QUANTITATION OF PROVIRAL LOAD AND THE DISTRIBUTION OF DRUG RESISTANT MUTATIONS IN HUMAN IMMUNODEFICIENCY VIRUS INFECTION

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October 2000

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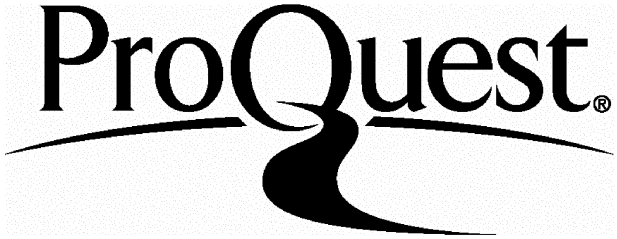

ABSTRACT

Infection with Human Immunodeficiency virus type 1 (HIV-1) induces a chronic and progressive disease process with a broad spectrum of clinical manifestations from acute primary infection to AIDS which is often associated with opportunistic infections and AIDS. The course of the disease is also associated with increasing levels of viral replication and destruction of the immune system. Treatment of HIV-1 infection with single antiretroviral drugs provides a limited duration of benefit due to the emergence of drug resistant strains of the virus during monotherapy. The aim of this study was to examine levels of HIV-1 proviral DNA load and the distribution of HIV-1 zidovudine resistance mutations in a group of 79 patients who were receiving zidovudine monotherapy and also in multiple post-mortem tissues samples from patients who died of AIDS.

A quantitative polymerase chain reaction (QPCR) assay was firstly developed to accurately measure HIV-1 proviral DNA load. The QPCR assay was based on coamplification of known copy number of a control sequence with patient HIV-1 DNA which allowed accurate quantitation. Measurement of proviral DNA load in post-mortem tissues from 11 patients who died of AIDS revealed high levels in lymph node and spleen samples, significant levels were also found in frontal lobe brain tissue. The sequence of the V3 loop of HIV-1 gp120 was obtained by direct sequencing of a selection of the tissues and 15 of the 20 sequences analysed showed a macrophage tropic, non syncytium inducing (NSI) phenotype. Mutations in HIV-1 reverse transcriptase which confer resistance to zidovudine were examined by a point mutation assay (PMA) which showed differences in levels of resistant genotypes between different tissues in individual patients. Separate evolution of drug resistant virus in different anatomical sites may reflect the efficiency of zidovudine in different tissues or the selective tissue tropism of HIV-1. The profiles of resistance at the five codons of HIV-1 RT examined were also different between patients and the presence of mutation at codon 41 and 215 was indicative of long term zidovudine monotherapy.

Monitoring the emergence of drug resistant HIV-1 plays an important role in successful antiretroviral therapy and rapid PCR based assays for resistant genotypes

have been demonstrated. In this thesis a Line Probe assay (LiPA) was evaluated for the detection of mutations at a number of codons of HIV-1 conferring Zidovudine resistance. Detection of resistant mutations by the LIPA test in PCR amplified DNA from a selection of PM tissues and whole blood samples showed 100% agreement with the sequence of HIV-1 RT obtained the samples. The LiPA test was both a rapid and sensitive assay for drug resistant mutations and wild type sequences.

HIV-1 proviral DNA load and the presence of zidovudine resistant mutation at codons 41 and 215 was examined in 79 individual patients who had received zidovudine monotherapy. Expressing proviral DNA load per milliliter (ml) of whole blood or per unit of CD4 cells demonstrated how measurement of proviral load levels can be confounded by variation in cell numbers during the course of infection. Expression of proviral load per ml of blood showed a slight increase over time from baseline, whereas proviral load expressed per 10^3 CD4 cells showed a more significant change over time which mirrored the change in CD4 levels over time. Kaplan Meier analysis of proviral DNA load demonstrated that individuals with levels of $> 4.0 \log_{10}$ DNA copies had a shorter time for progression to a 50% CD4 cell decrease and also a shorter time to death. Further analysis of proviral DNA load as a marker for disease progression using Cox proportional hazard models showed a strong relationship between proviral load expressed per 10^3 CD4 cells and progression to death. The relationship of zidovudine resistance to proviral load, CD4+ T-cell count and disease progression was also examined. A significant correlation was found between presence of resistance mutation and a low CD4 cell count, resistance mutation was also strongly associated with AIDS and with a progression to death in a Cox proportional hazard model.

The results presented in this thesis of measurements of proviral DNA load and drug resistance mutations combined with an in-depth statistical analysis provide an important insight into HIV-1 disease progression in a group of patients receiving zidovudine monotherapy.

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Acknowledgements

I am very grateful to my supervisor Dr Vincent Emery for his supervision, and also his constant support and advice during this work. I would like to thank Professor Paul Griffiths for enabling me to perform HIV research in the Department of Virology at the Royal Free Hospital School of Medicine. I would also like to thank Dr Margaret Johnson of the Ian Charleson Clinic and Professor Christine Lee of the Haemophilia centre and patients for provision of samples which were fundamental to this work. I am grateful to Dr James McLaughlin of the Histopathology department (RFH) for providing valuable autopsy tissue material. My thanks also go to Dr Steve Kaye (Dept of Virology, UCL) and Professor Clive Loveday (Dept of Retrovirology, RFH) for enabling me to learn the point mutation assay and for their helpful advice. I am grateful to Dr Amanda Morcroft (Dept of Population Sciences, RFH) for her excellent statistical analysis and advice. I would also like to thank Dr Peter Simmonds and Dr Jeanne Bell (Depts of Medical Microbiology and Neuropathology, respectively, University of Edinburgh) for allowing access to the LiPA assay and for their support and encouragement.

Many friends have been a great source of support and solace throughout this work. I would especially like to thank Dr Duncan Clark for constantly keeping me on the straight and level when I was determined to nose dive into apathy. Everybody in the Dept of Virology helped get this thesis off the ground and I thank all the crew, Mike Kidd for his help and patience, Mounir Ait Khalead, Lea Cope, Sidney James, Mark Atkins and especially Azeem Ansari for his friendship and great football skills. I would like to thank again Duncan and Charlotte for their kindness and fine wines and for providing a calming sanctuary during my many visits. Finally I would like to thank Liz for everything, her support, patience and love and the necessary threats of torture to get this thing finished.

Padraig Strappe.

Abbreviations

aa	Amino acid
7tm	Seven-transmembrane-spanning
Adc	AIDS dementia complex
AIDS	Acquired immunodeficiency syndrome
AOP	Amino-oxypentane
ARC	AIDS related condition
ARV	AIDS related virus
ATP	Adenosine 5'-triphosphate
AZdu	3'-Azido-2-deoxyuridine
AZT	3'-Azido-2-deoxythymidine
AZT-DP	3'-Azido-2-deoxythymidine diphosphate
AZT-MP	3'-Azido-2-deoxythymidine monophosphate
AZT-TP	3'-Azido-2-deoxythymidine triphosphate
BAFLs	Bands across four lanes
BHAP	bis(heteroaryl)piperazine
BCIP	5-bromo-4-chloro-3-inodylphosphate -p-toluidine
BSA	Bovine serum albumin
CA	Capsid associated
CCR2	C-chemokine receptor 2
CCR3	C-chemokine receptor 3
CCR5	C-chemokine receptor 5
CCR8	C-chemokine receptor 8
CIP	Calf intestinal phosphatase
cDNA	Complementary DNA
CMV	Cytomegalovirus
CDR2	Second complementary determining region
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTL	Cytotoxic T lymphocyte
CXCR4	CX-chemokine receptor 4
D4T	2',3'-didehydrothymidine
dATP	2'-deoxyadenosine 5'-triphosphate
ddC	2',3'-dideoxycytidine
ddU	2',3'-dideoxuridine
ddI	2',3'-dideoxyinosine
dGTP	2'-deoxyguanosine 5'-triphosphate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
DTT	Dithiothreitol (Clelland's reagent)
EDTA	Ethylenediamine tetraacetic acid
FCS	Foetal calf serum
FDC	Follicular dendritic cell
FIV	Feline immunodeficiency virus
GI	Gastrointestinal

GPCR	**g-protein coupled receptor**
HAART	**Highly Active Antiretroviral Therapy**
HBV	**Hepatitis B virus**
HEPT	**1-(2-hydroxyethoxymethyl)-6-(phenylthio)thymine**
HIV	**Human immunodeficiency virus**
HIVE	**HIV encephalitis**
HTLV	**Human T-cell leukaemia virus**
IDU	**Intravenous drug users**
IN	**Integrase**
IPTG	**Isopropyl-1-thio-beta-D-galactoside**
IQR	**Inter quartile range**
KDA	**Kilodalton**
LAV	**Lymphadenopathy-associated virus**
LB	**Luria-Bertani medium**
LiPA	**Line probe assay**
LMP	**Low melting point**
LTR	**Long terminal repeats**
MA	**Membrane associated**
Mabs	**Monoclonal antibodies**
MDC	**Macrophage derived chemokine**
MOPS	**(3-[N-Morpholino]propanesulfonic acid)**
MRNA	**Messenger RNA**
MSK	**Medium salt buffer**
MT	**Macrophage tropic**
NBT	**Nitroblue tetrazolium chloride**
NC	**Nucleocapsid**
NNRTI	**Non-nucleoside RT inhibitors**
NSI	**Non-syncytium inducing**
OD	**Optical density**
OH	**Hydroxy**
PAGE	**Polyacrylamide gel electrophoresis**
PBMC	**Peripheral blood mononuclear cells**
Pbs	**Primer binding site**
PBS	**Phosphate buffered saline**
PCR	**Polymerase chain reaction**
PFA	**Phosphonoformate**
PM	**Post mortem**
PMA	**Point mutation assay**
PMEA	**9-(2-phosphonylmethoxyethyl) adenine**
PMEApp	**9-(2-phosphonylmethoxyethyl) adenine diphosphate**
PMEDAP	**9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine**
PMEDAPpp	**9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine diphosphate**
PPT	**Polypurine tract**
QPCR	**Quantitative PCR**
REV	**Regulator of expression of the virion**
RNA	**Ribonuleic acid**
RNAseH	**Ribonuclease H**
RRE	**Rev response element**

RT	**Reverse transcriptase**
SAP	**Shrimp alkaline phosphatase**
SDF	**Stroma derived factor**
SDS	**Sodium dodecyl sulphate**
SDW	**Sterile distilled water**
SI	**Syncytium inducing**
SIV	**Simian immunodeficiency virus**
SQ	***Sma-1* plasmid competitor**
SU	**Surface protein**
TAR	**Transactivation responsive region**
TBE	**Tris borate EDTA**
TE	**Tris-EDTA**
TEMED	**N,N,N,N'-tetramethylethylenediamine**
TIBO	**Tetrahydroimidazol(4,5,1-jk)(1,4)benzodiazepin-2(1H)-one and thione**
TM	**Transmembrane**
TQ	**Wild type plasmid competitor**
TSAO	**2',5'-bis-O-(ter-butyldimethylsily)-3'-spiro-5''-4''-amino-1'',2''-oxathiole-2'',2''-dioxide)pyrimidine**
X-gal	**5-Bromo-4-chloro-3-indolyl-beta-D-galactoside**

CHAPTER 1

INTRODUCTION

1.1 Human Immunodeficiency Virus and Acquired Immunodeficiency Syndrome

Human Immunodeficiency Virus type 1 (HIV-1) is a member of the lentivirus subfamily of retroviruses, and Acquired immunodeficiency syndrome (AIDS) is the final stage of a persistent infection with HIV-1, or HIV-2 (Barre-Sinoussi et al., 1983; Clavel et al., 1986; Gallo et al., 1984; Levy et al., 1984). Primary infection with HIV-1 may cause an influenza-like or acute mononucleosis-like illness, known as seroconversion illness (Gaines et al., 1988; Tindall and Cooper., 1991). During this period infection is associated with high numbers of virions and virus infected cells in the blood, HIV-1 antigen may also be detected in the blood and a transient lymphocytopenia may occur (Clark et al., 1991; Cooper et al., 1988; Daar et al., 1991). Following seroconversion to detectable HIV-1 antibodies, the viral load decreases and a cytotoxic T lymphocyte (CTL) response is evident (Daar et al., 1991; Koup et al., 1994; Safrit et al., 1994). The clinically asymptomatic period may last from between a period of months to more than 10 years (Rutherford et al., 1990). During this time the viral load can remain stable and begin to increase as the disease enters the AIDS stage while the number of CD4 + T cells slowly declines. This gradual loss of both cellular and humoral immune control (Miedema et al., 1994) causes a generalised immunodeficiency which may result in a variety of late stage illnesses including chronic diarrhoea, opportunistic infections, neurological disorders, dementia or Kaposi's sarcoma (CDC., 1993).

The first cases of a 'new acquired cellular immunodeficiency,' later known as AIDS were reported in the United states of America in 1981 amongst a group of previously healthy homosexual men (Gottlieb et al., 1981). Following this initial report AIDS cases were identified among haemophiliacs, blood transfusion recipients, intravenous drug users, children born to infected mothers and sexual partners of infected individuals (CDC reports., a,b,c,d,e., 1982).

The causative agent of AIDS was first identified in 1983, with the isolation of a retrovirus from a patient suffering from chronic lymphadenopathy (Barre-Sinoussi et al., 1983). This virus was named Lymphadenopathy-associated Virus (LAV). The

following year two other groups reported the isolation of a retrovirus from patients suffering from AIDS. These were named Human T-cell Leukaemia Virus type III (HTLV-III) (Gallo et al., 1984) and AIDS Related Virus (ARV) (Levy et al., 1984). Publication of the complete nucleotide sequence of LAV, HTLV III and ARV (Wain-Hobson et al., 1985a., Ratner et al., 1985) in 1985 allowed comparisons to be made between isolates. This showed that LAV was closely related to HTLV III, with a difference at nucleotide level of 1.5% and a 2.25% difference at amino acid level. ARV showed greater diversity to HTLV III, 6.3% at the nucleotide level and 9.2% at the amino acid level. It was concluded that all three were variants of the same virus (Ratner et al., 1985b) which was then named as HIV-1 and accepted by the international committee of the taxonomy of retroviruses (Coffin et al., 1986a,b).

1.2 HIV-1 Virion Structure

The HIV-1 virion is a spherical particle (Figure 1.1) with a diameter of about 100 nm (Gelderblom et al., 1987). The outer envelope of the virion consists of a lipid bilayer derived from the host cell membrane and a virus encoded tetrameric envelope protein complex. The envelope protein complex consists of two membrane proteins; an outer envelope, or surface (SU) protein gp120 non-covalently linked to a transmembrane (TM) protein gp41 (Allan et al., 1985a; Montagnier et al., 1985). A myristoylated membrane associated (MA) protein p17 is located underneath the virion envelope and is required for incorporation of envelope glycoproteins into mature virions and is associated with gp41 (Gelderblom et al., 1987; Gelderblom et al., 1989; Yu et al., 1992). The cone shaped inner core of the virion consists of a core associated (CA) protein p24 (Mervis et al., 1988), a nucleocapsid (NC) protein p7/p9, two positive stranded RNA molecules, a p51pol reverse transcriptase (RT), p66pol RT-RNaseH, a p34pol integrase (IN) and the protease (Pro).

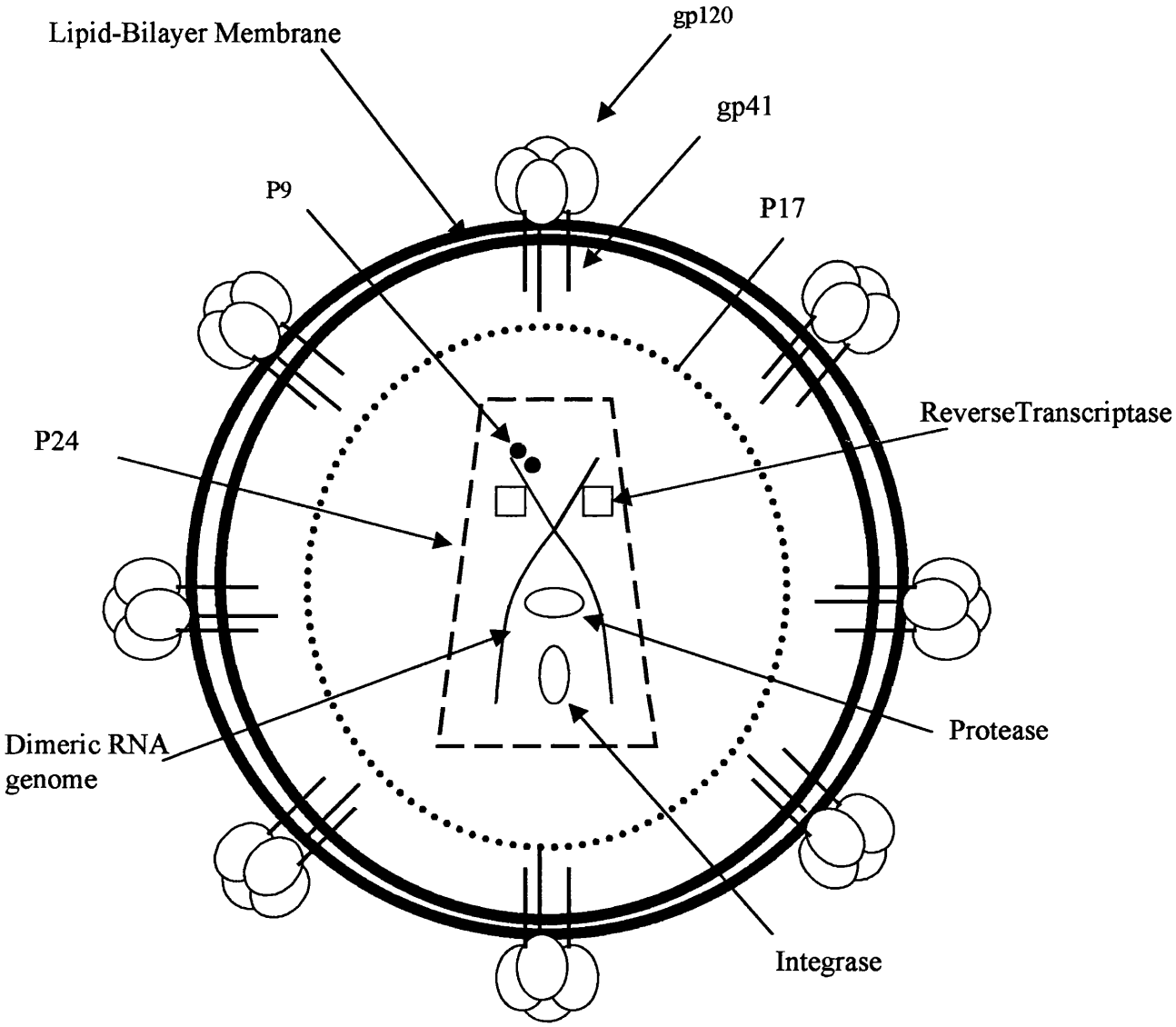


Fig 1.1 HIV-1 Virion structure.

The dimeric RNA genome is enclosed within the core matrix protein. Several enzymes such as Reverse Transcriptase, Protease and Integrase and small nuccleoccapsid proteins are associated with the RNA. The matrix is surrounded by the capsid protein. The lipid bi-layer membrane of HIV-1is composed of phospholipid and 72 knobs composed of heterodimers of the envelope glycoprotein. Each heterodimer is composed of a surface region (gp120) that interacts with a transmembrane protein (gp41).

1.3 HIV-1 genome organisation

The HIV-1 genome illustrated in Figure 1.2 is approximately 9.6kb in length and consists of three major structural genes gag, pol, and env, two long terminal repeats (LTRs) regions at the 5' and 3' regions, regulatory genes tat and rev and accessory genes vif, vpr, vpu and nef.

1.3.1 Long Terminal Repeats (LTRs)

The long terminal repeats exist solely in the proviral genome and consist of direct repeats of almost identical sequences as illustrated in figure 1.3. They are contained as two pieces in the RNA genome but not as a complete functional unit and play a crucial role in integration. The LTRs consist of a 5' U3 region followed by a repeat region and a 3' U5 region. The R region represents identical 5' and 3' ends of the RNA genome and the U region represents unique sections of retroviral RNA and are located adjacent to the R-regions at the 5' and 3' ends. During reverse transcription the full length LTR, U3RU5 is duplicated, the U3 region of the RNA genome is copied to the 5' end of the provirus and the U5 region is attached to the 3' end. The general functions of the LTR's include an important role in the integration of proviral DNA in the host genome and in the initiation and enhancement of retroviral transcription. The LTR's interact functionally with a wide variety of cellular proteins and larger multiprotein complexes such as polymerase II (Pol II), cellular transcription factors and polyadenylation complexes.

An important feature of the HIV-1 LTR is the presence of transcriptional regulatory elements both upstream and downstream of the transcription start site which is located at the junction between the U3 and R region. Upstream elements are contained within the U3 region and downstream elements are located in the R and U5 regions. The LTR can be divided into functional domains that include the negative regulatory element (NRE), the enhancer and basal promoter elements, the core promoter and the Tat activation response element (TAR). The NRE has a negative influence on transcription (Okamoto et al., 1990) and contains numerous binding sites for proteins which include TCF-1, USF, NF-AT, ILF-1, GRE and AP-1 (Gaynor, 1995). The enhancer region

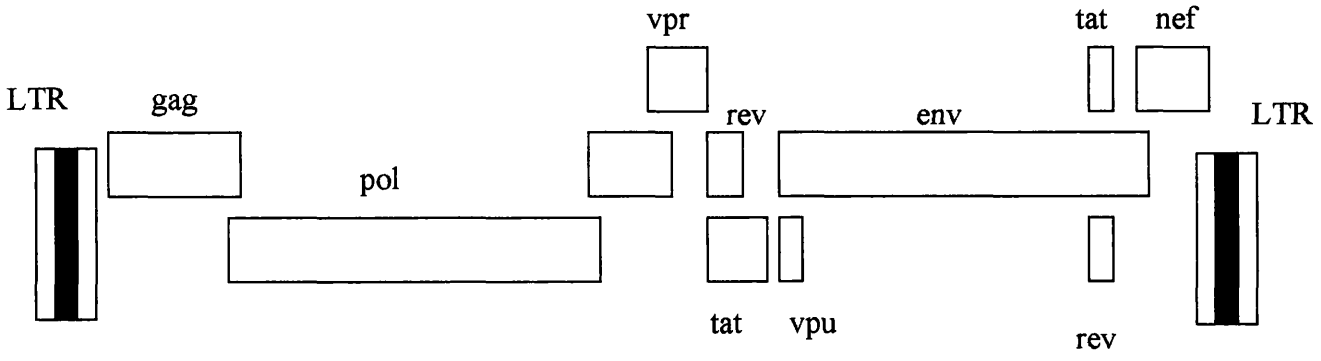


Fig 1.2 Genomic organisation of Human Immunodeficiency Virus type 1. Proviral DNA form.

contains two 10bp conserved binding sites for the nuclear factor kB (NF-kB)/relB family of transcription factors which upregulate transcription in response to a variety of stimuli including cytokines, mitogens and other viral activating proteins (Mallardo et al., 1996). The basal promoter region is a G+C rich region that contains 3 Sp1 boxes, which bind Sp1 transcription factors. The Sp1 sites function synergistically with the TATA box to ensure maximum activation of the promoter by Tat (Sune and Garcia-Blanco, 1995). Tat and Sp1 together form a complex that leads to the phosphorylation of Sp1 (Chun et al., 1998). The core promoter region contains a TATA box and an initiation region (InR). A number of transcription factors and RNA polymerase II bind to the TATA box and initiation of transcription is preceded by binding of TFIID to the basal to the basal promoter. A preinitiation complex consisting of RNA polymerase II and general initiation factors is assembled within the core promoter (Buratowski et al., 1989). The initiation region is a short pyrimidine rich region that interacts with TFII-I, USF and YY1 and is required for Tat responsive transcription (Rittner et al., 1995).

Termination of transcription and polyadenylation is mediated by a tripartite region in the 3' LTR, the major part is a hairpin structure around the polyadenylation signal sequence. The structural integrity of this hairpin has been shown to be crucial for polyadenylation (Berkhout et al., 1995). LTR activity is dependent on the chromatin structure and the active HIV-1 promoter/enhancer region has been shown to be nucleosome free while the enhancer is occluded by a nucleosome in the inactive promoter. A region containing binding sites for transcription factors SP-1 and AP-1 is crucial for chromatin rearrangement (Elkharroubi and Martin., 1996) and histone acetylation removes a nucleosome from the transcriptional start site in the inactive LTR (Vanlint et al., 1996).

1.3.2 The structural genes of HIV-1

1.3.2.1 gag (group specific antigens)

The gag gene of HIV-1 encodes the structural components of the virion. Expression of the gag gene products results from translation of a gag/pol full length mRNA

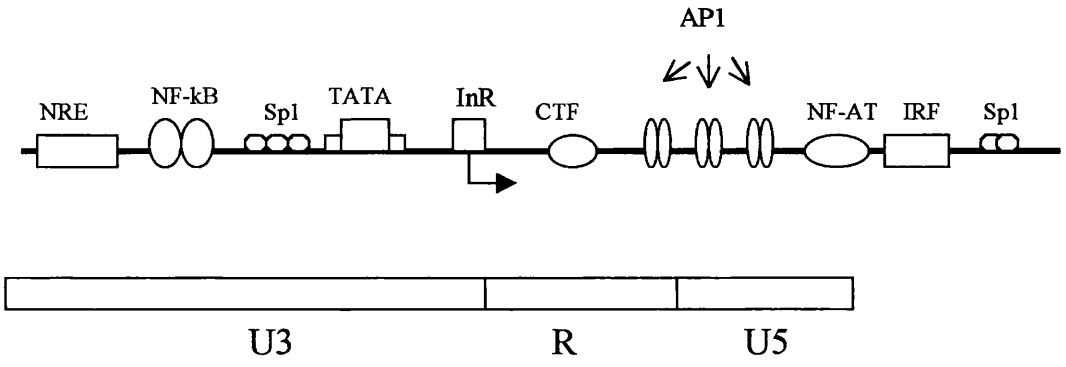


Fig 1.3 Structural organisation of the HIV-1 long terminal repeat.

transcript. Core proteins p24 CA, p17 MA, p7 NC and p6 are generated by proteolytic cleavage of the 55kDA gag precursor protein Pr^{55Gag} (Veronese et al., 1988). The membrane associated or matrix protein (p17 MA) is associated with the plasma membrane due to co-translational myristoylation and contains a highly basic region of the N-terminal which binds to phospholipids, essential for the formation of infectious virions (Bryant and Ratner., 1990, Zhou et al., 1994). MA is required for the self assembly of Gag and the Gag-Pol polyproteins (Yu et al., 1992) and also acts in targeting the pre-integration complex to the nucleus of non-dividing cells (Bukrinsky et al., 1993).

1.3.2.2 pol (polymerase)

The HIV-1 pol gene, which encodes the protease, reverse transcriptase and integrase viral enzymes, is also translated from a full length gag/pol transcript known as the p165 gag-pol precursor mRNA. Translation of the pol gene products requires a ribosomal frame shift, since the pol gene is in a +1 reading frame in relation to gag (Jacks et al., 1988). This frameshift occurs at a frequency of 5%. The resultant pol polyprotein is then cleaved to its individual products by viral protease to yield the p22 protease, the p66 and p51 reverse transcriptase subunits, and the p32 integrase. The C-terminus of the p66 subunit of reverse transcriptase has RNase H activity which is essential for proviral DNA synthesis (Hansen et al., 1987).

1.3.2.3 env (envelope)

Enveloped viruses are equipped with a transmembrane protein on their surface to facilitate binding to cellular receptors and subsequent entry into the cell. HIV-1 contains 72 envelope proteins composed predominantly of trimers and are essential for infectivity of the virus. The env gene of HIV-1 is transcribed into a singly spliced mRNA. When translated it yields a glycosylated polyprotein precursor (gp160) on ribosomes associated with the endoplasmic reticulum (ER). This 160 kDa precursor protein is transported from the ER to the Golgi complex where mannose-rich oligosaccharide side chains are added. In a late Golgi compartment such as the trans-Golgi network the precursor is cleaved by cellular proteases of the subtilysin family such as Furin and PC7 (Hallenberger et al., 1997) into two components, gp120 the external glycoprotein and gp41 the transmembrane glycoprotein. These mature

envelope proteins are then transported to the cell surface (Wiley et al., 1988) where gp120 is present as a multimeric unit, non-covalently linked to gp41 (Helseth et al., 1991). The gp120 envelope gene has a number of variable regions (V1, V2, V3, V4 and V5) and conserved domains (C1, C2, C3, C4 and C5). The variable regions play a role in viral tropism and determining virus phenotype (Groenink et al., 1993).

1.3.3 The Regulatory And Accessory Genes of HIV-1

In addition to the structural genes found in all retroviruses, the HIV-1 genome contains six additional genes encoding regulatory proteins p14 Tat, p19 Rev and accessory proteins p27 Nef, p23 Vif, P15 Vpr and p16 Vpu. The function of the Tat protein is to upregulate transcription from the viral promoter, the U3 region of the long terminal repeats (LTR) by binding to nascent RNA transcripts. The rev protein promotes the export from the nucleus of unspliced or singly spliced RNAs which act respectively, as genomic RNA/template for the translation of gag/pol proteins and template for envelope proteins. In the absence of rev no structural proteins are made. The nef protein appears to be required for efficient replication *in vivo*. Tat, Rev and Nef are not incorporated into virion particles but are the first viral components produced from multiply spliced viral mRNA. The Vif and Vpu proteins respectively influence the infectivity of cell-free virions and the release of virions from infected cells. Vpr seems to be involved in the replication of HIV in non-dividing cells.

1.3.3.1 tat (trans activator)

Tat is translated from three multiply spliced mRNA's and is essential for viral replication. The tat gene is encoded by two exons, the first of which precedes the env gene, and codes for approximately 76 amino acids while the second is within the env gene and codes for approximately 12 amino acids (Ayra et al., 1985). The most common form of Tat in infected cells is an 86 amino acid protein (Aldovini et al., 1986) which is localised in the nucleus (Hauber et al., 1987). The HIV-1 proviral genome is flanked by long terminal repeats (LTRs) which contain signals for integration, initiation, and regulation of transcription and poly-adenylation of mRNA. Tat acts as a powerful transactivator of LTR gene expression and the presence of Tat

has been shown to dramatically increase transcription of all genes linked to HIV-1 LTR's (Arya et al., 1985; Sodroski et al., 1985a,b).

Tat acts by binding to a 59 nucleotide RNA stem loop structure TAR (transactivation response element) located between +1 and +60 in the viral RNA (Rosen et al., 1985). Initiation of transcription occurs through binding of a stable RNA Pol II holoenzyme (which consists of RNA Pol II, general transcription factors TFIIF, TFIIH and a mediator) onto preassembled TFIID and TFIIB at the TATA box region. After incorporation of a few nucleotides the complex will pause. A critical regulatory step, which represents the transition from initiation to elongation, is the phosphorylation of the C terminal domain (CTD) of the largest subunit of RNA Pol II. Phosphorylation can occur by interaction of Tat with the p62 subunit of TFIIH and this increases the ability of TFIIH to phosphorylate the CTD of RNA Pol II. Tat can also interact through its activation domain with Tak (Tat associated kinase) which has also been described as the kinase subunit of the positive acting early elongation factor complex (P-TEFb). Tak/P-TEFb contains the CDC2-related catalytic subunit PITALRE (Cdk9) and this active kinase complex stimulates the hyperphosphorylation of the CTD of RNA Pol II, resulting in a highly processive RNA Pol II elongation complex (Jones and Peterlin, 1994). Analogous to other cyclin dependent kinases, Cdk9 has a cyclin related partner called cyclin T (CycT), which can bind the activation domain of Tat on its own and in the context of the Cdk9-cycT complex. Binding of cycT to Cdk9 is thought to induce a conformational change in Tat enhancing its affinity and specificity for the Tar RNA. The cycT gene maps to human chromosome 12 and can increase Tat trans-activation when introduced into murine cells (Wei et al., 1998).

1.3.3.2 rev (regulator of expression of the virion)

The second regulatory gene of HIV-1 is rev, which is essential for viral replication. Rev protein is translated from a multiply spliced transcript, the translation of which yields a 19 KDa protein composed of 116 amino acids (Cullen & Greene, 1990). It is found in the nucleus of infected cells and predominantly in the nucleolus (Cullen et al., 1988; Malim et al., 1989). The main function of Rev seems to be the promotion of nuclear export and cytoplasmic expression of the mRNA's which encode the viral structural proteins. Rev plays a crucial role in switching from "early regulatory" to

"late structural" gene expression (Cullen, 1991). Extensive mutagenesis studies have identified two functional domains within Rev, a multifunctional domain and an effector domain. The multifunctional domain is involved in localisation of Rev to the nucleus, binding of Rev to an RNA structure, the Rev Response element (RRE) located within the env gene and oligomerisation of Rev at the RRE (Malim et al., 1988, 1989, 1990; Daly et al., 1989). The RRE consists of a series of stem-loop structures (SLI-SLV) protruding from a long central stem. Stem II contains a high affinity binding site (SLIIB) for Rev. Binding of Rev to the RRE targets the attached mRNA to the nuclear export machinery. The effector domain of Rev contains nuclear export signals (NES's) which interact with cellular factors mediating nuclear export of Rev containing mRNA's. A working model of Rev action can be summarised as binding of REV to the high affinity site (SL IIB) in the RRE present in primary and singly spliced mRNA. REV oligomerises on the mRNA forming a ribonucleoprotein complex. Within this complex the Rev NES binds to the nuclear export factor Exportin 1 complexed with Ran GTP.

Exportin 1 was originally identified in yeast and named chromosome maintenance gene 1 (CRM1). The primary sequence of exportin 1/ crm1 showed it to be a member of the importin-beta (karyopherin-beta) superfamily of shuttling nuclear transport receptors (Ohno et al., 1998). This complex interacts with the nucleoporin homologue protein (Rab/hRIP) which localises the complex to the nuclear pore (Fritz and Green., 1996). The protein-RNA complex is translocated to the cytoplasm where GTP is hydrolysed to GDP and Ran GDP. Exportin 1 and REV are released and the HIV mRNA is available for translation.

1.3.3.3 nef (negative effector)

The nef gene is located as a single open reading frame at the 3' end of the viral genome. Nef is a 25 to 27 KDa protein (Franchini et al., 1986) which is translated from a multiply spliced mRNA transcript resulting in a polypeptide of 200 to 205 amino acids (Harris et al., 1992). Nef is a cytoplasmic protein which is myristoylated and is found on the cytoplasmic face of the plasma membrane (Allan ET al., 1985b). The exact function of Nef is unknown; however, Nef has been shown to reduce surface expression of the CD4 molecule (Garcia & Miller. 1991), to be a repressor of

viral replication and to down regulate HIV LTR gene expression (Ahmed & Vankatesan. 1988).

The nef gene has been shown to contain several conserved sequences including an invariant mrystoylation signal, an acidic region, a region with 4 Pxx repeat sequences and a potential site for protein kinase C phosphorylation (Shugars et al., 1993). The C-terminal end of Nef contains prominent T-cell epitopes, of which residues 73-97 are major targets for CTL's (Koenig et al., 1990). Nef is found primarily in the cytoplasm and associates via the N-terminus with the actin molecules of the cytoskeleton in a process that requires myristoylation (Fackler et al., 1997).

Nef plays a role in increasing viral replication and pathogenesis (Miller et al., 1994). Primate studies have shown that a nef deleted SIV fails to produce AIDS or death in adult primates compared to wild type SIV (Kestler et al., 1991). Sequence analysis of the nef gene in certain HIV infected individuals, known as long-term non progressors, has shown a deletion compared to patients with advanced disease progression (Deacon et al., 1995; Kirchoff et al., 1995), highlighting an important role for nef in natural infection.

A major effect on the HIV infected host cell attributed to Nef is the down regulation of cell surface CD4 molecules (Garcia et al., 1991; Mariani et al., 1993). The internalisation of CD4 is followed by accumulation of the molecules in early endosomes (Aiken et al., 1994) and requires phosphorylation of Nef. Further Studies have demonstrated an interaction between CD4 bound to Nef connecting to clathrin-associated adaptor complexes and have suggested that CD4 is targeted directly for degradation in late endosomes/lysomes (Mangasarian et al., 1997; Piguet et al., 1998).

Nef expression can also lead to an alteration of normal T-cell function by blocking IL-2 and NF-kB induction (Greenway et al., 1994). Nef has also been shown to downregulate MHC I on the cell surface possibly through an interaction with a clathrin associated adaptor complex (Le Gall et al., 1998). Downregulation of MHC I may allow HIV-1 infected cells to evade cytotoxic T cells (Collins et al., 1998). Nef can recruit cellular kinases and alter cellular activation via signal transduction pathways.

Nef recognises the SH3 domain of the Hck and Lyn nonreceptor protein tyrosine kinases (Saksela et al., 1995), interacts with the T cell specific Lck tyrosine kinase (Greenaway et al., 1996) and Nef can also recruit a member of the p-21 activated kinase (PAK) family (Nunn and Marsh., 1996).

A further role for Nef is enhancement of virion infectivity. Besides downregulation of CD4 and increasing envelope virion incorporation, Nef can increase HIV-1 infectivity in a CD4 independent manner. Nef can stimulate the efficiency of proviral DNA synthesis (Aiken and Trono., 1995) possibly through a mechanism of increasing viral uncoating or stabilising the reverse transcription complex and also recruits p-21 activated kinases (PAK) to sites of virion assembly for phosphorylation of Gag matrix protein (Swingler et al., 1997) which could affect virion infectivity

1.3.3.4 vpu (viral protein u)

Vpu is a 9 KDa transmembrane protein and is found only in HIV-1 and not in HIV-2 or SIV (Subbramanian et al., 1994). The two functions associated with Vpu are the degradation of CD4 in the endoplasmic recticulum and the enhancement of viral particle release, which occurs at the cytoplasmic membrane. The cytoplasmic tail of Vpu is 54 amino acids in length and consists of two alpha helical regions flanking a 12 amino acid acidic region (Chen et al., 1993). The acidic region is highly conserved and contains two serine residues at positions 52 and 56 that are phosphorylated by caesin kinase-2. This phosphorylation is important for Vpu function (Paul and Jabbar, 1997). Vpu is not a proteolytic enzyme, and degradation of CD4 is thought occur through activation of proteolytic pathways by interaction with a Vpu–responsive element present in the CD4 tail (Vincent et al., 1993). Vpu may also direct CD4 and possibly MHC class I molecules to endoplasmic recticulum associated degradation by recruitment into the cytosolic ubiquitin-proteasome (Schubert et al., 1998).

The second function of Vpu involves efficient viral particle release from infected cells. Vpu deleted mutant viruses show an inefficient release into culture medium (Klimkait et al., 1990), and have been shown to be produced in internal membrane compartments

or attached to the plasma membrane. The action of Vpu in virus release may be cell type dependent as a study has shown that Cos-7 cells were able to release particles both in the presence and absence of Vpu (Gottlinger et al., 1993). This may suggest that Vpu activity can be replaced by cellular factors and explain the ability of HIV-2 and SIV to release particles without Vpu.

1.3.3.5 vif (Viral infectivity factor)

The vif gene is also translated from a singly spliced mRNA and is situated between the pol and env genes. It encodes a highly basic 23 KDa protein (Kan et al., 1986) which is expressed late in the viral life cycle (Schwarty et al., 1991) and contains two cysteine residues at positions 114 and 133 that are highly conserved and necessary for Vif function (Ma et al., 1995). A virus particle contains 60 to 100 molecules of Vif (Camaur and Trono, 1996), and Vif co-localises with virally encoded Gag proteins (Simon et al., 1997) which may contribute to its role in virion assembly (Sakai et al., 1991; Fisher et al., 1987). Vif has been shown to be essential for cell free and cell to cell transmission of virus in cells that are permissive for infection (Sakai et al., 1993). The presence of the vif gene is also important for replication in non-permissive cells such as macrophages (Borman et al., 1995).Vif also plays a role in the transport of virions to the nuclear membrane via associations with the cytoskeleton intermediate filaments (Karczewski et al., 1996). The interaction of Vif with cellular and viral proteins involves phosphorlyation of specific residues at Ser 144, Thr 155 and Thr 188 of the Vif protein (Yang et al., 1996).

1.3.3.6 vpr (viral protein r)

The vpr gene encodes a protein of 96 amino acids with a predicted molecular weight of 14 KDa and is relatively conserved by lentiviruses (Cohen et al., 1990). Vpr is required for efficient replication in macrophages and is dispensable for replication in cell lines (Balliet et al., 1994). Incorporation of Vpr into the virion requires an anchor to associate with assembling capsid structures. This may arise through interaction with the p6 region of the C-terminal portion of the Gag precursor (Kondo et al., 1995; Paxton et al., 1993). Although no classical nuclear localisation signal has been clearly identified for Vpr, the protein primarily localises in the nucleus when expressed in the absence of other HIV-1 proteins (Mahalingam et al., 1995). Vpr may gain access to

the nucleus by interaction with host cellular proteins such as Rip-1 and RIP (Refaeli et al., 1995; Zhao et al., 1994), and may regulate the docking of the HIV-1 pre-integration complex to nucleoporins by binding to Karyopherin-alpha (Popov et al., 1998).

Vpr can play a role in viral transcription as a weak transactivator of HIV-1 LTR (Cohen et al., 1990) and also enhances the migration of the pre-integration complex in newly infected non-dividing cells (Heinzinger et al., 1994). Vpr induces cellular differentiation (Levy et al., 1993) and can inhibit cellular proliferation in T-cells and macrophages and blocks the cell cycle in G2/M phase, which is associated with phosphorylation of CDC2 kinase (Jowet et al., 1995; Re et al., 1995). Vpr has also been shown to regulate apoptosis by suppressing NF-kB activity by induction of IkB transcription (Ayyavoo et al., 1997).

1.4 HIV-1 replication

Infection of cells by HIV-1 requires the fusion of the cellular and viral membranes which is brought about by interaction of the viral envelope glycoprotein complex and receptors on the cell surface. HIV-1 specifically infects cells that express the HLA class II receptor (CD4) on their cell surfaces, such as CD4+ T lymphocytes and cells of the monocyte-macrophage lineage (Dalgleish et al., 1984; Klatzmann et al., 1984a,b; Maddon et al., 1986; McDougal et al., 1986). CD4 was shown to be the primary receptor for HIV-1 as introduction of the CD4 gene into non-susceptible cells such as fibroblasts conferred infectivity (Dalgleish et al., 1984). However, following the discovery of the CD4 molecule as the receptor for HIV and its interaction with the viral envelope glycoprotein, it was soon recognised that expression of CD4 was not always sufficient to permit infection. Rodent cell lines transfected with human CD4 did not permit viral entry mediated by the HIV envelope glycoprotein. However, entry was possible with heterologous envelopes that utilised different receptors on the same cells.

An example of restricted tropism was found in viral strains adapted for growth in transformed T-cell lines which could replicate well in activated primary T-cells but not in monocytes and macrophages even though these cells express CD4. Viral strains maintained in peripheral blood mononuclear cells replicated well in macrophages and T-helper cells but not in transformed T cell lines. The differences in tropism between these viruses were associated with sequence differences in the V3 region of the gp120 envelope glycoprotein. These observations suggested that in addition to CD4, other molecules would have important roles in species-specific and cell type-specific tropism of HIV. Figure 1.4 illustrates the essential steps in the HIV replication cycle beginning with binding and fusion of the vial particle to the cellular membrane, uncoating and reverse transcription of viral RNA to DNA and subsequent integration into the host genome. Viral mRNA expression within the nucleus is followed by export to the cytoplasm, translation, and finally assembly of components of the viral particle and budding from the cellular membrane.

Figure 1.4. General features of the HIV-1 replication cycle.

1. Binding of virion envelope gp160 complex to a CD4 molecule and a cellular chemokine co-receptor.

2. Fusion of virion particle with cell membrane and penetration into cell.

3. Uncoating of viral inner core and release of viral RNA.

4. Reverse Transcription of viral RNA to a double stranded DNA copy.

5. Transport of viral DNA to the nucleus as part of a preintegration complex and integration of viral DNA into the host chromosome.

6. Synthesis of unspliced and spliced mRNA transcripts

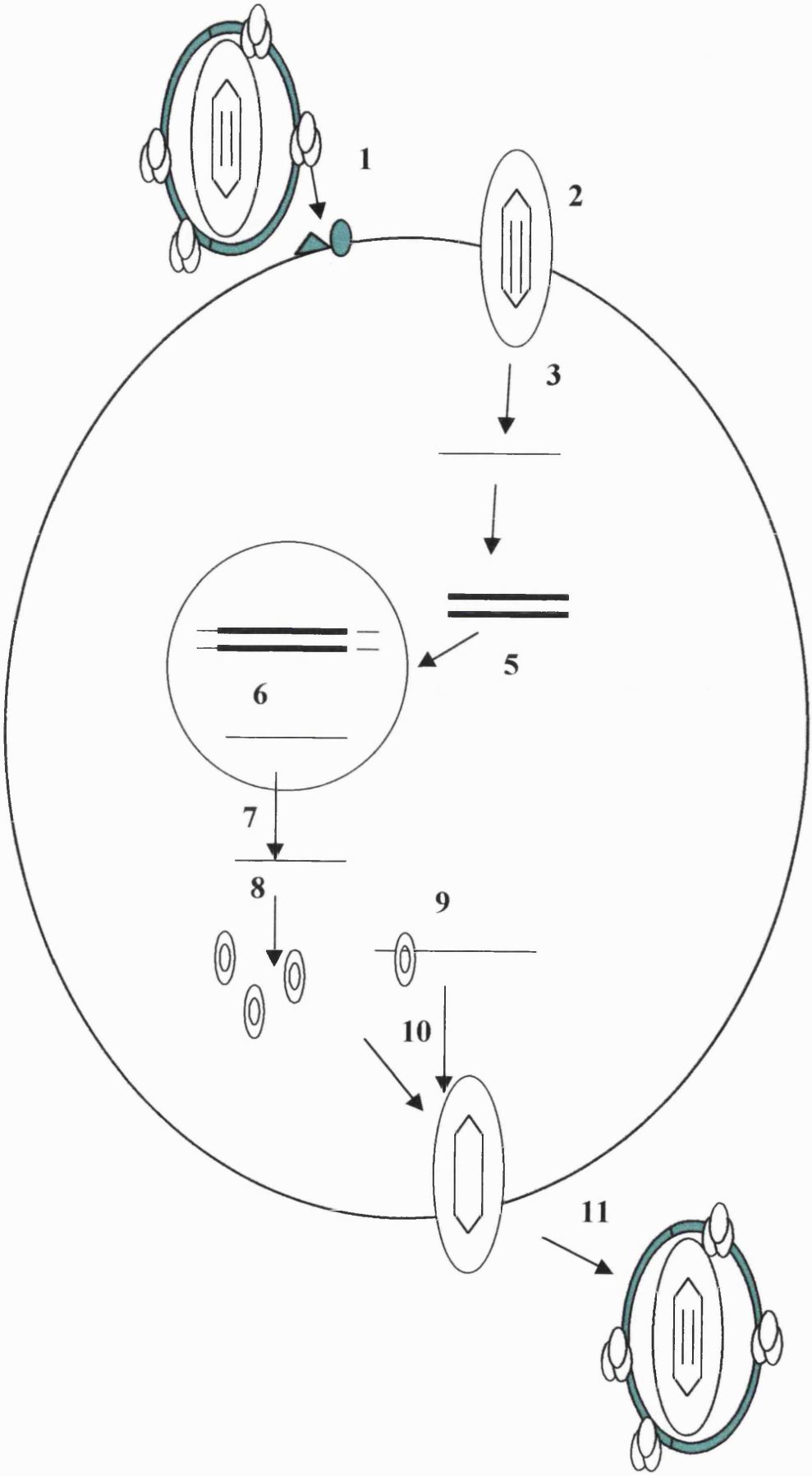
7. Transport out of the nucleus of RNA transcripts for translation.

8. Translation of unspliced and spliced mRNA into regulatory/accessory proteins and structural proteins respectively.

9. Encapsidation or packaging of the unspliced viral RNA by the Gag polyprotein.

10. Assembly of viral particle and targeting to the plasma membrane.

11. Budding of viral particle from the cellular plasma membrane and subsequent maturation.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1.4.1 Chemokine Receptors

Following the discovery of the CD4 molecule as the receptor for HIV and its interaction with the viral envelope glycoprotein, it was soon recognised that expression of CD4 was not always sufficient to permit infection. Rodent cell lines transfected with human CD4 did not permit viral entry mediated by the HIV envelope glycoprotein but entry was possible with heterologous envelopes that utilised different receptors on the same cells. An example of restricted tropism was found in viral strains adapted for growth in transformed T-cell lines which could replicate well in activated primary T-cells but not in monocytes and macrophages, even though these cells express CD4. Viral strains maintained in peripheral blood mononuclear cells replicated well in macrophages and T-helper cells but not in transformed T cell lines. The differences in tropism between these viruses were associated with sequence differences in the V3 region of the gp120 envelope glycoprotein. These observations suggested that in addition to CD4 other molecules would have important roles in species-specific and cell type-specific tropism of HIV.

An explanation for the findings of restricted cellular tropism came after the cloning and expression of a seven-transmembrane G protein - coupled receptor (GPCR), termed fusin which facilitated HIV entry of T-cell line tropic virus into CD4+ murine cells (Feng et al., 1996). Fusin was subsequently renamed CXC chemokine receptor 4 (CXCR4). A second major advance was the discovery of an activity in lymphocyte supernatants that inhibited HIV replication of three chemokines of the CC or beta class; RANTES, MIP-1 alpha and MIP-1 beta. These polypeptides were shown to inhibit replication of macrophage tropic but not T-cell tropic strains of HIV-1 (Cocchi et al., 1995). The receptor for the three beta-chemokines was demonstrated to be chemokine receptor five (CCR5) and a major cofactor for entry of macrophage tropic strains of HIV (Dragic et al., 1996). The current nomenclature for receptor tropism is R5 (for CCR5-tropic virus), X4 (for CXCR4-tropic virus) and R5X4 for dual-tropic virus. Soon after the discovery of CCR5 and CXCR4 coreceptors, CCR2b and CCR3 were shown to be functional coreceptors for a number of HIV-1 strains (Choe et al., 1996; Doranz et al., 1996).

Several chemokine and orphan receptors have since been shown to be functional corecptors of both HIV and SIV and these are summarised in Table 1.1. The recently discovered chemokine receptors including, CCR8 (Rucker et al., 1997), STRL33 (also referred to as Bonzo) (Liao et al., 1997) and Apj (Hoffman and Doms., 1998) function as coreceptors for a subset of T-cell and macrophage-tropic strains of HIV-1 and SIV. CX_3CR1 (also referred to as V28) functions as a co-receptor for a more limited number of T-cell tropic strains of HIV-1 and HIV-2 (Rucker et al., 1997; Reeves et al., 1997). The orphan receptors GPR1, GPR15 (also referred to as BOB) and ChemR23 appear to function as coreceptors mainly for SIV (Farzan et al., 1997; Deng et al., 1997; Samson et al., 1998). For SIV, both macrophage and T-cell tropic strains use CCR5 for entry (Edinger et al., 1997; Chen et al., 1997), CXCR4 and CCR3 are rarely used by SIV.

Macrophage tropic isolates of HIV-1 are prevalent during the early stages of infection (Connor et al., 1993; Zhu et al., 1993), while T-cell tropic strains emerge later in disease (Tersmette et al., 1988). This change in viral phenotype is associated with immune system decline and a progression to AIDS (Schuitemaker et al., 1992). These changes in viral tropism reflect changes in coreceptor use from CCR5 to CXCR4 as a consequence of mutations in the envelope glycoprotein (Connor et al., 1997). Evolution of coreceptor use from CCR5 to CXCR4 may be associated with disease progression.

The distribution of chemokine receptors in the brain has revealed insights into the neuropathogenesis of HIV-1. Within the CNS the alpha and beta chemokines have been localised to astrocytes, microvessels and microglia (Glabinski et al., 1995) and elevated expression of beta-chemokines has been seen in the brains of macaques with SIV encephalitis (Sasseville et al., 1996). Studies on brain tissues from patients with HIV encephalitis (HIVE) have demonstrated elevated MIP-1alpha and MIP-1beta and these were localised to microglia and astrocytes (Schmidtmayerova et al., 1996). CXCR4 has been localised on neurones and occasionally on microglial cells (Lavi et al., 1997). CCR5 has been demonstrated on macrophages, astrocytes and neurons

Coreceptor	Other names	Ligands	Viral usage	Expression Patterns
Major Receptors				
CCR3	CKR3	Eotaxin, MCP-3, MCP-4, RANTES	HIV-1 (minor for HIV-2)	CD4+ T cells (Th2), Eosinophils, Microglia
CCR5	CKR5	MIP-1 alpha, MIP-1 beta, RANTES	HIV-1, HIV-2, SIV	Monocytes, CD4+ T-cells
CXCR4	LESTR, FUSIN	SDF--1	HIV-1, (minor for HIV-2)	Lynphocytes, Macrophages, Neurons
GPR15	BOB	?	SIV, HIV-2, (minor for HIV-1)	Macrophages, T-cells, colon
STRL33	BONZO	?	SIV, HIV-2, (minor for HIV-1)	T-cells, Monocytes,Placenta
Minor Receptors				
CCR2	CKR2b	MCP-1 - 4	HIV-1, HIV-2	Monocytes, CD4+ T cells
CCR8	ChemR1	I 309	HIV-1, SIV	Monocytes, Thymocytes
CX3CR1	V28	Fractalkine / Neuroactin	HIV-1, HIV-2	Lymphocytes, Brain
GRP1	?	?	SIV	Macrophages

Table 1.1. Chemokine receptors for HIV and SIV entry.

(Rottman et al., 1997). CCR3 and CCR5 have been shown to act as coreceptors for HIV entry of primary fetal microglia (He et al., 1997).

1.4.2 Binding , fusion and entry

Recent studies have revealed both structural and functional information on the mechanism of the fusion process (Chan and Kim, 1998., Kwong et al., 1998) and a working model has been proposed that involves multiple steps. Initial binding of the gp120/gp41 envelope complex to the CD4 receptor and a CCR5 or CXCR4 chemokine co-receptor induces a conformational change in the envelope glycoprotein. A transient intermediate structure exists called the "Pre-Hairpin" in which gp41 exists as a membrane protein in both the viral and cellular membranes (Futura et al., 1998). The prehairpin intermediate resolves into a structure of a trimer of hairpins, which may represent the fusion active state of gp41 (Lu et al., 1995) allowing fusion of the viral and cellular membranes.

1.4.2.1 Binding

The HIV-1 surface envelope glycoprotein gp120 binds with high affinity to the V1 domain of the CD4 molecule (Arthos et al., 1989; Jameson et al., 1988). Analysis of the crystal structure of the CD4 molecule revealed that the V1 domain protrudes from the surface of the molecule and displays similarity to the second complementary-determining region (CDR2) of immunoglobulin domains (Ryu et al., 1990; Wang et al., 1990., Sattentau et al., 1989). Mutations in V1 resulted in reduced binding to gp120 and to certain CD4 directed monoclonal antibodies (Mabs) that can block gp120-CD4 binding. One hydrophobic and four positively charged amino acid residues in V1 may contribute to the high affinity for gp120 (Phe43 and Lys29, Lys35, Lys46 and Arg59) (Moebius et al., 1992; Moore et al., 1993a).

The CD4 binding site on gp120 was poorly defined for many years and mutational analysis and delineation of epitopes for anti-gp120 Mabs that block binding to CD4 demonstrated that amino acid residues critical for CD4 binding were scattered throughout gp120 (Kowalski et al., 1987; Olshevsky et al., 1990; Syu et al., 1990).

The prevailing view was that the CD4 binding site of gp120 was a complex folded structure in which amino acid residues from diverse regions were brought in close proximity (Moore et al., 1993a). Proper folding and functioning of gp120 was thought to be dependent on cysteine residues responsible for disulphide bridge loop structures (Leonard et al., 1990; Pollard et al., 1992; Tschachler et al., 1990). Information of the N- and O- linked oligosaccharides of gp120 also deemed them important for CD4 binding, syncytium formation and infectivity because they are required for proper folding of gp120 (Fenouillet et al., 1990; Gruters et al., 1987). Binding to hydrophobic and positively charged amino acid residues in V1 of CD4 was thought to involve amino acid residues Asp338, Glu340, Asp427 and Trp397 (Cordonnier et al., 1989; Kowalski et al., 1987).

With the elucidation of the crystal structure of gp120 however, a definitive concept of the gp120-CD4 interaction was available. The crystal structure of gp120 reported by several groups (Kwong et al., 1998, Wyatt et al., 1998, Rizzuto et al., 1998) confirmed details surmised from previous biochemical, mutagenic and immunochemical approaches and also revealed undiscovered features. The gp120 glycoprotein binds to the most amino-terminal of the 4 immunoglobulin-like domains of CD4, and residues of the envelope are brought together to form a broad area that associates with CD4. Many of the contacts are between residues in the peptide backbone of gp120 but the crystal structure revealed and important 'knob and socket' interaction between Phe 43 of CD4 and gp120. An inner and an outer domain of gp120 linked by a bridging sheet were also revealed and upon binding to CD4, a confomational change moves the V1/V2 loops away from the bridging sheet exposing the CXCR4 chemokine co-receptor binding site. The region of gp120 that binds the CXCR4 co-receptor is revealed within the highly conserved stem of the V1/V2 structure near the base of the V3 loop.

1.4.2.2 Fusion and Entry

The result of binding of gp120 to CD4 is fusion of virion and cell membrane. The variable domains V1, V2 and V3, have been demonstrated to be involved in HIV-1 fusion and infectivity. Amino acid substitutions in these regions affect syncytium formation and infectivity without affecting CD4 binding (Bergeron et al., 1992; Freed

et al., 1991; Fung et al., 1992). Studies have suggested that upon binding of gp120 to CD4, conformational changes in gp120 and subsequent shedding of the gp120 molecule from virions would result in exposure of the gp41 fusion domain to the plasma membrane (Moore et al., 1990). However, gp120 shedding has only been found to occur with lab adapted strains of HIV-1 and not with primary HIV-1 isolates (Moore et al., 1993b; Thali et al., 1992). The gp41 fusion domain is probably more exposed upon binding because of conformational changes in gp120 upon binding to CD4 (Sattentau et al., 1991) and involvement of the V2 and V3 domains in the fusion process.

