DETECTION AND GENETIC CHARACTERISATION OF HIV-1 VARIANTS INFECTING DIFFERENT SUSBSETS OF $CD4^+$ AND $CD8^+$ T LYMPHOCYTES IN VIVO.

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For my father and mother
with love and thanks.

'It was the best of times, it was the worst of times, it was the age of wisdom, it was the age of foolishness, it was the epoch of belief, it was the epoch of incredulity, it was the season of Light, it was the season of Darkness, it was the spring of hope, it was the season of despair.....'

Charles Dickens, *A Tale of Two Cities.*

DECLARATION

All of the procedures and investigations carried out in this thesis have been performed by the author

The contents of this thesis were composed by the author

Sarah McBreen
Edinburgh, 2001

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ABSTRACT

To investigate the mechanism and functional significance of infection of CD8 lymphocytes by human immunodeficiency virus type 1 (HIV-1) *in vivo*, frequencies of infection and genetic relationships between HIV-1 variants infecting naïve ($CD45RA^+$) and memory ($CD45RO^+$) CD4 and CD8 lymphocytes were determined. CD4 and CD8 lymphocytes were purified from peripheral blood mononuclear cells in 16 study subjects by negative selection, followed by positive selection of each of the two subsets with anti-CD45RA and -RO monoclonal antibody-coated beads. The contribution to total proviral load by the naïve and memory subsets was estimated by quantitiative PCR combined with measurement of their frequency in total T lymphocytes. In this analysis we found that HIV preferentially infects the naive subset of CD8+ lymphocytes along with the naive and memory populations of CD4+ cells.

Sequence comparisons of the V1/V2 and V3 region of the envelope gene were also carried out on the naive and memory subsets of CD4 and CD8 cells. Variants infecting CD8 lymphocytes were partially or completely genetically distinct in the V3 region from those recovered from CD4 lymphocytes, and showed a greater degree of compartmentalisation than observed between naïve and memory subsets of CD4 lymphocytes. A preferential distribution of syncytium-inducing, CXCR4-dependent variants was also observed within CD4 lymphocytes. Even more marked sequence differences were observed between CD4 and CD8 T lymphocytes upon sequence comparison of the V1/V2 hypervariable region, with some evidence for recombination between V1/V2 and V3 regions. Population differences may have

originated through different times of infection rather than necessarily indicating a difference in their biological properties.

Provirus-containing lymphocytes in PBMCs and in lymph nodes are not generally transcriptionally active and not destroyed by HIV infection, either because of infection with a defective virus or because virus expression is inhibited. A series of immunocytochemical techniques was used to determine whether any infected cells in the lymphoid population were in fact productive and to allow comparison with the frequencies detected by PCR. Sequential incubations with monoclonal antibodies and labelling with fast red, fast blue, and diaminobenzidine (DAB) allowed the distribution of three cell surface or internal markers to be determined. Staining of CD4, CD8 and HIV p24 antigen was achieved in cultured cells by this method. In-situ PCR was also used to detect HIV proviral sequences in lymph node and spleen tissue sections.

In this study we provide evidence for the widespread infection of CD4 and CD8 naive and memory cell populations within the peripheral blood of HIV seropositive individuals, indicating that HIV has a broader tropism for cell types *in vivo* than described previously. The preferential distribution of HIV-1 in naïve CD8 lymphocytes suggests that infection occurred early in T lymphocyte ontogeny, such as during maturation in the thymus. Destruction of cells destined to become CD8 lymphocytes may be a major factor in the decline of CD8 lymphocyte frequencies and function on disease progression, and contribute directly to the observed immunodeficiency in AIDS.

ABBREVIATIONS

Ab	Antibody
ABC	Avidin Biotin complex
ADCC	Antibody dependent cell mediated cytotoxicity
ADM	AIDS drug misuser
Ag	Antigen
AIDS	Acquired Immune Deficiency Syndrome
ALV	Avian leukaemia virus
AP	Alkaline Phosphatase
APCs	Antigen Presenting Cells
ARC	AIDS related complex
ARV	AIDS associated retroviruses
ATP	Adenosine tri-phosphate
AZT	Zidovudine
BSA	Bovine serum albumin
CA	Capsid Antigen
CD	Cluster determinant
CDC	Centers for disease control
cDNA	Complement DNA
CDR	Complimentary determining region
CMV	Cytomegalovirus
CNS	Central nervous system
CTLs	Cytotoxic T lymphocytes
CTLp	Cytotoxic T lymphocyte precursor

CTS	Central termination signal
d4T	Stavudine
DAB	Diaminobenzidine
DC	Dendritic cell
ddC	Zalcitabine
ddI	Didanosine
DEPC	Diethyly pyrocarbonate
DMSO	Dimethyl sulphoxide
DNA	Dideoxyribonucleic acid
DNAase	Dideoxyribonuclease
dNTPs	Dinucleotide tri-phosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminotetraacetic acid
EIA	Enzyme immunoassays
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscope
Env	Viral envelope
Env gp	Viral envelope glycoprotein
ER	Endoplasmic reticulum
Fc	Fragment crystalline
FDC	Follicular dendritic cell
FIV	Feline immunodeficiency virus
GC	Germinal centres
GI	Gastrointestinal system
GM-CSF	Granulocyte-macrophage colony stimulating factor

Gp120	External (or surface) envelope glycoprotein
Gp41	Transmembrane envelope glycoprotein
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HIVE	HIV-encephalitis
HLA	Human leukocyte group A
HRP	Horseradish-peroxidase
HTLV	Human T lymphotropic virus
IDU	Intravenous drug use
IF	Immunofluorescence
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL-2	Interleukin 2
IN	Integrase
Kb	Kilobase
KDa	Kilodalton
KS	Kaposi's sarcoma
LAS	Lymphadenopathy syndrome
LAV	Lymphadenopathy associated virus
LTR	Long terminal repeat
MA	Matrix protein
mAB	Monoclonal Antibody
MGCs	Multinucleated giant cells
MHC	Major histocompatibility complex

MIP	Macrophage infammatory protein
MRC	Medical research council
mRNA	messenger RNA
NC	Nucleocapsid protein
Nef	Negative Factor
NK	Natural Killer cell
NSI	Non-syncytium inducing
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCP	*Pneumocystis carinii* Pneumonia
PCR	Polymerase chain reaction
PGL	Persistent generalised lymphadenopathy
PIC	Pre-integration complex
PND	Principal neutralising domain
R	Repeat region
RANTES	Regulated-upon-activation normal T cell expressed
Rev	Regulator of viral gene expression
RNA	Ribonucleic acid
RNA-dep-DNA-pol	RNA dependent DNA polymerase
Rnase	Ribonuclease
RRE	Rev responsive element
RT	Reverse transcriptase
SA	Streptavidin
SDS	Sodium-*n*-lauroylsarcosine
SI	Syncytium inducing

SIV	Simian immunodeficiency virus
SSC	Sodium saline citrate
Taq	Thermus aquaticus
TAR	Tat responsive element
Tat	Transcriptional transactivator
TCR	T cell receptor
TNF	Tumour necrosis factor
tRNA	Transfer RNA
TSA	Tyramide signal amplification
UN-AIDS	Joint United Nations Programme on HIV/AIDS
UPGMA	Unweighted pair group method with arimetic mean
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U
Vpx	Viral protein X
WHO	World Health Organisation

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CHAPTER ONE: INTRODUCTION

1.1 BACKGROUND

Human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) are human lentiviruses belonging to the Retroviridae family. Infection with HIV-1, HIV-2 or in some cases co-infection with both viruses is now widely accepted to be causally associated with the subsequent progression to a state of severe immunodeficiency known as the acquired immune deficiency syndrome (AIDS). Type 1 HIV is recognised as the agent of the global AIDS pandemic, with HIV-2 mostly confined to the countries of West Africa (DeCock *et al.*, 1993).

The first recognised cases of AIDS occurred in the United States in 1981 when outbreaks of previously rare diseases, such as *Pneumocystis carinii* pneumonia (PCP) and Kaposi's sarcoma, were observed in a few homosexual men. Similar outbreaks of other immunodeficiency associated conditions such as mucosal candidiasis, disseminsated cytomegalovirus infection and chronic herpes simplex virus ulcers were described a short time later. All patients had evidence of a severe T-lymphotropic dysfunction manifested by hyperresponsiveness to mitogens and antigens. This outbreak was not limited to populations within the United States. By the end of 1982 reports of this immunodeficiency syndrome expanded to include Haitian immigrants, haemophiliacs, transfusion recipients, and sex partners of risk-group members and children born to mothers at risk. Epidemiological studies suggested that an infectious agent was responsible for this newly described immunodeficiency syndrome. In 1983 Montagnier and colleagues first isolated a retrovirus from the lymph nodes of a patient in Paris with multiple lymphadenopathy. This virus was designated lymphadenopathy-associated-virus (LAV). LAV was

shown to replicate and cause cytopathology in cultures of human peripheral blood lymphocytes (Barre-Sinoussi *et al.*, 1983). The following year, scientists at the National Cancer Institute in Bethesda confirmed the isolation of a cytopathic T-lymphotropic retrovirus, designated human T-lymphotropic virus III (HTLV-III) from peripheral blood mononuclear cells (PBMCs) in patients with acquired immunodeficiency syndrome (AIDS) (Gallo *et al.*, 1984). At the same time Levy and colleagues cultured an AIDS-associated retrovirus (ARV) from PBMCs of an AIDS patient (Levy *et al.*, 1985). Molecular cloning and sequence analysis revealed that the genomes of LAV and HTLV-III were almost identical. Electron microscopy demonstrated that these viruses from AIDS patients were morphologically similar to members of the lentivirus genus of the family *Retroviridiae*. As there was a certain degree of confusion regarding the nomenclature of this virus implicated in AIDS, it was renamed human immunodeficiency virus type 1 (HIV-1). In 1985, a lentivirus was isolated from captive Asian macaques with an AIDS-like illness (Daniel *et al.*, 1985). Because of morphologic similarity and serologic cross-reactivity with HTLV-III, this macaque virus was designated simian T-lymphotropic virus type III (STLV-III). It was later re-designated simian immunodeficiency virus (SIV).

In 1986, Clavel, Montagier and colleagues discovered a second HIV (HIV-2) in West Africa (Clavel *et al.*, 1986). Although both HIV-1 and HIV-2 can cause AIDS, individuals infected with HIV-2 exhibit a longer period of clinical latency and lower morbidity. At present the origins of HIV-1 and HIV-2 infection in man remains unclear although it is possible that both of these viruses arose from zoonotic transmissions from nonhuman primates (for more detailed discussion, see section 1.6).

1.2 CLASSIFICATION OF RETROVIRUSES

The Retroviridae comprise a large family of viruses, primarily infecting vertebrates, although they are also found in other animals such as insects and molluscs. All retrovirus isolates are quite similar in virion structure, genome organisation and mode of replication. They belong to seven distinct genera (Table 1.1) (Luciw *et al.*, 1996). The genome of retroviruses consists of two, usually identical, molecules of single-stranded RNA, and the enzyme reverse transcriptase which converts genetic information from single-stranded RNA to double-stranded DNA (Figure 1.1). Retroviruses also contain another enzyme, integrase, which is necessary for covalently joining virus to cell DNA to form the provirus.

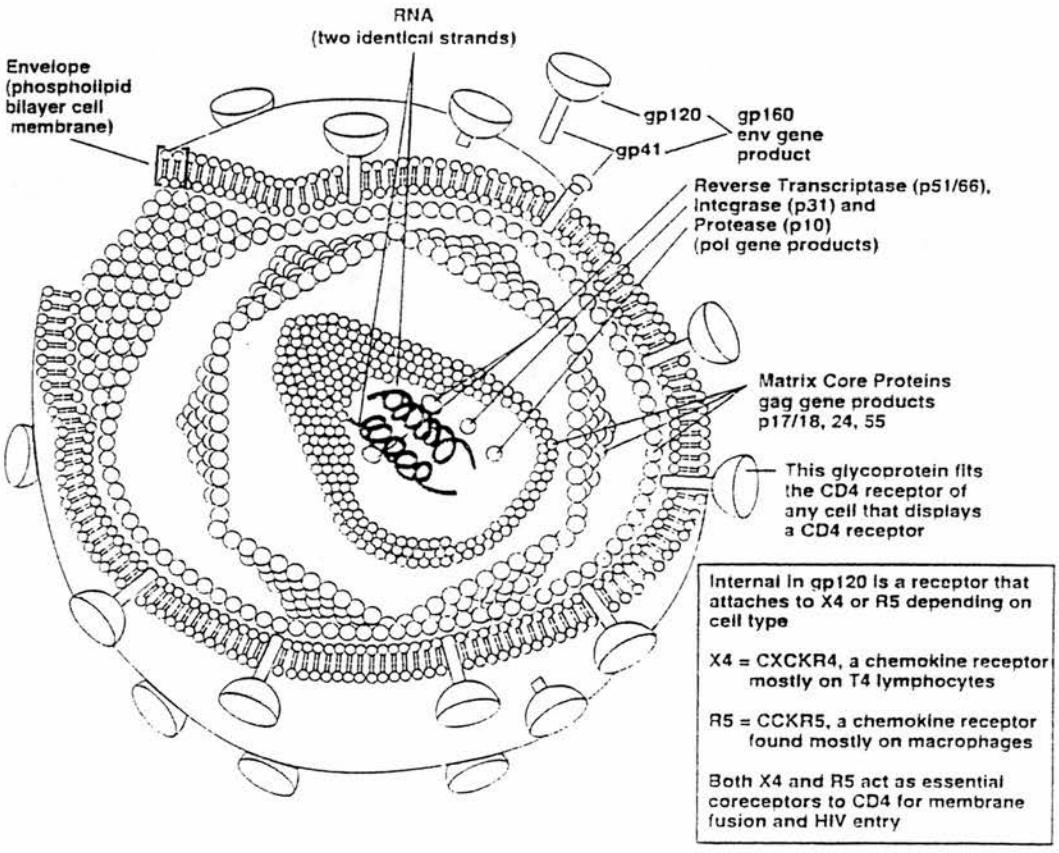


Figure 1.1 Simplified Structure of HIV-1.
(Modified from Fields Virology, 1996)

Table 1.1 Retrovirus Genera

Genus	Example Isolates
Avian-leukosis-sarcoma	Rous sarcoma virus (RSV) Avian myleoblastosis virus (AMV) Avian erythroblastosis (AEV) Avian myelocytomatosis (MC) Rous-associated virus (RAV)
Mammalian C-type	Moloney murine leukemia virus (Mo-MLV) Harvey murine sarcoma virus (Ha-MSV) Abelson murine leukemia virus (A-MuLV) AKR-MuLV Feline leukemia virus (FeLV) Simian sarcoma virus (SSV)s Reticuloendotheliosis virus (REV)
B-Type viruses	Mouse mammary tumor virus (MMTV)
D-Type viruses	Mason-Pfizer monkey virus (MPMV)
HTLV-BLV group	Human T-cell leukemia (or lymphotropic) virus (HTLV)-1 and –2 Bovine leukemia virus (BIV)
Lentivirus	Human immunodeficiency virus (HIV)-1 and -2 Simian immunodeficiency virus (SIV) Feline immunodeficiency virus (FIV) Visna/maedi virus Bovine immunodeficiency virus (BIV)
Spumavirus	Simian foamy virus (SFV) Human foamy virus (HFV) Feline syncytium-forminsg virus (FeSV)

HIV-1, HIV-2 and SIV belong to the genus lentiviruses. This genus includes complex exogenous viruses responsible for a variety of neurological and immunological diseases, but not directly implicated in malignancies. HIV and SIV, like the other retroviruses have two genomic forms: single-stranded RNA in the extracellular phase of the viral life cycle (i.e. virions) and double-stranded DNA (i.e. provirus) within the cell. The replication cycle initiates with entry of the virion core

into the cytoplasm, synthesis of double-stranded DNA using the single-stranded genome as template, transfer of the core structure to the nucleus, and integration of the DNA into the host genome. These steps are mediated by proteins found within the virion and proceed in the absence of viral gene expression. Next, mRNAs, viral genomes and proteins are synthesised and processed using host T-cell systems including RNA polymerase and specific viral gene products. Virion assembly proceeds by encapsidation of the genome by unprocessed precursors of the gag, pro and pol genes, association of the nucleocapsids with the cell membrane, release of the virion by budding and finally processing of the precursors to the finished products.

1.3 VIRION STRUCTURE

HIV-1 is an enveloped virion 100-110nm in diameter and spherical in shape. A lipid bilayer envelope surrounds a cone-shaped nucleocapsid, which is connected at the narrow end to the lipid bilayer. The region between the viral envelope and nucleocapsid has been termed the paranucleoid region, core shell, or lateral body (figure 1.1). Each mature virion is composed of two identical RNA strands encapsulated by the proteolytic proteins processed from the *gag* precursor polypeptide. The *gag* gene products are the matrix protein (MA), which is located between the nucleocapsid and the virion envelope (membrane); the main capsid protein (CA) , which forms the capsid shell, and the nucleocapsid protein (NC), which binds tightly to the RNA genome. In each virion the 5' ends of the two molecules of genomic single-stranded viral RNA interact with each other through hydrogen bonds between molecules aligned in the same polarity or through short antiparallel arrangements.

A transfer RNA molecule is positioned near the 5' end of each genomic RNA strand and serves as the primer for initiation of negative strand viral DNA synthesis by reverse transcriptase (RT). The *gag* gene encodes the precursor for several virion capsid proteins, the *pol* gene encodes the precursor for several virion enzymes [protease (PR), RT, RNase H, and integrase (IN)], and the *env* gene encodes for the envelope glycoprotein (env gp). The transcriptional transactivator (*tat*) and the regulator of viral expression (*rev*) genes are encoded by two overlapping exons and produce small nonvirion proteins, which are essential for viral replication. These include viral protein R (*vpr*), viral infectivity factor (*vif*), *vpu, vpx* and *tev*. The viral surface of HIV-1 is made up of 72 knob-like projections containing trimers or tetramers derived from the envelope glycoproteins (gp160). Proteolytic cleavage of gp160 produces gp120, which is the glycoprotein responsible for interactions of virions with the cellular receptors, and gp41, the transmembrane (TM) glycoprotein that anchors the complex gp120/gp41 to the membrane (reviewed in Levy *et al.*, 1993., Luciw, 1996 and references within)..

1.3.1 Genomic organisation of HIV-1

With their complement of 10 genes, HIV and related lentiviruses are genetically much more complicated than murine and avian C-type retroviruses. Every HIV particle has two identical positive strands of RNA, each of which is approximately 9.2 kb long. In the early steps of replication, the virus RNA is converted into double-stranded DNA, which is then integrated into the host genome. The reverse transcription and synthesis of a double-stranded DNA results in the formation of two identical long terminsal repeats (LTRs) flanking the main viral

genes. HIV and SIV have therefore two genetic forms, single-stranded RNA in virions and double-stranded DNA (provirus) within the cell. The provirus behaves as a cellular gene, with the promoter and transcriptional start site located in the 5' LTR, and a terminsation/polyadenylation site located in the 3' LTR. Viral replication depends on the activation of the LTR and on the expression from the 9.2 kb provirus, of the nine different open reading frames (ORFs) (Figure 1.2). Of these, three encode structural proteins, and six encode regulatory and accessory proteins. The three structural genes consist of *gag* (group antigen), *pol* (polymerase) and *env* (envelope) and are essential for virus replication. The three regulatory genes consist of *rev* (regulator of viral gene expression), *tat* (transcriptional transactivator), and *nef* (negative regulatory factor). In addition, HIV also encodes five accessory/auxillary proteins believed to be involved in virus maturation and release, virion infectivity factor (vif), viral protein U(vpu), viral protein R (Vpr), and vpx and tev as accessory genes. In spite of the difference in regulatory capacity, the basic life cycle of HIV is similar to that of avian and murine retroviruses.

In order to facilitate the synthesis of 11 distinct proteins from a genome of less than 10 kb, HIV displays great economy in its coding potential, employing a complex array of differential RNA splicing and overlapping translational reading frames. In T-cells or macrophages which support replication of HIV-1, three sizes of viral mRNAs are produced: unspliced (9.0kb), single-spliced (4.5kb) and multiply spliced, (1.8-2.2kb). The unspliced 9kb transcripts which represent the genomic mRNA, are homogenous in size and serve two purposes: they are the viral RNA which is packaged into new virions; and they translate the structural gag proteins and the enzymatic pol polyprotein, comprising reverse transcriptase, protease and integrase.

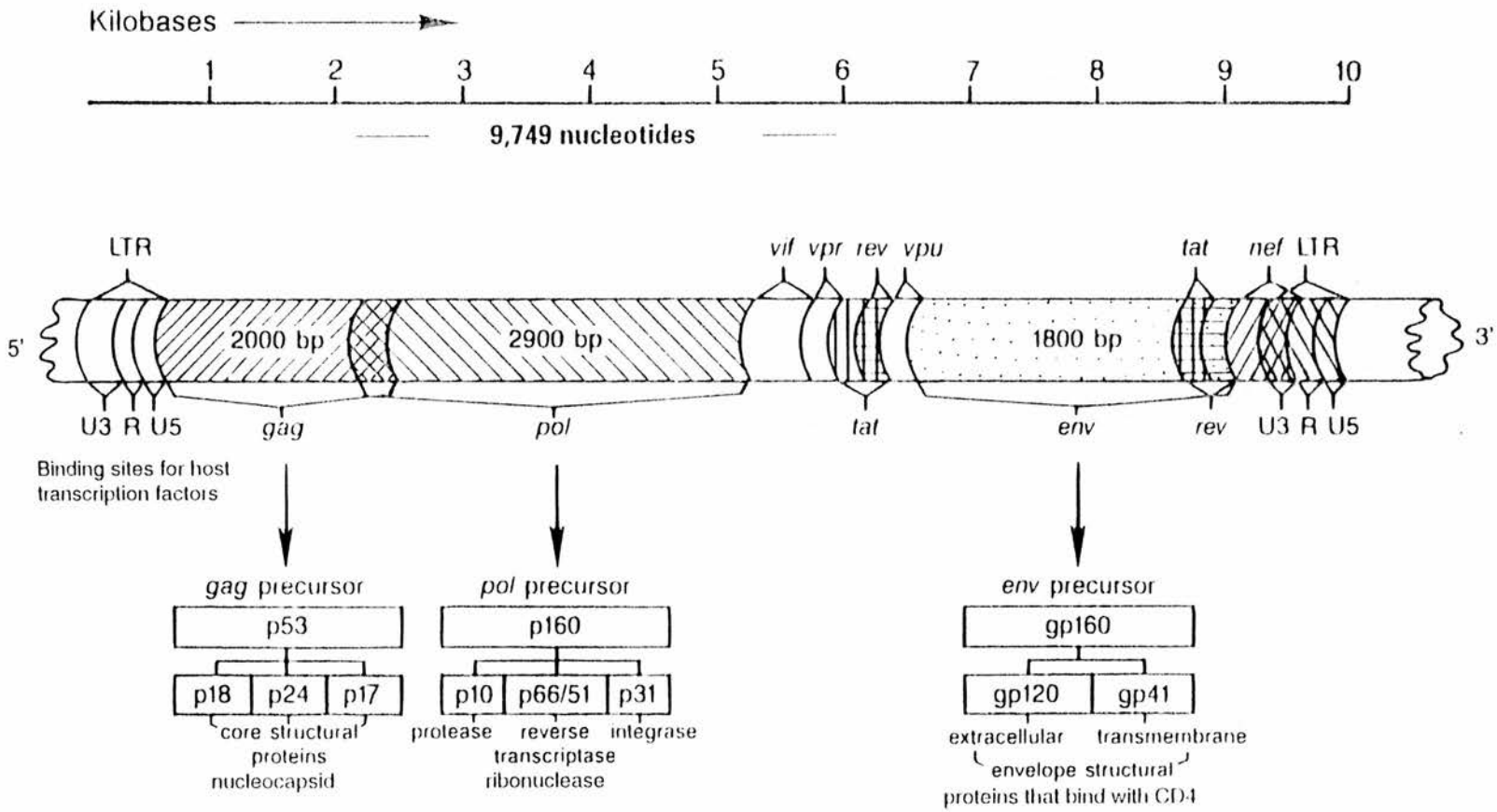


Figure 1.2 Genome of HIV-1 (Modified from Matthews et al, 1994).

The 4kb transcripts are singly spliced, heterogeneous in their size and code for *env*, *vif*, *vpr*, and *vpu* genes and the first exon of *tat*. The 2kb transcripts are multiply spliced and code for *tat*, *rev*, *nef* and *tev* genes. The appearance of the 2kb species precedes that of the 4kb and 9kb species: they are referred to as early and late transcripts respectively. Early transcripts such as the *tat* and *rev* genes control late events in viral replication. Mutations affecting *tat* or *rev* genes can abrogate viral replication as these proteins control the expression and localisation of viral transcripts.

1.3.2 Structural genes of HIV-1

The *gag* gene codes for the main non-glycosylated structural components of the virus particle. They are initially made as a 55 kilodalton precursor, which is translated from full length mRNA. Subsequent cleavage of the *gag* gene polyprotein (p55) by the viral protease gives rise to the more mature capsid proteins of HIV; the matrix protein (MA, p17), the capsid protein (CA, p24), p2, nucleocapsid (NC, p7), the nucleic acid binding protein (p9) and the proline-rich protein (p6).

The matrix protein (p17) contains about 130 aminso acids, has a molecular weight of 17 to 18 kDa and is located between the virion capsid and envelope. MA is cleaved from the N-terminsus of p55 by viral PR. MA is part of the viral nucleoprotein complex (i.e. preintegration complex), produced after reverse transcription and it can mediate nuclear import of this complex. Deletions in MA do not affect the processing of gag polyprotein, virion assembly or release; however these deletions can impair the incorporation of env gp into virions resulting in the production of non-infectious virus particles.

The capsid (p24) protein contains about 240 aminso acids, has a molecular weight of 24 to 27kDa, a high degree of hydrophobicity and is the major subunit of the capsid shell. The capsid protein is released from the central portion of the gag polyprotein by two cleavages mediated by the viral PR. Specific interactions of CA with the viral RNA genome, other gag proteins as well as posttranslational modifications are presumed to play important roles in CA function during capsid assembly. Small deletions in the C-terminsal half of CA reduce viral particle formation. Mutagenesis studies have shown that the domain governing nucleocapsid assembly is located in the C-terminsal portion of CA.

Protease-mediated cleavage from sequences in the C-terminsal portion of the Gag polyprotein produces the nucleocapsid (NC) region. NC contains approximately 70 aminso acids and has a molecular weight of 7 to 9kDa. This basic hydrophilic protein binds genomic viral RNA in the nucleocapsid and may function to condense the viral RNA genome for packaging into capsids during virion morphogenesis. NC has a globular central domain consisting of the two cysteine-histidine motifs and flanking basic residues. The first cysteine-histidine motif is essential for packaging and shows that binding of zinc ions induces conformational changes in the NC, while the second motif is dispensable. Because NC is an intrinsic component of the nucleocapsid, this protein is thought to play a role in virion uncoating during entry and may influence the reverse transcription process that takes place in a nucleoprotein complex containing viral RNA as well as capsid proteins.

Cleavage of the gag-pol polyprotein p160 by the viral protease produces the viral enzymes p10 (PR), p66/p51 (RT) (and RNase H), and p32 (IN). In HIV the *gag* and *pol* genes overlap by 241 nucleotides and the *pol* gene is in a –1 reading frame

with respect to *gag*. Expression of the *pol* gene is facilitated by a ribosomal frameshift which occurs at a low frequency and is directed by a short homopolymeric sequence located in the overlap between the *gag* and *pol* open reading frames that allows a 'slip back' of the ribosome into a –1 position. This results in the production of large quantities of the *gag* gene products and relatively small quantities of the *pol* gene products.

The mature form of PR is 99 aminso acids in length and displays a molecular weight of 10kDa. The gag-pol polyprotein dimerizes in the infected cell, and the mature PR is releases by an autocatalytic cleavage. The fully active PR then targets other sites in the viral polyproteins; altogether four and seven cleavage sites are recognised in the gag and gag-pol polyproteins. Site-specific mutagenesis demonstrated that PR is required for replication. Also, this enzyme produces non-infectious particles containing uncleaved gag and gag-pol polyproteins.

PR produces reverse transcriptase and RNase H in two steps from the gag-pol polyprotein during virus assembly. Firstly, p66 is cleaved and forms a homodimer. Next, one unit of p66 in this homodimer is cleaved by PR near the C-terminsus to yield a heterodimer composed of p51 and p66. Both the heterodimer and p66 homodimer display RT and RNase H activity. RT is an RNA-dependent DNA polymerase which synthesises DNA from RNA as well as DNA templates and requires an oligonucleotide primer. RNase H functions in reverse transcription by degrading the RNA moiety of RNA/DNA hybrids and thereby uncovering the template for viral DNA synthesis. RNase H also generates oligoribonucleotide primers for the reverse transcription process. RT and RNase are indispensable for viral replication.

The C-terminsus of the HIV gag-pol precursor polypeptide is proteolytically processed by PR to produce the 32 kDa integrase (IN). An integrase domain near the N-terminsus of HIV exhibits high sequence conservation with IN of other retroviruses. This domain also contains pairs of cysteine and histidine residues that adopt a similar structure to the metal-finger motif in several DNA-binding proteins. Integrase is a viral enzyme possessing both DNA cleavage and joining activities. IN mediates covalent linkage of linear double-stranded viral DNA into the host T-cell genome.

The HIV-1 envelope glycoprotein is encoded by a bicistronic mRNA containing the *env* open reading frame (ORF) just downstream of the *vpu* ORF. This mRNA is synthesised at a late stage of viral replication and its expression is dependent on the posttranscriptional function of the viral *rev* gene. The *env* genes of HIV and SIV encode proteins of 850 to 880 aminso acids; extensive glycosylation of the Env precursor polyprotein during synthesis produces gp160 which is the major form of the *env* gene product in infected cells. Oligopeptide mapping and aminso acid sequencing reveal that gp160 is cleaved to yield the N-terminsal subunit (gp120), which is about 550 aminso acids long and the C-terminsal subunit (gp41), which is about 350 aminso acids long, as mature envelope proteins. External viral gp120 is responsible for binding to the cellular receptors, while transmembrane gp41 anchors gp120 through noncovalent interactions and mediates membrane fusion with target T-cells (Veronese *et al*, 1985). Noncovalent binding between gp120 and gp41 produces the complex gp120/gp41 (Helseth *et al.*, 1991) as well as the formation of tetramers on the cell surface and the viral membrane (Popovic *et al.*, 1983; Pinter et al., 1989).

The external envelope glycoprotein, gp120 is a highly glycosylated hydrophilic protein positioned on the external surface of virion membranes as well as plasma membranes. The aminso acid residues in both the N-terminsus and C-terminsus of gp120 are critical for maintaining the association of this subunit with gp41. HIV gp120 contains 24 potential sites for N-linked glycosylation, 13 of which are conserved between isolates and 17 of these are modified with carbohydrate side chains. Because of the extensive glycosylation of gp120, few regions of the peptide backbone of gp120 protrude from the carbohydrate mass. Since, HIV gp120 has 18 cysteine residues, which are highly conserved, disulphide bonds are presumed to play more critical roles in the structure and function of this protein. A model for the gp120 subunit shows nine intra-chain disulfide bonds which delineates gp120 into several functional regions including five conserved domains (C1-C5), five hyper-variable domains (V1-V5) and a conformation-dependent domain that interacts with the CD4 receptor.

The transmembrane envelope glycoprotein (gp41) is hydrophobic and traverses the lipid bilayer membranes of both virions and cells. Gp41 is classified as a type 1 integral membrane protein. It contains four potential glycosylation sites and three cysteine residues. The main functions ascribed to gp41 are fusion activity (Freed *et al.*, 1990), interaction with gp120 (Bergeron *et al.*, 1992; Chen *et al.*, 1993), anchoring of gp120/gp41 to the membrane (Chen *et al.*, 1994), involvement in cytopathogenicity and participation in virion assembly (Bullough *et al.*, 1994). About 20 aminso acids at the N-terminsus of gp41 are hydrophobic and define the fusion peptide which is required for fusion of the virion membrane with the cell plasma membrane during the entry step in viral replication. A second hydrophobic domain

spans virion and cell membranes and thereby enables gp41 to serve as an anchor for the Env gp heterodimer. The region between these two hydrophobic domains is external to the membrane and contains a highly conserved sequence similar to the leucine zipper motif implicated in protein/protein interactions of a variety of viral and cellular proteins (Venable *et al.*, 1989).

1.3.3 Regulatory genes of HIV-1

Tat is encoded by two exons. The first exon is located in the central region of the viral genome between *vpr* and *env*. The second exon is located 3' of the *env* gene and is of variable size, from 14-29 aminso acids. The *tat* gene is translated from early multiply spliced transcripts and is considered to be an important factor in controlling late events in viral replication. The *tat* gene increases rates of transcription from the HIV-1 LTR (hence the name, transactivator of transcription). Tat allows RNA polymerase II to traverse the length of the viral genome, thus dramatically increasing levels of elongated transcripts. This effect of the *tat* gene is dependent on a viral cis-acting RNA sequence, called the transactivation response element (TAR) which is located precisely at the start of all viral transcripts. TAR extends from nucleotides +1 to +60 and forms a stable stem-loop structure. When the *tat* gene is present, it binds to nascent RNA, represses the synthesis of short transcripts and modifies the transcription complex allowing complete elongation of viral transcripts. The first exon of the *tat* gene suffices for high levels of transactivation, the second exon mediates cellular functions, such as lymphocyte activation (Lisziewicz *et al.*, 1995; Ott *et al.*, 1997). While Tat has a modular structure, the RNA-binding domain of the *tat* gene

cannot be separated from its activation domain for efficient interaction with TAR (Wimmer *et al.*, 1999).

Rev is also translated from early transcripts and is an important candidate in controlling late events in viral replication. When viral transcripts from cells infected with HIV-1 bearing mutations in Rev were analysed, a significant difference was apparent when nuclear and cytoplasmic RNAs were analysed. All three mRNA (9 kb, 4 kb, and 2 kb) transcripts were present in the nucleus, but only 2kb transcripts were observed in the cytoplasm. The appearance of 9kb and 4kb transcripts in the cytoplasm was restored only after co-expression of rev in the same cells. Thus, rev allows for the movement of unspliced and singly spliced transcripts from the nucleus to the cytoplasm. Rev derives its name, the regulator of viral gene expression, because these late transcripts encode the structural and enzymatic proteins needed for virion assembly.

Like *tat*, the *rev* gene is dependent on the presence of a cis-acting RNA element. This sequence, the Rev response element (RRE) is between 240-and 351 nucleotides long and is located just 3' to the junction between the gp120 and gp41 subunits of Env. Rev binds to the RRE, preventing the nuclear sequestration of incompletely spliced viral transcripts. Since, Rev interacts with the nuclear export pathway, these unspliced and singly-spliced transcripts are translocated to the cytoplasm through the RRE.

The HIV regulatory protein Nef is a myristylated 27kDa protein expressed early during virus infection from a multiply-spliced transcript. The *nef* gene of HIV-1 extends from the 3' end of *env* into the U3 domain of the 3' LTR. Initial studies characterised Nef as a downregulator of viral gene expression but more recently Nef

has been shown to down-modulate CD4 (Garcia *et al.*, 1991). Expression of Nef in a T-cell line appears to induce cell surface CD4 endocytosis which is dependent upon a dileucine motif in the cytoplasmic tail of CD4 (Aiken *et al.*, 1994). A second function of Nef is to increase viral replication rates (Miller *et al.*, 1994; Spina *et al.*, 1994); however this activity and its ability to down-modulate CD4 can be separated by specific point mutations in Nef (Saksela *et al.*, 1995). The mechanisms by which Nef affects cell surface CD4 levels and viral replication rates remain unclear.

1.3.4 Accessory genes of HIV-1

Viral protein U (Vpu) is translated from a singly-spliced mRNA, is dependent on *rev* function and is also produced late in infection. The *vpu* gene is located at the 5' end of the *env* gene and is a viral integral membrane phosphoprotein that was originally characterised as a 16kDa protein which increases viral particle release from infected cells (Strebel *et al.*, 1989). Subsequent studies demonstrated that expression of vpu could decrease the half-life of CD4 in experiments on transfected HeLa epithelial carcinoma cells (Willey *et al.*, 1992). In this system, the effect of Vpu on the stability of CD4 was dependent upon the presence of co-expressed gp160 capable of interaction with CD4. Although the mechanism of the effect of Vpu on CD4 is still unclear, recent studies have demonstrated direct *in vitro* binding of Vpu to the cytoplasmic tail of CD4 (Bour *et al.*, 1995).

The viral infectivity factor (Vif) is located immediately downstream of the *pol* gene. The *vif* gene is synthesised from an ORF encoding 193 aminso acids, has a molecular weight of 23 to 27kDa and accumulates in the cytosol and cytoplasmic membrane fractions of infected cells. The exact function of Vif is unclear but it has

been shown to promote infectivity of cell-free virus since Vif-negative mutants were shown to be 1000 times less infectious than virions with Vif. It has been suggested that Vif plays an important role at the end-stage of virus replication although the exact mechanism by which Vif affects viral infectivity is still unclear. The exact functional domain of Vif has yet to be defined and additional studies are required to determinse the early step of viral replication controlled by Vif and also whether Vif can modify virion proteins and/or virion structure.

Vpr is a 15kDa virion-associated protein translated from a singly-spliced mRNA, which is dependent on Rev function and thus accumulates late in infection. It is encoded by a single exon and overlaps with the *vif* gene. Vpr is assembled into virions and may play an important role in the early steps of the life cycle such as nuclear localisation of the viral pre-integration complex. In mature virions, Vpr is associated with the nucleocapsid and is as abundant as Gag-related proteins. Vpr may also influence viral gene expression by altering the host T-cell regulatory mechanism, which enhances viral replication. Vpr was also recently found to induce apoptosis. In addition, it can induce arrest of cells in the G2 phase of the cell cycle causing apoptosis in PBMCs, and fibroblasts (Bartz *et al.*, 1996).

Vpx is 14 to 16kDa protein, translated from a singly spliced, Rev-dependent transcript produced late in infection. Vpx has only been found in HIV-2 and SIV isolates but not in HIV-1. In mature virions there are the same number of Vpx molecules as there are molecules of the major Gag capsid protein. Mutagenesis studies have not identified a clear phenotype for this gene. A strong sequence homology exists between the viral protein Vpx of HIV-2 and Vpr of HIV-1 (Wu *et al.*, 1996). Vpx-negative isolates have been shown to infect primary lymphocytes with

reduced efficiency. Other studies have also revealed that Vpx is needed for efficient viral replication in PBMCs (Selig *et al.*, 1999).

1.4 LIFE CYCLE OF HIV-1

The life cycle of HIV-1 infection requires infection of a human cell. Studies in electron microscopy and X-ray crystallography reveal that direct fusion of the viral and cell plasma membranes should be the principal pathway for virion entry which is initiated by virion attachment (Ryu *et al.*, 1990; Wang *et al.*, 1990). A more complex picture of retroviral interference has emerged from work on HIV-1 infection of CD4+ T-cells and monocytes/macrophages, for which infection is mediated by high-affinity binding between viral gp120 and cell surface CD4 (McDougal *et al.*, 1986). Establishment of productive infection is generally accompanied by the disappearance of CD4 from the cell surface (Dalgliesh *et al.*, 1984; Maddon *et al.*, 1986; Stevenson *et al.*, 1988). Formation of complexes between CD4 and HIV env proteins occurs intracellularly (Hoxie *et al.*, 1986) and impairs CD4 maturation and transport to the cell surface (Kawamura *et al.*, 1989; Crise *et al.*, 1990; Jabbar & Nayak 1990; Bour *et al.*, 1991; Butera *et al.*, 1991). Other mechanisms of cell surface CD4 downmodulation involve non-env viral proteins that act at each of transcriptional, translational and post-translational levels (Hoxie *et al.*, 1986; Salmon *et al.*, 1988; Yuille *et al.*, 1988; Garcia & Miller 1991; Willey *et al.*, 1992,).

