins, the genomic RNA, reverse transcriptase (RT), protease (PR), integrase (IN), and $tRNA^{LYS3}$ (adapted from Morrow *et al*, 1994).

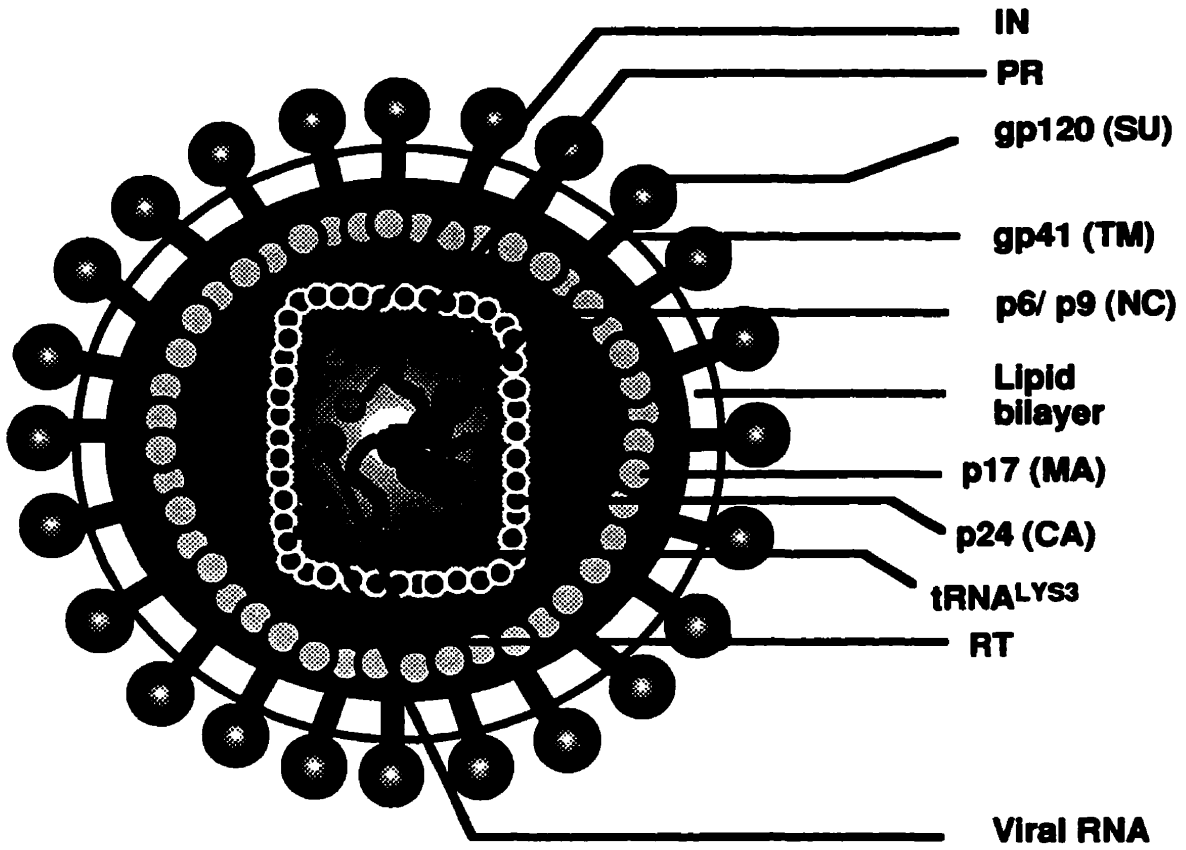
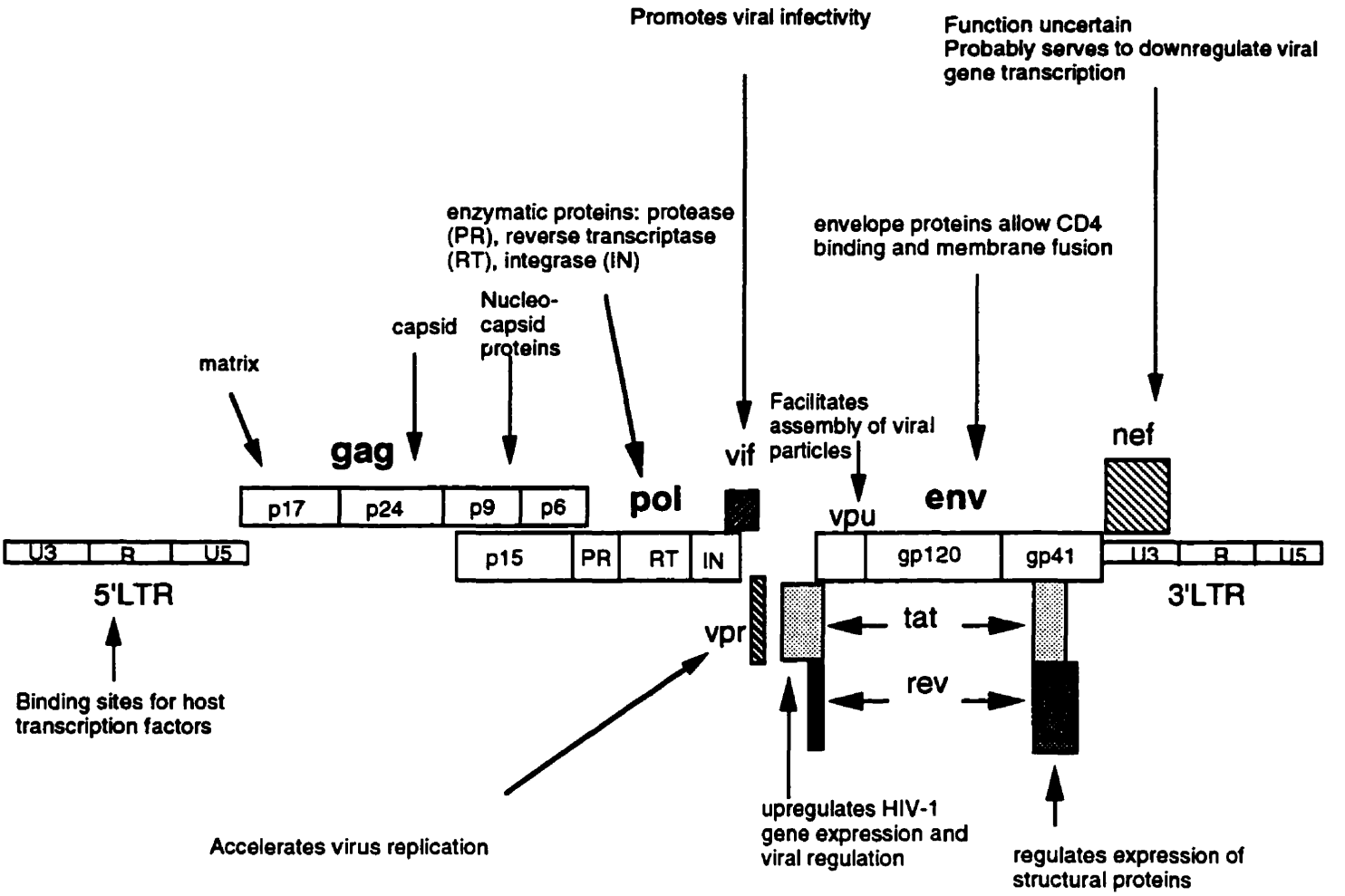

IN
PR
gp120 (SU)
gp41 (TM)
p6/ p9 (NC)
Lipid bilayer
p17 (MA)
p24 (CA)
tRNALYS3
RT
Viral RNA

Figure 2: The HIV-1 Genome

Schematic representation showing each of the known genes of HIV-1 and their primary function. In addition to the three standard retroviral genes *gag*, *pol*, and *env*, HIV-1 encodes for a number of smaller structural and regulatory proteins including: *tat*, *rev*, *nef*, *vif*, *vpu*, and *vpr*. The 5' and 3' LTR sequences are indicated as well (modified from Greene, 1993).


Promotes viral infectivity
Function uncertain
Probably serves to downregulate viral gene transcription
enzymatic proteins: protease (PR), reverse transcriptase (RT), integrase (IN)
envelope proteins allow CD4 binding and membrane fusion
capsid
Nucleo-capsid proteins
matrix
Facilitates assembly of viral particles
gag
vif
nef
pol
vpu
env
p17
p24
p9
p6
p15
PR
RT
IN
gp120
gp41
U3
R
U5
5'LTR
3'LTR
vpr
tat
rev
Binding sites for host transcription factors
Accelerates virus replication
upregulates HIV-1 gene expression and viral regulation
regulates expression of structural proteins

binding sites, polyadenylation signal sequences, cis-acting elements, and other specialized regions that are recognized by host and viral transcriptional factors (Starcich *et al*, 1984).

In addition to the structural genes, the HIV-1 genome also contains unique regulatory genes. These regulatory genes play an important role in gene expression and in viral replication. The *tat* gene encodes a 14-15 kDa transactivating protein that interacts with an area on the long terminal repeat (LTR) known as the Tat responsive element (TAR). This protein plays an important role in up-regulating HIV expression and viral replication (Rice and Matthews, 1988, Feinberg *et al*, 1991). Another regulatory gene, *rev*, encodes a 20 kDa protein that interacts with the rev responsive element (RRE) and is required for HIV replication and proper posttranscriptional splicing of *gag* and *env* proteins (Sodroski *et al*, 1986, Malim *et al*, 1989). The third regulatory gene, *nef*, encodes a 27 kDa protein that can downregulate HIV transcription by binding to downstream sites on the LTR known as Nef regulatory elements (NRE) (Ahmad and Venkatesan, 1988). While its function is not fully understood, *nef* does not interact with viral RNA (unlike *tat* and *rev*) and it is not a requirement for viral replication (Terwilliger *et al*, 1986, Niederman *et al*, 1989, Hammes *et al*, 1989).

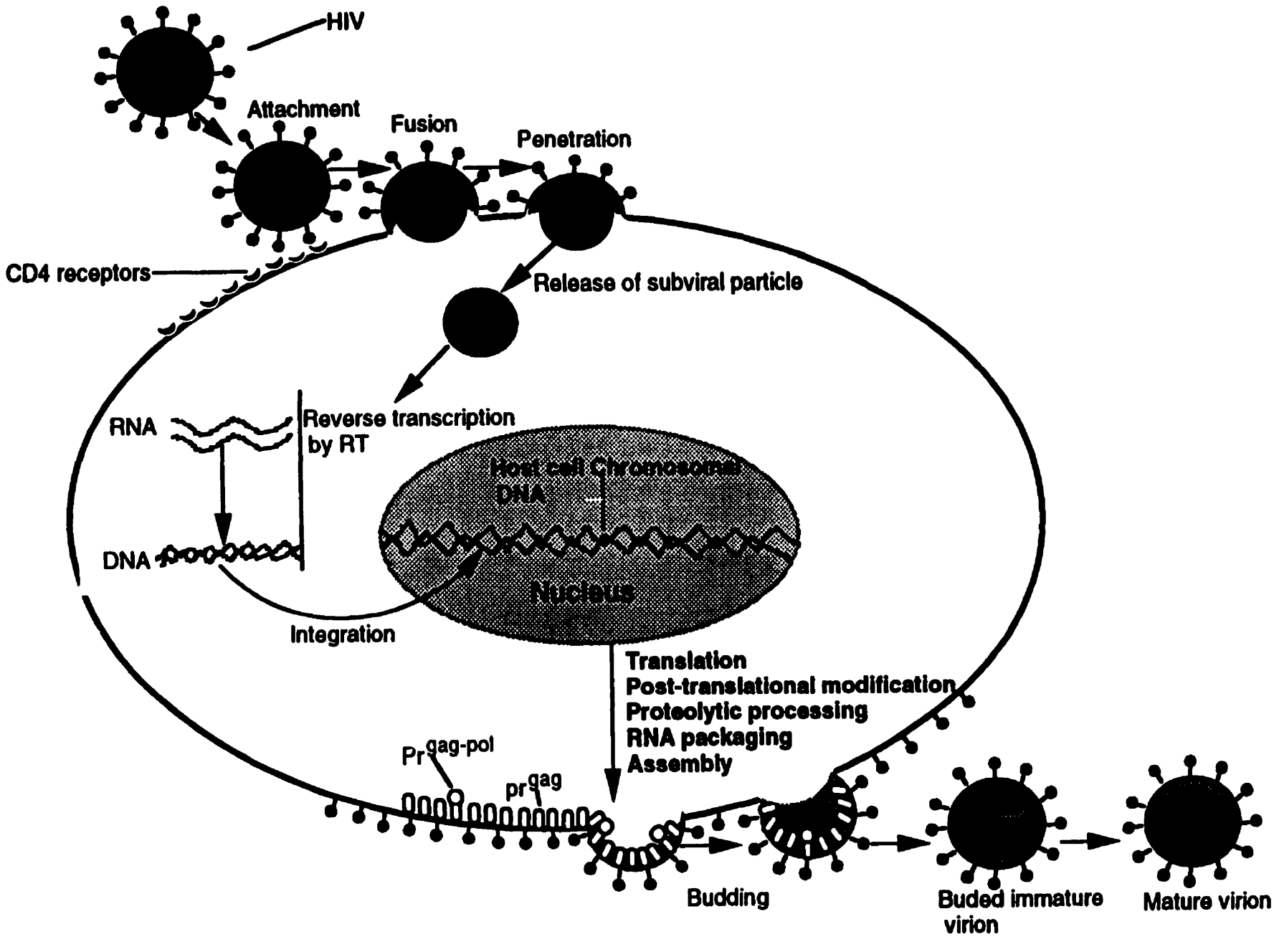
The HIV-1 genome also encodes numerous accessory proteins that are involved in several aspects of the viral life cycle. The *vif* (virion infectivity factor) gene encodes a 24 kDa protein that increases viral infectivity (Lee *et al*, 1986). The *vpu* gene (viral protein U) encodes a 16 kDa protein that increases the rate of virus export by facilitating the assembly of viral particles (Strebel *et al*, 1988, Klimkait *et al*, 1990). The *vpr* (viral protein R) gene encodes a 15 kDa protein that accelerates the replication and cytopathic effect of the virus (Cohen *et al*, 1990). While these genes are only considered to be accessory, their protein products appear to play a major role in determining the virulence of the virus and its ability to produce virions in a manner consistent with infectiousness (reviewed in Trono, 1995).

1.1.5 Replicative Cycle of HIV-1

The infectious cycle of HIV begins with the binding of the external SU-gp120 to the CD4 receptor on the surface of CD4 expressing lymphocytes, monocytes, and other cell types (Figure 3) (Dalgliesh *et al*, 1984). This interaction brings about a conformational change in gp120 leading to the TM-gp41 mediated fusion of the host-derived viral envelope with the target cell (Lifson *et al*, 1986a, Gallaher, 1987). HIV particles may also enter the cell by CD4-mediated endocytosis (Maddon *et al*, 1986). Although the CD4 molecule is the primary receptor for HIV, questions remain as to whether there are other low affinity receptors. The recent identification of a protein known as "fusin" suggests that accessory molecules apart from CD4 are utilized for successful binding and entry of the virus (Feng *et al*, 1996). Furthermore, more recent developments have shown that another receptor co-factor known as CC-CKR-5 may play a large role in this initial stage of the viral replicative cycle (Deng *et al*, 1996, Dragic *et al*, 1996). The discovery of these co-receptors suggests that the initial stages of binding and entry of HIV are not as simple as once imagined.

As soon as an HIV virion enters the cell, viral reverse transcriptase transcribes viral RNA into double-stranded linear DNA. This reaction is primed by a $tRNA^{LYS3}$, which by way of complex interactions with the primer binding site (PBS) of the viral RNA allows initiation of reverse transcription to occur (Jiang *et al*, 1994). This newly formed HIV-1 DNA, known as proviral DNA, is translocated to the nucleus where viral integrase acts by catalyzing its integration into genomic host DNA (Brown *et al*, 1987). Once the provirus is integrated into the host DNA, it can be transcribed into genomic mRNA and then translated into viral proteins. A number of host transcriptional factors may play a part in the initiation of proviral transcription (Ho *et al*, 1987). Following translation, viral proteins undergo a number of modifications, including cleavage, glycosylation, and phosphorylation. The assembly of infectious virions takes place at the plasma membrane with the packaging of

Figure 3:
Schematic representation of the stages of the HIV-1 life cycle. These include: attachment of HIV-1 virus and fusion to host CD4+ cell, uncoating of viral RNA and conversion via RT to double stranded DNA, translocation of proviral DNA into the nucleus and integration into host genome, production of viral RNA and proteins, modification and assembly of infectious virions, and release from the host cell. After its release, the budded immature virion matures and prepares to infect another cell (adapted from Levy, 1993).


HIV
Attachment
Fusion
Penetration
CD4 receptors
Release of subviral particle
RNA
Reverse transcription
by RT
DNA
Host cell chromosomal
DNA
Nucleus
Integration
Translation
Post-translational modification
Proteolytic processing
RNA packaging
Assembly
Pr gag-pol
pr gag
Budding
Buded immature
virion
Mature virion

viral RNA, modified viral proteins and enzymes (*Ibid*). During the final stage of virion release, the core virion buds from the plasma membrane, acquiring an outer lipid bilayer that contains the envelope glycoproteins. Once released from the infected and now lysed host cell, the HIV virion undergoes a number of morphological changes including cleavage of the polyprotein precursors into mature products by the viral protease (Leonard *et al*, 1988, Cann and Karn, 1989).

1.2 HIV and the Immune System

1.2.1 Immunopathogenesis of HIV Infection

HIV-1 displays specific tropism for CD4-expressing cells via the CD4 receptor on helper T cells. Host CD4 cells can block, contain or fuel infection depending on tropism, viral phenotype and/or biological situation. This results in latent, productive, or lytic stages of infection. In any case, the final outcome of CD4+ cell depletion is profound immunosuppression (reviewed in Fauci, 1988).

