THE INFLUENCE OF HIV-1 PROVIRAL BURDEN ON DISEASE PROGRESSION AND RESPONSE TO ANTIRETROVIRAL THERAPY

RODNEY S. RUSSELL

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THE INFLUENCE OF HIV-1 PROVIRAL BURDEN ON DISEASE PROGRESSION AND RESPONSE TO ANTIRETROVIRAL THERAPY

by

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ABSTRACT

When HIV-1 infects $CD4^+$ T cells, its RNA genome is reverse transcribed into double stranded proviral DNA which translocates to the nucleus and integrates into the host cell genome. This integrated proviral DNA can remain silent, but ultimately is the source of all HIV-1 replication. Therefore, we hypothesize that the size of the HIV-1 proviral pool determines the rate of disease progression and is a useful prognostic indicator of the durability of responsiveness to antiretroviral therapy. To test this hypothesis, we developed a non-radioactive quantitative PCR-based proviral load assay and measured the frequency of $CD4^+$ T cells containing HIV-1 proviral DNA in 78 HIV-1-infected individuals. Our results show that HIV-1 proviral load is a stable pool of virus that is unaffected by current antiretroviral therapies. We found that HIV-1-infected individuals with higher proviral loads displayed a lower mean $CD4^+$ T cell count and significantly higher mean peak plasma virus load. When we separated HIV-1-infected individuals into groups based on whether or not they responded to therapy, we found that non-responders had higher proviral loads than did responders. Taken together, these results indicate that the HIV-1 proviral DNA pool has a strong influence on the potential for immune reconstitution and viral suppression in response to antiretroviral therapy, suggesting that HIV-1 proviral load is much more important than originally believed. Current regimens of antiretroviral therapy are not going to be sufficient to eradicate HIV, and new therapies that target this pool of HIV-1 proviral load are needed.

The second aim of my study was to evaluate the effects of the CCR5Δ32 HIV-1 coreceptor mutation on the rate of HIV-1 disease progression, and to determine whether the slower rate of disease progression reported in CCR5Δ32 heterozygotes could be due to lower levels of HIV-1 proviral DNA. We found that the heterozygous individuals in our group displayed slower rates of disease progression than individuals homozygous for wild type CCR5. However when we compared HIV-1 proviral load between groups of CCR5Δ32 heterozygous and CCR5wt homozygous individuals, we saw only a slight difference in mean proviral load. This result indicates that the slower rates of disease progression observed in the CCR5Δ32 heterozygotes is not due to a decreased HIV-1 proviral load.

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LIST OF ABBREVIATIONS

A	Adenine
Ag	antigen
AIDS	acquired immune deficiency syndrome
APC	antigen-presenting cell
AZT	3'-azido-3'-deoxythymidine
bp	base pair
BSA	bovine serum albumin
C	Cytosine
CA	capsid: p24
Cβ	beta chain constant segment
CCR3	C-C chemokine receptor 3
CCR5	C-C chemokine receptor 5
CCR5Δ32	32 base pair deleted C-C chemokine receptor 5 gene
CCR5wt	wild type C-C chemokine receptor 5 gene
CDC	Center for Disease Control (USA)
cDNA	complementary DNA
CTL	cytotoxic T lymphocyte
CXCR4	C-X-C chemokine receptor 4
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid

dNTPs	deoxyribonucleoside triphosphates
DTT	dithiothreitol
EDTA	Ethylenediamine-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EU	exposed uninfected
FCS	foetal calf serum
G	Guanine
gp160	160 kDa envelope polyprotein
HAART	highly active antiretroviral therapy
HCl	hydrochloric acid
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HIV-1	human immunodeficiency virus type 1
HIV-2	human immunodeficiency virus type 2
HLA-DQα	human leukocyte antigen – DQ region – alpha chain
HLA-DR	human leukocyte antigen – DR region
ICAM-1	intercellular adhesion molecule-1
ID	identification
IL-2	Interleukin 2
IL-6	Interleukin 6
IN	integrase: p31
kb	kilobase pair
LFA-1	leukocyte-function associated antigen-1

LTNPs	long-term nonprogressors
LTR	long-terminal repeats
MA	matrix; p17
mAbs	monoclonal antibodies
Mg^{2+}	Magnesium ion
min	minute
MIP-1α	macrophage inflammatory protein-1-alpha
MIP-1β	macrophage inflammatory protein –1-beta
ml	millilitre
mM	millimolar
mRNA	messenger RNA
µg	microgram
µl	microlitre
µM	micromolar
n	sample size
NA	nucleic acid
NaCl	sodium chloride
NaOH	sodium hydroxide
NC	nucleocapsid; p7
Nef	negative regulatory factor
NF-κB	nuclear factor-kappaB
ng	nanogram

NK	natural killer cell
nm	nanometer
NNRTIs	non-nucleoside reverse transcriptase inhibitors
NRTIs	nucleoside reverse transcriptase inhibitors
NSI	non-syncytium-inducing
p	p-value
^{32}P	Phosphorus radioisotope
PBL	peripheral blood lymphoctyes
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PR	protease: p11
P/S	Penicillin / Streptomycin
P. t. troglodytes	Pan troglodytes troglodytes
R^2	coefficient of determination
R5	CCR5 tropic
R5X4	dual tropic
RANTES	regulated upon activation normal T cell expressed and secreted
Rev	regulator of expression of virion proteins
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute (culture medium)
RT	reverse transcriptase; p66/p51

RT-PCR	reverse transcription - polymerase chain reaction
SDS	sodium dodecyl sulfate
sec	second
SI	syncytium-inducing
SIVcpz	simian immunodeficiency virus – chimpanzee strain
SSC	sodium chloride-sodium citrate
SSPE	sodium chloride-sodium phosphate-EDTA
sssDNA	single-stranded salmon sperm DNA
SU, gp120	surface envelope protein
T	Thymine
Tat	transactivator
TBE	Tris-borate-EDTA
TCR	T cell receptor
TM, gp41	transmembrane protein
TNE	Tris-sodium chloride-EDTA
TNF-α	Tumor necrosis factor-α
uv	ultraviolet
Vβ8	beta chain variable segment 8
Vif	virion infectivity factor
Vpr	viral protein R
Vpu	viral protein U
X4	CXCR4 tropic

<	less than
>	greater than

1. INTRODUCTION

In this chapter I provide a brief history of how AIDS, and subsequently HIV-1, were discovered, followed by a description of the viral genome, structure, and life cycle. I will then review literature that is relevant to the work done in my MSc project, and describe my hypotheses.

1.1 Etiology of HIV

1.1.1 Epidemiology of HIV Infection

It has been estimated that there are now almost forty million HIV-infected individuals worldwide. Almost twelve million people have already died from AIDS, leaving over eight million orphans. In 1997 alone, there were almost six million new infections and 2.3 million deaths, of which 460,000 were children under the age of fifteen. Two thirds of the globe's HIV-infected population live in sub-Saharan Africa and Southeast Asia where even the minimum antiretroviral therapy is simply too expensive.[141] This bodes for an increasing number of deaths in the future.

These statistics paint a grim picture of the global status of HIV infection and AIDS. However, the body of knowledge gathered about HIV over the last two decades is quite astonishing. No single pathogen has ever received so much attention or inspired so much research interest. Since its discovery in 1983, more than 80,000 scientific articles

have been published on the topic of HIV. Together with the information about HIV came answers to other basic questions in areas such as molecular immunology and human virology.

1.1.2 Discovery of AIDS and HIV

In December 1981, *The New England Journal of Medicine* published three articles reporting the occurrence of *Pneumocystis carinii*, Kaposi's sarcoma, and multiple viral infections in groups of young males who were all either homosexuals, drug users, or both.[89,144,202] These first cases drew attention because such manifestations usually only occur in immune-compromised individuals or cancer patients receiving immunosuppressive therapy. Immunological testing revealed that these men also had T lymphopenia and depressed lymphocyte proliferation in response to mitogens and antigens,[144,202] depressed cell-mediated immunity, virtually no helper T cells, and an inversion of the T-helper to T-suppressor/cytotoxic cell ratio.[89] The absence of a history of recurrent infections or histologic evidence of lymphoproliferative or other neoplastic diseases suggested that these immune defects were acquired;[202] thus the term "Acquired Immunodeficiency Syndrome" (AIDS). It was initially proposed that this syndrome was related to some aspect of homosexual activity, but the same set of symptoms was noticed shortly thereafter in other populations such as intravenous drug users[17] and hemophiliacs.[49,61,184] The discovery of three more groups of AIDS patients: blood transfusion recipients;[44,115] adults from Central Africa;[32,183,210] and infants born to

mothers with AIDS,[191,164,198] finally convinced the medical and research communities that AIDS was caused by an infectious agent.[74] Since it was already known that T-helper cells were depressed or eliminated in AIDS, it was hypothesized that a T-lymphotropic retrovirus could be the causative agent.[5,62,63,80,87] Soon after, AIDS was shown to be linked to such a retrovirus that is now termed the human immunodeficiency virus (HIV).[79,185,194,197] We now know that HIV is a lentivirus of the *Retroviridae* family and that there are two main subtypes; HIV-1 and HIV-2, which was later discovered in West Africa.[30] Both viruses lead to AIDS but the pathogenic course of HIV-2 appears to be longer.[16,64,126,128]

1.1.3 Origin of HIV

Because of sequence similarities with primate lentiviruses, it has been suggested for a long time that HIV-1 and HIV-2 represent cross-species infections.[83,104,113,199] The primate reservoir of HIV-2 was quickly identified as the sooty mangabey,[19,20,82,83,104] while the origin of HIV-1 remains uncertain. However, viruses that appear to be closely related to HIV-1 have been isolated from the common chimpanzee.[177,178] In 1998 HIV-1 sequences representing what is believed to be the earliest known case of HIV infection were detected by PCR in stored plasma samples from 1959. Phylogenetic analysis of the sequence led the investigators to believe that the virus was introduced into humans sometime in the 1930s.[225] In February of 1999, it was found that all HIV-1 strains known to infect man are closely related to an SIVcpz lineage found in an African chimpanzee

subspecies called *P. t. troglodytes*. Since chimpanzees are commonly hunted for food in West Africa, they are a plausible source of such a species jump.[81] The remainder of this thesis is focused on HIV-1.

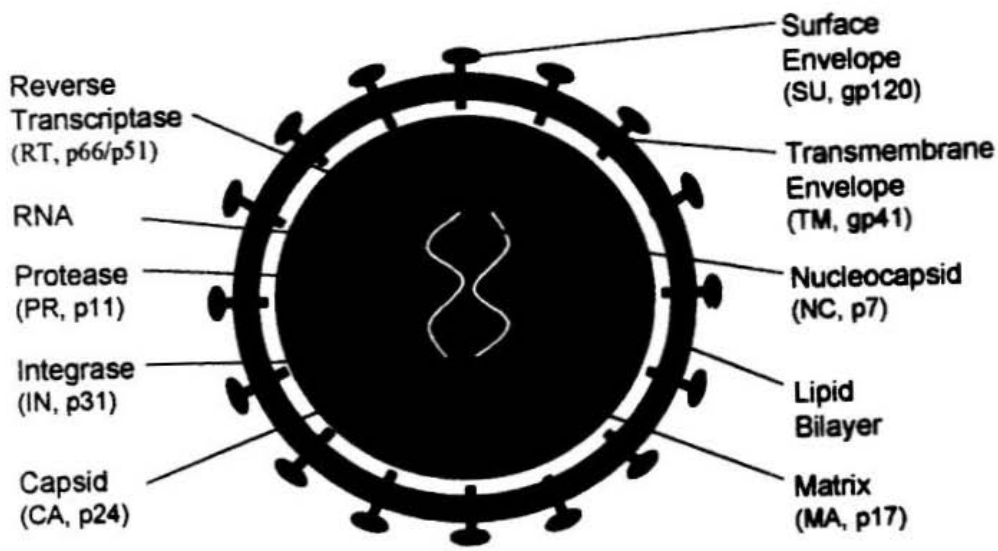
1.2 THE STRUCTURE OF HIV-1

1.2.1 Viral Proteins

As shown in Figure 1.1, HIV-1 has a cone-shaped core composed of the viral Gag capsid protein (CA, p24) containing two identical RNA strands closely associated with the viral reverse transcriptase (RT, p66/p51), and the nucleocapsid protein (NC, p7). The viral protease (PR, p11) and integrase (IN, p31) are also contained within the core. The inner portion of the viral membrane is surrounded by the matrix protein (MA, p17) which provides the viral structure and maintains the integrity of the virion.[85,86] HIV Vif and Nef proteins are closely associated with the core.[13,135,218] Vpr may also be located inside the virion but probably outside the core.[137] The surface of the virus is made up of trimers or tetramers of the envelope glycoprotein gp160.[65,86,167,182,215] gp160 consists of an external surface envelope protein (SU, gp120) and a transmembrane protein (TM, gp41)[147] which fit together in a knob-and-socket-like structure.[196] gp120 contains the binding sites for cellular receptors and the major neutralization domains.[18,46,162,193] Also contained within the viral envelope is an array of host cell-derived membrane proteins, including leukocyte-function associated antigen-1 (LFA-1) and intercellular adhesion molecule-1

Figure 1.1. Schematic representation of the HIV-1 virion structure. The HIV-1 virion, indicating the approximate location of the cleaved products of the Gag polyproteins, the Env glycoproteins, and the *pol* gene-encoded enzymes IN, RT, and PR. The colors indicated correspond to the precursor proteins shown in Figure 1.2. (Adapted from Frankel[75])

Figure 1.1


Surface
Envelope
(SU, gp120)
Transmembrane
Envelope
(TM, gp41)
Nucleocapsid
(NC, p7)
Lipid
Bilayer
Matrix
(MA, p17)
Capsid
(CA, p24)
Integrase
(IN, p31)
Protease
(PR, p11)
RNA
Reverse
Transcriptase
(RT, p66/p51)

(ICAM-1) which have been suggested to play a role in HIV-1 infection.[103]

1.2.2 Viral Genome

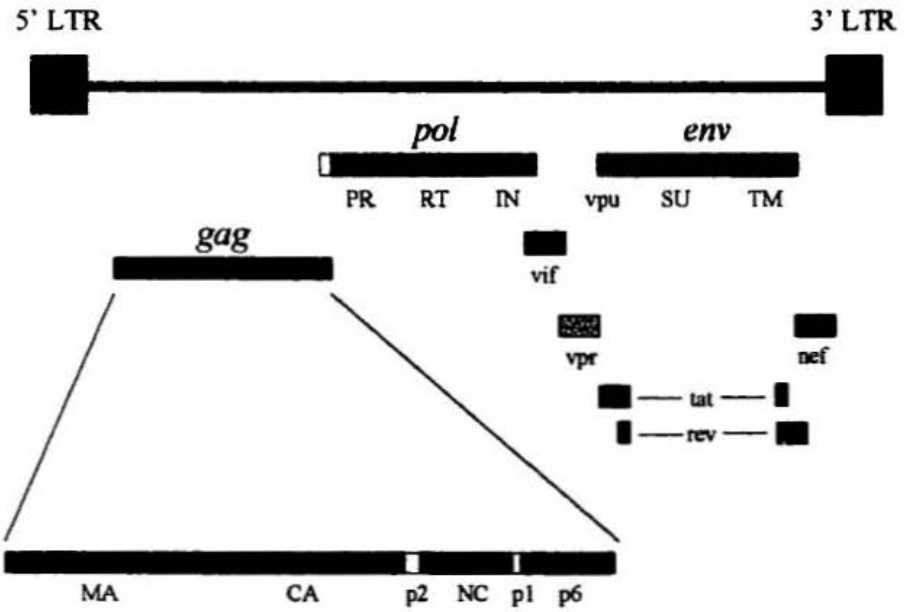
Figure 1.2 shows the genome of HIV-1 consisting of two identical copies of single-stranded positive polarity RNA molecules approximately 9.8 kb in length. The genome encodes nine open reading frames, three of which encode the Gag, Pol, and Env polyproteins that are subsequently proteolyticly cleaved into individual proteins common to all retroviruses. The Gag polyprotein gets cleaved into four structural proteins; matrix, capsid, nucleocapsid, and p6. The two outer membrane structural components, gp120 and gp41, are generated from the Env gp160 polyprotein. Finally, the three Pol proteins, protease, reverse transcriptase, and integrase, provide the essential enzymatic functions. HIV-1 also produces six additional accessory proteins by differential splicing and cleavage: Nef, Tat, Rev, Vif, Vpr, and Vpu.[75]

1.3 VIRAL LIFE CYCLE

The life cycle of HIV-1, as seen in Figure 1.3, is a complex multi-step process that begins at the surface of the host cell where the viral envelope protein, gp120, binds to the CD4 receptor and a coreceptor on the surface of $CD4^+$ T cells and macrophages.[138] The binding of gp120 to CD4 induces a conformational change in the gp120 molecule exposing a glycine-rich region of gp41 often called the "fusion peptide". This

Figure 1.2. The HIV-1 Genome. Schematic representation of the HIV-1 genome and precursor protein organization. The virus' 5'- and 3'- long terminal repeats (LTR) are shown, as well as the open reading frames. Adapted from Frankel.[75]

Figure 1.2


5' LTR
3' LTR
pol
env
PR
RT
IN
vpu
SU
TM
gag
vif
vpr
nef
tat
rev
MA
CA
p2
NC
p1
p6