The life cycle can be summarised as follows: (1) HIV-1 attachment requires binding of the virus to the surface of cells expressing a cellular receptor for HIV-1 (Figure 1.3).(Dalgleish *et al.*, 1984; Klazmann *et al.*, 1984; Maddon *et al.*, 1986).

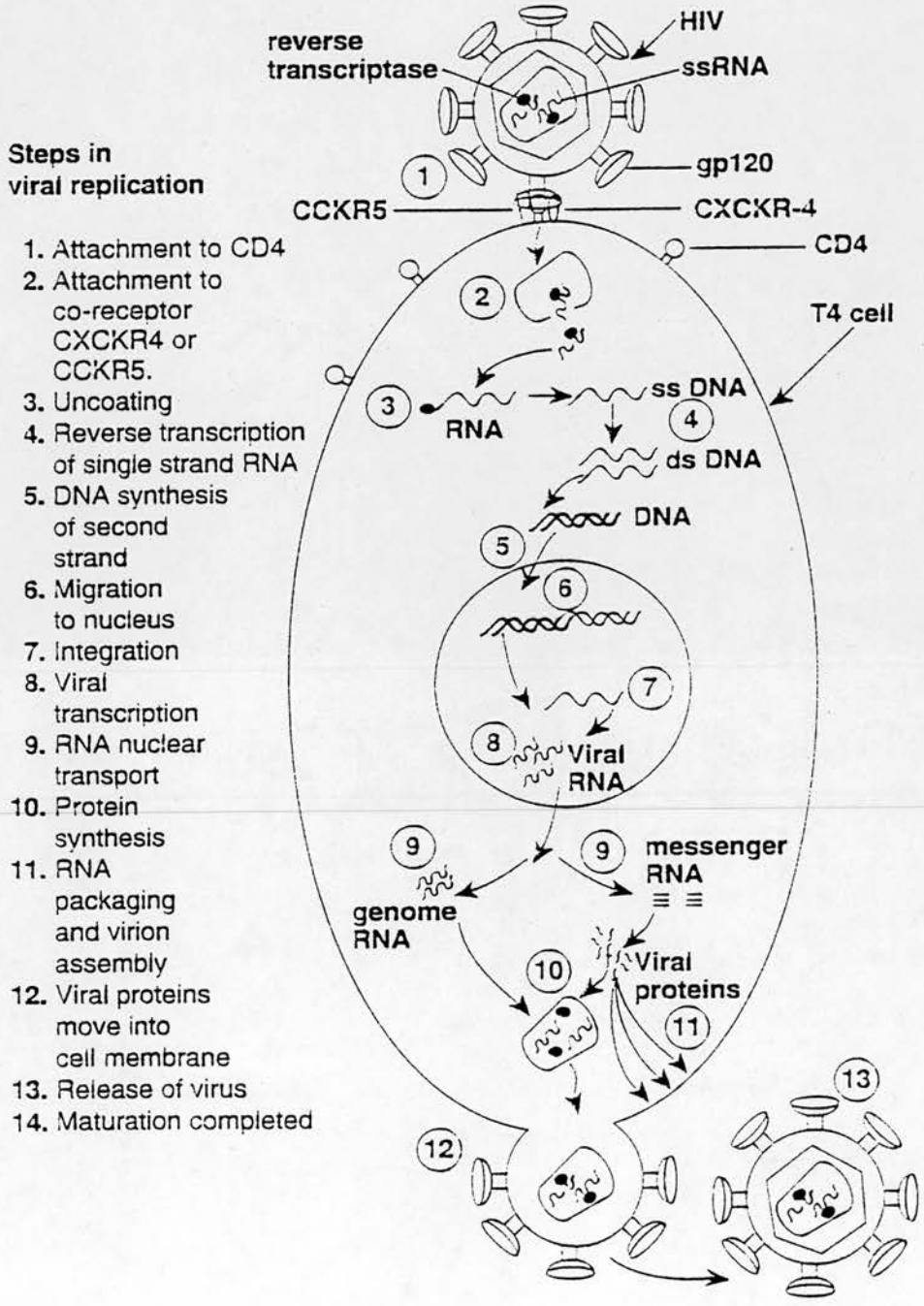


Figure 1.3 Life Cycle of HIV-1 (Modified from Stine, 2000).

Virus attachment involves the interaction of the gp120 envelope glycoprotiens with specific receptors – the CD4 glycoprotein and members of the chemokine receptor family (Sattentau *et al.*, 1991; Deng *et al*, 1996). (2) After attachment to the cell surface, the virus is internalised and uncoated (Farnet & Haseltine *et al*, 1991). (3) Following entry into the target cell, a double-stranded DNA copy of the HIV RNA genome is synthesised by the viral enzyme, reverse transcriptase (RT). (4) The newly synthesised complementary viral DNA is incorporated into the nucleus while still associated with viral Gag proteins and the viral integrase. (5) Integrase catalyses a concerted cleavage and ligation reaction in which the viral DNA genome becomes integrated into the host DNA (provirus). (5) A latent phase may follow viral integration restricting the life cycle until the infected cell is activated allowing the transcription of viral genomic RNA and mRNA. (6) Synthesis of virion proteins. The virally encoded Rev protein plays an important role in determinsing the relative amounts of spliced and unspliced HIV RNAs transported to the cytoplasm, for translation or packaging into the nascent HIV particles. The actions of the various regulatory factors determinse both the levels of HIV RNA synthesised and the qualitative nature of those RNAs (7) Assembly and budding of virions (8) Processing of capsid proteins (reviewed in Coffin, 1996, and Luciw, 1996).

1.4.1 Attachment

Like all viruses, retroviruses have a specific requirement for interaction with a cell-surface receptor molecule for infection. The HIV-1 receptor complex essential for virus entry consists of the CD4 molecule expressed on T-cells, macrophages, dendritic cells and microglia, and a member of the seven-transmembrane chemokine receptor

family, termed a co-receptor in the context of HIV and SIV infection. Early studies, however, still hold true today: that HIV-1 attachment to T-cells correlates with CD4 expression (McDougal *et al.*, 1986a; McDougal *et al.*, 1986b), is gp120-dependent (Bahraoui *et al.*, 1989; McDougal *et al.*, 1986b) and might be the rate-limiting step in viral entry into T-cells (Orloff GM *et al.*, 1991). Due to difficulties involving assaying virus-cell binding, HIV-1 attachment is still poorly understood. Virus binding has been shown to be rapid, saturable and temperature-dependent in murine retroviruses. It was also found to correlate qualitatively with infectivity, implying that specific receptor usage is manifest at the attachment level. An indirect analysis of ectotropic murine retrovirus attachment, in which virus infectivity was related to receptor expression, showed that viral attachment is limited, in a cell type-specific manner, by factors other than specific receptor expression level (Wang H *et al.*, 1991). Similar restrictions may apply to HIV-1 and related viruses. Thus, attachment specificity might determinse the tropism of these viruses.

1.4.2 The CD4 receptor

One of the first discoveries in HIV research studies was that the first major cellular receptor involved in virus-cell interactions was the CD4 molecule (Dalgleish *et al.*, 1984; Klazmann *et al.*, 1984; Maddon *et al.*, 1986). CD4 is a member of the immunoglobulin superfamily and functions as a T-cell co-receptor that increases the affinity of a helper T-cell for an antigen-presenting cell through its interactions with major histocompatibility (MHC) class II molecules. It is present on most T-helper cells, monocyte/macrophages, B cells, and specialised cells of the central nervous system. Early experiments found that insertion of the CD4 gene into Hela cells made

them permissive to productive infection and it was also shown that CD4 receptors could be blocked by specific monoclonal antibodies, thereby preventing infection of most T-cell types. The CD4 molecule has been implicated in signal transduction pathways involved in T-cell activation (Barre-Sinoussi *et al.*, 1883; Margolick *et al.*, 1987). CD4 is also crucial in the generation of many immunologic functions, such as the activation of macrophages, induction of cytotoxic or suppressor T-cell functions, secretion of growth or differentiation factors for lymphoid and hematopoietic cells and induction of B-cell function. The CD4 molecule is a transmembrane glycoprotein of 58kD and consists of an extracellular region of 370 aminso acids, a transmembrane region of 25 aminso acids, and a cytoplasmic tail of 38 aminso acids at the C-terminsal end (Janeway & Travers, 1996). The extracellular portion of CD4 is folded into four distinct domains designated D1-D4. The N-terminsal D1 domain shares extensive structural and sequence homology with the variable region of Ig light chains (Sullivan *et al.*, 1996). The other three domains are less closely related to Ig molecules at the level of primary structure but fold similarly to Ig family domains, confirminsg that CD4 is a member of the Ig-like superfamily (Moore & Ho *et al.*, 1993).

1.4.3 HIV tropism and CD4+ cells.

Early reports that HIV-1 infection in vivo was restricted to the CD4+ subset of T lymphocytes (Klatzmann *et al.*, 1984a) were followed by studies which showed that anti-CD4 mAb could block both infection of CD4+ target T-cells and subsequent formation of syncytia (Klatzmann *et al.*, 1984b; Dalgleish *et al.*, 1984). Immunoprecipitation with anti-gp120 antibodies revealed complexes at the cell surface between the HIV-1 envelope glyocprotein and a 58-kDA molecule identified

as CD4 (McDougal *et al.*, 1986). It was subsequently demonstrated that transfection of CD4 cDNA into human cells led to expression of CD4 at the cell surface and conferred susceptibility to HIV-1 (Maddon *et al.*, 1986). Mouse cells which expressed human CD4 could bring up gp120 but were not infectable by HIV-1 (Maddon *et al.*, 1986), indicating that additional unidentified cellular factors, absent in cells of mouse origin, are required for this process.

1.4.4 The envelope glycoprotein

The envelope glycoprotein regions on the surface of HIV particles bind to CD4 receptors, located on the plasma membrane of CD4+ T lymphocytes, monocytes, macrophages, and dendritic cells and thereby attach virions to cells. This viral glycoprotein is responsible for the induction of syncytia in tissue culture cells and is a major target for antiviral immune responses in the infected host. The *env* region of HIV encodes 850-880 aminso acids; extensive glycosylation of the *env* precursor polyprotein during synthesis produces gp160 which is the major form of the *env* gene product detected in infected cells (Moore & Ho. 1993; Levy. 1998). Intracelluar cleavage of gp160 yields the N-terminsal subunit (gp120), which is about 550 aminso acids long, and the C-terminsal subunit (gp41) which is about 350 aminso acids long (Capon *et al.*, 1991). Gp120 is a highly glycosylated, hydrophilic protein positioned on the external surface of virion membranes as well as plasma membranes of infected cells (Schneider *et al.*, 1986).

1.4.5 Binding site for gp120 on CD4

Through extensive mutagenesis studies, the HIV-binding site has been mapped to CD4 domain D1 (Ryu *et al.*, 1990; Wang *et al.*, 1990). Epitope mapping of CD4 substitution mutants with MAbs showed that a small region, containing aminso acids 41 to 52, was involved in gp120 binding. Four charged residues (Lys-29, Lys-35, Lys-46 and Arg-59) and one hydrophobic phenylalanine at position 43 were also essential for gp120 binding (Ashkenazi *et al.*, 1990; Moebius *et al.*, 1992). These five aminso acids are predicted to form a hydrophobic pocket by folding phenylalanine residue, a structure that may be involved in direct contact with gp120 (Moebius *et al*, 1992).

1.4.6 Binding site for CD4 on gp120

The HIV-1 gp120 envelope protein has a complex secondary structure stabilized by disulfide bonds between conserved cysteine residues. Extensive variability is apparent in five discrete gp120 domains termed V1-V5, as shown by sequence comparison among various HIV-1 isolates (Shioda *et al.*, 1991; Groenink *et al.*, 1992; Donaldson *et al.*, 1994; Broder & Collman, 1997). Conserved aminso acid sequences, termed C1-C5, separate these hypervariable regions (Willey *et al.*, 1986; Modrow *et al.*, 1987; Myers *et al.*, 1993). Several of these domains interact with each other to form the complex secondary structure of gp120. Consequently, probing the CD4-binding site by aminso acid deletions and substitutions in gp120 is often impeded by the extensive structural changes caused by the mutations. Although the N-terminsal part of gp120 was originally thought to be involved in CD4 binding, it is now accepted that the vast majority of residues important for CD4 binding are located

in the C-terminsal half of gp120, from the C2 to the C4 domains. They consist of residues 256-299 in the C2 domain, residues 368 to 389 in the C3 domain and residues 421 to 457 in the C4 domain. Additional residues in the C5 domain have been reported to also contribute to the formation or stability of the CD4-binding site. The C2 and C5 domains of gp120 are poorly exposed at the surface of the molecule and while they are important in the structure of the CD4 binding site they are not as involved in the direct contact of CD4 unlike the C3 and C4 regions which have proved to be of particular importance for interactions with CD4 (Olshevsky *et al.*, 1990; Pollard *et al.*, 1991).

1.4.7 Post-binding events following CD4 and viral envelope glycoproteins

Following binding to CD4, HIV-1 enters the cell by a pH-independent mechanism involving direct membrane fusion (Stein *et al.*, 1987; McClure *et al.*, 1988). The fusogenic capacity of HIV-1 is located in the extracellular N-terminsal sequences of gp41 proximal to the membrane-spanning region (Kowalski *et al.*, 1987; Freed *et al.*, 1990). Association of gp120 and gp41 appears to restrict the fusion activity of gp41 by masking the amphipathic regions of the transmembrane glycoprotein until the virus and cell membrane are in close apposition (Perez *et al.*, 1992). Both the C- and N-terminsal 30 residues of gp120, located in the C1-C5 domains, are involved in these gp41 associations. These regions form a molecular pocket that provides an anchor site for the cysteine loop present in the extracellular part of gp41. The fusogenic domain of gp41 may be exposed once gp120 associates with CD4 to trigger a conformational change resulting in its dissociation from gp41. In addition to the fusion activity provided by gp41, the V3 domain of gp120 may be

essential for the virus to come into close contact with the cell surface and for virus-cell membrane fusion to occur (Hart *et al.*, 1991; Sattentau *et al.*, 1991). The role of this envelope domain in viral infection is further supported by the highly neutralizing activity of antibodies against the V3 loop (La Rosa *et al.*, 1990; Robert-Guroff *et al.*, 1994).

1.4.8 Other receptors

The entry of HIV-1 into a cell is a multistep process. Env mediates binding of virus to the cell surface through a high affinity interaction with CD4, the primary virus receptor (Dalgleish *et al.*, 1984; McDougal *et al.*, 1986). However, it is the subsequent interaction with the appropriate chemokine receptor that is thought to trigger the final conformational changes in *env*, leading to fusion between the viral and cellular membranes, allowing infection to proceed. HIV-1 variants can differ in their cellular tropism (DeJong *et al.*, 1992; Hwang *et al.*, 1992): all variants are capable of replicating in primary T-cells (Tersmette *et al.*, 1988; Connor *et al.*, 1994) but only some can replicate in primary macrophages (Zhu *et al.*, 1993; Van't Wout *et al.*, 1994) or in permanent T-cell lines (Schuitemaker *et al.*, 1992). With the identification of the HIV-1 co-receptors (Alkhatib *et al.*, 1996; Bleul *et al.*, 1996; Choe *et al.*, 1996; Deng *et al.*, 1996; Doranz *et al.*, 1996; Dragic *et al.*, 1996; Feng *et al.*,1996; Oberlin *et al.*, 1996; Samson *et al.*, 1996a), the basis for the observed variation in HIV-1 cytotropism has been revealed. In addition to CD4, T-cell line adapted HIV-1 variants use the chemokine receptor CXC chemokine receptor CXCR4 as their co-receptor, macrophage tropic HIV-1 variants use the CC chemokine receptors CCR5, and primary SI HIV variants can use both (discussed in detail below).

Chemokines are described as a large family of cytokines that have chemo-attractant properties. They are small polypeptides that recruit leukocytes from the circulation to sites of infection. Chemokines are secreted by many cell types. The family can be divided into two major classes, α (also called CC) and β (also called CXC), based on minsor differences in structure and function. Chemokines activate cells by binding to specific cell-surface receptors that belong to a superfamily of serpentine G-protein coupled receptors (Horuk *et al.*, 1994; Powers and Wells, 1996). To date, eight CC and five CXC chemokine receptors have been identified, (Table 1.2). Structural distinctions of the different branches of the superfamily have been shown to parallel general distinctions in the biological activities of chemokines. For example, most CXC chemokines are chemoattractants for neutrophils but not monocytes, whereas CC chemokines generally attract monocytes and lymphocytes, but not neutrophils Basophils and eosinophils are also affected predominsantly by CC chemokines. The C chemokine appears thus far to be lymphocyte specific (Baggiolini *et al.*, 1994; Schall & Bacon, *et al.*, 1994).

1.4.9 CCR5

HIV-1 uses two chemokine receptors as its principal co-receptors: CCR5 is the chemokine used primarily during transmission and the asymptomatic phase of infection (Alkhatib *et al.*, 1996; Choe *et al.*, 1996; Deng *et al.*, 1996; Doranz *et al.*, 1996; Dragic *et al.*, 1996), whereas CXCR4 is generally used at later stages in the infection (Berson *et al.*, 1996; Feng *et al.*, 1996). The CCR5 receptor is the major co-receptor for macrophage-tropic non-synctium-inducing (NSI) isolates of HIV-1 . Rare strains are also able to use CCCR2b and CCR3 as co-receptors (Deng *et al.*, 1996;

Frade, 1997). Macrophage tropic NSI viruses are generally found within the first few months of infection and persist throughout the course of infection (Asjo *et al.*, 1986; Groenink *et al.*, 1991; Kuiken *et al.*, 1992; McNearney *et al.*, 1992; Roos *et al.*, 1992; Schuitemaker *et al.*, 1991). CCR5 is a seven transmembrane-domain protein containing four extracellular domains: an aminso-terminsal domain and three extracellular loops. The replication of primary, NSI HIV-1 isolates in CD4+ T lymphocytes is inhibited by β-chemokines, macrophage inflammatory protein 1α (MIP-1α), MIP-1β and regulated upon activation normal T-cell expressed and secreted (RANTES), the natural ligands for CCR5. While RANTES, MIP-1α and MIP-1β block infection of lymphocytes, they fail to block infection of macrophages or dendritic cells. It is not known how chemokines achieve their HIV suppressive effects at the molecular level. Chemokine binding could simply antagonize the gp120-chemokine receptor interaction, preventing the fusion reaction (Deng *et al.*, 1996). Env-CCR5 interactions are conformationally complex, involving all four extracellular domains of CCR5. There are two functionally redundant sites on CCR5: the N-terminsal end and one of the extracellular loops. Viruses particularly well adapted for CCR5 can often tolerate significant alterations in one of these sites. Interestingly, dual-tropic viruses that also use CXCR4 are much less tolerant of changes in CCR5 (Doranz *et al.*, 1996). Whether M- and dual-tropic viruses really use CCR5 in two somewhat different ways will require the analysis of a larger number of viral strains.

Subsequent to the discovery of CCR5 as the co-receptor, Samson *et al* and Liu *et al* described a polymorphism; a homozygous 32bp deletion in the CCR5 gene. This mutant allele CCR5 was demonstrated to play a critical role in the establishment of HIV-1 infection, because homozygosity for an allele encoding a fusion-defective

CCR5, was present in ~1% of the Caucasian population, and was strongly associated with resistance to HIV-1 infection (Dean *et al.,* 1996; Liu *et al.,* 1996; Samson *et al.,* 1996b). Although CCR5 Δ32 homozygotes have been found to be highly resistant to HIV-1 infection, some individuals homozygous for the defective CCR5 gene do get infected (Biti *et al.,* 1997). Because of the rapid course of the disease in these patients and the presence of SI variants at the earliest time that can be tested, it is most likely that infection is established using CXCR4 as co-receptor.

1.4.10 CXCR4

As well as influencing HIV-1 tropism, chemokine receptors also appear to determinse species specificity. Indeed, mouse cells expressing human CD4 cannot be infected with HIV-1, whereas co-transfection with human CXCR4 renders these cells susceptible (Berson *et al.*, 1996; Feng *et al.*, 1996). CXCR4 (previously known as LESTR/fusin) has been shown to mediate entry of the T-cell line-tropic (T-tropic) form of HIV-1 but not macrophage tropic isolates. CXCR4 is a receptor for the CXC chemokine stromal cell-derived factor 1 (SDF-1), which can inhibit CXCR4-dependant HIV-1 infection and halt the formation of syncytia by blocking the co-receptor. By contrast to CCR5, the N-terminsal domain of CXCR4 is considerably less important for co-receptor function. Rather the first and second extracellular loops of CXCR4, especially the second extracellular loop, are crucial determinsants for all viral strains studied to date. Both the T and dual-tropic viruses appear to interact with CXCR4 in a similar fashion.

The phenotypic switch from CCR5 use (R5 virus) to CCR5/CXCR4 (R5X4) or CXCR4 use (X4) predicts the progression to AIDS, but the dynamics of this

switch are not understood. Monomeric R5 gp120 binds CCR5 poorly in the absence of CD4, but competes well with macrophage inflammatory protein 1β (MIP-1β; a natural chemokine ligand for CCR5) for CCR5 binding as a gp120-soluble CD4 complex. Monomeric X4 gp120 binds CXCR4 on certain cell types with detectable affinity in the absence of CD4, suggesting that the CXCR4-binding surface on gp120 is constitutively partially accessible.

Primary SI variants, which have the intrinsic capacity to replicate in permanent T-cell lines, are able to use both CCR5 and CXCR4 as co-receptor (Simmons *et al.*, 1996). Both CCR5 and CCR3 can serve as co-receptors for HIV-1 on microglial cells (He *et al.*, 1997). The lack of T-cell line tropism of NSI variants is probably due to the lack of CCR5 expression on these cells. The lack of macrophage tropism by SI variants cannot be attributed to restriction by co-receptor availability as both CCR5 and CXCR4 are expressed on macrophages. Kozak and colleagues showed that the limiting factor in macrophage infection by SI variants is not co-receptor expression but CD4 expression. SI variants have a lower affinity for CD4 compared with NSI variants, and these variants may require much higher levels of CD4 expression to enter cells (Kozak *et al.*, 1997).

The HIV-1 co-receptors CCR5 and CXCR4 on CD4+ T-cells are differentially expressed on the surface of memory and naïve cells. Specifically, CCR5, the co-receptor for macrophage tropic viruses is largely restricted to memory cells while CXCR4, the dominsant co-receptor for T-cell line tropic viruses is expressed on both memory and naïve cells, although more so on naïve cells (Bleul *et al.*, 1997; Spina *et al.*, 1997). It is unknown how this differential expression of the major HIV-1 co-receptors correlates with the susceptibility of these subsets of cells to viruses of

various phenotypes (CCR5-using versus CXCR4-using) *in vivo* (Ostrowski MA, 1999).

The isolation of X4 strains of HIV-1 is correlated with a decline in peripheral CD4+ T-cell levels and the onset of clinical symptoms, and therefore X4 strains have been considered more pathogenic than R5 strains. This difference in pathogenicity may be due to differences in chemokine receptor usage and hence, target pool size *in vivo*. The pattern of expression of chemokine receptors on T-cell subsets and their regulation has important implications for AIDS pathogenesis.

CCR5 is expressed on 5 to 25% of peripheral cells, mostly CD4-CD45RA-memory/effector cells, primary T-cells and granulocyte precursors (Alkhatib *et al.*, 1996; Deng *et al.*, 1996) while CXCR4 is expressed on nearly all peripheral T-cells. CXCR4 is thought to be widely distributed in human cells and tissues (Feng *et al.*, 1996) and has been detected in a number of CD4+ T-cell lines (Loetscher *et al.*, 1994). Monocytes, the other peripheral blood cell types infected by HIV-1, express CXCR4 but do not appear to be infectable by X4 strains of HIV-1, perhaps due to a post-entry block. Monocyte differentiation to macrophages *in vitro* results in CXCR4 down-regulation and CCR5 up-regulation allowing entry of R5 strains of HIV-1. Wu *et al* also observed CCR5 on macrophage-like cells in sections of human lymph nodes (Wu *et al.*, 1996).

Individual isolates of HIV-1 display markedly distinct tropisms for infection of primary macrophages as compared with CD4+ T-cell lines. The viral determinsants for this cyto-tropism reside in the envelope glycoprotein, particularly in the gp120 subunit containing the third hypervariable loop (V3) (Cheng Mayer *et al.*, 1991; Shioda *et al.*, 1991; Westervelt *et al.*, 1991; Chesebro *et al.*, 1992). Exchange studies

of genomic fragments from different isolates showed that the Env region was indeed predominsantly responsible for differences in host range. For example, a 157bp fragment including the V3 and V4 domains but not the CD4 binding site was associated with the capacity to infect primary macrophages (Shioda *et al.*, 1991). Exchange of a 283bp domain, also including the V3 loop and only 2 of 11 aminso acids in the CD4-binding domain, conferred macrophage tropism from the macrophage-tropic variant ADA to the T-cell line adapted NL4-3 isolate (Westervelt *et al.*, 1991). The same genomic Env fragment was also identified as important for synctium induction (Groenink *et al.*, 1993). Exchange of part of the V1 domain together with the V2 and V3 domains conferred SI capacity to an NSI variant.

In addition to being more pathogenic in the periphery, X4 strains may be more effective than R5 strains in decreasing the output of new T-cells by the thymus. The predominsant population of $CD4^+CD8^+$ thymocytes expresses both CCR5 and CXCR4 and likely serve as the main target of each strain. However, CXCR4 but not CCR5 is expressed at high levels on immature $CD4^+CD8^-CD3^-$ intrathymic T-cell progenitors (ITTPs). Proliferation and maturation of X4-virus-infected thymocytes may contribute to the rapid spread of X4 through the thymus. However, Berkowitz *et al* found that R5 strains of HIV-1 can also infect and destroy intrathymic cells. R5 strains initially infect stromal cells including macrophages in the thymic medulla but after a short time infection spreads slowly throughout the thymocyte populations (Berkowitz *et al.*, 1998).

At least nine other chemokine receptors, or structurally related molecules, have been described as supporting HIV-1 *env*-mediated membrane fusion or viral entry *in vitro*. These include CCR2b, CCR3, CCR8, BOB/GPR15, Bonzo/STRL33,

GPR1, US28, APJ, V28/CX3CR1 and MDC. The chemokine receptor CCR3 can be used efficiently by a fraction of HIV-1 isolates *in vitro*, provided the expression of this protein in transfected cells is boosted (Alkhatib *et al.*, 1996; Rucker *et al.*, 1997; Bazan *et al.*, 1998; Ross *et al.*, 1998). Several studies have compared co-receptor usage by primary HIV-1 isolates of several genetic subtypes and in general, results were similar, other than the occasional use of CCR3, the majority of isolates used CCR5 and CXCR4 (Bjorndal *et al.*, 1997; Connor *et al.*, 1997). Another study looked at the use of all other receptors and found that most NSI isolates could only use CCR5. The SI isolates used primarily CXCR4, but some did employ V28, CCR8 and APJ (Zhang *et al.*, 1998).

Table 1.2. Chemokine receptors and their ligands involved in HIV-1 infection.

CKR	Previous name	Ligands	Distribution
CCR1	CC-CCKR1	MIP-1α, RANTES, MCP3	Monocytes, B/Tcells, eosinophils.
CCR2a	MCP-1Ra	MCP-1	Monocytes, T-cells, basophils
CCR2b	MCP-1Rb	MCP-1/3/4	Eosinophils
CCR3	CKR3	Eotaxin, RANTES, MCP-2-5, MPIF-2	Baso/eosinophils microglial cells
CCR4		TARC	T-cells, basophils
CCR5	CC-CKR5	MIP-1α/β, RANTES	Monocytes, dendritic
CCR6		MIP-3α	
CCR7		ELC,MIP-3β	
CCR8	ChemR1	1309	Activated monocytes, activated PBLs
CXCR1	IL-8RA	IL-8, GCP-2	Neutrophils,NK cells
CXCR2	IL-8RB	IL-8, MGSA/gro-α, MAP-2, IL-10, ENA-78, Mig, GCP-2	Monocytes, B/Tcells, baso/eosinophils
CXCR3		IP-10, Mig	Activated T-cells
CXCR4	LESTR/ HUMSTR, fusin, LCR-1	SDF-1	Monocytes,B/T-cells, dendritic cells

1.4.11 Interactions of chemokine receptors with hypervariable regions.

Cell tropism for HIV-1 is thought to be determinsed at the level of viral entry by the interaction of the viral envelope proteins with certain types of co-receptors that support either T-tropic and M-tropic HIV-1 infection. Generally, acquistion of basic charges in the V3 loop correlates with CXCR4 use (Fouchier *et al.*, 1992; Shioda *et al.*, 1992), perhaps because the regions of CXCR4 most important for co-receptor function are far more acidic than the corresponding regions of CCR5 (Brelot *et al.*, 1997; Lu *et al.*, 1997).

Studies of chimeric envelope glycoproteins demonstrated that the third variable (V3) loop of gp120 is a major determinsant of which chemokine receptor is used (Cocchi *et al.*, 1996; Deng *et al.*, 1996). Speck *et al* (1997) recently demonstrated that the specific aminso acids in the V3 loop of the envelope protein that determinse cellular tropism also regulate chemokine co-receptor preference for cell entry by the virus. Comparisons of variants with Q or E at position 25 in the V3 loop regulated the ability to use CXCR4 for cell entry. The use of CCR5 was influenced by mutations at position 30 (Speck *et al.*, 1997). It has been observed that V3-deleted versions of gp120 do not bind CCR5, even though CD4 binding occurs at wild-type levels (Trkola *et al.*, 1996). Anti-V3 neutralising antibodies may also interfere with gp120-CCR5 binding (Wu *et al.*, 1996). These recent findings support an involvement of the V3 loop in chemokine receptor binding. However, the possibility of the involvement of other domains of gp120, including the V1/V2 regions, cannot be excluded. Indeed, it has been speculated that subsequent to the binding of the CD4 molecule, gp120 changes its conformation and exposes the cryptic

V3 loop together with the V1/V2 loop embedded in the gp120 molecule (Sattentau *et al.*, 1991., Wyatt *et al.*, 1995).

Alterations in V1 and V2 sequence can alter viral tropism and co-receptor use (Boyd *et al.*, 1993; Groenink *et al.*, 1993; Koito *et al.*, 1994; Cho *et al.*, 1998) as can changes in the V3 loop (Hwang *et al.*, 1991; Shioda *et al.*, 1992; Cocchi *et al.*, 1996; Speck *et al.*, 1997). Antigenic sites on gp120 of HIV-1 that elicit strain-specific neutralising antibodies include the V1/V2 loop as well as the V3 loop. (Goudsmit *et al.*, 1988; Matsushita *et al.*, 1988; Palker *et al.*, 1988; Fung *et al.*, 1992; Davis *et al.*, 1993; McKeating *et al.*, 1993;). Construction of chimeric proviruses from SI and NSI molecular clones of HIV-1 has demonstrated that the V1/V2 and V4 regions may also be involved in SI capacity (Groenink *et al.*, 1993; Fouchier *et al.*, 1995). During the transition from NSI to SI phenotype, the hypervariable V2 region is thought to undergo increases both in length, mainly through insertion of potential N-linked glycosylation sites, and charge (Groenink *et al.*, 1993; Fouchier *et al.*, 1995).

Also complex structural interactions between V1, V2 and V3 loops and elements of CD4-binding site of gp120 control entry of the virus into cells. Stamatatos *et al* (1998) generated two mutant envelope proteins by making deletions in the V1 and V2 loops of a macrophage-tropic primary HIV-1 isolate SF162. Comparison of the immunochemical structure of the wild-type and mutant monomeric and virion associated gp120 molecules revealed that the V1 and V2 loop deletions differentially altered the structure of the V3 loop, the CD4-binding site, and epitopes within conserved regions of gp120. Regardless of structural differences, both mutated envelope proteins supported viral replication into peripheral blood mononuclear cells to levels comparable to those of the wild-type virus but had no

effect on co-receptor usage. It is likely that the structural changes resulting from the introduced deletions in the V1 and V2 loops affected the kinetics of the CD4-induced gp120 conformational changes that influenced the extent of gp120-corecptor interaction (Stamatatos *et al.,* 1998).

1.4.12 CD4-Independent Infection

Both HIV-1 and HIV-2 are capable of infecting CD4-negative cell lines. For HIV-1 this has been demonstrated *in vitro* for cell lines of nervous system, fibroblast, and liver origin (Chiodi *et al.*, 1987; Dewhurst *et al.*, 1987; Harouse *et al.*, 1989; Tatento *et al.*, 1989; Choe *et al.*, 1995). HIV infection of CD4-negative cell cultures *in vitro* has been extensively reported, including CD4-negative glioma cell lines which have been shown to be infectable (Cheng-Mayer *et al.*, 1987). Infection of some neuronal cell lines may have been mediated via a glycolipid, galactosyl ceramide (Horouse *et al.*, 1991), and sequences in either V3 or V4/V5 determinse this tropism (Horouse *et al.*, 1995). The relevance of CD4-independent entry *in vivo* and its influence on pathogenesis are not yet clear. However there is evidence that CD4-independent brain astrocytes in pediatric AIDS patients become infected by HIV-1 *in vivo* (Saito *et al.*, 1994). Infection of these astrocytes may occur through a CCR5-dependent mechanism (Berger *et al.*, 1997; Berger *et al.*, 1999), or through an efficient gp120/gp41-independent route, such as phagocytosis of other infected cells or by Fc receptor-mediated endocytosis of antibody-coated HIV virions as the HIV virion has been found sequestered in intracellular vacuoles (Gendelman *et al.*, 1989; Abbas *et al.*, 1999).

It has been shown that efficient CD4-independent infection has occurred for several HIV-2 isolates (Clapham *et al.*, 1992). The highly cytopathic nature of this infection indicates that HIV-2 can utilise one or more alternative receptors independently of CD4. The selection of the HIV-2 strain ROD/B (derived from the prototype HIV-2 strain ROD/A) was shown to utilise the CXCR4 receptor to efficiently enter CD4-negative target T-cells (Endres *et al.*, 1996; Reeves *et al.*, 1997). A number of other HIV-2 strains, including some primary isolates, are also able to infect T-cells which express CXCR4 or CCR5 but lack CD4. FIV infection of CD4-negative cell cultures *in vitro* has been reported to occur via the CXCR4 receptor (Willet *et al.*, 1997; Poeschla *et al.*, 1998). In contrast, a significant number of SIV isolates can use CCR5 as a primary receptor and can infect CCR5-positive, CD4-negative primary cells such as rhesus brain capillary endothelial cells whereas only low-level CD4-independent infection has been reported for a single R5 HIV-2 isolate (Chen *et al.*, 1998). The addition of CD4 in soluble or membrane-bound form can enhance infection for most CD4-independent viruses, suggesting that CD4 is required for optimal entry efficiency (Edinger *et al.*, 1999). Mutations located immediately upstream of a proposed coiled coil domain in the transmembrane protein and flanking the base of the V4 loop are crucial for the CD4-independent phenotype of HIV-2. These mutations can reduce the amount of sCD4 required to trigger CD4-independent T-cell-cell fusion, suggesting they lower the activation threshold for the fusion process. A lower activation threshold of CD4-independent envelope proteins may enable them to utilise other membrane molecules for entry which are not as efficient as CD4 in triggering the conformational changes required for the membrane fusion process (Reeves *et al.*, 1997).

1.5 STEPS IN VIRAL REPLICATION

1.5.1 Fusion and entry

The mechanisms by which retroviruses enter cells is one of the most poorly understood aspects of the virus life cycle. Enveloped viruses enter by either a pH-dependent or pH-independent process (Stein *et al.*, 1987; McClure *et al.*, 1988). The site of this fusion whether it is directly at the cell surface (pH-independent) or via endocytosis (pH-dependent) varies among retroviruses. Some retroviruses do not cause cell fusion, and infection by these is sensitive to high pH. Like many enveloped viruses, these seem to be internalised via receptor-mediated endocytosis (RME) into acidic endosomes, most likely provoked by the lower pH typical of endosomal contents. Enveloped animal viruses such as HIV-1 penetrate cells in a pH independent process (Stein *et al.*, 1987; McClure *et al.*, 1988), following direct fusion of the viral envelope with the plasma membrane of the cell and not through the relatively acidic conditions that are present following endocytosis.

It seems likely that the fusion process is mediated by a region of hydrophobic aminso acids at the aminso terminsus of the smaller transmembrane TM (gp41) region (Freed *et al.*, 1990; and can be modulated by the cytoplasmic domain (Ritter *et al.*, 1993; Owens *et al.*, 1994) and gp120 (Bergeron *et al.*, 1992; Chen *et al.*, 1993). Upon binding of HIV-1 to its receptor (the CD4 molecule), fusion of the viral and cellular plasma membrane takes place. The interaction of the chemokine receptors with gp120-CD4 complex can promote changes in the envelope glycoprotein complex. It has also been speculated that upon binding to sCD4 to gp120, conformational changes (important for fusion) occur by exposure of the cryptic V3

loop together with V1/V2 loop embedded in the gp120 molecule (Sattentau *et al.*, 1991; Wyatt *et al.*, 1995; Stamatatos *et al.*, 1998). Sakaida and colleagues also showed that T-tropic HIV-1 derived V3 loop peptides bind to CXCR4 and inhibit T-tropic HIV-1 infection (Sakaida *et al.*, 1998). By analogy with the influenza hemaglutinin, it has been suggested that the HIV gp41 ectodomian undergoes major conformational changes during virus entry (Carr *et al.*, 1997). The proposed result of these changes is the insertion of the hydrophobic gp41 NH_2-terminsus (the "fusion peptide") into the membrane of the target T-cells. Mutagenic analysis (Freed *et al.*, 1996) and the recently determinsed crystal structures of HIV-1 gp41 ectodomain fragments (Chan *et al.*, 1997) are in accordance with this proposed model. The gp41 ectodomain structures reveal an extended, trimeric coiled coil that could potentially bridge the viral and target T-cell membranes (Chan *et al.*, 1997). Interactions of other gp41 helical segments near the membrane-spanning region with the interhelical grooves of the internal coiled coil are important for fusion-related conformational changes in gp41.

Recent studies have shown that the V3 region of gp120 is involved in the early step of virion-cell interaction indicating that the cell tropism is thought to be determinsed at the level of viral entry by the interaction of viral envelope proteins with certain types of co-receptors that support T-tropic or M-tropic HIV-1 infection. Binding experiments using mutant gp120 molecules with a deletion at the V3 region or anti-V3 loop neutralising antibody have strongly suggested that the V3 region is crucial for interaction with CCR5 (Wu *et al.*, 1996).

