ROLE OF SEXUALLY TRANSMITTED INFECTIONS IN ACCELERATED HIV DISEASE PROGRESSION

BY

DR. AGGREY O. ANZALA

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

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AGGREY O. ANZALA

A Thesis/Practicum submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

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ABSTRACT

The course between initial infection with human immunodeficiency virus type 1 (HIV-1) and the development of acquired immunodeficiency syndrome (AIDS) is highly variable. We have previously shown that over 50% of the women in the Pumwani sex worker cohort in Nairobi, Kenya progress very rapidly to AIDS following HIV-1 infection with median duration of four years. We have also shown that more frequent condom usage within this cohort is associated with a slower disease progression. Several aspects of our study population could account for the more rapid disease progression including high incidence of sexually transmitted infections (STI) experienced by these women. The goal of this project was to determine whether STI influence parameters potentially related to accelerated disease progression including HIV-1 plasma viremia and CD4 T cell count. Thirty-two HIV-1 positive and 10 HIV-1 negative women in the Pumwani cohort were serially seen over a 1-5 month duration. Specimens for STI diagnosis, CD4 and CD8 T cell counts, quantitation of HIV-1 plasma viremia and plasma cytokine concentration were obtained at scheduled visits. Statistical analysis was performed with two sample T-tests. Acute bacterial STI resulted in increased plasma viremia, Th_2 type cytokines, TNF-α and sTNF-α receptor and decrease in CD4 T cell counts. Evidence from this study suggests that STI influence parameters potentially related to accelerated disease progression and may therefore alter the course of HIV-1 infection.

DEDICATION

This thesis is dedicated to the memory of Ruth Auma, James Nasio, Patrick Nyange, Tom Obongo and Edward Oyugi. All had been indefatigable in their work with AIDS in Kenya.

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Now that the writing is done, I am faced with the task which I fulfill with pleasure, but find by no means easy. I wish to acknowledge with thanks the help of those without whom I would not have undertaken this work. Many hands helped to make this thesis, and their degree of contributions varied. Acknowledgement itself appears casual and says little about the particular individuals whose criticisms, suggestions, research findings, technical assistance and ideas, often expressed in casual conversation, rendered me invaluable service. As I have said, I do not find it easy to record in full my indebtedness to friends and colleagues.

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INTRODUCTION

A. OVERVIEW

No group of viruses has received as much attention from scientists and the general public in recent years as retroviruses and, in particular, the human immunodeficiency virus type 1 (HIV-1), the etiologic agent of the acquired immunodeficiency syndrome (AIDS). The intense scrutiny given these agents reflects their importance as human pathogens.

It is estimated that by the year 2000, HIV-1 will have infected 40-110 million people worldwide (Greene WC, 1993). Ninety percent of all these infections will have occurred in developing countries (*Current and Future Dimensions of HIV/AIDS Pandemic*, WHO, 1992). The epidemiology of HIV-1 in developing countries reflects powerful social and economic factors. HIV-1 seroprevalence is generally highest in urban centres (Nzilambi N, et al., 1988), along trade routes (Carswell JW, et al., 1989), among commercial sex workers (CSW) (Plummer FA, et al., 1991; Simonsen JN, et al., 1990; Bonacci MA, 1992), and among male migrant workers.

According to current understanding, most of those infected will, on average, develop AIDS within ten years and die within 2-3 years of diagnosis (Rutherford GW, et al., 1990; Hessol NA, et al., 1994). Since the most economically productive portion of the population (ages 15-49) has the highest prevalence of HIV-1 infection (DeCock KM, et al., 1990), deaths due to HIV/AIDS will have dramatic economic and social effects, based on socioeconomic models (Lurie P, et al., 1995; Potts M, et al., 1991). Despite widespread public awareness, public health education, and control programmes,

the rate of HIV-1 spread worldwide has continued to increase, especially in subSaharan Africa and Southeast Asia (Blattner WA, 1991; Merson MH, 1993; Peter L, et al., 1995). The continuing spread of HIV-1 in poor countries highlights the need for effective prevention and therapeutic strategies. Currently, scientists are engaged in understanding the natural history of HIV-1 infection. The mechanisms involved in disease progression have been a major focus of research (Els Hogervorst, et al., 1995; Connor RI, et al., 1993; Saag MS, et al., 1991). Results of longitudinal cohort studies have shown that progression to disease is very variable and is associated with several immunologic and virologic parameters. The exact role played by the virus and the host in the course of HIV-1 infection is unclear. Identification of factors that correlate with, and possibly contribute to, the outcome of HIV-1 infection may be important in explaining the variable course between initial infection and the development of AIDS; and possibly enhance the management of those infected with HIV-1. In Nairobi, Kenya, we have been engaged in understanding the epidemiology of sexually transmitted infections (STI), including HIV-1, and the natural history of HIV-1 infection in a sex worker cohort. We have shown that the incubation time to AIDS in this cohort is very short (median 4 years) (Anzala AO, et al., 1995). This thesis addresses the question: Is the rapid progression to disease experienced by this cohort a result of the heavy burden of STI and is this effect a result of an altered immunoregulatory network in which uncontrolled HIV-1 replication occurs?

B. DISCOVERY OF THE AIDS VIRUS

In the early 1980s, the Centers for Disease Control and Prevention (CDC) in the United States started reporting of cases of *Pneumocystis carinii* pneumonia (PCP) and generalized persistent lymphadenopathy in otherwise healthy men (CDC, 1981). Initial cases were among homosexual men and intravenous drug users (Gottlieb MS, et al., 1981; Siegal FP, et al., 1981). By the end of 1982, PCP and other conditions associated with immunodeficiency appeared in Haitian immigrants, haemophiliacs, blood transfusion recipients, sex partners of these patients, and children born to mothers at risk. All these observations pointed to a transmissible agent spread through genital secretions and blood, and efforts at isolating and identifying an etiologic agent intensified.

The first indication that this syndrome could be caused by a retrovirus came in 1983 when Barre-Sinoussi, working with Montagnier at the Institute Pasteur, isolated a reverse transcriptase containing virus from the lymph nodes of a patient who had presented with persistent lymphadenopathy syndrome (LAS) (Barre-Sinoussi F, et al., 1983). In 1984, Robert Gallo (Gallo RC, et al., 1984; Popovic M, et al., 1984) and Jay Levy (Levy JA, et al., 1984) also isolated a retrovirus. Robert Gallo named it human T-lymphotropic virus type III (HTLV-III) while Jay Levy named his isolate AIDS-associated retrovirus (ARV). Thus, the three isolated, newly identified retroviruses had similar characteristics which included substantial growth in PBMC, the killing of CD4+ lymphocytes, and the inability to immortalize CD4+ lymphocytes. Most importantly, infection by these viruses, as described at that time, was restricted to AIDS patients. Over time, the three isolates (LAV, HTLV-III and ARV) were recognized as members

of the same group of retroviruses and their properties identified them as lentiviridae. Their genomic organization showed only remote similarities to that of HTLV and their proteins were also distinct from those of HTLV. For all these reasons, in 1986, the International Committee on Taxonomy of Viruses recommended giving the AIDS virus a separate name, the human immunodeficiency virus (HIV) (Coffin JM, et al., 1986). HIV isolates were subsequently recovered from the blood of many patients with AIDS, as well as from the peripheral blood mononuclear cells (PBMC) of several clinically healthy individuals (Levy JA, et al., 1985; Salahuddin SZ, et al., 1985). The widespread transmission of this agent was appreciated. Shortly after the discovery of HIV-1, a second type of the AIDS virus was recovered in Portugal from patients with AIDS who had been to West Africa (Clavel FD, et al., 1986). Sequence analysis showed that this new virus differed by more than 55% from the previous HIV-1 virus, thus it was designated as HIV-2. HIV-2 was subsequently isolated from individuals from Guinea Bissau, The Gambia, and the Ivory Coast.

C. THE HIV VIRUS

The human immunodeficiency viruses, HIV-1 and HIV-2, are members of the Lentivirus subfamily of retroviruses. HIV-1 and HIV-2 differ from the previously identified human retroviruses, human T cell leukemia virus types I and II (HTLV-I and HTLV-II), which are members of the oncornaviruses subfamily, in that the lentiviruses result in a characteristic infection and loss of CD4+ lymphocytes (Gottlieb MS, et al., 1981; Stahl RE, et al., 1982).

Other members of the lentiviruses subfamily include the primate lentiviruses, simian immunodeficiency virus (SIV) in African green monkeys (SIV/AGM) and SIV in macaques (SIV/MAC) (Payne SL, et al., 1987; Letvin NL, 1985) and four non-primate lentiviruses, including equine infectious anemia virus (EIAV) in horses (Rice NR, 1989), visna in sheep (Haase AT, 1986), caprine arthritis encephalitis virus (CAEV) in goats (Chin IM, 1985), and feline immunodeficiency virus (FIV) in cats (Pedersen HC, et al., 1987). Although these viruses are quite diverse, there is considerable genetic relatedness between isolates in their reverse transcriptase enzyme (Chin IM, 1985).

HIV is an enveloped virus with particles approximating 100 nm in diameter. Inside the capsid are two identical positive RNA strands. By electron microscopy, virions have a characteristic dense, cylindrical protein core that encases genomic RNA molecules and viral enzymes (reverse transcriptase, integrase and protease). Surrounding the core is the membranous lipid envelope derived, in part, from the host cell membrane. This ultrastructure is indistinguisable from that of other lentiviruses (such as visna virus or equine infectious anemia virus) but is quite different from that of HTLV. Figure 1 shows the HIV virion with the structural and other proteins identified. The genomic size of HIV-1 is about 9.8 Kb with open reading frames coding for several viral proteins. In general, the genome exhibits the (5'-LTR-*gag-pol-env*-LTR-3') organization as shown in Figure 2.

The primary transcript of HIV-1 is a full-length viral mRNA, which is translated into the *gag* and *pol* proteins. The *gag* precursor p55 gives rise by proteolytic cleavage to smaller proteins, capsid protein (p24), nucleocapsid proteins (p9, p6) and the matrix

FIGURE 1. An HIV virion with the structural and other virion proteins identified. gp120 is the major extracellular envelope glycoprotein, gp41 is the transmembrane envelope glycoprotein, p25 (24) is the major core antigen, p17 (18) is the major matrix protein, p66 (RT) is the reverse transcriptase, and p12 are the RNA binding protein.

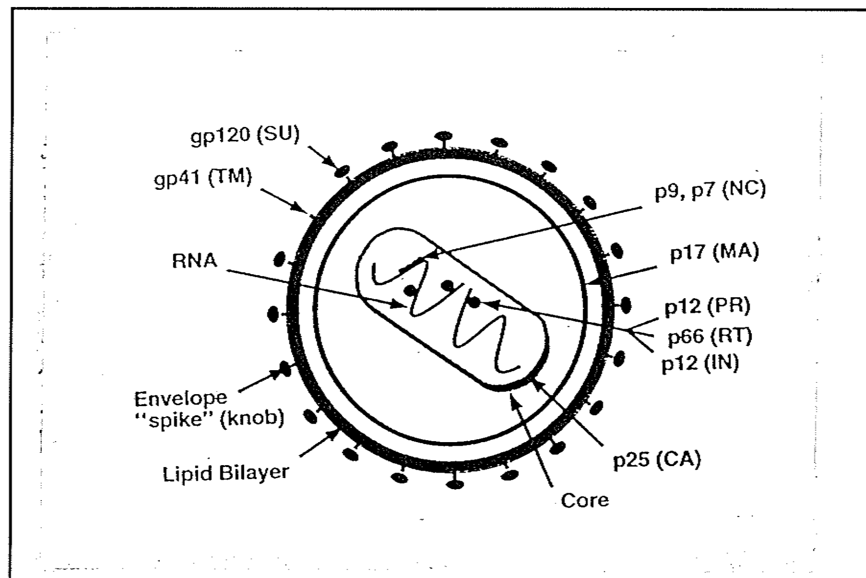
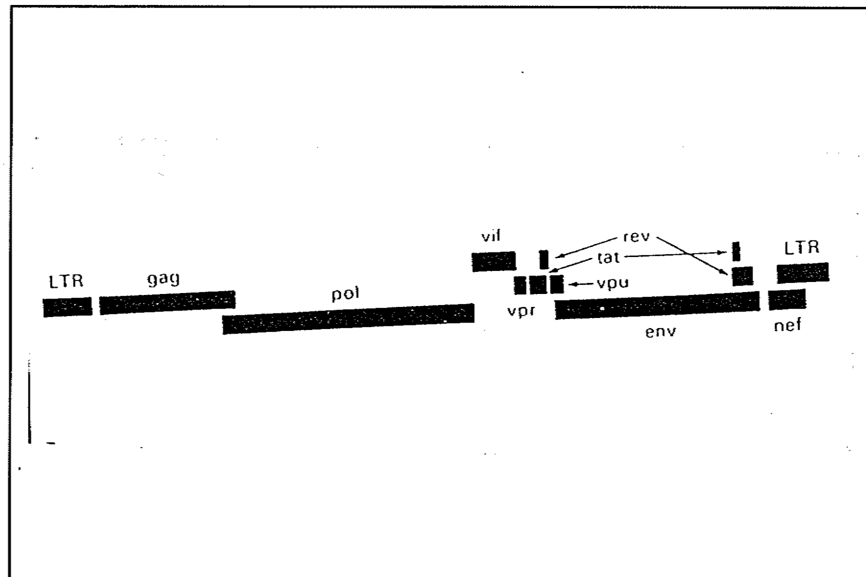


FIGURE 2. Genome organization of HIV-1. The nomenclature and the known properties of these genes are described in the text.



protein (p17). The *pol* precursor protein is cleaved into the reverse transcriptase (RT), the protease (PR) and integrase (IN) proteins. PR processes the *gag* and *pol* polyproteins and IN is involved in virus integration. The *env* precursor gp160 is proteolytically cleaved to generate the envelope gp120 and gp41 proteins (Vaishnav Y, 1992; Greene WC, 1991; Martin M, 1989; Cann AJ, 1989). In addition to the structural genes, HIV-1 has six nonstructural genes: *tat* (transactivator of transcription), *vif* (viral infectivity factor), *vpr* (viral protein R), *vpu* (viral protein U), *rev* (regulator of virion expression), and *nef* (negative effector function). These genes are located between the *env* gene and the 3' LTR region of the HIV genome. *Vif* increases viral infectivity (Strebel K, et al., 1987), *tat* regulates viral transcription (Arya SK, et al., 1985), *rev* regulates viral mRNA splicing, and *nef* functions as post-transcriptional regulator (Luciw PA, et al., 1987). *Vpu* may be important in viral assembly and the exact function of *vpr* remains a mystery (Cohen EA, et al., 1990; Limkait T, et al., 1990).

D. HOST RANGE

D1. Species Specificity

The AIDS pandemic is a new problem for humans, but it is unclear whether HIV-1 is also new to humans. HIV-1 has a very narrow host range, infecting and causing disease only in humans. Chimpanzees can be infected by inoculation and, although seroconversion and viremia readily occur, no infected chimpanzee has developed opportunistic infection, neoplasms or significant immunologic abnormalities (Derosiers RC, et al., 1987; Fultz PN, 1986). Several explanations have been provided for these

observations including nonpathogenicity of HIV for chimpanzees, insufficient time for disease to develop, and lack of possible cofactors. Attempts to develop more readily available host models are underway.

D2. Cellular Host Range

Human cells susceptible to HIV-1 infection (cellular host range) are numerous. The virus can be detected by recently improved techniques of cell culture, *in situ* hybridization procedures, immunohistochemistry, electron microscopy, and recently, polymerase chain reaction (PCR) in a wide variety of human cells, but the extent of the infection varies and depends on the particular virus strain used. In general, CD4 T lymphocytes are highly susceptible to HIV-1 infection and are thought to replicate HIV to the highest titres. In the haematopoietic system, studies conducted soon following the discovery of HIV demonstrated that CD4 helper T lymphocytes were the major targets of HIV infection (Klatzmann DE, 1984). Other cells were also found to be susceptible to HIV infection, in particular, the macrophages (Gartner RA, 1986; Levy JA, 1985; Nicholson JK, 1986). Infection of macrophage results in only low levels of virus production and lack of cell death, making macrophages reservoirs of HIV-1 virus in the body. Other cells now known to be infectable include stem cells in the bone marrow, monocytes, B lymphocytes, megakaryocytes, natural killer (NK) cells, eosinophils, thymic epithelial cells, and dendritic cells (Sakagnchi MT, 1991; Patterson S, 1987; Numazaki K, 1989; Freedman AR, 1991; Chehimi J, 1991; Castro BA, 1988).

One of the first breakthroughs in studies of HIV was the identification of CD4 differentiation antigen of the T lymphocytes as the major cell surface receptor for HIV,

which helps explain the depletion of CD4 T helper lymphocytes (Piatak JM, 1993). The CD4 molecule on the surface of a subset of T lymphocytes functions as a ligand for major histocompatibility class (MHC) II molecules during antigen presentation. This molecule belongs to the immunoglobulin supergene family. CD4 is a non-polymorphic 55 Kd glycoprotein consisting of four tandem extracellular domains, a short hydrophobic transmembrane region and a cytoplasmic tail. CD4+ T cells are the major regulatory cells of the immune system. Their regulatory function depends both on the cell surface molecules, such as CD4, the T cell receptor (TCR) induced upon activation, and on a wide array of small proteins (cytokines) that they secrete when activated. Although the CD4 molecule is essential for binding HIV particles, there is however mounting evidence to show that it is not sufficient for efficient viral entry and infection (Christian C, et al., 1993; Tamas O, et al., 1995). Many cell types that express the human CD4 molecule are resistant to HIV-1 infection, suggesting a requirement for other cellular cofactors or receptors. Studies addressing this and other specific cell cofactors that influence viral entry are ongoing. Both T lymphocytes and macrophages become infected through CD4 molecules, as shown by ability of monoclonal antibodies to CD4 to block HIV entry into these cells in culture (Clerici M, 1992; Salk J, 1993). Other mechanisms of HIV-1 entry into certain cells are possible. Under laboratory conditions, low concentrations of antibody may enhance HIV-1 entry into monocytes/macrophages via Fc receptor (Robinson WE, et al., 1988; Matsuda S, et al., 1988). It has been difficult to demonstrate either CD4 protein or its messenger RNA in some cells that appear to be

infected *in vivo* (eg., endothelial cells, astrocytes), suggesting that other receptors or mechanisms of entry do exist and HIV-1 has polytropic tissue/cellular infectivity.

E. LIFE CYCLE OF HIV

HIV uses the CD4 receptor and other, yet undefined, receptors to gain entry into the cells. HIV-1 binds to CD4 through the high affinity binding of envelope glycoprotein, gp120, to a specific region of the CD4 molecule (McDougal JS, 1986). The V1 region of the CD4 molecule interacts with the fourth conserved portion near the C-terminal end of the HIV envelop gp120. Once bound to CD4, HIV is internalized into the cytoplasm of the cell. The exact mechanism of internalization is still under intense study but it is thought to involve fusion of the cell membrane with another envelope glycoprotein, gp41, that is noncovalently associated with gp120. As previously noted, the CD4 molecule is essential for initiating HIV binding but is not sufficient for efficient viral entry and infection. One cell surface molecule that has been postulated to play a part in this process is dipeptidly peptidase IV (DPP IV), also known as CD26. It is a serine protease that cleaves its substrates at specific motifs; such motifs are highly conserved in the V3 loop of HIV-1 (Tamas O, et al., 1995; Christian C, et al., 1993). However, these data have been the subject of controversy due to the inability of other laboratories to confirm the results (Broder CC, et al., 1994; Patien C, et al., 1994). In the last five months, there have been numerous reports dealing with the co-receptors for HIV-1. The discovery of fusin, coupled with the observed effects of the CC chemokines RANTES, MIP-1α, and MIP-1ß on infection by macrophage-tropic HIV-1 viruses,

suggested that chemokine receptor family members might play a role as co-receptor for the macrophage-tropic viruses (Bates P, 1996; Liu R, et al., 1996; Samson M, et al., 1996; Bleul CC, et al., 1996). Five groups have reported that CC-CKR5 was the major co-receptor for macrophage-tropic strains of HIV-1; in addition, other CC-CKR5 appear to play a role also. Fusin, on the other hand, is thought to be the co-receptor for lymphotropic HIV-1 virus. After the virus has gained entry into the cell, the virion-associated RT, in conjunction with ribonuclease H, transcribes the viral RNA into double stranded DNA. The double-stranded HIV DNA is then translocated into the nucleus where it is randomly inserted into the host cell genome via action of the viral integrase. At this point in the HIV lifecycle, the HIV genome is designated a provirus (Varmus H, 1988).

Once the HIV provirus has been incorporated into the host cell genome, both cellular and viral factors are required to initiate expression of viral genes and viral progeny (Varmus H, 1988). The cellular factors may be constitutively expressed by the cell or may be induced by a variety of activating signals: antigens, mitogens, heterologous gene products, cytokines, UV light, and heat. Following activation of the HIV provirus, the first viral genes to be expressed are those that encode nonstructural proteins involved with regulatory functions (Rosenberg ZF, 1989; Greene WC, 1991). One of these regulatory proteins is *tat*, which is a powerful transactivator of HIV gene expression as discussed ealier. *Tat* exerts its effect by stimulating production of full-length RNA transcripts. These are then multiply spliced and translocated to the cytoplasm where regulatory proteins are expressed. At this phase in the viral lifecycle,

another regulatory protein, *rev*, is important. *Rev* effects the transport of unspliced and single spliced mRNAs from the nucleus to the cytoplasm. These unspliced and single spliced mRNAs encode the structural and enzymatic proteins of HIV required for the assembly of the infectious virion at the cell surface (Rosenberg ZF, 1989; Greene WC, 1991). The progeny virus is released from the host cell by budding at the cytoplasmic membrane and appropriates certain host cell molecules, including MHC class II, within its envelope. One unique feature of HIV and of the retroviral lifecycle is the ability of the provirus to persist in a quiescent state without production of progeny virus (cellular latency). It has been observed that in infected individuals, for every one HIV infected CD4 T cell that is expressing viral proteins, there are approximately nine infected CD4 T cells that harbour latent HIV proviral DNA (Schnittman SM, 1989). Several hypotheses have been advanced to explain the mechanism of cellular latency with HIV. It could be caused by methylation of certain portions of the integrated viral LTR needed for induction of the replicative process (Bednarik DP, 1989) or it could result from an inactivation of regulatory proteins (Asjo BJ, 1988; Cheng-Mayer CT, 1988; Schwartz S, 1988). As cellular factors are important for HIV expression, these latently infected cells presumably lack the critical cellular factors required for initiation of HIV RNA transcription. All these possible mechanisms of HIV latency are currently under study.

F. REGULATION OF HIV TRANSCRIPTION

Transcription of the HIV genome depends on the intracellular environment into which the virus integrates and is regulated by a complex interplay between viral

regulatory proteins and cellular transcription factors that interact with viral LTR (Antoni BA, 1994; Gaynor R, 1992). Sequences involved in viral gene expression are contained within U3 and R regions of the HIV 5' LTR. The DNA-binding activity of several transcription factors is modulated in response to cellular activation, differentiation, and the actions of cytokines and mitogens.

HIV LTR contains binding sites for several cellular transcription factors including the constitutively expressed protein SP-1 and the inducible factor NF-$_K$B, the basal transcription activity of this promoter is quite low in most cellular contexts (Jones KA, 1989; Jones KA, et al., 1986; Nabel G, et al., 1987). The *tat* gene of HIV encodes a transactivator protein that enhances LTR-derived gene expression, expression of the viral *tat* results in a large ($\approx$100-fold) increase in HIV LTR dependent gene expression (Sodroski J, et al., 1985; Arya SK, et al., 1985). Both the functional expression of the viral *tat* gene product and intact copy of the Cis-acting target sequence for *tat*, designated the transactivation response element (TAR), are essential for HIV replication (Rosen CA, et al., 1985; Fisher AG, et al., 1986). TAR DNA essentially serves as an anchor for *tat* and certain cellular factors to facilitate interactions with promoter elements such as TATA and Sp1, an HIV-1 enhancer.

The core promoter of the HIV 5' LTR resembles that of many eukaryotic genes transcribed by RNA polymerase II. The promoter contains a consensus TATA sequence and initiator elements to which general transcription factors bind (Berkhout B, et al., 1992; Pomerantz RJ, et al., 1990). A complex array of factors interacting either directly or indirectly with TATA region in combination with upstream DNA binding factors are

critical for regulation of HIV gene expression (reviewed in reference Pugh BF and Tjian R, 1992). Within HIV LTR, there is a region originally defined as the negative regulatory element. Within this distal enhancer element are binding sites for a number of positively and negatively acting cellular transcription factors; however, the mechanism by which this region down regulates HIV transcription is unknown.

The most widely studied element in the modulatory region of the HIV LTR is the enhancer region which consists of 10 bp conserved sequences known as NF-$_{\kappa}$B motifs. In 1987, Baltimore and Nabel first established the role of NF-$_{\kappa}$B in controlling transcription from the HIV LTR by demonstrating a direct correlation between increases in NF-$_{\kappa}$B DNA-binding activity and HIV LTR directed transcriptional activity during T cell activation. Mutation of NF-$_{\kappa}$B motifs in HIV LTR chloramphenicol acetyltransferase reporter constructs resulted in a marked decrease in gene expression following transfection into lymphoid cells, in both the presence and absence of the *tat* gene (Nabel G and Baltimore D, 1987). NF-$_{\kappa}$B is a ubiquitous transcription factor that takes part in various biological processes including immunological responsiveness, lymphoid cell differentiation and growth control (reviewed in reference Grimm S and Bacuevle P, 1993; Grilli M, et al., 1993). NF-$_{\kappa}$B exists in the cytoplasm in latent form as a complex consisting of a dimer of DNA binding subunits bound to an inhibitor, inhibitory kappa beta (IKB). NF-$_{\kappa}$B plays a critical role in T cell activation. In activated lymphoid cells, genes such as those encoding granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), the inflammatory cytokines, beta interferon (IFN-ß), tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6 and IL-2 are highly induced

as a result of regulatory control by NF-$_{\kappa}$B (Miller E, et al., 1993). The production of these immunomodulatory cytokines is an essential part of the host immune response to bacterial and viral pathogens.

As noted in the section "Life Cycle of HIV", *rev* is another important protein involved in the regulation of HIV replication. *Rev* is involved in modulating the expression of both unspliced ($\approx$9 Kb) and singly spliced ($\approx$4 Kb) species of mRNAs that encode the viron structural proteins *gag, pol* and *env*, and nonstructural proteins *vif, vpr* and *vpu*. *Rev* mutants of HIV are incapable of inducing the synthesis of the viral structural proteins and are, therefore, replication defective.

In addition to *tat* and *rev*, HIV-1 encodes a third gene product, *nef*. *Nef* is expressed early in the viral replication cycle from multiply spliced mRNA transcripts (Kim SY, et al., 1989; Schwartz S, et al., 1990). Much effort has been made to understand the functional properties of *nef* in viral replication and pathogenesis. This is the result of studies in simian immunodeficiency virus (SIV) infections which have shown that *nef* deletion resulted in low level viremia and lack of diseaes progression in monkeys (Colombini S, et al., 1989; Ratner L, et al., 1985; Shibata R, et al., 1990). Given the similarity of this profile to that observed in longterm survivors of HIV-1 infection, considerable efforts have been made to understand the functional properties of *nef* in viral replication and pathogenesis. Initial studies described *nef* as a negative regulatory factor, suppressing both viral replication and transcription of the LTR and helping to maintain viral latency (Luciw PA, et al., 1987; Niederman TM, et al., 1989; Cheng-Mayer C, 1989). Recent data have demonstrated a positive effect of *nef* on the rate of HIV

replication in primary peripheral blood mononuclear cells (Miller MD, et al., 1994; deRoude A, et al., 1992). Clearly, these findings suggest that *nef* is likely to play a significant role in viral replication. The net result is that the regulation of HIV transcription is highly complex and dependent on multiple viral and host factors, and their interactions.

G. CYTOKINE MODULATION OF HIV REPLICATION

Inflammatory mediators and cytokines are secreted by activated lymphoid and myeloid cells following a normal immune response to infection. Many of these cytokines have been shown to stimulate HIV replication, contribute to HIV pathogenesis and possibly hasten progression to AIDS (reviewed in references Farrar WL, et al., 1991; Poli G and Fauci AS, 1992; Poli G and Fauci AS, 1993). Cytokines stimulate the production of additional cytokines in an autocrine and paracrine manner. The lymph node provides an environment where B cells, T cells and myeloid cells are in close contact and therefore complex cytokine networks involving paracrine and autocrine effects may be important in such tissues with regard to replication of the virus and disease progression. Activated B lymphocytes, isolated from the lymph nodes of AIDS patients, are able to stimulate HIV expression in autologous T cells by secretion of IL-6 and TNF-α (Tesmer VM, et al., 1993). Cell-to-cell contact and cytokine gene expression is required to stimulate HIV-1 replication, therefore the effects of a given cytokine on HIV-1 replication are dependent on the mixture of soluble mediators present within the immediate environment of infected cells.

G1. Activating Cytokines

While virus replication has been shown to occur throughout the course of HIV-1 infection (Schnittman SM, et al., 1989; Ho DD, et al., 1989), it is clear that a large proportion of HIV-1 infected cells express HIV during the latter stages of AIDS than during the asymptomatic period. Current evidence suggests that immunologic activation of latently infected cells leads to the induction of HIV-1 expression, and that this induction can be brought about by antigenic activation of PBMC leading to cytokine secretion.

Tumor necrosis factor (TNF), originally defined by its anti-neoplastic activity, is now recognized as a polypeptide mediator of inflammation and cellular immune responses (Old LJ, 1987; Beutter B and Cerami A, 1988). TNF-α is produced by macrophages, natural killer (NK) cells and T cells, and plays a critical role in the acute phase response to bacterial pathogens. TNF-ß (lymphotoxin) is closely related to TNF-α ($\approx 30\%$ amino acid homology) but is produced exclusively by lymphocytes (Volcek J and Lee TH, 1991). Both TNF-α and TNF-ß effect their function by binding with high affinity to the same two receptors, TNFR-α and TNFR-ß expressed on virtually all cell types. Consistent with a common receptor, TNF-α and TNF-ß exhibit similar biological activities including induction of the cytokines (IL-1, IL-6, GM-CSF), B cell and myeloid cell differentiation. Overproduction of TNF-α and TNF-ß *in vivo* has been implicated in progressive wasting and septic shock (Tracey KJ, 1987). The stimulatory effect of TNF-α on HIV-1 expression is well documented for mononuclear phagocytes and T lymphocytes. TNF-α stimulates HIV-1 replication by activating the enhancer sequence

within the HIV-1 LTR. The signal transduction pathway leading to increased HIV-1 replication involves the TNF-α receptor leading to NF-$_{\kappa}$B DNA binding activity (Schutze S, et al., 1992). As TNF-α is produced by monocytes/macrophages and lymphocytes as a chemical mediator in response to various conditions such as infection and immunologic stimulation (vaccine administration), the biologic implications of excessive secretion of TNF-α and other cytokines in HIV-1 infected individuals includes viral replication and cellular proliferation which may shift the physiological environment in favour of HIV-1 replication and disease progression.