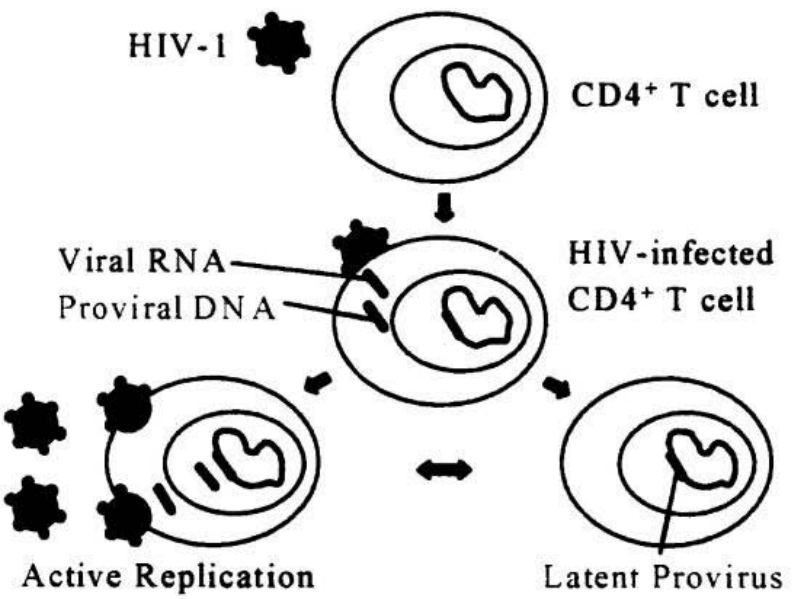
change is thought to bring the virus close enough to the plasma membrane to facilitate fusion of the viral envelope with the plasma membrane, and allow the HIV-1 core to enter the cell.[102] Once inside the cell, the virus uncoats in the cytoplasm and the viral reverse transcriptase (RT) makes multiple double-stranded DNA copies of the viral RNA genome.[166] The preintegration complex is transported to the host cell nucleus, probably with the help of the viral Vpr, Nef, and p17 matrix.[34] In the nucleus, the viral integrase (IN) catalyzes a series of reactions resulting in integration of the viral genome into a host chromosome, forming an integrated provirus.[120] The HIV-1 promoter is located within the 5' untranslated region of the provirus and contains a number of regulatory elements important for human RNA polymerase II transcription, including a binding site for NF-κB.[116] Once the infected cell becomes activated, the provirus is transcribed with both spliced and unspliced viral RNAs being produced. At first, only the smaller double-spliced mRNAs encoding for Tat, Nef, and Rev regulatory proteins get transported to the cytoplasm. Later, single-spliced and unspliced genomic-length RNAs get transported from the nucleus to the cytoplasm with the help of Rev.[111] Once in the cytoplasm, the viral mRNAs are translated producing larger Gag, Pol, and Env precursor proteins. Cleavage of these precursor proteins by HIV protease (PR) generates the structural and enzymatic proteins. The viral proteins localize to the plasma membrane where the core particle is assembled and an immature virion begins to bud from the cell surface. As the virus is released from the cell surface it undergoes its final maturation and becomes infectious.[118,229]

1.4 IMMUNOPATHOGENESIS

From the time that AIDS was discovered in 1981, it was clear that the condition involved a loss of immune competence and susceptibility to opportunistic infections. The hallmark of this condition was depletion of peripheral blood $CD4^+$ T cells.[89] The most obvious explanation was that HIV-1 infects $CD4^+$ T cells causing cell lysis, resulting in depletion of helper T cells and the eventual collapse of the immune system. However, this hypothesis was immediately questioned because the proportion of infected cells did not seem to explain the extensive decline in cell number.[200] AIDS and HIV infection are also associated with other immune abnormalities including polyclonal B cell activation and the production of autoantibodies, decreased cytolytic activity of NK cells and Ag-specific CTL, reduced T helper cell function, impaired Ag-presenting activity, and disruption of immunoregulatory cytokine production.[132] Simple viral cytopathicity was not sufficient to explain this broad effect on the immune system. Hence, it was suggested that immunopathogenesis might be responsible. Some of the models proposed include (1) HIV-1-specific $CD8^+$ CTL that kill HIV-1-infected $CD4^+$ T cells;[227] (2) autoimmune reactions;[200] (3) immune suppression by HIV proteins;[132] (4) activation of APC and/or T cells;[77,107] and (5) apoptotic T cell death.[70] Despite the vast amount of work that has been done to test these hypotheses, no single mechanism has been shown to be broadly responsible for the immunopathogenesis of AIDS.

Figure 1.3. Life cycle of HIV-1. After HIV enters the host cell, viral reverse transcriptase makes a double-stranded DNA provirus from the viral RNA. This provirus translocates to the nucleus and integrates into the host cell genomic DNA, where depending on the activation state of the cell, latent or active infection can result.

Figure1.3


HIV-1
CD4+ T cell
Viral RNA
Proviral DNA
HIV-infected
CD4+ T cell
Active Replication
Latent Provirus

1.5 DISEASE PROGRESSION

1.5.1 Primary HIV Infection

The course of HIV-1 infection involves three distinct phases: primary infection, clinical latency, and the symptomatic phase. HIV infected individuals generally fall into three categories with respect to disease progression: typical progressors, rapid progressors, and long-term nonprogressors. Primary infection in a typical progressor lasts approximately 12 weeks.[29,45,91] During this time, the virus reaches levels of up to one million copies of HIV-1 RNA per milliliter of plasma. Interestingly, viral levels decrease 10- to 1000-fold during the first few months, coincident with the development of an HIV-1-specific CTL response.[29,45,180] The significance of this decrease in plasma viral load was emphasized by studies showing that plasma HIV-1 RNA concentrations during chronic infection predict the rate of $CD4^+$ T cell decline and the risk of rapid progression to AIDS and death.[150,151] Furthermore, plasma HIV-1 RNA concentrations during the first year after seroconversion have been shown to independently predict disease progression.[43,52,150] However, by this time the virus has already established a latent pool of replication competent HIV-1 proviral DNA incorporated into the genomic DNA of resting $CD4^+$ T cells.[91]

1.5.2 Clinically Latent Period

As the levels of viremia decrease, the HIV-1-infected individual enters the chronic phase of infection, which typically lasts for 8 to 10 years.[12,68,134] Although once believed that viral replication was minimal or nonexistent during this period, the demonstration of virus replication in lymphoid tissue indicated that virus replication is continuous throughout the entire course of infection. During the asymptomatic phase, $CD4^+$ T cell counts decline slowly from approximately 500 to 200/μl, and virus load increases in the blood.[172] Disruption of lymph node tissue architecture also occurs over this period, leading to a decreased ability to trap virus.[171,172] Both $CD4^+$ and $CD8^+$ HIV-1-specific cell-mediated immune responses can be detected,[31,210] and a variety of antibodies against HIV-1 structural and regulatory proteins are generated during this stage of infection.[216,217] However, inhibition of these HIV-1-specific responses is usually observed during the progression from early to intermediate stages of disease.[99]

1.5.3 Advanced HIV-1 Infection

As an HIV-infected individual progresses to the final stage of disease, $CD4^+$ T cell counts fall to below 200/μl and viral load increases to levels similar to that of primary infection. By this time most of the lymphoid tissue has been replaced by fibrotic tissue.[171,172] During late stage AIDS the individual is prone to a variety of opportunistic

infections including *Candida albicans*, Epstein-Barr virus, cytomegalovirus, and herpes viruses.[190]

1.5.4 Rapid Progressors and Long-Term Nonprogressors

In the absence of effective treatment approximately 80-90% of HIV-1-infected individuals appear as "typical progressors" with a median survival time of 10 years.[12,68,134] The remaining 10-20% are either "rapid progressors" or "long-term nonprogressors" (LTNPs). Rapid progressors develop AIDS within 2 to 3 years after seroconversion.[201] Immune responses are usually defective with low levels of anti-HIV antibodies[181,186] and severely impaired $CD8^+$ T cell-mediated suppression of viral replication.[130,139] Virus load is usually very high in rapid progressors[95] and HIV-1 isolated from rapid progressors appears more homogeneous than that isolated from typical progressors.[54]

On the other hand, a small percentage of HIV-1-infected persons do not experience clinical progression of HIV-1 infection and maintain stable $CD4^+$ T cell counts for many years without antiretroviral therapy. The criteria for nonprogression typically include documented HIV-1 infection for more than 7 years, stable $CD4^+$ T cell counts higher than 600 cells/μl, absence of symptoms, and no antiretroviral therapy. Immune function is conserved in LTNPs with strong HIV-1-specific humoral and cell-mediated responses.[12, 28,134,201]

1.5.5 Markers of Disease Progression

Due to the variations in the rate of disease progression and the need to evaluate the efficacy of existing and newly developed antiretroviral therapies, HIV researchers are interested in identifying immunological and virological parameters of disease progression. Increased levels of circulating β_2-microglobulin[108] and $CD8^+$ $HLA\text{-}DR^+$ $CD38^-$ T cell numbers, for example, have been associated with progression to AIDS.[88] However, $CD4^+$ T cell counts and plasma HIV-1 RNA levels are the two best independent predictors of the rate of disease progression.[66,150] Decreased $CD4^+$ T cell counts and increased viral load levels are associated with advanced HIV-1 infection. Over the last 3-4 years, research on AIDS predictors has focused on plasma HIV-1 RNA levels. Studies have even suggested that an elevated or increasing viral load is considered to be the most accurate predictor of HIV disease progression.[42,84,150,151] During primary HIV infection, concentrations of piasma HIV RNA can exceed 1,000,000 viral copies/ml (500,000 virions/ml). However, with the emergence of antiviral immune responses, these high plasma concentrations decline precipitously, and after a period of time, maintain a steady-state value.[121,221] This steady-state concentration, which is set during the first year of infection, is a powerful predictor of the clinical outcome and allows identification of HIV-1-infected individuals at risk for rapid disease progression.[117,131] Further evidence for the predictive value of plasma viral load was obtained in studies of LTNPs, who were shown to have up to 20-fold lower HIV-1 RNA levels than progressors.[15,170] Because of the results of studies such as these, RT-PCR-

based methods of HIV-1 RNA measurement were quickly adopted as standard care and for evaluation of drug efficacy.[188]

1.6 HIV-1 ANTIRETROVIRAL THERAPY

1.6.1 Reverse Transcriptase Inhibitors

HIV-1 reverse transcriptase, protease, and integrase provide the essential enzymatic functions for the viral life cycle. As early as 1987, antiretroviral drugs that blocked reverse transcription by binding RT were being tested. The first of these compounds to be approved was 3'-azido-3'-deoxythymidine (AZT).[157] Today, RT inhibitors can be divided into two groups, depending on whether they are targeted against the enzyme's active site or binds other areas of the protein. Nucleoside analog RT inhibitors (NRTIs) are nucleoside derivatives that block reverse transcription by competitively binding to the active site of RT. These include AZT, ddI, ddC, d4T, 3TC and abacavir. Non-nucleoside RT inhibitors (NNRTIs) such as nevirapine, delavirdine, and efavirenz, binding to regions other than the active site and sterically block the incorporation of incoming nucleosides.[50] Various combinations of NRTIs and/or NNRTI's were used with significant benefit to the patient, but due to the high mutation rate associated with HIV's error-prone RT, viruses containing mutations that conferred resistance to these drugs were soon found in the blood of HIV-infected individuals.[132]

1.6.2 Protease Inhibitors

In the mid 1990's HIV protease was targeted by researchers. HIV protease inhibitors prevent cleavage of gag and gag-pol precursors, thereby arresting maturation and blocking infectivity of nascent virions.[119,189] There are currently five such drugs available by prescription, amprenavir, indinavir, nelfinavir, saquinavir, and ritonavir.[73,155] These protease inhibitors rapidly and profoundly reduced viral load, as indicated by a significant decline in plasma HIV RNA concentrations within a few days after the start of treatment.[106,214] Monotherapy with indinavir, nelfinavir, or ritonavir reduces plasma HIV RNA concentrations 100 to 1000 X in 4 to 12 weeks.[48,142] The inclusion of these compounds in the battery against HIV provided a more effective approach to the control of HIV replication. RT inhibitors block viral replication at an early step in the viral life cycle, just after viral entry, whereas the protease inhibitors exert their effects later in the life cycle, during the steps involved in production of new virus. Therefore, it seemed logical to give these drugs in combination, thus effectively targeting two different steps in the viral life cycle, at the same time. As expected, combination therapy, or highly active antiretroviral therapy (HAART) consisting of a protease inhibitor and two nucleoside analogues causes suppression of HIV-1 replication in infected persons to such an extent that the virus can become undetectable for more than two years.[94,96] This sustained reduction in viral replication improves immune function, delays disease progression, and prolongs survival.[3,4,169]

1.6.3 Integrase Inhibitors

Given the apparent success of RT and protease inhibitor combination therapy, it is not surprising that basic scientists and pharmaceutical companies are now targeting the third major enzyme involved in the HIV-1 life cycle. The integrase enzyme facilitates the integration of the viral genome into a host chromosome, forming an integrated provirus.[120] Although none are currently available for prescription, numerous compounds have been developed as potential integrase inhibitors and a few are now being tested.[110,146]

1.7 LIMITATIONS OF ANTIRETROVIRAL THERAPY

1.7.1 Drug Resistance

Unfortunately, the HIV-1 reverse transcriptase lacks proof-reading activity, making it highly error-prone and capable of mutating its genome as many as 10^4-10^5 times per day.[212] This high mutation rate allows the generation of mutant viruses that can replicate even in the presence of multiple drugs. The existing theory is that a wild type strain of virus usually dominates in the blood of an HIV-1-infected individual, but that slower replicating viral mutants exist at low levels. When antiretroviral therapy is initiated, the wild type viral replication is blocked. However, this selective pressure has no effect on the mutant strains which can then dominate the viral population. These

viruses can also acquire additional mutations that increase their resistance.[187] Even worse is the fact that patterns of drug resistance exist such that the acquisition of a point mutation which confers resistance to one drug, may also confer a cross-resistance to another within the same class.[40] Furthermore, it was shown recently that drug resistant strains of HIV occur in the semen of HIV-1-infected men and are transmittable even when HAART appears to be successful.[35,224] This, of course, means that a newly infected individual may already harbor a drug resistant strain of HIV. Thus, drug resistant strains of HIV-1 represent a difficult challenge for AIDS researchers. However, the idea of eliminating virus all together is still a greater challenge.

1.7.2 Latent HIV-1 Infection

The development of RT-PCR as a tool for measuring plasma HIV-1 RNA concentrations allowed HIV researchers to quickly evaluate the efficacy of newly developed antiretroviral drugs and to study the dynamics of viral replication. In 1995, two controversial papers in *Nature* reported that the half-life of free virus in the plasma of an HIV-1-infected individual was no more than six hours, and that productively infected cells also had a short half life of 1.6 days.[106,214] In 1997, a mathematical model based on the kinetics of viral decay seen shortly after initiation of HAART suggested that 2.3 – 3.1 years of a drug regimen that "completely inhibits virus replication" would be sufficient to "eradicate" HIV infection. This hypothesis was based on the rapid reductions of free HIV-1 and the significant increases in $CD4^+$ T cells in the blood of HIV-1-infected

individuals shortly after the initiation of HAART.[179] However, this hypothesis was based on two flawed assumptions. First, it was assumed that the changes seen in the $CD4^+$ T cells in the peripheral blood, which accounts for 2% of the body's T lymphocytes, reflected changes occurring in the total pool of T lymphocytes. This seems to have been an obvious mistake, since it was already known that the frequency of HIV-1-infected cells in unfractionated and sorted $CD4^+$ cell populations isolated from lymphoid tissues was as much as 10-fold higher than the frequency in peripheral blood.[173] The second mistake that this group made was to assume that the early increase in peripheral blood CD4 counts was due to newly produced naive T cells. This early increase was later shown to be due to the redistribution of HIV-specific T cells that had previously emigrated to peripheral sites of viral replication, such as the lymph nodes.[168] Another problem with the eradication hypothesis is that it required an antiviral drug regimen that "completely inhibits virus replication." Given the extent of viral mutation mentioned above, such a regimen is at present unavailable.