Fusion of the virus with the plasma membrane is induced by the HIV-1 transmembrane protein gp41 (Freed et al., 1991, 1993) Subsequent entry occurs primarily via a pH-independent mechanism (Stein et al., 1987, McClure et al., 1988), although endocytosis-mediated entry of HIV-1 has also been described (Pauza et al., 1988). The gp41 protein contains a single membrane spanning region, an internal domain and a hydrophobic external domain. The external domain contains a hydrophobic glycine rich fusion peptide that is essential for membrane fusion, and two regions containing a heptad repeat sequence motif characteristic of coiled coils. The internal domain of gp41 is associated with p17 MA protein molecules whereas the external domain is non-covalently associated with both N- and C- terminal domains of gp120 (Gelderblom et al., 1989; Yu et al., 1992). Mutational analysis revealed that the hydrophobic N-terminal part of gp41 contains target-cell specific fusion determinants, suggesting that gp41 may in part determine HIV-1 host range by recognition of a cell type-specific counterpart (Bergeron et al., 1992). The V2 and V3 domains of gp120 were also found to be determinants of HIV-1 host range and fusion capacity (Shioda et al., 1991; Sullivan et al., 1993). However, it remains unclear whether these domains of gp120 actively participate in the fusion process, or act solely in a conformation-dependent mechanism to induce the increased exposure of the fusion domain of gp41.

Information combined from structural studies, mutational analyses and studies with inhibitory peptides suggest a model of envelope-induced fusion that involves two interdependent conformational changes in gp41. Firstly, similar to Influenza virus, where the low pH environment of the endosome induces a conformational change in

the transmembrane protein (HA2A), binding of HIV-1 to the receptor complex frees the constrained N-terminal heptad repeat region of gp41 to assemble into the central coiled-coil, moving the fusion peptide in the process into a position where it would interact with the target cell membrane. The second stage would be the formation of the coiled-coil bundle. This structure is arranged such that the N-terminal fusion peptide and the C-terminal membrane spanning domain of gp41 are at the same end of the bundle. This formation would force the cell membrane and the viral membrane in close proximity, where the initiation of lipid mixing might occur.

1.4.3 Post-entry, Reverse transcription and Integration

After fusion of the virion with the plasma membrane, the inner core of the virion is released in the cytoplasm and rapidly uncoated (Thalli et al., 1992). The interaction with Gag polyprotein and the cellular protein, cyclophilin A (CyPA) is thought to play an important role in a step after virus entry. CyPA is incorporated into the virus core during assembly (Gamble et al., 1996) and is essential for the formation of infectious virions (Braaten et al., 1996). Viruses containing a mutation in Gag which do not interact with CyPA are not infectious and CyPA may act by destabilising Gag-Gag interactions and facilitate uncoating (Aiken, 1998). Phosphorylation of the Gag matrix protein (MA) by the cellular mitogen-activated protein kinase (MAPK) which is also virion associated, also acts post entry by releasing the MA-reverse transcription complex from the cell membrane and aiding nuclear translocation (Gallay et al., 1995; Jaque et al., 1998). In the remaining core particle, replication begins as for all retroviruses. The viral nucleic acids remain associated with a number of viral proteins required for converting the viral RNA genome to proviral DNA, transport of the complex to the nucleus and integration of the proviral DNA in the host genome (Bowerman et al., 1989). Conversion of viral RNA to proviral DNA is carried out by the virus encoded enzyme reverse transcriptase (RT).

Reverse transcription is initiated in the viral particle (Trono, 1992) and requires a single stranded template and a base paired primer with a free 3'OH end to initiate DNA synthesis (Baltimore et al., 1971). All retroviruses use a cellular-derived transfer RNA molecule (t-RNA) as a primer which is packaged along with the RNA genome in

virions (Verma et al., 1972). Figure 1.5 illustrates the individual events of reverse transcription of HIV-1 RNA to a proviral DNA copy.

1.4.4 Initiation of reverse transcription

For HIV-1 the primer is the t-RNA^{LYS}. An 18 nucleotide long region, the primer binding site (PBS, nucleotide 183-200) (Wain-Hobson et al., 1985; Raba et al., 1979), complementary to the 3' end of the t-RNA^{LYS} molecule has been identified on the HIV genome. The first 6 nucleotides of the PBS are the minimal requirement for the initiation of reverse transcription (Rhim et al., 1991), along with a proper secondary RNA structure around the PBS (Corbrinik et al., 1988) and a 27 - nucleotide long primer representing the 3' end of the tRNA (Cordell et al., 1979). HIV-1 RT binds to the tRNA, and to the duplex region of the primer template (Salafranque-Andreola et al., 1989; Huber et al., 1989). The nucleocapsid protein P6 of HIV-1 binds to HIV RNA through two conserved regions called Cys-His boxes (Gorelick et al., 1990) and is required for in vitro DNA synthesis (Barat et al., 1989).

1.4.5 Synthesis of the strong stop minus strand DNA

During the generation of the first DNA strand (minus strand) which is complementary to the plus strand RNA genome, synthesis ends at the 5' end of the RNA template. This short intermediate accumulates and is called "strong stop" DNA (Haseltine et al., 1976).

1.4.6 Degradation of RNA from the RNA-DNA hybrid

The RNA strand of the RNA-DNA duplex is removed by RT's RNAse H activity. RNAse H specifically degrades RNA present in the RNA-DNA duplex, and its activity is essential for retroviral replication (Tanese et al., 1991).

1.4.7 First transfer and elongation of the minus strand DNA

The transfer of the newly made DNA to the 3' end of the RNA template is facilitated by the complementary nature of the 3'R-region and the 5'R-region (Coffin et al., 1977). A transfer can occur before the complete R region is copied (Lobel & Goff, 1985). The first transfer is possibly an intermolecular event between the two RNA molecules and facilitated by a physical association of the two molecules (Panganiban & Fiore, 1988). The Strong stop DNA strand can be elongated after the transfer has taken place.

1.4.8 Generation of plus strand primer and initiation of "strong stop" plus strand DNA synthesis

An RNA fragment that functions as a plus strand primer is found at the 5' end of the plus strand DNA (Charneau & Clavel, 1991). This primer is generated by specific endonuclease activity of RT at a region containing multiple purines, the polypurine tract (PPT) (Omer et al., 1984). The PPT is essential for viral replication and is found at the 3' end of the retroviral genome of all retroviruses (Sorge & Hughes, 1982; Pullen & Champoux, 1990). Plus strand strong stop DNA synthesis is initiated before minus strand DNA synthesis is completed (Mitra et al., 1979). Plus strand synthesis is discontinuous, gapped positive strands are generated due to plus strand synthesis at additional sites (Kung et al., 1981). The strong stop primers are removed by RT-mediated cleavage at the primer- DNA junction (Huber & Richardson, 1990).

1.4.9 Removal of the tRNA primer and second transfer or jump

During strong stop plus strand synthesis only the first 18 nucleotides at the 3' end of the tRNA are transcribed (Rhim et al., 1991). RNAse H cleaves the tRNA from the minus strand DNA generating a free overhanging plus strand DNA segment facilitating the second transfer (Omer et al., 1982). The minus strand DNA becomes circular to facilitate the intramolecular transfer between the plus strand and minus strand of the partly double- stranded DNA (Panganiban & Fiore, 1988).

1.4.10 Completion of the minus strand and plus strand

Completion of the minus strand DNA requires strand displacement of a short stretch of double- stranded DNA and subsequent synthesis of the 5' end of the minus strand. The synthesis of DNA plus strand is completed in parallel to the completion of minus strand DNA. As a consequence of the two jumps, the provirus acquires additional flanking sequences, U_3 on the 5' side and U_5 on the 3' side.

1.4.11 Integration of HIV-1 proviral DNA

The HIV-1 proviral DNA is complexed with viral proteins into a high molecular weight nucleoprotein preintegration complex, and is transported to the nucleus by an active process, which requires ATP, but is independent of cell division. For other retroviruses, transport of high molecular weight preintegration complexes is dependent on mitosis (Burkrinsky et al., 1993a). The HIV-1 matrix and Vpr proteins present in the preintegration complex contain nuclear localisation signals that may facilitate nuclear transport in non-dividing cells (Burkrinsky et al., 1993b). However, efficient reverse transcription is still dependent on activation and proliferation of the host cell. Integrase (IN) protein is the only viral protein required for the integration of proviral DNA in the host genome. IN recognises specific sequences at both ends of the linear viral DNA, and cuts at the 3' terminii and also removes T and G nucleotides from the 3' end of the viral DNA. The HIV-1 integrase carries out an exonuclease step by mediating a random 5 base staggered cut in the chromosomal DNA. The processed 3' ends of the viral DNA are then ligated to the 5' ends of the 'open' chromosomal DNA. Finally both ends of the inserted viral DNA are repaired and joined to the 3' ends of the chromosomal DNA. The mechanism of integration was derived from analysis of both ends of the viral DNA and flanking host sequences (Vink et al., 1990; Pauza, 1990a) and from mutagenesis studies of HIV-1 integrase (Engelman et al., 1995). Integration of HIV-1 is essential for replication (Sakai et al., 1993). Non-integrated circular forms of proviral DNA, 1 and 2-LTR circles exist (Bukrinsky et al., 1993). The importance of unintegrated DNA in disease pathogenesis is unknown, however

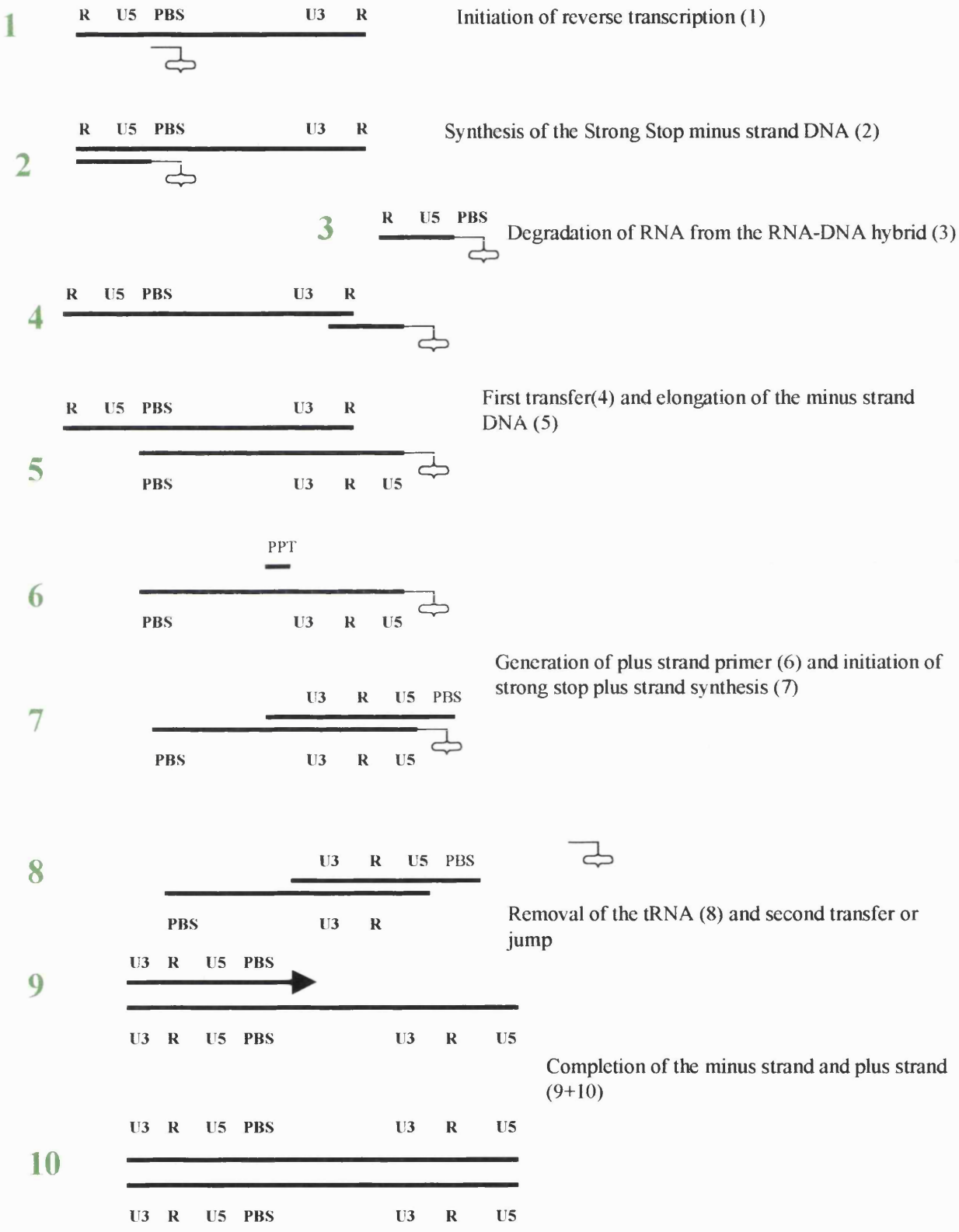


Figure 1.5 Outline of reverse transcription of HIV-1 RNA to a proviral DNA copy.

one study has demonstrated predominantly unintegrated DNA in T-cells derived from individuals who were asymptomatic, whereas in AIDS patients there was increase in integrated relative to extrachromosomal DNA forms.

1.4.12 HIV-1 gene expression

The integrated HIV-1 provirus is flanked by LTRs, generated during the process of reverse transcription. The 5' LTR functions to promote transcription, whereas the 3' LTR is required for efficient polyadenylation of the transcripts. Various ubiquitous host transcription factors that act via the 5' LTR such as Sp1 and TATA factor TFIID are required for HIV-1 gene expression (Garcia et al., 1987). HIV-1 expression is strongly dependent on cellular transcription factors such as NFAT-1, USF, TCF-1 alpha, AP-1 and most important NF-kB, whose activities are induced upon cellular activation by mitogenic or non-mitogenic stimuli and cytokines (Koyanagi et al., 1988; Poli et al., 1990). LTR - driven gene expression can also be activated by proteins from a variety of DNA viruses and retroviruses (Barry et al., 1990; Gendleman et al., 1986; Greene et al., 1989). The HIV-1 encoded protein Tat is a potent transactivator of HIV-1 transcription, which acts via the transactivation responsive region (TAR) in the 5' LTR. Tat binds to the TAR RNA stem - loop structure resulting in increased steady state levels of LTR - driven transcripts (Cullen, 1986). Tat induced transactivation may result from both increased transcription initiation and elongation. The Nef protein is dispensable for HIV-1 replication in transformed T cell lines *in vitro*, but is required for efficient replication in primary lymphocytes and macrophages (De Ronde et al., 1992), and is important in SIV in maintaining high viral load in *vivo* (Kestler et., 1991).

1.4.13 Viral RNA packaging, assembly and budding of the virion

Viral RNA represents only a small fraction of the total cellular RNA in an infected cell but it is specifically packaged into virions. The region of RNA responsible for direct packaging is referred to as the packaging signal (psi) or the encapsidation signal (E)

and this signal is composed of a series of specific sequences forming complex secondary structures. Deletions within the region between the splice donor and the Gag initiation codon of HIV-1 have reduced the incorporation of HIV-1 RNA into budding virions (Lever et al., 1989), and another region within the first 40 nucleotides of gag has also been shown to be important for RNA packaging (Luban and Goff., 1994). The RNA secondary structure of sequences that play a role in packaging has been mapped (Harrision and Lever, 1992) and mutations within the hairpins that flank the splice donor site have been shown to reduce packaging of RNA (Berkhout and van Wamel, 1996, Laughrea et al., 1997).

The myristoylated Gag and Gag-Pol precursor proteins aggregate at the inner cell membrane forming a spherical structure. Encapsidation of the viral genomic RNA in this structure is dependent on NC protein and the RNA packaging signal present between the major splice donor site of HIV-1 and the gag initiation codon. The assembled core-RNA complex buds through the plasma membrane where it acquires the lipid membrane, complete with viral envelope and host proteins. During this budding process, HIV-1 protease cleaves the Gag and Gag-Pol precursor proteins into functional proteins resulting in a mature virion. Vif is thought to play an essential role during virion formation since HIV-1 virions produced in the absence of Vif are less infectious. Vif itself is not packaged in virions but may play an essential role in virion assembly. The only accessory protein known to be incorporated into virions is Vpr. Vpr confers growth advantages to HIV-1 isolates expressing the protein, particularly in macrophages. Vpr may be required for early steps of the HIV-1 replication cycle such as nuclear transport of the preintegration complex. Vpr may also induce cell-differentiation and activation and in this way facilitate HIV-1 replication.

1.4.13.1 Cellular proteins in HIV-1 virions

HIV-1 contains both virus encoded and cellular proteins. The acquisition of host proteins could influence viral pathogenesis *in vivo* by providing a function early during infection of a cell, interacting with virion proteins during the assembly process or could be nonspecifically incorporated. Both the surface and the interior of the virion can contain cellular proteins.

Histocompatability leukocyte antigen class II (HLA class II) is the most commonly found host protein found on the surface of HIV-1. Virus grown in H9 cells can contain up to 600 molecules of HLA II which is greater than the estimated 220 molecules of gp120 present (Arthur et al., 1992). HLA class II has also been demonstrated on the surface of virions from patient plasma and virions produced *in vitro* from PBMC (Saarloos et a., 1997). The presence of HLA II can have a slightly positive effect on virus infectivity (Cantin et al., 1997) and it has been demonstrated that HLA II on HIV-1 can present superantigen to resting T cells which may result in apoptosis without the appropriate secondary signals (Rosio et al., 1995).

HLA class I is present on the HIV-1 virion at lower levels than HLA II. The role played by HLA class I on the virion surface is unclear as one study has demonstrated that human HLA I immunisation confers partial protection in macaques challenged with SIV (Chan et al., 1995), while a second study showed no protection with HLA class I immunisation in macaques (Arthur et al., 1992).

A number of adhesion molecules have also been found on the surface of HIV-1 including CD44, LFA-I and ICAM-I. The incorporation of ICAM-I has been shown to increase the infectivity of recombinant viruses possibly by binding to LFA-I on CD4+ cells (Fortin et al., 1997) and the presence of adhesion molecules may permit virus binding to CD4 negative cells (Castilletti et al., 1995).

Several cellular proteins have been identified inside the virus particle including cyclophilin A, actin cytoskeletal proteins and ubiquitin and these proteins play a role in the assembly of the virion. Cyclophilin A, an abundant cellular protein has been found inside the virion and also bound to the Gag protein (Thali et al., 1994, Ott et al., 1995). Cyclophilin A is thought to provide a chaperone activity by assisting in the folding of p24 capsid protein inside the cell and may also be involved in the uncoating of the virion in an early step during infection (Luban, 1996., Braten et al., 1996). Actin is a major structural protein in cells and forms filaments and lattice structures, it is associated with the cortical plasma membrane and may play a role in budding of retroviruses (Damsky et al., 1997). Assembling Gag proteins are thought to associate

with structural actin filaments and immunofluorescence and cell fractionation studies have demonstrated HIV-1 Gag polyprotein associated with internal actin filaments (Rey et al., 1996).

1.5 HIV-1 Phenotype, Variability and AIDS Pathogenesis

HIV-1 variability plays an important role in the pathogenesis of AIDS and due to rapid genetic changes HIV-1 *in vivo* is defined as a quasispecies, which is a population of highly related, yet genetically distinct viruses within the same individual (Wain-Hobson, 1992). The error prone nature of HIV-1 contributes to the high degree of genetic variation, this is due to the lack of a proof reading mechanism in HIV-1 reverse transcriptase, while mutation rate studies have demonstrated that the HIV-1 genome has a rate of 0.3 nucleotide change per cycle of virus replication (Mansky and Temin, 1995).

HIV-1 isolates obtained from infected individuals at the early asymptomatic stage of infection generally display low replication rates, lack syncytium inducing (SI) capacity and replicate efficiently in macrophages but not in transformed T-cell lines (Asjo et al., 1986; Tersmette et al., 1988). In the progressive stages of HIV-1 infection, the majority of virus isolates present in infected individuals display increased replication rates and decreased capacity to replicate in macrophages (Cheng-Mayer et al., 1988; Tersmette et al., 1988). In 50 to 60% of infected individuals, SI HIV-1 isolates emerge in the course of infection, which have the capacity to replicate in transformed T cell lines (Koot et al., 1992; Tersmette et al., 1989).

1.5.1 Cellular host range

HIV-1 replicates primarily in CD4 positive T cells and macrophages. Peripheral blood mononuclear cells are susceptible to both SI and NSI HIV-1 variants, whereas transformed T cell lines are relatively non-susceptible to NSI HIV-1 isolates (Koot et al., 1992; Schuitemaker et al., 1991, 1992). In contrast to the majority of SI HIV-1 isolates, NSI HIV-1 isolates replicate efficiently in macrophages (Schuitemaker et al., 1992). Macrophages are thought to be the primary target cells upon transmission of HIV-1 (Schuitemaker et al., 1994). Early after infection, the virus population present in infected individuals may be heterogeneous based on sequence variation in the gag-

gene of HIV-1, but limited variation in the V3 domain of gp120 molecule suggests a strong selection for replication of viruses with NSI, macrophage tropic phenotype (Chesebro et al., 1992; Fouchier et al., 1992; Kuiken et al., 1992; Zhang et al., 1993). Analysis of biological properties of HIV-1 isolates in donor recipient pairs indicated that the transmitted virus generally displays NSI, macrophage tropic phenotype, even if this virus represented a minority of HIV-1 variants in the donor (Scarlatti et al., 1993; Van 't Wout et al., 1994). This suggests that NSI, macrophage tropic isolates are either selectively transmitted as a result of macrophages being the first encountered target cells (Mann et al., 1990), or that there is a selective outgrowth of NSI, macrophage tropic isolates upon transmission of phenotypically diverse HIV isolates (Schuitemaker et al., 1994). Macrophages in a broad variety of tissues may become infected by HIV-1, disseminating the virus throughout the body (Folks et al., 1988; Gartner et al., 1986; Koenig et al., 1986). The HIV-1 infected macrophages are thought to function as a viral reservoir *in vivo* (Gendelman et al., 1985) and NSI, macrophage tropic HIV-1 isolates remain present in HIV-1 infected individuals during all stages of infection (Schuitemaker et al., 1991; 1992).

Other cells of lymphocytic origin such as Langerhans' cells, microglial cells and peripheral blood dendritic cells have been described to be susceptible to infection by HIV-1 and may thus represent important target cells for transmission (Braathen et al., 1987; Langhoff et al., 1991; Patterson & Knight. 1987). Other studies have demonstrated that skin dendritic cells are relatively non-susceptible to infection by HIV-1 but they trap and effectively deliver the virus to T cells (Pope et al., 1994). Other cell types may also be susceptible to HIV-1 infection *in vitro* including muscle cells, B cells, colorectal cells, fibroblasts and hepatocytes. However These cells lack CD4 expression, and there is no evidence that they are infected *in vivo* (Cao et al., 1990; Clapham et al., 1989; Levy et al., 1993; Tersmette et al., 1985).

1.5.2 Role of Chemokine Receptors in Disease Progression

The majority of HIV-1 strains that initiate infection and persist through the early phase of infection are macrophage tropic/non-syncytium inducing isolates. As disease progresses T-cell tropic / syncytium inducing isolates emerge. Studies have shown that

HIV isolates obtained from patients early after seroconversion use CCR5, whereas isolates obtained later in disease have expanded their coreceptor usage to include other members of both the CC and CXC chemokine receptors (Connor et al., 1997). Patients who remain long-term non-progressors demonstrate exclusive use of CCR5 (Xiao et al., 1998) and extensive use of chemokine receptors has been shown to be associated with a single variant species of HIV-1 rather than the presence of quasispecies. Dual tropism for both CCR5 and CXCR4 may evolve in CCR5 restricted viral strains through acquisition of the ability to utilise the first and second extracellular loops of CXCR4 (Benson et al., 1997), and these viruses may be seen as intermediate forms during disease progression. Disease progression and expansion of chemokine receptor usage can also be associated with viral resistance to CC chemokine (Rantes, MIP-1alpha, MIP-1beta) blocking (Connor et al., 1997). CC chemokines may exert a selective pressure *in vivo* allowing the emergence of virus that evade their control possibly through mutation of the V3 region of the envelope gene (Scarlatti et al., 1997). Chemokine receptor expression may change dramatically and upregulation and downregulation of individual receptors may occur through an immune modulating event influencing the cell type or number of cellular targets for infection.

1.5.3 Protective alleles of chemokine receptor genes

With the identification of CCR5 as the major coreceptor for macrophage-tropic strains, a polymorphism in CCR5 was discovered that renders homozygous individuals resistant to virus infection (Liu et al., 1996). This polymorphism, most commonly found in the Caucasian population is a 32 base pair deletion resulting in the production of a truncated molecule which is not expressed at the cell surface (Samson et al., 1996; Hunag et al., 1997; Rana et al., 1997). Resistance to infection conferred by the allele is not absolute as several reports have described HIV infected CRC deletion homozygous (Theodore et al., 1997; Bit et al., 1997) indicating that receptors other that CRC may play a role in HIV transmission under certain conditions. A slower progression to AIDS and death has been associated with CRC deletion heterozygous and this has been attributed to a reduced cell surface expression of the coreceptor (Dean et al., 1996; Wu et al., 1997). Another polymorphism associated with delayed disease progression is a single conserved amino acid change (Val - Ile) in the first

transmembrane domain of CCR2b (CCR2b-64I). CCR2b-64I is found in all ethnic groups (Smith et al., 1997) and is in strong linkage disequilibrium with the 32bp CCR5 deletion. Heterozygosity for either allele confers an independent and similar degree of protection from disease progression (Smith et al., 1997). Another much rarer mutation CCR5 - m303 with similar effects to the 32bp deletion has also been described (Quillent et al., 1998). Further support for the concept of protection or improved clinical benefit has been demonstrated in a mutation of the gene encoding the chemokine stromal derived factor 1 (SDF-1), individuals homozygous for a common allele in the 3' untranslated region of the SDF-1 gene transcript (SDF1-3'UTR-801G-A, abbreviated to SDF1-3'A) are also protected from progression to AIDS and death (Winkler et al., 1998). The SDF-1 chemokine is the principle ligand for CXCR4, the major coreceptor for T-cell tropic strains which occur later in disease. Homozygotes for SDF-3'A may express higher levels of SDF-1 which could delay the switch to T-cell tropic HIV strains by competing with the virus for CXCR4. Protection seen in SDF1-3'A homozygotes appears late in the course of disease (Winkler et al., 1998). However a recent study has shown contrasting results with individuals homozygous for the SDF-3'A genotype showing a more rapid progression to AIDS and subsequently followed by an elongated survival time after AIDS diagnosis (van Rij et al., 1998). The protection from disease progression associated with these mutations is longer than treatment with zidovudine and the therapeutic extension of their modes of action could hold promise.

1.5.4 Mechanisms of CD4 T-lymphocyte dysfunction and destruction

An early hallmark of HIV infection prior to the significant depletion in CD4 cells was the recognition of impairment of a variety of T-cell functions. Proliferative responses to a range of stimuli are diminished in all stages of disease. The earliest T-cell proliferative response to be lost is the response to recall antigens such as Tetanus toxoid, Influenza, *Candida albicans* and *Cryptococcus neoformans*. Further immune dysfunction has been attributed to a switch in cytokine production in CD4+ T cells from IL2 and interferon gamma (Th1) to secretion of IL-4 and IL-10 (Th2) (Clerici and Shearer., 1993). Abnormalities occur in both the presence and absence of direct infection, direct killing of CD4 cells may deplete certain functional cellular subsets.

Down modulation of CD4 expression by CD4 - gp120 complexes of other viral proteins reduces MHC class II-CD4 interactions, which could cause a decline in antigen specific responses. Cross-linking of CD4 by gp120 binding in the presence of anti-gp120 antibodies can also cause suppression of T-cell activation.

A number of mechanisms have been proposed for the *in vitro* killing of single cells and the predominant process acting *in vivo* is unclear. Direct killing of single cells could happen through a combination of massive budding of virions from the cell surface leading to disruption of the integrity of the cell membrane and also interference with cellular RNA processing and protein synthesis. Uninfected CD4 cells can be killed by fusion with an infected cell, leading to the formation of a multinucleated giant cell. Soluble gp120 shed from virions and infected cells can bind to CD4 on uninfected cells and following binding of anti-gp120 antibody, cells can be eliminated by antibody-dependent cell mediated cytotoxicity (ADCC).

A reduction in CD4 T-cell regeneration could be due to destruction of lymphoid precursor cells and disruption of the microenvironment required for efficient regeneration of immune competent cells. Apoptosis or programmed death of T-lymphocytes could occur through the direct effects of HIV-1 proteins or as a consequence of an activated immune system. Tat has been shown to sensitise cells to apoptosis triggered by a second signal such as CD95 (Westendorp et al., 1995) while Vpr induces apoptosis in T-cells following cell-cycle arrest at the G2 phase (Stewart et al., 1997). A relationship between increased lymphocyte apoptosis in AIDS and the activation state of the immune system was demonstrated by the majority of apoptotic cells exhibiting an activated phenotype (Meeyaard et al., 1997). The CD95 system plays an important role in termination of an immune response and hyperactivation of the CD95 pathway during HIV infection may result in elimination of infected and non-infected cells. Increased levels of CD95 and CD95L molecules have been demonstrated on lymphocytes of HIV infected patients accompanied with increased susceptibility to apoptosis (Gougeon et al., 1997). A strong downregulation of increased CD95L expression has been associated with a decrease in viral load following effective antiretroviral combination therapy (Bohler et al., 1997).

1.6 Pathogenesis of HIV in clinical terms

Clinical HIV disease is characterised by a slow progressive loss of immunocompetence. The first sign that a patient has developed HIV infection is a seroconversion illness, which may mimic many of the acute viral illnesses prevalent in the community. The illness develops between two to six weeks following exposure but does not occur in all patients, and is characterised by fever, fatigue, rash and headache. Development of a seroconversion illness has been shown to be indicative of a more severe HIV course (Lindback et al., 1994). Early diagnosis and possible intervention with anti-retroviral therapy may have an effect on the prognosis of the HIV infected patient since it has been demonstrated that HIV RNA viral load measurements taken four to six months post seroconversion may be predictive of subsequent clinical progression (Mellors et al., 1995). During the asymptomatic phase patients experience minimal clinical manifestations and CD4 count can be between 200-500/ul although the rate of HIV replication is ongoing with high levels of CD4 cells being produced and destroyed by the virus each day (Wei et al., 1995). During this phase the clinical emphasis is on initiating and optimising antiretroviral therapy. Over time, with the competence of the immune system gradually declining minor manifestations of immunodeficiency are evident such as shingles, lymphadenopathy, oral hairy leukoplakia often accompanied by weight loss, fever or diarrhea. As immunodeficiency progresses and CD4 count drops to below 200/ul more serious manifestations appear such as *Pneumocystis carinii* pneumonia, further complications such as cerebral toxoplasmosis, infection with CMV or development of *Mycobacteria avium* complex are accompanied with a decline in CD4 count to below 100/ul. The major opportunistic infections associated with a progressive HIV infection and a deteriorating immune system are described in the following sections.

1.7 Opportunistic Infections

The ability of opportunistic infections to take advantage of a weakened cell mediated immune function were a hallmark of the first AIDS cases described in early 1981 when an increase in *pneumocystis carinii* pneumonia was reported among gay men

(Masaur et al., 1981). Numerous other opportunistic infections of AIDS have been recognised including cytomegalovirus (CMV) retinitis, disseminated mycobacterium avium (MAC) infection, cryptococcal meningitis and toxoplasma encephalitis. Fundamental to the development of opportunistic infections in the HIV-1 infected patient is the relationship with immune dysfunction as measured by CD4 count. With an increased risk of opportunistic infection when the CD4 count drops below 200-250/mm^3 and the majority of infections developing when CD4 count is below 100/mm^3. In the present era of HAART and the restoration of immune function, the continued need for prophylaxis and maintenance therapy for opportunistic infections is under debate as it may be possible to discontinue anti-CMV and anti-PCP treatment for patients.

1.7.1 Protozoal Infections

Protozoal infections associated with HIV-1 include *Pneumocystis carinii* and *Toxoplasma gondii*. Prior to AIDS *Pneumocystis carinii* pneumonia (PCP) was mainly seen in children with acute lymphocytic leukemia and before the widespread use PCP prophlylaxis up to 80% of late stage HIV-1 infected patients in the USA developed PCP. The most common clinical manifestation of *Pneumocystis carinii* infection is pneumonia. Parenteral treatment can include cotrimoxizole or intravenous pentamidine, both of which have associated side effects such as rash and fever and pancreatitis respectively. An oral regimen of Dapsone and an oral regimen of Dapsone and trimethoprim has proved to be beneficial (Medina et al., 1990). *Toxoplasma gondii*, a ubiquitous intracellular protozoan parasite is the most common cause of opportunistic infection of the central nervous system in patients with AIDS (Luft and Remington., 1992). Cats serve as the major host for the organism, along with secondary hosts such as birds and other domesticated animals. The major means of transmission of *Toxoplasma gondii* is by ingestion of uncooked meats containing tissue cysts or vegetables contaminated with oocysts and also by direct contact with cat faeces. Toxoplasma seroprevalence in HIV-1 infected patients in the U.S. has been reported to be between 15% and 40% and incidence of Toxoplasma encephalitis in AIDS patients has been reported to be 5% (Grant et al., 1990). The broad tropism of Toxoplasma for various cell types in the central nervous system can cause clinical manifestations including neuro-psychiatric dysfunction. Diagnosis usually involves

neuroradiological imaging for characteristic lesions in the brain. The main therapeutic agent for treatment is pyrimethamine, which has an associated toxicity of bone marrow suppression (Dannemann et al., 1992). Protozoan agents such as *Cryptosporidium* and *Isospora belli* can cause gastrointestinal infections particularly diarrhea, which is one of the most common problems experienced by HIV infected patients. *Cryptosporidium* is an intracellular protozoan that can be spread through contamination of potable water or by animal to person. Treatment is usually with nitazoxanide, paromomycin or azithromycin. *Isospora belli* can cause severe diarrheal illness in AIDS and is most common in sub-saharan Africa, the agent is responsive to cortrimoxazole treatment.

1.7.2 Viral Infections

The principle clinical manifestation of Cytomegalovirus (CMV) in AIDS patients has been chorioretinitis which if untreated leads to blindness, while other complications include gastrointestinal disease and neurological disease. A high seroprevalence of CMV has been found among gay males with lesser rates associated with haemophiliacs and those infected heterosexually (Drew et al., 1981; Webster et al., 1989). CMV retinitis is the best characterised end organ disease and the virus causes a necrotising infection where retinal areas are permanently destroyed with accompanying visual loss, and retinal detachment is a frequent complication. Gastrointestinal disease is another frequent end organ manifestation, esophagitis and colitis are the two most common clinical syndromes. Other CMV syndromes include pneumonitis, adrenalitis and CMV encephalitis.

Ganciclovir was the first approved anti-CMV agent and competitively inhibits the viral DNA polymerase. The principle toxicity associated with ganciclovir is neutropenia when combined with zidovudine (Hochster et al., 1990). Another anti-CMV agent is foscarnet which also inhibits DNA polymerase and its associated side effects include proximal and renal tube toxicity (Palestine et al., 1991). Cidofovir which has a longer half life than ganciclovir or foscarnet has increased renal toxicity (Flores-Aguilar et al., 1994). Infection with Varicella zoster virus (VZV) is another complication seen in late stage disease the usual clinical manifestation is reactivation disease seen in the form of dermatomal zoster. Treatment is with one of the usual anti-

herpes agents such as acyclovir, valacyclovir or famciclovir. Herpes Simplex Virus (HSV) infection causes recurrent chronic mucocutaneous ulcers in and around the mouth (HSV-1) and perianal regions (HSV-2), treatment is with one of the anti-herpes agents and if drug resistance is suspected, treatment with foscarnet is also possible (Erlich et al., 1989).

Progressive multifocal leukoencephalopathy (PML) caused by JC virus (JCV) can affect patients in late stage disease (Berger et al., 1987). The disease is characterised by infection of oligodendrocytes in the brain leading to demyelination. These white matter lesions can be demonstrated by neuroradiological imaging, and patients suffer neurological deficits. No specific treatment is available but the incidence of PML has declined rapidly following the introduction of HAART.

1.7.3 Fungal Infections

Mucosal candidal infections were first seen as a significant clinical problem in the HIV epidemic, arising as a result of immune dysfunction. The most common location for candidiasis are oral, esophageal and vaginal sites with thrush (Pseudomembranous candidiasis) being the most frequent oral form (Katz et al., 1992). Treatment for oral candidiasis is with topical antifungal agents such as Nystatin, Clortrimazole and Fluconazole. A fungal infection previously seen in AIDS is *Cryptococcus neoformans* causing cryptococcal meningitis. Like *Histoplasma capsulatum* it is found in high concentrations in the faeces of birds and infection is thought to be by inhalation. The most common clinical manifestation is meningitis and Amphotericin B is widely used as treatment for both fungal infections (Kovacs et al., 1988; Sarosi et al., 1992).

1.7.4 Mycobacterium infections

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), can cause disease in both immunocompromised and non-immunocompromised persons. In the mid 1980's disease was increasing particularly in the USA after a significant period of declining incidence (Burwen et al., 1995). The increased incidence was particularly seen in HIV infected persons and in other socio-economic groups. In Africa, TB is one

of the most common opportunistic infections in HIV infected persons and most cases arise as a result of reactivation of latent infection (Mukadi et al., 1993). TB can manifest as both pulmonary and extrapulmonary disease and a further complication can be the development of multidrug-resistant TB (MDRTB). MDRTB isolates are found in newly acquired infection and are resistant to at least isoniazid and rifampin (Small et al., 1991). *Mycobacterium avium* complex (MAC), a non-tuberculosis mycobacterium, has also shown increased incidence in late stage HIV disease. The organism is ubiquitous in the environment, and the most common clinical manifestation of MAC is a disseminated disease with fever, anorexia and weight loss. Clarithromycin and azithromycin have shown good activity against MAC and in preventing disseminated infection (Havlir et al., 1996).

1.7.5 Bacterial Infections

Bacterial pneumonias have occurred in the late stage of HIV disease and it is thought that humoral immune dysfunction is the major predisposition. The two most common pathogens are *Streptococcus pneumoniae* and *Haemophilus influenzae* which are usually community acquired and their clinical manifestation includes fever, cough and variable pleuritic chest pain (Redd et al., 1990). Patients with bacterial pneumonia typically respond well to therapy such as cotrimoxazole and second and third generation cephalosporins have also been used.

The incidence of syphilis among gay men has declined significantly through the institution of safe sex practices (Rolfs et al., 1990). The disease is caused by the spirochete *Treponema pallidum* and is a complex and staged systemic illness. Primary syphilis is manifested as a chancre or painless ulcer at the site of initial infection which can heal within weeks. Secondary syphilis which can occur up to eight weeks later is more disseminated and consists of a non-vesicular rash beginning on the trunk which can spread to include the palms and soles. Mucosal lesions and CNS involvement are common. During the latent phase, the next progressive stage, there are no clinical manifestations. The final stage known as tertiary syphilis is a slowly progressive inflammatory illness that can affect any organ in the body and can be manifested as neurosyphilis, cardiovascular syphilis or gummatous syphilis.

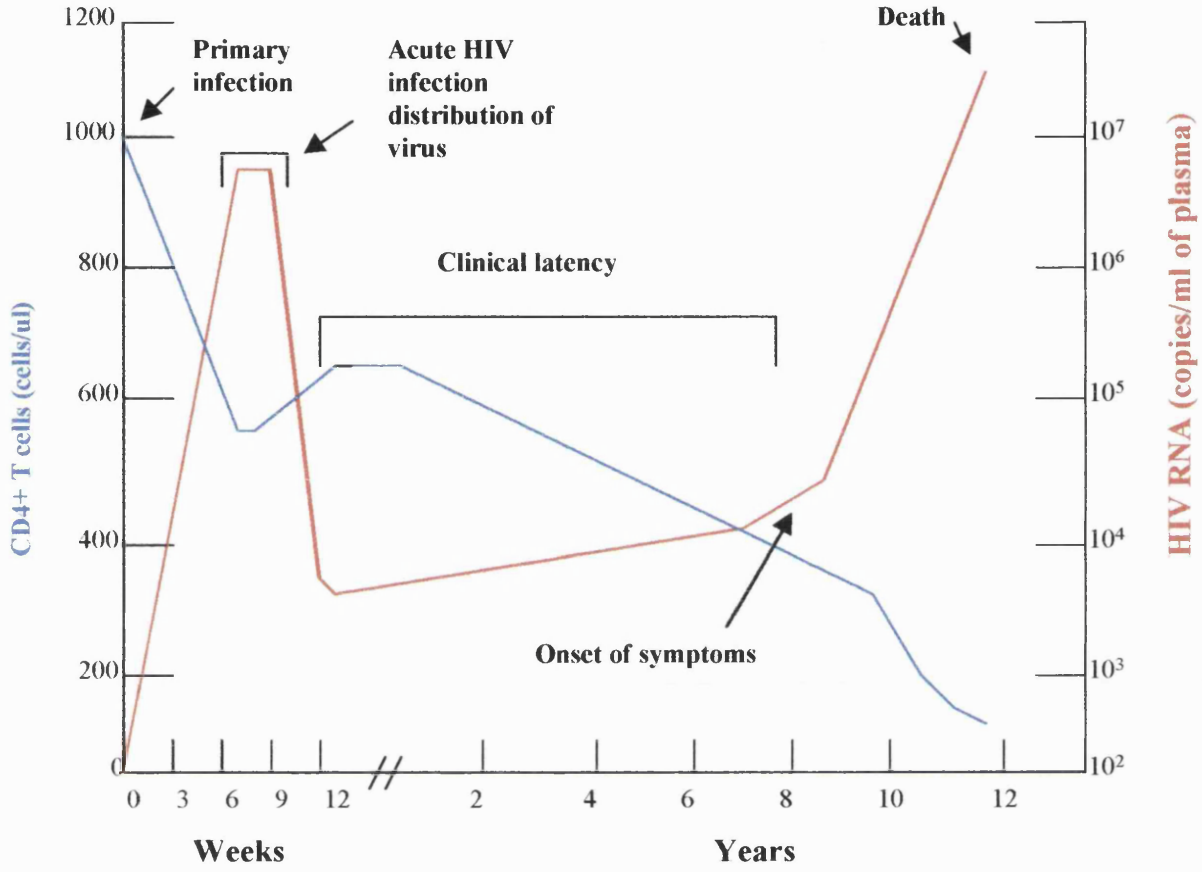


Figure 1.6 Schematic outline of a typical course of HIV infection demonstrating the initial primary infection followed by the acute infection stage or seroconversion illness. The pattern demonstrates the gradual decrease in CD4 T cell count (Blue) with an increase in HIV viral RNA load (Red).

1.8 Virus Load and HIV-1 disease progression

After the initial burst of virus replication in primary HIV-1 infection (Clark et al., 1991; Daar et al., 1991), the frequency of infected cells is relatively low in the early asymptomatic stage and increases at later stages of infection (Connor et al., 1994; Coombs et al., 1989; Ho et al., 1989). Gradual impairment of both cellular and humoral immune control at later stages of infection may allow an increase in the numbers of virus infected cells and free virions in plasma (Tepstra et al., 1989). The increase in virus load may reflect changes in the biological properties of HIV-1 isolates. Slow replication rates and limited T cell host range of macrophage tropic NSI isolates in early asymptomatic HIV-1 infection may limit virus load in the blood. As the disease progresses T cell tropic SI variants emerge which may be responsible for increased virus load (Connor & Ho., 1994; Schuitemaker et al., 1992; Tersmette et al., 1989). Figure 1.6 illustrates a typical course of HIV-1 infection, demonstrating a gradual decrease in CD4 cell count with increasing viral RNA load.

Lymphoid organs are a primary site for the establishment and propagation of HIV-1 infection over time (Pantaleo et al., 1993). The ability of HIV-1 to replicate in target cells is dependent on the activation of these cells. Since lymphoid organs contain substantial numbers of immune activated T cells, they are an important reservoir for replicating HIV-1 during the early stages of infection. In asymptomatic individuals a greater frequency of HIV-1 infected cells have been detected in the lymphoid organs compared with the peripheral blood. The number of cells containing HIV-1 DNA or yielding infectious virus was found to be 0.5-1 log higher in lymph nodes than in PBMC of HIV-1 infected individuals. Expression of both structural and regulatory HIV-1 specific mRNA was also detectable at high levels in lymph node mononuclear cells (Pantaleo et al., 1993). The quantity of HIV-1 RNA in the germinal centres was found to decrease with progression of the disease, as did the number of follicular dendritic cells. It has been suggested that active viral replication in PBMC would indicate that significant damage in the lymphatic tissues has already occurred. In the early stages of HIV-1 disease, large amounts of virus particles are found in lymph

nodes in association with follicular dendritic cells (FDCs), which play an important role in processing and presenting antigen to CD4 T lymphocytes. As HIV-1 disease progresses, there is gradual destruction of the FDC network and the normal lymph node architecture is disrupted, which may contribute to the deterioration of normal immune function (Pantaleo et al., 1993; Graziosi et al., 1993).

Initial efforts to devise a model of the pathogenic consequences of HIV-1 infection were based on the idea that very few cells in infected individuals contain or express HIV-1 and that virus replication is restricted during the period of clinical latency. However, more sensitive methods of virus detection and quantitation of HIV-1 RNA (Piatak et al., 1993a) have shown that HIV-1 replication is active throughout the course of infection and that the level of viremia, as measured by the amount of HIV-1 RNA in the plasma, accurately reflects the extent of virus replication in an infected person. Plasma HIV-1 RNA can be detected in virtually all HIV-1 individuals although its concentration can vary widely depending on the stage of infection.

During primary HIV-1 infection concentrations of plasma HIV-1 RNA can exceed 10^7 copies per ml (Piatak et al., 1993b) and after 8 to 12 weeks these levels can be reduced by approximately 100-fold with the emergence of an antiviral cytotoxic T-cell (CTL) immune response (Koup et al., 1994). Concentrations of HIV-1 RNA decline over a period of time to a so called 'set point'. The determinants of the 'set point' of plasma viral RNA concentrations are unknown, but may include the effectiveness of the host antiviral immune response, the number of target cells available for infection, the replicating capacity of the infecting strain of HIV-1 and the degree of immune activation or age of an individual. In most HIV-1 infected individuals the set point RNA concentrations are between 10^3 and 10^5 copies per ml and increasing plasma HIV-1 can indicate the development of advancing immunodeficiency and AIDS.

The steady state concentration of HIV-1 RNA present in the plasma is a function of virus turnover (rates of production and clearance of virus) in circulation. To assess the kinetic rate of virus turnover requires that some disturbance of the steady system is provided so that measurement of viral clearance, the magnitude of virus production and the longevity of virus-producing cells be determined. The initiation of an effective

antiviral therapy in persons with moderate to advanced HIV-1 disease has allowed virus turnover to be measured, revealing a very dynamic process of virus production and clearance (Wei et al., 1995; Ho et al., 1995). Measurement of the slope of the initial fall in viraemia after initiation of potent antiviral therapy permits the calculation of the half life of clearance of HIV-1 virions. The half life of virions in circulation is about 6 hours, or half the population of HIV-1 virions turns over every six hours. Clearance rates do not vary substantially between persons with different pre-treatment CD4 counts or plasma HIV-1 RNA concentrations. The main determinant of steady state concentrations of HIV-1 in an infected person is the amount of virus production taking place and to maintain the steady state level of virus in persons with moderate to advanced disease, 10^8 to 10^9 virions must be produced and cleared each day. The rapid decline of plasma HIV-1 RNA with the introduction of an antiviral therapy indicates that when new rounds of virus infection are blocked, virus production from infected cells continues for about 2 days, with the average half-life of an infected cell about 1.6 days. The estimated average generation time of HIV-1 (the time from release of a virion from one cell until it infects another cell and results in the release of a new generation of virions) is approximately 2.5 days. These studies have also shown that more than 99% of virus production comes from recently infected cells rather than from long lived chronically infected cells or from latently infected cells recently activated to produce virus.

Together with the rapid fall in HIV-1 RNA in treated patients, there is a large increase in the level of circulating CD4 cells which probably represents cells that were uninfected by HIV-1 and proliferated in an attempt to maintain levels of host T-cell numbers. Based on the rate of increased CD4 cell numbers in treated patients, it is estimated that 5% of the total body CD4 T-cell population are being destroyed each day. To maintain stable CD4 cell numbers, an equal number of T cells must be produced each day by the immune system. The importance of viral load measurement as a prognostic marker of HIV-1 infection, in particular the steady state viral load or set point seen at the end of the primary phase of infection, has been shown by Mellors et al (1996) to strongly predict the rate of decrease of CD4+ lymphocytes and also the progression AIDS and death. In this prospective study of a large cohort of HIV infected men, it was shown that individuals with viral loads in the highest quartile had

almost a three fold shorter time of progression to disease than individuals whose viral load was within the lowest quartile. Furthermore, viral load was a better indicator of disease progression than baseline CD4 count. Viral load measurements are now routinely used to monitor antiretroviral therapy.

1.8.1 Viral Dynamics

Important insights into the complex host - pathogen interaction in HIV infection have been revealed by direct measurement of circulating free virus. The rapid exponential drop in plasma virus levels by protease and reverse transcriptase inhibitors allowed Ho et al (1995) and Wei et al (1995) to demonstrate that HIV-1 infection is a dynamic process, with continual new rounds of viral particles produced by recently infected cells. In a further study by Perelson et al. (1996), plasma virus levels were examined early after the initiation of therapy so that the rate of clearance of free virions and the loss of the infected cells producing plasma could be calculated. The decay rate constant for free virus (t1/2<6hr) and for the cells that produce most of the plasma virus (t1/2 = 1-2 days) were similar in different patients, suggesting a constant decay rate for a particular compartment. This rapid turnover of productively infected cells has also been demonstrated by *in situ* hybridisation for viral RNA in tonsillar biopsies from patients starting antiretroviral therapy (Cavert et al., 1997). The rate of virus production has been suggested to be in excess of 10^{10} virions per day (Perelson et al., 1996) and that the rate of new infection of CD4 cells is substantial.

Further studies, using combinations of antiretroviral drugs reduced plasma virus levels to undetectable levels (Gullick et al., 1997) and analysis of virus decay rates revealed a rapid initial decay during the first 1-2 weeks of treatment followed by a slower decay. This second phase of decay may represent the turnover of a longer-lived viral reservoir with an estimated half-life of 1-4 weeks (Perelson et al., 1997). Macrophages may represent the viral reservoir responsible for the slower phase of decay and virions trapped on follicular dendritic cells have also been shown to decline in this time scale (Cavert et al., 1997). Analysis of the second phase of decay rates has allowed a prediction of treatment times required for virus eradication of 2-3 years of completely

suppressive antiretroviral therapy, assuming no additional reservoirs of HIV exist. Recent studies have shown that latently infected resting memory CD4+ T cells with integrated provirus represent a stable reservoir of HIV-1. The frequency of these cells with integrated DNA is low (< 0.05%) in both blood and lymph nodes (Chun et al., 1995; Chun et al., 1997) and these cells present with a CD45RO+ memory phenotype. Enhanced virus culture methods have also induced recovery of replication competent virus from these cells (Chun et al., 1997a). The frequency of latently infected CD4+ T cells have shown no detectable decrease during the first two years of antiretroviral therapy (Finzi et al., 1997), suggesting an extremely slow decay rate for this compartment. Further evidence for the long lived nature of this reservoir was demonstrated by sequence analysis of isolated viruses which showed little evidence for the evolution of drug resistance mutations (Finzi et al., 1997; Wong et al., 1997). Taken together these studies have shown that the viruses present in the resting CD4+ memory compartment are derived from long lived cells infected prior to the initiation of antiretroviral therapy and represent a major barrier to virus eradication.

1.9 Anti-viral Therapy of HIV-1 Infection

Since the identification of HIV as the causative agent of AIDS, research has been directed towards the development of an effective vaccine and antiviral therapy. Each step in the viral life cycle may potentially be targeted by antiretroviral agents. Table 1.2 lists the generic and chemical names of antiretroviral drugs used to treat HIV-1 infection.

1.9.1 Substrate Analogues

AZT, ddI, ddC, and D4T belong to the class of 2',3'-dideoxynucleoside analogues and are assumed to act as chain terminators of the reverse transcriptase reaction following intracellular phosphorylation to the 5'-triphosphate form (Furman et al, 1986; Huang et al., 1990; St Clair et al., 1987). Dideoxynucleosides differ from normal deoxynucleosides in that the 3' OH group on the ribose sugar ring is replaced by another group which cannot form diester linkages. In the case of AZT the hydroxyl (OH) group is replaced by an azido (N_3) group. The anti-HIV activity of 2',3'dideoxynucleosides is dependent on the initial intracellular phosphorylation by a cellular nucleoside kinase such as thymidine kinase (Hao et al., 1988; 1990). Many dideoxynucleosides have a low affinity for nucleoside kinases (e.g. 2',3'-dideoxyuridine, ddU) and the nucleoside kinase activity of some resting cells, such as monocytes or macrophages may be insufficient to completely phosphorylate some analogues, such as AZT. To overcome this problem special prodrugs such as aryl methoxyglycinyl derivatives (McGuigan et al., 1993) and bis(S-(2-hydroxyethylsulfidyl)-2-thioethyl) esters (Puech et al., 1993) have been developed to deliver the monophosphate forms intracellularly and bypass the first phosphorylation step. More recently, promising 2',3'dideoxynucleoside analogues have been described such as 3TC, the (-)-beta-enantiomer of 2',3'-dideoxy-3'-thiacytidine and FTC, the(-)-beta-enantiomer of 2',3'-dideoxy-3'-deoxy-5-fluoro-3'-thiacytidine (Cammack et al., 1992; Schinazi et al., 1992a,b,c). In both cases the (-)-beta-enantiomer was found to be less toxic and more potent than the (+)-beta-enantiomer. Both 3TC and (-)FTC act as DNA chain terminators in the HIV-1 RT reaction and also act against Hepatitis B virus

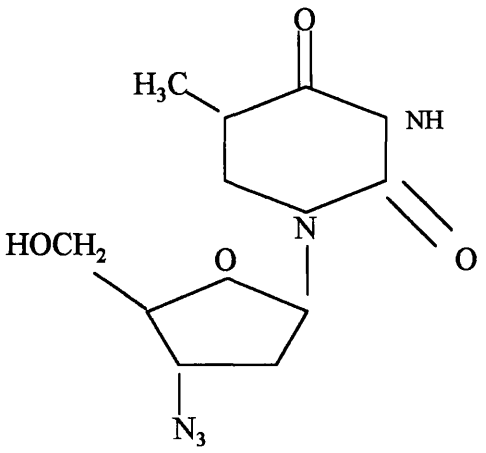
GENERIC NAME	CHEMICAL NAME
Zidovudine (AZT)	3'-Azido-2-deoxythymidine
Didanosine (ddI)	2',3'-dideoxyinosine
Stavudine (d4T)	2',3'-didehydrothymidine
Zalcitabine (ddC)	2',3'-dideoxycytidine
Lamivudine (3TC)	2'3'-dideoxy-3'-thiacytidine
PMEA	9-(2-phosphonylmethoxyethyl) adenine
PMEDAP	9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine
Nevirapine	11-cyclopropyl-5,11-dihydro-4-methyl-6H-dipyrido-(3,2-b:3'-e) (1,4) diazepin-6-one)
TIBO	Tetrahydroimidazo [4,5 1-jk] [1,4]- benzodiazepin-2 (1H)-one
HEPT	1-(2-Hydroxyethoxymethyl)-6-(phenylthio) thymine
BHAP	Bis (heteroaryl) piperazine
TSAO	2', 5'-Bis-O-(tert-butyldimethylsilyl) – 3'-spiro-5'-(4'-amino-1',2'-oxathiole-2',2'-dioxide
Alpha-Apa	Alpha-Anilinophenylacetamide
Ritonavir	(ABT-538, (2S, 3S, 5S)-5-[N[-[[N-methyl-N-[(2-isopropyl-4-thiazolyl) methyl] amino]-carbonyl]valinyl]amino]-2-[N[(5-thiazolyl) methoxycarbonyl] amino]-1,6-difenyl-3-hydroxyhexane)

Table 1.2 List of generic names of antiretrovitral drugs and associated chemical names.

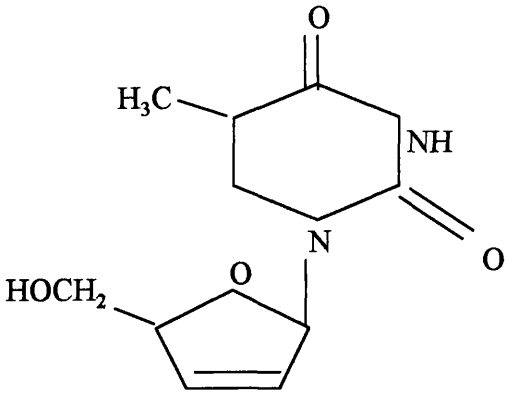
(HBV) in a similar fashion since HBV replicates through an RNA template-driven process (Doong et al., 1991; Furman et al., 1992).

The acyclic nucleoside phosphonates such as 9-(2-phosphonylmethoxyethyl) adenine (PMEA) and their 2,6-diaminopurine derivatives, such as (9-(2-phosphonyl-methoxyethyl)-2,6-diaminopurine (PMEDAP) represent another class of HIV inhibitors that act as chain terminators with the viral RT reaction (Pauwells et al., 1988; Balzarini et al., 1991a, 1991b). They act after they have been converted to their diphosphate form, i.e., PMEApp and PMEDAPpp. PMEA and its congeners have been proven to be effective in a wide variety of retrovirus cell systems, including HIV in monocytes/macrophages and human peripheral blood lymphocytes (Balzarini et al., 1991c), feline immunodeficiency virus (FIV) in feline peripheral blood lymphocytes (Hartman et al., 1994) and maedi or visna virus in sheep choroid plexus cells (Thormar et al., 1993). PMEA has been found to be efficacious in several animal models for retrovirus infection, including Friend leukemia virus, Moloney sarcoma virus and LP-BM5(murine AIDS infection in mice) (Gangemi et al., 1989; Balzarini et al., 1990) and feline immunodeficiency virus in cats (Egberink et al., 1990) and simian immunodeficiency virus infection in monkeys (Tsai et al., 1994). When PMEA was compared to AZT for *in vivo* effectiveness against retrovirus infection, PMEA proved superior in terms of potency and selectivity (Balzarini et al., 1989).

