HIV SUPPRESSOR FACTORS

HIV SUPPRESSOR FACTORS:
MODULATION OF HIV-1 TRANSCRIPTION AND REPLICATION
BY
HUMAN T LYMPHOCYTES

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Abstract

A variety of host factors influence the ability of Human Immunodeficiency Virus (HIV)-type-1 to access and subsequently replicate within the cellular immune system. Understanding these factors is a crucial step in the development of novel therapeutic strategies including both chemotherapeutic treatments and vaccines. Although it has recently been reported that the CC chemokines RANTES, MIP-1α and MIP-1β are the major HIV-1 suppressive factors derived from $CD8^+$ T lymphocytes, this work demonstrates that these factors are not active at the level of transcriptional control and do not share identity with HIV-1 suppressive factors as measured in a transcriptional control assay. These other remaining factors are produced not only by $CD8^+$ T lymphocytes, but by $CD4^-$ T lymphocytes and cell lines derived from the other major leukocyte subsets. These factors are fractionable by standard chromatographic methodologies, and are active in models of both replication and transcription of laboratory and primary HIV-1 isolates. This work should form the basis for several areas of research related to modulation of HIV-1 replication and transcription.

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List of Abbreviations

AIDS - Acquired Immune Deficiency Syndrome
CAF - Cellular Antiviral Factor
CAT - Chloramphenicol Acetyl Transferase
CD - Cluster of Differentiation
CTL - Cytotoxic T Lymphocyte
ELISA - Enzyme Linked Immunosorbant Assay
FCS - Fetal Calf Serum
G(M)-CSF - Granulocyte (Monocyte) - Colony Stimulating Factor
HIV - Human Immunodeficiency Virus
HPLC - High Performance Liquid Chromatography
HTLV - Human T cell Leukemia Virus
HVS - *Herpesvirus saimiri*
IκB - Inhibitor protein κB
IFN - Interferon
IL - Interleukin
LTR - Long Terminal Repeat
LW - Laboratory Worker
M - Macrophage
MHC - Major Histocompatibility Complex
MIP - Macrophage Inflammatory Protein
NFκB - Nuclear Factor κB
NFAT - Nuclear Factor of Activated T cell
NSI - Non-Syncytium Inducing
OMK - Owl Monkey Kidney
PBL - Peripheral Blood Lymphocyte
PBMC - Peripheral Blood Mononuclear Cell
PHA - Phytohemagglutinin
Pi - PMA and ionomycin
PMA - Phorbol Myristate Acetate
PTx - Pertussis Toxin
RANTES - Regulated on Activation Normal T Expressed and Secreted
RP- Reverse Phase
SDF - Stromal Derived Factor
SEC - Size Exclusion Chromatography
SI - Syncytium Inducing
SIV - Simian Immunodeficiency Virus
T - T cell
TCF - T Cell specific transcription Factor
TcR - T cell Receptor
TGF - Transforming Growth Factor
TNF - Tumour Necrosis Factor

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CHAPTER ONE

GENERAL INTRODUCTION

First identified in the early 1980s, Human Immunodeficiency Virus (HIV)-type-1, continues to present tremendous challenges, both in treatment of the virus itself and the associated Acquired Immune Deficiency Syndrome (AIDS) which usually accompanies its late stages of infection. Recent advances in pharmaceutical strategies to contain HIV have met with considerable success. However, due to both cost and likely poor compliance, elimination of this virus from infected individuals in undeveloped countries is unlikely to occur prior to development of a therapeutic vaccine.

In addition to emphasis placed on development of vaccination strategies, researchers world-wide continue to focus on the basic fundamentals of HIV-1 and its dramatic subversion of the human immune system. Indeed, in just the past few years, several exciting discoveries have prompted the development of new areas of HIV/AIDS research especially with respect to the interaction between the virus and the host leukocytes (white blood cells) which compose the cellular immune system. Although required to act in concert to generate an immune response against any foreign pathogen, the cellular immune system can be generally divided into cells that are targeted for infection by HIV-1 ($CD4^+$ T lymphocytes and macrophage lineage cell types) and those that are not ($CD8^+$ T lymphocytes and B lymphocytes). Historically, this has been credited to the dependence of HIV on the CD4 molecule as a receptor for the HIV envelope proteins gp120 and gp41. Attention of late, however, has been directed at a long-standing phenomenon related to differential susceptibility of $CD4^+$ T lymphocytes, macrophage lineage cell-types, and laboratory T cell lines to different strains of HIV-1. That is, HIV-1 isolated from naturally infected primary lymphocytes (taken from an HIV^+ patient), usually replicates most

efficiently in target cells that are likewise recently isolated (primary $CD4^+$ T lymphocytes and macrophages). These virus isolates are referred to as macrophage-tropic (M-tropic) HIV-1. In contrast, long-term culture of HIV-1 in laboratory T cell lines eventually leads to development of isolates which are found to be progressively incapable of efficiently infecting primary macrophages. Called T cell-tropic viruses, these HIV-1 isolates are restricted to targeting T cell lines and $CD4^+$ T lymphocytes. As will be discussed in this thesis, the molecules responsible for this distinction have recently been demonstrated to be the coreceptors utilized by HIV-1 (in addition to the CD4 molecule) to fuse with the target cell membrane. There are two major groups of these so-called coreceptors which are distinguished by the presence (CXC or alpha) or absence (CC or beta) of an intervening amino acid between the two N-terminal cysteine (denoted by C) residues in their respective chemokine ligands. Although the mechanisms involved in coreceptor utilization by HIV-1 are thus far poorly understood, to infect a target cell most T cell-tropic (T-tropic) viruses require expression of CXC chemokine receptors (eg. CXCR4), and most macrophage-tropic (M-tropic) viruses require expression of CC chemokine receptors (eg. CCR5, CCR3 and CCR2). Completing this story, the expression of CXC chemokine receptors is generally restricted to T cell lines and $CD4^-$ T lymphocytes, and the expression CC chemokine receptors is limited to $CD4^+$ T lymphocytes and macrophage lineage cell types. Thus specificity of T and M-tropic viruses is primarily determined by differential coreceptor expression on T cell lines, $CD4^+$ T lymphocytes and macrophages.

The complex interplay between HIV-1 and host factors is continued upon entry of the virus into the target cell. Once the single-stranded viral RNA genome has been reverse-transcribed into double-stranded DNA, the virus is then able to integrate into the host cell DNA.

From this position, the virus can either remain latent, or through a series of complicated steps hijack the host cell transcriptional machinery and produce progeny virions to perpetuate the infection of the infected individual's cellular immune system. This transcription is controlled by the 5' promoter element of the integrated dsDNA called the Long Terminal Repeat (LTR) which interacts with various host transcription factors and viral proteins.

As both coreceptor-mediated entry and subsequent generation of *de novo* virions present attractive therapeutic targets, many groups have concentrated on delineating weaknesses in these interactions. The research described in this thesis was initiated to examine factor(s) produced by $CD8^+$ T lymphocytes which suppress transcription driven off the HIV-1 5' LTR. This work was complicated by the abovementioned discovery of the relationship between chemokines and HIV replication. The following sections outline previous descriptions of the $CD8^-$ T lymphocyte derived suppressive activity, the role of the CC chemokine receptors and their respective ligands, and the complex transcriptional factors which may be targets for the suppressive activity observed in our assay system.

A. Non-cytolytic control of HIV transcription and replication by $CD8^-$ T lymphocytes

Although $CD8^+$ T lymphocytes are well established for mediating cytotoxic responses against virus infected target cells, and are also able to inhibit hepatitis B virus gene expression in transgenic mice (Guidotti, 1994 and Guidotti, 1996), it was Chris Walker and Jay Levy who first described the ability of this lymphocyte subset to powerfully suppress HIV replication *in vitro*

without eliminating the infected $CD4^+$ T lymphocyte targets (Walker, 1986). Additional work by Walker and Levy demonstrated that this effect is mediated by soluble factors capable of diffusing through a 0.45 micron filter (Walker, 1989), termed Cellular Antiviral Factor (CAF). Now over ten years after the initial report, with tremendous advances made in the understanding of the relationship between HIV and both the cellular and humoral immune systems, a clear and unequivocal identification of the factors responsible for this effect has not been made.

Accordingly, additional considerations with respect to the role of this activity in HIV pathogenesis are important to clarify. These primarily include: i) the relevance of *in vivo* CD8-derived suppression to clinical status; ii) whether or not the activity is a specific immune response elicited by HIV infection, and the corollary of which is whether or not the activity is present in HIV-negative individuals; iii) the specificity of this activity to HIV subtypes and pathogenic variants; and iv) the contribution that the CC chemokines Regulated on Activation of Normal T cell Expressed and Secreted (RANTES), Macrophage Inflammatory Protein (MIP)-1α and MIP-1β described by Cocchi et al.(1995), make to understanding of HIV pathology and the CD8-derived HIV suppressive effect.

CD8-derived suppression and clinical status

Although highly variable from patient to patient, cytotoxic (and humoral) immune responses specific to HIV develop within a few weeks of infection, concomitant with the typical drop in viral load signifying the end of the primary infection stage (Koup, 1994 and Borrow, 1994). Similarly, the emergence of anti-HIV noncytolytic activities in the early stages of primary HIV infection has been described as peaking well before development of neutralizing

antibodies (Mackewicz 1994). Interestingly, in one study (Daar, 1991), dramatic decreases in viral replication as measured by p24 antigenemia and HIV-1 mRNA in peripheral blood mononuclear cells (PBMC), were not matched by changes in viral burden (incidence of proviral HIV-1 DNA per PBMC). Such one sided changes imply that expression of integrated proviral genes is suppressed without removal of the infected source via lytic mechanisms.

If the CD8-derived suppressive activity is initiated in the early stages of HIV infection, there is even stronger evidence that it is maintained for many years following seroconversion (development of measurable specific antibody responses). Several groups have demonstrated anti-HIV responses mediated by either $CD8^+$ T lymphocytes isolated from the peripheral blood of asymptomatic individuals (Landay, 1993, Mackewicz, 1991, Kannagi, 1990, and Gomez, 1994) or from lymph node biopsies (Blackbourn, 1996). This latter result is especially important not only because most immune responses are either generated or maintained in the lymph node, but also because of the pivotal role that this tissue plays in HIV disease. Originally considered to remain latent following the acute viremia of primary infection (Clark, 1991), it is now apparent that HIV seeds the lymph nodes soon after infection, and continues to replicate rapidly without necessarily being detectable in the peripheral blood (Panteleo, 1993 and Embretson, 1993). As described by Panteleo et al., eventually the integrity of the follicular dendritic cell network, required for effective trapping of virus particles is lost (reviewed by Panteleo et al., 1994). This loss appears coincident with the decrease of $CD8^+$ T lymphocytes capable of anti-HIV activity as patients progress to AIDS (Landay et al., and Kannagi et al.). Although lymph nodes may provide an environment which increases suppressive efficiency (in terms of localization of

soluble mediators and target cells), there have been no demonstrations of the requirement for intact lymph nodes to allow for the CD8-derived HIV suppressive response.

Finally, in a cross-sectional study using suppression of transcription as the endpoint assay, our group has reported that anti-HIV-suppressive activity generated by phytohemagglutin (PHA) and Interleukin (IL)-2 stimulation of $CD8^+$ T lymphocytes isolated from HIV^+ patients does not correlate to any clinical markers of disease progression (Copeland, 1997). The obvious discordance with the abovementioned literature may be due to the powerful mitogenic stimulation which may make differences between patient groups difficult to distinguish. Alternatively, as will be discussed in this thesis, the HIV-1 LTR-suppressive factors may be partially or completely distinct from the factors active in other systems which only measure replication.

Elicitation of CD8-derived suppression

Conventional CTL require ligation of the T cell Receptor (TcR) by Major Histocompatibility Complex (MHC) Class I presenting viral peptides to occur prior to degranulation. This activity thus utilizes MHC matching at both the cognitive and effector phases of the response. In contrast, the presence of the CD8-derived factors in culture supernatants, the ability of $CD8^+$ T lymphocytes to suppress HIV replication in heterologous $CD4^+$ T lymphocytes (Mackewicz, 1992), and the inability of anti-Class I antibodies to abrogate the CD8-derived suppressive effect (Brinchmann, 1990) indicates that, at least at the effector phase, the non-cytolytic suppressive activity is non-MHC Class I restricted. However, one of the initial reports by Walker (1989) indicated that direct cell-cell (CD8:CD4) contact was required for maximal

suppression of virus replication. There are several possibilities which could account for this, including the need for costimulation; decreased diffusion of the active mediators; and differential requirements for recognition of MHC Class I-presented virus specific peptides in the cognitive, but not the effector phases (Yang, 1997). This latter report suggested that HIV-negative individuals, who presumably have low circulating levels of precursor $CD8^+$ T lymphocytes specific for HIV-derived peptides, do not mount vigorous non-cytolytic responses to HIV *in vitro*. Indeed, unstimulated $CD8^+$ T lymphocytes isolated from seronegative donors were unable to inhibit replication of HIV-1 in acutely infected autologous $CD4^+$ T lymphocytes (Kannagi et al., Mackewicz, 1991, and Walker, 1991). Nevertheless, when artificially stimulated with anti-TcR/CD3 antibodies (Brinchmann and Røsok, 1997), or PHA (Mackewicz, 1992), $CD8^+$ T lymphocytes from HIV-negative individuals are capable of mediating anti-HIV noncytolytic activities.

Specificity of CD8-derived suppression

There have been no reports of variability of suppression mediated by $CD8^+$ T lymphocytes based on changes of viral tropism (T cell line-tropic vs. macrophage/primary T cell-tropic) which are dictated by non-synonymous sequence alterations within the envelope (*env)* gene (Oravecz, 1996). This should not be surprising considering that although clearly active at blocking replication, CD8-derived suppression is, at least in part, mediated by interference with proviral transcription (Mackewicz 1995, Copeland 1995, 1996). Although the exact mechanism (s) has yet to be delineated, elements including NFAT sites within the HIV-1 LTR are required intact for mediation of the suppressive effect under stimulation with the HIV-1 transactivating

protein Tat (discussed below) (Copeland 1996). However, the signal leading to the suppressive effect does not influence proliferation of the target cells and is active against *trans*-activated promoters from Rous Sarcoma Virus and Human T cell Leukemia Virus type-1 (Copeland, 1995). In addition, the replication of HIV-2 and the related Simian Immunodeficiency Virus (SIV) are modulated by $CD8^+$ T lymphocytes (Kannagi, 1988, and Walker, 1991).

In another SIV model, replication of SIV in acutely and naturally infected $CD4^+$ T lymphocytes from African Green Monkeys were modulated by CD8-derived supernatants (Ennen, 1994). The principle mediator of this effect has subsequently been identified as the simian homolog of Interleukin-16 (IL-16) which also suppresses the replication of HIV-1 (Baier, 1995). Human IL-16 (hIL-16) which binds to the CD4 receptor, is also able to repress the HIV-1 promoter activity (Maciaszek, 1997), but is reported to be distinct from the CD8-derived factors described by Walker and Levy (Mackewicz, 1996).

A recent study examined the role that variances in nucleic acid sequence in the LTR have on reporter gene expression (Estable, 1996). There was considerable variation found in sequence and gene expression-mediated by LTRs cloned from different patients (n=42, 478 LTRs sequenced), and ongoing studies are assessing the effect of these changes on CD8-mediated suppression of gene expression (M. Estable, personal communication).

CC chemokines and CD8-derived suppression of HIV-1

Any discussion of CD8-derived suppressive activities related to HIV pathogenesis would be remiss without mention of the CC chemokines RANTES, MIP-1α and MIP-1β. Prior to December 1995, these low molecular weight (8-14kd) cytokines were known for their

chemotactic effects on $CD4^+$ and $CD8^+$ T lymphocytes (Schall, 1993, and Taub, 1993) and macrophages (Schall, 1990). However, the directions of both chemokine and HIV research changed immeasurably with the publication of a report (Cocchi, et al.) by a group led by Robert Gallo and Paulo Lusso claiming to identify "RANTES, MIP-1α and MIP-1β as the major HIV-suppressive factors produced by $CD8^+$ T cells". This report described the purification of these chemokines, by conventional biochemical techniques (multiple steps of filtration and reverse-phase HPLC), from supernatants of a $CD8^+$ clone which powerfully suppressed replication of macrophage-tropic HIV-1 laboratory isolates. Whether used independently, or in combination, these chemokines were capable of suppressing replication of HIV_{BaL} (macrophage-tropic laboratory isolate) or primary isolates of HIV-1, and laboratory isolates of HIV-2 and SIV. In addition, a combination of antibodies to RANTES, MIP-1α and MIP-1β was able to abrogate the HIV-suppressive effect mediated by the crude supernatant derived from their $CD8^-$ clone. Although the specificity of the CC chemokines for only M-tropic HIV-1 isolates differed from the reported broad activity of the anti-HIV CD8-derived factors, suppression of HIV-1 RNA in treated targets and the low-molecular weight of these molecules was consistent with previous descriptions of the anti-HIV suppressor factor(s) (Copeland, 1995, 1996, and Mackewicz, 1995).

In a seemingly unrelated study, a group led by Ed Berger at the National Institutes of Health, Bethesda, Ma, identified Fusin (renamed CXCR4), a seven-transmembrane domain protein responsible for fusion of T-tropic strains of HIV-1 (Feng, 1996). The sequence of this coreceptor was noted for its homology to the receptor for Interleukin-8 (IL-8r) which is a CXC chemokine, and subsequent identification of natural ligand of CXCR4 yielded Stromal Derived Factor (SDF)-1 (Oberlin, 1996), a CXC chemokine. Recognizing the importance of this

discovery and noting the suppressive effect mediated by the CC chemokines, several groups (including the Berger group), postulated that the CC chemokines were mediating their inhibitory effect by binding to their respective receptors. Indeed, in studies utilizing cell fusion-reporter gene assays, the CC chemokine receptors CCR5 (Deng, 1996, Choe 1996, Dragic, 1996, Alkhatib, 1996, and Doranz, 1996), CCR3 (Choe et al. and Doranz et al.), and CCR2b (Doranz et al.), were all identified as being coreceptors for M-tropic isolates of HIV-1. Although some of the data for CCR3 and CCR2b conflicted between these studies, CCR5 and Fusin have been accepted as having primary responsibility for interacting with CD4 and gp120 / gp41 to mediate fusion of most primary isolates of HIV-1 (Simmons, 1996). In addition, CCR5 may be especially important for establishment of infection in vivo, because individuals homozyogous for a 32 base-pair deletion appear to be resistant to sexually transmitted HIV (Liu, 1996, Huang, 1996, Samson, 1996, and Dean, 1996). This homozygous deletion causes premature termination of translation, which produces a protein lacking the last three transmembrane domains. This protein is not expressed on the cell surface, and thus is unable to interact with CD4 and gp120/gp41 (Liu et al.). In Caucasian populations, approximately 1-2% have this homozygous deletion, and 10-11% have the heterozygous deletion (Samson et al. and Dean et al.).

To mimic this natural resistance[1], attempts have been made to synthesize altered versions of RANTES capable of binding to CCR5 and preventing envelope-mediated fusion (Arenzana-Seisdedos, 1996 and Simmons, 1997), but without transducing activation signals in the form of Ca^{+2} fluxes (Turner, 1995). Although this avenue of investigation may eventually successfully

[1] There has been one report of HIV-1 infection in an individual with the homozygous 32-bp deletion (Biti, 1997)

produce powerful and specific therapeutics, the claim that the CC chemokines share identity with the CD8-derived factors first described by Walker and Levy in 1986 has since been disputed (Mackewicz, 1996, Palliard, 1996, and Rubbert, 1997).

B. Regulation of HIV-1 gene expression

The regulation of HIV-1 gene expression is mediated by complex interactions of *cis*-acting elements within the 5' LTR with cellular transcription factors (such as NFAT and NFκB), *trans*-acting factors such as the products of the *tat* gene and exogenously provided cytokines and other factors which may alter the activation state or environment of the host cell. Although many of these interactions are incompletely elucidated, or contested by different researchers, the basic mechanisms are well described. As this thesis is concerned with exogenous factors which regulate HIV-1 gene expression, a brief review of the key interactions and mechanistic relationships is important.

Nuclear Factor of Activated T cells (NFAT)

NFAT is a cellular transcription factor responsible for regulation of T lymphocyte activation by regulation of the Interleukin-2 (IL-2) gene (Crabtree, 1989). Two forms of NFAT are distinguishable: the cytoplasmic portion, NFATp which, in a phosphorylated state is unable to enter the nucleus; and the nuclear component, NFATn has been reported to be a dimer of cJun and cFos proteins which have a basic leucine zipper structure which allows for efficient dimer formation and DNA binding (McAffrey, 1993). Upon T cell activation, calcineurin, calcium-

dependent phosphatase is thought to dephosphorylate NFATp (also referred to as NFATc) , which then moves to the nucleus, and with NFATn binds to the NFAT element (Rao, 1994). Recognizing that sequences within the 5' HIV-1 LTR have significant homology to the NFAT sites in the IL-2 promoter, several researchers have attempted to prove that the NFAT proteins bind to this site and activate transcription driven off the HIV-1 LTR (Shaw, 1988, Greene, 1990, Lu, 1990 and Tong, 1990). Although DNase footprinting studies indicated that there are indeed binding sites for NFAT proteins in the HIV-1 LTR (Shaw, 1988) reports of a functional effect mediated by this interaction are controversial. Indeed, a recent report (Kinoshita, 1997) described the surprising activation of NFAT deletion mutants by NFATc provided in *trans*. Although NFATc was not active through non-HIV-1 LTR-derived consensus κB-elements (from immunoglobulin NFκB binding sites), DNase footprinting demonstrated that NFκB elements within the LTR are bound by NFATc and that this binding is dependent on additional elements adjacent to these sites. These sites were reported to be distinct from those occupied by the NFκB family of proteins. Thus, activation of the HIV-1 LTR can occur through mobilization of NFATc which then binds to a site relatively distant from the LTR sequences homologous to the NFAT binding sites in the IL-2 promoter.

Nuclear Factor κ B (NFκB)

The enhancer elements of the HIV-1 LTR, particularly the two NFκB 10bp sequences are well established in their role in HIV-1 gene expression. There are several different cellular transcription factors which bind to the NFκB sites, however most are in the *rel* family which are related to the cellular oncogene *v-rel* (reviewed by Gaynor, 1992). These proteins, particularly

the 50 and 65kd proteins (p50 and p65, respectively) form a stable dimer capable of binding strongly to the NFκB motifs (Ruben, 1991). Although originally recognized for their ability to regulate basal transcription of HIV-1 genes in the absence of *trans*-activation by viral Tat in activated $CD4^+$ T lymphocytes (Nabel, 1987), the interactions that lead to NFκB binding have become considerably complicated. The ability of the complex of NFκB proteins p65 and p50 to translocate to the nucleus is tightly controlled by its sequestration by Inhibitor protein κB (IκB). With respect to T cell activation, the phosphorylation state of IκB is regulated by a Protein Kinase C dependent kinase (Baeuerle, 1988, and Urban, 1990). However, phosphorylation of IκB is not the only mechanism that modulates the interaction with the NFκB proteins. Indeed, reactive oxygen species (Screck, 1991) and the HIV protease (Riviere, 1991) are able to degrade IκB which releases the p50/p65 complex. Thus, altering the levels of free radicals or proteases in the cellular environment could alter the activation state of the HIV-1 LTR.

Although binding of NFκB proteins leads to low levels of basal transcription, the HIV-1 LTR also has 3 adjacent SP-1 sites which are required intact for PMA-stimulated transcription (Li, 1994, and Perkins, 1993). In addition, the viral protein Vpr (a non-essential gene product) is also able to interact with the NFκB/SP-1 sites and *trans*-activate LTR-mediated gene expression (Wang, 1995). These reports are preliminary, however they do serve to illustrate the complicated interplay between NFκB cis-acting elements, and viral and host factors. Interestingly, the claimed absolute dependence of HIV-1 transcription on intact NFκB elements (Alcami, 1995) has recently been disputed by the identification of an HIV^+ patient who has replication competent HIV-1 which lacks NFκB binding sites (Zhang, 1997) in the 5' LTR. This patient did not demonstrate any clinical abnormalities as would be suggested by this attenuation, however there

was a duplication of the upstream T Cell-specific transcription Factor (TCF)-1 element. Although the function of the TCF-1 element is poorly characterized, this group has since described several so-called long-term survivors who also have a duplication of this element in primary isolate 5' LTRs (yet the NFκB sites are intact). Even though this phenomena requires additional clarification, the functional redundancy of the mechanisms which lead to transcription of HIV-1 genes cannot be over-emphasized.

Tat, TAR and the TATA box

The *cis* and *trans*-interactions of the TATA box, TAR RNA transcripts, and viral Tat which lead to stabilization of RNA polymerase II at the transcriptional start site, are best considered together. Mutagenesis studies have indicated that the integrity of the TATA box is crucial for both basal and Tat-induced transcription (Berkhout, 1992). This is most likely because the interactions of a cellular transcription factor, called TFIID, and RNA polymerase transcription factors are stabilized by the TATA element (Buratowski, 1989). This stabilization is sufficient to initiate basal transcription, including transcription of the TAR sequences (which are not subsequently translated). However, in absence of Tat, transcription pauses at the end of the TAR RNA sequences. All HIV genes have TAR sequences extending from +1 to +60, which when transcribed into nascent mRNA transcripts, form stem-loop structures which have distinct binding sites for viral Tat (Muesing, 1987) and a constitutively produced cellular transcription factor called TRP-185 (Sheline, 1991).

When both Tat and TRP-185 bind to TAR RNA, the binding of RNA polymerase II to the TAR RNA is altered, presumably allowing it to continue transcribing the full-length

transcript beyond the untranslated TAR RNA element (Wu-Baer, 1995).Whether provided in an autocrine or paracrine manner, Tat mediates from 10-50 fold enhancements of basal transcription (Verhoef, 1996). A recent report has also indicated elongation rates may also be increased by Tat directly phosphorylating RNA polymerase II (Parada, 1996). In these ways, Tat, in the presence of the TATA element, and nascent TAR RNA transcripts, stimulates not only increased rates of transcriptional initiation, but also increases elongation of HIV-1 transcripts (Laspia, 1989, Feinberg, 1991, and Marciniak, 1990).

Finally, as mentioned above, reactive oxygen species can increase IκB degradation and thus increase NFκB nuclear translocation. Interestingly, a recent study has documented the ability of Tat to also activate NFκB, an effect which is blocked in anti-oxidant conditions (Demarchi, 1996). Thus Tat may have alternative mechanisms for controlling HIV-1 transcription in addition to influencing transcriptional elongation or initiation.