The almost complete depletion of helper T cells occurs despite the fact that fewer than 1% of these cells harbor HIV-1 DNA. Moreover, numerous immunological abnormalities are present in HIV-infected individuals even before their CD4+ cells are reduced in number (Ho *et al*, 1989). Both direct and indirect mechanisms have been proposed to explain the depletion of CD4+ cells. Direct lytic infection may result from the following:

1. The direct production of virions leads to the death of infected T cells because of increased plasma permeability resulting in osmotic lysis (Lynn *et al*, 1988).
2. The accumulation of unintegrated viral DNA interferes with normal cellular functions thus causing cell death (Garry, 1989).
3. The binding of gp120 to the CD4 receptor may often take place within the cytoplasm causing lethal intracellular interactions (Hoxie *et al*, 1986, Capon and Ward, 1991).

Several mechanisms have also been proposed to account for indirect HIV-1 depletion of uninfected CD4+ cells:

1. HIV may block maturation of CD4+ precursor T cells by inhibiting cytokine production needed for T cell maturation (Rook *et al*, 1983).
2. The *in vitro* fusion of cell membranes of uninfected cells with the cell membrane of an infected cell results in the formation of giant, non-functional, multinucleated cells, known as syncytia, that die soon after they are formed (Lifson *et al*, 1986b).
3. Programmed cell death or apoptosis may be caused by an HIV peptide that acts like a superantigen by attaching to the CD4+ lymphocyte. Other factors including antibodies to CD4 and gp120-antibody complexes have also been found to potentially induce apoptosis (reviewed in Ameisen, 1993).
4. HIV-infection of some CD4+ cells may lead to autoimmune destruction of both infected and uninfected helper T cells. The expression of gp120 on infected cells, or the binding of gp120 to uninfected CD4+ cells, renders them susceptible to the host immune system (reviewed in Hermaszewski *et al*, 1993).
5. Antigen-specific responses of CD4+ cells require the interaction of the CD4 receptor with MHC class II molecule. Since the envelope of HIV binds to the CD4 receptor, it may impede interaction with class II MHC (Dalgleish *et al*, 1984, Klatzmann *et al*, 1984, Gay *et al*, 1987).

In all likelihood, a combination of several of these mechanisms contribute to depletion of CD4+ T cells and T cell dysfunction. HIV can also be transmitted during cellular contact. In this process, syncytia might form, and/or contact could take place without the fusion of cell membranes. This type of HIV transmission has been estimated to be up to 100 times more effective than infection by free virus particles (Dimitrov *et al*, 1993). Thus, the spread of HIV could result from both cell to cell transfer or from circulating free virus.

Other CD4+ cells besides helper T-cells are also susceptible to infection by HIV-1. HIV has been isolated from monocytes and macrophages of peripheral blood, lung, and the central nervous system (Ho *et al*, 1986, Weiss, 1993). Monocytes and macrophages

can become infected by either HIV attachment to the CD4 receptor, or by direct phagocytosis of HIV particles (reviewed in Haseltine, 1991). HIV infection of monocytes and macrophages does not result in cytopathic effects or syncytia formation (Crowe *et al*, 1987). Monocytes and macrophages, which have become infected by the virus, usually remain in tissues for extended periods of time and may serve as reservoirs that can spread the virus to other parts of the body (Weiss, 1993, Haseltine, 1991).

1.2.2 Immune Responses and HIV

HIV-specific humoral and cell-mediated immune responses are present in infected individuals. Most evidence suggests that a surprisingly vigorous immune response accompanies HIV-infection and manages to contain the virus over long periods without eradicating it. For example, infected individuals produce large amounts of HIV antibodies soon after infection (Robert *et al*, 1988). Furthermore, the appearance of cytotoxic T lymphocytes (CTL's), specific for epitopes in the envelope glycoprotein, *gag*-derived proteins, and others occurs early after viral infection and are sustained throughout infection (Walker *et al*, 1989).

1.2.2.1 Humoral Response

The main basis for HIV detection is the antibody response against HIV molecules (Levy, 1993). The most immunogenic molecules appear to be envelope glycoproteins. High titers of anti-gp120 and anti-gp41 antibodies have been found in most infected individuals. Other antibodies have also been found including those against p24, RT, and *gag* and *pol* products. Neutralizing antibodies that bind to gp120 have been identified in HIV-infected individuals. However, antibodies do not appear to effectively clear the virus (*Ibid*).

1.2.2.2 Cell-Mediated Response

In HIV infection, natural killer (NK) cell activity is often reduced, particularly in individuals who progress to AIDS (Cai *et al*, 1990). HIV-infected cells can be destroyed through antibody-dependent cellular cytotoxicity (ADCC). In this process, infected cells are killed through the recognition by NK cells of antibodies bound to viral envelope proteins on the cell surface (Brenner *et al*, 1991).

Another major cell type reactive with HIV-infected cells is the CD8+ subset of T-lymphocytes. Studies have shown that CD8+ lymphocytes can kill cells that express a variety of different HIV proteins, including RT, *env* proteins, p24, and even some accessory proteins like Vif and Nef (reviewed in Rowland-Jones and McMichael, 1993). The CTL response appears to be highest during the asymptomatic period of infection and then declines with disease progression (Carmichael *et al*, 1993). The role of these cells in combatting HIV-infection is not well understood and they may not neutralize the virus effectively. The ability of CTL's to lyse cells may be severely compromised in AIDS patients. Also, certain HIV strains may be able to successfully evade detection by these cells (Phillips *et al*, 1991b, Nowak and McMichael, 1995).

1.2.3 HIV and Immunosurveillance

The immune system may not confer adequate protection, in part because the CD4+ cells that initiate protective responses are killed or inactivated by the virus.

The HIV genome possesses a high degree of genetic variability which results in antigenic variations that serve to successfully evade the host (Hahn *et al*, 1986). While the *env* gene has been found to have the most antigenic variation in the virus, variation has also been observed in the *gag* region of the genome and elsewhere (Starcich *et al*, 1986). Thus, it has been proposed that the constant mutation of viral genes results in changes to viral protein epitopes. These mutations enable the virus to evade (to some extent) the host immune system (reviewed in Nowak and McMichael, 1995).

The possibility that other infections activate HIV by stimulating the lymphocytes that harbor the provirus has received increasing attention. *In vitro* studies have determined that if other viruses are co-infected along with HIV-1, the expression and production of HIV is greatly enhanced. It has been discovered that transactivating factors produced by the infecting virus may interact either directly or via intracellular factors with the HIV-1 LTR. The early gene products of herpesvirus, adenovirus, and hepatitis B virus may interact with the promoter region on the HIV LTR, thus activating increased viral production (reviewed in Gendelman *et al*, 1986, Nelson *et al*, 1990). Nonetheless, clinical studies have not conclusively determined if other viruses are cofactors in HIV pathogenesis (Levy, 1993).

Individuals may be infected with mixtures of high and low virulent HIV strains. The highly virulent variants may exert their cytopathic effect almost immediately and thus elicit rapid host immune responses. Lower virulent strains may persist for longer periods or be latent. As they strengthen in numbers, they may simultaneously increase in virulence, leading to depletion of CD4+ cells and progression to AIDS (Miedama *et al*, 1990, Weiss, 1993).

1.3 Regulation of HIV-1 by Cellular Factors

Clearly, the long latency periods of viral infection indicates that host-viral interaction plays an important role in determining controlled or activated viral expression. T cell-activating mitogens such as PHA and PMA have been shown to induce cellular proliferation and *in vitro* viral replication by inducing the T-cell activation factor NFκB, that binds enhancer elements on the LTR (Nabel and Baltimore, 1987). Other transcription factors such as NFAT, AP-1, SP-1, and TBP also enhance viral mRNA transcription by binding to the LTR (reviewed in Gaynor, 1992). Ultraviolet light also caused enhanced HIV gene expression (Valerie *et al*, 1988).

Cytokines are a unique group of regulators that play an important role in the upregulation of HIV expression. TNFα, in particular, has been shown to substantially increase HIV production,

by activating the cellular transcription factor, NFκB (Osborn *et al*, 1989, Folks *et al*, 1989, Duh *et al*, 1989). Some cytokines, including IL-1, IL-2, and IL-6 can also upregulate HIV gene expression, while others can influence HIV production at a posttranscriptional, posttranslational level (reviewed in Poli and Fauci, 1992). For example, IFN-γ can induce virus expression by increasing the efficiency of virion budding (Wong *et al*, 1988). However, only TNFα and TNFβ have been shown to trigger increased virus expression in both T cells and mononuclear phagocytes (Poli and Fauci, 1992).

Other cytokines may suppress HIV expression as well as have bifunctional effects. IFN-α and IFN-β are suppressors of the synthesis of viral proteins in both mononuclear and T-cell lines. On the other hand, TGF-B, has been shown to both block reverse transcriptase activity in monocytic cell lines as well as to upregulate HIV expression in the U937 monocytic cell line (Poli *et al*, 1991, Lazdins *et al*, 1991).

2. Heat Shock Proteins

2.1 The Historical Roots of the Heat Shock Response

Historically, heat has been shown to be implicated in the control of disease from the time of the ancient Greeks (Nover, 1989). The first recent evidence of an active heat shock response can be traced back to studies done by F. M. Ritossa on the fruitfly, *Drosophila Melanogaster*. In 1962, Ritossa discovered that heat treatment of *Drosophila* induced new sites of gene activity (observed by chromosomal puffing) (Ritossa, 1962). Characterization of the corresponding heat shock proteins of *Drosophila* occurred over a decade later, when chromosomal regions associated with heat-induced puffing were shown to contain the genetic code for a specific set of proteins, now known as heat shock proteins (HSPs) (Tissières *et al*, 1974). By the end of the 1970's, heat shock responses were found in all prokaryotic and eukaryotic cells (Nover, 1989).

In nearly all cells, metabolic and environmental toxins as well as viral or bacterial infection induce increased production of this set

of proteins (Schlesinger, 1990). Because so many toxic stimuli can be involved, the term heat shock proteins was replaced by the more accurate term, stress proteins. Many of these proteins are also present in organisms under standard conditions, and may be involved in normal cell function. The constitutive expression of HSPs in normal, unstressed cells indicates that their functions are complex and that they may often be necessary for cell survival. (Lindquist, 1986, Lindquist and Craig, 1988, Ang *et al*, 1991).

2.2. HSPs: Suspected Roles and Functions

2.2.1 Role in the Cellular Defence Against Heat Shock

A long-standing hypothesis about HSPs is that they protect cells from the toxic effects of heat and other stresses (Lindquist, 1986). Virtually every class of HSP has been implicated in studies on thermotolerance. Most evidence has pointed to two general ways in which HSPs prevent the accumulation of aberrant proteins that arise in response to stress. First, HSPs function as molecular chaperones by preventing unfolded proteins from aggregating, thereby restoring the normal structure and function of the substrate proteins. However, they also aid in protecting cells from the effects of stress by enhancing the movement of structurally damaged proteins through proteolytic mechanisms that will eventually lead to their degradation (reviewed in Lindquist, 1986, Parsell and Lindquist, 1994).

The heat shock response is induced in different organisms at different temperatures. In *Drosophila*, induction occurs between 33-37° C and HSPs like HSP70 have been found to play a major role in thermotolerance mechanisms of this organism (reviewed in Parsell and Lindquist, 1994). For example, thermoresistant cells that had been subjected to repeated lethal heat treatments, were found to overexpress HSP70 (*Ibid*). In thermophilic bacteria growing at 50°C, these proteins were only induced when temperatures were raised to 60°C. In mammals, inducible levels of HSPs are usually seen only under conditions of fever (>39° C) (*Ibid*, Lindquist and Craig, 1988). The ability of HSP70 to protect cells from thermal stress is not

dependent on its ATP-binding domain, an area necessary for several of this protein's chaperone functions (Li *et al*, 1992).

Other HSP families, such as HSP90 and HSP27, have also been seen in high levels in thermoresistant cell lines (Lindquist and Craig, 1988). Mild heat shock or stable transfection, leading to increased cellular levels of HSP27 and HSP70, may enable cells to overcome apoptotic cell death (Samali and Cotter, 1996). Thus, these proteins may protect cells by inhibiting synthesis of apoptotic-inducing agents. In this way, HSPs could protect cells from both normal hyperthermic challenge as well as prevent fatal cellular pathways like programmed cell death (*Ibid*).

Thermotolerance experiments have shown that the HSP response may be crucial to cell survival. Cells given a mild preheat treatment to induce HSPs undergo a much slower death than those whose temperature is elevated abruptly. Preheat treatment (to induce an HSP response) may also induce tolerance to other forms of stress (reviewed in Lindquist and Craig, 1988, Parsell and Lindquist, 1994).

The knockout of genes encoding for several bacterial and yeast HSPs eliminates the ability of these organisms to respond to hyperthermic challenge (Georgopoulos, 1977, Craig *et al*, 1987, Craig *et al*, 1994). Moreover, transfection of genes that encode a human HSP allows rodent cells to become thermotolerant (Landry *et al*, 1989).

2.2.2 Role as Molecular Chaperones

The cloning of the heat shock genes and the subsequent purification of the major HSPs by Welch and colleagues led to several important discoveries (Welch and Feramisco, 1982). First, heat shock proteins are highly conserved. For example, HSP70, the major heat shock protein family, was found to share over 50% homology between E. coli and human cells. Some domains were 96% similar. Also, many HSPs were found to be constitutively present and essential for normal cell function (reviewed in Schlesinger, 1990).

HSPs are directly involved in protein synthesis and translocation through intracellular compartments (Becker and Craig, 1994). Because of their involvement in these processes, HSPs are known as 'molecular chaperones'. This term was originally coined to describe an abundant protein, known as nucleoplasmin, in Xenopus oocytes, that promotes the active assembly of nucleosomes (Hendrick and Hartl, 1993). The term 'molecular chaperone' now includes a larger group of proteins that bind and stabilize an otherwise unstable protein to subsequently ensure its correct pathway, whether by folding, assembly or translocation. The majority of chaperones belong to three highly conserved protein families, HSP70, HSP90, and HSP60. However, other HSP families, such as HSP27 may also function in this way (Ellis and Van der Vies, 1991, Gething and Sambrook, 1992).

Many chaperone proteins are expressed constitutively and abundantly and are essential for cell viability (reviewed in Hendrick and Hartl, 1993). The participation of accessory components, or 'molecular chaperones' is vital in protein folding and assembly (reviewed in Gething and Sambrook, 1992).

2.2.3 Role in Transcriptional Regulation

The role of HSPs in transcriptional regulation of heat shock genes is crucial to their involvement in normal cell events (Morimoto, 1993). The expression of HSPs in response to physiological stress is dependent on the activation of heat shock transcription factor (HSF). The results of in vitro experiments implicate HSPs in general and HSP70, in particular, in regulation of HSF activation. HSP70 inhibits activation of HSF in a negative self-regulatory manner by binding to the monomeric form of HSF, thus ensuring its deactivation (Abravaya *et al*, 1992). Under conditions of stress, resulting in protein denaturation, the number of substrates competing for association with HSP70 increases, leading to dissociation of inactive HSF-HSP70 complexes (*Ibid*). After release from HSP70, HSF is transformed into a trimeric state, translocating to the nucleus and activating the transcription of the heat shock genes (Lis and Wu, 1993). HSPs, including HSP70, are then produced, which serve to regulate normal

protein conformation (Didomenico *et al*, 1982). In this way, when expressed in sufficiently high quantities, HSP70 acts to regulate HSP expression by binding to the transcriptional activator responsible for HSP production (Lis and Wu, 1993).

2.3 Structural and Functional Aspects of the HSP families

2.3.1 HSP70 Family

The HSP70 family is the most thoroughly studied heat shock family system (Lindquist and Craig, 1988). In most eukaryotes, a number of HSP70 subspecies are found in the nucleus, mitochondria, chloroplasts, endoplasmic reticulum (ER), and the cytosol. All mammalian HSP70 examined to date bind ATP with high affinity; this ATP binding is crucial to their function as molecular chaperones. Among the many suspected roles of this family of HSP is (i) their involvement in posttranslational protein folding, assembly, and translocation, and (ii) their involvement in binding to peptide regions that target intracellular proteins for lysosomal degradation (Chiang *et al*, 1989, Beckmann *et al*, 1990).

In addition, the chaperone function of HSP70 exists in numerous organisms. The binding of nascent chains on ribosomes, the translocation of proteins from target compartments, and the modulation of macromolecular peptide assemblies are but some of the many functions associated with HSP70 chaperone involvement (reviewed in Welch, 1991, Beckmann *et al*, 1992).

Evidence implicating mammalian HSP70 in protein folding and assembly was initially discovered when an HSP70-like protein in the ER, known as GRP78 (glucose related protein) was shown to be identical to a protein previously described as BiP, the immunoglobulin heavy chain binding protein (Bole *et al*, 1986, Munro and Pelham, 1986). Grp78, a protein located exclusively in the ER is about 60% homologous to HSP70 (Pelham, 1986). BiP had been found to play an important role in the folding and assembly of immunoglobulin heavy and light chains. In this way, BiP or GRP78

was identified as the first HSP to bind unfolded proteins. This led to the recognition of HSP70 as an important molecular chaperone (*Ibid*).

HSP70 exists in various intracellular compartments, besides the ER. All organisms have at least one HSP70 in the cytosol. Under various conditions of stress, mammalian cells produce large numbers of cytosolic HSP72, a highly inducible member of the HSP70 family. All healthy mammalian cells also produce another member of the HSP70 family known as HSP73, or HSC70 (for heat shock cognate), distinct from HSP72. Both HSP72 and HSP73 are functionally analogous and both possess a high degree (>90%) of sequence homology (Welch, 1991, Brown *et al*, 1993).