1.5.2 Viral DNA synthesis by reverse transcription

After entry of the core into the cytoplasm, the process of reverse transcription of the RNA genome into double-stranded DNA occurs (Figure 1.3). Reverse transcriptase requires two strand-transfers (or "jumps") to convert the dimeric single stranded viral RNA into a molecule of double stranded linear DNA. The viral RNA genome is synthesised using a host DNA-dependent RNA polymerase (DNA-dep RNA pol) and as a consequence contains cellular post-translational modifications, namely the 5' end of the genome is capped (Gppp) and the 3' end has a poly A tail. To enable efficient retroviral replication to occur there are three essential requirements: (a) two copies of the viral single-stranded RNA genome (template), (b) the host tRNA primer that is bound near the 5' end of the viral template (i.e. primer binding site, PBS), and (c) the heterodimeric protein that has both RT and RNase H activity.

The first step in reverse transcription occurs in the cytoplasm where DNA is synthesised from the tRNA primer which is bound to the viral RNA template by using the RNA-dependent DNA polymerase (RNA-dep-DNA-pol) activity of RT. The first strand synthesis (negative (-) strand) is extended by RT to the 5' end of the viral RNA genome; this is designated (-) strand strong stop DNA, which is a copy of the short region consisting of R and U5 lying between the primer-binding site and the 5' end of the genome. A second component of RT, RNase H degrades RNA in the DNA/RNA hybrid and thereby exposes newly synthesised strong stop DNA sequences which are complementary to the short repeat (R) at the 5' end of the viral genome. The newly synthesised DNA region acts as a second primer binding site to the complementary R at the 3' end of the genome. Initial strand-transfer, also known as the first "jump"

takes place. Synthesis of the (-) strand is then continued along the viral RNA template through U3 and into the viral genome using the RNA-dep-DNA-pol while RNase H degrades the remainder of the RNA template. Synthesis of (+) strand begins at the end of the (-) strand and continues through U5 and the tRNA primer. A second jump occurs, as the tRNA primer is complementary to the 5'PBS, completing the synthesis of the (+) DNA strand. Synthesis of the (+) strand can occur as an intermolecular or an intramolecular event.

1.5.3 Integration of viral DNA

Integration of a double-stranded DNA copy of the viral RNA into the host T-cell genome is a crucial step in a productive retroviral infection. Two regions of the viral genome are required for integration: the long terminsal repeats (LTR) ends of the viral DNA and the 3' region of the *pol* gene, which encoded the integrase protein. After viral DNA synthesis, the linear DNA, the CA, the integrase enzyme (IN) and the nucleocapsid (NC) protein enter the nucleus. Translocation of the preintegration complex into the nucleus is known to be mediated by nuclear localisation signals in the MA/Vpr proteins. Integrase cleaves the LTRs at conserved CA dinucleotides within the U3 and U5 (3' processing reaction) and subsequently joins the recessed viral ends to the 5' phosphates of a staggered double-strand cut in chromosomal target DNA (strand transfer reaction). The integrase reaction leaves single-stranded gaps and two mismatched nucleotides at each 5' terminsus. A cellular DNA repair system fills in the resulting gap in the molecule, displacing the two mismatched bases at the 5' end and ligating the remaining ends. The integrated HIV DNA genome is known as the HIV provirus.

1.5.4 Regulation of viral gene expression

Once the virus is integrated, it is treated as a normal cellular gene and the polymerase enzymes (cellular polymerase II) that transcribe cellular genes begin to produce viral RNA transcripts. Alternatively the virus may become transcriptionally silent and enter a latent stage, but may be reactivated subsequently. Viral RNA synthesis is complicated and involves several cellular proteins and viral regulators. Replication is mediated via transcription of the integrated DNA form of the virus (provirus) which behaves as a cellular gene with a promoter and transcriptional start site located in the 5' long terminsal repeat (LTR), and a terminsation/polyadenylation site located in the 3' LTR. The U3 domain of HIV-1 5' LTR contains basal promoter elements, including a TATAA box for initiation by host T-cell RNA polymerase II, and sites for binding the cellular transcription factor SP1. Initiation of viral RNA synthesis occurs at the U3/R border of the 5'LTR. First of all, an initiation complex for full-length viral RNA transcription is formed through interaction between HIV-1 Tat and nascent TAR RNA. Subsequently, an R-U5 segment is transcripted and serves as the leader sequences for RNA elongation. The 5' ends of the transcripts are post-transcriptionally capped with 7-methlyguanosine by cellular enzymes soon after elongation initiates. HIV-1 RNA is synthesised by cellular RNA polymerase II and the 3' end of the viral transcript is terminsated at the border of R/U5 in the 3' LTR. Signals in U3 region are recognised by cellular enzymes that add poly –A tails at the 3'-ends of viral transcript (Barre-Sinnoussi *et al.*, 1996; Luciw *et al.*, 1996). After transport to the cytoplasm, a fraction of these full-length RNA copies is reserved as the genomic RNA that is eventually assembled into progeny virions. The others serve

as precursors for alternatively spliced mRNAs that are translated in the cytoplasm to produce viral proteins (Barre-Sinoussi, 1996). High-level transcription from the provirus is regulated by the viral protein Tat. As described previously, Tat is typical of many transcriptional activators and includes an activation domain and a nucleic acid binding domain. Tat function is dependent on a bulged RNA-stem-loop structure, TAR (Tat activation region), that is present at the 5'-terminsus of all viral mRNAs. Although HIV-1 transcription is mediated by cellular RNA polymerase II, Tat acts mostly at the level of transcriptional elongation rather than at initiation itself, leading to the synthesis of fully spliced mRNAs. When sufficient mRNAs are produced, *rev* allows for the movement of the unspliced and singly spliced mRNA species from the nucleus to the cytoplasm. These late transcripts encode the structural and enzymatic proteins needed for virion assembly.

1.5.5 Virion assembly and release

The first event in virion assembly is the formation of the nucleoprotein complexes composed of p55 Gag as well as the Gag-pol polyprotein (p160) and genomic viral RNA. Cleavage of the Gag-containing polyproteins during assembly is mediated by the protease domain to produce a mature nucleocapsid composed of the fully processed Gag (MA, CA, NC, p6, p1 and p2) and Pol (PR, RT, RNase H, and IN) as well as two molecules of the viral single-stranded RNA genome. The viral glycoproteins are synthesised, glycosylated and processed by the endoplasmic reticulum and Golgi apparatus. The glycoprotein is cleaved into a membrane spanning and an extracellular region and associates to form dimers or trimers that migrate to the plasma membrane. These oligomers of the Env glycoprotein are inserted into the

cell plasma membrane, and the matrix domain of p55 is presumed to interact with the cytoplasmic tail of Env gp41 during virion assembly (Arroyo *et al.*, 1995). The viral nucleocapsid complex extrudes or buds through the plasma membrane to produce a virion with a nucleocapsid surrounded by a lipid bilayer membrane, which contains oligomers of Env gp. Selected host proteins are also incorporated into mature virus particles. Cell-to-cell spread of HIV is enhanced by the ability to form multinucleated giant T-cells, or syncytia. Syncytia are fragile and their formation enhances the cytolytic activity of the virus (Lifson *et al.*, 1986a; Lifson *et al.*, 1986b; Lifson *et al.*, 1986c) .

1.6 ORIGIN AND EVOLUTION

HIV-1 sequences pre-dating the recognition of AIDS may be crucial in defining the time of origin and the subsequent evolution of these viruses in humans. There are three principal groups of HIV-1: the main (M) group is responsible for the global AIDS epidemic and comprises by far the majority of HIV-1 isolates; an outlier (O) group is found in Cameroon, Gabon and Equatorial Guinea; and a new group (N) was recently identified in two people in Cameroon (Simon *et al.*, 1998). Group N is the least widespread of all HIV-1 lineages.

More extensive sampling of HIV-1 in Africa and elsewhere has revealed much greater diversity than previously recognised. Phylogenetic analyses of *gag* and *env* gene sequences have revealed up to ten clusters of the main, M, group of HIV-1 (Myers *et al.*, 1991; Louwagie *et al.*, 1993). This has led to the development of a subtype taxonomy (Louwagie *et al.*, 1993), with designations A to J applied to the phylogenetically clustered lineages (or clades). The subtypes can have complex

patterns of subclusters, sometimes associated with the geographic origin of the virus. The greatest diversity in terms of numbers of subgroups present is found in sub-Saharan Africa, in particular western Central Africa, including Zaire (Democratic Republic of Congo), Congo, Gabon, and neighbouring countries (Hu *et al.*, 1996). In East Africa, the D and A subgroups are most common, while in southern areas the C group is more predominsant. The major epidemics in other continents are mostly associated with a subset of these variants (Holmes *et al.*, 1992) with the B subtype characterising the epidemic in North America, Europe and Australia almost exclusively. Although the O group of viruses have lower numbers of infections, sequencing studies have revealed a startling level of diversity. Whereas most subgroups of the M group represent many thousands or even millions of infected individuals, most of the individual sequences from the O group are as different from each other as are the subgroups of group M (Loussertajaka *et al.*, 1995), possibly signifying an older, or even independent origin for the O group epidemic (Gurtler *et al.*, 1996).

The natural reservoir for HIV-1 remains unclear, although it is highly likely that known strains of HIV-1 have arisen in a similar fashion to HIV-2, namely following cross species transmission. Phylogenetic analysis has shown a close relationship between HIV-1 and SIV infecting chimpanzees (SIV_{cpz}), which may indicate the natural reservoir for HIV-1 is in fact the chimpanzee (Peeters *et al.*, 1992). Recently, Gao and colleagues provided new evidence which suggested that HIV-1 came to humans from the chimpanzee, *Pan troglodytes troglodytes*, which harbours a related simian immunodefiency virus, SIV_{cpz} and lives in the same part of central Africa where AIDS is thought to have arisen (Gao *et al.*, 1998). There are also

other chimpanzee subspecies infected with SIV_{cpz} that are able to transmit their viruses to humans (such inter-species transmissions of infectious disease are known as zoonoses (Gao *et al.*, 1998).

Four lines of evidence substantiate zoonotic transmission of primate lentiviruses; (1) similarities in viral genome organisation, (2) phylogenetic relatedness, (3) prevalence in the natural host and (4) geographic coincidence. Gao and colleagues' evolutionary analysis of the M, N and O groups in comparison to SIV_{cpz} isolates indicates that these HIV-1 groups represent separate transfers from chimpanzees to humans. Chimpanzees are commonly hunted for food, especially in west equatorial Africa (Teleki *et al*, 1989), and as a consequence represent a ready source for zoonotic transmissions. It was previously shown that several strains of HIV-2 in West Africa were independently derived from SIV_{sm} (sooty mangabey [*Cercocebus atys*]) (Chen Z *et al.*, 1997; Gao F *et al*, 1998). HTLV-1 also originated from related simian viruses, including STLV-1 of chimpanzees. Other explanantions for the origin of HIV-1 groups M, N and O, such as viral diversification within human populations or acquistion of virus from another primate species, are either inconsistent with phylogenetics or implausible (Gao *et al.*, 1998).

Since HIV-1 sequences first began to accumulate, researchers have been interested in estimating the age of the epidemic and the rate of viral evolution (Smith *et al.*, 1988; Li *et al.*, 1988; Gojobori *et al.*, 1990; Gao *et al.*, 1992; Louwagie *et al.*, 1993; Myers *et al.*, 1995). Estimates of HIV-1 divergence rates are highly dependent on the region of the genome under study (Leitner *et al.*, 1996). Divergence rates also have to allow for limitations such as recombination events and different evolutionary rates in different lineages. For example, the V3 variable domain of the envelope

protein is the most heavily sequenced region of the viral genome and an important functional and immunogenic region. In the D subtype, the V3 loop is mutating relatively rapidly, in the A and B subtypes it is mutating at a moderate pace, and in the C subtype it is changing slowly. Early analyses of HIV sequences have suggested dates for the separation of HIV-1 and HIV-2 from a common ancestor, which varied from as little as 40 years (Smith *et al.,* 1988) to as much as 1000 years (Gojobori *et al.,* 1990). The earliest sequence from a confirmed HIV-1 positive human sample was obtained in west-central Africa in 1959 (Nahmias *et al,* 1986). Phylogenetic analysis has placed this sequence close to the root of the major B and D subtypes, suggesting that these groups diverged only a short time earlier. This suggests that the major expansion/dispersion of HIV-1 M group occurred not long before 1959, perhaps 10 or 15 years earlier (Zhu *et al.,* 1998). The date of the nonhuman primate lentivirus precursor of HIV-1 is still unknown but it is an event which could have occurred hundreds or thousands of years earlier. The dynamics of the epidemic have never been regular, with the B subtype being the dominsant strain in the USA and Europe even though it is almost unknown in Africa (Hu *et al.,* 1996).

1.6.1 The natural history of HIV-1 infection

Infection with HIV-1 leads to a progressive impairment of cellular immune function, characterised by a gradual decline in peripheral blood CD4+ T lymphocyte levels which results in an increased susceptibility to a wide variety of opportunistic viral, bacterial, protozoal and fungal infections, and also to certain malignancies. The natural history of HIV-1 infection can be viewed as a progression of three distinct clinical stages: an initial stage associated with primary infection (CDC Category A), a

chronic stage resulting in a period of clinical but not virological latency (CDC Category B) and finally, a crisis stage representing a period of profound immunodeficiency manifest by the development of opportunistic infections and neurological disorders (CDC Category C).

Initial infection with HIV may be followed by an acute disease syndrome (Cooper *et al.*, 1985), similar, in some respects, to infectious mononucleosis. During this initial infection plasma HIV-1 titres increase manifested by high levels of viraemia and high levels of HIV replication; viral p24 antigen can be easily detected (Goudsmit *et al.*, 1987) and virus can be readily isolated from the blood (Barre-Sinoussi *et al.*, 1983; Gallo *et al.*, 1984; Coombs *et al.*, 1989; Ho *et al.*, 1989; Daar *et al.*, 1991; Saag *et al.*, 1991; Rouzioux *et al.*, 1992; Pan *et al.*, 1993). This florid HIV replication *in vivo* is likely to be associated with CD4 + T-cell death, although early in disease the ability to produce new naïve CD4+ cells allows for the maintenance of CD4+ cell levels within the normal range. The memory subset of CD4+ lymphocytes appears to be both readily infected *in vitro*, and more highly infected in vivo than naïve CD4 cells (Schnittman *et al.*, 1990). A second consequence of initial viremic infection is an apparent seeding of the lymph nodes with HIV, providing a continuing site of persistent viral replication (Pantaleo *et al.*, 1991; Saksela *et al.*, 1993; Embretson *et al.*, 1993; Pantaleo *et al.*, 1993). This acute stage of clinical infection is associated with an acute seroconversion illness characterised by fever, malaise, rash and myalgia (Ho *et al.*, 1985). CD4 counts fall dramatically during this stage of infection, although it is not clear whether this is a direct result of HIV cytopathicity or redistribution to extravascular sites (Fahey *et al.*, 1990; Ferbas *et al.*, 1996; Bofill *et al*, 1996).

Following the initial infection, HIV-infected individuals enter a stage of clinical latency, the asymptomatic period, during which the number of CD4+ cells remains within the normal range or slowly decreases over time (Ho *et al.*, 1985;). Studies by Wei *et al* and Ho *et al* have showed that although this is a clinically latent phase of HIV infection, it hides a very high rate of virus production (Ho *et al.*, 1995; Wei *et al.*, 1995). The increase in viral replication in infected individuals is largely the result of a dynamic process involving continuous rounds of *de novo* infection and replication in host T-cells, with rapid turnover of both free virus and virus producing cells. Recent evidence also suggests that significant replication persists in lymph node follicles (Embretson *et al.*, 1993) and other areas of lymphoid tissue (Graziosi *et al.*, 1993; Pantaleo *et al.*, 1991; Pantaleo *et al.*, 1993). Only low levels of plasma vireamia (30 TCID 50 per ml) (Ho *et al.*, 1989) is seen within this chronic stage of persistent infection and few productively infected CD4+ cells are present in the peripheral blood (1 in 10,000) (Harper *et al.*, 1986).

During the asymptomatic phase of HIV infection, there may be selective pressures favouring the emergence and persistence of non-syncytium inducing (NSI) strains (Asjo *et al.*, 1986; Tersmette *et al.*, 1988; Schuitemaker *et al.*, 1991). In the face of an effective host immune response, a HIV strain that replicates slowly and can persist in monocytes and macrophages may have a selective advantage over rapidly replicating strains (Wolfs *et al.*, 1992; Zhu *et al.*, 1993).

The development of symptomatic HIV infection is a continuum of progressive clinical states (Lane *et al.*, 1983). Initial symptomatic disease may be characterised by non-specific clinical presentations such as lymphadenopathy, diarrorhea, weight loss and recurrent candidal infections. Progression to clinically defined AIDS is

characterised by a dramatic loss of CD4+ lymphocytes and the development of opportunistic infections, AIDS encephalopathy, or characteristic malignancies. HIV can be isolated from as many as 1 in 100 peripheral blood mononuclear cells (PBMCs) (Ho *et al.*, 1989), and HIV DNA can be detected in up to 1% of CD4+ T-cells in late stages of disease (Schnittman *et al.*, 1990; Schnittman *et al.*, 1989). The course and pace of clinical progression are variable; epidemiological studies have suggested that before the advent of effective anti-retroviral therapy 50% of HIV-infected individuals developed full-blown AIDS by 8-11 years post-seroconversion (Rutherford *et al*, 1991, Blattner *et al*, 1991).

1.6.2 HIV-1 Serological diagnosis

Detection of infection with HIV-1 or HIV-2 can be accomplished by several techniques including the direct demonstration of virus (electron microscopy), virus isolation by culture techniques, demonstration of a virus-specific antibody response, and detection of viral antigen or the presence of the viral genome. Antibody tests combining a screening assay and a confirmatory test have been the most widely used techniques for establishing the presence of HIV infection. ELISAs are currently the preferred initial screening techniques, utilising inactivated virus or synthetic peptides as antigens. Some assays use recombinant HIV-1 proteins to *env* and *gag* products while the synthesised peptides correspond to conserved regions of *env* and p24 core proteins. Currently licensed ELISAs have a sensitivity and specificity of 98% to 99%. Western blot techniques are the most widely utilised confirmation assays for ELISA-reactive sera, although other methods such as fixed-cell immunofluorescence or RIPA have also been used.

HIV-1 may be isolated from peripheral blood leukocytes or genital secretions and may occasionally be isolated from plasma or a variety of tissue sites (e.g. brain, retina, bone marrow and lymph nodes). The virus is rarely isolated from saliva or urine. Diagnosis of HIV-1 infection is not always possible based on the presence of antibodies in the serum. For example, antibodies are not present in acute phase of infection (serological window period) and can be lost in the advanced stages of disease. Therefore diagnostic tests for either direct detection of the virus or its components may be important in monitoring the disease when no antibody is present. A relatively sensitive isolation technique is cocultivation of the test specimen with uninfected mitogen stimulated peripheral blood mononuclear cells. After incubation for several days to weeks, supernatant fluids are evaluated for reverse transcriptase activity or for HIV p24 antigens. Although virus culture can provide conclusive evidence for HIV-1 diagnosis, it is a costly, time consuminsg and labour intensive method with the potential for exposure to high concentrations of infectious virus.

Detection of circulating HIV-1 p24 antigens by ELISA appears to be a useful adjunctive test in certain situations. In the acute phase of infection, HIV p24 antigen is often present in serum before antibodies are detected. As antibodies appear, p24 antigen becomes undetectable and remains so until the development of AIDS when virus production increases dramatically. The low detection rate of p24 antigen in serum from asymptomatic patients may be due to low levels of antigen production or to the formation of immune complexes. Antigen assays, particularly those including acid dissociation which disrupt p24 antibody-immune complexes, have been found to be useful in improving the detection and quantitation of p24 antigen, allowing a more accurate assessment of *in vivo* viral replication. They have also proved important in

monitoring the progress of individuals receiving antiviral drugs, as demonstrated during trials of zidovudine and dideoxycytidine.

While HIV diagnosis remains serology-based (virus antibody/antigen), molecular techniques are gaining in use owing to advances in molecular biology. Traditional molecular approaches (Southern or dot-blot hybridisation and autoradiography) have been largely superseded by amplification techniques often using non-radioactive protocols. Amplification techniques, utilising PCR or other methodologies are becominsg increasingly utilised for the detection of viral RNA and cellular proviral DNA.

The branched DNA (bDNA) signal amplification assay (Urdea *et al.*, 1993) employs multiple DNA probes linked to an enzymatic system for visualisation of captured target molecules by sequential oligonucleotide hybridisation, and is an alternative to sequence amplification. The bDNA assay can employ a range of DNA probes, which recognise a wide spectrum of HIV sequence variants. The enhanced sensitivity (ES) modification of the prototype assay (Kern *et al.*, 1996) is capable of detecting as few as 400 copies per millilitre (copies per ml) of HIV-1 RNA in serum or plasma by using preamplification molecules and a range of target probes. This assay can suffer from inhibitory substances within the sample.

Detection of HIV proviral sequences in PBMCs by PCR remains the principal method of diagnosing HIV infection by molecular techniques. This is particularly relevant to HIV provirus detection where a potentially low peripheral load exists, consisting of rare sequences in a complex mixture of human genomic sequences, particularly in asymptomatic infection and neonatal infection where a molecular diagnosis would be of most use.

In the majority of individuals infected with HIV-1 not receiving antiretroviral treatment, HIV virion RNA is detectable in the cell-free fraction of peripheral blood (Piatak *et al.*, 1993; Mellors *et al.*, 1996; Bruisten *et al.*, 1997). The detection of HIV RNA may also be particularly appropriate during the acute phase of infection, before any other HIV markers become available. These assays included reverse transcriptase PCR (RT-PCR), bDNA and nucleic acid-based sequence amplification (NASBA). The presence of HIV-1 RNA in serum or plasma is readily detectable in these sensitive assays, (i.e. they measure down to at least 500 copies of HIV RNA) in the majority of individuals, and provides a clear diagnosis in the majority of cases.

HIV-1 replicates in lymph nodes and microglial cells, and the detection of HIV provirus in tissues at central sites of the body may further confirm the presence of HIV (Embretson *et al.*, 1993; Pantaleo *et al.*, 1993). While these are not front-line diagnostic procedures, the possible clearance of detectable virus from peripheral sites in HIV-infected individuals receiving potent combination antiretroviral therapy indicates that it may soon be a requirement to detect virus at central sites of the body.

The application of PCR to diagnose vertical transmission events in the perinatal and postnatal periods has several advantages over detection of virus-specific antibodies which may take 18 months to yield an unequivocal result, owing to the presence of passively acquired maternal antibodies. Transmission of HIV during pregnancy may be diagnosed by HIV provirus detection in infants as early as 6 weeks.

Recent estimates of virus production are of the order of $>10^9$ virions per day, and complete turnover of CD4+ cells occur in about 2 days (Ho *et al.*, 1995; Wei *et al.*, 1995). This represents an enormous replicative activity and together with the error-prone nature of the HIV reverse transcriptase, contributes to the tremendous

diversity of HIV. This diversity surely leads to competition for survival, the main driving force of Darwinian selection, and the consequent evolution of viruses that are best adapted to their changing environment.

1.6.3 Prognosis of HIV-1

Prognostic markers have helped distinguish the progressors from nonprogressors during the asymptomatic period. Decline of CD4+ T-cell counts is a universal characteristic of people progressing to AIDS and may be the hallmark of disease (Phillips *et al.*, 1991). Among the other parameters that are useful in predicting progression to disease are the high levels of β_2-microglobulin, soluble IL-2 or TNF receptors, and soluble CD8 in the blood; high levels of neopterin in the urine; low antibody titers to the p24 or p17 Gag proteins in serum; high levels of p24 or infectious virus in the blood; decreased dehydropiandrosterone levels in serum and the decreased delayed-type hypersensitivity reaction (Levy, 1993). During the asymptomatic phase of infection there appears to be an increase in CD8+ T-cells suggesting that this population may also contribute to the limiting of HIV-1 replication during this phase (Giorgi *et al.*, 1987; Lang *et al.*, 1989).

Although the accelerated viral replication characteristic of HIV infection is involved in the decline in CD4+ T-cells observed in disease progression (Ho *et al.*, 1995), factors not directly dependent on viral infection of target T-cells are also important in this process. Antigen-induced cell death (AICD) in HIV infection is mediated by at least two different components of the nerve growth factor/tumour necrosis factor receptor family: TNF-β (Clerici *et al.*, 1996) and APO-1/Fas (Kaisikis *et al*, 1995). Indeed, lymphocytes of HIV-seropositive individuals express Fas in large

quantities and are more prone to undergo programmed cell death upon ligation of this receptor and the magnitude of anti-Fas-induced death is inversely correlated with absolute CD4 cell counts (Debatin *et al.*, 1994, Kaisikis *et al*, 1995., McCloskey *et al*, 1995). Serum concentrations of TNF-β and sAPO-1/Fas are predictors of disease progression independent of HIV viral load (Medrano *et al.*, 1998).

Progression to HIV infection is characterised by complex dysregulation of Th that can involve the T-cells and/or affect antigen presenting cells (APCs) (Lane *et al*, 1985., Smolen *et al*, 1985; Giorgi *et al.*, 1987; Miedema *et al.*, 1988; Clerici *et al.*, 1989). Th defects (analysed as the ability of Th lymphocytes to proliferate and secrete IL-2) to soluble antigens appear early in disease. Soluble antigens are processed and presented to CD4+ Th on the surface of autologous APCs in association with class II molecules (Germain and Margulies, 1993). Thus, an alteration in the ability to recognise soluble antigens is the most sensitive and accurate index of dysfunction of CD4+ T lymphocytes. Because immune response to soluble antigens involves the collaboration between two cell type, APCs and Th (Noelle and Shaw, 1991), the primary defect could be due to an impaired ability of the APCs to process or present antigenic peptides or to provide other accessory functions. Phytohemagglutinin (PHA)-stimulated proliferation and IL-2 production become defective in HIV-seropositive individuals in whom Th defects to soluble antigens and alloantigens are already present. Because both CD4 and CD8 T-cells are stimulated by PHA and because the ability of this T-cell mitogen to stimulate lymphocytes is only marginally dependent on APCs, the inability to respond to mitogens is an index of a profound impairment in Th function in HIV-infected patients (reviewed in Clerici *et al.*, 1999).

The CD4 T-cell count is an important and reliable prognostic factor in disease progression and it can be used to monitor the effects of antiretroviral treatment. There are many methods available to assess the CD4+ T-cell count: the absolute CD4+ T-cell number in the blood, the percentage of CD4+ T-cells and the CD4+CD8+ T-cell ratio.

If HIV-specific CTLs are important in the control of viral replication, it may be expected that the presence or absence of such activity would have an influence on disease progression. Carmichael *et al* have showed that CTL precursor (CTLp) frequencies decline in parallel with the observed decline in CD4 counts as disease progresses (Carmichael *et al*, 1993). An initial inversion of the CD4:CD8+ T-cell ratio is another prominsent feature of HIV-1 infection although it is unclear as to whether this inversion is due to a decrease in the number of CD4+ T-cells or augmentation of CD8+ T-cells.

1.6.4 Classification system for HIV-1 infection (reviewed in Luciw, 1996).

Infection with HIV can lead to a range of clinical conditions, ranging from asymptomatic infection to severe immunodeficiency and the acquisition of numerous opportunistic infections and neoplasms. A number of classification systems for HIV-related illnesses have been proposed. The Centers for Disease Control (CDC) have constructed a system which classifies the manifestations of HIV-1 into three clinical categories, A, B and C (Table 1.3) (Centers for Disease Control, 1993). These categories respond to CD4+ T-lymphocyte counts per microliter of blood and guide clinical and therapeutic actions in the management of HIV-infected adolescents and adults.

Table 1.3: 1993 revised classification system for HIV infection and expanded AIDS surveillance case definition for adolescents and adults.

	Clinical Categories		
CD4 T-cell categories[b]	(A) Asymptomatic acute HIV or PGL[a]	(B) Symptomatic, not (A) or (C) conditions	(C) AIDS-indicator conditions
(1) >500/μl	A1	B1	C1
(2) 200-499/μL	A2	B2	C2
(3) <200/μL (AIDS indicator	A3	B3	C3

[a] PGL: Persistent generalised lymphadenopathy
[b] CD4 T-cell categories correspond to CD4 T lymphocytes per μl blood

1.6.4.1 Acute primary infection syndrome (Clinical Category A)

The CDC Category A group identifies patients who have recently undergone seroconversion, shortly following initial infection, which may be accompanied by an acute seroconversion illness, with clinical features such as a mononucleosis-like illness, fever, rigors, malaise, lethargy, anorexia, nausea, diarrhoea, sore throat and a truncal maculopapular, urticarial or vesicular rash. Neurologic signs and symptoms often predominsate, including headaches, stiff neck, neuritis, irritability, and depression. This illness may last from 2 to 3 weeks but usually result in clinical recovery. Incubation periods ranging from a few days to 3 months have been described and seroconversion usually occurs 1-10 weeks after the onset of acute illness.

1.6.4.2 Asymptomatic infection and persistent generalised lymphadenopathy (Clinical Category A).

Category A also includes asymptomatic patients showing no signs or symptoms of HIV-1 infection. This asymptomatic period may last from a few months to many years before any signs of clinical disease become apparent. In adults, mean incubation periods before development of disease has been estimated at 10 years in the absence of therapy. The most useful and available single measurement of prognosis appears to be the number or percent of CD4+ T lymphocytes in peripheral blood. The CD4 count gives an indication of the degree of immunosuppression and predicts the risk of developing AIDS. The ratio of CD4 to CD8 cells is also useful in reflecting the changes between the critically important T-helper cell and the cytotoxic-T suppressor cells. Plasma viral load measurements are also useful in predicting disease progression and together with CD4 measurements, they have proved to be very effective in determinsing an individual's response to therapy. Patients with persistent generalised lymphadenopathy are also grouped in clinical Category A. They are otherwise asymptomatic but have palpable lymphadenopathy (nodes >1cm) at two or more extrainguinal sites. Lymphadenopathy by itself is not a good predictor of disease progression.

1.6.4.3 Symptomatic HIV infection (Clinical Categories B and C)

The CDC Category B group consists of symptomatic conditions in an HIV-infected individual that are not included among conditions listed in clinical Category C. Some of these conditions may include oral hairy leukoplakia, herpes zoster, oropharyngeal candidiasis and listeriosis. After a variable period of asymptomatic

HIV seropositivity, a variety of symptoms may herald deterioration. Category C may consists of symptoms such as chronic fevers, night sweats, diarrhoea, weight loss, herpes zoster, oral thrush or hairy leukoplakia. These symptoms may occur individually, simultaneously, or sequentially. The term AIDS-related complex (ARC) is used when two or more symptoms or two or more laboratory findings are indicative of immune dysfunction. Once the diagnosis of AIDS is made, survival is often less than 2 years, although considerable variability exists, depending on factors such as age and therapy (Hirsch *et al.*, 1993).

1.7 EPIDEMIOLOGY (reviewed in UNAIDS/WHO 1998 and 1999 and references within).

After 15 years of study, much has been learned about the magnitude and distribution of AIDS and HIV-1 infection. Infection with HIV-1, HIV-2 or in some cases coinfection with both viruses is now widely accepted to be causally associated with the subsequent progression to severe immunodeficiency and acquired immune deficiency syndrome (AIDS). Type 1 HIV is recognised as the agent of the global AIDS pandemic, with HIV-2 mostly confined to the countries of West (DeCock *et al.*, 1993). The global pandemic of HIV infection comprises many separate epidemics in different geographic areas and is influenced by many factors including the time of introduction of the virus, population density and diverse cultural and social variables.

Estimates by the Joint United Nations Programme on HIV/AIDS (UNAIDS) and the World Health Organisation (WHO), indicated that at the end of 1999, some 33.6 million men, woman and children were infected with HIV and that 12 million people around the globe had already lost their lives to the disease. With 16,000 new

infections daily, the proportion of people living with HIV is set to grow even further. HIV/AIDS is one of the top ten killers worldwide, and may soon overtake well established causes of death such as diarrhoeal diseases. The overwhelminsg majority of people with HIV (95%) live in the developing world. It is clear that infection rates are rising rapidly in much of Asia, Eastern Europe and southern Africa.

Sub-Saharan Africa continues to bear the brunt of HIV and AIDS, with close to 70% of the global total of HIV-positive individuals. Life expectancy at birth in southern Africa, which rose from 44 years in the early 1950s to 59 in the early 1990s, is set to drop to 45 between 2005 and 2010. Heterosexual transmission is the main mode of transmission among African adults. New information suggests that between 12 and 13 African women are infected for every 10 African men. Female prevalence is higher due to the greater efficiency of male-to-female sexual transmission. HIV has also caused huge increases in death rates among young adults. This age factor makes AIDS uniquely threatening to children quite apart from the risk of vertical transmission. By the end of 1999, the epidemic had left behind a cumulative total of 11.7 million AIDS orphans.

HIV was a relative latecomer to Asia, but its spread has been swift, with over 4 million now thought to be infected (Quinn, 1996). Thailand was the first Asian country in which HIV was recorded. Transmission was generally concentrated in groups such as drug users and sex workers whose behaviour was known to put them at risk. (Morris *et al.*, 1996). Although no Asian country has reached anything like the prevalence in sub-Saharan Africa, by 1997 HIV was well established across the continent. The countries of South-East Asia, with the exception of Indonesia, the Phillipines and Laos, are comparatively hard hit, as is India. While prevalence remains

low in China, it was estimated at the end of 1996 that the number of people living with HIV/AIDS was over 200,000. Two major epidemics are underway in China; the first among increasing numbers of injecting drug users (Wu *et al.*, 1996) and the other newer epidemic, surfacing among heterosexuals, especially along the prosperous eastern region where prostitution is widespread. Thailand has recently shown a decrease in the number of new infections among sex workers and their clients. This is due to sustained efforts towards prevention aimed at increasing condom usage among heterosexuals and AIDS-awareness.

In Eastern Europe, HIV infection was not recognised until much later than in the USA or Africa. However, the pattern of consistently low prevalence began to change in 1995 in several of the countries of the former Soviet Union, Belarus, Moldova, the Russian Federation and Ukraine with new infections increasing six-fold in the past three years. Most of these new infections are related to intravenous drug users (IDUs) and their partners. In the Ukraine, 44 people from a survey of the whole population tested positive for HIV in 1994, roughly the same as in 1992 and 1993. But the number of diagnoses increased 30-fold in 1995 and exploded to over 12,000 in 1996. Now, just over four years later, more than 110,000 people are estimated to be infected. Injecting drug use gave the Eastern European and central Asian region the world's steepest HIV curve in 1999 (UNAIDS/WHO, 1999).

In 1997 it was estimated that 30,000 new infections occurred among Western Europeans. New infections are concentrated among IDUs in the southern countries of the continent, particularly Greece and Portugal. In Europe as a whole, the number of cases attributable to homosexual transmission decreased significantly from 62% to 36% between 1985 and 1992 (Quinn, 1996). The opposite trend for IDU has been

reported with an increase from 16 to 40% in recent years (UNAIDS?WHO). Similarly, transmission through heterosexual contact also increased. In Western Europe, the number of new AIDS cases decreased 38% between 1995 and 1997. This downturn is due to the new antiretroviral drug therapies, which postpone the development of AIDS and prolong the life of individuals living with HIV.

HIV infection amongst individuals in the Americas has been estimated at three million, with over one million in North America and two million in Latin America and the Carribean. There is a trend in these countries for a marked decline of HIV-1 among the heterosexual population (Heverkos *et al.*, 1995). In some disadvantaged sections of society in the USA, there is an increase in the number of AIDS cases. Among African –Americans, new AIDS cases rose by 19% among heterosexual men and 12% among heterosexual women in 1996. This is mainly due to unsuccessful prevention efforts in the minsority communities.

1.7.1 Transmission of HIV-1

HIV-1 transmission can be divided into four main groups depending on the mode of transmission (1) Sexually active individuals (homosexual and heterosexual), (2) intravenous drug users (IDUs) and their sexual partners (3) transfusion of infected blood products and (4) transmission from mother to infant. African-Americans and Hispanics are disproportionally represented in the HIV-1 positive population. Health care workers who may also come into contact with contaminsated blood or blood products are also at increased risk of infection.

Transmission through sexual contact accounts for 75 to 85 percent of the nearly 40 million worldwide infections with HIV that have occurred so far (Mann &

Tarantola *et al.*, 1998). HIV-1 is transmitted by both homosexual and heterosexual contact, and, as with other sexually transmitted infections, the likelihood of infections is related to the number of partners as well as to different sexual practices (Luciw, 1996). In the United States, anal intercourse between men has been the most common mode of transmission, whereas in Africa, the Caribbean, and Asia, heterosexual vaginal intercourse appears the dominsant mode of transmission. In both homosexual and heterosexual intercourse the receptive partner appears more at risk of infection than the insertive partner (Rodrigues *et al*, 1995). HIV-1 can be isolated from both semen and female genital secretions, and in both it appears to be largely cell associated. In cervical biopsies, HIV antigens are detectable in inflammatory cells, and genital ulcer disease has been associated with an enhanced risk of acquisition or transmission.

The primary risk of HIV transmission via blood is through intravenous drug abuse. HIV is transmitted among injecting drug users through the use of contaminsated needles and other equipment. Risk factors include frequency of needle sharing, frequency of injections, use of "shooting galleries", and prevalence of HIV infection in the area. There is also an increased risk of transmission to sexual partners of intravenous drug users (Ronald *et al.*, 1993). Similarly, frequent drug abuse is associated with other high-risk behaviours such as sexual promiscuity. For example, prostitution is frequently motivated by the cost of drug addiction (Celentano *et al.*, 1994).

Before 1985, individuals receiving blood transfusions or organ transplants, and haemophiliacs receiving clotting factors from pooled blood were at high risk of HIV infection. Screening of the blood supply has practically eliminsated transfusion-related

transmission of HIV. Haemophiliac recipients of pooled clotting factors are protected further by appropriate handling of the factor (prolonged heating) to inactivate the virus. A small number of health care workers have contracted HIV infection from accidental needle sticks, cuts, or exposure of broken skin to contaminsated blood. To date, there has only been one case where a health care worker has infected patients. This involved the transmission of HIV from a dentist to a number of patients (Ou *et al.*, 1992). Prospective studies of needle stick recipients indicate that fewer than 1% of those exposed to HIV-positive blood experience seroconversion. Tattoo needles, acupuncture and other invasive body piercing instruments that may have come into contact with contaminsated blood are other potential vectors of HIV transmission.

Most HIV-1 infections of children result from mother-to-infant transmission, which has been shown to occur *in utero* (intrauterine or transplacental), at the time of delivery through exposure to an infected genital tract (perinatal) or postnatally, as a consequence of breast feeding (Mofenson, 1998; Peckham *et al.*, 1995). The rate of perinatal transmission has ranged from 13 to 40%, with the highest rates being reported in Africa where rates of breast-feeding are highest and the severity of maternal HIV disease has been greatest.

A number of potential routes have been shown not be important in the transmission of HIV, including non-sexual personal contact such as touching and hugging, exposure to saliva, coughing, sneezing, insects, water, food or utensils, toilets, swimminsg pools and public baths.