Thesis Organization and Objectives

As described above, $CD8^+$ T lymphocytes have a well-established ability to produce soluble mediators (or factors) capable of suppressing HIV-1 transcription and replication. This thesis examines the hypotheses that these factors are distinct from the CC chemokines RANTES, MIP-1α and MIP-1β, that they are produced by other lymphocyte subsets, and that the active mediators are fractionable by standard biochemical purification methodologies.

The work described in Chapter 2 resulted from attempts to confirm recent discoveries relating to the identity of these factors and shows that the $CD8^+$ factors active in our system are distinct from the CC chemokines RANTES, MIP-1α and MIP-1β. This work was published in the journal *AIDS* in May of 1997. The work in Chapter 3, which has been submitted to *The Journal of Immunology*, documents the ability of the $CD4^+$ T lymphocyte subset to produce factors which suppress HIV-1 LTR-mediated gene expression in T cells, but enhance it in monocyte-lineage cell lines. Preliminary observations resulting from standard chromatographic purification of $CD8^+$ T lymphocyte-derived supernatants are outlined in Chapter 4. This chapter also contains several important preliminary observations suggestive of further areas of research. The key observations arising from these studies and their implications are then discussed in Chapter 5.

CHAPTER TWO

CD8+ T CELL-MEDIATIED SUPPRESSION OF HIV-1 LONG TERMINAL REPEAT-DRIVEN GENE EXPRESSION IS NOT MODULATED BY THE CC CHEMOKINES RANTES, MACROPHAGE INFLAMMATORY PROTEIN (MIP)-1α AND MIP-1β

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Preface

The work described in this chapter has been published in the journal *AIDS* in May of 1997, and demonstrates that the CC chemokines RANTES, MIP-1α and MIP-1β are not responsible for the $CD8^+$ T lymphocyte-derived suppression of HIV-1 transcription. This paper, and the experiments it describes were all completed by myself. Dr. K.F.T. Copeland was responsible for developing the HIV-1 LTR CAT reporter gene assay system in our laboratory and Paula McKay established the HVS-transformed $CD8^+$ T lymphocyte clone used in some of the experiments.

CD8+ T-cell-mediated suppression of HIV-1 long terminal repeat-driven gene expression is not modulated by the CC chemokines RANTES, macrophage inflammatory protein (MIP)-1α and MIP-1β

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Objective: To assess the role of RANTES, macrophage inflammatory protein (MIP)-1α and MIP-1β in modulation of HIV-1 long terminal repeat (LTR)-mediated gene expression and determine whether these chemokines share identity with CD8+ T-lymphocyte-derived HIV-1 LTR-suppressive factors.

Design: HIV-1 LTR-directed reporter gene expression is a model for transcription that is susceptible to inhibition by factors produced by CD8+ lymphocytes of HIV-1-infected individuals. The effect of recombinant chemokines on LTR-directed gene expression was examined. The ability of chemokines found to be present in CD8 supernatants to suppress HIV-1 LTR-mediated gene expression was determined by antibody inhibition assays.

Methods: The concentrations of RANTES, MIP-1α and MIP-1β in a panel of CD8+ T-lymphocyte-derived supernatants were determined by enzyme-linked immunosorbent assay. Recombinant chemokines were added to freshly transfected (pLTR-CAT and pSV40-*tat*) human Jurkat T cells. Excessive polyclonal neutralizing antibodies to these chemokines were added to transfected Jurkat T cells cultured in the presence of strongly inhibitory CD8+ T-cell-derived supernatants with known chemokine concentrations.

Results: The concentrations of RANTES, MIP-1α and MIP-1β in a panel of CD8+ lymphocyte-derived supernatants were found to correlate with their relative ability to suppress the LTR-mediated gene expression ($r = 0.679$, 0.764 and 0.48, respectively). The addition of recombinant CC chemokines had no effect over a broad range of doses on HIV-1 LTR-mediated gene expression. The CD8-suppressive effect on HIV-1 LTR-driven gene expression was not abrogated by a combination of antibodies to RANTES, MIP-1α and MIP-1β.

Conclusions: RANTES, MIP-1α and MIP-1β do not alter HIV-1 LTR-directed gene expression at doses up to 100 ng/ml. Although present in varying concentrations in supernatants derived from CD8+ lymphocytes from HIV-positive individuals, these chemokines are not responsible for the powerful CD8-derived suppressive effect on HIV-1 LTR-mediated gene expression observed in our system.

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Keywords: CD8, cellular factors, cytokines, cellular immunity, chemokines

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Introduction

Activated CD8+ T lymphocytes from HIV-positive patients are capable of suppressing replication of HIV-1 in CD4+ T lymphocytes through a non-cytolytic mechanism [1,2]. Although enhanced in ability by direct cell-to-cell contact, this suppression is mediated by soluble factors, and has been found to be active against related lentiviruses including HIV-2 and simian immunodeficiency virus [3–6]. Several groups have demonstrated that the suppression of HIV-1 replication occurs prior to mRNA transcription and that this effect is mediated via the long terminal repeat (LTR) [5,7–9]. We have demonstrated that CD8+ T-lymphocyte-mediated suppression of HIV-1 transcription is dependent on the integrity of both the nuclear factors NFκB and NFAT elements of the HIV-1 LTR [10,11].

A recent report has demonstrated that replication of HIV_{BAL} [macrophage-tropic non-syncytium-inducing (NSI) virus] in a human T-cell line *in vitro* can be strongly inhibited by RANTES, MIP-1α and MIP-1β [12]. These members of the β-chemokine family are major chemotactic molecules for CD4+ T lymphocytes, CD8+ T lymphocytes and macrophages [13–16]. Cell-surface receptors for these chemokines (CC chemokine receptors CCR5, CCR3 and CCR2b) have recently been identified as coreceptors on CD4+ T lymphocytes for infection by NSI and macrophage-tropic strains of HIV-1 [17,18], and primary isolates [19–21]. In addition, fusin, a molecule with homology to the CXC chemokine receptor, interleukin (IL)-8 receptor, has been described as necessary for infection of human CD4+ lymphocytes with syncytium-inducing T-cell-tropic laboratory strains of HIV-1 [22]. Taken together, these results suggest that CD8-derived chemokines may suppress *de novo* infection of CD4+ T lymphocytes via steric interaction with the HIV coreceptor and gp120/gp41.

We have previously described the ability of CD8+ T-cell-derived factors to suppress HIV-1 transcription using transient transfection of the human Jurkat T-cell line with HIV-1 LTR driving chloramphenicol acetyl transferase (CAT) [10,11,23]. To determine whether RANTES, MIP-1α and MIP-1β are responsible for the suppression of HIV-1 transcription observed in our system, we first compared the concentrations of the CC chemokines to the relative suppressive ability of a battery of CD8+ T-lymphocyte-derived supernatants. We then attempted to replicate the CC chemokine-mediated suppression observed by Cocchi *et al.* [12] by supplementing the culture media of the HIV-1 LTR-transfected Jurkat cells with comparable concentrations of recombinant RANTES, MIP-1α and MIP-1β. Finally, using polyclonal neutralizing antibodies, we attempted to block the suppressive effect of cell culture supernatants from both a *Herpesvirus saimiri* (HVS)-transformed CD8+ T-lymphocyte clone and a suppressive CD8+ supernatant.

Materials and methods

Subjects

HIV-1-infected asymptomatic individuals were referred by Dr F. Smaill at the McMaster Medical Centre Special Immunology Services Clinic (Hamilton, Ontario, Canada) and by Dr S. Walmsley at the Immunodeficiency Clinic of the Toronto General Hospital (Toronto, Ontario, Canada). Ethics approval for these studies was conferred by McMaster University Health Science Centre and the University of Toronto.

CD8+ T-cell cultures and supernatants

CD8+ T lymphocytes were isolated from the heparinized blood of HIV-positive patients and HIV-negative controls using Ficoll-Paque density gradients followed by positive selection using anti-CD8 immunomagnetic beads (Miltenyi, Auburn, California, USA). CD8+ T lymphocytes were then cultured for 3–4 days in RPMI-1640 medium (Canadian Life Technologies, Burlington, Ontario, Canada) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml phytohemagglutinin (PHA) and 20 U/ml IL-2 at an initial concentration of 1×10^6 cells/ml. The cells were then washed twice and resuspended in the same medium but lacking PHA and recultured for 3–4 days. Supernatants were collected and stored at −70°C.

Generation of HVS-transformed CD8+ T-cell clones and supernatants

HVS stock 488 (kindly provided by Dr R. Desrosiers, New England Regional Primate Research Center, Southborough, Massachusetts, USA) was prepared in owl monkey kidney cells as previously described [24]. Several HVS-transformed CD8+ T-lymphocyte clones were generated as previously described [10]. Clone 3-14, previously shown to potently suppress HIV-1 LTR-mediated transcription [10,11] was maintained in RPMI-1640 containing 20% FCS, 20 U/ml IL-2, 100 U/ml penicillin and 100 μg/ml streptomycin. Supernatants were removed and stored at −70°C.

Transfections and vectors

The vector pLTR-CAT incorporates the HIV-1 LTR of the BRU strain to position +77 driving CAT [25]. pSV40-*tat* (SV40 promoter driving HIV-1 *tat*) [26] was used to enhance HIV-1 transcription to a level that has the potential to be clearly inhibited. The human Jurkat T-cell line (30×10^6) cultured in RPMI-1640 (supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin), was transfected with 10 μg pLTR-CAT and 5 μg pSV40-*tat* using a diethy-

laminoethyl dextran procedure [27]. Transfected cells were cultured in CD8+ T-cell supernatant and medium for 24 h, then stimulated with phorbol myristate acetate (25 ng/ml) and ionomycin (2 μM) for 18 h. The cells were then lysed by repeated freeze/thaw cycles (3×). Lysates standardized for protein concentration were then assayed for the presence of CAT by enzyme-linked immunosorbent assay (ELISA; Boehringer Mannheim, Montreal, Quebec, Canada).

Chemokine ELISA

The CC chemokines RANTES, MIP-1α and MIP-1β were detected in CD8+ T-lymphocyte-derived culture supernatants using ELISA (R&D Systems, Minneapolis, Minnesota, USA).

Recombinant chemokines

Purified recombinant chemokines, including human RANTES, MIP-1α and MIP-1β, were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. The chemokines were added to the Jurkat cells immediately following transfection, to yield final concentrations in the range of 1–100 ng/ml.

Neutralizing antibodies

Polyclonal goat anti-human IgG neutralizing antibodies to RANTES, MIP-1α and MIP-1β were obtained from R&D Systems. Prior to addition to the transfected cells, the CD8+ T-lymphocyte supernatants were incubated with the antibodies for 1 h at either room temperature or 37°C, as indicated.

Results

RANTES, MIP-1α and MIP-1β are present in CD8+ T-lymphocyte-derived culture supernatants

The concentrations of RANTES, MIP-1α and MIP-1β were measured by ELISA in a battery of supernatants from CD8+ T lymphocytes derived from asymptomatic HIV-infected subjects (CD4 count 350×10^6/l). Table 1 shows the range of suppression of HIV-1 LTR-mediated gene expression by these supernatants and their corresponding chemokine concentrations. Correlations between the concentrations of RANTES, MIP-1α and MIP-1β and percentage suppression by patient CD8+ T-lymphocyte supernatants (supernatants from clone 3-14 excluded) varied considerably ($r = 0.679$, 0.764 and 0.48, respectively). Supernatant from the HVS-transformed clone 3-14, which inhibited 100% (at 1 : 1 dilution with medium) in comparison to media only control, was found to contain 16, 2.4 and 13 ng/ml of RANTES, MIP-1α and MIP-1β, respectively.

Table 1. Level of long terminal repeat (LTR)–chloramphenicol acetyl transferase (CAT) suppression and concentration of CC chemokines in CD8+ lymphocyte supernatants.

		ng/ml		
Patient	% Suppression*	MIP-1α	MIP-1β	RANTES
1	8.9	0.4	0.6	7.0
2	18	1.0	2.6	6.0
3	24	2.1	4.5	5.0
4	39	0.7	2.1	1.0
5	58	5.5	61.0	17.0
6	61	3.5	8.0	8.0
7	66	12.5	10.8	17.0
8	66	6.1	17.0	14.0
Clone 3-14	100	16.0	2.4	13.0

*Level of suppression is given as percentage suppression from media only control. MIP, Macrophage inflammatory protein.

RANTES, MIP-1α and MIP-1β do not inhibit HIV-1 LTR-mediated gene expression

To determine whether the CC chemokines were capable of modulating HIV-1 LTR-driven gene expression, recombinant RANTES, MIP-1α and MIP-1β were added to freshly transfected Jurkat T cells in doses ranging from 1 to 100 ng/ml (Fig. 1). At all doses, each individual CC chemokine had no effect on the level of LTR-mediated gene expression. A triple combination of chemokines (100 ng/ml each) was able to effect a moderate decrease of 37% relative to control. In a separate experiment, combinations of all three chemokines in equal doses ranging from 10 to 200 ng/ml did not suppress HIV-1 LTR-mediated gene expression relative to medium only control (data not shown). In comparison, the supernatant from the HVS-transformed CD8+ T-lymphocyte clone 3-14 (diluted 1 : 5 with media) was able to suppress reporter gene expression 75% (in other experiments 1 : 1 dilution yielded 100% suppression), even though the concentrations of RANTES, MIP-1α and MIP-1β in this culture supernatant (Table 1) were relatively low compared with the concentrations of the recombinant chemokines used in these experiments.

CD8 suppression of HIV-1 LTR-mediated gene expression is not abrogated by anti-chemokine antibodies

To confirm that the CC chemokines are not responsible for CD8+ lymphocyte-mediated suppression of HIV-1 LTR-driven gene expression, inhibitory supernatants from both CD8+ T-lymphocyte cultures from an HIV-positive patient, and HVS-transformed CD8+ T-cell clone 3-14 were treated with polyclonal goat anti-human IgG antibodies to RANTES, MIP-1α and MIP-1β (at 200, 50 and 100 μg/ml, respectively). In two separate experiments, excessive anti-chemokine antibodies were unable to abrogate the inhibition of HIV-1 LTR-driven CAT expression (Fig. 2).

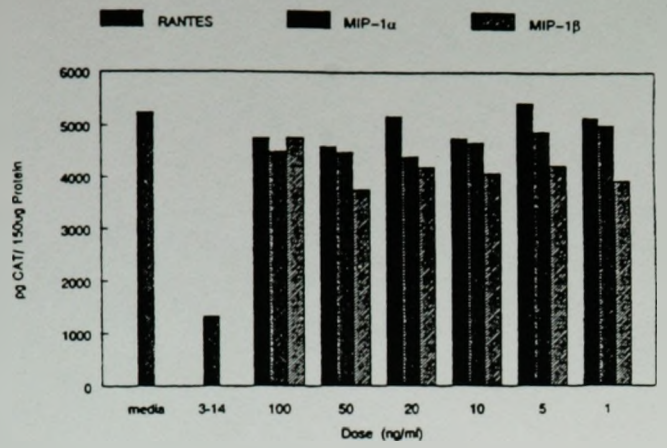

Fig. 1. CC chemokines do not affect long terminal repeat (LTR)-mediated reporter gene expression. Recombinant RANTES, macrophage inflammatory protein (MIP)-1α and MIP-1β were added to Jurkat T cells cultured in RPMI-1640 plus 10% fetal calf serum (FCS), immediately following transfection with pLTR-CAT and pSV40-*tat*. Positive control supernatant from CD8+ clone 3-14 was diluted 1 : 5 with medium (RPMI plus 10% FCS). All sample values are averages of duplicates, and this experiment is representative of two separate experiments. CAT, Chloramphenicol acetyl transferase.

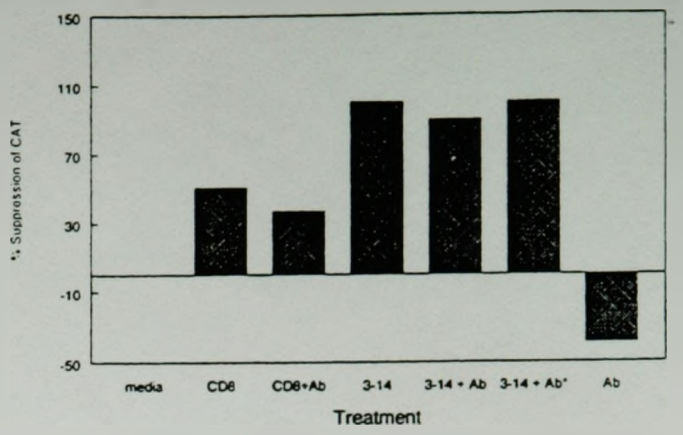

Fig. 2. Neutralizing antibodies (Ab) to CC chemokines are unable to abrogate suppression of long terminal repeat (LTR)-mediated reporter gene expression. Addition of the combination of polyclonal goat anti-human neutralizing Ab to RANTES, macrophage inflammatory protein (MIP)-1α and MIP-1β (200, 50 and 100 μg/ml, respectively) to Jurkat T cells cultured in equal parts inhibitory supernatant and RPMI plus 10% fetal calf serum, or to control media alone. Supernatants from CD8+ lymphocytes and clone 3-14 were incubated with anti-chemokine Ab for 1 h prior to addition to Jurkat cells, at room temperature or *37°C, as indicated. All sample values are averages of duplicates, and this experiment is representative of two separate experiments. CAT, Chloramphenicol acetyl transferase.

Discussion

The ability of CD8+ T lymphocytes from HIV-positive individuals to strongly suppress HIV replication *in vitro* via a non-cytolytic mechanism has been extensively studied [28]. Although direct cell-to-cell contact results in more efficient suppression, this mechanism is mediated at least in part by soluble factors [2]. These soluble factors presumably have the potential to act at any stage of the life cycle of HIV-1 to inhibit replication. We and others have reported that the suppression of replication of HIV-1 by CD8+ T-lymphocyte-derived factors occurs at the level of transcription [5,7–11].

Recently, Cocchi *et al.* [12] described the ability of the CC chemokines RANTES, MIP-1α and MIP-1β to inhibit replication of the macrophage-tropic NSI HIV-1_{BAL} strain in a human T-cell line. Indeed, here we report that these CC chemokines are present in varying concentrations in the supernatants of cultured CD8+ T lymphocytes from HIV-positive patients (Table 1). Statistical analysis comparing the ability of CD8-derived supernatants to suppress HIV-1 LTR-driven gene expression and the concentration of these chemokines to their respective ability to suppress yielded moderate-to-strong correlations (RANTES, $r = 0.679$; MIP-1α, $r = 0.764$; MIP-1β, $r = 0.48$). However, since the ability of CD8+ T lymphocytes isolated from different HIV-positive patients to produce HIV-1 inhibitory factors is dependent on *in vitro* activation in our system, the correlative production of chemokines may be reflective of the overall activated state of the cell population rather than a modulatory effect on the HIV-1 LTR. An alternative hypothesis is that specific transcriptional control mechanisms that regulate the production of the LTR-suppressive factors are shared with the CC chemokines. Thus, high levels of chemokines in CD8+ cell supernatants may only be representative of the presence of other inhibitory factors detectable by their inhibition of LTR-mediated gene expression.

To examine the LTR-suppressive potential of RANTES, MIP-1α and MIP-1β present in supernatants derived from CD8+ T lymphocytes, we first tested the effect of the recombinant CC chemokines on HIV-1 LTR-driven gene expression. In three separate experiments, in combination and at high doses, there were no observable effects of these chemokines on HIV-1 LTR-CAT expression (Fig. 1). In comparison, the control supernatant from HVS-transformed CD8 clone 3-14, diluted 1 : 5, was able to powerfully inhibit CAT expression even though the concentrations of RANTES, MIP-1α and MIP-1β in this supernatant (tested neat) were only 13, 16 and 2.4 ng/ml, respectively. Thus, even when diluted, factors present in this supernatant were able to inhibit expression of the HIV-1 LTR, whereas high concentrations of recombinant RANTES, MIP-1α or MIP-1β cannot.

The lack of a suppressive role for the CC chemokines in our system was further confirmed by the observa-

tions that polyclonal neutralizing antibodies to human RANTES, MIP-1α and MIP-1β (200, 50 and 100 μg/ml, respectively) are unable to abrogate LTR suppression mediated by supernatant from cultured CD8+ T lymphocytes from an HIV-positive patient and from CD8+ clone 3-14. The fact that the CC chemokines RANTES, MIP-1α and MIP-1β do not block HIV-1 LTR-mediated gene expression in our system, although they are able to inhibit the replication of a macrophage-tropic HIV-1 strain [12], should not be surprising considering the range of possible targets in the HIV-1 life cycle. Understanding mechanisms that block both viral entry and activation of proviral transcription is especially important considering the recent identifications of coreceptors for HIV-1 entry into the host cell. If the CC chemokines do indeed act at this level by binding to their respective receptors, and thereby preventing cofactor-assisted fusion, there is obvious hope for therapeutic use to prevent *de novo* infection of CD4+ lymphocytes expressing the appropriate chemokine receptor. However, the remaining CD8-derived factors should not be discounted, most importantly because as we have shown, unlike the CC chemokines RANTES, MIP-1α and MIP-1β, they exert their effects at the level of transcriptional control. Intracellular events leading to both accelerated virion production in primary infection and reactivation of latent infection seen late in HIV disease are therefore likely targets for these CD8-derived factors. One can foresee the potential role of these factors in combination with chemokines, nucleoside analogues and protease inhibitors in immunochemotherapies designed to block viral entry, reverse transcription, proviral transcription and finally cleavage and assembly of new viral particles. To this end, we are examining the biochemical characteristics and intracellular effects of the CD8-derived factors in hope that these powerful, but elusive, molecules may yet be identified.

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Note added in proof

Since the submission of this manuscript, we have become aware that other groups have demonstrated that the CC chemokines RANTES, MIP-1α and MIP-1β were not responsible for suppression of HIV replication mediated by both CD8+ T-cell supernatants from HIV-infected asymptomatic individuals and CD8+ T-cell clones.

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CHAPTER THREE

CD4-DERIVED SUPPRESSIVE ACTIVITY: EVIDENCE FOR AUTOCRINE NON-CYTOLYTIC CONTROL OF HIV-1 TRANSCRIPTION AND REPLICATION

Leith, J., K.F.T. Copeland, P.J. McKay, D.B. Bienzle, C.D. Richards and K.L. Rosenthal submitted to *The Journal of Immunology*, September 1997.

Preface

The contents of this chapter represent a collaborative effort between myself and Dr. K.F.T. Copeland, and have recently been submitted to *The Journal of Immunology*. This paper reports that $CD4^+$ T lymphocytes share the well-established ability of $CD8^+$ T lymphocytes to suppress HIV-1 transcription and replication. I was responsible for all experiments using T lymphocytes as an endpoint assay system, with the exclusion of the experiments reporting CD4-derived suppression of NFκB-mediated gene expression. All experiments involving monocyte-lineage cell lines were developed and carried out by Dr. Copeland. In addition, Dr. D. Bienzle developed the HVS-transformed $CD4^+$ T lymphocyte clone used in these experiments. Paula McKay provided invaluable assistance with HIV-1 replication assays and flow-cytometric analysis.

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CD4-Derived Suppressive Activity: Evidence for Autocrine Non-Cytolytic Control of HIV-1 Transcription and Replication

Running Title: CD4+ T Cell-derived HIV-1 Suppressive Factors

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Abstract

We previously demonstrated that soluble factors from a *Herpesvirus saimiri* (HVS)-transformed human $CD8^+$ T cell clone and $CD8^+$ T lymphocytes from HIV-infected individuals suppress HIV-1 Long Terminal Repeat (LTR)-mediated gene expression in $CD4^+$ T cells and enhance HIV-1 LTR-mediated gene expression in monocytic cells. Here we demonstrate that soluble factors produced by $CD4^+$ T lymphocytes mediate similar dichotomous effects on HIV-1 transcription and replication in T cells and monocytes. These effects were dose-dependent and mediated, at least in part, through the NFκB elements of the HIV-1 LTR. A panel of supernatants from $CD4^+$, $CD8^+$ and unfractionated peripheral blood lymphocytes (PBL) from both HIV-infected and uninfected individuals generated these activities, indicating that *in vivo* priming with HIV-1 was not necessary for production of these HIV-1 LTR-modulatory factors. Using $CD4^+$ Jurkat T cells infected with primary or laboratory strains of HIV-1 and transfected with an LTR-reporter gene construct, we show that suppression of virus replication occurred concomitant with suppression of HIV-1 LTR-mediated gene expression. Similarly, supernatants derived from $CD4^+$ and $CD8^+$ T lymphocytes enhanced both HIV-1 replication and LTR-mediated gene expression in U1 monocytes. Interestingly, enhancements induced by $CD8^+$, but not $CD4^+$ T lymphocyte-derived factors were pertussis toxin sensitive and associated with a significant induction of TNF-α production. Our results demonstrate that factors produced by both $CD4^+$ and $CD8^+$ T lymphocytes can regulate HIV-1 LTR-mediated gene expression and virus replication. $CD4^+$ T lymphocyte-derived autocrine regulation of HIV-1 is a powerful control mechanism with relevance to our understanding of HIV pathogenesis.

Introduction

A variety of host factors influence the ability of human immunodeficiency virus type 1 (HIV-1) to infect and replicate within $CD4^+$ T lymphocytes and macrophage lineage cell types [1]. These include receptor and coreceptor expression, the activation state of T cells and host immune responses to HIV, particularly the production of soluble factors or cytokines. Clearly, CD4 and coreceptor(s) expression is critical for HIV infection. Recently, virus-cell fusion was shown to be mediated by members of the chemokine receptor family. Macrophage-tropic strains of HIV-1 use CCR5 as a fusion cofactor [2][3][4][5][6], whereas T cell-tropic strains of HIV-1 use CXCR4/fusin as a coreceptor[7]. The importance of these coreceptors is supported by the recent observation that some individuals who are repeatedly exposed to HIV but remain uninfected have mutations of CCR5[8].

HIV-1 infection is also influenced by the host T cell activation state. Indeed, HIV-1 replication is more efficient in activated cells[9][10]. Levels of HIV consistently increase when the immune systems of HIV-infected individuals are activated by exogenous stimuli, such as opportunistic infections [11][12] or following immunization [13][14]. This increase in the rate of HIV replication is associated with cellular activation and the expression of HIV-inducing cytokines, as well as an acceleration in the course of HIV disease (reviewed in [1]). The impact of immune activation on viral replication and disease progression has been confirmed in SIV-infected monkeys and HIV-infected chimpanzees [13][14].

