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INHIBITORY EFFECTS ON HUMAN IMMUNODEFICIENCY VIRUS TYPE-1 BY INSULIN-LIKE GROWTH FACTOR-1

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Abstract

The spread of human immunodeficiency virus type-1 infection throughout the world has led to intensive multidisciplinary approaches to control and better understand the replication of this virus. Drugs such as 3'-azido-3'-deoxythymidine (AZT) have shown some efficacy but drug toxicity and resistance have directed research into new strategies to control AIDS and HIV infection.

Insulin-like growth factor-1 (IGF-1) is a member of a family of a family of structurally related peptides that also includes insulin. Insulin and IGF-1 are considered primarily as metabolic and growth modulatory hormones, respectively. The structure of human IGFs is very similar to that of human insulin (49% sequence homology) except that the IGFs are single chain polypeptides, since the 12-residue C-peptide linking the A- and B- chains is not removed during processing like that of insulin.

Inhibition of HIV-1 replication by insulin, albeit at supraphysiological concentrations (10^{-7} M) has previously been shown. The fact that this effect could only be seen at such high concentrations indicated that insulin might be acting via the IGF-1 receptor, which it may bind at highly elevated concentrations, but with much less affinity. We now show that IGF-1 can effectively inhibit HIV-1 replication in chronically infected U937 cells at concentrations similar to normal physiological concentrations. We have demonstrated that this inhibitory effect may be abrogated by the presence of anti-alpha-IR3 and anti-IGF-1 antibodies, directed towards the IGF-1 receptor and the IGF-1 protein, respectively. With

the use of HIV-LTR-CAT and pSV-tat plasmids in a COS-7 system, we demonstrate that the inhibitory effect of IGF-1 occurs at the level of transcription. Finally we also show in a human U38 target cell line stably transfected with HIV-LTR-CAT, that IGF-1 maintains its inhibitory effect, although to a lesser degree than that in the COS-7 system. Lastly, it is shown that TNF-α acts to augment transcription of the HIV-LTR and is able to over-shadow the inhibitory effects of IGF-1 on transcription.

Résumé

La propagation de l'infection par le virus de l'immunodéficience humaine de type-1 à travers le monde a mené à diverses stratégies multidisciplinaires pour contrôler et mieux comprendre la réplication de ce virus. Des médicaments tel 3'-azido-3'deoxythymidine (AZT) se sont montrés efficaces mais la toxicité et la résistance reliées à ces drogues ont orienté la recherche vers de nouvelles stratégies de contrôle du SIDA et de l'infection au VIH.

Le facteur de croissance insulinique-1 (IGF-1) fait partie d'une famille de peptides de structure semblable qui inclut aussi l'insuline. L'insuline et le IGF-1 sont principalement connus comme étant des hormones métaboliques et de croissance. La structure des IGFs humains est très similaire à celle de l'insuline humaine (49% d'homologie de séquence) sauf que les IGFs sont des polypeptides à chaîne unique. Ceci est dû à un peptide C-terminal de 12 restes, liant les chaînes A et B, qui n'est pas enlevé comme c'est le cas durant la transformation de l'insuline.

Il a déjà été montré que l'insuline inhibe la réplication du VIH-1 à des concentrations supraphysiologiques (10^{-7} M). Comme cet effet était seulement observé à des concentrations aussi élevées, cela démontrait que l'insuline pouvait agir via le récepteur de l'IGF-1 auquel elle peut se lier à des concentrations très élevées, mais avec beaucoup moins d'affinité. Nous démontrons ici que le IGF-1 peut effectivement inhiber la réplication du VIH-1 dans des cellules U-937 infectées de façon chronique à des concentrations semblables à la normale physiologique. Nous avons démontré que cet effet

inhibiteur pouvait être aboli en présence d'anticorps anti-alpha-IR3 et anti-IGF-1, dirigés contre le récepteur IGF-1 et la protéine IGF-1 respectivement. En utilisant les plasmides HIV-LTR-CAT et pSV-tat dans un système COS-7, nous avons démontré que l'effet inhibiteur de IGF-1 se situe au niveau de la transcription. De plus, nous montrons que dans des cellules cibles humaines U38 transfectées de façon stable avec HIV-LTR-CAT, le IGF-1 maintient son effet inhibiteur, toutefois à un niveau moindre qu'avec le système COS-7. Finalement, il est montré que le TNF-α agit en augmentant la transcription du HIV-LTR et est capable de camoufler les effets inhibiteurs du IGF-1 sur la transcription.

Introduction

Human immunodeficiency virus type-1 (HIV-1) is the etiological agent of the degenerative disease known as acquired immunodeficiency syndrome (AIDS) (Barre-Sinoussi 1983, Gallo 1984). Despite intensive investigation, much remains to be learned about the sequence of events leading to the emergence of AIDS following HIV-1 infection. The development of AIDS is the culmination of a progressive infection with HIV, whose course and outcome are determined by the interactions between numerous viral and host cellular factors, possibly influenced by additional agents or cofactors (Fauci 1988, Rosenberg 1991). HIV-1 belongs to a unique virus family, the *Retroviridae*, a group of small, enveloped, positive-strand RNA viruses. HIV-1 infects and destroys cells of the immune system rendering an infected individual immunodeficient and susceptible to subsequent opportunistic infections (Fauci 1984). Infection of target cells by HIV-1 is dependent on the surface expression of cluster determinant 4 (CD4), which serves as a specific virus receptor (Dagleish 1984, Klatzmann 1984, Maddon1986, Hwang 1991) as well as fusin and CC-CKR5 which serve as virus co-receptors for infection. Viruses from the family *Retroviridae* code for the enzyme reverse transcriptase, which enables them to replicate their RNA genome through a DNA intermediate. Simple retroviruses contain three long, contiguous open reading frames (ORF's) coding for the gag, pol, and env proteins, which constitute their structural and enzymatic repertoire, all packaged in the progeny virion. However, HIV belongs to the lentivirus subfamily, members of which are

characterized by several additional ORF's not found in simple retroviruses. These ORF's code for viral proteins usually packaged in the virion and readily detectable in the cells. These gene products, collectively referred to as auxiliary proteins, are capable of regulating viral replication and infectivity.

HIV contains two identical RNA strands each encoding its 9.7 kb genome. When the viral RNA is reverse transcribed to produce the provirus, repeated sequences from the extreme ends of the viral RNA and adjacent unique 5' (U5) and 3' (U3) sequences are duplicated in such a way as to produce a directly repeated structure with the composition U3-R-U5 at each end of the provirus, called the long terminal repeat (LTR). The LTR DNA contains *cis*-active sites that mediate the up-regulation and down-regulation of viral transcription. A key feature of the control of HIV-1 gene expression is that elements located both upstream and down-stream of the mRNA start site are required. The RNA starts at the beginning of the R region so that upstream control elements are contained within U3 and downstream elements within R and U5. The LTR can be divided into several functional regions with respect to the control of gene expression. These are, the negative regulatory element (NRE), the enhancer, the basal promoter elements, the core promoter, Tat-activation-response-element (TAR) and the inducer of short transcripts (IST). Most of these transcription control elements are shared by many promoters of eukaryotic genes, however, TAR and IST appear to be unique to the lentivirus. Both of these elements are found downstream of the RNA start site and it is probably these elements that endow the HIV-1 promoter with the flexibility of gene

expression that is required to meet the range of cellular environments faced by this virus. The IST is a DNA element that functions as a positive downstream enhancer that stimulates the production of abortive transcripts. The TAR element is a positive enhancer that stimulates the synthesis of productive transcripts but which is unique in terms of eukaryotic transcription control because it is only functional as an RNA element.

. The two coding exons specifying the known HIV regulatory genes, *tat* and *rev*, overlap each other. HIV Tat containing a nuclear localization signal (NLS) is an 86 amino-acid protein transcriptional activator that increases the expression of HIV-1 genes, whose structure has yet to be determined (Lespia 1989, Sodroski 1985). Other HIV genes with proposed functions in the regulation of HIV gene expression include *vpr* and *nef*. Vpr is a nuclear protein that is incorporated into the budding virus and is thought to play a role in viral production. It may also be involved in the active transport of the preintegration complex to the nucleus of an infected cell (Cohen 1990, Heinzinger 1994, Paxton 1993). The HIV accessory genes, *vif* and *vpu*, play a role in modulating the efficiency of virus infection. Vif (viral infectivity factor) apparently is important for efficient viral infection and has been shown to be absolutely essential for infection of peripheral blood mononuclear cells (PBMC) (Gabudza 1992, Sakai 1993, VonSchwelder 1993). Vpu plays a role in viral budding from an infected cell, possibly by affecting the stability and processing of the gp120 molecule. It is also thought to play a role in CD4 degradation, and in the induced cytopathic effect (CPE) (Gottlinger 1993). In addition to these specialized regulatory and accessory

genes, HIV encodes structural genes and enzymatic activities similar to those found in other retroviruses. The *gag* gene encodes viral structural proteins that form the viral core particle, attach the particle to the membrane, and package viral genomic RNA. The viral enzymes, protease, reverse transcriptase, and integrase, are encoded by the *pol* gene. The *env* gene encodes the viral gp160 glycoprotein precursor that is believed to be cleaved by the cellular protease furin into envelope proteins gp120 and gp41, critical for viral attachment and entry into CD4+ cells (Earl 1991, Hallenberger 1992, Ohnishi 1994).

Gag encodes for the p55Gag polyprotein precursor that is cleaved upon viral budding by the viral protease into nucleocapsid (NC or p15), matrix (MA or p17), and capsid (CA or p24) structural proteins. NC is a basic polyprotein that non-specifically binds nucleic acid and is associated with genomic RNA in the virion. It is essential for the production of infectious virus and is further cleaved by the viral protease into NCp7, NCp1, NCp2, and NCp6 proteins. NCp7 coats the viral RNA, protects it from nucleases, and promotes reverse transcription (DeRocquigny 1992, Henderson 1992). MA is a myristylated protein that exists in close association with the viral membrane or envelope. It has a nuclear localization signal (NLS) and plays a role along with vpr in the active transport of the HIV-1 preintegration complex into the nucleus (Bukrinsky 1993, VonSchwedler 1994). CA is a hydrophobic structural protein that constitutes the major internal structural feature of the virion core/shell. It forms a bullet shaped core that surrounds the mature virion (Haseltine 1991).

p160Gag/Pol fusion protein results from an infrequent (-)1 ribosomal frameshift that occurs with a frequency of about 10% during translation of the p55Gag polyprotein and results in a readthrough of the stop codon in the p55Gag protein (Jacks 1988, Parkin 1992). The p160 Gag/Pol polyprotein precursor encodes NC, MA, and CA proteins as well as the viral proteins protease (PR or p11), integrase (IN or p32), and reverse transcriptase (RT or p66/51). PR is a homodimeric protein that is responsible for the cleavage and subsequent maturation of viral proteins. RT is a heterodimeric protein that is responsible for the conversion of the single stranded (ss) (+) genomic RNA into double stranded (ds) proviral DNA. IN is responsible for integrating ds proviral DNA non-specifically into the host genome. Once all of these viral polyproteins are expressed, they, along with other cellular components such as $tRNA^{lys}$ and cellular cyclophilins, assemble beneath the plasma membrane and immature virus particles bud from the cell surface (Barat 1993, Berkowitz 1993, Gottlinger 1989, Mak 1994). HIV-1 buds from the infected cell membrane non-cytopathically as an immature form. At some uncertain point during this assembly and budding process, the viral PR self cleaves and then further cleaves the rest of the viral proteins into their mature forms, generating mature, infectious virus. Dimeric RNA undergoes maturation after the virus has been released from the cell and then condenses to a more compact conformation (DiMarzo-Veronese 1986, Fitzgerald 1992, Kohl 1988, LeGrice 1988)

In addition to the structural and enzymatic proteins encoded for by *gag*, *pol*, and *env* genes, HIV-1 possesses at least six known

auxiliary proteins; Vif, Vpr, Tat, Rev, Vpu, and Nef. The Tat, Rev, and Nef proteins are synthesized early from Rev-independent multiply spliced mRNAs (approximately 2 kb), while Vif, Vpr, and Vpu are expressed late from Rev-dependent singly spliced mRNAs (approximately 4 kb). The closely related HIV-2 does not code for Vpu, though it codes for another late protein, Vpx, not found in HIV-1. Mutations affecting either Tat or Rev severely impair viral replication. In contrast, mutations affecting other auxiliary proteins do not greatly perturb the viral replication kinetics, at least *in vitro*. Hence, these proteins have been dubbed dispensable or nonessential for in vitro replication and are usually referred to as accessory gene products.

Though accessory proteins are not required for viral replication, they are nonetheless capable of modulating replication events *in vitro*, and accordingly, phenotypes associated with their expression have been recognized. Importantly, accumulating evidence suggests that these proteins may modulate viral pathogenesis *in vivo*, thus affecting disease progression and outcome.