By far the greatest challenge facing HIV researchers today is the question of how to eliminate latently infected $CD4^+$ T cells that exist in the blood of HIV-1-infected individuals. During acute infection, HIV-1 preferentially infects activated $CD4^+$ T cells and reverse transcribes a double stranded DNA copy of its RNA genome. This provirai DNA in transported to the nucleus of the cell where it integrates into the host cell genomic DNA. These activated cells can immediately begin transcribing the provirus to produce new virus particles (reviewed by Levy[132]). However, a small proportion of these cells can revert to a resting memory state while still carrying the integrated

provirus.[27,28,72] Since the necessary transcription factors are not present in the resting $CD4^+$ T cells, the stably integrated HIV provirus is not transcribed, but sits silently, hidden from the immune system.[161] Contrary to earlier suggestions that these latently infected cells have a short half-life of only 0.5 – 2 weeks,[179] new evidence obtained in 1997 indicated that these cells constitute a long-lived pool of replication-competent HIV. Wong *et al* studied six patients who were receiving combination therapy and all had plasma HIV-1 RNA levels below 50 copies per/ml for up to two years. They showed that *in vitro* activation of $CD4^+$ T lymphocytes using immobilized antibodies to CD3 and CD28 enabled the isolation of HIV-1 from PBMC.[220] Similarly, Finzi *et al.* showed by a multi-step purification technique, that replication-competent virus could be routinely recovered from resting $CD4^+$ T lymphocytes of 22 patients receiving HAART. They reported that the frequency of these cells was low (0.2 to 16.4 per 10^6 cells) and interestingly, did not decrease with increasing time on therapy.[72] A third group reported similar findings in 13 patients receiving HAART using a PCR-based method that amplified only integrated HIV-1 DNA. Chun *et al.* found that highly purified $CD4^+$ T cells from 13/13 HIV-1-infected individuals with undetectable plasma viremia, carried integrated proviral DNA and were capable of producing infectious virus upon cellular activation *in vitro*.[27] These findings suggested that latently infected resting $CD4^+$ T cells are long-lived and that the time required for virus eradication, if at all possible, would be considerably longer than previously predicted.[27,28,72]

The use of combination antiretroviral drug regimens has dramatically decreased morbidity and mortality in HIV-infected individuals, and causes sustained reductions in

plasma viral load.[93,96,109,169] However, recent work has shown that viral load levels can rebound to pretreatment levels in as little as 21 days when treatment is halted.[97] The source of these newly produced virions is most likely the pool of latently infected $CD4^+$ T cells carrying stably integrated HIV-1 proviral DNA. Despite the earlier optimistic predictions, it now appears that this proviral load is established early in HIV-1 infection and is a stable reservoir, unaffected by HAART. Ibanez *et al.* reported that no significant change in integrated HIV-1 DNA copy number was seen in a group of HIV-1-infected individuals receiving 48 weeks of HAART.[114] In a similar study, Bruisten *et al.* started 42 antiretroviral naive HIV-1-infected individuals on combination therapy and monitored HIV-1 RNA, and DNA levels over an 80 week period. Although HIV-1 RNA levels declined by as much as 1.9 $\log_{10}$, HIV-1 DNA load never decreased significantly from baseline values.[11] The final nail in the 'eradication' coffin was driven in May of 1999, when Finzi *et al.* reported that the half-life of the latent reservoir of HIV-1-infected resting $CD4^+$ T cells could actually be as long as 43.9 months. The authors hypothesized that if this reservoir consists of as few as 10^5 cells, eradication could take up to 60 years.[71]

1.8 HYPOTHESIS 1

Since proviral load is a stable pool that is unaffected by antiretroviral therapy, we expect that this parameter will not be useful for monitoring disease progression, nor will it be a useful tool to measure the efficacy of HIV therapy. However, we propose that the

higher the frequency of $CD4^+$ T cells containing integrated HIV-1 proviral DNA in the blood of an HIV-1-infected individual, the faster the virus rebound after discontinuation of HAART, and the higher the set point reached. We also believe that since the greatest viral sequence diversification occurs at the step just prior to proviral DNA integration, the degree of HIV-1 sequence diversity should be directly proportional to the size of the integrated proviral pool. The greater the proviral load, the more sequence diversity available for HIV-1 and the easier it should be for the virus to mutate beyond both the immune response and the pressure of antiretroviral therapy.

In short, we hypothesize that proviral load ultimately determines the rate of disease progression and is a useful prognostic indicator of the durability of responsiveness to antiretroviral therapy and the development of drug resistance.

To test this theory, we first had to develop a method to measure HIV-1 proviral load. The development and standardization of which are described in Chapter 3. The results obtained using this method and the analysis of the effects of proviral load on disease progression and response to therapy are found in Chapter 4.

1.9 THE CCR5Δ32 CORECEPTOR MUTATION AND PROVIRAL LOAD

While the above work was under way, there was much interest within the HIV research community in the recently identified HIV coreceptors and the effects of coreceptor mutations on disease progression. However, no mechanism had yet been proven responsible for these proposed effects. In addition to the aforementioned project,

we set out to determine whether decreased proviral load might be the link between a heterozygous CCR5 coreceptor mutation and slower disease progression.

1.9.1 Background

It was known as early as 1981 that $CD4^+$ T cells were specifically depleted in AIDS patients.[89] Soon after HIV-1 was identified as the cause of AIDS, it was shown that monoclonal antibodies (mAbs) against CD4 could prevent HIV-1 replication *in vitro*, thus suggesting a role for CD4 as the cellular receptor for HIV-1.[47,125] This argument was strengthened in 1986 when it was found that HIV-1 surface glycoprotein gp120 could form a complex with CD4.[149] Transfection studies with the human CD4 gene demonstrated that human cells that were normally not infectable by HIV-1, could be rendered susceptible to HIV-1 infection after transfection with CD4. However, the idea that there might be a second receptor involved in the mechanism of HIV-1 infection came about when human CD4 was transfected into murine fibroblast cells. As it turned out, this was not sufficient to render the murine cells susceptible to HIV-1 entry. gp120 binding to these cells was demonstrated, but virus entry did not occur.[140] However, Landau *et al* showed that when murine cells expressing human CD4 were fused with human cells that did not express CD4, the resulting hybrids could bind to HIV-infected cells.[129] Thus, it was proposed that HIV-1 needs a species-specific cell surface factor other than CD4 for efficient entry into human cells.[21] However, this 'coreceptor' remained unidentified for over a decade.

I feel it is important at this point to clarify some tangled nomenclature that has been used to denote HIV-1 tropism. Due to HIV's extraordinarily high mutation rates in infected persons, with most variability occurring in *env*, HIV-1 exists as a 'quasispecies' that is continuously evolving within the host. It is important to note that ALL primary HIV-1 isolates can replicate in primary T cells, i.e. any freshly isolated strain of HIV-1 will infect and replicate in freshly isolated PBMC. However, once cultured *in vitro*, isolates of HIV-1 emerge that have selective tropisms based on the cell type in which they were cultured. Some isolates replicate efficiently in continuous $CD4^+$ T cell lines, but only poorly in primary macrophages. These so-called 'T-cell tropic' isolates replicate rapidly and can usually facilitate *in vitro* formation of multinucleated giant cells, or syncytia, and thus have also been referred to as 'syncytium-inducing' (SI) viruses. Due to the obvious confusion and ambiguities, these isolates are now more appropriately referred to as 'X4' isolates for reasons that will be explained below. Interestingly, other strains show the opposite selectivity, infecting primary macrophages much more efficiently than continuous $CD4^+$ T cell lines. These, misleadingly labeled, 'M-tropic' isolates, which replicate more slowly than X4 isolates *in vitro* and are non-syncytium-inducing (NSI), are now properly referred to as 'R5' isolates. Dual tropic virus strains that replicate equally well in macrophage-monoctye and T cell lines are referred to as R5X4 isolates (reviewed in 8, 60, and 158).

1.9.2 Discovery of the HIV-1 Coreceptors

Chemokines are specific immunological factors that have combined chemoattractant and cytokine properties. They are 70-90 amino acids in length and can now be subdivided into four groups, based on the chemokine receptor nomenclature currently in use, which uses CC, CXC, XC, and CX3C followed by R (for recpetor).[229] The α-chemokines, or CXCL chemokines, contain a single amino acid between the first and second cysteine residues, whereas the β-chemokines, or CCL chemokines have adjacent cysteine residues.[160] The pieces of the HIV-1 coreceptor puzzle began to fall into place in 1995 when Cocchi *et al.* showed that the β-chemokines macrophage inflammatory protein (MIP)-1α, MIP-1β, and RANTES (regulated upon activation normal T cell expressed and secreted), secreted from $CD8^+$ T cells, could inhibit the replication of R5, but not X4, strains of HIV-1.[33] At the same time, Paxton *et al* were finding that the $CD4^+$ T cells from some HIV "exposed uninfected" (EU) individuals were resistant to infection by R5 strains.[176] In 1996, Feng *et al.* devised an elegant application of gene-transfer technology to identify the first HIV-1 coreceptor.[69] They used recombinant vaccinia viruses to express Env on one cell population and CD4 on another. One of these cell populations also expressed vaccinia-encoded bacteriophage T7 RNA polymerase and the other contained the *LacZ* reporter gene linked to a T7 promoter, thus allowing the production of β-galactosidase in fused cells only. When murine cells were transfected with a plasmid cDNA library from HeLa cells in which the cDNAs were linked to the T7 promoter and mixed with cells expressing Env from an X4 isolate,

staining with X-gal allowed the detection of fused cells. Repeated experiments with a subdivided library eventually resulted in the isolation of a single plasmid that was capable of conferring the capacity for fusion. Subsequent sequencing identified a seven-transmembrane segment G-protein-coupled receptor, which the authors named fusin, that had already been cloned but was designated as an orphan receptor as its natural ligand was not yet known. Expression of recombinant fusin rendered CD4-expressing non-human cell types permissive for both Env-mediated fusion and productive infection by X4 HIV-1. Although fusin functioned efficiently for X4 Envs, its expression did not support the fusion of R5 Envs.

With the identification of fusin as the coreceptor for X4 HIV-1 strains, the G-protein-coupled receptor superfamily became the primary focus in the search for the cofactor for R5 isolates. Since it was already known that the β-chemokines RANTES, MIP-1α, and MIP-1β inhibited HIV-1 replication of R5 isolates, it was suggested that the second receptor for R5 isolates might be a β-chemokine receptor. Coincidentally in 1996, a group studying chemokine biology reported the cloning, sequencing, and functional characterization of a chemokine receptor that responded to RANTES, MIP-1α, and MIP-1β that they named CCR-5.[192] This finding started a frenzy in the HIV research community that led to the simultaneous finding by five different groups that CCR-5 was indeed the second receptor for entry of R5 isolates of HIV-1.[1,23,55,58,59] For simplicity, fusin was then referred to as CXCR4 and the confusing nomenclature of M-/T-tropic and NSI-/SI-isolates was later replaced with the more accurate designations of R5 and X4 isolates, based on coreceptor usage.[7]

1.9.3 Discovery of the CCR5Δ32 Coreceptor Mutation

It had been suggested that host genetic determinants influence susceptibility to HIV-1 infection, but identification of the specific genes involved remained elusive.[8] Naturally, it was hypothesized that genotypic differences in the coreceptors might explain the apparent resistance to HIV-1 infection seen in a group of homosexual men who remained seronegative despite numerous high-risk sexual encounters. *In vitro* assays indicated that $CD4^+$ lymphocytes from two of these individuals were resistant to infection by R5 HIV-1 isolates, but were readily infectable by X4 strains. This, along with the finding that activated PBL from these individuals secreted high levels of RANTES, MIP-1α, and MIP-1β, suggested the possibility that overproduction of these CC chemokines might be responsible for the non-infectability by R5 strains.[176] However, analysis of both cDNA and genomic DNA from these two EU individuals showed that both men were homozygous for a mutant CCR5 allele with a 32 base-pair deletion (Δ32) in the region corresponding to the second extracellular loop. This deletion causes a frame-shift that introduces a premature translation termination site resulting in a nonfunctional truncated protein that is not expressed on the cell surface.[136] The CCR5Δ32 mutation has a gene frequency of approximately 20% in populations of European descent. It is also found at frequencies of 2-5% throughout Europe, the Middle East, and the Indian subcontinent. Approximately 15-20% heterozygosity and 1% homozygosity is seen in Caucasian populations.[8]

Approximately 15-20% heterozygosity and 1% homozygosity is seen in Caucasian populations.[8]

Since it had already been established that initial infection involved transmission of R5 isolates (reviewed in 67 and 154), these results indicated that the EU individuals were missing the fusion coreceptor essential for infection by these isolates. The evidence for the role of the Δ32 mutation in resistance to HIV-1 infection is strengthened by the fact that amongst the thousands of HIV-1-infected individuals genotyped, there are only four reported cases of HIV-1 infection in CCR5Δ32 homozygous individuals to date.[9,127,163,208] The identification of these four individuals contradicted the idea that most viruses transmitted through sexual contact use CCR5 as a coreceptor, since CCR5Δ32 homozygous would not have any CCR5 on their cells and thus should not have been infected. However, it is possible that the original infecting virus in these individuals was capable of using another chemokine receptor, such as CCR3.[205] One of these individuals was a hemophiliac who received blood products, so he may have been initially infected intravenously with an X4 or dual tropic HIV-1 isolate.[163]

1.9.4 CCR5Δ32 Heterozygosity and Disease Progression

Although heterozygosity for the CCR5Δ32 coreceptor mutation offers no protection from HIV-1 infection,[53,112,226] there has been an ongoing debate regarding the effects of heterozygosity on the rate of disease progression. Despite a few reports stating that heterozygosity for the CCR5Δ32 coreceptor mutation has no effect on disease

progression,[38,159] several studies have reported an increased prevalence of heterozygotes in groups of LTNPs.[53,100,206,213] Heterozygosity has also been associated with increased survival time,[10,53,152] decreased viral loads,[10,51,112,153,213] and higher $CD4^+$[10,51,112] and $CD8^+$[206] T cell levels. However, as would be expected, these effects are lost when the infecting virus is an X4 strain of HIV-1.[10,152]

In 1997, Wu et al reported markedly reduced expression of CCR5 on T cells from CCR5Δ32 heterozygous HIV-1-infected individuals, which correlated with reduced infectability by R5 strains of HIV-1.[222] The authors suggested that the presence of only one functional copy of the *ccr5* gene in the $CD4^+$ T cells of these individuals would result in decreased cell surface expression of CCR5, and that this decreased coreceptor expression could explain the delayed disease progression seen in the heterozygotes. Furthermore, biochemical studies subsequently showed that mutant CCR5 produced by the deleted gene could form heterocomplexes with wild type CCR5 and that this interaction caused CCR5 to be retained in the endoplasmic reticulum resulting in decreased cell surface expression.[6] In 1998, the importance of the CCR5Δ32 mutation was confirmed when various other groups showed that $CD4^+$ T cells from heterozygous individuals had decreased levels of cell surface CCR5 and reduced susceptibility to infection with R5 isolates.[124,175] It was also shown that increased expression of CCR5 on $CD4^+$ T cells correlated with advanced disease.[165]

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At this point it was conceivable that decreased levels of CCR5 on $CD4^+$ T cells of CCR5Δ32 heterozygous HIV-1-infected individuals could indirectly account for their delayed disease progression. However, the specific mechanism responsible for this effect had not been identified. We hypothesized that if these decreased coreceptor levels also existed *in vivo*, and resulted in similar decreased susceptibilities to infection by R5 viruses, then CCR5Δ32 heterozygotes should have a reduced frequency of $CD4^+$ T cells containing incorporated HIV-1 DNA. In other words, heterozygotes should display lower proviral loads in comparison with individuals homozygous for the wild type *ccr5* gene (CCR5wt). We propose that the mechanism of delayed disease progression seen in the heterozygotes can be explained by their lower proviral loads as decreased proviral loads should facilitate a slower spread of HIV-1 within the $CD4^+$ T cell population resulting in a slower $CD4^+$ T cell decline and reduced rate of disease progression.

To test this hypothesis we performed PCR-based genotyping for the CCR5Δ32 coreceptor mutation on approximately 100 HIV-1-infected individuals. We then compared disease progression between CCR5wt homozygous and CCR5Δ32 heterozygous groups based on $CD4^+$ T cell and plasma HIV-1 RNA levels to determine whether the heterozygotes in our cohort experienced slower rates of disease progression. Finally we compared proviral load levels between the two groups. The results of these analyses are summarized in Chapter 5.

2. MATERIALS AND METHODS

In this chapter I will give a detailed description of the materials and methods used to carry out the work done in my Master's project. I will start by briefly describing my study group, and then follow with an explanation of the methods used for HIV-1 proviral load measurement and CCR5 genotyping.

2.1 Study Participants.

Our cohort consists of more than 100 HIV-1-infected individuals attending the St. John's General Hospital Infectious Diseases Clinic. HIV-1 infection was detected by HIV-1 p24 enzyme-linked immunosorbent assay (ELISA) and confirmed by Western Blot. Approximately 85 of these individuals make regular visits to the clinic and provide blood samples for our studies. Most of the individuals in our cohort are believed to have been infected through heterosexual transmission of a relatively homogeneous pool of virus. Healthy HIV-negative volunteers were recruited from laboratory personnel and students. All stages of HIV disease are represented within this cohort as plasma viral load levels range from undetectable to over one million HIV RNA copies per ml, $CD4^+$ T cell counts from 0-1300/μl, $CD8^+$ T cell counts from 150-3000/μl and duration of infection ranges from less than six months to greater than 15 years. Plasma HIV-1 RNA levels, $CD4^+$ T cell counts and $CD8^+$ T cell counts were monitored as part of the standard clinical care program. Levels of plasma HIV-1 RNA were measured using the Amplicor

HIV-1 Monitor RT-PCR assay (Roche Diagnostic Systems, Mississauga, Ontario) according to the manufacturers instructions. The sensitivity of the assay at this time was 400 copies/ml, so samples containing no detectable HIV-1 RNA were assumed to be 400 copies/ml. All HIV-1 RNA levels were transformed to $\log_{10}$ values before analysis. Peripheral blood $CD4^+$ and $CD8^+$ T cell levels were measured by standard flow cytometry (Becton Dickinson, Ontario) using commercially available monoclonal antibodies (DAKO, Mississauga, Ontario). Antiretroviral drug therapy varies with some subjects receiving none while others have received various combinations of 1-4 drugs. Clinical status ranges from asymptomatic to AIDS. We have confidential access to all relevant clinical and clinical laboratory information such as plasma viral load, $CD4^+$ and $CD8^+$ T cell counts, as well as each individual's symptoms and treatment histories. All subjects provided informed consent for participation in this study which was approved by the MUN Faculty of Medicine Human Investigation Committee.

2.2 Lymphocyte Isolation.

Whole blood was collected at the Infectious Diseases Clinic in two 10 ml heparinized vacutainers by venipuncture with a 21-gauge needle. The blood was diluted 1:1 with Phosphate-buffered saline (PBS, pH=7.2), layered over Ficoll-Hypaque density gradient centrifugation buffer (Pharmacia Biotech Inc., Baie d=Urfe, Quebec) and spun at 400 x *g* for 30 min. After centrifugation, the buffy layer at the interface containing the peripheral blood mononuclear cells (PBMC) was collected and washed three times with

lymphocyte culture medium (RPMI, FCS, HEPES, and P/S, all from Gibco BRL, Burlington, Ontario).