Effective T cell activation requires engagement of the T cell receptor/CD3 complex and at least one costimulatory signal. Recently, it was shown that activation of CD4+ T cells with immobilized antibodies to CD3 and CD28 specifically induced a potent anti-HIV effect [15].

The intrinsic resistance of $CD4^+$ T cells to HIV infection following anti-CD28 costimulation was shown to be specific for M-tropic isolates of HIV-1, and was due to either enhanced production of the β-chemokines, RANTES, MIP-1α and MIP-1β [16], which bind to CCR5 and block infection by M-tropic isolates of HIV-1[17][18], or a lack of transcripts encoding CCR5 [19].

Host immune responses, particularly the balance between HIV-inducing and HIV-suppressive cytokines control the net level of HIV replication [1][20]. In 1986, Walker et al.[21] demonstrated that $CD8^+$ T cells from HIV-infected individuals were able to nonlytically suppress the replication of HIV-1. This was subsequently shown to be mediated by a soluble factor(s) whose identity was unknown [22]. Recently, Cocchi et al [23] identified the β-chemokines RANTES, MIP-1α and MIP-1β as CD8-derived factors able to suppress infection by macrophage-tropic, but not T cell-tropic, strains of HIV-1. The potential importance of these findings was supported by Paxton et al. [24] who showed that $CD4^+$ T cells from HIV-exposed uninfected individuals produced higher levels of β-chemokines *in vitro.* More recently, IL-16, a chemoattractant cytokine produced by $CD8^+$ T cells that binds to CD4, was shown to suppress the replication of HIV-1[25]. Indeed, transfection of human $CD4^+$ T cells with the IL-16 gene markedly inhibited HIV replication[26].

We previously described the ability of $CD8^+$ T cell-derived factors to suppress HIV-1 long terminal repeat (LTR)-driven gene expression in human T cells [27][28]. This was shown using supernatant from a *Herpesvirus saimiri* (HVS)-transformed human $CD8^+$ T cell clone and from $CD8^+$ T lymphocytes from HIV-infected patients. Interestingly, CD8-derived supernatant that suppressed HIV LTR-driven gene expression in $CD4^+$ T cells caused an enhancement of HIV LTR-driven gene expression and replication in monocytic cells. Neither effect was mediated

by the β-chemokines [29][30]. During these studies, we found that supernatant from a HVS-transformed $CD4^+$ T cell clone was also able to markedly suppress HIV-1 LTR-driven gene expression. Here we demonstrate that similar to $CD8^+$ T cells, soluble factors produced by $CD4^+$ T lymphocytes mediate dichotomous effects on HIV-1 transcription and replication in T cells and monocytes. $CD4^-$ T lymphoyte-derived autocrine regulation of HIV-1 is a novel and powerful control mechanism with obvious relevance to our understanding of HIV pathogenesis and treatment.

Materials and Methods

Subjects

HIV-1-infected asymptomatic individuals were referred by Dr. Fiona Smaill at the McMaster Medical Centre Special Immunology Services Clinic (Hamilton, Ontario, Canada) and by Dr. Sharon Walmsley at the Immunodeficiency Clinic of the Toronto General Hospital (Toronto, Ontario, Canada). Ethics approval for these studies was conferred by McMaster University Health Science Centre and the University of Toronto.

Leukocyte Cultures and Supernatants

$CD4^+$ and $CD8^+$ T lymphocytes were isolated from the heparinized blood of HIV-positive patients and HIV-negative controls using Ficoll-Paque density gradients followed by positive selection using anti-CD8 immunomagnetic beads (Miltenyi, Auburn, CA). The T lymphocytes were then cultured for 3-4 days in RPMI 1640 (Canadian Life Technologies, Burlington, ON) supplemented with 10% FCS, 100 units/ml penicillin, 100ug/ml streptomycin, 5ug/ml PHA and 20 U/ml IL-2 (a generous gift from Chiron Corp., San Francisco, CA) at an initial concentration of $1x10^6$ cells/ml. The cells were then washed twice and resuspended in the same medium but lacking PHA and recultured for 3-4 days. Supernatants were collected and stored at -70°C.

Monocytic cell lines (U38 and U1) were cultured in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin. U38 cells were derived from U937 cells and are stably transfected with the HIV-1 LTR linked to the bacterial gene chloramphenicol acetyl transferase (CAT)[31][32] (AIDS Research and Reference Reagent Program, Division of

AIDS, NIAID, NIH, contributor Dr. B.K. Felber and Dr. G.N. Pavlakis). The U1 cell line [33] derived from U937 cells is chronically infected with HIV-1 (AIDS Research and Reference Reagent Program, contributor Dr. T.M. Folks).

Generation of Herpesvirus Saimiri (HVS) Transformed CD8$^+$ T Cell Clones and Supernatants

Herpesvirus saimiri stock 488 (kindly provided by Dr. Donald Desrosiers, New England Regional Primate Research Center, Southborough, MA, USA) was prepared in owl monkey kidney cells as previously described [34]. Several HVS-transformed CD4$^+$ and CD8$^+$ T lymphocyte lines and clones were generated as described [28]. The two used in these experiments, HVSCD4 clone and HVSCD8 line, were both >95% CD4$^+$ and CD8$^-$ by FACS analysis, respectively. All transformed lymphocyte cultures were maintained in RPMI 1640 containing 20% FCS, 20 U/ml IL-2 (Chiron), 100 U/ml penicillin and 100 ug/ml streptomycin. Supernatants were removed and stored at -70^{0}C.

Transfections and Vectors

The vector pLTRCAT incorporates the HIV-1 LTR of the BRU strain to position +77 driving CAT[35]. pSVtat (SV40 promoter driving HIV-1 tat)[36] was used to enhance HIV-1 transcription to a level that had the potential to be clearly inhibited. The CD4$^-$ Jurkat T cell line (30x10^6 cells) cultured in RPMI (supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin and 100ug/ml streptomycin), was transfected with 10ug pLTRCAT and 5ug pSVtat using a DEAE dextran procedure [37]. Uninfected transfected cells were cultured in test supernatant and media (1:1). Primary cells infected (20ng p24 / 5x10^6 cells) with the primary

isolate HIV_{419} were transfected 5-6 days post-infection using the above procedure. Infected/transfected cells were then cultured in test supernatant and media as before. After 24hrs, transfected or infected/transfected cells were stimulated with PMA (25ng/ml) and ionomycin (2μM) for 18hrs. Supernatants were then removed from infected/transfected cells and assayed for p24 by ELISA (Organon Teknika, Durham, NC). The cells were then lysed by repeated freeze-thaw cycles (3x). Lysates standardized for protein concentration were then assayed for the presence of chloramphenicol acetyl transferase (CAT) by ELISA (Boehringer Mannheim, Montreal,). Acute infections using laboratory isolate HIV_{IIIB} (obtained through the AIDS Research and Reference Program Division of AIDS, NIAID, NIH, contributed by Dr. R. Gallo) were conducted as above.

U1 and U38 monocytes (3×10^7) were also transfected using the DEAE dextran procedure. The vector pLTRCAT was provided to U1 cells at 10 μg per transfection and pSVtat was provided to U38 cells at 5 μg per transfection. Transfected cells were cultured with $CD8^+$ T cell supernatant at a ratio of 1:3 (supernatant : RPMI). Twenty four hours following transfection the cells were treated with PMA (25 ng/ml; Sigma) and ionomycin (2 μM; Sigma) for 18 hours. Supernatant of U1 cells was retained for measurement of p24. The cells were then lysed by 4 rounds of freeze/thaw and the chloramphenicol acetyl transferase (CAT) was measured as described above. In some experiments target cells were pre-treated with pertussis toxin (10ng/ml).

Detection of TNF-α in cell culture supernatants

TNF-α concentrations were measured in cell culture supernatants by ELISA according to the manufacturer's directions (Genzyme Diagnostics, Cambridge, MA).

Results

Suppression and enhancement of HIV-1 LTR-mediated gene expression by HVS-transformed human CD4$^+$ T cell supernatants is dose-dependent

In order to study the role that CD8$^+$ T cell-derived supernatants had on HIV-1 LTR-mediated gene expression, we developed Herpesvirus saimiri (HVS)-transformed lines from HIV$^+$ patient lymphocyte cultures. Several CD8$^+$ lines and one CD4$^+$ cloned line were noted for their ability to strongly suppress HIV-1 gene expression. Since there were no descriptions of CD4$^+$ T cell-derived factors capable of suppressing HIV-1 LTR-mediated gene expression, we conducted additional studies on this latter clone, termed HVSCD4. Fig.1A shows a typical dose response suppression of HIV-1 LTR-driven gene expression in Jurkat T cells treated with supernatant derived from HVSCD4. Maximal suppression occurred between a 1:1 and 1:4 ratio which is similar to that seen with CD8-derived supernatants [30].

We previously observed that CD8$^+$ T lymphocyte-derived supernatants from HIV$^+$ individuals were capable of exerting an enhancing effect on HIV-1 LTR-mediated gene expression in U38 monocytes which are stably transfected with HIV-1 LTR driving CAT [29]. This effect was also noticed when the U38 cell line was treated with HVS CD4-derived supernatant (Fig. 1B). As seen in Jurkat T cells described above, the enhancing effect was maximal at 1:1 dilution, and likewise appeared to lose activity as the dilution approached 100-fold. Thus, the ability of CD8$^+$ T lymphocytes to suppress HIV-1 LTR-mediated gene expression in Jurkat T cells and enhance expression in U38 monocytes is also shared by an HVS-transformed human CD4$^+$ T cell clone.

Suppression and enhancement of HIV-1 LTR-mediated gene expression by CD4$^+$ and CD8$^+$ T lymphocyte-derived supernatants

Having observed LTR-suppressive and enhancing effects of supernatants derived from a CD4$^+$ HVS-transformed clone, we screened a panel of culture supernatants from CD4$^+$, CD8$^+$ and unfractionated peripheral blood lymphocytes from both HIV$^-$ patients (P1-P5) and uninfected laboratory worker controls (LW1-LW4) for their effects on LTR-mediated gene expression in CD4$^+$ Jurkat T cells and U38 monocytic cells, respectively. Confirming our results using the HVS-transformed CD4$^+$ T cell clone, primary culture supernatants from stimulated CD4$^+$ lymphocytes demonstrated comparable levels of suppression to the CD8$^+$ T lymphocyte and unfractionated PBL-derived supernatants from 5 of 5 HIV$^+$ patients (Table I). Tested in the U38 monocyte system, these same supernatants were found to have an opposite, enhancing effect on LTR-mediated gene expression. Interestingly, similar suppressing / enhancing effects were found to be mediated by supernatants derived from CD4$^+$, CD8$^+$ and PBLs from HIV-negative laboratory worker controls LW1-LW4 (except PBLs from LW1 and LW2). That this LTR-modulatory capability is not specific to lymphocytes derived from HIV$^-$ individuals indicates that *in vivo* priming with HIV-1 is not necessary to induce production of these HIV-1 LTR-modulatory factors *in vitro*. Thus, factors present in cultures of both major T lymphocyte subsets, and in unfractionated PBL are capable of suppressing gene expression driven off the HIV-1 LTR in Jurkat T cells, but enhancing it in U38 monocytes.

Suppression of HIV-1 NFκB-mediated gene expression by CD4- and CD8-derived supernatants

To characterize the CD4-derived suppressive activity further, we examined the specificity of this control by using a construct containing a dimer of the HIV-1 NFκB enhancer element driving CAT. This element within the HIV-1 LTR has also been reported to be responsive to Nuclear Factor of Activated T cell (NFAT)-mediated upregulation of HIV-1 transcription [38] and thus may be influenced by binding of either NFAT, or NFκB proteins. To determine if the CD4-derived supernatants also mediated a suppressive effect through the NFκB elements we used $CD4^+$, $CD8^+$ and whole peripheral blood lymphocyte culture supernatants from the same HIV^- patient. As seen in Figure 2, there is no significant difference in the suppression mediated by either whole PBL or fractionated T cell subsets. However, in comparison to media alone, all three treatments mediate very significant suppressive effects on gene expression in this system ($p<.005$ for each treatment). This result indicates that like the CD8-derived effect [28], the suppression of HIV-1 LTR-mediated gene expression by CD4+ T lymphocytes is at least in part the result of suppression of activation through the NFκB enhancer elements.

Replication of primary and SI laboratory strains of HIV-1 is suppressed by $CD4^+$ T cell-derived supernatants

To determine if the observed suppression of HIV-1 LTR-mediated gene expression was related to modulation of virus replication, we developed a model of acute infection followed by transient transfection of the LTR-CAT reporter gene construct (see Materials and Methods).

Briefly, Jurkat T cells acutely infected with a non-syncytia-inducing (NSI) primary isolate HIV_{419} were transfected with the HIV-1 LTR-driving CAT. After 24 hrs of stimulation, the lysates were assayed for CAT and the supernatants for p24. As indicated in Table II, suppression of virus replication ($p<.05$) appears coincident with suppression of LTR-mediated gene by HVSCD4 and HVSCD8 T cell supernatants. Although not necessarily indicative of the same factors acting in both of these treatments, the suppression of both transcription and replication of a primary HIV-1 isolate mediated by the HVSCD4 supernatants was strikingly similar to that mediated by supernatants derived from HVSCD8.

To confirm the suppressive ability of the CD4-derived supernatants on another system of virus replication, primary $CD4^+$ T lymphocytes from an HIV^- donor were acutely infected with the laboratory strain HIV_{IIIB}. Data shown in Figure 3 indicates that with respect to replication alone, supernatants derived from the HVS $CD4^+$ clone ($p=.018$), but not a combination of RANTES, MIP-1 α, and MIP-1β (200 ng/ml each) suppressed p24 production. As expected, the β-chemokines did not block replication of the SI lab strain HIV_{IIIB}, but factors present in our HVS-transformed $CD4^+$ clone supernatant exhibited marked suppression of replication of this strain, and the NSI primary isolate HIV_{419} (with concommitant suppression of HIV-1 LTR-mediated gene expression)

Replication and transcription are enhanced in monocyte lineage U1 cells by $CD4^+$ T cell-derived factors

We previously described strong enhancing effects that CD8-derived supernatants have on HIV-1 replication and LTRCAT-mediated gene expression in transiently transfected U937-

derived U1 monocytes [29]. To further assess the functional characteristics of the factors present in CD4-derived supernatants, we screened culture supernatants derived from $CD4^+$ and $CD8^+$ lymphocytes from an HIV^+ individual. In this assay system, we again found enhancement of LTR-mediated gene expression occurred concomitant with increases in p24 production by treatment with either CD4- or CD8-derived primary patient supernatants (Fig. 4A and 4B). In agreement with previous findings, CD8-derived enhancement of gene expression and replication in U1 moncytes was pertussis toxin (PTx) sensitive (Fig 4A and 4B) (Copeland et al., submitted). However, there was no significant reduction of the CD4-derived enhancement by similar PTx treatment. The active factors in the supernatants from the $CD4^+$ T lymphocytes are further distinguished from those present in the CD8+ T lymphocyte cultures by their effect on TNF-α levels in the U1 culture media measured just prior to lysis. TNF-α has been well characterized for its ability to act in an autocrine manner to upregulate HIV-1 gene expression in monocytic cells through NFκB elements[39][40]. CD8-derived supernatants from this patient induced a two-fold increase in TNF-α levels over control which was susceptible to PTx treatment ($p=0.0001$, Fig. 4C). In contrast, the CD4-derived supernatant induced only a modest increase in TNF-α levels ($p<.05$), which was also abrogated by PTx treatment. Thus, the enhancement of HIV-1 LTR-driven gene expression and replication in monocytic cells by $CD8^+$ T cell supernatants, but not $CD4^+$ T lymphocyte-dervied supernatants, was PTx sensitive and the concurrent induction of TNF-α production was significantly lower in $CD4^+$ T lymphocyte-derived supernatant treated monocytic cells ($p<.005$).

Discussion

This study was initiated following our observation that soluble factors from a *Herpesvirus saimiri* (HVS)-transformed human T cell clone that strongly suppressed HIV-1 LTR-mediated gene expression was CD4 positive. Our results demonstrate that like $CD8^+$ T cells, $CD4^+$ T cells produce soluble factors that modulate HIV-1 LTR-mediated gene expression in T cells and monocytes. This activity measured was found in supernatants from positively selected $CD4^+$ and $CD8^-$ T lymphocytes, from both HIV-positive and HIV-negative individuals. Therefore, production of the LTR-modulatory factors by these cell types does not absolutely require prior exposure to HIV-1 *in vivo*.

Examination of these factors with respect to replication of the laboratory isolate HIV_{IIIB} and a primary isolate (HIV_{419}) in $CD4^+$ T lymphocytes also yielded strikingly similar suppressive effects, indicating that factors present in the CD4-derived supernatants are capable of modulating both gene expression and replication of HIV-1. Although not examined in this study, it is not unreasonable to speculate that expression of HIV-1 LTR-suppressive factors could markedly limit the active sources of *de novo* virion production from the $CD4^+$ T lymphocye population.

The similarities between the suppressive activity mediated by $CD8^-$ T lymphocytes, and that mediated by $CD4^+$ T lymphocytes were reiterated in studies of monocyte-lineage cells. Both U937-derived U38 and U1 cell lines demonstrated enhancements mediated by $CD4^+$ T lymphocyte-derived culture supernatants of gene expression, and concurrent gene expression and replication, respectively. In the U38 model, we observed consistent enhancements of gene expression induced by supernatants from both HIV^+ and HIV^- individuals. As seen in Table I, in

the latter group we observed discordance between enhancement mediated by supernatants from fractionated lymphocyte subsets and unfractionated PBL from LW1 and LW2. This may be the result of soluble factors produced by B lymphocytes and monocytes (found in the unfractionated PBL), but not $CD4^+$ and $CD8^+$ T lymphocytes alone.

We have previously described the enhancing effect related to CD8-derived supernatants in these systems [29], but as Fig. 4 demonstrates, this CD8-derived activity in U1 monocytes is distinguished from the CD4 effect by its susceptibility to Pertussis Toxin (PTx). The effect of PTx with respect to replication and gene expression is also apparent in the increases in TNF-α levels induced by treatment with CD8-derived supernatants. Interestingly, although significant enhancement of TNF-α is induced by the CD4-derived supernatants ($p<.05$), the effect is not nearly as powerful as that induced by the CD8-derived supernatants. Possibly indicative of differential expression of factors present in the respective supernatants (or cell-type specific receptor expression), this increase in TNF-α production could also be representative of the influence of the concentration of the active factors present in the lymphocyte culture supernatants. This question, along with the more interesting question of whether or not the enhancing effect in monocytic cells is mediated by the same factors responsible for the suppressing effect in $CD4^+$ T lymphocytes may only be answered by purification and identification of all active, involved molecular species. To this end, preliminary biochemical characterization does indicate that at least one of these factor(s) is an acid-stable molecule of low molecular weight (J. Leith et al., unpublished observations). Interestingly, a recent report has demonstrated that Interleukin-16 (IL-16) is capable of inducing down-regulation of the

HIV-1 LTR through the NFκB element [41]. IL-16 has not been found to share identity with CAF as described by Mackewicz et al [42], and kinetics [41] which differ from the activity of both the CD4- and CD8-derived factors active in our system. To confirm the identity of the factors active in our system and to further elucidate their respective mechanisms of control of HIV-1 gene expression, we are continuing with a standard protocol for biochemical purification of both CD4 and CD8-derived suppressive factors.

Although there is great interest in $CD8^-$ T cell-derived factors that nonlytically control HIV infection and replication, there has previously been little emphasis on factors produced by $CD4^+$ T cells that control HIV-1 in a similar manner. Recent descriptions of the interactions between RANTES, MIP-1α and MIP-1β, and their cognate receptor complexes [17][18][43][44] has shifted attention from exogenous mechanisms of control of HIV-1 replication and transcription to endogenous control of expression of both β-chemokines and their respective receptors. Paxton et al. [24] were the first to note that $CD4^+$ T cells from individuals who remain uninfected despite multiple high-risk sexual exposures to HIV-1 were relatively resistant to HIV-1 infection. This was suggested to be the result of autocrine production of the β-chemokines RANTES, MIP-1α and MIP-1β, but later found to be the result of the Δ32-base pair deletion in CCR5 [8]. Continuing the study of endogenous resistance mechanisms, one recent report characterized an antiviral state induced by ligation of CD28 and CD3, but not stimulation with PHA and IL-2 [15]. Based on the down-regulation of CCR5 (but not CXCR4) mRNA expression by the former treatment [19] or enhanced production of RANTES, MIP-1α and MIP-1β [16], this effect would appear to be the result of the inability of M-tropic isolates to bind to this receptor. Either mechanism has effectively the same result as the CCR5 Δ32 base-pair deletion

that may be significantly protective against infection *in vivo* [45]. Although using 5-fold greater concentrations of IL-2 than in our experiments, the endogenous HIV-suppressive effect of CD28 / CD3 costimulation (derived from conditioned media) was found to be considerably more powerful than the triple combination RANTES, MIP-1α and MIP-1β at up to 500 ng/ml, indicating that there are remaining, CD4-derived soluble mediators active in this system. We are currently studying the effect of CD28 / CD3 costimulation on HIV-1 suppression in our systems.

Noting that the β-chemokines do not influence HIV-1 gene-expression [29][30], $CD4^+$ T lymphocyte-derived modulation directly implicates an autocrine control pathway independent of co-receptor modulation. We have shown that factors produced by $CD4^-$ T lymphocytes are able to suppress concurrent HIV-1 gene expression and replication. Thus, this autocrine suppressive pathway is novel and distinct from other mechanisms of suppression of HIV-1 (coreceptor modulation or β-chemokine production). Such a pathway, triggered on activation (likely coincident with productive infection) could then act to partly suppress viral replication. This may appear to contradict the conventional view that the primary reservoir for highly replicating HIV-1 is the $CD4^+$ T lymphocyte. However, control of the viral replication by an autocrine pathway may begin to answer a long-standing paradox of advanced HIV disease: Given the total destruction of the cellular immune system, as witnessed by architectural ablation of the lymph nodes and loss of $CD4^+$ T lymphocytes apparent after several years of HIV-1 infection (for a review see [46]), what are the sources for the high titres of HIV-1 found in the peripheral blood in many end stage AIDS patients? An obvious explanation is that latently infected cells of the monocyte / macrophage lineage increase normally low levels of replication as $CD4^+$ T lymphocytes decline in number [47]. However, another option, as suggested by our preliminary

data, is that as HIV disease progresses the ability of $CD4^+$ T lymphocytes to control endogenous replication is lost, contingent on decreased production of autocrine suppressive factors. Such loss of autocrine control could lead to dramatically increased production of HIV-1 by the remaining naturally infected $CD4^+$ T lymphocytes.

An additional consideration is the extent to which the contribution to the pool of circulating virus is increased by the described enhancing effect of both $CD4^+$ and $CD8^+$ T lymphocytes on HIV-1 infected macrophages. This activity could encourage overall increased viral replication and possibly result in increased infiltration of the virus into neuronal sites heavily populated with monocyte / macrophage lineage cell types. Further studies are obviously necessary to clarify the roles of the abovementioned cell types in the network of suppression and enhancement of HIV-1 replication. And as the dynamics of this network are likely to continuously vary in response to changes in viral load and populations of lymphocyte subsets, our results serve to illustrate the importance of understanding the complex nature of the interactions between HIV-1 and its human host.

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Table I

Patient	HIV[b]	Jurkat (% Suppression)[a]			U38 (% Enhancement)		
		CD4	CD8	PBL	CD4	CD8	PBL
P1	+	75	90	99	100	176	172
P2	+	78	92	86	107	55	72
P3	+	69	90	91	128	168	161
P4	+	100	95	100	33	29	22
P5	+	43	42	ND	153	115	ND
LW1	-	95	99	88	118	76	18.5
LW2	-	71	81	100	212	112	0
LW3	-	97	84	97	117	42.5	160.5
LW4	-	100	ND	98	322	ND	256

[a] Measured as a percentage of averaged duplicates compared to treatment of media alone control
[b] Representative sample of 5 HIV-positive patients and 4 HIV-negative laboratory worker controls

Table II

Suppression of replication and transcription in infected / transfected Jurkats by CD4 and CD8-derived supernatants

Treatment	p24 (ng / ml)[a]	CAT (pg / 150ug protein)
Control	324 (40.4)	1255 (339)
HVS CD4	172 (9.0)	732 (135)
HVS CD8	202 (38.7)	622 (71)

[a] All values are show as mean of triplicates, +/- standard error. Experiment is representative of two experiments showing same result.