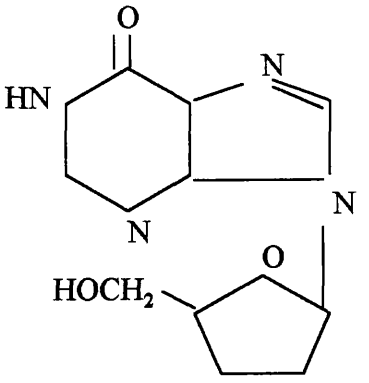
All acyclic nucleoside phosphonates demonstrate prolonged antiviral action, lasting up to a week after a single dose administration. This is thought to be due to the long half life of the active diphosphate metabolites, i.e., PMEApp and PMEDAPpp within the cells (Naesens et al., 1991). An additional advantage of the acyclic nucleoside phosphonates (PMEA and PMEDAP) is their dual action against both retroviruses and herpesviruses. The major drawback of acyclic nucleoside phosphonates are their slow cellular uptake and poor oral bioavailability which has led to the development of prodrugs (esters) such as the bis(pom) derivative of PMEA to increase uptake (Srinivas et al., 1993; Starrett et al., 1992). The chemical structures of some substrate analogues are illustrated in Figure 1.7.



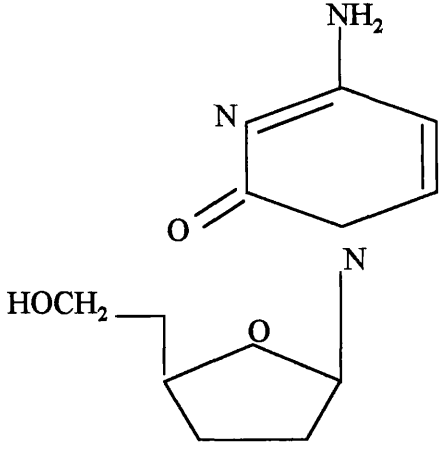
Zidovudine (AZT)



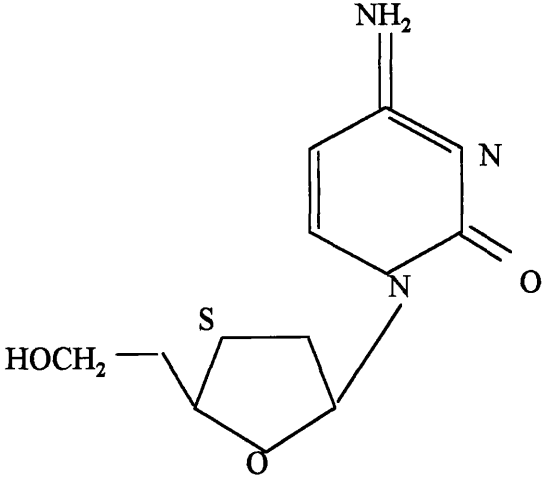
Stavudine (d4T)



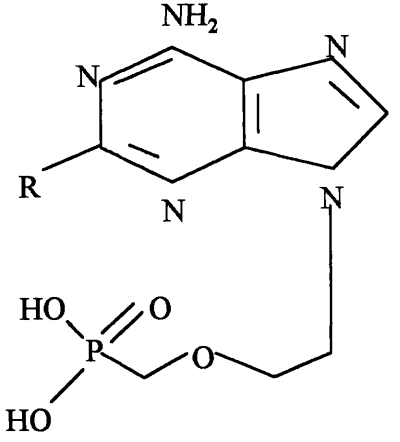
Didanosine (ddI)



Zalcitabine (ddC)



Lamivudine (3TC)



PMEA (R=H)

PMEDAP (R=NH_2)

Figure 1.7 Chemical structures of several substrate analogues, generic names of the drugs are indicated.

1.9.1.1 3'-Azido-2-deoxythymidine (zidovudine)

The first drug to be licensed for the clinical treatment of HIV-1 infection was Zidovudine (3'-Azido-2-deoxythymidine; AZT) which was first synthesised in 1964 as a potential cancer chemotherapy agent (Howitz et al., 1964) and was found to have antiretroviral activity against HIV-1 in 1985 (Mitsuya et al., 1985). It was first registered for clinical use in March 1987 in the USA as a result of the findings of the first large clinical trial in patients with advanced HIV disease. This showed significant improvement in survival of patients treated with AZT compared to patients treated with placebo (Fischl et al., 1987).

AZT is a dideoxynucleoside and causes termination of an elongating DNA. It has been shown to act against all polymerases and to have activity against HIV-1 *in vitro* (Mitsuya and Broder, 1986). AZT diffuses across human cell membranes in a non-facilitated manner (independent of the nucleoside carrier transport system within the cell membrane) and in a non-concentration dependent manner i.e. transport is not dependent on a difference in concentration on the two sides of the membrane due to the more lipophilic nature of AZT compared to thymidine (Zimmerman et al., 1987). Once AZT is in the cell it is phosphorlyated at the 5' position by a host cell thymidine kinase enzyme to a monophosphate (AZT-MP) form and then to a diphosphate (AZT-DP) form by a thymidylate kinase. AZT-DP is then phosphorylated again by other cellular enzymes to the active AZT triphosphate (AZT-TP) (Furman et al., 1986). The limiting step in the conversion of AZT to its active form is the conversion of the monophosphate to the diphosphate form. The conversion of AZT to AZT-MP takes place at 60% of the rate of thymidine phosphorylation but conversion of AZT-MP to AZT-DP takes place at only 0.3% of the equivalent thymidine rate.

Zidovudine acts at the site of reverse transcription (Vrang et al., 1988) and can inhibit viral replication in at least two ways. Firstly AZT-TP competitively inhibits the incorporation of thymidine into viral DNA by reverse transcriptase, with AZT-TP filling 1 of every 1.5 to 2.4 sites available for thymidine incorporation (St Clair et al., 1987). Secondly, AZT-TP acts as a chain terminator by joining to the end of the growing viral DNA strand and preventing the joining of other nucleotides by the usual

phosphodiester linkages, due to the replacement of the OH group with the azido group. However, AZT does not stop virus production from infected cells, or transmission to uninfected cells (Smith et al., 1987), as other steps in the virus life cycle dependent on cellular RNA polymerase are not affected by AZT.

1.9.1.2 Clinical Trials with Zidovudine

The results of an initial dose finding phase one study suggested that AZT might be a beneficial treatment for HIV-1 infection (Yarchoan et al., 1986). In this study 19 patients with AIDS or ARC were randomised to take four doses of AZT (1mg/kg or 2.5mg/kg every 8hours, 2.5mg/kg or 5mg/kg every 4 hours) for six weeks. The results showed a decrease in opportunistic infections, night sweats and fever and a gradual increase in CD4 lymphocyte counts and CD4:CD8 ratios. Following this initial trial a large multicenter double blind placebo trial of AZT therapy was begun in patients with AIDS or ARC (Fischl et al., 1987). Patients were stratified on the basis of CD4 cell count and given either 1500mg/day AZT or placebo. This trial was stopped early following the recommendations of an independent monitoring board because there was a significant decrease in the mortality rate of those taking AZT. At termination of the trial, 1 in 145 in the AZT group and 19 in 137 in the placebo group had died. Significant increases in body weight and CD4 cells and a reduction in p24 antigenemia were observed in the AZT group. The increases in CD4 counts in the AIDS patients were only transient with counts falling to pretreatment levels after 12 weeks. The results of this study and the need for an effective therapy for AIDS led to the licensing of AZT as an acceptable AIDS therapy.

The speed at which AZT was licensed for use and available for treatment of advanced HIV disease meant that several important questions such as timing of introduction, toxicity and long term effects remained unanswered. The first major study to investigate the use of AZT in asymptomatic patients with HIV infection and CD4 cell count of < 500cells /ul was the ACTG 019 study (Volberding et al., 1990). This study was designed to determine the safety and efficacy of AZT in prolonging survival and delaying the onset of AIDS or ARC. The study compared two different AZT doses (1500mg/day, n=457 ; 500mg/day, n=453) and a placebo group , (n=428); the mean

follow up was 55 weeks. Results showed that subjects in the AZT groups had a significant increase in CD4 cell count along with a significant decrease in p24 antigen levels compared to placebo. Progression rate to AIDS or ARC in the three groups (placebo, 500mg/day, 1500mg/day) were 7.6, 3.6 and 4.3/ 100 person years respectiviely. The study concluded that AZT was safe and effective in asymptomatic HIV infection with CD4 levels below 500/ul. Several other smaller studies investigated the role of AZT in patients with asymptomatic HIV infection and CD4 counts between 200 and 500 x 10^6 cells/L (Cooper et al., 1993; Hamilton et al., 1992) and demonstrated a significant clinical benefit from early initiation of AZT therapy, in terms of increase in CD4 cell counts, reduction in p24 antigen levels, disease progression and occurrence of opportunistic infections. These findings were also demonstrated in the largest and longest clinical trial of AZT in asymptomatic HIV infected individuals, the Anglo-French Concorde study (Seligmann et al., 1994). The Concorde study compared immediate treatment with AZT (1000mg/day) on study entry with AZT treatment when symptoms developed. In contrast to the findings of ACTG 019, the concorde trial did not encourage the early use of AZT as no statistically significant difference in death, clinical outcome or disease progression was demonstrated between the two groups.

At higher doses of AZT, 1500mg/day causes severe haematological toxicity such as neutropenia and anaemia. A number of studies have addressed the question of dosage and have demonstrated that a reduced daily dose of between 300 and 600mg/day is at least as effective as higher doses and is less toxic, with nausea being the most common reported toxicity (Fischl et al., 1990; Volberding et al., 1990).

1.9.1.3 Isolation of Zidovudine-Resistant Strains of HIV-1

The potential of HIV-1 to become resistant to anti-HIV drugs has become an increasing concern since it was first reported that HIV variants isolated from patients following prolonged AZT therapy show reduced susceptibility to AZT. Resistance of HIV-1 to antiviral drugs is generally associated with reduced sensitivity of the virus to the drug by *in vitro* cell culture studies and the emergence of a resistance mutation in the virus. Mutations in the RT gene on HIV-1 have been detected that are associated with nucleoside substrate analogue drugs and the specific RT inhibitors, resistance to

protease inhibitors is associated with specific amino acid substitutions within the viral protease. The clinical significance of resistance to anti-HIV drugs has not been resolved and the rapid emergence of drug resistant HIV-1 mutants is seen as a limitation of the drug and not an argument against the clinical usefulness of the drug.

The first report of isolates of HIV-1 from patients who had been undergoing AZT therapy for six months or more to show reduced sensitivity to the drug was by a HeLa cell CD4+ plaque reduction assay, which measures the production of distinct foci produced by cell free virus or HIV infected cells (Chesebro and Wehrly, 1988). Comparison was made between the ID_{50} values for isolates from 18 untreated individuals (ID_{50} 0.01-> 0.05uM) and those of AZT treated patients (ID_{50} range 0.06-> 6uM after 6 to 18 months therapy. 5 of the 15 treated patients showed a 100 fold or greater increase in ID_{50}, after a minimum of six months therapy. These isolates with reduced sensitivity to AZT were tested against other anti-retroviral drugs, and although they were not sensitive to 3'-azido-2'3'-dideoxyuridine(AZddu), they remained sensitive to 2'3'-dideoxycytidine (ddC), 2',3'-didehydrothymidine(D4T) and phosphonoformate (PFA). Several later studies of sequential HIV isolates from patients undergoing long term AZT therapy confirmed the finding that AZT resistance may emerge after only 6 months of therapy and that although sensitivity is reduced to AZT, isolates remain sensitive to other nucleoside analogues (Richman et al., 1990; Land et., 1990; Rooke et al., 1989).

The most plausible explanation for the emergence of drug resistant phenotypes of HIV-1 during AZT therapy is the development of mutations in the reverse transcriptase gene. The complete 1.7kb reverse transcriptase region was obtained from both drug sensitive and drug resistant isolates by PCR amplification of proviral DNA (Larder and Kemp., 1989). The amplified DNA was inserted into an M13 based vector mptac 18.1 and sequenced. Sequence analysis of the reverse transcriptase gene from five pairs of sensitive (ID_{50} 0.01) and reduced sensitivity (ID_{50} 2-5.6) clinical isolates, obtained before and during AZT therapy showed the presence of three common point mutations in all five reduced sensitivity isolates and a fourth mutation in three of the five isolates (Larder and Kemp, 1989). These mutational changes were Aspartate to

Asparagine at position 67, Lysine to Arginine at position 70, Threonine to Phenylalanine or Tyrosine at position 215 and Lysine to Glutamine at position 219.

To investigate these changes *in vitro*, a mutant HIV-1 clone was constructed by inserting a 2.55kb fragment of the HIV-1 pol gene from an infectious clone HXB2D, into the M13 vector mp19, using site directed mutagenesis to insert the four amino acid changes into the RT gene by use of synthetic oligonucleotides. A 1.9Kb fragment containing the inserted mutations in RT was removed from vector mp19 and transferred to the infectious molecular clone HXB2-D. DNA from wild type and mutant infectious clones were then transfected into the T-cell line MT-4 to create virions. Wild type virus and resistant virus were titrated by plaque assay in a HeLa CD4+ cell line and also assessed for sensitivity to zidovudine in a plaque reduction assay. The results showed that the mutant isolate containing the mutations was 100 fold more resistant to AZT than the wild type. This result was similar to the data obtained from resistant clinical isolates confirming that the four point mutations conferred resistance to AZT. The degree of resistance conferred by these mutations individually or in combination was investigated by the construction of a series of HIV-1 clones containing various combinations of the mutational changes and testing their sensitivity to AZT (Larder et al., 1991a, Larder. 1994).

Reduced sensitivity or resistance to AZT was seen in all cases when the amino acid changes at position 70 or 215 were present either individually or in combination. The changes at positions 67 and 219 confer little resistance on their own but in general confer greater resistance in combination with other changes. These findings confirmed that viral resistance to AZT increases with the accumulation of these four specific reverse transcriptase mutations. A number of clinical isolates were identified which showed high level AZT resistance but contained only three of the four previously implicated mutations, changes at positions 67, 70, and 215. A molecular clone previously constructed containing this genotype only gave partial resistance and this suggested the possibility that additional mutations existed in some clinical isolates. By use of an HIV marker rescue system a mutational change of Methionine to Leucine was identified at position 41 (Kellam et al., 1992). A molecular clone of HIV-1 was constructed containing changes at positions 41, 67, 70, and 215. This gave a highly

resistant virus after transfection of T-cells with a 179 fold higher resistance than wild type. This value was similar to levels seen in highly resistant clinical isolates.

It is now widely accepted that these five point mutations are responsible for development of reduced sensitivity isolates to AZT. It has been suggested that the change at position 70 may be a pre-existing polymorphism. An in-depth sequence study of treated and untreated patients showed the presence of the resistance conferring change at position 70 in 7/25 patients never treated with AZT (Najera et al., 1995).

Other mutations have been implicated as conferring AZT resistance by sequence studies of the RT gene. One study using an RNA:RNA hybridisation assay (Japour et al., 1991) which detects intracellular RNA and is a measure of AZT sensitivity demonstrates 8 amino acid changes in one resistant isolate. Three of the four changes at positions 125, 142, and 294 were in conserved amino acids. A change at position 215 was also detected. A second study by Sheehy and Desselberger (1993) identified ten amino acid changes at positions 108, 135, 173, 211, 245, 281, 334, 335, 379, and 464 in three isolates which showed reduced sensitivity to AZT. However none of the five mutations at positions 41, 67, 70, 215 and 219 which are strongly implicated in resistance to AZT were detected in the three sensitive isolates. It is possible that the amino acid changes identified in these studies may contribute to the development of resistance but until introduced into a defined genetic background by site directed mutagenesis and the resulting virus measured for drug sensitivity, their effect will remain unclear.

1.9.1.4 Acquisition of zidovudine Resistance Mutations

An investigation into the acquisition of AZT resistance mutations in HIV-1 isolates from treated individuals was carried out using selective PCR (Boucher et al., 1992). Serial isolates were obtained from 18 asymptomatic patients over 2 years of AZT therapy. Selective PCR was used to investigate amino acid positions 67, 70 and 219. The amino acid change at position 215 had been determined in a previous study and 16/18 isolates were mutant at position 215 between 48 and 112 weeks of AZT therapy.

All pretreatment isolates were wild type at codon 70. Between weeks 16 and 32, 11/18 isolates showed a mixed or mutant population at this codon, however only 7/18 isolates obtained between weeks 76 and 132 showed a mutation at this position. Seventeen of the isolates obtained between weeks 76 and 132 were wild type at codons 67 and 219, with one isolate showing a mixed population at codon 67 and wild type at codon 219. During the study three of the patients progressed to AIDS and these patients were analysed by selective PCR at all four codons (67, 70, 215, and 219). In all three cases the mutation at position 70 appeared first, and then disappeared with the appearance of the change at position 215. In 2/3 cases the change at codon 70 reappeared. In one patient the change at codon 67 appeared transiently and then reappeared with the appearance of the mutation at codon 219, following this all mutations were present in the RT gene. Mutations at positions 67 and 219 were not detected in the other patients.

A detailed genotypic analysis of virus from 17 HIV-1 infected patients was carried out by Kellam et al (1994) using selective PCR to look at all five codons associated with AZT resistance. This study proposed a model for the acquisition of the five implicated resistance mutations. The first mutation to appear is at position 70, although additional viral populations with single amino acid changes at positions 41 or 215 may coexist early in therapy. Different combinations of these three changes (41, 70, 215) may become linked to give double mutants with varying degrees of resistance. Eventually the double linked 41/215 mutant outgrows the other mutants and becomes fixed in the viral population. The appearance of the changes at positions 67, 70 and 219 with the double mutant occurred in only one of the two patients from this group studied sequentially, after 136 weeks therapy. The pattern of resistance development may be similar between patients, however the speed of mutation acquisition may differ. Figure 1.8 illustrates the ordered appearance of AZT resistance mutations proposed by Larder 1994, with prolonged AZT monotherapy.

1.10 HIV-1 Reverse Transcriptase Structure

The HIV-1 reverse transcriptase enzyme is a heterodimer of 66kDa and 51 kDa subunits (p66 and p51, respectively) derived from the lytic processing of a p66

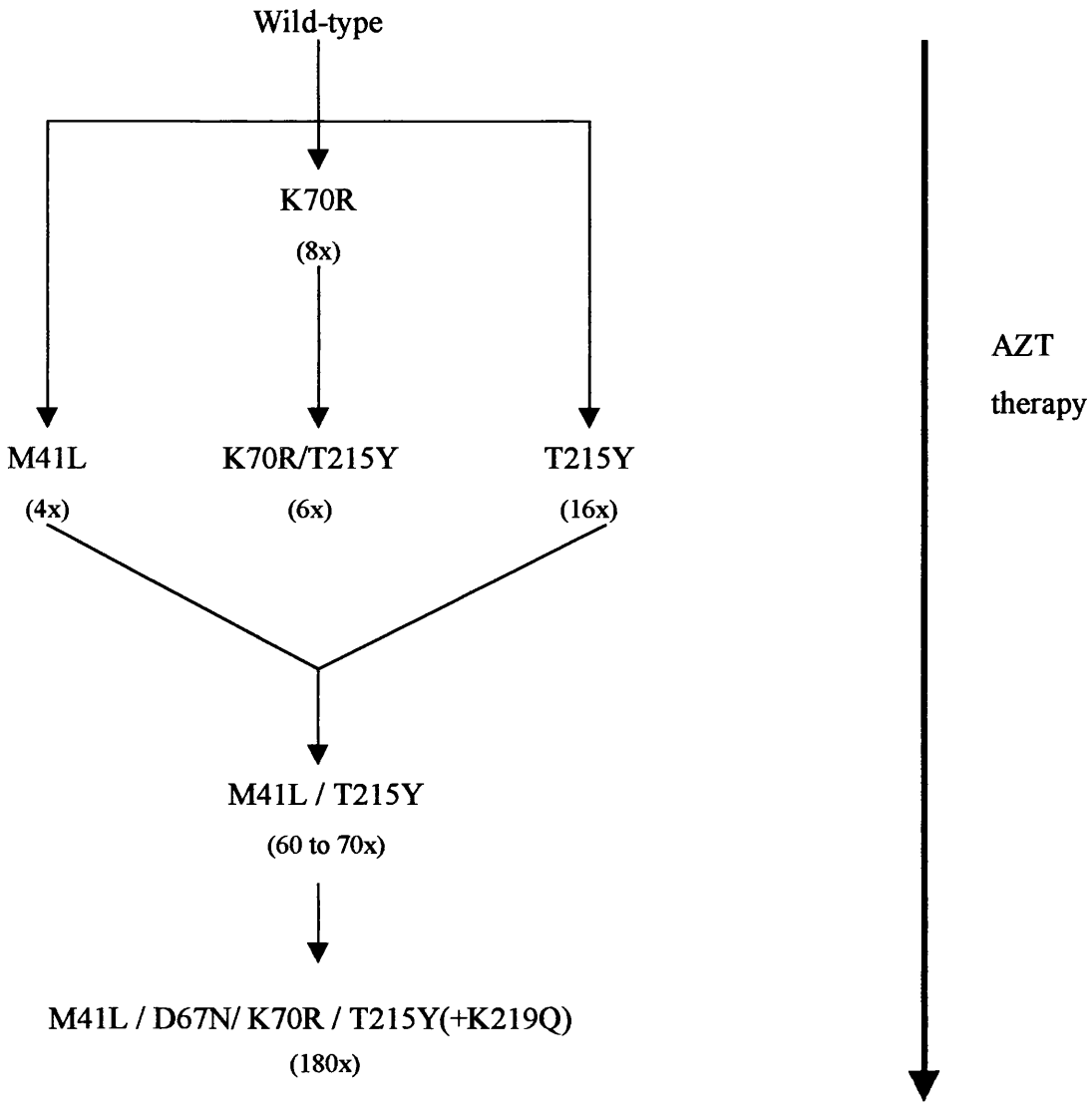


Figure 1.8 Illustration of the order of appearance and accumulation of AZT resistance mutations during therapy. Adapted from (Larder, 1994) beginning with a wild-type HIV-1 RT genotype. The approximate increase in the level of phenotypic resistance conferred by single or multiple mutations is given in parentheses

homodimer at the carboxyl terminus of one of the subunits. Information on the structure and function of HIV-1 RT has been gained from crystallographic studies, studies comparing the primary sequences of different retroviral RT with sequences of non-viral enzymes and studies relating changes in structure (mutations, deletions, insertions) to changes in enzyme activities.

The first 250 amino acids of the aminoterminal part of several retroviral reverse transcriptases show considerable homology with each other and with the alpha subunit of *Escherchia coli* (*E.coli*) polymerase (Johnson et al., 1986; Larder et al., 1987). The carboxyterminal region of retroviral RT genes shares homology with the ribonuclease H from *E.coli.* Functional studies have shown an interaction between the p51 and p66 subunits is required for optimal polymerase activity, since the most active form of HIV-1 RT is the p66-p51 heterodimer. P51 either as a monomer or dimer has zero to only very low polymerase activity and the p66 subunit as a monomer or in its dimeric form has less polymerase activity than the p66/p51 heterodimer (Starnes et al., 1988; Muller et al., 1989; Hansen et al., 1988). The carboxyterminal 15 KDa protein, generated during the cleavage of p66 into p51 and p15, has no detectable ribonuclease activity on its own (Davies et al., 1991). The addition of purified p51 subunit to p15 leads to the reconstitution of ribonuclease activity, indicating that the interaction of the carboxyterminal part with the aminoterminal part is required for ribonuclease activity (Hostomsky et al., 1991). As the p66 homodimer and the p66/p51 heterodimer have similar RNase H activities, the p66 homodimer was predicted to be asymmetric, with only one of its RNase H domains correctly folded for catalysis. Crystallographic studies have now resolved the probable structure of HIV-1 RT and RNase H and confirmation of the corresponding heteodimer by a 3.5A resolution crystal structure (Davies et al., 1991; Arnold et al., 1992; Kohlstaedt et al., 1992). The crystal structure has demonstrated that HIV-1 RT is a heterodimeric molecule consisting of a p66 subunit, which forms a cleft capable of containing a template, and the p51 subunit supporting the p66 subunit. The polymerase domain of the p66 subunit consists of four main subdomains, arranged to form a DNA/RNA-binding cleft in a way that resembles a right hand.

The thumb consists of a four helix bundle and the fingers are formed by a mixed beta sheet/alpha helix structure. The palm subdomain, containing the putative polymerase site, and the connection subdomain, which links the polymerase and RNase domains are formed mostly from a five stranded beta sheet. The structure of the p51 subunit, although assembled from the same primary sequence shows virtually no conformational similarities to the p66 subunit. The RNase H domain has been mapped to a region in the structure that contacts the connection subdomain of p66 and the thumb subdomain of p51. The RNase H domain is quite similar in overall structure to RNase H of *E.coli*, with two divalent metal cations necessary for catalysis located near a cluster of four acidic residues (Asp443, Glu478, Asp498, and Asp549). Important polymerase active site residues have been identified by analogy to the klenow fragment of DNA polymerase I: three key carboxlyate side chains, possibly involved in substrate binding or chemical catalysis, are donated by residues Asp185, Asp186, and Asp110 in the palm subdomain.

A highly conserved region or motif is found in almost all polymerases. This motif (YXDD) contains four amino acids (Y=tyrosine, X can represent several amino acids but in most reverse transcriptases it is a methionine (M), D=aspartic acid) and is necessary for polymerase activity. For HIV-1 this motif is represented by amino acids 183 - 186. Changes of the methionine at 184 (into leucine or tyrosine) reduce polymerase activity by more than 90% (Prasad et al., 1989). Changes of the two aspartic acid residues (into asparagine, histidine or glutamine) abolishes all polymerase activity (Larder et al., 1989; Boyer et al., 1992). A third important aspartic residue is located at position 110 and a change at this residue results in complete loss of polymerase activity (Barber et al., 1990).

The HIV-1 RT structure determined by Kohlstaedt et al (1992) contains nevirapine, a competitive inhibitor of the enzyme's polymerase activity bound at a site between the palm and thumb subdomains distal to the substrate - binding pockets. The nevirapine molecule is placed near the expected primer terminus in the p66 subunit and interacts with Tyr181 and Tyr188 side chains. The positions of the residues that mutate to confer zidovudine resistance are located in the 'fingers'. Whereas AZT, an inhibitor of DNA polymerisation, acts by chain termination, the mode of action of neviripine may

be to disrupt duplex binding and thereby affect nucleotide incorporation and/or DNA translocation.

1.11 Nonsubstrate analogues

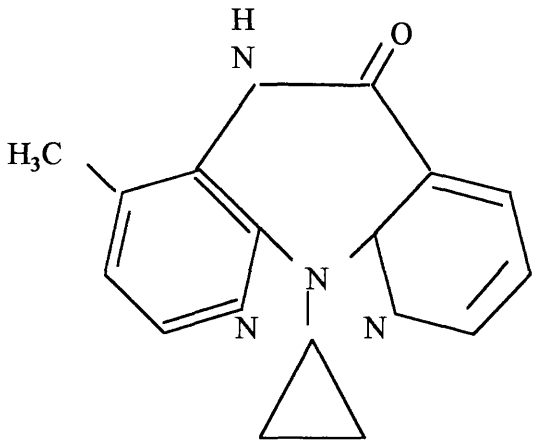
The clinical usefulness of the dideoxynucleoside analogues, AZT, ddI, ddC, and D4T are limited by their toxic side effects. The level of toxicity differs between compounds; anaemia or neutropenia for AZT, peripheral neuropathy for ddC and D4T, and acute pancreatitis for ddI. These toxic side effects may be related to the interference of the 2',3'-dideoxynucleosides metabolites with 2'-deoxynucleoside metabolism and the cellular DNA polymerisation process. Nonsubstrate analogues that do not interact with the substrate binding site of DNA polymerases may be expected not to cause the toxic side effects associated with the 2',3'-dideoxynucleoside analogues (De Clercq, 1992, 1993).

The first compounds shown to specifically inhibit HIV-1 but not HIV-2 replication were 1-(2-hydroxyethoxymethyl)-6-(phenylthio)thymine (HEPT) (Miyasaka et al., 1989) and tetrahydroimidazol(4,5,1-jk)(1,4)benzodiazepin-2(1H)-one and -thione (TIBO) (Pauwells et al., 1990). The specificity of the TIBO derivatives (R82150 and R82913) was attributed to a specific interaction with the HIV-1 RT (Debsyer et al., 1991; Pauwells et al., 1990). Following the discovery of HEPT and TIBO, several other compounds, i.e. nevirapine (BI-RG-587) (Merluzzi et., 1990), pyridone derivatives(L-696,229 and L697,661) (Goldman et al., 1991, 1992) and bis(heteroaryl)piperazine(BHAP) (Romero et al., 1993) were described as HIV-1 specific RT inhibitors. Neviripine, pyridone, and BHAP compounds emerged from a screening program for HIV-1 RT inhibitors, and their anti-HIV-1 activity was later confirmed in cell culture. The HEPT and TIBO derivatives, the 2',5'-bis-O-(tert-butyldimethylsilye)-3'-spiro-5''-(4''-amino-1'',2''-oxathiole-2'',2''-dioxide)pyrimidine (TSAO) derivatives (TSAO-T and TSAO-m^3 T) (Balzarini et al., 1992) and alpha-anilinophenylacetamides (alpha-APA R89439) (Pauwells et al., 1993) were discovered through the evaluation of anti HIV-1 activities in cell culture and then proved to act as inhibitors of HIV-1 RT. These nonsubstrate analogues are also referred to as "non-nucleoside RT inhibitors" (NNRTIs).

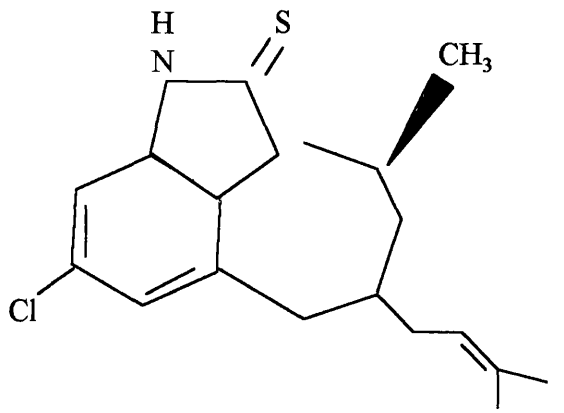
For a compound to be classified as an NNRTI it must inhibit HIV-1 by a specific interaction with HIV-1 RT and inhibit HIV-1 replication in cell culture at a concentration that is significantly lower than that concentration which affects normal cell viability. On this basis the compounds described would classify as NNRTIs as they were found to inhibit HIV-1 cytopathicity at a concentration that was 1,000-fold below the cytotoxicity threshold.

The RT inhibitory potency of NNRTIs is greatly influenced by the choice of template/primer, it is much greater with poly(C).oligo(dG) than with poly(A).oligo(dT) as the template/primer (Pauwells et al., 1990; Debyser et al., 1993). Inhibition of HIV-1 RT by the NNRTIs is non-competitive with respect to both the substrate (dGTP) and the template primer, this contrasts with the 2',3'-dideoxynucleoside 5'-triphosphates which competitively inhibit the incorporation of natural deoxynucleoside triphosphates into the growing DNA strand.

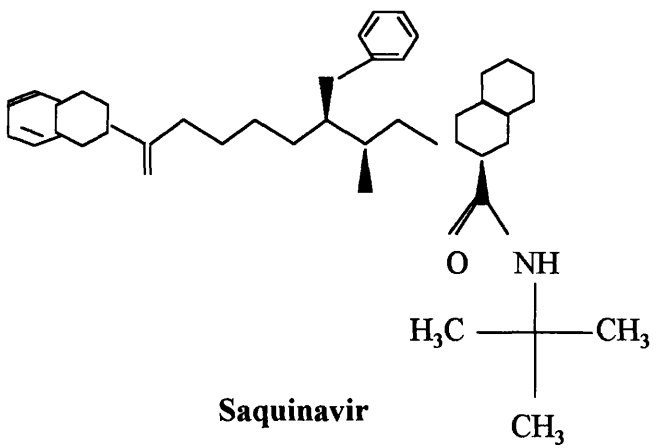
The non competitive inhibition of HIV-1 RT by the NNRTIs suggests that these compounds interact with a nonsubstrate binding site of HIV-1 RT. The binding site for nevirapine has been shown by photoaffinitty labelling to span the region 174 to 199, the tyrosine residues at positions 181 and 188 being crucial for binding of neviripine (Cohen et al., 1991; Wu et al., 1991). The importance of Tyr-181 and Tyr-188 in the binding of HIV-1 RT to nevirapine and TIBO was demonstrated by the use of chimeric RT constructs in which the tyrosine residues at position 181 or 188 were replaced by the HIV-2 counterparts Isoleucine and Leucine (Shih et al., 1991). The structural identification of the site was accomplished by Kohlstaedt et al (1992) who determined a low resolution crystal structure of the RT complexed with nevirapine. The drug bound to a hydrophobic pocket in a location adjacent to the enzymes active site at positions 183-186 containing the YMDD motif. The Y181I and Y188L RT constructs were resistant to nevirapine and TIBO but were susceptible to the 2',3'-dideoxynucleoside triphosphates (De Vreese et al., 1992). The chemical structures of two examples of non-substrate analogues, neviripine and 9-chloro-TIBO are illustrated in Figure 1.9.



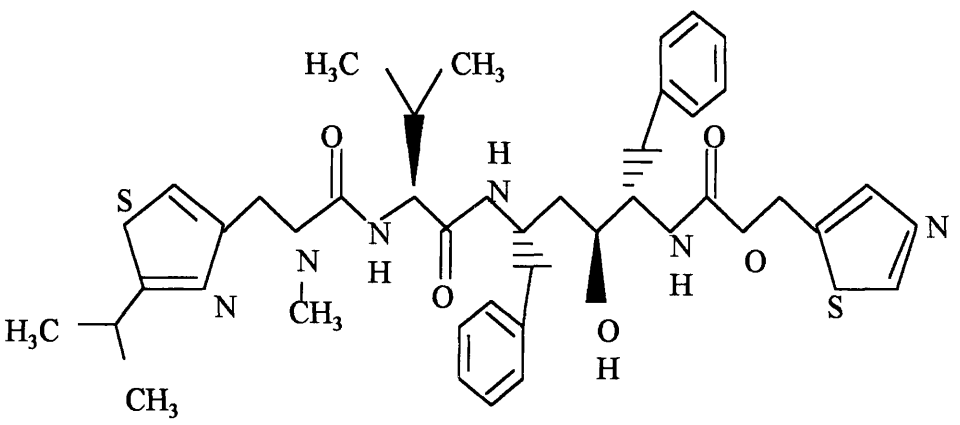
Neviripine



9-chloro TIBO



Saquinavir



Ritonavir

Figure 1.9 Chemical structures of two non-substrate analogue drugs, neviripine and 9-chloro TIBO and two protease inhibitors, saquinavir and ritonavir.

1.11.1 Clinical studies of nonsubstrate analogues

Several NNRTIs have been examined in phase I and phase II clinical studies. TIBO R82913 was administered daily by intravenous infusion for 2 to 20 weeks at daily doses of up to 300mg in a pilot study of 22 patients. The drug was well tolerated with no serious haematological, biochemical or clinical side effects (Pialoux et al., 1991). Another phase I dose finding study with oral TIBO R82913 indicated that the oral bioavailability was low (7 to 10%) and that improvement in bioavailability would be needed before more long term studies (De Wit et al., 1992). Studies with nevirapine indicated that the drug is rapidly absorbed and tolerated when tablets of 2.5mg to 400mg were taken daily.

Pyridone L-697,661 has been subject to a short term clinical evaluation with one of the following dosage regimens, 25mg taken orally twice a day, 100mg three times a day, or 500mg twice a day. The compound was well tolerated and exhibited a significant dose related activity against HIV-1 (Saag et al., 1993). This antiviral response subsided after 6 to 12 weeks, when drug resistant virus variants appeared. The characteristic pyridone resistance mutation at position 103 (Lys to Asn) was detected (Saag et al., 1993) along with the amino acid substitution at position 181(Tyr to Cys), which is responsible for resistance to virtually all of the NNRTIs.

The term 'convergent combination therapy' has been applied for combinations of several drugs that lead to different mutations and when accumulated in the same enzyme would cripple the enzyme to such an extent that it would no longer function (Chow et al., 1993). However this proposal has proved to be faulty since HIV can acquire co-resistance to multiple drugs such as zidovudine, ddI and nevirapine and also remain viable.

1.11.2 Resistance associated with non-substrate analogues

Resistance to the non-nucleoside reverse transcriptase inhibitors rapidly emerges in all patients receiving monotherapy with this class of drugs and single point mutations are sufficient to confer a 100 fold increase in resistance. Resistance was first demonstrated

for the pyridinones (Nunberg et al., 1991), mediated by the amino acid mutations 103 Lys-Asn. Substitutions at the 181 and 188 positions are common in isolates from patients receiving the TIBO inhibitor L-697,661 and nevirapine. A synoptic review of mutations conferring resistance to NNRTI's is presented by Schinazi et al (1997). With the exception of the 138 Glu-Lys mutation associated with TSAO derivatives, which is located on the p51 subunit of HIV-1 reverse transcriptase, all other mutations are located on the p66 subunit of the HIV-1 RT (Jonckheeve et al., 1994; Boyer et al., 1994). All of the mutations that confer NNRTI resistance appear to be clustered around the pocket site where the NNRTI's bind (Nanni et al., 1993; Tantillo et al., 1994).

1.12 Protease inhibitors

During HIV-1 replication, the gag and gag-pol gene products are synthesised as precursor proteins (Pr55 and Pr160 respectively), the latter as a result of a ribosomal frame shift. As the virus particle buds from a productively infected cell these polyproteins are processed with the maturing virion by the viral protease to yield structural proteins, p17, p24, p7 and p6 as well as essential enzymes, reverse transcriptase, integrase, and protease. The search for protease inhibitors was launched after it was ascertained that the HIV-1 protease is required for viral infectivity (Kohl et al., 1988).

The HIV-1 protease is an aspartyl protease composed of two identical 99-amino acid monomers, combining to form a C_2-symmetrical homodimer whose single active site contains the signature sequence Asp-Thr-Gly for aspartyl proteases at amino acids 25 to 27 and 125 to 127. Inhibition of the HIV-1 protease results in the budding of immature noninfectious particles, thus interrupting completion of the viral life cycle. The elucidation of the three dimensional structure at the 3-A [0.3nm] level (Navia et al., 1989) and the 2.8-A [0.28nm] level (Erickson, 1990) has allowed computer based design of inhibitors of HIV-1 protease.

The identification of the HIV protease cleavage sites has allowed protease inhibitors with high specificity to be designed using the "transition state peptidomimetic"

principle. The compounds are structurally similar to the gag and gag-pol polyprotein substrates by replacing the hydrolysable peptide linkage by a nonhydrolysable transition state isotere. There has emerged a variety of HIV-1 protease inhibitors: hydroxyethylamine derivatives (RO 31-8959) (Roberts et al., 1990), hydroxyethylene derivatives (U-81749) (McQuade et al., 1990) and C_2 symmetric dihydroxyethylene derivatives (A-77003) (Kempf et al., 1991). These compounds bind to the active site and competitively inhibit enzymatic function.

As an alternative to the peptide based approach, penicillin derived compounds have been pursued as HIV-1 protease inhibitors: (I) penicillin -C_2-symmetric dimers held together by an ethylenediamine linker (Humber et al., 1993) and (ii) monomeric penicillins linked to peptide isosteres (Holmes et al., 1993). X-ray crystal structure information of the HIV-1 protease dimer and the role of a structural water molecule in linking the protease inhibitor to the HIV-1 protease dimer has allowed the development of an entirely new class of HIV-1 protease inhibitors, the nonpeptide cyclic ureas (Lam et al., 1994). XM323 the prototype of this series has an *in vitro* inhibitory concentration of 0.036uM and in contrast to most peptide based protease inhibitors, XM323 also has good oral bioavailability (27% in rats and 37% in dogs) (Lam et al., 1994). The chemical structures of two protease inhibitors commonly used in treatment of HIV-1 infection, Saquinavir and Ritonavir, are illustrated in Figure 1.9.

1.12.1 Clinical studies with protease inhibitors

Early investigations with the HIV-1 protease inhibitors Ro 31-8959 (commercial name, Saquinavir) showed antiviral activity against acute and chronically HIV infected cells (Galpin et al., 1994). Ro 31-8959 is active against AZT-resistant strains and acts synergistically with ddC, it is not inhibitory to renin, pepsin, cathepsin, elastase, prolidase or collagenase (Galpin et al., 1994). Monotherapy with protease inhibitors have been shown to reduce RNA viral load rapidly with a few days from the start of treatment (Wei et al., 1995; Ho et al, 1995). Monotherapy with indinavir, nelfinavir or ritonavir can reduce HIV RNA load by a factor of 100 to 1000. These reductions in the viral load are paralleled by increases in the CD count (Danner et al., 1995; Markowitz et al., 1995). Combination therapy of protease inhibitors and nucleoside analogues

(zidovudine and lamivudine) have been shown in large clinical trials to slow the progression of disease and improve survival (Hammer et al., 1997; Guleick et al., 1997). Combinations of protease inhibitors and nucleoside analogues can suppress HIV for long periods of time. The combination of indinavir with zidovudine and lamivudine reduced plasma viral load to undetectable levels in 70% of patients after 24 weeks and in 60% after 2 years (Gullick et al., 1997). Combining a protease inhibitor with a nonnucleoside reverse-transcriptase inhibitor can also suppress viral load. In a small study of 12 patients receiving both indinavir and nevirapine, significant reductions in viral load were seen after 24 weeks (Murphy et al., 1997).

1.12.2 Resistant mutations associated with protease inhibitors of HIV-1

The limited duration of the anti-HIV response that occurs in most patients treated with protease inhibitor monotherapy is associated with the appearance of drug-resistant virus (Condra et., 1995; Molla et al., 1996). The major amino acid mutations associated with clinical resistance have been mapped (Schinazi et al., 1997). A substitution of phenylalanine for valine at position 82 is the initial mutation associated with resistance to indinavir and ritonavir, an aspartate to asparagine mutation at position 30 results in initial resistance to nelfinavir and mutations in glycine at position 48 and leucine at position 90 result in initial resistance to saquinavir (Schinazi et al., 1997). Initial single amino acid mutations yield only a slight change in drug sensitivity (Ho et al., 1994; el-Farrash et al 1994), however secondary mutations accumulate and can lead to high level resistance (Condra et al, 1995; Molla et al., 1996). The HIV protease can tolerate a number of mutations with altering function of the enzyme, up to one third of the 99 amino acids (Molla et al., 1996). Protease resistant HIV-1 is retained even after cessation of therapy. In patients with indinavir resistant virus who discontinued therapy and switched to indinavir plus nucleoside analogues, the initial reduction in plasma load was followed by an increase with four weeks due to the re-emergence of indinavir-resistant virus (Condra et al., 1995).

1.13 Alternative Strategies to inhibition of HIV-1 replication

Despite proven effiacies of highly active antiretroviral drugs, current therapy strategies can have intolerable side effects, high cost and demand strict patient compliance. Alternative approaches to inhibiting HIV-1 replication have been sought and can act at the level of viral entry or exit and also inhibit reverse transcription, integration and transcription/translation. Attempts have also been made to limit spread of HIV-1 by direct killing of cells harbouring HIV-1.

Three types of RNA molecules have been developed for gene therapy against HIV-1, antisense oligonucleotides, ribozymes and RNA decoys. Antisense oligonucleotides are complimentary to the target RNA sequence and work by blocking specific translation and also by possibly promoting degradation of RNA duplexes through RNAse H (Cohen, 1996). Incoming viral RNA can also be targeted before reverse transcription and the delivery of multiple RNA molecules can improve effectiveness and reduce the development of resistance. The activity and stability of antisense oligonucleotides has been improved by replacing inter-nucleoside phosphates with methlyphosphonates or phosphorothioates and by modifying the ends of the molecule (Kim et al, 1995; Sarin et al, 1989).

Ribozymes are antisense RNA molecules with catalytic activities for their substrate RNAs. A minimal hammerhead ribozyme cleaves target RNA containing a GUX motif where X can be C, U or A resulting in a 2' 3' cyclic phosphate and 5' hydroxy terminii. The efficiency of cleavage of hammerhead ribozymes can be improved by the presence of RNA binding proteins such as the HIV-1 p7 nucleocapsid that accelerates association and dissociation of RNA helices. Hairpin ribozymes are modelled after the minus strand of the satellite RNA of tobacco ringspot virus which cleaves target RNA containing BNGUC where B can be U, C or G and N can be any nucleotide ((Hampel and Tritz, 1989). Hairpin ribozymes are comprised of four helices separated by two internal loops, the RNA substrate binds to helices 1 and 2 and cleavage occurs at the 5' end of the guanosine within the conserved cleavage site in the substrate (Hampel et al, 1990). An advantage of ribozymes as therapeutic genes is that the ribozyme is not consumed in the enzymatic reaction and a single molecule can act on many substrates. Furthermore ribozymes and antisense oligonucleotides have a lack of immunogenecity.

Figure 1.10. Interference at different stages of the HIV-1 life cycle with anti-viral agents.

1. Binding: Blocking of CD4 with soluble CD4 and chemokine receptors with modified chemokines. Disruption of chemokine receptor expression by intra-antibodies.

2. Fusion and Entry: Use of specific conformation dependent neutralising antibodies against gp41. Disruption of adsorption into cellular membrane by polysulfonates and dextran sulphate.

3. Interference with the uncoating process with synthesised bicyclams.

4. Inhibition of reverse transcription by nucleoside and non-nucleoside analogs.

5. No specific inhibitors of transport of the preintegration complex to the nucleus have been described.

6. Use of anti-integration inhibitors such as ribozymes against integrase mRNA.

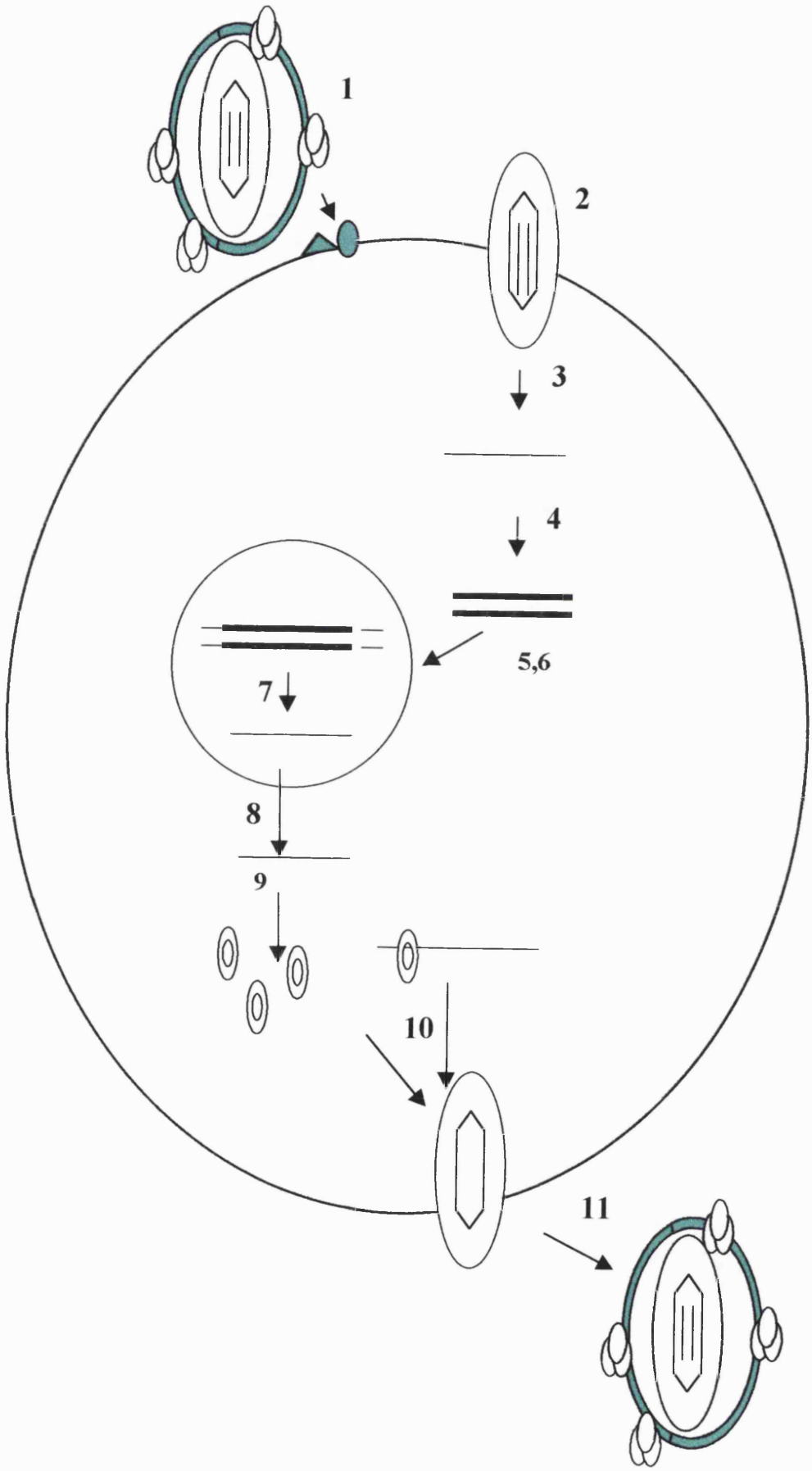
7. Blocking HIV gene expression by inhibition of trans-activation by Tat antagonists.

8. Interference with the transport of unspliced mRNA from the nucleus to the cytoplasm with transdominant mutants of Rev.

9. Inhibition of translation by antisense oligonucleotides, ribozymes and RNA decoys.

10. Use of HIV-1 protease inhibitors to inhibit cleavage of Gag and Gag-Pol precursor proteins. Inhibition of myristoylation of Gag protein and glycosylation of envelope glycoproteins gp120 and gp160.

11. Inhibition of budding or release of viral particles by interferon-alpha (IFN-alpha), aromatic polycyclic diones (Hypericin).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RNA decoys are RNA molecules that mimic viral RNA and can bind to specific viral proteins such as Tat and Rev and sequestration of the viral protein could be achieved to a level that would inhibit HIV expression. The tat responsive element TAR and the rev responsive element RRE have both been expressed under the control of an RNA polymerase III specific promoter and inhibition of viral gene expression has been demonstrated (Sullenger et al., 1991; Lee et al., 1992). A problem associated with RNA decoys is that they may sequester crucial cellular factors causing deleterious effects on cell function.

Protein centred strategies for inhibition of HIV-1 have included the use of mutant forms of viral proteins with partial homology to the wild type protein which by protein-protein interactions can interfere with the function of the wild type. This concept is known as a trans-dominant negative effect, where a small number of mutant proteins can disrupt the effect of a much larger number of wild type proteins by disrupting assembly or polymerisation of a viral protein complex. A dominant negative mutant of Rev known as Rev M10 has been shown to interfere with the function of wild type REV either through competitive binding to the RRE or formation of inactive multimers. Rev M10 has been shown to protect cells against HIV infection (Bevec et al., 1992) and also inhibit the production of infectious HIV-1 from chronically infected cells. Sequestration of HIV-1 structural proteins has been achieved using CD4 containing and endoplasmic reticulum retention signal (Buonocore et al., 1990. Single chain antibodies have also been used to bind and sequester HIV-1 envelope proteins to the endoplasmic reticulum (Marasco et al., 1993).

Autolytic destruction of infected cells has also been studied using potent toxins or conditional lethal genes. Toxic molecules used have included immunoconjugates containing ricin A and either CD4 or anti-gp41 (Till et al., 1990) and a pseudomonas exotoxin-CD4 hybrid protein (Ashorn et al., 1991). Delivering a gene that is cytotoxic only in the presence of another compound has been demonstrated by the Herpes Simplex Virus (HSV) thymidine kinase (TK) which, when expressed kills the cell only in the presence of ganciclovir or acyclovir. TK under the control of HIV-1 LTR is tat-

inducible has been shown to kill acyciclovir treated cells infected with HIV-1 (Caruso et al., 1995).

Inhibition of post-translational events such as myristoylation and glycosylation has also been attempted. The gag precursor protein (Pr55) and gag-pol precursor protein require attachment of myristic acid via an amide bond to the N-terminus to ensure production of mature infectious particles (Gottlinger et al., 1989). The myristoylation reaction is carried out by the cellular enzyme N-myristoyltransferase and several myristic acid inhibitors such as N-myristoyl glycinal diethylacetal (Tashiro et al., 1989) and 13-oxatetradecanoic acid (Bryant et al., 1991) have been shown to inhibit HIV replication *in vitro*, although very high concentrations are needed.

The envelope glycoproteins gp120 and gp41 are extensively glycosylated and a number of aminosugar derivatives have been developed to inhibit the alpha-glycosidase I enzyme that cleaves the terminal alpha-glucose unit and initiates trimming of the N-linked oligosaccharides. N-butyl-deoxynojirimycin (NBuDJN) has been shown to inhibit HIV replication in cell culture (Dedera et al., 1990) and has also been tested in patients at a dose of 1000mg every 8 hours in combination with zidovudine (Fischl et al., 1994). Gastrointestinal side effects such as diarrhea and abdominal pain were noted which were possibly due to the inhibitory effect of NBuDJN on intestinal alpha-glucosidases such as maltase and sucrase.

The Nucleocapsid (NC) protein of HIV-1 has also been targeted by novel antiviral agents. The NC protein contains two copies of an array of amino acids known as a zinc finger array consisting of Cys-X-Cys-X-His-Cys, where X is a variable amino acid residue. This region binds zinc to form a stable globular domain that is essential for recognition of the RNA genome and also plays a role in viral assembly. Nitroso-containing antiviral agents that eject zinc from the zinc finger array have been identified and used in clinical trials (Vandevelde et al., 1996).

1.13.1 Therapeutic approaches based on chemokine receptors

An important finding which preceded the discovery of the CCR5 coreceptor was that the chemokines for this receptor, RANTES, MIP-1alpha and MIP-1beta could inhibit infection by macrophage tropic strains of HIV in PBMC *in vitro* (Cocchi et al., 1995). It has also been shown that the chemokine ligands for CCR3, CCR5, CCR8 and CXCR4 can inhibit viral infection via their respective receptors (Amara et al., 1997; Alkhatib et al., 1996; Deng et al., 1996). Macrophage derived chemokine (MDC) secreted by CD8 cells is able to inhibit both macrophage and T-cell tropic viruses *in vitro* (Pal et al., 1997). Their usefulness as therapeutic agents in vivo may be limited as RANTES, MIP-1alpha and MIP-1beta are relatively ineffective at preventing infection of macrophages (Moriuchi et al., 1996). Modified form of RANTES have been shown to be chemokine receptor antagonists and to inhibit CCR5 - dependent virus infection. An eight amino acid truncation in the amino-terminus of RANTES, termed RANTES (9-68) has been shown to inhibit chemotaxis, leukocyte activation and virus infection through CCR5 (Arenzana-Seisdedos et al., 1996). Two amino-terminal derivatives of RANTES, met-Rantes and amino-oxypentane (AOP) RANTES also inhibit macrophage tropic strains of HIV and do not induce chemotaxis (Proudfoot et al., 1996; Simmons et al., 1997).

Small polypeptide inhibitors of coreceptors have recently been described which interact with CXCR4 and prevent T-cell tropic HIV entry (Donzela et al., 1998; Doranz et al., 1997). Two of these compounds ALX40-4C and T22 are small polypeptides while AMD3100 is a member of the bicyclam family of compounds. Each of the compounds interacts with CXCR4 and prevents ligand mediated signalling and viral entry. The effects of inhibiting CXCR4 function *in vivo* are not completely known, targeted deletion of the murine SDF-1 gene in genetic knockout experiments results in late fetal lethality, abnormal B cell and myeloid development and defective cardiac ventricular septum formation (Nagasawa et al., 1996).

Figure 1.10 illustrates the stages within the HIV-1 life cycle where interference with anti-virals is possible.

1.14 Aims

The aims of this thesis were to study the relationship between HIV-1proviral DNA load and the presence of Zidovudine drug resistance mutations in a cohort of infected individuals receiving anti-retroviral therapy and in a collection of post-mortem body tissues from individuals who had died of AIDS. To measure proviral DNA load accurately in both whole blood and PM tissues, a quantitative PCR was developed and validated. The presence of drug resistance mutations were detected by both sequencing PCR products and quantitated by use of a point mutation assay. The aim of studying PM tissues was to investigate the levels of proviral load and also the distribution of drug resistance mutations in various tissues and whether sites in the body could harbour HIV with differences in load and resistance profile. The clinical significance of HIV-1 proviral DNA load and its correlation with levels of drug resistance and prognosis for disease progression were assessed by studying a large cohort of seventy nine HIV-1 infected individuals and applying statistical models of survival.

CHAPTER 2

MATERIALS AND METHODS

This chapter describes in detail the laboratory methods used in this study. The first section describes the procedures used for the extraction of DNA from clinical samples and amplification by the polymerase chain reaction (PCR). Reaction conditions and thermocycling parameters are described for PCR amplification of the HIV-1 gag and reverse transcriptase genes. PCR product purification, ligation into pUC-18, screening of plasmid DNA mini preparations and DNA sequencing of clones is also described. Chemicals, enzymes and buffers used in procedures are described in tables 2.2 and 2.3. Methods used in the development and calibration of a quantitative PCR assay for HIV-1proviral DNA are described in chapter 3.

2.1 Clinical samples

All clinical samples were processed within a Class II microbiological safety cabinet (British Standard 5726: 1992), in a room dedicated to the manipulation of potentially infectious material.

2.1.1 Blood samples

Blood samples from HIV-1 positive individuals were obtained by the Ian Charleson Day Centre and by the Haemophilia Centre, Royal Free Hospital London. These patients had previously tested positive for HIV-1 antibodies using an enzyme-linked immunosorbent assay (ELISA, Abbott Labotatories). Peripheral venous blood was collected into sterile preservative (heparin , 50 units per 1 millilitre (UmL^{-1}) blood) and thoroughly mixed. Samples were received from patients recieving anti-retroviral therapy and from patients who were not receiving therapy.