HIV-1 Life Cycle

HIV-1 binds to the surface of susceptible CD4+ cells via the high affinity interaction of the HIV-1 surface envelope protein, gp120, with the cellular CD4 molecule. In addition to CD4, HIV requires the use of the co-receptors fusin in the case of T lymphocyte tropic virus and the chemokine receptor CC-CKR-5 in the case of

macrophage tropic virus (Dragic 1996, Deng 1996, Bates, 1996, Choe 1996) Postbinding conformational changes in the HIV-1 envelope proteins allow interactions of the gp41 transmembrane protein with the target cell membrane, resulting in fusion of the viral lipid envelope with the host cell membrane and internalization of the viral particle. Following HIV entry into the target cell, a double stranded DNA copy of the HIV RNA genome is synthesized by the viral enzyme, reverse transcriptase. The viral DNA is then transported to the nucleus while still associated with viral Gag proteins and viral integrase. Integrase catalyzes a concerted cleavage and ligation reaction in which the viral DNA genome becomes integrated into host DNA. Integrated viral DNA is the template for transcription of viral RNA in a process that is regulated by both viral and cellular factors, particularly the viral Tat protein, and cellular transcription factors (Cullen 1991, Steffy 1991, Rosen 1991, Gaynor 1992). HIV gene expression is also regulated at the level of RNA processing and transport (Cullen 1991, Steffy 1991, Rosen 1991). The virally encoded Rev protein plays a critical role in determining the relative amounts of spliced and unspliced HIV RNAs transported to the cytoplasm, for translation or packaging into the nascent HIV particles. The actions of the various regulatory factors determine both the levels of HIV RNA synthesized and the qualitative nature of those RNAs. Detailed studies of these control mechanisms have allowed the delineation of HIV gene expression into "early" and "late" phases. Early HIV gene expression involves the production of small multiply spliced RNAs that encode the various viral regulatory molecules. Late gene expression is responsible for production of

larger RNAs that encode the viral structural proteins and enzymatic activities, as well as the synthesis of the new viral genomic RNAs. The combined effects of the viral and cellular regulatory factors also influence the establishment of latent infection, or the progression to productive cytopathic HIV infection. Translation of HIV mRNA into viral proteins may also be subject to regulation by both viral and cellular proteins, further affecting the levels of production of HIV.

Translation of HIV mRNAs result in the synthesis of HIV regulatory molecules, the HIV structural proteins and enzymes, and the HIV accessory proteins. Newly synthesized HIV envelope proteins are inserted into the host cell membrane. Viral capsid proteins and enzymes undergo proteolytic processing by the viral protease. Assembly of new viral particles and packaging of the viral RNA genome occur adjacent to the cell membrane, and, as the newly synthesized particle buds from the cell, it acquires its lipid envelope, containing the HIV envelope proteins.

Pathogenesis of HIV Infection

The development of AIDS is the result of a chronic progressive infection with HIV (Fauci 1988, Pantaleo 1993). As the progressive events in HIV infection are more fully understood, some of the sequential steps in pathogenesis have become evident and may be correlated with molecular events involved in disease progression. Although each HIV positive individual exhibits a unique pattern of progression, a similar sequence of events may be observed.

Initial infection with HIV may be followed by an acute disease syndrome (Cooper 1985), similar in some respects, to infectious mononucleosis. During this initial infection there appear to be high levels of HIV replication; viral p24 can be easily detected and virus can be readily isolated from the blood (Daar 1991, Clark 1991). This florid HIV replication *in vivo* is likely the cause of much initial CD4+ cell death, although early in disease the ability to produce new naive CD4+ cells allows for the maintenance of CD4+ cell levels within the normal range. The memory subset of CD4+ lymphocytes appears to be both more readily infected *in vitro*, and more highly infected *in vivo* than naive CD4+ cells (Schnittman 1990). Thus even following initial infection, considerable damage may already be done to specific immune responses to recall antigens. A second consequence of the initial viral infection is an apparent seeding of the lymph node with HIV, providing a continuing site of persistent viral replication (Embertson 1993, Panteleo 1991, 1993).

Following an initial infection, HIV infected individuals enter a stage of clinical latency, the asymptomatic period during which the number of CD4+ cells remains within the normal range or slowly decreases over time. Viral burden in the peripheral bloodstream is markedly reduced, as measured by quantitative viral isolations (Ho 1989, Coombs 1989); only 1 in approximately 50,000 cells is expressing infectious HIV (Ho 1989). In addition, the percentage of cells containing HIV RNA or DNA (as detected by polymerase chain reaction (PCR)) is quite low; fewer than 1 in 10,000 CD4+ cells contain HIV DNA (Schnittman 1989, Simmonds 1990). These decreases in

viremia are temporally associated with the induction of cellular and humoral immunity to HIV (Fauci 1991).

Although evidence of viral replication in the blood is reduced during the clinical latency phase, HIV replication is not completely eliminated. Recent evidence suggests that significant replication persists in lymph node follicles (Embertson 1993, Panteleo 1993). Virus can be identified in T cells and in follicular dendritic cells, although it is not yet clear whether virus is actually replicating in these latter cells or is sequestered during antigen presentation. This sequestration may reflect active clearance of virus by the follicular dendritic cells (Panteleo 1993, Cameron 1992), as well as an effective humoral and cell-mediated response to HIV.

Even during the asymptomatic phase, immune function is already compromised. Despite normal levels of CD4+ cells, T cell responses to specific recall antigens are impaired, although response to nonspecific T-cell mitogens remain intact (Lane 1985; van Noesel 1990, Clerici 1989). This could in part, reflect the selective elimination of memory T cells, or could be a consequence of a more generalized nonspecific immune activation involving both T cells and B cells(Lane 1983). T cells that are non-specifically activated could be refractory to subsequent activation by specific antigens. Furthermore, inappropriately stimulated T cells would be candidates for programmed cell death, or apoptosis (Newell 1990). Increased apoptosis of T cells incubated with gp120 has been reported (Groux 1992, Miedma 1990, Terai 1991, Banda 1992), leading to the hypothesis that a significant proportion of the CD4+ cell depletion

observed with HIV infection may be mediated through apoptosis (Ameisen 1991).

In addition to an effective host immune response, virological features may also contribute to the lower levels of active HIV replication observed during the asymptomatic phase. HIV isolates obtained from asymptomatic patients often exhibit different biological phenotypes than those recovered from later-stage symptomatic infection (Tersmette 1989, Fenyo 1989, Cheng-Mayer 1988, Miedema 1990). HIV isolates derived from asymptomatic patients tend to replicate more slowly in tissue culture, to produce lower levels of virus, and not to induce the characteristic HIV-associated cytopathic effect of syncytium induction. These isolates are referred to as "slow-low" viruses (Fenyo 1988), or nonsyncytium-inducing (NSI) strains (Tersmette 1988). Further characterization has shown that these viruses possess other characteristics that differ from viruses isolated late in disease and from commonly studied tissue culture-adapted strains such as LAV/HTLV-III (lymphadenopathy-associated virus/ human T-lymphotrophic virus type III); the NSI strains infect both primary human T cells and monocytes in culture, but do not infect T-cell leukemia lines. During the asymptomatic phase of HIV infection, there may be selective pressures favoring the emergence and persistence of NSI strains. In the face of an effective host immune response, an HIV strain that replicates slowly and can persist in monocytes and macrophages may have a selective advantage over rapidly replicating strains. Highly replicating strains might be better targets for an effective cell-mediated immune response.

The development of symptomatic HIV infection is a continuum of progressive clinical states (Fauci 1991). Initial symptomatic disease may be characterized by nonspecific clinical presentations such as lymphadenopathy, diarrhea, weight loss, and recurrent candidal infections. Progression to clinically defined AIDS is characterized by a dramatic loss of CD4+ lymphocytes and the development of opportunistic infections, AIDS encephalopathy, or characteristic malignancies.

Accompanying the development of AIDS is a marked increase in active HIV replication detected in the blood (Coombs 1989, Ho 1989). HIV can be isolated from as many as 1 in 100 peripheral blood mononuclear cells (PBMCs) (Ho 1989), and HIV DNA can be detected in up to 1% of CD4 + T cells in late stages of disease (Schnittman 1989, 1990). This high level of HIV infection probably reflects alterations in both the host and the virus. There is some evidence that disease progression is associated with loss of an effective immune response against HIV (Fauci 1991). The tremendous variability of the HIV envelope gene (env), particularly the V3 loop (the site of the principal neutralizing determinant for anti-HIV antibodies (Javaherin 1989, Goudsmit 1988)), leads to the selection of viral variants resistant to previously effective neutralizing antibodies. The loss of an effective T-cell response may be a result of continued low-level HIV replication during the asymptomatic period, with a gradual depletion of CD4+ cell population and function, ultimately resulting in loss of cell-mediated immunity to HIV.

Concomitant with progression to late-stage HIV infection, phenotypic changes can be detected among HIV isolates. HIV isolates obtained late in disease replicate in both primary T cells and established CD4+ T-cell lines, but not in primary monocytes (Tersmette 1989, Fenyo 1989, Cheng-Mayer 1988, Miedema 1990). This is in contrast to virus isolated during the asymptomatic phase. These late isolates replicate rapidly and to high levels in culture ("fast-high"), and lead to the formation of syncytia (SI or syncytium inducing isolates) (Fenyo 1988, Tersmette 1988). This change in the viral phenotype may in part be responsible for the higher levels of HIV replication observed late in disease.

An important issue in the pathogenesis of AIDS is the distinction between clinical latency (i.e. an asymptomatic state with only low levels of viral replication in an infected patient) and virologic latency (a state of restricted viral replication within a single infected cell). According to one model, virological latency plays an important role in the persistence of the asymptomatic state (Bednarik and Folks, 1992). Activators of HIV expression might act as cofactors for disease progression (Bednarik 1992, Fauci 1988). Although there is no direct evidence for such a model, there is indirect evidence compatible with the existence of virological latency in HIV-infected individuals. At all stages of HIV infection, Schnittman et al. (1989) consistently found an approximately 10-fold higher frequency of CD4+ cells in peripheral blood containing HIV DNA as compared to cells expressing HIV RNA or proteins. In lymph nodes during the asymptomatic phase, in situ DNA PCR and RNA hybridization demonstrated high numbers of cells containing HIV

DNA, but only rare cells expressing viral RNA (Embretson et al 1993). It appears that, at any given stage of disease, the majority of integrated HIV proviruses are transcriptionally silent. Latently infected cells containing these silent proviruses may serve as reservoirs for the generation of high levels of infectious virus when appropriate activation signals (i.e. cofactors for the progression of AIDS) induce HIV expression.

The most critical questions in AIDS pathogenesis concern the processes that trigger the progression of disease from clinical latency to asymptomatic disease. The course and pace of clinical progression are variable; epidemiological studies have suggested that 50% of HIV infected individuals developed full blown AIDS by 8 - 11 years post-seroconversion (Rutherford 1991, Blattner 1991). The existence and effect of cofactors that may accelerate the progression of AIDS. Such cofactors could be endogenous (i.e. particular genetic traits conveying sensitivity or resistance to HIV, potentially linked to major histocompatibility locus determinants (Steel 1988, Kaslow 1990), or they could be exogenous agents such as other infectious agents (Nelson 1990), or even the presence of continued immune stimulation from any cause (Simmonds 1991). Such cofactors could activate virologically latent HIV. Activation of HIV transcription may play a direct role in activation of virologically latent HIV and could contribute to HIV disease progression.

HIV-1 Trans-activation

Replication of HIV is critically dependent on two viral regulatory proteins, tat and rev. Tat is required early in the viral life cycle to increase the rate of transcription from the viral LTR. The tat protein is an 8 kDa protein produced from different multispliced mRNAs and present at low abundance in HIV infected cells. As the level of transcription first rises, spliced mRNAs that encode tat and rev are made. Tat further stimulates transcription, and then Rev, after reaching a critical level, acts to suppress splicing and help transport unspliced viral mRNAs to the cytoplasm. These unspliced mRNAs encode the viral structural proteins, which are translated and assembled into virus particles.

Tat increases expression of extracellular matrix proteins (Taylor 1992), inflammatory cytokines (interleukin-1(IL-1), IL-6, tumor necrosis factor alpha (TNF-α) and TNF-β) (Buonaguro 1992, 1994, Sastry 1990, Scala 1994), transforming growth factor β1(Cupp 1993, Lotz 1994, Zauli 1992), IL-4 (Puri 1992) and IL-10 (Masood 1994). In contrast, the expression of other genes, including those for manganese-dependent superoxide dismutase (Westendorp 1995), IL-2 (Wetendorp 1994), the HLA class I proteins (Howcroft 1993), and p68 kinase, is down regulated by Tat (Roy 1990)).

Transcription from the HIV LTR is controlled through several upstream regulatory elements, including the TFIID components TBP (Kashanchi 1994), Sp1-, and NF-κB-binding sites. The activation of NF-kB plays a major role in stimulating HIV-1 gene expression in response to a variety of stimuli of chemical, physical, and biological

nature. Induction of HIV-1 gene expression by TNF-α (Antoni 1994, Vlach 1992) phorbol-12-myristate-13-acetate (PMA) (Demarchi1993, Vlach 1992), UV radiation (Ursini 1993), or cellular differentiation (Griffin 1989) is coupled to NF-kB activation. In addition, mutations in the NF-kB binding sites result in the loss of LTR activation in several cell lines (Duh 1989, Nabel 1987, Osborn 1989) as well as in peripheral blood CD4 T lymphocytes (Alcami 1995). An additional element, the trans-activating response element (TAR), is located just 3' to the start of transcription and is required for Tat transactivation. Unlike a traditional enhancer element, the natural position and orientation of TAR must be maintained. TAR forms a stable RNA hairpin at the 5' end of nascent viral transcripts (Berkhout 1989), and it is now clear that Tat binding to TAR RNA is essential for Tat functioning (Roy 1990, Dingwall 1990, Calnan 1991).