IL-6 was first characterized as a B cell stimulatory factor. It is secreted by macrophages, B cells, T cells, endothelial cells and bone marrow stromal cells. Like IL-1 and TNF, IL-6 is multifunctional and produced as an acute phase response factor (Arai KI, 1990). IL-6 induces HIV-1 expression and replication in chronically infected monocytic cell lines. IL-6 does not seem to affect viral replication in T cells. Of interest is the fact that IL-6 induces HIV-1 expression at a post-transcriptional level by enhancing translation of viral proteins (Poli G, et al., 1990).

IL-1 is a pleiotropic cytokine produced by activated macrophages, fibroblasts, and T and B lymphocytes. With very diverse biological activities, including inducing growth and differentiation of T and B cells, IL-1 also stimulates HIV-1 replication at the transcriptional level by inducing NF-$_{\kappa}$B DNA binding activity in T cells (Krasnow SW, et al., 1991).

G2. Inhibitory Cytokines

Type 1 IFNs possess broadspectrum of antiviral, immunoregulatory and

antiproliferative activites (reviewed in reference Samuel CE, 1991). Type 1 IFNs are produced in leukocytes (IFN-α) and fibroblasts (IFN-ß) in response to viral infection. IFN-α and IFN-ß bind to a common ubiquitously expressed cell surface receptor leading to the induction of specific genes, the interferon-stimulated genes. They are responsible for cellular antiviral effects and immunoregulatory activities. Two of the best characterized interferon-stimulated genes encode 2',5'-oligoadenylate synthetase and protein kinase R (PKR). 2',5'-oligoadenylate synthetase exerts antiviral activity by activating an RNAse that degrades viral RNA, whereas PKR phosphorylates the eukaryotic initiation factor 2α thus preventing efficient translation of viral proteins (Samuel CE, 1991). IFN-α/ß exert similar anti-HIV-1 effects on replication in monocytes and macrophages. When viral infection and IFN treatment occur simultaneously, replication is blocked prior to the formation of proviral DNA indicating that IFN may affect HIV-1 receptor binding, fusion, uncoating or reverse transcription (Haseltine WA, 1991; Meylan PRA, et al., 1993).

Two additional cytokines, IL-10 and IL-13, have also been shown to inhibit HIV-1 replication. Both of these cytokines are members of the IL-4 superfamily. IL-10 is produced under different conditions of immune activation by Th_0 and Th_2 subset of helper T cells as well as monocytes, macrophages and B cells (de Waal Malefty R, et al., 1991; Fiorentino DF, et al., 1992). IL-10 is thought to inhibit HIV-1 replication at the level of virus assembly (Kootstra NA, et al., 1994). There is also data to show that IL-10 has the potential to inhibit Th_1 cell functions (Moore KW, et al., 1993) and therefore bias the immune response to Th_2, a response associated with HIV-1 disease progression

(Clerici M, et al., 1993). The exact role of IL-10 in the cytokine network in HIV infection remains to be clarified. IL-13 is expressed by activated lymphocytes. It has been shown to inhibit production of HIV-1 by tissue culture differentiated macrophages. The mechanisms by which IL-13 inhibits HIV-1 replication are not yet clear (Montaner LJ, et al., 1993).

G3. Th_1/Th_2 Dichotomy

Since its introduction, the concept of T helper type 1 (Th_1) and type 2 (Th_2) immune responses has come to play a central role in the field of immunity to infection (Modlin RL, et al., 1993; Locksley RM and Louis JA, 1992). Antibody production and cell-mediated immunity are often reciprocal immune responses associated with two patterns of cytokines originally identified in mouse CD4 T cells (Mosmann TR, et al., 1986). Th_1 cells secrete IL-2, IFN-γ and TNF-ß (lymphotoxin); these promote macrophage activation (which results in delayed type hypersensitivity). Th_2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13; these provide help for humoral immune responses, including IgE and promote mast cell and eosinophil differentiation and activation. These patterns have also been shown to occur in humans (DelPrete AF, et al., 1991; Wierenga EA, et al., 1990; Yamamura M, et al., 1991). In murine models of Leishmaniasis and leprosy, the protective role of the Th_1 type response and the deleterious effects of its counterpart, Th_2 type response, have become classic examples of this probably simplistic, yet complex, concept (Maggi E, et al., 1994; Gianfranco Del Prete, et al., 1995). The Th_1 and Th_2 patterns probably represent the most extreme functional differences among CD4 T cells.

In general terms, infections by viruses and intracellular pathogens are often better controlled by cytotoxic (Th_1 type) responses, whereas infections by extracellular parasites and bacteria may be controlled more effectively by antibody (Th_2 type) responses, with cross-regulation between the two responses. HIV infection may potentially be controlled at two levels. Antibody may be useful for preventing initial infection by free virus but the high levels of antibodies produced by HIV-1 infected individuals do not eradicate an ongoing infection or prevent progression to AIDS. There are suggestions that cytotoxic/cell-mediated response may be more important in the immune response to infection. Clerici, Shearer and others have suggested that during the early stages of HIV infection, there is a selective loss of immune response against recall antigens; that is, antigens of common infectious agents to which individuals are normally immune (Clerici M, et al., 1993; Clerici M, et al., 1993). This is followed by transient bias toward Th_2 like responses including a decreased ability to produce IL-2 and IFN-γ. These selective immune defects are followed by a decline in CD4 cells and increased susceptibility to frequent infection by common pathogens. Great interest has therefore been generated by the suggestion that a Th_2 bias and hence Th_1 inhibition contributes to the loss of control of the immune system over HIV-1 infection, resulting in progression to AIDS (Salvator TB, 1993; Clerici M, et al., 1993; Maggi E, et al., 1994). The recently discovered cytokine, IL-12, has been noted to increase NK and CTL activity, and production of IFN-γ, suggesting a bias towards Th_1 response (Wong HL, et al., 1988; Stern AS, et al., 1990). Mario Clerici and others have gone further and shown that IL-12 can restore HIV-specific cell mediated immunity *in vitro*, demonstrating a Th_1-like function (Clerici

M, et al., 1993; Chyi-Song H, et al., 1993). Taken together, these results do support the model of switch from Th_1 to Th_2 cytokine phenotype during progression to AIDS. In spite of reports to the contrary (Cecilia G, et al., 1994; Romagnani S, et al., 1994), on balance there is more evidence to indicate that a switch from Th_1 to the Th_2 cytokine phenotype does occur during progression of HIV disease. This switch is influenced not by HIV-1, but by concurrent infections in HIV-1 infected subjects (Molina JM, et al., 1990; Gendelman HE, et al., 1990; D'Addano M, et al., 1990).

H. COURSE OF HIV INFECTION

HIV infection is transmitted sexually; through intravenous contact with contaminated blood products; or across broken mucosal or epidermal epithelial cell barriers. HIV can also be transmitted from mother to child *in utero* or through lactation after birth (McCune JM, 1991). The typical course of HIV disease starts with a primary infection that is followed in 50 to 70% of individuals by an acute viral syndrome characterized by generalized lymphadenopathy, high plasma viremia and significant decreases in CD4 T cells. This occurs 3 to 6 weeks after the initial exposure and infection (Tindal B, 1991). HIV is widely disseminated during this early stage of infection to such tissues as the lungs, bone marrow, liver, central nervous system (CNS) and particularly to the lymph nodes (Tindal B, 1991). At these sites, an immune response to HIV that is responsible for the suppression of plasma viremia is mounted. As a result, the acute syndrome resolves, the CD4 T cells return to normal and the infection may enter a period of clinical latency that can last for 10 years or longer

(Pantaleo G, 1993). During this period, HIV persists in the lymph nodes where it continues to replicate and infect circulating T cells. There is probably never a true state of complete viral latency (ie., no viral expression) during the course of HIV infection. Ho, Wei and others have shown that HIV-1 replication *in vivo* occurs continuously at high rates throughout the course of HIV-1 infection (Ho DD, et al., 1995; Wei X, et al., 1995). Virus is detectable in the plasma of virtually all patients regardless of the clinical stages (Piatak M, et al., 1993; Winters MA, et al., 1993). From these studies, the kinetics of virus and CD4 lymphocyte turnover required to sustain steady-state levels of CD4 cells and plasma viremia are extraordinary; it is estimated that 30% or more of the total virus population in plasma must be replenished daily for a typical HIV-1 infected individual; this amounts to 1.1×10^8 virions per day. As for the CD4 lymphocytes, approximately 2×10^9 cells turn over per day (Ho DD, et al., 1995; Alan SP, et al., 1996; Wei X, et al., 1995). The kinetics of virus and CD4+ lymphocyte production reported have important biological and clinical implications. They are indicative of a dynamic process involving continuous rounds of *de novo* virus infection, replication and rapid cell turnover that probably represents the primary driving force underlying HIV-1 pathogenesis. The result, over a time course that encompasses years, is the net decline of the number of CD4 T cells and gradual deterioration of multiple aspects of the immune response. In the late stage of HIV disease, large amounts of virus are detectable in the peripheral blood (Pantaleo G, 1993). This is thought to be due to the breakdown in the ultrastructure of the lymph nodes that permits virus to escape into circulation (Fauci AS, 1993). When the circulating CD4 T cell level drops below 200 cells/mm^3,

individuals become increasingly susceptible to opportunistic infections and malignancies. Recognition of the critical nature of the events that affect the course of HIV disease has important implication not only for a better understanding of HIV pathogenesis, but also in the design of HIV/AIDS management.

I. COFACTORS IN HIV DISEASE PROGRESSION

One important feature of HIV infection is the variable duration from initial infection with HIV to the development of AIDS (the incubation period) or clinical latency. Some individuals infected with HIV may remain asymptomatic for years before progressing to AIDS while others progress to AIDS very rapidly following HIV infection (Melby M, et al., 1986; Quinnan GV, et al., 1984; Anzala AO, et al., 1995; Pantaleo G, et al., 1995; Buchbinder SP, et al 1994; Lifson AR, et al., 1991). The precise pathogenic mechanism which determines the development of the disease in HIV infected people is unknown. HIV infected individuals are subject to multiple infections, including viral, bacterial, fungal and protozoan pathogens; the frequency of exposure to these infectious agents to the HIV infected host could indirectly or directly favour HIV replication and accelerate progression to AIDS.

Several mechanisms have been advanced by which viruses can increase HIV gene expression in HIV infected individuals. Firstly, DNA viruses commonly found in HIV infected individuals can encode transactivator proteins capable of increasing expression of the HIV LTR. These include cytomegalovirus (CMV) (Davis MG, et al., 1987; Nelson JA, et al., 1990), Epstein-Barr virus (EBV) (Kenney S, et al., 1988), *Herpes*

simplex virus (HSV) (Feng CP, et al., 1993; Mosca JD, et al., 1987) as well as adenoviruses, papovaviruses and hepatitis B virus (Gendelman HE, et al., 1986; Klien S, et al., 1989). The CMV and HSV transactivator proteins involved in increased expression of HIV-LTR are encoded by the immediate early gene products (Tevethia MJ, et al., 1987; Ostrove JM, et al., 1987). These gene products act by binding to promoter regions of HIV-LTR. Secondly, several viral gene products including hepatitis B virus X and HTLV-I Tax, can activate NF-$_{\kappa}$B DNA binding activity which, as indicated earlier, directly enhances HIV LTR regulated transcription (Baeuerle PA, et al., 1994). Thirdly, viruses may play an indirect role in increasing viral spread through the body and susceptibility of cells to infection. For example, some strains of human herpes virus 6 infect CD8 T cells. This infection, among other things, results in upregulation of CD4 surface expression on CD8 T cells, expanding the repertoire of cells permissive to HIV infection (Lusso P, et al., 1991). Herpesvirus also upregulate Fc receptors on myeloid cells providing an alternate route of HIV entry in a wide range of cells. HIV upregulation may occur indirectly in viral infected cells through the production of activating cytokines, including IL-1 and TNF. The net result is that, for an HIV infected individual, concurrent infection with viral pathogens such as those discussed above may alter the course of HIV infection by leading to increased expression of HIV, dissemination of HIV and accelerated progression to disease.

As a sexually transmitted infection, HIV is often found in the presence of bacterial STI, including *Treponema pallidum*, *Neisseria gonorrhoeae, Haemophilus ducreyi* and *Chlamydia trachomatis.* One of the major components of the outer

membrane, lipopolysaccharide (LPS) of gram-negative bacteria has been shown to potently stimulate HIV LTR CAT constructs transfected into monocyte/macrophage-like cell lines (Perno CF, et al., 1989; Kornbluth RS, et al., 1986). LPS binds to a 55 KDa cell surface protein (CD14) expressed on monocytes, macrophages and B cells. Exposure of these cells to LPS leads to the induction of genes associated with cellular activation and differentiation (Ronslston A, et al., 1992). LPS has been shown to be able to rapidly stimulate NF-$_{\kappa}$B binding activity in monocytes and macrophages (Műller JM, 1993). The upregulation of HIV transcription by LPS appear to function through the activation of NF-$_{\kappa}$B which binds to two 10 bp motifs in the HIV LTR (enhancer region) (Bagasra O, 1992; Pomerants RJ, et al., 1990). Factors that stimulate HIV replication in cell culture may also regulate HIV expression in lymphocytes and monocytes/ macrophages *in vivo*. The effect of LPS on HIV replication occurs at picogram per milliliter concentrations, thus recurrent exposure of HIV infected individuals to gram-negative bacteria (for example, *N. gonorrhoeae*) may result in increased HIV replication and thus alter the natural history of HIV infection.

Physical factors causing cellular stress, such as UV light and heat, enhance HIV replication during *de novo* infection and chronically infected cells (Stanley SK, et al., 1989). A transcriptional mechanism mediated by stress-induced activation of NF-$_{\kappa}$B is implicated in the process, since UV light and heat both induce expression of HIV LTR driven reporter constructs in an NF-$_{\kappa}$B dependent manner (Valerie K, et al., 1988). However, the relationship of physiological stresses to *in vivo* progression of HIV disease are difficult to prove. Similarly, malnutrition is associated with immunologic

impairment. Indicators of malnutrition such as low albumin and transferrin levels in serum correlate with poor clinical outcome in AIDS patients (Sűttmann U, et al., 1995).

As dicussed above, multiple cofactors may be responsible for HIV disease progression either acting directly on HIV LTR to bring about increased HIV expression or indirectly through cytokines and NF-$_{K}$B. The importance of understanding the type and variety of infections that alter the course of HIV infection may lead to improved treatment strategies for those infected with HIV-1. Multiple cofactors are therefore likely to be involved in the development of the disease (Anne R, et al., 1995; Voth R, et al., 1990; Silvija IS, et al., 1995).

J. HYPOTHESIS

The AIDS epidemic in Africa is distinct from the disease that is present in North America and Europe. It is primarily a heterosexually transmitted disease with a male-to-male ratio of 1:1, it is probably transmitted more easily (Plummer F, et al., 1994; Nagelkerke NJD, et al., 1990; Lepage P, et al., 1993), the progression of infection and disease onset is probably faster (Anzala AO, et al., 1995; N'Galy B, et al., 1988; Colebunders RL, et al., 1991). It is probable because of these features that the AIDS epidemic in Africa has reached such alarming proportions with a 30% prevalence of HIV infection in antenatal clinics in some African countries (Piot P, et al., 1994; Merson MH, 1993). The average African host is exposed to a large number of infectious diseases. These include various bacterial, viral and parasitic infections. There are also widespread helminth infections. Also of major importance is the high prevalence of STIs which play

a significant role in facilitating the dissemination of HIV and may also play a role in HIV disease progression.

Since 1985, we have been engaged in a study of the epidemiology of STIs, including HIV, in a large population of women involved in commercial sex work in a slum area of Nairobi, Kenya (Kreiss JK, et al., 1986; Simonsen JN, et al., 1990; Plummer FA et al., 1991). In the course of our studies on the natural history of HIV infection, we have shown that the rate of progression from asymptomatic HIV infection to AIDS is very rapid with a median duration of four years (Anzala AO, et al., 1995) compared to reports from North America and Europe of 8 to 10 years (Angela BM, et al., 1992; Bacchetti P, et al., 1989). Bwayo, Nagelkerke and others compared the CD4 cell declines among sex workers from Pumwani and HIV-1 positive women from the general population in Nairobi. A Markov model was used to analyze transitions between HIV-1 disease stages as defined by CD4 counts (Bwayo JJ, et al., 1995). The sex workers experienced a rapid decline in CD4 counts, consistent with earlier findings of rapid disease progression (Anzala AO, et al., 1995). The rapid progression of HIV infection experienced by this cohort is in marked contrast to progression rates documented in others. Clearly, multiple factors may be responsible for this finding including gender, multiple HIV-1 infections, repeated infections with STIs, genetic factors, environmental factors, and/or differences in HIV-1 virus virulence. However, given the enormous burden of STIs experienced by these women, averaging 4.5 STIs per year (Plummer FA, et al., 1991; Simonsen JN, et al., 1990), it seems logical that STIs could be playing a role in the course of HIV infection.

HIV infection does not generally result in constitutive cytokine gene transcription and secretion (D'Addano M, et al., 1990; Gendelman HE, et al., 1990; Molina JM, et al., 1990). However, the cytokine response of HIV-1 infected cells to subsequent antigenic challenge by viral and bacterial antigens are perturbed and lead to increased expression of cytokines and increased expression of HIV-1. It is feasible, therefore, that cytokine release in HIV-1 infected persons with recurrent viral and bacterial infections (in this case, STIs) potentiates HIV-1 expression and, in turn, accelerates the course of HIV-1 infection. Many individuals infected with HIV are also infected with other infectious agents. The response of the HIV infected host to these organisms could indirectly alter the course of HIV infection. Identification of factors that contribute to the outcome of HIV infection may be of important in explaining the variable course of HIV infection and possibly enhance the management of those infected with HIV. My hypothesis is that the rapid disease progression experienced by this cohort is related to the heavy burden of STIs experienced by the women and that these infections, either directly or through the induction of cytokines which alter HIV-1 replication or modulate controlling immune responses, have a significant role in HIV-1 pathogenesis.

K. STATEMENT OF OBJECTIVES AND APPROACH

The goal of my research project was to determine whether STIs accelerate HIV-1 disease progression in HIV-1 positive women in the Pumwani sex worker cohort in Nairobi, Kenya. The approach taken was to: a) enroll women in a prospective study and follow them for a period of six months; b) evaluate them clinically every 14 days during

the follow-up period; c) determine at each visit the presence or absence of STIs; d) obtain specimens at each visit for STI culture, quantitation of HIV plasma viremia, plasma cytokine levels, and CD4 and CD8 T cell counts; and e) correlate changes in these parameters with STI.

MATERIALS AND METHODS

A. STUDY SUBJECTS

Women attending the Pumwani-Majengo Clinic in a slum district of Nairobi, Kenya were enrolled into this study. They were the source of all the clinical data and the biological material. These women are part of a large open cohort involved in the study of the epidemiology and prevention of sexually transmitted infections (STIs) including HIV-1. In January-March of 1985, the cohort was established through a public community meeting attended by the local government representatives, women working as prostitutes from the Pumwani-Majengo district, and members of the Departments of Medical Microbiology, University of Nairobi (Kenya), University of Washington (Seattle, USA), and University of Manitoba (Winnipeg, Canada). At the meeting, the project was discussed with the women, and community representatives were elected from each region of Pumwani-Majengo to interact with the women in the community and encourage them to enroll in the project. The enrollment requirements were that the women must reside within the Pumwani-Majengo district and earned their living through prostitution. Free management of STIs and other general health care were offered to those who accepted and were willing to participate in the intervention programs. At enrollment, each woman, having been assigned a unique study number, was interviewed and basic demographic information was obtained. Information pertaining to prostitution, sexual practices, and medical/obstetrical history was obtained. A complete physical examination including a pelvic examination was performed. Specimens were obtained for STIs including *Neisseria gonorrhoeae, Haemophilus ducreyi* and *Chlamydia trachomatis* for

culture as described by D'Costa et al. (D'Costa LJ, et al., 1985). Specimens for syphilis and HIV-1 testing were also obtained. Rapid plasma reagin test (RPR) was used as the screening test for syphilis, and confirmation was performed using the *Treponema pallidum* haemagglutination (TPHA) test. The presence of HIV-1 antibodies was detected using commercial ELISA kits which are described later in the ***Methods II*** section. After mid-1989, absolute lymphocyte counts were performed at 6-month intervals by a FACScan (Becton Dickinson) flow cytometer. After enrollment, women were scheduled for follow-up at 3-month intervals, but in the interim, women were free to attend the clinic anytime they wished. At each follow-up visit, interval symptoms were reviewed, genital examinations were performed, and specimens obtained. Women with obvious clinical signs and symptoms of STIs were treated according to national guidelines or clinic protocol. All other women returned to the clinic 3-4 days later for results at which time treatment for positive cultures was administered. These women averaged 6-7 visits to the clinic per year for reassessment, counselling, management of their STIs, and general health care. To date, over 1800 women have enrolled in the cohort.

B. STUDY DESIGN

A subset of 32 HIV-1 positive women and 10 HIV-1 negative women from the Pumwani sex worker cohort were enrolled in this study. Women presenting to the clinic with history of STIs were interviewed and informed consent to participate in the study was obtained. A complete physical examination including a pelvic examination was performed. Women who had other underlying infections (such as pneumonia,

tuberculosis, fever) were excluded from the study. Women whose only illness was an STI were enrolled into the study. As a control group, HIV-1 positive women with no opportunistic infection and no STI were also enrolled and followed at 14 day intervals. Women were seen serially over a 1-5 month period during which, at various time points in the follow-up period, the women may or may not have had an actue episode of STI. At enrollment, medical history was reviewed including sexual activity, obstetrical history, clinical symptoms related to HIV-1 infection, and STIs. A complete general physical examination, including a pelvic examination, was performed. Women who had other concurrent infections such as tuberculosis or those with advanced HIV-1 disease (CDC stage IV) were excluded from the study. A summary of the study design is shown below:

TIME 0		
Entry in Study	----------→	Acute STI episode - specimen for STI culture - blood for: HIV-1 plasma
Rx		plasma cytokines CD4/CD8 counts
TIME (14 days)	----------→	Test of cure culture - blood for: HIV-1 plasma plasma cytokines CD4/CD8 counts
TIME (14 + N)	----------→	Another STI episode - specimen for STI culture - blood for: HIV-1 plasma
Rx		plasma cytokines CD4/CD8 counts
TIME (14 days)	----------→	Test of cure culture - blood for: HIV-1 plasma plasma cytokines CD4/CD8 counts

Rx denotes treatment for STI was administered.

Specimens for culture of *N. gonorrhoeae* and *H. ducreyi* were obtained and cultures performed as described (D'Costa LJ, et al., 1985). *C. trachomatis* was detected from endocervical secretions using the enzyme immunoassay (Chlamydiazyme®). Specimens were also obtained for quantitation of HIV-1 plasma viremia by the reverse transcription-polymerase chain reaction (RT-PCR) method described in detail later in the ***Methods II*** section, plasma cytokines including interferon gamma (IFN-γ), interleukin-2 (IL-2), IL-4, IL-6, IL-10, tumor necrosis factor (TNFα) and soluble tumor necrosis factor-α receptor type II (sTNFαRII); quantitations were performed by commercially available enzyme-linked immunosorbent assays described later in the ***Methods II*** section.

Specimens were also obtained for CD4 and CD8 T cell counts by flow cytometry. Women who had obvious signs and symptoms of a STI were treated. At enrollment, women were scheduled for follow-up visits every two weeks. At these visits, interval symptoms were reviewed, a pelvic examination was performed, and specimens for STI culture, HIV-1 plasma and plasma cytokine quantitation, and CD4/CD8 counts were obtained.

C. SPECIMEN COLLECTION

Specimens for *N. gonorrhoeae* and *H. ducreyi* were obtained by cotton swabs and immediately inoculated onto modified Thayer-Martin media for *N. gonorrhoeae* culture and on GC agar supplemented with bovin hemoglobin and activated charcoal for *H. ducreyi* culture. The specimens were transported to the laboratory within three hours of collection, incubated and identified as described by D'Costa et al. (1985). Specimens for *C. trachomatis* were obtained by cotton swabs, and the cotton tip was transferred to a transport bottle, and transported to the laboratory where the identification of *C. trachomatis* was performed using a serovar chlamydia antigen detection test (Abbott Laboratories).

Blood samples for cytokine and HIV-1 plasma quantification were obtained from the women at enrollment and at subsequent follow-up visits. Samples were collected in plastic vacutainer tubes with ethylene-diamine-tetraacetic acid (EDTA; Becton Dickinson Vacutainer Systems). The specimens were transported to the laboratory within three hours of collection. Plasma was separated by centrifugation at 400 g (Beckman GS-6R

Centrifuge) for 10 minutes at 4^0C. Contaminating platelets were removed from the plasma by centrifugation (Microspin 245 Sorvall Instruments Dupont) for five minutes at 10,000 g. The plasma was aliquoted into 500 μl vials, stored at -70^0C and thawed once before analysis. Specimens for CD4 and CD8 T cell counts were collected in 2 ml purple top vacutainer tubes with EDTA (Becton Dickson Vacutainer Systems). The T lymphocyte subsets were measured in whole blood by the FACScan flow cytometry system. The tests for laboratory diagnosis of STIs were selected because these are standard tests used in our laboratory. For the quantitation of CD4 and CD8 T cells, the FACScan flow cytometry is the most accurate and standard test.

D. DATA ENTRY AND STATISTICAL ANALYSIS

Raw data were computer-coded and entered on a statistical data base. The analysis was done using a paired t-test. The test compares the difference between two paired parameters. Since we were comparing HIV-1 viral levels, CD4/CD8 counts at various time points within an individual during the presence of an STI and when free of an STI, the test most appropriate for analysis was the paired t-test. Cytokines, HIV-1 virus load and CD4/CD8 T cell counts were compared within individual patients at each time point during the presence and absence of STIs. Mean parameter levels for time points with and without particular STIs were calculated. Differences between plasma cytokine levels, plasma virus load, CD4 and CD8 counts compared to the presence or absence of an acute episode of an STI was accomplished using a paired sample T-test. The association of the presence or absence of an elevated cytokine level with outcome

of STI was examined by Chi-square test with calculations of odds ratio. The paired sample T-test was used because this is the best statistical test when comparing the difference of one parameter at two different time points.

E. CHEMICALS AND BUFFERS

Unless otherwise stated, the sources of the chemicals used were: Fisher Scientific, Sigma Chemical Company, and Mallinckrodt Chemical Company.

E1. Molecular Biology Reagents

LB Broth - 10 g tryptone, 5 g yeast extract, 10 g NaCl bring up to 1 litre and heat sterilized.

20X SSPE - 3M NaCl, 172.5 mM Na_2HPO_4, 20.0 mM EDTA, pH 7.5 and heat sterilized.

10% SDS (sodium dodecylsulfate) - 100 g SDS, ddH_2O up to 1 litre.

10% SSC - 1.5 M sodium chloride, 0.15 M sodium citrate.

PBS plates - 4 g tryptone, 2.5 g yeast extract, 2.5 g NaCl, 7.5 g agar, ddH_2O up to 500 ml, autoclaved for 20 minutes at 120^0C and allowed to cool but not solidly, then the following were added: 50 mg ampicillin (final concentration of 100 μg/ml), 20 mg X-gal dissolved in 1 ml dimethyl formamide (final concentration of 40 μg/ml), and 590 mg isopropyl-ß-Dthiogalactopyanoside (IPTG) dissolved in 2 ml sterile ddH_2O (final concentration of 5 mM). Poured into sterile plastic plates and once hardened, stored at 4^0C in foil to avoid light damage.

Boiled Prep Buffer (BPB) - 8% sucrose, 0.5 Triton X-100, 5 mM EDTA pH 8.0, 10 mM Tris-HCl pH 8.0, then filter sterilized and stored at room temperature (22-25^{0}C).

Chloroform:Isoamyl Alcohol (24:1) - a mixture of chloroform and isoamyl alcohol (24:1 v/v).

10X Kinase Buffer I - 0.5 M Tris Cl (pH 7.6), 0.1 M $MgCl_2$, 50 mM dithiothreitol, 1 mM superuridine.

SOB Medium - 20 g bacto-tryptone, 5 g yeast extract, 0.5 g NaCl, adjust pH to 7.5 with potassium hydroxide and sterilize by autoclaving; prior to use, add 20 ml of 1 M $MgSO_4$, sterilized separately by autoclaving.

Ethidium bromide (10 mg/ml) - 1 g of ethidium bromide were added to 100 ml of H_2O; stirred for several hours to ensure dye dissolution. Wrapped the container in aluminum foil and stored at 4^{0}C.

Denhardt's Solution - 50X - 5 g Ficoll, 5 g polyvinylpyrrolide, 5 g BSA (Pentax Fraction V); added 500 ml ddH_2O and filtered through a disposable Nalgene filter. Dispensed into 25 ml aliquots and stored at -20^{0}C.

E2. DNA Molecular Weight Standards

Molecular weight III (Boehringer Mannheim) - lambda phage DNA digested with EcoRI and HindIII: fragment sizes in base pairs were as follows: 21,226, 5148, 4973, 4277, 3530, 2027, 1904, 1584, 1330, 983, 831, 564, and 125.

Molecular weight V (Boehringer Mannheim) - pBR322 digested with HaeIII: fragment sizes in base pairs are as follows: 587, 540, 504, 458, 434, 207, 234, 213, 192, 184, 124, 123, 104, 89, 80, 64, 57, 51, 21, 18, 11, and 8.

E3. <u>RNA Molecular Weight Standards</u>

Molecular weight III (Boehringer Mannheim) - fragment sizes in base pairs are as follows: 1600, 1000, 600, 400, and 300.

E4. <u>Plasmid</u>

pBluescribe II (Stratagene) is a cloning and sequencing phagemid (plasmid with a phage origin of replication) with multiple advantages. It had a multiple cloning site (MCS) located in the N-terminal portion of the lacZ gene that encodes for ß-galactosidase. This allowed for recombinants cloned into MCS to be detected by the interruption of the ß-galactosidase activity making colour selection on PBS plates possible. Selection for the presence of the plasmid was by ampicillin resistance gene. The MCS has 21 unique restriction enzyme sites and it is flanked by T3 and T7 DNA polymerase promoter sites for the generation of the sense or anti-sense RNA transcripts of the insert from either T3 or T7 promoters.