The plasma and peripheral blood mononuclear cells (PBMC's) were separated from 10 ml of whole blood by density centrifugation (400xg for 30 minutes) on Ficoll (Pharmacia). The buffy coat obtained was washed twice in 20ml of RPMI (GIBCO) and centrifuged at 1500rpm for 10 minutes. The purified PBMC's were resuspended in 2 ml of freezing medium (foetal calf serum (FCS), 10% DMSO) and two samples of 1ml each were stored at -70°C. 2ml of the separated plasma was also stored at -70°C.

Inhibition of Taq polymerase by substances such as heparin and hemin has been demonstrated (Izraeli et al., 1991; Panaccio et al., 1991). The use of the Qiagen blood extraction kit described in section 2.2.2 has been shown to eliminate inhibitors of PCR amplification (Klein et al., 1997). Another study by Lin et al. (1997) recommended pretreatment of plasma samples with Heparinase I. This avoids inhibition of the amplification of HIV-1 and hepatitis C virus (HCV) RNA following a reverse transcription reaction. In that study, 50µl of eluate was mixed with 50µl of RT-PCR reaction mix. In the amplification procedure described in section 2.2.2 of this thesis, 2ul of a 200ul eluate is mixed with a 50µl PCR reaction mix, significantly reducing any effect of inhibitors by a dilution factor of 1:100.

2.1.2 Extraction of DNA from PBMC's

One ampoule of stored PBMC's, approximately 2.5 x 10^6 cells was slowly thawed at room temperature and the cells were then gently centrifuged at 1000x g for 10 minutes. Two methods were used to extract the DNA.

The cell pellet was resuspended in 500µl of extraction buffer (50mM EDTA, 100mM Tris-HCl pH 7.5, 50 mM NaCl, 100ug/ml proteinase K, 0.5% SDS) and incubated overnight at 37°C. Following incubation the DNA was extracted twice with 500µl of phenol and once with 500µl of chloroform. The aqueous phase was removed and mixed with 1ml of 100% ethanol and 50µl of 3M sodium acetate and the DNA was allowed to precipitate overnight at -70°C. The DNA precipitate was centrifuged at 14000xg for 30 minutes. The supernatant was discarded and the DNA pellet was washed twice with 70% ethanol and centrifugation at 14000xg for 10 minutes. The supernatant was discarded and the DNA pellet was dried under vacuum before being resuspended in 100µl of distilled water.

A second method for extraction of HIV-1 DNA from whole blood was also used following the manufacturers protocol of the QIAGEN WHOLE blood DNA extraction kit/QIAamp DNA blood kit (QIAGEN, Catalogue No 51104). Extraction of nucleic acid from clinical samples with the QIAGEN kit has been shown to be suitable for

PCR amplification of Hepatitis B virus (HBV) DNA (Klein et al., 1997), HCV RNA, HIV-1 RNA and DNA (Lin et al., 1997). The principle of the kit is based on binding of nucleic acids to a silica-gel membrane in the presence of a high salt buffer followed by washing to remove impurities, with subsequent elution of DNA in water or a low salt buffer. Most of the formulations and concentrations for the buffers in these kits were deemed *commercially sensitive* by the manufacturer, and were not available to be quoted. 1ml (2 x 500µl aliquots) was centrifuged at 10000rpm, through a QIAGEN column. Following two washes with WASH buffer A, 500µl of LYSIS buffer was added and the column was left to stand for 20 minutes. Following two washes with WASH buffer B , 200µl of elution buffer was added and the column was centrifuged at 10000rpm. The 200µl DNA eluate was stored at -70°C.

2.1.3 Extraction of DNA from post-mortem body tissue samples

Samples of post-mortem human body tissue were obtained at autopsy and placed in plastic Bijoux or universal containers and stored at -70°C until further use. The DNA was obtained by the proteinase K digestion and phenol-chloroform extraction method as described for PBMC DNA extraction (section 2.1.2). The amount of DNA was calculated by measuring the optical density (OD) at 250 nm and the DNA was stored in aliquots of 1µg/ml at -70°C.

2.2 Amplification of HIV-1 DNA by the polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a powerful *in vitro* method of DNA amplification. Target DNA is amplified through a series of repetitive cycles involving template denaturation, primer annealing, and the extension of the annealed primers by a heat stable DNA polymerase. This leads to an exponential increase in the amount of target sequence. To prevent the detection of previously - amplified specific sequences and also cloned material, precautions were taken to minimise contamination. A physically separate and distant room was used for the storage of PCR reaction buffers and reagents and the preparation of PCR reaction mixtures known as 'clean PCR set up'. Nothing entered this laboratory that had been present in any other laboratory

within the department. A separate room was also used for the processing, storage and addition of clinical samples to the PCR reactions. A further separate room was used for the thermal cycling process, gel electrophoresis and other analysis of PCR products. Gloves were used at all times and disposable laboratory coats were used in each room and not transferred. When nested PCR was performed a designated area in a separate room was available for transfer of an aliquot from the first round reaction mix to the second round reaction mix. Designated pipettes and sterile filter tips (Anachem, UK) were used at all stages of sample preparation and addition to PCR reaction mixtures. The addition of clinical samples to PCR tubes was also performed in a class II safety cabinet to minimise cross contamination. Multiple negative controls were performed with each batch of reactions including, open tubes containing reaction mix that controlled for aerosol contamination and closed tubes containing reaction mix that controlled for contamination of PCR reagents.

The use of separate rooms for each stage proved sufficient to minimise contamination, however other steps can be included to further minimise contamination levels. Irradiation with ultraviolet light can damage DNA and has been used to prevent contamination by irradiation of the reaction mix prior to the addition of template and the area used for PCR set-up (Sarkar and Sommer., 1990). Control of post-PCR contamination with amplified products has been demonstrated with the use of PCR products containing deoxyuracil which cannot be re-amplified in subsequent PCR reactions (Longo et al., 1990). The procedure involves incorporation of dUTP in place of dTTP in the PCR reaction mix and pretreating all reaction mixes with uracil-N-glycosylase (UNG), followed by inactivation of UNG with heat treatment. The uracil base of any uracil-containing DNA (contaminating DNA) is cleaved from the phosphodiester backbone by UNG and the resulting apyrimidinic sites are degraded by heat treatment. Normal DNA template containing thymine is unaffected and the UNG enzyme is inactivated by heat, preventing degradation of newly amplified DNA containing uracil.

2.2.1 Oligonucleotide primers used for the amplification of HIV-1 DNA

All primers used in this work were synthesised by R&D systems (Oxford) on an Applied Biosystems 394 Synthesiser. After synthesis all primers were purified by High Power Liquid Chromatography (HPLC). The positions of the primers is based on the HXB2 HIV-1 prototype sequence (Ratner et al., 1985a) and indicated in brackets.

2.2.1.1 HIV-1 gag primers

GE1, (Outer nested, S) 5'-GAGGAGCCACCCCACAAGATT-3' (1318-1339)

GE2, (Outer nested, AS) 5'-TAGGTGGATTATTTGTCATCCA-3' (1534-1575)

GI1, (Inner nested, S) 5'-TGCTAAACACAGTGGGGGGA-3' (1349-1368)

GI2, (Inner nested, AS) 5'-CCTGAAGGGTACTAGTAGTT-3' (1503-1522)

2.2.1.2 HIV-1 pol primers

SPP1 (outer nested, S) 5'-AGGACCTACACCTGTCAACAT-3' (2483-2503)

MH6A (Outer nested, AS) 5'-ACTCAGGAATCCAGGTGGCTT-3' (3769-3789)

SPP2 (Inner nested, S) 5'-GTTGACTCAGATTGGTTGCACTTT-3 ' (2519-2542)

SPP6 5'(Inner nested, AS) 5'-TGGAGTTCATAACCCATCCAAAG-3' (3231-3253)

SPP6B (Inner nested, AS) 5'Biotin-TGGAGTTCATAACCCATCCAAAG-3' (3231-3253)

2.2.1.3 HIV-1 envelope primers

V3.1 (Outer nested, S) 5'-TACAATGTACACATGGATT-3' (6958-6976)

V3.2 (Outer nested, AS) 5'-ATTACAGTAGAAAAATTCCCC-3' (7362-7382)

V3.3 (Inner nested, S) 5'-TGGCAGTCTAGCAGAAGAAG-3' (7001-7029)

V3.4 (Inner nested, AS) 5'-CTGGGTCCCCTCCTGAGG-3' (7315-7332)

S: Sense orientation

AS: Anti-sense orientation

2.2.2 PCR reaction reagents and conditions

In this study two approaches were taken for the amplification of HIV-1 DNA. The first involved a single round of amplification using two primers (GE1 and GE2) specific for the gag gene of HIV-1. The second approach was a nested PCR which comprised of two rounds of amplification using an outer set of primers (GE1 and GE2) for the first round and an inner set of primers (GI1 and GI2) for the second round of PCR. Both sets of primers were specific for the gag gene.

Nested PCR was used for the amplification of the pol gene of HIV-1 which includes reverse transcriptase (RT) region of HIV-1 encompassing zidovudine resistance mutations. The final nested PCR product of the RT gene was 735bp in length and included codon 41 to 74 at the 5' end and codons 215 and 219 at the 3' end. The same PCR reaction components and cycling conditions were used for HIV-1 gag and pol regions. Initially the optimisation of primer concentration was carried out with primer concentrations ranging from 2.0μm to 0.5μM concentration of primer per 50μl reaction volume. The optimum magnesium concentration was established by carrying out duplicate reactions with $MgCl_2$ ranging from 1mM to 5mM. The results from both optimisation procedures were visualised following electrophoresis of reaction products in ethidium bromide - stained 2.5% w/v agarose gels (See section 2.3).

2μl of QIAGEN extract or 5μl of proteinase K extract were added to a 50μl volume PCR mix containing 50mM KCl, 10mM Tris-HCl(pH 8.8), 1.5mM $MgCl_2$, 0.1% Triton X-100, 3.3μM each of dGTP, dATP, dTTP, dCTP, 0.5μM of each of the outer primers, and 3 units/reaction of Taq polymerase. The PCR solution and target DNA were subjected to thirty heat cycles, each consisting of 2 minutes at 94°C, 1 minute at 60°C and 1 minute at 72°C. This was followed by a final cycle of 7 minutes at 72°C. For a nested PCR 2μl of the first reaction were transferred to a second tube containing an identical buffer but with two inner primers replacing the outer primers in 50μl reaction volume. This was subjected to thirty cycles as described above.

2.3 Agarose gel electrophoresis of PCR products

Visualisation of PCR products was carried out using an ethidium bromide stained 2.5% gel. Five grams of agarose (Flowgen) were dissolved in 20ml of 10x TBE buffer (0.89M Tris-borate, 0.89M boric acid, 0.1M EDTA pH 8.5) and 180mls of distilled water and heated until boiling in a microwave oven on full power for 5 minutes (5 x 1 minute intervals) and mixed gently every minute. The gel was allowed to cool to approximately to 50°C, 2µl of ethidium bromide (stock concentration: 10mg/ml) was added (Final ethidium bromide concentration 100µg/ml) and the gel was poured onto a prelevelled electrophoresis tray with well-forming combs and allowed to set for thirty minutes at room temperature.

25µl of PCR product was added to 2.5µl of 10x loading dye and dispensed into a well of the agarose gel and electrophoresed at 150 volts for thirty minutes in 1x TBE buffer. Molecular weight markers (phiX-174 Hinf III) were also electrophoresed with the PCR products. After electrophoresis was completed the gel was placed on a transilluminator and the positive PCR products visualised by UV light. If required, a photograph was taken using a polaroid camera and Polaroid 667 film.

2.4 Phosphorylation of HIV-1 gag primers for blunt ended ligation

50 pmoles of primer in a volume of 5µl was added to 5µl of 10x kinase buffer (0.5M Tris (pH 7.6), 0.1M $MgCl_2$, 50mM Dithiothreitol (DTT), 1mM spermidine, 1mM EDTA), 5 µl of 10 mM ATP, 3µl (10 units) of polynucleotide kinase and 22µl of distilled water to make up a reaction volume of 50µl. The reaction was incubated in a water bath at 37°C for 30 minutes. The reaction was then stopped by adding 2µl of 0.5M EDTA (pH 7.4). 5µl of each phosphorylated primer was used in a PCR reaction.

2.5 Purification of PCR products

Prior to ligation of phosphorylated PCR products into a dephosphorylated vector excess unincorporated nucleoside triphosphates and primers were removed from the PCR products by using the GENE CLEAN (BIO 101 Inc) nucleic acid purification kit.

To 50µl of the PCR product amplified using phosphorylated primers, 150µl of 6M NaI solution was added. The resulting solution was mixed thoroughly, before 5µl of Glassmilk (prepared silica matrix) was added. This was mixed vigorously and incubated at room temperature for 20 minutes on a rotating drum to allow the formation of the Glassmilk\DNA complex. Following incubation the Glassmilk\DNA complex was pelleted by centrifugation for 5 seconds at 10,000rpm. The supernatant was removed and discarded and the pellet washed three times with Newash (NaCl,ethanol,water). After the third wash the DNA was dissociated from the Glassmilk in 20µl of 1x TE buffer (10mM Tris-HCl, pH 7.4; 1mM EDTA pH 8.0) by incubation at 55°C for five minutes. The Glassmilk was pelleted by centrifugation at 10,000rpm for 10 minutes, and the supernatant was transferred to a separate tube and stored at -20°C.

2.6 Dephosphorylation of pUC-18 vector

The pUC-18 vector (Pharmacia) was first linearised with S*ma-1* restriction endonuclease (Boehringer Mannheim), 2µl (20 units) of S*ma-1* added to 1µg (1µl) of pUC-18 vector 2µl of 10x restriction buffer (one phor-all buffer, GIBCO) and 15µl of distilled water. The reaction was carried out at 37°C for 3 hours. Following digestion of the plasmid the *Sma-1* enzyme was inactivated by heating at 65°C for 10 minutes. The plasmid was then dephosphorylated by adding 1µl (10 units) of calf intestinal phosphatase (CIP) and 5µl of 10x CIP buffer (500mM Tris-HCl, pH 9.0, 10mM $MgCl_2$, 1mM $ZnCl_2$ 10mM spermidine) and 24µl of distilled water directly to the restriction enzyme reaction mix. The dephosphorylation reaction was carried out at

37°C for 30 minutes and the phosphatase enzmye was inactivated by heating at 85°C for 15 minutes.

2.7 Filling in of 3' ends of PCR products using a 5' to 3' Polymerase enzyme (Klenow Fragment)

The products of PCR amplification (amplicons) have staggered ends which can be filled in by using a 5' to 3' polymerase enzyme, the Klenow Fragment, and deoxynucleotide triphosphates. The Klenow reaction consisted of 500ng of the purified PCR products, 2mM dNTP's, 10 units of Klenow Polymerase (Promega), 5x Klenow buffer (0.25M Tris-HCl, pH 7.2, 50mM $MgSO_4$, 0.5M DTT. The reaction was incubated at room temperature for 30 minutes. Following the reaction the extended product was purified by a phenol-chloroform extraction. The DNA was precipitated with 100% ethanol and 3mM sodium acetate and stored at -70°C. The precipitated DNA was pelleted by centrifugation at 14,000 rpm, and the pellet was washed with 70% ethanol, air dried, and resuspended in sterile distilled water (SDW).

2.8 Blunt ended ligation of Klenow filled-in PCR products

Ligation of PCR products into a *Sma-1* digested dephosphorylated pUC 18 vector was carried out using the protocol outlined in Sambrook *et al* (1989). The ligation reaction mixture consisted of 10 mM ATP, 10 x ligation buffer (300mM Tris-HCl pH 7.8, 100mM $MgCl_2$, 100mM DTT), 10 units of T4 DNA ligase (Amersham), and varying amounts of dephosphorylated vector. The reaction was incubated at 16°C for 24 to 48 hours or at 4°C overnight. Successful ligations were also achieved after incubation at 16°C for 72 hours.

2.9 Production of competent JM109 *E.coli* bacteria for transformation of ligation reaction products

Transformation of bacterial cells with ligated vector and DNA insert requires a host strain that is compatible with the method used to select transformants. The method of selection was the standard blue/white colour screening with standard ampicillin selection. The pUC-18 plasmid contains an ampicillin resistance gene that allows bacterial cells containing the plasmid to grow in medium containing the antibiotic. Blue/white colour screening refers to the ability to identify recombinant clones utilising the *lac* operon of *E.coli.* The pUC-18 plasmid also contains a segment of DNA derived from the E.coli *lac* operon that codes for the amino-terminal fragment of the enzyme beta-galactosidase. Synthesis of the enzyme can be induced by isopropylthio-beta-D-galactoside (IPTG), which is capable of intra-allelic complementation with a defective form of beta-galactosidase encoded by the host bacterium. Bacteria exposed to IPTG synthesise both fragments of the enzyme and form blue colonies when grown on media containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl-beta-D- galactoside (X-gal). Insertion of DNA into the polycloning site of the plasmid inactivates the amino-terminal of the beta-galactosidase gene and bacteria containing recombinant plasmids develop white colonies.

The JM109 bacterial strain was used for colour selection and was first grown on minimal (M-9 medium) plates, supplemented with thiamine-HCl prior to the preparation of competent cells and transformation. The JM109 strain carries lacA M15 and $lacI^Q$ on an F' episome. Growth of the JM109 strain on supplemented minimal plates (M-9) selects for the presence of the F' which carries a nutritional requirement for growth (proline biosynthesis) and decreases the number of false positives. The JM109 strain of bacteria is made 'competent' to receive plasmid DNA by transformation. Treatment of bacteria with an ice-cold solution of $CaCl_2$ and a brief heat-shock treatment allows DNA from a variety of sources to be taken up by the bacteria. The efficiency of transformation can be increased by treating bacteria with reducing agents and hexaminecobalt chloride (Sambrook et al., 1989). *E.coli* can also

be transformed by high voltage electroporation also known as electrotransformation. Electroporation is carried out at low temperatures in a chilled cuvette and requires high electrical field strenghts for introduction of DNA.

Ten microlitres of JM109 stock cells (stored in 80% glycerol at -70°C) were inoculated into 10ml of Luria broth (LB) medium and incubated overnight in a 37°C shaking incubator. The next day, 500µl of the overnight culture was inoculated into 400ml of LB medium and incubated at 37°C in a shaking incubator until the optical density (OD) of the culture at 600nm was approximately 0.2. To reach this OD value the culture was incubated for approximately 4.5 hours. The bacterial cells were harvested by low speed centrifugation at 1000rpm for 15 minutes. The cells were then resuspended in 200ml of chilled transformation buffer, TFB (10mm 2-[N-morpholino]ethanesulfonic acid (MES), 45mM $MnCl_2$, 10mM $CaCl_2$, 100mM KCl, 3 mM Hexaminecobalt chloride) and left on ice for 20 minutes. The cells were centrifuged again at 1000rpm and resuspended in 20mls of TFN buffer. 400µl of the competent JM109 bacterial cells were aliquoted into 1.5ml eppendorfs, snap frozen in dry ice\ethanol and stored at -70°C until required.

2.10 Transformation of competent *E.coli* bacterial cells with pUC-18 vector

An aliquot (200µl) of competent cells (JM109) was thawed on ice. Ten to 20ng of plasmid DNA (approximately half the ligation reaction) was added to the competent cells and then incubated on ice for 30 minutes. A heat shock treatment of 42°C for 2 minutes was then carried out in a water bath to increase the efficiency of transformation. The cells were then cooled on ice for 1 minute. 2ml of LB medium was added and the cells were incubated for 1 hour at 37°C to allow the cells to recover. The cells were concentrated prior to plating out by low speed centrifugation (1000rpm) and resuspended in 100-200µl of LB medium. The cells were plated on an LB plate containing 50µg/ml ampicillin, 0.5mM IPTG and 40µg/ml X-GAL and incubated at 37°C overnight. Recombinant colonies containing the insert appear white, whereas non-recombinants containing beta galactosidase activity are blue as outlined above.

2.11 Isolation of plasmid DNA

2.11.1 Mini-preparation of plasmid DNA

5ml of LB medium containing 50μg/ml ampicillin was inoculated with a single recombinant bacterial colony and incubated at 37°C overnight with vigorous shaking. Following the overnight incubation the cells were pelleted by centrifugation at 1000rpm for 15 minutes. The bacterial cell pellet was resuspended in 200μl of Solution 1 (50mM glucose, 10mM EDTA, 25mM Tris-HCl pH 8.0, 5mg/ml lysozyme (Sigma)) and incubated at room temperature for 5 minutes. 500μl of freshly prepared Solution 2 (0.2N NaOH, 1% SDS) was added and mixed by inversion (care was taken not to vortex or shake vigorously) and incubated on ice for 5 minutes. 375μl of Solution 3 pH 4.8 (60ml of 5M potassium acetate, 11.5ml of glacial acetic acid, and 28.5 ml of double distilled water, stored at 4°C) was added, mixed by inversion and incubated on ice for 5 minutes. The chromosomal DNA was pelleted by centrifugation (14000rpm) for 5minutes and supernatant was transferred to a separate Eppendorf tube and extracted once with phenol-chloroform. The supernatant was transferred to a fresh tube treated with 20μg/ml RNase A (Sigma) for 30 minutes at 37°C. The RNase A treated sample was extracted again with phenol-chloroform and the upper aqueous phase was transferred to a fresh tube containing 2 volumes of 100% ethanol and 10% volume of sodium acetate. The plasmid DNA was precipitated at -70°C for 1 hour. The DNA was pelleted by centrifugation (14000rpm) for 30 minutes and the pellet was washed with 70% ethanol by centrifugation (14000rpm). The DNA pellet was dried under vacuum and dissolved in 10-20μl of sterile deionised water.

2.12 Restriction Endonuclease Digestion of Plasmid DNA

The presence of a ligated DNA insert was confirmed by digestion of the plasmid DNA with two restriction enzymes (double digestion) to release the ligated insert from the plasmid DNA.

1μg of purified plasmid DNA was digested with 10 units of EcoR1 and Hind III restriction enzymes in buffer B (10mM Tris-HCl, 100mM NaCl, 5mM $MgCl_2$, 1mM beta-mercaptoethanol) by incubation at 37°C for 2 hours. The restriction enzyme digestion products were separated on a 2% agarose gel in 1 X TBE buffer by electrophoresis at 150 volts for 1 hour with molecular weight markers consisting of lambda Hind III and phix 174 Hinf III DNA fragments.

2.13 Large scale preparation and purification of closed circular plasmid DNA by equilibration centrifugation in Cesium Chloride-Ethidium Bromide gradients, and by silica matrix columns

A large scale preparation of plasmid DNA from recombinant clones was made from a 50ml overnight culture of the plasmid in LB supplemented with 50ug/ml ampicillin in a shaking incubator at 37°C overnight. DNA was isolated from the plasmid preparation by the same method described in section 2.6.2, with buffer volumes and reagents increased by a factor of ten.

For quantitative PCR it was necessary to use, pure closed circular plasmid DNA was used as a template. The plasmid was purified by centrifugation in a cesium chloride (CsCl)-Ethidium Bromide gradient (Sambrook, *et al* 1989). For every millilitre of the DNA solution exactly 1g of solid CsCl was added and the solution was warmed to 30°C to dissolve the salt. 0.8ml of a solution of ethidium bromide (10mg/ml in water) was added for every 10ml of DNA/CsCl solution. The solution was centrifuged at 800rpm for 5 minutes, and the red solution under the top scum was transferred to an ultracentrifuge tube (Beckman Quick-Seal). The remainder of the tube was filled with light paraffin oil and balanced with a second Beckman centrifuge tube. The tubes were then sealed with a metal cap using the Beckman sealing device. The density gradient was centrifuged for 24 hours at 60,000rpm (500,000 x g) using a Ti65 rotor at 20°C. After centrifugation the lower of the two bands containing closed circular DNA, located in the centre of the gradient was removed using a 21-gauge hypodermic needle. The upper band contains linear bacterial (chromosomal) DNA and nicked

circular plasmid DNA. Following collection of the plasmid band, the ethidium bromide is removed from the DNA solution by extraction with organic solvents. An equal volume of water-saturated butan-1-ol was added to the DNA solution in an Eppendorf tube and mixed by vortexing. The mixture was centrifuge for 10 minutes at 14000rpm (11,000 x g) in a microcentrifuge. The lower aqueous phase was transferred to a clean Eppendorf tube and the extraction procedure was repeated until all the pink colour had disappeared from both the aqueous and organic phases. The CsCl was removed from the DNA solution by dilution with 3 volumes of water and precipitating the DNA with 2 volumes of 100% ethanol for 15 minutes at 4°C followed by centrifugation at 14000rpm (11,000 x g) in a microcentrifuge for 15 minutes. The DNA pellet was dissolved in sterile distilled water. The OD_{260} of the final DNA solution was measured and the concentration of the DNA was calculated. The DNA was stored in aliquots at -20°C.

An alternative and simpler method of large scale plasmid DNA preparation was carried out using the Qiagen Maxi prep kit (Qiagen Inc) according to the manufacturers instructions. The type of purification column used was a Qiagen - tip 500 which is appropriate for high copy number plasmids with a typical yield of 300 - 500μg DNA from 100ml bacterial culture grown in LB. A 100ml culture of LB was set up containing a 5ml subculture of the plasmid, and incubated at 37°C overnight. The bacterial cells were harvested by centrifugation at 6,000rpm in a IECPR-7000 centrifuge for 15 minutes at 4°C. The bacterial pellet was completely resuspended in 10ml buffer P1 (50mM Tris-HCl, pH 8.0, 10mM EDTA, 100μg/ml RNase A), 10ml of cell lysis buffer P2 (200mM NaOH, 1% SDS) was added and the lysate mixed gently and incubated at room temperature for 5 minutes. 10ml of chilled buffer P3 (neutralisation buffer, 3.0M potassium acetate pH 5.5) was added, the lysate mixed gently and incubated on ice for 20 minutes in 50ml Falcon tubes. The cell lysate was centrifuged at 20,000g for 30 minutes at 4°C and the supernatant re-centrifuged for a further 10 minutes to ensure removal of all particulate material. Meanwhile a Qiagen Tip - 500 for each DNA preparation was equilibrated with 10ml equilibration buffer QBT (750mM NaCl, 50mM MOPS, pH 7.0, 15% (v/v) propan-1-ol. 15% (v/v)Triton X-100) by allowing the buffer to flow through the column by gravity. The cleared supernatant was placed onto the column and allowed to enter the resin by gravity flow.

The flow through was discarded and the column washed twice with 30ml QC wash buffer (1.0M NaCl, 50mM MOPS, pH 7.0, 15% (v / v) isopropanol). DNA was eluted off the column by the addition 15ml QF elution buffer (1.25M NaCl, 50mM Tris-HCl, pH 8.5, 15% (v/v) isopropanol), precipitated with 0.7 volumes (10.5 ml) 95% isopropanol at room temperature and centrifuged at 15,000g at 4°C for 15 minutes. The DNA pellet was washed twice in 70% (v/v) ethanol, the pellet was allowed to air dry and then resuspended in 500µl of SDW. The stock DNA was stored at -70°C until use.

2.14 Sequencing of cloned PCR products in the pUC-18 vector

Manual sequencing was carried out using the SEQUENASE VERSION 2, sequencing kit from United States Biochemicals (USB). Purified plasmid DNA containing PCR product insert isolated by the mini-prep method was first denatured to single stranded DNA by alkali treatment prior to sequencing. Five micrograms of DNA template (approximately 20µl of mini-prep DNA) was treated with 5µl of denaturing solution (1M NaOH, 1mM EDTA) and incubated at room temperature for 5 minutes. The DNA was then desalted by centrifugation through a Sephadex CL6B column (PHARMACIA) for 3 minutes at 3000xg. The column eluate was then used for sequencing.

The DNA was sequenced in both directions using the M13 forward and reverse primers (Pharmacia), the corresponding sequences of the M13 primers are present in the pUC-18 vector located either side of the multiple cloning site. 8.5µl (5ng) of template DNA was added to a primer annealing mix of 1µl of 10x TM buffer, 2µl (50 pmols) of forward or reverse primer, and incubated at 37°C for 15 minutes. Following the primer annealing step the sequencing reaction was carried out. The following reagents were added to the template plus primer: 1µl of 0.1M DDT, 2µl of labelling mix (diluted 1:5) containing 7.5µM dGTP, dCTP, dTTP, 0.5µl (5µCi) of radiolabelled ^{35}S dATP (Amersham), the solution was mixed by pipetting, before 2µl (2 units) of diluted SEQUENASE enzyme (diluted 1:8 in enzyme dilution buffer). The reaction was incubated at room temperature for 5 minutes. During this time the termination

mixes were prepared by adding 2.5µl of each dideoxynucleotide (ddGTP,ddATP,ddTTP,ddCTP) in to four tubes labelled G,A,T and C for both forward and reverse primer reactions. The tubes were incubated at 37°C on a PCR thermocycler to ensure constant temperature control.

The termination step was then carried out by adding 3.5µl of the sequencing reaction into each of the ddNTP tubes. The termination reaction was incubated at 37°C for 5 minutes. The termination reactions were stopped by adding 4µl of STOP solution (95% Formamide, 20mM EDTA, 0.1% Bromophenol blue, 0.1% Xylene Cyanol FF) into each tube. The sequenced DNA products were then either stored at -20°C or heated to 95°C prior to loading on 8% polyacrylamide denaturing gel.

2.15 Direct sequencing of PCR products

A consensus sequence of a PCR product can be achieved by direct sequencing instead of cloning the product into a plasmid vector. Two methods of direct sequencing are described in this thesis. The first involves purification of PCR products by magnetic beads and separation to single strands by alkali denaturation and then manual sequencing using the SEQUENASE VERSION 2 sequencing kit from United States Biochemicals (USB). The second method for direct sequencing was by direct cycle sequencing of PCR products using the thermo sequenase radiolabelled terminator cycle sequencing kit (Amersham).

2.15.1 Biomagnetic purification and separation of PCR products by Dynabeads (Dynal AS)

This method involves the use of streptavidin-coated magnetic beads (Dynabeads) to prepare single-stranded DNA from double stranded PCR products for solid phase DNA sequencing. M-280 streptavidin-coated magnetic beads are supplied as a suspension containing 6.7 x 108 Dynabeads/ml in PBS (pH 7.4) containing 0.1% BSA and 0.02% sodium azide as a preservative). The final PCR reaction volume was 100µl. A biotinylated prime at the 5' end and a non biotinylated primer at the 3' end, were used in a ratio of 10pmol : 30pmol respectively. For each PCR product sample, 20µl of streptavidin-coated M-280 beads were washed once in an equal volume of binding and washing buffer (10mM Tris-HCl, pH 7.5, 1mM EDTA, 2.0M NaCl). After magnetic separation the dynabeads were resuspended in twice the volume of binding and washing buffer to give a working concentration of 5µg/µl. To allow the immobilisation of the PCR product, 40µl of PCR product was added to an equal volume of washed dynabeads (approximately 13.4 x 10^6 magnetic beads per PCR product aliquot) and incubated at room temperature for 20 minutes. Following incubation, the tubes were placed in a magnetic separator and the supernatant was removed and discarded. The beads were then washed in 50µl of binding and washing buffer, before being incubated for 10 minutes in 0.1M NaOH to separate the strands. The tubes were then placed back in a magnetic separator and the NaOH supernatant containing the non biotinylated strand was removed. The dynabeads with the immobilised biotinylated strand were washed once with 50µl of 0.1M NaOH, once with 50ul binding and washing buffer and once with 50µl Tris-EDTA (TE) buffer (10mM Tris-HCl pH 9.0, 1mM EDTA) before being resuspended in 20µl of SDW.

2.15.2 Direct manual sequencing

Direct sequencing was carried out using the SEQUENASE VERSION 2, sequencing kit (USB) in conjunction with a modified method described by Winship (Winship, 1989). The modification consists of the inclusion of 10% tissue culture grade DMSO which was added to the annealing and termination reactions. This was found to enhance the intensity of the signal and reduce background interference. The

sequencing reaction involved the addition of approximately 100ng of purified PCR product (Gene-Clean) to the annealing mix (10% DMSO, 200mM Tris-HCl pH 7.5, 100mM $MgCl_2$,250 mM NaCl, 10ng oligonucleotide primer), which was then boiled for three minutes to denature the template. Following boiling, the mix was placed on ice thus preventing template renaturation, and extension mix (0.025mM DTT, 2µl of diluted labelling mix, 1uci of radiolabeled ^{35}S dATP, 2 units of sequenase enzyme) was added. The sequencing reaction mixture was then divided between four prewarmed (40°C) termination mixes containing: 80µM dNTPs (dGTP,dATP,dTTP,dCTP); 8µM ddNTPs (ddGTP,ddATP,ddCTP,ddTT); 50mM NaCl; 10% DMSO and left to incubate for five minutes. The reaction was then stopped by the addition of 4µl of Stop solution (95% Formamide, 20mM EDTA, 0.1% Bromophenol blue, 0.1% Xylene cyanol FF). Prior to loading on a 8% polyacrylamide gel the samples were heated to 95°C for two minutes.

2.15.3 Direct cycle sequencing

Direct cycle sequencing of PCR products was carried out by the Thermo Sequenase radiolabelled terminator cycle sequencing kit (Amersham). Thermo Sequenase is a thermostable DNA polymerase used in cycle sequencing which generates stronger signals due to multiple rounds of DNA synthesis. The amplification process employs a single primer so the amount of product DNA increases linearly with the number of cycles. Synthesis is initiated at the site where the primer anneals to the template and elongation proceeds from the 3' end of the annealed primer by the DNA polymerase in the presence of dNTPs, and is terminated by the incorporation ^{33}P labelled ddNTPs.

PCR products were first treated with 1µl (10 units) of Exonuclease 1 and 1µl (2 units) of shrimp alkaline phophatase (SAP) and incubated at 37°C for 15 minutes and then at 80°C for 5 minutes. The Exonuclease I removes residual single stranded primers and any single stranded DNA produced by the PCR. The shrimp alkaline phosphatase removes the remaining dNTPs from the PCR mixture, which would interfere with the sequencing reaction. The exonuclease and shrimp alkaline phosphatase are inactivated by heating to 80°C for 15 minutes. For each sample four eppendorf tubes were

labelled G, A, T and C and the termination mixes were prepared by adding 2µl of each termination mix (G, A, T or C) to 0.5ul of the appropriate ^{33}P radiolabelled dideoxynucleotide (ddGTP, ddATP, ddTTP or ddCTP). A reaction mix was prepared with 2µl of reaction buffer, 0.5µl of sequencing primer (50ng), 2µl of sequenase enzyme (10 units/µl) and 12.5µl of SDW. 3µl of DNA was added to the reaction mixture (17µl) and 5µl of this mix was aliquoted into each of the tubes labelled G, A, T and C. 2.5µl of the appropriate termination mix was added to each of the tubes, mixed and covered with 50ul of mineral oil. The tubes were then placed in a thermocycler and subjected to 35 cycles of 94°C for 30 secs, 55°C for 1 min, and 72°C for 1 min. Following thermocycling, 6ul was removed from each tube and aliquoted into the wells of a microtitre plate, 4µl of Stop solution was added to each well to terminate the reaction. The microtitre plate was sealed with a cellophane cover and stored at -20°C until use. Prior to electrophoresis the microtitre plate was heated to 90°C for 5 minutes on a heating block.

2.16 Polyacrylamide gel electrophoresis of sequencing products

Polyacrylamide gel electrophoresis was carried out using 8% wedge gels (0.4mm at the top and 1.2mm at the bottom). For 100ml of 8% gel, 50g Urea, 20ml of 40% bis-acrylamide, 0.1g ammonium persulfate, 10ml of 10X TBE buffer, and 30ml of distilled water were mixed together. Prior to pouring 20µl of TEMED (N,N,N',N',-Tetramethylethylenediamine) was added and after pouring the gel between two glass plates, a well forming comb was put in place. The gel was left to polymerise at room temperature for 30 to 45 minutes.

Approximately half of the sequencing reaction (7µl) was electrophoresed at 75 watts and maximum current in 1x TBE buffer for 2.5 hours until the bromophenol blue dye reached the bottom of the gel. Following electrophoresis, the gel was fixed by soaking in 7% methanol, 7% acetic acid solution. The gel was then dried for 2 to 3 hours at 80°C on a vacuum drier, placed in an autoradiography cassette containing Kodak XAR-5 film and exposed overnight. Exposure of films at –70°C was not necessary given the high sensitivity and resolution of Kodak XAR-5 film.

2.17 Detection of zidovudine resistance mutations using the Point Mutation Assay

The Point Mutation Assay (PMA) for the analysis of the distribution of zidovudine resistant mutations was performed as essentially described in Kaye et al (1992) and is outlined in Figure 2.1. Firstly a 1306bp fragment of HIV-1 RT was amplified using nested primer sets, SPP1 and MH6A in the first round and a 734bp fragment by primers SPP2 and SPP6 in the second round (section 2.2.2). The 734bp amplified product included codons 41 to 74 of the reverse transcriptase gene at the 5' end and codons 215 and 219 at the 3' end. In the second round PCR one of the two primers was biotinylated depending on which codon was being analysed. Primer SSP2 was biotinylated for use with probe ARP1 (codon 67), ARP3 (codon 219), ARP4C (codon 215) and primer SPP6 was biotinylated for use with probe ARP 2B (codon 70). The PCR reaction was carried out as described in section 2.2.3. Prior to performing the PMA, the PCR products were bound to a streptavidin coated microtitre plate (See section 2.17.2).

2.17.1 Probe sequences used to assay point mutations

Sequences of the probes are on the HXB2 protype sequence and number and type of base etended from the probe is indicated.

Probe A41 (codon 41, antisense) AAATTTTCCCTTCCTTTTCCA (2670-2691). Mutant adds A or G x 2

Probe ARP1 (codon 67, antisense) TTTTCTCCATTTAGTACTGT (2749-2768). Wild-type adds C, mutant adds T x 7.

Probe ARP2B (codon70, sense) AAAGAAAAAAGACAGTACTA (2738-2757). Wild type adds A x 2, mutant adds G.

Probe ARP4C (codon 215, antisense) CTGATGTTTTTTGTCTGGTGTG (3194-3215). Wild-type adds G, mutant adds A x 2 (Phe) or T (Tyr).

Probe ARP3. (codon 219, antisense) AGGTTCTTTCTGATGTTTTT (3205-3224). Wild type adds T, mutant adds G.

2.17.2 Streptavidin coating of microtitre wells

A solution of 25μg/ml streptavidin was prepared in 100mM Tris-HCl, pH 7.6 containing 0.1% sodium azide. 25μl of the streptavidin solution was added to each well of the microtitre plate and incubated overnight at room temperature in a moist box. The wells were then washed twice with PBS (Dulbecco A) and filled with blocking solution (1% BSA in PBS containing 0.1% azide), and incubated at room temperature for 1 hour. The blocking solution was then partially removed leaving 100μl in each well and the microtitre plates then stored at 4°C for up to 6 months.

2.17.3 Point Mutation Assay procedure

Following PCR of the HIV RT gene, 10µl aliquots of PCR product were diluted to 25µl with 10mM Tris-HCl pH 7.6 containing 0.05% Tween 20 (Buffer TTB) and added to each of the four microtitre plate wells (Nunc, U-well) coated with 25µl/well streptavidin (25µg/ml), and incubated at room temperature for 15 minutes to capture the biotinylated PCR product. The microtitre plate wells were then washed for 3 x 1 minutes with TTB buffer and then 40µl of 0.15M NaOH was added to each of the wells for 5 minutes at room temperature to denature the PCR product. The wells were then washed with TTB buffer for 4 x 1 minutes and 25µl of anneal mix (40mM Tris-HCl, pH 7.6, 20 mM $MgCl_2$, 50mM NaCl) and 2.5µM of the appropriate oligonucleotide probe was added to each well. The microtitre plate was sealed with a plastic disposable plate sealer and heated in a water bath to 65°C for 2 minutes and then left to cool for 30 minutes to room temperature on a metal block that was previously heated to 65°C, allowing the oligonucleotide probe to anneal. A 6µl aliquot of labelling mix (40mM Tris-HCl, pH 7.6, 16.6 mM dithiothreitol, 0.016 BSA, 0.15 units Klenow DNA polymerase and 0.16uM of one ^{35} S-labelled dNTP, 1,200 Ci/mmol) was added to each of the four wells and incubated for 2 minutes. The wells were then washed for 5 x 1 minutes with TTB buffer to remove unincorporated label. 40µl of 0.1M NaOH solution was added to each of the wells and incubated for 5 minutes. The content of each well was then added to a 5ml scintillation cocktail (Sigmafluor, Sigma UK) and samples were counted for 1 minute in a scintillation counter.

2.17.4 Calculation of Point Mutation Assay results

1. The ambient background is subtracted from all readings. This is found by counting a vial containing scintillation cocktail only.

2. The readings for the following bases are then divided by the numbers indicated. This is required because where these bases occur in a sample being analysed an

identical base or bases follows it and the Klenow polymerase adds more than one labelled base.

Codon 41	divide the G signal by 2
Codon 67	divide the T signal by 7
Codon 70	divide the A signal by 2
Codon 215	divide the A signal by 2

3. The mean of the signals of the bases which do not occur at the point being analysed is then calculated as follows:

Codon 41	C only
Codon 67	mean of A and G
Codon 70	mean of C and T
Codon 215	C only
Codon 219	mean of A and C

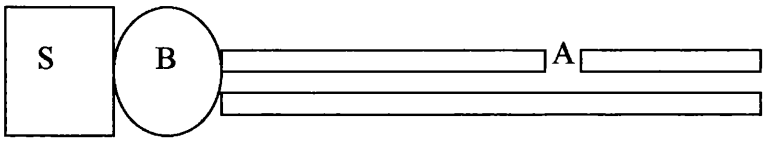
4. The background signal (calculated in 3) is then subtracted from the wild type and mutant signals as follows:

Codon 41	Wild type = T	Mutant = A or G
Codon 67	Wild type = C	Mutant = T
Codon 70	Wild type = A	Mutant = G
Codon 215	Wild type = G	Mutant = A or T
Codon 219	Wild type = T	Mutant = G

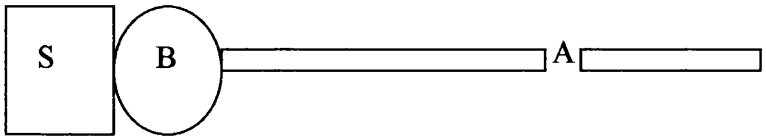
5. The percentage of wild type or mutant at the codon being analysed is then calculated using the following formulae:

% Mutant (M) = (Corrected M signal / Corrected M signal + Corrected WT signal) x 100.

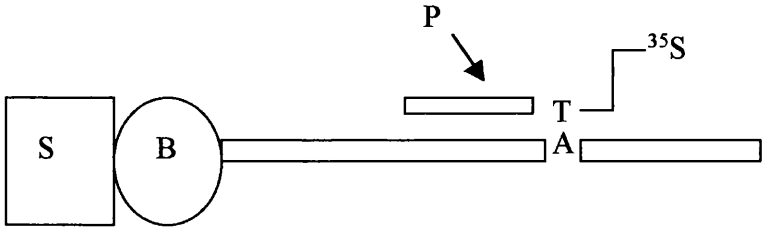
% Wild type = (Corrected WT signal / Corrected WT signal + Corrected M signal) x 100.



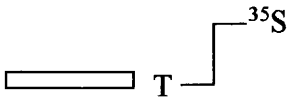
1. Binding of biotinylated PCR product (B), containing mutated base A, to streptavidin coated microtitre well (S)



2. Denaturation of PCR product to obtain single stranded DNA



3. Annealing of appropriate probe (P) and extension with ^{35}S labelled base



4. Denaturation of radiolabelled extended probe and addition to scintillation cocktail for radioactivity counting

Figure 2.1. Schematic outline of the Point Mutation Assay

2.18 Line Probe Assay for the detection of drug-induced mutations in the HIV-1 RT gene

The Line Probe Assay (LiPA) is a genotyping assay from Innogenetics (INNO, Belgium) for drug induced mutations at codons 41, 69, 70, 74, 184 and 215 based on reverse hybridisation. The assay described in this thesis was provided by Innogenetics to evaluate ease of use in a laboratory setting and reproducibility of results. A region of the HIV-1 RT gene was amplified in a nested PCR reaction using primers SPP1 and MH6A (first round) and SPP6 and biotinylated SPP6 (second round) as described in sections 2.2.4 and 2.2.5. The biotinylated PCR product was denatured and hybridised to a LiPA membrane strip containing oligonucleotide probes specific for the drug induced mutations, wild type sequences and polymorphism's at 20 individual lines on the strip. A streptavidin alkaline phosphatase conjugate was then added followed by substrate components (NBT and BCIP). A purple brown precipitate was then developed on the membrane strip at the appropriate line. The components of the solutions provided in the LiPA assay were not described.

2.18.1 Line Probe Assay procedure

Following PCR amplification of the HIV-1 RT gene, 10µl of the biotinylated second round product was added to an equal volume of denaturation solution in a test trough and incubated for 5 minutes at room temperature. 2ml of prewarmed hybridisation solution (39°C) was added to the trough together with a membrane strip and incubated at 39°C in a shaking water bath (approximately 80 rpm) for 30 minutes with the lid closed. The hybridisation solution was then aspirated from the trough and the membrane strip was washed with 2ml of prewarmed stringent wash solution (39°C) twice at room temperature for 20 sec and at 39°C for 10 minutes, to remove unbound and nonspecifically bound PCR product. The membrane strip was further washed for 2 x 1 min with 2ml of rinse buffer. 2ml of streptavidin alkaline phosphatase conjugate solution was added to each membrane strip and incubated for 30 minutes at room temperature while agitating on an orbital shaker. Each strip was then washed for 2 x 1 min with rinse solution and for 1 minute with substrate buffer. 2ml of substrate

solution (containing NBT and BCIP) was then added to each strip and incubated for at least 5 minutes while agitating at room temperature until the colour reaction was complete. The strips were then removed from the troughs and allowed to dry on absorbant paper before reading the results. The developed strips were then stored in the dark.

Table 2.2 Description of chemicals and enzymes and associated suppliers

CHEMICAL	SUPPLIER
Adenosine triphosphate (ATP)	Sigma
Agar (Bacteriological grade)	Oxoid
Bis-Acrylamide	Severn-Biotech
Agarose	Flowgen
Agarose (Low melting point, LMP)	Flowgen
Ammonium persulfate (APS)	Sigma
Ampicillin	Sigma
Boric acid	Sigma
Bovine serum albumin (BSA)	Sigma
Butan-1-ol	BDH
RPMI Medium	Gibco-Brl
Calcium Chloride ($CaCl_2$)	Sigma
Calf Intestinal Phosphatase (CIP)	Pharmacia
Cesium Chloride (CsCl)	Sigma
Chloroform	BDH
Deoxy nucleotide triphosphates (dNTPs)	Promega
Dimethylsulfoxide (DMSO)	Sigma
Dithiothritol (DTT)	Sigma
T4 DNA Ligase	Amersham
EcoR I restriction enzyme	Promega
Ethanol	BDH
Ethidium Bromide	Sigma
Ethylenediaminetetra acetic acid (EDTA)	Sigma
Ficoll	Pharmacia
Foetal Calf Serum (FCS)	Gibco-Brl
Formaldehyde (37% solution)	Promega
Glucose	Sigma
Glacial acetic acid	BDH
Hind III restriction enzyme	Promega
Hexaminecobalt chloride ($Co(NH_3)_6Cl_3$	Sigma
Isopropanol	BDH
Isopropylthio-beta-D-galactoside (IPTG)	Sigma
Klenow polymerase	Promega
Lysozyme	Sigma
Magnesium Chloride ($MgCl_2$)	Bioline
Magnesium sulphate ($MgSO_4$)	Sigma
Manganese Chloride ($MnCl_2$)	Sigma
beta-Mercaptoethanol	Sigma
Methanol	BDH
Molecular weight markers (phiX-174 Hinf III)	Promega
(2-[N-Morpholino]ethanesulfonic acid (MES)	Sigma
(3-[N-Morpholino]propanesulfonic acid (MOPS)	Sigma
Phenol	Sigma

Phenol-Chloroform	Sigma
Phosphate buffered saline (PBS)	Oxoid
Polynucleotide kinase	Boehringer Mannheim/Roche
Potassium acetate (CH_3.COOK)	Sigam
Potassium chloride (KCl)	Sigma
Proteinase K	Gibco-Brl
pUC-18 vector	Pharmacia
RNase A	Sigma
Scintillation cocktail (Sigmafluor)	Sigma
Silver Nitrate	Promega
Sodium Azide (NaAz)	Sigma
Sodium Acetate (NaAc)	Sigma
Sodium Carbonate	Sigma
Sodium Chloride (NaCl)	Sigma
Sodium dodecyl Sulphate (SDS)	Sigma
Sodium hydroxide (NaOH)	Sigma
Sodium Thiosulfate	Promega
Sma-1 restriction enzyme	Boehringer Mannheim/Roche
Spermidine	Sigma
Streptavidin	Sigma
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Sigma
Tris-borate	Sigma
Tris-HCl	Sigma
Triton X-100	Sigma
Tryptone	Oxoid
Tween 20	Sigma
Urea	Merck
Yeast extract	Oxoid
Zinc Chloride ($ZnCl_2$)	Sigma
5-bromo-4-chloro-3-indolyl-beta-D-galactoside (X-Gal)	Sigma

Table 2.3 Description and composition of reaction buffers

Buffer	
DNA extraction buffer	50mM EDTA, 100mM Tris-HCl, 50mM NaCl, 100ug/ml proteinase K, 0.5% SDS
PCR buffer (10x)	500mM KCl, 15mM $MgCl_2$, 1.0% Triton X-100
TBE (10x)	0.89M Tris-borate, 0.89M boric acid, 0.1M EDTA pH 8.5
Kinase buffer (10x)	0.5M Tris-HCl, 0.1M $MgCl_2$, 50mM DTT, 1mM spermidine, 1mM EDTA
CIP buffer (10x, dephosphorylation)	500mM Tris-HCl pH 9.0, 10mM MgCl2, 1mM $ZnCL_2$, 10mM spermidine
Klenow buffer (5x)	0.25M Tris-HCl pH 7.2, 50mM $MgSO_4$, 0.5M DTT
Ligation buffer (10x)	300mM Tris-HCl pH 7.8, 100mM $MgCl_2$, 100mM DTT
Transformation buffer (TFB)	100mM (2-[N-Morpholino]ethanesulfonic acid (MES), 45mM $MnCl_2$, 10mM $CaCl_2$, 100mM KCl, 3mM $(Co(NH_3)_6Cl_3$
Solution 1 (Alkali lysis)	50mM glucose, 10mM EDTA, 25mM Tris-HCl pH 8.0, 5mg/ml lysozyme
Solution 2 (Alkali lysis)	0.2N NaOH, 0.1% SDS
Solution 3 (Alkali lysis)	3.3M Kac pH 4.8, 7.8% glacial acetic acid
Buffer B (enzyme digestion buffer, 10x)	10mM Tris-HCl, 100mM NaCl, 5mM $MgCl_2$, 1mM beta-Mercaptoethanol
Binding and Washing buffer (Dynabeads)	10mM Tris-HCl pH 7.5, 1mM EDTA, 2M NaCl
TE buffer	10mM Tris-HCl pH 9.0, 1mM EDTA

Anneal mix (Point mutation assay)	40mM Tris-HCL pH 7.6, 20mM $MgCl_2$, 50mM NaCl
Labelling mix (Point mutation assay)	40mM Tris-HCl pH 7.6, 16.6 mM DTT, 0.016% BSA
TTB buffer (PMA assay)	10mM Tris-HCl pH 7.6, 0.05% Tween 20

CHAPTER 3

Development of a Quantitative PCR Assay for HIV-1 DNA

3.1 INTRODUCTION

The polymerase chain reaction (PCR) is a highly sensitive and specific method for the detection and quantitation of nucleic acid in a variety of samples (Kwok et al., 1987; Mullis et al., 1987; Ou et al., 1988). Viral load measurements have been central to understanding the pathogenic mechanisms of many viruses (Hagiwara et al., 1993; Ho et al., 1995., Mellors et al., 1996; Stagno et al., 1975; Wei et al., 1995). Quantitative PCR methods to study HIV-1 viral load during infection have been developed that demonstrate varying degrees of sensitivity, specificity and technical flexibility. The importance of viral load for disease development has been demonstrated for a number of viruses, including human cytomegalovirus (HCMV), human herpes virus 8, and HIV-1 in studies using quantitative PCR assays (Connor et al., 1993; Fox et al., 1992; Lock et al., 1997).

The simplest quantitative PCR is a measurement of the amount of amplification product by reference to a series of externally amplified known standards. Methods to relate the amount of amplified product to the initial concentration of the target have used a standard curve generated with plasmid DNA (Aoki-Sei et al, 1992; Bettens et al, 1991), HIV-1 cells with a known copy number (Connor et al., 1993; 1990; Roques et al., 1993), or *in vitro* transcribed RNA standards (Holodniy et al., 1991; Winters et al., 1993). Methods based on limit dilution of DNA or cDNA are effective but very labour intensive as they require 5 to 10 replicate PCRs to be performed on each sample (Simmonds et al., 1990; Donaldson et al., 1994a,b). At the limit of dilution, positive and negative reactions exist and the number of DNA copies present can be calculated from the proportion of negative reactions by calculating Poisson distributions.

Various factors may reduce the efficiency of the amplification process, such as the presence of *Taq* polymerase inhibitors copurifying with DNA from clinical specimens which may result in an inaccurate viral load measurement (De Franchis et al., 1988). The inherent variability of this approach can be overcome by co-amplification of the specific template with an internal reference template such as the beta-globin gene in the same reaction tube (Kellog et al., 1990). A semi-quantitative measure of viral load

can be obtained with limiting dilution of template DNA and using a nested primer methodology in combination with Poisson distribution calculations for evaluation of the results (Simmonds et al., 1990).

Competitive PCR methods based on the co-amplification of two similar template species, the wild type sequence to be quantified and a reference template introduced in a known amount, provide a fully quantitative measurement of viral load. Mathematical models have shown that quantitative assays based on co-amplification of internal standards are superior to other methods, provided that the amplification efficiencies of target and standard are equal (Nedelman et al., 1992a & 1992b). Co-amplification of an endogenous sequence allows for control of the extraction efficiency and recovery of nucleic acid from each sample (Kellog et al., 1990; Lee et al., 1991). However, the thermodynamics and amplification efficiency may substantially differ from those of the viral target sequence.

More accurate results are obtained when an exogenous reference template is used which has an equal or similar size and shares primer recognition sequences with the wild type template. To achieve this, mutant templates have been generated which only differ from the target by a unique restriction site and can be distinguished after amplification by restriction enzyme digestion (Becker-Andre & Halbrock., 1989; Steiger et al., 1991). Mutant templates can also be generated by the introduction of an insertion (Bruisten et al., 1993; Telenti et al., 1992), or a deletion (Bagnarelli et al., 1994; Menzo et al., 1992; Piatak et al., 1993). An alternative approach to create internal standards is the randomization of a small part of the target sequence (Van Gemen et al., 1993), which ensures equal efficiency of amplification, but requires hybridisation of specific probes to discriminate between target and mutant amplification products (Van Gemen et al., 1994). Under these conditions the similar templates compete for the same primer pair during amplification, ensuring that amplification occurs at the same rate and is independent of any variable that is influencing the PCR reaction. Quantitation is performed by comparing the PCR signal of the specific template with the PCR signals from known concentrations of the reference template or competitor.

Competitors identical in size to the wild type template but bearing a novel restriction enzyme site, have been used in quantitative PCR assays for viral DNA (Clark et al 1996; Fox et al 1992). The unique restriction enzyme site is introduced by PCR based mutagenesis with specific primers containing the restriction enzyme site sequence. The amplification products are recognised by digestion with the specific restriction enzyme followed by gel electrophoresis. Accurate quantitative measurement of the separated PCR products can be by use of radiolabelled primers in the competitive PCR reaction.

PCR products of different sizes are usually separated by conventional gel electrophoresis (Fox et al., 1992; Piatak et al., 1993b). PCR products can also be separated by capillary gel electrophoresis (Fasco et al., 1995) or HPLC. Following electrophoretic separation on a polyacrylamide gel and exposure to X-ray film, the intensity of the bands can be measured using a scanning densitometer.

Alternative methods include the use of primers labelled with a fluorescing hapten in a competitive PCR, PAGE separation and quantitation using an automated laser fluorescent DNA sequencer. More automated quantitation methods involve the capture of biotinylated PCR products on streptavidin labelled solid supports (microplates). Quantitation of the PCR products can performed by measuring incorporated radioactivity, immunoenzymatic or chemiluminescence detection (Semple et al, 1993; Whitby et al., 1995).

An alternate approach to the use of a radiolabelled primer in the QPCR assay is to silver stain the separated products following enzyme digestion. In this chapter the commercially available 'Promega' silver stain system was adapted for use with separated PCR products and assessed for accurate quantitation using scanning densitometry. Silver staining was introduced more than a decade ago as a sensitive procedure to detect trace amounts of proteins in polyacrylamide gels (Switzer et al., 1979) and has been extended to detect other biological molecules that have been separated by electrophoresis (Merril, 1990; Rabilloud, 1990), and more recently for the analysis of complex DNA profiles generated in DNA amplification (Caetano-Anolles et al., 1993) and DNA sequencing.

Two approaches to silver staining can be taken, one uses diamine or ammonical silver solutions for gel impregnation and dilute acid solutions of formaldehyde for image development. The other method impregnates with silver nitrate in a weakly acid solution and uses formaldehyde to reduce silver under alkaline conditions. The 'Promega'silver staining system is adapted from the methods of Bassam et al (1991 and 1993), and is based on a photochemical-derived silver stain (Goldman and Merril, 1982), in which silver nitrate is the impregnating agent and formaldehyde (in an alkaline environment) is the reducer. With this method, impregnation uses relatively low concentrations of silver in a solution containing formaldehyde. Image development occurs using formaldehyde at higher concentrations compared to other methods (Blum et al., 1987) and the reaction is performed at a low temperature (8-12°C) in the presence of thiosulfate. Thiosulfate chemically dissolves silver salts by complexation (Blum et al., 1987), which alters the kinetics of silver reduction and helps minimise background staining.