As in the case of BiP/grp78, all HSP70's exhibit strong affinity for unfolded proteins. Mitochondrial HSP70 binds unfolded proteins as they are translocated into the matrix from the cytosol (Becker and Craig, 1994). The ability of HSP70, to bind to peptides and ensure proper folding and assembly contrasts with earlier results that showed protein folding to be a spontaneous event, dictated exclusively by primary amino acid sequence (Beckmann et al, 1990, Anfinsen, 1973). Numerous *in vivo* studies have shown that protein folding requires the active participation of accessory components like HSPs (Beckmann *et al*, 1990, Welch, 1992).

HSP70 proteins function by preventing aggregation of newly synthesized polypeptides. This is mediated by tight binding of HSP70 to hydrophobic surfaces whose interactions are necessary to promote aggregation. The HSP70's then mediate protein folding to a native state, a process fuelled by ATP hydrolysis (Beckmann *et al*, 1990). It is still unclear how HSP70 molecules recognize and bind unfolded proteins or release their target. In cell free systems, HSP70 binds to the hydrophobic surfaces of unfolded proteins and not to properly folded proteins (Welch, 1991). Thus, HSP70 acts to change protein conformation, weakening interactions with other proteins needed for folding (reviewed in Frydman and Hartl, 1994).

Since stably folded proteins are generally unable to traverse the membranes of subcellular organelles (nucleus, mitochondria, chloroplasts, ER), it makes sense that HSPs might aid in translocation of protein. HSP70 is one of a number of factors required to maintain

precursor proteins ready for translocation (*Ibid*). HSP70 translocates protein to the nucleus, ER, and mitochondria by unfolding the partially folded polypeptide so that it can be translocated into the membrane pore (Shlesinger, 1990, Shi and Thomas, 1992). HSP70's are also involved in the stabilization of unfolded protein precursors before their assembly into multimolecular complexes. For example, HSP70 in the cytosol can promote the proteolytic processing of proteins by facilitating their transport into lysosomes (Chiang *et al*, 1989). They are also involved in the rearrangement of protein oligomers and in the resolution of protein aggregates (Becker and Craig, 1994).

2.3.2 HSP60 Family

The family of HSP60 proteins, another distinct family of HSP, also bind unfolded polypeptides and play a crucial role in the folding and assembly of protein structures. In contrast to the unfolding and stabilization role of most forms of HSP70, HSP60's participate actively in the folding and assembly of polypeptides from non-native proteins to their native state. Members of this family exist in all prokaryotes. In eukaryotes, they are mainly present in the organelles of endosymbiotic origin, like the mitochondria and chloroplasts (reviewed in Schlesinger, 1990, Gething and Sambrook, 1992, Frydman and Hartl, 1994). Although this family is stress inducible in bacteria, mitochondria, and in plant chloroplasts, they are also constitutively expressed. These proteins are highly conserved and like the HSP70 molecules, their main function is a chaperone one (Frydman and Hartl, 1994). Based on these properties, this family of HSP are commonly referred to as Chaperonin (Nover 1989).

The first evidence that eukaryotic HSP60 possessed chaperone functions came from the discovery that there were sequence similarities between it and a 57-kDa protein known as tailless-complex polypeptide1 (TCP1) (Gupta, 1990, Becker and Craig, 1994). TCP1, a protein found in high concentration in developing sperm cells, was soon found to have a similar sequence and structure to

other cytoplasmic chaperonin (*Ibid*). The HSP60 family consists of a large 60-kDa subunit (groEL) and a smaller, similar 10-kDa subunit (groES), which interact together in an ATP-dependent manner (Welch, 1991). They consist of double-ringed structures and in E. coli, they have been found to facilitate the ordered assembly of the bacteriophage head complex in infected bacteria (*Ibid*).

A role in translocation has also been proposed for this class of stress protein. HSP60 may trigger the complete unfolding of a protein, thereby easing its movement across the membrane. However, it is clear that in translocation into mitochondria, proteins interact first with an HSP70 molecule and then with the HSP60 one. Once these proteins traverse the membrane, HSP60 becomes an essential component in the refolding of these proteins (Ostermann *et al*, 1989). This process, originally thought to be spontaneous, is in fact catalyzed by HSP60. HSP60 may achieve its function by interacting with unfolded proteins in the same way as HSP70. However, unlike their 70-kDa counterparts, HSP60 do not bind to hydrophobic surfaces but rather to some other unknown area (*Ibid*).

Other similarities with the HSP70 family include their mutual dependence on ATP activity and their weak ATPase activity. Moreover, despite sequence differences, with members of the HSP70 family, chaperonin-60 proteins function similarly to HSP70 i.e. they bind to structural elements present during the intermediate folding steps of protein substrates. Although their function is ATP-dependent, it is unclear what role ATP hydrolysis plays in the folding and release of target polypeptides (Gething and Sambrook, 1992). Both protein families are also hyper-expressed, when cells are exposed to environmental stress (*Ibid*).

2.3.3 HSP90 Family

The HSP90 family is another family of HSP that bind to and stabilize folded proteins. HSP90 is the most abundant constitutively expressed stress protein in the cytosol of eukaryotic cells (Wiech *et al*, 1992). Members of this family possess a high degree of sequence homology. In mammalian cells, two genes, designated α and β, encode

for HSP90. The protein products they encode are present in high numbers under normal growth conditions, but can be further induced by environmental stress (reviewed in Lindquist and Craig, 1988).

HSP90 is a molecular chaperone that prevents non-native proteins from participating in aberrant molecular interactions. This is achieved by the binding of an HSP90 dimer to one or two molecules of the substrate protein to stabilize the target proteins in an inactive form (Wiech *et al*, 1992). In mammalian cells, HSP90 associates with a large number of cellular proteins, including retroviral-transforming proteins, steroid hormone receptors, and cellular protein kinases (Oppermann *et al*, 1981, Gething and Sambrook, 1992). The Rous sarcoma virus protein, pp60src, is maintained by HSP90 in an inactive form until it reaches its final destination at the cell membrane (Lindquist and Craig, 1988). Other viral proteins including those of Sindbis virus and vesicular stomatitis virus (VSV) are also associated with HSP90 (reviewed in Craig, 1986).

Currently, most of what is known about HSP90 *in vitro* relates to its interaction with steroid hormone receptors. All steroid hormone receptors have been found to associate with proteins of the 90-kDa variety. Binding of HSP90 to the receptor maintains it in an inactive state (in the form of an aporeceptor complex), unable to bind to its intended substrate. This complex remains folded and inactive as long as HSP90 is bound to it. However, with increasing hormone concentrations, a shift of the receptor occurs, resulting in the dissociation of HSP90 and the subsequent binding of the hormone to the receptor. In this way, HSP90 prevents the receptor from interacting with inappropriate targets until it can be bound by the hormone. Once the steroid is bound, the complex can migrate to the nucleus to become active (Bohen *et al*, 1994, 1995). Thus, HSP90 keeps the receptor inactive until the proper signal for activation is received. Furthermore, dissociation of HSP90 from the hormone-receptor complex correlates with activation of the receptor for DNA binding. Therefore, until the hormone displaces HSP90 from the complex, it cannot bind DNA or influence transcription (*Ibid*).

HSP90 differs from HSP70 and HSP60 in several ways. First, it is highly phosphorylated at serine and threonine residues (Ferris *et al*, 1988, Haire *et al*, 1988). More importantly, unlike the other two major HSP families, HSP90 is not dependent on ATP hydrolysis. Association of HSP90 with other chaperone molecules, such as HSP70, suggests that the two proteins may act in conjunction (Rose *et al*, 1989, Gething and Sambrook, 1992).

One of the most studied members of the HSP90 family is GRP94, a protein found mainly in the ER. GRP94 is implicated in the sorting of proteins destined for transport (Gething and Sambrook, 1992, Kulomao *et al*, 1986). HSP90 are also involved with other cellular phenomena including thermotolerance in mammalian cells, prevention of aggregation of denatured polypeptides in vitro, and the chaperoning of proteins (Bansal *et al*, 1991, Ellis and Van der Vies, 1991).

2.3.4 HSP27 Family

Small molecular weight HSPs range in mass from 15 to 30 kDa. Although this family of HSPs was among the first to be identified, little is known about their function (Arrigo and Welch, 1987, de Jong *et al*, 1993). This group of stress proteins exhibits less cross-species homology than do higher molecular weight HSPs (reviewed in de Jong *et al* 1993). The expression of HSP27 is observed under heat shock conditions and may have a protective effect on cell viability at elevated temperatures (Jakob *et al*, 1993). HSP27 are present in the cytosol of most cells and are expressed in the absence of heat shock or other stress conditions (de Jong *et al*, 1993).

The two isoforms of α-crystallin, αA and αB are expressed in abundance in the lens of the eye where they make up around 35% of total protein. This protein is largely responsible for ensuring the unique properties of the eye lens (Craig, 1986). The C-terminal region of the αB isoform of this protein has extensive (>70%) homology with mammalian HSP27; it may thus be part of the HSP27 family (Hickey *et al*, 1986, Ingolia and Craig, 1982). Furthermore, both α–B-crystallin and HSP27 are found in detergent-insoluble

fractions during heat shock, and both possess a similar sequence in their respective heat shock elements (HSE), the area of the promoter to which HSF binds during transcription (Klemenz *et al*, 1991).

As in the case of most other HSPs, HSP27 functions as a molecular chaperone (Horwitz, 1992). Expression of HSP27 and α-crystallin led to an ATP-dependent increase in the refolding of denatured substrate proteins (Jakob *et al*, 1993). Thus, both HSP27 and α-B-crystallin function as molecular chaperones by influencing the folding of proteins and binding to unfolded proteins to prevent non-specific protein interactions (*Ibid*, Horwitz, 1992). It is not known whether HSP27 acts with other molecular chaperones or alone (Jakob *et al*, 1993).

Prior to heat shock, HSP27 and α-B-crystallin exist in large macromolecular complexes that disassociate under stress. These proteins may function differently when complexed than when separate (Zantema *et al*, 1992). Since HSP27 has been identified as the p29 estrogen receptor-associated protein, chaperone duties may be performed by it in this context as well (Mendelsohn *et al*, 1991). Thus, HSP27 may cooperate with other HSPs, like HSP90 (already known to interact with steroid receptors), in chaperoning these receptors before the hormone binds to the receptor complex (Ciocca *et al*, 1993).

HSP27 also confers thermoresistance to cells that are subject to stress. The presence of HSP27 provides protection against heat shock that was administered shortly after transfection of the pHS2711 gene, known to encode for human HSP27 (Landry *et al*, 1989). Heating hamster cells for 10 minutes led to an immediate increase in phosphorylated forms of HSP27. Early phosphorylation of HSP27 (immediately following heat shock) may establish a protective effect that limits heat-induced reactions that ordinarily lead to hyperthermic damage. Other HSPs may act to repair damage that has already been caused and that have escaped the effect of HSP27 (*Ibid*).

Several agents that affect phosphorylation of HSP27 may prepare this protein for its chaperone role. TNFα increases HSP27 phosphorylation by inducing a mitogen-activated (MAP) kinase.

Phosphorylation of HSP27 may also affect signal transduction pathways (Kaur *et al*, 1989, Arrigo, 1990). However, the constitutive expression of HSP27 in L929 fibrosarcoma cells confers cellular resistance to TNFα. This is in contrast with reports that HSP27 is twice as effective as α-B-crystallin at protecting cells against TNFα cytotoxicity. Thus, HSP expression may not always be consistent with thermotolerance. In this way, HSP27 may act as both a molecular chaperone as well as enhance cellular resistance to cytokines (Mehlen *et al*, 1995).

- Proteins that are phosphorylated at serine and threonine residues may themselves be downstream targets in signalling pathways. Therefore, HSP27 may be involved in the signal transduction pathways of several cell regulators (*Ibid*, Ciocca *et al*, 1993). HSP27 is phosphorylated at several serine residues early after heat shock. The same residues are phosphorylated regardless of stress condition (Landry *et al*, 1992). It is not known whether phosphorylation of HSP27 is required for chaperone activity or the thermoprotective effect (Lavoie *et al*, 1993, Knauf *et al*, 1994). Initially, rapid phosphorylation of HSP27 by MAPKAP kinase 2 was thought to contribute to both its thermotolerance and chaperone duties. However, since non-phosphorylated HSP27 can exert the same chaperone and thermoprotective functions, phosphorylation of the small HSP may not be essential for either function (Knauf *et al*, 1994).

Recent identification of a novel protein kinase cascade that is activated in response to heat shock and leads to phosphorylation of human HSP27 has raised new questions (Zhou *et al*, 1993). The signal transduction pathway that results in phosphorylation of HSP27 may also be involved in mediating responses to cytokines including TNFα, a known stimulator of HSP27 phosphorylation (Rouse *et al*, 1994).

2.4 HSPs and the Immune Response

Interleukins and mitogens which activate lymphocytes and macrophages also bring about increased expression of HSPs. Members of the HSP gene family have been implicated in

lymphocyte differentiation, homing, antigen processing and presentation, and immunoglobulin assembly (Kaufmann, 1990, Young, 1990, Born *et al*, 1990). Because cells that have been infected by invading pathogens produce protective stress proteins, they are able to escape cytotoxicity by monocytes, natural killer cells (NK), and lymphokine activated killer (LAK) cells (Kaufmann, 1990, Jäättela and Wissing, 1993). Aspects of the immune system for which a role for HSPs has been hypothesized are discussed below.

2.4.1 HSPs and Antigen Presentation

Antigen recognition by T cells is achieved through the T cell receptor (TCR). This requires intracellular processing of certain components and subsequent presentation on the cell surface by specialized molecules, i.e. class I and class II proteins that comprise the major histocompatibility complex (MHC). In general, antigenic peptides do not bind to TCRs but require association with the MHC on the surface of an antigen presenting cell (APC) to activate antigen-specific T cells. This is true for both helper (CD4+) T cells which recognize antigen bound to MHC class II molecules and for cytolytic (CD8+) T cells which recognize antigen in association with MHC class I molecules (Pierce *et al*, 1991).

Native antigens are converted to peptides prior to presentation by MHC molecules. Several peptide binding proteins can facilitate the binding of peptides to the MHC and to the TCR, and thus play an important role in antigen presentation (Lakey *et al*, 1987). For example, the protein PBP72/74, a member of the HSP70 family, can bind to processed antigenic peptides (Van Buskirk *et al*, 1989). The function of this protein may be similar to the chaperone role of other members of the HSP70 family. It may bind to peptides to prevent their proteolysis, thus enabling them to bind to the MHC. They may also bind to exposed sites on the MHC, allowing for more appropriate folding and binding of peptides (reviewed in Pierce *et al*, 1991). The peptide binding site of HSP70 is similar to peptide-binding domains of histocompatibility proteins (Rippmann *et al*, 1991). Furthermore, the gene that encodes HSP70, is located within the MHC locus,

suggesting that the properties are genetically linked (Sargent *et al*, 1989, Gunther, 1991). HSPs might be an evolutionary forerunner of MHC molecules (Polla, 1991).

Stress proteins made by bacteria and parasites are often the major target antigens reacted against by the immune system (Newport, 1991). HSPs are considered important in some vaccine strategies for their ability to prevent microbial infections (*Ibid*). However, in order to serve as effective vaccines, stress proteins must be able to elicit an appropriate cellular and/or humoral response that will be effective at preventing the establishment of these pathogens. Efforts to map the specificity of host immune responses to stress proteins are ongoing.

2.4.2 HSPs and γ/δ T cells

Most T cells express a TCR composed of α and β chains but a minority (5-10%) express a receptor composed of γ and δ chains. It is well known that those expressing the α/β chains recognize antigenic peptides when presented by MHC class I or class II molecules. While the mechanism by which γ/δ T cells recognize antigen is still under review, these cells have been found to lyse targets in a broad, non-restrictive fashion (reviewed in Kaufmann, 1990). Generally, these T cells lack both the CD4 and the CD8 surface molecules, but the functions of γ/δ T cells may be similar to those of their more abundant relatives (Kaufmann and Schoel, 1994). Many of the γ/δ cells characterized appear to be specifically attracted to the microbial forms of stress proteins (Kaufmann and Kabelitz, 1991).

In the immune response to mycobacterial infections, such as *Mycobacterium Tuberculosis* or *Mycobacterium Leprae*, γ/δ T cells are specifically attracted to HSP65, an epitope of the HSP60 family (Haregewoin *et al*, 1989, Kaufmann, 1990, O'Brien *et al*, 1991). T lymphocytes from patients with mycobacterial infections specifically recognize the mycobacterial stress proteins, HSP70 and HSP60. This suggests that these HSPs are major targets of the immune system (Suzue and Young, 1996). γ/δ T cells may recognize HSPs expressed on

the bacterial surface or in the context of MHC molecules (Kaufmann and Schoel, 1994).

2.4.3 HSPs and Immunosurveillance Mechanisms

Recognition of stressed host cells by T cells attracted to HSPs could technically provide a means of immune surveillance, by focussing on a marker present after all types of stress (Young and Elliot, 1989). γ/δ T cells may precede other types of T cells to the point of infection. Second, they are activated more rapidly than other immune cells in response to a variety of bacterial and viral infections (reviewed in Kaufmann and Kabelitz, 1991, Kaufmann and Schoel, 1994).