1.8 IMMUNE RESPONSE IN HIV-1 INFECTION

After a burst of virus production during primary infection by HIV-1, the plasma viral load declines, which may indicate either a diminsishing pool of susceptible cells or an effective immune response. Subsequently, HIV-1 undergoes antigenic drift, especially in epitopes that lead to generation of neutralising antibodies, bind selective co-receptors, or participate in cytotoxicity which indicates a continous and effective immune response by the human host. It is not known exactly which components of the immune system are necessary for protection from natural infection. The immune system must elicit effector T-cell responses to eliminsate virally infected cells, and produce antibody responses to neutralize cell-free virus. Despite the devasting effects of HIV-1 on host immunity, infected individuals develop both humoral and cell-mediated responses against HIV-associated antigens. These include virus-specific neutralizing antibodies, antibody-dependant T-cellular cytotoxicity (ADCC), cytotoxic T lymphocytes (CTL), natural killer (NK) cells, and complement-dependent lysis (Pantaleo *et al.,* 1995). In most patients, antibodies develop within 4-6 weeks post infection. They are detected by an enzyme-linked immunosorbent assay (ELISA) and their specificity for envelope, gp120 (*env*), p17 (core) and p24 (matrix) from the *gag* protein and p53 and p64 from the polymerase (*pol*) proteins can be confirmed by Western blotting. Antibodies against *env* persist throughout infection while antibodies against *gag* decline during advanced infection. Antibodies to the transmembrane glycoprotein, gp41, are often not detected until several weeks later. Virtually all neutralising activity in the sera of infected individuals is directed against these proteins and particularly against regions of gp120. Within 2-3 months of primary HIV infection, neutralising antibodies develop that typically recognise

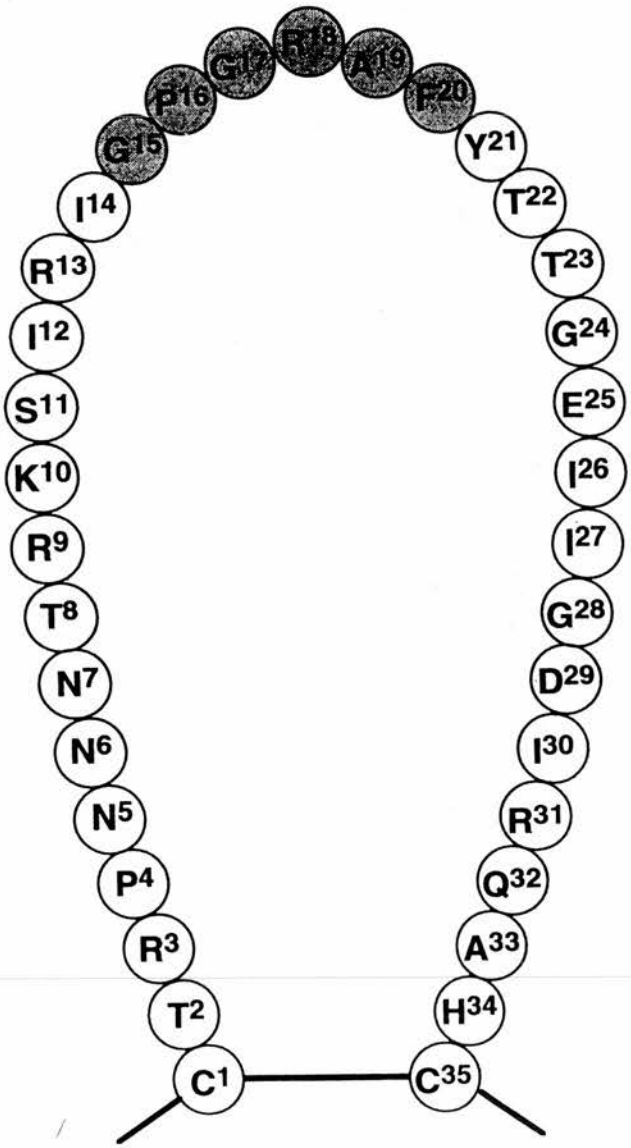


Figure 1.4 The V3 Loop (modified from Fields Virology, 1996).

epitopes on the V3 loop (Figure 1.4), an exposed 38-aminso acid segment of the viral envelope protein, gp120. Unfortunately, the aminso acid sequence of the V3 loop differs between HIV strains; thus V3-specific antibodies that neutralise one virus strain often fail against others. Some investigators have noted a correlation between progressive disease and low neutralising antibody titers in serum (Ho *et al.*, 1985), whereas others have not (Groopman JE *et al* , 1987., Robert-Guroff M *et al*, 1987,). The V3 hypervariable region has been found to be the principal neutralising domain in HIV-1 infection. V3 neutralising antibodies can prevent HIV-1 entering target T-cells, although they do not necessarily abrogate binding to the cell via the gp120-CD4 interaction. The V3 loop can present both linear and conformational determinsants for antibody recognition. Later in infection, other neutralising antibodies develop and recognise the CD4-binding site located in the carboxy-terminsal region of gp120. This CD4-binding region appears to be a complex folded epitope that is relatively conserved between different virus strains: antibodies that are directed against the CD4-binding region can neutralise a broad range of HIV strains. Neutralising antibodies directed against V1 and V2 hypervariable regions of gp120 have also been detected. As with the V3 loop, linear and conformational determinsants appear to be involved.

1.8.1 Humoral responses to HIV-1 infection

HIV-specific humoral responses can be detected readily during primary infection, but are mostly comprised of low-avidity Env-specific IgG antibodies that possess little or no neutralising activity. In fact, emergence of significant neutralising titers does not generally take place before transition to chronic HIV infection. Delays

in antibody response might be due to the dysfunstion and depletion of CD4+ Th cells which in turn interfere with the T-cell-B-cell interaction, altering the antibody response such as avidity maturation and neutralizing activity (Soudeyns *et al.*, 1999). The principal neutralisation mechanism for HIV-1 involves coating the virus by antibody obstructs the close approach of virion and target T-cell, thereby effectively inhibiting virus-cell attachment. The HIV envelope is the major target for humoral antibody responses. Six regions of the viral envelope are involved in HIV-1 neutralisation. These have been localised to the envelope gp120 and the external portion of gp41. It was originally documented that the principal neutralising domain (PND), was the central portion of the third variable region (V3 loop; aa308-322), located in the N-terminsal portion of the gp120. More recently however, it has become increasingly clear that the V3 loop of HIV-1 primary isolates is not an important target for neutralising antibodies generated by infection or by candidate HIV vaccines.

Although V3 is a variable region, the PND is a linear epitope conserved among many strains or differing only slightly in aminso acid structure. Recent studies concerning the molecular events driving HIV-1 neutralisation suggest that the dominsant mechanism may be simpler than originally thought: occupation by antibody of a minsimum number of binding sites on the envelope glycoproteins may be the principal factor determinsing neutralisation, implying that antibody affinity and concentration are more important criteria than epitope specificity. The V3 loop is not a significant epitope for primary virus neutralisation; any neutralisation achieved by V3-directed antibodies, however, appears to be mediated by inhibition of virion

attachment, not later stages of the fusion process (Spenlehauer *et al.*, 1998; Stamatatos *et al.*, 1995). The V3 loop can have both neutralising and non-neutralising epitopes, since sera with high-titer antibody to V3 peptides do not always neutralise the homologous HIV-1 strain (Kostrikis *et al.*, 1996; Moore *et al.*, 1998). The lesser role of the V3 loop in primary virus neutralisation appears to be a consequence of its decreased accessibility in the mature oligomeric envelope of primary isolates, leading to a lower affinity to this region. Work with escape mutants has indicated that sites within and outside the V3 loop can be involved in efficient antibody neutralisation (Yoshiyama *et al.*, 1994). The V3 region can be both a linear and conformational determinsant for antibody recognition. In this regard, glycosylated forms of the HIV-1 envelope can be better recognised than nonglycosylated proteins by immune sera (Moore *et al.*, 1996; Wyatt *et al.*, 1998). Another major neutralising region on gp120 is the CD4-binding domain. Antibodies that cross-neutralise a large number of different strains, including those with different V3 regions, are directed against this region. The determinsant is generally conformation dependent. Finally, the V2 region is also another neutralising region of gp120 (Yoshiyama *et al.*, 1994). Similar to the V3 loop, both linear and conformational determinsants are involved in this region which is also recognised by antibodies to both glycosylated and non-glycoylated regions. The clinical relevance of these neutralising antibodies remains uncertain since AIDS patients often elicit antibodies that enhance rather than neutralise infection by the virus found in the patient. The virus changes under immunologic responses to escape neutralisation (Burns *et al.*, 1993; Wei *et al.*, 1995; Nyambi *et al.*, 1997). Thus, the induction of neutralising antibodies would appear to be most beneficial early

in the course of HIV infection and to have less influence in later (Wei *et al.*, 1995; Lathey *et al.*, 1997).

Antibodies that bind effectively to HIV-1 envelope glycoprotein on virions or infected cells may either inactivate or enhance viral infectivity through Fc-mediated effector mechanisms. These include: antibody-dependent T-cell-mediated cytotoxicity (Evans *et al.*, 1989; Koup *et al.*, 1989; Ljunggren *et al.*, 1987; Ljunggren *et al.*, 1988; Ljunggren *et al.*, 1989), antibody-dependent complement-mediated virolysis (Wahren *et al.*, 1987), and antibody-dependent enhancement of infection (Takeda *et al.*, 1988; Homsy *et al.*, 1989; Laurence *et al.*, 1990; Reisinger *et al.*, 1990; Robinson *et al.*, 1990; Tremblay *et al.*, 1990).

1.8.1.1 Antibody dependent T-cell mediated cytotoxicity

Antibody-dependent T-cell-mediated cytotoxicity (ADCC) directed against HIV-1 infected cells has been demonstrated by a number of investigators. Such activity appears to reside in IgG fractions and is directed predominsantly against epitopes on gp120 and gp41 (Evans *et al.*, 1989; Koup *et al.*, 1989; Ljunggren *et al.*, 1987; Ljunggren *et al.*, 1988; Ljunggren *et al.*, 1989). Antibody-antigen-coated cells are recognised by effector NK cells bearing the Fc receptors or by other monocytes and killed by a cytotoxic mechanism, most probably cytokine mediated (Yagita *et al.*, 1992). Only certain epitopes on HIV envelope proteins induce this response, since not all anti-Env antibodies produce this activity and they can be distinguished from neutralising antibodies (Bottiger *et al.*, 1988). A detrimental effect of ADCC could be the release by cell destruction of large quantities of infectious particles with subsequent spread in the host. Antibodies active in ADCC are present in substantial

titers during the course of infection (Evans *et al.*, 1989) but eventually decline with progression of disease. The extent of effector-cell activity is the important parameter influencing the ability of the ADCC process (Brenner *et al.*, 1991; Szelc, *et al.*, 1992; Tyler *et al.*, 1990).

1.8.1.2 Antibody-dependent complement mediated virolysis

Antibody bound to HIV-1 virions activates complement in human plasma. HIV-1, however, carries complement-regulatory molecules, which are acquired from the host T-cell membrane during budding, or recruited from plasma by attachment to HIV-1 envelope glycoproteins. These regulatory molecules promote the resistance of virions to virolysis by inhibiting full activation of the complement cascade (Wahren *et al.*, 1988). Primary isolates of HIV-1 have been found to be more resistant than TCLA viruses to complement-mediated virolysis (Spear *et al.*, 1990; Saifuddin *et al.*, 1995; Sullivan *et al.*, 1996). The reistance of primary isolates to virolysis was found to directly correlate with the poor exposure of antibody binding sites on the primary virus envelope (Takefman *et al.*, 1998).

1.8.1.3 Antibody-dependent enhancement of infection

Binding of neutralising antibody does not always result in a decrease of virus infectivity. The clinical importance of antibody dependent enhancement (ADE) in still relatively unknown, but its association with disease suggest that it has a role in pathogenesis. Both Fc receptor mediated (FcR-ADE) and complement-mediated (C'ADE) antibody dependent enhancement of infectivity have been described for HIV-1 (Robinson *et al.*, 1988; Takeda *et al.*, 1988; Homsy *et al.*, 1989; Laurence *et*

al., 1990; Reisinger *et al.*, 1990; Tremblay *et al.*, 1990). It has been shown that FcR-ADE is CD4 dependent; it acts through stabilisation of the virus-cell interaction rather than via Fc receptor-mediated internalisation of HIV-1 (Connor et al., 1991). Complement receptor 2 (CD21) can mediate C'ADE, either by acting as a receptor for HIV-1 opsonised with antibody and complement, or by increasing virus binding to the cell due to an interaction of CD21 with opsonised virus (Boyer *et al.*, 1991).

1.8.2 CELL MEDIATED IMMUNE RESPONSES TO HIV-1 INFECTION

1.8.2.1 CTL Response

Cellular responses in HIV-infected individuals were first described more than 10 years ago. High frequencies of cytotoxic T-lymphocytes (CTLs) have been detected in peripheral blood and a general loss of CTLs has been observed with disease progression (Skolnick *et al.*, 1993). These cells recognise virus-infected cells by a direct interaction with the T-cell receptor (TCR) of the CTL and a viral peptide presented by MHC class I molecules on the surface of the infected cell (Goulder *et al.*, 1999). *In vitro* models using cloned CTLs have shown that they can dramatically inhibit HIV replication and under idealised conditions can clear infectious virus from cultures (Yang *et al.*, 1996). In such circumstances, inhibition of viral replication can be cytolytic and non-cytolytic (Yang *et al.*, 1997). CTLs react with *env*, *gag*, and *pol* regulatory gene products. *Env*-specific CTL reactivity is seen in nearly all infected individuals while *Gag* and *Pol* reactivity appears less constant. CD8+ cells have been shown to inhibit HIV-1 replication in CD4 cells *in vitro*. Immune reconstitution and CTL depletion studies indicate that HIV replication and the level of viraemia are controlled by HIV-specific CTLs (Brodie *et al.*, 1999; Jin *et al.*, 1999) and the

correlation of strong, HIV-specific responses mediated by CD4+ T-cells with viral load indicates that Th cells may also play an important role in immune control of HIV-1 (Rosenburg *et al.*, 1997). Because neutralising antibody responses do not appear until several weeks after viraemia has subsided; it is possible that CTL responses are the only mechanism involved in viral clearance during primary HIV infection.

CTLs have also been reported in persistently uninfected people who have been exposed to HIV or in transiently infected individuals, suggesting that these persons may have generated a protective CTL response. Studies of long-term non-progressors infected with HIV-1 suggest that low viral load is associated with apparently effective neutralizing and CD8+ lymphocytes responses. (Pantaleo *et al.*, 1995). CD8+ T-cells seen in peripheral blood in chronic infection has been shown to represent clonally expanded, antigen specific CTLs (Tan *et al.*, 1998) and CTLs can be readily demonstrated in peripheral blood, lymph node, skin and semen of infected persons (Bachelez *et al.*, 1998; Quayle *et al.*, 1998). It is only recently that the contribution of these responses to immune control of HIV has been widely appreciated. Advances in the development of more sensitive and quantitative assays (Altman *et al.*, 1996) have provided the first link between CTLs and control of viraemia (Ogg *et al.*, 1998). Natural killer (NK) cells can also display cytotoxic reactivity against HIV-1 gp120 (Tyler *et al.*, 1989; Szelc *et al.*, 1992). NK cells recognise and kill virally infected cells in a non-MHC-directed manner. In HIV infection NK cells are found to have decreased function, particularly as individuals progress towards disease.

1.8.2.2 T-helper cell response

Studies by Clerici have suggested that specific subsets of CD4+ T-helper cells are instrumental in determinsing the extent of cell-mediated and humoral immune responses (Clerici *et al.*, 1993). One T-helper cell subset (Th1) produces interferon-γ and IL-2 which increase cellular immunity while the other subset (Th2) produces IL-4 and IL-10 which enhance antibody production. The interaction of the Th1 and Th2 cell subsets is competitive and over-expression of cytokines by one cell type can suppress the activity of the other. A predominsance of Th2 cells could be responsible for the decrease in Th1 activity. This would be reflected by a high antibody production in the absence of a strong CD8+ cell response. IL-2 produced by Th1 cells increases CD8+ cell anti-viral activity, whereas IL-10 produced by Th2 blocks this activity (Levy *et al.,* 1993). How well HIV-specific CD4+ T-cell responses can be restored in chronic infection has yet to be determinsed. Moreover, the greater the pretreatment CD4+ T-cell loss, the more difficult it may be to fully recover a functional T-cell repertoire. Th1 responses are found primarily in healthy, asymptomatic individuals and in high-risk individuals who do not show evidence of HIV infection. It has been suggested that this type of response helps induce cell-mediated immunity. It has also been suggested that virus-specific T-helper cell responses are critical to the maintenance of CTL function. In the absence of ongoing helper cell function, CTL responses decline and there is a lack of control of viremia during the chronic phase of infection (Goulder *et al.*, 1999). Rosenburg *et al* revealed that a unique subset of long-term non-progressors could control viremia in the absence of antiviral therapy indicating that HIV can induce robust T-helper cell responses in some individuals. In this small subset of persons controlling viremia, both

strong CTL responses as well as strong Gag-specific T-helper cell responses are detected, consistent with the murine data which indicates a need for T-helper cell responses in order to maintain control of viremia (Rosenbug *et al.*, 1997). Other studies have indicated an association between strong T-helper cell responses and strong CTL responses (Kalams *et al.*, 1998), indicating that these two arms of the cellular immune response are coordinately linked, and providing support for the hypothesis that T-helper cells may mediate their antiviral effect through facilitation of CTL responses.

1.8.2.3 Natural killer cells

Cytotoxic reactivity against HIV-1 gp120 can also be displayed by non-T-effector cells phenotypically resembling those with natural killer (NK) activity (Tyler *et al.*, 1989; Szelc *et al.*, 1992). NK cells recognise and kill virally-infected cells in a non-MHC-directed manner. In HIV infection, this cell type has found to have decreased function, particularly as infected individuals progress to disease. NK cells most probably play the important role in ADCC (Szelc *et al.*, 1992); polymorphonuclear leukocytes could aslo be involved. CD16+ NK cells carrying the anti-gp120 antibodies can be detected in the blood (Tyler *et al.*, 1989).

1.8.2.4 Monocytes

Monocytes-macrophages are crucial in the phagocytosis and eliminsation of foreign organisms, the presentation of antigen to lymphocytes and the secretion of regulatory cytokines. Hence, infection of these cell types would be expected to result

in a panoply of immunologic abnormalities and these have been amply demonstrated in AIDS patients.

It is not clear what host responses are important in protection against HIV-1 infection or disease, and it is also not clear whether such a protective response can be elicited. Overall, HIV-1 has evolved to express an envelope structure of minsimal antigenicity and immunogenicity in its oligomeric form, a strategy which enables it to evade humoral immunity. The virus has also an ability to rapidly escape any minsimal neutralising antibody response that does develop (Burns *et al.*, 1993; Wei *et al.*, 1995; Nyambi *et al.*, 1997). Furthermore, it may spread through a cell-to-cell route against which antibody is relatively inefficient. Neutralising antibody effective against this challenge virus can protect against infection but may not affect the course of an established infection. Neutralising antibodies may play a role in the critical early phase of infection, since by reducing the viral inoculum, they may buy cell-mediated immunity time to mature and to clear cells that do become infected.

1.9 ANTI-RETROVIRAL TREATMENT FOR HIV-1 INFECTION.

The need for highly active antiretroviral therapy regimens (HAART) is highlighted by the lack of an effective anti-HIV vaccine in the foreseeable future and the limited possibility of altering human behaviour to control the HIV pandemic (Luciw *et al.*, 1996). HIV poses several difficult challenges for the development of effective antiviral therapies. Firstly, proviruses have a chance of remaining latent within the cell (Chun *et al.*, 1997; Finzi *et al.*, 1997; Wong *et al.*, 1997; Finzi *et al.*, 1999; Zhang *et al.*, 1999). Although anti-HIV drugs inhibit replicating virus and reduce viral load in infected individuals, proviruses within quiescent T-cells will not be

affected. Secondly, HIV is disseminsated in T-lymphocytes and monocyte/macrophages throughout the body, including the CNS. Delivery of the antiviral agent to all target T-cells is difficult to achieve. Thirdly, reverse transcription is a process that does not incorporate proof-reading to eliminsate misincorporated nucleotides; therefore variant genomes including antiviral resistant strains are readily generated (D'Aquila *et al.,* 1995; Welles *et al.,* 1996).

In individuals with HIV-1 infection, recent antiretroviral drug combinations have proven remarkably successful at suppressing virus production and reducing lymphoid tissue viral reservoirs to levels near or below detection levels. To understand how to interpret changes in plasma HIV RNA and other viral parameters after the institution of antiretroviral therapy it is important to know exactly how the drugs act. The two main classes of antiretroviral drugs, reverse transcriptase inhibitors and protease inhibitors, both prevent infection of new cells. For reverse transcriptase inhibitors the viral RNA is unable to reverse transcribe fully into DNA and hence the virus is unable to infect the cell (Larder & Kemp, 1989; Larder et al., 1991; Kellam et al., 1992; 1994). Protease inhibitors (PIs) prevent infected cells from producing replication-competent virions and hence new infection of cells is prevented owing to the lack of infectious particles (Kozal *et al.*, 1996). There are currently 15 antiretroviral agents approved for the treatment of HIV infection (table 1.4). These drugs may be used alone, or as components of double or triple therapy.

Table 1.4 Current Antiretroviral therapy

Drug Therapy		
HIV Protease Inhibitors	Indinavir	Abbreviation
	Nelfinavir	
	Ritonavir	
	Saquinavir	
Nucleoside Analogues	Didanosine	ddI
	Lamivudine	3TC
	Stavudine	d4T
	Zidovudine	AZT
	Zalcitabine	ddC
Nonnucleoside-reverse transcriptase inhibitors	Delaviridine	
	Nevirapine	

The first treatment available was Zidovudine (ZVD/AZT), a nucleoside reverse transcriptase inhibitor (Yarchoan *et al.*, 1986). It acts as a competitor for nucleotides used by the polymerase and is a potent chain terminsator of viral DNA synthesis. In 1990 two studies showed that zidovudine delayed the progression to severe AIDS related complex (ARC) and AIDS in patients with early symptomatic disease and in those who were symptomless (Fischi *et al*, 1990., Volberding *et al*, 1990). A number of early studies suggested that early adminsistration was more effective in slowing progression to disease (Yarchoan *et al.*, 1986; Graham *et al.*, 1992; Cooper *et al.*, 1993; Fauci *et al.*, 1993; Volberding *et al.*, 1995). However, the Concorde study found that no important clinical benefit was observed in symptomless patients when immediate and deferred treatment policies were compared (i.e. starting AZT after randomisation, or delaying it until the onset of symptomatic disease or a CD4 count at which the onset of symptomatic disease was imminsent) (Concorde coordinating committee 1994).

The beneficial effects of AZT may persist for months to years, but eventually failures occur, in part related to the development of viral resistance. The rapid turnover of the virus population, which has a half-life of 1-2 days, coupled with the high rate of HIV-1 mutation, provides the ideal machinery for producing drug-resistant variants (Ho *et al.*, 1995; Perelson *et al.*, 1996; Wei *et al.*, 1995). The advantages of AZT monotherapy have been limited with the emergence of drug-resistant strains, especially after prolonged therapy, and this associated with mutations arising at four positions in HIV RT (Boucher *et al.*, 1992). Other nucleosides that have shown promise in HIV-1 infection are didanosine (ddI), zalcitabine (ddC), lamivudine (3TC) and stavudine (d4T). They are all less effective than AZT as initial therapy for advanced infection but are useful as alternatives for patients who have failed or are intolerant of AZT.

A number of studies have been carried out to investigate the therapeutic potential of combination therapies using two and three combined drug regimens (Caliendo *et al.*, 1994). In the first trial, patients were randomised to receive either AZT alone, AZT plus didanosine or AZT plus zalcitabine (Delta Coordinating Committee, 1996). Combination therapy was shown to improve survival over monotherapy while also significantly delaying progression to AIDS or death. A similar double blind trial comparing the combination of three drugs (AZT, saquinavir, zalcitabine) was found to result in a considerably more favourable outcome than with combinations of AZT and saquinavir or AZT and zalcitabine (Collier *et al.*, 1996). Katlama and Staszewski also showed that the effects of double nucleoside combinations on viral load were superior in the short term to those seen with monotherapy (Katlama *et al.*, 1996; Staszewski *et al.*, 1996). Other phase II clinical

trials are also showing the superior effects of triple combination regimens including PI inhibitors or NNRTIs over double and monotherapy arms in terms of change of CD4 counts and viral load over 24-52 weeks (Gulick *et al.*, 1997).

Combination therapy with one or more nucleoside analogue drugs may produce additive or synergistic effects; have activity in different T-cell populations; reduce the emergence of resistance; decrease viral fitness; decrease toxicity by the use of lower doses of drugs with non-overlapping toxicity profiles; and perhaps advantageously exploit pharmacokinetic interactions (Knox *et al.*, 1996; Larder., 1994). It has become apparent from recent studies that HIV-1 has the capacity to mutate in order to become resistant to antiretroviral agents. Monotherapy with nucleoside analogues or non-nucleoside reverse transcriptase (RT) inhibitors (NNRTIs) has, without exception, resulted in the emergence of drug-resistant virus. This may only take a matter of weeks with NNRTIs or months to years in the case of AZT, ddI and ddC (Larder *et al.*, 1989a; Larder *et al.*, 1989b; Boucher *et al.*, 1992; Boucher *et al.*, 1993; Tisdale *et al.*,1993; Schinazi *et al.*, 1993).

The limited success of nucleoside inhibitors (ddI, ddC, AZT and d4T) in the treatment of HIV infection led to the development of drugs that use an alternative target. Inhibitors of HIV-encoded protease, combined with nucleoside analogues with antiretroviral activity, cause profound and sustained suppression of viral replication, reduce morbidity, and prolong life in patients with HIV infection. Recent guidelines recommend that initial treatment of all HIV-infected patients include the adminsistration of an HIV protease inhibitor. HIV protease inhibitors prevent cleavage of Gag and Gag-Pol protein precursors in acutely and chronically infected cells, arresting maturation and thereby blocking the infectivity of nascent virions.

Protease inhibitors have no effect on cells already harbouring integrated proviral DNA. Protease inhibitors (PIs) have become the first-line antiviral agents for the control of HIV-1 infection and are widely used in HAART regimens. The four approved HIV-protease inhibitors are based on aminso acid sequences recognised and cleaved in HIV proteins, indinavir, nelfinavir, ritonavir and saquinavir. In three large clinical trials, protease inhibitors with nucleoside analogues slowed the progression of disease and improved survival (Cameron *et al.,* 1999; Hammer *et al.,* 1996; Mclaren *et al.,* 1996).

A number of aminso acid mutations in the protease protein are associated with the emergence of resistant clinical isolates (Schinazi *et al.,* 1997). Single or primary mutations do not lead, in the main, to high levels of resistance, but subsequent secondary mutations may arise in order to restore viral replicative fitness (Roberts *et al,* 1998). Furthermore, resistance may arise not only to the protease inhibitors being given but also to other protease inhibitors (Condra *et al.,* 1995). No firm recommendations are presently possible for patients who fail PI-containing regimens.

Treatment failure has been defined in virological terms as the inability to completely suppress viral replication. Once multidrug resistance is present, regaining control of vireamia becomes difficult because no effective 'salvage' strategy has been devised. The factors leading to this type of failure are straightforward: poor adherence to HAART, prior exposure to antiretroviral drugs in mono- or bi-therapy, the sequential addition of drugs to a failing regimen, the phenomenon of 'quasispecies' (the simultaneous presence in a patient of a swarm of viral variants), and the extent of cross-resistance among antiviral drugs used . More recently, the transmission of viruses resistant to inhibitors of both RT and protease have been reported (Hecht *et*

al., 1998., Boden *et al.*, 1998). The optimal initial treatment strategy to avoid the emergence of drug resistance is the use of a maximally suppressive antiviral regimen. Most physicians now begin therapy with a triple combination consisting of two nucleosides and either a PI or an NNRTI. However, it cannot be assumed that this is applicable to all infected individuals. Those individuals with more advanced disease may need to be treated with four or even five drug combination regimens to attempt maximum suppression of virus replication. Once a course of treatment is initiated, it is essential to monitor the response by following general health, the CD4 count, the plasma viral load and the emergence of resistant viral strains. Viral eradication is not achievable with current strategies (Wong *et al.*, 1997), and the shift in treatment paradigm to one of long-term suppression has led to the challenge of ensuring continuous treatment benefit and avoiding failure (Carpenter *et al.*, 1997).

1.10 MOLECULAR EVOLUTION

The complex evolutionary process of HIV-1 is marked by a high level of genetic variation. RNA viruses show high mutation rates. Because of this high mutation rate and high rates of replication, the rates of RNA genome evolution can be more than a million-fold greater than the rates of the DNA genome of their hosts (Holland *et al.*, 1982). Molecular evolution involves the reconstruction of the evolutionary history of genes and organisms, also known as molecular phylogeny, inferred from molecular data.

1.10.1 Genetic distance estimation

The evolutionary distance between a pair of sequences is usually measured by the number of nucleotide or aminso acid substitutions between them. The mutation rate of a virus can be defined as the probability that during a single replication of the virus genome a particular nucleotide position is altered through substitution, deletion, insertion or recombination. There are many methods for estimating evolutionary distances, and these vary in their accuracy and in the extent to which they have been applied to different viruses (Jukes-Cantor, Tajima-Nei, Kimura 2-parameter model, etc). In this study the Jukes-Cantor method was used (Gojobori et al, 1986; Nei et al, 1987). Like the other methods it is relatively simple and frequently used by molecular evolutionists.

The Jukes-Cantor method was developed under the assumption that the rate of nucleotide substitution is the same for all pairs of four nucleotides A, T, C and G, and it gives a maximum likelihood estimate of the number of nucleotide substitutions between two sequences (Jukes-Cantor, 1969). Nucleotide substitutions in coding genes can be subdivided into two classes, i.e., synonymous (ds) and nonsynonymous substitutions (dn). Synonymous (or silent) substitutions are the nucleotide substitutions that do not result in aminso acid changes, whereas nonsynonymous substitutions are those that change aminso acids. HIV-1 envelope genes are highly variable between and often within individuals. Part of this variability is thought to be the result of immune-mediated positive selection for sequence diversity. To measure positive selection it has become customary in HIV research to calculate the ratio of the proportions of synonymous and nonsynonymous substitutions per potential synonymous and nonsynonymous site, respectively. The Jukes-Cantor one parameter

distance method can be used to calculate distances for synonymous and nonsynonymous substitution as this parameter does not take into account differences in the frequency of transitions (purine to purine, pyrimidine to pyrimidine substitutions) or transversions (purine to pyrimidine). Unlike the Jukes-Cantor model, the Kimura two-parameter model takes into account the rate of transitional and transversional substitutions per site.

1.10.2 Nucleotide substitution

The rate of nucleotide substitution is assumed to be the same for all nucleotide sites but this assumption rarely holds true, and the rate varies from site to site. It can be calculated by dividing the pairwise distance calculated for any two sequences with twice the time of divergence between the two sequences. The divergence time is assumed to be the same for the two sequences considered. The rate of nonsynonymous substitution may be extremely variable among genes as the majority of nonsynonymous substitutions are subject to purifying selection from external factors such as host immune response. Consequently, nonsynonymous substitutions which improve protein function may be selected resulting in a greater rate of nonsynonymous to synonymous substitution. Reciprocally, nonsynonymous substitutions resulting in a deleterious effect on protein function will be eliminsated by selection, reducing the rate of nonsynonymous substitution. However, synonymous substitutions do not cause changes in aminso acids and will not be subject to purifying selection. These substitutions are thought to reflect the underlying mutational rate allowing the estimation of the rate of nucleotide substitution.

1.10.3 Molecular phylogeny

1.10.3.1 Tree-Building Methods

Genetic distance is represented in a phylogenetic tree as a branch length and is an estimate of how many mutational events actually occurred between two sequences. Phylogenetic methods use different ways to estimate the genetic distance between sequences and to organise a set of sequences into a hierarchy of ever more distantly related sequences. There is a general trend toward greater evolutionary distances between viral sequences over time. However, estimates of HIV-1 divergence rates are dependent on the region of the genome under study. Different alignments of the same sequences can yield different divergence rates and depend on the evolutionary model used to calculate the genetic distances. Phylogenetic trees can be rooted or unrooted. A rooted tree indicates the direction of evolution, and the root is the common ancestor of all the operational taxonomic units (OTUs) studied. An unrooted tree specifies the relationship among the OTUs but does not define the evolutionary path. In practice, the majority of tree-making methods produce unrooted trees. However, in order to root an unrooted tree an outgroup can be added which is evolutionarily related to the OTUs under study, having diverged from the other OYUs prior to their divergence from on another.

There are numerous methods for constructing phylogenetic trees from molecular data and they can be classified into distance methods and discrete character methods (maximum parsimony). In distance methods, a pairwise evolutionary distance (pairwise distance) is computed for all pairs of taxa (e.g nucleotide sequences) to be studied and a phylogenetic tree in constructed by certain principles and algorithms. In the construction of phylogenetic trees, the principle of minsimum evolution or

maximum parsimony is often used. Character methods involve data with discrete character states such as nucleotide states in DNA sequences). The standard algorithim of the tree-making methods based on this principle is to examinse all possible topologies (branching patterns) or a certain number of topologies that are likely to be close to the true tree and to choose one that shows the smallest amount of total evolutionary change as the final tree.

Recent computer simulations (Nei et al, 1991) have shown that one of the most efficient distance methods in recovering the correct topology is the neighbour-joining method proposed by Saitou and Nei (1987). This neighbour-joining (NJ) method produces a unique final tree under the principle of minsimum evolution (Saitou and Nei, 1987). The method involves finding pairs of operational taxonomeric units (OTUs [=neighbours]) that minsimise the total branch length at each stage of clustering OTUs. The branch length as well as the tree topology can quickly be obtained using this method. In the neighbour-joining method the smallest value of the sum of all branches is not calculated for all the topologies. Instead, the examinsation of different topologies is embedded in the algorithim, so that only one final tree is produced. This method produces an unrooted tree, and usually requires an outgroup to find the root.

Another distance method is the unweighted pair-group method with arithmetic means (UPGMA). This method employs a sequential clustering alogrithm. Clustering of OTUs starts with the two OTUs with the smallest distance, and more distantly related OTUs are gradually added to the cluster. The two OTUs that are most similar among all the OTUs are identified and subsequently treated as a new single OTU. New distances between this composite OTU and other OTUs are calculated similarly,

until only two OTUs are left. This method assumes a constant rate of evolution and hence a rooted tree is produced.

1.10.3.2 Statistical significance of tree topologies

There are two different types of methods available for testing the reliability of a tree obtained. These are the maximum likelihood method (Felsenstein, 1988) and the bootstrap test (Felenstein, 1989). The maximum likelihood method examinses the reliability of every interior branch of the tree. This method can be very time consuminsg and is generally limited to small data sets. The bootstrap test involves randomly resampling the data from which the tree was constructed, producing a new tree with the resampled data. This process is repeated several hundred times and the frequency at which particular branches are observed in the newly constructed trees is calculated giving a probability to each branch in the original tree. This statistical method is suitable for use in neighbour-joining and maximum parsimony methods.

AIMS

The aims of this study were to investigate further the dynamics of HIV replication and the functional significance of CD8+ T lymphocyte infection *in vivo*. Resolving the mechanism of HIV infection in CD8+ T lymphocytes is relevant to evaluating the contributory effect of infection of this cell type on immunodeficiency. In this study we examinsed the frequency of infection and the relation of virus load in the naïve and memory subsets of CD4+ and CD8+ T lymphocytes *in vivo*. Changes in virus distribution were also investigated in the cells from individuals receiving antiretroviral therapy. This study also aimed to characterise genetically HIV-1 variants obtained from the naïve and memory CD4+ and CD8 T lymphocytes, assess whether separate populations existed, the degree of compartmentalisation and whether such differences reflected variation in biological properties. Finally, immunocytochemical techniques and in-situ PCR were used to detect the presence of HIV-1 DNA in CD4+ and CD8+ T lymphocytes *in vitro* and *in vivo*.

CHAPTER TWO: MATERIALS AND METHODS

2.1 SAMPLES AND CLINICAL DETAILS OF STUDY PATIENTS

20-30ml samples of whole blood were collected in ethylenediaminsetetraacetic acid (EDTA) blood tubes (Sarstedt, Leicester, UK) from 16 seropositive individuals. The individuals in this study group were attending the Genito-Urinary Medicine clinic (The Royal Infirmary, Edinburgh) and the Western General Hospital in Edinburgh. CD4 counts, viral load information and risk groups from all patients were available (please refer to Chapter 3, table 3.1 for this infomration). Risk factors for infection included intravemous drug use and sexual contact with an HIV-positive individual. Seven individuals were receiving anti-retroviral therapy during the course of this study.

CD4, CD8 and total lymphocyte counts were available for all the samples studied. CD4 counts ranged from 15 to 850 CD4 lymphocytes per µl. Four individuals had CD4 counts associated with late stage disease (CD4< 200/µl), whereas the other 12 had no symptoms and a CD4 count above 200/µl. Of the individuals with CD4 counts less than 200/µl, 2 had an AIDS defining illness. Absolute CD8 counts ranged from 280 to more than 2500/µl.

2.2 BIOLOGICAL RISKS OF WORKING WITH HIV-1.

With no cure or vaccine for HIV, prevention of infection is of paramount importance. Universal precautions are a set of infection control practices, developed by the Centers for Disease Control and Prevention (CDC), in order for all health care

workers, teachers and other persons who may become exposed to blood-borne pathogens to appropriately utilise barrier protection (gloves, gowns, masks, eyewear etc.) for anticipated contact with blood and certain body fluids of all patients. HIV-1 is a blood borne virus and transmission of HIV infection may occur via contact with any body fluids of an infected individual which might contain virus particles or infected cells, such as cerobrospinal fluid, peritoneal, pleural, pericardial fluids, semen, vaginal secretions, breast milk and blood. Unfixed tissues, organs and any other body parts from HIV positive patients are also included in this high-risk categorisation.

HIV-1 has been classified as a dangerous pathogen of the hazard group 3. All work with live HIV-1 must be carried out within a specifically designed Containment Laboratory. It is recommended that the growth and manipulation of HIV in research laboratories must be carried out in Containment Level 3 facilities. Needle stick and scalpel blade injuries with contaminsated blood are the major route of all laboratory based infections. Special care must therefore be taken when dealing with all needles, blades, scissors and syringes. Universal safety measures include wearing double gloves, a plastic apron over a specific coat, and a disposable plastic cuffs being worn at all times. Chain mail gloves between two layers of rubber gloves should be worn when handling cutting of fresh tissues. A face shield is also required to protect the eyes and mouth from splashes. All work involving blood tissue and body fluids from HIV-1 positive individuals, must be undertaken in a Containment Level 3 laboratory using a Class 1 microbiological safety cabinet. Unfixed high-risk specimens must not be exposed outside the Class 1 safety cabinet. All work surfaces must be disinfected regularly with either 70% ethanol, 0.5%

Nonidet, 0.5% Sodium hypochlorite or 1:1 ethanol/acetone. Regular autoclaving should be enforced to decontaminsate experimental waste such as gowns, gloves and remaining tissue residues, blood clots and small pieces of equipment. Fumigation is regularly carried out for decontaminsating working spaces and larger pieces of equipment.

2.3 DETERMINATION OF INDIVIDUAL ABSOLUTE CELL COUNTS

Absolute cell counts were determinsed by flow cytometry in the HIV Immmunology laboratory, The Royal Infirmary, Edinburgh. An Ortho Trio patient summary report (Ortho-Clinical Diagnostics, Herts, UK) was produced illustrating the CD3-positive T-cells, CD4-positive T-cells, CD8-positive T-cells, CD19-positive B cells, CD16-positive, CD3-negative natural killer cells as cells per microlitre of whole blood. The numbers of CD4 and CD8 memory and naïve cells were determinsed using a panel of antibodies against CD4, CD8, CD45RA (naïve cells) and CD45RO (memory cells). (FACS analysis was carried out by Helen Cerutti).