F. METHODS I

F1. <u>HIV-1 Serology</u>

All individuals in the study were tested for the presence of HIV-1 antibodies with commercial enzyme immunoassays (detect HIV, IAF Biochem and Enzygnost HIV-1/2 EIA, Boehring). All HIV-1 positives were confirmed by immunoblot (Novapath

Immunoblot, BioRad) until 1991 when they were confirmed by a third ELISA test, Recombigen HIV-1/2 EIA (Cambridge Biotech). These commercial tests were used to detect the presence of HIV-1 antibodies according to the manufacturer's instructions.

F2. Cytokine Assays

Tumor necrosis factor-α (TNF-α), soluble tumor necrosis factor-α receptor type II (sTNFαRII), interferon gamma (IFN-γ), interleukin-2 (IL-2), IL-4, IL-6, and IL-10 were measured in plasma by commercial enzyme immunoassays (Quantikine, R&D Systems). Duplicate determinations were performed for each plasma sample. These assays were carried out according to the manufacturer's instructions.

F3. Polymerase Chain Reaction (PCR)

Separate rooms were used for PCR preparations and post PCR analysis in an effort to prevent contamination. Positive diplacement micropipetters with disposable pistons (Microman Pipettes, Mandel Scientific Ltd.) were used. Novel primers and probes used were developed by Drs. Keith Fowke and Magdy Dawood (Dawood MR, et al., 1992) in our laboratory. They were chosen for HIV-1 PCR because of their high degree of conservation among the various strains of HIV-1, especially the African isolates, at these positions. The primer positions are in accordance to HIV-1_{HXB2} (Ratner L, et al., 1987). A cocktail containing all of the PCR reagents, with the exception of water and the sample DNA, were prepared for all samples to be tested on any given day. 36.5 μL of the cocktail was aliquoted into each individual reaction tube 0.5 ml (BioRad). The final concentration of the reaction after the addition of ddH_2O and sample DNA were: 1x PCR buffer (1 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% [w/v]

gelatin), 0.2 mM deoxynucleotide triphosphate (dNTP; Pharmacia), 0.5 μM of primers [sense: 5'-ACCTCAGGTACCTTTAAGACCAATG-3'; antisense: 5'-TGTGTAGTTCTGCCAATCAGGGAA-3'] and 2.5 units AmpliTaq DNA polymerase (Perkin Elmer). Enough ddH_2O was added to each tube to bring the volume up to 100 μL. The reaction was overlaid with two drops of mineral oil, the tubes were sealed and placed in the thermal cycler (Perkin Elmer Cetus). For each set of PCR reactions, one positive and one negative control were included. The amplification protocol for HIV-1 PCR was: denaturation for one minute at 94^{0}C, primer annealing for one minute at 56^{0}C, extension for one minute at 72^{0}C; repeated for 30 cycles followed by seven minutes at 72^{0}C to complete all extensions.

F4. Post PCR Analysis

Following the amplification, the tubes were moved to the second room, opened and the aqueous layer removed from beneath the mineral oil and transferred to a fresh 0.5 ml tube. The PCR products were ethanol precipitated by adding 10 μl 3M sodium acetate and 200 μL cold 95% ethanol. The tube was kept at -70^{0}C for 15 minutes and then spun in a microcentrifuge at 4^{0}C for 15 minutes at 12,000 rpm. The supernatant was then poured off, the pellet air dried, and then resuspended in 20 μL $T_{10}E_1$. Ten μL of the preciptated product and 2 μL tracking dye (0.1% bromophenol blue plus 10% glycerol in $T_{10}E_1$) were loaded onto an ethidium bromide stained agarose gel (Nusieve agarose, Mandel Scientific Company) and the electrophoresed. Electrophoresis was stopped when the dye reached the bottom of the gel. Molecular weight standards (DNA Molecular Weight Standards V, Boehringer Mannheim) were included in each gel to

allow for determination of the size of the PCR product. The determination of a positive reaction was achieved by the size of the band on the gel or by Southern blotting and probing with radiolabelled internal oligonucleotide corresponding to 9051-9086 nucleotide position of HIV-1 HSB2 strain.

F5. Radiolabelling Oligonucleotides

The oligonucleotide primers were resuspended at 1 pmol/uL in ddH_2O. Ten μL (10 pmol) of each primer (sense and antisense), 2 μL 10x polynucleotide kinase buffer (Pharmacia LKB Biotechnology), 7 μL γ-^{32}P adenosine 3' triphosphate (ATP) (3000 Ci/mmol NEN Dupont) and 1 μL T4 polynucleotide kinase (5.3 units/UL; Pharmacia LKB) were added in a reaction tube and incubated at 37^{0}C for one hour. After the incubation, the γ-^{32}P-ATP labelled primers were cleaned by bringing the volume to 100 μL with $T_{10}E_1$ and passing it through a 1 ml tuberculin syringe spin column of sephadex G-50 (Pharmacia) by centrifuging for two minutes. The specific activity of the purified ^{32}P labelled primers was determined by placing 1 μL of the primer into 5 ml scintillation cocktail and counting on a liquid scintillation counter (L5 500 CE, Beckman). Primers were used only if their specific activity was greater than 1 x 10^8 counts per minute (CPM)/μg of primer.

F6. Extraction of HIV-1 RNA From Plasma

To eliminate possible ribonuclease contamination, all the glassware was cleaned with detergent, thoroughly rinsed and oven baked at >210^{0}C for at least three hours. All centrifuge tubes and pipette tips were autoclaved before use. All solutions had been prepared in diethyl pyrocarbonate (DEDC)-treated ddH_2O. The method used for the

extraction of HIV-1 RNA from plasma was by the use of oligo(dT) cellulose resin that binds mRNA (Macro-FastTrack mRNA Kit, Invitrogen Corporation). Two ml of plasma was mixed with 1 ml of lysis buffer and the mixture incubated at 45^{0}C for 20 minutes in slow shaking water bath. After the incubation, one oligo(dT) cellulose tablet was added to the reaction tube. The tube was sealed and the tablet allowed to swell for two minutes. The tube was then gently rocked by hand until the tablet was dispersed. The tube was then agitated in a horizontal position at room temperature for 20 minutes using a rotator (New Brunswick Scientific Edison). Following the agitation, the oligo(dT) cellulose was pelleted at room temperature at 4000 xg in a microcentrifuge for 8 minutes. The supernatant was aspirated taking extra care not to disturb the oligo(dT) cellulose pellet. The oligo(dT) cellulose pellet was gently resuspended in 1.3 ml of binding buffer (Invitrogen) and pelleted by centrifugation. This was repeated until the buffer was clear. After the last wash, the pellet was resuspended in 0.3 ml of binding buffer (Invitrogen). The sample was then transferred into a spin-column (inside the spin-column/ microcentrifuge tube set). The sample was spun at room temperature for 20 seconds at 5000 xg. The spin-column was removed from the microcentrifuge tube and the liquid inside the tube was discarded. This process was repeated three times each time the pellet was resuspended in binding buffer. After the last wash, the spin-column was placed into a new sterile and RNAse-free microcentrifuge tube, 100 μL of elution buffer was added into the cellulose bed and mixed with a sterile pipette. The suspension was centrifuged for 20 seconds, additional 100 μL of elution buffer was added into the cellulose bed, mixed and centrifuged collecting a total of 200 μL of eluent that contained HIV-1 RNA.

The RNA was precipitated by adding 10 μl of glycogen (2 mg/ml; Invitrogen), 30 μl of 2M sodium acetate and 600 μL of 100% ethanol and the mixture frozen at -70^{0}C until ready for use. To recover the HIV-1 RNA, the sample was spun at 4^{0}C in microcentrifuge at 16,000 xg for 20 minutes, the superantant was aspirated, the pellet air dried and resuspended in 20 μL of diethyl pyrocarbonate (DEPC)-treated ddH_2O.

F7. Reverse Transcription (RT) and PCR

HIV-1 RNA was reverse transcribed in 20 μL volume containing RT master mix of 2 mmol/L (each) of dATP;, dGTP, dCTP, dTTP, 100 pmol random hexamers (Boehringer Mannheim, Germany), 20 units of RNAsin (Promega, Madison, Wisconsin), 200 units of recombinant moloney murine leukemia virus reverse transcriptase (BRL) in RT buffer (50 mmol/L Tris HCl pH 8.2, 6 mmol/L $MgCl_2$, 10 mmol/L dithiotreitol, 100 mm/L NaCl). The reaction mixture was incubated for one hour at 42^{0}C. The reaction product (10 μL) was added to 36.5 μL of a cocktail containing all the PCR reagents in addition to 0.1 pmol each of ^{32}P end-label primers (sense/antisense described in ***PCR*** section). The mixture was placed in thermocycler and PCR performed as previously described in the ***PCR*** section.

F8. RT-PCR Product Analysis

Amplified DNA was electrophoresed through an ethidium bromide stained 3% agarose (Nusieve agarose, Mandel Scientific Company) and visualized by UV light, the resultant bands were harvested and Cherenkov counted in a liquid scintillation counter (LS, 500 CE Beckman).

G. METHODS II

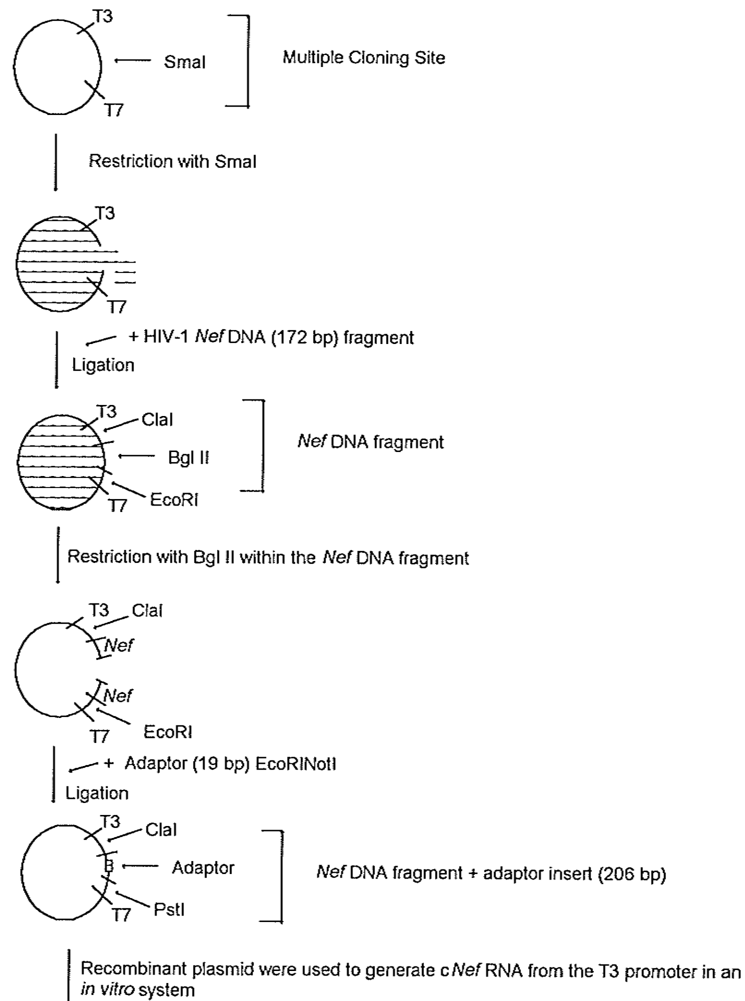
G1. Quantitation of HIV-1 Plasma Viremia

To gain more insight into the role of STIs in the pathogenesis of HIV-1 infection, an assay that quantitates HIV-1 RNA in human plasma was required. We therefore developed a method that combines the use of reverse transcription followed by polymerase chain reaction (RT-PCR). In this assay, an altered synthetic RNA transcript containing identical priming sites for reverse transcription and amplification compared to the native transcript was engineered. It was used as an internal standard for reverse transcription, amplification and quantitation of HIV-1 RNA in human plasma. The internal standard and native amplification products differ in length by virtue of a small nucleotide sequence inserted in the internal standard. Parts II and III of the ***Methods*** section describes the procedures used to generate the internal standard. Figure 3 shows the steps that were followed.

G2. Plasmid Preparation

G2a. **Transformation of DHFα.** The plasmid used was the pBluescript II and the strain of the bacteria used was *Escherichia coli* DH5α (Strategene). The competent cells were thawed on ice, an aliquot of 20 μL was transferred to 1.5 ml eppendorf tube prechilled, 1 μL of the plasmid containing about 5 ng of DNA was then added to the competent cells and incubated on ice for 30 minutes. The cells were then heat shocked by placing them at 42^{0}C for 45-60 seconds, then immediately placed on ice. Eighty μL of SOC (2 g tryptone, 0.5 g yeast extract, 1 ml IM NaCl, 0.25 ml 1M KCl, 1 ml 2M $MgCl_2$, 1ml 2M $MgSO_4$ bring up to 99 ml ddH_2O, autoclave, let it cool and then add 1

FIGURE 3. Summary of the process used to generate the internal standard.



ml of 2 ml glucose and filter sterilized) were added, the mixture was incubated with shaking for one hour at 37^{0}C. 10 μL and 90 μL were plated on ampicillin (35-50 μg/ml) selective plates and incubated at 37^{0}C overnight.

G2b. **Plasmid Amplification**. Blue colonies containing the plasmid DNA were picked from the overnight culture. A single colony was inoculated into 5 ml Luria-Bertani (LB) liquid media (10 g bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl, all in 1 litre pH adjusted to 7.5 with sodium hydroxide) with ampicillin 100 mg/L. The bacteria was grown overnight with shaking at 37^{0}C. After the overnight culture, 1 ml was added to 500 ml of brain heat infuion (BHI) broth (Gibco) plus 100 mg/L ampicillin and incubated at 37^{0}C with rigorous shaking to an $OD_{\lambda 600}$ of 0.6-0.8. Chloramphenicol was then added to a final concentration of 25 μg/ml and incubated overnight.

G2c. **Small Scale Plasmid DNA Preparation**. Blue colonies with initial plasmid DNA or white colonies representing plasmid with potential DNA inserts were picked from PBS plates and grown overnight at 37^{0}C in BHI with shaking as already discussed. From the total volume of 5 mls, 1½ ml of the culture were removed to a 1.5 ml microcentrifuge tube and spun for one minute at 12,000 g in a microcentrifuge (Microspin 245 Sorvall Instruments, Dupont). The supernatant was removed and the pellet containing the cells resuspended in 350 μL of boiled prep buffer (BPB; 8% sucrose, 0.5% Triton X-100, 50 mM EDTA, 10 mM Tris-HCl, pH 8.0 filter sterilized and stored at room temperature). Twenty-five μL of freshly made lysozyme (Sigma) solution (10 mg/ml) in BPP was added to the resuspended cells and briefly mixed by vortex. The tube was incubated for one minute at room temperature and then transferred

to 100^{0}C waterbath or sandbath for one minute. The tube was then spun for 10-15 minutes. A sterile toothpick was then used to remove the insoluble slimy pellet from the bottom of the tube which contained mostly genomic DNA and cellular debris from the bacteria. To recover the plasmid DNA, 200 μL of 7.5 M NH_4OAc and 600 μL of isopropanol was added to the supernatant and briefly mixed by vortex. The mixture was incubated for 15 minutes at room temperature, then centrifuged at 12,000 xg for 15 minutes at room temperature, the supernatant was removed and allowed to air dry for 10 minutes. The pellet was resuspended in 50 μL of $T_{10}E_1$ supplemented with 10 μg/ml RNAse A and 2 mM spermidine to remove contaminating RNA. The isolated plasmid was of sufficient quality to permit restriction analysis to identify the plasmid and also identify the plasmids with HIV-1 *Nef* DNA insert.

G2d. **Large Scale Plasmid DNA Preparation**. The 500 ml BHI broth overnight culture was divided into two aliquots and spun down for 20 minutes at 5K rpm at 4^{0}C (Beckman J2-HS; JA-10 rotor). The pellet was resuspended in 5 ml of solution I (50 mM gluscose, 25 mM tris HCl pH 8.0, 10 mEDTA, 5 mg/ml lysozyme). The suspension was transferred to polyallomer tubes and incubated at room temperature for 5 minutes. Ten ml of freshly made solution III (0.8 g NaOH, 5 ml 20% SDS, add ddH_2O to 100 ml) was added to each tube, shaken to mix and then incubated on ice for 10 minutes. The samples then spun at 15K rpm for 30 minutes at 4^{0}C (Beckman J2-H5). The supernatant was transferred to another polyallomer tube, 0.6 volumes of isopropyl alcohol was added and let to stand for 15 minutes at room temperature. The sample was centrifuged at 12.5K rpm for 20 minutes at room temperature. The pellet from each tube

was resuspended in 4 ml of $T_{10}E_1$ and the two combined to make 8 ml. To the 8 ml suspension, 8.4 g cesium chloride (1.05 g/ml), 500 μL ethidium bromide and 250 μL of 10% sarkosyl was added. The sample was transferred to a 15 ml ultracentrifuge tube, heat sealed and spun at 50K (Beckman: Ti75 rotor) at room temperature for 18-24 hours. The bottom band containing the plasmid DNA was extracted using an 18 guage needle. The volume was adjusted to 5 ml with ddH_2O. An equivalent volume of isoamyl alcohol was added and the sample mixed by vortex. The sample was centrifuged and the organic layer removed. This process was repeated until both phases were clear. Plasmid DNA was then precipitated with 2 vlumes of 100% ethanol at -20^0C for 30 minutes. The sample was then centrifuged at 8K for 15 minutes at 4^0C, the pellet was air dried and then resuspended in 5 ml of ddH_2O, 1/10 volume 3M NaOAc, 2 volumes of ethanol and kept at -20^0C overnight. The overnight sample was spun at 8K for 15 minutes, air dried and then dissolved in 1 ml of $T_{10}E_1$. The isolated plasmid DNA was of sufficient quality to allow restriction analysis, ligation of foreign DNA fragments, and transcription from either the T3 or T7 promoters.

H. METHODS III

H1. Preparation of the Controls

The amount of HIV-1 in plasma was measured by a reverse transcription polymerase chain reaction (RT-PCR) method. The comparisons of the amount of specimen derived (RT-PCR product) to the amount of product from a separately amplified external control standard does not provide rigorous basis for absolute

quantitation. Normalization based on co-amplification of a heterologous "internal control" target sequence (such as ß-globin) does not optimally address this problem, owing to different priming efficiencies for different primer-target combinations. In order to avoid the problems addressed above, an altered synthetic RNA transcript containing identical priming sites for reverse transcription and amplification, compared to the native transcript, was engineered as described below.

H2. Preparation of the RNA Standard

H2a. **Confirmation of the Plasmid**. Following the transformation of *E. coli* DH5α with the plasmid (pBScIISK; Stratagene) and amplification, the plasmid DNA was extracted as previously described. To confirm the presence of the plasmid, 5 μL of plasmid extract was added to 2 μL medium-salt buffer (50 mM NaCl, 10 mM Tris-HCl, pH 7.5; 10 mM $MgCl_2$, 1 mM dithiothreitol), 12 μL ddH_2O and 1 μL PvuII (restriction enzyme). The mixture was incubated at 37^0C for one hour. The enzyme was inactivated with 0.5 M EDTA, pH 7.5 after one hour. The digested products were resolved on 1% agarose gel. The plasmid obtained was then used in the preparation of the RNA standard.

H2b. **RNA Standard**. The HIV-1 *nef* region targeted by the oligonucleotide primer pair [sense: 5'-ACCTCAGGTACCTTTAAAGACCAATG-3'; antisense: 5'-TGTGTAGTTCTGCCAATCAGGGAA-3'] (Dawood MR, et al., 1992) was targeted in the PCR protocol previously described in the ***PCR*** section. The amplified DNA was electrophoresed through 2% preparatory agarose gel stained with ethidium bromide, visualized by UV light and the resultant band harvested. The HIV-1 *nef* DNA fragment

was recovered from the agarose gel by electroelution. The resultant DNA fragment was blunt end ligated into the plasmid pBScII SK+ (Stratagene). The resultant plasmid was determined to be a satisfactory template for the amplification of the HIV-1 *nef* region targeted by the primers described above. Synthetic *nef* RNA was generated using T3 polymerase (Stratagene); the *nef* mRNA was purified and resuspended at a concentration determined by spectrophotometry as described in detail below.

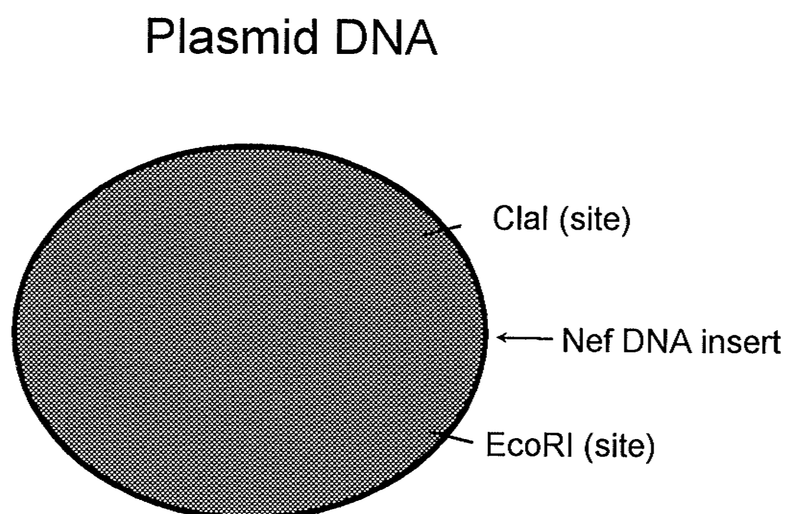
H2c. **Ligation of HIV-1 *Nef* DNA Fragment into the Plasmid**. Plasmid DNA was extracted using the large scale plasmid DNA preparation method. The plasmid was restricted within the multiple cloning site with a single cutter: 20 μL of the extracted plasmid was added to 2 μL of ddH_2O, 2 μL low-salt buffer (10 mM Tris-HCl pH 7.5; 10 mM $MgCl_2$, 1 mM dithiothreitol), SmaI (restriction enzyme; Gibco BRL) and incubated at 25^0C for one hour. After the digestion, the plasmid DNA was recovered by phenol-chloroform extraction and ethanol precipitation. The restricted plasmid was then dephosphorylated; 20 μL of restricted plasmid DNA was incubated with 1 μL (0.01 units) of calf intestinal alkaline phosphatase (CIP), 3 μL CIP buffer (10x 0.5 M Tris Cl, pH 9.0; 10 mM $MgCl_2$, 1 mM $ZnCl_2$, 10 mM spermidine), and 6 μL ddH_2O at 37^0C for 30 minutes. After dephosphorylation, the plasmid DNA was recovered by phenol-chloroform extraction and ethanol precipitation. A preparative 0.8% agarose gel electrophoresis to the plasmid DNA. The bands containing the restricted plasmid DNA were cut out of the gel and the plasmid DNA recovered by electroelution [unidirectional electroelutor (Kodak Company IBI)]. The recovered plasmid DNA was cleaned once with phenol and once with chloroform. The DNA was recovered by ethanol

precipitation. The plasmid DNA obtained was then used in a blunt-end ligation reaction. One μL (0.4 μg) of blunt-ended plasmid DNA was added to 2 μL (20 μg) of HIV-1 *nef* DNA fragment, 0.5 μL 10 mM ATP, 4 μL ligase buffer (5X, 0.5 M Tris, pH 7.4; 0.1 M $MgCl_2$, 0.1 M dithiothreitol, 10 mM spermadine, 10 mM ATP, 1 mg/ml BSA), and 0.5 μL (10 units) of T4 DNA ligase. The volume was adjusted to 20 μL with ddH_2O and the mixture incubated at 16^0C overnight. Following the overnight ligation, 0.5 μL (1 μg) of tRNA was added and the ligated plasmid DNA and *nef* DNA fragment (172 bp) recovered by ethanol precipitation, air dried and resuspended in 15 μL of $T_{10}E_1$.

H2d. **Amplification of the Recombinant Plasmid**. After the ligation, the recombinant plasmid was transfected into *E. coli* DH5α as previously discussed in the ***Methods II*** section. Following the transfection of *E. coli* DH5α, the *E. coli* was then cultured to yield large amounts. The plasmid (pBScII) has LacZ gene within the multiple cloning site. Following ligation of the *nef* DNA fragment within the multiple cloning site, the LacZ gene was disrupted. On the X-gal IPTG plates, the colonies carrying the recombinant plasmid were white. A single colony was picked and amplified overnight in LB-ampicillin media.

H2e. **Confirmation of Ligation of *Nef* DNA Fragment in the Recombinant Plasmid**. Plasmid DNA (recombinant plasmid) was prepared from the overnight LB-ampicillin media by boiled preps for plasmid as described in the ***Small Scale Plasmid DNA Preparation*** section. Two restriction enzymes, Cla I and EcoRI (Gibco BRL), were used. The two enzymes restrict *nef* DNA fragment as shown in Figure 4. After the digestion, the DNA fragments were resolved on 1% agarose gel and the *nef* DNA

FIGURE 4. Summary of specific restriction sites flanking *nef* DNA fragments.



fragment confirmed by size. Following the confirmation, a large scale recombinant plasmid preparation was performed as described in the section ***Large Scale Preparation of Plasmid DNA*** to generate large amounts of recombinant plasmid.

H2f. **Ligation of an Adaptor into the HIV-1 *Nef* DNA Insert Within the Recombinant Plasmid**. Having inserted *nef* DNA fragment (172 bp) into the plasmid (pBScIISK) to form a recombinant plasmid, the next step was to insert an adaptor within the *nef* DNA in the recombinant plasmid. The aim was to increase the length of *nef* DNA fragment so the two *nef* (without adaptor) and *nef* δ (with adaptor) could easily be differentiated on agarose gels. The adaptor used was EcoRINotI (Boehringer Mannheim), the adaptor had a 5' phosphorylated blunt end and non-phosphorylated EcoRI half site: 5'-GTCGACGCGGCCGCG-3'

3'-CAGCTGCGCCGGCGCTTAA-OH5'

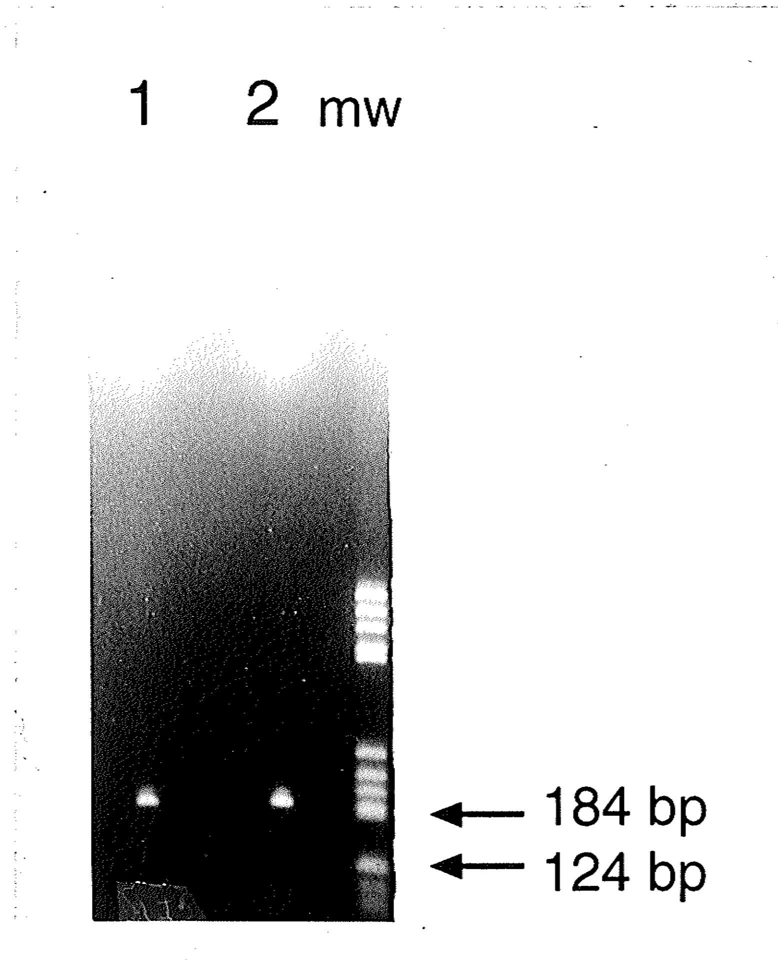
A site within *nef* DNA was identified making sure it did not interfere with primer binding sites (PCR primers) for *nef* DNA fragment. The restriction enzyme used was BglII (Boehringer Mannheim). The enzyme restricts sites consistent with the adaptor for easy ligation. The digestion of the recombinant plasmid with BglII and ligation of the adaptor EcoRI (not I) was carried out as previously discussed.

H2g. **Confirmation of Adaptor Ligation in the HIV-1 *Nef* DNA Fragment**. Following an overnight culture, a boiled plasmid preparation was performed and plasmid DNA obtained. The confirmation of adaptor ligation in the recombinant plasmid (plasmid + *nef* DNA insert) was performed by restriction analysis and PCR analysis. Confirmation by restriction analysis was achieved by two restriction enzymes, ClaI and

PstI, which just restrict flanking the *nef* DNA insert as shown in Figures 5 and 6. The two recombinant plasmids with *nef* DNA insert (*nef*) and *nef* DNA + adaptor (*nef* δ) were individually restricted and confirmation achieved by 3% agarose gel by virtue of their size differences. The aim of confirmation by PCR of the adaptor ligation in the recombinant plasmid was 2-fold: 1) to confirm the ligation; and 2) to show that *nef* (*nef* DNA) and *nef* δ (*nef* DNA + adaptor) could be co-amplified in the same reaction tube using similar primers and that the two could be distinguished by agarose gel electrophoresis due to differences in length, *nef* (172 bp) and *nef* δ (206 bp).

H2h. **PCR Co-Amplification**. The plasmid *nef* (*nef*) and the plasmid *nef* delta (*nef* δ) with the adaptor insert were used to transform *E. coli* DH5α separately. Once inside the bacteria, the plasmid were amplified by large scale plasmid preparation as previously discussed. The two plasmids were then co-amplified by PCR using similar primers in the same reaction tube. The aim was to ensure that the two (the original *nef* and the *nef* with the adapter insert) could be co-amplified using similar primers but following agarose gel electrophoresis; the two would appear as distinct bands due to differences in length (Figure 7). The experiment was performed as follows: one tube had plasmid *nef* DNA, a second tube had plasmid *nef* δ, a third tube had plasmid *nef* DNA plus plasmid *nef* δ, and the fourth and fifth tubes were positive and negative controls, respectively. All the tubes had equal amounts of primer, PCR buffer, and Taq polymerase enzyme. The PCR was carried out as discussed in the section ***PCR Methods***. The PCR products were resolved on a 3% agarose gel.