HSPs can also play regulatory roles in immune responses to a number of tumours and virally-infected cells. Several types of T cells (and not just the γ/δ subset) have been shown to recognize stress proteins. Therefore, it has been postulated that self-reactive T cells may provide a first level of defense against infection by recognizing stressed cells by virtue of HSPs and thus help to eliminate them (Kaufmann, 1990). HSPs may be induced in many viral infections (see 2.5). The CTLs that combat these viruses may limit viral spread by killing infected cells.

T cells that recognize epitopes on these proteins may also attack uninfected host cells that contain constitutive levels of these proteins. Several HSPs can induce autoimmunity, and some autoimmune diseases, including rheumatoid arthritis, systemic lupus and others involve antibodies to HSPs in sera and at sites of immune-mediated tissue damage (reviewed in Winfield and Jarjour, 1991). Thus, lymphocytes that specifically recognize stress protein determinants must be able to differentiate between normal and stressed cells, perhaps by distinguishing between substimulatory levels and higher levels of HSP production. Upon stress, the number of HSPs and their location on both the cell surface and within cellular compartments increase (Lindquist and Craig, 1988, Jarjour *et al*, 1991). In autoimmunity, the immune system may not be able to adequately discern between HSPs that are normally present versus

those induced by invading microorganisms (Winfield and Jarjour, 1991).

2.5 HSPs and Viral Infection

Both DNA and RNA viruses have been reported to induce increased expression of at least one subset of HSPs in human cells *in vitro*. Different HSP70 subspecies are induced following infection by: vaccinia virus (Jindal and Young, 1992), Epstein-Barr virus (Mannick *et al*, 1995), polyoma virus (Cripe *et al*, 1995), rabies virus (Sagara and Kawai, 1992), adenovirus (Niewiarowska *et al*, 1992, Nevins, 1982), Newcastle disease virus (Collins and Hightower, 1982), and herpes simplex virus (Notarianni and Preston, 1982, Macnab *et al*, 1985). HSP90 has also been found in host cells following infection by: Epstein-Barr virus (Cheung and Dosch, 1993) and herpes simplex virus (LaThangue and Latchman, 1988). However, the presence of HSP90 does not interfere with viral infection (Bansal *et al*, 1991). HSP90, a protein essential in several signal transduction mechanisms, may be required for the activity of hepatitis B virus reverse transcriptase (Hu and Seeger, 1996). HSP27 may be involved in infection by adenovirus (Zantema *et al*, 1989), human papilloma virus (Ciocca *et al*, 1992), and hepatitis B virus (Ciocca *et al*, 1991, Delhaye *et al*, 1992).

In addition to the modulation of HSPs in the afore-mentioned infections, stress proteins have also been implicated in aspects of viral replication (Burdon, 1986). Many viruses rely on host factors to replicate; viral protein folding and assembly might depend on host HSPs. These proteins might be induced during viral infection because their presence is vital to the infectious process. HSPs may be present in intact virions, as well as bind to virus components (Santoro, 1994).

2.6 HSPs and HIV-1 Infection

An HSP60 related protein has been found to have a physical association with both HIV and SIV (Bartz *et al*, 1994). *In vitro*, heat shock resulted in elevated levels of virus production (Santoro, 1994).

Furthermore, human cells chronically infected with HIV-1 had increased levels of viral gene transcripts (*Ibid*). Heat shock may also transcriptionally activate both the long terminal repeats (LTR) of HIV-1 as well as the HSP70 promoter, albeit through different mechanisms (Geelen *et al*, 1988, Kretz-Remy and Arrigo, 1994).

Continuous hyperthermia, at 3°C-4°C above normal physiological temperatures, results in disruption of virus expression with cellular death (Santoro, 1994). While levels of natural killer cells increased during hyperthermia, numbers of circulating CD4+ cells decreased substantially (Kappel *et al*, 1991). That several groups have shown an increase of an HSP responsive T cell subset (γ/δ T cells) in HIV-1 infection indicates that HSPs may play a role in immune responsiveness (DePaoli *et al*, 1991, De Maria *et al*, 1992).

Stress protein induction may reflect a defensive response of host CD4-expressing lymphocytes to numerous physiological disruptions, including those caused by HIV-1 (Hershkoviz *et al*, 1993, Brenner *et al*, 1995). HIV-1 associated disease is characterized by cytokine imbalances, immune cell activation, fever, and opportunistic infections, which may directly and indirectly affect HSP production. Over-expression of stress proteins in infected cells may alert the immune system to viral infection. In this way, immunity directed against HSPs or HSP complexes may be involved in the massive T cell depletion observed in AIDS. Conversely, induction of stress responses may render infected cells resistant to immune based cytolysis and apoptosis (Santoro, 1994, Andrews *et al*, 1995).

Injection of HSP70 conjugated to viral proteins elicited strong humoral and cell-mediated immune response to specific HIV-1 proteins (Suzue and Young, 1996). This identified HSPs as potential immune targets when associated with HIV-1. Young and colleagues constructed a vector containing mycobacterial HSP70 fused to HIV-1 gag p24. Mice inoculated with the vector, mounted both a humoral and cellular anti-viral immune response. Covalent linkage of HSP70 to p24 was essential, suggesting that HSP70 had enhanced the immunogenicity of p24 (Suzue and Young, 1996).

Moreover, given that HIV-1 relies on many host factors for certain aspects of its replicative cycle, it is not unreasonable to

assume that HIV utilizes host HSPs to mediate viral protein folding, maturation, and assembly (Santoro, 1994). Viral components have been shown to bind to host stress proteins during the viral life cycle (Franke *et al*, 1994, 1995, Thali *et al*, 1994). Recent findings have shown that a host chaperone factor, cyclophilin A, is specifically incorporated into HIV-1 virion particles (Cullen and Heitman, 1994, Thali *et al*, 1994). The recent determination that this factor is required for certain clades of the virus but not others, suggests that it may play an important role in inhibiting stages of virus replication (Bratten *et al*, 1996b). Along with several HSP-related proteins, cyclophilin A has been found to be necessary for assembly and infectivity of HIV-1 virions (Bartz *et al*, 1994, Franke *et al*, 1994, 1995). Other host factors, including HSPs may similarly be involved in the production of infective virions and their deletion may also result in the virus's impaired ability to produce virions in a manner consistent with infectiousness.

Project Rationale

Heat shock proteins are induced during HIV-1 infection (Di Cesare *et al*, 1992, Poccia *et al*, 1993). Since HSPs are cellular chaperones, they may play an important role in multiple aspects of the viral life cycle. Moreover, HSPs represent primary antigenic targets of the immune response (Kaufmann, 1990). Therefore, their use as potential co-antigenic stimulants with HIV-proteins may have relevance in regard to therapeutic strategies (Suzue and Young, 1996). Immune responses against virally-induced HSPs may contribute to elimination of virus, CD4+ T cell depletion and/or autoimmunity in infected individuals.

This study was designed to determine whether changes in stress protein expression in HIV-infected CD4+ cells occur in tissue culture. To test whether or not HSPs are modulated in HIV-1 infection, we compared constitutive and stress-inducible levels of HSP production in uninfected CD4+ lymphocytic cells, acutely HIV-1 infected cells, and chronically infected cells. Furthermore, we examined the effect of other external stresses including heat shock and TNFα. We also determined whether any constitutive, heat-inducible or TNFα inducible HSP subspecies might be present in CD4+ cells concomitant with HIV-1 infection.

Materials and Methods

1. Cell Culture

The lymphocytic cell lines, CEM.NKR, H9, MT-2, and Jurkat were obtained from the American Type Culture Collection (Rockville, Maryland, USA) (Mann et al, 1989). Cell cultures were passaged twice weekly in complete RPMI 1640 medium (Gibco Laboratories, Toronto, Ontario, Canada) that was supplemented with 10% fetal calf serum (Flow Laboratories, Toronto, ON., Canada), 2 mM L-glutamine, 100 I.U./ml penicillin, 100 µg/ml streptomycin, and 10 mM HEPES. The cell lines were maintained in incubators at 37°C and under 5% CO_2 atmosphere.

Chronically-infected H9 and CEM cells were established in our laboratory as outgrowth lines surviving acute infection. The CEM cell line has been maintained in incubators for >4 years and cells consistently show 100% infectivity. Cell viability was assessed by trypan blue exclusion and cell counts over time were used to determine cellular proliferation.

2. HIV-1 Stock and titration of viral infectivity

The IIIB isolate of HIV-1 was kindly supplied by Dr. R. C. Gallo of the National Institute of Health (NIH), Bethesda, Maryland, USA. The virus was isolated from clarified supernatants of chronically infected H9 cells. Stock virus titers were quantified using the infectivity assay described by Johnson and Byington (1990). Four-fold serial dilutions of the viral stock were added to wells of a 96-well plate containing 0.4 x 10^6 MT-2 cells/well at a total volume of 150 µl. The plate was incubated at 37°C in 5% CO_2 for 3-4 days. On the third and fourth day, individual wells were checked for formation of syncytia and cell death. The 50% tissue culture infective dose ($TCID_{50}$) was then calculated using the method of Reed and Muench (Dulbecco, 1988). The $TCID_{50}$ of the original serial stocks range from 1-4 X 10^6 $TCID_{50}$ units/ml.

3. Cell infection

Virus stock preparations, 1-4 x 10^6 $TCID_{50}$/ml, incorporated approximately 1-4 x 10^6 cpm/50 µl, as measured in reverse transcriptase assays, and contained approximately 100-400 ng/ml viral p24 as measured in p24 antigen capture assays (see below). Aliquots of cell lines (5 x 10^6 cells/ml in complete growth medium) were centrifuged and cell pellets were separately treated as follows: (i) resuspended in 1 ml infectious virus stock at a multiplicity of infection (m.o.i.) of 0.2 (i.e 10^6 $TCID_{50}$ units/5 x 10^6 cells); (ii) resuspended in the same amount of virus stock that was inactivated by heating at 56°C for 1h; (iii) resuspended in 1 ml of conditioned clarified medium from uninfected H9 cells (mock treated controls); and (iv) resuspended in serial dilutions of virus stock (m.o.i.=0.2, 0.067, and 0.02). All the preparations were incubated for 5h at 37°C and then washed twice by low speed centrifugation, resuspended to the original 5 x 10^6 cells/ml aliquots in fresh growth medium and incubated at 37°C for different time intervals (5h, 12h, 24h, 48h, 5, 6, 7, 9, or 22 days after infection). The cells were routinely split on a weekly basis on days 4 and 7 post-infection.

4. Induction of stress responses in CD4-expressing cell lines

In most of the experiments, the cells were divided into several groups. Uninfected and chronically-infected CD4-expressing CEM cells were stressed by exposure to heat shock or the stress-inducing cytokine, TNFα. Unstressed, uninfected and chronically-infected cells were aliquoted and maintained in complete RPMI 1640 medium. The heat-stressed group was treated much the same except for an initial heat shock of 42°C for 2h followed by a recovery at 37°C. The TNFα groups were exposed to 10, 25 or 50 ng/ml of this cytokine for a 24h period.

5. Extracellular HIV-1 detection by reverse transcriptase (RT) assay

Measurement of HIV-1 activity in cell-culture supernatants by the RT assay was performed as described by Lee *et al* (1987). In brief, 50 µl of clarified aliquots of supernatants from cell cultures

were spun (3000 rpm, 30 min, 4°C) in a Beckmann TL-100 tabletop ultracentrifuge. 50 µl of the resulting supernatant was then added to 50 µl of reaction mixture containing: 50 mM Tris-HCL (pH 7.9), 5 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 150 mM KCl, 0.5 mM ethylene glycol-bis-(b-aminoethyl ether) N, N, N', N-tetraacetic acid, 0.05% Triton X-100, 2% ethylene glycol, 0.3 mM reduced glutathione (GSH), 20 µCi [^{3}H] thymidine triphosphate, and 50 µg/ml of poly (rA) oligo (dT) template primer. The tubes were then mixed and incubated at 30°C for 22h. The reaction was stopped by adding 1 ml of cold 10% TCA (in 0.02 M NaPPi). The resulting DNA was then precipitated on ice for 2h and then collected on GF/A disks (Gelman Sciences, Ann Arbor, Michigan, USA). It was then thoroughly rinsed twice with cold 10% TCA followed by one rinse with absolute ethanol. The disks were then dried for 20 min and counted in a beta liquid scintillation counter to determine levels of incorporated radioactivity.

6. Extracellular HIV-1 detection by p24 antigen capture ELISA assays

Virus production was monitored by detection of p24 HIV-core antigen in cell-free culture supernatants by enzyme linked immunoabsorbent assay (ELISA) using a murine monoclonal antibody against HIV core antigen (Abbott Diagnostics Laboratory, Mississauga, ON., Canada).

7. Cell extraction

5 ml aliquots of cell cultures at the designated time intervals were harvested and counted. Cell viabilities in all experiments were always found to be greater than 80%. Total cytoplasmic extracts were prepared by lysing 5 x 10^6 cells in 100 µl lysis buffer containing 20 mM Tris (Tris hydroxymethyl aminomethane)-buffered saline (TBS, pH 8.0), 2% sodium dodecyl sulphate (SDS), 2 mM ethylenediaminetetraacetic acid, 0.5% Nonidet P-40, phenylmethylsulphonyl fluoride (100 µg/ml), aprotinin (1 µg/ml), and leucopeptin (1 µg/ml). Protein concentrations in cellular extracts were determined using a modified Lowry procedure (Lowry *et al*, 1951).

8. Determination of intracellular levels of stress and HIV-1 viral proteins

In order to determine the levels of heat shock and viral protein expression, one and two-dimensional Western blot analysis was performed. Single-dimension protein analysis of cellular extracts (25 µg of protein) was performed using SDS-polyacrylamide gel electrophoresis in 12% acrylamide gels as described by Laemmli (1970), using the Novex system (Helixx Technologies, Scarborough, ON, Canada). Cell extracts were solubilized in 2x SDS-sample buffer containing: 0.5M Tris (pH 6.8), 4% SDS, 4% *B*-mercaptoethanol, DTT, 20% glycerol and 0.1% bromophenol blue. SDS-polyacrylamide electrophoresis was then performed using a 12% Tris/glycine gel (Laemmli, 1970).

Two-dimensional electrophoretic analyses were performed as described by O'Farrell (1975). In brief, isoelectric focussing (pI 3 to 10) was performed in the first dimension, and SDS-polyacrylamide electrophoresis in 12% acrylamide gels in the second dimension. Cellular extracts were maintained in their native state by diluting the extract 1:1 with 2x sample buffer which contained 30% glycerol, 0.2 mM arginine (free base), and 0.2 mM lysine (free base). After the gel was run in the first dimension, it was placed in a staining solution containing 0.1% coomassie blue in 40% methanol, and 10% glacial acetic acid for 30 minutes. The gel was destained in 25% ethanol and 8% glacial acetic acid for 20 minutes. After destaining, the IEF gel was placed in 20% ethanol for 1h, and then the lane to be transferred was cut out and run in the second dimension on 12% acrylamide gels.

Following electrophoresis, the gels were transferred to nitrocellulose membranes, blocked overnight with 5% milk in TBS (pH=7.5), containing 0.05% Tween 20 (TBST), and probed for various HSPs using HSP27, HSP60, HSP90, and HSP70 class-specific monoclonal antibodies (SPA-800, SPA-806, SPA-820, and SPA-830, StressGen Biotechnologies Corp., Victoria, British Columbia, Canada). The blots were also probed for HIV-1 using anti-viral p24 monoclonal antibodies (clone 39/5.4A, Cellular Products Inc., Buffalo, New York, USA). This antibody not only reacted with p24 but also could detect the viral p55 gag-precursor. All antibodies were

prepared in TBS/milk at the recommended concentrations and were exposed to the blots for the recommended times and temperatures. The blots were then washed in TBST for 3 x 15 min. and placed in a 1:1000 dilution of horseradish peroxidase-conjugated sheep anti-mouse antibody (Amersham Canada Ltd., Oakville, ON, Canada) for 1h. The blots were then washed again in TBST for 2 x 15 min and in TBS for 10 min. The enhanced chemiluminescent (ECL) detection procedure was then used to detect HSP and viral protein expression on radioautographic film (Amersham Canada Ltd.). Chemiluminescence was quantified using the Bio-Rad GS-250 molecular phosphorimager (Bio-Rad Inc., Mississauga, ON, Canada).

Results

1. Stress protein expression in CEM.NKR cells following acute HIV-1 infection

One of our primary aims was to determine whether any significant modulation in HSPs arose at various stages of primary lytic HIV-1 infection. The CEM.NKR cell line was chosen because these cells show little or no detectable levels of cognate HSP27, unlike other CD4-expressing cell lines (Brenner *et al*, 1995). Chronic HIV-1 infection, however, resulted in a ten-fold increase in levels of heat-inducible HSP27 expression as compared to uninfected cells. This cell line was thus the most ideal to distinguish between cognate and inducible HSP27 signals. Cells were mock-infected or acutely infected with HIV-1 and followed for the periods between 5h and 22 days in a number of different experiments. Western blot analysis was used to monitor intracellular levels of HSPs (HSP27, HSP60, HSP70, and HSP90) as well as HIV-1 viral (p24 and p55 *gag* precursor) proteins. Furthermore, levels of extracellular virus were monitored by p24 antigen-capture and reverse transcriptase assays at all times.

Figure 4 shows relative levels of the HSPs in CEM cells following acute HIV-1 infection. An induction of HSP27 was observed following infection. This was not seen in uninfected cells. The *de novo* appearance of intracellular hsp27 peaked at 24h following infection and declined thereafter. In contrast, HSP60, HSP70, and HSP90 levels were similar in viral-infected and mock-infected cell extracts regardless of the time post-infection.