2.4 ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC).

Blood samples were diluted with an equal volume of Phospate buffered saline (PBS) (GIBCO BRL, Paisley, UK) and peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation over a Ficoll-Hypaque gradient (Nycoprep, Nycomed). 10 mls of diluted whole blood were carefully layered over 3mls of Nycoprep and centrifuged at 2000rpm in Sorvall H-1000B for 30 mins with no brake. The denser red blood cells pelleted while the less dense PBMCs and platelets remained on top of the

Ficoll-Hypaque gradient. Plasma was collected at the top layer and stored in liquid nitrogen for further use. The PBMC'c were collected and washed twice in 1 x PBS for 10 mins at 1400rpm in Sorvall H-1000B with the brake on. This ensured full removal of leftover platelets and any remaining Ficoll-Hypaque gradient. Using a haemocytometer and Trypan Blue exclusion all viable cells were counted. All PBMC's were then used for subsequent T-cell separation protocols. There are two general approaches to magnetic cell sorting: positive selection and depletion. Positive selection implies that the wanted cells are labelled and isolated directly as the magnetic positive fraction. Depletion involves labelling the unwanted cells thus eliminsating them from the desired fraction. In this project a combination of depletion and positive selection was used. PBMC's were initially separated into CD4+ and CD8+ lymphocytes by negative selection and then further enriched by positive selection into the naïve and memory subsets using the MACS system (Miltenyi Biotec, Bergish-Gladbach, Germany).

2.4.1 Negative selection (depletion).

In this project a CD4+ T-cell Isolation Kit and a CD8+ T-cell Isolation kit were used to obtain the CD4+ and CD8+ T-cells. The T-cell Isolation kits are individual magnetic labeling systems for the isolation of untouched T-cells from peripheral blood. T-helper cells, dendritic cells, monocytes, granulocytes, B cells, platelets, early erythroid precursor cells and natural killer cells are labelled using a cocktail of hapten-modified antibodies. All cells except the targetted CD4+ T-cells or the CD8+ T-cells are magnetically labelled using Microbeads. Excellent recoveries of highly pure CD4+ cells

and CD8+ cells are achieved by retaining the magnetically labelled non-CD4+ and non-CD8+ cells on a depletion column while the non-labelled cells pass through. Both labelled and non-labelled fractions can be completely recovered.

2.4.2 Positive selection.

The enriched CD4+ and CD8+ cells were positively selected to obtain the CD45RA+ (Naive) and CD45RO+ (Memory) cells. The enriched fractions of CD4+ cells and the CD8+ cells were directly labelled with antibody specific for CD45RO (anti-CD45RO) cells. Microbeads were then added to the antibody labelled cells and they were passed through a column. When the column was removed from the magnetic field, the magnetically retained CD45RO+ cells are eluted. The depleted fraction was labelled with anti-CD45RA and again the Microbeads were added to the antibody labelled cells. When the column was removed from the magnetic field the CD45RA+ cells were eluted.

Cytotoxic T-cells and T-helper cells were isolated separately by incubating with a cocktail of monoclonal antibodies directed against CD4 or CD8 (depending on which subset is being isolated), CD11b, CD16, CD19, CD36 and CD56 (20µl per 10^7 cells) followed by incubating on ice for 15 mins. Next, the antibody labelled cells were washed and resuspended ($1x10^7$ cells/80µl incubation buffer; PBS/2% inactivated foetal calf serum (FCS)/5mM EDTA). Magnetic beads (20µl per $1x10^7$ cells; Miltenyi Biotec) were added to the cells and they were incubated for a further 15 mins on ice. After a final wash the cells were resuspended in 500µl incubation buffer. In order to remove the magnetically labelled non-CD4+ and non-CD8+ cells, enrichment was performed with

pre-washed BS+ depletion columns (capacity: $1x10^8$ cells) and the VarioMACS magnet. The magnetically labelled non-CD4+ and non-CD8+ cells were retained in the column whilst the CD4+/CD8+ cells were washed through with 15ml of incubation buffer.

The CD4+ and CD8+ enriched T-cell fractions were then further selected for CD45RA+ (Naive) and CD45RO+ (Memory) cells using directly labelled microbeads (Miltenyi Biotec). CD4+ and CD8+ cells were stained with CD45RA and CD45RO microbeads (20µl /$1x10^7$ positive cells) for 15 mins at 4°C. After one washing step, cells were resuspended in incubation buffer (400 µl/$1x10^7$ cells) and enrichment was performed with a pre-washed MS+ positive selection column (capacity: $1x10^7$ cells) The positively selected cells (RA+ /RO+) retained in the column were eluted by removing the column from the magnet and washing with 1ml of incubation buffer.

Each isolated cell fraction was divided in two and frozen in liquid nitrogen. One part was snap frozen as a cell pellet for DNA extractions while the other was resuspended in 0.5 mls of freezing medium containing 90% RPMI and 10% DMSO (dimethyl sulfoxide). This aliquot was frozen slowly over ice-cold ethanol (100%) and then stored in liquid nitrogen. 1ml aliquots of plasma from each sample were also stored in liquid nitrogen. In order to show that the cell separation procedure did not interfere with the polymerase chain reaction (PCR), all separations were carried out in parallel with PBMC's isolated from buffy coat leucocytes derived from HIV-negative blood (Regional Blood Transfusion Service, Edinburgh).

2.5 ANALYSIS OF THE PURITY OF ISOLATED SUBSETS BY FLOW CYTOMETRY

The purity of the separated cell subsets was assessed from five HIV-1 negative controls and one HIV-1 positive sample. Flow cytometry was carried out under the supervision of Dr. Sarah Howie, Dept. of Pathology, University of Edinburgh. The following combinations of monoclonal antibodies were used on 100µl aliquots (minsimum of $1x10^5$ cells) of each of the isolated cell suspensions; CD4+CD45RA+, CD4+CD45RO+, CD8+CD45RA+ and CD8+CD45RO+ (10µl per $1x10^5$ cells; 30mins at 4°C); (a) fluorescein conjugated (FITC) CD8, phycoerytherin (PE) CD3 and CyChrome (Cy5-PE) CD4; (b) FITC CD45RA, PE CD45RO; (c) FITC rabbit anti mouse CD45RA, PE CD45RO, Cy5 CD4; (d) FITC anti mouse CD45RO, PE-CD45RA, Cy5 CD4; (e) FITC rabbit anti mouse CD45RA, PE CD45RO, Cy5 CD8; (f) FITC anti mouse CD45RO, PE-CD45RA, Cy5 CD8 (DAKO, Glostrup, Denmark). Next, cells were washed twice in PBS with 2% FCS to remove any unbound antibody and fixed in 200µl 2% formalin for FACS analysis. The purity of the isolated CD4+CD45RA+, CD4+CD45RO+, CD8+CD45RA+ and CD8+CD45RO+ cell populations was assessed by FACS (Fluorescent Activated Cell Sorter) on a Coulter Epics Elite after gating on live lymphocytes based on a standard light scatter histogram.

2.6 EXTRACTION OF DNA FROM CELL SUBSETS

DNA was extracted from PBMC or specific subsets of PBMC's by incubating the cells at 37°C for 20 mins with 400µl of lysis buffer (0.11M sodium chloride, 55mM Tris

pH8, 1.1mM EDTA pH8, 1mg/mL proteinase K, 0.55% sodium dodecyl sulphate (BDH laboratory Supplies, Poole, UK) and 40µl µg/mL poly A). The lysed cells were then shaken vigorously for 5 mins with 450µl of water saturated with phenol (Rathburn Biochemicals, Walkerburn, UK) and spun for 5 mins at 15,000rpm (14000xg) (Heraeus benchtop centrifuge). The upper aqueous layer was transferred to a fresh Eppendorf containing 450µl of chloroform/iso-amyl alcohol (50:1) (BDH laboratory supplies). The centrifugation step was repeated and the extracted DNA contained within the upper aqueous layer was then mixed with 0.1 volume of 3M sodium acetate (pH5.2) (Sigma) and 800µl of ethanol (-20°C) (Rathburn Biochemicals). After thorough mixing the DNA was left to precipitate overnight at -20°C and then centrifuged for 20mins at 15,000rpm in Sorvall H – 1000B. After washing once with 80% (v/v) ethanol the DNA was left to dry at 42°C for 15mins. The nucleic acid was re-dissolved in 25µl nuclease-free water and left for 10mins before using in any further reaction to ensure the entire pellet is dissolved.

2.6.1 PCR Quantification

HIV virus and cDNA were quantified by a limiting dilution nested PCR (Simmonds et al 1990). The first and second round PCR were firstly subjected to a thermal cycle of 18 seconds at 94°C to allow denaturation of the dsDNA, 21 seconds at 50°C to allow the primers to anneal to the ssDNA, and 72°C for 1.5 mins to allow strand extension. Each template strand was allowed 25 cycles of amplification. At the end of the last cycle samples were heated to 72°C for 6 mins to allow terminsation of uncompleted

strands. The initial (primary) PCR was carried out in a 50μl volume, containing DNA in an adjusted volume of 1μl , 5μl of 10 x PCR buffer (50mM KCI, 10mM Tris-HCL, pH 9.0, Triton X-100 and 1.5mM $MgCl_2$), 30μM each of dGTP, dATP, dTTP and dCTP, 0.25μl of sense primer (approximately 20μM), 0.25μl of antisense primer (approximately 20μM), 42μl of pyrogen-free water and 1μl of *Taq* polymerase (1μl is equal to 5 units). The second, nested PCR was carried out in a 20μl volume, for each sample, containing 1μl of primary PCR product, 2μl of 10 x PCR buffer, 0.2μls of dNTP's, 0.1 μl of sense primer (approximately x 8 μM), 0.1μl of antisense primer (approximately x 8 μM), 15.6μl of pyrogen-free water and 0.4 units of *Taq* polymerase (Promega). Prior to transfer to the thermal cycler each sample was covered with a drop of minseral oil to prevent loss of sample due to evaporation. Quantification was performed using primers spanning the V1/V2 and V3 region. The nucleotide sequences of the primers and the position of the 5' basc in the HXB2 genome (Myers et al 1993) are given in table 2.2.

Table 2.2: Primer sequences

Primer	Sequence (5′-3′)	Position of 5′ base
V1/V2	5′- ATTGTACTGTGCTGACAT T-3′	(-6944)
V1/V2	5′- CAATAATGTATGGGAATT GG-3′	(-6857)
V1/V2	5′- GA TCAAAGCCTAAAGCCATG-3′	(+6560)
V1/V2	5′- GAGGATATAATCAGTTTATGG-3′	(+3539)
V3	5′ - TACAATGTACACATGGAATT-3′	(+6957)
V3	5′- TGGCAGTCTAGCAGAAGAAG-3′	(+7009)
V3	5′- CTGGGTCCCCTCCTGAGG-3′	(-7331)
V3	5′ - ATTACAGTAGAAAATTCCCC-3′	(-7381)

After preliminsary quantifications using serial ten fold dilutions of DNA and subsequently (using V3 primers), on two-fold dilutions in triplicate around the end-point, between 8 and 10 replicates were then carried out at the end-point in order to accurately determinse the virus load. The end point was taken as the lowest dilution with at least one positive replicate. All separations, extractions and amplifications were carried out with parallel samples of PBMC's isolated from buffy coat leukocytes derived from HIV-negative blood to serve as negative controls.

2.6.2 Visualization of amplified PCR products

Amplified PCR products were visualized on 2% Agarose gels containing ethidium bromide. Ethidium bromide is an intercalating agent which exhibits fluorescence under UV light, allowing any positive PCR products to be detected as fluorescent bands. The gel was run for 10-15mins at 150V and analysed under UV light.

2.7 CLONING OF THE PCR PRODUCTS

Amplified DNA was ligated into a plasmid vector prior to nucleotide sequencing using 50ng of the pGEM-T Vector System (Promega, UK). 20µl of PCR product and 3Weiss units/µl of T4 DNA ligase (Promega). After overnight incubation at 4°C, the ligation product was used to transform JM109 competent T-cells (Promega) and plated on L-Agar plates (200µg/ml Ampicillin). Cloned PCR products were analysed by PCR amplification of the cloned insert using primers complementary to the plasmid sequences flanking the polylinker, which were also used for the sequencing of the cloned inserts. Next, the plasmid DNA was denatured by incubating with 1/10 volume of 2M sodium hydroxide/2mM EDTA at 37°C for 30 mins. DNA was precipitated by adding 1/10 volume of 3M sodium acetate/2 volumes of ethanol and incubating at -20°C overnight. DNA was collected by pelleting at 13K for 10 mins.

2.8 DNA SEQUENCING OF PCR PRODUCT

2.8.1 Solid-phase sequencing

Dideoxynucleotide sequencing was carried out using the Sequenase Version 2.0 DNA sequencing kit (United States Biochemical) with Sequenase Version 2.0 T7 DNA polymerase (Amersham). For each template strand of ssDNA a single annealing reaction was used to allow the primer strand to bind to the ssDNA. For each template, 2µl of the appropriate primer, 2µl of 5x Sequenase reaction buffer, 1µl of dimethylsulfoxide (DMSO) and 5µl of the cloned DNA were pipetted into an Eppendorf tube and placed in a heating block. The PCR parameters were one cycle at 65°C for five mins after which the mixture was allowed to cool slowly to room temperature over the next 30 mins. During this time 2.2µl of the appropriate dideoxy terminsation mixture were added to a microtitre plate containing wells labelled G, A, T, and C for each sample. This was then incubated at 37°C in a water bath. To each annealed template-primer the following extension (labelling) mix was added: 2µl of diluted labelling mix (1/20), 1µl of Dithiothreitol (DTT, 0.1M), 0.5µl of [α-^{35}S] dATP and 2µl Sequenase polymerase (1:8, approximately 3.25 units). After thorough mixing, 3.5µl of the annealing reaction and extension mix was added to the corresponding wells containing the terminsation mix and incubated for approximately 5 mins at 37°C . Next, 4µl of stop solution (95% formamide, 20mM EDTA, 0.05% Bromophenol blue, and 0.05% xylene cyanol FF) was added to each terminsation reaction stopping the terminsation reaction. The plate was heated to 95°C for denaturation prior to loading on a 6% acrylamide gel. The gel mix was made up of the following: 21g urea (AnalaR), 6 mls of sequagel XR concentrate, 5mls 10x TBE

(0.089 M Tris, 0.089 M Boric acid, 0.02 M EDTA), 0.05g ammonium persulphate (Sigma) and 20µl TeMed (Sigma). The gel mix was then dissolved in a final volume of 50mls with distilled water. The gel was poured between clean glass plates and left to polymerise for 1 hour. The gel was then pre-run at 70 volts for 10 mins using 1x TBE as the electrophoresis buffer after which it was run for 1-2 hrs at 75 volts allowing approximately 300 bases to be read. The gel was then fixed, dried and developed on BioMAX film overnight after which the bands were analysed.

2.8.2 Cycle sequencing

Direct sequencing of PCR products uses double-stranded product directly as a template for sequence analysis of either strand. Cycle sequencing involves repeated cycles of thermal denaturation, primer annealing and polymerization to produce a greater amount of product in a DNA sequencing reaction. Internal primers used in the preceeding PCR were used to initiate the sequencing reaction. This amplification employs a single primer so the amount of product DNA increases linearly with the number of cycles. The first step in sequencing PCR products consists of treating the PCR sample with a combination of 1µl of shrimp phosphatase and 1µl exonuclease per sample. This mixture is incubated at 37°C first, followed by 15mins at 80°C. This process removes phosphatases from the ends of PCR products and forms blunt ends before the sequencing process starts. Preparation of the terminsation mix involves adding 2µl of the nucleotide dGTP master mix to 0.5µl of each α-33^P ddNTP (G, A, T, or C - one of each per sequence). Four tubes are labelled ('G', 'A', 'T' and 'C') and 2.5µl of each terminsation

mix was added. 4.5µl of the reaction mix (2µl of reaction buffer, 3µl of DNA, 0.5µl of primer, 12.5µl of H_2O and 2µl of Thermo Sequenase polymerase) was transferred to each terminsation tube ('G', 'A', 'T' and 'C'), mixed well and covered with 10µl of oil. The cycling program parameters involved denaturating at 94°C for 30 secs, 55°C annealing for 30 secs and an extension temperature of 72°C for 1 mins. This was repeated for 30 cycles. Next, 6µl of each terminsation reaction was transfered to a fresh tube containing 4µl of stop solution. Samples were then heated to 95°C for 2mins and loaded immediately on to a sequencing gel. Gels were run, fixed and developed on BioMAX film overnight.

2.8.3 Extraction of plasma RNA

RNAases are difficult to inactivate and only minsute amounts are sufficient to destroy RNA. Therefore, it was necessary to create a Ribonuclease-free environment. This was achieved by using proper microbiological aseptic techniques, latex gloves, sterile reagents, plastics and utensils. The laboratory area used was first treated with RNAase away (Molecular Bio-Products Inc, San Diego, U.S.A). In order to avoid co-purification of DNA, plasma samples were filtered with a 0.2µM filter before use. RNA was extracted from three patient plasma samples using the Qiagen RNA extraction kit according to the manufactuers instructions (Qiagen, UK). Buffer solutions were prepared and used in accordance with the volume of plasma RNA being extracted.

2.8.4 RT-PCR

RT-PCR is a technique which couples reverse transcription (RT) of an RNA template with PCR amplification. RT-PCR is a sensitive and versatile method which can be used to determinse the presence of a transcript, to estimate expression levels and to clone cDNA products. RNA was detected by the Access RT-PCR System (Promega) which was designed to incorporate RT and PCR in one amplification in one reaction step. The reaction mix was prepared as follows: 1x AMV/T*fi* (*Thermus flavus*) buffer, 0.2mM of each dNTP (dATP, dCTP, dGTP and dCTP), 1mM outer sense and anti-sense primers, 1mM $MgSO_4$, 0.1U/µl AMV-RT, 0.1U/µl T*fi* DNA polymerase and 10µl RNA. This was made up to a final volume of 50µl with nuclease-free water. Positive control RNA, primers and a negative control were also supplied. The T*fi* polymerase is a thermostable DNA polymerase from *Thermus flavus*. The RT-PCR reaction conditions are shown in Table 2.3.

Table 2.3: Reaction conditions for Access RT-PCR

Reverse Transcription	1 Cycle	48°C	45 mins
RT Inactivation and Denaturation	1 Cycle	94°C	2 mins
Denaturation		94°C	2 mins
Primer Annealing	40 Cycles	55°C	1 mins
Extension		68°C	2 mins
Final Extension	1 Cycle	68°C	7 mins
Soak	1 Cycle	4°C	10 mins

1µl of primary product was used in a secondary reaction and the product was visualised on a 2% Agarose gel stained with ethidium bromide. See section 2.x (Gel Visualisation).

2.9 IMMUNOSTAINING TECHNIQUES

Cell medium was prepared using sterile instruments and a sterile still air cabinet. It was prepared as follows: 100 mls of RPMI 1640 (GIBCO BRL), 10 mls of heat inactivated FCS (GIBCO BRL), 1% streptomycin (GIBCO BRL), 1% pencillin (GIBCO BRL), 1% L-glutaminse (GIBCO BRL), 300µl phytohaemaglutinin (Murex, Dartford, UK) and 100µl interleukin-2 (IL-2) (MRC, NIBSC). PBMC's were isolated by Ficoll-Hypaque centrifugation as described earlier from a HIV-1 negative whole blood pack supplied by the Regional Blood Transfusion service. The cells were washed and resuspended in complete RPMI (1ml/10^6 cells). After incubation for 72 hours at 37°C in a plastic cell culture flask, the cells were passaged and more medium was added to the new flasks of cells.

Viral SI and NSI isolates came from two patient isolates (23 and 36 - SI and NSI respectively). These isolates were originally cultured in the PBMC's for three days and the viral supernatant was stored in 5ml aliquots in the -70°C freezer. Once defrosted these 5ml aliquots of SI and NSI isolates were placed in individual 10ml culture flasks containing 5mls of fresh PBMC'S. They were subsequently grown up at 37°C for 3-5 days. These procedures were carried out in a Category 3 area with a sterile still air flow cabinet, plastic pipettes and autoclaved tips.

2.9.1 Monoclonal ABC procedure

Slides of infected and uninfected cells were prepared once the presence of cells and syncytia were viewed in the culture flasks. Once syncytia had formed, 5ml aliquots from each flask (uninfected control cells, SI and NSI infected) were taken out and washed twice with 10mls of 1x PBS. They were centrifuged for 5 minsutes at 2000rpm in Sorvall H-1000B with the brake on. The pellet was then resuspended in 2ml of 1x PBS. Next, 20µl suspensions of liquid were spotted on to each well of a 12 well multispot microscopic slide (Hendley-Essex). The slides were dried in the Category 3 cabinet and once dry they were immersed in ice-cold acetone (Merck, BDH) to permeabilize the cell membranes. The slides were then rinsed and washed in Tris Buffered Saline (TBS) (AnalaR) to remove any excess acetone. They were then blocked for any non-specific staining with normal serum. The normal serum used in each experiment was determinsed by the species of the secondary antibody e.g. if the secondary antibody was rabbit anti-mouse, normal rabbit serum was used. CD4 and CD8 monoclonal antibodies were supplied by SAPU and were incubated on the slides for 30 minsutes at 37°C.

After washing the slides twice in 1x TBS they were then incubated in a Biotinylated secondary antibody (rabbit anti-mouse, DAKO) for 30 minsutes at 37°C so that it would bind to the primary antibody. At this stage the AB (Avidin-Biotin) Complex (DAKO) was prepared according to the manufacturer's protocol. Next, the antibody was rinsed off and the slide was washed twice in 1x TBS. Excess buffer was washed off and the AB Complex was applied to the slide for 30 mins at 37°C.

The fast red/fast blue complex (Sigma, UK) was prepared using powdered forms of fast red, fast blue with Napthol AS MX phospate (Sigma, UK) and N,N dimethylformamide (Sigma, UK) and Tris buffer (pH 8.2) (AnalaR). The slides were incubated with this complex for 30 minsutes at 37°C and then rinsed in water, dried, mounted (with 50% PBS in glycerol) and viewed under the light microscope. Double label stains were prepared on the same wells of the slide by simply subjecting the cells to a different monoclonal antibody and a different colour complex, for example; CD4 monoclonal with fast red and CD8 with fast blue on a single well or CD8 monoclonal with a p24 monoclonal (Dupont) in conjunction with either Diaminsobenzidine or FITC (Sigma, UK). Triple label incubations were tried with a p24 monoclonal antibody using the same monoclonal ABC procedure.

2.10 *IN-SITU* PCR

Brain, lymph nodes and spleen tissue sections were obtained at autopsy between 24 and 48 h after death from individuals with HIV-1 who had died of AIDS related conditions. These organs were fixed in 10% formalin for 2-3 weeks, sliced for examinsation and selected tissue blocks were then processed in a tissue-tek VIP (Miles Scientific) before paraffin embedding. Five μm sections of frontal lobe tissue were floated onto aminso alkylsilane-coated in situ PCR glass slides (Perkin Elmer) and incubated at 55-60 °C for 36-48 h. The presence of HIV-1 in the selected tissues was confirmed by tube PCR using primers to the gag region of HIV-1, GE1 and GE2, which were also used

in the in situ PCR, and by immunohistochemistry for HIV-1 p24 antigen. Control tissue was taken from lymph node and spleen uninfected with HIV-1.

2.10.1 Pretreatments of tissue sections.

The sections were deparaffinised in xylene for 30 mins at 37 °C and then two further xylene treatments of 10 mins each at room temperature. Tissue sections were placed in 100% ethanol for 10 mins and then rehydrated through 75, 50 and 25% ethanol (5 mins each) to fresh molecular grade water (Sigma) for 5 mins. Next, sections were incubated in 0.02 M HCL for 10 mins and then rinsed in PBS. The slides were then immersed in 0.01% Triton X-100 (Boehringer Mannheim) preheated to 40 °C for 90s and then washed in PBS. The slides were transferred to a humidified chamber and treated with 10 μg/ml proteinase K (GibcoBrl) in 0.1 M HCL pH 7.5, 5 mM EDTA for 10 mins at 55 and 37 °C for 20 mins. The tissue sections were then placed in a staining rack in a plastic container containing 500 ml of 0.01 M citric acid buffer ph 6.0 and microwave irradiated at full power in a 600 W microwave oven for 10 mins. The slides were removed from the oven and incubated in 20% acetic acid at 4 °C for 15 s and then rinsed in water for 2 x 5 mins and in PBS for 5 mins. Tissue sections were fixed in paraformaldehyde made in PBS for 5 mins and then rinsed in PBS for 2 x 5 mins and water for 5 mins. The slides were then dehydrated through graded alcohols 25, 50 and 75% for 2 mins each and then stored in 100% ethanol until required for in situ PCR.

2.10.2 *In-situ* PCR

In-situ PCR was performed using a solution that contained 100 pmol of each HIV-1 Gag primer GE1 5'GAAGGAGCCACCCCACAAGATT; and GE2 5'TAGGTGGATTATTTGTCATCCA which hybridised to positions 1317-1337 and 1553-1554 of the HX13 2 prototype sequence (Oswell DNA), 1.2 µl of a 1:10 dilution of 50 nmol stock of Biotin-16-dUTP (Boehringer Mannheim), and the following reagents which are part of the GeneAmp in situ PCR core kit (Perkin Elmer), 200 µM of each dNTP, 10 x PCR buffer, 4.5 mM MgCl2, and 10 units of AmpliTaq DNA polymerase, IS.

The tissue sections were removed from 100% ethanol and allowed to air dry completely. A slide was placed on the assembly tool (Perkin Elmer) preheated to 70 ^{0}C to facilitate a hot start and 50 µl of reaction mix was enclosed onto the section with a clip and a silicon rubber disc. The slide was then placed in the *in-situ* PCR machine (Perkin Elmer) and held at 70 °C until all slides were in place. The *in-situ* PCR was performed using the following conditions, 94 °C for 2 mins 30s and 55 °C for 1 mins 30 s for 1 cycle, 94 °C for 55 s and 55 °C for 45 s, for 30 cycles. Controls included reactions without primers to demonstrate that DNA repair was not creating false positives, a reaction containing just one HIV-1 gag primer (GE1) and an irrelevant primer to demonstrate that a prins type amplification was not evident, a reaction without TAQ polymerase and a reaction without Biotin dUTP. A positive control for in-situ amplification with b-globin primers 5′ ACACAACTGTGTTCACTAGC and 5′ CAACTTCATCCACGTTCACC was also carried out.

2.10.2 Post *in situ* PCR treatment

The slides were removed from the in situ PCR machine and washed in 2 x saline sodium citrate (SSC) for 2 mins and in PBS for 2 mins. The sections were then post-fixed in fresh 4% paraformaldehyde made in PBS for 5 mins and then washed in PBS for 3 x 5 mins.

2.10.3 Detection of *in situ* PCR product by tyramide signal amplification (TSA).

The tissue sections were washed in water for 2 mins and endogenous peroxidase was quenched by treatment with 3 % aqueous H_2O_2 (BDH) for 10 mins. The slides were then washed in water for 2 mins and in TBS buffer for 2 mins before being attached to disposable immunostaining chambers (Shandon) and then placed in a SEQUENZA immunostainer (Shandon). The sections were washed for 3 x 5 mins with TNT buffer (0.1 M Tris-HCL pH 7.5, 0.15 M NaCl, 0.05% Tween-20) and then incubated in 300µl of TNB buffer (0.01 M Tris-HCL pH 7.5, 0.15 M NaCl, 0.5% DuPont blocking reagent) for 30 mins. One hundred µl of StreptAvidin-HRP diluted 1:500 in TNB buffer was applied and the sections were incubated for 30 mins at room temperature. The sections were then washed in TNT buffer for 3 x 5 mins and 100µl in Biotinyl tyramide (diluted 1:50 in amplification buffer) was added and the tissues were incubated for 10 mins at room temperature. The slides were washed in TNT buffer for 3 x 5 mins and 100µl of streptAvidin-HRP or streptAvidin-Texas red (diluted 1:500 in TNB buffer) was applied and incubated for 30 mins at room temperature. The sections were washed in TNT buffer

for 3 x 5 mins. Chromogenic substrate (diaminsobenzidine; Vector) was then applied to each section and monitored between 3 and 5 mins until the colour fully developed.

2.10.4 Diffusion of *in situ* PCR amplicons.

To ensure that the positive *in situ* PCR signal was not due to diffusion of amplified DNA to an uninfected cell, the post *in situ* PCR amplification solution was run on a 2 % agarose gel with molecular weight markers and stained with ethidium bromide. An aliquot of the post *in situ* PCR solution was also subjected to nested PCR using primers GI1 5'TGCTAAACACAGTGGGGGGA; and GI2 5'CCTGAAGGGTACTAGTAGTT which hybridised to positions 1346-1365 and 1521-1540 of the HX13 2 prototype sequence.

CHAPTER THREE: INTRODUCTION

3.1 MECHANSIMS OF CD4 T-CELL LOSS

The progression of human immunodeficiency type 1 (HIV-1) infection is clearly associated with an increase in the viral load in plasma and a progressive depletion of CD4+ T-cells. One explanation for this depletion is the exhaustion of T-cell turnover, which must occur at a very high rate to replace the CD4+ lymphocytes permanently destroyed during HIV infection. The mechanisms for T-cell depletion during HIV infection are more complex than originally thought. The depletion observed in the blood compartment may result from a combination of cell destruction in lymph nodes and inflamed tissues or it could be due to a failure of T-cell regeneration in primary lymphoid organs such as the thymus (Chene *et al.*, 1999). Some observations have suggested that HIV-1 infection causes major destruction to this organ and is also associated with a profound disorganisation of the epithelial network (Rosenzweig *et al.*, 1993). This deterioration was also confirmed by the inability to restore thymopoiesis in infected thymuses from SCID-hu mice treated with HAART (Wilthers-Ward *et al.*, 1997). It has been suggested that age-related involution may also affect the ability of the thymus to reconstitute T-cells expressing CD4 cell-surface antigens that are lost during HIV infection (Pantaleo *et al.*, 1993). Antiretroviral therapies reverse the loss of naïve cells to an extent through expansion of pre-existing cells and thymic production of new cells. However, this recovery is poor and it has been suggested to be the result of severely impaired T -cell progenitors (Hengel *et al.*, 1999).

Theories about the causes of the observed loss of CD4 cells range from direct destruction or dysfunction by cytopathic infection with HIV-1 to apoptosis resulting from defects in antigen presentation (Levy *et al.,* 1993). Apoptotic cell death of CD4 + T-cells occurs through the activation of a complex cell-intrinsic suicide program. Apoptosis plays a major role during development, homeostasis and in many disease including cancer, AIDS and the neurodegenerative disorders (Stellar *et al.,* 1995). HIV can cause direct cytopathic events in activated CD4+ T-cells in culture, either in single cells or by synctium induction. The cytopathic effect of HIV in CD4 T-cell cultures, manifested by ballooning of cells and formation of syncytia, was shown to be associated with apoptosis. Syncytia are giant multi-nucleated cells and may include as many as 500 CD4 expressing cells. These syncytia produce virus for a short time after which they die. By incorporating non-infected cells into syncytia, a single gp160 expressing cell can eliminsate many uninfected CD4 cells. This apoptosis event is a late process occurring when cells are actively involved in the synthesis of viral proteins (Laurentcrawford *et al.,* 1993; Weiss, 1993).

3.2 REASONS AND MECHANISMS FOR HIV-INDUCED CELL KILLING

HIV-1 can be generally grouped into three clinical phases (i) the acute infection period, which in some ways presents similarly to a mononucleosis-like syndrome: (ii) an asymptomatic period of variable duration where initial bursts in viremia fall dramactically, often as much as 10-100 fold: (iii) the period of clinical disease during which multiple opportunistic infections and neoplasms are manifested. Recent estimates of virus

production are of the order of $>10^9$ virions per day, and complete turnover of $CD4^+$ cells occurs in about 2 days (Bonhoeffer *et al.*, 1995; Ho *et al.*, 1995; Wei *et al.*, 1995). Uninfected individuals show a mean level of 1100 CD4 T-cells/μl whole blood, while individuals with AIDS can sometimes have CD4 T-cell counts less than 1/μl.. This represents an enormous replicative capacity and, together with the error prone reverse transcriptase, contributes to the tremendous diversity of HIV. This diversity surely leads to competition for survival, the main driving force of Darwinian selection, and the consequent evolution of viruses that are best adapted to their changing environment. The success of HIV in establishing chronic disease and in promoting a slow but progressive deterioration of the different components of the immune response is somewhat difficult to explain. Potent virus-specific cell-mediated and humoral immune responses can already be detected during the early days of primary HIV infection, and can persist for years without either preventing the establishment of chronic infection or blocking HIV disease progression (Soudeyns *et al.*, 1999).

During mucosal infection, HIV primarily targets Langerhans cells (LCs) and or tissue macrophages (Zaitseva *et al.*, 1997). As part of the physical process of antigen processing and presentation, LC's carrying HIV migrate towards the T-cell areas of lymphoid tissue. Hence, macrophage tropic (R5) and T-cell tropic (X4) strains of HIV are directly carried to the site where immune responses are generated. Importantly, R5 and X4 viral envelope can mediate biologically relevant transmembrane signals to $CD4^+$ T-cells. In the case of R5 envelope, this interaction results in activation and chemotaxis of these cells. The active recruitment of susceptible target T-cells to the site of initial virus

localisation certainly leads to rapid spreading and amplification of HIV infection. As well as the rapid spreading of the virus, the strategies of establishing chronic infection are already operative, with the formation of a pool of latently infected $CD4^+$ T-cells containing replication-competent HIV proviral DNA. This enables HIV to establish viral latency before the appearance of an HIV-specific immune response in the host. Furthermore, these latently infected $CD4^+$ T-cells constitute a stable viral reservoir in which HIV remains sheltered from the effects of host immune response and anti-retroviral therapy (Chun *et al.*, 1997; Chun *et al.*, 1998). HIV-specific $CD4^+$ T-cell proliferative responses are rapidly lost during primary HIV infection, as a result of direct and/or indirect virus induced cytopathology. These include accumulation of nonintegrated circular DNA copies of the genome, increased permeability of the plasma membrane, and synctium formation. Preservation of virus-specific $CD4^+$ responses have been shown to be dependent upon the time of initiation of anti-retroviral therapy at the time of primary infection (Rosenburg *et al.*, 1997). Although viral load increases with disease progression, direct killing of $CD4^+$ lymphocytes by HIV most probably cannot account for the magnitude of the loss of these cells during the course of HIV infection.

Other cell types of the hematopoietic system are also susceptible to infection by the virus, particularly macrophages although they produce low levels of HIV and sequester virus in intracellular vacuoles. Stem cells, monocytes, B-lymphocytes, megakaryocytes, natural killer (NK) cells, eosinophils, thymic epithelial cells and dendritic cells are all the other hematopoietic cells known to be infected (reviewed in Levy, 1998).

3.3 T-LYMPHOCYTE RESPONSES IN HIV-1 INFECTION.

Several observations have associated cell death with direct toxicity from the virus or viral envelope proteins (Levy *et al.,* 1993). Ayyavoo et al and Zauli et al showed that several HIV gene products could induce or modulate T-cell apoptosis through dysregulation of intracellular signalling pathways, leading to depletion and functional impairment of $CD4^+$ and $CD8^+$ T-cells (Ayyavoo *et al.,* 1997; Zauli *et al.,* 1999). These apoptotic pathways could be mediated either directly by virus replication as a consequence of viral gene expression, or indirectly through priminsg of uninfected cells to apoptosis when triggered by different agents. Tat, a viral transcriptional factor, was shown to affect transcription of genes involved in cell survival. In recent studies, Tat alone was insufficient to induce apoptosis but it appeared to sensitize cells to apoptosis by triggering a second signal, such as CD95 or T-cell receptor (TCR) signalling (Li *et al.,* 1995). The Vpr gene was also recently found to induce apoptosis (WithersWard *et al.,* 1997). Vpr is required for productive infection of non-dividing cells and it was shown to induce arrest of cells in the G_2 phase of the cell cycle (Bartz *et al.,* 1996). Following the arrest of cells in the G_2, Vpr induces apoptosis in human T-cells, peripheral blood lymphocytes and fibroblasts. In addition to these pathways, a complementary cytopathic effect is provided by the immune system, since infected cells may be killed by HIV-specific CTLs or antibody-dependent T-cell-mediated cytotoxicity (ADCC) (Gougeon *et al.,* 1996).

A phenotypic study of apoptotic cells on a large cohort of HIV-positive patients revealed that not only T-cells but all mononuclear cells, including B cells, natural killer

cells, granulocytes and monocytes, have an increased fragility upon short-term culture (Gougeon *et al.*, 1996). Recent analyses of lymph nodes from patients demonstrated that apoptosis occurred *in vivo* and was detected not only in CD4 but also in CD8, B cells and dendritic cells (Amendola *et al.*, 1996).

Moreover, the cell fusion that often leads to cell death has been associated with gp120. The ability of HIV to kill the target T-cell correlates with the amount of CD4 expressed by the cell. The gp120 binds tightly to CD4, can prevent its cell surface expression and immunological function and promotes cell-cell fusion leading to cell lysis. Addition of gp120 to peripheral blood mononuclear cells (PBMC) or cultured brain cells caused cell killing in a dose-dependant manner. Changes in the N-linked glycosylation sequences of gp120 produce a cytopathic variant. Only a small proportion of circulating T-cells actively express HIV *in vivo*, indicating that the killing of single infected cells is unlikely to account solely for the profound immunodeficiency associated with this disease. Therefore, HIV may indirectly alter the immunological competence of noninfected CD4+ T-cells. In this context, gp120 and MHC II compete for a distinct but overlapping binding site on CD4. In addition, the CD4 and MHC II interaction is considerably weaker than CD4 and gp120. It is thus likely that gp120 can displace MHC II interactions with CD4. Binding of soluble gp120 on the surface of noninfected CD4+ cells can thus render them unable to participate in immunological responses against a variety of pathogens, including HIV-1. Binding of purified gp120 to CD4+ T-cells can not only deliver intracellular signals that inhibit subsequent T-cell proliferation but it can also transduce inhibitory signals that lead to T-cell anergy characterised by resistence to

subsequent T-cell activation. The ability of gp120 to induce CD4 internalization may involve mechanisms whereby noninfected T-cells which have cell-surface CD4-gp120 complexes are targetted for destruction by cytotoxic cells (Bour *et al.*, 1995).

Regulation of cell survival and death is essential for T-cell homeostasis during precursor cell development and terminsation of an immune response in the periphery. The blind homeostatis hypothesis states that only T-cell levels, regardless of CD4 or CD8 phenotype are regulated in the peripheral blood and peripheral lymphoid tissues. According to this hypothesis, as CD4+ T-cell numbers decrease, peripheral CD8+ T-cell numbers increase. This homeostatic mechanism is maintained for long periods after HIV-1 infection is established but is lost in the last two years preceeding AIDS (Margolick *et al.*, 1996). The observed depletion of T-cells may result from a combination of trapping of these cells in lymph nodes and inflamed tissues, as well as a failure of mature T-cell regeneration in primary lymphoid organs and in particular, the thymus (Chene *et al.*, 1999). T-cell development can occur via extrathymic pathways but generation of the mature T-cell repertoire that is required for immune system function is dependent on the thymus. HIV infection is accompanied by a decrease in thymic function. T-cell progenitors in the thymus or peripheral pools may be infected with HIV and therefore fail to proliferate and replenish the mature T-helper cell population.