The RNA binding domain of tat is nine amino acids long and contains six arginines and two lysines. Short peptides spanning just this region bind specifically to TAR (Calnan 1991a, Weeks 1990, Cordingley 1990). The RNA binding domain is unusually flexible both in amino acid sequence and in structure (Calnan 1991a), with a single arginine residue providing the only sequence specific contact (Calnan 1991b). In contrast to the protein, the structural requirements for TAR are more rigid. Mutagenesis experiments have shown that a three nucleotide bulge and particular nucleotides surrounding the bulge are essential for Tat binding and transactivation (Weeks 1991, Sumner-Smith 1991). Modification of two phosphates at the base of the bulge interferes with binding (Calnan 1991b), leading to a model

in which arginine forms a network of hydrogen bonds with two structurally distinct phosphates.

In addition to Tat binding, it appears that TAR also binds cellular proteins whose function may be to stabilize Tat binding or to modulate Tat activity. Mutations within a six nucleotide loop in TAR reduce transactivation but do not directly affect Tat binding (Roy 1990, Dingwall 1990, Calnan 1991a, Cordingley 1990). Several proteins have been shown to bind the loop and some partially purified proteins have to been shown to stimulate HIV transcription *in vitro* (Marciniak 1990, Wu 1991, Sheline 1991).

Insulin-like Growth Factor-I (IGF-I)

Insulin-like growth factor 1 (IGF-1) and insulin are closely related mitogenic peptides, generally considered to be metabolic hormones thought to regulate cellular proliferation and other anabolic processes in many tissues (Froesh 1987). In addition, IGF-1 has been shown to increase CD4+ T cell and B cell populations , resulting in enhanced immunoglobulin synthesis (Clark 1993).

IGF-1 is one member of a family of structurally and evolutionarily related peptides that also includes insulin, IGF-II, the relaxins, the invertebrate bombyxins and the molluscan insulin-like peptides (1). The structure of the human IGFs is very similar to that of human insulin (49% sequence identity for IGF-1, 47% for IGF-II) except that the IGFs are single chain polypeptides, since the 12-residue C-peptide (8 for IGF-II) linking the A- and B-chains is not

removed during processing like that of insulin. In the IGFs, the equivalent of the A-chain is extended at A21 by a D region of 8 (IGF-1) or 6 (IGF-II) amino acids. Insulin and the IGFs contain three identical disulfide bridges.

Three structurally related receptors for members of the insulin and IGF-1 peptide family have been identified; the insulin receptor, the type I IGF receptor (or the IGF-1 receptor), and orphan receptor known only as the insulin receptor-related receptor or IRR. These three receptors belong to the receptor protein tyrosine kinase family where they constitute a subgroup with distinct features. They are encoded by a single gene located in humans on chromosomes 19, 15, and 1, respectively.

The insulin receptor binds insulin with high affinity but also IGF-II (with 10-fold lower affinity) and IGF-1 (with a 50- to 100-fold lower affinity) (Nissley 1991). The insulin receptor is thought to be primarily involved in metabolic signaling but may also play a role in mitogenic signaling in some cell types in culture as well as in some phases of fetal development. The weak cross-reactivity of IGF-I and IGF-II with the insulin receptor explains in part their insulin-like metabolic effects, hence, their names. Most, if not all cells express IGF receptors although in varying degrees as it is essential in metabolic signalling and growth.

The IGF-I receptor binds IGF-I with high affinity and IGF-II with an equally high or slightly lower affinity depending on the cell type. The IGF-I receptor binds insulin with a 500-1000 times lower affinity, explaining in part the well known role of insulin as a growth factor at high concentrations. The IGF-1 receptor is thought of as

primarily being involved in mitogenic signaling. While epidermal growth factor (EGF) and platelet derived growth factor (PDGF) are competence factors that make quiescent cells (G_0) enter the G1 phase of the cell cycle, progression factors such as insulin and IGF-I are required in the late G1 phase to enter the S phase (Pledger 1977).

IGF-I and IGF-II also bind to a family of structurally related circulating binding proteins (unrelated to the receptors) of which six members have been identified and cloned (Shimaski 1991, Rechler 1992). The circulating binding proteins play a role in the modulation of the IGFs endocrine, paracrine and autocrine functions (Clemmons 1991) and limit the *in vivo* hypoglycemic potential of the IGFs which circulate at levels high enough to bind to insulin receptors if they were free.

The insulin receptor gene (>120 kb pairs) is much larger than the 4.2 kb mRNA that encodes the insulin receptor molecule, and consists of 22 exons ranging in size from 36 to >900 bp. The structure of the IGF-I receptor gene is strikingly similar (Abbott 1992) except for the lack of an equivalent of the exon 11 alternatively spliced in the insulin receptor, suggesting that the two genes arose from gene duplication.

The cDNAs encoding the insulin and IGF-1 receptor precursors have been cloned and sequenced (Ulrich 1985, 1986, Ebina 1985) giving valuable insights into the receptor structure and organization and generally confirming structural concepts derived from biochemical approaches (Czech 1982). The receptors are synthesized as single chain precursors of 1355, 1337, and 1271 amino acids, respectively, for the insulin, IGF-I and IRR receptors. The precursor

is cleaved at a tetrabasic sequence into separate α- and β-subunits. The α-subunit contains the ligand binding domain and is entirely extracellular, while the β-subunit contains a single transmembrane domain so that about a third is extracellular and two thirds (containing the tyrosine kinase domain) are intracellular. The receptor is organized as a functional dimer with an α2β2 structure, with a small number of α-α and α-β disulfide bridges. The α-subunit contains multiple putative N-glycosylation sites while the β-subunit contains only a few (Siddle 1992).

Ojectives

Wasting is a common complication in patients with AIDS. Unlike protein-colorie malnutrition, the weight loss associated with AIDS is characterized by a greater depletion of lean body mass (Kotler 1985). Although nutritional intervention may produce weight gain in many patients, body fat remains to be repleted preferentially to lean body mass, particularly in patients without overt malabsorption (Kotler 1990). Failure to correct the loss of lean body mass highlights the need for therapies that have specific anabolic effects on protein metabolism.

Treatment with human growth hormone (GH) has been shown to improve nitrogen balance in a variety of catabolic states in humans (Wilson 1992). Many of the biological effects of GH are known to be mediated through IGF-1, which is anabolic in animals

models of catabolism and in healthy human subjects fed a hypocaloric diet (Clemmons 1992).

In 1993, Geffner et al. reported that poor growth is a common feature of symptomatic children infected with HIV-1, and that more severe HIV-1 infection in children was associated with *in vitro* resistance to the growth promoting actions of IGF-1, GH, and insulin. More recent studies found that both insulin (at supraphysiological concentrations) and IGF-1 (at physiological concentrations) were able to inhibit the replication of HIV-1 in vitro (Germinario 1995). The objective of the following experiments were to confirm the inhibitory action of insulin, and primarily of IGF-1 on HIV-1 replication, and to demonstrate that this inhibitory effect was occurring at the transcriptional level. The fact that IGF-1 was able to inhibit HIV-1 replication, and that a common observance in HIV infected individuals is a resistance to the actions of IGF-1, provides for a means by which it is beneficial for HIV-1 to somehow render an individual insensitive to IGF-1 action and thus allow for the continual replication of itself.

Materials and Methods

A. Cell Culture

Cord blood mononuclear cells (CBMC) were obtained from umbilical cord blood (kindly provided by the Department of Obstetrics and Gynecology, Jewish General Hospital) and isolated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient centrifugation. The cells were collected, washed, and stimulated with 10 ug phytohemagglutinin (PHA) (Difco, Detroit, Michigan) per ml in RPMI-1640 media (GIBCO Laboratories, Mississauga, Ontario). HIV-1 Chronically infected U937 monocytic cells (100% were positive for p24 antigen), H9, U38, and ACH-2 cell lines were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, Bethesda, Maryland, U.S.A. All cells were maintained in suspension culture at a concentration of 3 x 10^5 to 5 x 10^5 cells/ml in RPMI-1640 medium containing 10% heat- inactivated fetal calf serum (FCS) (Flow Laboratories, Toronto, Canada), 2mM L-glutamine, 100 U/ml penicillin, and 100 ug/ml streptomycin. Cos-7 monkey-kidney cells were kindly provided by Dr. Larry Kleiman (Lady Davis Institute, Jewish General Hospital) and were maintained in DMEM medium containing 10% heat- inactivated fetal calf serum (FCS) (Flow Laboratories, Toronto, Canada), 2mM L-glutamine, 100 U/ml penicillin, and 100 ug/ml streptomycin. All cell lines were maintained in water-jacketed incubators at 37°C and under 5% CO2. Cell viability was measured using trypan blue exclusion, and cells were analyzed to be mycoplasma-negative, as assessed by

fluorescence microscopy using 4',6-diamine-2-phenylindole (DAPI) dye.

B. Virus Culture and Infection

The HIV-IIIB laboratory strain of HIV-1 was kindly supplied by R.C. Gallo (National Institutes of Health (NIH), Bethesda, Maryland). Virus was concentrated by ultracentrifugation from chronically infected H9 cell cultures. Stock virus titers were quantified by an infectivity assay. direct infection of PHA-stimulated CBMCs was performed with a viral titer of TCID50 (50% tissue culture infective dose) of 2000 in a maximum volume of 0.5 ml (viral stock = 5 x 10^3 TCID50/ml). After a 2 hour infection at 37°C, cells were washed free of unattached virus and resuspended in fresh medium (i.e. RPMI-1640 containing 1% heat-inactivated fetal calf serum for CBMCs and 5% heat inactivated serum for U937 cells). This procedure was designated as an acute infection for CBMCs. In the case of CBMCs, 10 units of interleukin 2 (IL-2) (human recombinant; Boehringer Mannheim, Indianapolis, IN) per milliliter was added to the medium.

C. Virus Replication Assays

Reverse transcriptase assays were carried out by a modification of a previously published procedure (Lee et al. 1987). Briefly, 50 ul of clarified culture supernatant was added to 50 ul of reaction cocktail counting 50 mM Tris hydrochloride (pH=7.9), 5 mM magnesium chloride, 150 mM potassium chloride, 0.5 mM ethylene

glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 0.5% Triton X-100, 2% ethylene glycol, 5 mM dithiothreitol, 0.3 mM reduced glutathione, 20 uCi of tritiated thymidine triphosphate, and 50 ug of template primer (poly(rA)oligo(dT)) per milliliter in polypropylene tubes. The tubes were then incubated at 20°C for 22 hours. The reaction was stopped by the addition of 1 ml of cold 10% (w/v) trichloroacetic acid (TCA). Newly synthesized DNA was precipitated on ice for atleast 2 hours, and then collected on Whatman (Clifton, New Jersey) GF/C glass fiber filters and rinsed two times with cold 10% TCA and absolute ethanol. Filters were dried for 20 minutes and counted for the incorporated activity.

Virus production was monitored by detection of p24 HIV core antigen in cell-free culture supernatants, using an EIA diagnostic kit (Abbott Laboratories, Mississauga, Ontario, Canada).

D. Experimental Reagents: Insulin, IGF-1, TNF-α, Plasmids, & Antibodies

Insulin (Sigma Co., St. Louis, MO) was dissolved in a 1x solution of phosphate buffered saline to make a stock solution, filter sterilized, and then kept at -20°C until required. IGF-1 (GIBCO Laboratories) was dissolved in a 0.1N solution of Acetic Acid to bring it into solution, and then diluted 1 in 20 with RPMI-1640 (0% FCS) to a final concentration of 100 ng/ml, and then stored at -20°C until required. TNF-α (Boehringer Mannheim) was stored at -20°C until required. HIV-LTR-CAT and pSV-tat plasmids were kindly provided by Dr. Emanuelle Faust (Lady Davis Institute, Jewish General

Hospital) and Dr. Steve Zeichner (NIH, Bethesda, Maryland). α-IR3 and anti-IGF-1 antibodies (Oncogene Science), specifically directed against the IGF-1 receptor and the IGF-1 protein molecule, respectively, were kept at 4°C until required.

E. Transfection Protocol

Cos-7 cells were transiently transfected by the $CaCl_2$-hepes-phosphate precipitation method as previously described (Maniatis 1982). Briefly, 10^6 cells were plated in tissue culture dishes 24 hours prior to transfection. 10 ug of plasmid DNA and 50 ul of 2.5 M $CaCl_2$ were mixed and brought to a final volume of 500 ul with ddH_2O. This was then added to 500 ul of 2x Hepes buffered saline solution drop by drop until a slightly cloudy mixture was formed. The total mixture was then added to the COS-7 cells drop by drop and samples incubated at 37°C for 16 hours. Plates containing COS-7 cells were washed twice and incubated in fresh DMEM media containing 10% FCS for 60 hours. Cells were then harvested and lysed and the extracts were assayed for CAT activity. The percent acetylation was determined by ascending thin layer chromatography as previously described (Garoufalis 1994) and quantified using the BioRad Gelscan Phosphoimager and the Molecular Analyst (BioRad) software program.