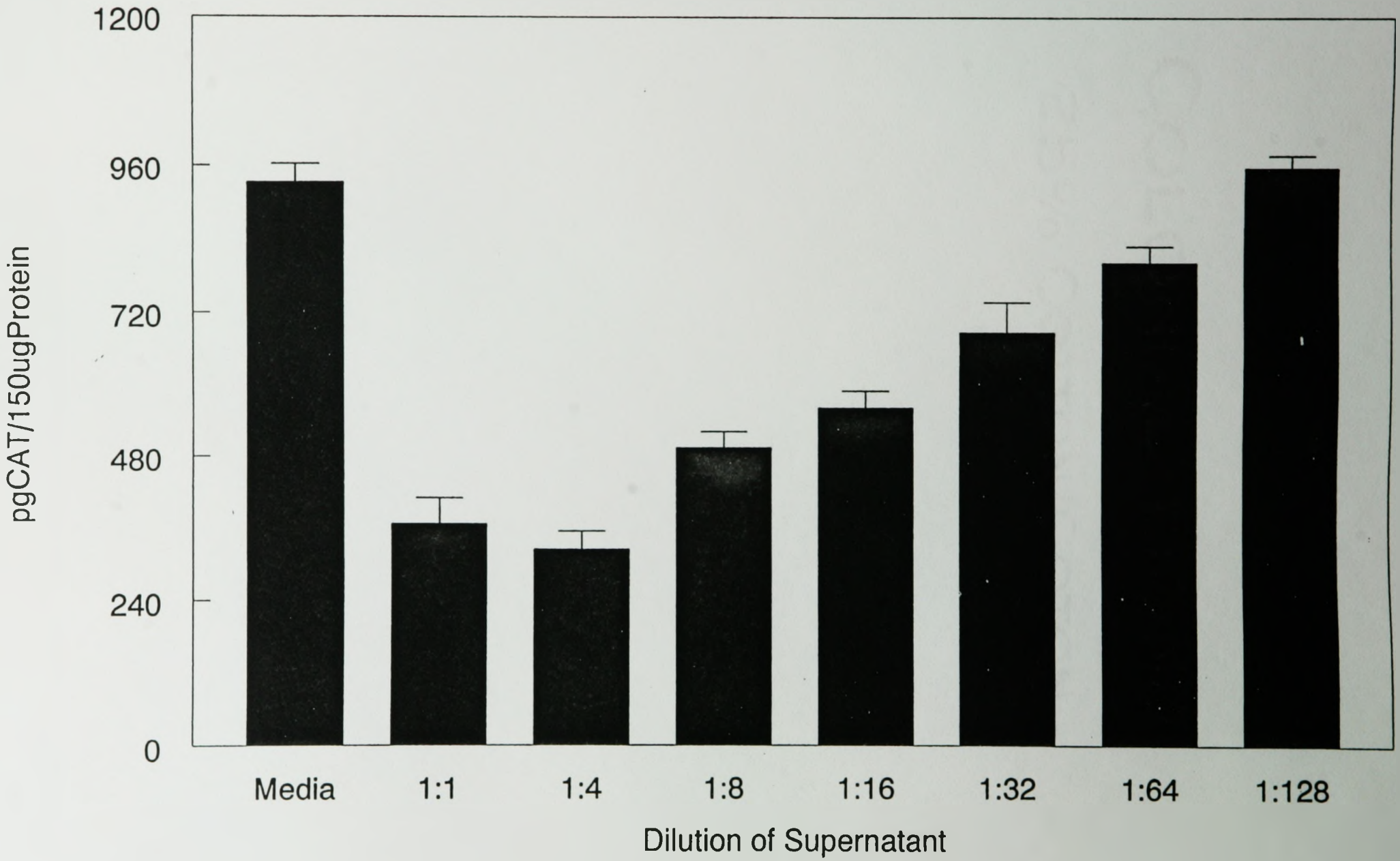
Captions

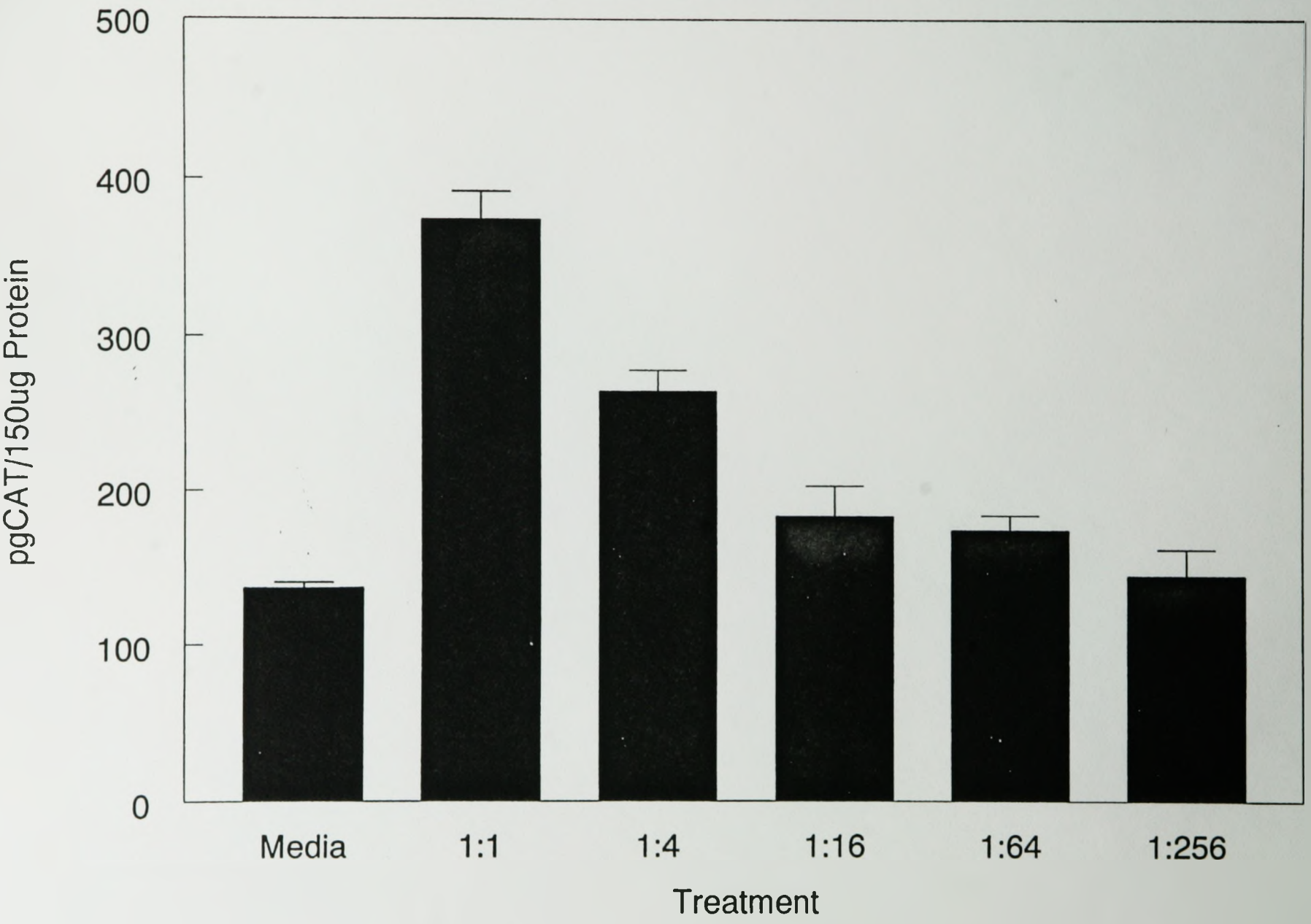
Figure 1. HIV-1 transcription is controlled in a dose-dependent manner by HVS-transformed CD4-derived supernatants in T cells and monocytes. HIV-1 LTR-mediated gene expression (compared to media alone control) is suppressed in Jurkat T cells (A) and enhanced in U38 monocytic cells (B). These data are shown as means of triplicates (+/- standard error) of CAT in lysates normalized for concentration of protein, and are each representative of two separate experiments.

Figure 2. Suppression of HIV-1 NFκB-mediated gene expression by $CD4^-$, $CD8^+$ and PBL-derived supernatants from an HIV^+ patient. Reporter gene expression driven off the NFκB enhancer of the HIV-1 LTR is suppressed (all p values <.005) by the treatment with supernatants derived from $CD4^-$-, $CD8^+$-, and PBL-derived supernatants. Data are shown as means of triplicates (+/- standard error).

Figure 3. Suppression of T-tropic isolates of HIV-1 by CD4-derived supernatants. Replication of HIV_{IIIB} in acutely infected primary CD4+ T lymphocytes is suppressed by HVS-transformed $CD4^-$ T lymphocyte-derived supernatants (p=.018), but not the CC chemokines RANTES, MIP-1α and MIP-1β (200ng/ml). Data is shown as means of triplicates (+/- standard error).

Figure 4. Differential pertussis toxin (PTx) sensitivity of CD4- and CD8-mediated suppression of HIV-1 replication and transcription in U1 monocytes. PTx sensitivity of CD8- (p<.05), but not CD4-derived enhancements of HIV-1 LTR-mediated gene expression (A) and p24 production (B) in U937-derived chronically infected U1 monocytes transiently transfected with the HIV-1 LTR-driving CAT. CD8-derived supernatants induce a PTx-sensitive enhancement of TNF-α concentrations (p<.005) measured in culture supernatants removed prior to lysis (C). CD4-derived supernatant induction of TNF-α is also sensitive to PTx, but not to the same degree (p<.05). Data are representative of at least two experiments showing the same result and are shown as means of triplicates or quadruplicates, (+/- standard error). The same patient lymphocyte-derived supernatants were used throughout these experiments.


1200
960
720
480
240
0
pgCAT/150ugProtein
Media
1:1
1:4
1:8
1:16
1:32
1:64
1:128
Dilution of Supernatant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400
300
200
100
0
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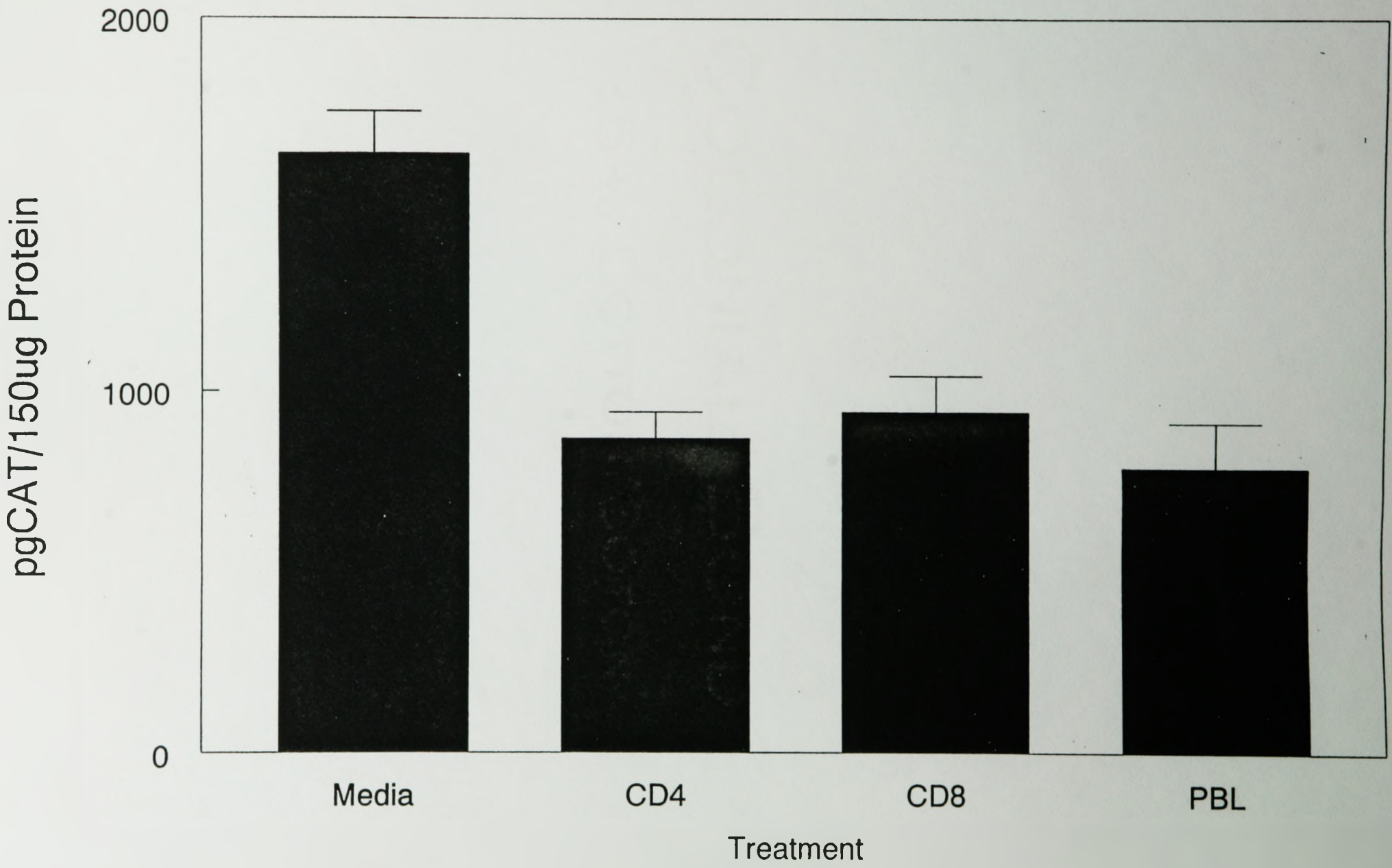
Media
1:1
1:4
1:16
1:64
1:256
Treatment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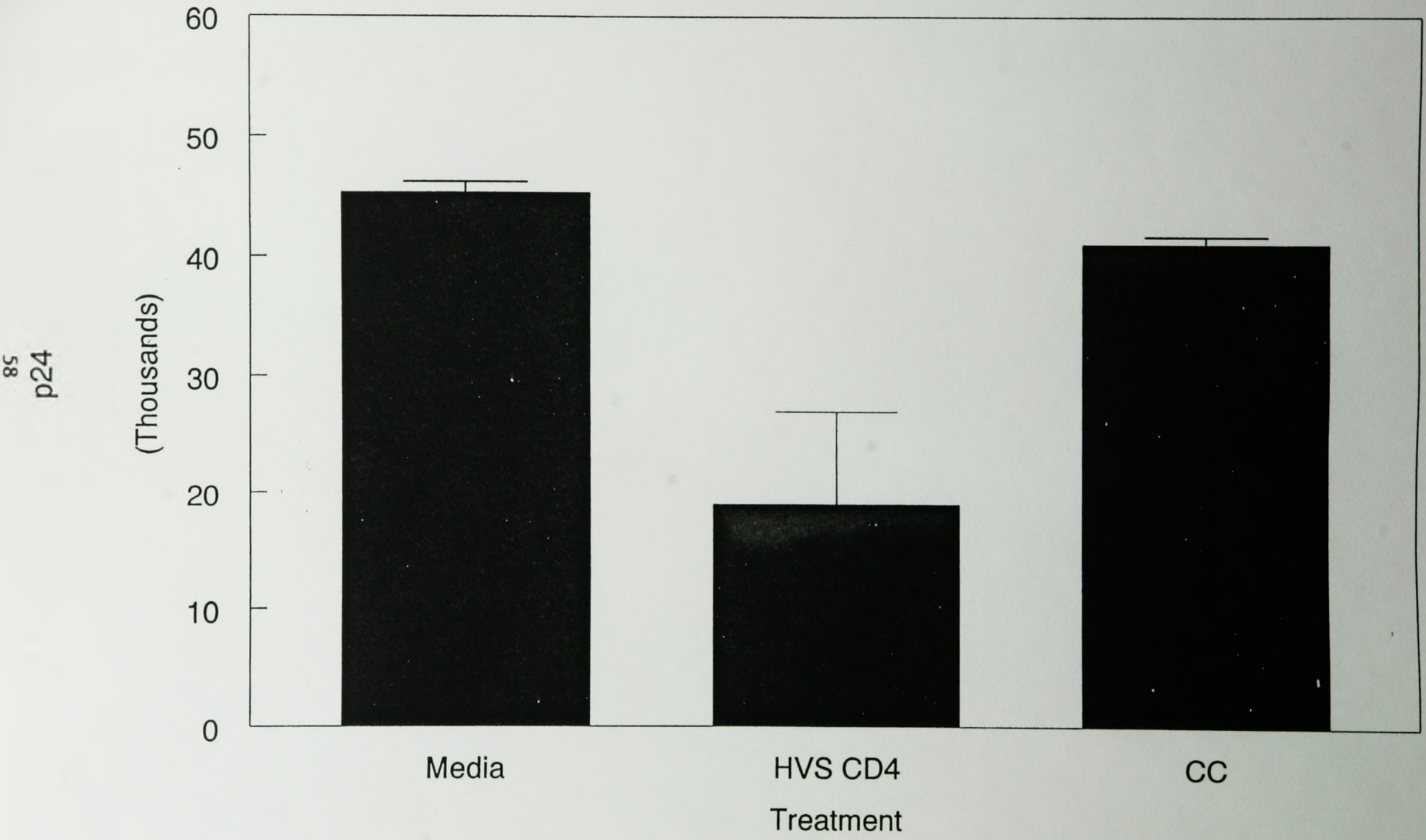
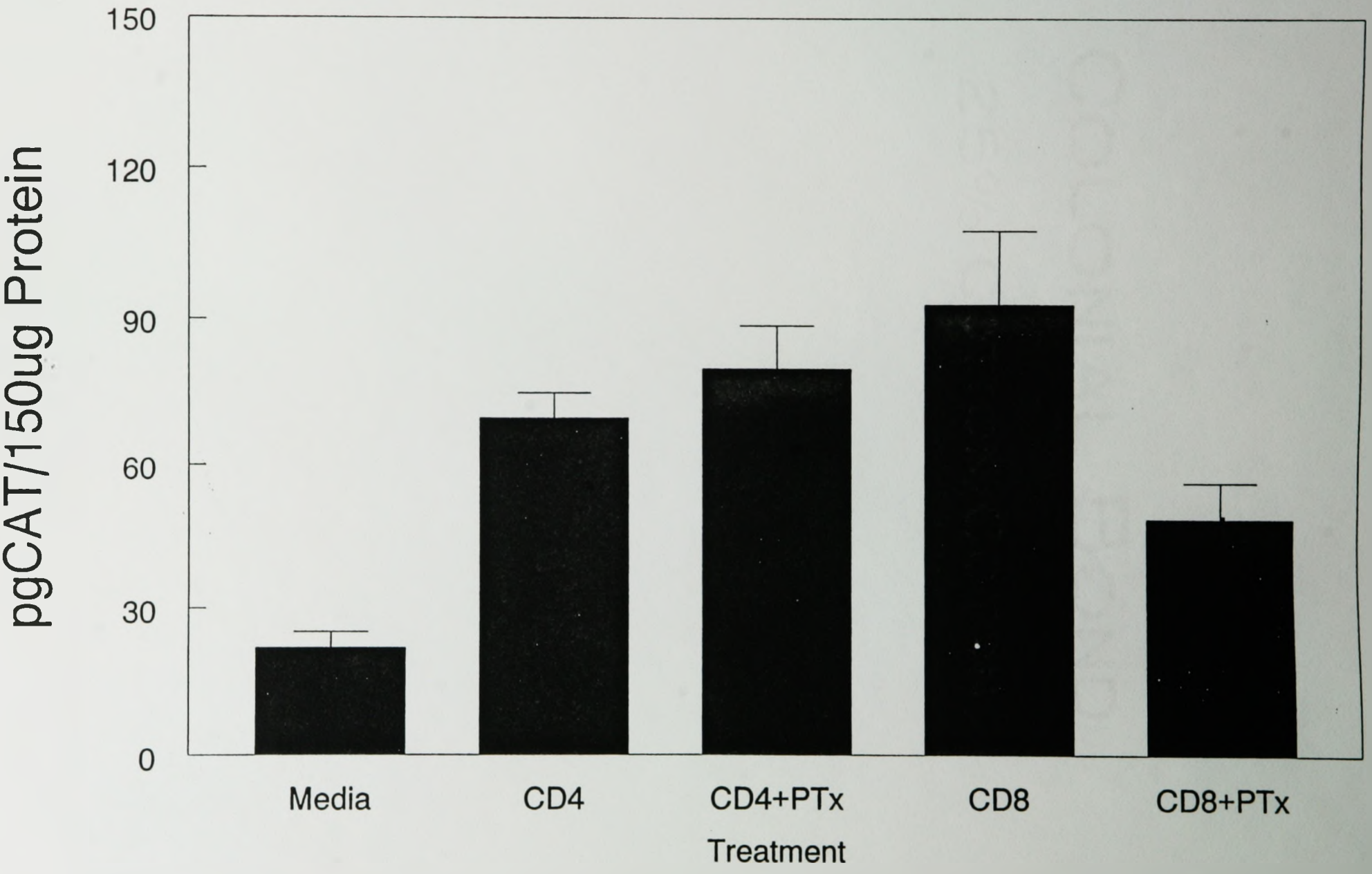
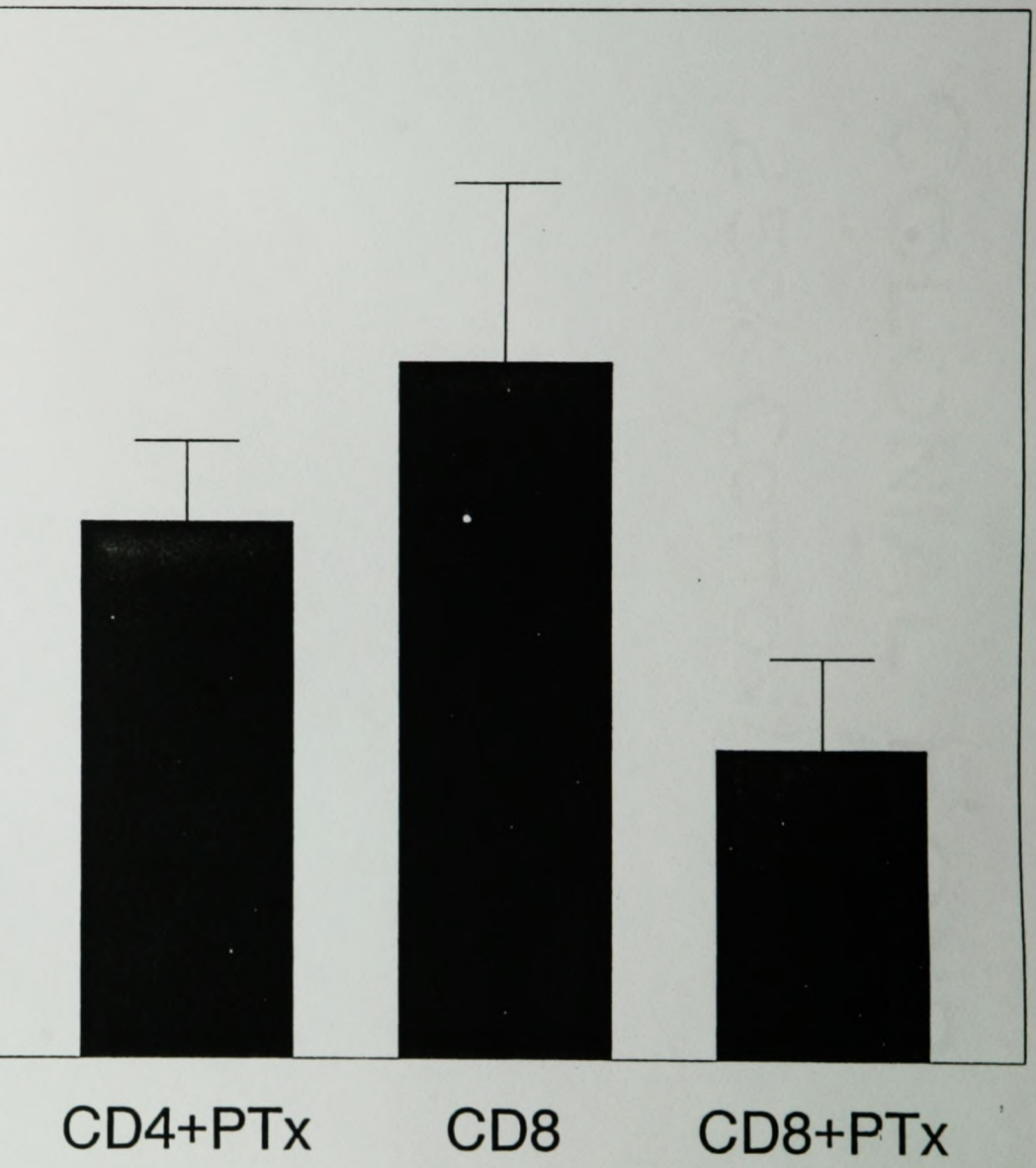
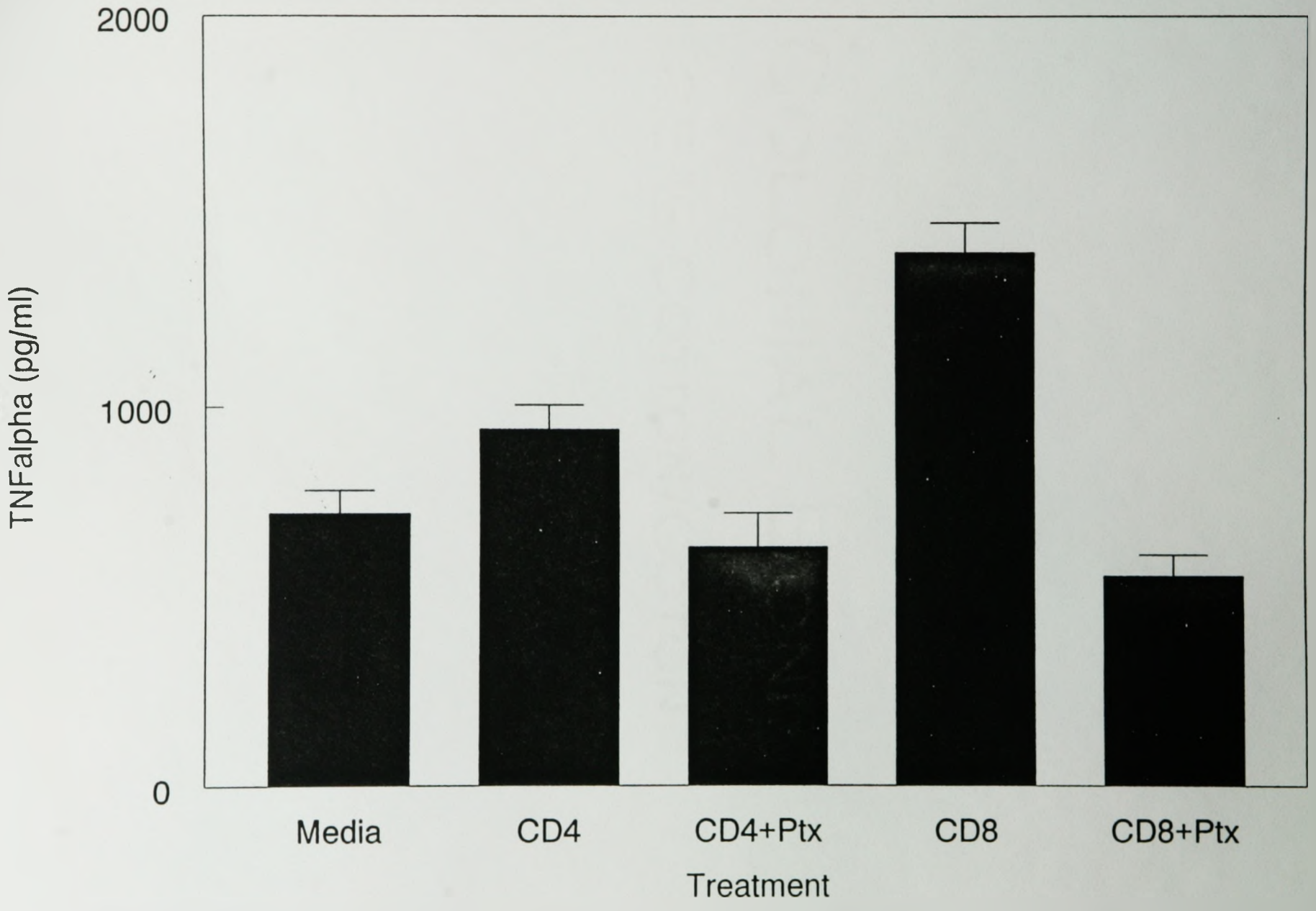
1000
0
pgCAT/150ug Protein
Media
CD4
CD8
PBL
Treatment


60
50
40
30
20
10
0
(Thousands)
^{35}S
p24
Media
HVS CD4
CC
Treatment


pgCAT/150ug Protein
150
120
90
60
30
0
Media
CD4
CD4+PTx
CD8
CD8+PTx
Treatment

p24 (pg/ml)
(Thousands)
20
10
0
Media
CD4


CD4+PTx
CD8
CD8+PTx
Treatment


2000
1000
0
TNFalpha (pg/ml)
Media
CD4
CD4+Ptx
CD8
CD8+Ptx
Treatment

CHAPTER FOUR

BIOCHEMICAL FRACTIONATION OF CD8-DERIVED SUPERNATANTS AND CHARACTERIZATION OF HIV-1 REPLICATION AND TRANSCRIPTION-SUPPRESSING FACTORS

Preface

This chapter describes preliminary studies of fractionation of the factors HIV-1 LTR-suppressive factors by chromatographic methods and characterization of the suppressive activity and the cell types from which it is produced. All work in this chapter was done by myself excluding the generation of the HVS-transformed $CD8^+$ T cell line CD8290 by Paula McKay, and Dorothee Bienzle provided assistance with ^{51}Cr release assays. In addition, special thanks goes to Dr. Jack Rosenfeld and Dr. Susan Breckenridge who provided not only the chromatography equipment, but excellent assistance and suggestions which were invaluable to these studies.