FIGURE 5. Plasmid pBScII SK with 172 bp HIV-1 *nef* insert was restricted by ClaI and EcoRI. The results show two different clones giving similar results.



Lane 1 Recombinant plasmid restricted with EcoRI and ClaI showing a 192 bp *nef* insert.

Lane 2 Recombinant plasmid restricted with EcoRI and ClaI showing a 192 bp insert.

FIGURE 6. Summary of restriction sites used to confirm the presence of *nef* DNA and *nef* δ DNA insert in the plasmid.

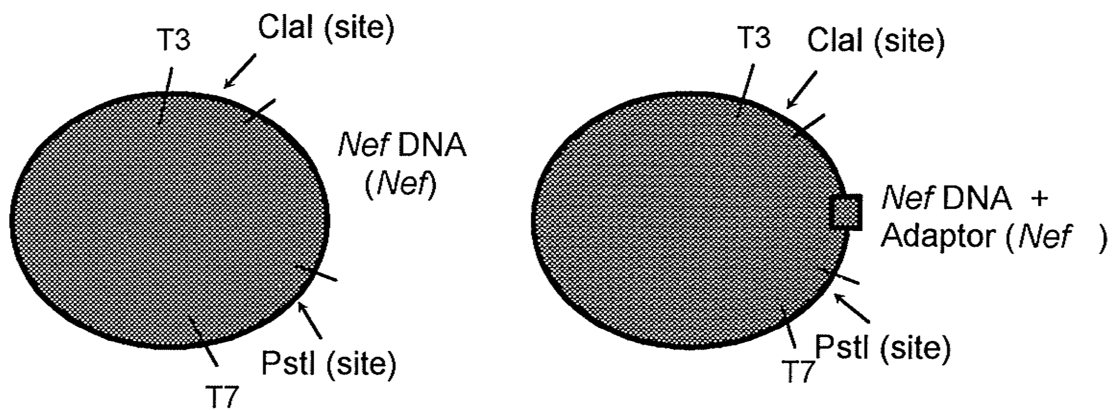
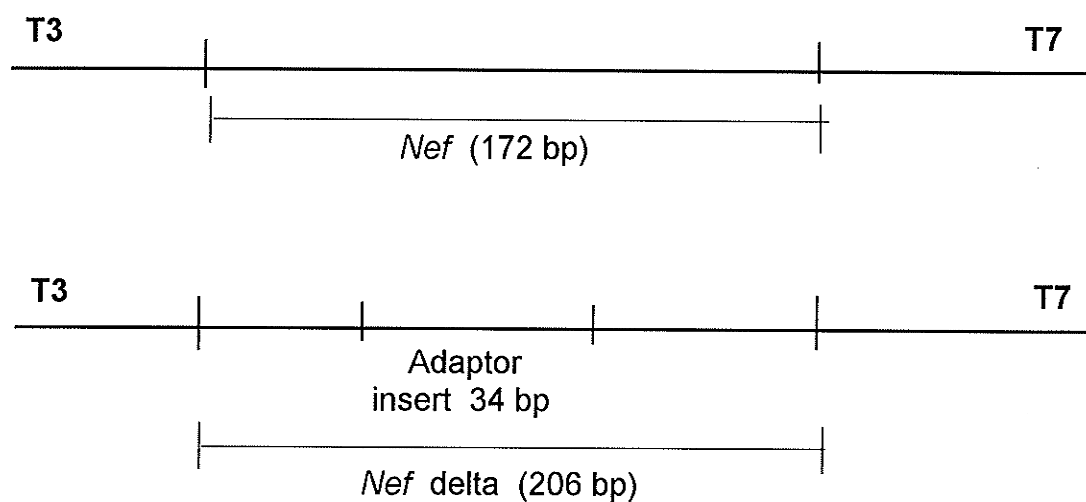


FIGURE 7. Original *nef* (172 bp) and engineered *nef* δ (206 bp), both flanked by T3 and T7 promoters.



The two recombinant plasmids obtained, one with *nef* DNA insert (*nef*) and the other with *nef* DNA + adaptor DNA (*nef* δ) were of good quality and were subsequently used for the generation of synthetic *nef* RNA and synthetic *nef* δ RNA, respectively.

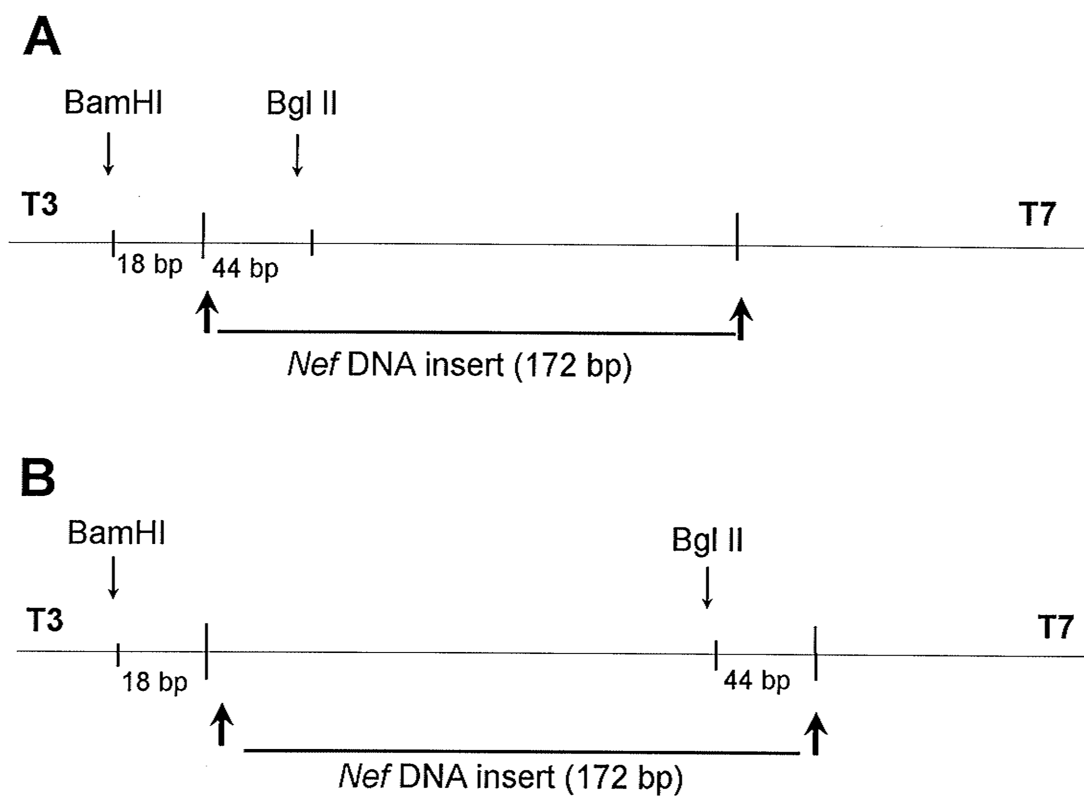
H2i. **Orientation of the *Nef* DNA Insert**. The plasmid pBScII as previously discussed had two promoters, T3 and T7, flanking the multiple cloning site. There was need to know the orientation of the *nef* DNA insert within the multiple cloning site to allow the use of appropriate promoter during the generation of synthetic *nef* RNA using *in vitro* transcription. The method used for the determination of the *nef* DNA insert orientation was restriction analysis and agarose gel electrophoresis. The 10 μL (20 μg) recombinant plasmid (plasmid + *nef* DNA insert) was digested with two restriction enzymes, BglII and BamHI. Their specific restriction sites with respect to the plasmid DNA and *nef* DNA is shown in Figure 8. After the digestion, the products were resolved on 1% agarose gel, the fragment observed on the gel was 146 nucleotide in length consistant with the orientation in Figure 8b which was antisense with respect to the T7 promoter.

I. METHODS IV

I1. *In vitro* Transcription

As explained, the purpose of cloning the *nef* DNA and the *nef* δ DNA was to eventually generate RNA that could serve as an internal standard for the quantification of plasma HIV-1. After obtaining both the plasmid *nef* and plasmid *nef* δ in pure form, both of them were then quantified by spectrophotometric determination (as outlined in

FIGURE 8. Specific restriction sites within *nef* DNA with respect to the plasmid and enzymes used to arrive at the orientation of the cloned *nef* DNA.



Maniatis; Beckman DU-62 Spectrophotometer). The plasmid was linearized by ScaI restriction digestion, then extracted with phenol chloroform. This linear plasmid was used to generate synthetic *nef* RNA by *in vitro* transcription. The *in vitro* transcription was carried out using T3 RNA polymerase. Eighty μg of plasmid *nef*, T3 transcription buffer (5X-200 mM Tris, pH 8.0; 40 mMg Cl_2, 10 mM spermidine, 250 mM NaCl), 60 μL νNTPs (25 mM ATP, GTP, CTP, UTP), 25 μL enzyme mix and 315 μL nuclease free water were mixed and incubated at 37^0C for 4 hours. Following the transcription, the template DNA was removed by addition of 1 unit/ug of DNA template RNAse-free DNAse I (Stratagene) and further incubated at 37^0C for 15 minutes. The reaction was stopped by addition of 115 μL of RNAse-free dH_2O and 15 μL of ammonium acetate solution and mixed thoroughly. The reaction was extracted once with buffered-saturated phenol/chloroform, and once with an equal volume of chloroform. The RNA was precipitated by the addition of 1 volume of isopropyl alcohol followed by incubation at -20^0C for 15 minutes. The reaction was then centrifuged at 12,000 g in the microfuge for 20 minutes to pellet the RNA. The supernatant was carefully removed and RNA resuspended in 20 μL RNAse-free dH_2O. The concentration of the RNA recovered was determined spectrophotometrically as outlined in Maniatis (Beckman DU-62 Spectrophotometer).

To ensure that the RNA obtained was free of any contaminating DNA template, 10 μL (10 μg) of the RNA sample was divided into two. On one, RT-PCR was performed and on the other, only PCR was performed. Both RT-PCR and PCR were performed simultaneously as previously described. The reaction products were resolved

on 3% agarose gel, and transferred to a nylon membrane and probed with a radiolabelled internal oligonucleotide to the *nef* DNA fragment corresponding to nucleotide positions 9051-9086 of HIV-1 HSB2 strain. The RNA obtained was free of DNA template contamination and of good quality to be used as the internal standard for plasma HIV-1 quantification.

I2. Equivalent Amplification of *Nef* RNA and *Nef* δ RNA

The *nef* δ RNA was evaluated as a template for PCR using the primer pairs described in the ***PCR*** section and directly compared with the wild type HIV-1 *nef*. Equal amounts of wild type *nef* RNA and *nef* δ RNA were reverse transcribed in the same reaction tube. After RT, ^{32}P end-labelled primer was added to the PCR reaction mixture and PCR performed in the following manner: there were a total of 30 tubes; no PCR was performed on the first tube, the second tube had five PCR cycles, the third tube had 10 PCR cycles, and each of the subsequent tubes were subjected to PCR reactions in additions of five until the last tube. The PCR products were analyzed by 3% agarose gel electrophoresis. The yield of DNA was quantitated at different cycle numbers of PCR by counts per minute present in the appropriate bands isolated from the resolving gels.

I3. Determination of Sensitivity

Having demonstrated that *nef* δ was readily distinguished from the wild type HIV-1 *nef* and yet functions as an equivalent target in PCR, the sensitivity was tested within a linear range of PCR cycles. Known amounts of *nef* δ RNA were diluted and used as starting material for RT and PCR reactions. The PCR products were analyzed by 3% agarose gel electrophoresis followed by Southern transfer. The transferred DNA was

probed with a ^{32}P end-labelled *nef* specific probe as described in the ***PCR*** section. The membrane was exposed to x-ray film (Kodak X-O-Mat AR5) and after 12 hours at -70^{0}C, the film was developed with automated film processor (MiniMed/90 X-ray Film Processor, AFP Imaging Corp.).

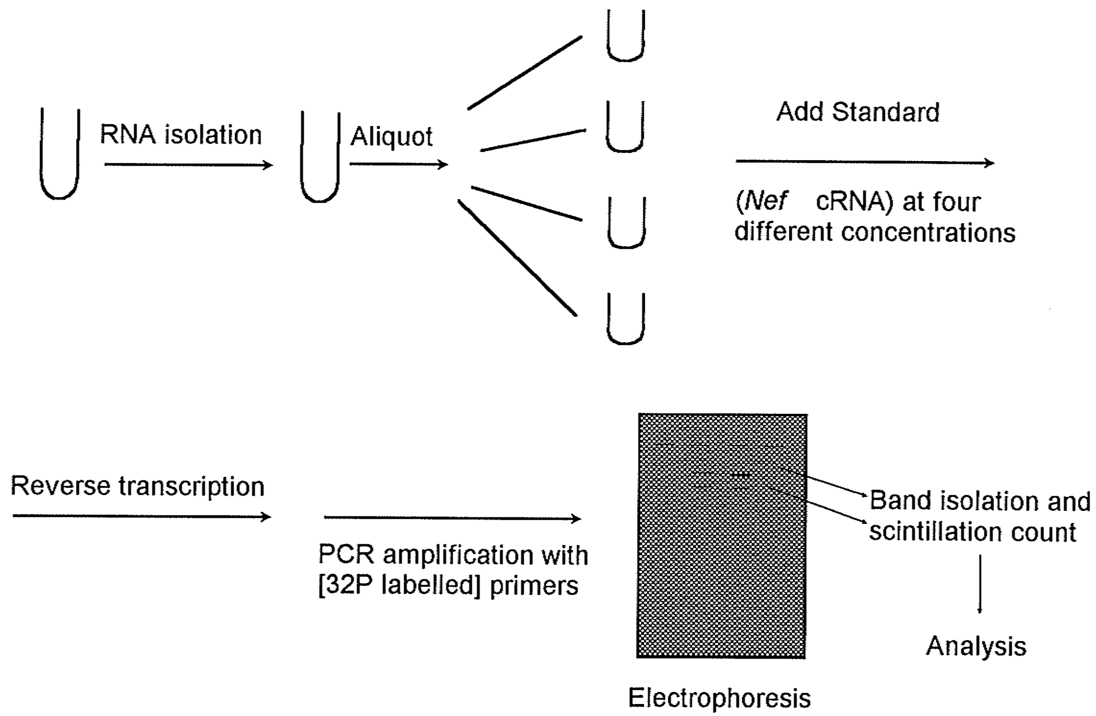
J. METHODS V

J1. RT-PCR Quantitation

The relationship of end product of PCR to the starting quantitites in a co-amplification PCR strategy is dependent on the ratio of the two templates at the time of initiation of the amplification protocol (Gilliland G, et al., 1990; Wang AM, et al., 1989). Two ml of human plasma specimen were processed as described in the ***RNA Extraction*** section. The extracted RNA was aliquoted (2 μL) into four different reaction tubes. Four 10-fold dilutions of *nef* δ RNA (standard; 10^3 to 10^6 copies *nef* δ cRNA) were made and then added to the plasma RNA aliquoted. These dilutions were reverse transcribed and amplified as previously described. The PCR products were electrophoresed through an ethidium bromide stained 3% agarose gel and visualized by UV light; the resultant bands were harvested. The specific activity of each band was determined by placing the harvested band into 5 ml scintillation cocktail and counting on a liquid scintillation counter (L5 500 CE, Beckman). Figure 9 is a summary of the RT-PCR method used.

At equivalent levels of incorporation of the radiolabelled primer, the input (amount of wild type RNA) and the standard (*nef* δ cRNA) was considered to be one.

FIGURE 9. Summary of sample processing and analysis scheme of HIV-1 plasma viremia.



The quantity of input RNA could thereby be extrapolated and the number of HIV-1 RNA copies derived. To verify the reproducibility of the assay, duplicate samples were obtained from two different HIV-1 positive individuals. The samples were independently processed and analyzed. The results of these are shown in the ***Results*** section.

Having demonstrated that *nef* δ RNA could readily be distinguished from wild type *nef* RNA and yet function as an equivalent target in RT-PCR, and having also shown that the starting wild type *nef* and the standard (*nef* δ cRNA) must approach one for equal amplification efficiency and that the assay was reproducible, this protocol was used to quantitate HIV-1 RNA in plasma. The results obtained are described in detail in the ***Results*** section.

RESULTS

A. OVERVIEW

Part I of the *Results Section* presents the results of the cloning and the preparation and standardization of the internal control (*nef* δ) that was used in the quantification of HIV-1 in plasma samples. Part II of the results section presents the clinical data, CD4 and CD8 counts, plasma cytokine levels, HIV-1 plasma viremia, and the analysis of all these data combined.

B. PART I - PREPARATION OF THE RNA STANDARD

B1. Confirmation of Ligation of HIV-1 *nef* DNA Fragment in the Plasmid

The HIV-1 *nef* region bracketed by the oligomer described in the *Methods Section* was amplified and ligated in the plasmid pBSc SK+ (Strategene). To confirm the presence of the insert in the plasmid, plasmid DNA (recombinant plasmid) was prepared from the overnight LB-ampicillin media by boiled preparations. The plasmid DNA was restricted with two restriction enzymes - ClaI and EcoRI. The two enzymes restrict flanking the HIV-1 *nef* insert as shown in Figures 3 and 5. The results obtained following electrophoresis of the restricted recombinant plasmid are shown in Figure 5.

B2. Ligation of the Adaptor into the HIV-1 *Nef* DNA Insert

After cloning the HIV-1 *nef* region, an adaptor was ligated into the HIV-1 *nef* DNA insert within the recombinant plasmid. The confirmation of the adaptor ligation into the HIV-1 *nef* region was achieved by restriction analysis and by PCR. The enzymes used for the restriction were ClaI and PstI, which flank the HIV-1 *nef* insert as

shown in Figure 6. Two recombinant plasmids, plasmid with HIV-1 *nef* DNA insert (*nef*) and plasmid with HIV-1 *nef* DNA insert plus adaptor (*nef* δ) were individually restricted with ClaI and PstI. Results obtained are shown in Figure 10 and confirmed the ligation of the adaptor into the HIV-1 *nef* DNA insert.

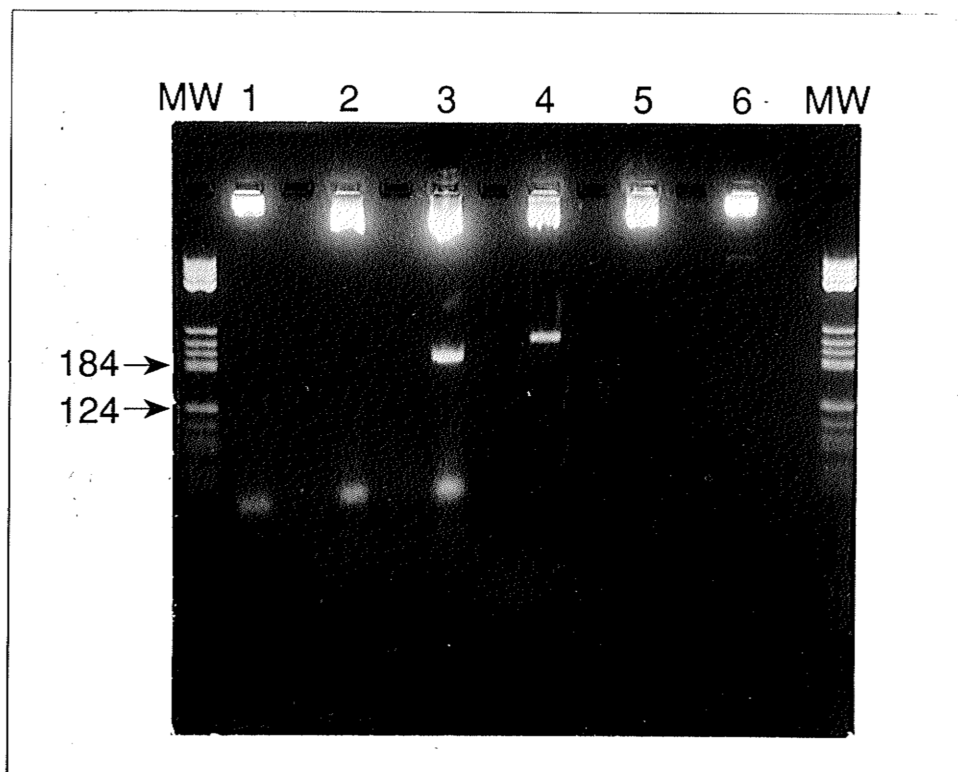
B3. PCR Co-Amplification

The second method used to confirm the ligation of the adaptor into the cloned HIV-1 *nef* DNA fragment was PCR. This experiment was also used to confirm that the cloned HIV-1 *nef* (*nef*) and HIV-1 *nef* plus adaptor (*nef* δ) could be co-amplified in the same reaction tube using similar primers and that the two could be readily distinguished by agarose gel electrophoresis due to differences in length of *nef* (172 bp) and *nef* δ (206 bp). The results are shown in Figure 11.

B4. Orientation of the Cloned HIV-1 *Nef* DNA Fragment

As previously noted, the plasmid pBScII SK+ had two RNA polymerase promoters, T3 and T7, flanking the cloned HIV-1 *nef* DNA fragment. There was need to know the orientation of the cloned HIV-1 *nef* DNA to allow us to use the appropriate promoter during the generation of the synthetic HIV-1 *nef* RNA. Ten μL (20 μg) of the recombinant plasmid was digested with two restriction enzymes, BglII and BamHI, their specific restriction sites with respect to the plasmid and HIV-1 *nef* DNA are shown in Figure 8. The digestion products were resolved on 1% agarose gel. A fragment size of 146 bp was obtained as shown in Figure 12. This fragment size is consistent with orientation in Figure 8b. From this result, the promoter used to generate the correct sense HIV-1 *nef* cRNA was T3 promoter.

FIGURE 10. Restriction of plasmid *nef* and plasmid *nef* δ with ClaI and PstI, and resolved on 3% agarose gel.



Lane 1 Unrestricted plasmid *nef*.

Lane 2 Plasmid *nef* restricted with EcoRI.

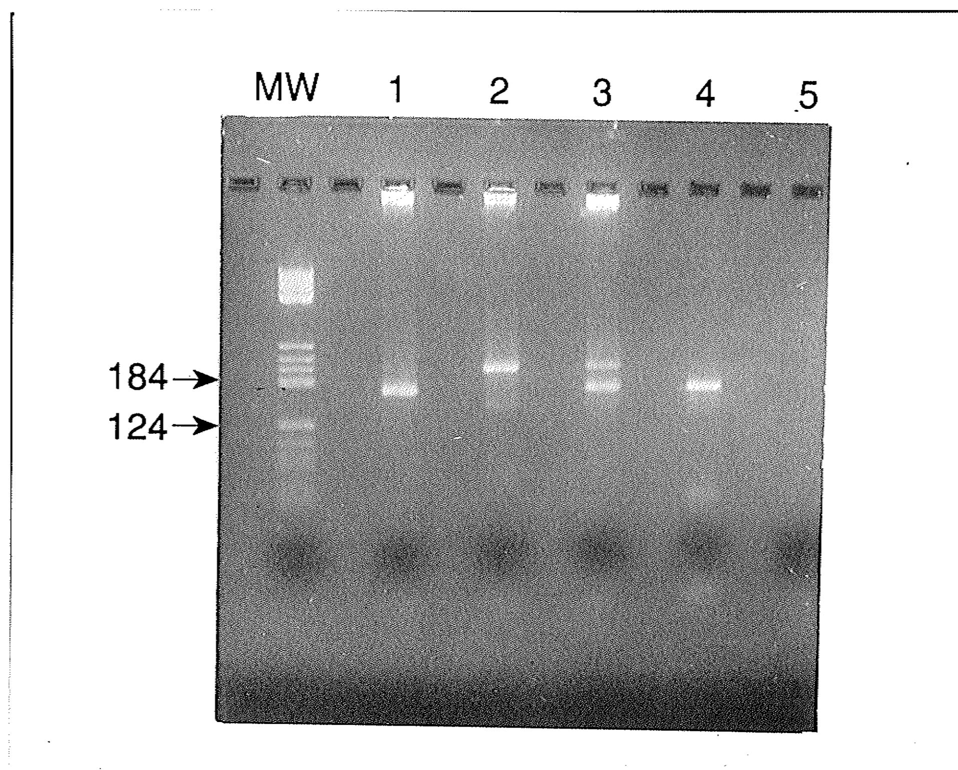
Lane 3 Plasmid *nef* restricted with ClaI and PstI, showing fragment of 197 bp.

Lane 4 Plasmid *nef* δ restricted with ClaI and PstI, showing a fragment of 235 bp.

Lane 5 Plasmid *nef* δ restricted with EcoRI.

Lane 6 Unrestricted plasmid *nef* δ.

FIGURE 11. PCR amplification of *nef* and *nef* δ. Products resolved on a 3% agarose gel.



Lane 1 PCR products of cloned HIV-1 (*nef*).

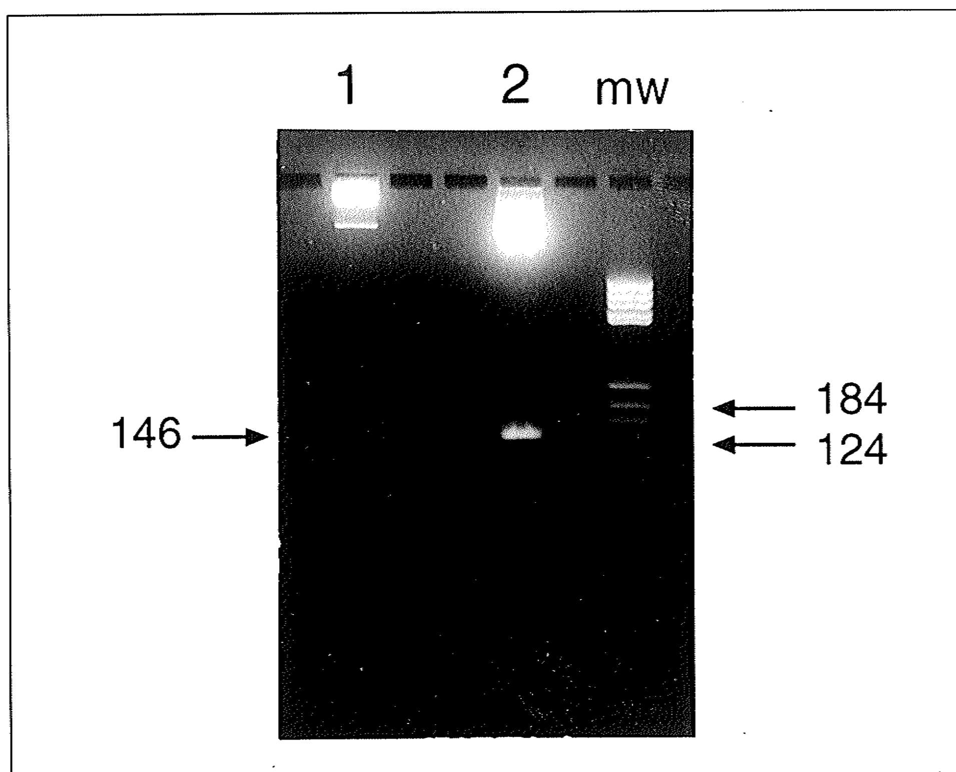
Lane 2 PCR products of cloned HIV-1 (*nef* δ).

Lane 3 PCR products of co-amplification of cloned *nef* and *nef* δ showing two distinct bands (*nef* δ top and *nef* lower).

Lane 4 Positive control (HIV-1 *nef*).

Lane 5 Negative control (H_2O).

FIGURE 12. Restriction of the recombinant plasmid with Bgl II and BamHI digestion products resolved on 1% agarose gel.



Lane 1 Unrestricted recombinant plasmid.

Lane 2 Recombinant plasmid restricted with Bgl II and BamHI, a 146 bp fragment was obtained.

B5. *In vitro* Transcription

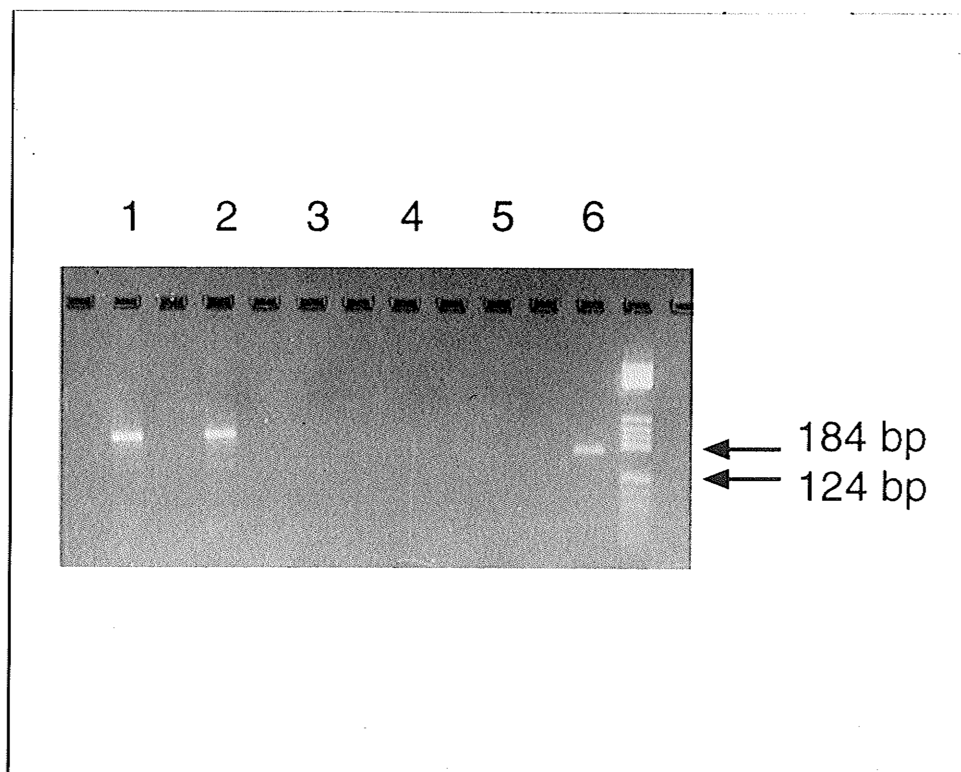
As explained, the purpose of cloning the HIV-1 *nef* DNA and the HIV-1 *nef* δ DNA was to eventually generate synthetic HIV-1 *nef* cRNA that could serve as an internal standard for the quantification of plasma HIV-1. Both HIV-1 *nef* plasmid and HIV-1 *nef* δ plasmid were obtained in quantity by large scale plasmid DNA preparation. The plasmid DNA was purified and quantified by spectrophotometric determination. Eighty μg of each plasmid were used independently in an *in vitro* system to generate large amounts of HIV-1 synthetic *nef* RNA and HIV-1 synthetic *nef* δ RNA.