The work presented in this chapter describes the development and calibration of a quantitative PCR assay for HIV-1 DNA. The assay was then used to measure proviral load in whole blood specimens and post-mortem tissues of individuals infected with HIV-1, and this work is presented in chapters 4 and 7.

3.2 METHODS

3.2.1 Construction of a Sma-1 enzyme restriction site using PCR mediated mutagenesis

A PCR mutagenesis approach (Fox et al., 1992) was used to create a unique Sma-1 enzyme restriction site in the middle of a 233bp sequence of HIV-1 gag using oligonucleotide primers containing the enzyme site sequence (*cccggg*). Figure 3.1 outlines the procedure of amplifying two smaller fragments (A+B) of the gag region, denaturation and annealing together of single strands of PCR products A and B containing the enzyme site, and subsequent Klenow filling in to produce a double

stranded product, which is reamplified and ligated into a pUC-18 vector. Following denaturation during stage 3 of the procedure, some reannealing of fragments A and B, will occur. However, following Klenow extension in stage 4 the selective PCR with primers P1 and P2 will only amplify the extended product containing the Sma-1 site. Quantitation of the Klenow extended product prior to PCR was not possible due to the low amounts of DNA in the reaction.

3.2.2 PCR of intermediate fragments A and B

Using 1µg of HIV-1 DNA from plasmid BH10 as a template, 2 PCR reactions were performed using 50 pmols each of primers P1 gaaggagccaccccacaagatt (1318-1339, sense) and P4 ccctgcatg***cccggg***atgactcta (1428-1452, anti-sense) in reaction A and primers P2 taggtggattatttgtcatcca (1534-1575, antisense) and P3 tagagtcat***cccggg***catgcagg (1428-1451, sense) in reaction B. The positions of the primers are based on the HXB2 HIV-1 prototype sequence (Ratner et al., 1985a) and indicated in brackets. The 134bp PCR product of reaction A contained a cccggg sequence at the 5' end of one strand incorporated by primer P4. The 147bp product of PCR reaction B contained a similar sequence incorporated by primer P3. The PCR was performed for 30 cycles as described (Materials & Methods, section 2.2.2) with increased magnesium concentrations of 4.0mM for reaction A and 6.0mM for reaction B.

3.2.3 Purification of fragments A and B

50µl of fragment A and B PCR products were electrophoresed in duplicate in a 1.1% low melting point (LMP) agarose gel at 60 volts for 1 hour. The gel was examined on an ultraviolet light (UV) transilluminator and each band was excised from the gel with disposable scalpel blades and placed in two separate Eppendorf tubes labelled A and B. 900µl of TE buffer (20mM Tris-HCl, 1mM EDTA, pH 7.6) was added to each of the two tubes and heated to 65°C for 10 minutes with occasional vortexing. 400µl of saturated phenol was added to each tube and vortexed for 1 minute. After centrifugation at 14,000g, 250µl of the upper aqueous layer was removed to a fresh tube. 25µl of 3mM NaAc pH 6.0 was added and mixed by pipetting. 500µl of 100%

ethanol was then added and the DNA was precipitated by incubation at -70°C for 2 hours. The DNA precipitate was recovered by centrifugation at 14,000g for 30 minutes and the DNA pellet was washed twice with 70% ethanol, air dried and dissolved in 50µl of SDW.

3.2.4 Annealing of fragments A and B and Klenow polymerase filling in of single stranded DNA

The DNA concentration of the purified fragments A and B was calculated by measuring absorbance at 260 nm using a UV spectrometer. Equal amounts of fragments A and B (250ng) were combined in the same tube and overlaid with 50µl of mineral oil. The tubes were heated to 95°C for 10 minutes to denature the double stranded PCR products and then left to cool at room temperature for 1 hour to allow annealing of single strands containing the Sma-1 restriction enzyme site.

The annealed fragments were extended, or filled in by adding the total volume (10µl) of the annealed reaction to 40µl of Klenow filling-in reaction mix (10U Klenow polymerase enzyme (Pharmacia), 300µM dNTPs (Pharmacia), 10x MSK buffer (Medium salt buffer)), and incubated at room temperature for 45 minutes. 10µl of the filled in reaction was then amplified by PCR using 50 pmols of the outer HIV-1 gag primers P1 and P2 and the parameters were as described in Materials and Methods, section 2.2.2.

3.2.5 Confirmation of presence of Sma-1 enzyme restriction site

Amplification by PCR produced a 233bp product and the presence of the introduced Sma-1 enzyme restriction site was confirmed by digestion of the PCR product with 10 units of Sma-1 enzyme for 2 hours at 37°C. Enzyme digestion produced two fragments of approximately 110bp and 113bp, which were visible following agarose gel electrophoresis. Further confirmation of the presence the Sma-1 site was achieved by ligation of the mutated fragment SQ into a pUC-18 vector and subsequent DNA sequencing.

3.2.6 Generation of wild type HIV-1 gag fragment (TQ)

A non mutated fragment (TQ) of the same size as SQ was also generated by PCR amplification of HIV-1 BH10 DNA with outer gag primers P1 and P2 and this fragment was also ligated into a pUC-18 vector.

3.2.7 Large scale plasmid preparation of SQ and TQ vectors and DNA purification

A large scale plasmid preparation of both SQ and TQ DNA was prepared and purified using 'Qiagen' column separation (Materials and Methods, section 2.13). Both SQ and TQ purified DNA was dissolved in 500µl of fresh SDW and aliquoted and stored in separate laboratories to minimise the risk of cross contamination.

The purity of the plasmid DNA was checked by agarose gel electrophoresis and double digested with Hind III and EcoR I restriction enzymes to yield a fragment of the correct size. The concentration of the purified plasmid DNA was calculated by measuring the OD_{260} of three dilution's (1:50, 1:100, 1:250) in triplicate with a UV spectrometer. A mean absorbance value was used to give an accurate DNA concentration value.

3.2.8 Calculation of plasmid DNA copy number

The plasmid copy number was calculated using the DNA concentration value and Avogadro's number. The following is a sample calculation for plasmid SQ:

Concentration of SQ DNA	= 718µg/ml
Size of PCR product insert	= 239bp
Total size of plasmid SQ	= 3129bp
1µg of 1000bp double stranded DNA	= 1.52 pMoles
1µg of SQ	= 0.49 pMoles
1µg of SQ	= $6.022 \times 10^{11} \times 0.49$
	= 2.95×10^{11}
Number of copies per microlitre	= $2.95 \times 10^{11} \times 0.718$
	= 2.11×10^{11} copies / µl
	= 4.22×10^{11} copies / 2µl

The SQ plasmid maxi-prep DNA was first diluted 1:4 to give a concentration of 10^{11} copies / 2µl and serially diluted 1:10 to give a range of copy number aliquots from 10^{11}/2µl to 10 copies / 2µl. SQ copy number aliquots were stored in 10µl aliquots at -70°C. The TQ plasmid copy number was calculated using the same formula and TQ DNA was diluted to give the same range of copy number dilution's as SQ and were stored at -70°C.

3.2.9 Sensitivity of the PCR for HIV-1 gag DNA using the SQ and TQ plasmid DNA aliquots

To determine the limit of detection and optimal reaction conditions of the PCR for HIV-1 gag DNA, PCR was carried out on a range of SQ copy number aliquots from 5 to 1000. The limit of detection was determined as 5 plasmid copies, and the optimal

$MgCl_2$ concentration was 2.5mM. This was repeated on a range of TQ DNA aliquots and the limit of detection was also found to be 5 copies.

A nested PCR was performed with inner primers GI1 and GI2 to improve the specificity of the reaction. To ensure that the nested PCR remained in the linear phase of the reaction, a titration of TQ copy number aliquots from 5 copies to 10^6 copies was amplified in the first round reaction for 30 cycles. Second round amplification of 2µl of a 1:5 dilution of the first reaction demonstrated a linear amplification of the copy number aliquots.

3.2.10 Calibration of quantitative PCR assay

To determine the range of accurate quantitation of the competitive PCR a plasmid mixing experiment was performed which is outlined in Figure 3.2. A set of PCR reactions containing 10^3 copies of SQ plasmid and increasing copy numbers of TQ plasmid (10 to 10^5) were carried out with primers P1 and P2 (25 pmols each) and approximately 1pmol of ^{32}P radiolabelled primer P1 under optimised reaction and thermocycling conditions (Materials & Methods, section 2.2.2). Following the PCR, 15µl of product was digested with 10 units of Sma-1 enzyme at 37°C for 2 hours and then electrophoresed on a 4% polyacrylamide gel at 100 volts for 1 hour. The gel was then stained with ethidium bromide and visually examined by a UV transilluminator.

The polyacrylamide gel containing the radiolabelled PCR products was then covered in saran wrap and exposed to autoradiography film at -70°C overnight. The film was then developed using an automated X-RAY film processor (X-Ray dept, RFH) and the autoradiograph of the PCR products was examined by a scanning densitometer (CS-9001PC scanner; Shimadzu, Tokyo). The intensities of signals for digested and undigested bands were determined and the calculated plasmid copy number was compared to the actual input copy number to produce a calibration curve. The range of accurate quantitation of the assay was between 40 and 10,000 copies of HIV-1 gag plasmid DNA when 1000 copies of SQ DNA was used as an internal control.

3.2.11 Silver staining of enzyme digested PCR products

Following Sma-1 enzyme digestion of HIV-1 PCR products and separation by polyacrylamide gel electrophoresis, the gel was placed in a plastic tray and treated with fix solution (10% glacial acetic acid) and agitated for 20 minutes at room temperature. The gel was then washed for 3 X 2 minutes in ultrapure water with agitation. The gel was then stained with staining solution (1g of silver nitrate and 1.5ml of 37% formaldehyde in 1L of ultrapure water) for 30 minutes at room temperature with agitation. During staining the developing solution was prepared by adding 1.5ml of 37% formaldehyde solution and 200ul of a sodium thiosulfate solution (10mg/ml) to a prechilled (10°C) sodium carbonate solution (30g of sodium carbonate in 1L of ultrapure water). The gel was removed from the staining solution and rinsed briefly in water for 5 to 10 seconds. The gel was placed immediately into the chilled developing solution and agitated for 2 to 3 minutes until the PCR product bands appear. To terminate the developing reaction, 1L of fix solution (10% glacial acetic acid) was added and the gel was agitated for 2 to 3 minutes. The gel was then rinsed 2 x 2 minutes each in ultrapure water. The resulting silver stained bands were scanned directly by densitometry or a permanent record of the stained gel was made by exposing the gel to Kodak EDF film on a white fluorescent lightbox for 10 to 30 seconds. The film was then processed manually or with an automatic film processor.

3.3 RESULTS

3.3.1 Generation of the HIV-1 gag control plasmid SQ

A PCR mutagenic approach, using primers containing the Sma-1 sequence ***cccggg*** was used to produce the HIV-1 control sequence. The presence of the inserted enzyme restriction site and its position compared to the wild type sequence was confirmed by DNA sequence analysis (Fig 3.3).

3.3.2 Calibration of QPCR for HIV-1 DNA

Known copy numbers of plasmid containing wild type sequence (TQ) were co-amplified with constant control sequence (SQ) copy numbers and performed in triplicate as described in materials and methods, section 2.2.2. The quantitative PCR system for the production of a standard curve used a radiolabelled primer to produce an autoradiograph (Fig 3.4). The rightmost lane of Fig 3.4 represents 1000 copies of SQ alone amplified and digested with Sma-1, demonstrating complete digestion. Correlation analysis of the relationship between calculated and actual wild-type sequence copy number was analysed by regression using the method of least squares. This yielded a calibration curve (Fig 3.5) with a correlation co-efficient of $r = 0.997$ ($p < 6 \times 10^{-11}$).

The competitive nature of the quantitative PCR assay is demonstrated in Figure 3.6, where extracted DNA from the whole blood of four individuals was co-amplified with either 400 or 2000 copies of internal control template. Co-amplification with 400 copies gave bands of equal intensity for the patient DNA and for the internal control sequence, demonstrating equal amplification efficiency of both patient DNA and control template. When patient DNA was co-amplified with 2000 copies of internal control, bands of less intensity are seen for the patient DNA compared to the greater amount of internal control template. Densitometric analysis of the bands revealed that patients A,B and D contained approximately 400 copies of DNA per ml of blood and Patient C contained approximately 300 copies per ml of blood.

3.3.3 Semi-nested quantitative PCR

To improve the specificity and sensitivity of the quantitative PCR assay, a semi-nested approach was taken using inner gag primers GI1 and GI2. The second round PCR was limited to 15 cycles to maintain linear amplification and ensure accurate quantitation. Figure 3.7(A) illustrates nested PCR amplification of a range of copy number aliquots of internal control sequence (SQ). Following a first round amplification of 30 cycles and a second round of 15 cycles the PCR products were electrophoresed on a 2% agarose gel. The results show that linear amplification was maintained in the limited second round.

Figure 3.7(B) illustrates the results of semi-nested co-amplification of 1000 copies of internal control template with a range of copy number aliquots of wild type gag template. Following PCR and digestion with Sma-1, the results demonstrate electrophoresed on a 2% quantitation over a wide dynamic range. The lower double bands in figure 3.7B represent the Sma-1 digested PCR product resulting in two fragments.

3.3.4 Silver staining of PCR products for quantitative PCR

Staining of a polyacrylamide gel containing separated PCR products following semi-nested PCR, where a duplicate plasmid experiment was performed is illustrated in Figure 3.8. Five hundred copies of internal control template (SQ) were co-amplified with a range of wild type plasmid copy numbers (50, 100, 500 and 1000 copies). The silver stained gel was exposed to EDF film in the presence of white light to produce a permanent record. The intensity of the silver stained bands was equal to that of an autoradiograph and permitted accurate densitometric measurements. Figure 3.8 also illustrates that linear amplification is maintained in the limited second round, allowing accurate quantitation through the ratio of wild type DNA signal versus internal control sequence signal and also by knowing the number of input control template copies.

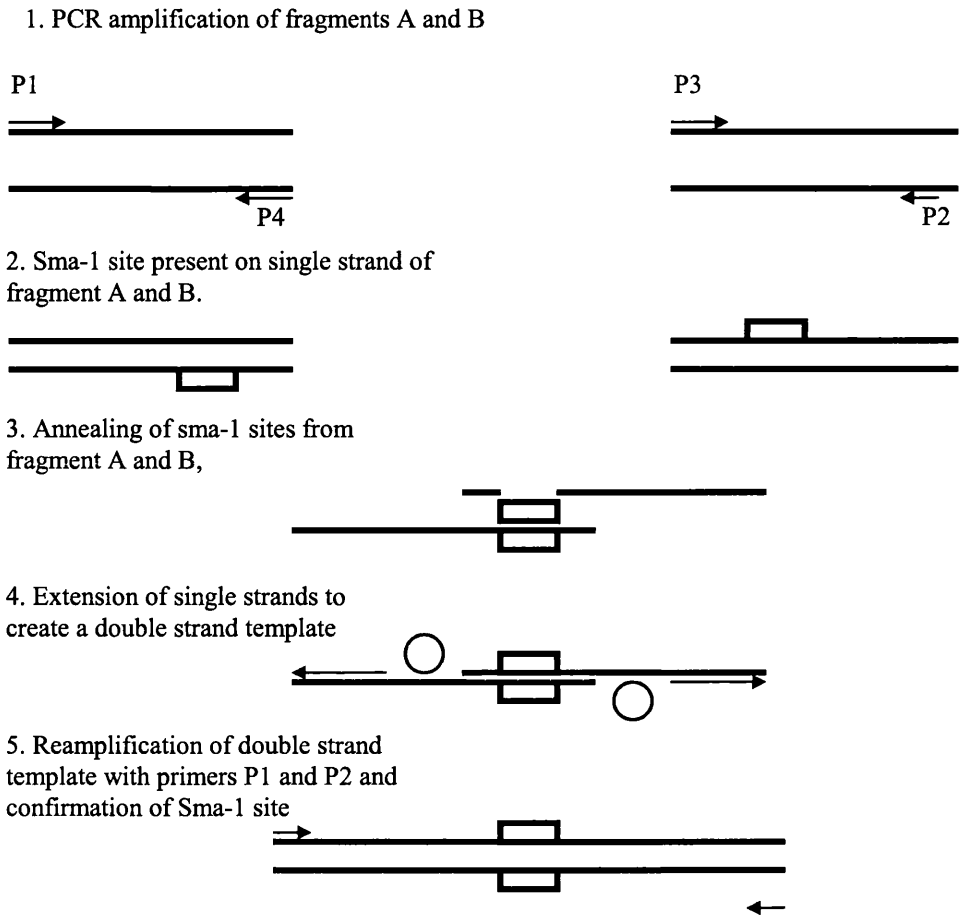


FIG 3.1. PCR mutagenesis approach to create a Sma-1 enzyme restriction site. The symbol ▭ represents the Sma-1 enzyme restriction site sequence *cccggg* and the symbol ○ represent Klenow polymerase and the filling in reaction.

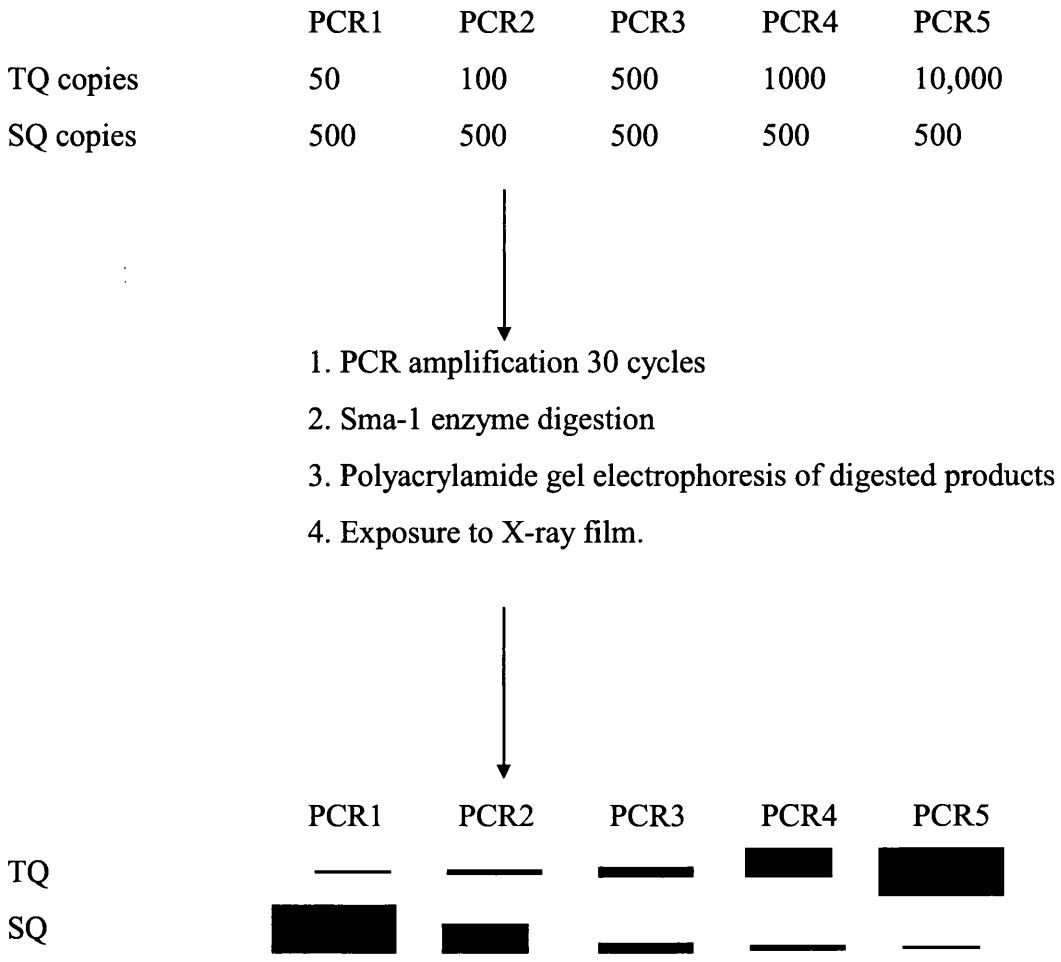


Figure 3.2. Schematic outline of the mixed plasmid coamplification approach taken to the calibrate the quantitative PCR by coamplifcation of known copy numbers of wild type template (TQ) and competitor template (SQ), the densitometry readings were then used to construct a calibration curve.

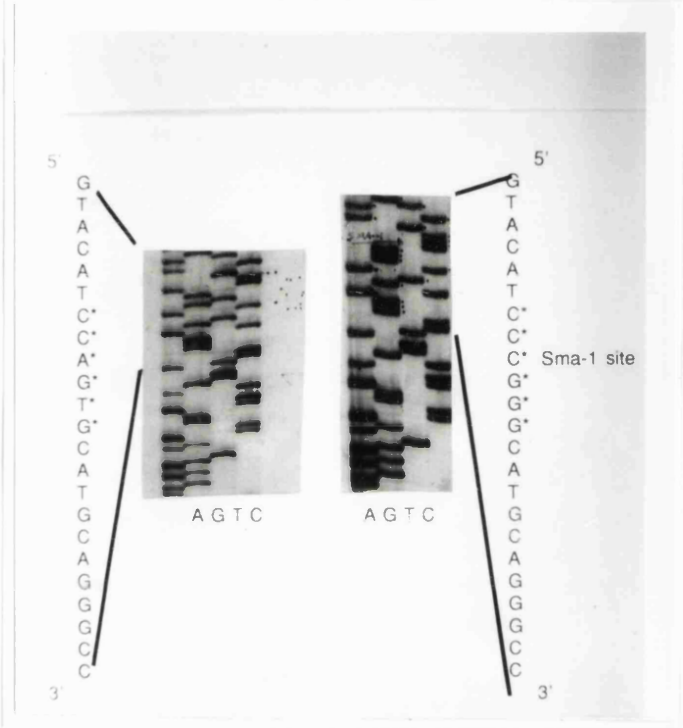
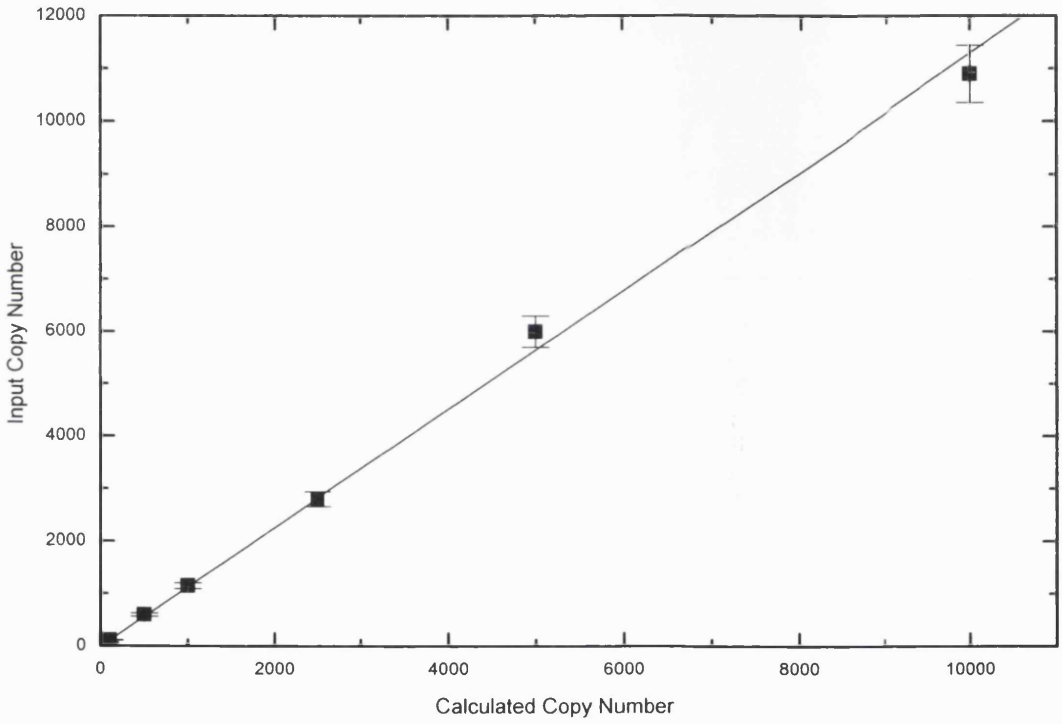


Figure 3.3. DNA sequence of the internal control template (right sequence) demonstrating the presence of the introduced sma-1 enzyme restriction site (*cccggg*). The corresponding region of the wild type template was also sequenced for comparison (left sequence).

Figure 3.4. Autoradiograph showing results of plasmid mixing experiment demonstrating the dynamic range of the quantitative PCR assay. A range of wild type template copy number (upper bands, 50 - 10^5) were co-amplified with a constant copy number (1000 copies) of internal control template (lower band). Following PCR, sma-1 enzyme digestion and PAGE separation of products, the gel was exposed to X-Ray film to produce the autoradiograph.

Figure 3.5. Calibration curve of the quantitative PCR assay. Following densitometry scanning of the autoradiograph, calculated wild type copy numbers were obtained and plotted against the known input copy numbers. Each point represents the mean of triplicate determinations with standard deviation plotted as error bars and the line of best fit was plotted through the points.


50
100
500
10^3
2.5 x 10^3
5 x 10^3
10^4
10^5


12000
10000
8000
6000
4000
2000
0
Input Copy Number
0
2000
4000
6000
8000
10000
Calculated Copy Number

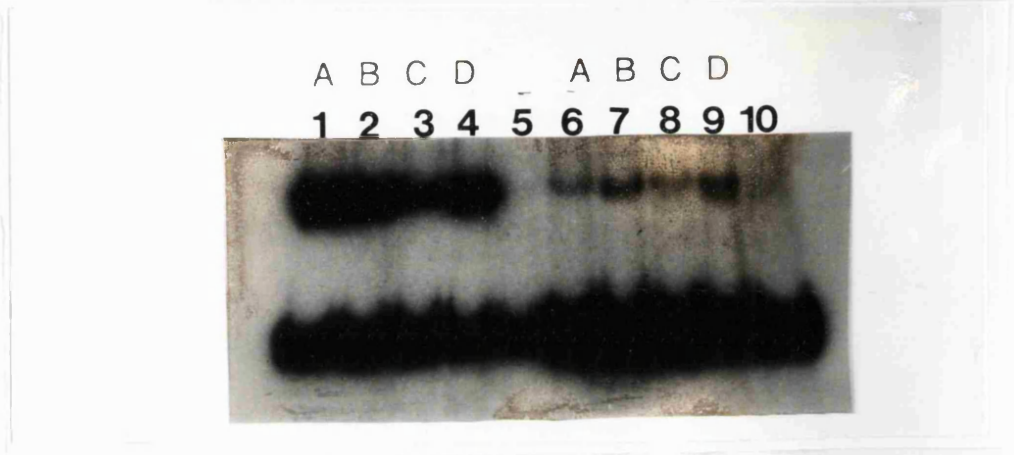
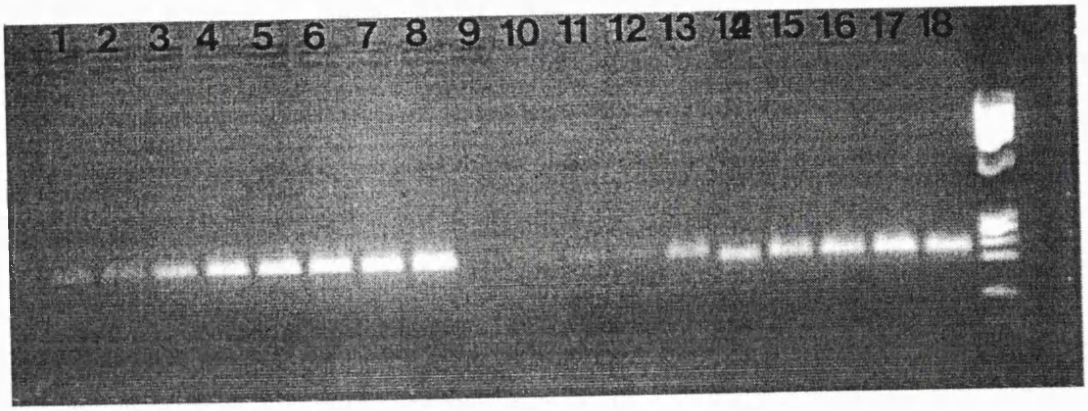


Figure 3.6. Competitive nature of the quantitative PCR assay illustrated by co-amplification of extracted DNA from 4 patients (A,B,C,D) with 400 copies of internal control sequence (lanes 1 - 4) and 2000 copies of internal control sequence (Lanes 6 - 9). Lanes 5 and 10 illustrate complete digestion of 400 and 2000 copies of internal control template, respectively.

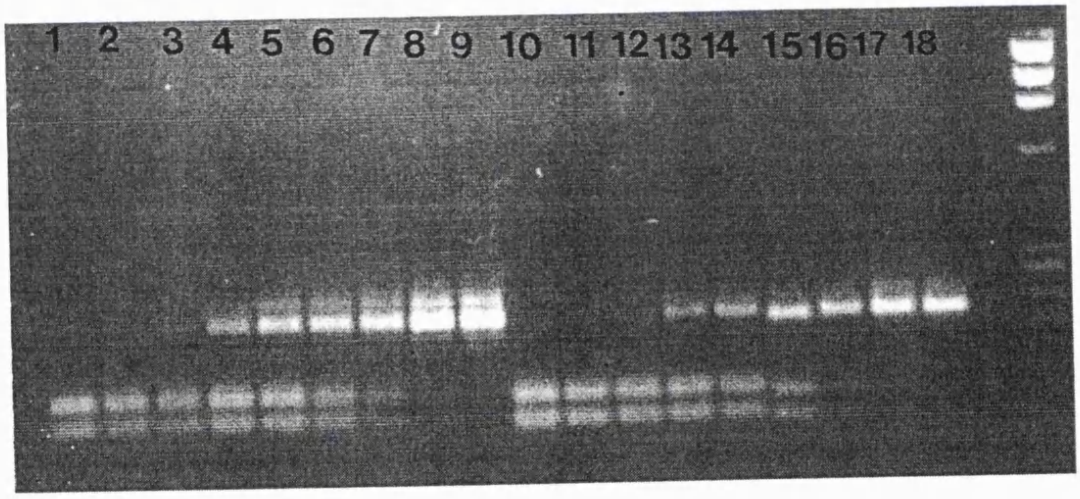
Figure 3.7 (A). Semi nested amplification of a range of internal control copy numbers 5,10, 50, 100, 500, 1000, 10,000 and 100,000 copies (Lanes 1 - 8 and 11 to 18, respectively). The first round PCR was for 30 cycles and the second round PCR was for 15 cycles. Lane 9 is empty and lane 10 contains a duplicate of lane 11. Molecular weight markers phiX174 DNA/Hae III.

Figure 3.7(B). Semi-nested quantitative PCR showing results of co-amplification of a range of copy number aliquots of wild type template 50, 100, 500, 1000, 10,000 and 100,000 copies with a constant 1000 copies number of internal control (Lanes 3 - 9 and Lanes 12 - 18). Lanes 1,2 and 10, 11 demonstrate complete digestion of 1000 copies of internal control to two bands of 91bp and 82bp. Molecular weight markers phiX174 DNA/Hae III.

A


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

B


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

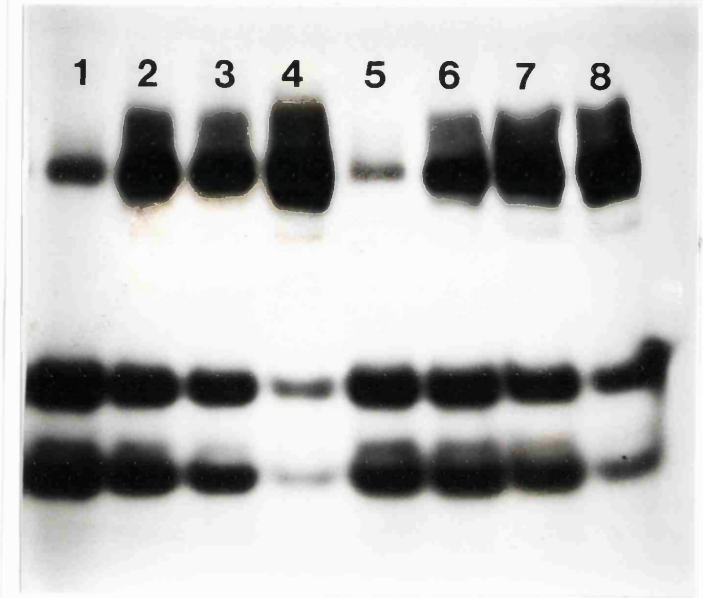


Figure 3.8. Silver staining of quantitative PCR products following sma-1 restriction enzyme digestion and PAGE separation . Aliquots of wild type template copy number, 50, 100, 500 and 1000 copies were co-amplified with a constant 500 copies of internal control sequence (lanes 1 - 4 and 5-8 respectively). The separated PCR products were stained with the 'PROMEGA' silver staining system.

3.4 DISCUSSION

A quantitative PCR for the measurement of HIV-1 proviral DNA load has been developed. The method of incorporation of an enzyme restriction site by PCR based mutagenesis has been employed in PCR quantitative assays for CMV, HHV6, HHV7 and HHV8 (Fox et al., 1992; Clark et al., 1996; Kidd et al 1996; Lock et al., 1997). The use of a control sequence in quantitative PCR ensures that each PCR tube is internally controlled for variations in amplification efficiency (Becker-Andre and Hahlbrock., 1989; Henco and Heiby, 1990) and such assays have been shown to be accurate in the quantitation of a number of different viruses including hepatitis C (Gretch et al., 1994), HIV-1 (Bruisten et al., 1993), HCMV (Fox et al., 1992), varicella-zoster virus (Mahalingam et al., 1993), HHV6 (Clark et al., 1996) and HHV7 (Kidd et al., 1996). Differences in amplification efficiency may greatly influence the quantitative evaluation of PCR products particularly when the template DNA is low or incompletely purified. These differences can arise from the methods used for nucleic acid purification, the presence of inhibitors and thermocycler performance.

Following PCR, the standards containing the restriction sites are digested with respective restriction enzyme, separated from the wild type template by electrophoresis and analysed separately. The mixed plasmid co-amplification experiment demonstrated accurate quantitation of HIV-1 by the QPCR assay with a broad dynamic range of 50 to 10^5 copies of target DNA when using 1000 copies of competitor. A semi -nested quantitative PCR improved the specificity of the assay and maintained accurate quantitation. A previous study describing an HIV-1 plasma RNA quantitative assay has illustrated the importance of limiting the number of cycles in the second round for accurate results (Semple et al., 1993).

Using 30 cycles of amplification in the first round and 15 cycles in the second round ensured that the PCR remains in the exponential phase and that exhaustion of reaction components, which can limit the accumulation of PCR products, does not occur (Mullis, 1991). The semi-nested second round PCR was applied to essentially improve specificity and this approach has also been shown to improve sensitivity when small numbers of specific copies have to be detected in a high background of non-specific

cellular DNA (Zimmermann et al., 1994; Brustein et al., 1993).The use of *pfu* DNA polymerase in a quantitative PCR assay for Hepatitis C (Whitby et al., 1995) has been shown to generate higher product yields than amplification with Taq polymerase and eliminate the need for a second round of amplification.

The quantitative competitive PCR assay described in this chapter is both sensitive and reproducible and applicable over a wide range of DNA copy number input. Previous studies have used limited-dilution polymerase chain reaction to determine HIV-1 levels (Simmonds et al., 1990; Dondaldson et al., 1994a,b), or the use of a cellular gene as an internal reference standard (Lee et al., 1991). The advantage of the quantitative PCR over limiting dilution is that tube-to-tube variation is controlled for, as well as amplification inhibiting factors which may co-purify with DNA from clinical specimens (De Franchis et al., 1988; Coutlee et al., 1991).

Using an internal reference template such as the HLA DQ alpha gene (Lee et al., 1991) has the disadvantage that amplification efficiencies may differ between the HIV template and the control template. The quantitative PCR assay discussed in this chapter uses the same HIV-1 primer binding sites for both the internal control and patient DNA, producing a product of the same size, ensuring amplification efficiency. Several quantitative PCR assays have used an internal control that employs a size difference between wild type template and internal standard (Brusiten et al., 1993), in such assays it is important to minimise this size difference, as an inverse exponential relationship exists between template size and amplification efficiency (McCulloch et al., 1995).

The quantitative assay described in this chapter may over-estimate target copy number by the formation of heterodimers of wild type template and internal control strands which would be resistant to restriction enzyme digestion. However, the formation of heterodimers can be prevented by ensuring that amplification is completed in the exponential phase (Becker-Andre and Hahlbrock, 1989). In this work the quantitative PCR was performed for 30 cycles in the first round and the plasmid co-amplification experiments showed accurate quantitation over a broad dynamic range indicating that the formation of heterodimers was not a confounding variable.

Silver staining of polyacrylamide gels following electrophoresis of enzyme digested PCR products combined with densitometry, is an accurate and reliable method for quantitative PCR. The silver stained gels can be analysed directly by scanning densitometry or exposed to positive film to obtain a permanent record. The stained gels produced bands of equal intensity to an autoradiograph when exposed to EDF film (Fig 3.8). Silver staining is cost efficient compared to autoradiography and reduces the risk of exposure to radiochemicals. A quantitative result can be achieved in less than two hours following enzyme digestion, whereas autoradiography can take between 24 and 48 hours of exposure of the radiolabelled PCR products to film.

In summary the quantitative PCR has been demonstrated to be accurate and have a broad dynamic range with a low inter- and intra- test variability that is similar to previously described quantitative PCR assays. This assay was used to quantitate proviral DNA load in post-mortem tissues and blood of HIV-1 infected individuals, and these results are presented in chapters 4 and 7 of this thesis.

CHAPTER 4

Quantitation of Human Immunodeficiency Virus type 1 (HIV-1) DNA in post-mortem tissues from AIDS patients by PCR

4.1 INTRODUCTION

Infection with Human Immunodeficiency Virus type 1 (HIV-1) is associated with progressive destruction of host immune functions culminating in the development of acquired immunodeficiency syndrome (AIDS). Many AIDS deaths can be attributed to secondary infections or neoplasms that develop as a consequence of immunodeficiency; however, to what extent direct infection with HIV-1 contributes to the observed pathogenesis is unclear. The tissue distribution of HIV-1 infection may play an important role in the pathogenesis of AIDS.

The onset of the symptomatic stage appears to be correlated with HIV-1 spread in various organs with lymphoid tissues, representing an important virus reservoir throughout the course of HIV-1 disease (Levy., 1993; Fauci., 1993; Pantaleo et al., 1993; Donaldson et al., 1994a). HIV infects a wide variety of tissues *in vivo*, and has been detected in a number of cell lines in culture. HIV-1 was initially isolated from CD4 lymphocytes (Klatzmann et al., 1984; Coffin et al., 1986; Dalgleish et al., 1984); however, infection of other cell types has been demonstrated and may contribute to the pathological abnormalities. HIV-1 can infect cells of the mononuclear-macrophage system *in vitro* and cells of this lineage, such as microglial cells in the brain have been shown to be infected *in vivo*. Using various techniques such as cell culture, *in situ* hybridisation, *in situ* PCR, immunohistochemistry, electron microscopy and PCR, HIV has been detected *in vivo* in cells of the haematopoietic lineage (Cullen et al., 1991; Freedman et al., 1991; Gartner et al., 1986; Levy et al., 1986; Livingstone et al., 1996; Scottalgara et al., 1993; Shattock et al., 1996; Tsubota et al., 1989; Uittenbogart et al., 1996;).

Pathological examination of tissue from AIDS patients, combined with the above techniques, can reveal lesions or cellular changes, particularly in brain (Gartner et al., 1986; Koenig et al., 1986; Moses et al., 1993; Price et al., 1988; Wiley et al., 1986; Wiley et al., 1990;), gastrointestinal tissue (Gill et al., 1992a,b; Kotler, 1993;), heart (Grody et al., 1990; Luginbuhl et al., 1993), lungs (Chayt et al., 1986; Dolei et al., 1992; Plata et al., 1990), kidneys (Cohen et al., 1989), adrenals (Barboza et al., 1992), eye (Cantril et al., 1988), salivary glands (Qureshi et al., 1995), cervix (Nuovo et al., 1993), prostate (da Silva et al., 1989), testes (Bagasra et al., 1994; da Silva et al., 1989) and skin (Tschachler

et al., 1987). Pathological examination of tissue from AIDS patients reveals abnormalities particularly in the brain, liver and lymphatic system (Gray et al., 1988; Schneiderman et al., 1987; Fox et al., 1991; Pantaleo et al., 1993). These lesions could be a result of direct infection of the tissue, or due to reactivation of other viruses, bacterial infection due to immunosuppression, or the over-production of molecules such as cytokines e.g. tumour necrosis factor-alpha (Poli & Fauci., 1992).

The quantitative measurement of HIV-1 DNA, or viral load by PCR in post-mortem tissues, reveals information about the distribution and virus-specific pathology in different organs in terms of association of viral load with infection of certain cell types, e.g the relationship between high viral load in the brain and the incidence of HIV encephalitis (Bell et al., 1996). *In vivo* studies of the tropism of HIV-1 are limited to examination of organs post-mortem or tissues which are available through biopsy e.g. lymph node tissue. Studies using quantitative PCR have previously revealed that the lymph node contains the highest levels of HIV-1 during the asymptomatic phase of disease, with very low or undetectable levels of viral DNA found in non lymphoid tissue (Pantaleo et al., 1993; Donaldson et al., 1994a). As disease progresses to AIDS and viral load increases in the peripheral blood, HIV-1 is then detected in tissues outside the lymphoid system, e.g. gastrointestinal tract, lung, liver, kidney and brain (Donaldson et al., 1994a).

HIV-1 may infect non-lymphoid tissues during the asymptomatic or pre-AIDS stage at a very low or undetectable levels; indeed, studies using PCR have demonstrated HIV-1 DNA in tissues such as brain before the onset of AIDS. HIV-1 was initially isolated from CD4 lymphocytes and destruction of these cells is almost certainly the main cause of the observed immunodeficiency (Ho et al., 1987). However, HIV-1 infection of other cell types may contribute to the pathological abnormalities.

Quantitative PCR for HIV-1 DNA extracted from post-mortem tissue, whilst not indicating the exact cell type infected can provide an accurate measure of viral load in various tissues. This may correlate with gross and microscopic pathological changes and symptoms in the infected individuals before death (Donaldson et al., 1994a).

4.1.1 Role of the V3 region of gp120 in cellular tropism

Regions located in the env gene of gp120 outside the CD4 binding domain (carboxy terminal) have been associated with viral tropism. These include the V1, V2 and V3 hypervariable regions. With the elucidation of the crystal structure of HIV-1 gp120 (see introduction section 1.4.1.1), information of precise interactions between envelope regions and cellular receptors has been available. While most HIV-1 strains are capable of replication in primary lymphocytes, replication in other cell types is more restricted (Schuitemaker et al., 1991). HIV-1 isolates capable of infecting macrophages are generally unable to infect T cell lines and vice versa (Cheng-Mayer et al., 1988).

The restriction of HIV-1 isolates for replication in macrophages is thought to be at the level of virus entry, with the critical determinant being within the V3 region of gp120. The sequence variation that occurs within the V3 region and also the V1 and V2 hypervariable, domains has been shown to affect the biological phenotype of the virus during *in vitro* culture (Andeweg et al., 1993; Boyd et al., 1993; de Jong et al., 1992; Groenink et al., 1993; Sullivan et al., 1993). Specific amino acid changes have been shown to influence viral characteristics such as cell tropism, the ability to induce syncytia and the ability to replicate *in vitro* (Chesebro et al., 1992; Ivanoff et al., 1992; Shioda et al., 1992; Tersmette et al., 1988). In particular, substitutions of basic amino acids in the V2 and V3 regions change virus isolates from non syncytium -inducing (NSI) isolates, to syncytium - inducing (SI) isolates (de Jong et al., 1992; Fouchier et al., 1992), and may confer a reduction in the ability of the virus to replicate in macrophages (Chesebro et al., 1992; Schuitemaker et al., 1992).

In early infection, HIV variants with an NSI / macrophage tropic (MT) phenotype predominate, whereas virus isolated later in infection may exhibit a rapid high replicating SI / T cell tropic phenotype (Koot et al., 1993; Tersmette et al., 1988). This switch in phenotype has been shown to precede an accelerated loss of CD4+ lymphocytes in the peripheral circulation, and a more rapid onset of AIDS compared to individuals whose isolates retain an NSI phenotype (Koot et al., 1993). This switch to an SI phenotype in the peripheral blood during late stage disease does not reflect the more commonly found NSI variant in a variety of post-mortem tissues (Donaldson et al., 1994b). More recent studies

have examined the V3 loop sequence in brain tissue of patients with HIV encephalitis (Reddy et al., 1996; Chang et al., 1998) and a similar NSI / macrophage tropic strain of the virus was demonstrated by sequence analysis. Only a subset of patients with HIV encephalitis have syncytial or multinucleated giant cells within the CNS (Wiley et al., 1994) and the relationship between SI strains and HIV encephalitis is unclear as there appears no evidence of such strains in brain tissue demonstrating giant cell encephalitis (Donaldson et al., 1994b).

4.2 METHODS

In this chapter proviral DNA load was measured by quantitative PCR (see chapter 3), in a variety of post-mortem tissues from 11 patients who died of AIDS-defining illnesses. The phenotypic strain of HIV-1 from a selection of the patients was examined by direct sequencing of the V3 loop region of gp120 described in materials and materials, section 2.15.2 and assessing the overall charge of the V3 loop by the presence of substituted amino acids in certain positions.

The V3 loop of HIV-1 gp120 was amplified by a previously described nested PCR approach (Donaldson et al., 1994b). The nucleotide sequences of the primers and the position of the 5' base in the HXB2 genome (Myers et al., 1991) were as follows. V3 (1) 5'- TACAATGTACACATGGATT, sense , 6957; V3 (2) 5'- ATTACAGTAGAAAAATTCCCC, anti-sense, 7361; V3 (3) 5'- TGGCAGTCTAGCAGAAGAAG, sense, 7009; V3 (4) 5'- CTGGGTCCCCCTCCTGAGG, anti-sense, 7314. Direct sequencing of V3 amplified DNA was achieved by using a solid-phase sequencing method (Hultman et al., 1989), as described in section 2.9.4. For solid-phase sequencing , the second PCR was performed in a 100μl volume by using one biotin labelled and one unlabelled primer (5 - 10 pmol of primer per reaction), generating a PCR product with one strand having a biotin moiety at the 5' end. PCR products were immobilised on streptavidin coated magnetic beads (Dynal), and single strands of DNA were purified by magnetic separation as described in section 2.9.3 of chapter 2.

Patient (sex, age at death	Treatment regimen and duration	CD4*	CD4(at death)	Opportunistic infections / Malignancies
1 (F 38y)	ZDV 250 mg BD 14 months	60	0	PCP X3, MAI, NHL
2 (M 63y)	ZDV 200 mg TDS 29 months	250	10	MTB, HCMV
3 (M 53y)	ZDV 400 mg TDS 36 months	200	10	Toxoplasma, NHL
4 (F 38y)	ZDV 200 mg TDS 47 months	180	0	HCMV
5 (M 46y)	ZDV 250 mg BD 36 months	120	0	PCP, wasting
6 (M 42y)	ZDV 250 mg BD 15 months	50	10	HCMV, KS
7 (M 38y)	ZDV 250 mg QDS 33 months	205	0	HCMV, KS, PCP
8 (M 55y)	ZDV 200 mg TDS 32 months	130	10	wasting, Cryptosporidiosis Cerebral atrophy
9 (M 32y)	ZDV 100 mg QDS 8 months	50	20	ITP, Haemorrhagic Cystitis
10 (M 36y)	NONE	N/A	10	N meningitidis infection
11 (M 37y)	NONE	N/A	10	MAI, HCMV

Table 4.1. Demographic and clinical characteristics of the patients studied. CD4* = CD4 count at the start of ZDV therapy (cells/ul), ZDV = Zidovudine, PCP = Pneumocystis carinii pneumonia, MAI = Mycobacterium avium intracellulare, NHL = Non-Hodgkin's lymphoma, MTB = Mycobacterium tuberculosis, HCMV = Cytomegalovirus, KS = Kaposi's sarcoma, ITP = idiopathic thrombocytopenic purpura.

PATIENT	LN	SPLEEN	BRAIN	LUNG	HEART	KIDNEY	ADRENAL
01	3.26	2.30	2.59	3.07	0	2.76	NA
02	3.71	3.31	2.42	2.72	1.30	1.83	2.35
03	2.15	1.65	1.39	0	0	NA	1.30
04	3.01	2.46	0	2.70	0	1.79	0
05	3.30	3.09	1.59	2.78	2.57	1.30	3.12
06	3.07	2.73	2.67	1.30	0	NA	1.30
07	2.73	3.74	0	2.13	1.30	2.53	0
08	3.17	0	0	0	1.30	0	4.14
09	3.09	2.3	2.30	1.30	2.24	0	NA
10	3.17	3.83	2.82	3.89	1.30	1.30	3.79
11	3.02	2.07	0	1.30	0	0	0

Table 4.2. HIV-I proviral DNA load in post mortem tissues. Results expressed as copies (log 10) per μg of DNA.

LN: Lymph node.

PATIENT	OESP	DUOD	COLON	PANCR	LIVER	STOMA	SAL GLAN
01	NA	NA	NA	NA	1.30	NA	NA
02	NA	1.30	NA	NA	0	NA	NA
03	0	NA	0	0	0	NA	NA
04	0	0	0	NA	0	NA	NA
05	NA	NA	NA	NA	3.36	NA	NA
06	0	0	0	0	1.85	NA	1.30
07	NA	NA	0	NA	1.30	NA	NA
08	0	NA	0	0	1.30	0	NA
09	NA	2.30	0	NA	NA	NA	NA
10	NA	0	2.91	0	0	0	NA
11	0	0	NA	NA	0	NA	0

Table 4.2 continued. Oesp: Oesophagus, Duod: duodenum, Pancr: Pancreas, Stoma: Stomach, Sal Glan: Salivary gland.

4.3 RESULTS

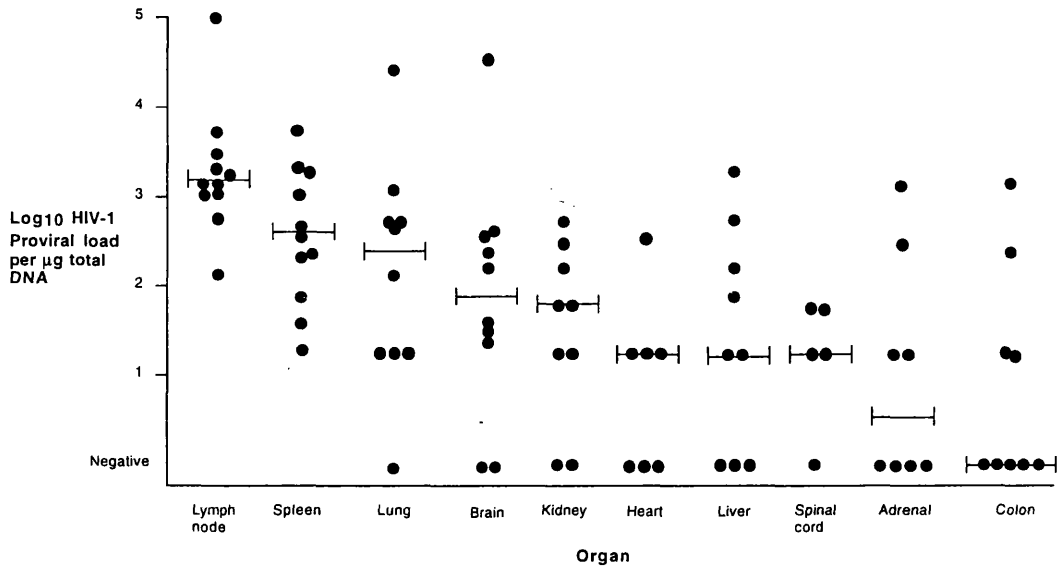
Ninety organs from 11 patients dying with AIDS were examined for HIV-1 proviral DNA load using a quantitative-competitive PCR method. The details of the patients are summarised in Table 4.1. Nine patients had been treated with ZDV for between 8 and 47 months (mean duration 27.25 +/- and SD of 13.5 months). The mean age at death was 42.3 years (range 32-63 years). Two patients had never received ZDV treatment and were assessed for viral load in a similar fashion.

Proviral burdens in lymph node and spleen were significantly higher than all other tissues ($p<0.05$ and $p<0.01$), with the exception of lung, for which only the lymph node burden was significantly higher. All patients in this study died of AIDS and HIV-1 was detected to varying amounts in non-lymphoid tissue such as brain, duodenum and liver. Patient 4 demonstrated a high viral load in lung and liver which was comparable to levels found in the lymph node. A high viral load in the lymph node did not necessarily correspond with a high load in the spleen as shown in patients 1,2, 6 and 7. The proviral load in brain was significantly lower than demonstrated in lymph node but was in the same range as shown by previous studies (Donaldson et al., 1994a). Figure 4.1 illustrates the relationship in terms of the median proviral DNA load between the various tissues examined showing that the viral DNA load for lymph node and spleen is higher than other organs. These results agree with previous studies that have shown that HIV-1 accumulates in the lymphoid organs and is actively replicating.

This study was not accompanied by a pathological investigation of the tissues, previous work has shown a correlation between high viral load in the brain and the presence of giant cell encephalitis which is a hallmark of HIV-1 infection of the brain.

The sequence of the V3 loop of HIV-1 gp120 was obtained by direct sequencing from a selection of tissues from 8 of the patients analysed. The V3 loop amino acid sequences from the tissues, compared to the HXB2 subtype B consensus sequence are shown in Tables 4.3 A to 4.3. G.

Figure 4.1. Distribution of HIV-1 proviral DNA load among the post-mortem tissues examined. The median values of load are shown.


5
4
3
2
1
Negative
Log10 HIV-1 Proviral load per µg total DNA
Lymph node
Spleen
Lung
Brain
Kidney
Heart
Liver
Spinal cord
Adrenal
Colon
Organ

A total of 19 sequences were obtained showing amino acid differences between patients, whereas, within each patient very similar amino acid sequences were found. None of the nucleotide sequences contained inactivating substitutions such as stop codons or frameshifts, and G to A hypermutations were not observed. A G to A hypermutation which has been observed in the HIV-1 genome (Vartanian et al., 1994) is thought to be the result of induced mutation whereby the viral reverse transcriptase is forced into making errors by imbalances in the intracellular dCTP concentration. In patients such as 05, 02 and 06 where several different tissues were analysed, a very similar V3 sequence was found within each patient.

A well defined relationship exists between V3 sequences and the properties of macrophage tropism and syncytium induction for HIV-1 (de Jong et al., 1992 and Fouchier et al., 1992), and also between the net charge on the V3 loop and similarity to the subtype B consensus V3 sequence (Chesebro et al., 1992). A similarity exists between this set of sequences and sequences characterised *in vitro*, showing macrophage tropic and non syncytium inducing (NSI) phenotype in both overall consensus sequence, and in the position and nature of amino acid substitutions. A frequent substitution of asparagine (N), with a lesser frequency proline (P) at position 305 of the V3 loop was found in 9 of the sequences. A change to arginine and threonine was also found at position 305.

The consensus sequence motif of GPGRAF seen at the tip of the V3 loop was found in 17 of the 19 sequences. Variations of this motif were found in 3 separate lymph nodes and these were GPGRAP and GPGRAV. An amino acid change from threonine (T) to isoleucine (I) was seen at position in 3 separate patients. The entire V3 loop charge was calculated by assigning a unitary positive charge to arginine (R) and lysine (K) residues, and a unitary negative charge to glutamtic acid (E) and aspartic acid (D) residues. The potential charge contributed by histidine residues was discounted. Overall the V3 loop sequences showed a low average positive charge of 4 and a lesser degree of amino acid sequence change compared to sequences of previously published syncytium inducing (SI) variants of HIV-1. A comparison of the V3 loop sequences on the basis of predicted overall charge and the number of amino acid changes from the subtype B consensus is illustrated in Figure 4.2.

umber of
nino acid
anges from
e subtype B
nsensus
quence

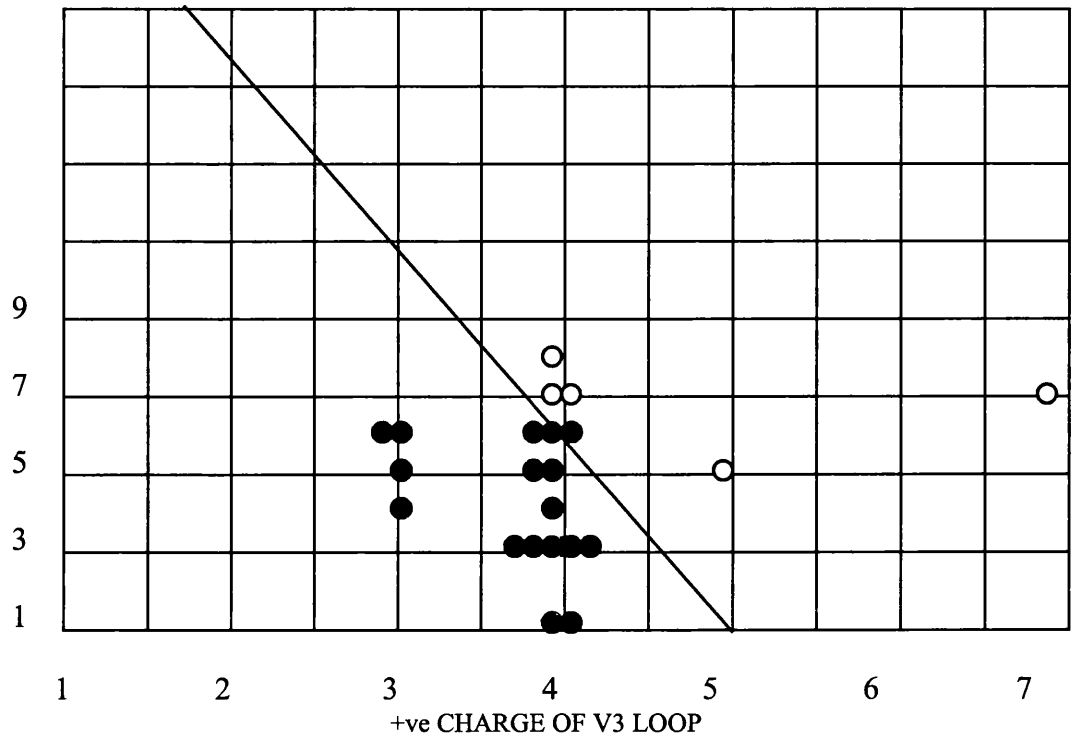


FIG. 4.2. Comparison of V3 loop sequences from post-mortem tissue samples on the basis of predicted overall charge (x-axis) and the number of amino acid changes from the subtype B concensus (y-axis).The symbol ● : represents NSI and macrophage tropic sequences, and the symbol○ : represents SI and T-cell tropic sequences. A diagonal line separates the two populations. The overall charge of the entire V3 loop (35 amino acids from nucleotide position 7,110 to nucleotide position 7127, based on the HXB2 sequence, Ratner et al., 1985) was calculated by assigning a unitary positive charge to Arginine and lysine residues and a unitary negative charge to glutamate and Aspartate residues. The potential partial charge contributed by Histidine residues was discounted.