Introduction

Preliminary biochemical characterization of the factors present in CD8-derived supernatants responsible for suppression of HIV-1 LTR-mediated gene expression required a continuous source for large volumes of supernatants containing the active constituents. Our laboratory has previously utilized *Herpesvirus saimiri* (HVS)-transformation of $CD8^+$ T lymphocytes, followed by selective subcloning to generate cloned lines capable of continuous Interleukin-2- dependent growth. However, noting difficulties in long-term viability of these cultures, we decided to avoid the sub-cloning step, favouring development of a more heterogeneous population of HVS-transformed $CD8^+$ T lymphocytes.

Protocols to fractionate the HIV-1 LTR-suppressive activity from supernatants derived from the two HVS-transformed lines, CD8290 and CD8106 were developed based on reverse phase and size exclusion liquid chromatography (RP- and SEC-HPLC, respectively). Concurrently, several separate experiments were conducted to determine the specificity of the LTR-suppressive activity within leukocyte populations; the kinetics of the suppressive activity; and the cytolytic potential of suppressive populations of $CD8^+$ T lymphocytes.

It is important to note that although the experiments described in this chapter are interesting and novel, the results presented should be considered preliminary, and thus warrant further study.

Materials and Methods

Generation of CD8-derived supernatants via Herpesvirus-transformation of $CD8^+$ T lymphocytes from an HIV^+ patient

A new stock of HVS was prepared from an original stock 488 (donated by Dr. R. Derosiers) by inoculating owl monkey kidney (OMK) cells grown to confluence in DMEM media with 1ml of stock 488. Cytopathic effects of HVS denoted by plaque formation on the OMK lawn occurred by day 7 of culture. Supernatant was removed and frozen at -70° C. $CD8^+$ T lymphocytes from an HIV positive patient (# 92-290 and 91-016) were inoculated with 0.5ml of new HVS stock for two hours, at which point 1.5ml of RPMI 1640 (supplemented with 20% Fetal Calf Serum; 100U/ml penicillin, 100ug/ml streptomycin, and 25mM HEPES) was added. After three weeks of culture, these cells were supplemented with IL-2 (20U/ml). The two lines developed by these methods are referred to as CD8290 and CD8O16 in the following studies, and are both >95% $CD8^+$ by flow-cytometric analysis. After several months of culture, these lines were cycled through AIM-V serum free media (Gibco, Burlington, ON).

Preliminary Filtration of HVS-derived supernatants

To make crude fractionations prior to chromatographic methods, CD8290 and CD8016 supernatants were clarified by passing through a 100kD filter and filtered on a 30kD molecular weight cut-off filters (Amicon, Beverly, MA). Fractions (filtrates and retentates) were diluted back to the original volume and tested separately for suppression of HIV-1 LTR-mediated gene expression in Jurkat T cells.

Reverse-Phase High Performance Liquid Chromatography (RP-HPLC)

Preliminary separation of the active constituents of the HVS-transformed $CD8^+$ T cell supernatants was completed by clarifying 200mls of CD8290 pooled supernatant which was then concentrated on a 30kD filter. The concentrate (100X) was then heat treated at 56° C for 40 minutes to eliminate any remaining infectious HVS. Samples of 200ul were run on a C18 column using a linear gradient starting from 80% ddH_2O and 20% acetonitrile plus 0.008% trifluoroacetic acid (TFA) and moving to 20%:80% over 30 minutes, at a flow rate of 2mls/minute. Fractions were collected at 1 min. intervals and then lyophilized. Samples were then reconstituted in 1/2 original volume of media (RPMI1640+10%FCS), and tested for suppressive capability. To determine the concentration of protein in the active fraction, after lyophilzation, the sample was brought up in phosphate buffered saline (PBS) and one portion was tested for [protein] using the BIO-RAD protein assay (Bio-Rad Labs, Hercules, CA). A second portion was boiled with 2-Mercaptoethanol and loading buffer and run on a 12% SDS-polyacrilamide gel. The gel was silver stained according to standard protocols, (destained with 5% methanol, 7% glacial acetic acid, and 88% ddH_2O) and then dried.

Size Exclusion Chromatography (SEC)

To ascertain an approximate molecular weight, CD8290 and CD8016 serum free supernatants were filtered as above, and then applied to a SEC column (Shodex, Waters) which has highest resolution from 800-60,000 daltons. Each 200ul sample was run in PBS, pH 7.3, mobile phase at 1ml/min, under ~350p.s.i. Fractions were collected at 30-120 second intervals, lyophilized and reconstituted in media (to original fraction volume). For this procedure

molecular weight standards of albumin, chymotrypsin, ovalbumin and RNase were run twice after test samples. Ultraviolet light absorbance (280nm) was used to measure the individual protein peaks. The resulting standard curve was used to estimate the approximate molecular weight of the active fractions. These experiments were repeated (along with MW standards) using a mobile phase consisting of acetonitrile and water (v/v 1:1), pH 1.6.

Cytokines and anti-cytokine antibodies

The cytokines Interferon (IFN)-γ (Sigma-Aldrich, Oakville, ON) and Tumour Necrosis Factor (TNF)-α (R&D Systems) were diluted to appropriate concentrations in media (RPMI 1640 + 10%FCS) and provided to the HIV-1 LTRCAT system. Polyclonal neutralizing antibodies directed against anti-human soluble TNF-receptor I and II were incubated with 500µl of suppressive supernatants for 1hr at 37° C prior to addition to the HIV-1 LTRCAT system. The final concentration of each antibody was 10µg/ml.

Transfection of primary $CD4^+$ T lymphocytes and HIV-1-infected Jurkat T cells

All transfections were conducted as described in Chapters 2 and 3. However, to transfect primary $CD4^+$ T lymphocytes, the standard protocol was modified as follows: immediately following DEAE-dextran transfection, cells were washed once, and then pooled by being brought up in media plus lyophilized PBS (same volume of media), and treated with 20U/ml of IL-2. The pooled cells were then split into the required number of aliquots in a 24-well plate, containing control or test supernatants.

Acute infection of primary CD4⁺ T lymphocytes

$CD4^+$ T lymphocytes polybrene-treated PHA blasts were incubated with 5ng p24 / $1x10^6$ cells for three hours, washed once, and then recultured at $1x10^6$ / ml (plus 10U/ml IL-2 from Chiron, San Francisco, CA), in media or media plus test supernatant or effector cells at a 1:1 ratio. After 6-7 days, 100ul of supernatant was removed for p24 analysis by ELISA (Organon Teknika, Durham, NC).

Redirected cytoxicity assays

Approximately $5x10^6$ Fc receptor-expressing P815 murine mastocytoma cells (American Type Culture Collection, Rockville, MD) were incubated 60 min. with 200μCi $Na^{51}CrO_4$ (DuPont, Mississauga, ON), washed three times in PBS containing 1%FCS, counted, and resuspended in media at $1.5x10^6$/ml. Cells were then incubated with 1ug anti-CD3 monoclonal antibody for 45min. at 37°C. These targets cells were then washed once and resuspended in media at $1x10^5$/ml, and 50ul was aliquoted to each well of a round-bottom 96-well plate. Effectors (CD8016 or CD8290) were then added to wells at indicated E:T ratios, and media was added to make the final volume 300μl. For each experiment, maximum ^{51}Cr release was determined by adding 1M HCL to untreated targets. Targets without effectors were set as minimum ^{51}Cr release. In addition, to make sure that release was specific to TcR ligation, targets were also incubated in absence of anti-CD3 antibody. Following 5hr incubation at 37°C, 100μl from each well was removed and counted in a γ-counter. Percent specific lysis was calculated as follows:

(Sample ^{51}Cr release - minimum release) ÷ (maximum ^{51}Cr release - minimum release) x 100

All samples and controls were tested in triplicate.

Results

HIV-1 LTR-suppressive factors do not pass through a 30kD molecular weight cut-off filter

As seen in Figure 1, factors which suppress HIV-1 LTR-mediated gene expression found in pooled HVS-transformed supernatants from CD8290 and CD8016 did not pass through a 30kD molecular weight cut-off filter. These filters should not be considered accurate for assessment of molecular weight, but this result does support the utility of 30kD filters as a preliminary step to concentrate the active factors prior to initiation of methods of chromatography.

RP-HPLC of HVS-transformed $CD8^+$ T lymphocyte supernatants yields fractions with LTR-suppressive activity

Fractionation of supernatants concentrated on a 30kD filter by a linear gradient of ddH_2O and acetonitrile run through a C18 RP-HPLC column produced one fraction with >50% suppression of HIV-1 LTR-mediated gene expression compared to media control (labelled M) (Figure 2). Fractions #1 (80% ddH_20, 20% acetonitrile) and #41 (20% ddH_2O, 80% acetonitrile) were spiked with 200ul of concentrated supernatant (are denoted by *). This fraction, #17, when lyophilized and reconstituted in PBS, had [protein] of 1.3 mg/ml. This high concentration of protein was confirmed by silver staining of an SDS-PAGE gel on which three samples were run (10, 20 and 50ul) (Figure 3).

Activity losses using this protocol were calculated to be less than 50%. That is, the full activity of a 200ul (equivalent to 20mls unconcentrated supernatant) sample collected over 4 fractions (8mls), concentrated 2-fold (to 4mls total) has thus been diluted 20-fold total (4 mls /

200ul). Thus each 1ml fraction is equivalent to 1ml of original unconcentrated supernatant. As 50% suppression of the HIV-1 LTR is mediated by a sample diluted by 1/2 (all test samples are used at 1:1 with media), this demonstrates a <50% loss of activity (1:1 dilutions of neat HVS-transformed $CD8^+$ T lymphocyte supernatants typically suppress between 50 and 100%). Further experiments (such as a titration curve) are required to confirm the accuracy of the above estimate.

SEC-HPLC of HVS-transformed $CD8^+$ T lymphocyte supernatants yields fractions with LTR-suppressive activity

Separation of pooled CD8290 and CD8016 supernatants by SEC - HPLC in PBS mobile phase yielded two successive fractions, (#7 and 7.5) which suppressed HIV-1 LTR-mediated gene expression (Figure 4a). In these experiments, CAT is expressed by UV absorbance at 405nm, instead of pgCAT / 150ug total protein because the PBS mobile phase causes an increase in basal expression of LTR-mediated gene expression over media control. Accordingly, all fractions from SEC-HPLC run with PBS mobile phase cause baseline increases in CAT over media alone, although unconditioned media run through the same conditions did not have suppressive effects on HIV-1 LTR-mediated gene expression. The molecular weight of the active fraction was estimated based on standards to be 50-60kD (Fig. 4b).

To confirm these results without the enhancing influence of PBS components, the mobile phase was changed to a 1:1 v/v ratio of ddH_2O:acetonitrile which in RP-HPLC was approximately the point on the linear gradient at which the active factors eluted (#17, pH 1.6). Shown in Figure 5a, as percent suppression in comparison to media alone control (and ddH_2O:acetonitrole - labelled H2O/Ac), fractions denoted 6.5 and 7 demonstrated peak

suppressive activity. These fractions correspond to a molecular weight range of approximately 20-30kD based on standards run in these conditions (Fig. 5b).

Using either mobile phase (representative chromatograms shown in Fig. 4a and 5a) , the loss of activity (estimated by similar means as described in section on RP-HPLC) was approximately 50%, indicating that this method is also useful for preparative fractionation.

Suppression of HIV-1 LTR-mediated gene expression is shared by $CD8^-$ leukocyte cell lines

The capability to suppress transcription of HIV-1 is demonstrated not only by HVS-transformed $CD8^+$ T lymphocyte lines CD8290 and CD8016, HVS-transformed $CD4^+$ T lymphocyte clone HVSCD4 (as described in Chapter 3), but also by the $CD4^+$ Jurkat T cell line, and the U937-derived U38 monocytic cell line (Figure 6). In both cases (U38 and Jurkat) supernatants were removed after 3-4 days of culture and tested neat.

HIV-1 LTR-mediated gene expression is suppressed by the cytokines IFN-γ and TNF-α

TNF-α and IFN-γ are cytokines produced by $CD8^+$ T lymphocytes and have been reported to be distinct from the factor CAF (Cellular Antiviral Factor) as described by Levy's group (Mackewicz, 1994). Separate reports have described HIV-1 replication enhancing and suppressive effects mediated by IFN-γ. To determine the effects of these cytokines on HIV-1 LTR-mediated gene expression, media alone treatments were supplemented with 10ng/ml and 100U/ml doses of TNF-α and IFN-γ, respectively. As shown in Figure 7, both IFN-γ and TNF-α (73% and 47% suppression vs. control, respectively) are able to significantly suppress HIV-1 LTR-mediated gene expression ($p<.005$ and .05, respectively). At this dose of 100U/ml, the

suppression mediated by IFN-γ is approximately equivalent to that mediated by HVS-transformed $CD8^+$ T cell line CD8290 shown in the same experiment ($p>.05$ between CD8290 and IFN-γ).

Effect of antibodies to human TNF-α soluble receptors on CD4- and CD8-derived suppression of HIV-1 LTR-mediated gene expression

Dependent on the dose used, TNF-α has been reported to have a biphasic effect (suppressing at high, but enhancing at low doses) on HIV-1 replication (Mackewicz, 1994). Although the complete array of effects that TNF-α may have on an HIV-1 infected $CD4^+$ T lymphocyte is incompletely elucidated, enhancement of HIV-transcription by TNF-α binding to its cognate surface receptor (which indirectly causes increased binding of NFκB elements in the HIV-1 LTR; Kruppa, 1992) is one mechanism that may lead to increased HIV-1 replication. Soluble mediators which interfere with this mechanism, could lead to decreased TNF-α-induced HIV-1 production.

Human soluble TNF-α receptors I and II (hsTNF-RI and II) may be two such molecules. Both are able to inhibit PMA-induced activation of the HIV-1 LTR in monocyte (Howard, 1993, and Granowitz, 1996) and T cell lines (Howard); down-regulate TNF-α receptor surface expression (Kalinkovich, 1995); and (hsTNF-RI only) have been found in high levels in the plasma of HIV-1-infected individuals. To preliminarily determine if hsTNF-RI and RII share identity with the HIV-1 LTR-suppressive factors, high concentrations of anti-TNF-RI and RII antibodies (five fold greater than ND_{50} - 50% of dose required to neutralize 300ng/ml of hsTNF-RI and RII) were pre-incubated with supernatants selected for their ability to suppress HIV-1

LTRCAT-mediated gene expression. These antibodies were unable to abrogate LTR-suppression mediated by a patient-derived $CD8^+$ T lymphocyte supernatant (Fig. 8a) and HVS-transformed $CD4^+$ and $CD8^+$ T lymphocyte-derived supernatants (Fig. 8b and 8c). Control treatments of antibodies alone (in combination) had observable suppressive effects in each experiment, which may be due to competition with TNF-α for binding to the TNF-R surface receptor (with which these antibodies are cross-reactive). Although these experiments do support the hypothesis that the active factors in these supernatants are distinct from the hsTNF-Rs, further experiments are necessary to confirm these preliminary results.

HVS-transformed $CD8^+$ T lymphocyte supernatants mediate suppression of the HIV-1 LTR in primary $CD4^+$ T lymphocytes

Due to unknown reasons, the DEAE-dextran transfection method that we have utilized to insert the HIV-1 LTRCAT construct into Jurkat T cells and U1 monocytes has not been successful in transfecting primary $CD4^+$ T lymphocytes. However, the observations of increased LTR-mediated gene expression following treatment with lyophilized PBS fractions from size exclusion chromatography experiments suggested that modifications to the DEAE-dextran method might make possible transfecting primary $CD4^+$ T lymphocytes. Figure 9 demonstrates that this is not only possible, but also that gene expression driven off the HIV-1 LTR in this population is suppressed by supernatants derived from an HVS-transformed $CD8^+$ T cell line (CD8016). Although further attempts were made to utilize this protocol for measurement of LTR-mediated gene expression in acutely infected primary $CD4^+$ T lymphocytes, these experiments were unsuccessful due to undetectable CAT expression. This may be because of

unknown cyto-pathological effects of the viral infection. Clearly, this methodology is important, but requires further experimental development.

Time-course of HIV-1 LTR-mediated gene expression in Jurkat T cells

To separate studies of the kinetics of the suppression of HIV-1 gene expression were conducted. The first, where LTRCAT-transfected cells cultured with or without a patient $CD8^-$ T lymphocyte-derive supernatant were removed and lysed every six hrs (Fig. 10a) show that HIV-1 LTR-mediated gene expression is induced 6-12hrs after PMA and ionomycin (Pi) stimulation, and increases linearly until lysis (18hrs after Pi stimulation). The second study (Fig. 10b), demonstrates that longer pre-incubation (prior to Pi stimulation) with $CD8^+$ T lymphocyte-derived supernatants from an HIV^+ patient to causes increased levels of suppression of HIV-1 LTRCAT-mediated gene expression. This result was confirmed in a separate experiment (with a different $CD8^-$ T lymphocyte supernatant from an HIV^+ patient), where the time-course was extended by 12hrs. Taken together, these time-course experiments indicate that although HIV-1 LTR-mediated gene expression occurs only after Pi stimulation, suppressive effects induced by factors in CD8-derived supernatants are maximized by pre-incubation of supernatants with the Jurkat T cell targets.

Increased efficiency of suppression of HIV-1 replication by direct cell-cell contact: evidence for a lytic mechanism

A recent report outlined in Chapter 1 described the requirement for Major Histocompatibility Class I recognition to suppress HIV-1 replication non-cytolytically (Yang,

1997). To confirm these results, a comparison was made between suppression of replication of HIV_{B-al} in acutely infected primary $CD4^+$ T lymphocytes mediated by culture supernatants from HVS-transformed $CD8^+$ T cell lines, and co-cultures (of the same cell lines) allowing direct cell-cell contact. As shown in Figure 11a, supernatants from CD8290, but not CD8016 suppressed HIV_{B-al} replication. This variable effect was seen again in Figure 11b, where 1:1 effector:target (E:T) ratios dramatically suppressed p24 production in co-cultures of CD8290 ($p<.005$) and HIV-1-infected primary $CD4^+$ T lymphocytes. This effect was decreased in 1:2 E:T ratios, but still greater than that mediated by CD8016 cells at a 1:1 E:F ratio. For both cell lines suppression mediated by cell-cell contact was significantly greater than suppression mediated by supernatants alone. Observations made during these experiments indicated that lytic activity of the these HVS-transformed $CD8^+$ T cell lines might be responsible for the decreased HIV-1 production noticed in co-culture treatments. To confirm the lytic potential of these cell lines, re-directed cytotoxicity assays were conducted as described (Grant, 1994). This assay measures non-specific lysis generated against a target cell line (P815) which has anti-CD3 bound to cell surface expressed Fcγ receptors. This assay does not indicate that killing is HIV-specific. As shown in Figure 12, both cell lines were lytically competent, with cell line CD8290 demonstrating significantly higher killing at 10:1 ratios than CD8016. It is important to also note that because both CD8290 and CD8016 are not cloned lines, the heterogenous population could contain distinct non-cytolytic and cytolytic populations which could exert separate mechanisms of modulating HIV-1 gene expression and replication. Further cloning based on lytic and non-cytolytic end-points is important to clarify the relationship between these two virus control mechanisms.

Figure 1. *Effect of 30kD filter on suppressive supernatants*

Factors produced by HVS-transformed T cell lines CD8016 and HVS-transformed $CD4^+$ T cell clone HVSCD4 responsible for suppressing HIV-1 LTR-mediated gene expression do not pass through a 30kD molecular weight cut-off filter. Experiment is representative of two experiments showing the same result, and means of triplicates are shown (+/- standard error).

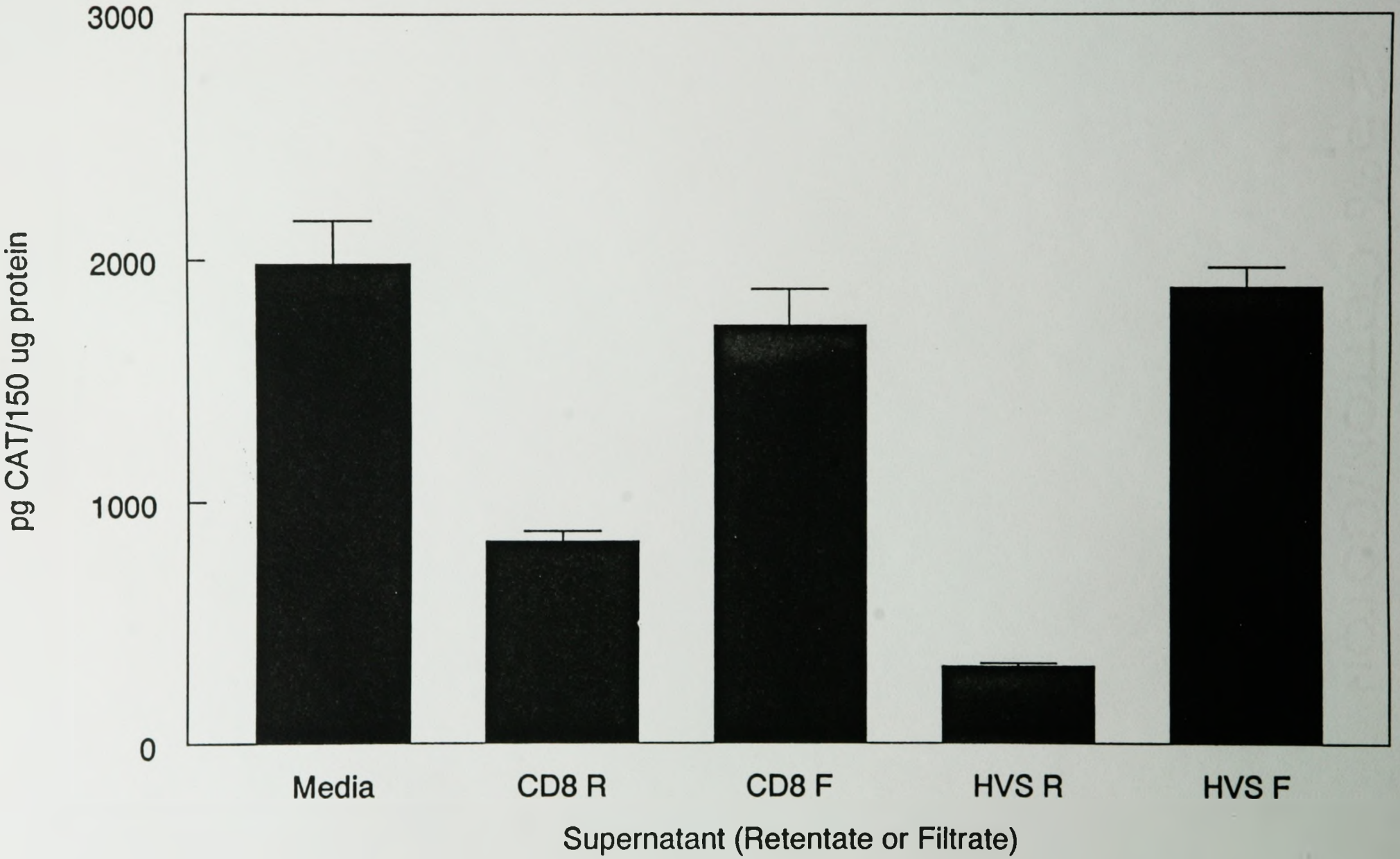

3000
2000
1000
0
pg CAT/150 ug protein
Media
CD8 R
CD8 F
HVS R
HVS F
Supernatant (Retentate or Filtrate)

Figure 2. *HIV-1 LTR-suppressive activity of RP-HPLC fractions.*

Suppression of HIV-1 LTR-mediated gene expression by RP-HPLC fractions peaks at fraction #17 (taken 17 minutes after flow initiated). Control fractions are shown with an asterix (1^{*} and 41^{*}, and were spiked with 200ul suppressive supernatant. Experiment is representative of two experiments showing the same result as single data points.