B6. Determination of Contaminating DNA Template

The HIV-1 synthetic *nef* RNA obtained had to be highly pure and free of any contaminating DNA template. The *in vitro* transcription product was treated with 1 unit/μg of DNA template RNAse-free DNAse 1. To confirm the absence of any contaminating DNA, 10 μL (20 μg) of the HIV-1 synthetic *nef* RNA sample was divided into two. One one sample, RT-PCR was performed and on the other, only PCR was performed. The PCR products were resolved on 3% agarose gel and the resutls are shown in Figure 13. Samples 3 and 4 are the same as 1 and 2, respectively, but no RT was performed on 3 and 4.

As shown on the figure in lanes 3 and 4 where no RT was performed, there was no PCR product, indicating no contaminating DNA template. To further ensure that the generated HIV-1 synthetic *nef* RNA was free of DNA, the RT-PCR and PCR products resolved on 3% agarose gel were transferred to a nylon membrane and probed with a

FIGURE 13. Determination of contaminating DNA template: 3% agarose gel. Samples 3 and 4 are the same as 1 and 2, respectively; no RT was performed on 3 and 4.



Lane 1 RT-PCR product of HIV-1 *nef* cRNA.

Lane 2 RT-PCR product of HIV-1 *nef* cRNA.

Lane 3 PCR product of HIV-1 *nef* cRNA.

Lane 4 PCR product of HIV-1 *nef* cRNA.

Lane 5 Negative control (H_2O).

Lane 6 Positive control (HIV-1).

radiolabelled internal oligo to the HIV-1 *nef* region. The results obtained are shown in Figure 14. The RNA obtained was free of any DNA template contamination.

B7. <u>Determination of HIV-1 Synthetic *nef* RNA Concentration</u>

500-fold dilution of the recovered HIV-1 synthetic *nef* RNA was performed in duplicate and the absorbance was read at wavelength of 260 and 280 nanometers. The results obtained were as follows:

dilution factor 1:500

A_{260} = 0.031 OD

A_{280} = 0.017 OD

an OD of 1 corresponds to approximately 40 μg/ml of single stranded RNA

(0.031 x 40)

= 1.24 μg/ml

the dilution factor was 500, the concentration of the original RNA sample was

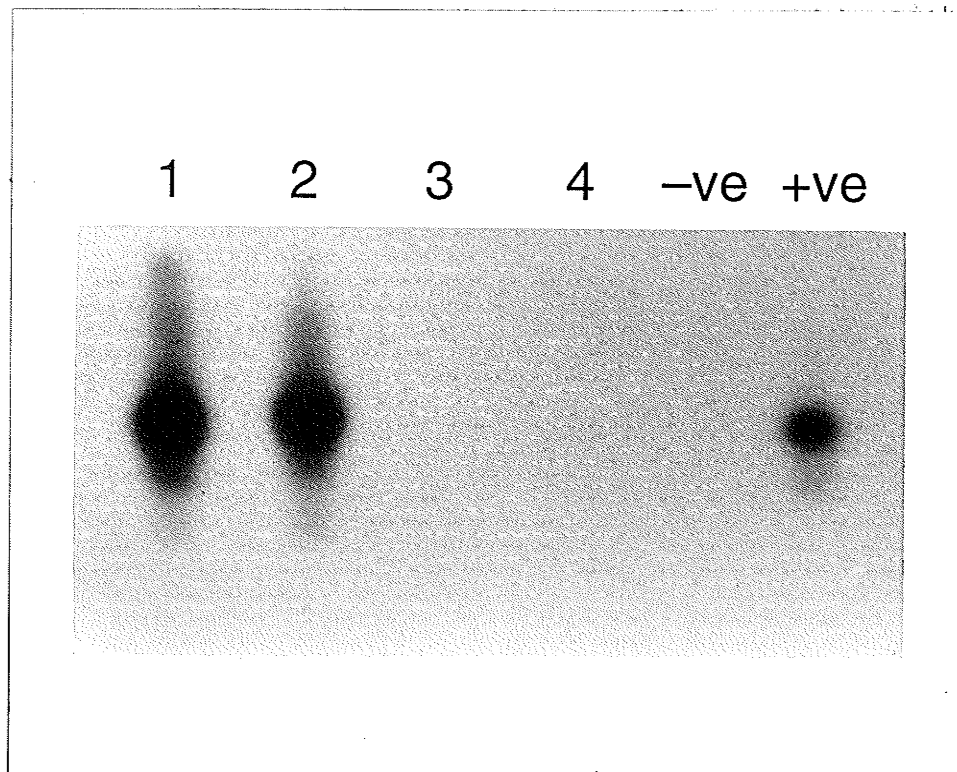
(1.24 x 500)/ml

= 620 μg/ml

B8. <u>Determination of the Number of RNA Copies in the HIV-1 Synthetic *nef* RNA and HIV-1 Synthetic *nef* δ RNA Transcripts</u>

As previously noted, the *in vitro* transcription was performed from the T3 promoter of the plasmid consistent with linearization of the plasmid with restriction enzyme ScaI, the length of the HIV-1 synthetic *nef* RNA transcript was 345 nucleotides and HIV-1 synthetic *nef* δ RNA was 379 nucleotides. The number of RNA copies in the RNA samples was obtained by the following calculation:

FIGURE 14. Determination of contaminating DNA by radiolabelled internal oligo to HIV-1 *nef* region.



Lane 1 RT-PCR product probed with internal oligo.

Lane 2 RT-PCR product probed with internal oligo.

Lane 3 PCR product probed with internal oligo.

Lane 4 PCR product probed with internal oligo.

1 mole of base = 0.33 ng per base of length

there are 6.022×10^{23} copies/mole

the length of the *nef* δ RNA transcript was 379 nucleotides

$$(379 \times 0.33)$$

$$= 125.07 \text{ ng/mole}$$

$$1 \text{ mole} = 125.07 \text{ ng}$$

$$6.022 \times 10^{23} \text{ copies} = 125.07 \text{ ng}$$

$$1 \text{ ng} = \frac{6.022\times10^{23}}{125.07}$$

$$= 4.8 \times 10^{21} \text{ copies/ng}$$

The concentration of *nef* δ RNA was 620 μg/ml and the concentration of *nef* δ RNA copies in the sample was therefore 2.9×10^{21} copies/μL.

B9. Determination of RT-PCR Sensitivity

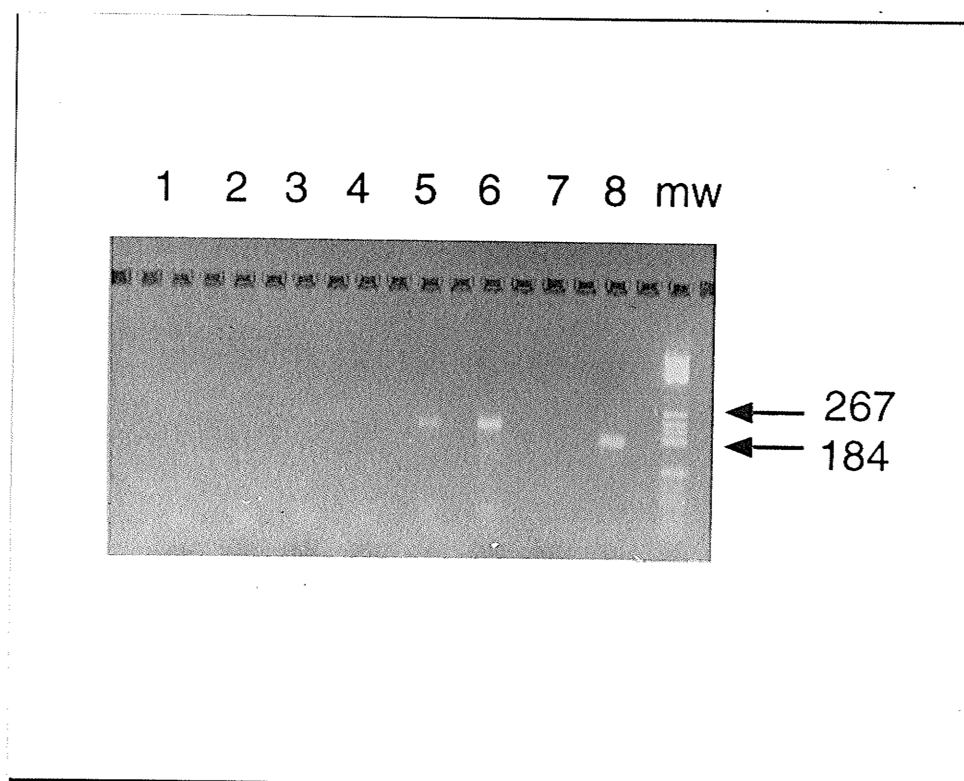
Known amounts of *nef* δ RNA were diluted and used as starting material for RT-PCR reactions. The PCR products were resolved on 3% agarose gel electrophoresis followed by southern transfer. Figure 15 is that of the PCR products resolved on 3% agarose gel and Figure 16 is that of the southern probed with ^{32}P labelled internal oligo.

The combined result of the 3% gel and the southern transfers showed that the sensitivity of the assay was limited to 40-60 copies of RNA in the starting material.

B10. Equivalent Amplification of *nef* RNA and *nef* δ RNA

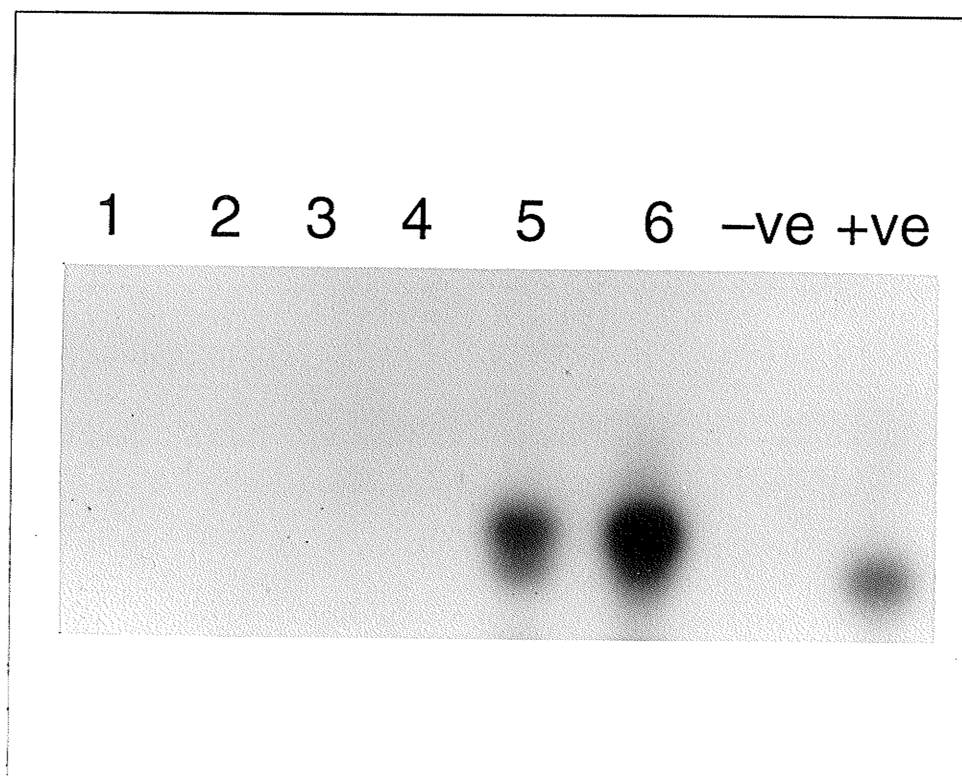
nef δ RNA was evaluated as a template for RT-PCR using the primer pair for HIV-1 *nef* region described in the *Methods Section* and directly compared with *nef* RNA. After reverse transcription, ^{32}P-end-labelled pairs were added to the PCR reaction

FIGURE 15. Determination of RT-PCR sensitivity; 3% agarose gel.



Lane 1	0 copies *nef* δ RNA.
Lane 2	5 copies *nef* δ RNA.
Lane 3	10 copies *nef* δ RNA.
Lane 4	20 copies *nef* δ RNA.
Lane 5	40 copies *nef* δ RNA.
Lane 6	60 copies *nef* δ RNA.
Lane 7	Negative control (H_2O).
Lane 8	Positive control (HIV-1).

FIGURE 16. Determination of sensitivity southern probed with internal oligo. The positive control was of *nef* RNA.



Lane 1 0 copies *nef* δ RNA.

Lane 2 5 copies *nef* δ RNA.

Lane 3 10 copies *nef* δ RNA.

Lane 4 20 copies *nef* δ RNA.

Lane 5 40 copies *nef* δ RNA.

Lane 6 60 copies *nef* δ RNA.

mixture and the PCR products were analyzed by electrophoresis. The yield of DNA was quantitated at different cycle numbers of PCR by the counts per minute present in the appropriate bands isolated from the electrophoresic gel. Figures 17 and 18 indicate that at equal amounts of starting material (100 copies each of *nef* cRNA and *nef* δ cRNA) in each reaction tube, the rate of rise of incorporated ^{32}P-labelled primer is the same with either *nef* RNA or *nef* δ RNA. These results also suggest that reverse transcription was equally efficient for both templates.

B11. <u>The Relationship of the End Product PCR to the Starting Quantitites</u>

In co-amplification PCR strategy using similar pairs, the end products of the PCR were dependent on the ratio of the two templates at the time of initiation of the amplification protocol. At ratios of sample template to standard template of less than or greater than 1.0, the amplification of the two templates is not equal. Figure 19 represents RT-PCR products of reactions containing a fixed amount of *nef* δ RNA (200 copies of RNA) standard with decreasing concentration of *nef* RNA (400 copies, 200 copies and 100 copies of RNA). The variable intensity of the visible bands in lanes 1 and 3 indicates the inconsistent amplification in the presence of different concentrations of the tamplate. In lane 2 where they were equivalent, *nef* RNA and *nef* δ RNA at the initiation, the band intensity is equal indicating equal amplification of the template. This was also confirmed by radioactive counting.

B12. <u>RT-PCR Quantitation</u>

As previously noted, the relationship of the end product of PCR to the starting material in a co-amplification PCR strategy is dependent on the ratio of the two templates

FIGURE 17. Equivalent amplification of *nef* RNA and *nef* δ RNA at different PCR cycle numbers. The starting material in each reaction tube was 100 copies of *nef* cRNA and 100 copies of *nef* δ RNA.

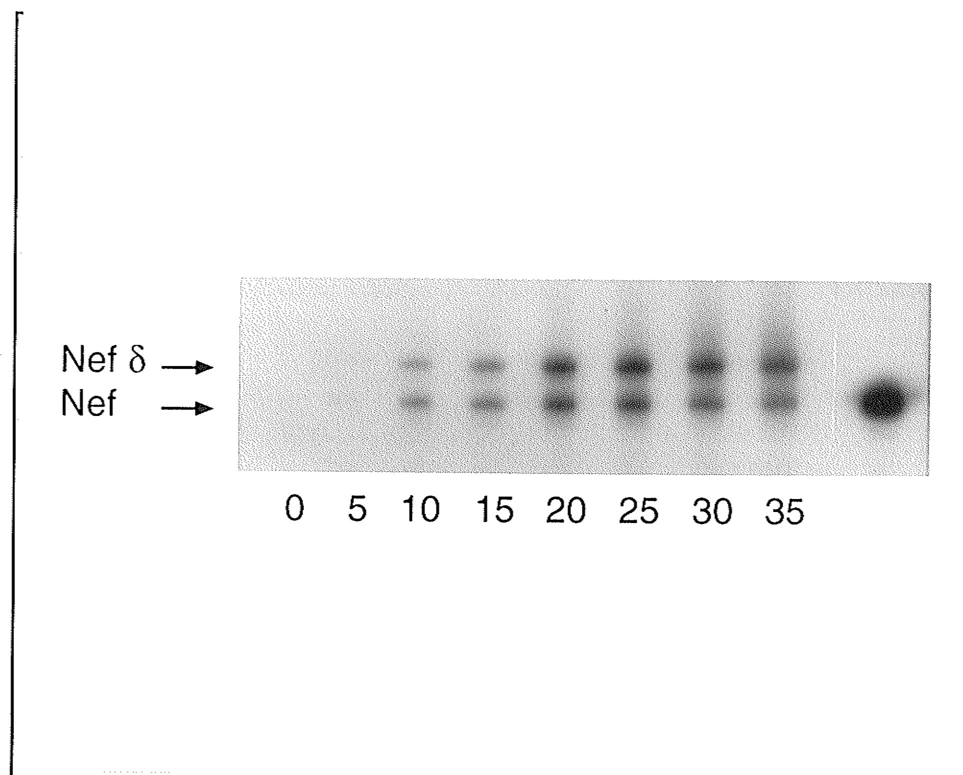
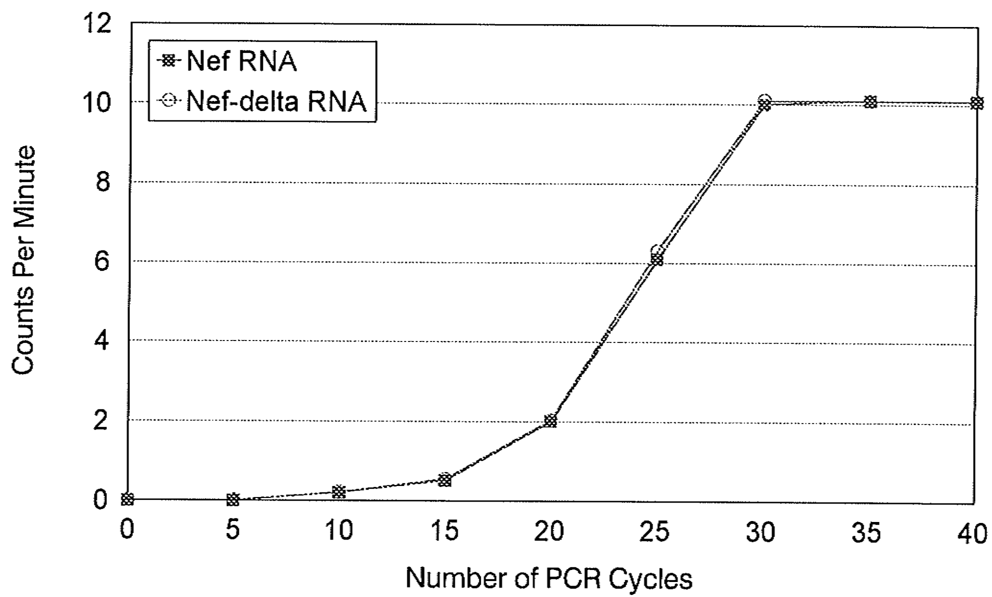
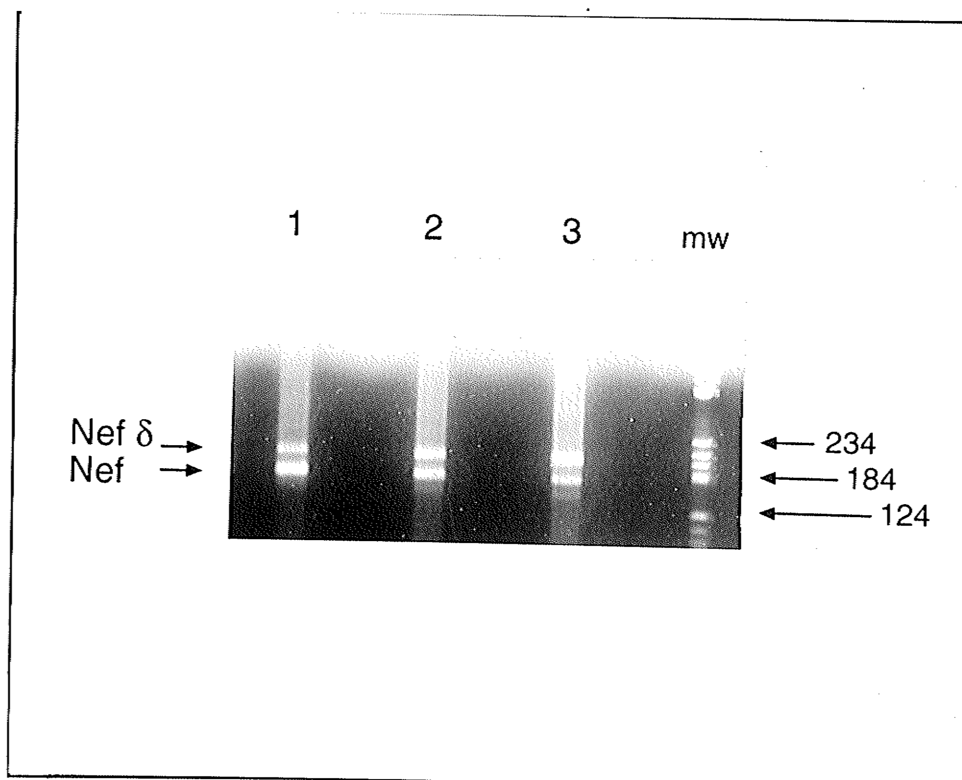


FIGURE 18. Equivalent amplification of *nef* and *nef* δ at different PCR cycle numbers. *Nef* and *nef* δ were independently analyzed for incorporation of ^{32}P-labelled oligonucleotide primer after RT-PCR by scintillation counting of isolated agarose gel slices of electrophoresed reaction products.



<u>FIGURE 19.</u> Co-amplification of *nef* RNA and *nef* δ RNA at different ratios of starting material.



Lane 1 200 copies of *nef* δ RNA and 400 copies of *nef* RNA in the starting reaction tube.

Lane 2 200 copies of *nef* δ RNA and 200 copies of *nef* RNA.

Lane 3 200 copies of *nef* δ RNA and 100 copies of *nef* RNA.

at the time of initiation of the amplification protocol demonstrating that the starting (standard template: sample template) ratio must approach unity for equal efficiency of amplification. At equivalent levels of incorporation of radiolabelled primer, the input standard template:sample template were considered unity. Since the amount of standard template in the initial reaction were known, the quantity of the input sample could thereby be extrapolated and the number of RNA of HIV-1 RNA plasma copies derived.

To verify the reproducibility of the assay, duplicate samples were obtained from five HIV-1 positive individuals. 2 ml human plasma were processed. Six 10-fold dilutions of the *nef* δ RNA standard (10^3 copies to 10^8 copies) were added to a fixed amount of plasma RNA. These dilutions were reverse transcribed and co-amplified with radiolabelled pairs as previously described and the PCR products were resolved on 3% agarose gel and the resultant bands analyzed as above. The results, shown in Table 1, demonstrate that the assay was reproducible.

To ensure that the results did not reflect contaminating DNA, 30 cycles of PCR were done on all patient extracted plasma RNA without first submitting the samples to RT-PCR assay. No amplified products were detected, indicating that the extracted RNA was free of HIV-1 DNA contamination. The specificity for HIV-1 of the bands assayed in this method was supported by absence of bands in control plasma.

B13. Quantitation of HIV-1 in Human Plasma

Having demonstrated that HIV-1 *nef* δ cRNA could readily be distinguished from wild type *nef* RNA and yet function as an equivalent target in RT-PCR and having also demonstrated that the starting wild type *nef* RNA and the standard synthetic *nef* RNA

must approach unity for equal amplification efficiency and that the assay was reproducible, this protocol was used to quantitate HIV-1 RNA in plasma.

C. PART II - HIV-1 DISEASE PROGRESSION AMONG SEROCONVERTING SEX WORKERS

C1. Disease Progression

The results presented in this section are from the initial project I conducted looking at the natural history of HIV-1 among 163 seroconverting prostitutes (Anzala AO, et al., 1995).

One of the notable features of HIV-1 infection is the variable duration from initial infection to the end-stage disease of AIDS. One hundred and sixty-three women who seroconverted while enrolled in our programme were followed for the development of HIV-1 related disease. A Markov model estimate of the mean time for transition from seroconversion to CDC stage IV-A was 3.5 years. A plot of the estimated survival curves is shown in Figure 20.

The characteristics of women who progressed to CDC stage IV-1 were compared with those women who remained disease free as shown in Table 2.

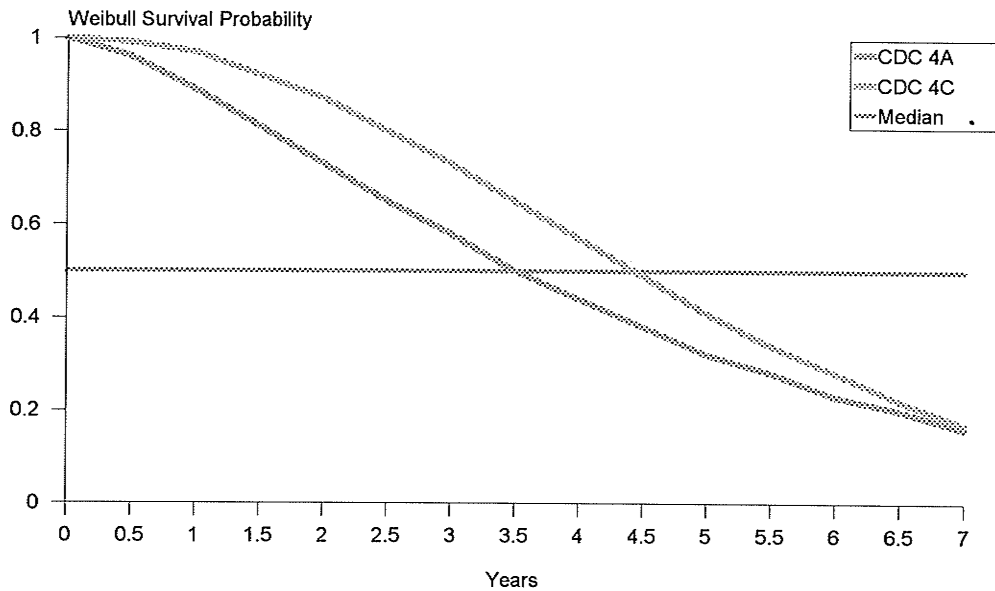
The HIV-1 disease progression was explored in terms of declines in CD4 counts. Two samples from Nairobi, Kenya were studied; one from the sex workers and the other, as a comparison group, from mothers enrolled in an HIV-1 vertical transmission study. The total number of sex workers was 485 and the total number of mothers was 231. The aim of the analysis was to determine the duration of time for

TABLE 2. Risk factors for CDC stage IV disease in seroconverting women.

Risk Factors	Disease Free	CDC Stage IV	P
Age (years)	31.6 ± 7.6	29.9 ± 6.6	0.16
Duration of prostitution (years)	4.7 ± 4.7	4.6 ± 5.3	0.87
Sex partners/day	5.3 ± 4.5	4.5 ± 3.7	0.23
Months without condom use	2.6 ± 5.4	9.0 ± 14.6	<.0001
Condom use before seroconversion	65	52	0.13
Oral contraceptives	36	41	0.59
Pregnancy	10	10	1.00
Gonococcal infections	2.8 ± 6.7	1.6 ± 1.7	0.14
Genital ulcers	1.6 ± 3.8	1.7 ± 2.0	0.84
Syphilis	25	41	0.053
CD4 count	423 ± 281	236 ± 221	<.01

Data are shown as mean ± SD.

FIGURE 20. Time to HIV disease in seroconverting prostitutes.



Survival curve is Weibull:S(t)=exp(-(at)^b)

the CD4 T cell count to drop below 200 (estimate of the first passage time). The Markov model was used for this analysis as it is a probability model that estimates the time it takes to move from one phase of disease to another, and does not require the knowledge of the time of infection. The first time passage for the sex workers was 37.4 months and for the mothers was 89.5 months (Figures 21 and 22).

D. PART III - EXPLORING THE EFFECTS OF SEXUALLY TRANSMITTED INFECTIONS ON MARKERS OF DISEASE PROGRESSION

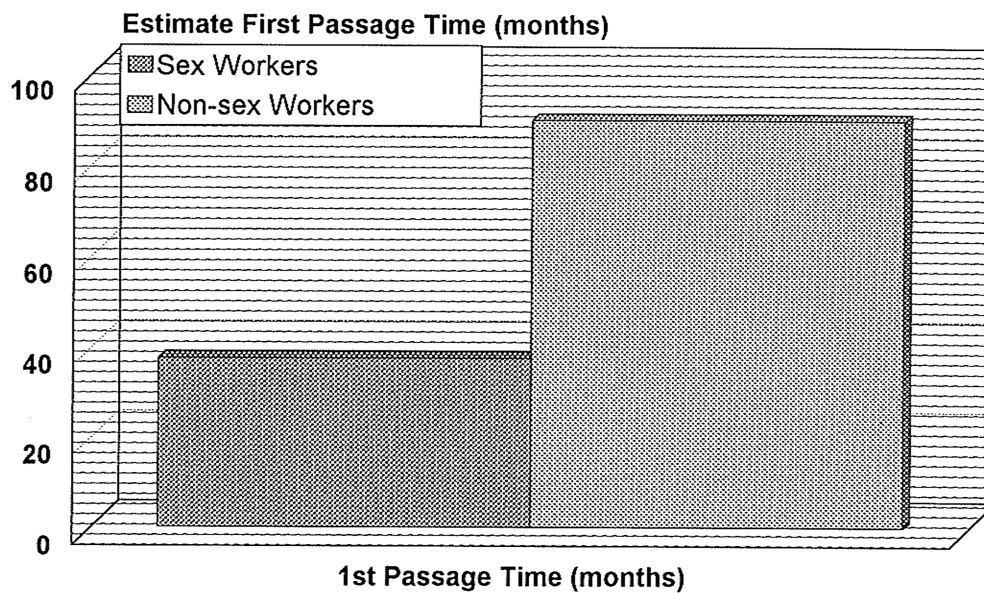
D1. Clinical and Laboratory Data

A nested subset of 42 women were enrolled in this study and followed for 1 to 5 months. Appendix II presents all the data for 42 consecutively studied HIV-1 positive and HIV-1 negative women with their corresponding clinical and laboratory parameters.