2.3 Purification of $CD4^+$ Cells.

Isolated PBMC were washed once with $CD4^+/CD8^+$ separation buffer (PBS + 0.5% bovine serum albumin [BSA]). $CD4^+$ T cells were positively selected by rotating the PBMC in a 15 ml tube at 4°C for 45 min at a concentration of $3x10^6$ cells/ml with metallic beads (10 beads/$CD4^+$ cell) coated with anti-CD4 antibody (Dynal Inc., Lake Success, New York). Then to isolate the $CD4^+$ T cells, the cell/bead suspension was placed in a rack containing a magnet on one side and allowed to stand for 2 min. The beads and $CD4^+$ cells form rosettes which are pulled to the side of the tube adjacent to the magnet allowing the supernatant, containing $CD4^-$ PBMC, to be removed. The beads containing only $CD4^+$ cells were then washed twice with PBS and stored at -70°C until DNA extraction was performed. This method is reported to yield >95% $CD4^+$ T cells.[76,145]

2.4 DNA Extraction from Cells.

Cell/bead samples were removed from -70°C and thawed at room temperature for no more than 5 min. 1 ml of DNAzol (Gibco BRL, Burlington, Ontario) was added to each tube using a 1 ml pipette and re-pipetted 5 times to homogenise. Cell/bead/DNAzol

homogenates were immediately transferred to a 1.5 ml Eppendorf tube and incubated for 2 min at room temperature. Tubes were then spun in a microcentrifuge for 10 min at 12,000 x *g* in order to pellet the beads. Homogenates were transferred to a new 1.5 ml tube with care not to transfer any beads. 0.5 ml of 99% ethanol (Sigma-Aldrich Canada, Oakville, Ontario) was added to each tube, inverted 20 times, and incubated for 5 min at room temperature. Tubes were spun again at 7500 x *g* for 2 min, and washed twice with 99% ethanol. Ethanol was removed and the pellets were air dried for approximately 10 min. DNA pellets were re-dissolved in 20-100 µl of TNE (Tris-NaCl-EDTA, pH=7.6; Sigma-Aldrich Canada, Oakville, Ontario) and incubated at 56°C for 2-3 hours to dissolve. A "Mock" extraction using a tube containing no cells was included with every set of extractions in order to test for HIV DNA contamination of extraction materials. DNA samples were stored at 4°C until needed, optical density was read at 260 nm to determine DNA concentration on the same day as the sample was to be used as evaporation during storage can affect the concentration.

2.5 DNA Extraction from Plasma.

A few of the earlier participants in our cohort had dropped out of the study before we were able to get fresh or frozen cell samples from them. However, plasma samples from these individuals were available to us. We were able to isolate genomic DNA from these plasma samples using the "Nuclisens – Boom Method" from Organon Teknika (Scarborough, Ontario). The source of this genomic DNA is assumed to be either from

cells that get accidentally taken up during Ficoll extraction, or possibly free DNA from cells that may have been sheared during the lymphocyte separation. Conceivably, this DNA was released into the plasma, but the plasma was collected and frozen before the DNA was degraded. This DNA was only used for the purpose of CCR5 genotyping and not for proviral load measurement.

Plasma samples were thawed on ice and the supplied wash and lysis buffers from the kit were pre-warmed at 37°C for 30 min, with mixing every 10 min. The remainder of the procedure was done at room temperature unless otherwise specified. 200 µl plasma was combined with 900 µl of lysis buffer, and vortexed for 10 sec. Tubes were then incubated for 5 min, inverted 5X, and incubated for another 5 min. Samples were spun at 10,000 x *g* for 30 sec to collect any homogenate from the top of the tube. 50 µl of a silica suspension was added to each tube, vortexed for 10 sec, and incubated for 10 min with vortexing at 2 min intervals. Tubes were spun at 10,000 x *g* for 30 sec and supernatants removed with a 1 ml pipette. The pelleted silica was washed with 1 ml of wash buffer, vortexed, spun at 10,000 x *g* for 30 sec, and the supernatant removed. This washing procedure was then repeated four times, once more with the wash buffer, 2X with 70% ethanol, and once with acetone. Pellets were dried with the caps open in a 56°C heating block for 10 min. Nucleic acids were eluted in 50 µl of the supplied elution buffer with light vortexing for 5 sec and a 10 min incubation at 56°C. The silica/buffer suspension was spun at 10,000 x *g* for 2 min and 30-35 µl of supernatant containing the nucleic acids (NA) was transferred to a new tube. NA samples were stored at -70°C.

2.6 CCR5 Genotyping.

Oligonucleotides flanking the putative deleted region were used to amplify a portion of the CCR5 gene by polymerase chain reaction (PCR) from genomic DNA isolated from PBMC. The primers CCR5c (5'-CAA CCC GAA GGT CTT CAT TAC ACC-3' and CCR5d (5'-CCT GTG CCT CTT CTT CTC ATT TCG-3') generate a 189 bp PCR product from the wild-type gene and a 157 bp product from the CCRΔ32 mutant gene.[112] As a positive control, one DNA sample from each set of extractions was also run in a separate reaction with *β-globin* primers ARP894.1 (14-33) LA1-N (5'-ACA CAA CTG TGT TCA CTA GC-3') and ARP894.2 (104-123) LA2-C (5'-CAA CTT CAT CCA CGT TCA-3') to give a 115 bp PCR product.[14] A tube without DNA was run as a negative control with every set of samples. The PCR was performed in a 20 µl reaction containing 100 ng of template, 0.2 µM CCR5 primers (Medicorp Inc., Montreal, Quebec), 0.16 µM β-globin primers (AIDS Reagent Project, Hertfordshire, United Kingdom), 1.5 mM Mg^{2+}, 0.25 mM dNTPs, and 1.25 units of *Taq* polymerase in 1X PCR reaction buffer (Gibco BRL, Burlington, Ontario). Amplification of plasma-derived nucleic acids was done in 50 µl reactions using 5-10 µl of NA. Reactions were run for 5 cycles of 60 s at 94°C, 60 s at 55°C, and 90 s at 72°C followed by 35 cycles of 30 s at 94°C, 30 s at 60°C, and 45 s at 72°C using a PTC-100 thermal cycler (MJ Research, Inc., Fisher Scientific, Nepean, Ontario).

2.7 RNA Extraction.

Total RNA was extracted from 5 x 10^6 cells using the TRIzol method (Gibco BRL, Burlington, Ontario). Cells were pelleted at 400 x *g*, supernatant removed and 1 ml of TRIzol was added. The mixture was re-pipetted 5X to homogenise and incubated at room temperature for 5 min. 200 µl of chloroform was added and the tubes shaken vigorously by hand for 15 sec, then incubated again at room temperature for 3 minutes. Tubes were then spun at 12,000 x *g* for 15 min at 4°C to allow phase separation. After centrifugation, the upper aqueous layer was transferred to a new tube and 500 µl of isopropanol added to precipitate the RNA. Samples were incubated at room temperature for 10 min and spun at 12,000 x *g* for 10 min at 4°C. Supernatants were removed and RNA pellets washed with 1 ml of 75% ethanol with vortexing for 5 sec. Finally the pellets were spun at 7,500 x *g* for 5 min at 4°C, supernatants removed and samples air-dried for 5-10 min. The RNA was redissolved in 20 µl of DEPC-treated water with slow re-pipetting and heated at 60°C for 10 min to allow complete solubilization. RNA samples were stored at -70°C until needed.

2.8 Synthesis of First-Strand cDNA.

First-strand cDNA was synthesised from total RNA using the "First-Strand cDNA Synthesis" kit from Pharmacia (Baie d=Urfe, Quebec). 5 µg of total RNA was diluted in 20 µl of DEPC-treated water and heated at 65°C for 10 min followed by 5 min on ice. A

13 µl reaction mix was prepared consisting of 11 µl of Bulk First-Strand reaction mix, containing the Moloney Murine Leukemia Virus reverse transcriptase, 1 µl of 0.2 µg/µl Not *1-d(T)*$_{18}$ primer, and 1 µl of 200 mM DTT. The heat-denatured RNA added to the reaction mix and reverse transcribed for 1 hr at 37°C, followed by 5 min at 65°C to inactivate RT, then stored at -70°C.

2.9 Preparation of HIV DNA Standards.

HIV DNA standards of known copy number were prepared by spiking various amounts of a pBR322 plasmid containing the full length HIV genome (interrupted in the *pol* region for safety reasons) (Perkin Elmer, Mississauga, Ontario)[98] into 500 ng of genomic DNA from PBMC of HIV-negative donors diluted in TNE buffer (pH = 7.6). Two sets of standards were prepared in order to accurately measure both high and low copy number samples. A low set consisted of 0, 1, 2, 5, 10, 25, 50, 75, and 100 copies of HIV-1 DNA/500ng of total DNA and a high set consisted of 0, 50, 100, 250, 500, and 1000 copies of HIV DNA/500 ng of total DNA.

2.10 Amplification of HIV Proviral DNA.

HIV proviral DNA was amplified in duplicate reactions by quantitative multiplex PCR using a set of oligonucleotide primers that bind to a highly conserved region of the HIV HXB2 *gag* gene (Los Alamos Database). The primers ARP872 HG1214N (5'-GGT

ACA TCA GGC CAT ATC ACC-3') and ARP873 HG1686C (5'-ACC GGT CTA CAT AGT CTC-3') generate a 473 bp PCR product.[203] Primers ARP894.1 and ARP894.2 (sequence above) were used to co-amplify a 115 bp portion of the human β-globin gene in the same reaction as an internal positive control for DNA integrity and loading consistency. Mock extractions and water controls were run as negative controls. Whenever possible, sequential samples from the same individual were run on the same day to minimise the effects of PCR variability. The PCR was done in a 50 µl reaction containing 500 ng of genomic DNA from $CD4^+$ cells of HIV-positive individuals (or prepared HIV DNA standards) with 0.2 µM *gag* primers (Medicorp Inc., Montreal, Quebec), 0.025 µM β-globin primers (AIDS Reagent Project, Hertfordshire, United Kingdom), 1.2 mM Mg^{2+}, 0.2 mM dNTPs, and 1.25 units of *Taq* in 1X PCR reaction buffer (Gibco BRL, Burlington, Ontario). Reactions were run for 32 cycles (high copy standards) or 35 cycles (low copy standards) with cycle #1 consisting of 1.5 min at 94°C, 2 min at 55°C, and 3 min at 72°C. Remaining cycles were 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C with the final cycle including an extra 7 min at 72°C. In some cases, nested PCR was performed on 1/10th of the original PCR reaction using a set of primers internal to the above *gag* primers, ARP8021.1 HG1366N SK145 (5'-AGT GGG GGG ACA TCA AGC AGC CAT GCA AAT-3') and ARP8021.2 HG1507C SK150 (5'-TGC TAT GTC ACT TCC CCT TGG TTC TCT C-3') (AIDS Reagent Project, Hertfordshire, United Kingdom) which generate a 142 bp product.[122] Reaction conditions for the nested PCR were the same as for the direct except that the Mg^{2+} concentration was reduced to

0.8 mM (optimal for the nested primers) and, β-globin primers were omitted, and the reaction was run for 40 cycles.

2.11 Gel Electrophoresis.

All PCR products were run at 100 V on 1-2% agarose (Gibco BRL, Burlington, Ontario) gels. For CCR5 genotyping, 5 µl of the 20 µl reaction was run for 50 min and visualised by UV illumination with 0.5 µg/ml ethidium bromide (Sigma-Aldrich Canada, Oakville, Ontario) in the gel only. For proviral PCR products, 7.5 µl of the 50 µl reaction was run for 30 min and visualised by uv illumination with 0.5 µg/ml ethidium bromide in the gel and running buffer (0.5X TBE). Molecular weights of PCR products were estimated by comparison with a 100 bp DNA ladder (Gibco BRL, Burlington, Ontario). All gels were visualised by exposure to ultraviolet light and the image captured using a "ChemiImager" (Alpha Innotech Corporation, San Leandro, California) and stored on disk for later analysis.

2.12 Proviral Load Determination.

Gel images were opened using the "AlphaEase 3.24" (Alpha Innotech Corporation, San Leandro, California) software supplied with the "ChemiImager." With this program a box of a desired size can be placed around each band on the gel. The program then determines the intensity of each selected band by measuring the average

intensity of the pixels within the box. To account for differences in background fluorescence amongst various regions of the gel, the "AUTOBACKGRD" option determines a separate background value for each selected box and subtracts that background from the respective measured intensities of each band. Then within the "alphaEase" program the band intensities corresponding to the HIV DNA standards can be used to generate a standard curve representing 'band intensity' versus 'copy number'. However this program does not give any statistics regarding the accuracy of the regression line generated. So the measured band intensity values were exported to "Corel Quattro Pro" (Corel Corporation, Ottawa, Canada) to construct a standard curve on which a linear regression analysis was done to determine how linear (i.e. useful) the standard curve really is. In "Quattro Pro" then the points that represent the linear range of the PCR program can be determined. Only lines of best fit having R^2 values greater than 0.90 and utilising at least 6 points are accepted. Then in "AlphaEase", only these points are used to draw a standard curve from which the copy numbers of the samples can be estimated. The HIV-1 DNA copy number is the average copy number of the duplicate values for each sample. Proviral load is then converted to 'X' copies of HIV DNA/10^5 $CD4^+$ cells based on the estimate that 144,560 human diploid cells contain 1µg of genomic DNA.[57]

2.13 Southern Blot Analysis.

A 1% agarose gel containing PCR products from amplification of HIV proviral DNA was placed in a shaking denaturation buffer (1.5 M NaCl, 0.5 M NaOH (Sigma-Aldrich Canada, Oakville, Ontario)) for 30 min and then in a neutralisation buffer (1 M Tris-HCl, pH=8.0, 1.5 M NaCl) for another 2 x 45 min. PCR products were transferred overnight onto a nitrocellulose membrane (Hybond, Pharmacia Biotech Inc., Baie d=Urfe, Quebec) by capillary action. After the transfer the membrane was baked at 65°C for 2 hr then pre-hybridised at 65°C for 1 hr in 5X SSPE, 5X Denhardt=s solution, 1% SDS (Sigma-Aldrich Canada, Oakville, Ontario), and 0.1 mg/ml single-stranded salmon sperm DNA (Pharmacia Biotech Inc., Baie d=Urfe, Quebec). The HIV-specific probe, ARP8021.3 HG1403P SK102 (5'-GAG ACC ATC AAT GAG GAA GCT GCA GAA TGG GAT-3') (AIDS Reagent Project, Hertfordshire, United Kingdom)[122] was labelled with radioactive ^{32}P using a T4 kinase kit (Gibco BRL, Burlington, Ontario) and the membrane incubated at 37°C overnight in hybridisation buffer (same as pre-hybridisation buffer but without the sssDNA). Next day the blot was subjected to a medium stringency wash (2 x 5 min in 2X SSC / 0.1% SDS at room temperature, 2 x 5 min in 0.2X SSC / 0.1% SDS at room temperature, and 2 x 15 min in 0.2X SSC / 0.1% SDS at 42°C (Sigma-Aldrich Canada, Oakville, Ontario), exposed to film and visualized using a PhosphorImager (Canberra Packard Canada, Montreal, Quebec).

2.14 Statistical Analysis.

Analysis of variance, F-tests, t-tests, and advanced linear regression analyses were all performed using the statistical analysis options included with "Corel Quattro Pro 7.0". Correlation analyses were done with "SPSS 7.5" (SPSS Inc., Chicago, Illinois), and contingency testing was performed using the "Instat 2.0" statistical analysis program (GraphPad Software, Inc., San Diego, California). Depending upon the number of individuals for which the necessary data was available, the sample sizes differ with each analysis.

3. DEVELOPMENT OF THE PROVIRAL LOAD ASSAY

In Chapter 2, I gave a detailed description of the methods used throughout my study. Since a significant proportion of the work done in this project focused on the development of a method for measuring HIV-1 proviral load, I have included here a separate chapter describing development and standardisation of this method.

3.1 Background for the Method

The first objective of my study was to develop a PCR-based assay for measuring HIV-1 proviral load. Numerous techniques have already been developed for this purpose, but most of these involve complex molecular biology and/or the use of radioisotopes.[22,39,90,174,207,219] We wanted to develop a simple quantitative PCR-based method that did not require radioactive isotopes. Appropriate samples were available as we were already collecting whole blood samples from HIV-1-infected individuals attending the local infectious diseases clinic. PBMCs and $CD4^+$ T cells were isolated as described in chapter 2. We chose the DNAzol method of DNA extraction because it had previously been shown in our lab to precipitate only large genomic DNA. By using this method of extraction, we should avoid the problem of isolating and amplifying unintegrated cytoplasmic circular HIV-1 DNA and distinguishing this from integrated HIV-1 DNA.

3.2 Optimization of HIV-1 *gag* PCR Amplification

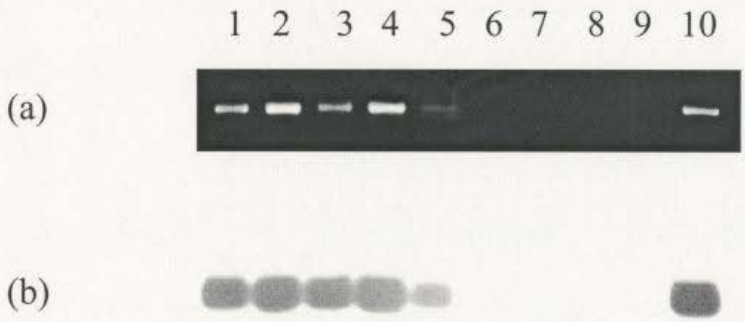
Due to the high degree of sequence variation that exists amongst clinical isolates from HIV-1-infected individuals, we selected a set of PCR primers that amplify a highly conserved 473 bp sequence located within the p24 capsid region of the HIV *gag* gene (Fig. 3.1a). We then worked out the optimal PCR conditions for the amplification of HIV-1 proviral DNA from positive controls and test samples. The specificity of these primers in our system was confirmed by Southern blot (Fig. 3.1b).