U38 cells were transiently transfected by DEAE-dextran method (Lacoste 1990). The precipitated DNAs (0.5 to 10 ug), representing pSV-tat plasmids were resuspended in TS solution (8 mg/ml NaCl, 0.38 mg/ml KCl, 0.1 mg/ml Na_2HPO_4 7 H2O, 3 mg/ml

Tris, 0.1 mg/ml CaCl2, pH 7.4). After resuspension, 0.05 mg of DEAE-Dextran (Pharmacia) was added. For each transfection 1x 10^7 cells in exponential phase were washed once in TS, resuspended with the DNA solution and incubated at room temperature for 20 min. Cells were then incubated at 37°C for 30 min in 10 ml with medium containing 10% serum and 0.1 mM chloroquine (Sigma Chemical Co.) after which they were centrifuged and resuspended in fresh medium and serum. At 32 hours after transfection, cells were induced with 5 ng/ml TNF-α (Boeringer Mannheim). At 16 hours after induction, cells were harvested and lysed and the extracts were assayed for CAT activity. The percent acetylation was determined by ascending thin layer chromatography as previously described (Garoufalis 1994) and quantified using the BioRad Gelscan Phosphoimager and the Molecular Analyst (BioRad) software program.

Results

A. Inhibition of HIV-1 Replication by Varying Concentrations of Insulin

Cord blood mononuclear cells were infected with the IIIB strain of HIV for 2 hrs at a $TCID_{50}$ of 2000. Cells were then washed twice of residual virus and resuspended to a final concentration of 1.6 x 10^6 cells/ ml in RPMI 1640 media supplemented with 1% heat-inactivated fetal calf serum. Cells were seeded in a 96 well plate with 4.0 x 10^5 cells / well in the presence of increasing amounts of insulin (6.7 x 10^{-8} to 6.7 x 10^{-6} M). The plates were then incubated in 37°C incubatators with 5% CO2 and allowed to proceed. At days 4 and 7, 100 ul of supernatant from each well was collected and subjected to both (50 ul per reaction) the reverse transcriptase reaction and p24 quantitation in order to determine the amount of viral replication per sample. Figure 4 demonstrates the inhibitory effect on virus replication by insulin proceeded in a dose dependent manner as a function of reverse transcriptase activity. Figure 5 also shows a dose dependent inhibitory effect by insulin as a function of the amount of p24 present in viral supernatant. These data reconfirmed earlier findings (Germinario et al.1994), and showed that the inhibitory effect was most effective at supraphysiological concentrations.

B. Inhibition of HIV-1 Replication by Varying Concentrations of Insulin-like Growth Factor-1 (IGF-1)

Chronically infected monocytic U937 cells which actively and continuously produce HIV were washed twice with PBS and resuspended to a final concentration of 4.0 x 10^5 cells/ml in RPMI 1640 media supplemented with 5% heat-inactivated fetal calf serum, antibiotics, and L-glutamine. Cells were seeded in 96 well plates with 1.0 x 10^5 cells / well in the presence of increasing amounts of IGF-1 (0 to 100 ng/ml). Cells were then incubated in 37°C incubators with 5% CO_2 and allowed to proceed. At 24 hours post exposure to the varying amounts of IGF-1, 50 ul of viral supernatant was collected, centrifuged and then subjected to the reverse transcriptase reaction. Figure 6 illustrates the inhibitory effect of IGF-1 on HIV-1 replication as measured by the amount of active reverse transcriptase in the culture media. HIV replication is significantly inhibited in the presence of IGF-1. The inhibitory effect is observed to be dose dependent and is optimal at normal physiological concentrations of approximately 50-100 ng/ml or 6.5 x 10^{-9} M to 1.3 x 10^{-8} M.

C. Inhibition of HIV-1 Replication by IGF-1 is Specific and Can Be Abrogated by α-IR3 and anti-IGF-1 Antibodies

Chronically infected monocytic U937 cells were washed twice with PBS and resuspended to a final concentration of 4.0 x 10^5 cells/ml in RPMI 1640 media supplemented with 5% heat-inactivated fetal calf serum. Cells were seeded in 96 well plates with 1.0 x 10^5 cells / well in the presence of increasing amounts of IGF-1 (0 to 50 ng/ml). The cells subjected to IGF-1 were then incubated in the presence of α-IR3 (5ug/ml) and anti-IGF-1 (5ug/ml) antibodies against the IGF-1 receptor and the IGF-1 molecule, respectively. Cells were then incubated in 37°C incubators with 5% CO_2 and allowed to proceed. At 24 hours post exposure to IGF-1 and the respective antibodies, 50 ul of viral supernatant was collected and subjected to the reverse transcriptase reaction. Figure 8A) demonstrates the effect of IGF-1 alone on chronically infected U937 cells as well as in the presence of the antibodies specific to the IGF-1 receptor figure 8B) and the IGF-1 molecule itself figure 8C). Figure 8 is plotted as a percentage of control . The first lane in each graph is taken as 100% (no IGF-1). From figure 8B) we see that αIR3, the antibody directed against the IGF-1 receptor, is capable of abolishing the inhibitory effects of IGF-1 observed in figure 8A). In figure 8C) anti-IGF-1, the antibody directed against the IGF-1 molecule itself is also capable of abolishing the observed inhibitory effects of IGF-1, to a somewhat greater extent than the αIR3 antibody. The inhibitory effect of IGF-1 on viral replication is shown , as well as the specificity of the inhibitory effect as it is abolished by the presence of

antibodies to the IGF-1 receptor in the presence of IGF-1 , and in the presence of antibodies to the IGF-1 molecule itself in the presence of IGF-1.

D. Inhibition of HIV-1 transcription by varying concentrations of IGF-1

Cos-7 cells were plated at a concentration of 10^6 cells/ plate 24 hours prior to transfection. Cells were transiently cotransfected with 9 ug HIV-LTR-CAT and 1 ug pSV-tat. HIV-LTR-CAT plasmid contains the HIV long terminal repeat linked to the chloroamphenicol acetyl transferase reporter gene. pSV-tat plasmid contains the HIV-tat gene which when transcribed and translated is capable of activating the HIV-LTR. Cells were transfected using the $CaCl_2$-hepes-phosphate precipitation method. Samples were maintained in DMEM supplemented media after transfection in water-jacketed incubators at 37°C and 5% CO_2. 16 hours post-transfection, samples were washed and replenished with fresh media in the presence of increasing amounts of IGF-1, and incubated for an additional 60 hours. Cells were then harvested and lysed and the extracts were assayed for CAT activity. As can be observed in figure 9, the transcriptional activation of the HIV-LTR by tat in this system can be influenced by the presence of IGF-1. IGF-1 inhibition of HIV-1 transcription is observed to occur in a dose dependent manner as assessed by CAT activity. Optimal inhibition is observed at a concentration of 50 ng/ml, comparable to normal physiological concentrations. Figure 10 is a plot of percent acetylation versus IGF-1 concentration. Quantitation was carried out by phosphoimager analysis of the chromatograph illustrated in figure 9.

E. Transcriptional Effects on U38 cells by IGF-1

U38 cells contain stably transfected HIV-LTR-CAT. These cells were transiently transfected with pSV-tat by the DEAE-dextran method. 5 ug tat was resuspended in TS solution. To this 0.05 mg of DEAE-dextran was added. For each transfection 1 x 10^7 U38 cells were washed with PBS and then resuspended in the TS solution containing the DNA and DEAE-dextran and incubated at room temperature for 20 minutes. Cells were then incubated at 37°C for 30 min in 10 ml RPMI containing 10% FCS and 0.1 mM chloroquine. After 30 min the cells were centrifuged, washed, and resuspended in fresh media containing varying amounts of IGF-1. At 48 hours post-transfection, cells were harvested and lysed and the extracts assayed for CAT activity. Figure 11 illustrates the effect of pSV-tat transfection on U38 cells. There is significant CAT activity upon pSV-tat transfection, however, as both figures 11 and 12 demonstrate, the effects by IGF-1 are not as substantial in this system. Figure 12 is a plot of % acetylation versus increasing amounts of IGF-1. Although the inhibitory effect of IGF-1 is not as dramatic as observed in previous experiments, there is still an observable difference between 10 and 50 ng/ml of IGF-1.

F. *Transcriptional Effects on the HIV-LTR by IGF-1 and TNF-α*

Cos-7 cells were plated at a concentration of 10^6 cells/ plate 24 hours prior to transfection. Cells were transiently cotransfected with 9 ug HIV-LTR-CAT and 1 ug pSV-tat. HIV-LTR-CAT plasmid contains the HIV long terminal repeat linked to the chloroamphenicol acetyl transferase reporter gene. pSV-tat plasmid contains the HIV-tat gene which when transcribed and translated is capable of activating the HIV-LTR. Cells were transfected using the $CaCl_2$-hepes-phosphate precipitation method. Samples were maintained in DMEM supplemented media after transfection in water-jacketed incubators at 37°C and 5% CO_2. 16 hours post-transfection, samples were washed and replenished with fresh media in the presence of increasing amounts of TNF-α, and incubated for an additional 60 hours. Cells were then harvested and lysed and the extracts were assayed for CAT activity. Figure 13 demonstrates the TNF-a does affect the level of tat induced transcription in the HIV-LTR. It has an additive effect with that of HIV-tat in this system as can be observed at a concentration of 20 ng/ml.

U38 cells were transiently transfected with pSV-tat by the DEAE-dextran method. 5 ug tat was resuspended in TS solution. To this 0.05 mg of DEAE-dextran was added. For each transfection 1 x 10^7 U38 cells were washed with PBS and then resuspended in the TS solution containing the DNA and DEAE-dextran and incubated at room temperature for 20 minutes. Cells were then incubated at 37°C for 30 min in 10 ml RPMI containing 10% FCS and 0.1 mM

chloroquine. After 30 min the cells were centrifuged, washed, and resuspended in fresh media containing varying amounts of either IGF-1 or TNF-α or both. At 48 hours post-transfection, cells were harvested and lysed and the extracts assayed for CAT activity. Figure 14 demonstrates the effect of the addition of both IGF-1 and TNF-α on tat transcription on the HIV-LTR. In the absence of any tat there is no transcriptional activation. The addition of Tat allows for transcription to occur, however, the effects of IGF-1 and TNF-α in this system are not as dramatic as those observed in the previous COS-7 transfection system. Figure 15 is a plot of the amount of acetylation as quantitated by phosphoimager analysis. It is observable that the effect of TNF-α alone with tat augments the amount of transcription as compared to tat alone. The addition of IGF-1 alone with tat inhibits slightly the effect of tat transcription. Interestingly, however, is the overall increase in transcription in the presence of both IGF-1 and TNF-α in addition to tat. TNF-α seems to be capable of overcoming the effects of IGF-1.

Figure 1. Schematic representation of the HIV life cycle from receptor binding, reverse transcription, integration, assembly, to virion release..

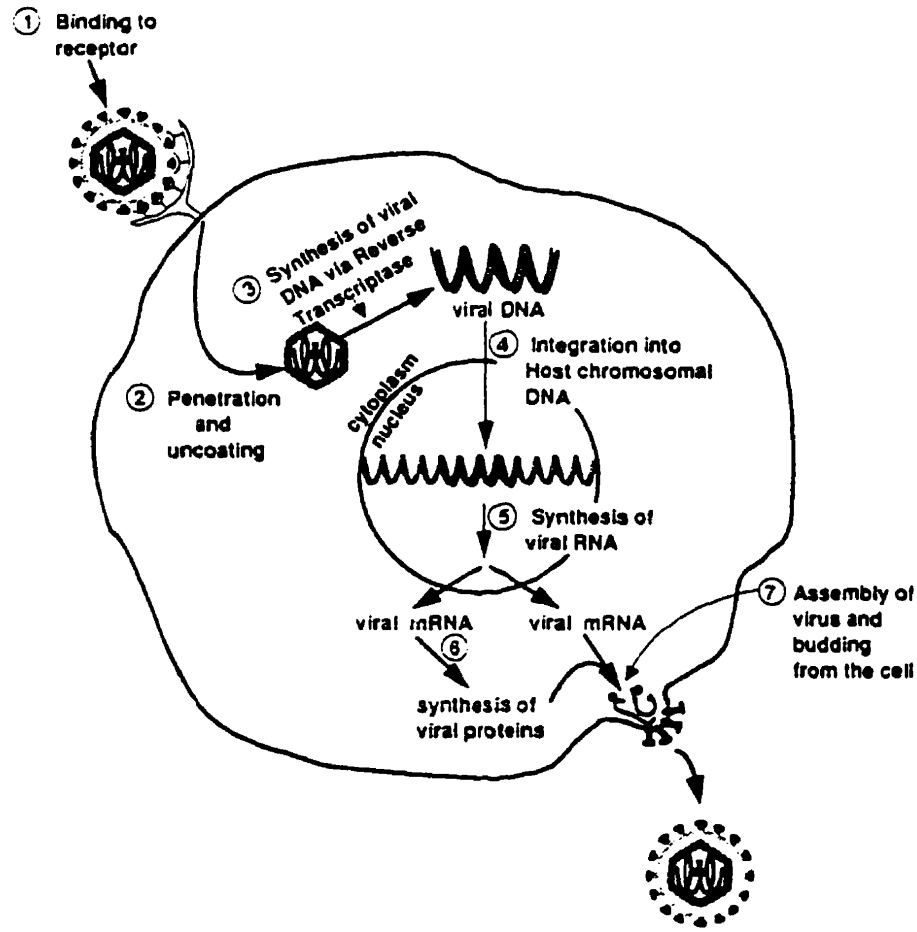

1 Binding to receptor
2 Penetration and uncoating
3 Synthesis of viral DNA via Reverse Transcriptase
viral DNA
4 Integration into Host chromosomal DNA
cytoplasm
nucleus
5 Synthesis of viral RNA
viral mRNA
viral mRNA
6
synthesis of viral proteins
7 Assembly of virus and budding from the cell

Figure 2. Schematic representation of HIV early and late gene expression and protein production.