D2. Characteristics of the Study Population

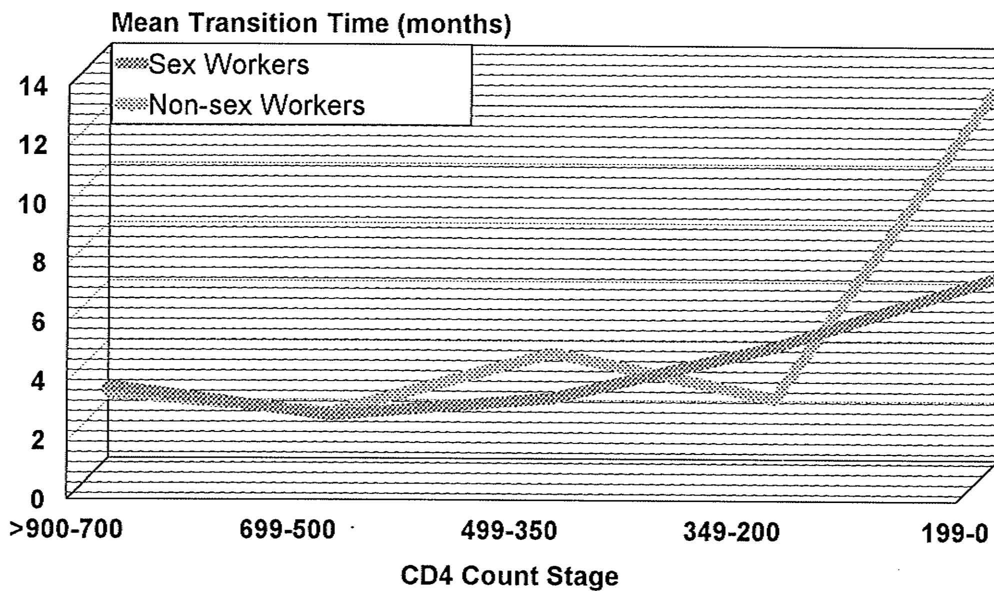
All 32 HIV-1 positive women in the study were in CDC stage III. Table 3 is a summary of the characteristics of the 42 women who comprised the study population.

FIGURE 21. Markov model estimates of time to CD4 count <200 among sex workers and non-sex workers.



p<.001 Wilks Maximum Likelihood

FIGURE 22. Markov modelling of transition time between CD4 count stages.



$p < .001$ Wilks Maximum Likelihood

TABLE 3. Characteristics of the 42 women comprising the study population (data are shown in mean ± SD [mean episode of individual STI during the 5 month study period]).

Age (years)	34.4 ± 5.8
Duration of prostitution (years)	9.9 ± 4.9
Mean duration of HIV-1 positive	5.0 ± 3.7 (range 0-9.9)
Mean CD4 count (on HIV positive women only)	332 ± 250
Mean CD8 count (on HIV positive women only)	1126 ± 579
Episodes of gonococcal infection	.88 ± .71 (0-3)
Number of subjects with gonococcal infection	30
Episodes of genital ulcers	.33 ± .61 (0-2)
Number of women with genital herpes	7
Episodes of *H. ducreyi* infection	.14 ± .35 (0-1)
Number of women with *H. ducreyi* infection	3
Episodes of PID	.5 ± .59 (0-2)
Number of women with PID	19
Episodes of *C. trachomatis* infection	.07 ± .26 (0-1)
Number of women with *C. trachomatis* infection	3
Episodes of positive syphilis serology	.17 ± .38 (0-1)
Number of women with positive syphilis serology	7
Number of women with no STI	8
Study visits	2.83 ± .73 (2-4)

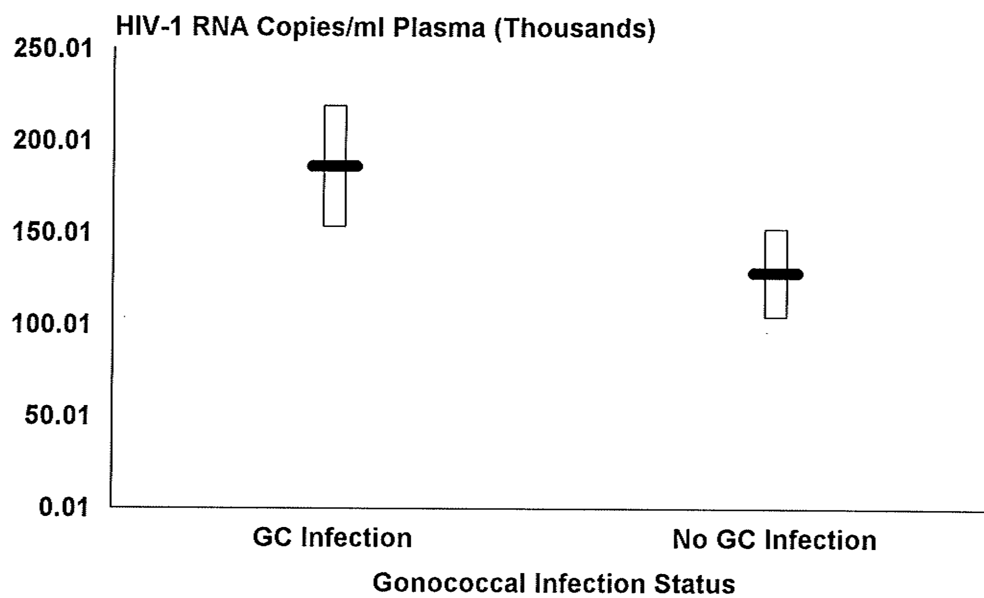
D3. Association Between Plasma Viremia and Gonococcal Infection

Little is known about the factors that govern the level of HIV-1 replication in infected individuals. One of the major components of the outer membrane lipopolysaccharide (LPS) of gram-negative bacteria has been shown *in vitro* to potentially stimulate HIV-1 replication. We sought to determine whether gonococcal infection of HIV-1 infected women leads to activation of virus replication. HIV-1 infected women were studied by measurement of plasma HIV-1 RNA copies at time of presentation with an acute episode of gonococcal infection and at intervals after the infection. Of the 30 HIV-1 positive women who presented with gonococcal infection, the majority of them experienced an increase in plasma viremia at the time of presentation with gonococcal infection. This increase in plasma viremia was observed to decline following treatment of the gonococcal infection (Figure 23).

D4. Association Between Plasma Viremia and Pelvic Inflammatory Disease

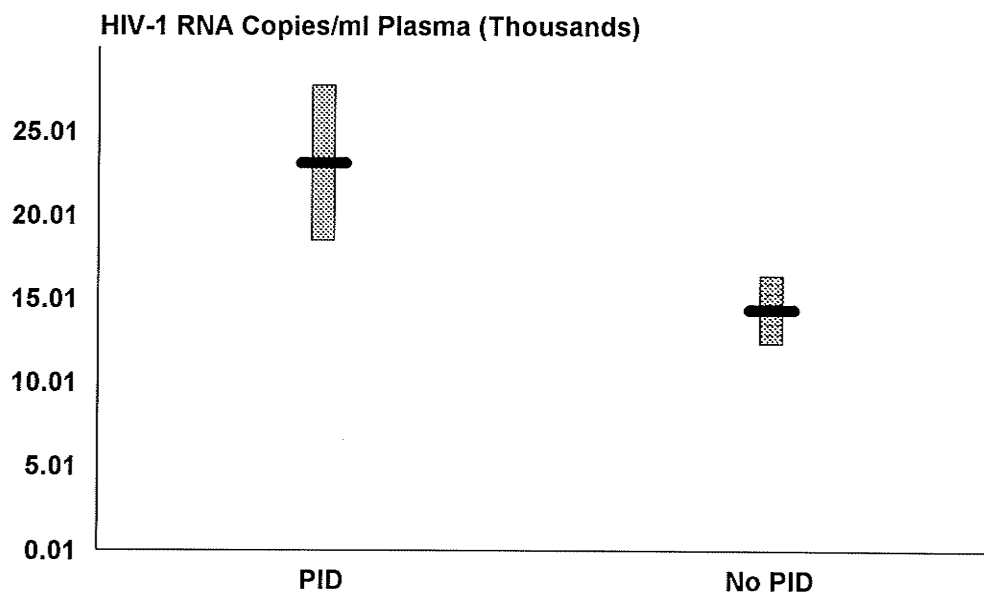
Pelvic inflammatory disease (PID) is the most common serious complication of genital infection in women. The most likely organism causing PID appears to vary in different geographic areas. However, gonococci and *C. trachomatis* are the most frequently isolated pathogens. We sought to determine whether PID in HIV-1 infected women resulted in an increase in HIV-1 viral replication. Of the 19 HIV-1 positive women who presented with PID, the majority (14 of 19) were observed to show an increase in plasma HIV-1 viremia at the time of presentation with acute PID, which declined following treatment of the acute PID (Figure 24).

FIGURE 23. Plasma HIV-1 RNA response during gonococcal infection. Plasma HIV-1 RNA response during gonococcal infection (GC infection) and following treatment of gonococcal infection (no GC infection). A total of 30 women were observed. Gonococcal infection was positively associated with an increase in plasma HIV-1 RNA.



p<.02 paired sample t-test

FIGURE 24. Plasma HIV-1 RNA response during pelvic inflammatory disease (PID) and following treatment of acute pelvic inflammatory disease. In this paired sample T-test, there were a total of 19 HIV-1 positive women presenting with PID. The increase in plasma viremia was associated with PID as shown.



p<.05 t-test

D5. HIV-1 Plasma Viremia and Sexually Transmitted Infections

Gonococcal infection resulted in readily demonstrable but trainsient increases in plasma HIV-1 RNA levels, indicative of activation of viral replication, as shown in Figure 25. This phenomenon was also observed with PID and other STIs studied. However, the episodes of other STI were too few to allow for statistical analysis.

D6. Plasma Cytokines and Gonococcal Infection

It has become evident that the immune response to pathogens is largely dependent on the preferential activation of particular CD4+ T helper cells able to secrete defined patterns of cytokines. Different cytokine patterns imply distinct effector functions. We sought to determine the pattern of cytokines secreted in HIV-1 infected individuals with an acute episode of a STI. Plasma cytokines were measured in HIV-1 infected individuals at presentation with an acute episode of STI and at 14 day intervals thereafter during the treatment of the acute STI. Of the 30 HIV-1 positive women presenting with acute gonococcal infection, the majority of them showed demonstrable increases in IL-4, IL-6, IL-10, TNFα and soluble TNFα receptor type II (Figure 26). Interferon gamma (IFNγ) and IL-2 were not detected.

D7. Association Between CD4 Lymphocytes and Sexually Transmitted Infections

To evaluate the impact of STI on CD4 lymphocytes, we measured CD4 lymphocytes in the study subjects at the time of presentation with an acute STI and at 14 day intervals thereafter during the course of treatment of the respective acute STI. A significant decrease in CD4 lymphocyte count was observed in the majority of the 30 individuals who presented with acute gonococcal infection at the time of

FIGURE 25. HIV-1 plamsa viremia in women during an episode with and without a STI (GC or PID) compared to women with no STI.

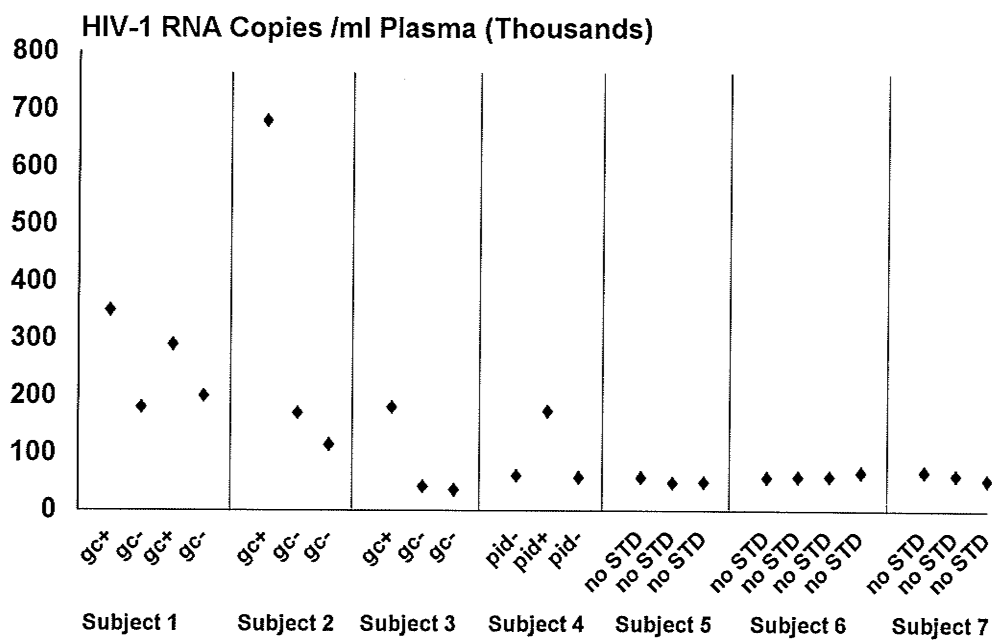
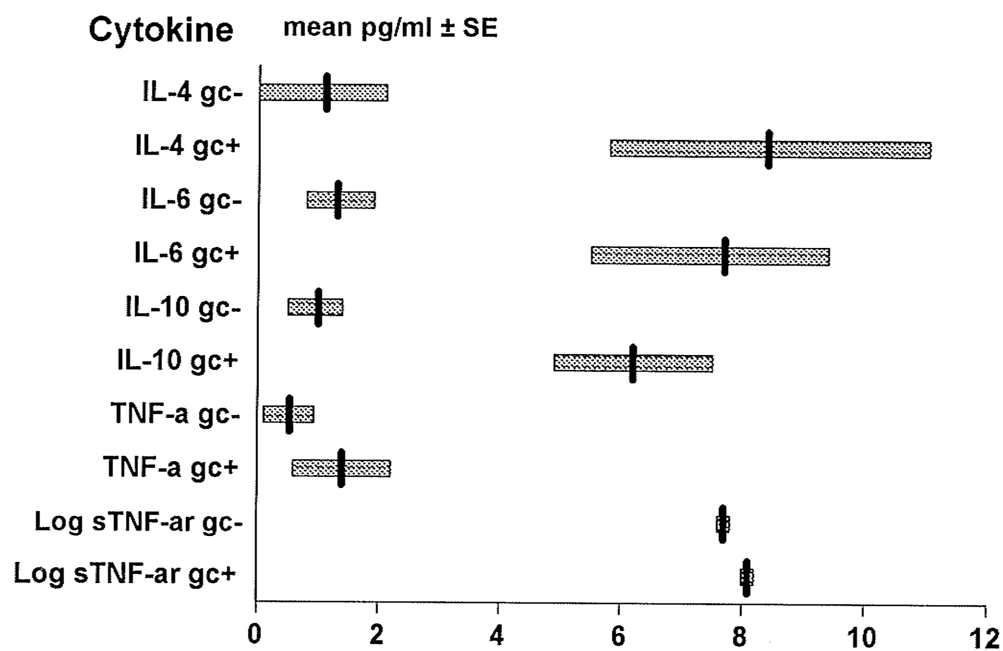
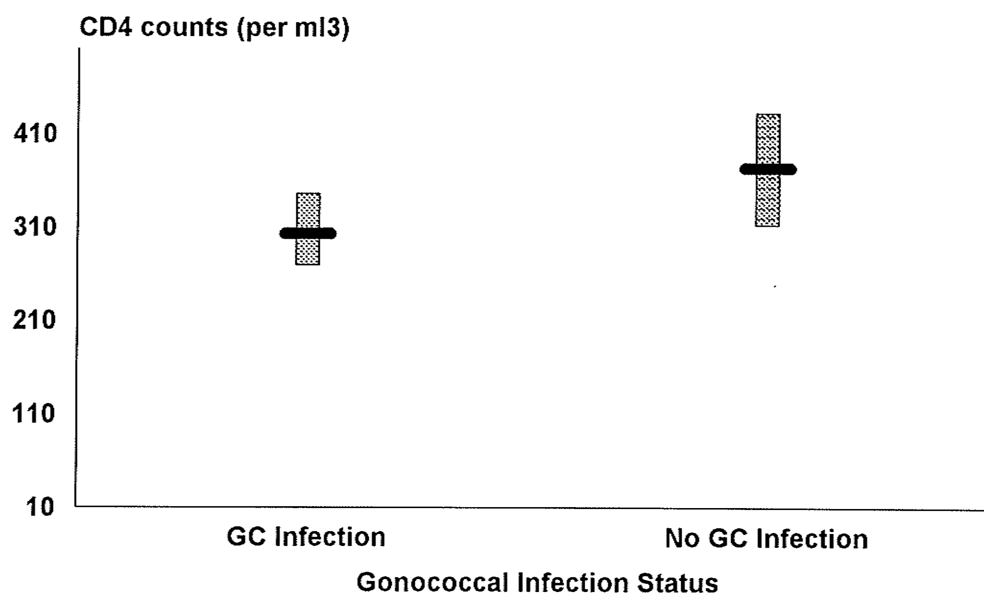


FIGURE 26. Measurement of plasma cytokines during acute gonococcal infection (gc+) and after treatment of gonococcal infection (gc-).



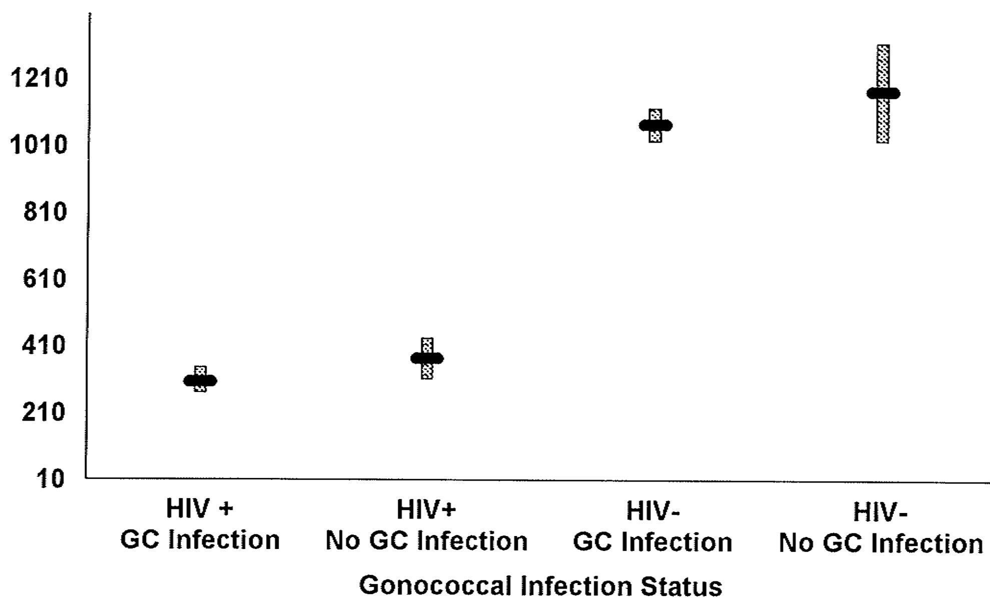
presentation with acute gonococcal infection ($p < 0.02$ paired sample t-test) (Figure 27). Although CD4 lymphocyte count decreased in HIV-1 negative women during episodes of acute gonococcal infection, this decrease was not statistically significant (Figure 28).

FIGURE 27. Change in CD4 lymphocyte count in HIV-1 positive women with acute gonococcal infection. Comparisons were made in CD4 lymphocyte counts in HIV-1 positive women at the time of presentation with acute gonococcal infection (GC infection) and at 14 days following treatment of the gonococcal infection (no GC infection). The comparisons were in 30 HIV-1 positive women presenting with acute gonococcal infection. Gonococcal infection was associated with a decrease in CD4 lymphocytes ($p<0.02$ paired sample t-test).



p<.02 paired sample t-test

FIGURE 28. Changes in CD4 lymphocyte counts in HIV-1 positive and HIV-1 negative women with gonococcal infection. Comparisons were made in CD4 lymphocyte counts in HIV-1 positive women at the time of presentation with acute gonococcal infection (GC infection) and at 14 days following treatment of the gonococcal infection (no GC infection). There was a significant decrease in CD4 lymphocyte counts during GC infection in HIV-1 positive women ($p<0.05$ t-test). Similar comparisons were made in HIV-1 negative women who showed a decrease in CD4 lymphocytes, although not statistically significant.



p<.05 t-test

DISCUSSION

A. PROGRESSION TO AIDS

A1. Natural History of HIV-1 Infection

The typical course of HIV-1 disease starts with a primary infection that is followed in 50 to 70% of individuals by an acute viral syndrome characterized by generalized lymphadenopathy, high plasma viremia, and a significant decrease in CD4 T cells. This occurs 3 to 6 weeks following initial exposure and infection (Tindal, 1991). HIV-1 is widely disseminated during this early stage of infection to such tissues as the lungs, bone marrow, liver, central nervous system (CNS), and particularly to the lymph nodes. At these sites, an immune response to HIV-1 that is responsible for suppression of plasma viremia is mounted. As a result, the acute syndrome resolves, the CD4 T cells return to normal, and the infection may enter a period of clinical latency that may persist for 10 years or longer (Pantaleo, et al., 1993). However, some individuals infected with HIV-1 progress to AIDS very rapidly (Melby et al., 1986; Quinnan, et al., 1984; Buchbinder, et al., 1994; Anzala, et al., 1995). The precise pathogenic mechanisms which determine the development of disease in HIV-1 infected individuals are not known.

Since 1985, we have been engaged in a study of the epidemiology of sexually transmitted infections (STI), including HIV-1, in a large cohort of sex workers. In the course of our studies on the natural history of HIV-1 infection, we have observed a very rapid rate of progression from asymptomatic HIV-1 infection to AIDS (Anzala, et al., 1995), with a median duration of four years (Figure 20). The characteristics of

women who progressed to AIDS were compared within the same cohort to those women who remained disease free (Table 2). By parametric proportional hazards modelling, no significant association was found between any of the time dependent covariables and disease progression. Of the "constant" covariables, lack of condom use was associated with increased risk of progression. This suggests that unprotected sexual exposure results in accelerated disease progression through such events as STI and multiple infections with HIV-1. Alternatively, rapid disease progression could be related to the gender or environment of these women. To better understand the factors influencing the course of disease, two samples from Nairobi were studied; one from a cohort of CSW and another, as a comparison group, from mothers enrolled in an HIV-1 vertical transmission study. A Markov model was used to analyze transitions between HIV-1 disease stages, as defined by CD4 counts (Figures 21 and 22). The sex workers experienced a rapid decline in CD4 counts compared to mothers from the HIV-1 vertical transmission study and this was consistent with earlier findings of rapid clinical disease progression among sex workers. We sought to determine whether the high burden of STI experienced by sex workers could explain the rapid disease progression.

A2. Cofactors in HIV-1 Disease Progression

Rates of HIV-1 disease progression vary widely between individuals who are infected with HIV-1. CD4 T cell count has been the principal marker used to assess risk of progression to AIDS. Several studies have reported a correlation between viral load, CD4 T cell counts, and HIV-1 disease stage (Coombs, et al., 1989; Ho, et

al., 1989; Aoki-Sei, et al., 1992; Pantaleo, et al., 1993). Based on these results, the progressive immune deficiency that is the hallmark of HIV-1 infection is thought to result primarily from a direct cytopathic effect of HIV-1 itself, meaning that the rate of viral replication and viral burden play a significant role in disease progression.

Little is known about the cofactors that govern the level of HIV-1 replication in infected individuals. Although HIV-1 replication in culture is known to be greatly facilitated by T cell activation, the ability of specific antigenic stimulation to augment HIV-1 replication *in vivo* has not been widely studied. Having observed a rapid disease progression in our cohort of women with a high burden of STI, we sought to determine whether STI in HIV-1 infected women leads to activation of viral replication. To begin to address these issues, an RT-PCR based strategy to quantitate HIV-1 in human plasma was developed as described in the ***Methods II and III*** sections and in the ***HIV-1 Quantiation*** section of the ***Discussion***, which follows.

B. HIV-1 QUANTITATION

B1. Markers of Disease Progression

Human immunodeficiency virus induces a persistent infection that progresses from an asymptomatic infection to AIDS. The identification of factors or markers that correlate with progression or regression of HIV-1 infection is important for understanding the pathogenesis and prognosis, and for the monitoring of treatment. Circulating CD4 lymphocyte counts, serum HIV-1 p24 antigen, serum neopterin, serum β_2-microglobulin, soluble CD8, and soluble interleukin-2 receptor levels have

been used as indicators of HIV-1 disease activity (Fahey, et al., 1990; Lacey, et al., 1987; Funches, et al., 1988). However, they are limited by sensitivity and specificity. Quantitation of plasma viremia using cultures of limiting dilutions have been designed but suffer from being time consuming, expensive and also coupled with difficulty of getting certain strains of HIV-1 to replicate *ex vivo*. With reports showing that the natural history and pathogenesis of HIV-1 infection is closely linked to the replication of virus *in vivo* (Ho, et al., 1989; Saksela, et al., 1994), we developed a method that combines the use of reverse transcription followed by polymerase chain reaction (RT-PCR) to quantitate individual HIV-1 RNA molecules in plasma. The method is detailed in Parts II and III of the ***Methods*** section. The sensitivity of RT-PCR makes it possible to detect minor changes in HIV-1 RNA molecules in plasma and the procedures requires only a few hours to perform. However, it can be difficult to obtain quantitative information. This is due to the exponential nature of the PCR amplification where small variations in amplification efficiency results in dramatic changes in product yields. In addition, the amount of product generated plateaus during the late stages of the reaction (Figure 18) due to consumption of necessary components and generation of inhibitors. These characteristics can obscure differences in the initial amounts of target sequences during the course of amplification. These problems can be overcome by the use of an internal control in the PCR, a procedure referred to as co-amplification RT-PCR.

B2. Co-Amplification RT-PCR

The general concept of co-amplification RT-PCR is the co-amplification in the

same tube of two different templates of equal or similar lengths and with the same primer recognition sequences, thus ensuring identical thermodynamics and amplification efficiency for both template species. The amount of one of the templates introduced must be known (Gilliland, et al., 1990). For our project, this was achieved by the generation of synthetic HIV-1 *nef δ* RNA as described in parts II and III of the ***Methods*** section and summarized in Figure 3. These HIV-1 synthetic *nef δ* RNA contained identical priming sites for reverse transcription and amplification compared to the native transcript. Following co-amplification, products of both template species (the internal standard and the target) must be clearly distinguished by gel electrophoresis analysis to allow evaluation of the relative intensities of the bands for both species. For our project, this was achieved by altering the HIV-1 synthetic *nef δ* RNA by insertion of an adaptor as described in part III of the ***Methods*** section. The internal standard (HIV-1 *nef δ* mRNA) and the target amplification products differed in length and could readily be distinguished on gel electrophoresis as shown in Figures 7 and 19. Because the templates were co-amplified in the same reaction tube using similar primers, any variable affecting amplification had the same effect on both templates. Consequently, the ratio of PCR products, which were calculated using scintillation counting of isolated agarose gel slices of electrophoresed reaction product following PCR with ^{32}P labelled oligonucleotide primers, reflected the ratio between the initial amount of the two sequences, thus allowing the evaluation of the amount of HIV-1 RNA template in plasma. In fact, according to the principle of PCR amplification, the product yield follows the well known function $\mathbf{P = P'.(1+e)^n}$

and is directly dependent on the starting template copy number (P'), amplification efficiency (e) and the number of cycles (n). In co-amplification PCR, the function can be written for both as:

$$\text{Standard DNA} = \mathbf{S = S'.(1+e)^n}$$

$$\text{Target DNA} = \mathbf{T = T'.(1+e)^n}$$

Since **e** and **n** were identical for both templates, the relative product ration (S/T) was dependent on their initial concentration and, where the product ratio was 1, the starting quantities must have been very close to equal. At ratios of target template to standard template of less than or greater than 1, the amplification of the two templates was not equal as shown in Figure 19, lanes 1 and 3. The variable intensity of the bands indicated the inconsistent amplification of the templates in the presence of different concentrations of the target template; differential amplification of the two RNAs occurred unless the standard template and the target template approached 1.

Having demonstrated that our standard could be co-amplified in the same reaction tube as the target and be readily distinguished, and having also demonstrated that the starting ratio of the standard template to target template must approach 1 for equal efficiency of amplification, this method was used to quantitate HIV-1 plasma viremia. The procedure used is summarized in Figure 9. To verify the reproducibility of the assay, duplicate plasma samples were obtained from five HIV-1 individuals and analyzed as described in the ***Methods*** section. The results obtained are shown in Table 1 and demonstrate that the assay was reproducible. The major limitations of the assay are the requirement for multiple dilutions of RNA, the use of

radioactive probes and the issue of crossover contamination common to all PCR-based strategies.

A direct measure of circulating free HIV-1 in plasma, as was done in our project, provides a tool for measuring rapid changes in the amount of free virus circulating *in vivo*. The ability to determine viral burden is essential in understanding the natural history of HIV-1 infection, predicating disease progression, and assessing therapeutic potentials for new antiviral agents and vaccines.

C. SEXUALLY TRANSMITTED INFECTIONS AND PLASMA VIREMIA

The precise pathogenic mechanism which determines the development of the disease state in HIV-1 infected individuals is unknown. The diversity of responses to HIV-1 infection suggest that multiple cofactors likely play a significant role in the pathogenesis of disease. These cofactors include numerous viral, bacterial, fungal and protozoal pathogens which, individually or together with HIV-1, may acclerate the progression of disease by a variety of mechanisms. As previously discussed, the sex workers in our study cohort had a rapid rate of disease progression compared to the general population. Given the enormous burden of STI experienced by these women, we sought to determine whether STI in HIV-1 infected women led to activation of virus replication and decline in CD4 T lymphocytes. HIV-1 infected and uninfected women were studied by measurment of HIV-1 plasma virus load during an acute episode of STI and at intervals of 14 days following treatment.

A large increase in plasma HIV-1 RNA was observed in subjects during acute episodes of gonococcal infection (Figure 23). A similar observation was noted with pelvic inflammatory disease (PID) as shown in Figure 24. The correlation between the increase in plasma viremia and gonococcal infection was significant (Figure 23, $p < .02$ paired sample t-test). Similar correlations were also found between plasma viremia and PID (Figure 24, $p < .05$ t-test). Thus, it appears that gonococcal infection and PID stimulate HIV-1 replication *in vivo*. The broad range of plasma viremia levels that we found in our HIV-1 infection patients (Appendix II) is consistent with previously reported data (Coombs, et al., 1989; Ho, et al., 1989; Holodniy, et al., 1991).

It is interesting to note that, after substantial increase in plasma viremia during acute episodes of gonococcal infection and PID (Figure 25), the levels of plasma viremia dropped following treatment and recovery from the STI. This suggests that the steady state may result from the number of new cycles of viral infection which is, perhaps, determined by the host's immune activation status.