3.3 Internal Controls

In order to properly compare proviral load in different samples, we had to ensure that any differences in the intensity of the bands on the gel reflected real differences in HIV-1 DNA copy number, and not artifacts of the assay or differences in the quality of the DNA samples. Two samples with the exact same HIV-1 DNA copy number could result in two very different band intensities if, for example, DNA concentrations were the same but one sample was mostly fragmented DNA. In this case the absorbance reading at 260 nm could be the same but the efficiency of amplification would be very different. Another source of error could be simple pipetting errors in reaction preparation or gel loading. One way to control for these problems is to run separate PCR reactions on all samples with a housekeeping gene and assume that if there are no differences in the intensities of the control gene amplification, then the differences seen in the target gene

Figure 3.1. Specificity of HIV-1 *gag* PCR primers. (a) Digital image of an agarose gel showing the 473 bp PCR product generated from amplification of $CD4^+$ T cell genomic DNA from 5 HIV-1-infected individuals (lanes 1-5), 4 uninfected controls (lanes 6-9), and an HIV-1 plasmid positive control (lane 10). (b) Digital image of a Southern blot performed on the gel shown in panel (a).

Figure 3.1

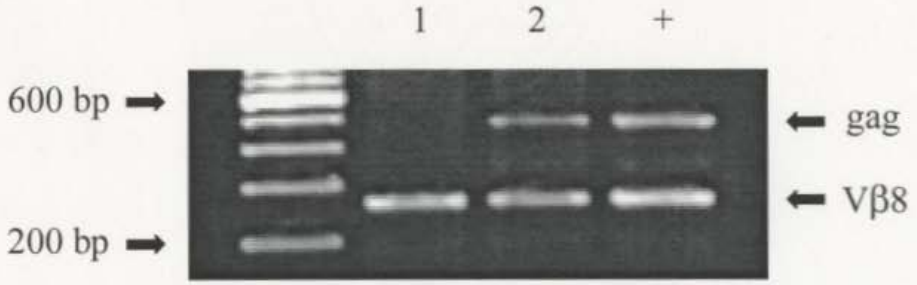


amplification are real. However, a better way to perform accurate quantitative PCR is to include an internal control. That is, to develop a system in which the target gene and the housekeeping gene are amplified in the same reaction. This method allows the levels of target gene amplification to be compared more confidently, since any conditions affecting the target gene amplification would also affect the housekeeping gene amplification and would then be obvious upon comparison of the band intensities of the housekeeping gene. Our first idea was to add to the reactions, a separate cDNA template and PCR primers. Total RNA was extracted from the Jurkat T cells and used to synthesize first-strand cDNA, which was then added to the reactions along with the TCR Vβ8 and Cβ primers (Vβ8: 5'-ATT TAC TTT AAC AAC AAC GTT CCG-3'; Cβ: 5'-TTC TGA TGG CTC AAA CAC-3'). This system allowed the simultaneous amplification of a 250 bp segment of the rearranged TCR Cβ/Vβ8 gene which served as an internal control. Figure 3.2 shows PCR products from the amplification of genomic DNA from two of our study subjects, with the Jurkat cDNA included as an internal control. Lane 1 was negative for the *gag* PCR product, whereas lane 2 shows a clear band at 473 bp. Since the 250 bp band is visible in both lanes, we can conclude that reactions 1 and 2 both worked efficiently since the internal control was positive. Therefore, the difference in the amount of *gag* DNA seen in these samples is likely to be real.

Although this internal control system allowed determination of whether amplification reactions worked efficiently in different tubes, there were some limitations with this system. The major problem was that this internal control really gave no

Figure 3.2. PCR amplification of HIV-1 with an internal control. Digital image of an agarose gel showing simultaneous amplification of HIV-1 *gag* (473 bp) and Jurkat TCR Vβ8 (250 bp) PCR products. 100 copies of an HIV-1 DNA plasmid was run as a positive control.

Figure 3.2



indication of the quality or quantity of target genomic DNA added to the reaction, as the amplification of *gag* and the internal control was from two different templates. Therefore, we decided to develop an internal control system in which the housekeeping gene was amplified from the same template source as the target gene. The advantage of this type of system is that it allows the comparison of *gag* levels, while also monitoring the integrity of the genomic DNA template. Similar studies had used various housekeeping genes such as HLA-DQα and β-globin as internal controls.[2,207,219] HLA-DQα and TCR-Cα were tried, but simultaneous amplification of *gag* with these genes resulted in the production of nonspecific bands, possibly due to cross-primer interactions. Since β-globin resulted in the cleanest PCR products, we began to standardize optimal conditions for amplification of *gag* with β-globin as the internal control. According to Henegariu *et al.*, when standardizing quantitative multiplex PCR, it is best to first optimize the amplification of the target gene, then introduce the housekeeping gene primers and optimize again.[101] Since optimal conditions had already been worked out for the *gag* amplification, various conditions were tested for amplification of *gag* and β-globin together. Figure 3.3a shows a series of reactions, set up with a fixed amount of genomic DNA, *gag* primers, and decreasing concentrations of β-globin primers (0.125 – 0.0125 μM). The aim of this experiment was to determine the optimal concentration of β-globin primers to be used together with the *gag* primers. Interestingly, the higher the concentration of β-globin primers, the lower the amount of *gag* PCR product made. This effect has been previously reported and is to be expected when co-amplifying segments of different sizes.[101] Apparently, the reaction will preferentially amplify the smaller PCR

product in the presence of similar primer concentrations. In Figure 3.3b, the intensities of the *gag* and β-globin PCR products are compared in order to determine the optimal β-globin primer concentration. To optimize the reaction, I chose the β-globin primer concentration that allowed maximal *gag* amplification, with a clearly visible β-globin band (Figure 3.3a, lane 9, 0.025 μM).

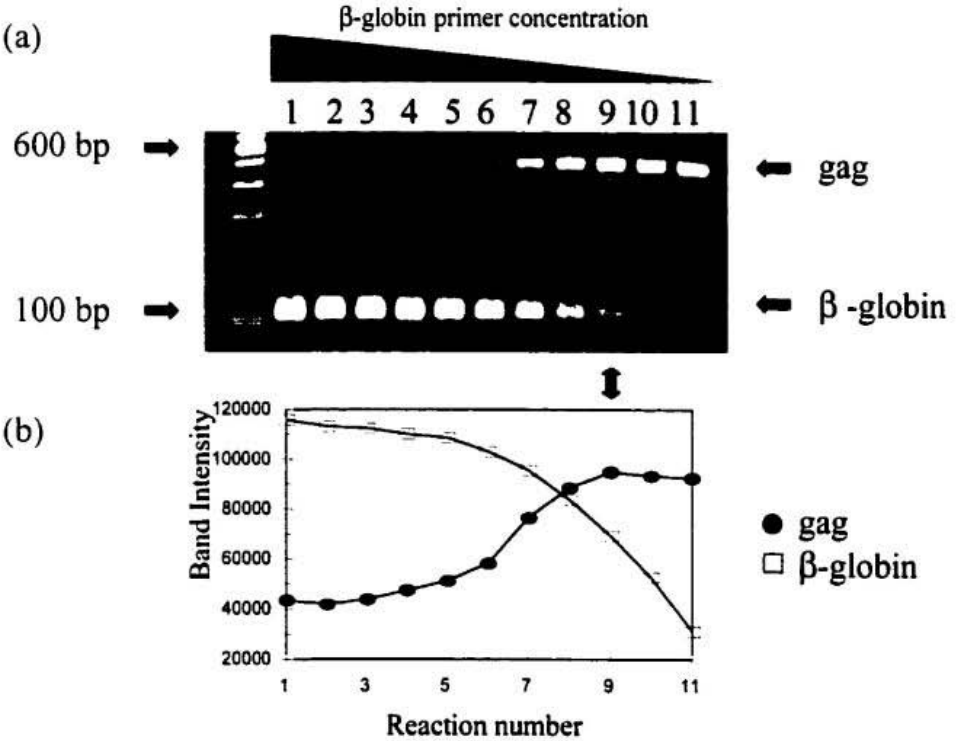
Once optimal primer concentrations are established, it is also worthwhile to test different concentrations of Mg^{2+} and dNTP's, in case these may not be optimal now for the multiplex system.[101] A series of reactions were set up with Mg^{2+} concentrations ranging from 0.8 – 1.6 mM, with increments of 0.2 mM. It was found that 1.2 mM was still the optimal Mg^{2+} concentration (data not shown). In a similar experiment, a series of reactions were set up with various concentrations of dNTP's to determine whether the amplification of both products simultaneously would require higher concentrations of nucleotides. It was found that 0.2 mM dNTP concentrations were still the optimal concentration (data not shown).

3.4 Establishing the Linear Range of the Proviral Load PCR

For accurate quantitative PCR, it is critical that measurements are taken within the linear range of the cycling program. To determine the linear range of our program, a series of 16 PCR tubes were set up, and one removed at the end of every cycle from cycles 25 – 40. Eight microlitres of each PCR product were then run on an agarose gel, a digital photograph taken, and the band intensities measured using the "AlphaEase"

Figure 3.3. Optimization of β-globin primers in HIV-1 proviral PCR. (a) Digital image of PCR products from amplification of subject DNA with decreasing concentrations of β-globin primers. (b) Graph comparing the band intensities of *gag* (•) and β-globin ( ) bands at different β-globin primer concentrations. The β-globin primer concentration of 0.025μM (lane 9) was chosen for future work as this concentration gave maximal *gag* amplification with a clear β-globin band.

Figure 3.3


(a)
β-globin primer concentration
1 2 3 4 5 6 7 8 9 10 11
600 bp
100 bp
gag
β -globin
(b)
Band Intensity
120000
100000
80000
60000
40000
20000
1
3
5
7
9
11
Reaction number
gag
β-globin

software. Figure 3.4 shows a graph of the band intensities plotted against the corresponding cycle numbers. The *gag* PCR bands as seen on the gel are shown below their corresponding bar on the graph. From the graph, the PCR cycling program appears to be linear between cycles 30 and 35. Linear regression performed on these points gave an R^2 value of 0.96, indicating that the line through these points is almost perfectly linear. Next, I determined the dynamic range of the method, i.e. the range of copy numbers of HIV-1 DNA that could be measured under these conditions. Various HIV-1 DNA standards of copy numbers ranging from 1 – 1000 and PCR programs of cycles 28, 30, 32, and 35 were tested. The band intensities were measured and plotted on a graph. Unfortunately, due to the broad range of proviral loads that existed within the study group, I was not able to establish one set of standards or cycle number that would allow me to measure proviral load on all my samples.

3.5 Quantitative Proviral PCR Protocol

Finally, to attain the required dynamic range needed, it was decided that all samples would first be run with a low set of standards (0, 1, 2, 5, 10, 25, 50, 75, and 100 copies of HIV-1 DNA) in a 35 cycle PCR program. Then any sample producing a band intensity that was higher than the highest standard in the linear range of the 35 cycle PCR would be re-run with a high set of standards (0, 50, 100, 250, 500, and 1000 copies of HIV DNA) for 32 cycles. Figure 3.5 shows 2 gels, panel (a) showing a set of samples run along with the high copy standards, panel (b) showing more samples run with the low

copy standards. Standards and samples were always run in duplicate. The 473 bp *gag* and 115 bp β-globin bands are clearly visible on both gels.

Figure 3.4. Linear range analysis. Graph showing the amplification of the *gag* product with 25 – 40 PCR cycles. Band intensity was quantitated and plotted against cycle number to find the linear range of the PCR program. The bands themselves are presented below their corresponding bars showing image intensity. The increase in intensity is linear between cycles 30 and 35 ($R^2 = 0.96$).

Figure 3.4

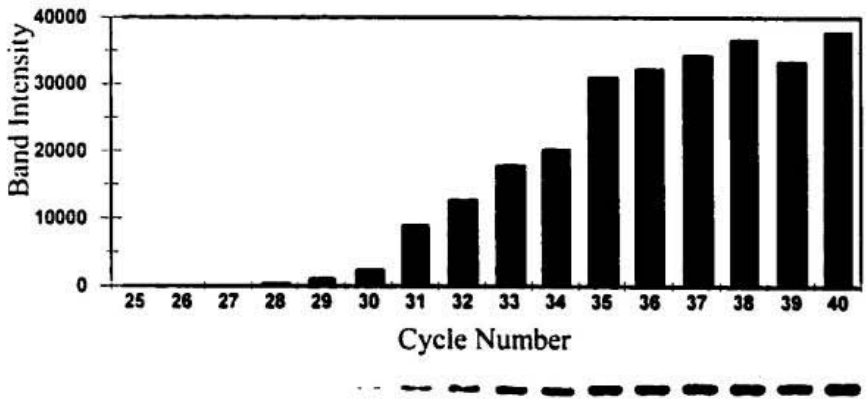
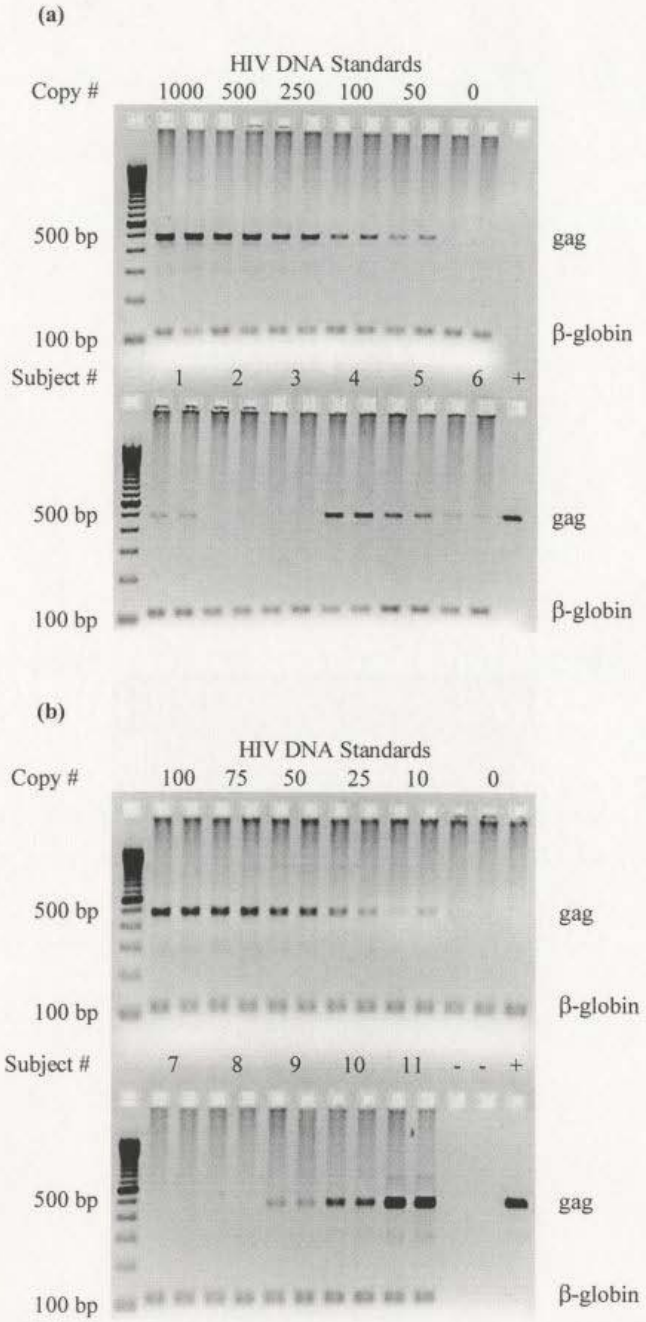

Band Intensity
40000
30000
20000
10000
0
25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
Cycle Number

Figure 3.5. Representative gel used to determine HIV-1 proviral load. Quantitative proviral PCR is done at 32 cycles with the 'high copy number HIV-1 DNA standards' (a) or 35 cycles with the 'low copy number HIV-1 DNA standards' (b) and band intensity measured by digital image analysis. Copies of HIV proviral DNA are calculated based on a standard curve generated from the band intensities of the standards.

Figure 3.5


(a)
HIV DNA Standards
Copy # 1000 500 250 100 50 0
500 bp
gag
100 bp
β-globin
Subject # 1 2 3 4 5 6 +
500 bp
gag
100 bp
β-globin
(b)
HIV DNA Standards
Copy # 100 75 50 25 10 0
500 bp
gag
100 bp
β-globin
Subject # 7 8 9 10 11 - - +
500 bp
gag
100 bp
β-globin

4. THE EFFECTS OF HIV-1 PROVIRAL LOAD ON DISEASE PROGRESSION AND RESPONSE TO THERAPY

As stated in Chapter 1, we hypothesize that proviral load ultimately determines the rate of disease progression and could be a useful prognostic indicator of the durability of responsiveness to antiretroviral therapy. Conversely, proviral load should also be associated with the likelihood of developing drug resistance. In this chapter, I present the results of research testing this hypothesis.