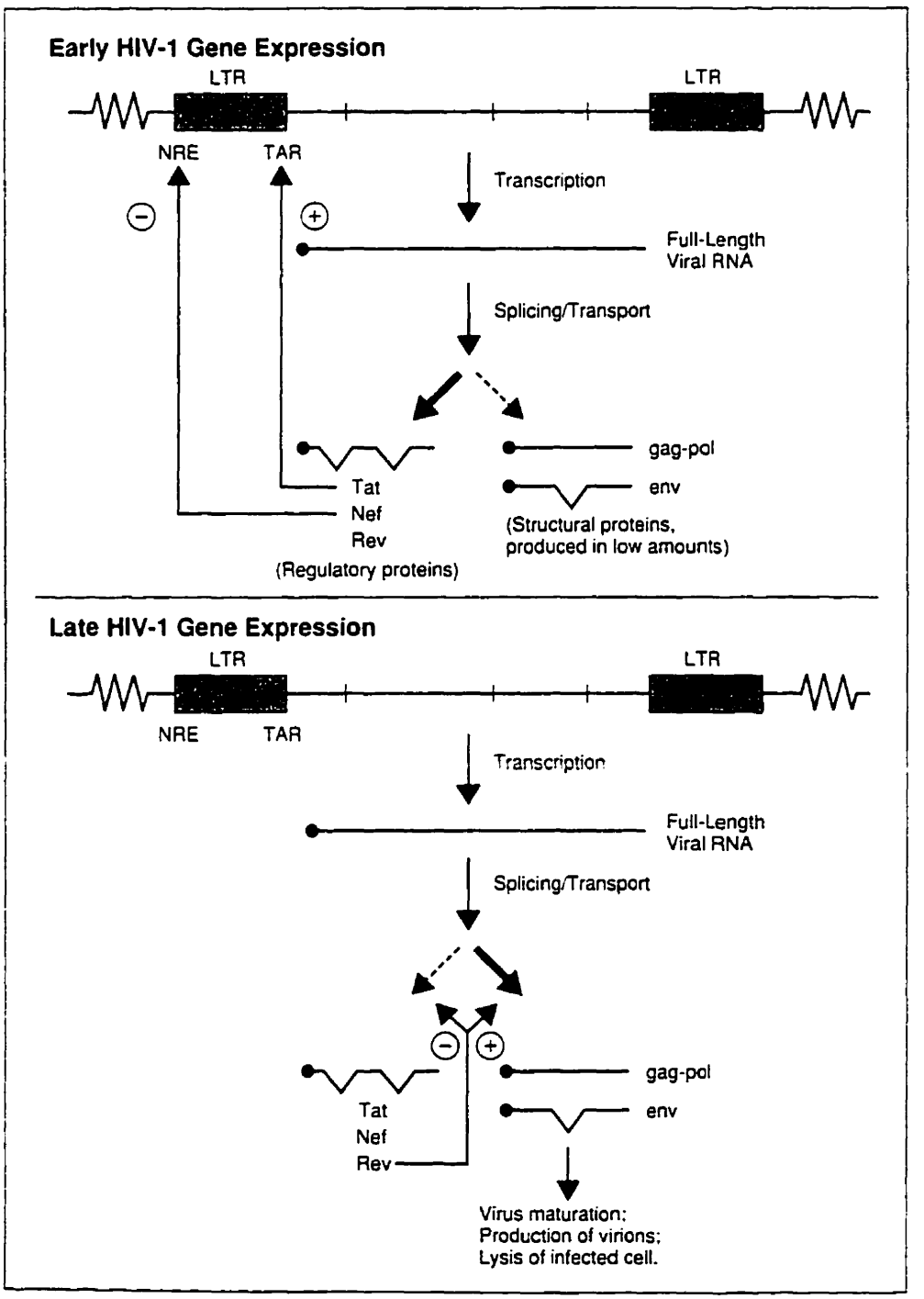

Early HIV-1 Gene Expression
LTR
LTR
NRE
TAR
Transcription
Full-Length
Viral RNA
Splicing/Transport
gag-pol
env
Tat
Nef
Rev
(Structural proteins,
produced in low amounts)
(Regulatory proteins)
Late HIV-1 Gene Expression
LTR
LTR
NRE
TAR
Transcription
Full-Length
Viral RNA
Splicing/Transport
gag-pol
env
Tat
Nef
Rev
Virus maturation:
Production of virions;
Lysis of infected cell.

Figure 3. Molecular model representation of the IGF-1 molecule. (Modified from De Meyts 1994)

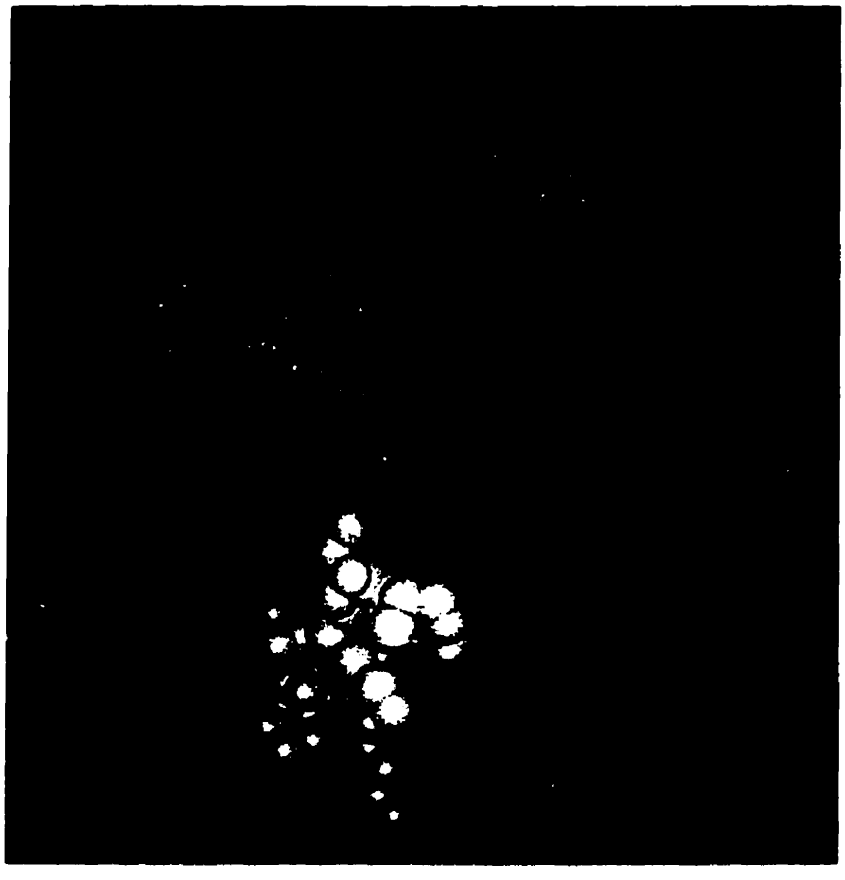


Figure 4. Effect of varying concentrations of insulin on HIV-1 infected cord blood mononuclear cells as determined by the reverse transcriptase assay. Results are the averages of experiments performed in duplicate.

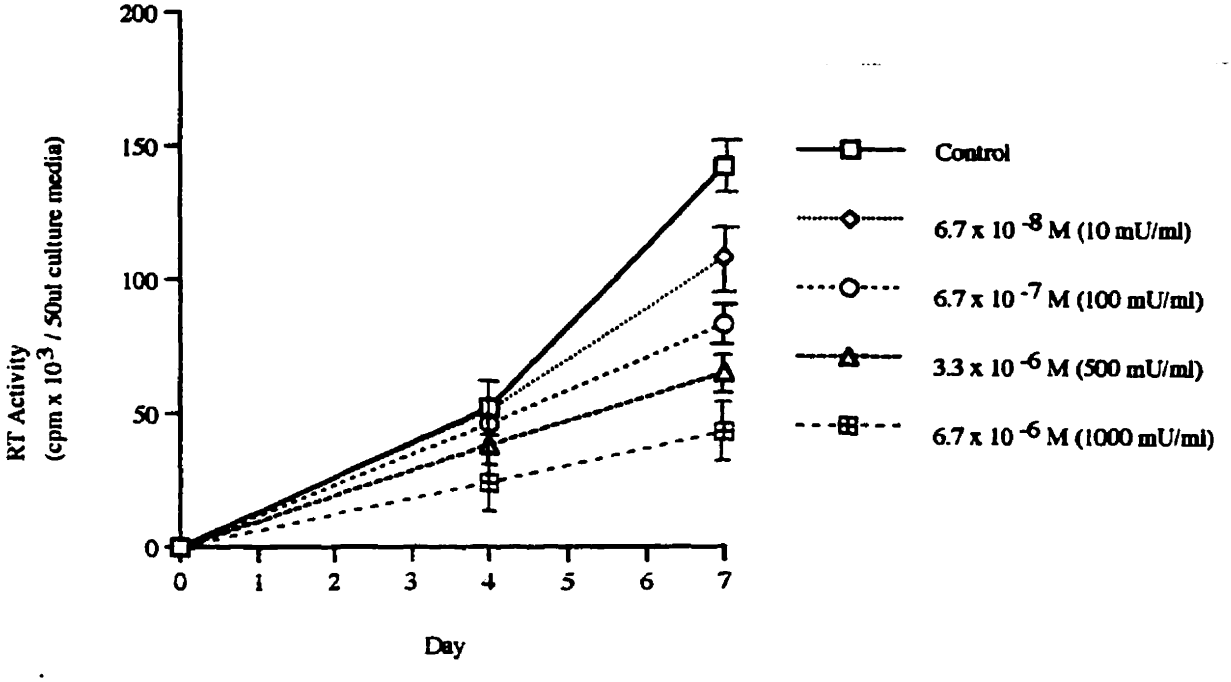

200
150
100
50
0
0
1
2
3
4
5
6
7
Day
RT Activity
(cpm x 10^3 / 50ul culture media)
Control
6.7 x 10 -8 M (10 mU/ml)
6.7 x 10 -7 M (100 mU/ml)
3.3 x 10 -6 M (500 mU/ml)
6.7 x 10 -6 M (1000 mU/ml)

Figure 5. Effect of varying concentrations of insulin on HIV-1 infected cord blood mononuclear cells as determined by the p24 assay. Results are the averages of experiments performed in duplicate.

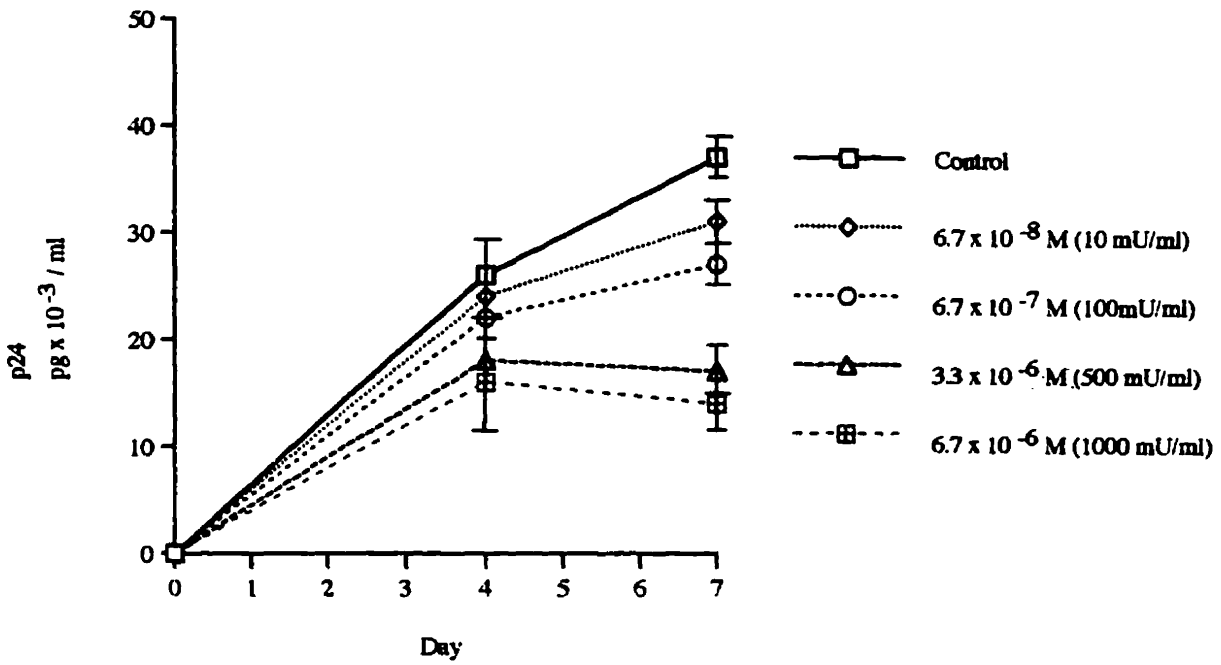

p24
pg x 10^{-3} / ml
50
40
30
20
10
0
0
1
2
3
4
5
6
7
Day
Control
6.7 x 10^{-8} M (10 mU/ml)
6.7 x 10^{-7} M (100mU/ml)
3.3 x 10^{-6} M (500 mU/ml)
6.7 x 10^{-6} M (1000 mU/ml)

Figure 6. Effect of varying concentrations of insulin-like growth factor I (IGF-I) on HIV-1 chronically infected U937 monocytic cells as determined by the reverse transcriptase assay. RT activity was measured 24 hrs post exposure. Results are the averages of experiments performed in triplicate.