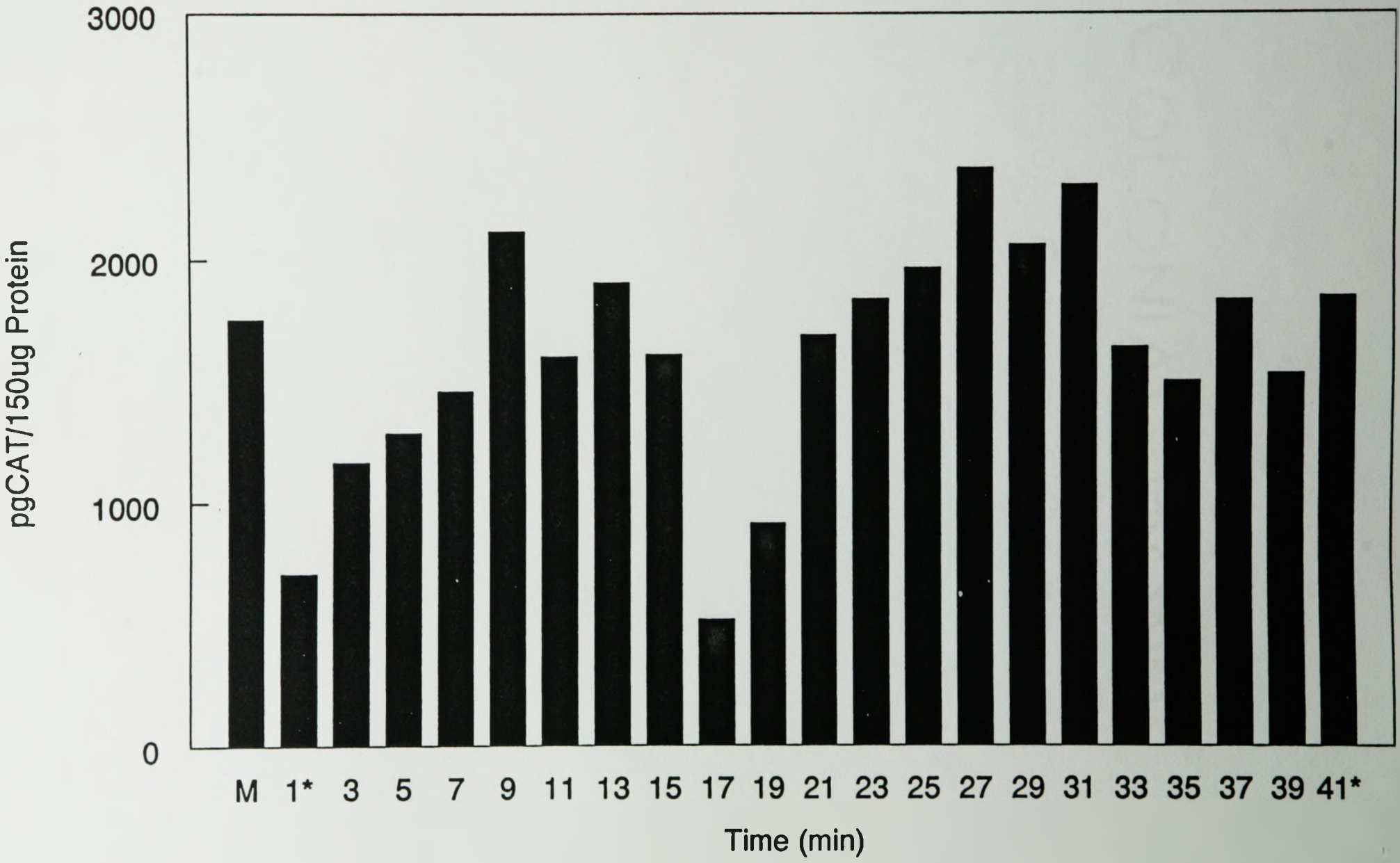

3000
2000
1000
0
pgCAT/150ug Protein
M 1* 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41*
Time (min)

Figure 3. *Silver stain of SDS-PAGE gel loaded with suppressive fraction #17.*

SDS-PAGE of 10, 20 and 50ul (lanes 1, 2 and 3, respectively) aliquots from HIV-1 LTR-suppressive fraction #17 demonstrate multiple high and low molecular weight species. As indicated, molecular weight standards were run in lane 5. An empty lane (lane 4) is indicated by B (blank).

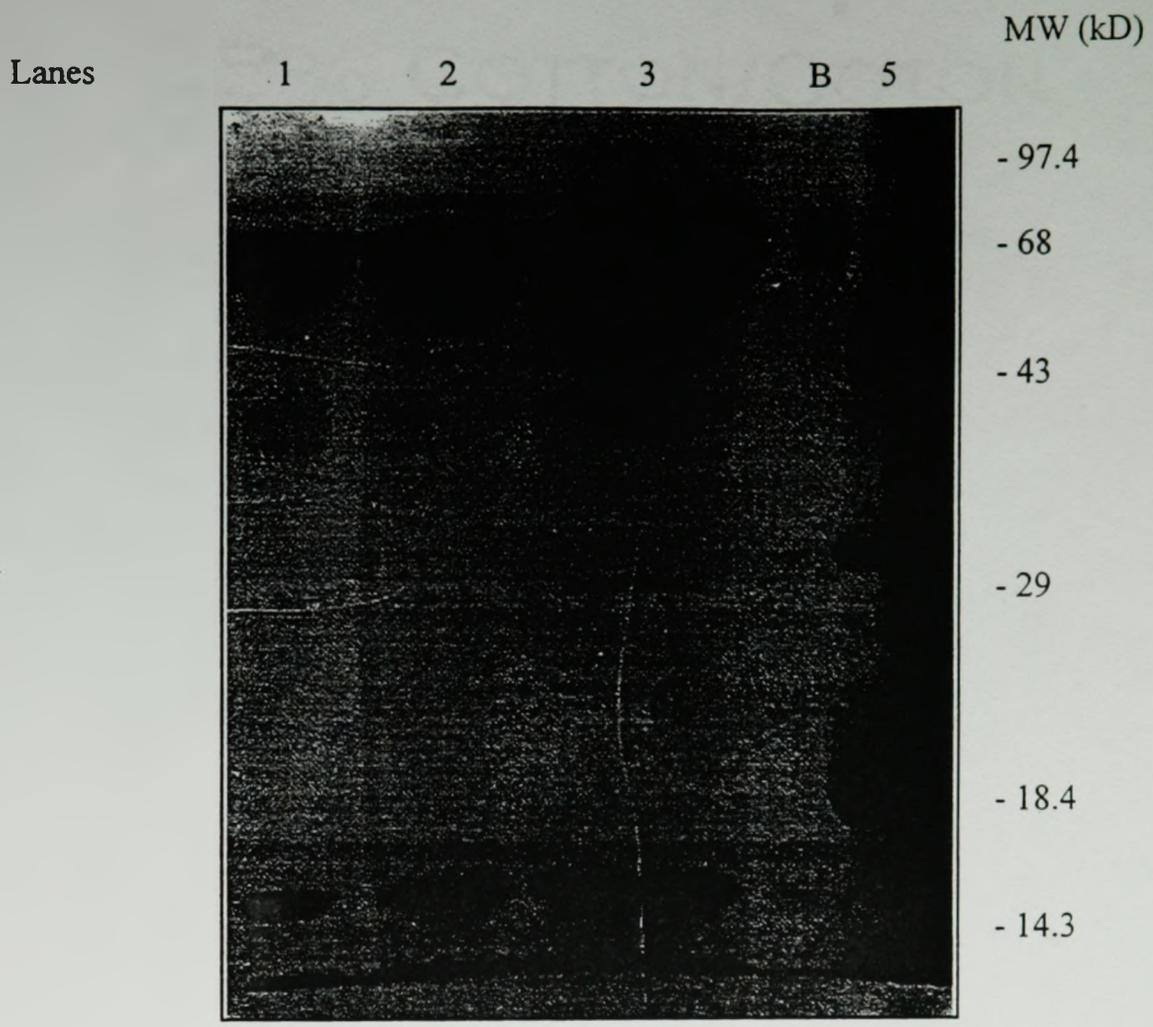
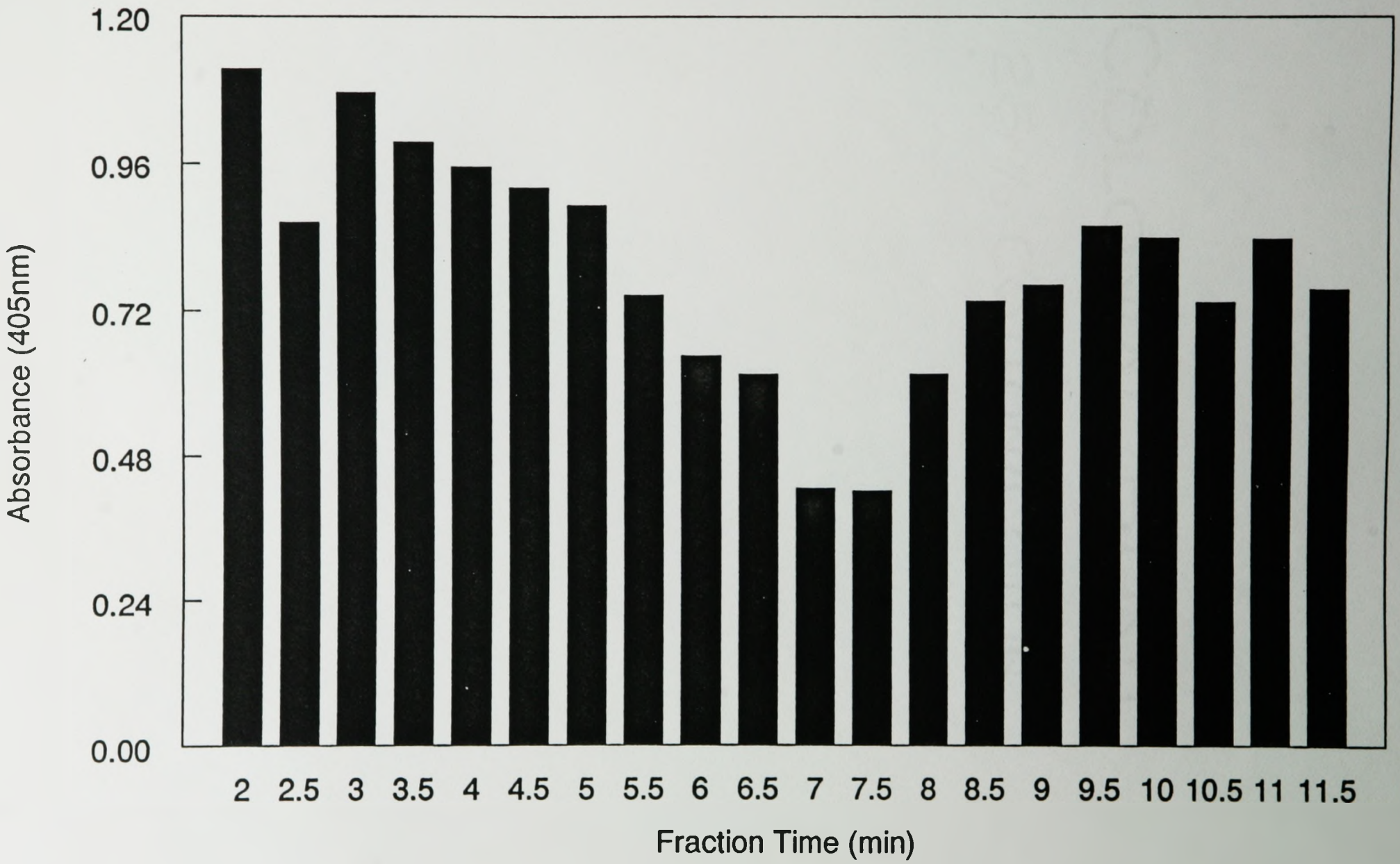

MW (kD)
Lanes
1
2
3
B
5
- 97.4
- 68
- 43
- 29
- 18.4
- 14.3

Figure 4. *Effect of SEC Fractions on HIV-1 LTR-mediated gene expression: PBS mobile phase.*

Size exclusion chromatography (SEC) in a PBS mobile phase yielded peaks in fractions #7 and 7.5 when assayed for suppression of HIV-1 LTR-mediated gene expression (Fig. 4a). This activity, indicated by a rectangular box superimposed on the chromatogram (Fig. 4b) lies between the molecular weight standards of 43 and 67kD (as indicated). For all chromatograms, absorption was measured at 254nm and standards were run immediately following test sample. Experiment is representative of two experiments showing the same result as single data points.


1.20
0.96
0.72
0.48
0.24
0.00
Absorbance (405nm)
2
2.5
3
3.5
4
4.5
5
5.5
6
6.5
7
7.5
8
8.5
9
9.5
10
10.5
11
11.5
Fraction Time (min)

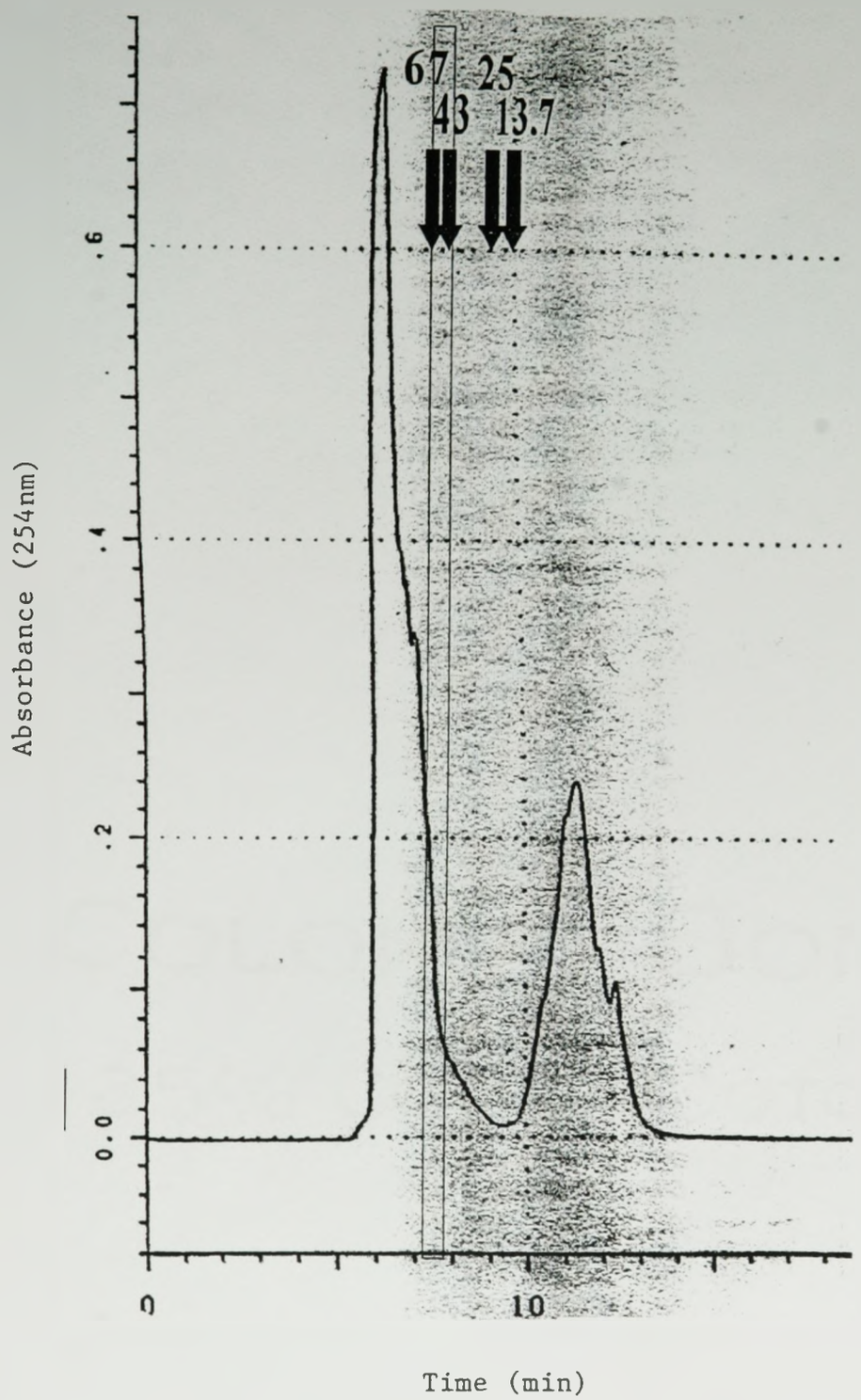

67
43
25
13.7
.6
.4
.2
0.0
Absorbance (254nm)
0
10
Time (min)

Figure 5. *Effect of SEC Fractions on HIV-1 LTR-mediated gene expression:* ddH_20 / acetonitrile mobile phase.

Size exclusion chromatography (SEC) in a ddH_20:acetonitrile (1:1) mobile phase yielded peaks in fractions #6.5-7.5 when assayed for suppression of HIV-1 LTR-mediated gene expression (Fig. 5a). Suppressive activity elutes between the 13.7 and 25kD molecular weight standards (Fig. 5b). Experiment is representative of two experiments showing the same result as single data points.

Percent Suppression vs. Control
0
10
20
30
40
50
60
6
6.5
7
7.5
8
8.5
9
9.5
H20/Ac
Fraction Time

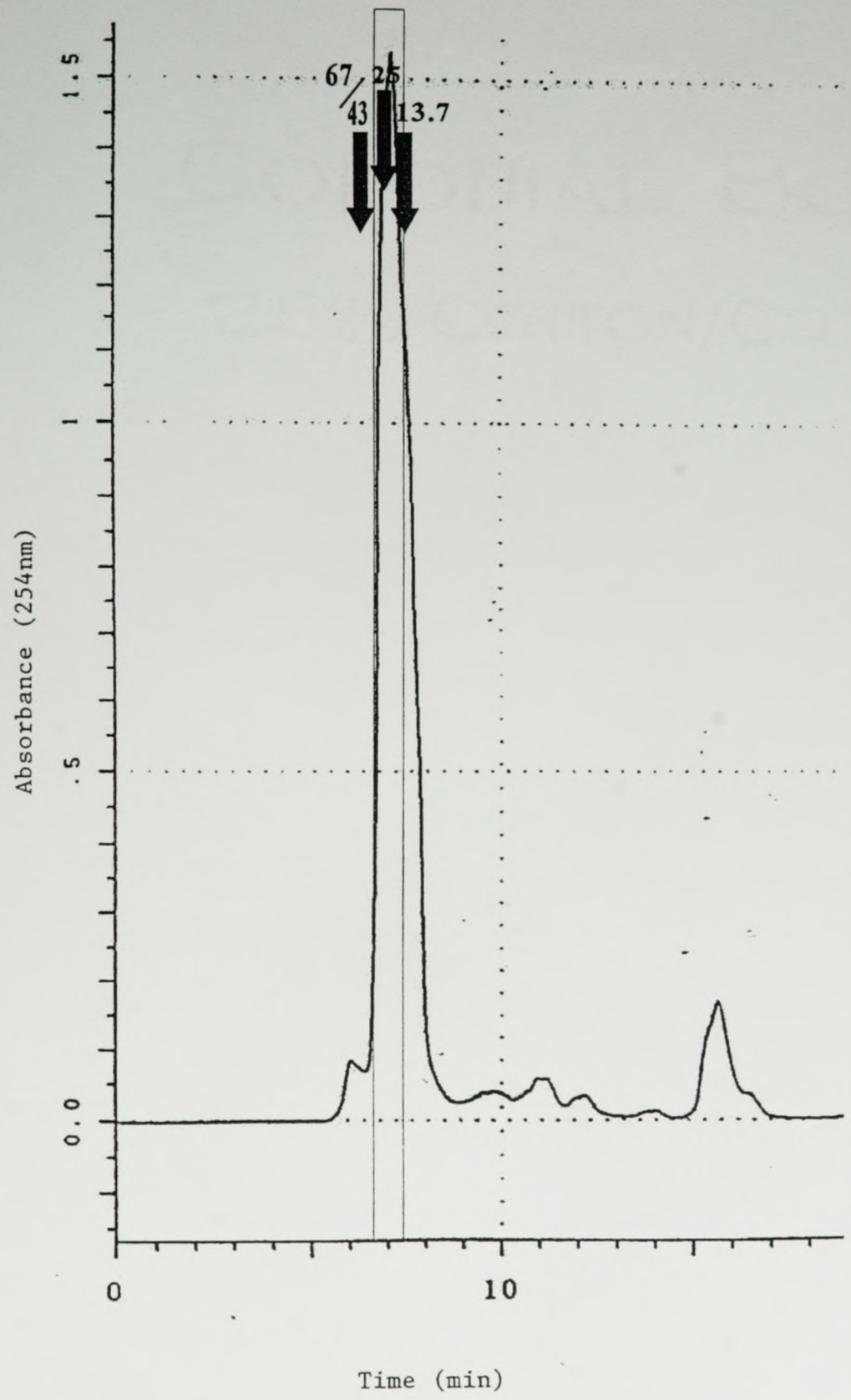

67
43
25
13.7
1.5
1
.5
0.0
Absorbance (254nm)
0
10
Time (min)

Figure 6. *Suppression of HIV-1 LTR-mediated gene expression by leukocyte-derived supernatants.*

In comparison to media alone control (Media) and supernatants from $CD8^+$ T cell lines (CD8290, CD8016), HIV-1 LTR suppressive activity is also mediated by $CD4^+$ T cell clone HVSCD4 and HTLV-1 transformed Jurkat cell line, as well as the U937-derived U38 monocytic cell line. Experiment is representative of two experiments showing the same result, and means of triplicates are shown (+/- standard error).

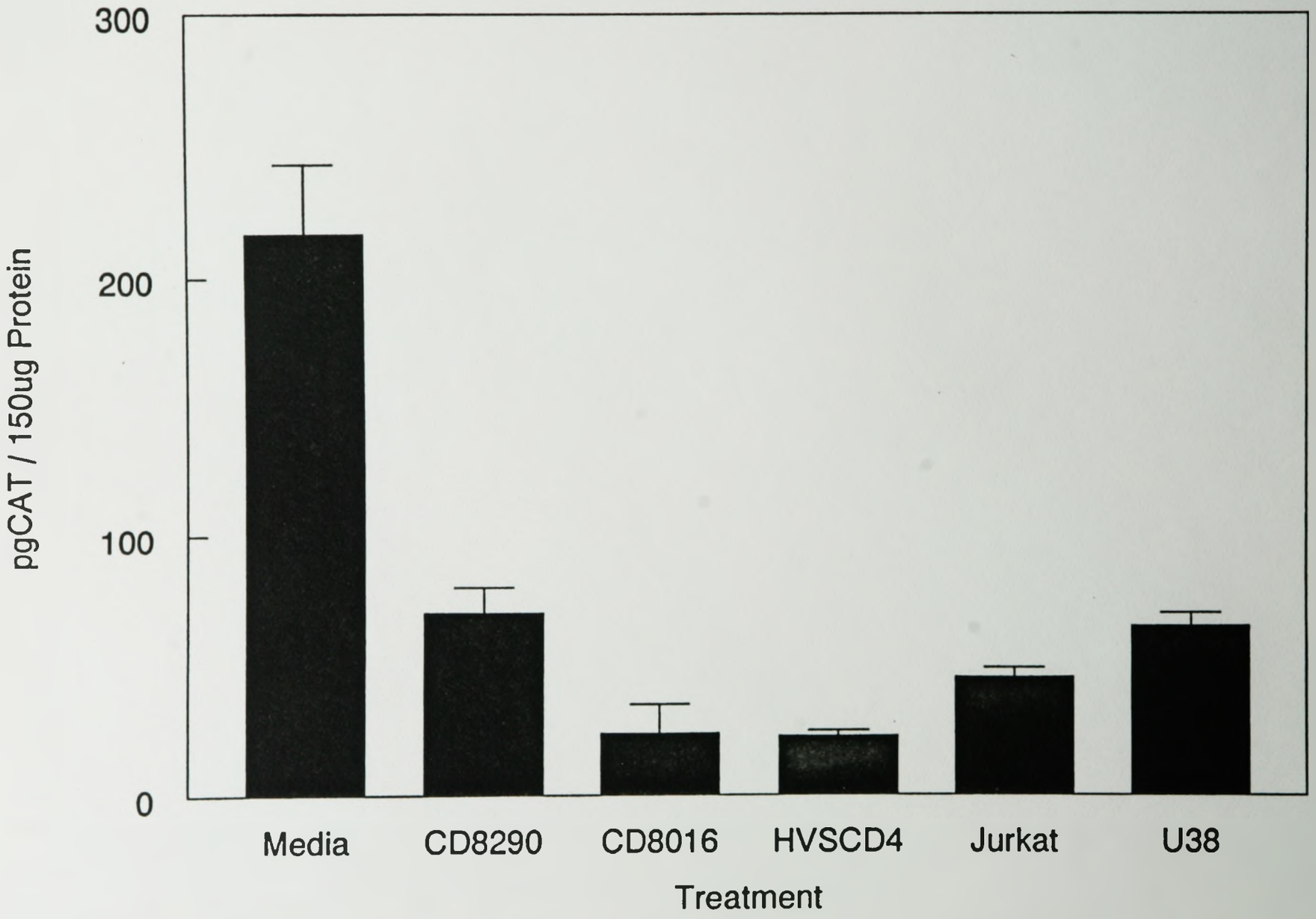

300
200
100
0
pgCAT / 150ug Protein
Media
CD8290
CD8016
HVSCD4
Jurkat
U38
Treatment

Figure 7. *Suppression of HIV-1 LTR-mediated gene expression by TNF-alpha and IFN-gamma.*

Compared to media alone control (Media) IFN-gamma and TNF-alpha suppress HIV-1 LTR-mediated gene expression. At this dose (100U/ml) suppression mediated by IFN-gamma is approximately equivalent to that mediated by CD8290 (HVS-transformed $CD8^+$ T cell line). This experiment is representative of two showing the same result, and data points are indicated as means of triplicates (+/- standard error).