4.4 DISCUSSION

4.4.1 HIV-1 infection of the liver

HIV-1 DNA was detected in 5 of the 10 liver samples studied and a low viral load of 20-70 copies/μg DNA was measured in 4 of the samples. One sample demonstrated a very high load of > 2000 copies/μg DNA, which was higher than the average load found in lymph node samples. HIV-1 has been detected in the mononuclear inflammatory cells, Kupffer cells and in endothelial cells (sinusoidal lining cells) of the liver by immunohistochemistry for viral antigen, and *in situ* hybridisation for viral RNA (Schmitt et al., 1989; Housset et al., 1990; Housset et al., 1993). A previous quantitative PCR study for HIV-1 DNA in 14 liver biopsies (Cao et al., 1992), demonstrated that the HIV-1 load in the liver was 4.5 fold lower than lymphocytes, and 2.5 lower than peripheral monocytes. HIV-1 mRNA , p24 and gp41 antigens were also detected in Kupffer cells and hepatocytes. These findings not only suggest that the liver is an important site for HIV-1 replication, but also supports previous *in vitro* studies showing that hepatoma cells, Kupffer cells and endothelial cells from liver sinusoids can be productively infected by HIV-1 (Cao et al., 1990; Housset et al., 1990).

HIV-1 infected individuals can present with clotting abnormalities and thrombi which suggest the existence of an endothelial dysfunction. Despite difficulties in growing human liver tissue *in vitro*, studies have examined whether HIV-1 induces functional alterations in human liver endothelial cells *in vitro*. A study by Lafon et al, 1994, examined the storage and release of endothelial-specific factors such as von Willebrand's factor. The pinocytic and phagocytic properties of the infected endothelial cells were examined using acetylated low density lipoproteins and latex beads. Synthesis of the von Willebrand factor and endothelin 1 were markedly decreased with pinocytic and phagocytic properties preserved suggesting that HIV-1 can trigger non lethal alterations in cultured endothelial cells.

4.4.2 HIV-1 infection of the intestine

The gastrointestinal (GI) tract is an important site of entry of entry for HIV-1 and the GI epithelial mucosa may support HIV growth (Fox et al., 1989). Cramping, abdominal pain, weight loss and sporadic or persistent diarrhoea commonly occur in HIV infected patients (Gerberding., 1989; Gelb & Miller., 1987). These features can be explained by either infection with opportunistic pathogens, or neoplastic involvement in many, but not all patients. In this study HIV DNA was not detected in any of the oesophageal samples, and only 2 patients who were receiving zidovudine demonstrated HIV-1 DNA in the duodenum, with a relatively low load of between 19 and 194 copies per μg/DNA. The colon of one patient who was not receiving zidovudine contained 820 copies per μg/DNA. This low rate of HIV-1 detection differs from a previous study where HIV was found in approximately 40% of oesophageal and duodenal biopsies (Gill et al., 1992).

HIV-1 RNA has been detected mainly in lymphocytes and macrophages of the lamina propria (Fox et al., 1989), and macrophage tropic strains of HIV have been shown to replicate in primary cultures of normal human ileal and colonic epithelial cells. It has been suggested that HIV-1 induced cytopathology of even a small subpopulation of GI epithelial cells, might directly compromise the barrier and transport function of the GI mucosa, and contribute to the increased incidence of opportunistic infection, and the poor nutritional status and cachexia that is common in AIDS patients (Ullrich et al., 1992).

4.4.3 HIV-1 infection of the lymph node

In all of the patients studied, proviral DNA load was significantly higher in the lymph nodes compared to the spleen and all other non lymphoid tissue (Table 4.2). These findings are similar to other studies that have demonstrated that lymphoid organs function as major reservoirs for HIV-1 (Pantaleo et al., 1993). The lymphoid system harbours 98% of the total body lynphocytes, and HIV-1 has been demonstrated in lymph nodes by electron microscopy, *in situ* hybridisation (ISH) and *in situ* PCR (IS-PCR).

The lymph node was first shown to play an important role in the pathogenesis of HIV, with the demonstration by immunohistochemistry of abundant viral antigen (Tenner-Racz et al., 1986; Biberfeld et al., 1986), and the presence of HIV particles in immune complexes on the surface of follicular dendritic processes (Tenner-Racz et al., 1989). Further work with ISH, using radiolabelled HIV-1 RNA probes, demonstrated high concentrations of viral mRNA in the germinal centres of lymphoid tissue (Fox et al., 1991). A second study using a semi-quantitative PCR approach indicated that a significantly higher viral load was present in lymphoid tissue, compared to peripheral blood during early and intermediate stages of disease, whereas in late stage disease the viral load was equal (Pantaleo et al., 1993). This dichotomy in viral burden between peripheral blood and lymphoid tissue is related to the histopathological abnormalities associated with different stages of disease.

Extracellular virus is trapped on the surface of follicular dendritic cells (FDC) within the activated centres of lymph nodes. The function of FDC during the normal immune response is to trap antigens that remain on their surface for long periods of time, this allows the induction and maintenance of immune responses. Histopathological modifications (follicular hyperplasia and expansion of the FDC network) are the primary mechanisms of sequestration of the virus in the lymphoid organs. As HIV disease progresses, the viral burden and the levels of replication both increase but are still preferentially retained within the lymphoid tissues as long as the structure of germinal centres remains intact. When high levels of active replication again become detectable in the blood, follicular hyperplasia is replaced by fragmentation and involution, which reduces the efficiency of HIV removal by the FDC network and results in an increase in HIV plasma levels (Pantaleo et al., 1993; Pantaleo et al., 1994). These histopathological abnormalities may not only explain the changes in viral distribution observed in the lymphoid tissue in different stages of disease, but may also reflect different functional states of the immune system during disease progression (Graziosi et al., 1993).

4.4.4 HIV-1 infection of the brain

Central nervous system disease is a significant complication of later stages of HIV infection (Budka et al., 1991a,b; Price et al., 1991). It is manifested in a variety of neurological, psychological and psychiatric disturbances, the most severe of which is dementia occurring in a least 10% of patients with AIDS (Price, 1994). A range of pathological abnormalities has been reported in the CNS of AIDS patients (Sharer, 1992), including HIV encephalitis characterised by the presence of giant cells, particularly in the central white matter and deep grey matter (Budka et al., 1991b; Sharer et al., 1985). HIV leukoencephalopathy is also seen in some patients and is manifested by severe white matter gliosis and myelin loss in presence of HIV-1 antigen demonstrated by immunohistochemistry (Budka et al., 1991a).

The major target cells for HIV-1 infection of the CNS are macrophages and microglia (Wiley et al., 1986), while astrocytes and endothelial cells may harbour a low level of infection (Moses et al., 1993). In this chapter HIV-1 DNA was detected in 7 of the 11 frontal lobe brain tissues analysed, with a range in viral load of 23 copies/μg DNA to 662 copies/μg DNA. The viral load of the brain was lower than that in the lymph nodes. This study was not accompanied by a pathological assessment of the brain, but previous studies have demonstrated a correlation between high viral load and the presence of giant cell encephalitis (Donaldson et al., 1994b; Bell et al., 1996).

4.4.5 HIV infection of the lung, kidney, heart and adrenals

The lung is a major target for infection by opportunistic pathogens in AIDS patients and is also frequently infected by HIV. In this study significant levels of HIV DNA were found in lung tissue of both zidovudine treated and untreated patients, with a viral load range of 19 copies to 768 copies/μg DNA. A previous study which examined HIV proviral load in PM lung material showed a viral load range of between 53 and 2504 copies per 10^6 cells (Donaldson et al., 1994a). Early reports on HIV-1 isolation from the lung showed that alveolar macrophages were infected with HIV-1 *in vivo* and that the virus could be recovered by co-cultivation with human peripheral blood mononuclear cells *in vitro* (Salahuddin et al., 1986). HIV is capable

of infecting a variety of lung cells including alveolar macrophages and fibroblasts (Plata et al., 1990).

The kidney showed a relatively high viral load comparable to levels in the brain, and HIV has been detected in the kidney epithelium of individuals with tubular glomerulopathy (Cohen et al., 1989). The highest viral load of 13,960 copies /μg DNA was detected in an adrenal gland tissue, this load was significantly higher than any of the lymph node tissues. Fetal adrenal cells and a human adenocortical carcinoma cell line have been found to be susceptible to HIV (Barboza et al., 1992), but most evidence suggests that *in vivo*, it is CMV that causes the cell destruction noted in this tissue (Pulkahandam et al., 1990; Greene et al., 1984).

A low viral load of between 19 copies and 365 copies per μg / DNA was found in post-mortem heart tissue, and HIV has previously been detected in the heart of patients with cardiomyopathy (Lipschultz et al., 1990). Dysrhythmias, hemodynamic abnormalities and congestive heart failure are serious complications of HIV infection (Lipschultz et al., 1989). A study of cardiac morbidity and mortality in children with HIV infection has shown that AIDS patients with encephalopathy had a higher risk of the most adverse cardiac outcomes, and that Epstein-Barr virus co-infection was the strongest correlate of chronic congestive heart failure (Luginbuhl et al., 1993). In this study 5 of the 6 patients demonstrating HIV DNA in the heart also showed the presence of HIV DNA in the brain.

4.4.6 Zidovudine treatment and the distribution of HIV-1 viral load in post mortem tissues

The HIV load present in the brain samples showed a significant difference between the zidovudine treated and untreated patients. 6 of 9 patients receiving zidovudine demonstrated HIV-1 DNA by PCR and 2 of the treated patients showed a low viral load of 19 to 38 copies/μg DNA. Of the 2 untreated patients one was negative by PCR and the second demonstrated the highest viral load of the brain tissues examined with 662 copies/μg DNA. Previous studies have documented an association with zidovudine therapy and a decline in the incidence of HIV dementia (Portegies et al.,

1993, 1995), and also a significant reduction of encephalitis and cognitive impairment (Bell et al., 1996; Gray et al., 1994).

HIV-1 DNA was not detected in the colon of the zidovudine treated patients, and was present in one of the untreated patients with a load of 822 copies/μg DNA. It is unclear whether the absence of HIV in the colon and the low detection rate in the duodenum of the treated patients is due to prolonged zidovudine therapy, but previous studies have a shown a gain in body weight in patients receiving zidovudine (Fischl et al., 1987), and a lower frequency of mucosal HIV infected cells (Ullrich et al., 1992). Mucosal HIV infection is associated with a hyporegenerative atrophy of the small intestine and enterocyte dysmaturation. Improved enterocyte maturation indicated by increased brush border enzyme activity may contribute to the clinical benefit of HIV infected patients from zidovudine therapy (Ullrich et al., 1992).

4.4.7 Sequence variation in the V3 loop of proviral HIV-1 present in post-mortem tissues

The V3 loop region of the subtype B env gene consists of 35 amino acids bound in a loop structure by a disulphide bridge between two cysteine residues (Rusche et al., 1988; Palker et al., 1988; Javaherian et al., 1989). This region is highly variable, although it has a highly conserved amino acid tetrapeptide, Gly-Pro-Gly-Arg at the tip of the loop (LaRossa et al., 1990), which is likely to be maintained for structural reasons. The importance of conserving the Gly-Pro-Gly-Arg motif has been demonstrated in studies where proviruses with mutations or deletions within the Gly-Pro-Gly motif were not infectious however, normal synthesis and processing of the gp160 of these mutants was observed (Grimalia et al., 1992). Some mutations within the Gly-Pro-Gly motif have also been shown to affect the ability of virus to form syncytia (de Jong et al., 1992), and mutation of the conserved Arginine has caused a change in a cleavage site in the envelope glycoprotein for a cellular protease (Freed et al., 1991). The consensus of V3 sequences found by LaRossa et al, predicted that the loop structure is a cysteine-beta strand-type II beta turn-beta strand-alpha helix cysteine.

Analysis of V3 loop sequences obtained from cloned and primary HIV-1 isolates, has revealed a distinct pattern of amino acid substitutions within this region that correlated with virus phenotype (Millich et al., 1993). It was found that a combination of non conservative basic amino acid substitutions in positions 11, 24 and 32, plus a basic or uncharged amino acid residue at position 25 were predictive of an SI phenotype. The presence of an acidic amino acid at position 25 was found to correlate with an NSI phenotype. These findings are consistent with previous analyses of this hypervariable loop (de Jong et al., 1992; Fouchier et al., 1992; Shioda et al., 1992; Shioda et al., 1994., Morris et al., 1994). In this study, substituted basic amino acids were found at positions 11 an 24 of the V3 loop in 2 lymph node samples, suggesting the presence of SI strains in those tissues. The nucleotide sequence of these samples showed a mix of consensus sequence and a sequence change to either Arginine or Lysine, suggesting that the SI variant was part of a minor population.

To investigate the relationship between *in vitro* phenotype and V3 sequence the overall charge on the V3 loop was plotted against the degree of sequence divergence from the subtype B consensus (Fig 4.2). In agreement with previous studies (Donaldson et al., 1994b; Milich et al., 1993), which used a similar method for sequence analysis of V3 from isolates of known biological phenotye, NSI/MT isolates showed a lower charge and greater similarity to the subtype B consensus than did SI and non-MT variants. A diagonal line separates the two populations showing that the majority of HIV variants from PM tissues examined are NSI/MT. The position of the diagonal line in figure 4.2 is in the same position as shown in two previous studies using this method of analysis (Donaldson et al., 1994b; Millich et al., 1993). The separation of NSI and SI sequences and the position of the diagonal line is based on the comparison of overall V3 loop charge and sequence divergence from subtype B consensus sequence in fifty-nine isolates of *in vitro* phenotype (Donaldson et al., 1994b). 5 of the variants were located to the right of the dividing line suggesting an SI/ non-MT variant. 5 of the SI variants were from lymph node or spleen tissue where high HIV load was present (average 942 copies/μg DNA), the brain sample containing an SI variant also showed a high viral load of 491 copies/μg DNA. SI variants are predominantly isolated from patients with AIDS and low CD4 count, and the presence of SI variants in the 4 lymph node and spleen tissues may reflect the high viral load

and replication rate in these tissues (Pantaleo et al., 1993) and an environment rich in both T-cells and macrophages. The one SI variant detected in brain may reflect the high grade lymphocytic infiltrate that was observed in this tissue.

Populations of HIV variants infecting different tissues *in vivo* have been shown to be generally distinct in the V3 loop region (Ball et al., 1994; Korber et al., 1994; Power et al., 1994; Dondaldson et al., 1994b). In this study some amino acid differences were seen between different tissues for individual patients. Patient 03 demonstrated a highly charged SI variant with four basic amino substitutions and a deletion at position 22 in the lymph node which greatly differed from the low charge NSI variant found in the lung. Patient 05 demonstrated the same V3 loop sequence in both lymph node and spleen and in non lymphoid tissues such as liver, kidney and ganglion with the latter containing an amino change from Asparagine to Isoleucine at position 5. Patient 06 contained an amino acid deletion at position 24 which was found in all 4 of the tissues from this patient. Changes were also seen in the normally conserved tip of the V3 loop particularly in Patient 02 who contained a GPGRVY motif in the lymph node compared to the conserved GPGRAF motif in spleen and lung. Changes in the apical tip have been described in previous studies and a particular tissue tropic variant has been suggested (Ball et al., 1994; Chang et al., 1988).

The importance of macrophage tropism in HIV pathogenesis is seen in animal models such as SCID-Hu mice, where a more rapid CD4+ lymphocyte depletion is observed with a MT variant (Mosier et al., 1993), and lack of disease progression in chimpanzees infected with HIV was attributed to an inability of HIV to infect chimpanzee macrophages (Schuitemaker et al., 1993).

V3 CONSENSUS	C T R P N N N T R K S I H I G P G R A F Y T T G E I I G D I R Q A H C	CHARGE	N
02 SPL	- - - - - - - - - - - G - P M - - - - - - - F - - - D - - - - - - - K - - -	4	6
02 LN	- - - - - - - - - - - G - T M - - - - V Y - - - - D - - - - - - - K - - -	4	7
02 LG	- - - - - - - - - - - G - P M - - - - - - - - - - - D - - - - - - - K - - -	4	5

Table 4.3 A. Amino acid sequence of V3 loop of patient 02 compared to consensus subtype B sequence. Charge: overall charge of the V3 loop; N: number of amino acid changes from the subtype B consensus sequence.

V3 CONSENSUS	C T R P N N N T R K S I H I G P G R A F Y T T G E I I G D I R Q A H C	CHARGE	N
PM 9 05GANGLION	- - - - I - - - - - - - - N - - - - - - - - - R - - - - - - - - - - - - - - -	4	3
PM 10 05 SPLEEN	- - - - - - - - - - - - - N - - - - - - - - - R - - - - - - - - - - - - - - -	4	2
PM 11 05 KIDNEY	- - - - - - - - - - - - - N - - - - - - - - - R - - - - - - - - - - - - - - -	4	2
PM 12 05 LIVER	- - - - - - - - - - - - - N - - - - - - - - - R - - - - - - - - - - - - - - -	4	2
PM 13 05 LYMPH	- - - - - - - - - - - - - N - - - - - - - - - R - - - - - - - - - - - - - - -	4	2

Table 4.3 B. Amino acid sequence of V3 loop patient 05

V3 CONSENSUS	C T R P N N N T R K S I H I G P G R A F Y T T G E I I G D I R Q A H C	CHARGE	N
PM 22 06 BLOOD	- I - G - D * - - - N - - - - Y -	3	5
PM 23 06 LN	- I - G - D * - - - N - - - - Y -	3	5
PM 24 06 SPLEEN	- I - - - - - - - - - - - R - - - - - - - - G - D * - - - N - - - - Y -	4	6
PM 20 06 BRAIN	- I - A - R * - - - - - - - - - R -	5	4

Table 4.3 C. Amino acid sequence of the V3 loop of patient 06. *represents a deletion in the sequence to preserve alignment.

V3 CONSENSUS	C T R P N N N T R K S I H I G P G R A F Y T T G E I I G D I R Q A H C	CHARGE	N
PM 36 04 COLON	- - - - - - - - - - R -	4	1
PM 38 04 SP CH	- - - - - - - - - - R -	4	1

Table 4.3 D Amino acid sequence of the V3 loop of patient 04.

V3 CONSENSUS	C T R P N N N T R K S I H I G P G R A F Y T T G E I I G D I R Q A H C	CHARGE	N
PM 14 03 LUNG	- - - - - - - - - - - - - N M - - - - - - - - - - - D - - - - - - - - - -	3	3
PM 16 03 LN	- - - - - - - - - - - R - R - - - - - - - V S * - R K - - - - - - - - - -	7	6

Table 4.3 E. Amino acid sequence of the V3 loop of patient 03.

V3 CONSENSUS	C T R P N N N T R K S I H I G P G R A F Y T T G E I I G D I R Q A H C	CHARGE	N
PM 25 01 LN	- I - - - - - - I - G - - - - - - - - - - - - - - K - - - - - - - - - -	4	4
PM 26 01 BRAIN	- I - - - S - - V - G -	3	4

Table 4.3 F Amino acid sequence of the V3 loop of patient 01.

V3 CONSENSUS	C T R P N N N T R K S I H I G P G R A F Y T T G E I I G D I R Q A H C	CHARGE	N
PM 30 09 LN	- I - - - - - - I - G - - - - - - - - - - - - - - K - - - - - - - - - -	4	4
PM 42 AZ LN	- - - - - - - - - R - - P - - - - - - P - G - - D - - - - - - - - - - -	4	5

Table 4.3 G. Amino acid sequence of patient 09.

CHAPTER 5

Distribution of Zidovudine Resistance Mutations in Multiple Post-Mortem Tissues from AIDS Patients

5.1 INTRODUCTION

Replication of HIV introduces errors into the genome which are responsible for conferring a growth advantage over wildtype virus when antiretroviral drugs such as zidovudine exert a selective pressure (Larder et al., 1989). The molecular basis for HIV-1 resistance to zidovudine has been mapped to codons 41, 67, 70, 215, and 219 of the reverse transcriptase gene in both *in vitro* and clinical blood samples (Larder et al., 1989, Boucher et al., 1992a). A mutation at codon 210 (leucine to tryptophan) has also been shown to be associated with high level resistance to zidovudine (Hooker et al., 1996). A study by Sheehy and Desselberger (1993), described the presence of twenty seven novel mutations, ranging from codon 60 to codon 553 in the reverse transcriptase gene, which were associated with *in vitro* resistance to zidovudine, particularly at positions 173 and 211. Three isolates which demonstated *in vitro* resistance to zidovudine and did not contain the previously published mutations contained a mutation at codon 211 (arginine to lysine) and two of the three isolates showed a mutation at codon 173 (lysine to glutamic acid). These novel mutations may represent transient or temporary mutations associated with resistance, which may exist prior to the dominant mutations associated with zidovudine resistance.

As HIV-1 can infect a variety of tissues *in vivo* the aim of this study was to examine the distribution of zidovudine resistance mutations in the RT gene of HIV-1 using a novel point mutation assay (PMA) in samples of post-mortem body tissues from patients who died of AIDS.

Whether drug resistant populations arise concordantly between separate anatomical compartments is unclear. This has important implications for clinical treatment strategies, and the understanding of virus trafficking from the peripheral blood to various organs. HIV-1 has been shown in a variety of tissues *in vivo* (discussed in chapter 4), and divergent viral populations could be maintained in distinct sites by differences in local immune selection pressures, constraints on viral entry and replication due to differing target cell expression of co-receptors (He et al., 1997; Shieh et al., 1998).

The relationship of strains circulating in the periphery to strains present in organs is complex and undefined. Studies have shown the presence of phylogenetically and phenotypically distinct variants at a number of HIV-1 genetic loci in different organs including blood, and within the same organ or organ system (Dellasus et al., 1992; Smith et al., 1993; Ait-Khaled et al., 1995; Wong et al., 1997). Differences in local anti-retroviral drug selection pressure and intracellular concentration (Perno et al., 1992), could also combine to influence tissue specific evolution of pol sequences and in particular, drug resistant mutations.

A previous study has examined reverse transcriptase (RT) sequences from PBMC and cerebrospinal fluids (CSF) of patients treated with zidovudine and demonstrated the presence of resistant genotypes in both compartments (Di Stefano et al, 1995). However, it is unclear whether virus resident in CSF reflects the population present in the brain parenchyma (Koyanagi et al, 1987). A study by Sheehy et al. (1996) of *env* and *pol* sequence variation in post-mortem tissue and blood from a patient who received zidovudine for 9 months prior to death revealed differences in different organs. This study demonstrated a reduction in genetic heterogeneity in *env* regions during therapy with an increased *pol* gene heterogeneity. Futhermore, the brain population of HIV-1 in this patient consisted of zidovudine sensitive genotypes and genetically distinct *env* sequences. However, the previously described novel mutations associated with zidovudine resistance (Sheehy and Desselberger, 1993) may have been present in the brain sample, as one single molecule of reverse transcriptase gene contained a mutation at codon 211 (arginine to lysine).

A more recent study investigating *pol* sequence variation in brain, spleen and lymph node tissue demonstrated that brain-derived sequences were phylogenetically distinct, adding support to the concept of anatomically distinct, independently evolving quasispecies or virodemes (Wong et al, 1997). Further evidence of different variants of HIV-1 co-existing within different organs and body fluids at the same time and within the same host has been demonstrated by Ball et al. (1994). This study used envelope length polymorphisms and nucleotide sequences from the variable regions V1 to V5 to demonstrate differences in envelope heterogeneity between different organs of an individual. V3 loop sequence variants present in the lymph node could be found in the

plasma population at autopsy and in sequential blood samples prior to death. Envelope sequence variants found in the brain were not detected in either plasma or sequential blood samples and high viral loads and encephalopathy was found to be associated with a mutated V3 apical loop sequence.

The issue of zidovudine resistance within the CNS is clinically important since zidovudine remains the only the antiretroviral agent of proven benefit in the treatment of AIDS dementia complex (ADC) (Portegies, 1995), and drugs such as ddI and ddC have been shown to have poor CNS penetration (Yarchoan et al., 1988; 1990). An early phase 1 study of zidovudine reported an improvement in neurological function in 3 of 4 patients, and a difference in neuropsychological test performance was demonstrated between participants in treatment and placebo groups (Schmitt et al, 1988). Similar neurocognitive improvement was seen in zidovudine treated children with AIDS and progressive encephalopathy (Pizzo et al, 1988). A retrospective study also demonstrated that the incidence of ADC decreased in the Netherlands in 1987, following the introduction of zidovudine treatment (Portegies et al, 1993). Placebo controlled dose response trials of zidovudine in AIDS patients with mild to moderate ADC have shown significant improvement in neuropsychological tests with a higher drug dose (Sidtis et al, 1993).

5.2 RESULTS

The point mutation assay (PMA) used in this study not only demonstrates the presence of resistance mutations, but also reveals quantitative information about the ratio of wild type sequence to mutated sequence at the various codons conferring drug resistance (Kaye et al., 1992). The method is described in full in chapter 2, section 2.11.3.

Post-mortem tissue from 11 patients who died of AIDS were examined by the PMA for the distribution of mutant/wild type alleles at each amino acid position associated with zidovudine resistance. Figure 5.1 provides the details of mutant/wildtype alleles found in the different tissues examined for each patient in the study.

In two patients who had not been exposed to zidovudine (patients 10 and 11), there was no evidence of a genotype conferring resistance at positions 41, 67, 70 or 219. However, small but reproducible quantities of resistance genotype at position 215 were found in brain and lung of Patient 10 and in spinal cord lung and lymph node of Patient 11. The highest amount of mutant genotype detected in these patients was 16% in the lung of patient 10 and 15 % in the lymph node of Patient 11. The other tissues in these two patients showed an amount of mutant genotype between 1 and 9%. This level of detection in the two treatment naïve patients was considered as background and used to normalise by subtraction, the levels of resistant genotype found in patients 1 to 8.

The remaining 9 patients possessed a resistance profile involving mutations at all codons, although mutations at codons 41 and 215 were predominant. However, careful inspection of the results from individual patients indicated that the distribution of mutations at each codon was not uniform in all organs. In all patients exposed to zidovudine, subtle or major differences between the distribution of mutant and wild type alleles at RT amino acids positions segregating with zidovudine resistance were observed. Differential distributions were most apparent in patients 1, 3, 5 and 7.

5.2.1 Patient 1

In Patient 1 the resistance profile in cardiac blood, lymph node, spleen, adrenal and lung were similar, showing high levels of genotypic resistance at codons 67, 70, 215 and 219. In contrast, the brain sample possessed 100% wild type sequence at codons 41, 67, 70, 219 and 99% mutant sequence at codon 215. The sub-mandibular gland showed a similar wild type genotype at codons 41, 67, 70 and 215. Codon 219 was not examined in this tissue. The PMA results in this patient reveal a proviral DNA sequence that has a completely wild type genotype in the brain and sub-mandibular gland, whereas the other tissues examined demonstrated a highly resistant genotype at all codons.

5.2.2 Patient 2

All of the tissues examined from Patient 2 displayed a predominantly mutant genotype at codons 41 and 215 and a wild type genotype at codons 67, 70 and 219. Lymph node, spleen and lung showed almost 100% mutant genotype at codons 41 and 215. Kidney and thyroid were approximately 75% wild type for codon 41 and 100% mutant for codon 215, suggesting that about 25% of proviral DNA in these tissues contained the linked 41 and 215 genotype. Duodenum contained 32% of wild type genotype at codon 41 and 100% mutant genotype at codon 215, showing a higher proportion (70%) of proviral DNA containing linked resistant mutations at positions 41 and 215.

5.2.3 Patient 3

Nine different tissues were examined by the PMA for Patient 3. All tissues showed approximately 100% mutant genotype at codons 215 and 219. Lymph node contained a 5% wild type population at codon 215 and lung contained a 5% wild type population at codon 219. There were approximately equal amounts of resistant and wild type genotypes at codon 41 in kidney, lymph node, heart, adrenal and spleen. No tissue demonstrated 100% mutant genotype for codon 41 and lung and liver demonstrate the lowest amount of mutants with 25% and 15% respectively. Interestingly, brain showed

the highest amount of resistant genotype at codon 41 with 82%, while the dorsal root ganglion, part of the peripheral nervous system, showing only 30% resistant genotype. The distribution of resistant genotypes at codons 67 and 70 fluctuated between different tissues with brain the only tissue showing 100% resistant genotype at codons 67 and 70.

5.2.4 Patient 4

Only three tissues were examined for Patient 4, with lymph node, spleen and lung demonstrating 100% resistant genotype at codons 41 and 215. Codon 219 was 100% wild type and codon 70 was predominantly wild type with only lymph node showing a 2% mutant genotype. The lymph node also contained equals amounts of wild type and resistant genotype at position 67. This patient received zidovudine for 4 years at 800mg per day and the PMA results show a genotype with 100% mutation at codons 41 and 215 which *in vitro* displays a highly resistant virus to zidovudine.

5.2.5 Patient 5

Six different post-mortem tissues and three whole blood samples taken prior to death were examined by PMA. 100% resistant genotype was evident at codon 215 and codon 41 for all tissues except the kidney, which was 100% wild type at codons 41, 67 and 70. No data was available for codon 215. Codon 70 was predominantly wild type for all tissues examined. A slightly different pattern was seen at codon 67 with lung and kidney showing 88% and 100% wild type genotype respectively. The lymph node also contained approximately 10% wild type codon 67.

5.2.6 Patient 6

Codon 215 was predominantly mutant in lymph node, spleen and kidney, while codon 41 was mutant in lymph node and kidney. Codon 70 was 100% wild type in all tissues and codon 67 was 100% mutant in spleen and kidney. The lymph node demonstrated an equal mix wild type and mutant genotypes at codon 67.

5.2.7 Patient 7

Resistant mutation at codon 215 was found in all tissues examined, while the liver and brain showed a wild type genotype at codon 219. Only Patient 1 showed a mix of wild type and mutant at codon 219, with brain showing wild type.

5.2.8 Patient 8

Predominantly mutant at codon 41 and 215 for all tissues and wild type at codon 219. A low level of mutant sequence at codons 67 and 70 was seen in the adrenal and cardiac blood respectively.

5.2.9 Patient 9

No significant levels of zidovudine resistant mutations were detected in Patient 9, who received the drug for eight months. At codon 215 the PMA detected a low level of mutation of between 2 and 6%.

5.2.10 Patients 10 and 11

These patients received no zidovudine antiretroviral therapy and served as controls for the specificity of the PMA.

5.4 DISCUSSION

This study utilised a sensitive point mutation assay to establish the quantitative prevalence of zidovudine resistance in multiple organs of patients dying with AIDS. The results clearly demonstrate that the evolution of zidovudine resistance is not uniform between different organs. The presence of high level resistance, as determined by the pattern of resistance mutations in the periphery or lymphoreticular system, is not always predictive of high level resistance in other organs, or even the same pattern of resistance mutations. For example Patient 1 demonstrated a profile of mutations associated with high level zidovudine resistance in lymph node, spleen, cardiac blood, adrenal and lung, whereas completely wild type sequences were detected in the brain and submandibular gland. Previous studies of HIV-1 RT sequence in brain and lymph node tissue has also revealed a wild type RT sequence in brain tissue from a patient who was receiving zidovudine and demonstrated resistance mutations in the lymph node (Ball et al., 1996).

Persistence in the brain of a completely wild type zidovudine sensitive strain may reflect the cell types infected: cells of the monocyte/macrophage lineage are the predominant target for infection in the brain (Wiley et al., 1986). *In vitro* studies have shown that zidovudine is ineffective against chronically infected monocyte cultures and this might be due to inefficient phosphorylation. Zidovudine penetration into multiple organs and/or its phosphorylation, differs so that lower intracellular levels of zidovudine triphosphate result in a reduced selective advantage for strains carrying the resistant genotype (Perno et al., 1992). A second explanation for the existence of zidovudine sensitive variants in the brain may be due to the infection of non-lymphoid tissues early in disease (with strains sensitive to zidovudine). These latent genomes persist at a low level of replication but reactivation of these strains in the target organ does not occur until the later stages of infection, leading to a phylogenetic disparity between strains resident in different organs (Coffin, 1995). A combination of these scenarios may exist, as previous studies have demonstrated that zidovudine anabolism can differ between organs (Perno et al, 1992), and compartmentalised evolution of HIV-1 strains can occur (Ait-Khaled et al., 1995; Wong et., 1997).

The inherently high rates of mutation and replication of HIV-1 allow for the continuous generation of diverse genetic variants *in vivo*. The question of whether HIV-1 populations arise concordantly between anatomic compartments has important implications for clinical treatment strategies and to the understanding of virus tropism and distribution. The replication of all single-stranded RNA viral genomes is subject to error due to the absence of proof-reading mechanisms (Holland et al, 1992). A mutation rate in the range of 3 X 10^{-4} mutation per nucleotide, per replication cycle has been estimated (Mansky and Temin, 1995), and this rate translates to an average of one mutation in every three 10 000-base pair genomes of HIV. This overall mutation rate is an accumulation of mutations formed during RNA-dependent DNA synthesis, DNA-dependent DNA synthesis, DNA replication and transcription and consists of substitutions, frameshifts and deletions. The high replication rate of HIV-1 compensates for the production of lethal mutations and provides a multitude of genetic variants that permit adaptation in the face of selective pressures such as the development of neutralising antibodies and cell-mediated immune responses, varying types of host cells and drug therapy (Koup et al., 1994 and Richman, 1995).

With the high virus production rates (10^8 to 10^{10} virions daily), and a high mutation rate, it is likely that any given mutation, or combination of mutations exist when a selective pressure, such as drug treatment is introduced. Previous studies have shown that variants containing the mutation in the RT gene at codon 70 (Lys-Arg), which confers low level resistance (Larder, 1994), have been found in previously untreated patients (Najera et al., 1995). Resistant mutations to ddI, ddC, and 3TC and to an NNRTI have also been detected, along with various mutations that could act as intermediates in the development of resistant genotypes in drug naive patients (Wong et al., 1997). Based on a mutation rate of $3x10^{-4}$ per bp per replication cycle and a replication rate of approximately 240 cycles of replication per year (Ho, 1997), a 1% increase in the growth rate of a mutant results in fixation of the mutant within 1000 replication cycles.

The accumulation of zidovudine resistance mutations is not random, but follows an ordered pattern leading eventually to a highly resistant virus (Boucher et al., 1992a).

Patient 8 who received zidovudine for 8 months, displayed no resistant mutations at codons 41, 67, 70 or 219. A small amount of resistant mutation was seen at codon 215, which was equal to that seen in the two patients who did not receive zidovudine. In a previous study, *pol* sequence variation in post-mortem tissue (brain and spleen) of an individual who received zidovudine for less than six months, showed no resistance mutations (Wong et al., 1997).

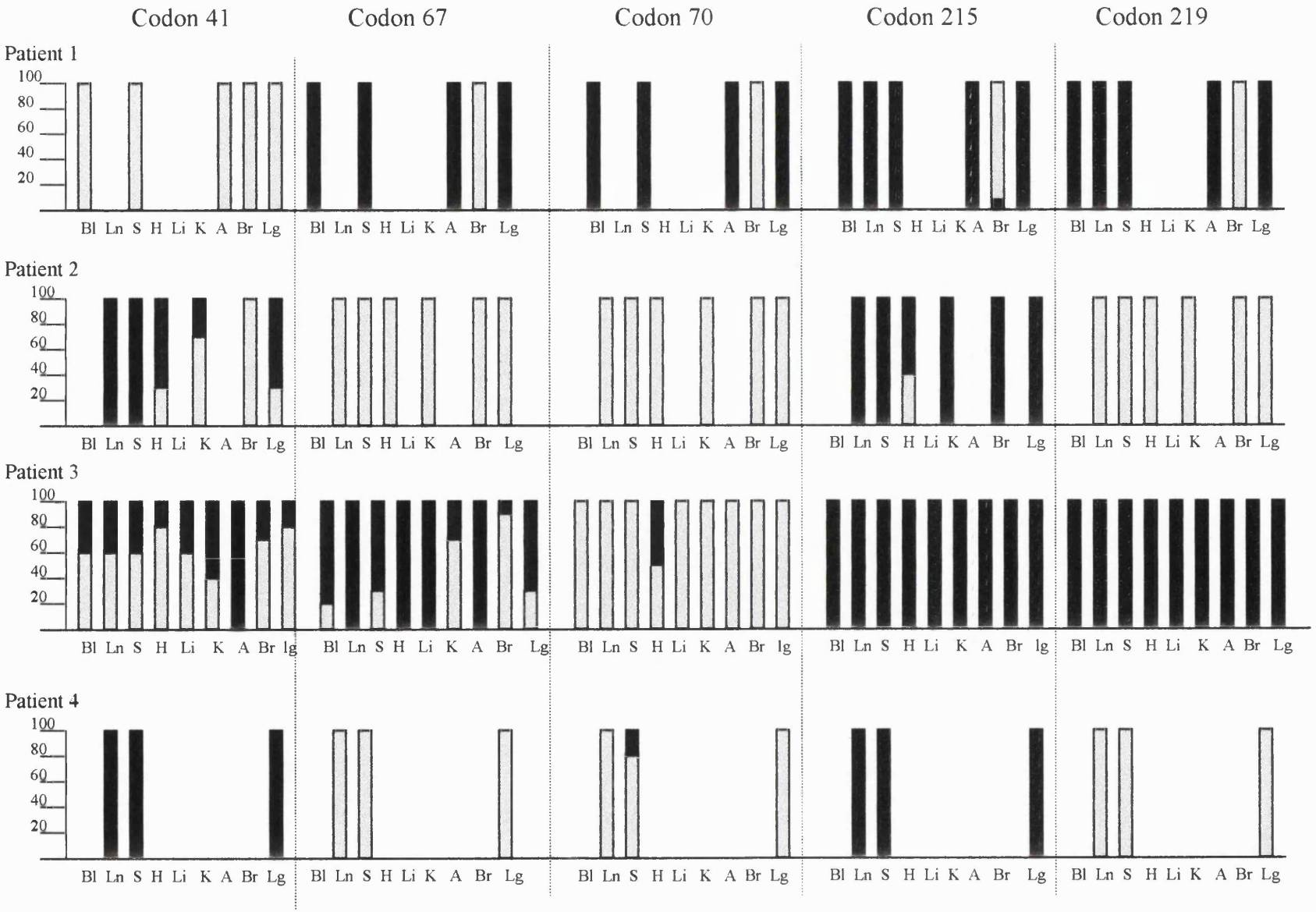
The first zidovudine resistance mutation is the replacement of lysine by an arginine at codon 70 (K70R). This is followed by a codon 215 mutation (usually T215Y), however the K70R population seems to decline with the simultaneous appearance of the M41L mutation. Genetic linkage of the codon 41 and 215 mutations is a landmark in the development of significantly resistant virus strains (Kellam et al., 1994). Prolonged zidovudine therapy results in the emergence of virus strains with four or five of the resistance mutations. Patient 1 who received zidovudine for 14 months demonstrated approximately 100% resistance mutation at codons 67, 70, 215 and 219 with approximately 10% mutation at codon 41. It is likely in this patient, over time, that resistance at codon 41 would have eventually emerged, with resistance at codon 70 decreasing to a greater extent. This is evident in patients 2, 4, 5, 6, 7 and 8 who were receiving zidovudine for longer periods of time and show decreased resistance at codon 70 and codon 219.

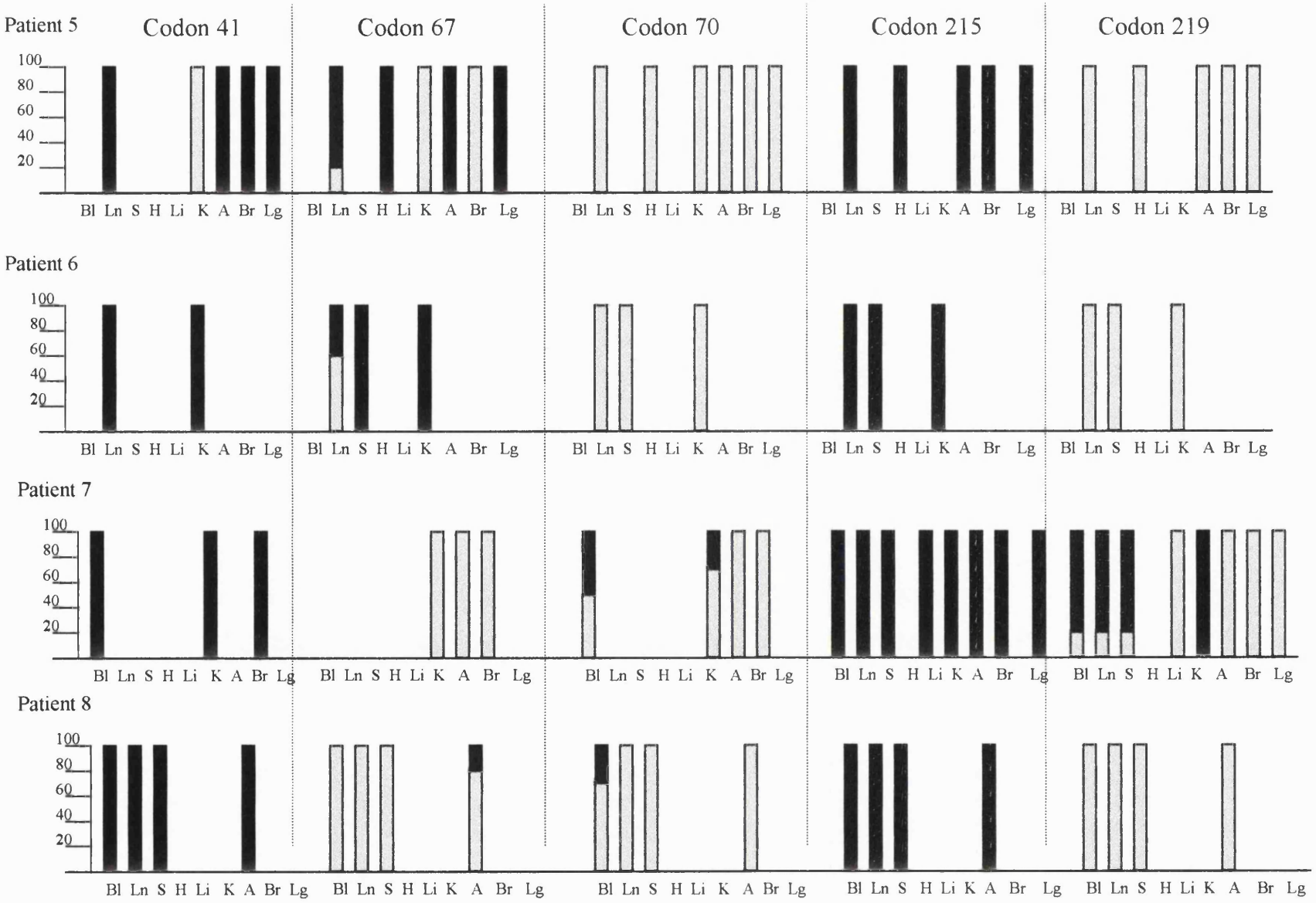
The point mutation assay used in this study for the detection and quantitative evaluation of zidovudine resistance mutations has been developed and validated in previous studies (Kaye et al., 1992). The assay has also been used to assess the contribution of a novel mutation at codon 210 of HIV-1 RT (Leucine to Tryptophan) and its association with high level resistance to zidovudine (Hooker et al., 1996). An alternative approach to study mixed populations of wild type and zidovudine resistance sequences is the selective amplification of HIV-1 DNA by oligonucleotide primers that allow selective priming of DNA synthesis from wild type or mutant nucleotides at codons 70 or 215 (Larder et., 1991; Sheehy et al., 1996). This approach although rapid can give only limited quantitative information.

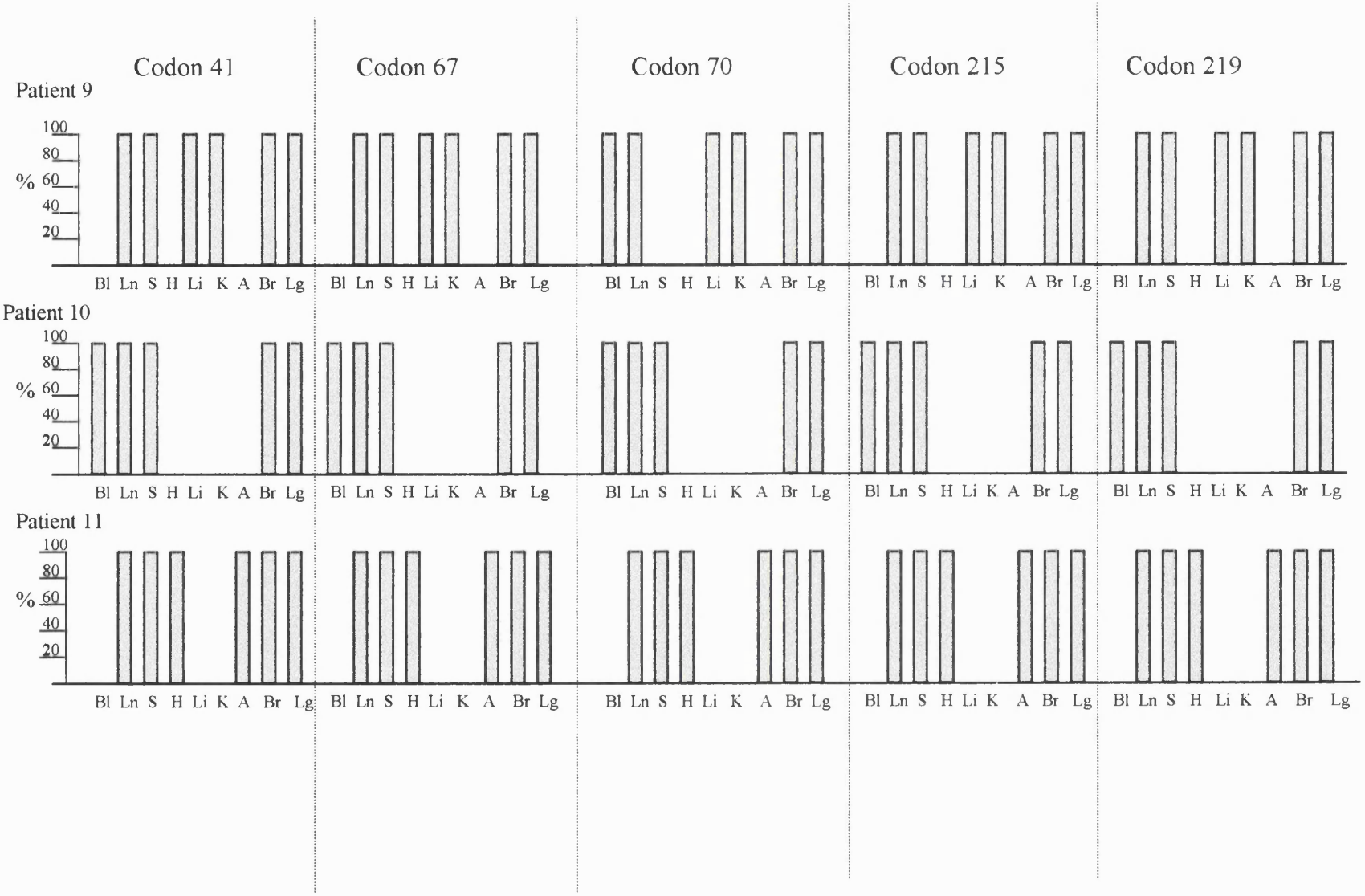
The data in this chapter demonstrate that divergent populations of drug resistant and sensitive virus can exist in separate organs of individuals, and that functional or anatomical barriers must play a role in maintaining these populations. This is important for the treatment of patients once zidovudine resistance has appeared in the peripheral blood, as continued therapy may still suppress virus populations in other anatomical sites.

Figure 5.1. Distribution of wild type (grey bars) and mutant (black bars) sequences at codons 41, 67, 70, 215 and 219, of HIV-1 RT gene in multiple organs obtained from 11 patients dying with AIDS. The % of wild type or mutant sequence is indicated for each codon within each tissues analysed.

A	Adrenal gland
Bl	Cardiac blood
Br	Brain
H	Heart
K	Kidney
Lg	Lung
Li	Liver
Ln	Lymph node
S	Spleen


Codon 41
Codon 67
Codon 70
Codon 215
Codon 219
Patient 1
Patient 2
Patient 3
Patient 4
100
80
60
40
20
Bl Ln S H Li K A Br Lg


Patient 5
Codon 41
Codon 67
Codon 70
Codon 215
Codon 219
100
80
60
40
20
Bl Ln S H Li K A Br Lg
Patient 6
Patient 7
Patient 8


Codon 41
Codon 67
Codon 70
Codon 215
Codon 219
Patient 9
Patient 10
Patient 11
%
100
80
60
40
20
Bl Ln S H Li K A Br Lg

CHAPTER 6

Detection of mutations associated with anti-retroviral drug resistance by Line Probe Assay, Point Mutation Assay and Direct Cycle Sequencing

6.1 INTRODUCTION

Reverse transcriptase inhibitors such as the antiretroviral nucleoside analogs AZT, ddI, ddC, 3TC, and d4T, are currently approved for treatment of advanced HIV-1 infection (De Clerq, 1995; Schinazi et al., 1992). These compounds act in a similar manner to chain terminators of the RT reaction after phosphorylation by intracellular kinases (Schinazi et al., 1993). However, upon prolonged treatment with these compounds resistance has been observed (Richman. 1992) Viral variants emerge with the ability to escape the inhibitory effects of these antiviral agents and these strains show nucleotide changes in the RT gene of HIV-1 (Richman, 1992; Richman. 1993). Amino acid (AA) changes at particular positions have been found to be associated with gradually increasing resistance and changes at positions 41(M to L), 69(T to D), 70 (K to R), 74 (I to V), 184 (M to V) and 215 (T to Y or F), are of particular importance (Mellors. 1995). Mutations at AA's 62, 75, 77, 116 and 151 encoding multidrug resistance (Iversen et al., 1996) may also be of significance.

The number of available anti-retroviral drugs, and the large number of possible combinations of these drugs, makes the use of a sensitive and rapid assay for the emergence of drug resistance a necessity. Currently, the best prognostic markers of survival for HIV infected individuals undergoing antiviral therapy are obtained by monitoring changes in HIV RNA load and CD4+ cells (Mellors et al., 1996). Genotypic resistance can be determined qualitatively by means of several molecular biology applications: Southern blotting (Richman et al., 1991), primer specific PCR (Larder et al., 1991), PCR ligase detection reaction (Frenkel et al., 1995), RNase A mismatch (Galandez-Lopez et al., 1991), hybridisation against labeled probes (Eastman et al., 1995), automated DNA sequencing (Larder et al., 1993), point mutation assay (PMA) (Kaye et al., 1992) and the Line Probe assay (LiPA) (Stuyver et al., 1997a,b). Only the PMA and automated DNA sequencing can reveal quantitative information of virus populations carrying mutations associated with drug resistance.

The Innogenetics (INNO) line probe assay (LiPA) detects wild type sequences and mutations, or polymorphisms, at codons 41, 69, 70, 74, 184, 214 and 215 of the HIV reverse transcriptase (RT) gene which are associated with AZT / ddI / ddC / 3TC resistance. The INNO-LiPA test is based upon the reverse hybridisation principle and has been adapted from previously developed LiPA tests for Hepatitis C virus genotyping (Stuyver et al., 1993; 1996). The HIV RT gene is amplified by nested PCR, using a biotinylated primer in the second PCR reaction. The biotinylated DNA is first denatured by alkali and hybridised with specific oligonucleotide probes immobilised as parallel lines on membrane-based strips. Following hybridisation and a stringent wash at 39°C to remove unbound and non-specifically bound PCR product, streptavidin labelled with alkaline phosphatase is added and binds to any biotinylated hybrid previously formed. Incubation with BCIP/ NBT chromogen results in a purple/brown precipitate.

Each typing strip contains 18 parallel DNA probe lines and two control lines. The control lines consist of an amplification control which demonstrates that biotinylated PCR product has hybridised to the strip, and a chromogen control which controls for the addition of reactive conjugate and substrate solution during the detection procedure. A total of 48 specific oligonucleotide probes to detect nucleotide polymorphisms at resistance-related codons, as well as several third letter polymorphisms in the direct vicinity of these target codons are contained in a single LiPA membrane strip (Stuyver et al., 1997a). Figure 6.1 contains a schematic outline of a LiPA strip illustrating the interpretation of the individual lines and resistance mutations associated with each drug. Figure 6.2 shows an example of LiPA membrane staining for six individual patients.

The direct sequencing approach used in this study involved amplification of two smaller sections (POL A and POL B) of the RT gene encompassing all of the drug resistance mutations analysed by both the LiPA test and the PMA. A sensitive cycle sequencing kit (Amersham) was used, which allows sequencing with the primers used in the PCR reaction. The kit is designed to eliminate sequencing artifacts such as stops (also known as Bands Across Four Lanes or BAFLs) and background bands. BAFLs

M	Line	Codon	Amino acid		Interpretation
-	1	conj ctrl			
-	2	Amp ctrl			
-	3	41	M41(ATG)		Wt
-	4	41	L41(TTG)		ZDV Mut
-	5	41	L41(CTG)		ZDV Mut
-	6	69/70	T69(ACT) K70(AAA)		ddC Wt, ZDV Wt
-	7	69/70	T69(ACT) R70(AGA)		ddC Wt, ZDV Mut
-	8	69/70	D69(GAT) K70(AAA)		ddC Mut, ZDV wt
-	9	69/70	D69(GAT) R70(GAT)		ddC mut, ZDVmut
-	10	69/70	N69(AAT) R70(GAT)		ZDV mut
-	11	74	L74(TTA)		Wt
-	12	74	V74(GTA)		ddI-ddC mut
-	13	184	M184(ATG)		Wt
-	14	184	V184(GTG)		3TC mut
-	15	214/215	F214(TTT)	T215(ACC/ACT)	Wt
-	16	214/215	L214(CTT/TTA)	T215(ACC/ACT)	Wt
-	17	214/215	T215(ACC/ACT)		Wt
-	18	214/215	F214(TTT)	Y215(TAC)	ZDV mut
-	19	214/215	L214(CTT/TTA)	Y215(TAC)	ZDV mut
-	20	214/215	F214(TTT)	F215(TTC)	ZDV mut

Figure 6.1 The LiPA HIV-1 RT format (adapted from Stuyver et al., 1997b).. Interpretation of the individual lines of a membrane strip (M). Lines 1 and 2 are a conjugate control and an amplification control respectively. The conjugate control contains biotinylated DNA and the amplification control contains three specific probes, selected in the conserved regions of the RT gene. The nucleotide sequence in brackets corresponds to the amino acid. Wt: wild-type, mut: mutant, blue corresponds to a wild-type sequence and red corresponds to a mutant sequence.

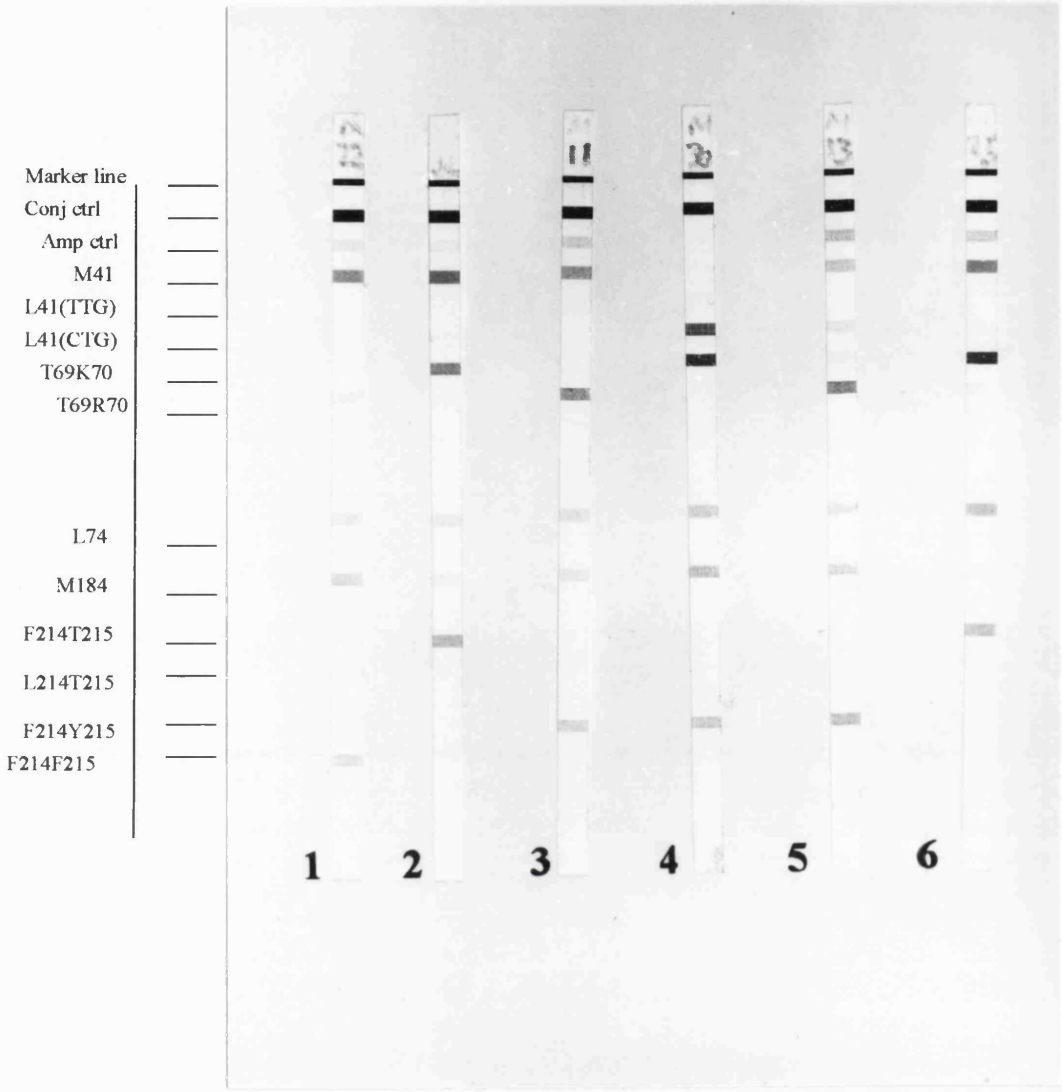


Figure 6.2 LiPA membrane staining for six individual patients. Positive hybridisation results correspond to the displayed key. Conj ctrl: conjugate control; Amp ctrl: amplification control.

can result from enzyme pausing at regions of secondary structures in G-C rich templates, while background bands can be caused by primer extensions aborting prematurely at random positions when a template is rich in a certain base and the complementary nucleotide in the reaction becomes depleted. The radioactive label is incorporated into the reaction products at the 3'-end by the ^{33}P labelled ddNTP, ensuring that only properly terminated DNA strands are labelled.