4.1 Stability of HIV-1 Proviral Load with Time

To measure the proportion of $CD4^+$ T cells containing incorporated HIV-1 DNA, we isolated $CD4^+$ T cells from HIV-1-infected individuals, extracted genomic DNA, and determined HIV-1 copy number as described in Chapters 2 and 3. Proviral load levels for the group ranged from 3 – 1270 copies of HIV-1 DNA / 10^5 $CD4^+$ T cells (Table 4.1). Samples where HIV-1 proviral DNA was undetectable by direct PCR were all positive by subsequent nested PCR. All samples were run in duplicate and the average proviral load was reported. Samples were re-measured if duplicates differed by more than 5%. The DNA content of any sample giving a weak β-globin band was re-measured and the proviral load test was repeated.

Pairwise analysis of serial samples from 46 HIV-1-infected individuals over a 6 - 12 month period showed no significant change in mean proviral load for the group; 63 ±

Table 4.1. Peak proviral load measurements for each subject. Listed are the study subject ID numbers with their respective 'peak proviral load' values that was used for most analyses. The 'peak proviral load' is the highest proviral load recorded over the period of study (6 – 12 months) for each subject, given as "X" copies of HIV-1 DNA / 10^5 $CD4^+$ T cells.

Table 4.1. Peak proviral load measurements for each subject.

Subject #	Peak Proviral Load**	Subject #	Peak Proviral Load
001	40	065	7
003	46	067	32
006	3	068	14
007	3	069	14
011	3	070	32
012	3	071	12
013	32	072	141
015	80	073	103
016	1270*	075	3
017	28	076	3
018	210	077	44
020	92	078	181
022	228	081	3
026	83	083	25
027	3	084	57
028	3	085	97
030	52	090	32
031	3	091	218
035	3	092	43
036	68	095	3
039	87	096	36
040	39	098	47
041	3	101	22
042	270	103	207
043	3	105	15
044	3	106	3
045	128	108	47
046	23	109	3
050	6	110	3
051	50	111	21
053	214	112	3
055	65	115	15
057	77	116	76
059	18	117	58
060	58	118	3
061	3	119	351
062	40	122	3
063	3	123	3
064	92	126	30

* Subject 016 produced a band that was more intense than even the 1000 copy standard, so half the normal amount of DNA was run and the measured copy number doubled.
** The sensitivity of the proviral load assay is 3 copies of HIV-1 DNA per 10^5 $CD4^+$ T cells.

99 copies of HIV-1 DNA / 10^5 $CD4^+$ T cells at the first timepoint available compared to 61 ± 190 after 6-12 months.

4.2 The Effects of HIV-1 Proviral Load on Disease Progression

Since plasma virus load gives a clear indication of the risk of disease progression,[150] we analyzed the correlation between proviral load and plasma HIV-1 RNA levels as one way to assess the relationship between HIV-1 proviral load in $CD4^+$ T cells and disease progression. It was found that proviral load was positively correlated with plasma HIV-1 RNA levels (Figure 4.1, Pearson's 0.45, $p < 0.001$, $n = 69$). Since plasma HIV-1 RNA levels are a good marker of disease progression, these results support a relationship between proviral load and disease progression.

To test the possibility that HIV-1 proviral load may serve as a prognostic indicator of disease progression, 64 HIV-1-infected individuals were grouped on the basis of either a low (<20 HIV-1 DNA copies / 10^5 $CD4^+$ T cells, $n = 30$) or high peak proviral load (>20 HIV-1 DNA copies / 10^5 $CD4^+$ T cells, $n = 34$). When these two groups were compared with respect to peak plasma HIV-1 RNA, we found that the 'low proviral load' group had a lower mean peak plasma virus load (12,900 ± 6600 copies of HIV-1 RNA/ml), than the 'high proviral load' group (67,600 ± 34,000 copies) (Figure 4.2a, $p < 0.01$). Another useful marker of HIV-1 disease progression is $CD4^+$ T cell decline.[66,150] When we compared the two groups with respect to mean $CD4^+$ T cell count, we found that the 'low proviral load' group also had a higher mean $CD4^+$ T cell level than did the 'high proviral load' group (Figure 4.2b, 436 ± 58 / µl versus 307 ± 32 / µl, $p < 0.05$).

Figure 4.1. Relationship between proviral load and plasma viral load. Proviral load is positively correlated with plasma HIV-1 RNA levels (Pearson's 0.45, $p < 0.001$, $n = 69$).

Figure 4.1

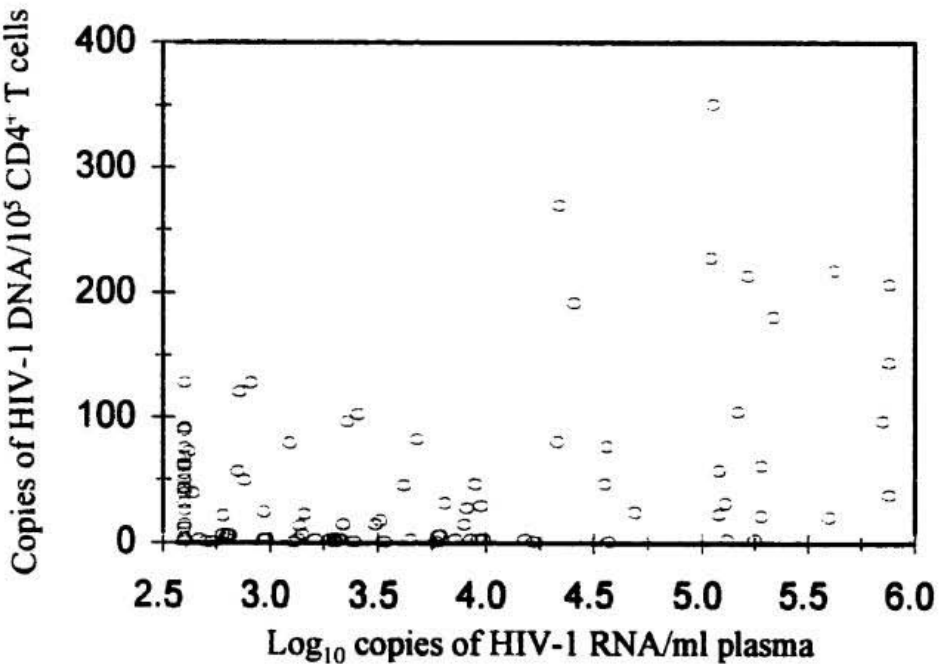
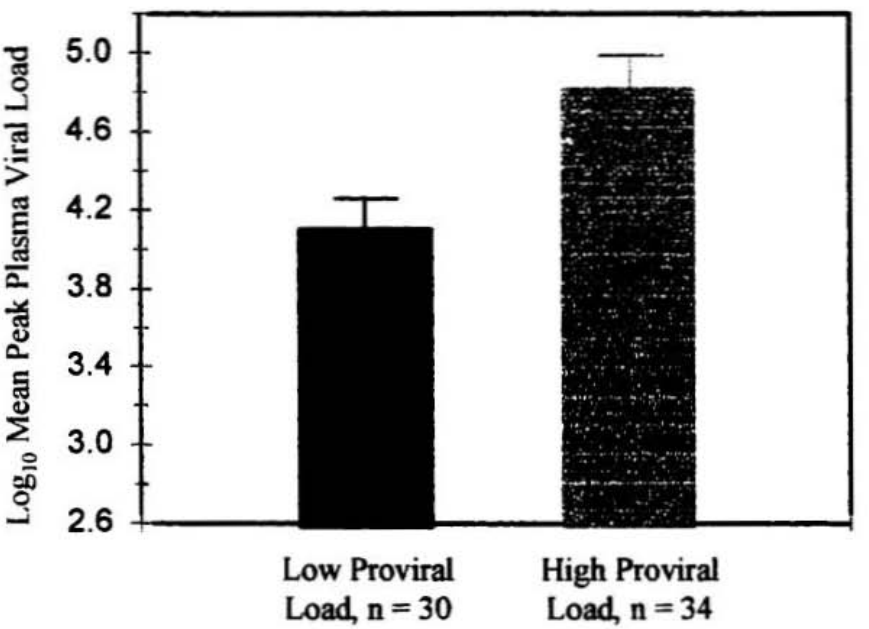

Copies of HIV-1 DNA/10^5 CD4$^+$ T cells
400
300
200
100
0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
Log$_{10}$ copies of HIV-1 RNA/ml plasma

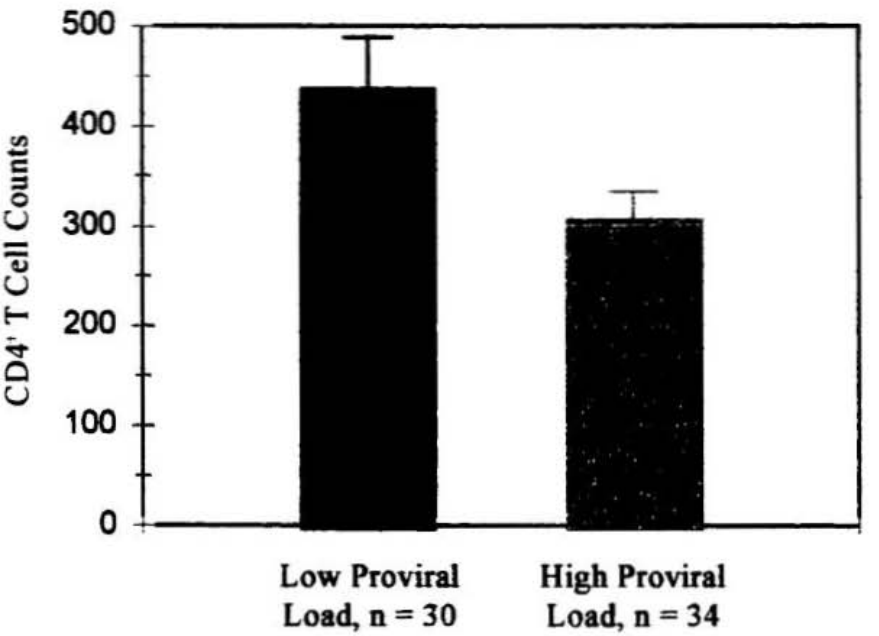
Figure 4.2. Proviral load and disease progression. (a) The 'low proviral load' group had a lower mean peak plasma viral load (12,900 copies per ml) than did the 'high proviral load' group (67,600 copies per ml), $p < 0.01$. (b) The 'low proviral load' group (black) also had higher mean $CD4^+$ T cell levels (436 cells / μl) than did the 'high proviral load' group (gray; 307 cells / μl), $p < 0.05$.

Figure 4.2

(a)


Log10 Mean Peak Plasma Viral Load
5.0
4.6
4.2
3.8
3.4
3.0
2.6
Low Proviral Load, n = 30
High Proviral Load, n = 34

(b)


CD4+ T Cell Counts
500
400
300
200
100
0
Low Proviral Load, n = 30
High Proviral Load, n = 34

These results clearly show that proviral load is associated with plasma viral load and $CD4^+$ T cell levels, suggesting that high proviral load is a good predictor of disease progression.

4.3 The Effects of Proviral Load on Response to Therapy

To test the hypothesis that proviral load may be a predictor of response to antiretroviral therapy, we grouped 50 individuals, receiving equally potent drug treatment, on the basis of their response to therapy as indicated by changes in plasma HIV-1 RNA levels. Subjects were designated as non-responders, if over 2 years of therapy their plasma virus load was high (>5.00 $\log_{10}$ copies of HIV-1 RNA / ml) and either remained high or increased (n = 10). Subjects designated as responders had a plasma virus load that was low (2.60 – 3.00 $\log_{10}$) and stayed low or significantly decreased (n = 40). Figure 4.3 shows that the responders had lower proviral loads with a group mean of 36 ± 7 HIV-1 DNA copies / 10^5 $CD4^+$ T cells compared to a group mean of 130 ± 31 for the non-responders (p = 0.02).

To further investigate the relationship between HIV-1 proviral load and response to antiretroviral therapy, we examined $CD4^+$ T cell counts in 2 groups of individuals over two years of antiretroviral therapy. The 'undetectable' group contained 10 HIV-1-infected individuals who consistently had proviral loads of less than 3 copies of HIV-1 DNA / 10^5 $CD4^+$ T cells by our assay. The 'high proviral load' group consisted of 11 individuals who all had proviral loads greater than 100 copies of HIV-1 DNA / 10^5 $CD4^+$

T cells. Both groups were receiving at least 2 reverse transcriptase inhibitors with the majority (7 / 11) of the 'high proviral load' group also receiving a protease inhibitor (Table 4.2), and all subjects had <500 $CD4^+$ T cells / μl at baseline. When the overall change in $CD4^+$ T cell counts was compared between the two groups, the 'undetectable' group experienced a mean increase in CD4 counts of 170 ± 33 / μl, whereas the 'high proviral load' group actually had a mean decrease of 54 ± 41 / μl (Figure 4.4a, $p < 0.001$). In Figure 4.4b, changes in plasma HIV-1 RNA were compared over the same period of time. Interestingly, 9 / 10 of the individuals in the 'undetectable' group had a decrease in plasma HIV-1 RNA of at least 1 $\log_{10}$, while only 1 / 11 of the 'high proviral load' group showed such a decrease (Chi-square, $p < 0.001$). Therefore, changes in $CD4^+$ T cell number and plasma viral load suggest that the size of the HIV-1 proviral DNA pool has a strong influence on the potential for immune reconstitution and viral suppression in response to antiretroviral therapy.

Figure 4.3. HIV-1 proviral load predicts response to antiretroviral therapy. Responders, as defined on page 72, had lower proviral loads with a group mean of 36 copies of HIV-1 DNA / 10^5 $CD4^+$ T cells, compared to 130 copies for the non-responders ($p = 0.02$).

Figure 4.3

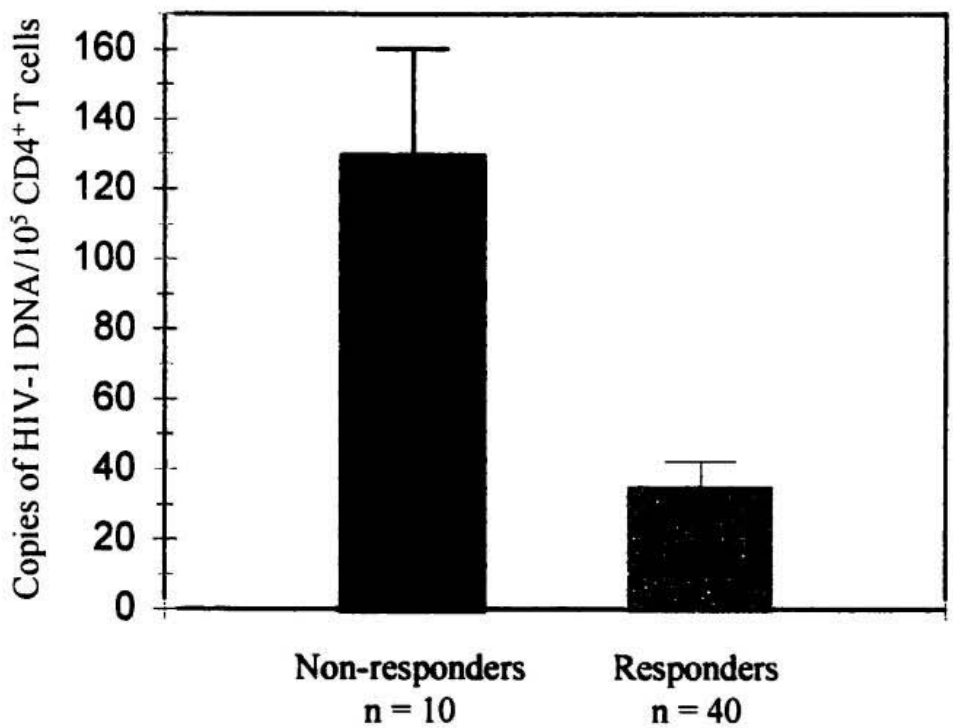

Copies of HIV-1 DNA/10^5 CD4$^+$ T cells
160
140
120
100
80
60
40
20
0
Non-responders
n = 10
Responders
n = 40

Table 4.2. Comparison of antiretroviral therapy. Shown are the individual anti-HIV-1 drug profiles for the 'high proviral load' (upper panel) and 'undetectable' (lower panel) groups over the two year period of study. Nucleoside analog RT inhibitors (NRTIs); AZT (Zidovudine); D4T (Stavudine); DDI (Didanosine); DDC (Zalcitabine); 3TC (Lamivudine); Non-nucleoside RT inhibitors (NNRTIs); NEV (Nevirapine); SUS (Efavirenz); Protease Inhibitors; SAQ (Saquinavir); IND (Indinavir); RIT (Ritonavir).