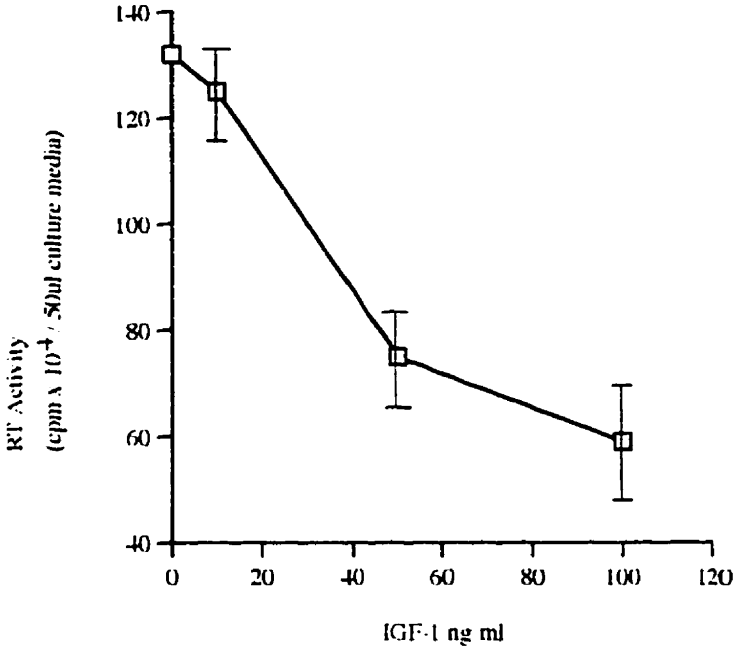

140
120
100
80
60
40
0
20
40
60
80
100
120
RT Activity
(cpm x 10^{-4} / 50ul culture media)
IGF-1 ng ml

Figure 7. Effect of varying concentrations of insulin-like growth factor I (IGF-I) on HIV-1 chronically infected U937 monocytic cells as determined by the reverse transcriptase assay plotted as a percentage of control. Control was designated as being 100%. RT activity was measured 24 hrs post exposure. Results are the averages of experiments performed in triplicate.

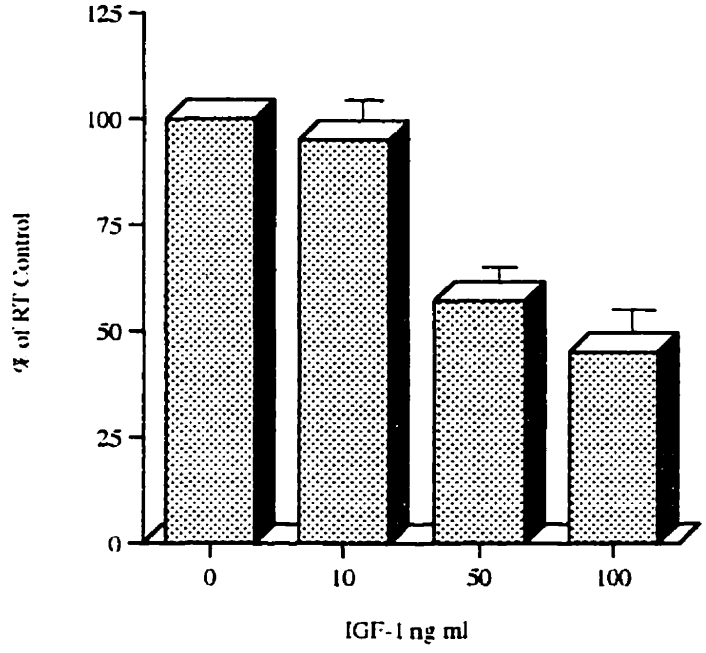

125
100
75
50
25
0
% of RT Control
0
10
50
100
IGF-1 ng ml

Figure 8. Effect of αIR-3 antibody specific to the IGF-1 receptor and anti-IGF-1 antibody specific to the IGF-I molecule in the presence of varying concentrations of IGF-I in chronically infected U937 monocytic cells as determined by the reverse transcriptase assay. RT activity was measured at 24 hrs post exposure to IGF-I and Abs. A) no antibodies B) Presence of 5 ug/ml of αIR3 C) Presence of 5 ug/ml of anti-IGF-1. Results are the averages of experiments performed in duplicate.

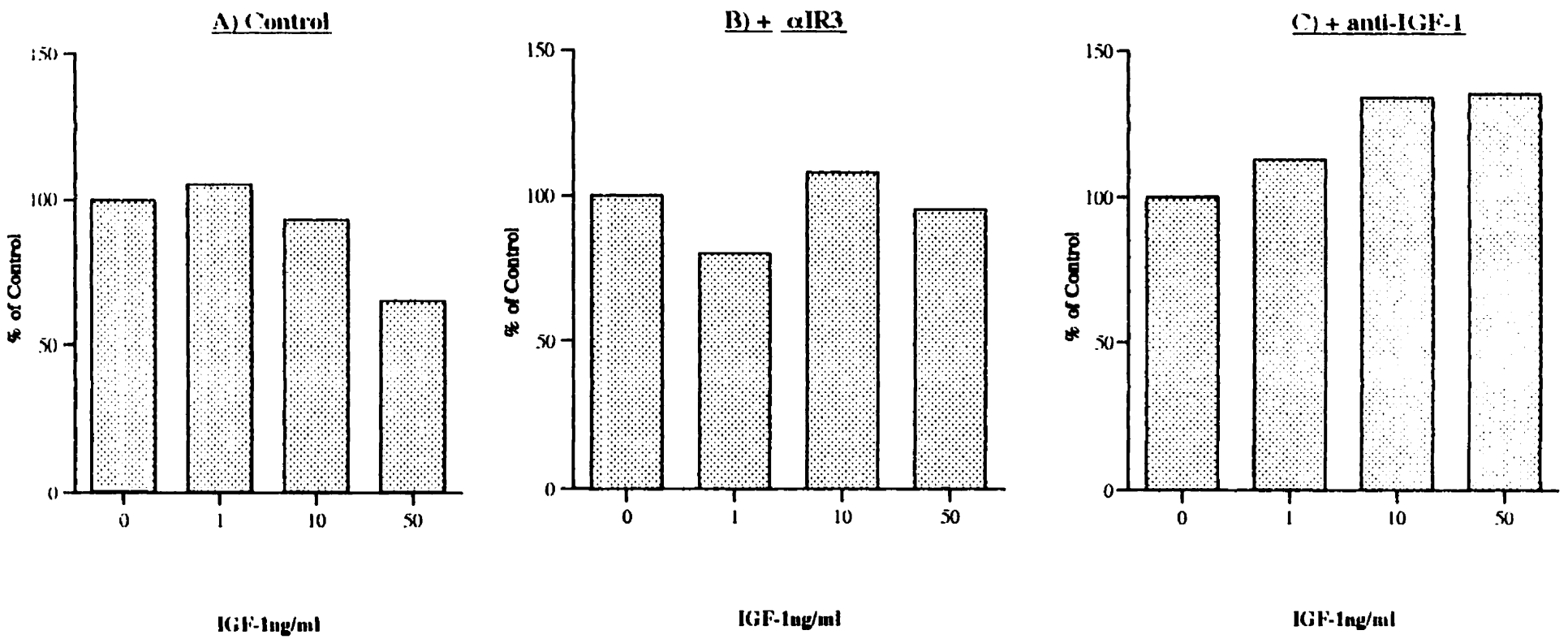
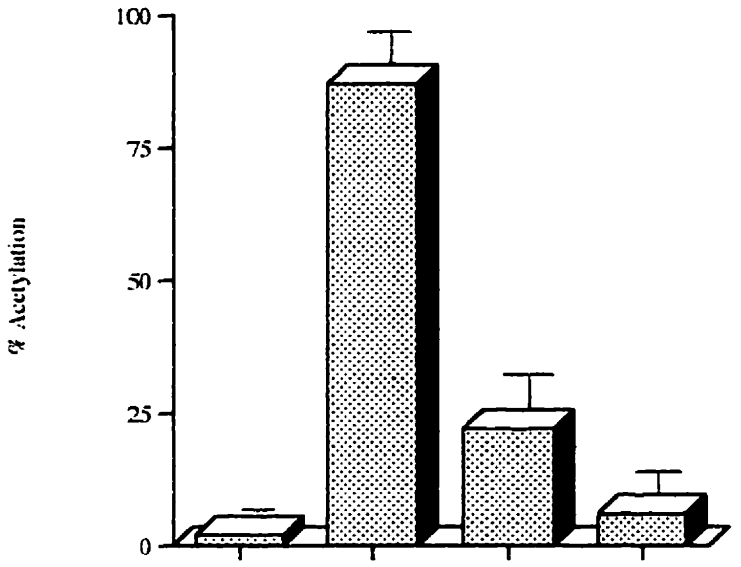

A) Control
B) + αIR3
C) + anti-IGF-1
% of Control
150
100
50
0
0
1
10
50
IGF-1ng/ml

Figure 9. Effect of varying concentrations of IGF-I on COS-7 cell transiently cotransfected with HIV-LTR-CAT and PSV-tat plasmids as determined by the Chloroamphenicol Acetyl Transferase Assay.

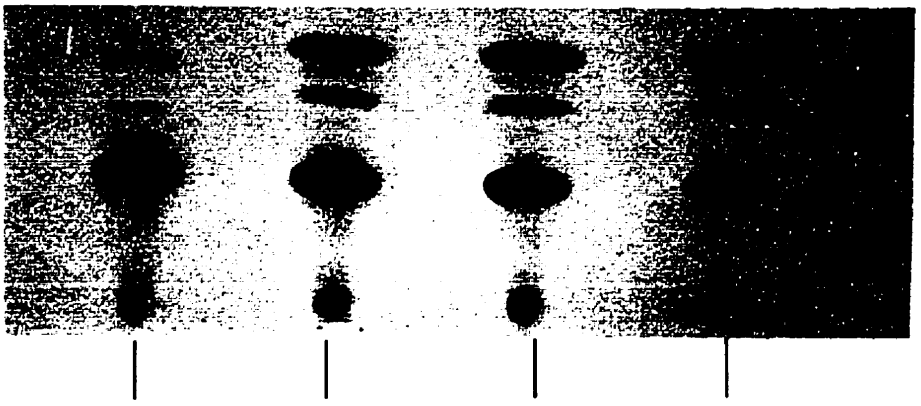

IGF-1 ng/ml 0 0 10 50
Calf Thymus DNA + - - -
HIV-LTR-CAT/tat - + + +

Figure 10. Effect of varying concentrations of IGF-I on COS-7 cell transiently cotransfected with HIV-LTR-CAT and PSV-tat plasmids as determined by the Chloroamphenicol Acetyl Transferase Assay represented as a percentage of acetylation. Results are the averages of experiments performed in duplicate.



IGF-1 ng ml	0	0	10	50
Calf Thymus DNA	+	-	-	-
HIV-LTR-CAT tat	-	+	+	+

Figure 11. Effect of varying concentrations of IGF-I and pSV-tat on U38 cells stably transfected with HIV-LTR-CAT. Results are representative of measurements taken 24 hrs post transient transfection with pSV-tat and exposure to IGF-I.



pSV-tat	-	+	+	+
IGF-1 ng/ml	-	-	10	50

Figure 12. Effect of varying concentrations of IGF-I and pSV-tat on U38 cells stabily transfected with HIV-LTR-CAT. Results are representative of measurements taken 24 hrs post transient transfection with pSV-tat and exposure to IGF-I. Chloroamphinicol Acetyl Transferase Assay represented as a percentage of acetylation. Results are the averages of experiments performed in duplicate.