As discussed in the ***Introduction***, lipopolysaccharide (LPS), a major constituent of the outer membrane of gram-negative bacteria, activates HIV-1 replication in monocyte/macrophage cells. The increase in plasma viremia observed in our study, during acute episodes of STI, may be due to stimulation of HIV-1 infected immune cells by specific bacterial antigens, including LPS. LPS may indirectly mediate or contribute to the observed plasma viremia through cytokine production following immune activation within the local lymph nodes. The

importance of these observations for AIDS pathogensis may lie in the fact that these bursts of HIV-1 plasma viremia may pertub the steady state of HIV-1 turnover which appears to be under immunologic control and thus may induce destruction of either a specific antigen reactive component of the T cell repertoire or a more general non-antigen specific component, or both. As shown in Figure 27, acute episodes of gonococcal infection were associated with a decline in CD4 T cell counts with $p < .02$ in the paired sample t-test. A decline in CD4 T cell counts was also observed in HIV-1 seronegative sex workers with acute episodes of gonococcal infection (Figure 28). The decrease in CD4 lymphocytes during acute gonococcal infection in HIV-1 positive individuals was an interesting observation. Various mechanisms to explain CD4 T cell loss have been advanced including intrinsic cytopathic effect of the virus, the immune system, cytotoxic T lymphocytes (CTL), consummate HIV-1 infected cells, and apoptosis. The immune activation and high level replication of HIV-1 during gonococcal infection may lead to virus and immune mediated killing of CD4 lymphocytes. These results are consistent with what has been documented following administration of antiviral agents, a decline in plasma viremia is associated with increased CD4 T cell counts (Ho, et al., 1995; Wei, et al., 1995), suggesting that the dynamics of HIV-1 replication and hence the plasma viremia are critical and play a major role in the evoluation of HIV-1 infection. Our results suggest that for HIV-1 infected individuals, recurrent STI may have adverse consequences as a result of STI-induced activation of HIV-1 replication.

D. SEXUALLY TRANSMITTED INFECTIONS AND CYTOKINE PATTERNS

It has become evident that the immune response to pathogens are largely dependent on the preferential activation of particular CD4 helper T cells able to secrete defined patterns of cytokines. As discussed in the ***Introduction***, at least two distinct murine CD4 T helper (Th) cell subsets, Th_1 and Th_2, showing mutually exclusive patterns of cytokine secretion, have been defined (Mosmann, et al., 1989). There is now general consensus for the existence of human CD4+ Th cells that resemble murine Th_1, Th_0 and Th_2 cells in both cytokine profiles and functional properties. Evidence for this dichotomy came from analysis of human T cell clones specific for a variety of bacterial and parasitic antigens (Romagnani, 1991; Del Prete, et al., 1991; Romagnani, 1994). Many viruses, including HIV-1, rely on the liberation and cellular action of host immune cytokines to expand their host cell range, which allow for viral replication. Because the HIV-1 lifecycle is so linked to the cytokine networks, as discussed in the ***Introduction***, we sought to determine the types of cytokines secreted by HIV-1 infected subjects with acute STI. HIV-1 infected individuals were studied by measuring cytokines in plasma at the time of presentation with an acute STI, and at intervals thereafter as discussed in the ***Methods*** section. As shown in Figure 26, acute gonococcal infection resulted in demonstrable, but transient, increases in IL-4, IL-6, IL-10, TNF-α and soluble TNF-α receptor type II (sTNFαRII). In all the plasma samples assayed, interferon gamma (IFN-γ) and IL-2 were not detected. The number of other STI in the study, including *H. ducreyi, C.*

trachomatis, syphilis and genital herpes (Table 3), were too few to allow for analysis. As shown in Appendix II, not all individuals with STI had demonstrable cytokine levels. The reason for this could be associated with physiologic changes of cytokine secretion. Cytokines are very labile proteins; they peak within hours of infection and then begin to decline. We therefore hypothesize that the duration between onset of an STI and presentation to the clinic influenced the demonstrability of the cytokines. A stimulus for cytokine production is LPS, which has been documented as a major stimulus for cytokine production (Pomerantz, et al., 1990; Dinarello, 1989). Our results herein clearly indicate the presence of increased Th_2 type cytokines, suggesting a bias towards a Th_2 response, which has previously been associated with risk of disease progression (Clerici, et al., 1993; Maggi, et al., 1994). Although the episodes of other STI were too few to make a meaningful observation, our results suggest that they are likely to influence the immune system in a similar manner as seen in gonococcal infections, with an increased secretion of Th_2 type cytokines.

Finally, HIV-1 infection does not generally lead to constitutive cytokine gene transcription and secretion (D'Addario, et al., 1990; Gendelman, et al., 1990; Molin, et al., 1990), suggesting that the increased expression of these cytokines was a result of antigenic challenge by STI.

E. SEXUALLY TRANSMITTED INFECTIONS AND DISEASE PROGRESSION

Markers providing evidence of HIV-1 replication have been used to assess the

relationship between HIV-1 viremia and clinical progression of HIV-1 infection. Scientific literature is replete with studies showing correlation between viral load, CD4+ lymphocyte counts, and HIV-1 disease stage (Coombs, et al., 1989; Ho, et al., 1989; Aoki-Sei, et al., 1992). Progressive immune dysfunction, which is the hallmark of HIV-1 infection, is thought to result primarily from direct cytopathic effects of HIV-1 itself and immune responses directed against surface exposed HIV-1 antigens (Pantaleo, et al., 1993; Wei, et al., 1995; Ho, et al., 1995). The viral burden is at least 100-fold higher in AIDS patients than in asymptomatic subjects, suggesting that increasing viremia may be an important marker of CD4 lymphocyte depletion.

The purpose of our study was to correlate STI and markers of disease progression, CD4 T cells and cell free HIV-1 RNA, and the patterns of cytokine secretion. Gonococcal infection and PID resulted in readily demonstrable increases in plasma HIV-1 RNA levels, indicative of activation of viral replication. Gonococcal infection also resulted in CD4 T cell decrease. Both the increase in plasma viremia and decline in CD4 T cell counts were transient and returned to baseline following the treatment of STI. These data indicate that important aspects of the host-virus relationship, underlying HIV-1 infection, may be obtained from careful analysis of interventions that perturb, either positively or negatively, the steady state equilibrium of virus replication *in vivo*. Recurrent STI in HIV-1 infected persons may result in bursts of cytokine production by infected monocytes and lymophocytes. An area of extreme importance is the mechanisms regulating cellular infection with and without

expression of HIV-1. The natural prolonged course of HIV-1 disease in the majority of the HIV-1 infected individuals suggests that several stages of the viral life cycle at the cellular level may be controlled by extracellular intervention. Furthermore, target cells must be in an activated state to permit complete reverse transcription and integration of the virus, the two steps essential for productive infection. Once integrated, HIV-1 may remain dormant until the right combination of extracellular influences are encountered to induce viral replication. From our data, the combination of extracellular influence may be the recurrent STI, which results in bursts of cytokine secretion and immune activation. Secretion of cytokines in the lymph nodes of infected individuals may increase viral production, spread, CD4 cell decline, and progression to disease. Potentially, the increased plasma viremia may induce destruction of either a specific STI reactive component of the T cell repertoire or a more general non-STI specific component, or both. Other infections that have been documented to increase viral load includes *Pneumocystis carinii* and *Mycoplasma tuberculosis* (Perriens, et al., 1991). Tuberculosis has been found to increase significantly with HIV-1 infection and to influence its course.

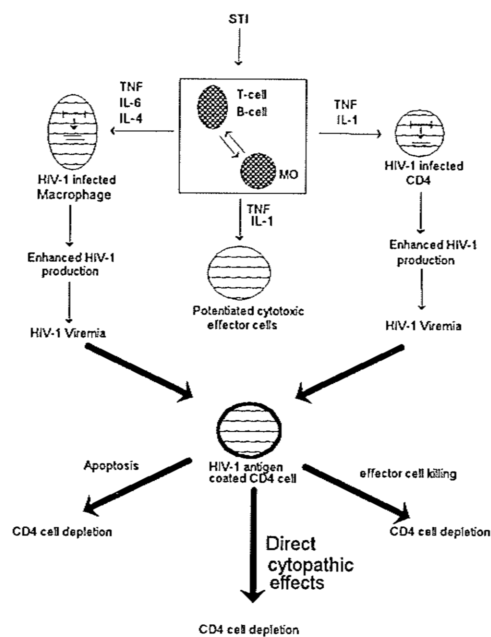
In addition to STI resulting in increases in plasma viremia, the cytokine secreted during acute episodes of STI were those of Th_2 phenotype. This suggests a shift in the immune cell population that predominantly secrete HIV-1 activating cytokines and promote the humoral arm of the immune response which has been associated with disease progression. Taken together, these results indicate that recurrent or frequent STI in HIV-1 infected subjects has potential adverse

consequences of increased plasma viremia, CD4 T cell decline, and bias to Th_2 immune response. The mechanism whereby bacterial STI results in increased plasma viremia is unclear from this study. One potential mechanism of increased plasma viremia observed in our study, during acute episodes of STI, is through direct activation of HIV-1 replication through LPS within the confines of the regional lymph nodes. It is important to note that HIV-1 replication occurs within the lymph nodes. A second possible mechanism is the perturbation of the immune responses controlling HIV-1 replication by secretion of Th_2 type cytokines as observed during infection with bacterial STI. These perturbations may result in the escape of HIV-1 from the host protective immune responses.

FUTURE STUDIES

Figure 29 graphically summarizes the hypothetical model for the interactions occurring between STI and the immune response in HIV-1 infected individuals. Potentially, increased plasma viremia may induce the destruction of either a specific antigen-reactive component of the T cell repertoire or a more general non-antigen specific component. To understand the mechanism of the CD4 T cell loss observed, further studies to elucidate whether STI results in antigen-specific constriction of the T cell repertoire and progression of HIV-1 disease is required. To test the hypothesis that STI result in antigen specific constriction of the T cell repertoire and disease progression, the effects of STI should be observed in a longitudinal study of newly infected sex workers.

FIGURE 29. Proposed effects of STI on HIV pathogenesis.



CONCLUSIONS

These observations are significant because they suggest that STI stimulate the immune system which may trigger HIV-1 production and thereby potentially exacerbate disease progression. These observations also support the concept that the burst of plasma HIV-1 RNA did not directly reflect AIDS progression but rather was a transient condition resulting from the STI, possibly by increasing the number of activated CD4 lymphocytes capable of supporting production of HIV-1 infection. A second possible mechanism is a transient perturbation of immune responses controlling HIV-1 replication. It is possible, however, that multiple bursts in HIV-1 production caused by recurrent STI that stimulate immune system may enhance HIV-1 disease progression and thereby exacerbate immune decline. If this is true, it is feasible that suppression or avoidance of these bursts could have important clinical impacts on the health of these women and the rate of disease progression.

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APPENDIX I

ABBREVIATIONS USED

HIV-1	Human Immunodeficiency Virus Type 1
AIDS	Acquired Immune Defiency Syndrome
cRNA	Complementary Ribonculeic Acid
CPM	Counts Per Minute
LTR	Long Terminal Repeat
IL-1	Interleukin-1
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-10	Interleukin-10
TNFα	Tumor Necrosis Factor - α
sTNFR	Soluble Tumor Necrosis Factor Receptor
RNA	Ribonucleic Acid
PBMC	Peripheral Blood Mononuclear Cells
STI	Sexually Transmitted Infections
PHIV	Plasma (HIV-1) Viremia
CD4	CD4 T Lymphocyte
CD8	CD8 T Lymphocyte

ABBREVIATIONS USED (continued)

GC	*Neisseria gonorrhoeae*
HD	*Haemophilus ducreyi*
CT	*Chlamydia trachomatis*
PID	Pelvic Inflammatory Disease
HSV	Herpes Simplex Virus
GUD	Genital Ulcer Disease
RPR	Rapid Plasma Reagin Test

APPENDIX II

Summary of patient study visits and the corresponding results of all the parameters analyzed.

APPENDIX II. Summary of patient visit dates and corresponding cytokine measurements (pq/ml), plasma viremia and STI diagnosis.

Patient #	Date	TNF	sTNFr	IL-6	IL-4	IL-10	CD4	CD8	sIL-22	PHIV	GC	HD	CT	PID	RPR	HSV	GUD	HIV
17	03.02.95	10.1	3,291.0	4.3	.0	.0	210	940	1,920	79,000	0	0	0	0	0	0	0	1
17	21.03.95	14.4	4,986.0	6.2	.0	7.8	190	960	1,825	82,000	1	0	0	0	0	0	0	1
17	04.05.95	10.2	2,435.0	.0	.0	18.6	180	870	2,841	72,000	0	0	0	0	0	0	0	1
30	21.03.95	.0	1,767.0	.0	.0	.0	540	940	571	76,000	1	0	0	0	1	0	0	1
30	20.04.95	.0	1,302.0	.0	.0	.0	830	1740	761	38,000	0	0	0	0	0	0	0	1
30	11.05.95	.0	1,600.0	.0	.0	.0	1240	1870	609	40,000	0	0	0	0	0	0	0	1
57	30.03.95	.0	3,874.0	25.0	.0	.0	120	1280	2,011	100,000	1	1	0	0	0	0	1	1
57	06.04.95	.0	2,911.0	.0	.0	.0	460	3420	2,354	74,000	0	0	0	0	0	0	0	1
57	18.04.95	.0	2,932.0	.0	.0	.0	520	3330	2,113	120,000	0	0	0	0	0	0	0	1
57	06.05.95	.0	2,920.0	.0	.0	.0	420	3820	1,919	59,000	0	0	0	0	0	0	0	1
79	14.02.95	11.2	4,141.0	12.6	.0	18.6	270	1270	1,590	88,000	1	0	0	1	0	0	0	1
79	17.02.95	.0	3,164.0	4.7	.0	.0	220	1150	1,291	56,000	0	0	0	0	0	0	0	1
79	21.02.95	.0	2,822.0	.0	.0	.0	270	1080	1,014	64,000	0	0	0	0	0	0	0	1
170	08.02.95	.0	3,896.0	4.3	34.6	15.6	50	920	171	120,000	1	0	0	0	0	0	0	1
170	15.02.95	10.8	2,718.0	3.3	.0	7.8	50	760	201	30,000	0	0	0	0	0	0	0	1
170	21.03.95	.0	2,142.0	.0	.0	.0	50	880	265	42,000	0	0	0	0	0	0	0	1
170	06.04.95	.0	1,840.0	.0	.0	.0	50	840	240	80,000	0	0	0	0	0	0	0	1
303	10.02.95	.0	3,476.0	.0	.0	.0	150	480	1,523	225,000	0	0	0	1	0	0	0	1
303	17.02.95	.0	2,990.0	.0	.0	.0	240	590	1,719	220,000	0	0	0	0	0	0	0	1
303	06.04.95	.0	3,910.0	.0	.0	.0	230	570	1,787	158,000	1	0	0	0	0	0	0	1
406	20.02.95	.0	3,286.0	10.2	.0	10.6	640	1010	1,179	42,000	1	0	0	1	0	0	0	1
406	20.03.95	.0	2,550.0	9.4	.0	8.2	590	810	958	48,000	1	0	0	0	0	0	0	1
406	18.04.95	.0	2,010.0	.0	.0	.0	.	.	980	40,000	0	0	0	0	0	0	0	1
406	02.05.95	.0	3,351.0	6.2	.0	9.6	590	910	1,113	54,000	1	0	0	0	0	0	0	1

NB. TNF = Tumor Necrosis Factor; sTNFr = Soluble tumor necrosis factor receptor; IL-6 = interleukin 6; IL-4 = interleukin 4; IL-10 = interleukin 10; sIL-22 = soluble interleukin 2 receptor (all cytokine levels are in picograms per ml); PHIV = plasma HIV viremia; GC = *Neisseria gonorrhoeae* infection; HD = *Haemophilus ducreyi* infection; CT = *Chlamydia trachomatis* infection; PID = pelvic inflammatory disease; RPR = syphilis serology; HSV = *Herpes simplex* virus infection; GUD = genital ulcer disease; and HIV = human immunodeficiency virus serology.

Patient #	Date	TNF	sTNFr	IL-6	IL-4	IL-10	CD4	CD8	sIL-22	PHIV	GC	HD	CT	PID	RPR	HSV	GUD	HIV
79	06.02.95	.0	3,884.0	.0	.0	7.8	450	560	682	180,000	1	0	0	0	0	0	0	1
579	17.02.95	.0	3,079.0	.0	.0	.0	620	650	543	42,000	0	0	0	0	0	0	0	1
579	27.02.95	.0	2,396.0	.0	.0	.0	600	690	679	36,000	0	0	0	0	0	0	0	1
612	06.03.95	.0	4,432.0	4.7	.0	16.3	240	1280	417	420,000	1	0	0	1	1	0	0	1
612	10.04.95	.0	3,614.0	3.8	.0	.0	230	1250	880	500,000	0	0	0	0	0	0	0	1
767	28.03.95	.0	1,608.0	.0	.0	7.8	480	900	566	0	0	0	0	0	0	0	0	0
767	25.04.95	.0	1,621.0	.0	.0	.0	900	1460	558	0	0	0	0	0	0	0	0	0
795	09.02.95	.0	2,813.0	8.0	.0	.0	310	1970	789	350,000	1	0	0	0	1	1	1	1
795	13.02.95	.0	2,840.0	11.8	.0	10.8	290	1330	659	180,000	0	0	0	0	0	0	0	1
795	28.03.95	.0	5,065.0	12.6	.0	7.8	300	1630	655	290,000	1	1	0	0	0	0	1	1
795	04.04.95	.0	2,180.0	.0	.0	.0	280	1340	670	200,000	0	0	0	0	0	0	0	1
825	21.03.95	.0	2,354.0	.0	.0	.0	210	1130	1,177	58,000	0	0	0	0	1	0	0	1
825	10.04.95	.0	2,176.0	.0	.0	.0	160	740	1,125	49,000	0	0	0	0	0	0	0	1
825	03.05.95	.0	2,427.0	.0	.0	.0	180	860	1,167	50,000	0	0	0	0	0	0	0	1
864	21.03.95	.0	2,663.0	.0	.0	.0	360	1170	960	59,000	0	0	0	0	0	0	0	1
864	05.04.95	.0	2,739.0	.0	.0	.0	370	1320	1,020	60,000	0	0	0	0	0	0	0	1
864	25.04.95	.0	2,354.0	.0	.0	.0	280	810	904	67,000	0	0	0	0	0	0	0	1
864	02.02.95	.0	2,430.0	.0	.0	.0	300	960	1,016	58,000	0	0	0	0	0	0	0	1
915	15.03.95	.0	2,226.0	.0	.0	.0	420	1320	651	61,000	0	0	0	1	0	0	0	1
915	27.03.95	.0	3,031.0	.0	.0	.0	900	1400	831	173,000	0	0	1	1	0	0	0	1
915	08.05.95	.0	2,379.0	.0	.0	.0	280	950	705	58,000	0	0	0	0	0	0	0	1
1158	17.02.95	.0	3,712.0	3.9	.0	.0	220	1860	883	460,000	0	1	0	0	0	0	1	1
1158	22.02.95	.0	2,901.0	.0	.0	.0	190	2020	748	600,000	0	0	0	0	0	0	0	1
1158	01.03.95	.0	2,515.0	.0	.0	.0	140	1650	634	540,000	0	0	0	0	0	0	0	1

NB. TNF = Tumor Necrosis Factor; sTNFr = Soluble tumor necrosis factor receptor; IL-6 = interleukin 6; IL-4 = interleukin 4; IL-10 = interleukin 10; sIL-22 = soluble interleukin 2 receptor (all cytokine levels are in picograms per ml); PHIV = plasma HIV viremia; GC = *Neisseria gonorrhoeae* infection; HD = *Haemophilus ducreyi* infection; CT = *Chlamydia trachomatis* infection; PID = pelvic inflammatory disease; RPR = syphilis serology; HSV = *Herpes simplex* virus infection; GUD = genital ulcer disease; and HIV = human immunodeficiency virus serology.

Patient #	Date	TNF	sTNFr	IL-6	IL-4	IL-10	CD4	CD8	sIL-22	PHIV	GC	HD	CT	PID	RPR	HSV	GUD	HIV
1192	19.01.95	.0	2,142.0	6.6	31.8	.0	1340	920	1,337	0	1	0	0	1	0	0	0	0
1192	21.02.95	.0	1,644.0	.0	.0	.0	1320	980	1,230	0	0	0	0	0	0	0	0	0
1192	27.02.95	.0	1,430.0	.0	.0	.0	1430	1080	1,148	0	0	0	0	0	0	0	0	0
1192	06.03.95	.0	1,054.0	.0	.0	.0	1030	640	1,175	0	0	0	0	0	0	1	1	0
1293	08.02.95	.0	2,109.0	.0	.0	.0	1020	700	531	0	0	0	0	1	0	0	0	0
1293	28.02.95	.0	1,592.0	.0	.0	.0	1160	950	497	0	0	0	0	0	0	0	0	0
1337	27.02.95	.0	2,248.0	.0	.0	.0	.	.	1,137	270,000	0	1	0	0	0	1	1	1
1337	20.03.95	.0	2,222.0	.0	.0	.0	120	960	1,027	230,000	0	0	0	0	0	0	0	1
1337	10.05.95	.0	2,844.0	.0	.0	.0	170	1310	1,384	300,000	1	0	0	0	0	1	1	1
1404	24.02.95	.0	2,879.0	.0	32.6	.0	550	1190	627	680,000	1	0	0	1	0	0	0	1
1404	06.03.95	.0	1,773.0	.0	.0	.0	610	1150	561	170,000	0	0	0	0	0	0	0	1
1404	13.03.95	.0	1,741.0	.0	.0	.0	760	1420	663	115,000	0	0	0	0	0	0	0	1
1423	27.02.95	.0	1,479.0	4.2	.0	.0	300	1120	759	60,000	1	0	0	1	0	1	1	1
1423	06.03.95	.0	2,209.0	.0	.0	.0	340	1210	482	32,000	0	0	0	0	0	0	0	1
1423	15.03.95	.0	2,298.0	.0	.0	.0	300	1110	777	35,000	1	0	0	1	0	0	0	1
1515	27.02.95	.0	5,064.0	18.1	.0	7.8	800	640	640	0	1	0	0	1	1	0	0	0
1515	06.03.95	.0	3,291.0	.0	.0	.0	1230	1110	510	0	0	0	0	0	0	0	0	0
1533	21.04.95	14.3	2,076.0	6.9	.0	14.4	0	160	1,637	125,000	0	1	0	0	0	0	1	1
1533	25.04.95	.0	908.0	.0	.0	.0	10	320	700	120,000	0	0	0	0	0	0	0	1
1533	03.02.95	.0	1,020.0	.0	.0	.0	10	330	878	150,000	0	0	0	0	0	1	1	1
1540	16.03.05	.0	2,992.0	.0	.0	.0	250	690	1,840	580,000	1	0	0	0	0	0	0	1
1540	28.03.95	.0	3,796.0	.0	.0	.0	260	790	2,000	430,000	0	0	0	0	0	0	0	1
1540	04.04.95	.0	2,610.0	.0	.0	.0	290	960	1,300	.	0	0	0	0	0	0	0	1

NB. TNF = Tumor Necrosis Factor; sTNFr = Soluble tumor necrosis factor receptor; IL-6 = interleukin 6; IL-4 = interleukin 4; IL-10 = interleukin 10; sIL-22 = soluble interleukin 2 receptor (all cytokine levels are in picograms per ml); PHIV = plasma HIV viremia; GC = *Neisseria gonorrhoeae* infection; HD = *Haemophilus ducreyi* infection; CT = *Chlamydia trachomatis* infection; PID = pelvic inflammatory disease; RPR = syphilis serology; HSV = *Herpes simplex* virus infection; GUD = genital ulcer disease; and HIV = human immunodeficiency virus serology.

Patient #	Date	TNF	sTNFr	IL-6	IL-4	IL-10	CD4	CD8	sIL-22	PHIV	GC	HD	CT	PID	RPR	HSV	GUD	HIV
1669	17.03.95	10.2	3,183.0	3.3	.0	12.4	80	480	1,740	300,000	1	1	0	1	0	0	1	1
1669	21.04.95	.0	3,369.0	.0	.0	.0	90	410	960	260,000	0	0	0	0	0	0	0	1
1669	17.05.95	.0	2,520.0	.0	.0	.0	.	.	940	140,000	0	0	0	0	0	0	0	1
1765	02.03.95	.0	2,889.0	30.6	.0	.0	440	1480	517	280,000	1	0	0	1	0	0	0	1
1765	06.03.95	.0	1,969.0	.0	.0	.0	700	2230	287	230,000	0	0	0	0	0	0	0	1
1765	15.03.95	.0	1,989.0	.0	.0	.0	660	2260	530	170,000	0	0	0	0	0	0	0	1
1772	07.02.95	.0	3,914.0	45.0	.0	14.8	200	400	741	280,000	1	0	1	0	0	0	0	1
1772	15.02.95	.0	2,670.0	.0	.0	.0	470	690	637	200,000	0	0	0	0	0	0	0	1
1772	22.02.95	.0	2,493.0	.0	.0	.0	520	680	749	180,000	0	0	0	0	0	0	0	1
1772	05.05.95	.0	3,049.0	.0	.0	12.6	440	630	791	240,000	1	0	0	0	0	0	0	1
1785	28.02.95	.0	1,293.0	.0	.0	.0	1320	1130	540	0	1	0	0	1	0	0	0	0
1785	06.03.95	.0	840.0	.0	.0	.0	1440	1190	450	0	0	0	0	0	0	0	0	0
1785	13.03.95	.0	900.0	.0	.0	.0	.	.	566	0	0	0	0	0	0	0	0	0
1766	07.04.95	.0	1,448.0	.0	.0	.0	960	590	.	0	0	0	0	0	0	0	0	0
1766	26.04.96	.0	1,314.0	.0	.	.0	940	600	.	0	0	0	0	0	0	0	0	0
1536	04.04.95	.0	1,200.0	.0	.0	.0	890	550	.	0	0	0	0	0	1	0	0	0
1536	03.05.95	.0	1,092.0	.0	.0	.0	910	530	.	0	0	0	0	0	0	0	0	0
1437	10.04.95	.0	1,051.0	.0	.0	.0	850	750	.	0	0	0	0	0	1	0	0	0
1437	10.05.95	.0	960.0	.0	.0	.0	820	750	.	0	0	0	0	0	0	0	0	0
41	16.01.95	.0	4,340.0	8.1	25.0	15.6	70	730	2,241	120,000	1	0	0	0	0	0	0	1
41	30.01.95	.0	3,120.0	3.1	.0	.0	80	780	1,663	65,000	0	0	0	0	0	0	0	1
41	10.03.95	.0	2,510.0	.0	.0	.0	90	670	.	63,000	0	0	0	0	0	1	1	1
41	25.04.95	.0	3,962.0	5.0	30.0	10.0	90	650	1,310	54,000	1	0	0	1	0	0	0	1
13	16.02.95	.0	2,601.0	.0	.0	.0	150	670	600	68,000	0	0	0	0	0	0	0	1
13	10.03.95	.0	2,505.0	.0	.0	.0	100	500	402	62,000	0	0	0	0	0	0	0	1
13	03.05.95	.0	2,896.0	3.1	.0	.0	120	640	476	54,000	0	0	0	0	0	0	0	1

NB. TNF = Tumor Necrosis Factor; sTNFr = Soluble tumor necrosis factor receptor; IL-6 = interleukin 6; IL-4 = interleukin 4; IL-10 = interleukin 10; sIL-22 = soluble interleukin 2 receptor (all cytokine levels are in picograms per ml); PHIV = plasma HIV viremia; GC = *Neisseria gonorrhoeae* infection; HD = *Haemophilus ducreyi* infection; CT = *Chlamydia trachomatis* infection; PID = pelvic inflammatory disease; RPR = syphilis serology; HSV = *Herpes simplex* virus infection; GUD = genital ulcer disease; and HIV = human immunodeficiency virus serology.

Patient #	Date	TNF	sTNFr	IL-6	IL-4	IL-10	CD4	CD8	sIL-22	PHIV	GC	HD	CT	PID	RPR	HSV	GUD	HIV
1481	10.02.95	.0	1,499.0	6.7	.0	.0	1320	2080	.	220,000	1	0	0	1	0	0	0	1
1481	24.02.95	.0	1,015.0	.0	.0	.0	1290	2540	.	165,000	0	0	0	0	0	0	0	1
1481	19.05.95	.0	2,096.0	7.3	.0	14.2	920	1450	.	170,000	1	0	0	0	0	0	0	1
391	16.02.95	.0	5,100.0	24.4	30.0	15.6	170	2410	2,628	57,000	1	0	0	0	0	0	0	1
391	22.02.95	.0	3,660.0	10.6	.0	8.6	110	1950	1,868	39,000	0	0	0	0	0	0	0	1
781	24.03.95	.0	2,163.0	4.8	.0	.0	390	1160	2,660	82,000	1	0	1	1	0	0	0	1
781	31.03.95	.0	1,231.0	.0	.0	.0	460	1370	1,980	54,000	0	0	0	0	0	0	0	1
1756	23.03.95	.0	1,396.0	.0	33.5	10.6	290	480	800	67,000	1	0	0	0	0	1	1	1
1756	20.04.95	.0	800.0	.0	.0	.0	370	710	680	32,000	0	0	0	0	0	0	0	1
1275	08.03.95	.0	917.0	.0	.0	.0	1310	870	.	0	0	0	0	0	0	0	0	0
1275	30.03.95	.0	1,096.0	.0	.0	.0	1230	960	.	0	0	0	0	0	0	0	0	0
631	28.03.95	.0	3,962.0	15.6	20.0	.0	400	480	.	90,000	1	0	0	0	0	0	0	1
631	04.04.95	.0	1,254.0	.0	.0	.0	330	750	.	70,000	0	0	0	0	0	0	0	1
45	30.03.95	.0	3,976.0	.0	15.0	10.0	210	1020	.	76,000	1	0	0	1	0	0	0	1
45	20.04.95	.0	2,216.0	.0	.0	.0	220	1060	.	42,000	0	0	0	0	0	0	0	1
673	31.03.95	.0	3,962.0	12.6	25.0	.0	120	850	.	120,000	0	0	0	1	0	0	1	1
673	04.04.95	.0	2,216.0	.0	30.0	.0	130	970	.	82,000	0	0	0	0	0	0	0	1
673	11.04.95	.0	2,838.0	10.6	26.0	.0	120	930	.	90,000	1	0	0	0	0	0	0	1
1788	28.03.95	.0	2,254.0	.0	.0	.0	840	580	.	0	1	0	0	1	0	0	0	0
1788	05.04.95	.0	1,264.0	.0	.0	.0	760	580	.	0	0	0	0	0	0	0	0	0
1208	05.04.95	.0	2,840.0	.0	.0	.0	330	1230	.	30,000	0	0	0	0	0	0	0	1
1208	25.04.95	.0	3,640.0	.0	.0	.0	280	910	.	58,000	1	0	0	0	0	0	0	1

NB. TNF = Tumor Necrosis Factor; sTNFr = Soluble tumor necrosis factor receptor; IL-6 = interleukin 6; IL-4 = interleukin 4; IL-10 = interleukin 10; sIL-22 = soluble interleukin 2 receptor (all cytokine levels are in picograms per ml); PHIV = plasma HIV viremia; GC = *Neisseria gonorrhoeae* infection; HD = *Haemophilus ducreyi* infection; CT = *Chlamydia trachomatis* infection; PID = pelvic inflammatory disease; RPR = syphilis serology; HSV = *Herpes simplex* virus infection; GUD = genital ulcer disease; and HIV = human immunodeficiency virus serology.