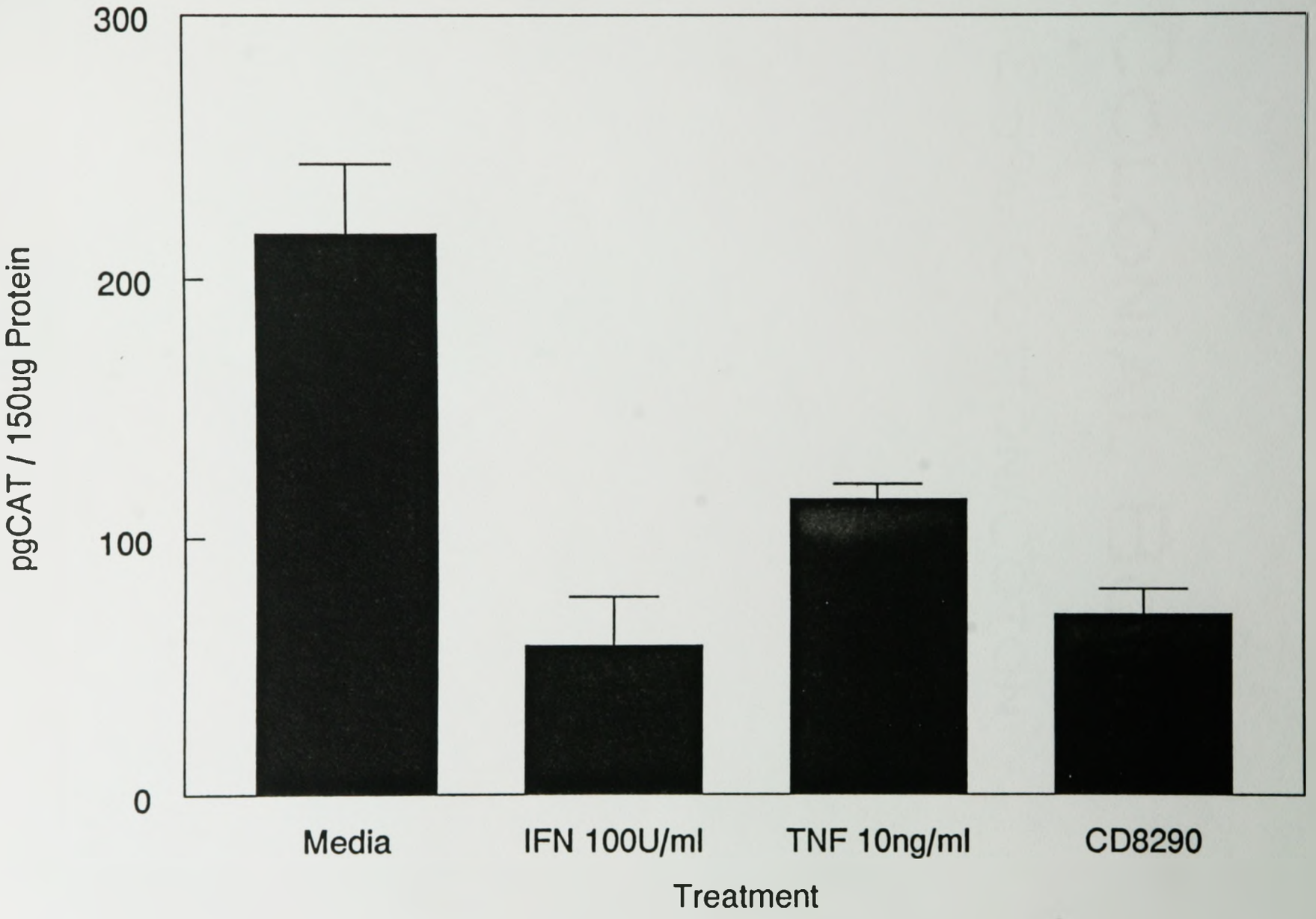
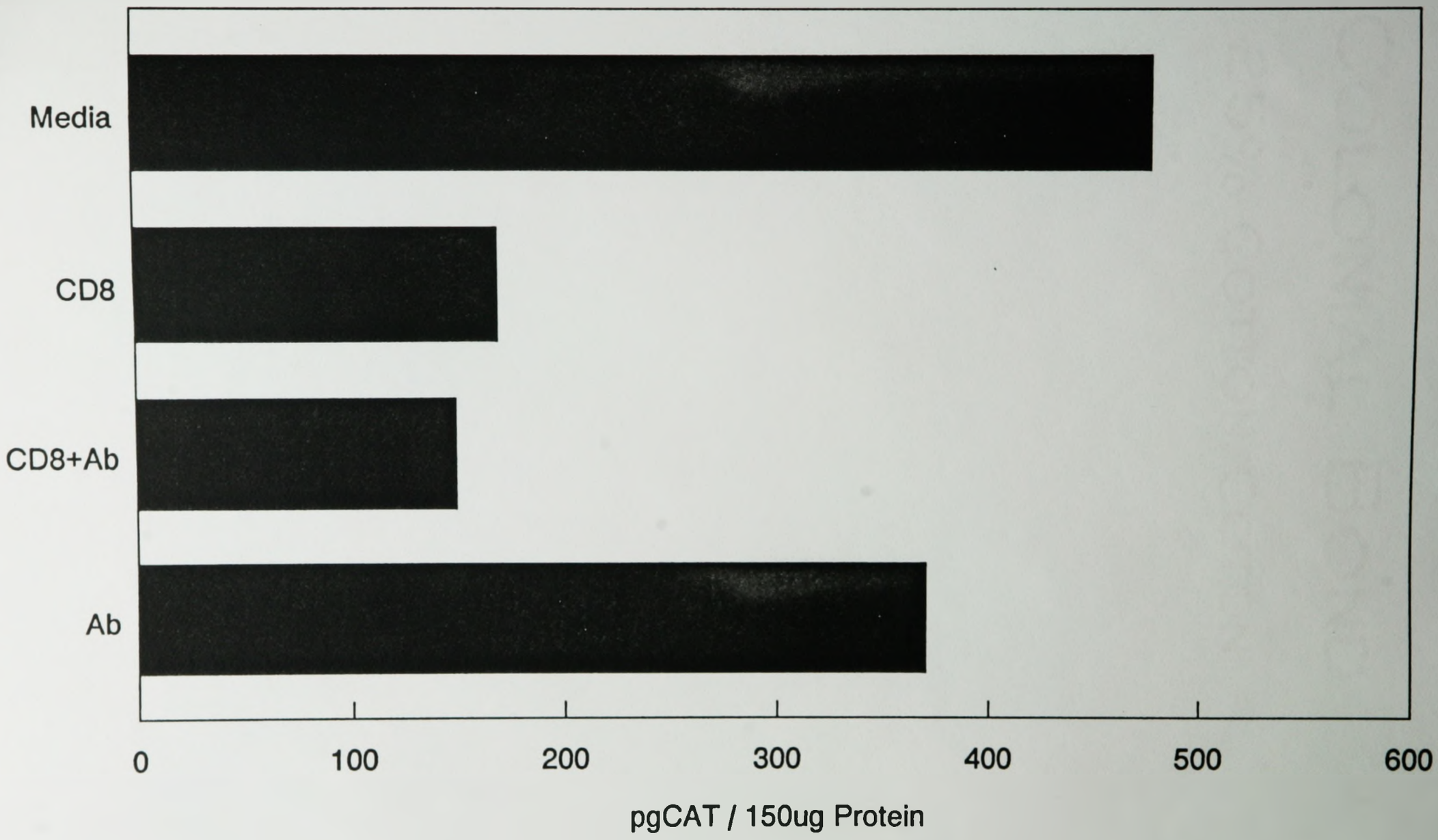
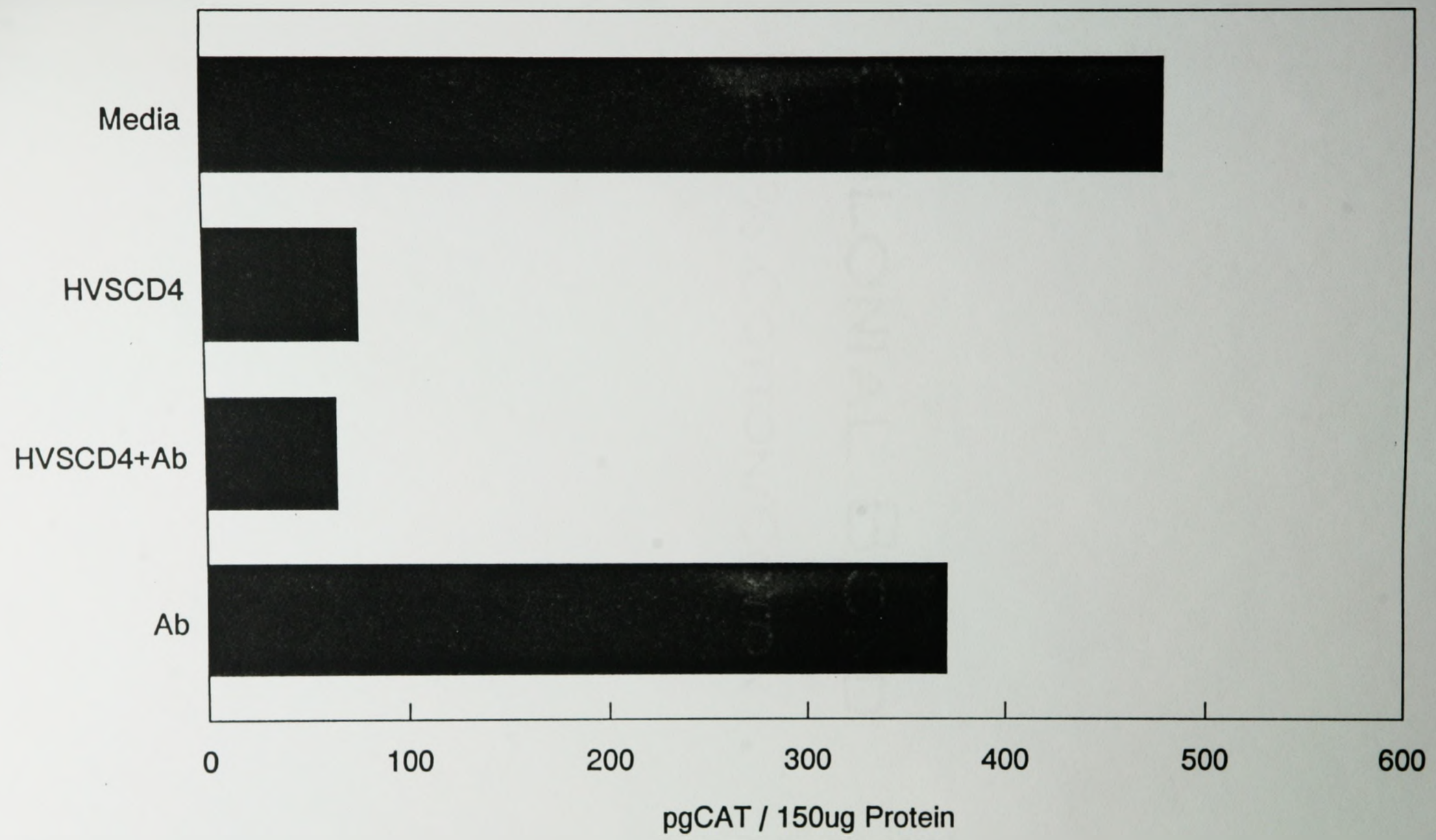

300
200
100
0
pgCAT / 150ug Protein
Media
IFN 100U/ml
TNF 10ng/ml
CD8290
Treatment

Figure 8. *Effect of anti-hsTNF-RI and RII antibodies on $CD8^+$ T lymphocyte mediated suppression of HIV-1 LTR-driving CAT.*

Pre-incubation of $CD8^+$ T lymphocyte-derived supernatants from an HIV^+ patient (Fig. 8a), a HVS-transformed $CD4^+$ T cell clone (Fig. 8b) and a HVS-transformed $CD8^+$ T cell clone (Fig 8c) with antibodies directed against hsTNF-RI and RII (10ug/ml each) dose not abrogate the respective HIV-1 LTR-suppressive effects of these supernatants. Each experiment is representative of two showing the same effect, and data points are averages of duplicates.


Media
CD8
CD8+Ab
Ab
0
100
200
300
400
500
600
pgCAT / 150ug Protein


Media
HVSCD4
HVSCD4+Ab
Ab
0
100
200
300
400
500
600
pgCAT / 150ug Protein

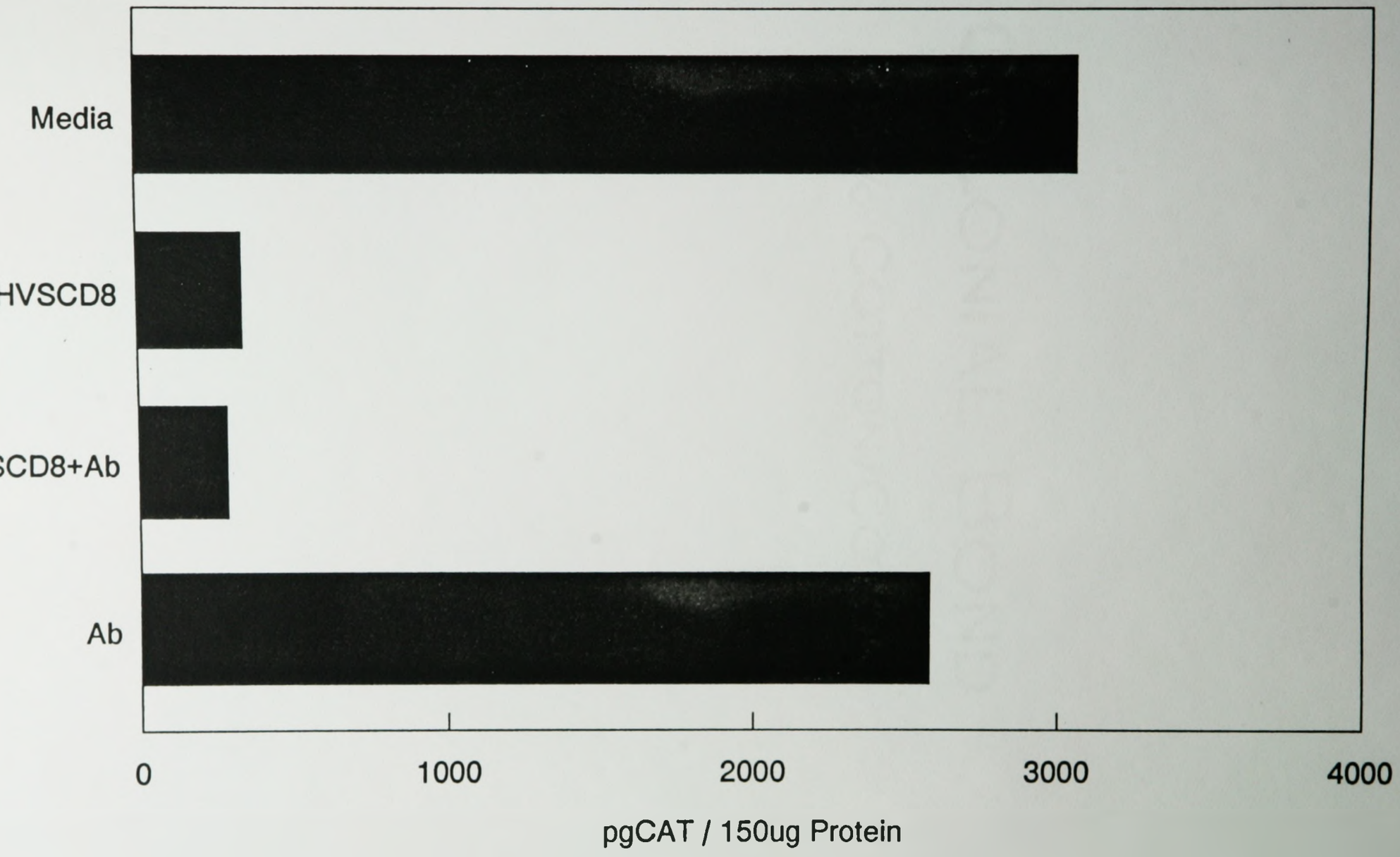

Media
HVSCD8
HVSCD8+Ab
Ab
0
1000
2000
3000
4000
pgCAT / 150ug Protein

Figure 9. *CD8-derived suppression of HIV-1 LTR-mediated gene expression in $CD4^+$ primary cultures.*

$CD4^+$ T lymphocytes from an HIV^- laboratory worker were transfected with HIV-1 LTR-driving CAT using a modified DEAE dextran method. The expression of HIV-1 LTRCAT was significantly suppressed ($p<.05$) by treatment with HVS-transformed T cell line-derived supernatants. Means of triplicates (+/- standard error).

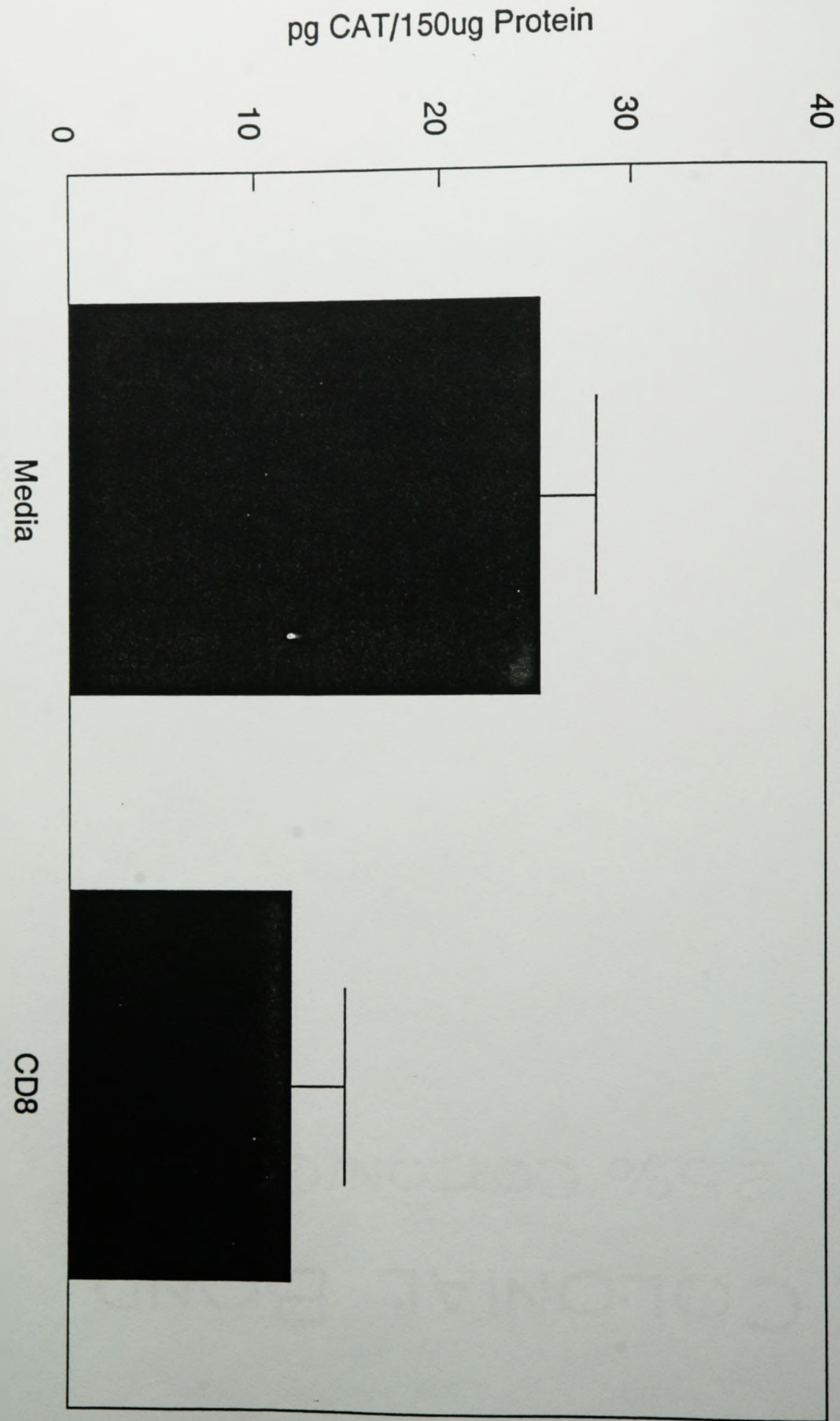
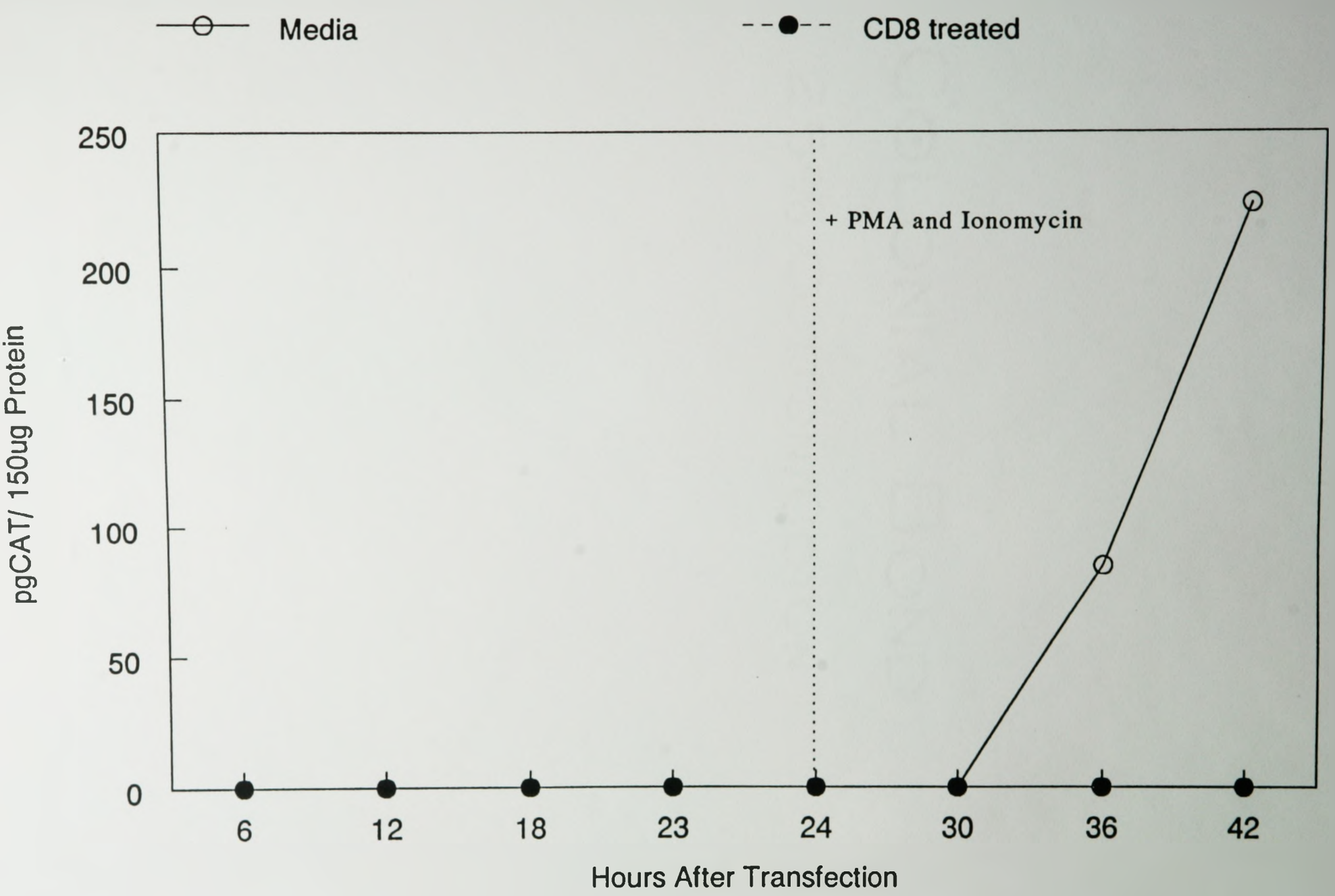

pg CAT/150ug Protein
0
10
20
30
40
Media
CD8

Figure 10. *Time-course of HIV-1 LTR-mediated gene expression in Jurkat T cells*

Jurkat T cells transfected with HIV-1 LTR-driving CAT were removed from culture every six hours and assayed for expression of CAT(Fig. 10a). Jurkat T cells were also treated with suppressive supernatant at various time points prior to (Exp. 1 and 2), and after (Exp. 2 only), stimulation with PMA and ionomycin (Fig. 10b). Cultures treated with $CD8^+$ T lymphocyte-derived supernatants from an HIV^+ patient are indicated in the open circles; untreated in closed circles. All data points are averages of duplicates.