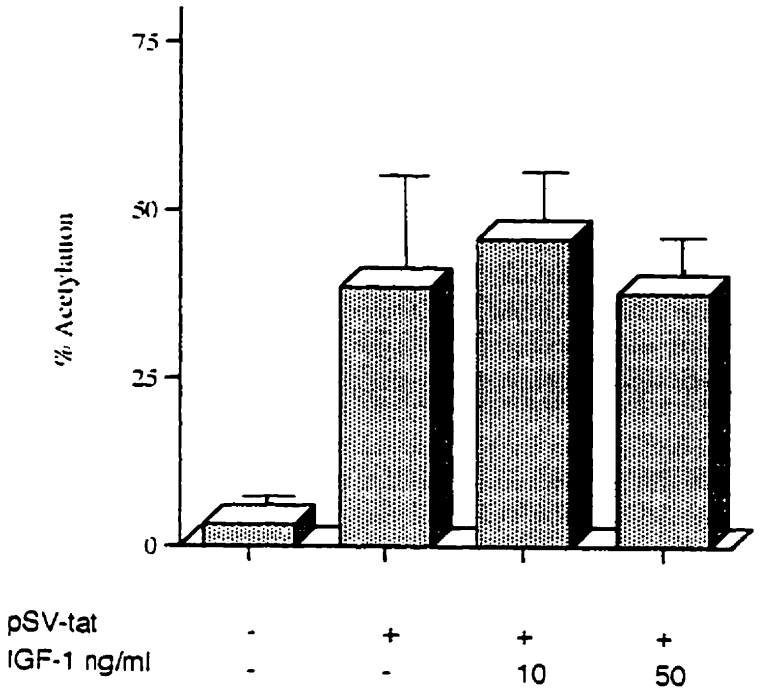
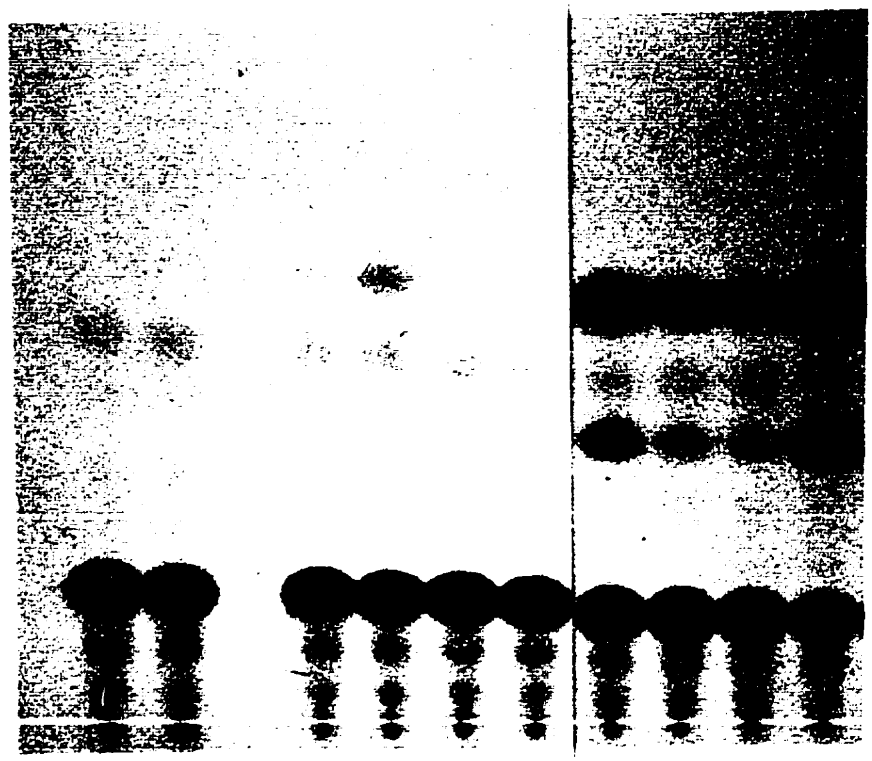
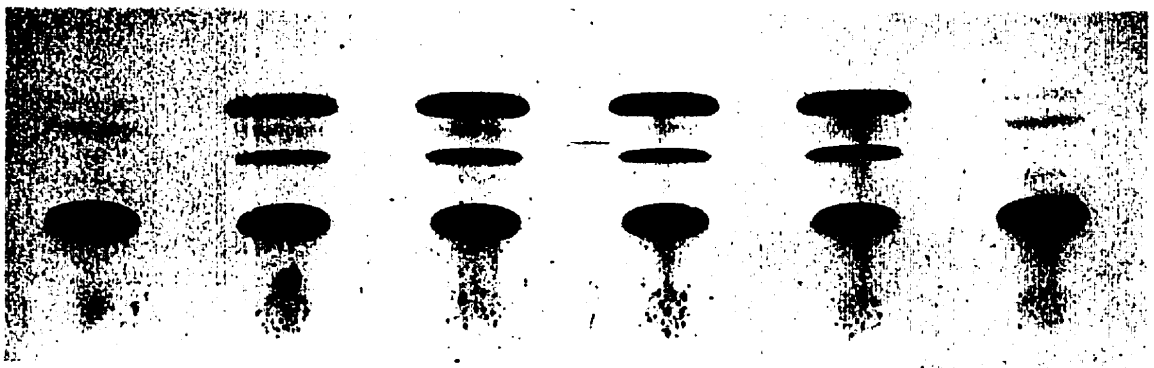

75
50
25
0
% Acetylation
pSV-tat - + + +
IGF-1 ng/ml - - 10 50

Figure 13. Effect of TNF-α on COS-7 cells transiently transfected with HIV-LTR-CAT and pSV-tat plasmids as determined by the Chloroamphenicol Acetyl Transferase Assay.



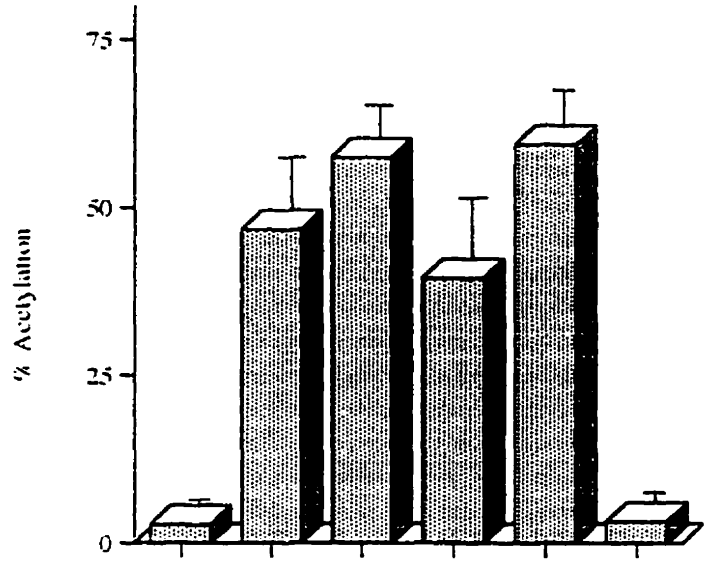
Calf Thymus DNA	+	+	-	-	-	-	-	-	-	-
HIV-LTR-CAT	-	-	+	+	+	+	+	+	+	+
pSV-tat	-	-	-	-	-	-	+	+	+	+
TNF- ng/ml	-	-	-	1	10	20	-	1	10	20

Figure 14. Effect of IGF-I and TNF-α in U38 cells stably transfected with HIV-LTR-CAT and transiently transfected with pSV-tat as measured by the Chloroamphenicol Acetyl Transferase Assay.



pSV-tat	-	+	+	+	+	-
TNF- 10ng/ml	-	-	+	-	+	+
IGF-1 50ng/ml	-	-	-	+	+	-

Figure 15. Effect of IGF-I and TNF-α in U38 cells stably transfected with HIV-LTR-CAT and transiently transfected with pSV-tat as measured by the Chloroamphenicol Acetyl Transferase Assay represented as a percentage of acetylation. Results are the averages of experiments performed in duplicate.


75
50
25
0
% Acetylation
pSV-tat - + + + + -
TNF- ng/ml - - + - + +
IGF-1 ng/ml - - - + + -

Discussion

A common complication in individuals with acquired immunodeficiency syndrome (AIDS) is the phenomenon of wasting. Unlike protein-calorie malnutrition, the weight loss associated with AIDS is characterized by a greater depletion of lean body mass than fat mass (Kotler 1985). Lieberman et al. in 1994 reported that insulin-like growth factor-1 (IGF-1) levels are lower and growth hormone (GH)-induced IGF-1 production is significantly lower in AIDS patients. Furthermore, GH levels are observed to be significantly elevated in AIDS patients implicating a scenario of resistance to growth hormone induced IGF-1 production. Thus, it is not hard to conceive that the wasting disease observed in AIDS suggests a relationship between increased viral burden in AIDS patients and low circulating IGF-1 levels.

IGF-1 is normally considered to be a metabolic hormone which mediates many of the anabolic effects of growth hormone (Froesch 1987). IGF-1 was once referred to as a somatomedin to indicate that it mediated the whole-body growth-promoting activity of GH (Daughaday 1972), but was later named IGF-1 in recognition of it's insulin-like metabolic activities (Daughaday 1987). The anabolic activities of IGF-1 were initially quantitated in GH-deficient or hypophysectomized rats. A common finding has been that recombinant human IGF-1 increases spleen and thymus weight disproportionately to its effects on whole body size (Froesch 1987, Guler 1988, Skottner 1989, Binz 1990). However, direct comparisons of the anabolic activities of IGF-1 and GH suggest that they have

differential effects on organ growth. For example, GH has a large effect on epiphyseal cartilage growth while IGF-1 has a greater effect on splenic weight (Skottner 1989).

In addition to these established anabolic effects, there is a growing body of evidence that links growth hormone and IGF-1 to the immune system (Kelley 1990, Ammann 1988, Weigent 1990). For example, mice with hereditary GH deficiency have been observed to develop an impaired immune system associated with thymic atrophy, immunodeficiency, and tissue wasting which is believed to contribute to the shortened life expectancy of these mice (Fabris 1971). Conversely, administration of exogenous GH induces T-cell proliferation in the thymus (Murphy 1992, Timsit 1992) and can alter the activity of all major immune cell types, including T cells, B cells, natural killer (NK) cells, and macrophages (Kelley 1989).

There have been fewer reports on the direct effects of IGF-1 on immune parameters despite the possibility that the actions of GH on lymphocytes may be mediated by the local induction of IGF-1, which then acts through the IGF-1 receptor (Geffner 1990). Further, at nanomolar concentrations, IGF-1 has been shown *in vitro* to be a growth-promoting factor for lymphocytes and to be chemotactic for resting and activated T cells (Schimpff 1983).

In addition to these effects on lymphocytes, IGF-1 has been reported to enhance the maturation of morphologically recognizable granulocytic and erythroid progenitors in suspension cultures of marrow cells (Merchav 1988). *In vivo*, infusions of IGF-1 increase thymus and spleen weight in hypophysectomized rats (Froesch 1987, Guler 1988) and dwarf rats (Skottner 1989), and induce repopulation

of the atrophied thymus in diabetic rats (Binz 1990). These reports suggest that IGF-1 may have endocrine, paracrine, as well as autocrine effects on the immune system.

The effects on lymphoid tissue after *in vivo* administration of IGF-1 have been described only in gross anatomical terms as an increased weight of spleen or thymus (Froesch 1987, Guler 1988), whereas the studies *in vitro* have focused primarily on the effects on T cells. There are few data describing the effects on *in vivo* administration of IGF-1 on the number, phenotype, or responsiveness of lymphocytes. Short term administration of IGF-1 to normal adult retired breeder mice has shown to have profound effects on lymphoid tissues owing to increases predominantly in CD4+ T cells and, quite unexpectedly, B cell populations resulting in enhanced immunoglobulin synthesis. Interestingly, there is an associated but delayed increase in T and B cell responsiveness to mitogens after IGF-1 administration.

Of relevance to this work were the prior observations that IGF-1 serum concentrations are altered in individuals with AIDS (Lieberman 1994), that administration of IGF-1 to symptomatic children with AIDS demonstrated marked *in vitro* resistance to the growth promoting action of IGF-1 compared with asymptomatic children (Geffner 1993), and that both insulin and IGF-1 displayed inhibitory effects on HIV-1 replication *in vitro* (Germinario 1995). On the basis of this, the objective of this work followed three themes. Firstly, to demonstrate and confirm the inhibitory effects of both insulin and IGF-1 on HIV-1 replication. Secondly, to confirm the specificity of this observed inhibitory effect, and lastly, to determine

the level and possibly the mechanism at which this inhibitory effect was occurring.

Varying Concentrations of Insulin and IGF-1 Inhibit HIV-1 Replication

A frequent but not absolute observance in symptomatic children with AIDS is that their plasma IGF-1 levels are relatively low as compared to asymptomatic, as well as healthy children (Lepage 1991, Schwartz 1991). In addition patients with AIDS associated cachexia have partial resistance to growth hormone and treatment with recombinant IGF-1 produces a transient anabolic effect. Because the effect of GH on normal erythropoiesis appears to be mediated through local IGF-1 production and action (Merchav 1988), one might expect there to be GH resistance in situations in which there is IGF-1 resistance. The disordered metabolism accompanying HIV infection may render the host resistant to the effects of IGF-1.

Clearly their seems to be a relationship between HIV infection and an alteration in IGF-1 plasma levels and action. Figures 4 through 7 show an inhibitory effect of insulin, and more importantly of IGF-1 on HIV-1 replication. The inhibitory effect proceeds in a dose dependent manner. The optimal effect is observed at supraphysiological and physiological concentrations for insulin and IGF-1, respectively. The inhibitory effects of both insulin and IGF-1 are most likely to be occurring via the IGF-1 receptor. This may be

supported in part by the fact that the inhibitory effect for insulin is observed at supraphysiological concentrations and it is well known that insulin can activate the IGF-1 receptor at such elevated concentrations. It should be noted that all concentrations shown to affect the replication of HIV in these experiments were previously observed not to be cytotoxic to cells involved. These findings are important in that both growth hormone and IGF-1 stimulate thymulin secretion by, and proliferation of, thymic epithelial cells. Thus, IGF-1 resistance which has been observed in AIDS patients, could contribute to the immunodeficiency observed.