STUDENT NAME ______________________ STUDENT NUMBER ____________

LAB SECTION ______________________

EXPERIMENT NO. 2
WHEATSTONE BRIDGE AND STEFAN'S LAW

PRELAB READING TEST

1. A Wheatstone bridge provides a very sensitive method of measuring ____________.

2. A resistance is measured by ____________ the bridge.

3. Stefan's law says the power radiated by any object at an absolute temperature, T, goes as the ____________ power of the temperature.

4. The multimeter has an uncertainty of ____________ on all ranges.

5. The lab pot has a maximum resistance of ____________ ohms.

6. Each time that you change the voltage across the lamp you should first ____________ the galvanometer.

7. The temperature of the lamp will be determined by ____________ the calibration graph in Fig. 3.

8. When you plot log P versus log T the slope should be equal to____________.

2

EXPERIMENT NO. 2
WHEATSTONE BRIDGE AND STEFAN'S LAW

OBJECTIVE

The purpose of this experiment is to (1) construct a Wheatstone Bridge; (2) to use the bridge to measure the resistance of the tungsten filament of a small electric lamp as a function of temperature; and (3) to verify Stefan's Law.

INTRODUCTION

The Wheatstone bridge is a very sensitive means for determining the value of a resistance when three other resistances are well known. The bridge consists of an array of resistors in two parallel branches as shown in figure 1, plus a galvanometer (G) which is a sensitive device for measuring small currents. Current through the branches comes from a common source, the power supply shown in the circuit.

The Wheatstone bridge is used by "balancing" it, that is, adjusting the resistances so that no current flows through the galvanometer. The voltage between R_1 and R_2 must therefore be same as between R_3 and R_4. This can occur only when the resistors R_1...R_4 are in a precise ratio, and since 3 of the resistances are known, the 4th may be calculated. For this to happen, we must have

$$R_1 / R_2 = R_3 / R_4 \quad (1)$$

If we know the values of R_1, R_2, and R_4, but R_3 is unknown (ie: $R_3 = R_x$), then,

$$R_x = R_1 R_4 / R_2 \quad (2)$$

In a Wheatstone bridge, two of the resistances, R_2 and R_4, are fixed, while one, R_1, is variable. By adjusting R_1 until the galvanometer shows no deflection, we may determine R_x using the above equation.

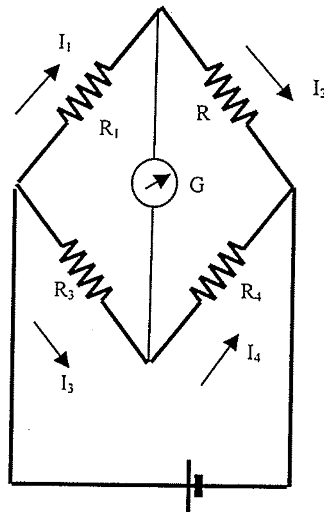


Figure 1

Stefan's Law

The electrical resistance of a pure metal such as tungsten varies linearly with the absolute temperature, T, measured in Kelvin (K). Let R_o be the resistance of tungsten at room temperature (about 300 K) while R is the resistance at some higher temperature T. Then we can calculate the resistance ratio $F = R/R_o$, and use the graph in Fig. 3 (page 10) to find the temperature T.

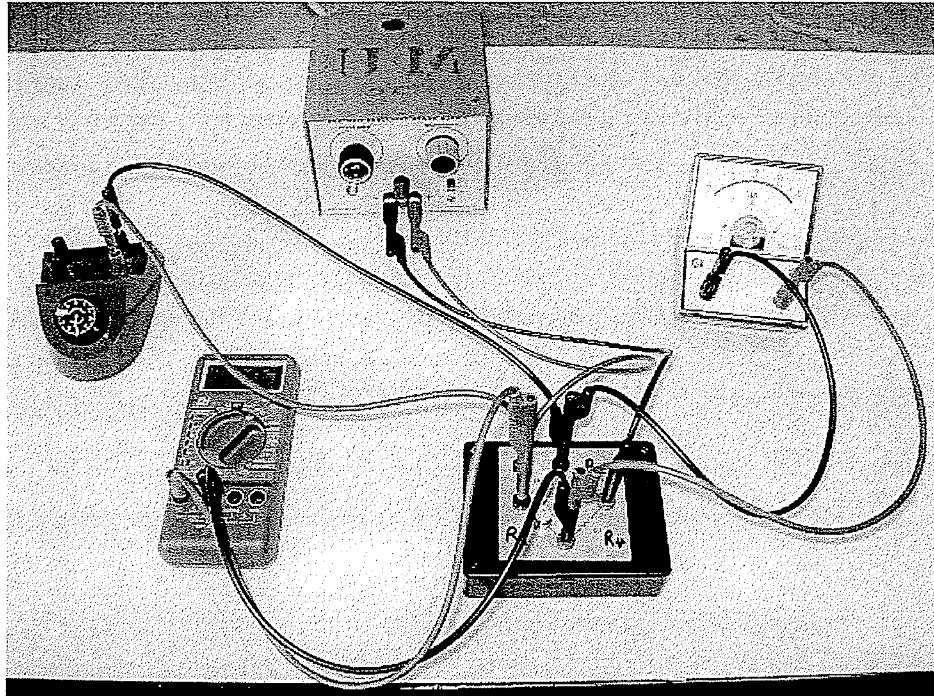
In this experiment, we will be heating tungsten, in the form of the filament of a 6-volt electric lamp. As the current through the lamp is changed, the temperature of the filament changes, and so also the resistance of the filament changes.

If a voltage V is applied across a resistance R, electrical energy is supplied to the resistance at a rate $P = V^2/R$. This energy is lost from the filament by conduction and radiation. According to Stefan's law, the power radiated from a hot source is proportional to the fourth power of the absolute temperature. If the source (the filament of the lamp) is hot enough, the rate of radiation of heat is so much more important than the conduction rate, that the latter can be neglected. Therefore we have

$$P = V^2 / R = BT^4 \tag{3}$$

where B is a constant. Eq. (3) is Stefan's Law which you will attempt to verify.

In the experiment, we will connect the electric lamp at position R_3, i.e.: the resistance of the lamp is the unknown R_x. R_2 and R_4 will have fixed values and R_1 will be varied to balance the bridge. Then R_x can be calculated from eq.(2).



THEORY

In your Theory section you should discuss both Eqs. (2) and (3) and **note that Equation (2) is valid only when the Wheatstone Bridge is balanced (R_1 adjusted so that the galvanometer reads "zero").** You should also explain what it means for a bridge circuit to be balanced – include a sketch of Fig. 1 to assist you with this.

Preliminary equipment checks

1. If you do not know how to use a multimeter read pp. 3-4 of this manual.

2. Identify the resistors:
   R_x is the unknown resistance of the lamp filament.
   R_1 is the resistance of the lab pot. Its resistance is read from the dial and has a maximum value of 1K.
   R_2 is a 1.5 K resistor
   R_4 is 75 Ω.

3. Use the ohmmeter function on the multimeter to measure the resistance of R_2 and R_4. The multimeter has an uncertainty of ±1% on all ranges. Record the measured values of

R_2 and R_4 with an uncertainty on the data page (page 11). Use the measured values of R_2 and R_4 in your calculations.

Note: when you use the multimeter to measure resistance, make sure the circuit is not constructed so that you measure only the resistance desired. For help in using the multimeter, refer to pages 3-4 of the Introduction section of this manual.

4. The lab pot is connected using the terminals labelled "2" and "4". If you don't know which two terminals on the lab pot to use, you can again use the multimeter to check. Set a reading on the lab pot and connect the multimeter between pairs of terminals, until you find the pair that gives the value on the meter you expect from the pot setting. You can also use this to check on the accuracy of the lab pot.

   The lab pot dial can be adjusted through ten full rotations for a maximum resistance of 1000 ohms. Therefore the lab pot resistance is 100 ohms/rotation and 1 ohm/dial division.

5. The lab pot contains a fuse which has a finite resistance which is in series with the lab pot setting. Measure the resistance of the fuse by first setting the lab pot dial to zero and using the multimeter to measure the resistance across terminals "2" and "4". **This fuse resistance should be added to each reading of the lab pot resistance, R_1, recorded.**

6. If, during the experiment, things don't work properly, use the multimeter to check for poor electrical connections.

7. Leave the power supply turned off, and not plugged in. **Set the voltage and current controls on the power supply to their minimum settings (fully counter clockwise).** Set the lab pot to read 100 Ω, one clockwise turn from 0. Construct the circuit as shown in the diagram (Fig. 2). It is already partly wired in a small connector box - resistors R_2 and R_4 and the bulb, R_x, are already connected. Plugs are provided on the connector box to connect the power supply, lab pot, and galvanometer. Eight stacking "banana plug" leads are provided to make the necessary connections. **The multimeter (set to the 2 volt range on the DC volts function) leads are connected directly across the lamp.**

   Attach these leads as shown in Fig. 2.

   Warning: leave one of the leads from the galvanometer unconnected, to avoid damaging the galvanometer by supplying too large current to it, in case something is wrong.

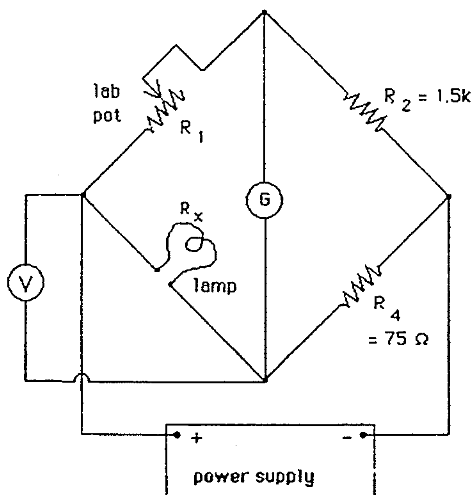


Figure 2

8. Have your circuit checked.

9. Plug in the power supply and turn it on. The voltmeter should read zero on the two volt range (or nearly zero). If it does not, disconnect the power supply and seek help!

10. If the voltmeter reads near zero, take the unconnected galvanometer lead and touch it quickly to the appropriate terminal; – if the deflection of the galvanometer needle is less than full-scale, it is safe to connect this lead securely.

PROCEDURE

The circuit should now be functioning correctly and ready to use.

1. Increase the current control on the power supply by one turn clockwise. Then increase the voltage control on the power supply until the voltmeter reads about 0.03 volts. This voltage is sufficient for the bridge circuit to work, but will not heat the lamp.

2. Now adjust the lab pot to make the galvanometer reading zero; that is, balance the bridge. Note the lab pot reading, R_1, and add the fuse resistance to it, then use equation (2) to calculate the resistance of the lamp, which will be R_0, the **room-temperature resistance** of the lamp. Record the data in Table 1.

3. Now begin to heat the filament by first increasing the current control on the power supply by about half a turn clockwise and then *slowly* increasing the voltage setting on the power supply to obtain the desired voltage across the lamp. Try not to overshoot. If you overshoot by a little on the voltage, take the measurement at this voltage. In this case V(actual) will be different than V(set). Suggested voltage values are given in Table 1 on page 11.

4. Then adjust the lab pot to balance the bridge again (zero reading on the galvanometer). **Remember, each time before changing the voltage and current settings on the power supply, to disconnect one lead of the galvanometer, to avoid too large currents through it while changing these settings.** Touch the lead lightly to the galvanometer terminal as you adjust the lab pot to balance the bridge. Only for the final fine adjustment to get a zero reading of the galvanometer should you secure the lead onto the terminal.

   Record the values of V across the lamp, from the multimeter, and R_1 from the lab pot and enter these in the table provided (page 11).

5. Now turn up the power supply again and repeat these measurements and calculations, to obtain the 8 readings between 0 and 6V on the multimeter. When the voltage reaches 2 volts you will have to switch the multimeter to the 20 volt range; otherwise you will get an "Overload" indication from the meter which simply tells you that the quantity that you are trying to measure is larger than the range set – change the setting to the next larger range. "Overload" with this multimeter is represented by a "L [][][]" on the display.

   At the highest current and voltage settings, when the lamp is glowing brightly, you may find the circuit a bit unstable; that is, after you think you have balanced the bridge, the galvanometer may drift a little away from zero. This is presumably due to changes in the resistances of R_4 and R_x as they heat up. (R_4 will get quite HOT!) Readjust for a zero reading again.

ANALYSIS

1. For each voltage, V, calculate the resistance R_x of the lamp using eq.(2) and calculate the power, V^2/R_x, being dissipated by the lamp. For both R_x and the power, calculate the % uncertainty. Indicate the % uncertainty at the top of the column in Table 1 – **you do not have to calculate the actual uncertainty.** Remember, the multimeter has an uncertainty of ±1% of the reading on all ranges and the labpot has an uncertainty of 3% of the reading. Use the rules for the Combination of Errors to determine the required uncertainties. For example, the uncertainty in R_x is 5%; show this. We will not determine any errors beyond the power.

   Reminder: Based on Rule #2 the equation for the Error in R_x written in terms of fractional errors would be

$$\frac{\Delta R_x}{R_x} = \frac{\Delta R_1}{R_1} + \frac{\Delta R_2}{R_2} + \frac{\Delta R_4}{R_4}$$

   Note that to convert a fractional error to a percentage error you simply multiply by 100.

2. Calculate the resistance ratio $F = R_x/R_o$, and use this along with the calibration graph (Fig. 3 – page 10) to find T, the temperature of the filament. Enter these values in Table 1 on page 11.

   In your lab report you should describe in detail how you read (interpolated) the calibration graph (Fig. 3) to obtain an accurate temperature for a given resistance ratio, F. Neither F nor T will generally be a round number and therefore this will require interpolation of the axes, either 2 x 8 = 16 interpolations **or,** alternatively, you could determine the equation of the calibration line

$$T = mF + b$$

   and then calculate T by substituting the value of F into the equation. This latter method is considerably less work since it requires only two interpolations to determine the slope, m, and the intercept, b, of the calibration line. To determine "b" you will have to first use a ruler to carefully extend the calibration line to the T axis, then interpolate the value of "b".

   To interpolate a value from the T axis, note that each division on the T-axis is 200 K and, if you measure a division with a ruler, it is about 10 mm. If the intersection is, for example, 7 mm above 400 K then the temperature would be (7/10x200 + 400) K. The F-axis would be interpolated the same way. Include Fig. 3 in your Analysis to show how you determined T – it will have a number of lines drawn on it as part of the interpolation procedure.

3. You will now have at least 8 sets of values of the power, $P = V^2/R$ and the temperature T, which you will use to verify Stefan's law, which states:

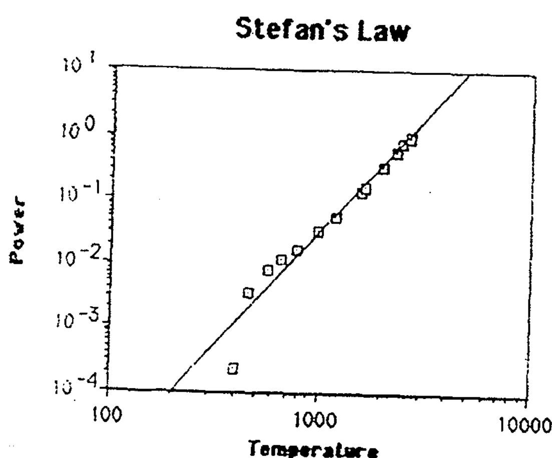
$$P = BT^4$$

If we take logarithms (base 10, or natural it doesn't matter) of both sides of this equation, we have

$$\log P = \log B + 4\log T \quad (4)$$

since B is a constant, then so is logB, so a graph of logP vs logT should be a straight line with slope 4 and intercept logB. Complete Table 2 as part of your analysis. Because the power being dissipated by the lamp is relatively small (less than one watt), when you take the log of the power you will get a negative number – this simply means that the data is in the 4th quadrant of your graph.

4. Plot logP vs logT, using the data you obtained in steps 2 and 3 of the procedure. Your graph may look something like the following:



5. Find the slope of the "best-fit" line to your graph. Also draw a "worst-fit" line – you can use the scatter in the data points as a guide since you likely will not have plotted any error bars. To draw the worst-fit line remember that you take one point above the best fit line and one point below the best fit line which are quite far apart and you connect them. The uncertainty in the slope will be the magnitude of the difference between the slopes of the best and worst lines.

See pages 18-22 of the Introduction to this lab manual to review how to draw a graph.

DISCUSSION

Does the value of the slope of your graph agree with the theoretical value of 4 predicted by Stefan's Law (draw Overlap Graph)? If not, why not? Can you identify possible sources of any discrepancy?

Also calculate the % Difference – should be less than 10%

$$\% \text{ Difference} = |m_{best} - 4| / 4 * 100$$

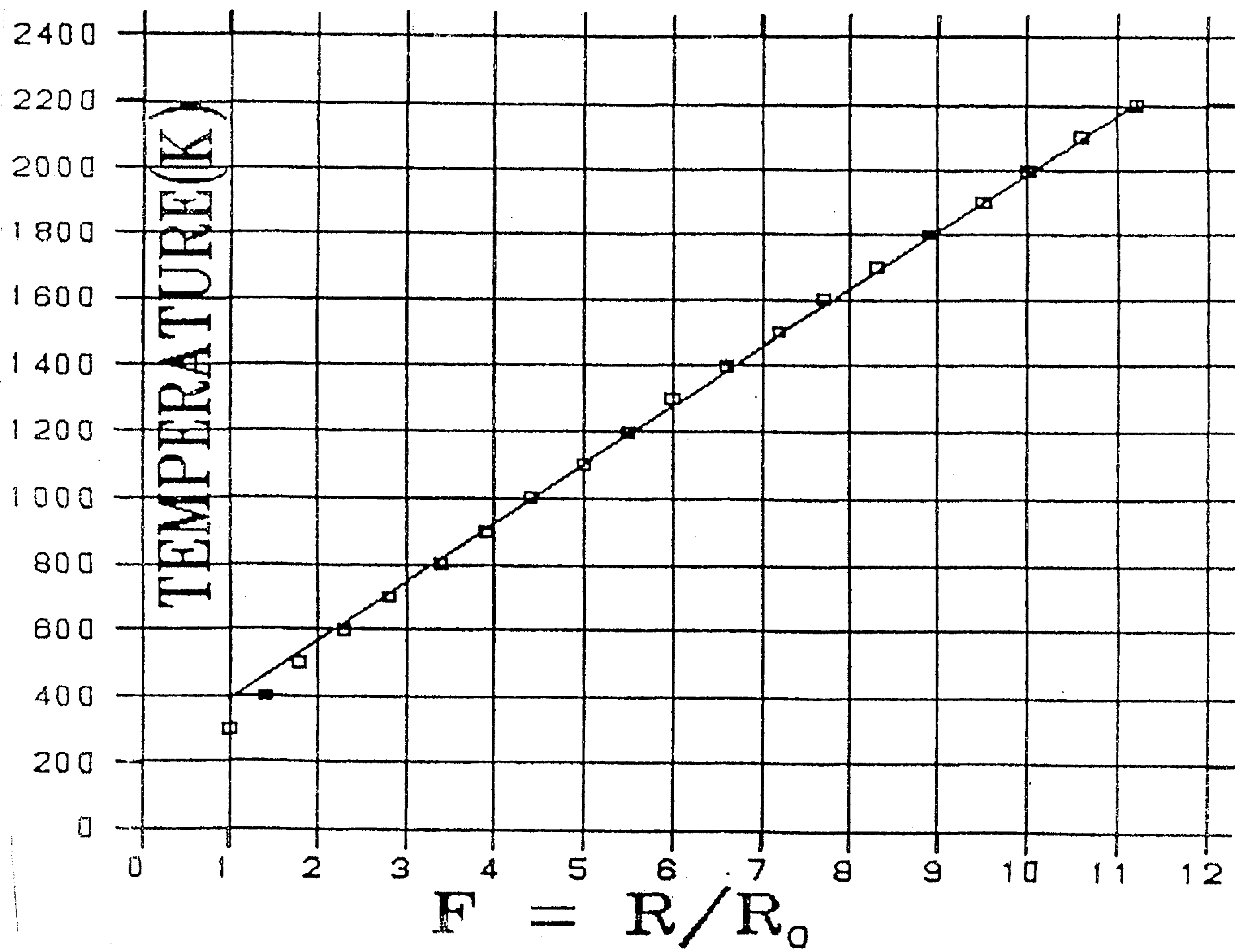


Figure 3

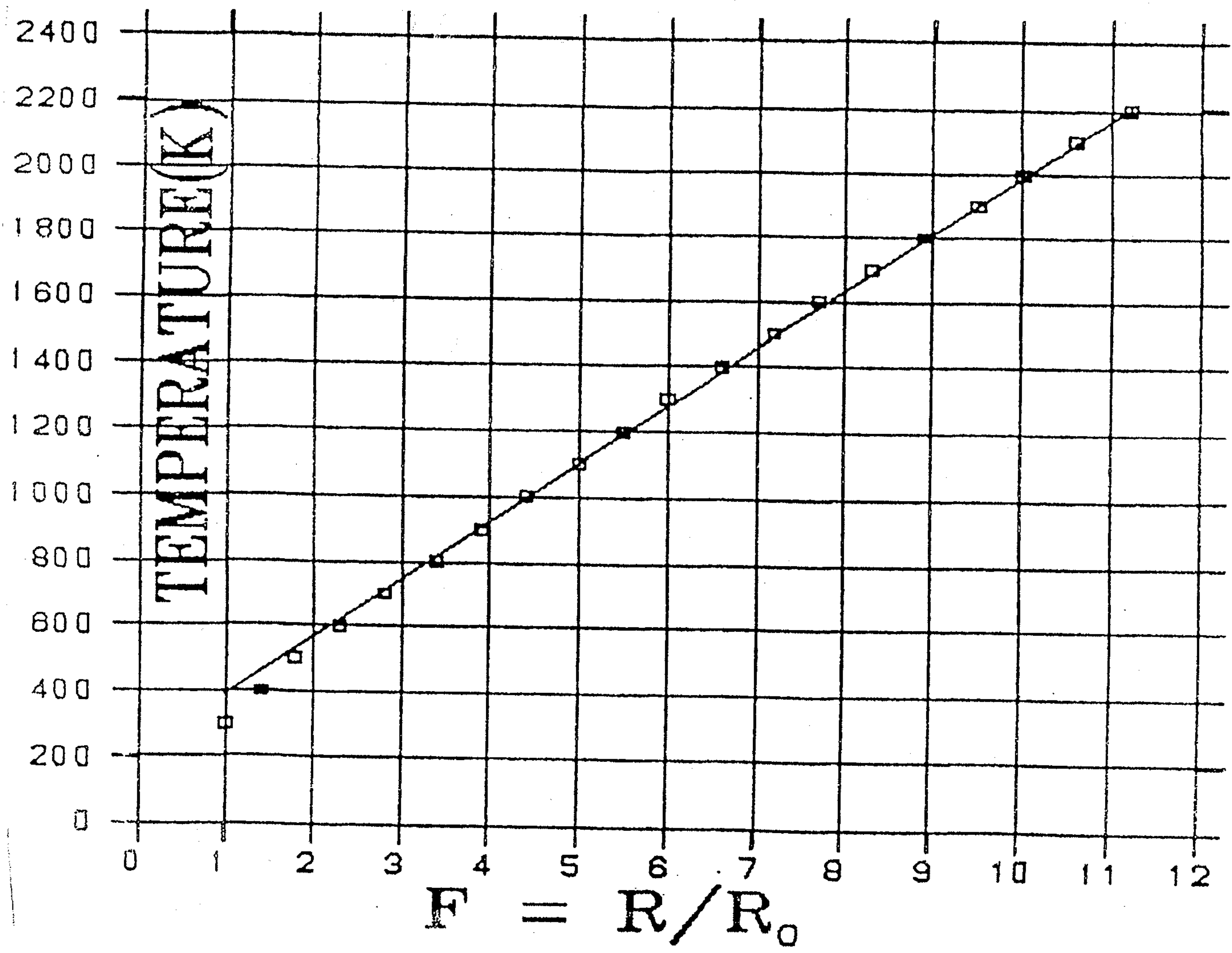


Figure 3

Student Name: ______________________________

Measured resistance of R_2 =

Measured resistance of R_4 =

Measured resistance of lab pot fuse =

Table 1

Voltage #	V (set)	V (actual) (V) ± 1%	R_1(ohm) ± 3%	R_x(ohm)	V^2/R_x (W)	F = R_x/R_0	T(K)
1.	~0			R_0 =			
2.	0.5						
3.	1.0						
4.	1.5						
5.	2.0						
6.	3.0						
7.	4.0						
8.	5.0						
9.	6.0						

Table 2

Voltage #	V^2/R_x (W)	log (V^2/R_x)	T (K)	log T
2.				
3.				
4.				
5.				
6.				
7.				
8.				
9.				

Student Name: ______________________________

Measured resistance of R_2 =

Measured resistance of R_4 =

Measured resistance of lab pot fuse =

Table 1

Voltage #	V (set)	V (actual) (V) ± 1%	R_1(ohm) ± 3%	R_x(ohm)	V^2/R_x (W)	F = R_x/R_0	T(K)
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8.	5.0						
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Table 2

Voltage #	V^2/R_x (W)	log (V^2/R_x)	T (K)	log T
2.				
3.				
4.				
5.				
6.				
7.				
8.				
9.				

Faculty of Science
Honesty Declaration
Individual Work

This form must be completed and attached to your completed report upon submission to your T.A. Only reports accompanied by this Honesty Declaration will be accepted for marking.

I, the undersigned, declare that the attached report, with the exception of the collection of data which was jointly completed with my lab partner(s) ____________________________,
is wholly the product of my own work, and that no part of it has been:

- **copied** by manual or electronic means from any work produced by any other person(s), present or past, including tutors or tutoring services,
- based on laboratory work that **I did not complete** due to unexcused absence(s),
- produced by several students working together as a team (this includes one person who provides any portion of an assignment to another student or students),
- **copied** from any other source including textbooks and web sites
  or
- **modified** to contain falsified data,

except as directly authorized by the Instructor.

I understand that penalties for submitting work which is not wholly my own, or distributing my work to other students is considered an act of Academic Dishonesty and is subject to penalty as described by the University of Manitoba's Student Discipline Bylaw*.

Please PRINT all information:

Last Name: ______________________ First Name: __________________________

Student Number: __________________ UM Email: __________________________

Course: ___________ Section: ______ T.A. Name: __________________________

Experiment Title and No.: ____

Date Experiment Completed: ____

Signature: ____

(*) Penalties that may apply, as provided for under the University of Manitoba's Student Discipline By-Law, range from a grade of zero for the assignment, failure in the course to expulsion from the University. The Student Discipline By-Law may be accessed at:
http://umanitoba.ca/admin/governance/governing_documents/students/discipline/index.html

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STUDENT NAME______________________ STUDENT NUMBER ______________

LAB SECTION _________________________

EXPERIMENT NO. 3
CHARGE TO MASS RATIO FOR THE ELECTRON

PRELAB READING TEST

1. The force on an electron moving in a magnetic field is at ____________ angles to both the direction of the field and the motion.

2. The magnetic field in Exp. #3 is produced by two identical coils of wire known as ____________ coils.

3. The SI units for the magnetic field, B, is ____________.

4. Inside the tube at its base there is an ____________.

5. Large Errors in the measurement of the radius of the electron beam path can occur due to ____________.

6. The radius of the electron beam path will be kept constant at about ____________.

7. Acceleration voltages of up to ____________ volts will be applied to the electron gun.

8. The value of e/m will be obtained from the best fit slope of a graph of voltage versus ____________.

3

EXPERIMENT NO. 3
CHARGE TO MASS RATIO FOR THE ELECTRON

OBJECTIVE

The objective of this experiment is to measure the ratio of the charge e to the mass m of the electron.

DESCRIPTION

A beam of electrons of known energy is produced in a special tube. A magnetic field in a direction at right angles to the direction of motion of the electrons is produced by current flowing through a pair of coils of wire. The magnetic field causes the electrons to move in a circle whose radius can be measured. With a knowledge of the strength of the magnetic field it is possible to calculate the charge to mass ratio, e/m, for the electron.

THEORY

An electron which is moving in a magnetic field at right angles to it experiences a force which is given by:

$$F=Bev \tag{1}$$

where F is the force in newtons, B is the magnetic field in tesla, e is the charge on the electron in coulombs, and v is the speed of the electron in metres per second. The direction of this force is always at right angles to the magnetic field and to the velocity of the particles, and thus can supply the centripetal force which is required for the charged particles to move in a circular orbit.

Hence,

$$mv^2/r=Bev \tag{2}$$

The kinetic energy acquired by an electron which is accelerated through a potential difference of V is given by

$$Ve=\tfrac{1}{2}mv^2 \tag{3}$$

Combining equations (2) and (3) we obtain

$$e/m=2V/(B^2r^2) \tag{4}$$

where e/m is the required charge-to-mass ratio of the electrons in coulombs/kg, V is the potential through which the electrons were accelerated in volts, B is the magnetic field in tesla and r is the radius of curvature of the electron path in metres. If we can measure V, B and r we can calculate the value of e/m.

To obtain a more accurate measurement, however, we will do a series of measurements at different voltages, with the radius, r, maintained at a constant value. If we then plot V vs B^2 we should obtain a straight line with slope $\frac{r^2}{2}$ (e/m) from which we will be able to determine e/m.

$$V = \frac{r^2}{2}\left(\frac{e}{m}\right)B^2$$

The magnetic field, B, which bends the electron beam into a circular path is supplied by two identical coils of wire known as Helmholz coils. It can be calculated from the formula

$$B = 9x10^{-7}\frac{NI}{R} \qquad (5)$$

where B is the magnetic field in tesla, N is the number of turns of wire in each of the coils, I is the current in amperes in each of the coils and R is the radius of each of the coils in metres.

In your Theory section derive equation (4) starting with Equation (1) (the Lorentz Force Equation). Show the details of the derivation summarized above. Also discuss Eq. (5).