3.3.1 Failure of CTLs

It is generally held that a progressive decline in HIV-specific cytolytic activity occurs in parallel with the development of HIV-related disease, so that it is rare to be able

to demonstrate HIV-specific CTLs in patients with fullblown AIDS. Such decline may be due to a general impairment of CD8+ cell cytolytic activity through lack of CD4+ T-cell help.

Another possible cause of progressive infection is the specific or nonspecific loss of CTLs over time. Even with a vigorous virus-specific CD8 response there is still the persistence of HIV viremia, and high levels of CTL activity to HIV peptides does not prevent the development of disease. In acute infection, CTL responses have been linked to the development of viral sequence variation within the targetted epitope; disease progression has also been observed coincident with the emergence of CTL-escape variants *in vivo* (Borrow *et al.*, 1997; Richard *et al.*, 1993). The possibility that CTL function may also be impaired *in vivo* may help to reconcile observations that class I restricted CTL clones can persist at high precursor frequencies in adults for many years without achieving immunologic control (Kalams *et al.*, 1994; Moss *et al.*, 1995). HIV-1 Tat and Nef proteins have also been shown to downregulate HLA class I expression, and thus allowed the virus to escape T-cell recognition (Collins *et al.*, 1998; Weissman *et al.*, 1998). The loss of immune function may contribute to the rapid fall in lymphocytes observed as an individual approaches AIDS (CD4< 200/μl).

Additional mechanisms for CD8+ cell impairment and deletion have been proposed: antigen presentation on B cells has been described to have the potential for direct tolerization of CTL responses and macrophages seem to be responsible for the induction of apoptosis of CD8+ cells by triggering TNF receptor II signalling after interaction with HIV gp120 (Brander *et al.*, 1999).

3.3.2 Effects of HIV-1 infection on CD8 T-cell function

Despite the broad spectrum of HIV-1 targets, the mechanisms by which the virus interacts with and affects CD8 T-cells as well as the pattern of infection (latent vs productive) have yet to be established. CD8+ T-cell infection has been previosuly described for several *in vitro* and *in vivo* in animal models (Stanley *et al.*, 1993). While CD4 lymphocytes are considered to be the principal target HIV-1 (refer to section 1.4.2 a number of more recent studies have shown the *in vivo* infection of other lymphoid cell types including CD8 T lymphocytes (DeMaria A *et al.*, 1994; Flamand *et al.*, 1998; Livingstone *et al.*, 1996; Mercure *et al.*, 1993; Yang *et al.*, 1998). CD8+ T-cells are a major immunological defence against HIV-1 infection. In most individuals, HIV mediates a strong specific cytotoxic activity that eliminsates productively infected cells. This response also blocks intracellular viral replication in CD4+ cells by production and secretion of a number of soluble inhibitory factors such as macrophage inflammatory protein (MIP) -1α, MIP-1β, RANTES, IL-16 and as yet other unidentifiable factors (Haynes *et al.*, 1996; McMichael *et al.*, 1998). During the course of infection, there is a gradual loss of CD4+ cells; after an initial increase in the number of cytotoxic T-cells, the CD8+ cell number similarly falls during disease progression (Levy *et al.*, 1993). The observed decline in both the number of CD8+ cells and specific HIV cytotoxic activity coincides with an increase in viral load and may ultimately be a contributory factor to the eventual collapse of the immune system and the development of AIDS.

The clinical significance and ability of HIV-1 to infect peripheral blood mononuclear cells other that CD4 T lymphocytes *in vivo* is controversial. Livingstone *et al*, previously reported that CD8+ cells accounted for the majority of the proviral load amongst PBMC's of individuals with advanced HIV-related disease. One of the factors contributing to the high frequency of CD8-positive lymphocytes occurring only in late-stage disease is the loss of CD4 T-cell population from the circulation. On the other hand, a substantial increase in CD8 T-cell infection on disease progression may possibly be related to the phenomenon by which HIV-1 spreads to non-lymphoid tissues during the later stage of disease with loss of immune control (Livingstone *et al.*, 1996). The exact mechanisms leading to $CD8^+$ T-cell dysfunction and depletion are still unclear. CD8 T-cell function may be indirectly influenced by a defective HIV-1 specific CD4 T-helper response that is necessary for the maturation and function of cytotoxic T-cells. It has also been demonstrated that CD8+ T-cells are subject to apoptotic events. After antigenic stimulation some CD8+ T-cells develop a state of unresponsiveness and eventual death which is mediated via apoptosis (Lewis *et al.*, 1994). Herbein et al showed that apoptosis of CD8+ T-cells was mediated by macrophages through the interaction of gp120 with the chemokine receptor CXCR4. SI HIV strains can be recovered in almost half of HIV-infected individuals, usually in the late course of infection and these are associated with accelerated disease progression. Therefore, SI/X4 viruses appear to exert their deleterious effect on the immune system not only by direct cytopathic effects on CD4+ T-cells, but also by indirect killing of CD8+ T-cells (Herbein *et al.*, 1998).

The peripheral interaction between CD4 and CD8 cells occurring *in vivo* as part of the immune response may allow direct transmission of infection to the CD8 lymphocytes. Alternatively, it is possible that damage or deficiency to the thymus may also contribute. HIV-1 may infect and destroy both intrathymic T progenitor cells ($CD3^-$ CD4+$CD8^-$), double positive thymocytes (CD3+CD4+CD8+) (McCune *et al.*, 1991; Su *et al.*, 1995) and mature $CD3^{hi}$CD8+ thymocytes (Lee *et al.*, 1997). The targetting of such progenitor cells would impair the production of both CD4+ and CD8+ naïve cells. Using the SCID-hu mouse which characterises thymic abnormalities after HIV infection, Lee *et al* found that HIV-1 can infect a population of mature $CD3^{hi}$CD8+ thymocytes wherein viral mRNA transcription occurs (Lee *et al.*, 1997). Recent studies have offered an alternative explanation as to why CD8 cells may become infected. They suggest that infection is mediated through the CD4 molecule whose gene is expressed following activation through the T-cell receptor (TCR) complex. HIV DNA sequences could be detected in uncultured and sorted mature CD3+ CD8+ T-cells from HIV+ individuals. Additionally, activated neonatal CD8+ cells were shown to express chemokine co-receptors and to be infectable by M-tropic HIV-1 isolates. These results suggest a new mechanism by which HIV could attack the immune system and may help to explain the CD8+ T-cell defects in AIDS patients (Flamand *et al.*, 1998; Su *et al.*, 1995; Yang *et al.*, 1998).

In addition to $CD4^+$ and $CD8^+$ HIV-specific T-cells, HIV might interfere with other cell types such as monocytes/macrophages and dendritic cells whose function is critical for the generation of immune responses. These specialized antigen-presenting cells

(APCs) are central to the induction of humoral and cell-mediated immune responses. Interference with APC function might be the result either of quantitative depletion by direct cytopathogenicity or suboptimal formation of MHC-antigenic peptide complexes. Another major component of the cellular immunity are NK cells which recognise and kill target T-cells in a non-MHC-directed manner. Impaired activity and a decline in NK cell numbers is one of the many immunological defects seen in AIDS patients. Indeed it has been demonstrated that HIV-1 tat released by infected cells can inhibit NK cell cytolytic function by acting on the intracellular signalling pathways of infected cells.

3.3.3 *In vivo* and *in vitro* infection of naïve and memory cells.

CD4+ and CD8+ T-cells are characteristically made up of two functionally different phenotypes, the naïve (CD45RA+) and memory (CD45RO+) cells. Naïve cells are those T-cells that have not been exposed to stimulatory signals before. Functionally the naïve cell subset has a lower cytokine response after mitogenic stimulation. They express low levels of adhesion molecules and when activated, they differentiate into effector/memory cells and express the RO isoform of CD45. Memory cells are generated after exposure to an antigen and have much broader cytokine expression profiles and express higher levels of adhesion molecules. After activation and in the absence of future immune stimulation they shrink in size and become inactive "resting" cells, capable of surviving for long periods in the lymphoid population (Sanders *et al.*, 1988). CD4 and CD8 naïve lymphocytes are lost preferentially as total CD4 counts fall (Rabin *et al.*, 1995; Roederer *et al.*, 1995). Stimulation of these cells may lead to HIV-infection. As the

naïve cell subset disappears the host becomes less able to control opportunistic infections and new virus variants. Naïve cells and their loss will inevitably lead to a reduction in the number of circulating T lymphocytes.

During HIV disease progression, naïve T-cells of the CD4+ and CD8+ subsets seem to be preferentially lost compared with cells of the memory phenotype. A decrease in thymic production of new T-cells would also decrease the diversity of the T-cell receptor, because a smaller number of T-cell receptor clones would be produced, further contributing to immune dysfunction (Roederer *et al.,* 1995). Naïve T-cells are phenotypically defined as coexpressing CD45RA and CD62L. Early depletion of thymus-derived naïve (CD45RA+ CD62L+) CD4+ and CD8+ T-cells is a fundamental feature of HIV-1 infection (Rabin *et al.,* 1995; Roederer *et al.,* 1995; Roederer *et al.,* 1997). Since these cells are resistant to productive infection by HIV-1 (Roederer *et al.,* 1995; Schnittman *et al.,* 1990; Woods *et al.,* 1997), it is unlikely that they are being destroyed by direct viral infection. It is possible that they are not being produced-that is, that thymic failure is a primary pathologic lesion in HIV-1 disease. Although there is substantial evidence that HIV-1 infection *per se* does not trigger T-cell activation, the virus may preferentially target a subset of T-cells that are easily activated subsequently. Such a sub-population of memory cells may be 4 to 10 times more susceptible to HIV infection *in vitro* than are naïve T-cells. This may be related to the selective depletion of memory T-cells in patients. Infected memory cells have been shown to contain integrated provirus, suggesting that their state of activation provided the necessary requirements for establishment of productive infection (Bour *et al.,* 1995). Ostrowski et al demonstrated

that both memory and CD45RA+/CD62L+ naive CD4+ T-cells are infected in HIV-1 infected individuals. HIV-1 provirus could be detected in the memory and naïve subsets from 11 HIV-1 infected individuals; in addition replication competent HIV-1 provirus could be isolated from these cells upon stimulation in tissue cultures (Ostrowski *et al.*, 1999).

To investigate the mechanism of CD8 depletion further we determinsed the distribution of infected cells in the naïve and memory cell subsets of CD4 and CD8 lymphocytes in 16 HIV+ individuals using a combination of monoclonal antibody cell separation techniques. HIV proviral sequences within these cell populations was quantified by limiting dilution nested PCR. HIV preferentially infected the naïve subset of CD8 lymphocytes along with the naïve and memory populations of CD4+ cells. The preferential distribution of HIV in the naïve subset of CD8 lymphocytes has implications for the mechanism of CD8 lymphocyte infection, and these hypotheses are discussed.

3.4 RESULTS

3.4.1 Cell separation and FACS analysis.

Analysis of HIV infection of CD4 and CD8 lymphocytes was carried out on samples from 16 HIV seropositive individuals (HIV-1 subtype B) attending the Genitourinary Medicine clinic and the Regional Infectious Diseases unit at the Western General Hosptial, Edinburgh. CD4 cell counts ranged from 10-850/μl blood. Four individuals had CD4 cell counts of less than 200 and 12 had CD4 counts greater than 200. Risk groups included intravenous drug users (IVDU), and homosexual and heterosexual conduct. The general characteristics of the 16 subjects included in this study together with their plasma virus measurements at the time of study entry are listed in table 3.1.

3.4.1.1 Experimental design.

$CD4^+$ and $CD8^+$ T naïve and memory cell subsets were isolated from samples using a combination of magnetic bead depletion and flow cytometric analytical techniques. Magnetic cell separations involved were carried out using a VarioMACS and a MinsiMACS magnetic system (Miltenyi Biotec) either by negative or positive selection. To isolate the naïve and memory subsets of CD4 and CD8 lymphocytes, the negative selection procedure was used initially to isolate the $CD4^+$ T-cells and $CD8^+$ T-cells. This isolation technique involved using direct $CD4^+$ and $CD8^+$ T-cell isolation kits and a VarioMACS magnet, which allowed for selection of up to $1x10^8$ cells. The $CD4^+$ and

TABLE 3.1

CLINICAL PROFILE OF PATIENTS IN THIS STUDY; RISK GROUP, CD4 AND CD8 COUNTS, THERAPY STATUS AND BLOOD VIRUS LOAD.

Patient Number	Sex	Risk Group illness	AIDS -defining	CD4 count	CD8 count	Viral load RNA copies/ml	Therapy	Duration
P01	F	IVDU	PCP	10	382	~14,000	HAART	12mths
P02	M	H		29	416	514,500	None	---
P03	M	H		74	772	116,000	HAART	8 mths
P04	M	H		130	524	>18,000	HAART	14mths
P05	M	H		232	675	<400	None	---
P06	F	Het		265	652	52,000	None	---
P07	M	IVDU/Het	Stage 4C2	267	1032	>48,000	None	---
P08	M	IVDU		280	1185	>460,000	None	---
P09	M	H		282	1388	>750,000	HAART	7mths
P10	M	H		301	1998	~81,540	HAART	22mths
P11	M	H		311	755	661,000	None	---
P12	F	IVDU		329	380	>64,680	HAART	6mths
P13	M	H		354	1317	67,980	None	---
P14	F	Het	Stage 4C2	360	442	<513	HAART	11mths
P15	M	H		748	1016	~60,589	None	---
P16	M	H		850	996	1,680	None	---

$CD8^+$ T-cells were then positively selected for the naïve and memory subsets by a direct labeling technique involving CD45RA and CD45RO MACS microbeads and a minsiMACS magnet which selected for a maximum of $1x10^7$ cells. The minsiMACS and varioMACS proved to be very efficient at isolating specific cell subsets while the beads allowed direct FACS (Fluorescent Activated Cell Sorter- Coulter EPICS machine) analysis of the selected cell subsets.

3.4.1.2 Purity of isolated cell subsets.

The purity of the isolated $CD4^+CD45RA^+$, $CD4^+CD45RO^+$, $CD8^+CD45RA^+$ and $CD8^+CD45RO^+$ cell populations was assessed by FACS. Directly conjugated fluorescent antibodies were used to check the purity of the depleted cell fractions from five HIV-negative individuals and one HIV-1 positive individual (table 3.2). Samples from the HIV-1 negative individuals showed that the purity of $CD4^+$ naïve T-cells was 91.7% with only 1.3% contaminsating $CD8^+$ cells. The purity of the sample from the HIV-positive individual was similar (95.1% $CD4^+$ naïve cells, 1.6% contaminsating $CD8^+$ cells). Equivalent results were also observed with the CD4+ memory cells with less than 1% contaminsating CD8 cells in both the negative and positive control samples. The mean purity of the $CD8^+$ naïve T-cells in the HIV-1 negative individuals was 83.3% with 1.7% contaminsating $CD4^+$ cells, while in the HIV-1 positive individual, CD8+ naïve cells were 92.8% pure with only 0.4% contaminsating $CD4^+$ T-cells. Median values for the naïve and memory cell subsets of $CD4^+$ and $CD8^+$ T lymphocytes were $CD4^+CD45RA^+$ 95.6%; $CD4^+CD45RO^+$ 95.3%; $CD8^+CD45RA^+$ 92.6%; $CD8^+CD45RO^+$ 79.2%.

TABLE 3.2

PURITY OF ISOLATED SUBSETS

Lymphocyte Subset	**CD4+ RA+**	**CD4+ RO+**	**CD8+ RA+**	**CD8+ RO+**	**CD4+ RA+**	**CD4+ RO+**	**CD8+ RA+**	**CD8+ RO+**	**CD4+ RA+**	**CD4+ RO+**	**CD8+ RA+**	**CD8+ RO+**
Sample	**CD3 expression (%)**				**CD4 expression (%)**				**CD8 expression (%)**			
HIV-negative												
1	79.1	98.7	98.7	68.5	73.4	93.9	2.5	2.4	2.3	1.9	93.1	60.9
2	97.2	93.8	99.2	97.6	95.6	90.9	0.4	1.3	0.6	0.3	98.4	97.5
3	98.9	99.9	98.4	91.8	96.1	97.1	0.9	2.6	1.6	1.3	92.6	79.2
4	99.4	99.2	88.4	96.8	95.2	96.5	0.1	0.3	0	0	84.2	91.0
5	99.2	97.6	88.8	50.2	98.1	95.3	0	0	1.7	0	80.9	45.6
Mean	94.7	97.8	94.7	81.0	91.7	94.7	1.6	1.3	1.2	0.7	89.8	74.8
HIV-positive	98.1	97.6	94.5	92.8	95.1	93.9	0.4	1.4	1.6	1.0	92.8	94.9

Satisfactory FACS analysis and PCR testing could not be carried out on the same samples due to the limitations in the sizes of blood samples available from the study group patients. However, estimated levels of CD4 contaminsation in the CD8 lymphocytes were calculated using the mean purity FACS data from control samples (Table 3.2) and were found to range between 1% and 12% of the total proviral load in the CD8 naïve cell subset. This level was insufficient to account for the detection of HIV-1 proviral sequences in the isolated CD8, $CD45RA^{+}$ lymphocytes in six patients. In one patient (p8) however, it is likely that CD4 contaminsation could account for the sequences detected in the naïve CD8 lymphocytes, although a very low level proviral load was detected in this subset.

3.4.2 Quantitation of HIV proviral sequences in lymphocyte subsets.

Once the purity of the separated cell subsets was determinsed nucleic acid was extracted and proviral DNA was quantified in each cell type by limiting dilution and nested PCR (Simmonds et al., 1990). The quantitation was performed using primers corresponding to the V1/V2 and V3 region. A nested PCR approach was used because this method can detect one single molecule of target DNA, and allow quantitation by dilution of DNA to an end-point (refer to section 2.6.1). The PCR product was visualized on a 2% agarose gel containing ethidium bromide.

HIV sequences in DNA from each of the purified lymphocyte subsets of the 16 study subjects was quantified, and expressed as proviral copies per million cells. All separations, extractions and amplifications were carried out with parallel samples of

mononuclear cells isolated from buffy coat leucocytes derived from HIV-1 negative blood, which serve as negative controls. HIV-1 DNA could be detected in CD4 T lymphocytes and CD8 T lymphocytes but was never detected in any negative controls.

The virus load in the naïve and memory subsets of CD4 and CD8 cells from 16 HIV-1 positive individuals, and their frequency in total T lymphocytes, was used to calculate the proportion of virus in each subset isolated (Table 3.4). Using cell counts from each sample and the number of copies of provirus detected it was possible to express the results as proviral copies per million cells. HIV-1 proviral DNA was detected in all the individuals studied, 4 with CD4 counts less than 200 and 12 with CD4 counts greater than 200. CD4 lymphocytes were infected at all stages of disease progression. Infection was detected in the CD4 naïve and CD4 memory subsets of all patients (between 55 and 4307 provirus copies per 10^6 cells) except in the CD4 memory subset of patient 15.

HIV DNA was also detected in 9 samples of separated CD8+CD45RA+ and in 3 CD8+CD45RO+ subsets from 10 of the 16 individuals. The contribution of contaminsating CD4 lymphocytes to the measured virus loads was small or insignificant in 10 of these samples (Table 3.1) and these were considered to represent infection of CD8 lymphocytes. In eight individuals, HIV preferentially infected the naïve subset of CD8 T lymphoctes, with proviral frequencies ranging from 25 to 1444 provirus copies per 106 cells. There was a tendency for higher frequencies of infected $CD8^+$, $CD45RA^+$ cells to be found in individuals with lower CD4 counts, although this difference did not reach statistical significance ($p = 0.286$). Frequencies of HIV-infected CD8 lymphocytes

TABLE 3.3

CLINICAL PROFILE AND FREQUENCIES OF INFECTION* OF NAIVE AND MEMORY SUBSETS OF CD4 AND CD8 T LYMPHOCYTES.

Study Subject	Sex	Risk† group	Viral load (geq. /ml)	CD4 count (μl)	CD4+ lymphocytes CD45RA+	CD4+ lymphocytes CD45RO+	CD8+CD45RA+ (naive) cells Proviral load	CD8+CD45RA+ (naive) cells CD4‡ contamination	CD8+CD45RO+ (memory) cells Proviral load	CD8+CD45RO+ (memory) cells CD4‡ contamination
p1	F	IDU	14,000	10	98	834	<10	0.9	<10	11
p2	M	MH	514,500	29	1106	587	107	10 (9.4%)	<10	8.0
p3	M	MH	116,000	74	133	278	1444	1.2 (0.1%)	<10	3.8
p4	M	MH	18,000	130	596	4272	<10	5.4	<10	58
p5	M	MH	<400	232	686	457	422	6.2 (1.5%)	67	6.2 (17%)
p6	F	Het	52,000	265	866	1566	<10	7.9	<10	21
p7	M	IDU/Het	48,000	267	95	520	<10	0.9	<10	7.1
p8	M	IDU	460,000	280	4307	2137	22	39 (180%)	259	29 (21%)
p9	M	MH	>750,000	282	575	2321	40	5.2 (13%)	<10	32
p10	M	MH	81,540	301	1307	464	622	12 (1.9%)	<10	6.3
p11	M	MH	661,000	311	834	3916	<10	7.6	20	53 (270%)
p12	F	IDU	64,680	329	55	115	<10	0.5	<10	1.6
p13	M	MH	67,980	354	115	222	71	1.0 (1.5%)	<10	3.0
p14	F	Het	<513	360	533	980	119	4.9 (4.1%)	<10	13
p15	M	MH	60,589	748	653	Neg.	31	5.9 (19%)	<10	<0.1
p16	M	MH	1,680	850	143	25	<10	1.3	<10	0.3

* Proviral copies per 10^6 cells.

† Abbreviations: IDU: injecting drug user; MH: male homosexual; Het: infection acquired through heterosexual contact

‡ Estimated contribution of contaminating CD4 cells to proviral load (% of total) detected in CD8 lymphocytes, using mean purity data from controls (Table 4).

did not correlate with disease status, risk group, antiviral therapy or total CD4 lymphocyte counts. There was no correlation between frequency of infection of CD8 cells with either CD4 subset.

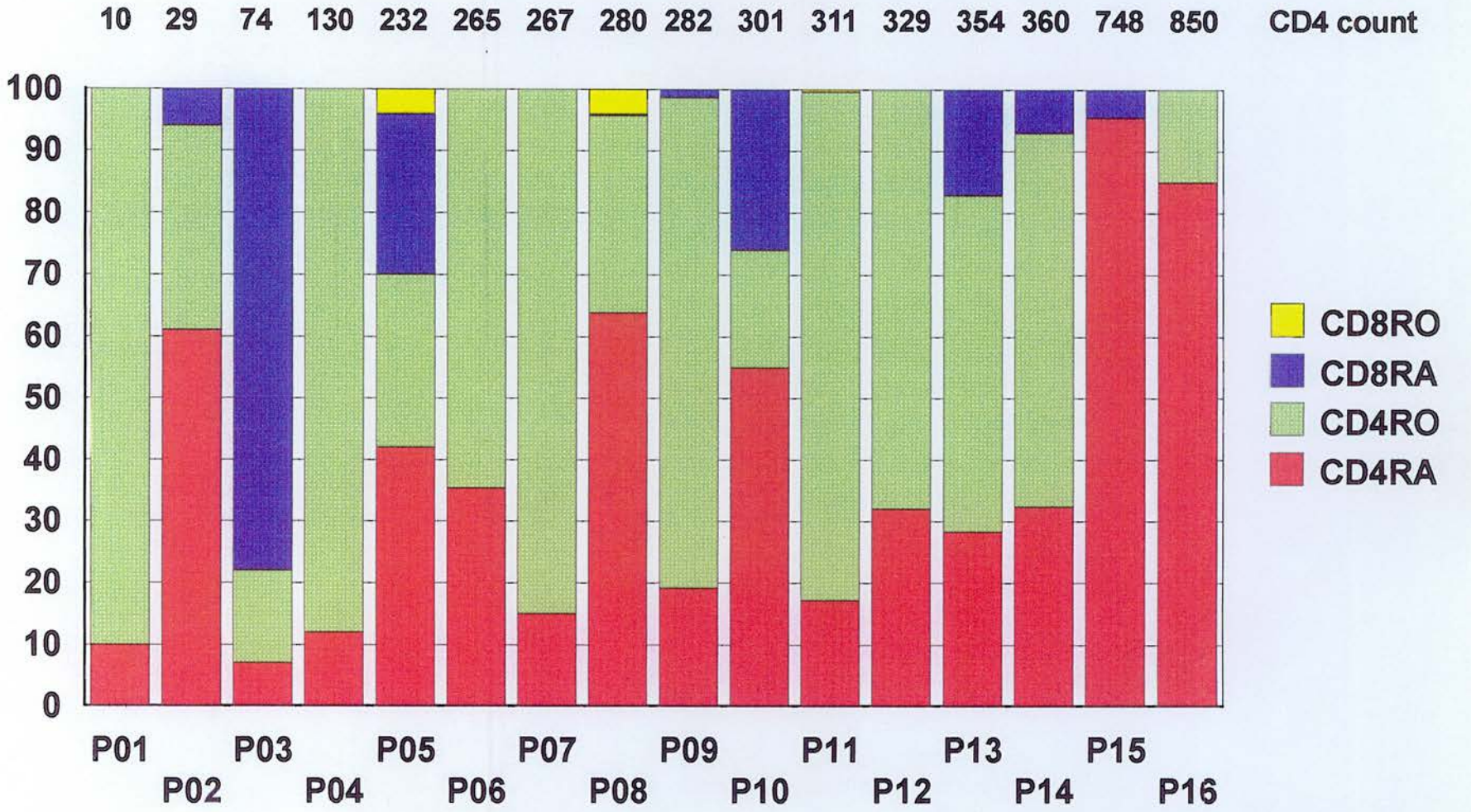
The frequency of infection and proportion of virus load contributed by naïve and memory subsets of CD4 lymphocytes varied with absolute CD4 count. CD4 naïve lymphocytes contributed the majority of infected cells in those with high CD4 counts ($r = 0.703$, $p = 0.02$), while memory cells were predominsantly infected in individuals with low CD4 counts ($r = -0.450$, $p = 0.08$). These results are also illustrated on a bar graph (figure 3.1).

The absolute cell counts for each cell fraction and the frequencies of infection were combined to determinse the contribution of HIV-1 DNA from each cell subset to the proviral load in total T lymphocytes. The contribution of each cell subset to the overall proviral load in T lymphocytes (% total load in T lymphocytes) were also illustrated on a bar graph with individual patients plotted along the X-axis in ascending order according to their CD4 count (cells/μl).

In order to investigate the percentage distribution of memory and naïve CD4 and CD8 lymphocytes, PBMCs from 11 individuals were stained with monoclonal antibodies directed against CD4, CD8, CD45RA and CD45RO. These values were plotted against CD4 counts (figure 3.2-pie charts). In these pie charts, the CD4 and CD8 naïve and memory cells are shown as percentages of the total lymphocytes present in each individual. In individuals with CD4 counts of greater than 350cells per μl, the majority of CD4 and CD8 lymphocytes expressed the naïve cell marker CD45RA. However as CD4

Figure 3.1 The relative distribution of CD4 and CD8 naive and memory T lymphocytes isolated by VarioMACS to proviral load in total T lymphocytes.

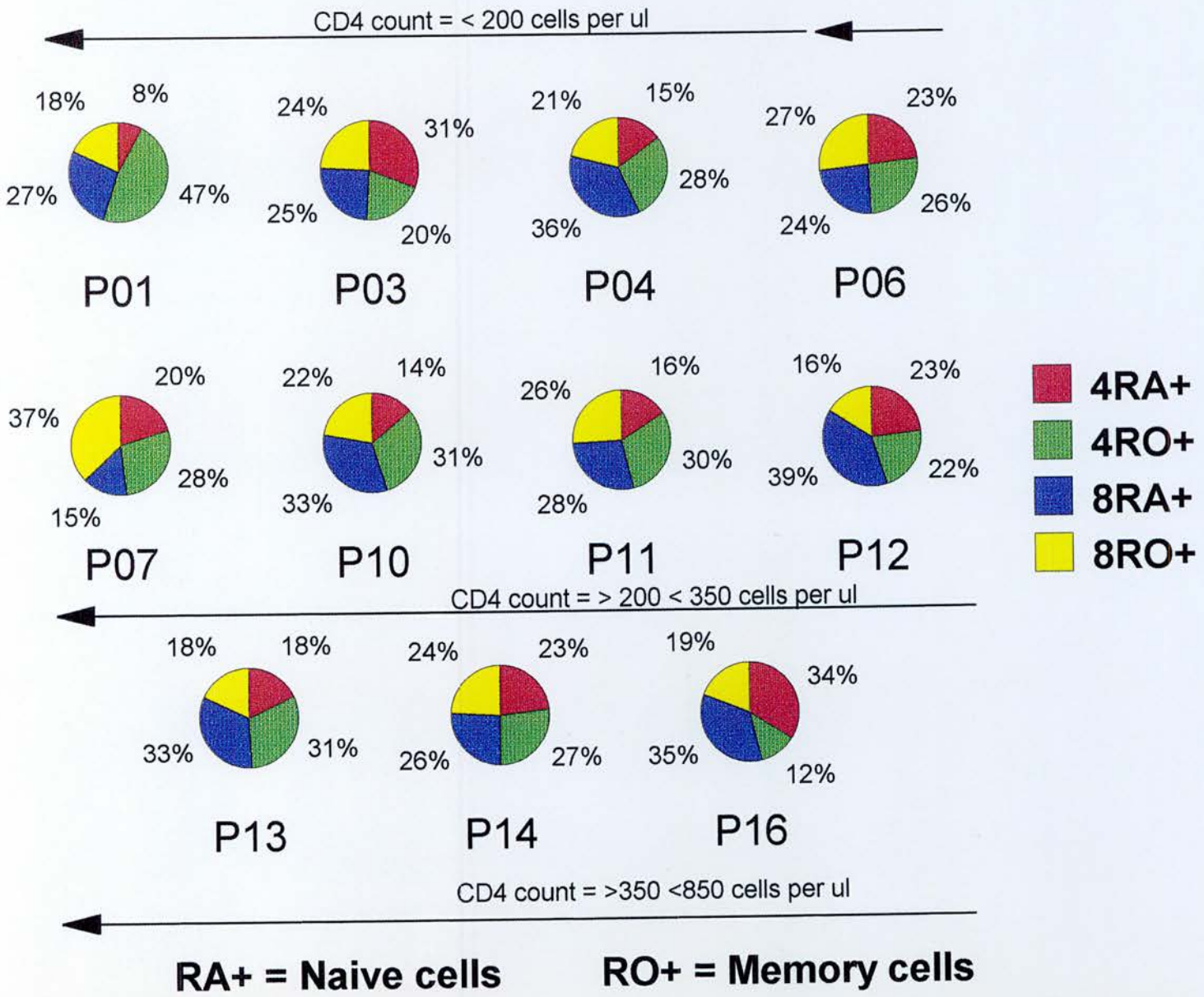
Fig 3.1 The relative distribution of CD4 and CD8 naive and memory T lymphocytes isolated by VarioMACS to proviral load in total T lymphocytes.



RA = Naive cells
RO = Memory Cells

Figure 3.2. Percentage distribution of naive and memory cells with disease progression.

Fig 3.2 Percentage distribution of naive and memory cells wth disease progression .



counts fall to 200-350 cells per μl, there is a modest decline in the number of naïve cells with the memory cell subsets becominsg the major cell population in both the CD4 and CD8 lymphocytes.

3.4.3 Effect of antiviral therapy on CD4 and CD8 lymphocyte infection.

In order to investigate the turnover of the infected cell populations during the period of HAART treatment, where virus replication is suppressed, the frequency of infected (proviral DNA positive) cells was analysed. CD4 and CD8 naïve and memory lymphocyte subsets were separated from pre- and sequential post-treatment samples collected from five individuals receiving combination antiviral treatment, and analysed for proviral sequences (Table 3.5). Treated individuals showed rises in circulating CD4 counts, and reductions in circulating viraemia. Despite the effectiveness of the antiviral therapy as indicated by the clearance of plasma RNA, the distribution and frequency of infected cells in each of the subsets remained relatively stable, in all cases remaining within a ± one $\log_{10}$ range of the levels detected in the initial samples. Infection of CD8 lymphocytes remained detectable in the three individuals (p9, p10 and p14) whose first samples were positive (figure 3.3a, figure 3.3b & figure 3.3c), while the two individuals (p1 and p12) with negative CD8 lymphocytes in the pre-treatment samples remained negative during treatment (figure 3.3d and figure 3.3e). There was evidence for a decline in the frequency of infected $CD4^+$, $CD45RA^+$ and $CD45RO^+$ lymphocytes over the course of treatment ($r = -0.428$; $p = 0.098$ and $r = 0.545$; $p = 0.029$ respectively) with half-lives of infected cells approximately 6 months, in agreement with previous studies (Ibanez,

TABLE 3.5

INFECTION OF LYMPHOCYTE SUBSETS OF STUDY SUBJECTS RECEIVING COMBINATION ANTIVIRAL THERAPY.

Patient	Therapy*	Time† (days)	CD4 Count	Plasma Load‡	$CD4^+$ RA^+	$CD4^+$ RO^+	$CD8^+$ RA^+	$CD8^+$ RO^+
p01	Pre¶	0	10	14,000	98	834	Neg.	Neg.
	+	27	150	1,700	34	239	Neg.	Neg.
p09	Pre¶	0	283	750,000	575	2321	40	Neg.
	+	48	-	3,000	72	1807	215	Neg
p10	Pre¶	0	301	81,540	-	-	-	-
	+	49	-	Neg.	1307	464	622	Neg.
	+	62	466	Neg.	533	533	697	Neg.
	+	100	-	Neg.	878	364	1049	Neg.
	+	115	-	±	667	577	2764	Neg.
	+	154	428	±	1498	1390	489	Neg.
	Stop	360	349	16,000	734	2884	435	Neg.
p12	Pre¶	0	329	64,680	55	115	Neg.	Neg.
	+	14	477	Neg.	15	188	Neg.	Neg.
	+	41	540	Neg.	19	119	Neg.	Neg.
	+	100	442	Neg.	9	48	Neg.	Neg.
	+	340	207	Neg.	18	56	Neg.	Neg.
p14	Pre¶	0	360	Neg.	533	980	11	Neg.
	+	27	418	Neg.	158	1307	203	Neg.
	+	153	465	Neg.	351	183	144	Neg

* Combination treatment over study period; Pre: Sample collected pre-treatment, Stop: Treatment stopped because of side effects.

† Time since start of treatment

‡ Circulating plasma virus load (genome equivalents of RNA /ml); Neg.: <400 geq. /ml

¶ Samples tested and recorded on Table 3.1

Figure 3.3 (a) and Figure 3.3(b). Percentage of HIV proviral load per T lymphocyte cell number in the CD4 and CD8 naive and memory cell subsets of P09 and P14.

Fig 3.3(a) and fig 3.3(b). Percentage of HIV proviral load per total T lymphocyte cell number in the CD4 and CD8 naive and memory cell subsets of P09 and P14.

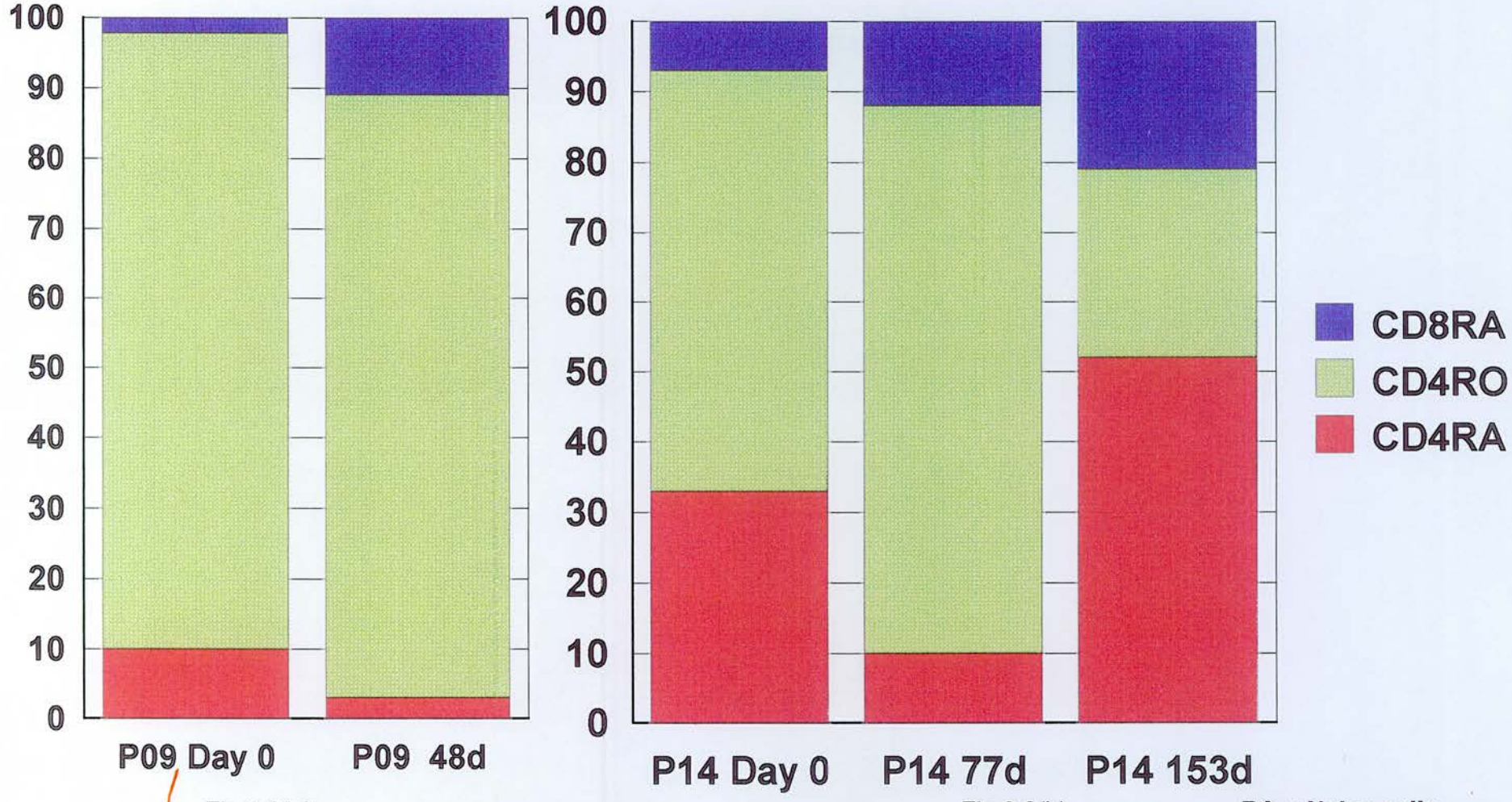


Fig 3.3(a)

Fig 3.3(b)

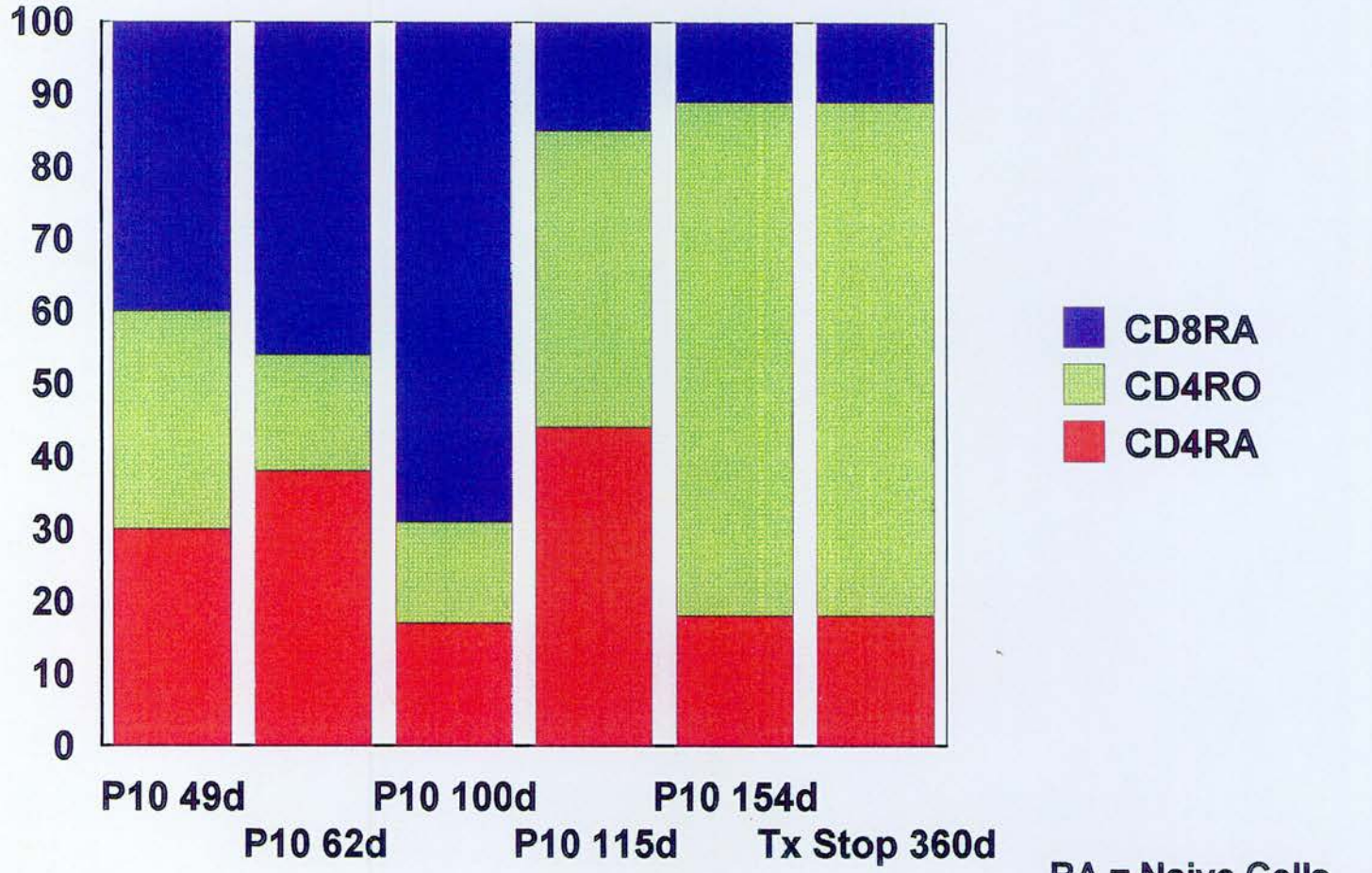
RA = Naive cells

RO = Memory cells

d = days since the start of treament

Figure 3.3 (c). Percentage of HIV proviral load per T lymphocyte cell number in the different cell subsets of P10.