Media
CD8 treated
250
200
150
100
50
0
pgCAT/ 150ug Protein
+ PMA and Ionomycin
6
12
18
23
24
30
36
42
Hours After Transfection

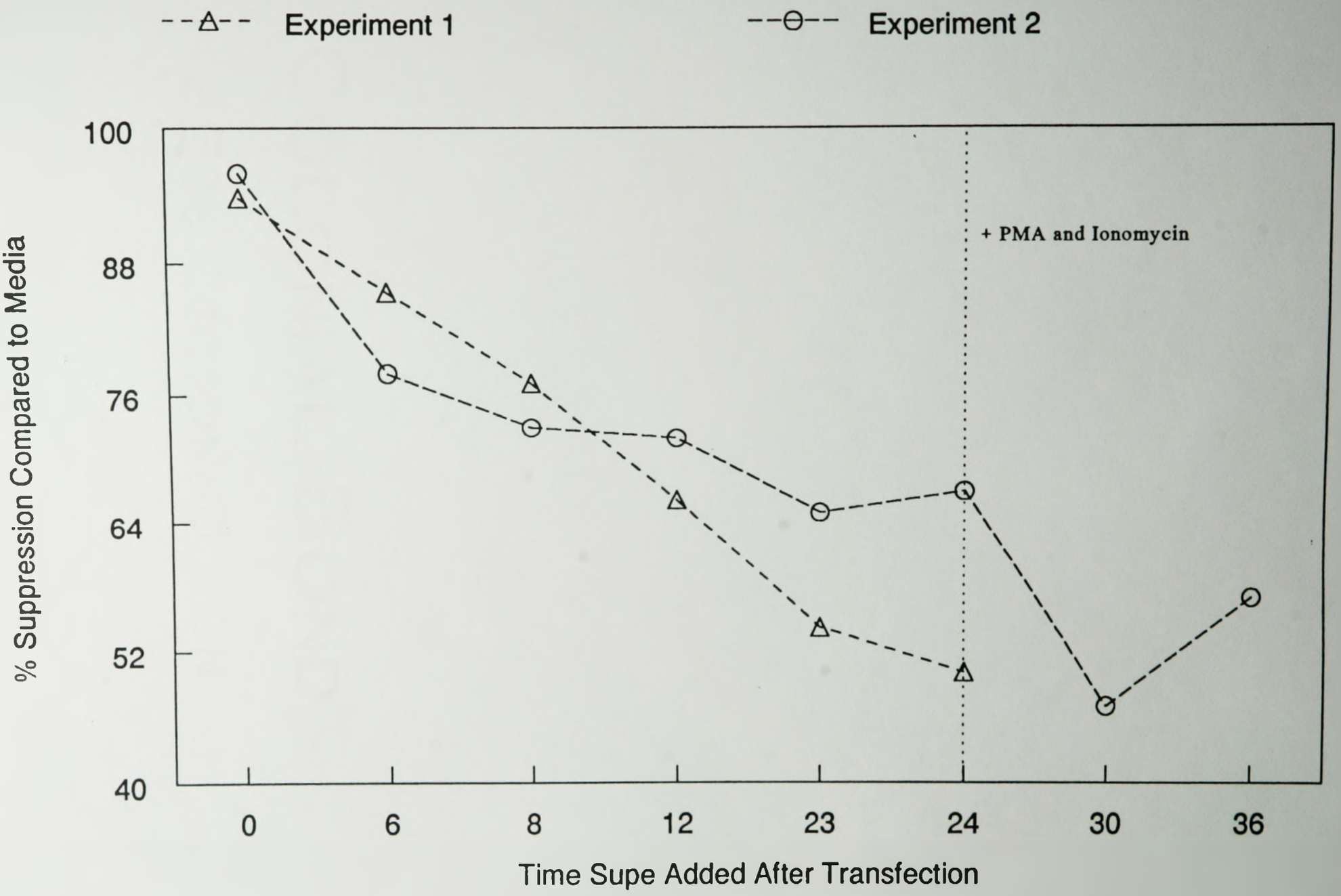
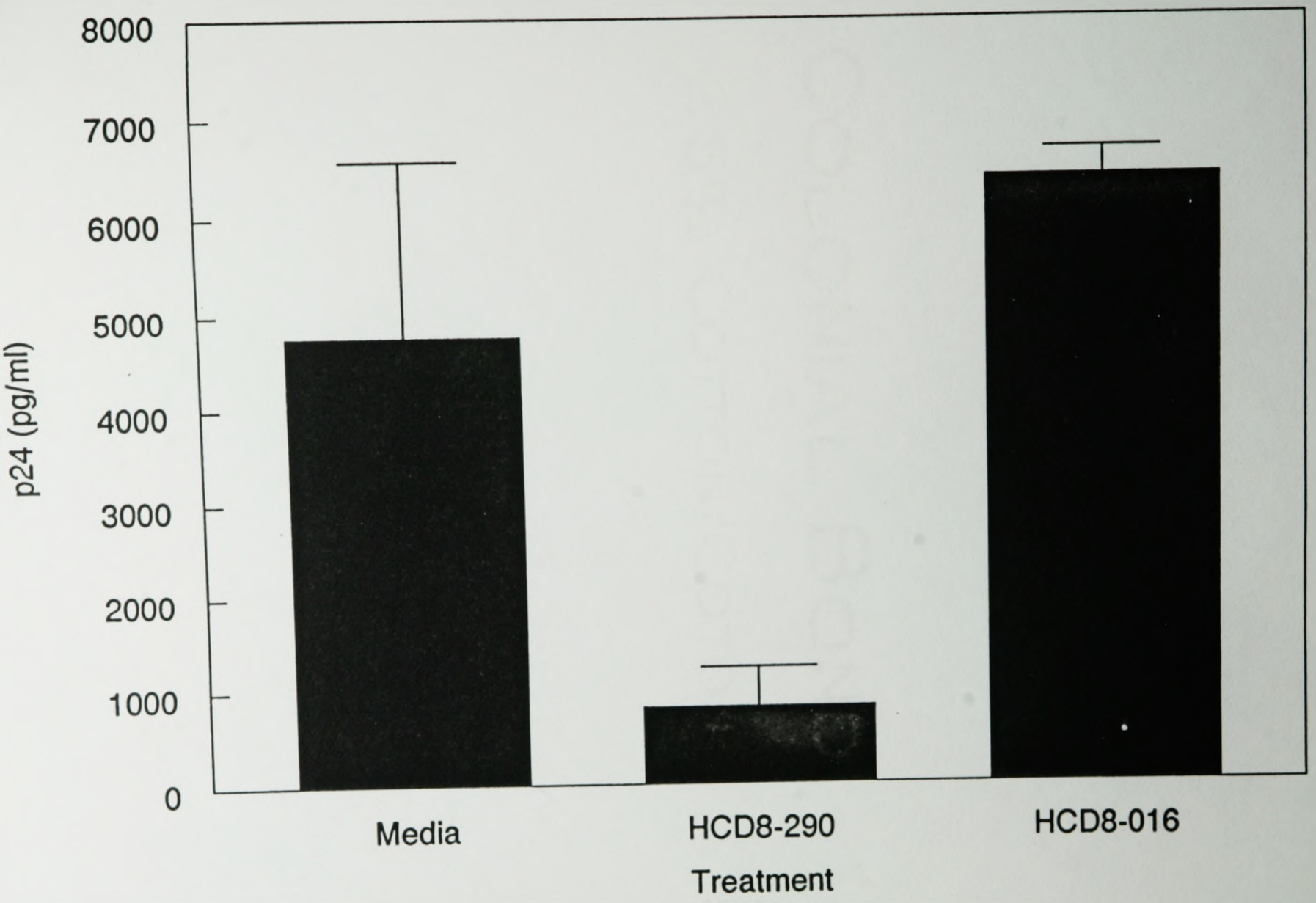

Experiment 1
Experiment 2
100
88
76
64
52
40
% Suppression Compared to Media
+ PMA and Ionomycin
0
6
8
12
23
24
30
36
Time Supe Added After Transfection

Figure 11. *Suppression of replication of HIV-1 by CD8-derived supernatants and direct cocultures.*

Replication of HIV-$1_{B\text{-}al}$ was suppressed by supernatants derived from HVS-transformed lines CD8290, but not CD8016 (Fig. 11a). Suppression of replication by coculture at ratios of 1:1 or 1:2 (Fig. 11b) resulted in 100-1000 fold greater suppression of p24 than supernatants alone. Data points are means of triplicates (+/- standard error).


8000
7000
6000
5000
4000
3000
2000
1000
0
p24 (pg/ml)
Media
HCD8-290
HCD8-016
Treatment

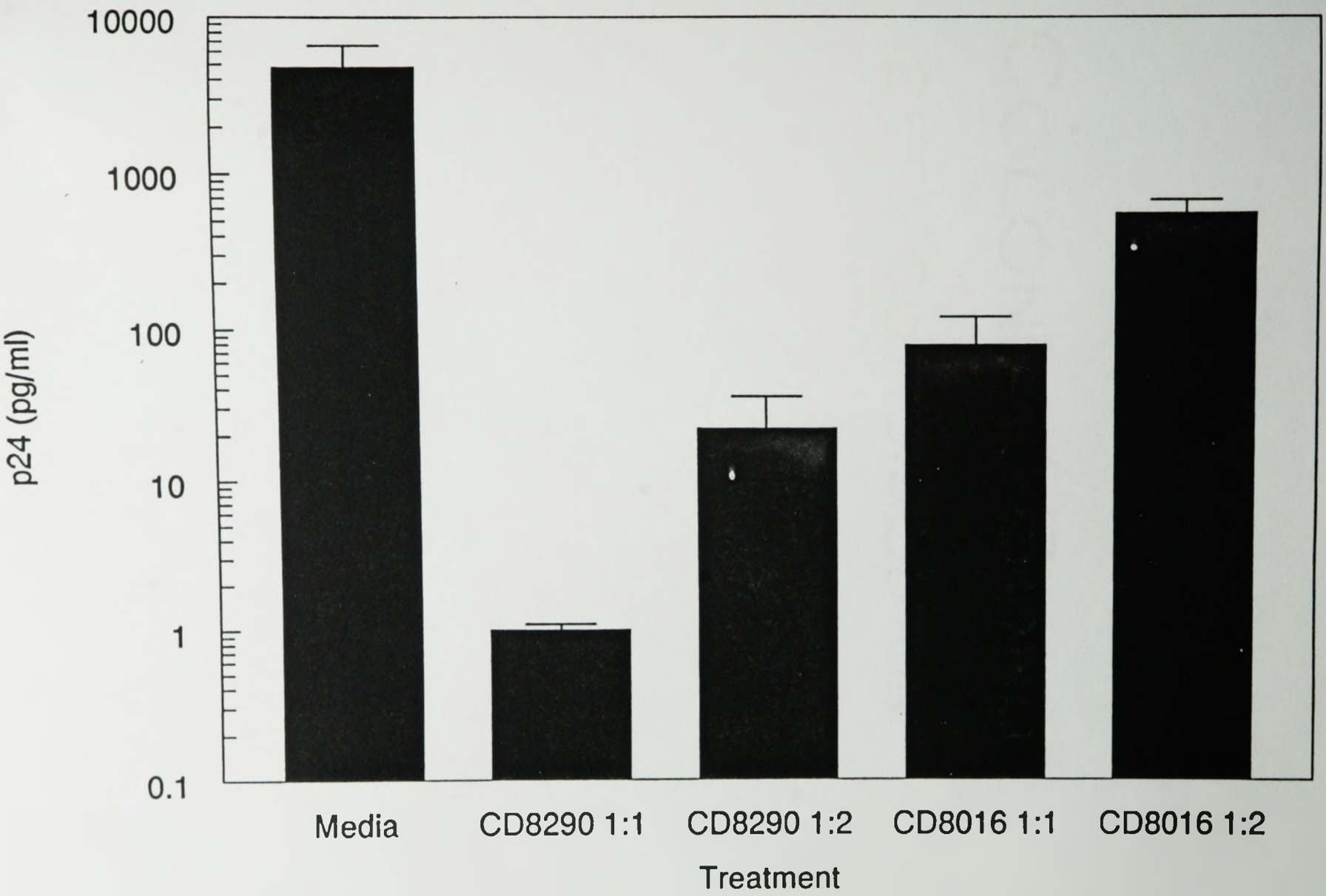
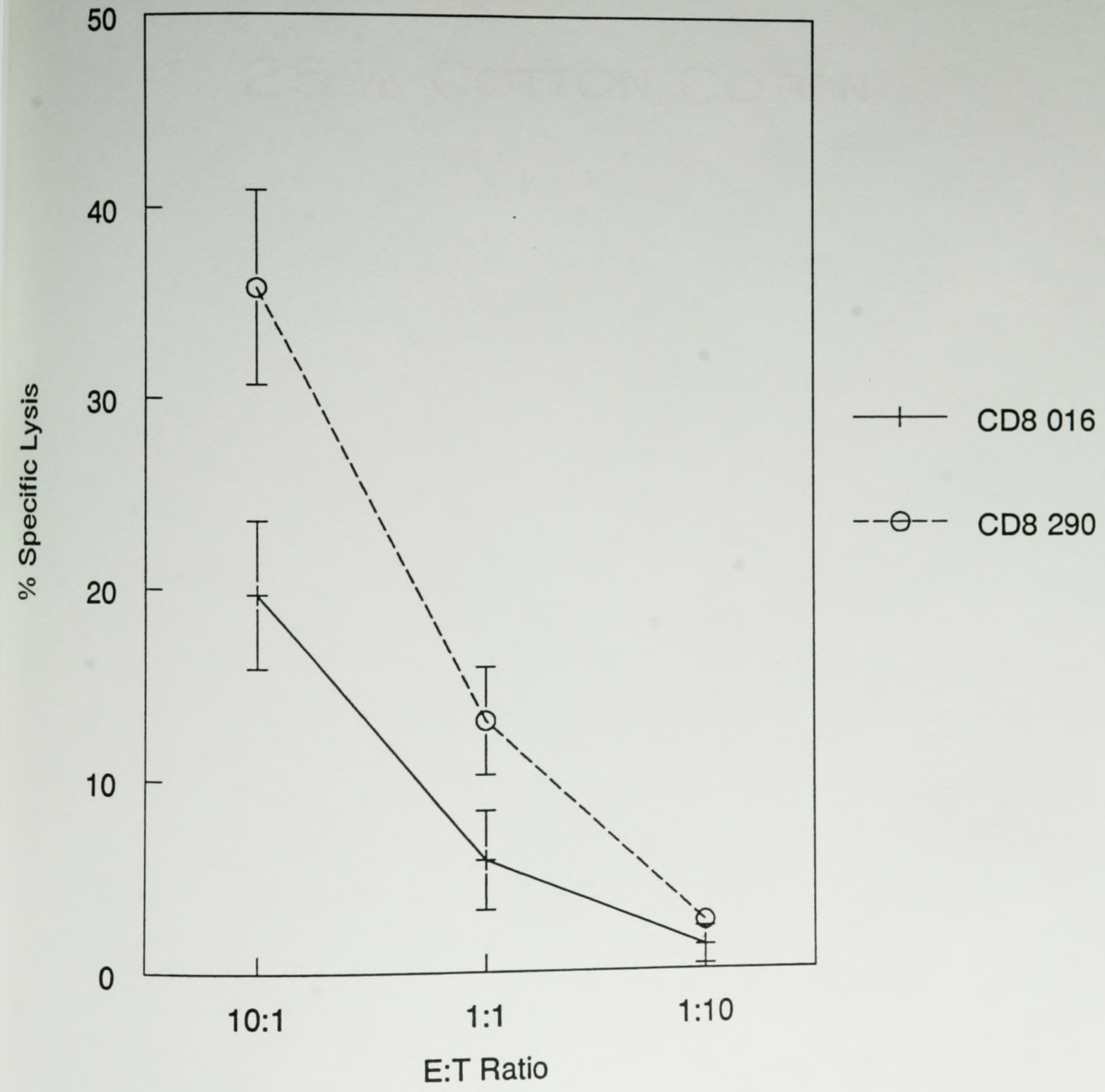

10000
1000
100
10
1
0.1
p24 (pg/ml)
Media
CD8290 1:1
CD8290 1:2
CD8016 1:1
CD8016 1:2
Treatment

Figure 12. *Redirected lysis by HVS-transformed $CD8^+$ T cell lines.*

Specific lysis of P815 mastocytoma target cells at various effector target ratios (10:1, 1:1 and 1:10) shows that CD8016 and CD8290 HVS-transformed T cell lines (as indicated) are capable of cytolytic activity. This experiment confirms results obtained in one preliminary experiment, and data points are shown as averages of triplicates (+/- standard error).


50
40
30
20
10
0
% Specific Lysis
10:1
1:1
1:10
E:T Ratio
CD8 016
CD8 290

CHAPTER FIVE

DISCUSSION

An understanding of the importance of non-cytolytic suppression of HIV-1 gene expression and replication to *in vivo* pathogenesis has become increasingly important, especially as we become more aware of the limits to classical cytolytic and humoral immune responses. This thesis, in contributing to the ever expanding pool of information concerned with non-cytolytic suppression of HIV-1, has likely posed more questions than it has answered. However, several key findings have been made and are thus important to highlight and further discuss.

$CD8^+$ T lymphocyte-derived HIV-1 LTR-suppressive factors are distinct from the CC chemokines RANTES, MIP-1α and MIP-1β

Since submission of the paper forming Chapter 2, several additional studies have helped to clarify the role of RANTES, MIP-1α and MIP-1β in abrogating HIV-1 replication. Initially reported by Cocchi et al. to be active at the level of RNA transcription, these CC chemokines now appear to be restricted in their effector functions to extracellular steric hindrance of virus envelope-mediated fusion events with target cells (Rucker, 1996, and Picard, 1997) . Thus, in the HIV-1 LTRCAT system (described in this thesis) confirmation of these results did not occur. As Chapter 2 discusses, although present in comparatively low (<100ng/ml) concentrations in HIV-1 LTR-suppressive $CD8^+$ T lymphocyte supernatants, RANTES, MIP-1α and MIP-1β do not suppress HIV-1 LTR-mediated gene expression, and polyclonal neutralizing antibodies do not abrogate LTR-mediated effects by either patient-derived $CD8^+$ T lymphocyte or HVS-transformed $CD8^+$ clone supernatants. Following submission of this manuscript (Ch. 2) two separate groups led by the original pioneers in this field, Jay Levy and Chris Walker, reported that the CC-chemokines are able to mediate HIV-1 suppressive effects, but that there are HIV-1

strains which are susceptible to suppression by $CD8^+$ T lymphocytes, but not RANTES, MIP-1α and MIP-1β (Mackewicz, 1996, Palliard, 1996).

Other groups have since demonstrated that, in a variety of virus replication systems (both monocyte / macrophage and $CD4^+$ T lymphocyte), the β-chemokines are able to block replication of some, but not all M-tropic isolates, and no T-cell tropic viruses (Oravecz, 1996, Barker, 1996, Kinter, 1996, Moriuchi, 1996, and Rubbert, 1997). Thus, unlike the broad specificity of the remaining unidentified CD8-derived factors, the CC-chemokines exert their HIV-suppressive effects by blocking tropism-specific regions involved in target cell-virus fusion events, and not (as Chapter 2 confirms) gene expression of HIV-1.

CD8 and CD4-derived suppressive factors are active against both transcription and replication of HIV-1, in Jurkat T cells and primary $CD4^+$ T lymphocyte cultures (CD8-derived factors only)

Although seemingly a reasonable assumption, suppression of transcription of HIV-1, as measured by model systems, is not necessarily related to modulation of whole virus replication. In addition to the artificial nature of reporter gene systems, the multiple targets of inhibition or enhancement found in systems of virus replication make this assumption important to prove when considering the effects of suppressive factors derived from $CD8^+$ or $CD4^+$ T lymphocytes. The data shown in Chapter 3 (Table II) confirms an observation similar to that made by Mackewicz, et al. (for HIV^+ patient $CD8^-$ T lymphocyte-derived supernatants; Mackewicz, 1995), that HVS-transformed lymphocyte-derived supernatants contain factor(s) capable of suppressing HIV-1 gene expression and replication. Although further work is required to

determine if the same active factor(s) concurrently suppress virus replication and gene expression, if the opposite result had been obtained from these experiments, the relevance of the HIV-1 LTRCAT system would have been in doubt. It should also be noted that due to the multiple targets (outlined in Ch. 1) for suppression of PMA and ionomycin induced transcription including the nuclear factors NFAT and NFκB, and trans-activating Tat, it is quite possible that the observed suppression of replication and transcription are coincident. Again, clarification of questions relating to this complex mechanism first requires isolation of all suppressive factors active in both systems of replication and transcription.

We have also demonstrated (Ch. 4, Fig. 9) that the HIV-1 LTRCAT construct can be transfected into primary $CD4^+$ T lymphocytes, and that subsequent *trans*-activation by viral Tat plus Pi stimulation is suppressed by supernatants from an HIV^+ patient $CD8^-$ T lymphocyte culture. As there have been no previous descriptions of suppression of HIV-1 LTR-driven gene expression in primary $CD4^-$ T lymphocytes, this result demonstrates for the first time that similar to Jurkat T cells, a pathway exists in these cells, allowing transduction of negative-regulatory signal(s) to the HIV-1 LTR. This preliminary experiment shows the potential feasibility of transfecting HIV-1 infected primary $CD4^+$ T lymphocytes, which could then serve as a more physiologically relevant model of HIV-1 gene expression and replication.

HIV-1 LTR-suppressor factors are produced by CD8-negative leukocyte subsets

The limited number of factors which are capable of modulating HIV-1 LTR-mediated gene expression (compared to those active at all steps in the virus life-cycle) would imply that the number of active factors, and thus their source cell-type is limited as well. However, observations outlined in Chapter 4 (Fig. 6) indicate that in addition to the cell types mentioned in Chapter 3 ($CD4^+$, $CD8^+$, and PBL from HIV^-/- patients), conditioned media taken from cultures of Jurkat T cells and U38 monocytes also suppress HIV-1 LTR-mediated gene expression. This should not be surprising because many cytokines are produced by both activated T lymphocytes ($CD4^-$ and $CD8^+$) and macrophages. Nevertheless, considering the apparent ubiquity of the HIV-1 LTR-suppressive factor(s), and that their activity is neither specific to lentiviruses (Copeland, 1995), nor induced by *in vivo* priming by HIV-1 (Ch.3, Table I), all would suggest that the unidentified factor(s) have other important, conserved roles in the immune system. These roles will be important to ascertain before development of a therapeutic form of these HIV-1 LTR-suppressive factor(s).

Suppression of the HIV-1 LTR is mediated by an acid-stable molecule of variable molecular weight

Initial chromatographic studies indicated that fractionation of HVS-transformed $CD8^+$ T lymphocyte supernatants yielded a peak of HIV-1 LTR-suppressive factors appearing at ~1:1 ratio of ddH_2O:acetonitrile, which co-migrated with large concentrations of protein. Changing columns (from C18 reverse phase to size-exclusion) and mobile phases (to PBS), again yielded recoverable activity, and demonstrated to have an approximate molecular weight of 50-60kd. In

acid conditions (pH1.6), this estimate, based on molecular weight standards, is two-fold lower, from 20-30kd. This effect is similar to that noted for size exclusion chromatographic separation of IL-16 (Baier, 1995), but presumably reducing conditions would cause similar changes in the migration (through a gel-matrix) of any dimeric / trimeric protein of this approximate molecular weight. As mentioned in Chapter 1, IL-16 has been discounted by Mackewicz et al., as sharing identity with the replication suppressive factor(s) active in their system; and unlike the LTR-suppressive factor(s) measured in our system (Chapter 4, Fig. 10a and 10b), IL-16 requires pre-incubation with the target cell for 24 hrs to exert its effects.

A final consideration when judging the success or failure of a separation protocol is the yield of active factor(s). Although experiments directly examining the loss of activity at each point in the fractionation protocols have not been conducted, a conservative estimate of 50% activity loss supports the utility of these materials and methods for preliminary fractionation of HIV-1 suppressive factors from other components of HVS-transformed $CD8^-$ T lymphocyte-derived supernatants. Further experiments to determine the maximum load volume of this column (200ul was the injector limit) could increase the yield of active factor(s) using these systems.

HIV-1 LTR-suppressive activity is shared with known cytokines

Clearly the most important question to resolve before continuing with a large-scale purification protocol is whether or not the factor(s) active in the HIV-1 LTR reporter gene system are known cytokines. To answer this question with respect to the factor(s) active in their system, Carl Mackewicz and Jay Levy conducted an extensive study of all major cytokines: IL-1 to 12,

TNF-α,β, IFN-γ,α,β, Transforming Growth Factor (TGF)-β, and Granulocyte and Granulocyte Monocyte -Colony Stimulating Factors (G-CSF and GM-CSF). These studies have been rextended to include the CC-chemokines, hsTNF-RI and II, IL-16 and other cytokines, but the data thus far has been reported only as "unpublished data" (Levy, 1996). The original study not only did not consider combinations of cytokines (as would be expected in a culture supernatant), but measured replication, which as we have demonstrated is susceptible to multiple levels of regulation not necessarily shared by proviral gene expression. Nevertheless, there are a few candidates which may contribute partially or wholly to the effect seen in our system. These include IL-16, TNF-α and IFN-γ.

As mentioned in Chapters 1 and 3, at first described for its ability to suppress HIV-1 LTR by blocking a CD4-dependent binding step (Baier, 1995), IL-16 has more recently has been reported to act through the HIV-1 LTR NFκB elements to suppress HIV-1 replication (Maciaszek, 1997). If it were not for differences in the kinetics of this activity (requirement for 24hr pre-incubation), this activity is very similar to that noted in our system. In addition, although not constitutively produced by $CD4^+$ T lymphocytes, IL-16 is produced under mitogenic stimulation (Center, 1996). However, this same group , along with Mackewicz and Levy, have reported that antibodies to human IL-16 did not abrogate the suppressive effect mediated by $CD8^-$ T lymphocytes, and very high doses of recombinant IL-16 were required to cause only a moderate reduction in reverse-transcriptase activity (1μg protein for 45% decrease from control) (Mackewicz, 1996). Thus, although it is possible that IL-16 may be active in some systems measuring HIV-1 LTR-mediated gene expression, it may not have as powerful effects in

some systems of whole virus replication. Clearly, the results reported by these two groups would be important to confirm.

The role that TNF-α has in the overall suppression mediated by both $CD4^+$ and $CD8^+$ T lymphocytes observed in our system is much more complex. TNF-α is found in elevated levels in the lymph nodes of HIV^+ individuals (Graziosi, 1994) and is well established to induce HIV-1 LTR-mediated transcription (Duh, 1989) and synergize with IL-6 to upregulate HIV-1 replication (Poli, 1990). In addition, TNF-α production is increased by infection of PBMC with HIV-1 (Vyakarnam, 1990), a mechanism which also leads to autocrine up-regulation of HIV-1 replication. However, the observation that 10ng/ml doses of TNF-α suppressed HIV-1 LTR-mediated gene expression made in Chapter 4 is not without precedent. In their study of cytokine control of HIV-1 replication, Mackewicz and Levy reported a dose-dependent dichotomous effect of TNF-α (suppressing at high, enhancing at low doses, Mackewicz, 1994). In addition, Guidotti et al. have observed that through an indirect mechanism, TNF-α in synergy with IFN-γ suppresses Hepatitis B virus gene expression in infected hepatocytes. Although in our system the mechanism of suppression of the HIV-1 LTR appears to be direct blocking of nuclear translocation of cellular transcription factors, it is possible that TNF-α may induce autocrine production of other HIV-1 LTR-suppressive factor(s).

Similarly, IFN-γ has been reported to have either enhancing or suppressing effects on HIV-1 replication, depending on the assay system (reviewed by Poli, 1994, and Mackewicz, 1994). Although there have been no reports of direct HIV-1 LTR-suppressive effects, IFN-γ has been reported to reduce the infectivity of HIV particles by stimulating oxygen metabolite production by monocytes (Ennen, 1993). Thus, the suppressive effect observed in Chapter 4 may

be the result of indirect changes to the oxidation state of the target cell. Since IFN-γ is also found increased in the lymph nodes of HIV^+ individuals (Graiziosi, 1994), the role that it, along with TNF-α, is important to elucidate. However, such a undertaking would be complicated due to indirect effects mediated by these two cytokines. In addition, with respect to replication, the suppression of NSI and SI isolates of HIV-1 may also be modulated by the chemokines RANTES, MIP-1α and MIP-1β, and Stromal Derived Factor (SDF)-1 in mechanisms which could occur concurrent with, but independent of, suppression of LTR-mediated gene expression (by IL-16, TNF-α or IFN-γ). In this case, suppression of HIV-1 replication, although apparently concurrent with decreased LTR-mediated gene expression, might be coincident rather than the result of factor(s) suppressing replication via a transcriptional mechanism.

As mentioned in the previous section, the variable molecular weights determined by SEC, suggest that the factor(s) active in our system may form dimeric or trimeric molecules dependent on pH. However, more accurate assessments of this characteristic are difficult to make due to the inaccuracy of the SEC system. Thus, matching a known molecule to the factor(s) active in our system based solely on SEC-estimated molecular weight is not possible based on these data. Further fractionation protocols, using ion-exchange chromatography will be useful to clarify the identity of the factor(s) active in our system.

Summary and Conclusions

The data described in this thesis support the primary conclusion that there are as yet unidentified factor(s) which are produced by $CD8^+$ T lymphocytes which act via the HIV-1 LTR to suppress gene expression. The active factor(s) are fractionable by standard biochemical

techniques and have an apparent molecular weight of 50-60kd at physiological pH, but 20-30kd at acidic pH. Although a few known cytokines can mediate suppression of the HIV-1 LTR, this effect is not mediated by the CC-chemokines RANTES, MIP-1α and MIP-1β. In addition, $CD4^+$ T lymphocytes produce factor(s) which also suppress LTR-mediated gene expression and replication of SI and NSI HIV-1 isolates. Finally, $CD8^+$ T lymphocyte cell lines produce soluble HIV-1 LTR-suppressive factor(s) which also suppress HIV-1 replication by more powerful, lytic mechanisms.

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