Thus, as shown previously with chronically-infected cells, HSP27 expression was modulated selectively following acute HIV-1 infection. We next determined the kinetics of intracellular virus expression as related to this inducible HSP27 response. At five hours post-infection, residual intracellular HIV-1 virion p24 was present in HIV-1 infected cells. This declined and almost completely disappeared at 48h (Fig. 5). In contrast, a peak of HSP27 induction occurred at 12 to 48h following infection, at the same time

Figure 4: The effect of acute HIV-1 infection on *de novo* levels of stress (HSP27, HSP60, HSP70, and HSP90) protein production.

Cells were either mock or acutely infected (at an m.o.i of 0.2) as described under Materials and Methods, and were then extracted at the indicated times. Protein extracts were electrophoresed on uni-dimensional SDS-polyacrylamide gels and Western blot analysis was performed using antibodies to the appropriate stress proteins as probes. A chronically-infected CEM extract, harvested at the same time, is presented for comparative purposes.

Uninfected (lanes 1,3,5,7), acutely infected (2,4,6,8) and chronically infected (lane 9) cell extracts at times of 5h (lanes 1-2), 24h (lanes 3-4), 48h (lanes 5-6) and 6 days (7-9) are shown.

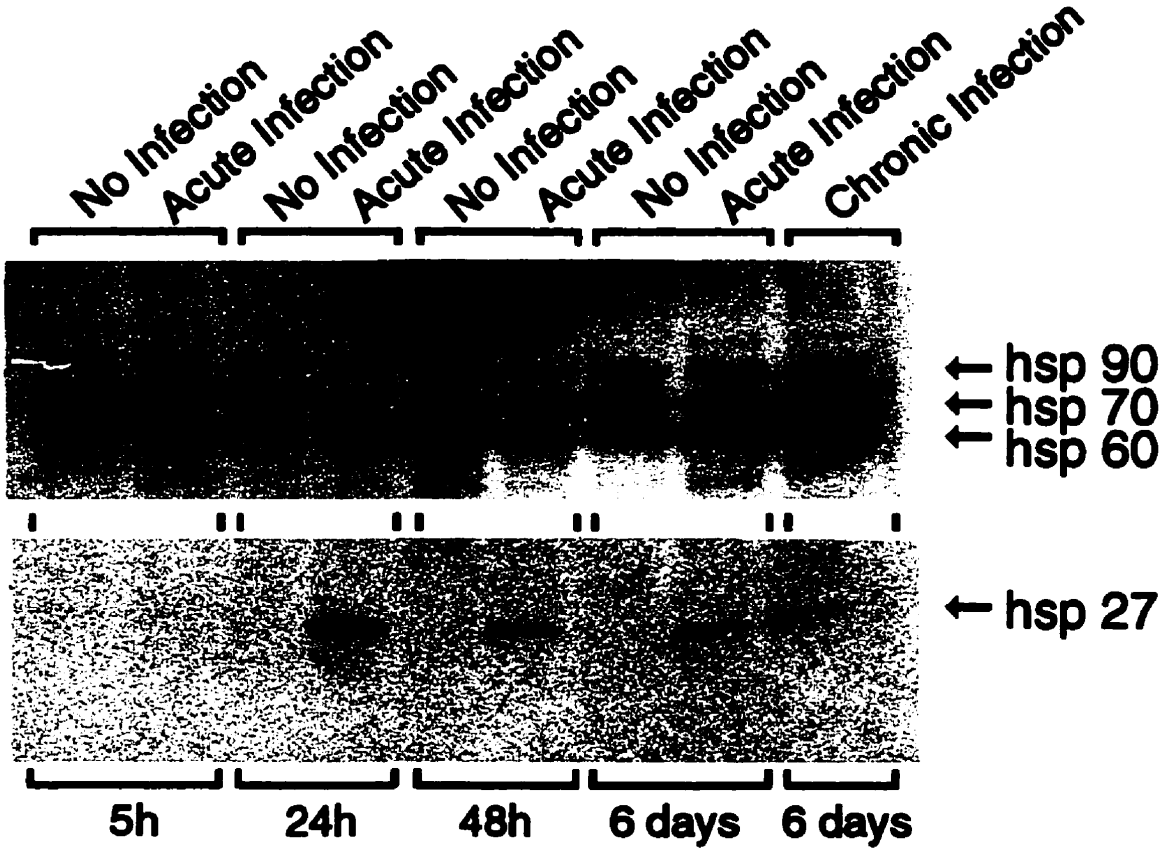
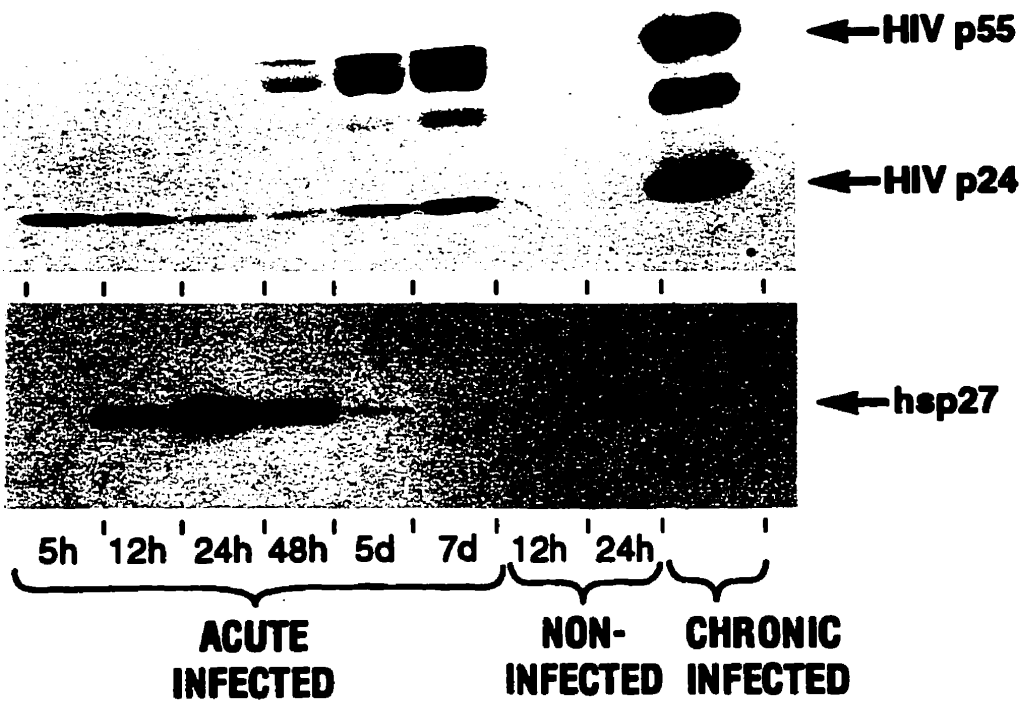

No Infection
Acute Infection
No Infection
Acute Infection
No Infection
Acute Infection
No Infection
Acute Infection
Chronic Infection
hsp 90
hsp 70
hsp 60
hsp 27
5h
24h
48h
6 days
6 days

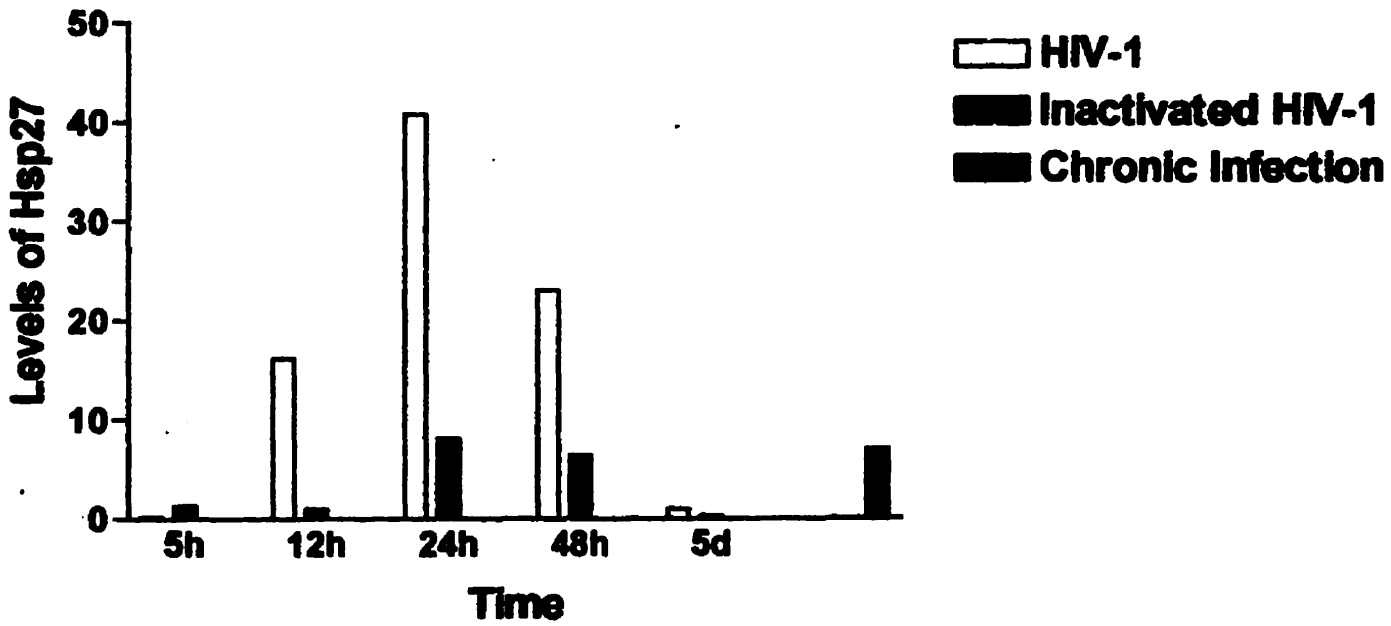
Figure 5: The effect of acute HIV-1 infection on levels of HSP27 and viral (p24 and p55-*gag* precursor) protein expression.

Western blot analysis indicates the relative levels of HSP27 and viral protein expression during the course of infection with live (m.o.i=0.2) and heat-inactivated (56°C for 1h) virus. Cellular extracts were isolated at designated times and stained for HSP27 or HIV-1 determinants. Chronically-infected CEM cell extracts are presented for comparative purposes. Extracts of cells exposed to heat-inactivated virus did not contain HSP27 at times other than indicated (not shown).

Extracts of cells exposed to heat-inactivated virus (mock-infected) (lanes 7,8), live HIV (1-6), or chronically infected cells (lane 9) at 5h (lane 1), 12h (lanes 2,7), 24h (lanes 3,8), 48h (lane 4), 5d (lane 5), and 7d (lanes 6,9) are shown.

Figure inset shows phosphorimager data for hsp27 intensity.


HIV p55
HIV p24
hsp27
5h
12h
24h
48h
5d
7d
12h
24h
ACUTE INFECTED
NON-INFECTED
CHRONIC INFECTED


Levels of Hsp27
50
40
30
20
10
0
5h
12h
24h
48h
5d
Time
HIV-1
Inactivated HIV-1
Chronic Infection

that levels of intracellular viral p24 began to decline. At this stage, there was no active viral synthesis, as reflected by the absence of intracellular viral p55 *gag* precursor protein. Levels of HSP27 induced at 12h and 24h were significantly less in extracts of cells exposed to heat-inactivated virus (Fig. 5). HSP27 induction with inactivated virus did not occur in all our experiments, and peak expression of this stress protein after 24h in three experiments was 15% on average of that observed with live virus. It should be noted that heat inactivation is often incomplete and suppresses but does not completely abrogate viral synthesis (unpublished results).

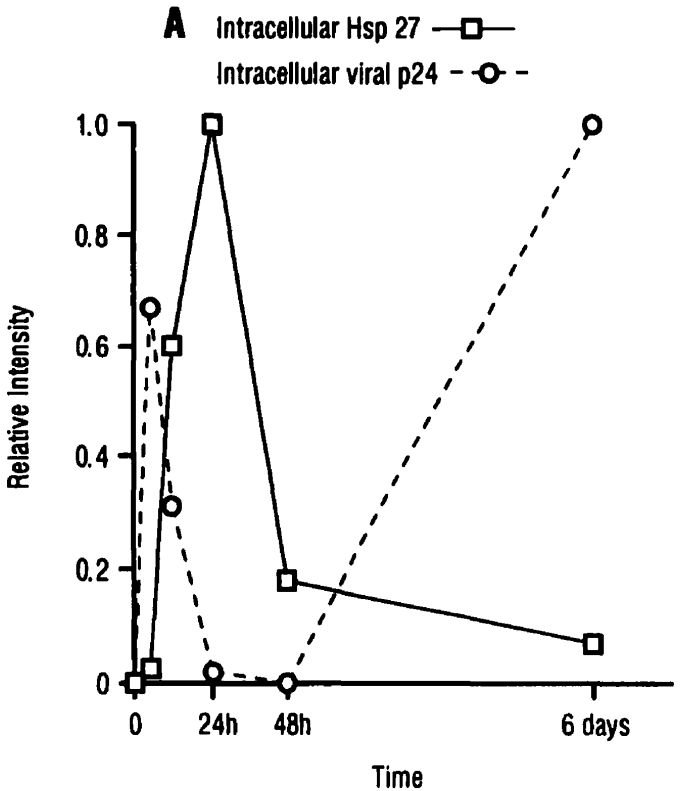
The absence of notable viral replication at the time of peak HSP27 expression was also determined by extracellular p24 and RT assays as shown in Figure 6B. HSP27 induction declined at 48h concomitant with the first appearance of intracellular viral protein and low levels of extracellular p24 and RT. Thus, there appeared to be an inverse relationship between the HSP27 response and that of viral p24 at early phases of infection (Fig. 6A).

Comparative levels of HSP70 and viral protein expression over the entire viral lytic cycle are depicted in Figure 7. Quantitative levels of HSP70 were relatively invariant throughout the course of infection (confirmed by phosphorimage analysis), in contrast to the variable levels detected of HSP27.

Figure 8 displays summary data from seven independent experiments, showing variations in cell number, intracellular HSP27 and viral p24 over time. HSP27 levels were modulated early during primary HIV infection. There was a 6.4±1.7-fold induction of HSP27 in infected as compared to uninfected cells, based on five experiments in which HSP27 levels were quantifiable in mock-infected controls. In all experiments, peak HSP27 expression was down-regulated by 2 or 3 days following infection concomitant with increased viral synthesis. HSP27 modulation was not associated with any significant differences in cell proliferation or cell viability in either mock-infected or acutely-infected cells for 6 days. Thereafter, a significant decline in the number of infected cells was accompanied by maximal increases in intracellular viral p24 and extracellular virus. There was a 50% reduction in cell survival in infected relative

Figure 6: Relationship between intracellular levels of HSP27 and viral p24 (A). Levels of extracellular viral p24 and RT (B).

Summary data depicting HSP27 expression and viral incorporation and synthesis over time. A) Western blot analysis of relative levels of intracellular viral p24 and HSP27. Levels of protein were analyzed using a molecular phosphorimager. B) Relative levels of extracellular p24 antigen (pg/ml) and RT (cpm/5µl) as measured over time.


A
Intracellular Hsp 27
Intracellular viral p24
Relative Intensity
1.0
0.8
0.6
0.4
0.2
0
0
24h
48h
6 days
Time

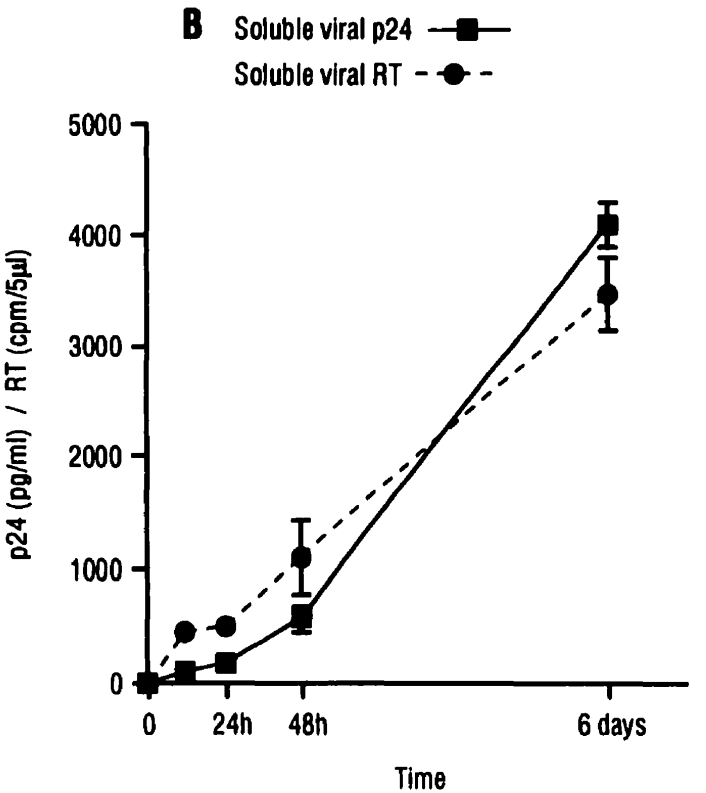

B
Soluble viral p24
Soluble viral RT
p24 (pg/ml) / RT (cpm/5µl)
5000
4000
3000
2000
1000
0
0
24h
48h
6 days
Time

Figure 7: The effect of acute HIV-1 infection on levels of intracellular HSP70 and viral (p24 and p55 *gag*-precursor) protein

Western blot analysis indicates relative levels of HSP70 and viral protein expression during infection at an m.o.i of 0.2. Cellular extracts were isolated at designated times and stained for HSP70 or HIV-1 determinants. Extracts of chronically-infected CEM cells are presented for comparative purposes.

Extracts of acutely infected (lanes 1-8), and chronically infected (lane 9) cells are shown at 5h (lane 1), 12h (lane 2), 24h (lane 3), 48h (lane 4), 6d (lane 5), 9d (lane 6), 12d (lane 7) and 29d (lane 8).