Fig 3.3(c). Percentage of HIV proviral load per total T lymphocyte cell number within the different cell subsets of P10.



RA = Naive Cells

RO = Memory Cells

d = days since start of treatment

Figure 3.3 (c) and Figure 3.3(d). Percentage of HIV proviral load per T lymphocyte cell number in the CD4 and CD8 naive and memory cell subsets of P01 and P11.

Fig 3.3(d) and fig 3.3(e). Percentage of HIV proviral load per total T lymphocyte cell number in the CD4+CD45RA+ and CD4+CD45RO+ cell subsets of P11 and P01.

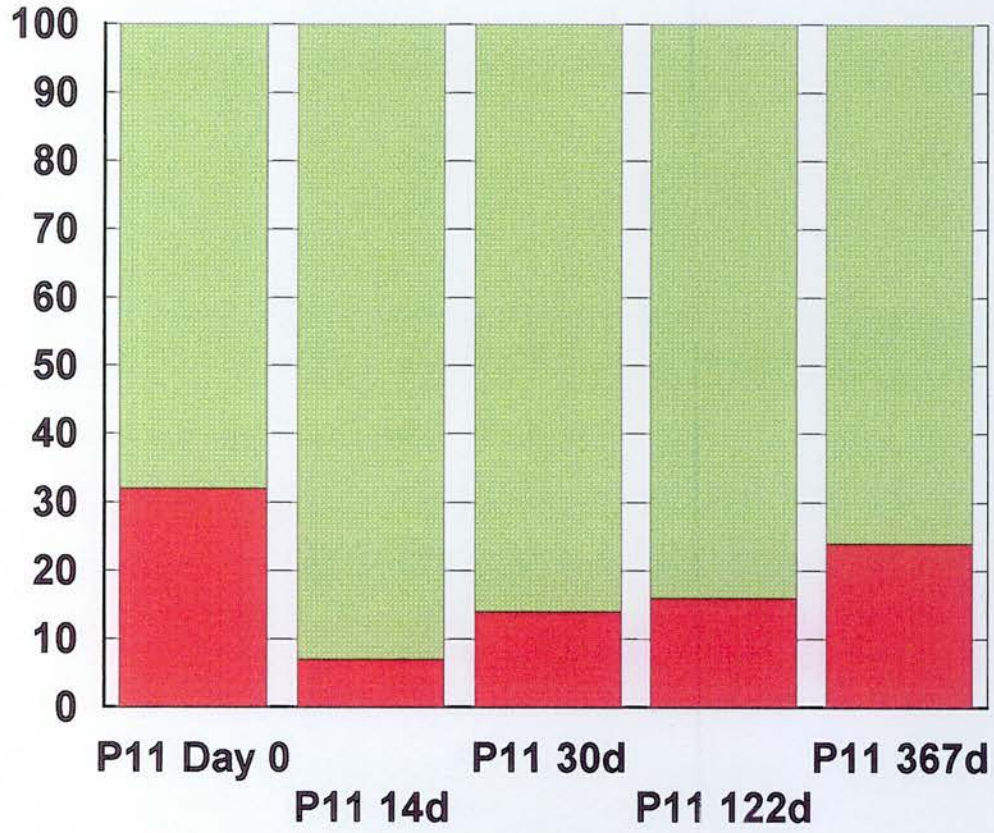


Fig 3.3(d)

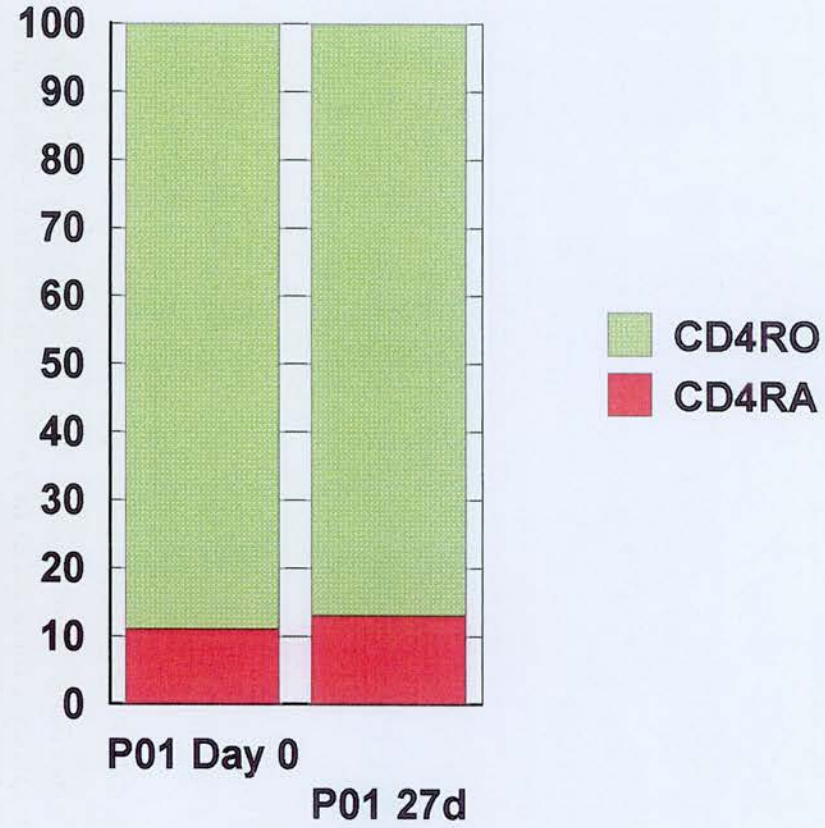


Fig 3.3(e)

RA = Naive
RO = Memory

d = days since start of treatment

1999; vanHarmelen, 1997; Izopet, 1998; Bruistein, 1998). A similar decrease in the frequencies of infected $CD8^+$, $CD45RA^+$ cells was not observed, although fewer observations were made ($r = 0.070$; $p = 0.870$).

3.5 DISCUSSION

To investigate the mechanism and functional significance of infection of CD8 lymphocytes by human immunodeficiency virus type 1 (HIV-1) *in vivo*, the frequency of infection in the naïve ($CD45RA^+$) and memory ($CD45RO^+$) CD4 and CD8 lymphocytes was carried out in 16 HIV-1 positive individuals during different stages of disease progression. This study shows that it is the $CD45RA^+$ subset of CD8 lymphocytes that is predominsantly infected with HIV-1 *in vivo*, in contrast to the distribution of proviral sequences in the RO and RA subsets of $CD4^+$ lymphocytes. Frequencies of infected CD8 cells in this study were comparable to those previously reported in a similar cross-sectional study of CD4 and CD8 lymphocytes (Livingstone *et al.*, 1996), although the correlation between CD8 infection with disease progression (or CD4 lymphocyte count) was not apparent from this study group.

The current study used microscopic magnetic beads for cell separation, and this allowed a direct assessment of the purity and degree of CD4 or CD8 lymphocyte contaminsation of isolated cells. Investigations were also carried out into whether cross-contaminsation could produce false-positive results as contaminsating CD4+ lymphocytes in purified CD8 T lymphocytes may potentially contribute to the observed proviral load. From FACS analysis, the mean contaminsation by CD4 cells in CD8 lymphocytes separated was calculated (Table3.2). Using the mean contaminsation level per subset in conjunction with the proviral loads detected per sample, the mean frequency of proviral sequences detected in CD8 lymphocytes that originate from contaminsating CD4 lymphocytes were estimated. In only 3-4 samples, where proviral load in CD8 lymphocytes was in any case very low, could CD4 contaminsation account for the sequences detected. Previous estimates of the

contribution of contaminsating cells to the detection of HIV-1 in CD8 lymphocytes have come to similar conclusions (Livingstone *et al.*, 1996). Furthermore, there was no correlation between frequencies of infected CD4 lymphocytes and detection of proviral sequences in either the CD8, $CD45RA^+$ or RO^+ populations (Table 3). For example, the sample with the highest frequency of infected CD4 lymphocytes (p4) contained undetectable frequencies of infected CD8 lymphocytes; similarly the sample with the highest CD8 proviral load contained relative low frequencies of infection of both subsets of CD4 cells. Thirdly, although there was considerable inter-subject variability in the detection of CD8 lymphocyte infection, longitudinal sampling of the same individual over time revealed remarkable stability in proviral loads in each of the lymphocyte subsets. For example, pre-treatment samples from p1 and p12 contained undetectable frequencies of infected CD8 lymphocytes, while samples subsequently remained negative. The remaining individuals with infected CD8 lymphocytes pre-treatment, remained PCR-positive subsequently. The consistency of provirus detection in the lymphocyte subset argues against sporadic contaminsation by CD4 lymphocytes as a reason for our observations.

As negative selection was used in this study to separate CD4 and CD8 lymphocytes, there is a possibility that purified CD8 T lymphocytes may contain null (CD4-, CD8-) cells. While acknowledging this fact, it was not possible to carry out positive selection of CD4 and CD8 T lymphocytes prior to separating naïve and memory cells as positive selection affects the activation status of the cells, and consequently the isoform expressed on the cell surface. Since completing this thesis however, further work was carried out (McBreen *et al,* 2000, manuscript attached) on a new group of 7 individuals in which CD4 and CD8 T lymphocytes were

positively selected from PBMCs. Purified CD8 lymphocytes in this way showed a range of proviral loads (Table 3.3, attached manuscript) similar to those found in CD8 lymphocytes isolated by negative selection. Having established that HIV proviral sequences can be detected in CD8-positive lymphocytes, it is unlikely that the results obtained from the negative selection procedure used here were the result of null cells.

In this study, expression of the RA and RO isoforms of CD45 was used to select between two functionally different subsets of T lymphocytes (naïve and memory /effector respectively) in the peripheral circulation. Separate identification of HIV-1 infection in naïve and memory/effector cells provides information on the time of infection of T-cells relative to maturation and activation, which in turn provides indirect information on the likely mechanisms underlying infection. While expression of CD45RA and CD45RO has been the most commonly used method for separating naïve and memory/effector cells, expression of other markers in combination with CD45 has been used to more strictly define functionally distinct subsets. Markers of cellular activation such as CD38 expression and down-regulation of CD27 and CD28 have been used to identify effector from memory and naïve $CD8^+$ lymphocytes (Burgisser *et al.*, 1999; Giorgi *et al.*, 1994; Hamann *et al.*, 1997; Ho *et al.*, 1993; Ogg *et al.*, 1999). Expression of CD62L in combination with CD45RA has been used to define purer populations of naïve CD4 cells, as there is evidence that a proportion of memory CD4 cells of HIV-positive individuals express CD45RA (Roederer *et al.*, 1997; Ostrowski et al, 1999). Whether abnormal expression of CD45RA on the CD8 lymphocytes of HIV-positive lymphocytes also occurs has not been determinsed. Recently it has been reported that the isoform of CD45 expressed on memory cells

may ultimately revert to RA, potentially producing a subset of $CD45RA^+$ cells functionally distinct from the naïve phenotype (Bell *et al.*, 1998).

While it is possible that a proportion of the $CD45RA^+$ CD4 and CD8 populations analysed in our study may have originally been memory cells, the predominsance of HIV-1 infection in the CD8 $CD45RA^+$ subset argues empirically that the main target CD8 lymphocyte is the naïve subpopulation. For example, it is unlikely that the infected CD8 cells detected are memory revertants as it would be likely that a similar frequency of non-revertant $CD45RO^+$ lymphocytes would also be infected.

Infection of naïve cells has been documented extensively in CD4 lymphocytes (Ostrowski *et al*, 1999; Schnittman *et al.*, 1990; Sleasman *et al.*, 1996), and is confirmed in the current study although in contrast to our findings, frequencies of infected $CD45RA^+$ have been reported to be lower than in the $CD45RO^+$ effector/memory population. The latter cells are considered to be the main contributors to the pool of infected lymphocytes *in vivo*, where cellular activation on antigenic stimulation produces cells susceptible to infection and destruction by HIV-1 (Stevenson *et al*, 1990; Chun *et al.*, 1997; Farnet *et al.*, 1997; Zack *et al.*, 1990; Zack *et al.*, 1992). In contrast, productive infection of naïve CD4 lymphocytes *in vitro* by laboratory isolates of HIV-1 is demonstrably inefficient, most likely through lack of T-cell activation necessary for efficient reverse transcription and integration of the HIV genome after entry (Stevenson et al, 1990; Chun *et al.*, 1997; Farnet *et al.*, 1997; Roederer *et al.*, 1997; Spina *et al.*, 1997; Zack *et al.*, 1990; Zack *et al.*, 1992) and because they do not express the CCR5 chemokine co-receptor required for the entry of primary (non-syncytium inducing) variants (Bleul *et al.*, 1997; Mo *et al.*, 1998).

Hypotheses developed to account for the infection of naïve CD4 lymphocytes may also be relevant for the infection of naïve CD8 lymphocytes *in vivo* reported here.

3.5.1 Loss of naïve CD8 T-cells

The preferential loss of naïve CD8 cells has important consequences for the development of immune responses in HIV infected individuals. As naïve subsets disappear the host becomes less able to control opportunistic infections and new virus variants. As yet it is not fully understood why CD8 naïve cells are selectively depleted. Destruction of cells destined to become CD8 lymphocytes may be a major factor in the decline in CD8 lymphocyte frequencies and function on disease progression, and contribute directly to the observed immunodeficiency in AIDS. Preferential distribution of HIV-1 in naïve CD8 lymphocytes suggests that infection occurred early in T lymphocyte maturation, perhaps during maturation in the thymus. Kitchen and colleagues found that infection of an immature $CD8^+/CD4^+$ thymocyte further differentiated into a $CD8^+/CD4^-$ cell and resulted in $CD8^+$ thymocytes that expressed HIV-1 (Kitchen *et al.*, 1997). Infection of such progenitor cells would impair the production of both $CD4^+$ and $CD8^+$ naïve T-cells. Damage to or deficiency of the thymus might also contribute. There are several potential mechanisms for the infection of CD8 lymphocytes, although most would not predict the preferential distribution of infection in the naïve subset. Peripheral CD8 lymphocytes express low levels of CD4 on mitogenic and antigenic stimulation *in vitro*, and it has been hypothesised that this allows productive infection of CD8 lymphocytes *in vivo* to occur (Flamand *et al.*, 1998; Kitchen *et al.*, 1998; Yang *et al.*, 1998).

These findings suggested that when CD8 cells are stimulated out of their quiescent state, as they would be when responding to antigenic stimulation, they make CD4 (Flamand *et al.*, 1998; Kitchen *et al.*, 1998). In HIV-infected individuals, cytotoxic T-cells recruited to kill HIV-infected cells would, as a consequence of specific antigenic activation, express the CD4 antigen and become targets for HIV infection (Flamand *et al.*, 1998). It is not known how frequently CD8+ T-cells express CD4 *in vivo*, although it has been speculated that this phenomenon may occur more often in lymphoid tissue, where costimulation occurs. The requirement for cellular activation suggests that the CD45RO+ population would be the main reservoir of infected CD8 lymphocytes.

The overall decline of naïve and memory T-cells in infected individuals has been influenced by the rates of production of each population, the frequency of infection of each population, the susceptibility of each population to the cytopathic effects of infection, and the suspectibility of each population to the indirect effects of cell killing mechanisms.

3.5.2 Hypotheses for CD8 T lymphocyte infection.

The interaction between CD4 and CD8 T-cells in the periphery as part of the immune response may perhaps transmit infection to CD8 T-cells *in vivo*. In the later stages of disease, the architecture of the lymph node collapses and these organs lose their ability to trap the virus. The spillover from the lymphoid organs may to some extent explain the increase in viral burden as disease progresses.

The failure of the lymphohematopoietic system during HIV progression may be due to a primary pathogenic event rather than simply T-cell destruction and

exhaustion. Either the bone marrow or the thymus is rendered dysfunctional during HIV-1 disease, resulting in low rates of replenishment of mature T-cells, which would ultimately result in immune system collapse. This would cause an overall decrease in the number of naïve T-cells, a distorted level of $CD8^+$ T-cell populations and a restricted TCR repertoire (reviewed in Hellerstein *et al.*, 1997). Donaldson and colleagues found that HIV could spread to non-lymphoid tissues during the later stage of HIV disease (Donaldson *et al*, 1994). These findings may possibly be related to the observed increase in CD8 T-cell infection upon disease progression (Livingstone *et al*, 1996). On the other hand, a defective HIV-1 specific CD4 T-helper response may indirectly influence normal CD8 T-cell function. CD8 T-cell depletion has been also been attributed to apoptotic and anergic events (Herbein *et al.*, 1998; Lewis *et al.*, 1993).

The thymus represents a major target for HIV-1 infection *in vivo* and destruction of thymopoietic areas is observed on autopsy examinsation of AIDS cases. Functional HIV proviruses have been found in thymocytes lacking surface CD4 expression. Neither HIV-mediated CD4 downregulation nor CD4-independent infection contributed to the localisation of HIV in cells lacking the primary virus receptor. Instead, infection of a CD4-positive precursor cell (CD4positive/CD8 positive) with subsequent differentiation into a mature CD4-negative phenotype resulted in productively infected CD4-negative cells (Kitchen *et al.*, 1997). HIV-1 infection of $CD4^+$, $CD8^+$ immature thymocytes destined to become CD8 lymphocytes during thymic maturation (Kitchen *et al.*, 1997; Lee *et al.*, 1997) would provide both a plausible mechanistic explanation for their infection, and would also explain the presence of proviral sequences in the naïve subsets of both CD4 and CD8

lymphocytes in peripheral blood. Infection of an immature progenitor cell followed by differentiation into a mature productively infected single positive CD8 cell is a novel mechanism of HIV-1 entry into a non-CD4 expressing cells.

Destruction of CD8 precursor cells would also explain the eventual failure of CD8 homeostasis and decline in circulating numbers of first naïve and then memory CD8 lymphocytes on disease progression (Roederer *et al.*, 1996; Roederer *et al.*, 1995), and the recovery in numbers of naïve $CD8^+$ (and $CD4^+$) lymphocytes generally observed after commencement of antiviral treatment (Arno *et al.*, 1998; Bohler *et al.*, 1999).

Evidence for the quiescent nature of HIV-1 infection in CD8 naïve lymphocytes, consistent with thymic infection, is provided by the great stability of the infected CD8 population on anti-retroviral therapy and indirectly by the genetic relationship between the CD8 and CD4 populations. Combination treatment achieved a complete clearance of circulating viremia in the majority of study subjects (Table 4), while over the period of virus suppression ranging from 1-11 months, there was no consistent reduction in frequencies of infected CD8 lymphocytes. Over the same period, frequencies of infected naïve and memory subsets of CD4 lymphocytes showed a modest decline, consistent with previous kinetic studies reporting half-lives of proviral sequences from 21-58 weeks (Bruisten *et al.*, 1998; Izopet *et al.*, 1998; Vanharmelen J *et al*, 1997; Ibanez *et al*, 1999).

Since $CD8^+$ T-cells mainly control viral replication, it seems reasonable to assume that infection of CD8 naïve cells, a cell type normally resistant to infection would have major pathogenic consequences, including increasing the reservoir of infected cells and disturbing the CD8 arm of the immune response. Our evidence for

infection of the CD45RA$^+$ population of peripheral CD8 lymphocytes highlights the need for future work in assessing the exact mechanism of peripheral CD8 lymphocyte infection *in vivo*. In the future, phenotypic characterisation of variants infecting the two cell types should indicate whether CD4-independent entry of HIV-1 can occur. Investigating the biological properties of the proviral sequences detected in CD8 lymphocytes would prove beneficial. This could be assessed by analysing virus or PCR-generated fragments of DNA sequence obtained directly from different T-cell types in PBMCs and constructing them into infectious clones which can then be further characterised for individual cellular tropism. Future in-depth analysis of the effects of HIV-1 infection within both mature and immature CD4 and CD8 T lymphocytes in fresh human thymus and lymph nodes samples could also provide clearer information about the thymic abnormalities that are known to occur upon disease progression. Determinsing the cellular distribution of HIV-1 proviral sequences within the thymus and lymphoid tissues may give rise to potentially interesting investigations. Increasing studies on the turnover of HIV-1 infected CD8 lymphocytes *in vivo*, such as the acquisition of antiviral resistance during treatment relapse, will provide information on the dynamics and consequences of CD8 infection in the peripheral circulation, and potentially, on the mechanisms underlying the loss of CD8 lymphocytes on disease progression.

3.5.3 Effects of HAART on infected CD4 and CD8 T lymphocytes

Treatment of HIV infection with anti-retroviral drugs involves blocking virus replication so detailed observations in individuals treated with powerful anti-retrovirals can provide further information on the role of viral replication in HIV

pathogenesis and the dynamics of this process. Combinations of drugs that inhibit viral reverse transcriptase and protease control infection with HIV-1 in many individuals by reducing the levels of RNA in plasma and depleting the pools of virus in lymphoid tissue. This sustained reduction in viral replication delays disease progression and prolongs survival. In this study we analysed the effect of antiviral treatment on the distribution of infected lymphoid cells.

In clinical practice, the most useful marker of progressive deterioration has been the absolute number of $CD4^+$ T-cells. Lymphocyte subsets change in absolute number and character over the course of HIV infection. An increase in CD4+ cell counts has been noted in HIV-infected individuals following HAART over several months. Although HARRT treatment dramatically decreases plasma viral load and effectively suppresses viral replication to undetectable levels (<400 RNA copies per ml), the plasma concentration of HIV-1 proviral DNA has been found to decrease only slowly. This could provide a basis for continued virus replication, and the rapid rebound of viral replication occurring after cessation of drug therapy.

So far, there is little information on the dynamics of infection in the peripheral blood of individuals undergoing anti-retroviral therapy. While most viral production is believed to take place in the lymphoid tissue, there is a real need to examinse the levels of productively infected cells in the periphery. In this study we analysed the effects of antiretroviral therapy on five study subjects and found that while particular individuals were undergoing HAART there was an appreciable difference in the distribution of infected cells *in vivo*. Our observations indicate that many individuals harbor HIV provirus in all populations of CD4 and CD8 cells while undergoing therapy. There was remarkable individual variation in the frequencies of infected cells

in each subset. Infected naïve and memory cells were observed to decline during HAART. While we were not able to detect any HIV-1 DNA in the CD8 memory cell population of individuals analysed we did detect HIV DNA in the CD8 naïve cell subset. No correlation was observed between the decrease in viral load observed with the CD4 subsets and the CD8RA cells during the course of anti-retroviral therapy.

Despite the apparent success of antiretroviral therapy in suppressing plasma HIV-1 RNA for long periods, long-lived reservoirs of infectious virus remain in CD4+ T-cells and perhaps other cells, suggesting the need for continued long-term treatment. In this study, frequencies of infected naïve and memory subpopulations of CD4+ T lymphocytes declined slowly during HAART treatment with half-lives of infected cells of approximately 6 months, in agreement with previous studies (Ibanez *et al.*, 1999; van Harmelen *et al.*, 1997; Izopet *et al.*, 1998; Bruistein *et al.*, 1998). Frequencies of infected naïve CD8 lymphocytes were more stable, with no observable decline over 6-12 months, although a larger number of patients are required to substantiate this observation.

After completion of this thesis project, further work was recently carried out to examinse the quiescent nature of HIV-1 infection in CD8 naïve lymphocytes (McBreen *et al.*, 2000). A method was designed to quantify complete proviral sequences (i.e that span the primer binding site [OBS} and U5 region; C-LTR-primers) and initial transcripts (primers amplifying the region immediately downstream from the PBS (pan-LTR). No observations were made between the two sets of primers, concluding that there is no evidence for the existence of significant numbers of incomplete transcripts that might be associated with abortively infected cells (Stevenson *et al.*, 1990; Zack *et al.*, 1990; Zack *et al.*, 1992). This stability of

the proviral load in the CD8 cells during effective antiretroviral therapy is also consistent with stably integrated proviral sequences in the this lympocyte subset. Indeed, CD8 lymphocytes themselves could represent a reservoir for HIV-1 which may persist for long periods at low levels in many treated individuals despite the use of maximally suppressive antiretroviral regimens.

The availability of quantitative data on plasma viral load in patients entering potent antiviral therapy has revealed that the half-life of plasma virions is only 6 hours and the great majority of the virus population turns over within a few days (Ho *et al.*, 1995; Wei *et al.*, 1995, Perelson *et al.*, 1996). Even so viral replication continues at a rate of 150 replication cycles a year (Coffin *et al.*, 1995) and underlies the rapid evolution of the population in the plasma. Despite this, phylogenetic studies show that lineages of related sequences can be detected over long periods in the circulation (Holmes *et al.*, 1992; Leigh Brown and Cleland *et al.*, 1996).

Regardless of their mechanism of decline, the absence of naïve-phenotype T lymphocytes theorectically limits immune responses to new antigens. In persons with late-stage HIV-1 infection who have immune collapse and loss of all $CD4^+$ T-cell subsets, concern over preferential loss of specific $CD4^+$ T-cell subsets may be irrelevant. In the era of effective antiretroviral therapy, concern over the restorative capacity of essential $CD4^+$ and CD8+ T-cell subsets may become more relevant. Without HAART HIV-1 infection leads to progressive deterioration of the immune system and eventual death from opportunistic infection or AIDS-related malignancy in the majority of cases.

For further understanding of HIV pathogenesis during disease progression, it is important to analyse whether the suppression of virus replication caused by

combinations of antivirals results in the reappearance of variants containing resistance mutations. Investigations into the replacement of the original proviral population by resistant variants will provide new information on the turnover of CD4 and CD8 lymphocytes *in vivo*.

CHAPTER FOUR

4.1 INTRODUCTION

Like other retroviruses, HIV-1 is characterised by a high degree of genetic variablility. HIV-1 infection is associated with a progressive deterioration of the host immune system as a consequence of chronic viral replication in susceptible host T-cells. The destruction of the host immune system should predict a progressive loss of host adaptive pressures over the course of HIV disease. The spectrum of genetically diverse variants found in a viral population within an infected individual is responsive to the selective forces of evolution. In primary infection, there is a relatively narrow distribution of genetic variants (Zhang *et al.*, 1993). The complex mixture of genetic variants that subsequently arise during the course of an infection (Holmes *et al.*, 1992; Kuiken *et al.*, 1992; Wain-Hobson *et al.*, 1989) results from competition and selection among the variants in response to immunologic pressure for change and alterations in cell tropism and replication efficiency (Coffin *et al.*, 1992., Hwang *et al.*, 1992).

Selection due to cell tropism and replication efficiency could result in discrete populations of viruses with distinct genetic and biologic features found within specific tissue types (Epstein *et al.*, 1991; Keys *et al.*, 1993). HIV-1 is susceptible to genetic recombination and has an error-prone reverse transcriptase enzyme, which combined with the absence of proofreading leads to a misincorporation rate of 10^{-4} to 10^{-5} per base, or approximately one misincorporation per genome per replication cycle (Pathak *et al.*, 1990; Preston *et al.*, 1988) and (Ricchetti *et al.*, 1990., Roberts *et al.*, 1988). With 10^{9} new virions produced each day and a half-life of approximately 6h, an HIV-1 infected individual harbours a swarm of closely related viruses that comprise the so called HIV-1 quasispecies. These HIV-1 variants have been shown to differ in the

biological properties such as replication rate, cell tropism, and synctium-inducing (SI) capacity (Asjo *et al.*, 1986; Choe *et al.*, 1996; Tersmette *et al.*, 1989).

HIV-1 shows considerable sequence diversity between different isolates, particularly those from geographically distinct regions, where divergence has taken place over a number of years. Sequence diversity is seen within isolates from the same individual as well as between HIV strains infecting different individuals (Levy, 1993). The degree of sequence variation is not uniform throughout the genome; for example *gag* and *pol* are more conserved than *env* (Levy, 1993). In particular, the region of the viral genome that encodes gp120 shows an extremely high degree of diversity, concentrated into five hypervariable domains (V1-V5) interspersed among less variable regions (C1-C4) (Levy, 1993).

4.2 The third variable (V3) domain of the envelope glycoprotein.

V3, the third variable domain in the gp120 subunit of HIV-1 determinses several biological properties of the virus such as cell tropism, cytopathicity and fusogenicity. The V3 domain forms an exposed, accessible loop on the surface of the viral particles (Moore *et al.*, 1994) and induces the production of V3 antibodies detectable either after natural infection or following specific immunisation (Zwart *et al.*, 1991). Moreover this region is a determinsant for cellular tropism and viral infectivity (Hwang *et al.*, 1991; Shioda *et al.*, 1992). It contains 35 aminso acids arranged in a disulphide loop. The tip of the loop is considered the principal neutralising domain because virus neutralising antibodies directed against this region block HIV-1 infection of primary lymphocytes and macrophages. V3 is less variable than is V1 or V2 and contains a relatively conserved motif Gly-Pro-Gly, at the central

tip of the loop. In general, the GPG motif is highly conserved among HIV-1 isolates, and sequences near the cysteine residues at the bottom of the loop also show little variability (Luciw *et al.*, 1996). The most extensive variation is observed in the regions flanking the GPG sequence at aminso acids 10 to 14 and 25 to 29. In addition sequence variation is higher in the group of SI-inducing isolates than in the NSI-inducing isolates (Fouchier *et al.*, 1992).

Over the course of infection, the virus an individual can carry broadens in tropism and biological variability. Small changes in the envelope glycoprotein aminso acid composition can lead to large differences in phenotype and biological properties such as replication rate, cellular tropism and syncytium-inducing capacity (Asjo *et al.*, 1990; Tersmette *et al.*, 1989). After seroconversion, sequence diversity expands, yielding HIV-1 variants with distinct characteristics such as slow or rapid replication kinetics, syncytium induction, and tropism for particular cell types in addition to primary T-helper lymphocytes (Asjo *et al.*, 1986; Evans *et al.*, 1987; Gartner *et al.*, 1986; Roos *et al.*, 1992; Schuitemaker *et al.*, 1992). Virus isolated in the early asymptomatic phase of infection is predominsantly slowly replicating, macrophage tropic, and nonsynctium-inducing (NSI) *in vitro* (Connor *et al.*, 1993; Roos *et al.*, 1992; Schuitemaker *et al.*, 1992). During disease progression HIV-1 isolates display increased replication rates and reduced macrophage tropism (Koot *et al.*, 1993; Schuitemaker *et al.*, 1992; Tersmette *et al.*, 1989). Although initial infection is limited to macrophage-tropic virus incapable of causing syncytia *in vitro* (NSI), sequence variation occurs rapidly. As a predominsant HIV species is maintained over time, swarms of quasispecies, of subtly altered viruses emerge with broadened tropism (lymphocytes and macrophages) and increased cytopathogenicity (synctium-inducing,

SI). Initially the SI capacity of HIV-1 isolates was determinsed by scoring syncytium formation upon inoculation of PBMC's with virus or cocultivation of PBMC with virus-infected cells. Alternatively, the SI capacity of HIV-1 was determinsed by using MT2 cells as an indicator, since MT2 cells selectively support replication of SI variants (Schuitemaker *et al.*, 1992).

The V3 domain of HIV-1 gp120 has been demonstrated to be a major determinsant of the SI capacities of virus isolates (Dewolf *et al.*, 1994). Comparison of V3 domains from a panel of SI and NSI HIV-1 variants resulted in a clear correlation between viral phenotype and V3 conformation. In SI isolates, positively charged aminso acid residues were found at position 11 and/or position 25 in V3, whereas in NSI isolates both residues were either negatively charged or uncharged. Basic aminso acids are known to play an important role in the SI phenotype. Analysis of natural variants of HIV-1 coupled with studies on point mutations introduced into V3 molecular clones of virus, indicates that basic aminso acids in one or more positions 11, 24, 25, 29 and 32 (see figure 4.1) confer an SI phenotype, whereas hydrophobic aminso acids in these positions correlate with a NSI phenotype (Chesebro *et al.*, 1992; de Jong *et al.*, 1992; Fouchier *et al.*, 1992; Milich *et al.*, 1993). Conversion within the NSI V3 loop of a negatively charged aspartate into a positively charged arginine (D to R) at position 25, or an aspartate switch to asparagine (D to N) at position 29 is sufficient to gain the SI phenotype (de Jong *et al.*, 1992).

The appearance of SI viruses often heralds worsening disease and heightened replication rates, with a decrease in the number of circulating CD4 lymphocytes. The rates at which these events occur vary among individuals, but increased viral burden

and increased viral cytopathogenicity appear closely correlated with CD4 decline and poor prognosis. The SI phenotype correlates with increased V3 sequence heterogeneity with a broad range of substitutions, insertions and deletions at many positions between the disulphide-bridged cysteine residues (Chesebro *et al.*, 1992; Milich *et al.*, 1993). In contrast, V3 sequences from NSI isolates show few sequence differences from each other or from a consensus sequence of 133 North American isolates (LaRosa *et al.*, 1990) that comprise predominsantly subtype B variants of HIV-1 (Louwagie *et al.*, 1993).

Changes in the *in vitro* phenotype of virus isolated from PBMCs can occur during different stages of disease progression. Donaldson *et al* found a highly restricted distribution of HIV in the body preceding the onset of AIDS with proviral sequences recovered from PBMCs, spleen and lymph nodes. In contrast, proviral sequences from patients who died from complications associated with AIDS were also distributed in the CNS and in lung and bowel tissues in addition to the lymphoid tissue (Hien *et al.*, 1994). The apparent redistribution of virus upon disease progression occurs at the same stage of disease as the change from an NSI to an SI phenotype (Donaldson *et al.*, 1994).

Although isolates become more cytopathogenic and often non-MT *in vitro*, the redistribution of HIV *in vivo* involves organs such as brains and lungs and other tissues in which the main targets of infection are reported to be tissue macrophages, microglia (in the CNS), and other non-lymphocyte cell types (Koenig *et al.*, 1986). V3 loop sequences from lymphoid and non-lymphoid tissues showed a highly restricted sequence variability and a low overall charge of the encoded aminso acid sequence compared with those of standard laboratory isolates of HIV-1. The low

charge and the restriction in sequence variability was similar to isolates with an NSI and macrophage-tropic phenotype *in vitro*. All patients, irrespective of disease progression, were found to be infected with the predicted NSI/MT phenotype.

Most of the published sequences of SI variants used for sequence comparisons were derived from isolates of HIV-1 that were passaged extensively in cell culture, therefore it is possible that whatever selection constraints restricts sequence diversity *in vivo* are absent in the conditions used for virus culture ands that virus is free to drift away from the subtype B consensus sequence. It is also possible that a V3 or V2 loop with a large positive charge may confer a growth advantage *in vitro*, leading to selective isolation of variants bearing such divergent sequences from a heterogeneous population. Perhaps designation of V3 as a hypervariable region may be unduly influenced by the characteristics of cultured isolates of HIV-1 and may not reflect the relative homogeneity of sequences in this region *in vivo* (Donaldson *et al.*, 1994).

Phenotypic differences may underlie differences in *in vivo* cellular tropism. HIV-1 variation between different tissues, such as blood, spleen and brain has indicated that distinct quasispecies have evolved independently (Ball *et al.*, 1994; Epstein *et al.*, 1991; Pang *et al.*, 1991) with considerable variation even within a single organ (Delassus *et al.*, 1992). Morris *et al* have recently shown the complexity of viral populations infecting non-lymphoid tissues such as the brain. These complex viral populations were rendered more diverse by recombination, and could provide a powerful adaptive mechanism for the spread of virus with new phenotypes, such as antiviral resistance or escape from cytotoxic T-cell recognition, into existing tissue-adapted virus populations (Morris *et al.*, 1999).

In addition, escape from anti-V3 loop neutralising antibodies resulting from aminso acid substitutions within and outside the V3 loop has been demonstrated both *in vivo* and *in vitro* (McKeating *et al.*, 1989; Nara *et al.*, 1990).