6.2 METHODS

Proviral DNA was extracted from whole blood and a variety of post-mortem tissue samples, using proteinase K and phenol-chloroform extraction followed by ethanol precipitation (section 2.1.2 and 2.1.3). 500ng was then used in a nested PCR reaction using primers to the RT gene of HIV-1.

The two approaches were taken to amplify HIV-1 RT are illustrated in Figure 6.3. Firstly, a large region (760bp) was amplified by nested PCR for use in both the point mutation assay (PMA) and line probe assay (LiPA), which encompassed the codons associated with resistance to AZT at positions 41, 67, 70, 215 and 219. The second approach involved amplification of two smaller separate regions, Pol A and Pol B for use in direct sequence analysis. The Pol A region of 183bp (nucleotide positions 2654 to 2837, based on HXB2 sequence, Ratner et al., 1985) encompassed codons 41, 76 and 70, whereas the Pol B region of 300bp (nucleotide positions 2946 to 3246, HXB2 sequence), included codons 184, 215 and 219.

Direct cycle sequencing and the point mutation assay were carried out as described in sections 2.9.5 and 2.11.3 of chapter 2. The protocol outlined in the LiPA kit (Innogenetics) was followed as described in chapter 2, section 2.18.1.

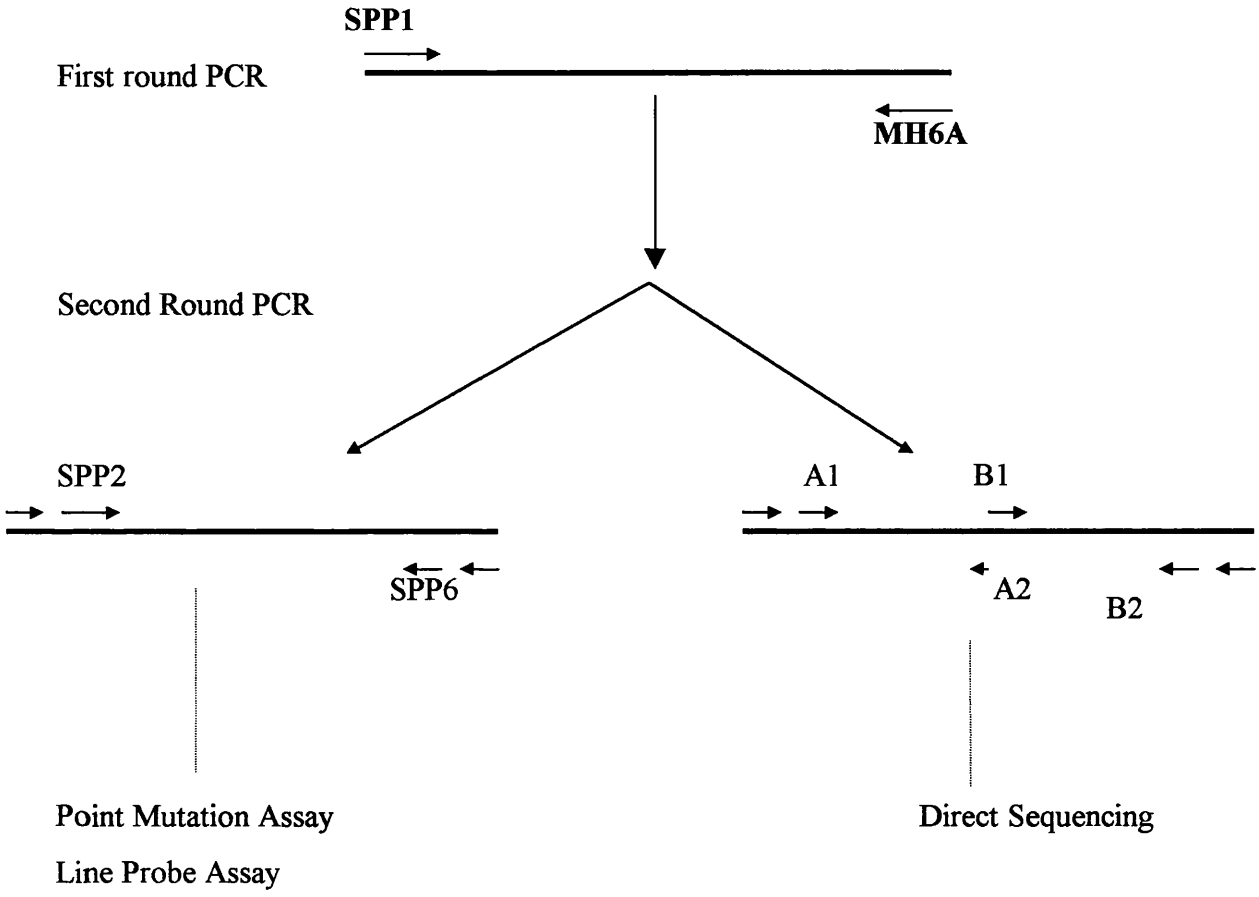


Figure 6.3. Strategy for amplification of the RT gene of HIV-1. A1:Primer ARP 892.1, A2:Primer ARP 892.2, B1:Primer ARP 892.4, B2:Primer 892.5.

6.3 RESULTS

19 different post-mortem tissues and 23 whole blood samples were examined for the presence of mutations associated with drug resistance. Three methods of detection were compared, direct cycle sequencing, the line probe assay, and the point mutation assay. The sensitivity of the LiPA test and direct sequencing is described in Table 6.1, where the number of samples positive for a certain codon by the LiPA test is compared to the result obtained by direct sequencing. Overall the LiPA test is highly sensitive compared to sequencing and can detect low numbers of mutant and wild type sequences which are below the threshold of detection by direct sequencing. The most discordant results are at codon 41 where there is a very low detection rate of the mutant sequence for Leucine CTG. Of the 19 samples that were positive by the LiPA test for L (CTG), only 5 of those samples showed this change by sequencing. A second anomaly at this position is that 14 samples showed by sequence analysis to be wild type (ATG) but did not show a positive LiPA test for the wild type codon M. An explanation for this might be the selective amplification of wild type sequences by the PCR primer ARP 892.1, which spans codon 41 and contains the wild type sequence at the 3' end. This primer could have a bias for wild type sequences and selectively change a mutant sequence CTG or TTG to ATG.

At codon 70 the LiPA test also demonstrated increased sensitivity compared to direct sequencing, by detecting mixes of wild type and mutant sequences in 25 of the 43 samples tested, whereas the direct sequencing method revealed only the dominant sequence type in these samples. Three samples which showed a mixed population of mutant and wild type sequences by LiPA demonstrated how the degree of staining intensity on the LiPA strip could reveal the proportion of wild type to mutant genotypes in the population. The corresponding autoradiographs of direct sequencing for these samples revealed two bands at the same position, one of greater intensity than the other. At codons 69, 74, 184 and 214, the LiPA test and direct sequencing were equally sensitive in detecting the wild type sequence. However, LiPA detection of the relatively rare mutant sequences at positions 69, 74 and 184 in the samples analysed was more sensitive than direct sequencing. A position 215 the mutant

sequence TAC coding for the amino acid tyrosine was detected in 31 of the 39 samples analysed by both LiPA and direct sequencing. 3 samples contained the mutant sequence TTC for phenylalanine and this was detected in 2 of the 3 samples by sequencing.

6.3.1 Comparison of PMA, LiPA and direct sequencing

22 samples were examined by the point mutation assay for the presence of drug resistant mutations at positions 41, 67, 70, 215 and 219. The results for these samples are described in Table 6.2. For comparison of sequence of PMA with sequence analysis the ambiguities discussed in the previous section at position 41 also apply and make interpretation at this codon difficult. Table 6.2 describes the number of samples analysed at each of the four codons that gave the same result by either direct sequencing and PMA or LiPA and PMA. 11 of the 19 samples analysed at codon 41 showed agreement between the PMA and the LiPA test. 7 samples contained a mixture of wild type and mutant sequences at codon 41 by LiPA, whereas the PMA demonstrated 100% wild type or mutant for these samples. Codon 67 was analysed by direct sequencing and the PMA only and 15 of the 19 samples tested showed agreement between the two tests. A mixture of wild type and mutant genotypes at codon 67 was detected by PMA in only one sample.

Agreement between sequencing and the PMA at codon 70 occurred in 15 of 20 samples analysed and 16 of 20 samples comparing the LiPA and the PMA. Six of the samples analysed at codon 70 by PMA and LiPA demonstrated a mixture of wild type and mutant genotypes by LiPA and the dominant genotype demonstrated by PMA agreed with the sequence information. In 4 of these samples, the PMA revealed a level of mutant genotype of between 89% and 96% which suggests that the LiPA test can detect very low amounts of wild type genotype in a mixed population. All of the 21 samples analysed at codon 215 showed complete agreement in results obtained by PMA, LiPA and sequencing. The specificity of the PMA at codon 215 was demonstrated by the detection in two samples of the phenylalanine mutant (TTC) and also by LiPA and direct cycle sequencing.

Codon No	Samples Tested*	Number of positive samples					
41	43	LiPA M 27	Seq ATG 27 (100%)	LiPA L 21	Seq TTG 16 (76%)	LiPA L 19	Seq CTG 5 (26%)
69	41	LiPA T 37	Seq ACT 37 (100%)	LiPA D 7	Seq GAT 6 (85%)		
70	43	LiPA K 28	Seq AAA 19 (67%)	LiPA R 31	Seq AGA 23 (74%)		
74	40	LiPA L 40	Seq TTA 40 (100%)	LiPA V 3	Seq GTA 2 (66.6%)		
184	41	LiPA M 41	Seq ATG 41 (100%)	LiPA V 4	Seq GTG 1 (25%)		
214	40	LiPA F 40	Seq TTT 40 (100%)	LiPA L 3	Seq CTT 1 (33.3%)		
215	39	LiPA T 12	Seq ACC 9 (75%)	LiPA Y 31	Seq TAC 31 (100%)	LiPA F 3	Seq TTC 2(66%)

Table 6.1. Comparison of RT mutation detection by the LiPA test and direct cycle sequencing. Numbers in brackets represent the percentage of the number of samples where both LiPA test and sequence detection agree.

* The total number tested at each codon on the LIPA strip consisted of samples that scored wild-type alone, mutant alone and a mix of wild-type and mutant. At codon 41, the 43 samples tested consisted wild-type, 11 (M), wild-type and mutant 16 (L+M) and mutant 16 (L), the mutant sequence is further separated into TTG or CTG. At codon 69, 3 of the samples consisted of a mix of T+D. Codon 70, 16 of the samples consisted of a mix of K+R. Codon 74, 3 of the samples consisted of a mix of L+V. Codon 184, 4 samples consisted of a mix of M+V. Codon 214, 3 samples consisted of a mix of F+L. Codon 215, 1 sample T+F, 8 samples T+Y, 23 samples Y, 2 samples F and 5 samples T.

Test compared	Number of samples showing agreement between tests			
	Codon 41	Codon 67	Codon 70	Codon 215
Sequence + PMA	19 / 19	15 / 19	15 / 20	21 / 21
LiPA + PMA	11 / 19	NA	16 / 20	21 / 21

Table 6.2. Comparison between assays, direct sequence analysis compared to PMA amd LiPA compared to PMA. Number of samples analysed at each codon which showed agreement between (I) Sequence and PMA and (II) LiPA and PMA.

6.3.2 Sequence analysis of HIV-1 RT from post-mortem tissue and whole blood samples

All nucleotide sequences were obtained from proviral DNA derived from whole blood PBMC's or from various post-mortem tissue samples. A total of 47 sequences were obtained. The corresponding amino acid sequences from a selection of post-mortem tissues and blood samples before time of death are presented in Tables 6.4.1A to 6.4.7.B. Direct cycle sequencing of the Pol A and Pol B fragments of the HIV-1 RT gene revealed nucleotide changes at codons 41, 67, 69, 70, 74, 184, 215 and 219 which have been previously associated with resistance due to 2',3'-dideoxynucleoside analog monotherapy. No nucleoside changes were observed at position 151 where a change from Q 151 M is associated with multidrug resistance (Iversen et al., 1996). Other mutations such as A62V, V75I, F77L and F116Y are also associated with resistance to AZT, ddI, d4T and 3TC when present with mutation Q151M. None of these nucleotide changes were observed in the samples studied. The mutation L210W has been shown to contribute to high level AZT resistance (Hooker et al., 1996) and this change was found in 8 of the samples studied (2 post-mortem tissues and 6 whole blood samples). Mutation L210W was found in association with a resistant mutation at codon 215. A number of amino acid changes which have previously been described to be associated with resistant strains of HIV-1 *in vitro* were also observed. These amino acid changes at positions 60 (V-I), 98 (A-S), 102 (K-R), 162 (S-C), 207 (Q-E) and 211 (R-K) were also found in association with mutation at codon 215.

	41 … 67 … 70
CONCENSUS HXB2	E **M** E K E G K I S K I G - E N P Y N T P V F A I K K K **D** S T **K** W R K L V D F R E
HLY 2-3-93	- - - - D R - - - Y - R - - - - - - - - - - - - - - - N - - - - - - - - - - - -
HLY 3-4-93	- - V L - N - - R - - - - - - - - -
HLY BRAIN	- L - R - - - - - - - - -
HLY LYMPH NODE	- - - - - - D - - - - - - - - - - - - - - - - - - R - - - - R - - - - - - - - -
HLY LUNG	- L - - - - - - - - - - - - - - - - - - I - - - - - - - - - - - - - I - - - - -

CONCENSUS HXB2	L N K R T Q D F W E V Q L G I P H P A G L K K K
HLY 2-3-93	- -
HLY 3-4-93	- -
HLY BRAIN	- - - - - - - - - - - P - - - - - - - - - - - -
HLY LYMPH NODE	- -
HLY LUNG	- -

TABLE 6.4.1A. Amino acid sequence of Pol A fragment for pateint 02.

	162
concensus HX B2	Y N V L P Q G W N G S P A I F Q **S** S M T N I L Q - F R N Q N P D I V I Y Q
HLY 2-3-93	- - - - - - - - - - - - - - - - R -
HLY 3-4-93	- - - - - - - - - - - - - - - - C -
HLY BRAIN	- - - - - - - - - - - - - - - - C -
HLY LYMPH NODE	- - - - - - - - - - - - - - - - C -
HLY LUNG	- - - - - - - - - - - - - - - - C -

	215
concensus HX B2	Y M D D L Y V G S D L E I G Q H R T K I E E L R Q H L L R W G L **T** T P D K K H
HLY 2-3-93	- - - - - - - - - - - - M - - - - - - - - - - - - Y - W K - - F Y - - - - - -
HLY 3-4-93	- - - - - - - - - - - - - - - - - A - - - - - - - Y - - K - - F Y - - - - - -
HLY BRAIN	- E Y - - K - - F Y A - - E - -
HLY LYMPH NODE	- E Y - - K - - F Y A - - - - -
HYY LUNG	- Y - - K - - F Y A - - - - -

Table 6.4.1B. Amino acid sequence of Pol B fragment for patient 02.

Position	41 (M); 67 (D); 70 (K)
CONCENSUS HXB2	E **M** E K E G K I S K I G - E N P Y N T P V F A I K K K **D** S T K W R K L V D F R E
val 18-3-92	- - - - - R - - L - - - - - - - - - - - - - - - - - - N - D R - - - - - - - - -
VAL LYMPH NODE	- - - - - - R - N - D R - - - - - - - - -
VAL LUNG	- N - - R - - - - - - - - -
CONCENSUS HXB2	L N K R T Q D F W E V Q L G I P H P A G L K K K
VAL 18-3-92	- - - - - - - - - - - - - - - - P - - - - - - Q
VAL LYMPH NODE	- Q
VAL lUNG	- R -

TABLE 6.4.2A. Amino acid sequence for Pol A fragment for patient 03.

concensus HX B2	Y N V L P Q G W N G S P A I F Q S S M T N I L Q - F R N Q N P D I V I Y Q
val 18-3-92	- -
VAL LYMPH NODE	- -
VAL LUNG	- -
Position	215 (T)
concensus HX B2	Y M D D L Y V G S D L E I G Q H R T K I E E L R Q H L L R W G L T T P D K K H
VAL 18-3-92	- - - - - - - - - - - - L E - - - - - - - - - - E Y - - K - - F Y - - - - - -
VAL LYMPH NODE	- - - - - - - - - - - - L E - - - - - - - - - - E Y - - K - - F Y - - - - - -
VAL LUNG	- G - - - - - - Y K - - F Y - - - - - -

TABLE 6.4.2B Amino acid sequence of Pol B fragment B fragment for patient 03.

Sample	Sequence
	41 (M), 67 (D), 70 (K)
CONCENSUS HXB2	E M E K E G K I S K I G - E N P Y N T P V F A I K K K D S T K W R K L V D F R E
HX KIDNEY	- I - - - - - - N - - R - - - - - - - - -
HX SPLEEN	- L - - - - - - - - - - - - - - - - - - I - - - - - - N - - R - - - - - - - - -
HX GANGLION	- L - - - W - N - D R - - - - - - - - -
HX LIVER	- L - - - - - - - - - - - - - - - - - - I - - - - - - N - - R - - - - - - - - -
HX LYNPH NODE	- L - - - - - - - - - - - - - - - - - - I - - - - - - N - - R - - - - - - - - -
CONCENSUS HXB2	L N K R T Q D F W E V Q L G I P H P A G L K K K
HX KIDNEY	- - - K - - - - - - - - - - - - - - S - - - R
HX SPLEEN	- - - - - - - - - - - - - - - - - - S - - - -
HX GANGLION	- - - - - - - - - - - - - - - - - - S - - - -
HX LIVER	- - - - - - - - - - - - - - - - - - S - - - -
HX LYMPH NODE	- - - - - - - - - - - - - - - - - - S - - - -

Table 6.4.3A Amino acid sequence of Pol A fragment for patient 05.

Sample	Sequence
concensus HX B2	Y N V L P Q G W N G S P A I F Q S S M T N I L Q - F R N Q N P D I V I Y Q
HX KIDNEY	- -
HX SLEEN	- -
HX GANGLION	- -
HX LIVER	- -
HX LYMPH NODE	- -
	215 (T), 219 (K)
concensus HX B2	Y M D D L Y V G S D L E I G Q H R T K I E E L R Q H L L R W G L T T P D K K H
HX KIDNEY	- - - - - - - - - - - - - Q - - - - - - - - - - E - - - K - - F Y - - - Q - -
HX SPLEEN	- - - - - - - - - - - - - Q - - - - - - - - - - E - - - K - - F Y - - - Q - -
HX GANGLION	- - - - - - - - - - - - - Q - - - - - - - - - - E - - - K - - F Y - - - Q - -
HX LIVER	- - - - - - - - - - - - - Q - - - - - - - - - - E - - - K - - F Y - - - Q - -
HX LYMPH NODE	- - - - - - - - - - - - - Q - - - - - - - - - - E - - - K - - F Y - - - Q - -

Table 6.4.3B. Amino acid sequence of Pol B fragment for patient 05.

		41																									67			70									
CONCENSUS HXB2	E	**M**	E	K	E	G	K	I	S	K	I	G	-	E	N	P	Y	N	T	P	V	F	A	I	K	K	K	**D**	S	T	**K**	W	R	K	L	V	D	F	R	E
SH 23-10-92	-	L	V	M	-	C	-	-	-	Y	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	R	-	-	-	-	-	-	-	-	-
SH 22-12-92	-	L	V	Q	-	W	Q	-	-	Y	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	N	-	-	R	-	-	-	-	-	-	-	-	-
SH BRAIN	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-
SH LIVER	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-
SH C.BLOOD	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	R	-	-	-	-	-	-	-	-	-
SH SPLEEN	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	R	-	-	-	-	-	-	-	-	-

CONCENSUS HXB2	L	N	K	R	T	Q	D	F	W	E	V	Q	L	G	I	P	H	P	A	G	L	K	K	K
SH 23-10-92	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SH 22-12-92	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SH BRAIN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SH LIVER	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SH C.BLOOD	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SH SPLEEN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 6.4.4A Amino acid sequence of Pol A fragment for patient 06.

																	162																				
concensus HX B2	Y	N	V	L	P	Q	G	W	N	G	S	P	A	I	F	Q	**S**	S	M	T	N	I	L	Q	-	F	R	K	Q	N	P	D	I	V	I	Y	Q
SH 23-10-92	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SH 22-12-92	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SH BRAIN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SH LIVER	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SH C.BLOOD	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SH SPLEEN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

																																	215						
concensus HX B2	Y	M	D	D	L	Y	V	G	S	D	L	E	I	G	Q	H	R	T	K	I	E	E	L	R	Q	H	L	L	R	W	G	L	**T**	T	P	D	K	K	H
SH 23-10-92	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	F	F	-	-	-	Q	-	-
SH 22-12-92	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-	K	A	-	-	-	-	-	-	-	-	-	-	-	-	-	F	F	-	-	-	Q	-	-
SH BRAIN	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	K	-	-	-	-	-	-	-	-	-	-	F	-	-	-	-	Q	-	-
SH LIVER	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	F	F	-	-	-	Q	-	-
SH C.BLOOD	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	F							
SH SPLEEN	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	F	Y						

Table 6.4.4B. Amimo acid sequence of Pol B fragment for patient 06

	41																									67		70												
CONCENSUS HXB2	E	**M**	E	K	E	G	K	I	S	K	I	G	-	E	N	P	Y	N	T	P	V	F	A	I	K	K	K	**D**	S	T	**K**	W	R	K	L	V	D	F	R	E
LYMPH NODE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BRAIN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

CONCENSUS HXB2	L	N	K	R	T	Q	D	F	W	E	V	Q	L	G	I	P	H	P	A	G	L	K	K	K															
LYMPH NODE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-																
BRAIN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-																

Table 6.4.5A. Amino acid sequence of Pol A fragment for patient 10.

																			162																						
concensus HX B2	Y	N	V	L	P	Q	G	W	N	G	S	P	A	I	F	Q	S	S	M	T	K	I	L	Q	-	F	R	K	Q	K	P	D	I	V	I	Y	Q		
LYMPH NODE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BRAIN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

																															215									
concensus HX B2	Y	M	D	D	L	Y	V	G	S	D	L	E	I	G	Q	H	R	T	K	I	E	E	L	R	Q	H	L	L	R	W	G	L	**T**	T	P	D	K	K	H
LYMPH NODE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	F	-							
BRAIN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	F	-							

Table 6.4.5B. Amino sequence of Pol B fragment for patient 10.

		41																									67			70										
CONCENSUS HXB2	E	**M**	E	K	E	G	K	I	S	K	I	G	-	E	N	P	Y	N	T	P	V	F	A	I	K	K	K	D	S	T	K	W	R	K	L	V	D	F	R	E
BL 5-4-93	-	-	-	L	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SPLEEN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LIVER	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

CONCENSUS HXB2	L	N	K	R	T	Q	D	F	W	E	V	Q	L	G	I	P	H	P	A	G	L	K	K	K
BL 5-4-93	-	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SPLEEN	-	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LIVER	-	-	-	-	-	-	-	-	R	-	-	P	-	-	-	-	-	-	-	-	-	-	-	-

Table 6.4.6A. Amino acid sequence of Pol A fragment for patient 11

																	162																				
concensus HX B2	Y	N	V	L	P	Q	G	W	N	G	S	P	A	I	F	Q	S	S	M	T	K	I	L	Q	-	F	R	K	Q	K	P	D	I	V	I	Y	Q
SPLEEN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	N	-	-	-
LIVER	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-
BL 5-4-93	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-

																																	215						
concensus HX B2	Y	M	D	D	L	Y	V	G	S	D	L	E	I	G	Q	H	R	T	K	I	E	E	L	R	Q	H	L	L	R	W	G	L	**T**	T	P	D	K	K	H
SPLEEN	-	-	-	-	-	-	-	-	-	-	-	-	-	Q	-	-	-	A	-	A	-	-	-	-	E	-	-	-	-	-	-	F	-	-	-				
LIVER	-	-	-	-	-	-	-	-	-	-	-	-	-	Q	-	-	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	F	-	-	-				
BL 5-4-93	-	-	-	-	-	-	-	-	-	-	-	-	-	Q	-	-	-	A	-	A	-	-	-	-	E	-	-	-	-	-	-	F	-	-	-				

Table 6.4.6B. Amino acid sequence of Pol B fragment for patient 11

	41 67 70
CONCENSUS HXB2	E **M** E K E G K I S K I G - E N P Y N T P V F A I K K K D S T K W R K L V D F R E
BL 20-3-92	- - V - - C - S - - V - - - - - - - - - - - - - H - - N - D R - - - - - - - - -
BL 16-10-92	- L Y - D - - S - Y A - - - - - - - - - - - - - H - - N - D R - - - - - - - - -
LYMPH NODE	- L - - D W - - - - - - - - - - - - - - - - - - H - - - - D R - - - - - - - - -
GANGLION	- L - - D W - - - - - - - - - - - - - - - - - - H - E - - D R - - - - - - - - -
LUNG	- L - - D R - - - - V - - - - - - - - - - - - - H - E E - D R - - - - - - - - -
CONCENSUS HXB2	L N K R T Q D F W E V Q L G I P H P A G L K K K
BL 20-3-92	- -
BL 16-10-92	- -
LYMPH NODE	- -
GANGLION	- -
LUNG	- -

Table 6.4.7A. Amino acid sequence of fragment Pol A for patient 12.

	162
concensus HX B2	Y N V L P Q G W N G S P A I F Q S S M T K I L Q - F R K Q K P D I V I Y Q
BL 20-3-92	- - E - L - - - -
BL 16-10-92	- - - - - - - G - D - - -
LYNPH NODE	- L - - - -
GANGLION	- L - - - -
LUNG	- L - - - -
	215
concensus HX B2	Y M D D L Y V G S D L E I G Q H R T K I E E L R Q H L L R W G L **T** T P D K K H
BL 20-3-92	- - - - - H - - - - - - - E - - - I - - - - - - - - - - - - - F Y - - - Q - -
BL 16-10-92	- - - - - - - - - - - - - E - - - A - - - - - - E - - - - - - F Y - - - Q - -
LYMPH NODE	- - - - - - - - - - - - - E - - - I - - - - - - - - - - - - - F Y - -
GANGLION	- - - - - - - - - - - - - E - - - I - - - - - - - - - - - - - F Y - -
LUNG	- - - - - - - - - - - - - E - - - I - V - - - - - - - - - - - F Y - -

Table 6.4.7B. Amino acid sequence of Pol B fragment for patient 12

6.4 DISCUSSION

The INNO LiPA test for the detection of drug induced mutations in the HIV-1 RT gene was tested on 24 whole blood samples from HIV-1 positive individuals receiving antiviral therapy, and 22 samples of various PM body tissues from patients who died of AIDS and were also undergoing antiretroviral therapy. Results from the LiPA test were compared with direct sequencing of the samples and analysis of the samples by the Point Mutation Assay (PMA) which gives a quantitative measure of the ratio of wild type sequence to a mutated sequence conferring drug resistance. Direct sequencing of PCR products is the most widely used approach to determine the presence of drug resistance mutations in the reverse transcriptase gene of HIV-1. However, this can be laborious and time consuming and can involve the use of radioactive isotopes. Direct sequencing using an automated sequencer with fluorescent primers is a second approach which can give a semi-quantitative measurement of the amount of wild type and drug resistance mutations but involves considerable expense in terms of equipment and reagents.

Specific mutations of the HIV-1 Pol gene confer resistance to nucleoside analogs such as zidovudine and also to the non-nucleoside reverse transcriptase inhibitors (NNRTIs). Drug resistant mutations have been identified by sequencing clinical HIV-1 strains showing *in vitro* resistance, while the impact of specific mutations on drug resistance has been subsequently confirmed by determining the drug susceptibility of molecular HIV-1 clones containing mutations inserted by site-directed mutagenesis. Accumulated mutations at codons 41, 67, 70, 215 and 219 of HIV-1 pol confer progressively increased levels of zidovudine resistance (Larder et al., 1989; Larder, 1994). Mutation at codon 215 induces a 16-fold decrease in zidovudine susceptibility and is the most common mutation associated with resistance to the drug (Boucher et al., 1992).

Virological assays for HIV-1 drug resistance can be broadly divided into culture based and molecular biology based methods. Culture based methods give a direct measure of infectious virus and associated phenotypic drug resistance, whereas molecular biology

based methods are indirect, assaying nucleic acids to determine point mutations associated with drug resistance. Several molecular biology based methods have been applied to the detection of resistant mutations, such as primer specific PCR (Larder et al., 1991), Southern blotting (Richman et al., 1991), PCR-ligase detection reaction (Frenkel et al., 1995), RNase A mismatch (Galendez-Lopez et al., 1991), hybridisation against labelled probes (Eastman et al., 1995), Point Mutation Assay (Kaye et al., 1992) and Line Probe Assay (Stuyver et al., 1997).

This study describes three methods, the Line Probe Assay, the Point Mutation Assay and direct cycle sequencing for the detection of mutations in the RT gene of HIV-1 associated with antiretroviral drug resistance and compares the sensitivity and reliability of each assay. The principle of the Line Probe assay is based on reverse hybridisation of a biotinylated PCR fragment of the relevant part of HIV RT with short immobilised oligonucleotides. The latter hybrid is detected via biotin-streptavidin coupling with a colourimetric system. The LiPA test is essentially qualitative but staining intensity can give a semi-quantitative measure of resistant to wild type genotypes. The equipment needed to perform the assay is basic, a heated water bath fitted with an orbital shaker and the assay can be completed in less than two hours. Line probe assays have been previously used to type Hepatitis C virus (Stuyver et al; 1993) and also to characterise mutations in the *rpoB* gene associated with rifampicin resistance in *Mycobacterium tuberculosis* (Cooksey et al; 1992).

The PMA is a semi-quantitative assay which can measure the ratio of HIV drug resistant genotypes to wild type genotypes and is based on the annealing and extension of specific oligonucleotide probes (Kaye et al; 1992). The specificity of the LiPA test was confirmed by direct cycle sequencing which demonstrated complete concordance between the two tests (Table 6.1). This specificity is similar to the application of LiPA for Hepatitis C virus genotyping, where the assay detected signature motifs in seven variable regions with 100% reliability (Stuyver et al; 1996). A LiPA test for mutations associated with rifampicin-resistant *Mycobacterium tuberculosis* showed 100% concordance with automated sequencing (Cooksey et al; 1997). However, in this study which involved samples containing minor variants, the LiPA test was superior to

sequencing with an ability to detect variants which were between 4 and 10% of the total population. This sensitivity agrees with overall detection limit of the LiPA test of 4% for a minor variant in a mixture containing different sequence variants (Stuyver et al., 1997a,b). DNA sequence analysis detected the major variant in all samples and in some cases two variants when both were of equal quantity.

The Point Mutation Assay possessed a similar sensitivity to the LiPA test with the ability to detect a minor variant present in 10% of the population. However, the specificity of the PMA was not as strong as LiPA compared to direct sequencing, particularly at codons 67 and 70. At codon 215 the PMA, LiPA and direct sequencing showed complete concordance in both specificity and sensitivity. It would appear from this study that when a mutation at codon 215 is present, it comprises almost the entire population, which highlights the importance of maintaining this mutation for complete resistance to zidovudine. A codon change to Tyrosine at position 215 was the predominant amino acid change for resistance, a change to Phenylalanine was found in only 3 of the 29 samples analysed.

Direct sequencing, although less sensitive than LiPA or PMA has the advantage of detecting mutations other than those described previously that may contribute to phenotypic resistance. The strategy for amplification of the Pol A and Pol B fragments from an initial primary amplification of a larger 780bp region of RT resulted in linkage between the Pol A and Pol B sequences. Sequence analysis of both PM and blood samples in this study revealed mutations at positions 60 (V-I), 98 (A-S), 102 (K-R), 162 (S-C), 207 (Q-E) and 211 (R-K) that have previously been shown to be associated with in vitro phenotypic resistance (Sheehy and Desselberger. 1993). The exact contribution of these mutations to the overall resistance profile of the virus has not been elucidated by the use of molecular HIV-1 clones containing the mutations by site directed mutagenesis.

A mutation at position 210 (L-W) was also detected by direct sequencing in samples that contained a mutation at position 215. The role of mutation 210 (L-W) has been investigated by the use of molecular clones with various combinations of RT

mutations at codons 41, 67, 70 and 215 (Hooker et al., 1996). W210 alone did not increase zidovudine resistance, whereas in conjunction with L41 and Y215 the W210 contributed to high level resistance. The sequence analysis of the RT gene also revealed a great similarity between post-mortem tissues of individual patients and whole blood samples preceding death.

CHAPTER 7

Quantitation of HIV-1 Proviral DNA load in peripheral blood. Relationship with immunological parameters, disease progression and zidovudine resistance

7.1 INTRODUCTION

The presence of HIV-1 in infected individuals has been measured in a number of ways. These include; assays for p24 antigen, infectious virus carrying cells, HIV-1 proviral cellular DNA, HIV-1 mRNA expression in peripheral blood cells and HIV-1 RNA in serum or plasma. The levels of infectious virus in plasma or PBMC can be measured using end point dilution culture (Daar et al., 1991; Ho et al., 1989), which is time consuming and may preferentially lead to the isolation of T-cell tropic or SI variants of the virus. A more direct and sensitive marker of viral load is the measurement of HIV-1 nucleic acid using quantitative amplification procedures.

Quantitative cultures of patient PBMC have been used to determine the number of infectious virus carrying cells in the peripheral blood. Several cross-sectional studies have shown that infectious titres of HIV-1 in PBMC are significantly higher in symptomatic patients than in asymptomatic patients (Ho et al., 1989; Lu et al., 1993). Longitudinal analysis of HIV-1 infected individuals has demonstrated increased viral infectivity over time and a temporal association with the emergence of a more cytopathic syncytium inducing (SI) viral phenotype (Connor et al., 1993).

Whether the quantitative detection of HIV-1 DNA in PBMC can be used to determine the number of circulating infected cells is a matter for debate. Proviral DNA measurement can only directly relate to the number of infected cells in circulation if it is assumed that an infected cell contains only one copy of provirus. A study by Simmonds et al (1990), using limiting dilution PCR, demonstrated strong evidence for one copy of provirus per HIV-1 infected cell in the 4 patients examined. However a study by Schnittmann et al (1989), demonstrated a level of up to 20 copies of proviral DNA per infected cell by titrating cells before DNA extraction and PCR, suggesting that a small number of infected cells may contain multiple copies of DNA.

The principle reservoir for HIV-1 in infected individuals is the CD4+ T-cell (Psallidopoulos et al., 1989; Schnittmann et al., 1989). Several cross sectional studies have shown a correlation between proviral DNA load and disease progression (Bagasra et al., 1993; Escaich et al., 1992; Genesca et al., 1990; Hsia et al., 1991;

Schnittmann et al., 1990; Yerly et al., 1992). A longitudinal study has also shown that the level of proviral DNA did not change significantly as disease progressed but an overall higher DNA load was associated with individuals who developed AIDS, compared to those remaining asymptomatic (Gupta et al., 1993).

HIV-1 can exist as a fully integrated provirus in the cell, or as an extrachromosomal unintegrated DNA form. Unintegrated DNA can exist in three forms, in the linear form, a 1-LTR form that circularises by intramolecular recombination, yielding a circular DNA with one LTR and a 2-LTR form that circularises by ligation of the terminii. Asymptomatic individuals have been shown to harbor HIV-1 DNA as a full length unintegrated DNA in quiescent T-cells and integration could be stimulated in these cells upon *in vitro* activation (Bukrinsky et al., 1991). Unintegrated viral DNA is transient in lymphoblastoid cells and does not play a significant role in the productive infection pathway (Pauza et al., 1990b), and high levels of unintegrated DNA can accumulate by reinfection of cells (Robinson and Zinkus, 1990). The 2-LTR circular form has been shown to have a rapid rate of decay *in vitro* and is indicative of recent productive infection of cells (Pauza et al., 1994). Levels of unintegrated DNA have also been used as a marker for efficacy of antiretroviral therapy. Bush et al. (1993) showed a 46% decrease of unintegrated DNA in a group patients who received zidovudine monotherapy. A rapid decrease in unintegrated DNA to 3% of the total cellular HIV-1 DNA has been demonstrated following initiation of nucleoside antiretroviral therapy with zidovudine, didanosine and zalcitabine (Donovan et al., 1994). Similar reductions in unintegrated DNA have also been shown in pediatric patients receiving zidovudine (Bush et al., 1996). A more recent study by Panther et al. (1999) demonstrated a reduction in 2-LTR circle DNA when acutely infected cells were treated with zidovudine or saquinavir, and Sharkey et al. (2000) demonstrated, also *in vitro*, a 90% reduction in 2-LTR DNA load in infected cells treated with the reverse transcriptase inhibitors zidovudine or nevirapine. This study also showed the presence of 2-LTR DNA in CD4 cells of patients receiving HAART, who demonstrated undetectable levels of virion RNA highlighting the persistence of episomal HIV-1 in DNA intermediates and ongoing virus replication. Detection of unintegrated HIV-1 DNA has also been linked with disease stage and immunodeficiency (Nicholson et al., 1996) and the measurement of the number of 1-

LTR DNA circles has been suggested as a marker of the transition from clinical latency to active replication (Jurriaans et al., 1995).

Cross-sectional studies of HIV-1 viral RNA expression in PBMC's of infected individuals have demonstrated a rise in viral RNA expression in late stage HIV disease (Bagnarelli et al., 1994; Gupta et al., 1993; Michael et al., 1992; Saksela et al., 1994). A splicing pattern of HIV-1 mRNA transcripts exists within the infected cell, the gag and gag-pol mRNAs are unspliced, mRNAs for the early genes, tat, rev and nef are doubly spliced and mRNAs for the late genes, vpu, env and vif are singly spliced (Pavlakis et al., 1992). *In vitro* studies have demonstrated that early in infection, multiply spliced transcripts (tat and rev) predominate and following accumulation of Rev protein in the nucleus, unspliced and singly spliced transcripts are exported to the cytoplasm and translated to produce virion structural polyproteins and accessory proteins (Michael et al., 1991). A change in expression from a spliced mRNA pattern to a predominantly unspliced pattern has been shown to be predictive of disease progression (Michael et al., 1992). In a larger study of the splicing patterns of 31 patients, rapid disease progression was associated with a 3-fold reduction in the ratio of spliced to unspliced mRNA (Michael et al., 1995). However there are conflicting data on the dynamic ratio of spliced to unspliced viral RNA throughout disease progression. Saksela et al (1994) demonstrated a lack of multiply spliced RNA in a group of 11 nonprogressors and abundant expression of either multiply spliced or unspliced mRNA appeared to be predictive of active disease progression. Quantitation of unspliced and multiply spliced mRNAs in 6 patients, following treatment with a combination of Ritonavir, AZT and ddC, revealed a dramatic decrease in both cell free virion RNA and unspliced cellular mRNA. Multiply spliced mRNA fell to undetectable levels in all the patients with 2 to 12 weeks of treatment (Bagnarelli et al., 1996).

HIV-1 virion-associated RNA detected in serum or plasma of infected individuals can reflect the total number of viral particles produced by infected PBMC's or the lymphoid organs. Early cross-sectional studies of viral RNA load in plasma showed significant differences in load between asymptomatic and symptomatic patients and a correlation with CD4 T cell decline (Semple et al., 1993; Yerly et al., 1992; Piatak et

al., 1993b). Longitudinal studies have shown little change in RNA load over time during the asymptomatic stage of HIV infection (Bagnarelli et al., 1994; Piatak et al., 1993). Kinetic studies have demonstrated that viral replication occurs in a highly dynamic way with a rapid turnover of plasma virions (Ho et al., 1995; Wei et al., 1995). In addition, levels of plasma HIV-1 RNA early in infection are predictive of the rate of disease progression (Mellors et al., 1996) and HIV-1 RNA levels can also predict the rate of development of clinical disease, independent of the CD4 count (Phillips et al., 1996). Plasma RNA load is now an established progression marker in HIV disease and also a marker for initiation and evaluation of antiviral therapy.

Following the introduction of zidovudine as an antiviral therapy for HIV-1, investigations by Larder et al (1989a) found that HIV isolates from patients with advanced HIV disease became less sensitive to the drug in the course of treatment. Sequence comparisons of the RT gene from resistant virus with those from sensitive virus revealed specific mutations (Larder et al., 1989b). These mutations occur at five locations in the RT gene (codons 41, 67, 70, 215 and 219) and have been shown to cause the resistant phenotype and to accumulate in a stepwise manner with prolonged exposure to zidovudine (Larder et al., 1989a; Kellam et al., 1992; Boucher et al., 1992a).

Several studies have attempted to relate the development of zidovudine resistance and disease progression, either by phenotypic analysis of the resistant virus, or monitoring the presence of resistance mutations. A study of children infected with HIV-1 found a significant correlation between susceptibility to zidovudine and a poor clinical prognosis (Tudor-Williams et al., 1992). A strong association has also been found between the presence of a resistant mutation at codon 215 and a decline in CD4 count (Kozal et al., 1993), patients in this study with a resistant mutation at codon 215 on average had a ninefold higher provirus burden in the PBMC. Clinical studies have also revealed that patients with less severe disease and higher CD4 counts tend to develop resistance more slowly than those with more advanced disease (Land et al., 1992; Richman et al., 1990). However, other results have demonstrated that high level resistance is not necessarily a pre-requisite for progression to AIDS. In a study of 24

initially asymptomatic patients, only partially resistant virus was detected in five individuals who progressed to AIDS (Boucher et al., 1992b).

In this chapter, 79 patients receiving zidovudine monotherapy were recruited to a study to assess the levels of proviral DNA load and resistance mutations at codons 41 and 215 of reverse transcriptase and to determine their relationship with CD4 T-cell numbers and disease progression or death by statistical methods.

7.2 METHODS

Methods for zidovudine resistant mutation quantitation and viral load measurement were performed as described in chapter 2 and chapter 3 respectively.

Comparison of proviral loads between groups was performed using the Mann-Whitney U test. The population was separated according to the median CD4 count, or the median proviral DNA load. Proviral load data was also used to compute the median viral load at each twenty day period post-enrollment. Statistical significance was tested using non-parametric tests such as Mann-Whitney, Wilcoxon or the Kruskall-Wallis test. The population was separated according to the median CD4 count or the median for proviral DNA load.

In the longitudinal analysis individual profiles of viral load were plotted and the data used to compute the median viral load at each twenty day period post enrollment. The gradient of the increase in viral load, or decrease in CD4+ T-cell count was computed by linear regression curve fitting. The prognostic value of proviral DNA loads was analysed using the Cox proportional hazard model, which explores the effects of several variables on survival. The model is used in multifactorial analysis to investigate several variables at the same time, the probability of an endpoint is termed a 'hazard' with the 'hazard ratio' being the relative risk of an endpoint occurring at any given time. Kaplan Meier analysis was performed to establish probability of time to death and also to a 50% decrease in CD4+ T cell count. Kaplan Meier analysis produces a survival curve which is a method calculating the probability of surviving a given length of time by considering time in many small intervals. The proportion of individuals surviving a given length of time is calculated by multiplying the probabilities of surviving each day up to that time, when the exact times of death and censoring are known. In both the Cox proportional hazard analysis and the Kaplan Meier analysis the proviral load was expressed per ml of blood or as genomes per 10^3 CD4 cells.

7.3 RESULTS

7.3.1 Relationship between proviral load and CD4 levels, zidovudine resistance and demographic variables.

Table 7.1 illustrates the characteristics of the 79 patients investigated with respect to baseline proviral DNA load, CD4 cell count and baseline date of recruitment. Analysis of the proviral load in 79 patients separated according to the median CD4 level of the group (160 cells/ul) illustrated that proviral DNA load per ml of blood was comparable between patients above or below the median CD4 level (Figure 7.1). In contrast, expression of the proviral load per 10^3 CD4 cells showed that patients with CD4 levels below the median of the group had a significantly increased proviral load when compared to patients with CD4 levels above 160 cells / ul ($P< 0.0001$, Figure 7.2). Table 7.2 illustrates the correlation between variables and their degree of significance. Table 7.3. shows the comparison between different variables, a weak correlation of borderline significance was seen between proviral load (per ml of blood) and age ($p = 0.054$). No significant difference was found between proviral load (per ml of blood) and gender, risk factors (homosexual or haemophiliac) or death (Table 7.3). There was also no significant correlation between CD4 levels and gender, risk and age. As expected a strong correlation was seen between CD4 levels and AIDS and also death ($p < 0.0001$).

7.3.2 Longitudinal fluctuations in proviral load

In 20 patients of the group, 2 or more consecutive samples were available for analysis of the temporal modulations in proviral load, the median follow-up time for these individuals was 200 days (range 45-480 days). The results shown in Figure 7.3A illustrate that the majority of patients on zidovudine therapy had a static, or gradually increasing load when expressed as genomes / 1ml of blood (Fig 7.3A). Five patients exhibited a different profile resulting in reductions in proviral load which were sustained in some individuals for up to 320 days. Similar analysis using proviral load

expressed as genomes / 10^3 CD4 cells showed that all proviral loads were increasing over time. (Fig 7.3B).

The median changes in proviral load were then used to compute a rate of increase of proviral load over time. The computed change in CD4 level for all patients from baseline showed a significant decrease over time (Fig 7.4A) and the mean rate of change over time for all of the patients examined was 28 cells per 100 days. Computing the change in viral load (per ml of blood) from baseline showed a very slight increase over time with a mean rate of increase of 0.08 $\log_{10}$ genome copies per 100 days (Fig 7.4B). A more significant change in the computed viral load from baseline over time was seen when load was expressed per 10^3 CD4 cells with viral load increasing at a rate of 0.43 $\log_{10}$ genome copies per 100 days (Fig 7.4B).

7.3.3 Analysis of the impact of zidovudine monotherapy and the presence of drug resistance mutations

The median duration of zidovudine use for patients in this study at the first proviral DNA measurement was 24.7 months (IQR 12.0-37.6). No relationship was found between the length of zidovudine use and proviral DNA levels (p= 0.37; Kruskall-Wallis test). The presence of a zidovudine resistance mutation was analysed at codons 41 and 215 using the point mutation assay which gives a quantitative measure of the drug resistant population. 48 individuals had a mutation at codon 41 (median amount of resistant genotypes 14%, range 10-41.5%) and 49 individuals had a mutation at codon 215 (median amount of resistant genotypes 26%, range 1-92%). Table 7.6 illustrates the correlation between CD4 count, proviral DNA (log 10 genomes/ml blood), duration of zidovudine therapy, the presence of a mutation at codon 41 and codon 215. A significant correlation was seen between CD4 count and a mutation at codon 41 ($p = 0.0009$) and codon 215 ($p = 0.0018$). A weak but non-significant correlation was seen between duration of zidovudine exposure and a mutation at codon 41 ($p = 0.085$) and a mutation at codon 215 ($p = 0.061$). In addition a correlation which did not reach statistical significance was seen between proviral load and CD4 cell count but proviral load did not correlate with zidovudine resistance mutations. Table 7.6 illustrates the comparison of % mutation at codon 41 and codon 215 with individual variables. A further significant correlation was also seen between mutation at both codons 41 and 215 with AIDS ($p = 0.018$) and death ($p = 0.023$, $p = 0.026$).

7.3.4 Prognostic value of HIV-1 proviral DNA load as a marker for disease progression

The relationship between proviral load and progression to a CD4 count of below 50% of baseline value and also progression to death was examined by Kaplan Meier analysis. Using death as an end point, patients with a proviral load of > 4.00 log 10 DNA copies (per ml of blood) had a median survival time of 28.9 months, compared to a median survival time of 48 months for patients with a proviral load of < 4.00 log10 DNA ($p = 0.019$, Figure 7.5). Kaplan - Meier analysis of progression to a CD4

count of below 50% baseline value showed that patients with a proviral load of > 4.00 log 10 DNA per ml of blood had a median time of 6.5 months before a 50% reduction in CD4 cell count compared to a median time of 18.9 months for patients with a proviral load of < 4.00 Log 10 DNA.per ml of blood ($p = 0.04$, Figure 7.6).

Three alternative methods of evaluating the prognostic value of proviral DNA load were investigated using Cox proportional hazard models. (A) Long term progression to a CD4 lymphocyte count of less than 50% of baseline values i.e. at measurement of proviral DNA load expressed as genomes / ml of blood. In this analysis 58 events occurred during median follow up of 16.7 months (range 5.6-38 months). (B) Long term progression to death using baseline values of CD4 cells and proviral DNA load (expressed per ml of blood). In this analysis 46 deaths occurred during a median follow up of 34.5 months (5.6 - 38.0 months). (C) Long term progression to death using of proviral load expressed per 10^3 CD4 cells.

Table 7.5 illustrates the Cox proportional hazard values for a long term progression to a 50% decrease in CD4 levels, using both a univariate and a multivariate logistic regression analysis. Proviral load expressed per ml of blood, age or the presence of resistance mutations had no significant relative hazard. The only significant prognostic value for a decline in CD4 level was an AIDS diagnosis in a univariate analysis with a relative hazard of 2.87 (95% CI, 1.63-5.06, $p = 0.0003$). However, in a multivariate analysis this significance is lost, implying that other variables such as proviral load partially accounted for the significance in the univariate model. When analysis was performed for a long term progression to death (Table7.6), proviral DNA load (measured per ml of blood) has a more significant value in both a univariate and a multivariate analysis ($p = 0.07$ and $p = 0.046$ respectively). CD4 count, AIDS diagnosis and age are significant for a progression to death in both univariate and multivariate analysis. The significance of a resistance mutation at codons 41 and 215 is reduced in the multivariate analysis compared to the univariate analysis.

When proviral load values are expressed per 10^3 CD4 cells (Table 7.7), the significance of load as a prognostic factor for progression to death is increased in both univariate and multivariate analysis ($p = 0.0001$ and $p = 0.032$ respectively).

7.4 DISCUSSION

The determination of host viral burden is fundamental to the study of HIV-1 pathogenesis. The accurate measurement of proviral DNA or virion RNA is essential for understanding the natural history of HIV disease, predicting disease progression in individuals and assessing the efficacy of antiretroviral therapies. Viral antigen detection is a poor measure of disease progression (Cao et al., 1987), and quantitative viral cultures, while showing a direct correlation with disease progression (Coombs et al., 1989; Ho et al., 1989), are influenced by the strain-selective nature of *in vitro* culture systems (Meyerhans et al., 1989).

The quantitative detection of HIV-1 DNA in PBMC's has been used as a measure of viral burden (Schnittman et al., 1989). Increased levels of proviral DNA have been shown by several cross-sectional studies to correlate with disease status. Wood et al., (1993), Escaich et al.(1992), Genesca et al.(1990) and Schnittman et al (1989), demonstrated that the CD4+ T-cell was a reservoir for HIV-1 in peripheral blood and a further study showed increased proviral load in symptomatic individuals compared to patients who were asymptomatic (Schnittman et al., 1990). A cross sectional analysis of proviral load in 25 zidovudine untreated and 30 zidovudine treated seropositive patients (Wood et al., 1993) demonstrated proviral DNA levels ranging from 1 copy per 10,000 cells in early disease, to one copy per 10 cells in advanced disease.

Some studies utilising *in situ* RNA hybridisation (Harper et al., 1986) and DNA PCR (Simmonds et al., 1990) have demonstrated very low HIV expression and low proviral load in PBMC of AIDS patients, while other studies have demonstrated higher levels using DNA PCR and *in situ* PCR (Ferre et al., 1992; Poznanski et al., 1991; Bagasra et al., 1993; Patterson et al., 1995). A detailed longitudinal study by Connor et al (1993), has demonstrated an increasing DNA proviral load (expressed as DNA copies per 10^6 CD4 cells) with a decline in CD4 cells. Although varying levels of proviral load have been reported, the general findings suggest that increasing viral burden is associated with clinical and immunological deterioration.

The previous studies of proviral DNA load described have frequently expressed DNA load per unit of CD4 cells. Proviral load can also be measured per ml of blood reflecting the total reservoir of HIV in the periphery. In this chapter an initial analysis of computing the change in proviral load per ml of blood in the group of patients studied showed a gradual increase over time of 0.08 Log_{10} genomes/ 100 days. Expressing proviral load per 10^3 CD4 cells and computing the change in load gave a greater rate of increase of 0.43 Log_{10} genomes/ 100 days, which reflects the mean decline in CD4+ T-cell count in the group of patients of 28 cells per 100 days.

A cross sectional study by Yerly et al (1992), of proviral DNA load measured per 10^6 CD4 cells in 56 patients showed that an increase in proviral load was associated with progression of HIV infection and a decrease in CD4+ cell concentration. High levels of proviral DNA copies were detected in patients who had higher concentrations of genomic RNA per ml of serum, increasing RNA load correlated with decreased CD4 cell count. When levels of proviral load were expressed per ml of blood, little variation in load was seen among categories of patients with various levels of CD4 cells. During the progression of HIV infection of the patients in this study, the mean concentration of proviral DNA load expressed per ml of blood increased by only a factor of three for patients with the lowest concentration of CD4 cells.

In order to ascertain the statistical significance of proviral load (expressed per ml of blood or per 10^3 CD4 cells) as a risk factor for a decline in CD4+ cells and progression to death, the Cox proportional hazard models and Kaplan Meier analysis were used. Such statistical analysis of the measurement of HIV-1 proviral DNA load per ml of blood or per 10^3 CD4 cells has not been reported before and provides an important insight into HIV pathogenesis.

Kaplan Meier analysis of proviral load, separated according to the median proviral load of the group showed that high levels of proviral load were significantly associated with a faster progression to a 50% decrease in baseline CD4 cell count among the patients studied, with a median time of 6.5 months to a 50% reduction in CD4 count ($p = 0.045$, Figure 7.3). Kaplan Meier analysis of progression to death in individuals with a proviral load >10,000 genomes per ml of blood, showed a median time of 28.9

months to death (p = 0.019. Figure 7.4), compared to 48 months for patients with proviral load below 10,000 genomes per ml of blood. This data extends previous studies of viral load showing a correlation of high load with declining CD4 count and disease progression as has previously been described by comparing DNA of asymptomatic with symptomatic individuals.

Cox proportional hazard models were used to the test the significance of HIV-1 proviral load as a risk for progression to a 50% decrease in baseline CD4 count and also as a risk for progression to death. Expressing proviral load per ml of whole blood showed little effect in the model for a decrease in CD4 count using both a univariate and multivariate analysis (Table 7.5). Proviral load expressed per ml of blood was associated with increased risk in the model for long term progression to death with a risk hazard of 1.68 (95% CI 0.96-2.97) in a univariate analysis. When proviral load was assessed in association with other factors in a multivariate analysis, the relative hazard was increased to 2.07 (95%CI 1.01-4.23, p=0.04, Table 7.6). Indicating that proviral load was an independent factor for progression to a 50% decrease in CD4 count

Expressing proviral load per 10^3 CD4 cells in the Cox proportional model showed a relative hazard of 1.06 for progression to death in the univariate analysis (95%CI 1.04-1.08, p = 0.0001), however the significance with this risk was reduced in a multivariate analysis that controlled for drug resistance, AIDS and age. Applying the Cox proportional model, proviral load was again shown to be a significant factor for disease progression. The significance is greatest when proviral load is expressed per 10^3 CD4 cells, reflecting the relationship between DNA load and declining CD4 cell count. In the multivariate analysis (Table 7.7) this significance was reduced when other parameters were included in the model, highlighting that much of the effect is due to HIV disease progression. Measurement of plasma HIV-1 RNA has been shown to be the strongest predictor of clinical prognosis in infected individuals (Mellors et al., 1996) and changes in plasma RNA levels during therapy have been shown to correlate with improvements in clinical prognosis (O'Brien et al., 1996; Katzenstein et al., 1996). The measurement of plasma HIV RNA levels is now the recommended laboratory standard for assessment of HIV infection (Carpenter et al., 1997). While

previous studies have shown an association between high levels of proviral DNA load and decreased CD4 cell count (Schnittman et al,. 1990; Yerly et al., 1992), dramatic changes in proviral DNA load in response to antiretroviral therapy have not been reported for both monotherapy and HAART and the use of proviral DNA load as a predictor of clinical prognosis has not been demonstrated.

Since the introduction of zidovudine as an antiviral therapy for HIV-1 infection, several studies have been conducted to determine its effects and the duration of the benefits achieved. Controlled randomised clinical trials have reported that one short term effect of zidovudine is a reduction in morbidity and mortality in HIV-1 infected individuals (Fischl et al., 1987; Volberding et al., 1990; Cooper et al., 1993). These studies also suggested that early pre-AIDS zidovudine treatment may delay the date of diagnosis of AIDS by nearly 12 months (Volberding et al., 1990; Mulder et al., 1994). However, other randomised studies have shown that zidovudine has little effect (Concorde coordinating committee; 1994).