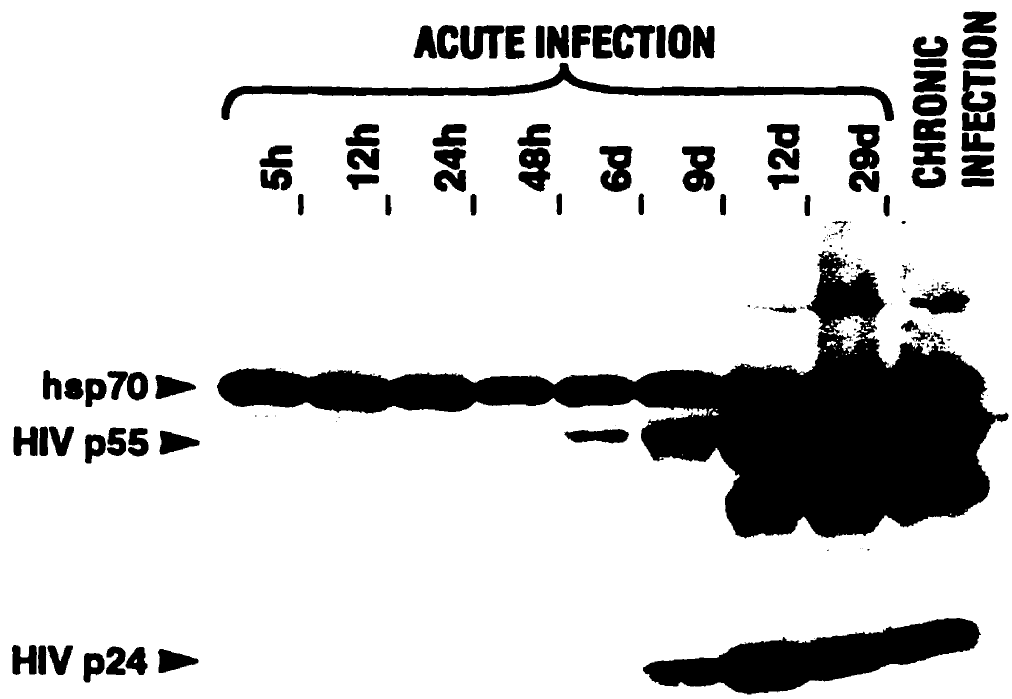
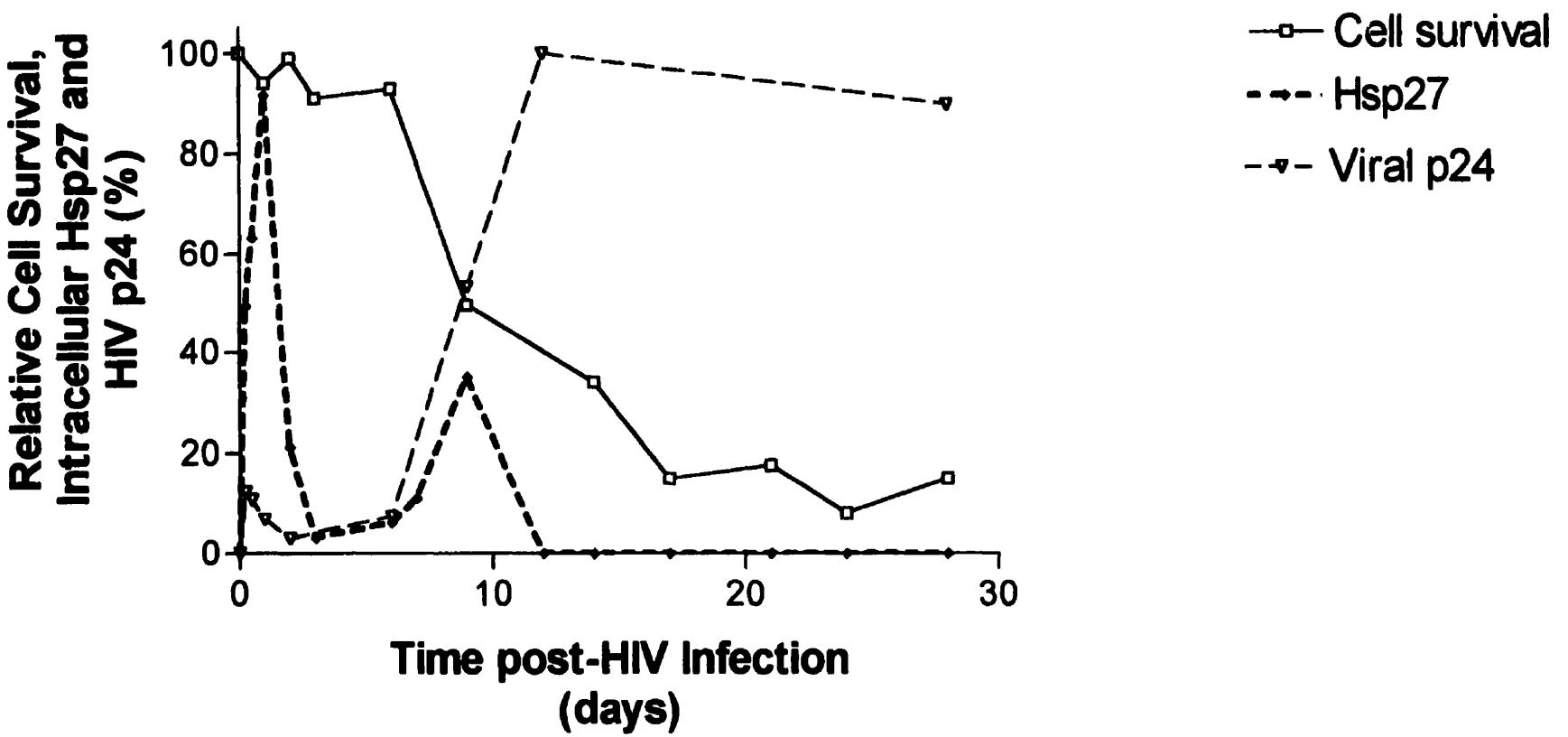

ACUTE INFECTION
CHRONIC INFECTION
5h
12h
24h
48h
6d
9d
12d
29d
hsp70
HIV p55
HIV p24

Figure 8: The effect of acute HIV-1 infection on levels of intracellular HSP27, viral p24, and cell survival

Average data based of seven independent experiments in which cell survival, intracellular levels of HSP27 and viral p24 were assessed. Cells were mock-infected or infected with HIV-1 (m.o.i=0.2). Numbers of cells at each time are expressed as a percentage of corresponding numbers observed following exposure to mock-infection. HSP27 levels are the average of quantified phosphorimage intensities expressed as a percentage of the HSP27 densities at 24h. Similarly, viral p24 levels are expressed as a percentage of maximal quantified phosphorimage densities.

Changes in Hsp27 during the course of acute/lytic HIV-1 Infection



to mock-infected cells between 6-9 days post-infection. A transient reemergence of HSP27 was observed at this lytic stage of infection, when virion protein synthesis and cell losses were maximal. In some experiments, complete loss of cell viability occurred two weeks following infection; in others, breakthrough of chronically-infected cell lines was observed.

2. Viral specificity of induction of HSP27

CEM.NKR cells were infected at different m.o.i's using the IIIB strain of HIV-1. Cells were divided into four groups: the first was mock-infected without virus, while the second-fourth were infected at m.o.i's of 0.02, 0.067, and 0.2 respectively. Figure 9 depicts levels of intracellular HSP27 and viral p24 at 24 and 48h after infection. At both 24 and 48h, HSP27 induction increased with increasing viral dose. As expected, intracellular virus increased with the viral dose used for infection. Therefore, levels of HSP27 induction seemed to be virus concentration-dependent. Figure 10 shows that HSP27 induction was not only reduced but also attenuated in dependent on the m.o.i of infection (Fig. 10A). Decreasing the m.o.i led to dose-dependent lags in intracellular viral protein synthesis and reductions in extracellular viral protein at seven days after infection (Fig. 10B).

Several experiments were performed to further ascertain the viral specificity of HSP27 induction (Fig. 11). As shown above, dilution of viral resulted in a concentration dependent reduction in HSP27 induction. Uninfected cells incubated with soluble CD4 failed to induce HSP27, showing that cross-linking of CD4 was insufficient to induce HSP27 (Fig. 11). Pre-treatment of a viral isolate (m.o.i. = 0.2) with neutralizing antibody abrogated HSP27 induction (Fig. 11). In addition, heat inactivation of virus leads to a substantial reduction of infectious titer and a near complete loss of HSP27 induction (Fig. 11). These cumulative findings suggest a viral-associated induction of HSP27 that is not simply attributable to endotoxin or cytokines in the viral inocula.

Figure 9: The effect of various viral inocula on synthesis of intracellular HSP27

Cells were mock-infected or infected at m.o.i's of 0.02, 0.067 , and 0.2. Protein extracts were analyzed by Western blot method and probed for HSP27 and viral p24.

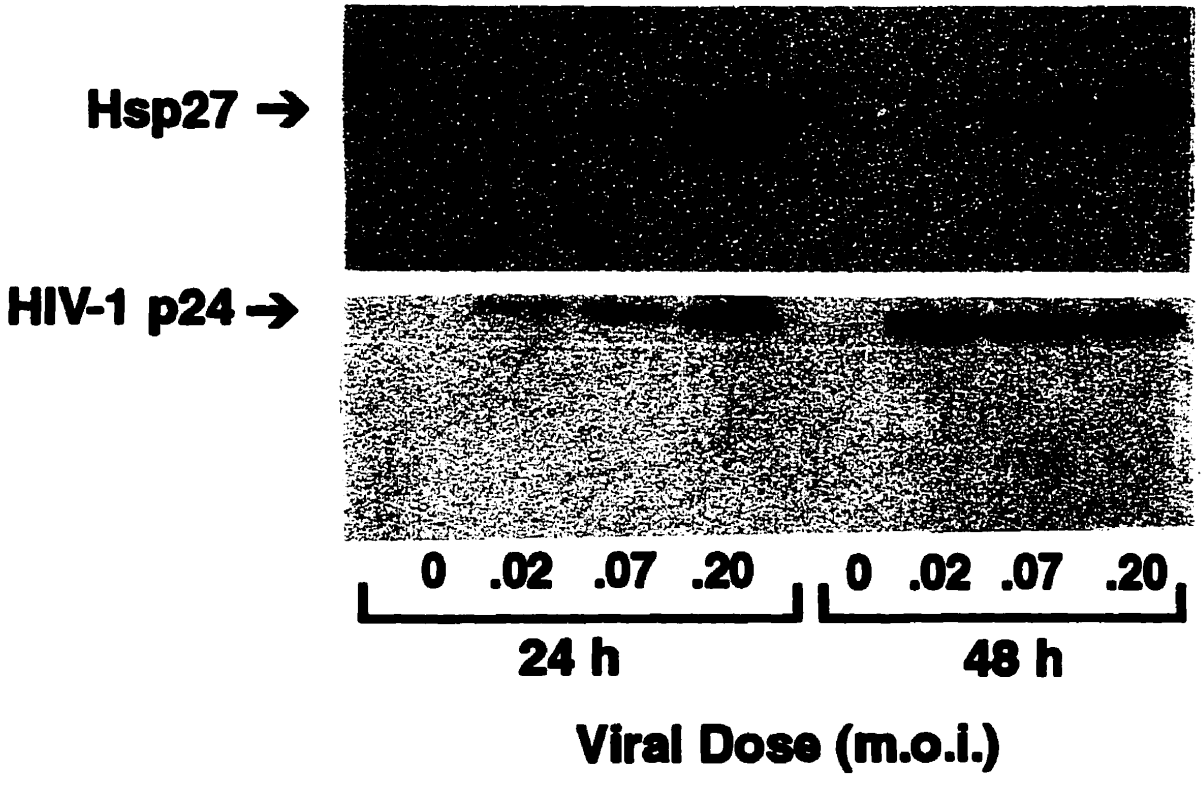
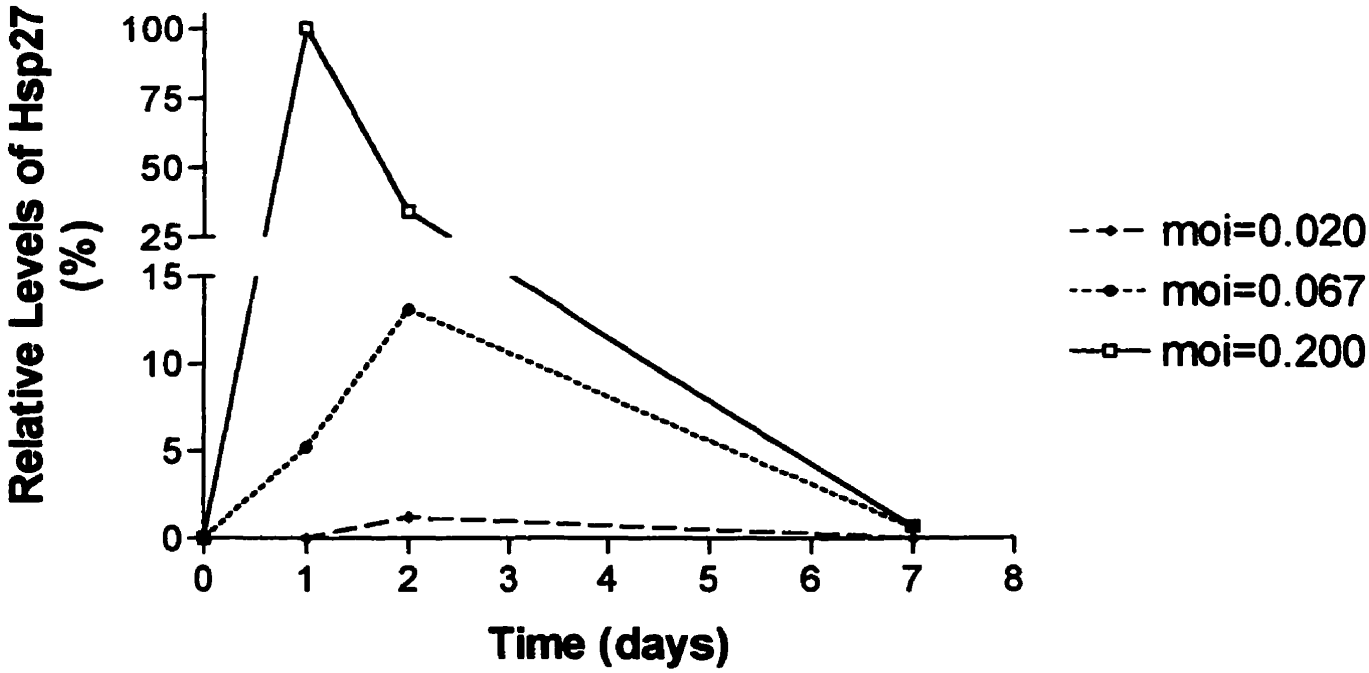

Hsp27 →
HIV-1 p24 →
0 .02 .07 .20
0 .02 .07 .20
24 h
48 h
Viral Dose (m.o.i.)

Figure 10: The effect of viral dilutions on intracellular HSP27 and extracellular virus replication

Cell extracts were monitored at various times post-infection for presence of HSP27 and viral p24. The levels of HSP27 were quantified by phosphorimage intensity and were expressed as a percentage of the HSP27 intensity observed 24h after infection at an m.o.i of 0.2 (Fig. 10A). Levels of extracellular p24 were assayed as described under Materials and Methods (ng/ml) (Fig. 10B).