Table 4.2. Comparison of antiretroviral therapy

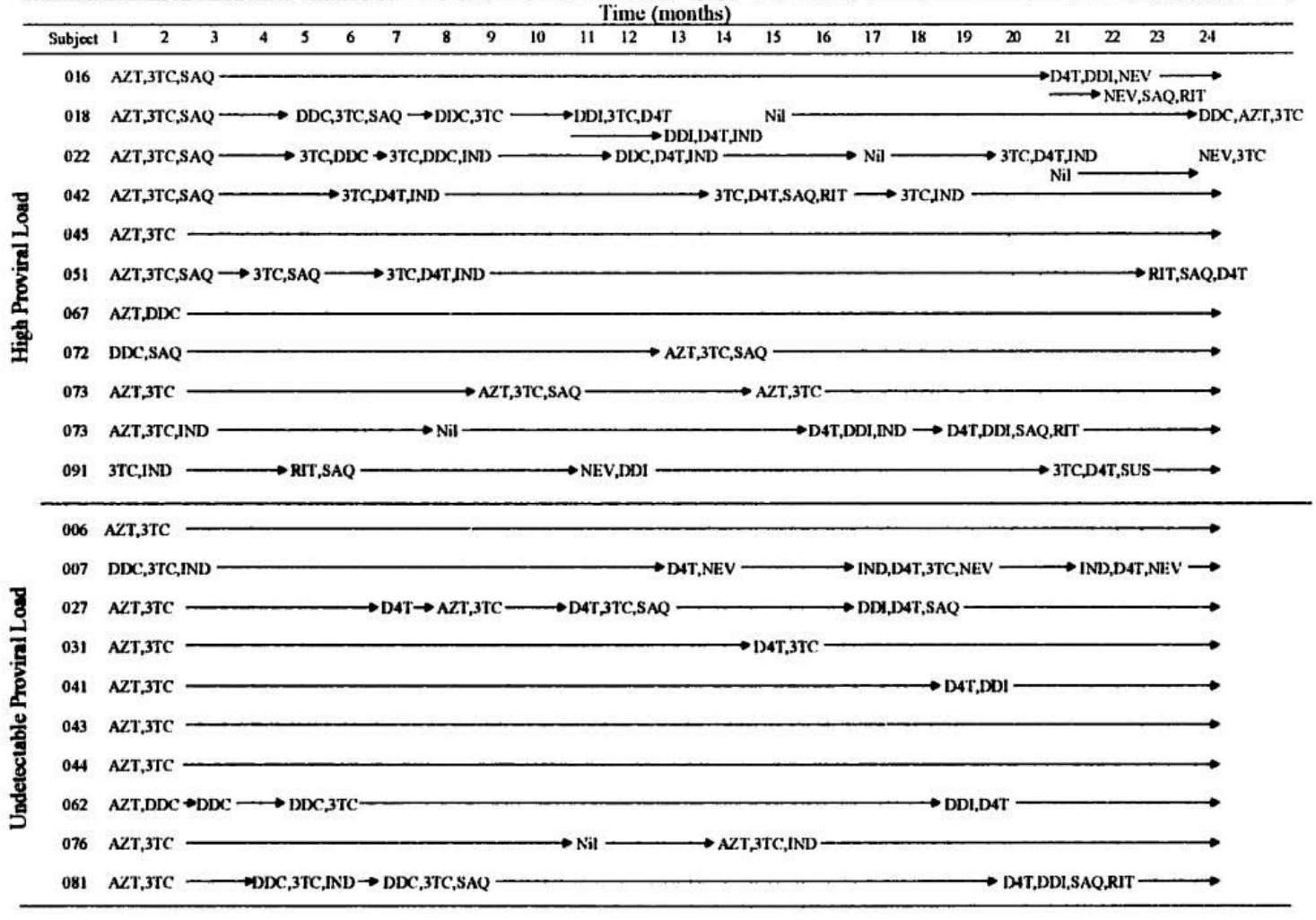
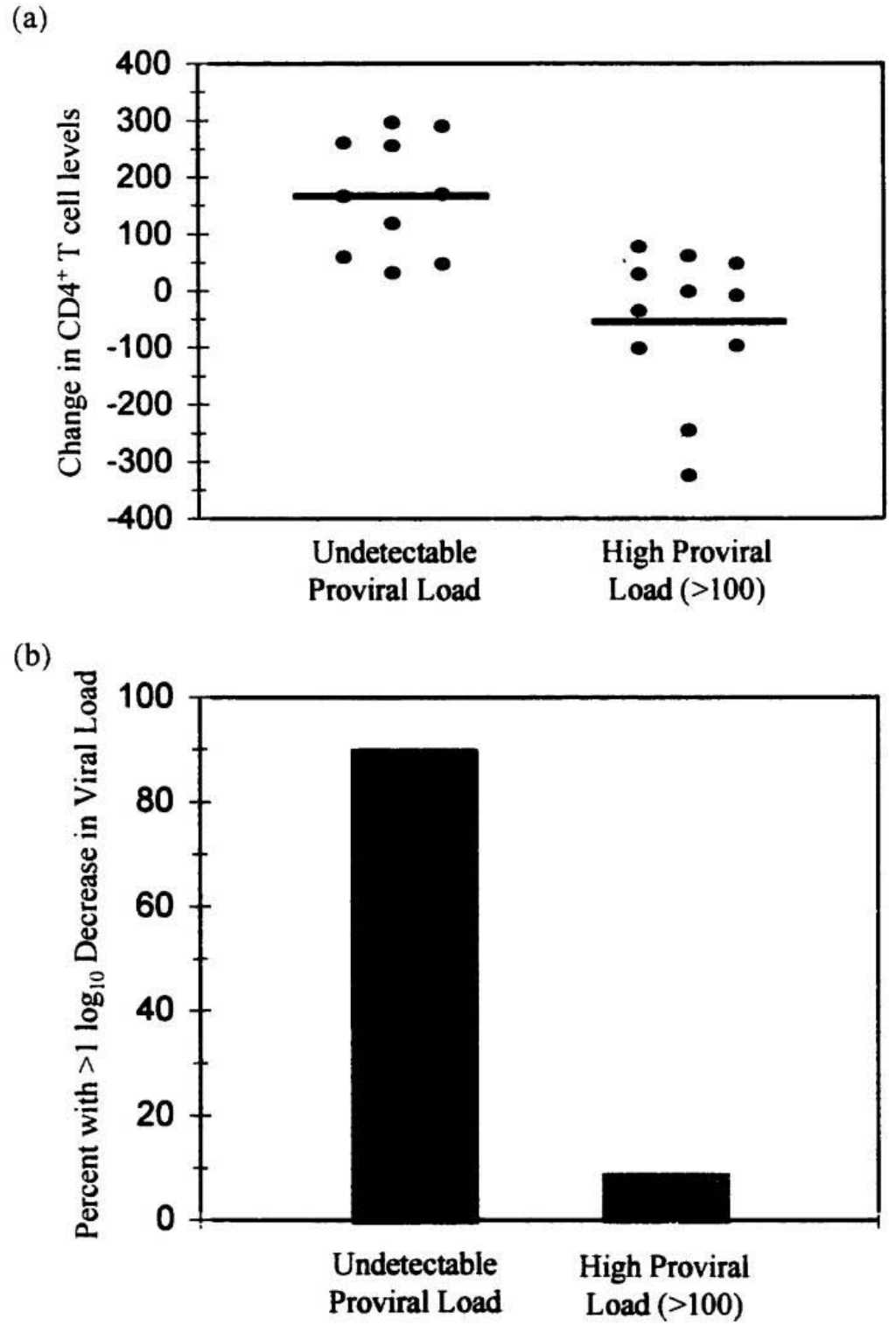


Figure 4.4. HIV-1 proviral load and response to antiretroviral therapy. (a) Subjects with undetectable proviral load by our method showed a greater mean increase in $CD4^+$ T cell counts when compared with a group with high proviral loads ($+170 \pm 33$ vs. -54 ± 41 / μl; $p < 0.001$) in response to therapy. (b) After initiation of antiretroviral therapy, a greater proportion of the undetectable group experienced significant decreases in plasma viral load than did the high proviral load group (90% vs. 9% respectively; Chi-square $p < 0.001$).

Figure 4.4


(a)
Change in CD4+ T cell levels
400
300
200
100
0
-100
-200
-300
-400
Undetectable Proviral Load
High Proviral Load (>100)
(b)
Percent with >1 log10 Decrease in Viral Load
100
80
60
40
20
0
Undetectable Proviral Load
High Proviral Load (>100)

5. THE EFFECTS OF THE CCR5Δ32 CORECEPTOR MUTATION

In recent years, there has been increasing interest in newly identified HIV coreceptors and also in mutations in these coreceptors that may be associated with slower rates of HIV disease progression. Previous studies had shown that HIV-1-infected individuals heterozygous for the CCR5Δ32 coreceptor mutation experienced slower rates of disease progression than individuals that were homozygous for the wild type gene.[10,51,53,100,152,153,206,213] We hypothesized that CCR5Δ32 heterozygotes should have lower proviral loads in comparison with CCR5wt homozygous individuals, due to a possible decrease in cell surface expression of CCR5 caused by the mutation, and that this could account for their slower rates of disease progression. In this Chapter I describe the results obtained from this study.

5.1 CCR5 Genotyping

To test our hypothesis, we performed PCR-based genotyping for the CCR5Δ32 coreceptor mutation on 107 HIV-1-infected individuals by the method outlined in Figure 5.1. Briefly, genomic DNA is amplified by PCR using a set of primers that span the putative deleted region. The primers are positioned so that amplification of the wild type gene produces a 189 bp PCR product. Amplification of genomic DNA from individuals homozygous for the wild type gene (CCR5wt) results in a single band at 189 bp (Figure 5.1, lane 1), since both copies of the gene would give the same PCR product.

Amplification of genomic DNA from a CCR5Δ32 heterozygous individual results in the production of the 189 bp band, but also an additional band 157 bp in size (Figure 5.1, lane 2), resulting from amplification of the gene lacking 32 bases within the amplified region. These PCR products are easily separated by size on a 1% agarose gel allowing simple identification of heterozygous individuals. Amplification of DNA from a CCR5Δ32 homozygous individual would produce a single band at 157 bp, however, none were identified in my study.

Twenty-two of the 107 HIV-1-infected individuals typed were heterozygous for the CCR5Δ32 mutation (Figure 5.2). The frequency of the heterozygous genotype (black) in our group was 20.6%, which is consistent with that reported in other studies of similar populations.[8] The remaining 79.4% were homozygous for the wild type CCR5 gene (gray). As mentioned earlier, no CCR5Δ32 homozygotes were identified, which is not surprising since homozygosity has been shown to confer a high degree of protection from HIV-1 infection.[136] Twenty-seven HIV-negative individuals were also typed and 18.5% (5 / 27) of these were found to be heterozygotes. The proportion of heterozygotes in the HIV-positive and negative groups was not significantly different.

5.2 CCR5Δ32 Heterozygosity and Disease Progression

In order to test whether lower proviral load could underlie slower disease progression in CCR5Δ32 heterozygotes, we first had to determine whether the heterozygotes in our cohort progressed more slowly than homozygous wild type

Figure 5.1. CCR5 genotyping. Genomic DNA is amplified using primers (green) that span the putative deleted region (red box). Homozygous wild type individuals (upper left) show a single band at 189 bp (lane 1), whereas individuals heterozygous for the CCR5Δ32 mutation (upper right) show the 189 bp band, and a smaller band at 157 bp (lane 2).

Figure 5.1

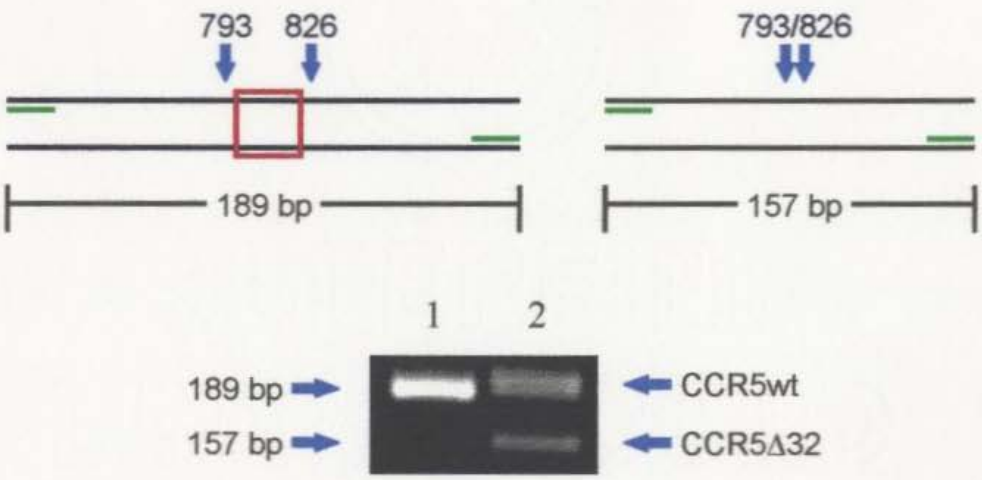
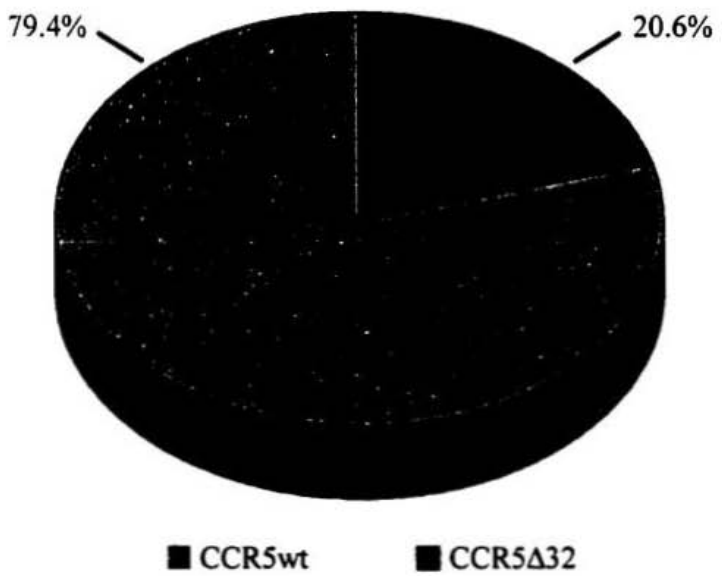


Figure 5.2. Frequency of the CCR5Δ32 mutation. 107 HIV-positive individuals were typed for the CCR5Δ32 HIV-1 coreceptor mutation. Twenty-two (20.6%) were heterozygotes (black), which was not different from that of the HIV-negative individuals tested (5/27;18.5%).

Figure 5.2

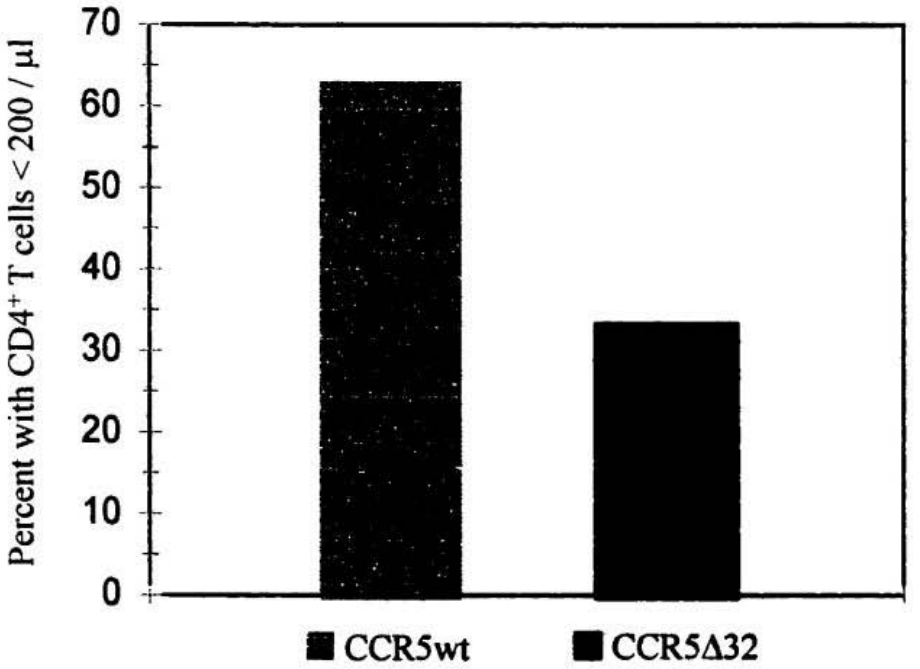


individuals. The best way to study rates of disease progression would of course be to use a defined endpoint, such as 'time to developing AIDS' or 'time to death'. However, these parameters require that we know the time of initial infection. As is often the case in similar studies, this information is not known for most of the subjects enrolled in our study. Therefore, we relied on identified markers, such as $CD4^+$ T cell counts, to study disease progression. As mentioned in chapter 1, peripheral blood $CD4^+$ T cell decline is a reliable marker of disease progression. One common measure of disease progression in HIV-1-infected individuals is the "nadir" $CD4^+$ T cell count, which is defined as the lowest recorded $CD4^+$ T cell count for an HIV-infected individual. The idea is that since $CD4^+$ T cell decline is a marker of disease progression, then individuals that have had at some point, a very low $CD4^+$ T cell count, have a more advanced disease status and higher relative risk for progression.[156] One advantage to using this parameter is that it does not require knowing the time of infection.

To study disease progression in our cohort, I defined a $CD4^+$ T cell count of 200 / μl as an endpoint, since this value is used by the CDC to define AIDS.[132] I then compared the proportion of CCR5Δ32 heterozygotes and CCR5wt homozygotes with nadir $CD4^+$ T cell counts of less than 200 / μl. Figure 5.3 shows that 63.0% (51 / 81) of the CCR5wt homozygotes (gray bar) had previously had a nadir $CD4^+$ T cell count of less than 200 / μl, whereas only 33.3% (6 / 18) of the CCR5Δ32 heterozygotes (black bar) met this criteria (Chi-square, $p < 0.005$). These results indicate that the CCR5Δ32 heterozygotes have slower rates of $CD4^+$ T cell decline, and therefore have slower rates of disease progression than the CCR5wt homozygotes.

Figure 5.3. CCR5Δ32 heterozygosity and $CD4^+$ T cell levels. The proportion of HIV-infected individuals with nadir $CD4^+$ T cell counts below 200 / μl was compared between CCR5wt homozygous and CCR5Δ32 heterozygous groups. A lower proportion with $CD4^+$ T cell counts below 200 / μl occurred in the heterozygous group, 33.3% (6 / 18) compared to 63.0% (51 / 81), $p < 0.005$.