The reverse transcriptase mutation most commonly associated with zidovudine resistance is a Thr to Tyr or Phe substitution at codon 215 and in many cases a Met to Leu substitution at codon 41 is also present. *In vitro* studies have shown that the mutation at codon 215 confers a 16 fold reduction in zidovudine susceptibility, and when combined with a mutation at codon 41, a 60 fold reduction in zidovudine susceptibility (Kellam et al., 1992). In this chapter genotypic analysis of zidovudine resistance was determined by the point mutation assay, which gives a quantitative measure of mutation at codons 41 and 215, and differs from the nested PCR approach (Larder et al., 1991), which gives a qualitative result. The significance of a mutation at codons 41 or 215 with factors such as proviral DNA load, CD4+ cell count, age, AIDS, death and duration of zidovudine use was investigated. Using the Cox model analysis, no correlation was found between proviral load and presence of either mutation, unlike a study by Kozal et al (1993), which demonstrated a ninefold increase in proviral load in patients with a resistance mutation at codon 215. The presence of zidovudine resistance and an AIDS defining incident in the patients studied was significant as was a low CD4+ cell count. These results illustrate the relationship between zidovudine resistance and declining immune status. A previous study has also

shown a strong association between the presence of mutation at codon 215 and a decline in CD4 count (Kozal et al., 1993) and studies have also shown that patients with less severe disease and higher CD4 counts tend to develop resistance more slowly and those with more advanced HIV-1 disease (Land et al., 1992; Richman et al., 1990).

The clinical significance of the zidovudine resistance mutations and their association with disease progression were examined by a Cox proportional hazard model, with univariate analysis for the mutations alone, or multivariate analysis including proviral DNA load, AIDS defining illnesses and age. There was no significant increased relative hazard associated with mutation at either codons 41 or 215 and long term progression to a 50% reduction in CD4+ cell count. The presence of a mutation at codon 41 conferred an increased risk of progression to death (RH 1.22, 95% CI 1.09 - 1.37, p = 0.0005), however, this was not significant in the multivariate analysis when the other factors were included (Table 7.6). An increased risk of progression to death was associated with resistance mutation at codon 215 in the multivariate analysis (RH 1.15, 95% CI 0.99 - 1.33, p = 0.060) when proviral DNA load was measured as genomes per ml of blood (Table 7.6).

In summary, mutations at reverse transcriptase codons 41 and 215 were associated with an increased risk progression to death in a univariate analysis, but only resistance at codon 215 remained weakly significant in a multivariate analysis. Previous studies using a Cox proportional analysis have shown a similar increased risk for progression to death conferred by the combined presence of mutations at codons 41 and 215, when genotypic resistance was measured (Japour et al., 1995), and when phenotypic resistance was measured (D'Aquila et al., 1995). The relative hazards calculated from the studies of Japour et al (1995) and D'Aquila et al (1995) for a progression to death in a multivariate analysis (5.42 and 2.78 respectiviely) were higher than the relative hazard of 1.15 for each 10% increase resistance at codon 215 illustrated in Table 7.6.

In this chapter the relative hazard of resistance at codon 41 or 215 was analysed, whereas in the previous reported studies, the relative hazard of the two combined mutations was calculated. Also, the Cox proportional model used in this chapter

included proviral load in the multivariate analysis, whereas the other two studies excluded this value, but controlled for syncytium inducing (SI) phenotype of the virus. The analysis of zidovudine resistance and its relation to disease progression presented in this chapter agrees with previous findings of poor clinical outcome and zidovudine resistance in adults (Richman et al., 1990; Boucher et al., 1992b) and in children (Tudour-Williams et al., 1992) and highlights the importance of monitoring drug resistance.

Demographic factors such as age, gender, ethnic origin, exposure category and socioeconomic status may play a role in survival after an AIDS diagnosis. Previous studies have questioned whether age is an important cofactor in HIV infection (Jacobson et al., 1993; Swanson et al., 1994), however most agree that older people are more likely to experience shortened survival following a diagnosis of AIDS (Chang et al., 1993). This may relate to a poorer capacity for lymphocyte production in older persons (Mackall et al., 1995). In this chapter no relationship was found between age and CD4 count, or age and proviral DNA load.

Early epidemiological studies suggested a poorer survival after AIDS diagnosis in women than in men (Rothenberg et al., 1987), however more recent studies have shown no difference in survival between the sexes (Lundgren et al., 1994). In the data presented in this chapter women had a lower median CD4 count of 110 cells per ul (range 10 - 180) compared to men (median 160, range 20 - 300). No significant difference in median proviral load was found between men or women. The majority of patients in this study were homosexual men (n = 51), who had a lower median CD4 count of 160 cells per ul, compared to 205 CD4 cells per ul for haemophiliacs (n=18).

Comparison of survival in different exposure groups is often complicated by factors, such as geographical differences, or the AIDS defining diseases, which may be preferentially diagnosed in some exposure groups. For example Kaposi's sarcoma has been shown to occur more commonly in homosexual men (Beral., 1991), while injecting drug users (IDU) have been reported to experience a faster rate of CD4 lymphocyte decline (Alcabes et al., 1993). It has also been suggested that IDU experience a greater incidence of giant cell encephalitis of the brain compared to

homosexual men who demonstrate increased lymphoma of the brain (Bell et al., 1996).

In summary this chapter presents results of statistical analysis of the significance of proviral DNA load, and the presence of zidovudine resistance with disease progression. The association of proviral DNA load with disease progression may be coupled with increased virulence of HIV strains (Connor et al., 1993), or may also reflect the emergence of HIV-1 variants that can escape immune surveillance (Nowak et al., 1991).

Measurement	Mean	SD	Median	IQR (Inter Quartile Range)
DNA (copies/ml blood)	17550	22052	10000	2500-23000
Log_{10} DNA	3.91	0.60	4.0	3.4 - 4.36
CD4	184	171	160	20 - 290
Baseline Date	28.10.92		4.11.92	13.10.92-8.1.93

Table 7.1. Values at baseline for proviral DNA load and CD4 count for all 79 patients. IQR: interquartile range.

Figure 7.1. Separation of proviral DNA load (expressed per ml of blood) according to median CD4 level of 160 cells per ul. Column 1: CD4 cell count > 160 cells/ul. Column 2: CD4 cell count < 160 cells/ul.

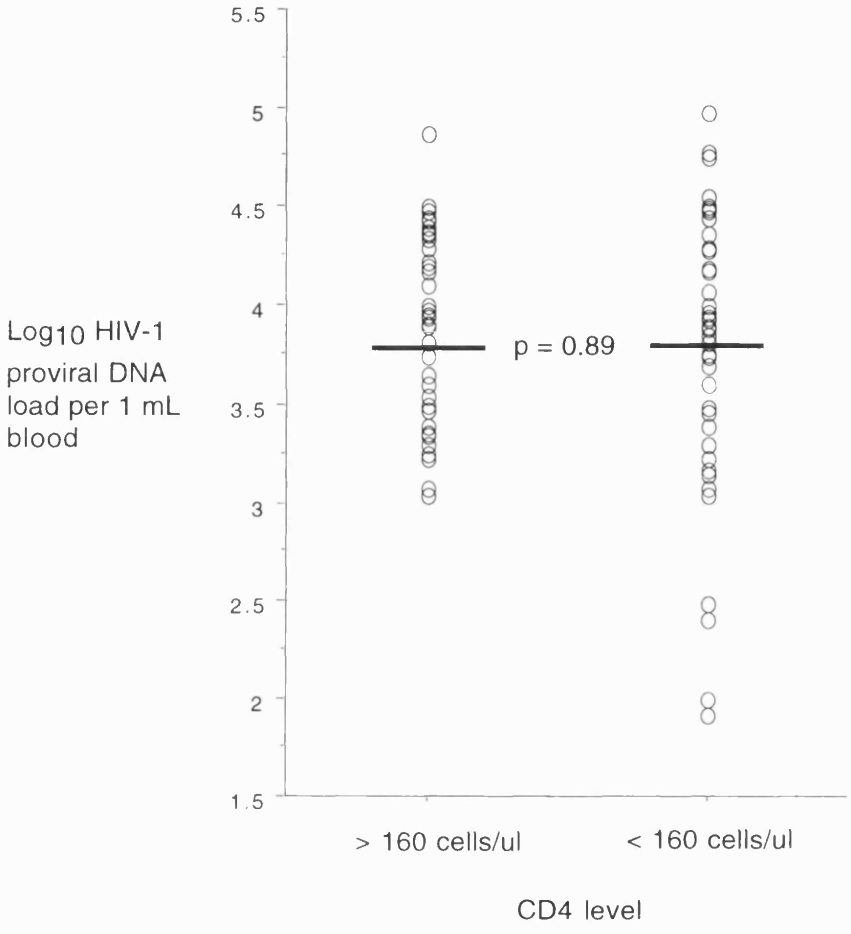

Log10 HIV-1 proviral DNA load per 1 mL blood
5.5
5
4.5
4
3.5
3
2.5
2
1.5
p = 0.89
> 160 cells/ul
< 160 cells/ul
CD4 level

Figure 7.2. Separation of proviral DNA load (expressed per 10^3 CD4 cells) according to median CD4 level of 160 cells / ul. Column 1: CD4 cell count > 169 cells/ul. Column 2: CD4 cell count < 160/ul. ($P<0.0001$).

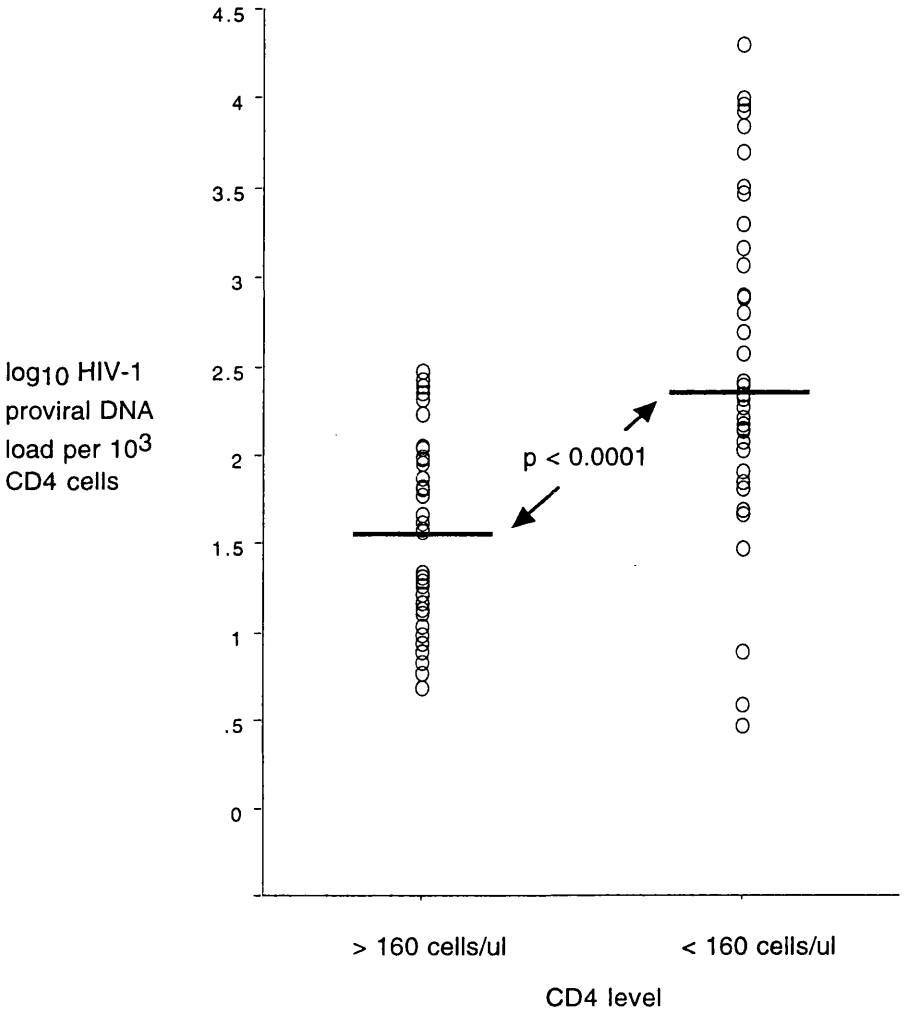
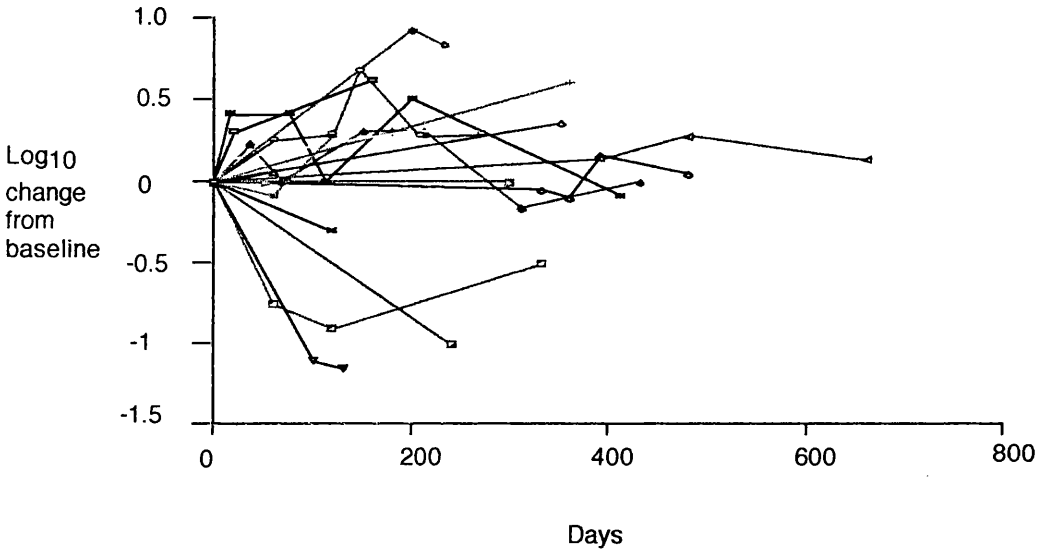

log10 HIV-1 proviral DNA load per 10^3 CD4 cells
4.5
4
3.5
3
2.5
2
1.5
1
.5
0
p < 0.0001
> 160 cells/ul
< 160 cells/ul
CD4 level

Figure 7.3A. Longitudonal fluctuations in proviral DNA load expressed per ml of blood.

Figure 7.3B. Longitudonal fluctuations in proviral DNA load expressed per 10^3 CD4 cells.

A: Log change in HIV DNA load per 1ml blood



B: Log change in HIV DNA load per 10^3 CD4 cells

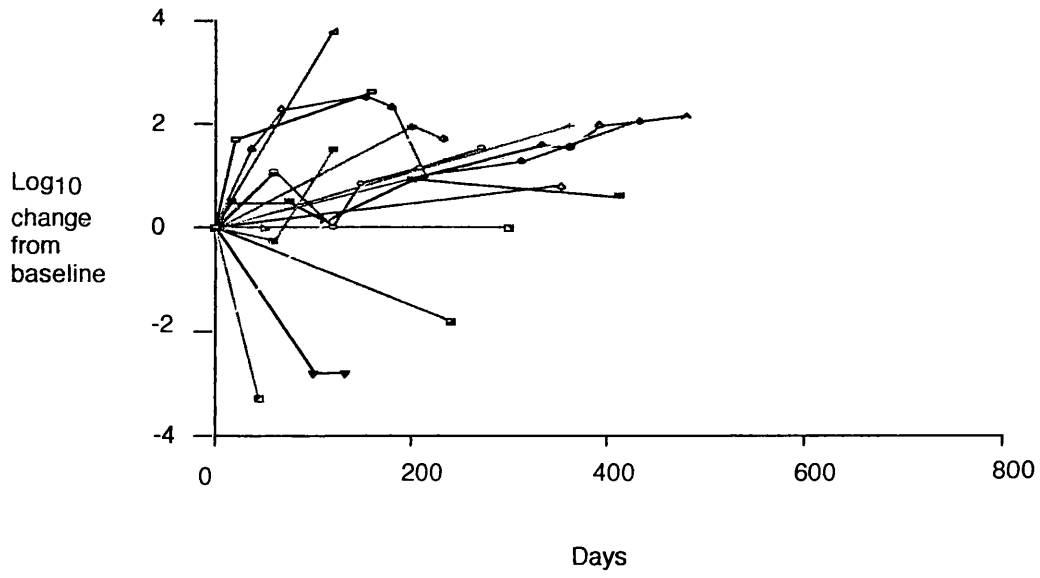
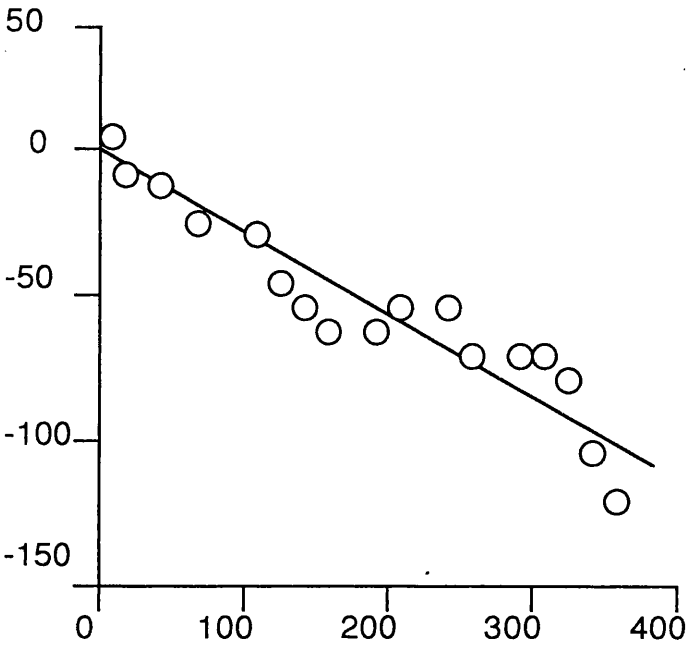


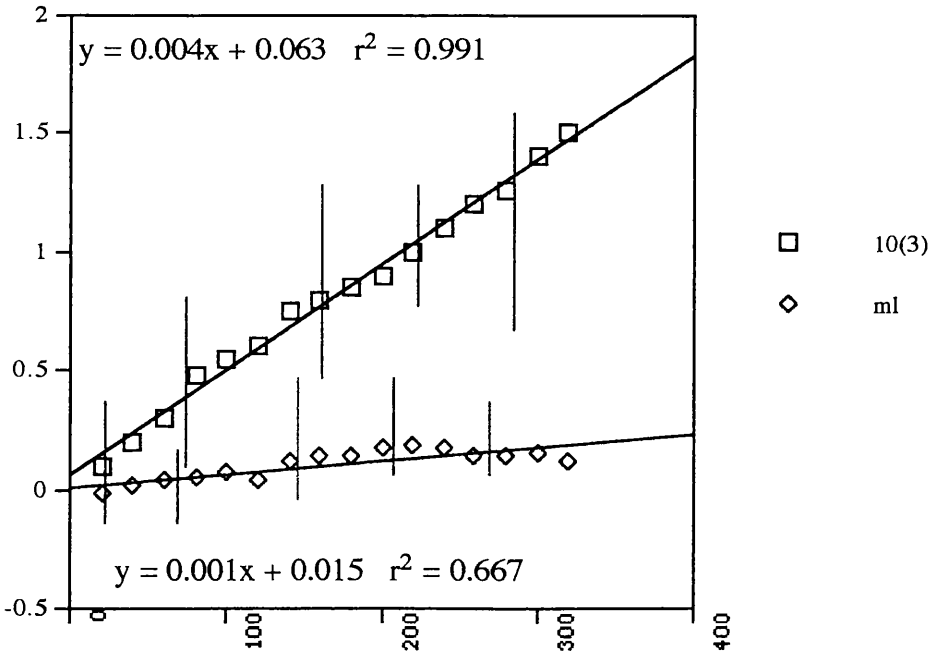
Figure 7.4A. Computed change in CD4 cell level for all patients in group.

Figure 7.4B. Computed change in proviral DNA load expressed per ml of blood and per 10^3 CD4 cells.

CD4 cell decline from baseline (cells/ul)



Log change in viral load from baseline



days

Figure 7.5. Kaplan - Meier analysis of proviral load and Progression to death

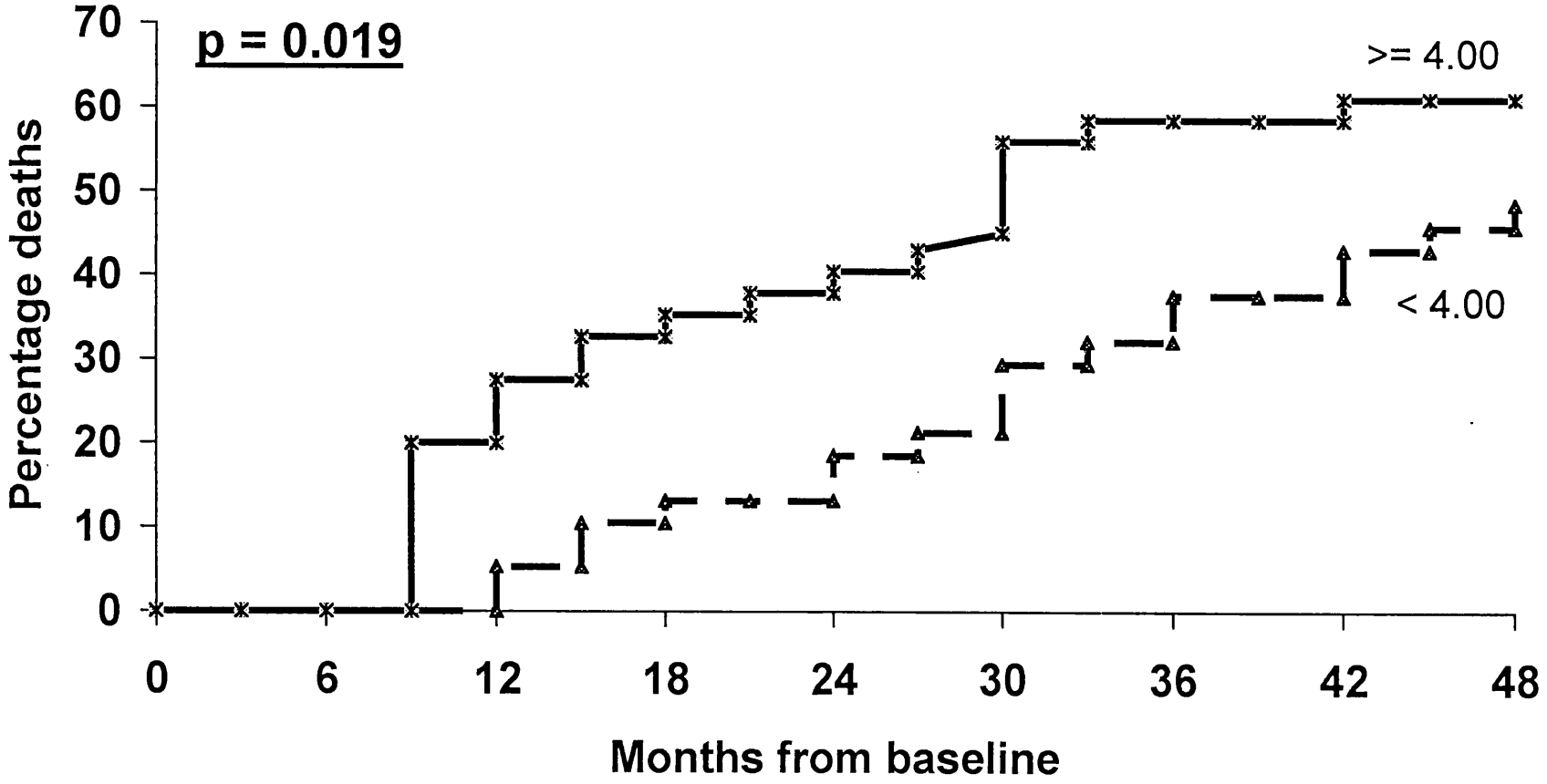
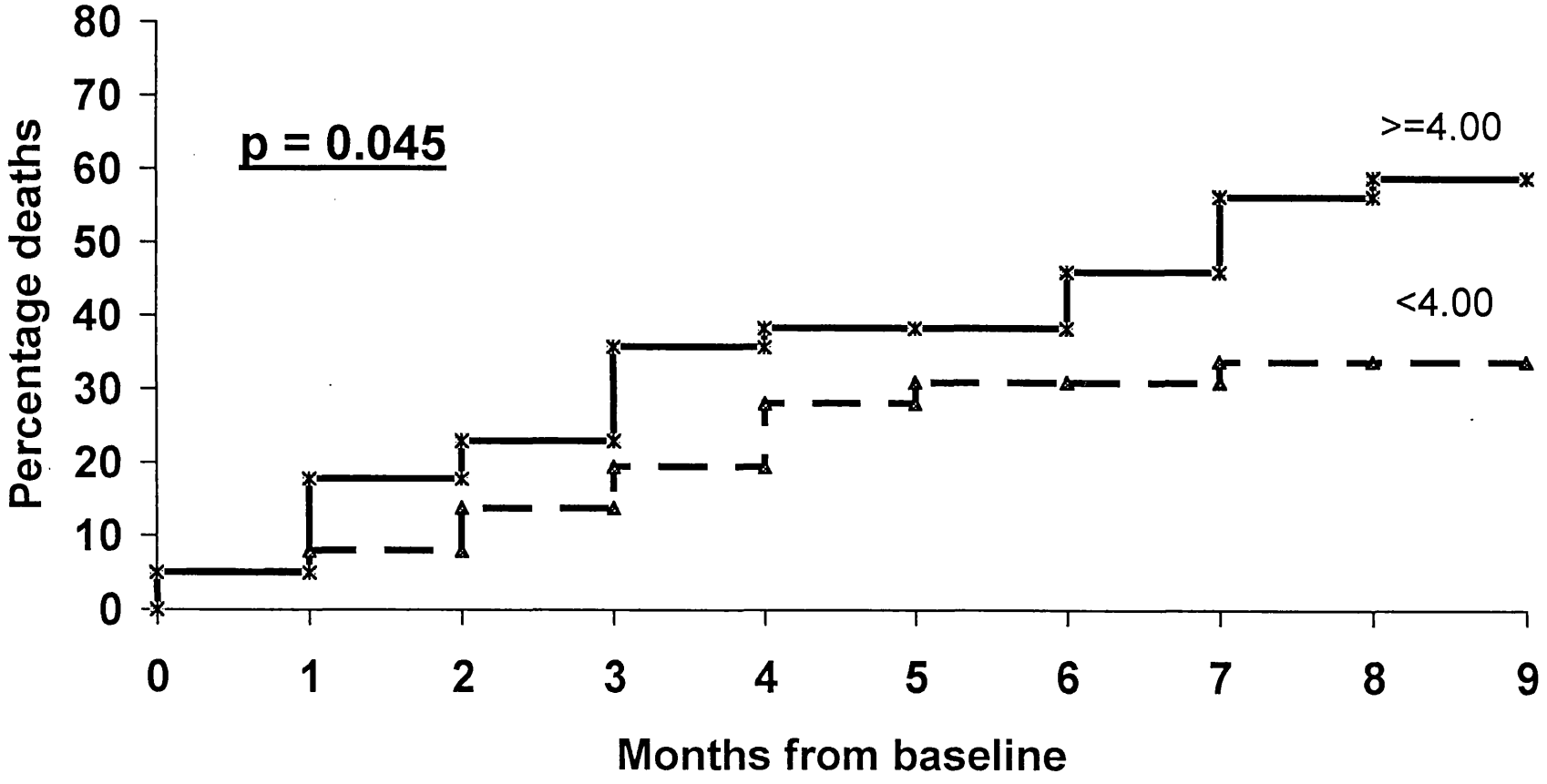


Figure 7.6. Kaplan - Meier analysis of proviral load and Progression to CD4 < 50% baseline



	CD4	Log 10 DNA	ZDV time	41 mut	215 mut
Age	- 0.0473 (0.68)	0.1599 (0.20)	-0.0453 (0.72)	0.0525 (0.72)	-0.1081 (0.46)
CD4		-0.1917 (0.091)	-0.1395 (0.27)	-0.4628 (0.0009)	-0.4346 (0.0018)
Log10DNA			-0.0592 (0.64)	-0.1450 (0.33)	-0.0163 (0.91)
ZDV time				0.2830 (0.085)	0.3065 (0.061)
41 mutation					0.7063 (0.0001)

Table 7.2. Correlation between variables and their degree of significance (shown in brackets) associated with the respective correlation coefficient.

		N	%	CD4 Median	CD4 IQR	CD4 p-value	Log 10 DNA Median	Log 10 DNA IQR	Log 10 DNA p-value
Gender	Male	73	92.4	160	20-300	0.2	4.00	3.40-4.36	0.95
	Female	6	7.6	110	10-180		3.90	3.46-4.36	
Risk	Homosexual	51	64.6	160	20-300	0.34	4.00	3.40-4.38	0.82
	Haemophiliac	18	22.8	205	40-350		3.94	3.40-4.36	
	Other	10	12.7	90	10-180		3.90	3.46-4.68	
Age	< 30.1	26	32.9	155	10-350	0.81	4.00	3.49-4.30	0.054
	30.1 - 36.9	27	34.2	160	40-360		3.91	3.36-4.08	
	> = 37	26	32.9	150	20-230		4.20	3.46-4.49	
CD4	< 60	27	34.2				4.28	3.48-4.58	0.12
	60-234	26	32.9				4.00	3.46-4.18	
	> = 235	26	32.9				3.93	3.40-4.18	
Log 10 DNA	< 3.56	27	34.2	150	50-290	0.044			
	3.56 - 4.19	26	32.9	195	120-420				
	> = 4.2	26	32.9	40	10-210				
AIDS	No	47	59.5	240	160-380	0.0001	3.97	3.46-4.20	0.36
	Yes	32	40.5	20	10-100		4.06	3.40-4.54	
Death	No	33	41.8	260	180-330	0.0001	3.93	3.40-4.18	
	Yes	46	58.2	55	10-180		4.13	3.46-4.46	

Table 7.3. Comparision of proviral DNA load (genomes/ ml blood) and CD4 lymphocyte count according to demographic variables

		Mutation 41 (%)				Mutation 215 (%)			
		N	Median	IQR	p-value	N	Median	IQR	p-value
Proviral load (genomes/ml)	< 3.56	16	14	9.5 - 61.5	0.86	17	47	1 - 98	0.87
	3.56-4.19	17	12	10 - 26		17	17	1 - 51	
	>=4.20	15	14	9 - 43		15	26	1 - 100	
CD4	< 60	17	43	12 - 87	0.039	17	98	26 - 100	0.0027
	60-234	13	12	11 - 33		13	31	2 - 59	
	>= 235	18	11.5	9 - 15		19	1	1 - 47	
Age	< 30.1	17	12	9 - 28	0.48	18	24	13 - 73	0.78
	30.1 - 36.9	18	14.5	11 - 87		18	1	1 - 99	
	> = 37.0	13	24	10 - 33		13	10	2 - 51	
AIDS	No	31	11	9 - 24	0.018	32	11.7	1 - 55	0.018
	Yes	17	33	12 - 89		17	92	17 - 99	
RIP	No	22	11	9 - 15	0.023	23	4	1 - 47	0.026
	Yes	26	27	11- 87		26	53	2 - 99	
ZDV use	< 23.3 mths	18	12.5	9 - 22	0.067	18	35	2 - 60	0.14
	>= 23.3 mths	20	34	11.5 - 78		20	66	8.5 - 99	

Table 7.4. Comparision of the significance of a mutation at codon 41 or codon 215 with variables.(Proviral load is expressed as Log_{10} genomes/ml blood

	Univariate			Multivariate		
	RH	95% CI	p-value	RH	95% CI	p-value
Baseline proviral DNA load	1.47	0.86 - 2.52	0.16	1.55	0.78 - 3.08	0.21
AIDS at baseline	2.87	1.63 - 5.06	0.0003	3.44	1.01 - 11.70	0.48
Age (per 10 yr.)	1.04	0.79 - 1.36	0.78	0.95	0.65 - 1.39	0.78
Mut 41 (per 10%, baseline)	1.10	0.97 - 1.26	0.15	1.10	0.90 - 1.36	0.34
Mut 215 (per 10%, baseline)	1.00	0.91 - 1.10	0.97	0.97	0.85 - 1.12	0.71

Table 7.5. Long term progression to CD4 < 50% baseline using a Cox proportionate hazard model. Proviral DNA expressed per ml of blood.

	Univariate			Multivariate		
	RH	95% CI	p-value	RH	95% CI	p-value
Baseline Proviral DNA load	1.68	0.96 - 2.97	0.070	2.07	1.01 - 4.23	0.046
CD4 level (Log_2)	1.48	1.32 - 1.66	0.0001	1.39	1.11 - 1.75	0.0042
AIDS (baseline)	6.57	3.47 - 12.44	0.0001	12.73	3.60 - 44.99	0.0001
Age (per 10 yr.)	1.30	0.95 - 1.76	0.097	2.30	1.39 - 3.80	0.0011
Mut 41 (per 10%, baseline)	1.22	1.09 - 1.37	0.0005	1.00	0.84 - 1.19	0.97
Mut 215 (per 10%, baseline)	1.13	1.03 - 1.25	0.0087	1.15	0.99 - 1.33	0.060

Table 7.6. Long - term progression to death using a Cox proportional hazard model. Proviral load expressed as Log_{10} genomes per ml of blood.

	Univariate			Multivariate		
	RH	95% CI	p-value	RH	95% CI	p-value
Baseline Proviral DNA load (genomes / 10^3 CD4 cells)	1.06	1.04 - 1.08	0.0001	1.04	1.00 - 1.07	0.032
AIDS (baseline)	6.57	3.47 - 12.44	0.0001	8.26	2.35 - 29.31	0.0011
Age (per 10 yr.)	1.30	0.95 - 1.76	0.0001	1.90	1.12 - 3.22	0.017
Mut 41 (per 10%, baseline)	1.22	1.09 - 1.37	0.0005	1.12	0.88 - 1.41	0.36
Mut 215 (per 10%, baseline)	1.13	1.03 - 1.25	0.0087	1.02	0.83 - 1.24	0.87

Table 7.7. Long term progression to death using Cox proportional hazard model. Proviral load expressed per 10^3 CD4 cells.

CHAPTER EIGHT

GENERAL DISCUSSION.

The natural history of HIV infection and the clinical manifestations of the disease have been studied in detail since AIDS was first recognised in 1981 (Gottlieb et al., 1981). HIV infection induces a chronic and progressive disease process with a broad spectrum of clinical manifestations from an acute primary infection to AIDS, which is often associated with opportunistic infections and malignancies. The course of disease progression is associated with increasing levels of viral replication and the destruction of the immune system, primarily due to the resulting dysfunction and depletion of CD4 cells. Direct destruction of CD4 cells by infection with HIV-1 is considered as the main reason for CD4+ T-cell depletion, which is a central pathological feature of the disease. However, it is uncertain how the virus persists in the presence of a vigorous immune response. Important insights into the complex aspects of host-pathogen interaction have been demonstrated by the measurement of viral load by both viral RNA and cellular proviral DNA. Early studies of quantitation of HIV in plasma and PBMC's by co-culture of isolates revealed that viral titres correlated with clinical status, with a reduction in titre from AIDS to asymptomatic patients (Ho et al., 1989; Coombs et al., 1989).

Non culture based quantitative assays using PCR technology have become established as methods for the quantitation of viral RNA and proviral load, with increasing viral load being closely correlated with disease progression (Jurrians et al., 1992; Piatak et al., 1993). Plasma HIV-RNA levels have been shown to possess a prognostic value in persons with both early and established HIV infection (Henard et al., 1995; Mellors et al., 1996) and also to reflect the risk of peripartum vertical transmission in pregnant women (Sperling et al., 1996; Cao et al., 1997). Large clinical trials of antiretroviral therapies have also demonstrated that a reduction in plasma RNA load associated with therapy correlates with eventual clinical endpoints (O'Brien et al., 1996; Katzenstein et al., 1996). These triple therapy regimens have reduced viral RNA levels to below detectable levels. At the time this thesis was initiated monotherapeutic treatments were only available and techniques to measure viral RNA load were being developed. Subsequent to the success of combination therapy in reducing viral RNA load, it is now important to know the levels of proviral load in infected individuals

To investigate the levels of proviral DNA load in individuals who had received long term zidovudine monotherapy and also to examine the distribution of HIV-1 DNA in various tissue compartments, a quantitative PCR assay was first developed and evaluated. A competitive quantitative PCR assay was developed which contained a sma-1 enzyme restriction site in a 230bp fragment of the HIV-1 gag gene introduced via mutagenesis. The assay was shown to be reproducible and to have a dynamic range of between 50 and 10^5 copies of HIV-1 DNA. This was an acceptable range for quantitation of HIV-1 DNA in whole blood and post-mortem body tissues and was equivalent to other quantitative assays developed using the same technique (Fox et al., 1992; Kidd et al., 1995; Clark et al., 1996; Lock et al., 1997). The accuracy of quantitation was further improved by performing three separate QPCR reactions for the same sample, using the same amount of input DNA, and different amounts of competitive control plasmid (100, 500 and 1000 copies). The specificity of the QPCR assay was also improved by using a semi nested PCR approach, with an inner set of gag primers and restricting the second round PCR to 15 cycles. All QPCR assays for both whole blood and tissue samples were performed using a radioactive primer with subsequent quantitative analysis of an autoradiograph using densitometry. An alternative to the use of a radiolabelled primer was investigated by silver staining of polyacrylamide gels following separation of patient amplified HIV-1 DNA and enzyme digested competitive control sequence. Silver staining produced a strong intense stain of the PCR products that could be readily measured by densitometry after exposure of the gel to EDF film in the presence of white light. Such an approach reduced the need for radioactivity without a reduction in the dynamic range and sensitivity of the assay.

In chapter four, the quantitative PCR was applied to the measurement of proviral DNA load in multiple post-mortem tissue samples from 11 individuals infected with HIV-1. 9 of the 11 patients examined had received zidovudine monotherapy for various periods of time. The highest proviral DNA loads were found consistently in the lymph node tissue samples, similar to previous studies that had demonstrated the lymphoid organs function as major reservoirs for HIV-1 (Pantaleo et al., 1993; Embretson et al., 1993). Application of quantitative *in situ* hybridisation methods for HIV-1 RNA have clarified the size of this lymphoid pool and its cellular subcompartments (Hassse et al.,

1996; Calvert et al., 1997). Infected secondary lymphoid tissues possess HIV in mononuclear cells (CD4+ T-cells and macrophages) and also whole virions adhering passively to the surface of follicular dendritic cells (FDC) within germinal follicles (Fox et al., 1991; Heath et al., 1995). Hasse et al., (1996) estimate that the FDC harbour approximately 100 million virus particles per gram of lymphoid tissue and mononuclear cells contain three million copies of HIV-1 RNA per gram. The high levels of HIV-1 DNA found in all lymphoid tissues examined, although not a measure of productive infection, represent the large reservoir of HIV provirus available for production of viral RNA and virions. No difference in proviral load was seen between the nine zidovudine treated individuals and the two untreated patients, the mean duration of zidovudine therapy was 27 months so any antiviral effect of reducing proviral load would have diminished. A previous study has shown that early zidovudine monotherapy had no effect in reducing HIV-1 DNA or RNA load in lymph node tissue samples (Cohen et al., 1996), however the combination of didanosine (ddI) and zidovudine resulted in a reduction in lymph node RNA but without a decrease in proviral DNA (Cohen et al., 1995). More recent studies investigating the effects of triple combination therapy of nucleoside analogs (zidovudine, didanosine, lamivudine or zalcitibine) and protease inhibitors (saquinavir or ritonavir) have shown reductions in both productively infected cells and extracellular virus, but not in proviral DNA (Cavert et al., 1997; Stellbrink et al., 1997). The latent pool of proviral DNA found in the lymph node represents a long lived reservoir for HIV and plays a role in the rapid increase of viral RNA that is observed following cessation of combination therapy.

Significant levels of HIV-1 proviral DNA were found in 7 of the 11 brain tissue samples (frontal lobe) examined by quantitative PCR, with a range of viral load from 23 copies/ug DNA to 662 copies/ug DNA. Infection of the brain is a significant complication of HIV disease, with AIDS dementia complex (ADC) being the most severe manifestation. An attempt was made to examine the significance of duration of zidovudine therapy and viral load in the brain of the infected individuals. Three of the nine patients receiving zidovudine and one of the untreated controls contained no detectable DNA by PCR in the brain. Patients who had received zidovudine for less than 16 months had a mean proviral DNA load of 100 genomes/ug DNA, whereas patients who had received zidovudine for greater 16 months had a mean DNA load of

approximately 10 genomes/ug DNA. The patients described in chapter 4 did not undergo neurological assessment for dementia, but previous studies have shown an association with zidovudine therapy and a decline in the incidence of HIV dementia (Portegies et al., 1989) and also lower proviral DNA load and reduction of encephalitis (Bell et al., 1996). HIV-1 proviral DNA has been detected in 90% of cerebrospinal fluid (CSF) samples from patients with encephalitis (Schmid et al., 1994) and recently high levels of HIV RNA in CSF have been shown to correlate with the presence of neurological disorders (Di Stefano et al., 1998). These findings taken together with the results of combination therapy reducing HIV-1 RNA to undetectable levels (Gisslen et al., 1997) suggest that antiretroviral therapy significantly reduces neurological disorders during HIV infection and that viral load in the CSF may act as a biological marker of neurological disease.

In the present era of highly active antiretroviral treatment of HIV-1, speculation exists as to the potential of the CNS to act as a reservoir of latent virus. Control of HIV-1 replication in the CNS is evidenced by a reduction in the incidence of HIV-1 associated dementia and improvements in neuropsychological performance following the introduction of HAART (Sacktor et al., 1999). However, given the existence of a latent and replication competent population of HIV infected CD45R0+ (memory) cells in the periphery (Chun et al., 1997), a similar situation may exist in macrophage/microglial cellular environment of the brain. The kinetics of viral suppression may differ in the brain compared to the CSF and plasma. Studies among HAART treated patients have shown differences in the dynamics of CSF and plasma RNA responses. Decreases in plasma and CSF HIV RNA load is similar in some patients but others show a slower decline in the CSF compartment than in plasma (Price and Staprans, 1997). Local populations of the virus may be maintained in different regions of the brain during antiretroviral treatment due to low activity or poor penetration of antivirals across the blood-brain-barrier (Groothuis and Levy, 1997). Protease inhibitors have been shown to have a high degree of protein binding which may reduce levels in the CNS and they also a display a high affinity for the multidrug transporter Pgp, which is present on the endothelial cells of the blood brain barrier (Kim et al., 1998). Affinity for Pgp may reduce a drugs oral bioavailability and its penetration into the CNS.

The sequence of the V3 loop of HIV-1 gp120 was obtained by direct cycle sequencing of a selection of tissues from eight of the patients analysised. The phenotype of the provirus was inferred from the net charge on the V3 loop and similarity to the subtype B consensus V3 sequence. A relationship has been described between macrophage or T-cell tropism or the ability to form syncytia and V3 loop charge and sequence change (de Jong et al., 1992; Fouchier et al., 1992; Chesebro et al., 1992). 15 of the 20 sequences analysed showed a macrophage tropic, non syncytium inducing (NSI) phenotype which is consistent with a previous study of HIV-1 V3 sequence diversity in post-mortem tissue (Donaldson et al., 1994). Five sequences showed a T-cell tropic syncytium inducing phenotype (SI) and four of these five tissues were of lymphoid origin. Post-mortem sequences with a macrophage tropic phenotype may reflect a selection for such variants particularly in non-lymphoid tissues where the beta-chemokine co-receptor CCR-5 predominates. The SI variants found in lymphoid tissue could potentially be dual macrophage and T-cell tropic variants utilising CXCR4, CCR5 or CCR3 co-receptors reflecting the cellular environment.

Chapter 5 describes the distribution of zidovudine resistance mutations at codons 41, 67, 70, 215 and 219 in a selection of the post-mortem tissue samples that were analysed for proviral DNA load in chapter 4. The point mutation assay was used to measure the percentage ratio of wild type sequence to resistance mutation at individual codons. A small quantity of resistant mutation (< 10%) at codon 215 was detected in brain and lung tissues of the two individuals who had received no zidovudine therapy. This may reflect unreported, or illicit use of zidovudine, or may provide evidence of a low frequency of mutant 215 virus in the HIV-1 quasispecies of AIDS patients in the absence of selection by zidovudine. One patient in this study revealed no resistant genotypes at codons 41, 67, 70 or 219 in body tissues after 8 months of zidovudine therapy, suggesting that resistance had not yet developed in this patient, or compliance to the therapy protocol was low. A small quantity of resistant genotype was seen at codon 215 in patient 9 which was equal to that seen in the two untreated patients however the first zidovudine resistance mutation observed in blood is normally a replacement of lysine by an arginine at codon 70 (K70R), followed by a codon 215 mutation.

A study by Wong et al (1997b) showed no resistance mutations in the brain and spleen of an individual who had received zidovudine for less than six months. The pattern of resistance mutations differed between organs of different patients and perhaps more importantly within individual organs of the same patient. Distinct tissue compartments could maintain divergent populations of HIV due to constraints on viral entry and replication, differences in local immune pressure and tissue differences in zidovudine anabolism. Genetic analysis has indicated that HIV-1 within the CNS can evolve independently of the peripheral virus (Hughes et al., 1997) and one study has suggested the concept of anatomically distinct, independently evolving quasispecies (Wong et al., 1997). Patients on long term zidovudine therapy (15 - 47 months) demonstrated approximately 100% mutation at codon 215 and this was linked to a mutation at codon 41. Not all of the patients studied who demonstrated resistance at both codons 41 and 215 showed mutation at codons 67, 70 or 219. This might reflect the redundancy of resistance at these codons once a linked, highly resistant genotype at codons 41 and 215 is achieved. In a study of the fitness of zidovudine resistant HIV-1 variants, the linked 41 and 215 resistant combination rapidly outgrew viruses with single 41 or 215 mutations due, to the higher degree of zidovudine resistance conferred by the combination (Harrigan et al., 1998).

The use of a line probe assay (LiPA) for the detection of drug resistance mutations, described in chapter 6, can provide an alternative means of rapid assessment. The specificity of the LiPA test was confirmed by direct cycle sequencing which demonstrated complete concordance between the two tests in detecting resistance mutations and wild type sequences in the RT gene amplified from 24 whole blood samples and 22 samples of various post-mortem body tissues. The LiPA test is essentially qualitative, but staining intensity can give a semi-quantitative measure of resistant to wild type genotypes. The LiPA test was superior to direct cycle sequencing with the ability to detect minor variants which were between 4 and 10% of the total populations.

Monitoring the appearance of drug resistant variants of HIV-1 during antiretroviral therapy is important for maintaining the effectiveness of the therapy and also provides

information for rational changes in therapy and patient management. At present there are two approaches for detection of drug resistant mutations. Firstly, direct sequencing of PCR amplified reverse transcriptase or protease genes. Secondly, assays which determine whether a wild type or mutant amino acid is present at a certain codon by hybridisation of specific oligonucleotide probes to the PCR product, such as the Line probe assay described in chapter six. There are advantages and disadvantages to both approaches. Direct sequencing offers the advantage of examining the complete sequence of the amplified gene and detecting unknown mutations, particularly important in drug development. However, the difficulty arises in assessing the magnitude of the data and interpreting the contribution of genetic variation and natural polymorphisms. A codon specific based assay is more sensitive than sequencing in detecting minority species. The Line probe assay has been shown to detect mutant populations that are present at a level of 2-5% of a mixed population (Stuyver et al., 1997a). Sequencing methodologies can only detect mutant populations that are present a level of 20-50% of a mixed population (Gunthard et al., 1998). A codon specific assay is simpler to perform and also simpler to interpret, however, the assay will only detect the mutations it is designed to look for. Bulk PCR sequencing or a codon specific can indicate the degree of linkage of mutations, as is the case with resistance to zidovudine. Only clonal analysis can detect the linkage of multiple mutations. The interactive effect of mutations that may lead to reversal or suppression of resistance also complicates the sole use of genotypic analysis of resistance. When lamivudine (3TC) is administered as a monotherapy or in combination with zidovudine, a mutation at codon 184 (M-V) conferring resistance to 3TC emerges. However, if zidovudine and lamivudine are continued after the emergence of 184V, the virus may evolve further, reversing or suppressing zidovudine resistance. In this case a phenotypic analysis of the virus would reveal the correct resistance profile. The original assays to evaluate HIV susceptibility were labor intensive, slow and imprecise, their use is also inappropriate in the context of patient management. A more rapid and reproducible phenotypic assay is now available which involves amplification of a region that includes the protease and reverse transcriptase genes and incorporating this into a recombinant HIV virus which can then be assessed.

Proviral DNA load was examined in 79 individual patients who had received zidovudine monotherapy. The statistical significance of proviral load expressed per ml of blood was examined in relation to CD4+ T-cell count and demographic variables such as gender, age, AIDS or death. High levels of proviral DNA load were associated with lower levels of CD4 counts, but this relationship was not highly statistically significant statistically. In patients from the study group for whom two or more samples were available for quantitative analysis, a computed change in viral load (per ml of blood) from baseline showed a very slight increase over time. However, when viral load was expressed per 10^3 CD4 cells, there was a more significant change over time, which mirrored the change in CD4 levels from baseline over time. This study highlights how the measurement of HIV-1 proviral DNA can be confounded by variation in cell numbers during the course of infection. Expressing proviral DNA load levels per unit of CD4 cells may be appropriate for short term assessment where CD4 levels remain relatively stable, but when CD4 levels fluctuate dramatically, e.g. following combination antiretroviral therapy, it may be inappropriate. Proviral load expressed per ml of blood reflects the absolute concentration of the provirus and may be a better estimate of the HIV reservoir in CD4+ T-cells.

Kaplan-Meier analysis of the relationship between proviral load and progression showed a shorter time to death associated with proviral levels > = 4.00 log 10 DNA ($p = 0.019$). The relationship between proviral load and progression to a 50% decrease in CD4 count was not significant by Cox proportional hazard analysis, however Kaplan - Meier analysis showed that proviral load > = 4.00 log 10 DNA equated to a median time of 6.5 months before a 50% CD4 cell decrease, compared to 18.9 months for proviral load < 4.00 log 10 DNA.

Further analysis of proviral load as a marker of disease progression using Cox proportional hazard models showed a weak but significant relationship between proviral load expressed per ml of blood and progression to death in both a univariate and a multivariate analysis. A highly significant relationship between proviral load and progression to death was seen when load was expressed per 10^3 CD4 cells in a univariate analysis, but was reduced in a multivariate analysis. In general the study described in chapter 7 shows a relationship between increased proviral load levels and

disease progression which has been demonstrated in previous studies (Schnittman et al., 1990; Yerly et al., 1992), where high proviral load levels were associated with symptomatic individuals.

The relationship of zidovudine resistance mutations in the RT gene of HIV-1 to proviral DNA load, immunological status (CD4 T-cell count), and disease progression was also examined in chapter 7. The presence and distribution of zidovudine resistance mutations were detected and quantitated by the point mutation assay (PMA), which has been used in previous studies of viral resistance genotypes (Kaye et al., 1992; Loveday et al., 1996). The molecular basis for HIV-1 resistance to zidovudine has been mapped to codons 41, 67, 70, 215 and 219 and these mutations have been shown to appear in an ordered stepwise manner (Larder et., 1989; Boucher et al., 1992). In Chapter 7 mutations at codons 41 and 215 were analysed by PMA as these linked together have been shown to confer high level resistance *in vitro* (Kellam et al., 1994). The median duration of zidovudine use of the 64 patients studied was 24.7 months and no relationship was found between length of zidovudine use and proviral DNA or the presence of mutation at either codon and proviral load. A significant correlation was found between a resistance mutation and a low CD4+ T-cell count which has been shown in a previous study (Kozal et al., 1993). Resistance mutation was also strongly associated with AIDS and death and in a Cox proportionate hazard model (univariate analysis), resistance was associated with a long-term progression to death.

The development of resistance and its association with disease progression in the group of patients studied could be further complicated by the transition of virus phenotype from NSI to SI which has been considered as a prognostic indicator of disease progression (Koot et al., 1993). The emergence of a highly replicating SI variant could lead to the rapid development of zidovudine resistance mutations and clinical studies have revealed that zidovudine therapy does not prevent conversion to the SI variant (Boucher et al., 1992). Previous studies have given conflicting results as to the relationship between drug resistance and SI variants. One study found a stronger association between SI phenotype and disease progression than between zidovudine resistance and progression (St Clair et al., 1993). Results from the AIDS Clinical trials

Group (ACTG) protocol 116B/117 showed that phenotypic high level resistance of HIV-1 to zidovudine predicted a more rapid clinical progression and death and that resistance was not associated with an SI phenotype (D'Aquila et al., 1995). A further study has also revealed independent evolution of the envelope and polymerase genes during zidovudine therapy demonstrating that the appearance of drug resistance mutations had no effect on sequence diversity in the V3 region (Leigh Brown and Cleland, 1996). More recent work by Van't Wout et al., (1998), has shown that lamivudine (3TC) has similar potency on inhibition of replication of both SI and NSI viruses.

Levels of proviral DNA load do not necessarily reflect active replication of HIV-1 and changes in DNA load are not significant or prolonged following zidovudine monotherapy (Donovan et al., 1991). Results in this thesis have shown that levels of proviral load remain relatively stable over time and higher levels are associated with a decline in CD4 T-cell count and a progression to death. The eventual outcome of long term zidovudine therapy is a high level of resistance to the drug which is also associated with a low CD4 T-cell level (Chapter 7). Recently, the significance of sanctuary sites of HIV-1, which survive the presence of combination therapy, have demonstrated the importance of monitoring proviral load in separated cell populations and in other body tissues (Wong et al., 1997a,b; Finzi et al., 1997). The presence of long lived cell populations with a much longer half life (Perelson et al., 1996), which can in some instances be activated to yield infectious virus have important implications for eventual HIV eradication with long term combination therapy (Ho et al., 1998).

The most fundamental discovery of the natural history of HIV-1 infection has been the elucidation of the replication kinetics of the virus. The rapid rate of virus production of between 10^9 to 10^{10} virions produced daily, the high reverse transcription error rate of between 10^4 to 10^5 per nucleotide per cycle of replication, have significant implications for the generation of genetic diversity of HIV-1. Given the length of the HIV-1 genome of approximately 10^4 nucleotides, it is possible that every single nucleotide point mutation and most double mutations will occur at least once in an infected individual each day (Coffin, 1995). A consequence of increased genetic

variability is the generation of viral variants with mutations in the protease and reverse transcriptase genes that confer resistance to antiretroviral drugs, and such mutations have been shown to exist even in the absence of the selective pressure of the drug. In some drug naïve patients and patients experiencing primary HIV-1 infection, viral isolates with mutations associated with drug resistance have been identified. Recent data has shown a prevalence of drug resistant mutations of up to 11% in a study population of treatment naïve patients (Grant et al., 1999). With the widespread use of HAART and evidence for transmission of drug resistant HIV-1 (Yerly et al., 1999), the presence of such mutations could predispose treatment naïve individuals to a rapid acquisition of high level resistance and subsequent therapeutic failure.

While reduction of HIV-1 plasma RNA to undetectable levels has been achieved with HAART, the ultimate aim of eradication of HIV-1 from an infected individual is still a problem. Recovery of replication competent virus from resting CD4+ memory cells in patients with low plasma RNA levels of HIV-1, reveals a population of cells with a low turnover rate containing latent virus. This latent reservoir remains a major obstacle to virus eradication, with estimates of at least ten years of continued and inhibitory therapy needed for elimination (Ho, 1998). Strategies of elimination of latently infected resting CD4 cells have focussed on the use of immune activating agents such as anti-CD3 antibodies and interleukin 2 (IL-2) to activate resting cells and in combination with HAART to inhibit the spread of virus. The use of anti-CD3 antibodies has proved toxic, but intermittent use of IL-2 results in expansion of the pool of CD4 cells with reduced toxicity. IL-2 in combination with TNF-alpha and interleukin 6 (IL-6) has been shown *in vitro* to induce the activation of virus from latently infected resting CD4 cells and *in vivo* studies have shown a marked reduction in this pool of cells in patients receiving IL-2 and HAART (Chun and Fauci, 1999). However it is still unknown if complete eradication of the virus is possible, as continued successful therapy would be needed for a long period of time and problems with viral rebound can arise with discontinuation of therapy after a long period of time (Harrigan et al., 1999). More recent data has indicated that the genotype of rebounding virus following discontinuation of HAART does not always reflect that of the latent reservoir of resting CD4 cells and that other sanctuary sites or reservoirs of latent virus maybe implicated (Chun et al., 2000).

The studies described in this thesis on the association of viral load and drug resistance with disease progression and also studies of the evolution and distribution of drug resistance mutations from patients on zidovudine monotherapy still has relevance in the present era of combined anti-retroviral therapy. Use of zidovudine is prominent among triple-drug regimens containing a combination of two nucleosides and a protease inhibitor and many patients receiving combination therapy today may have a previous history of zidovudine monotherapy and acquisition of resistance mutations. The use of lamivudine (3TC) with zidovudine in these triple drug regimens actually enhances the effectiveness of zidovudine when the mutation conferring resistance to 3TC appears. The development of side effects such as lipodystrophy and other metabolic disorders due to protease inhibitors may necessitate a switch to a regimen containing zidovudine plus 3TC plus a non-nucleoside reverse transcriptase inhibitor or the carbocyclic guanosine analogue abacavir. Zidovudine monotherapy has proved effective in reducing transmission of HIV-1 during pregnancy and is still a recommended treatment for both mothers and infants. It is also hoped that zidovudine will become more widely available in sub-Saharan African countries to help reduce perinatal HIV transmission.

The distribution of HIV DNA in multiple post-mortem tissues outside the lymphoid system (Chapter 5), and the relatively high levels found in brain, lung, liver and gastrointestinal tissues provide an insight to the tropism and invasiveness of HIV when therapy fails. Recent data on viral RNA rebound to pre-HAART levels in patients who discontinued therapy (Hatano et al., 2000), highlights the need for controlling of HIV-1 replication and adherence to therapy regimens to restrict the ability of the virus to establish productive infection in other body sites.

The persistence of integrated proviral DNA in resting memory CD4 T-cells after combination therapy suggest that other sanctuary sites of HIV besides cells of the periphery may exist and be potential sources of infectious virus. Future requirements for virological analysis and long term management of patients may include assessing proviral DNA load in separated cells of the peripheral blood particularly memory CD4 cells and other body fluids such as semen or CSF.

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