Relative Levels of Hsp27 (%)
100
75
50
25
15
10
5
0
0
1
2
3
4
5
6
7
8
Time (days)
moi=0.020
moi=0.067
moi=0.200

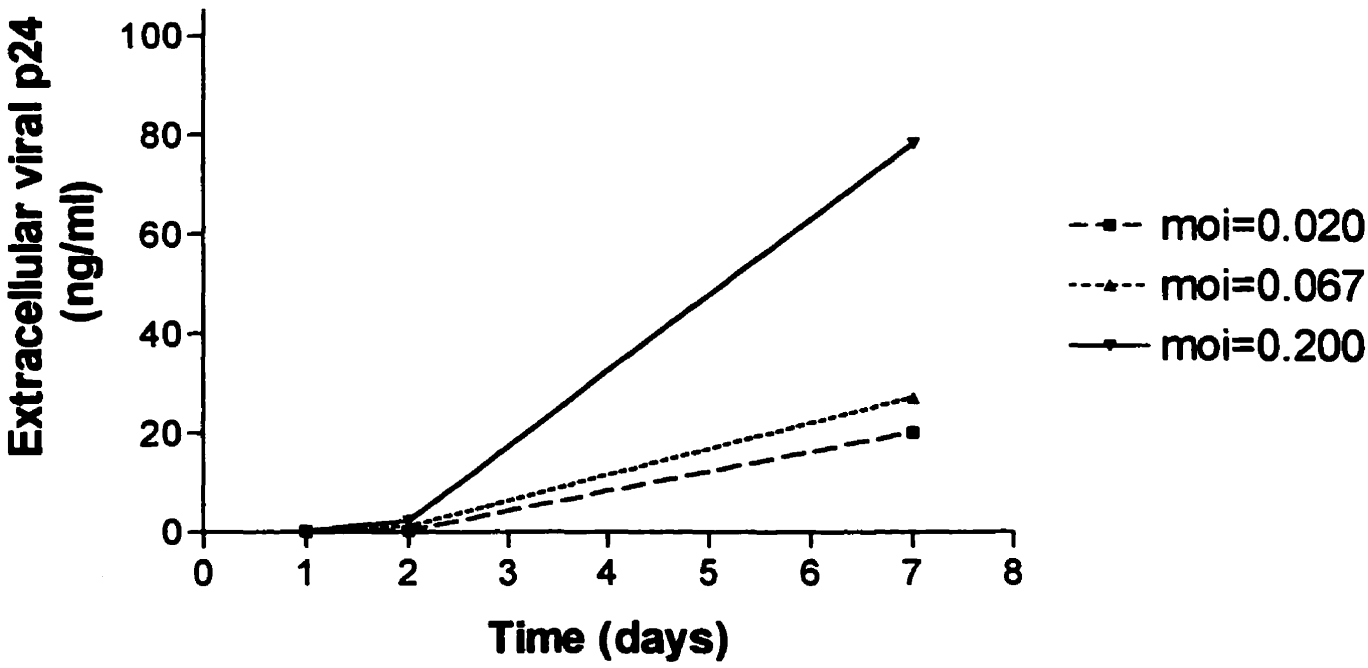
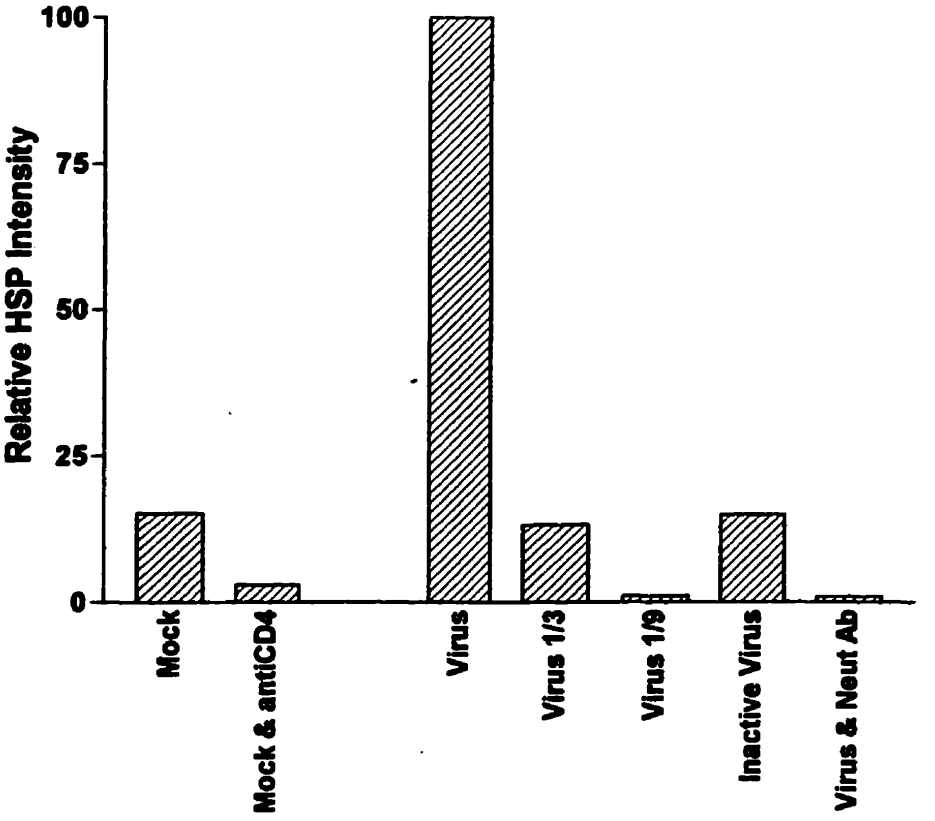

Extracellular viral p24 (ng/ml)
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
Time (days)
moi=0.020
moi=0.067
moi=0.200

Figure 11: Viral Specificity of the HSP27 Response.
The relative levels of HSP27 in mock infected cells, mock infected cells pretreated with soluble CD4, viral infected cells at different m.o.i. of the viral inocula, cells infected with virus that had been neutralized with a pooled HIV-1 antibody stock from seropositive individuals. The HSP27 induction at 24h of the different infection samples were expressed as a percentage of the viral infected (m.o.i. = 0.2) run on the same day.



Viral Specificity of the HSP27 Response

CEM cells were a) mock infected; b) mock infected after a pre-incubation with soluble CD4; c) virally infected at different dilutions of a viral inocula (m.o.i. = 0.2); d) infected with heat-inactivated (56°C, 1h)) virus (m.o.i. = 0.2); infected with a viral stock (m.o.i. = 0.2) that was pre-treated with a high titer neutralizing HIV-1 antibody from seropositive individuals. Levels of HSP27 signals induced at 24 hours were quantified on the phosphorimager and data are expressed as a percent of the signal obtained with the viral stock (m.o.i. = 0.2).

3. Determination of HSP isoforms in CEM.NKR and Jurkat cells following acute HIV-1 infection

Although only HSP27 levels appeared to be quantitatively altered following HIV-1 infection, we wanted to determine whether the other HSP subspecies were modulated during the viral life cycle. Stress protein expression was monitored using two-dimensional Western blot analysis as described under Materials and Methods. At 24h after acute HIV-1 infection, the transient expression of novel HSP27 and HSP70 homologues arose that were absent in mock-infected cells (Fig. 12). However, these isoforms disappeared by 48h. Uninfected CEM cells showed no cognate HSP27. In contrast, HSP70, which is constitutively expressed in CEM cells, shows marked novel viral-induced homologues that are present at 24h and begin to decline at 48h post-infection.

To demonstrate that this selective induction of HSP27 and HSP70 isoforms was not restricted to CEM.NKR cells, Jurkat cells were also acutely infected with HIV-1. As shown in Figure 13, similar *de novo* expression of HSP27 and HSP70 was evident. As in the case of CEM, uninfected Jurkat cells showed no detectable HSP27 but constitutive expression of HSP70 was apparent. With acute infection, new isoforms of HSP70 were detected in addition to the emergence of HSP27.

4. HSP isoforms produced after mock-infection, exposure to heat-inactivated virus, and infection by live virus

Figure 14 shows HSP70 homologues present after mock-infection, exposure to heat-inactivated virus, and active infection. Uninfected cells show a major constitutive (pI 3-5.5) and minor basic (9-10) form of HSP70 (Fig. 14). Viral infection resulted in increases in levels of these constitutive acidic and basic HSP70 isoforms. In addition, novel homologues were present between pI 5.5-6.5. Inactivated virus also resulted in increased levels of cognate (pI 3-5.5) but viral-induced HSP70 isoforms (pI 5.5-6.5) were substantially less abundant than those observed with live virus. In so far as heat inactivation does not abrogate viral synthesis, leading to an absence of infection in the window time frame of 0-9 days

post-infection and infection emerging at 10 days post-infection, the bands observed with inactivated virus may represent early viral-associated effects.

5. The effect of heat shock on intracellular HSP expression in chronically HIV-1 infected cells

There are no discernible differences in levels of expression of cognate or heat-inducible HSP60, HSP70, and HSP90 in uninfected vs chronically HIV-infected CEM.NKR cells (Brenner *et al*, 1995). Neither uninfected nor chronically infected CEM cells expressed detectable levels of cognate HSP27. We determined whether prior exposure of cells to a heat shock for 2h at 42°C followed by recovery for 24h at 37°C, might affect their ability to produce HSP subspecies. Figure 15 depicts constitutive and heat-inducible HSP27, HSP60, and HSP70 expression in chronically infected cells at 3h, 24h, 48h, and 72h following heat shock. No discernible differences between cognate and heat-inducible levels of HSP60 and HSP70 expression were observed in chronically-infected cells. In contrast, HSP27 expression was seen only following heat stimulus. Thus chronic HIV-1 infection alone was not sufficient to induce a HSP27 response. Unlike early acute HIV-1 infection, chronically infected CEM cells expressed no detectable HSP27 until exposed to further stimulus. Thus, HSP27 expression induced by acute HIV-1 infection is down-regulated following active viral replication in both acutely and chronically-infected cells. Heat stress in chronically-infected cell lines, can reduce both cellular proliferation and viral replication, causing a restoration of the HSP27.

6. Determination of HSP isoforms after heat shock of chronically-infected cells

To identify whether novel HSP isoforms exist in chronically-infected extracts, Western blot analysis of two-dimensional gels of relevant cell extracts was performed. The two-dimensional patterns showed that uninfected CEM cells exposed to heat shock expressed two HSP27 subspecies, whereas only a single dominant heat-inducible HSP27 homologue was present in untreated, chronically

Figure 12: The effect of acute HIV-1 infection on novel HSP isoforms in CEM.NKR cells

CEM cell extracts were harvested at various times following mock or acute HIV-1 infection (m.o.i=0.2). Extracts were subjected to isoelectric focussing (left to right, pI=10 to 3) in the first dimension and SDS-PAGE in the second dimension. (A) Mock-infected extracts harvested 24h following infection and probed for HSP27 and HSP70. Similar patterns were obtained at 48h and 6 days (unpublished results). (B and C) Extracts were probed for HSP27 and HSP70 expression at 24 and 48h following acute infection. By 6 days, the pattern of HSP27 and HSP70 induction paralleled that observed with mock-infected cells.

hsp70 [

CEM
No Infection

hsp27 [

hsp70 [

CEM 24h Post-
Acute Infection

hsp27 [

hsp70 [

CEM 48h Post-
Acute Infection

hsp27 [

Figure 13: The effect of acute HIV-1 infection on HSP isoform expression in Jurkat cells

Jurkat cell extracts were harvested at 24h following mock or acute-infection (m.o.i=0.2). Extracts were subjected to two-dimensional gel electrophoresis (left to right pI=3 to 10) and Western blots were analyzed for expression of HSP27 and HSP70.

Jurkat
24h Mock Infection

Jurkat
24h HIV-1 Infection

Figure 14:The effect of mock-infection, exposure to heat-inactivated virus, and acute-infection on novel HSP isoforms

CEM cells were mock-infected, exposed to heat-inactivated virus (56°C for 1 h), or acutely-infected with HIV-1. Cell extracts harvested after 24h were subjected to two-dimensional gel electrophoresis (left to right pI=3 to 10) and Western blots were analyzed for HSP27 and HSP70 expression.

CEM.NKR- 24h Post-Infection
2-D SDS PAGE, (pI: 3-10)

Hsp 70→

Uninfected

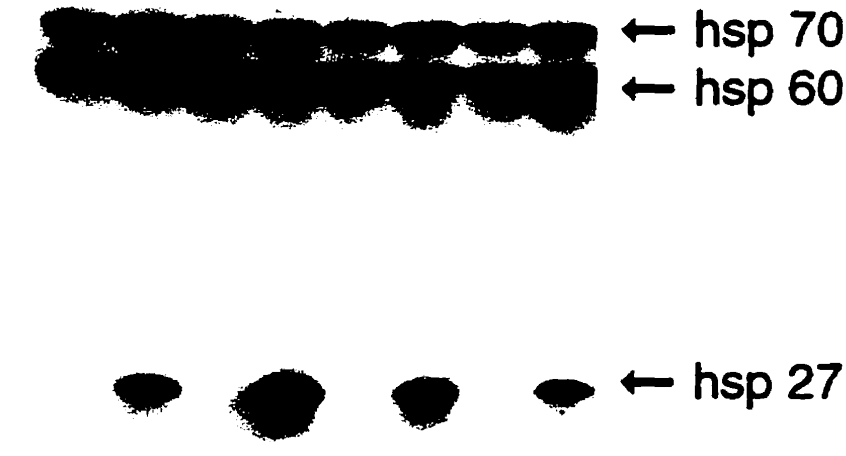
Inactivated HIV-1

HIV-1 Infected

Figure 15: The effect of chronic HIV-1 infection and heat shock on intracellular HSP production

Chronically infected CEM cells were either untreated or were exposed to a heat shock of 42°C for 2h prior to recovery for various times. Cellular extracts were harvested at designated times and stained for HSP27, HSP60, and HSP70.

Extracts of chronically infected cells (lanes 1-8) at times of 3h (lanes 1-2), 24h (lanes 3-4), 48h (lane 5-6), and 72h (lanes 7-8) were either left untreated (lanes 1,3,5,7) or exposed to a mild heat shock of 42°C for 2h (lanes 2,4,6,8).


← hsp 70
← hsp 60
← hsp 27
- + - + - + - +
3h 24h 48h 72h
- no heat shock
+ heat shock 42°, 2h

HIV-infected CEM cells (Fig. 16). Similarly, two HSP60 isoforms were seen in the case of stressed, uninfected cells, while one of these isoforms was absent in chronically-infected, heat-stressed cells (Fig. 16). In this experiment, antibodies to HSP27 stained bands in the regions of 27 and 54 kDa, while anti-HSP60 antibodies stained bands in the regions of 60 and 120 kDa. Thus, HSP27 and HSP60 probably exist as dimers in their native state. When DTT is added to these reactions, only single bands are observed. This experiment shows the loss of some HSP27 and HSP60 species during the heat shock response in chronically-infected cells.

No quantitative differences in cognate or heat-inducible HSP70 expression were seen when comparing uninfected and chronically infected CEM cells (Brenner *et al*, 1995). To determine whether qualitative differences in HSP70 isoforms might be present, two-dimensional blots were incubated with antibodies that recognized both constitutive and inducible HSP70 forms. Figure 17 shows an array of HSP70 isoforms that varied slightly in molecular weight and markedly in isoelectric mobility. Only one major and one minor HSP70 homologue were observed in unstressed, uninfected cells, whereas unstressed, chronically-infected cells showed an increase in intensity in the minor constitutive HSP70 moiety. Moreover, transient heat stress resulted in the additional expression of several inducible HSP70 homologues. There was at least one additional major HSP70-inducible homologue in heat-stressed infected CEM cells as compared to heat-stressed uninfected CEM cells. Thus, at least two novel, constitutive and inducible HSP70 homologues can arise in CEM.NKR cells concomitant with HIV-1 infection (Fig. 17).

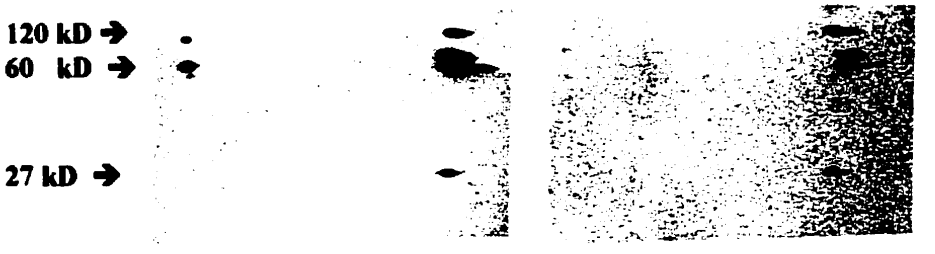
7. The effect of TNFα on HSP isoforms produced after chronic HIV-1 infection

CEM cells were treated with TNFα, a potent stress-inducer as described in Materials and Methods. When uninfected cells were treated with TNFα, novel HSP70 homologues, similar to those obtained after heat shock were observed (Fig. 18). Treatment of chronically HIV-infected cells with TNFα resulted in the induction of major homologues that were absent in uninfected cells. These species

Figure 16: The effect of chronic HIV-infection and heat shock on HSP27 and HSP60 isoforms

CEM cells were mock-infected, or chronically infected with HIV-1. Sample extracts were either untreated or were exposed to a 42°C heat stimulus for 2h as described. Cell extracts harvested 24h post-infection were subjected to two-dimensional gel electrophoresis (left to right pI=10 to3) and Western blots were analyzed for HSP27 and HSP60.

Heat -inducible Hsp 27 and Hsp 60



Non-infected CEM **Chronically-Infected CEM**

Heat-inducible Hsp 27

Non-infected CEM **Chronically-Infected CEM**

Figure 17: The effect of chronic HIV-1 infection and heat shock on HSP70 isoforms

CEM cells were mock-infected, or chronically infected with HIV-1. Cells were either untreated or exposed to a 42°C heat stimulus for 2h. Cell extracts harvested after 24h were subjected to two-dimensional gel electrophoresis (left to right pI=10 to3) and Western blots were analyzed for HSP70.