Inhibitory Effects of IGF-1 on HIV-1 Replication are Specific and May be Abrogated by the Presence of Antibodies Against the IGF-1 Molecule and Receptor

Resistance to IGF-1, GH, and other growth factors has been previously suggested as a potential cause for growth failure in children with AIDS (Schwartz 1991, Laue 1990). Such resistance can result from pre-receptor, receptor binding, or post-receptor-binding abnormalities.

The IGF-1 receptor (IGF-1R) is a member of the tyrosine kinase class of cell surface receptors which become autophosphorylated on tyrosyl residues upon ligand binding (Czech 1989). It has striking homology to the insulin receptor, however, each receptor maintains a unique specificity for its own ligand (Schumacher 1991). The autophoshorylation of the IGF-1R via subunit transphosphorylation is

clearly a necessary requisite for transmission of an intracellular signal, as is also found for the insulin receptor (Sweet 1987, Ulrich 1990). In the case of other growth factor receptors with tyrosine kinase domains such as the EGF and PDGF receptors, receptor activation results in phospholipase C activation leading to 1,2-diacylglycerol and inositol 1,4,5-triphosphate production and a corresponding increase in protein kinase C (pkC) and calcium mobilization, respectively (Berridge 1993).

To demonstrate that the observed inhibitory effects of IGF-1 on HIV-1 replication were indeed specific to IGF-1, we employed the use of specific antibodies. αIR-3 and anti-IGF-1 antibodies specific to the IGF-1 receptor and the IGF-1 molecule, respectively, were introduced in our system in the presence of IGF-1 and HIV chronically infected U937 cells. Our results, as seen in figure 8, indicate that in the presence of 50 ng/ml IGF-1 and in the absence of any antibodies we observed an inhibition of approximately 30%, as compared to the control without any IGF-1 or antibody treatment. In the presence of an antibody specific to the IGF-1 receptor, the percent of inhibition dropped to approximately 10%. More interesting was the effect of administering an antibody specific to the IGF-1 molecule, which actually abrogated the inhibitory effect of IGF-1 above control levels. This is not totally surprising and may be explained by the fact that even our control, which had no added IGF-1, may have contained some background low levels of IGF-1 normally present in the FCS which is supplemented to the culture media. Thus, addition of αIR-3 antibodies not only negated the effect of the administered IGF-1, but also the baseline levels of IGF-1

normally present in FCS supplemented culture media resulting in reverse transcriptase values slightly above those of our control.

Inhibition of HIV-1 Replication by IGF-1 Occurs at the Transcriptional Level

Circulating IGF's, produced predominantly by the liver, traditionally were believed to have primary endocrine roles in growth. It is now clear that these peptides have endocrine actions that include growth promotion as well as insulin-like effects on glucose and fat metabolism (Froesch 1985, Rechler 1990, Sherwin 1994, Zapf 1986). In addition, the production of IGF's in most cells and tissues has led to the hypothesis that the IGF's also act in an autocrine/paracrine fashion to affect cell proliferation and differentiation (LeRoith 1991, 1993). IGF-1 is expressed at low levels embryonically but is induced postnatally in the liver as a result of GH production by the pituitary (Rechler 1990, Streck 1992a, 1992b). In the adult, IGF-1 is found at high levels in the liver, where it is tightly regulated by GH, and at lower levels in most tissues, where its expression is less GH dependent (Rechler 1990).

Previous studies have revealed that the IGF-1 receptor is a tyrosine kinase receptor which upon ligand binding self-phosphorylates and subsequently goes on to phosphorylate other cellular factors which in turn can translocate to the nucleus. On the basis of this observed action, we proposed that the inhibitory effect of IGF-1 may be acting by somehow inhibiting HIV-1 replication at the level of transcription. Indeed, in our system where we employed

COS-7 monkey cells transiently co-transfected with an HIV-LTR-CAT and a pSV-TAT plasmid and exposed to varying levels of IGF-1 concentrations. We found a dramatic effect on transcription as measured by the chloroamphenicol acetyl transferase assay. Figures 9 and 10 both demonstrate that the IGF-1 effect is dose dependent and does occur optimally at normal physiological concentrations. As can be seen in figure 10, an inhibition of approximately 65% occurs in the presence of 10ng/ml of IGF-1 and is further increased to approximately 85% at 50ng/ml IGF-1. These effects were observed in transiently transfected COS-7 cells. In our quest to demonstrate the same effect in a human target cell line, we utilized U38 cells stabily transfected with an HIV-LTR-CAT construct which required only the transient transfection of pSV-TAT in order to achieve transcriptional activity. Though the effects are not as clear in this system, the observed results, as seen in figures 11 and 12 maybe owing to the fact that these cells in particular are not as responsive to the effects of IGF-1 ligand binding, or that the signaling mechanism in these cells is somehow altered. It seems that IGF-1 binding to it's receptor and signaling downstream events is clearly affecting the transcriptional activity of the HIV-LTR in the COS-7 system, but to a lesser extent in the U38 system. It is interesting to note that our results seem to result in a slight paradox in that most agents that stimulate the cellular machinery are usually potent activators of HIV-1 expression. However, one must always keep in mind the unusual complexity of cellular pathways and the potential for intervening effects at multiple levels, both in a stimulatory and

inhibitory manner. Thus, although it is unusual that IGF-1 and insulin seem to inhibit HIV-1 replication, it is not all that surprising. Recently, Sharma et al. 1997 have shown that IGF-1 may actually increase the level of HIV-1 transcription. However, the study by Sharma et al. does not present results in a statistically significant manner, as in our study.

Effect of IGF-1 and TNF-α on HIV-1 Replication

Treatment with human growth hormone (GH) has been shown to improve nitrogen balance in a variety of catabolic states in humans (Wilson 1992). In addition, GH and IGF-1 have a number of immune-stimulating activities (Kelley 1990) and, therefore, may be of immunological as well as nutritional benefit in HIV infected patients.

The mechanism by which IGF-1 resistance develops could be related to increased production of cytokines, which occurs in patients with AIDS. Specifically, TNF-α production is increased in some AIDS patients, particularly in the setting of opportunistic infections, progressive encephalopathy, and wasting (Remick 1991). Whereas IGF-1 has been shown to promote human adult and embryonic erythropoiesis, TNF-α inhibits erythroid colony formation at a concentration of approximately 10 U/ml (Migliaccio 1988). Chronic TNF-α induced inhibition of erythropoiesis has been suggested as a cause for the hypoplastic anemia that occurs in AIDS patients (Odeh 1990).

Due to the observation that IGF-1 levels are decreased, whereas TNF-α levels are increased in some AIDS patients, we set out to determine how the use of these two factors would affect HIV replication independently of one another or in combination, and in which manner. TNF-α upon binding to its receptor is known to activate nuclear factor kappa B (NF-κB). NF-kB, a well known transcriptional activator, enhances the transcriptional level induced by tat. This observation was confirmed in our COS-7 system (figure 13) where transcriptional activation of the HIV-LTR was augmented in the presence of 20 ng/ml of TNF-α. The effects of using both TNF-α and IGF-1 in combination are somewhat less clear in our U38 system (figures 14 and 15). The effects of TNF-α alone are clear in that we observed a slight increase in transcriptional activity, whereas a slight decrease was observed in the presence of IGF-1 alone (fig 15). The application of both factors revealed a slight overall increase in transcriptional activity. This may be explained by the fact that the TNF-α effect is much stronger and over-shadows any effect by IGF-1 in our system. This may have to do with which cellular events are involved post binding of these two factors to there receptors and whether or not these pathways may intersect at some interval.

The most prominent nuclear protein to become tyrosine phosphorylated in response to IGF-1 action is the product of the *c-jun* proto-oncogene. c-Jun is a member of the immediate early gene family so designated because their transcription is activated within minutes of growth factor addition (Sheng 1990). This increase is rapid, transient and independent of new protein synthesis. mRNA for

these components typically are present at low to undetectable levels in quiescent cells. In the nucleus c-Jun, via leucine zipper interaction (Bos 1988) either forms a homodimer with a second molecule of c-Jun or forms a heterodimer with c-Fos. The heterodimeric complex is more stable and as a result more transcriptionally active (Nakabeppu 1988). This functional dimer is referred to as the activator protein-1 (AP-1) complex. Activated AP-1 binds to a specific DNA sequence, the AP-1 binding motif, also called the TRE sequence for TPA Response Element.

The precise mechanism by which IGF-1 hinders the replication of HIV-1 remains to be solved. The results observed in these experiments indicate that the effects of IGF-1 on HIV replication are dose-dependent and occur at the level of transcription. The IGF-1 signalling pathway involved in inhibiting the replication of HIV may intersect with other signalling pathways such as those elicited by TNF-a or even IL-4. Thus, the number of cellular or nuclear proteins involved in the resulting cascade may be numerous, and all may act to somehow alter the level of HIV transcription. It will be interesting and hopefully beneficial to observe the role of IGF-1 in future studies involving HIV replication as well as the role that IGF-1 plays in individuals with AIDS.

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IMAGE EVALUATION
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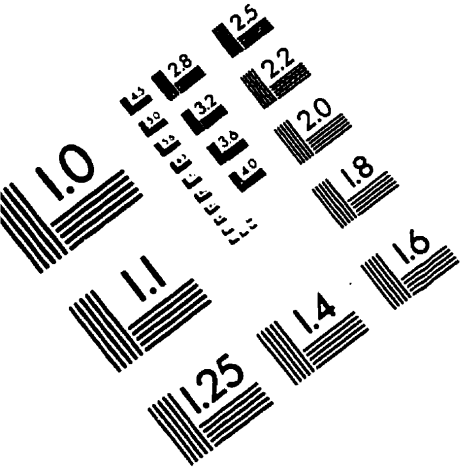

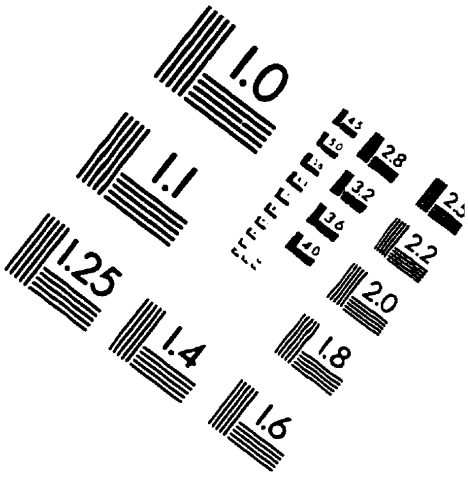

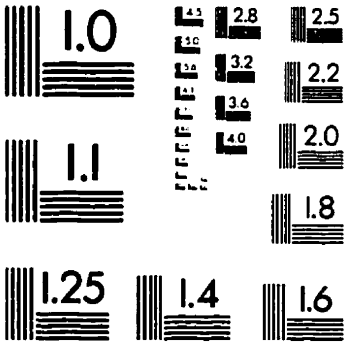

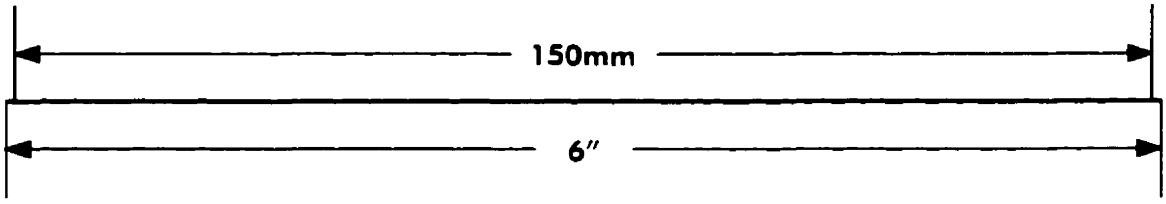

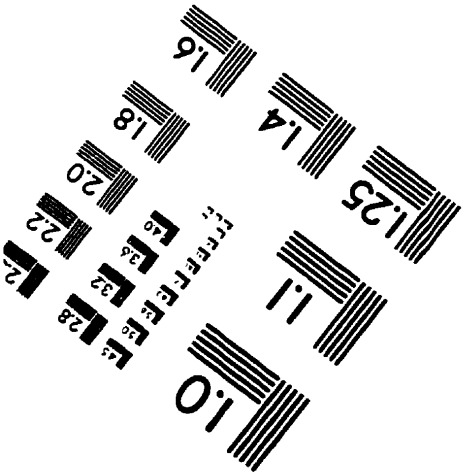

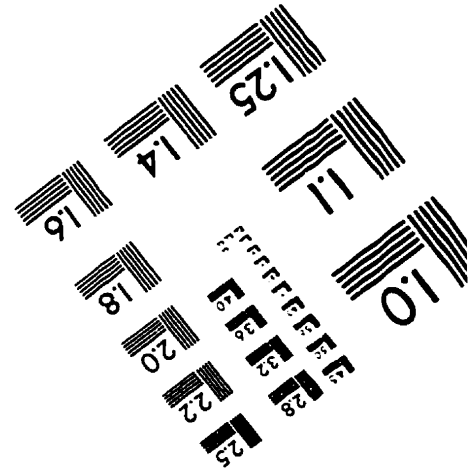

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