Figure 5.3

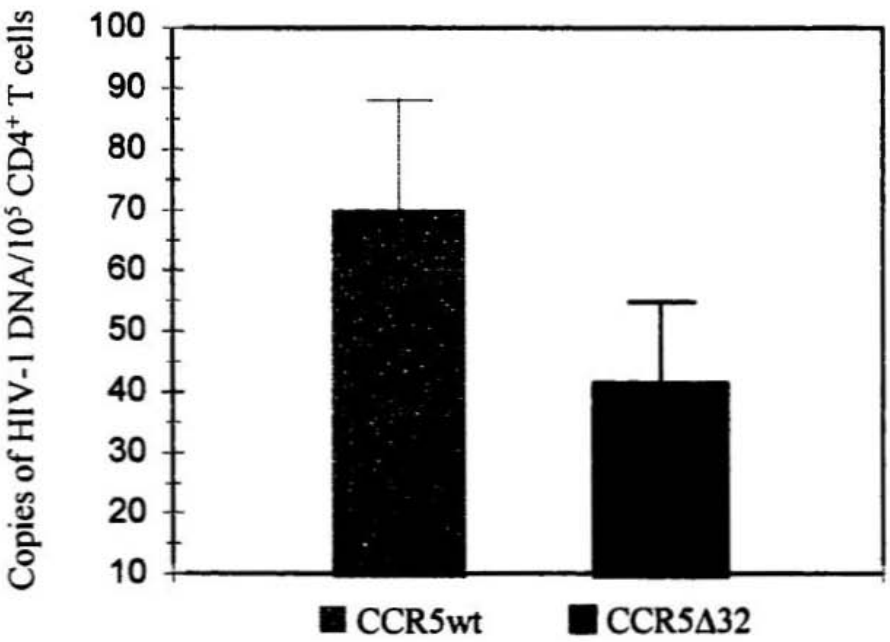

Percent with CD4+ T cells < 200 / µl
70
60
50
40
30
20
10
0
CCR5wt
CCR5Δ32

5.3 HIV-1 Proviral Load and CCR5 Genotype

Once the CCR5Δ32 heterozygotes were identified and a slower rate of disease progression confirmed in this group, I then wanted to test the hypothesis that these individuals had lower proviral loads than the CCR5wt homozygotes. Using the proviral load data obtained from the work described in chapter 4, I compared mean proviral load in the two groups. Figure 5.4 shows that the CCR5wt homozygous group had a slightly higher mean proviral load of 70 ± 21 copies of HIV-1 DNA / 10^5 $CD4^+$ T cells than did the CCR5Δ32 heterozygotes who had a group mean of only 41 ± 14 copies. However, this difference was not statistically significant ($p = 0.26$). Thus the slower disease progression observed in the heterozygotes does not appear to be due to a major difference in proviral load, in comparison to CCR5wt homozygotes.

Figure 5.4. HIV Proviral load and CCR5Δ32 heterozygosity. HIV proviral load was compared between CCR5wt homozygous (gray) and CCR5Δ32 heterozygous (black) groups. The CCR5wt homozygous group had a slightly higher mean proviral load than the heterozygous group, 70 ± 21 copies of HIV-1 DNA / 10^5 $CD4^+$ T cells compared to 41 ± 14 copies, but this difference was not statistically significant, p = 0.26.

Figure 5.4


Copies of HIV-1 DNA/10^5 CD4$^+$ T cells
100
90
80
70
60
50
40
30
20
10
CCR5wt
CCR5Δ32

6. DISCUSSION AND CONCLUSIONS

6.1 The Significance of HIV-1 Proviral Load

The first goal of this study was to determine whether the size of the pool of incorporated HIV-1 proviral DNA in the peripheral blood $CD4^+$ T cells of an HIV-1-infected individual affects the durability of the response to antiretroviral therapy. Therefore, we developed a non-radioactive PCR-based method for measuring the frequency of $CD4^+$ T cells containing HIV-1 proviral DNA and measured this in 78 HIV-1-infected individuals. The proviral loads obtained using this method were comparable to those found by other groups using methods that measure only 'integrated HIV-1 proviral DNA' as opposed to 'total cellular HIV-1 DNA' which includes the labile pool of unintegrated HIV-1 proviral DNA in recently infected cells.[114] All 78 individuals had detectable proviral loads, including fifteen with undetectable plasma viral loads, thus confirming the presence of a pool of latently infected cells in the peripheral blood of HIV-1-infected individuals responding well to antiretroviral therapy with respect to plasma viral load.[11,26,41,71,72,114] In the individuals with detectable plasma virus loads, proviral load levels correlated with plasma HIV-1 RNA levels. In agreement with others, we found that HIV-1 proviral load remained relatively stable over 6 – 12 months of observation and was unaffected by antiretroviral therapy.[11,26,41,71,72,114] Due to the stability of this pool, proviral load is unlikely to be of any relevance for monitoring the efficacy of currently available antiretroviral therapies. However, we provide evidence

here suggesting that proviral load in an HIV-1-infected individual is an underlying determinant of the rate of disease progression. When we separated our subjects into groups based on a high versus low proviral load, the group with higher proviral loads displayed a lower mean $CD4^+$ T cell count and significantly higher mean peak plasma virus load. We believe that the higher proviral loads facilitate higher peak viral loads indicating higher 'set points' of plasma viral load. Based on the work of Mellors *et al*, this would link proviral load to rate of disease progression.[150]

Latently infected $CD4^+$ T cells harboring incorporated HIV-1 proviral DNA are now believed to be the source of all new viral replication when antiretroviral therapy is stopped.[71] We believe that this pool of latent virus is not only the source of new virus but that the size of this pool may be a major determinant of the long-term success of antiretroviral therapy. When we separated HIV-1-infected individuals into groups based on whether or not they responded to therapy, we found that non-responders had higher proviral loads than did responders. These results corroborate the work of McDermott *et al* who showed that responders had almost a 5-fold lower mean HIV-1 DNA copy number than non-responders.[148] Based on these results the authors concluded that HIV-1 DNA levels reflect therapeutic efficacy, whereas we assert that the lower proviral loads in the responders group were actually the reason why they responded. Thus, a low proviral load should predict a positive response to therapy. To further investigate this possibility, we took two extreme groups of patients and retrospectively analyzed changes in $CD4^+$ T cell counts and HIV-1 plasma viral load over two years of antiretroviral therapy. We found that the group of individuals with proviral loads of <3 copies of HIV-1 DNA / 10^5

$CD4^+$ T cells had significantly greater increases in $CD4^+$ T cell counts than did the group of individuals all with proviral loads > 100 copies of HIV-1 DNA / 10^5 $CD4^+$ T cells. The low proviral load group also had a greater proportion of individuals experiencing a significant decrease in plasma viral load over the same period. It was also noted that half of the HIV-1-infected individuals in the high proviral load group were also in the non-responder group of the previous analysis. These results suggest that in general, an HIV-1-infected individual with a high proviral load is less likely to benefit from antiretroviral therapy than one with a low proviral load.

If we consider the fact that the virus' sequence diversity is generated during reverse transcription which takes place just before the integration of the provirus,[220] it seems that a larger pool of incorporated proviral DNA would necessarily harbour a broader range of sequence diversity. This broad range of sequence diversity would increase the likelihood of a resistance mutant arising that is capable of replicating in the presence of HAART. This diversity would also be a stress on the existing immune response which would face multiple mutations in both antibody and cytotoxic T cell epitopes. Therefore, we believe that the level of HIV-1 proviral load is also a good prognostic indicator of the potential for immune reconstitution and viral suppression achievable with antiretroviral therapy.

From these studies we conclude that the level of HIV-1 proviral load in the peripheral blood $CD4^+$ T cells of HIV-1-infected individuals is relatively stable throughout the course of disease and is a useful prognostic indicator of the durability of response to antiretroviral therapy. We believe that the size of this pool of integrated

HIV-1 proviral DNA is a major determinant of the long-term success of antiretroviral therapy and of the natural history of disease progression. Measuring the size of this pool in the blood of HIV-1-infected individuals may help explain why some patients rapidly fail antiretroviral therapy. It should also be informative to study the relationship between proviral load levels and the frequency of, or time to emergence of a dominant drug resistant HIV-1 variant.

The major implication of this work is that the success of antiretroviral combination therapy in reducing plasma virus load to undetectable levels will not easily translate into viral eradication. The latent pool of HIV-1 proviral DNA literally hiding in the DNA of resting $CD4^+$ T cells is probably the main reason that HIV-1 establishes a persistent infection. Not only is this pool the source of new virus in the absence of therapy, but the size of this pool can be a major determinant of whether, or how much an individual will benefit from antiviral therapy. Unfortunately, the initial success of HAART fueled the false hope that HIV-1 could be eradicated from an individual by antiretroviral drugs alone. The presence of a pool of latent provirus was already apparent but practically ignored because of the drastic reductions in plasma virus load seen in HIV-1-infected individuals receiving HAART. We now know however, that the half-life of these latently infected cells is much longer than originally believed,[71] and recent work has shown that HIV-1 transcription persists in PBMCs of HIV-1-infected individuals who have had undetectable levels of plasma HIV-1 RNA for 20 months or more.[78] It was recently proposed that HIV-1 replication, in the presence of HAART, occurs in multiple local bursts, associated with immune activation in response to antigens.[92] Many

investigators now agree that eradicating HIV-1 by drugs alone will be a formidable task, and that the long-term efficacy of drug combinations presently available has been overestimated.[78,92] Already a great deal of work has gone into the development of new antiviral strategies that specifically target the latent pool of HIV-1 proviral DNA. One area receiving a great deal of attention is the idea of combining HAART with cytokine therapy. A variety of cytokines, including interleukin (IL)-2, tumor necrosis factor (TNF)-α, and IL-6 can induce *in vitro* expression of HIV from latently infected, resting $CD4^+$ T cells obtained from HIV-infected individuals.[25] Thus, it has been proposed that HIV might be deliberately purged from these cells by treating infected individuals simultaneously with HAART and agents that activate cells to express HIV. The hope is that after being activated, these latently infected cells might die from cytopathic effects and/or immune effector mechanisms, such as HIV-1-specific CTL, while at the same time HAART would prevent new rounds of infection by any new virions released.[36,37,105,195] In one such study, Chun *et al* gave intermittent IL-2 plus HAART to a group of HIV-1-infected individuals and showed that this treatment decreased the size of the pool of resting $CD4^+$ T cells containing replication competent HIV.[24] Two subjects enrolled in the study underwent lymph node biopsies, and surprisingly, HIV could not be cultured from these tissues. The authors detected low levels of HIV-1 proviral DNA in these cells, but suggested that this was "replication-incompetent provirus." A few weeks later, these 2 individuals were taken off HAART, but within 3 weeks of the discontinuation of therapy, their plasma HIV-1 RNA levels rebounded. These results were explained by suggesting that there must be reservoirs of virus that the IL-2 and HAART could not

reach. However, I believe that the source of the new virus was the HIV-1 proviral DNA that they detected, but assumed was replication-incompetent. Our method of HIV-1 proviral load quantitation would be useful in the evaluation of such studies, as testing the efficacy of these new therapies will require monitoring of HIV-1 proviral load.

6.2 HIV-1 Proviral Load and the CCR5Δ32 Coreceptor Mutation

The second goal of my study was to evaluate the effects of the CCR5Δ32 HIV-1 coreceptor mutation on the rate of HIV-1 disease progression, and to determine whether the slower rate of disease progression reported in CCR5Δ32 heterozygotes could be due to lower levels of HIV-1 proviral DNA. Using the protocol described by Huang *et al*,[112] we typed 107 HIV-1-infected individuals for the CCR5Δ32 mutation and identified 22 heterozygotes (20.6%). The frequency of heterozygosity found in our cohort was consistent with that reported for other groups of similar ethnic background.[8] We then tested whether the CCR5Δ32 heterozygous individuals in our study group experienced slower rates of disease progression than the CCR5 wild type homozygous individuals. Measuring rates of disease progression is a difficult task in itself that becomes even more difficult when the exact dates when each individual was actually infected with HIV-1 are not known. To circumvent this problem we made use of accepted markers of disease progression, such as $CD4^+$ T cell levels. The "nadir CD4 count" or the lowest recorded $CD4^+$ T cell count for an HIV-1-infected individual, is one parameter that is clearly associated with disease progression in HIV-1 infection.[156] We compared nadir CD4

counts between CCR5Δ32 heterozygous and CCR5wt homozygous HIV-1-infected individuals and found that a significantly greater proportion of homozygotes had nadir CD4 counts below 200 / μl when compared with heterozygotes (63.0% versus 33.3% respectively, $p < 0.005$). Since the rate of $CD4^+$ T cell decline is a reliable measure of the rate of HIV-1 disease progression,[66,150] we believe that these results indicate that, for the most part, the heterozygous individuals in our group have slower rates of disease progression than do the CCR5wt homozygous individuals.

The mechanism by which CCR5Δ32 heterozygosity facilitates slower rates of disease progression has not yet been defined. However, $CD4^+$ T cells from heterozygous individuals have lower levels of cell surface CCR5 and are less susceptible to infection with R5 isolates of HIV-1 *in vitro*.[124,175] We hypothesized that if these decreased coreceptor levels also exist *in vivo*, and similarly decrease susceptibility to HIV-1 infection, then CCR5Δ32 heterozygotes should have a reduced frequency of $CD4^+$ T cells containing incorporated HIV-1 DNA. We proposed that the heterozygotes should display lower proviral loads in comparison with CCR5wt homozygous individuals. If this were true, the decreased proviral loads should facilitate a slower spread of HIV-1 within the $CD4^+$ T cell population resulting in a slower $CD4^+$ T cell decline and reduced rate of disease progression.

When we compared HIV-1 proviral load between groups of CCR5Δ32 heterozygous and CCR5wt homozygous HIV-1-infected individuals, we saw only a slight difference in mean proviral load between the two groups. The mean proviral load for the CCR5Δ32 heterozygous group was 41 copies of HIV-1 DNA / 10^5 $CD4^+$ T cells,

compared to a group mean of 70 copies for the CCR5wt homozygous individuals ($p = 0.26$). This result does not support our hypothesis. Thus, we conclude that the slower rates of disease progression observed in the CCR5Δ32 heterozygotes is not due to a decreased HIV-1 proviral load. However, there are many factors that could affect the outcome of this result. First of all, although there were 107 HIV-1-infected individuals typed for the CCR5Δ32 mutation, proviral load data could only be obtained from 63 homozygotes and 15 heterozygotes. With such a difference in sample sizes it is difficult to obtain statistically significant results, and with so few heterozygotes, one or two high proviral loads can have drastic effects on the outcome of the statistical analyses. This was indeed true in our case, as subjects 073 and 103 had very high proviral loads of 103 and 207 copies respectively, much higher that the rest of the heterozygotes. Another complicating factor is that we do not have proviral load data on some of the homozygotes that died before we began measuring proviral load. In Chapter 4, I showed that high proviral loads are predictive of faster rates of disease progression. Since most of the individuals in our cohort are thought to have been infected for longer than 10 years, the subjects that have already exited our study by death probably had higher proviral loads, and therefore progressed much faster. Had data from these individuals been included in the above analysis, the results may have been different.

Despite the fact that we did not find an association between CCR5Δ32 heterozygosity and HIV-1 proviral load, this coreceptor mutation does appear to have an effect on disease progression. This finding has identified the HIV-1 coreceptors as new targets for antiretroviral therapy. The idea is that it might be possible to decrease the

availability of these coreceptors on the cell surface of $CD4^+$ T cells through the use of small molecule inhibitors or gene therapy, thus mimicking the effects of the CCR5Δ32 mutation. Given the limitations of current antiretroviral therapy mentioned in the earlier pages of this chapter, it is not surprising that HIV researchers have acted quickly in developing novel therapeutic strategies that target the coreceptors. Some of the ideas that are being developed and tested include compounds such as T-20, a synthetic peptide corresponding to a region of the transmembrane subunit of the HIV-1 envelope protein that blocks virus-cell fusion and viral entry.[123] T-22 is a similar compound developed to specifically inhibit replication of X4 strains of HIV-1.[143] Another novel strategy under development is production of "intrakines" which are modified versions of chemokines like RANTES, that have been structurally modified so that they bind to CCR5 intracellularly and anchor the receptor in the endoplasmic reticulum, thus reducing its expression at the cell surface.[223] Other chemokine analogs, such as aminooxypentane-RANTES, have been designed that block HIV-1 entry by competitively binding to chemokine receptors at the cell surface.[204]

Finally, I would like to summarize the major results and findings of the work performed during the course of my MSc project. In Chapter 3, I describe the development of a non-radioactive, PCR-based method for measuring HIV-1 proviral load and used this method to generate proviral load data from seventy-eight HIV-1-infected individuals. This method would be useful for monitoring the efficacy of newly developed HIV therapies designed to reduce the size of this pool of latent virus. In Chapter 4, I confirmed that HIV-1 proviral load is a stable pool of virus that is unaffected

by current antiretroviral therapies. I also showed that proviral load levels predict the rate of disease progression and influence the degree of immune reconstitution and viral suppression achievable with highly active antiretroviral therapy. Finally, in Chapter 5, I used my proviral load data to attempt to determine the underlying mechanism of the slower rates of disease progression reported in HIV-1-infected individuals that are heterozygous for the CCR5Δ32 HIV-1 coreceptor mutation. The results of the work showed that a decreased proviral load was not responsible for the slower rates of disease progression seen in the CCR5Δ32 heterozygotes enrolled in our study.

6.3 Final Summation

In conclusion, if I was asked to summarize in one sentence what the work of my MSc project really meant, I would have to say, HIV proviral load is much more important than it has previously been considered, and that finding ways to address HIV proviral load should be the ultimate goal of antiretroviral therapy in HIV infection.

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