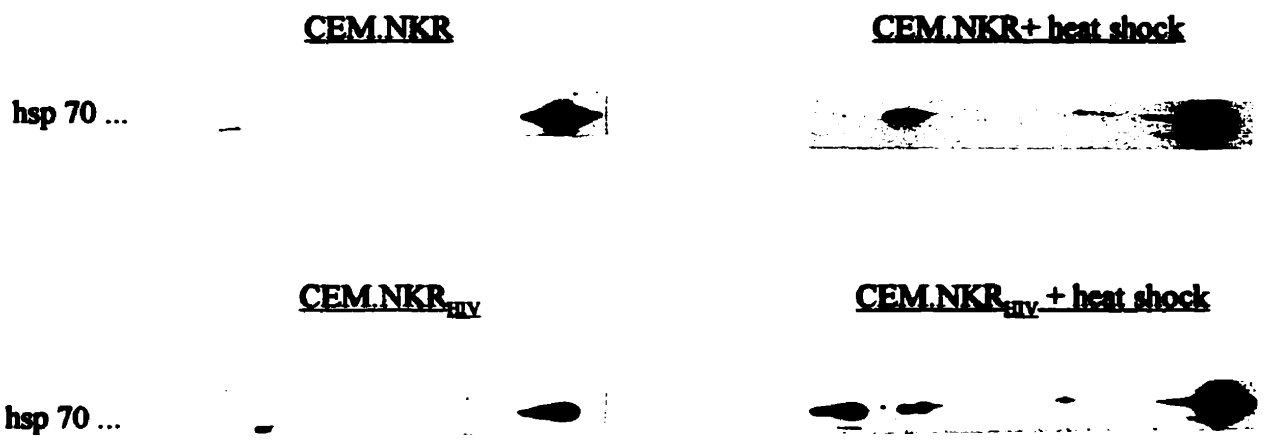
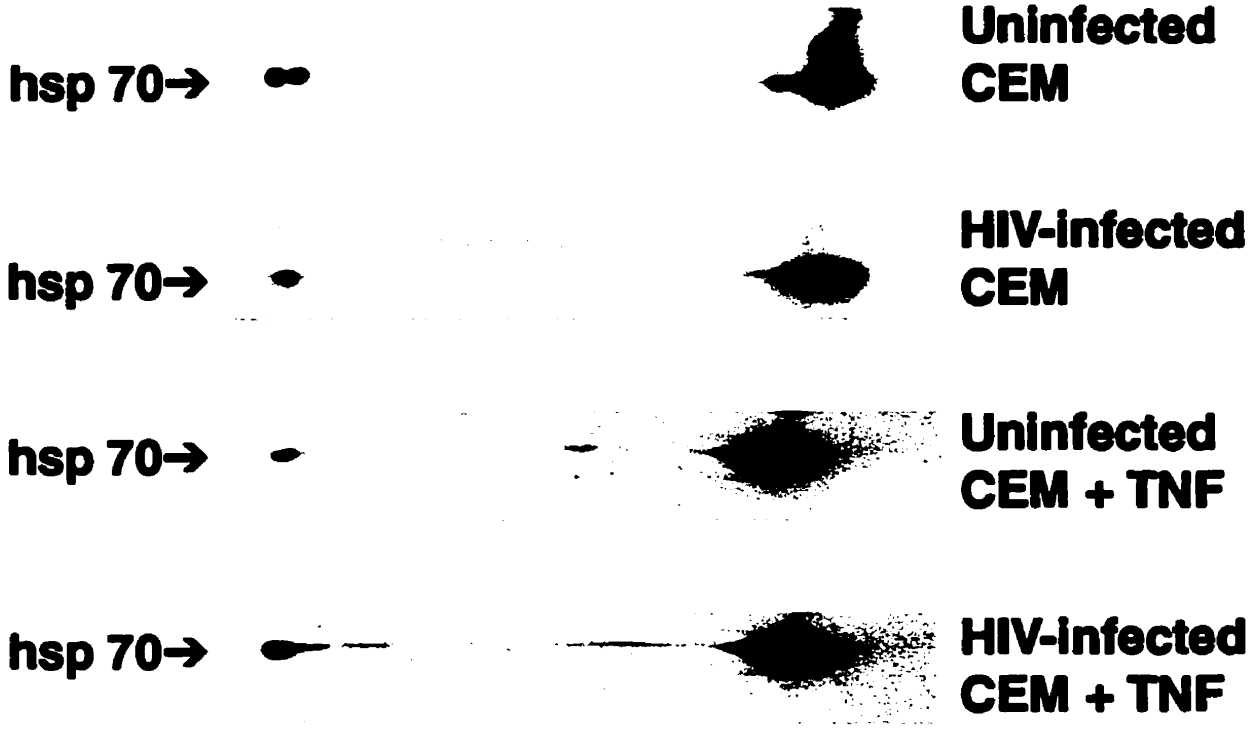

CEM.NKR
CEM.NKR+ heat shock
hsp 70 ...
CEM.NKR$_{HIV}$
CEM.NKR$_{HIV}$ + heat shock
hsp 70 ...

Figure 18: The effect of TNFα in uninfected and chronically-infected cells on HSP70 isoforms

CEM cells were mock-infected or chronically infected with HIV-1. Cells were either untreated or exposed to 25 ng/ml of TNFα. Western blot two-dimensional analysis (pI=10-3) was used to compare HSP70 homologues in uninfected and chronically-infected cells, treated or not with TNFα.


hsp 70→
Uninfected CEM
hsp 70→
HIV-infected CEM
hsp 70→
Uninfected CEM + TNF
hsp 70→
HIV-infected CEM + TNF

were similar to isoforms induced after the treatment of uninfected cells with TNFα. This demonstrates that treatment of CEM cells with TNFα has a marked effect on the expression of HSP70 isoforms. Furthermore, the effect of TNFα on uninfected and chronically infected CEM cells is similar to that observed after heat shock. TNFα also induced HSP27 expression but no differences were observed between extracts of chronically-infected and uninfected cells.

Discussion

The aim of our work was to determine the impact of acute (lytic) and chronic (non-lytic) HIV-infection of CD4-expressing T cells on inducible and constitutive stress protein expression. To this end, levels of cytoplasmic HSP27, HSP60, HSP70, and HSP90 subspecies were measured in uninfected, acutely, and chronically-infected cells.

Previous work in our laboratory showed a marked increase in levels of heat-inducible HSP27 with chronic HIV-infection (Brenner *et al*, 1995). In that study, short febrile stimulation (45°C for 15 min or 42°C for 2h) of chronically-infected cells triggered HSP27 responses similar to those observed here. During this period of sustained HSP27 synthesis (2-4 days), cellular proliferation and intracellular viral synthesis was suppressed (*Ibid*). In the present study, we confirmed these results (Fig. 15). However, in order to further characterize changes in stress response with chronic-infection, we performed two-dimensional Western blot analysis and found that chronic HIV-infection induced the expression of novel HSP70 homologues in addition to changes in HSP27 and HSP60 (Figs. 16-17). TNFα also induced similar *de novo* expression of HSP70 subspecies (Fig. 18).

We then attempted to monitor changes in stress protein production at various stages of acute HIV-1 infection. We found that the stress response, at early stages, was characterized by 6.4±1.1 fold increases in HSP27 expression (Figs. 4-5). This inducible response was unique to HSP27, as no quantitative differences were seen in HSP60, HSP70 or HSP 90 expression (Figs. 4, 7). However, there were qualitative changes in the forms of HSP subspecies produced concomitant to acute HIV-infection. There was a transient emergence of novel HSP27 and HSP70 isoforms at early stages that were absent in uninfected cells (Figs. 11-12). Thus, both chronic and acute viral infection selectively alter inducible stress responses.

Differences in HSP subclass expression in CD4-expressing cells, following viral infection or other types of stress, have been identified. Several groups have previously reported that mitogens or

stress induce the phosphorylation of HSP27 to yield multiple homologues (Saklatvala *et al*, 1991, Freshney *et al*, 1994, Rouse *et al*, 1994, Belka *et al*, 1995). Our findings indicate that only one or two HSP27 isoforms emerge following acute or chronic HIV-1 infection (Figs. 11, 12, 16). This suggests that our observations represent either the *de novo* synthesis of HSP27 or the release HSP27 from large macromolecular complexes (>200 kDa), although no evidence for such complexes was obtained in our Western blot experiments (Arrigo and Landry, 1994). Furthermore, the fact that heat-enhanced chronically-infected cells expressed only one HSP27 homologue, as compared to two in uninfected cells, indicates that HSP27 induction may be a direct consequence of CD4+ T cell thermotolerance (Fig. 16) (Mehlen *et al*, 1995). However, expression of novel HSP27 isoforms also occurred 12-24h following acute HIV-1 infection and prior to viral synthesis and assembly (Fig. 11-12). This suggests that HSP27 may play a role at early stages of the HIV-1 replicative cycle.

HSP27 is modulated in several other types of viral infections. In the case of human papilloma virus (HPV), HSP27 was increased only in the basal cells of the cervix and vagina of those women sampled (Ciocca *et al*, 1992). During HPV infection, most cells that manifest viral, cytopathic effects may lose their capacity to express "defensive" proteins, including HSP27 (*Ibid*). Expression of HSP27 may also be inversely correlated with the oncogenicity of adenovirus-transformed rodent cells (Zantema *et al*, 1989).

Differences in HSP27 modulation associated with HIV-1 infection and cell tropism, heat, or mitogens may suggest an important role of the HSP stress response in viral-host cell interactions. Our results showed that the highest level of expression of HSP27 occurred at 24h post-infection, a time when progeny virions are not yet expressed (Fig. 5). Furthermore, we found that expression of HSP27 in acutely-infected cells was dose-dependent (Figs. 9-10). Thus, an increased viral presence in cells may produce a more active defence, i.e. increased production of HSPs. Therefore, stress-regulated and HSP subclass expression could conceivably affect the course of HIV-1 infection.

We determined whether qualitative differences might exist in HSP isoform production in acutely-infected cells. No quantitative differences in levels of constitutive HSP70 expression were apparent (Fig. 7). But, several novel HSP70 homologues were induced by 24h after acute infection or exposure to heat-inactivated virus (56°C for 1h) (Fig. 13). Quantitative increases in HSP70 subspecies were observed by two-dimensional protein electrophoresis, consistent with reports that showed nuclear HSP70 translocation at early times following either HIV-1 infection or gp120 binding to the CD4 receptor (Furlini *et al*, 1994). This may explain why inactivated virus can trigger weak HSP27 and HSP70 stress responses. Our results show differences in the isoforms of HSP70 induced by live and heat-inactivated virus (Fig. 13-14). Moreover, the homologues of HSP70 induced at days 6-9 following infection do not appear to be qualitatively different from those associated with cognate HSP expression. This suggests that HSP27 and HSP70 may be differentially modulated at different stages in the viral replicative cycle.

The fact that novel HSP27 and HSP70 subspecies are only generated between 6 and 24h following acute infection is rather surprising. Although cells were not synchronized prior to infection, the early events of infection may have caused cultures to behave in a synchronized fashion. This raises the possibility that late viral gene products may be involved in the down-regulation of the early host stress response. This may directly or indirectly contribute to the ability to produce infectious virions. The observed weak modulation of selective HSP isoforms by inactivated virus suggests that some early events, triggered by viral binding but independent of the replicative process, are responsible for aspects of stress modulation in CD4 cells.

We also attempted to discover what effect external stimuli might have on HSP production in HIV-infected cells. There have been conflicting views regarding the role of heat shock in viral infections. Heat shock has been shown to elevate viral levels in cell lines that had been chronically-infected with a number of different viruses

(Santoro, 1994). Human cells with integrated HIV-1 have shown increased viral gene transcription following heat shock (Stanley *et al,* 1990). Furthermore, heat shock transcriptionally activates both the long terminal repeats (LTR) of HIV-1 as well as the HSP70 promoter (Geelen *et al,* 1988, Kretz-Remy and Arrigo, 1994). Conversely, continuous hyperthermia at 3° C to 4°C above the physiological range can prevent cellular proliferation and active replication of several RNA and DNA viruses during primary infection of cultured cells (Santoro, 1994). Levels of host viral immunity can be stimulated during transient hyperthermia (Kappel *et al,* 1991, Hershkoviz *et al,* 1993), and heat-stress has also been shown to elevate CD4 cell secretion of TNFα, a stimulus of HIV production (Hershkoviz *et al,* 1993). Moreover, heat shock has been found to impair CD4 cell adhesion and migration (*Ibid*).

Our findings show different effects of heat shock on the kinetics of HSP27 and HSP70 synthesis in chronically HIV-infected cells. Heat shock caused the induction of *de novo* HSP27 synthesis, with the greatest expression at 24h. There were 2-10 fold increases in heat-inducible HSP27 homologues in heat-stressed HIV-infected cells as compared to stressed, uninfected cells (Fig. 16). HSP27 novel induction lasted for up to 3 days, with its greatest expression at 24h. HSP27 induction was associated with inhibition of cell proliferation and suppression of viral replication that also continued for up to 3 days. In contrast, no quantifiable differences in levels of HSP60, HSP70, and HSP90 after mild heat stimuli were noted (Fig. 15). However, heat-inducible HSP60 and HSP70 isoforms were produced after chronically-infected cells had been exposed to heat shock (Figs. 16-17). Our lab has previously identified novel heat-inducible HSP27 and HSP70 isoforms in stressed, infected U937 and H9 cells, that were absent in stressed, uninfected cells (unpublished data). Thus, heat shock alters the kinetics of cellular proliferation and viral expression in chronically-infected CD4+ cells.

We also determined the effect of TNFα, a potent cytokine, on HSP expression. A correlation between production of HSPs and cellular resistance to TNFα has been previously observed. Several groups found that the induction of stress proteins by heat shock

prevents against cell lysis by TNFα (Jäättela *et al*, 1992, Kusher *et al*, 1990, Müller *et al*, 1993). We showed that heat shock and TNFα produce similar stress responses. The isoforms produced after both heat shock and TNFα had similar densities as determined by molecular phosphorimage analysis (Figs. 17-18). At 24h, both TNFα and a mild heat shock (42°C for 2h) induced expression of several HSP70 isoforms that were not present in either uninfected or chronically-infected cells. These isoforms were uniquely induced by these external stimuli. While HSP27 may become rapidly phosphorylated after exposure of cells to TNFα (Kaur *et al*, 1989, Arrigo, 1990), we found no variation in either quantity of HSP27 or types of HSP27 homologues produced after treatment with this cytokine.

Total amounts of cognate, heat-inducible and TNFα-inducible HSP70 did not appear to vary in either uninfected or chronically HIV-1 infected CEM cells. Only two-dimensional Western blot analysis was able to resolve unique HSP70 subspecies that arose with TNFα or heat shock treatment in infected and uninfected cells (Fig. 17-18). Thus, novel HSP70 induction may arise to allow CD4+ cells to cope with chronic HIV-1 infection.

Different HSP70 subspecies are induced by infection with different DNA viruses including adenovirus, HSV, SV40, and vaccinia in both human and animal cells (Phillips *et al*, 1991a). The induction of HSP moieties is virus-specific. We found that several HSP70 moieties produced after infection by HIV-1 were only present at certain times (Fig. 11). The lack of novel HSP isoforms at late stages of infection suggests that these moieties may not be directly involved in viral assembly. It is more likely that these HSP homologues represent a host response to viral infection with implications for lytic, immunocytopathic or apoptotic events (Suto and Srivastava, 1995, Li *et al*, 1996, Samali and Cotter, 1996).

Increased cell surface expression of HSP27 and HSP70 in heat-stressed and chronically-infected cell lines may contribute to host immune cell elimination of HIV-infected cells (Di Cesare *et al*, 1992). For example, T lymphocytes that bear the γ/δ receptor, known to recognize HSP, are increased during HIV-1 infection, suggesting that

HSPs might mediate the neutralization of infected host cells (DePaoli *et al*, 1991). This may provide a possible therapeutic approach for reducing the number of infected cells that spread the HIV virus. In fact, HSPs may be involved in modulating antigenic presentation of viral antigens, making them more easily recognizable by T cells (De Nagel and Pierce, 1992).

Since HSPs are generally considered to be cytoplasmic and nuclear-translocatable molecules, the fact that they are localized on the cell membrane is surprising (Poccia *et al*, 1993). Further evidence that HSP surface expression exists in PBMCs as well as monocytic and lymphocytic cell lines indicates that HSPs may be a surrogate marker of HIV-1 infection. In fact, cell damage related to oncogene activation or viral infection may be necessary for HSPs to travel from intracellular compartments to the cell membrane (*Ibid*). In our experiments, the appearance of HSPs was found to be greater after external stress, which is thought to be involved in the increased production of HSPs within cells as well as on the cellular surface. Efforts are ongoing to discover the precise location of these HSPs to determine their potential use as a surrogate immune marker.

Chaperone proteins in cells are responsible for the correct folding and trafficking of proteins. In HIV-1 infection, cyclophilin A serves as a host chaperone protein and is incorporated into virions through contacts with Gag (Franke *et al*, 1994, Thali *et al*, 1994, Colgan *et al*, 1996). Mutation of a single *gag* proline disrupts the *gag*-cyclophilin interaction, blocking incorporation of cyclophilins into virions thereby reducing infectivity (Franke *et al*, 1994). Although cyclophilin A is not required for HIV-1 assembly, it is essential for functions that occur after viral entry but before reverse transcription (Braaten *et al*, 1996a). We showed that HSP27 and novel HSP70 isoforms, other chaperone proteins, were present at their highest levels 24h after infection but prior to assembly (Fig. 5). Thus, HSP27, HSP70, and cyclophilin A are present at similar times in the HIV-1 life cycle. Perhaps HSPs, like cyclophilins may bind to host cells and play a role in efficient virion assembly.

Cyclophilin expression may differ between the M and O clades of HIV. This may help to explain some unique aspects of HIV-1 replication (Braaten *et al,* 1996b). Determining a more precise mechanism of the function of stress proteins in the HIV-infected cell will surely provide further insight into their function during the HIV-1 life cycle.

Several anti-viral agents, including cyclosporins, prostaglandins, and deoxyspergualin may affect HSP-mediated pathways, thereby inhibiting viral replication (Nadler *et al*, 1992, Santoro, 1994, Thali *et al*, 1994). For example, cyclosporin A reduces virion infectivity by inhibiting binding of cyclophilin to virus particles (Thali et al, 1994, Huss, 1996). Only those A and J prostaglandin compounds that induce HSP70 can exert anti-viral activity and prevent certain viral infections, including those by HTLV-1 and HIV-1 (Amici and Santoro, 1991, Hughes-Fulford *et al*, 1992, Amici *et al*, 1994, D'Onofrio *et al*, 1994, Rozera *et al*, 1996). The mechanisms by which these agents allow HSPs to interfere with viral protein synthesis remain undetermined. However, the relationship between HSPs and virus is complex and may depend on whether host cells were acutely or chronically infected (Santoro, 1994). Our results indicate that acute and chronic HIV-1 infection can affect HSP production in different ways. Thus, variations in HSP modulation (depending on infection conditions) may provide information on intracellular defense against active viral replication. Further studies of HSPs may yield important information on the role of these proteins in HIV and other viral disease.

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IMAGE EVALUATION TEST TARGET (QA-3)

150mm

6"

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