4.3 Sequence variation in selected cell subsets

Populations of HIV variants infecting PBMCs *in vitro* are variable at different stages of disease progression. On the basis of this, it is clearly important to investigate the *in vivo* association between virus distribution, phenotype and nucleotide sequence diversity during different stages of disease progression. Comparisons of sequences from the *env* gene of variants in different lymphocyte subsets will allow inferences concerning the rate of turnover within the peripheral circulation. In this study, detailed sequence comparisons of the V3 loop and flanking regions of virus variants were carried out in CD4 and CD8 naïve and memory cells from seven HIV-1 seropositive individuals. Sequences from the V1/V2 loops and flanking regions were also analysed from three seropositive individuals in the same cell populations. Plasma RNA was extracted, amplified and sequenced from another three individuals and compared with V1/V2 and V3 sequences derived from the corresponding individuals' cell subsets.

Comparisons of V3 charge and sequence diversity of the inferred aminso acid sequences of the V3 region were made between different T-cell subsets and individuals in this study. Rooted trees were constructed using the MEGA (Kumar *et al.*, 1993) package with the sequences HIV MN and HIV HXB2 as an outgroup and the Jukes Cantor method to account for multiple substitutions. The bootstrap resampling method was used (100 replicates) to assess the robustness of each branch in the tree constructed.

4.5 RESULTS

To investigate the possible genetic partitioning and differences in predicted phenotype of HIV infecting CD4 and CD8 lymphocytes, proviral sequences of the V3 hypervariable region of *env* were compared. From seven individuals previously analysed (p2, p3, p8, p12, p13, p14 and p15) in Chapter 3, approximately 10 clones derived from amplified DNA extracted from the separated lymphocyte subsets were sequenced. Further genetic partitioning analysis in the V1/V2 hypervariable region of *env* was also carried out in two individuals p3 and p8.

The majority of the study subjects were male homosexuals while the remainder were in the risk group of intravenous drug users. Their ages ranged from 24 to 51 at the time of sampling. General information for each study subject is listed in Table 4.1. DNA from each cell subset was extracted according to the protocol described in section 2.5. Extracted DNA was amplified in the V3 and V1/V2 regions using primers as previously described (refer to section 2.5.2). PCR amplified DNA was then used for further molecular investigations.

Table 4.1 Clinical information for study subjects*.

Study Subject	Gender/ Age	Risk Group[a]	Viral Load	Rx[b]	CD4 count[c]	CD8 count[c]
P02	M/49	AH	514,5000	No	29	416
P03	M/34	AH	116,000	Yes	74	772
P08	M/45	IDU	460,000	No	280	1185
P12	F/38	IDU	64,680	Yes	329	380
P13	M/51	PAH	67,980	No	354	1317
P14	F/24	P-Het	<513	Yes	360	442
P15	M/33	PAH	60,589	No	748	1016

* These samples were also analysed in Chapter 3.

[a] : Risk Groups: AH = AIDS Homosexual; PAH = Pre-AIDS Homosexual; IDU = Intravenous Drug User; P-Het = Pre-AIDS Heterosexual

[b]: Anti-retroviral therapy. Details of duration are listed in table 2.1.

[c]: CD4 and CD8 counts at the time the sample was taken.

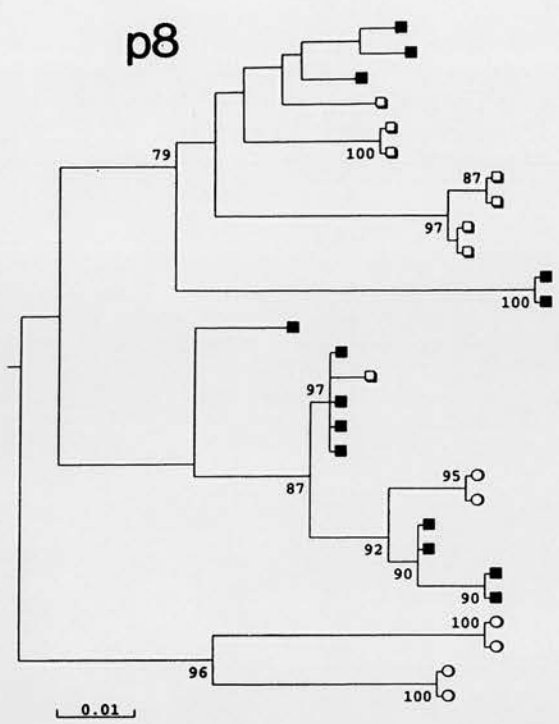
4.5.1 Estimation of Viral Load

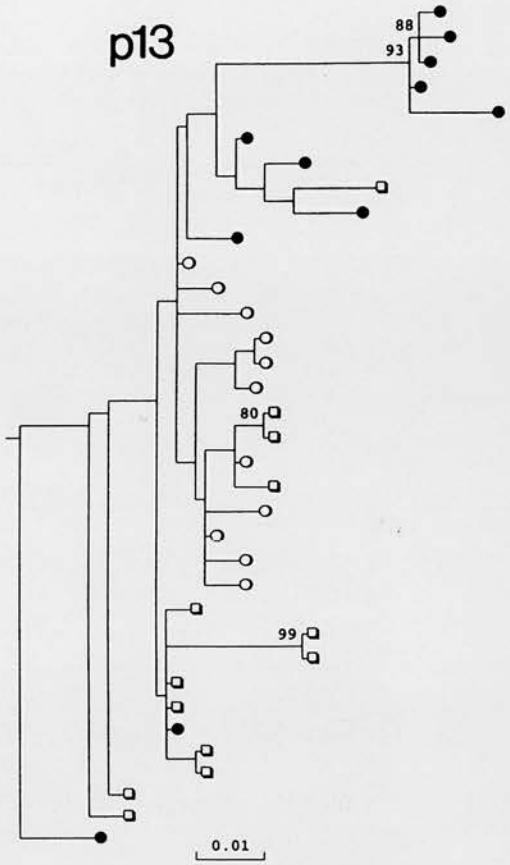
PCR amplified DNA was cloned into pGEM-T vector using poly (T) overhangs (refer to section 2.7). Proviral DNA from clones were sequenced using the Sequenase version 2.0 kit (USB) following the manufacturers protocol (refer to section 2.8.1). The sequence dataset for the V3 region extended from positions 6957-7381 in the HIVMN genomic sequence (Genbank accession number M17449), while the V1/V2 sequences were compared from positions 3539-6944. Sequences were then aligned and diversity estimated using the Simmonic 2000 Sequence Editor package. To investigate the possible genetic partitioning and differences in predicted phenotype of HIV infecting CD4 and CD8 lymphocytes, proviral sequences of the V3

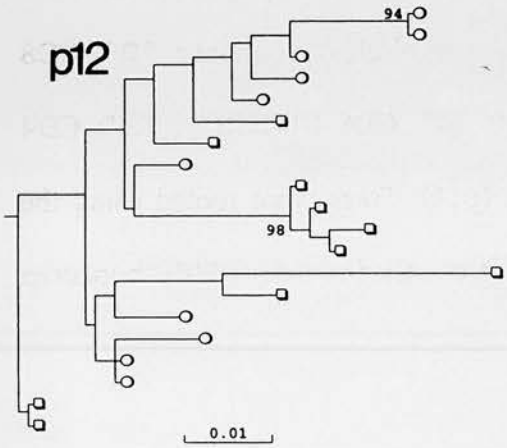
hypervariable region of *env* were compared (Figs. 4.1). From 7 study subjects, approximately 10 clones derived from amplified DNA extracted from the separated lymphocyte subsets were sequenced. The combined set of V3 sequences and V1/V2 sequences from each individual were monophyletic, and distinct from those of previously published V3 region sequences of clade B isolates: HIVSF2 (K02007), HIVRF (M17451), HIVOYI (M26727), HIVLAI (K02013), HIVJRFL (M74978), HIVYU2 (M93258), HIVCAM1 (D10112), HIVNY5CG (M38431), HIVHAN (U43131), HIVWMJ22 (M12507), and HIVSFAAA (M65024). This comparison provided no evidence for inter-sample or exogenous laboratory contaminsation. Phylogenetic analysis was carried out using the MEGA program. Distribution of pairwise distances were carried out using the Systat statistical package.

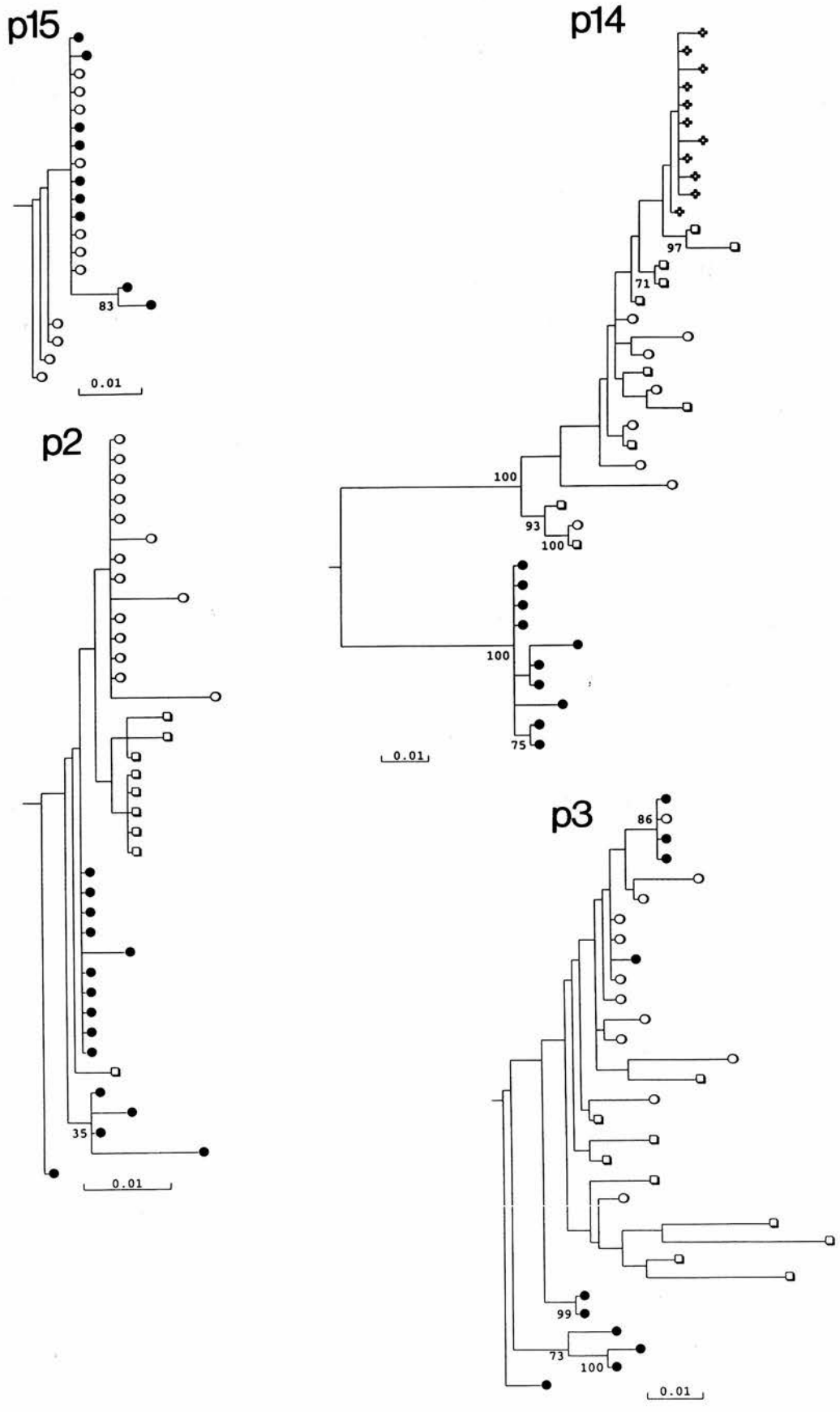
Two regions of HIV-1 proviral DNA, V1/V2 and V3 were successfully amplified by nested PCR from the naïve and memory CD4 and CD8 T lymphocytes of p3 and p8 (V1/V2 region) and p2, p3, p8, p12, p13, p14 and p15 (V3 region), respectively. Each PCR amplified DNA was cloned and ten clones from each library sequenced. The proviral loads in the samples analysed were sufficiently high to ensure that multiple templates were sampled in the 1μg aliquots used for sequencing. The exception was the naïve subset from p8; combined with the evidence for potential contaminsation (Table 3.2), sequences from this subset in the phlyogenetic analysis shown in Fig 4.3 have been omitted. No association between the detection of HIV-1 proviral DNA and the disease progression or risk groups was observed within samples from the naïve and memory subsets (data not shown).

Fig. 4.1. Phylogenetic analysis of V3 region sequences from different lymphocyte subsets of seven study subjects (study numbers in circles). Symbols: "●": CD8 CD45RA+ (naïve) cells; "❍": CD4 CD45RA+; "■": CD8 CD45RO+; "❑": CD4 CD45RO+; "_" cDNA sequences from plasma (p14). Trees were rooted using the HIV-1_{MN} sequence; scale bar indicated under tree. Clades with >70% bootstrap supported indicated by number on branch.


p8
79
100
87
97
100
97
87
95
92
90
90
100
96
100
0.01


p13
88
93
80
99
0.01


p12
94
98
0.01


p15
83
0.01
p14
97
71
100
93
100
100
75
0.01
p2
35
0.01
p3
86
99
73
100
0.01

Sequence diversity within lymphocyte subsets, and the degree of partitioning between them varied considerably between the study subjects. Phylogenetic analysis was carried out using sequences from the V1/V2 and V3 regions from a range of CD4 and CD8 naïve and memory cells from a number of patients. Divergence between nucleotide sequences was estimated using Jukes-Cantor distances (scale indicated below tree), and the phylogentic tree constructed from the distance matrix by the neighbour-joining method. The robustness of groupings was indicated by bootstrap resampling of 100 datasets, with values of $\geq$70% indicated on branches. Phylogenetic analysis of nucleotide for the V1/V2 and V3 regions revealed a variety of relationships between variants recovered from different subsets within different individuals. By comparing the sequences observed with published V1/V2 and V3 sequences of clade B isolates, no evidence was provided for inter-sample or exogenous contaminsation.

4.5.2 Phylogenetic analysis of V3 aminso acid sequences from CD4 and CD8 naïve and memory cells.

V3 sequences from the naïve CD4 and CD8 subsets of p15 grouped together and showed mean diversities of 0.002 - 0.0064, similar to the mean pairwise distance between subsets (0.0071) (Fig 4.1). In marked contrast, sequences from CD8 lymphocytes of p14 were distinct from all other cell types, forminsg a separate clade supported in 100% of bootstrap re-samplings. The mean pairwise distance between the CD8 and CD4 lymphocytes ranged from 0.113 ($CD4^+$, $CD45RA^+$) to 0.119 ($CD4^+$, $CD45RO^+$), much greater than the diversity with the CD8 population

(0.0076). There was no correlation between sequence diversity in any of the subsets with disease progression, nor with proviral load (data not shown).

Amongst the six study subjects from whom CD8 lymphocyte sequences were obtained, five showed either partial or complete separation from CD4 virus populations. In contrast only one of six study subjects showed any evidence for separation of CD4 naïve and memory subsets (p8) (Fig 4.2). Viral phenotype was predicted according to the translation of the nucleotide between positions Cys301 and Cys336 in the V3 region. This method can be used to predict *in vitro* phenotype and co-receptor usage of variants from the different lymphocyte subsets (Fig. 4.3). In general, if the positively charged aminso acids arginine (R) and lysine (K) were observed at positions 11 or 25 counted from the first cysteine (Cys 301) in the V3 loop, variants were predicted as SI strains, otherwise they were predicted as NSI strains. In general, SI strains were also known as T-cell tropic using CXCR4 as co-receptors whereas NSI strains are preferentially macrophage-tropic and use CCR5 as co-receptor.

Most sequences contained neutral or acidic residues at these sites, indicative of an NSI, CCR5-dependent phenotype. The aminso acid residue at position 11 was uncharged (in general S) and the residues at position 25 and 29 were either negatively charged (E or D) or uncharged (Q). The exceptions were p8 and p14. Changes in phenotype and tropism of virus isolates are known to occur during disease progression. In these individuals V3 sequences from certain subsets (p8: $CD4^+$, $CD45RA^+$ lymphocytes; p14: $CD4^+$ lymphocytes and plasma) contained arginine residues at position 306 along with several other aminso acid changes in V3 and

Fig. 4.2. Consensus V3 and flanking region sequences of lymphocyte subsets from seven study subjects. Separate consensus sequences were calculated for the two phylogenetic groupings of CD8 sequences from p3 and p8. Amino acid residues numbered according to HIV-1_{HXB2}; positions 306 and 320 indicated; basic residues contributing to an SI phenotype indicated in bold. Symbols: ".": sequence identity with HIV-1_{MN}; "?": variable sites without 75% majority consensus residue.

306 320

HIV-MN	DNAKTIIVHLNESVQIN	CTRPNYNKRK**R**IHIGPGRAFYTTKNIIGTIRQAHC	NISRAKWNDTLRQIVSKLKE
p2 CD4 RA	N.....	.....N.T.RS.....-E...K.....	...SL...N..E...K..R.
CD4 RO	N..R.....	.....N.T.RS.....-E...K.....	...SL...N..E...K..R.
CD8 RA	N.....	.....N.T.RS.....-E...K.....	...SL...N..E...K..R.
p3 CD4 RA	N...I...Q..S..S..	.....N.T..G.....?...GE...D.....	.?..T?..N..E.V.E..R.
CD4 R0	N...I...Q..?..S..	.....N.T..G.....GE...D..?...	.?..T...N..E.V.E..R?
CD8 RA A	N...I...Q.....S.H	.?...N.T..G?.....?...GE...D.....	.?..T?..N..E.?.E..R.
CD8 RA B	N...I...Q..S..S..	.....N.T..G.....A.GE...D.....	....TE..N..E.V.E..R.
P8 CD4 RA	..T.S...Q.K.P...?	.....NKT.R**R**.....??.?.D.....	.L...T..N..K???...R.
CD4 RO	..T.?...Q.K.P.?.T	.....N.T.RS.?....?.L??.GA...D.....	...?T...N..K?..?..R.
CD8 RA A	..T.?...Q.K.P.E.T	.....N.T.RS.....S.L...GA...D.....	..TET...N..K...?..R.
CD8 RA B	..T.I...Q.K.P.K.T	.....N.T..S.....A.E....D.....	.L..TG..N..K...M..R.
p12 CD4 RA	N...I...Q.....E..	.....N.T.?S.N.....GE...D.....	.?..T.....KK.AI..R.
CD4 RO	N...I...Q.....E..	.....N.T.?S.?.....GE...D.....	.L..?...?..KK.?I..R.
p13 CD4 RA	N.....?.Q.K.P....	.....N.T.RS.....A.GQ...N.....	.L.ET?..N..K.V.T..R.
CD4 RO	N...A...Q.K.P....	.....N.T.RS.....A.GQ...N.....	.L.?TA..N..K.V.T..R.
CD8 RA	N.....Q.K.P....	.....N.T.?S.?.....A.GQ...N.....	.L.ETA..N..K.?.T..R.
p14 CD4 RA	....S...Q.....	.....N.T..**R**.T.....VY...GE...D..R.?.	T.?KKA..N..G...E..R.
CD4 RO	....S...Q.....	.....N.T..**R**.T.....VY...GE.?.?..R.Y.	T.?K?A..N..G...E..R.
Plasma	....S...Q.....	.....N.T..**R**.T.....VY...GE.V.D..R.Y.	T.N.EA..N..G...K..R.
CD8 RA	?...N...Q...T.E..	.....N.T..S.P....K...A.GD...N.....	....TR..N..K...I..R.
p15 CD4 RA	N.....Q.K.A.P..	.....N.T..S.P.....?.GE...D.....	.L.SKT..K..H.VAI..RK
CD8 RA	N.....Q.K.A.P..	.....N.T..S.P.....A.GE...D.....	.L.SKT..K..H.VAI..R.

flanking regions. These sequences formed phylogenetically distinct clades that grouped separately from the predicted NSI, R5 variants from the other subsets.

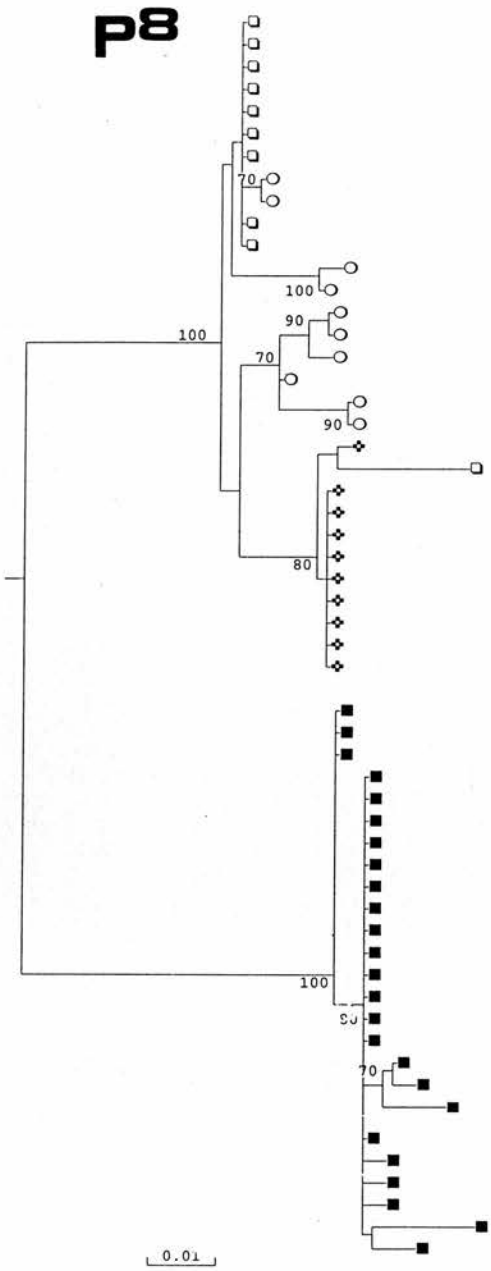
4.5.3 Phylogenetic analysis of V1/V2 nucleotide sequences from CD4 and CD8 naïve and memory cells.

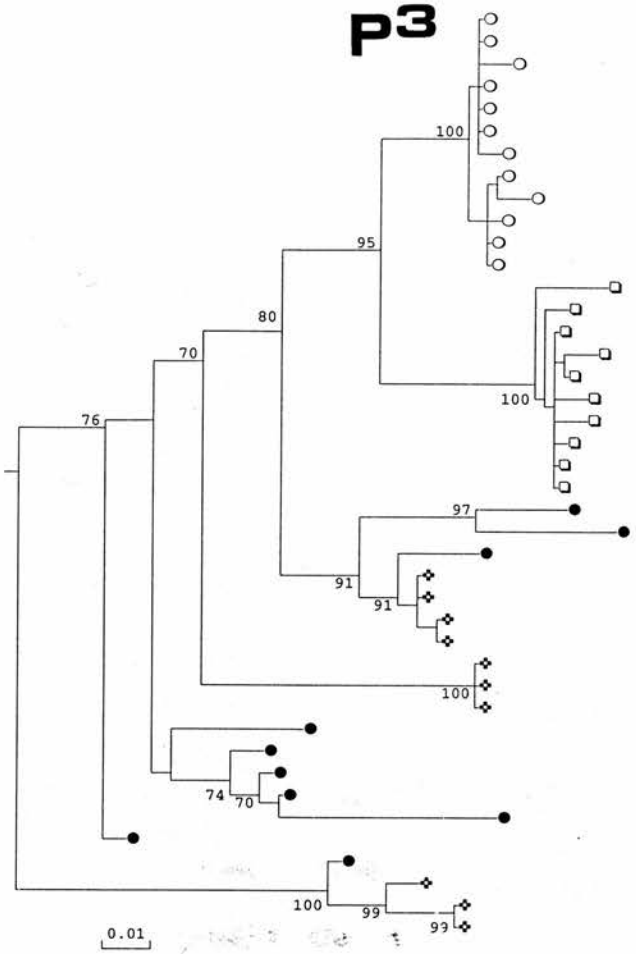
More extensive and different patterns of partitioning of variants from CD4 and CD8 lymphocytes were observed on sequence comparison of the V1/V2 hypervariable region (Fig. 4.3). Sequences from p3 showed complete separation of $CD4^+$, $CD45RA^+$ and $CD45RO^+$ subsets, each of which were distinct from CD8 sequences (which formed two distinct clades. Although there were two clades of CD8 sequences in V3 (Figs. 4.1, 4.2), there was no evidence for partitioning of the different memory phenotypes of the CD4 lymphocytes. In p8, $CD8^+$ $CD45RO^+$ cells contained variants that were distinct from every other cell type analysed. Although there was little partitioning of CD4 naïve and memory subsets in V1/V2, $CD4^+$, $CD45RA^+$ cells contained a distinct population of variants in the V3 region which had a predicted SI, X4 phenotype (Figs. 4.1, 4.2).

4.5.4 Comparison of lymphocyte and plasma derived RNA sequences.

To investigate which of the lymphocyte subsets contained the most actively replicating virus population, we compared lymphocyte and plasma-derived RNA sequences from three individuals (Figs. 4.1, 4.3), who showed clear compartmentalization of CD4 and CD8 sequences in V3 (p14) or V1/V2 (p3, p8). Different sequence relationships were observed in each case. For p14, sequences in the V3 region from plasma grouped most closely with those from CD4 naïve and

Fig. 4.3. Phylogenetic analysis of V1/V2 hypervariable region sequences from different lymphocyte subsets of two study subjects (study numbers in circles). Symbols: "●": CD8 CD45RA+ (naïve) cells; "❍": CD4 CD45RA+; "■": CD8 CD45RO+; "❑": CD4 CD45RO+; "_" cDNA sequences from plasma. Trees were rooted using the HIV-1_{MN} sequence; scale bar indicated under tree. Clades with >70% bootstrap supported indicated by number on branch.


p8
70
100
90
100
90
70
80
100
70
0.01


p3
100
95
80
70
76
100
97
91
91
100
74
70
100
99
99
0.01

memory subsets; the presence of an arginine residue at position 11 predicted an SI, X4 phenotype. Similarly, sequences from p8 in the V1/V2 region were most similar to the CD4 population, particularly the naïve subset, and were particularly distinct from those detected in the CD8 memory population. However, in p3, plasma sequences formed at least 3 groups in V1/V2, at least one of which was more closely related to CD8 sequences than to CD4, while others were dissimilar to any of the lymphocyte-derivedsequences.

4.6 DISCUSSION

Human immunodeficiency virus type-1 phenotype variability plays an important role in AIDS pathogenesis. In early asymptomatic HIV-1 infection slow-replicating macrophage-tropic NSI

HIV-1 variants are predominsant (Schuitemaker *et al.*, 1992; Van't Wout *et al.*, 1994). In the course of infection, HIV-1 isolates display increased replication rates (Tersmette *et al.*, 1989; Connor *et al.*, 1994) and reduced tropism for macrophages (Schuitemaker *et al.*, 1992). In 50% of infected individuals syncytium-inducing (SI) isolates emerge in the course of infection coinciding with an accelerated loss of CD4-positive T lymphocytes, rapid disease progression, and reduced survival time following AIDS diagnosis (reviewed in van't Wout *et al*, 1998). Variable domains of the HIV-1 envelope molecule gp120 play a prominsent role in determinsing viral phenotype. Both V1/V2 and V3 domains were found to be important determinsants for HIV-1 tropism (O'Brien *et al.*, 1990; Hwang *et al.*, 1991; Cheng-Mayer *et al.*, 1990; Shioda *et al.*, 1990; Koito *et al.*, 1994; Westervelt *et al.*, 1992) and SI capacity (DeJong *et al.*, 1992; Freed *et al.*, 1995; Fouchier *et al.*, 1992; Groenik *et al.*, 1993; Sullivan *et al.*, 1993) although other parts of the viral genome were shown to be involved as well (Cheng-Mayer *et al.*, 1991; Fouchier *et al.*, 1994). To investigate whether differences in the envelope region of HIV-1 reflected specific cellular adaptation, we compared nucleotide sequences in the V1/V2 and V3 regions from infected CD4 and CD8 naïve and memory T lymphocytes between HIV-1 infected individuals.

4.6.1 Phenotypic diversity of HIV-1 within the CD4 and CD8 naïve and memory T-cells.

The third variable domain (V3) of the human immunodeficiency virus type 1 external envelope contains determinsants of cell tropism, cytopathicity, and infectivity and neutralising V3 antibodies are able to block attachment of the virus or subsequent post-binding events. This study encompassed sequence comparisons of the V3 region in the CD4 and CD8 naïve and memory cell subsets from six study subjects. Cloning and sequencing of the entire V3 region revealed in five patients variants infecting CD8 lymphocytes were partially or completely genetically distinct in the V3 region from those recovered from CD4 lymphocytes, and showed a greater degree of compartmentalization than observed between naïve and memory subsets of CD4 lymphocytes. In addition, residues at positions 306 and 320 predicted a preferential distribution of synctium-inducing, CXCR4-dependent variants in CD4 lymphocytes (p14).

Even more marked sequence differences were observed upon sequence comparison of the V1/V2 hypervariable region, with evidence for recombination between these regions in at least one study subject (p8). The complexity of viral populations within T lymphocytes produced by recombination could provide a powerful adaptive mechanism for the spread of virus with new phenotypes, such as antiviral resistance or escape from cytotoxic T-cell recognition.

Cell-free HIV-1 circulating in plasma more closely resembled variants infecting CD4 lymphocytes. Viruses isolated from $CD4^+$ memory/effector cells have been found to be more genotypically related to those found in the plasma (Ostrowski *et al.*, 1999).

Sequences recovered from the CD8 lymphocytes were frequently distinct from those from CD4 lymphocytes, whereas the $CD45RA^+$ and RO^+ subsets of the latter were generally undifferentiated from each other. Although there was great individual variation in the sequence relationships between different T-cell types, the observation that CD8 lymphocytes of p14 retained a low charge, likely CCR5-dependent V3 loop sequence when both subsets of CD4 lymphocytes and the plasma population switched to a probable CXCR4 phenotype argues for slower turnover of the CD8 population in this case. The temporal differences, rather than a difference in cellular tropism, may therefore underlie the genetic differences between CD4 and CD8 lymphocytes in this and the other study subjects.

4.6.2 HIV-1 tropism in vivo.

Peripheral CD8 lymphocytes express low levels of CD4 on mitogenic and antigenic stimulation *in vitro*, and it has been hypothesised that this allows productive infection of CD8 lymphocytes *in vivo* to occur (refer to Chapter 3 introduction). However, it is possible that genetic variants of HIV-1 evolve during persistent infection to infect T-cells by a non-CD4 dependent mechanism; high levels of virus replication and extensive depletion of the CD4 target population may contribute to this switch.

Briefly summarising, these observations provide the phylogenetic evidence for infection of the CD45RA+ population of CD8 lymphocytes while additionally identifying the complexity of individual variants infecting CD4 and CD8 T lymphocytes *in vivo*. In this study we have observed consistent genetic differences in the V1/V2 and V3 hypervariable regions of all samples analysed. However, it is

difficult to know whether the different sequences obtained represent genuine diversity of HIV within thaT-cell type or whether each cell type purified consists of a heterogenous population of cells at different stages of activation. Divergent genetic differences observed between the CD4 and CD8 lymphocytes analysed may also explain the alternating frequency of turnover between the naïve and memory subsets studied earlier (refer to Chapter 3 introduction). While phenotypic characterisation of HIV variants infecting CD4 and CD8 lymphocyte would clearly be of value, and the genetic differences observed in V3 and V1/V2 provide evidence for a genetic difference between the CD4 and CD8 populations, CD4-independent infection would likely remain cell-cycle dependent or perhaps become more so if based on expression of high levels of co-receptors.

In the future, phenotypic characterisation of variants infecting the two cell types should indicate whether CD4-independent entry of HIV-1 can occur. This may be achieved by phenotyping HIV populations *in vivo* without prior virus isolation by amplification and expression of partial or whole *env* sequences. *Env* sequences can be substituted into a proviral backbone, or cloned into a separate expression vector which can be transfected with an *env*-deleted proviral clone to generate pseudotypes. Designing pseudotypes which accurately reflect the properties of HIV-1 encoded proteins *in vivo* will provide further information on the mechanisms of viral infectivity and cellular tropism. Comparing sequences from the amplified sequence molecules will also allow accurate measurement of population diversity including the extent *of in vivo* recombination. It is hypothesised that minsor changes in the *env* sequence could mask subtle adaptive changes associated with HIV replication. This work is currently

ongoing at the Laboratory for Clinical and Molecular Virology, University of Edinburgh.

CHAPTER FIVE: INTRODUCTION

5.1 Overview

Human immunodeficiency virus, the aetiological agent of AIDS, can establish productive or latent infections in CD4+ T lymphocytes and monocytes in culture, and these alternative states of viral-gene expression could readily account for the damaging consequences of infection and the difficulties in developing a protective vaccine. Provirus-containing lymphocytes in PBMCs and lymph nodes are generally transcriptionally inactive and are not destroyed by HIV infection, either because of infection with a defective virus or because virus expression is inhibited. Latently infected cells could escape detection and destruction by host defences and disseminsate infection in and between individuals in the face of natural or vaccine-induced immunity. A series of immunocytochemical techniques was used to determinse the distribution of infected cells in the lymphoid population to allow comparison with the frequencies detected by PCR. The combination of cell sorting and immunocytochemical identification may extend the knowledge of the distribution of infected cells in the lymphoid population to a wider range of cell types, and allow the frequencies of infected cells in PBMCs to be compared with those in lymphoid tissue.

The distribution of actively and latently infected T-cellsallows a more accurate evaluation of the in vivo tropism of HIV, reveals more about the dynamics of infected cell turnover, and provides information on the mechanism of lymphocyte depletion upon disease progression. Sequential incubations with monoclonal antibodies and labelling with fast red, fast blue, and diaminsobenzidine (DAB) allowed the

distribution of three cell surface or internal markers to be determinsed. Staining for the CD4 and CD8 cell surface markers from cultured cells was achieved using this method and allowed infection to be detected by analysing expression of the p24 antigen within the identified cells. In-situ PCR was also used to detect HIV proviral sequences in selected lymph node and spleen tissue sections.

5.2 IMMUNOCYTOCHEMISTRY

Immunocytochemical techniques, invented by A.H Coons (Coons *et al.*, 1941; Coons *et al.*, 1955) have gradually, with modifications and improvements, contributed hugely to our understanding of human pathology. The continued refinement of these tools has significantly expanded the capabilities of the pathologist in diagnostic procedures. Immunocytochemistry is the identification of a tissue constituent or cells in situ by means of a specific antigen-antibody reaction, tagged by a microscopically visible label. Immunocytochemical techniques have been shown to be useful for the localisation of viral proteins present in individual cells. The detection of HIV-1, HIV-2 and SIV viral proteins has been reasonably successful and this technique has allowed our further understanding of the natural history and host-virus interactions in patients with these infections. Immunocytochemistry on tissue sections is also an important diagnostic and research tool in all branches of pathology. In neuropathology, the localisation of cell types or structural proteins by immunocytochemisry in human post-mortem tissues is of major importance in determinsing the aetiology or mechanisms of disease processes.

The presence of HIV-1 in brain tissue has been demonstrated by in-situ hybridisation for specific HIV-1 mRNA (Saito *et al.*, 1994; Ranki *et al.*, 1995),

immunohistochemical staining for viral antigen (Wiley *et al.*, 1986), amplification of extracted DNA by PCR (Achim *et al.*, 1994) and in-situ PCR for proviral DNA (Nuovo *et al.*, 1994; Bagasra *et al.*, 1996, Strappe *et al.*, 1997). HIV-encephalitis (HIVE) is a direct effect of HIV-1 infection of the CNS and occurs in a proportion of untreated AIDS patients. In 1991, a consensus study defined the term HIVE as the presence of a constellation of histopathologic findings such as microglial nodules, multi-nucleated giant T-cells (MGCs) and/or immunocytochemically detected HIV antigens in brain parenchymal cells (Budka *et al.*, 1991). The presence of HIV-1 antigens or nucleic acid must be demonstrated by immunohistochemistry staining or nucleic-acid-based detecting techniques. These techniques are a helpful adjunct to the diagnosis of HIVE even though HIVE can be diagnosed by the presence of giant T-cells alone. Microglial nodules are focally concentrated collections of microglial cells, macrophages and lymphocytes and have been observed in both grey and white matter (reviewed in Bell *et al.*, 1998).

HIV-1 replicates in lymph nodes (LN) and microglial cells and the detection of HIV provirus in tissues at central sites of the body may further confirm the presence of HIV. Other potential mechanisms for persistence involve the survival of an inactive form of the virus that retains the potential to give rise to infectious virus. Potential reservoirs for HIV infection include anatomical sites such as the gut, the male genital tract, and the CNS in those cells which do not display productive infection. A substantial fraction of the total lymphocyte pool is located in the gut associated lymphoid tissues (GALT). There is evidence that HIV-1 is trapped by follicular dendritic cells within LN germinsal centres (GC), which subsequently infect CD4+ T-cells as they migrate through GC to the paracortex (Embretson *et al.*, 1993; Pantaleo

et al., 1993). Several investigators have also demonstrated the presence of HIV virions (Ludewig *et al.*, 1995; Rappersburger *et al.*, 1988; Stingl *et al.*, 1990), proviral DNA (Zambruno *et al.*, 1991) and RNA transcripts (Giannetti *et al.*, 1993) within Langerhans cells. Langerhans cells infected with HIV-1 are defective in their ability to induce a primary immune response (Blauvelt *et al.*, 1995) and may transmit the virus to the T-cell compartments of lymph nodes (Stingl *et al.*, 1990).

HIV-1 infection of the central nervous system (CNS) was thought to be limited to the microglial cells (Lipton *et al.*, 1992) but recently there has been accumulating evidence that astrocytes are also a target for infection (Dow *et al.*, 1992; Genis *et al.*, 1992; Epstein *et al.*, 1993; Benos *et al.*, 1994; Tornatore *et al.*, 1994; Nuovo *et al.*, 1994; Balluz *et al.*, 1996; Takahashi *et al.*, 1996; Niikura *et al.*, 1996). The level of HIV-1 replication in astrocytes is low but a persistent restricted infection clearly develops (Tornatore *et al.*, 1994; Brack-Werner *et al.*, 1992).

In addition to CD4+ and CD8+ HIV-specific T-cells, HIV might interfere with other cell types whose function is crucial for the generation of effective immune responses (i.e. monocytes/macrophages and dendritic cells). These specialised antigen presenting cells (APCs) are central to the induction of humoral and cell-mediated immune responses. Interference with APC function might be the result either of quantitative depletion of direct cytopathogenicity or suboptimal formation of major histocompatibility complex (MHC)-antigenic peptide complexes.

Besides lymphocytes, cells of the macrophage lineage are major target T-cells for HIV-1. HIV-1 infected macrophages persist in tissues for extended periods of time containing latent proviral DNA or large numbers of infectious particles within cytoplasmic vacuoles (Meltzer MS *et al.*, 1990; Embretson *et al.*, 1993). Furthermore

monocytes/macrophages may be important as vehicles for viral disseminsation throughout the body. In tissues such as the lung and the brain, HIV-1 is located primarily in macrophage-like cells (i.e. alveolar macrophages and microglia). Macrophages are also believed to be a vehicle for the transmission of the virus between individuals because of mucosal infection, and in haemophiliacs it was found that a crucial property of the HIV-1 variant which was transmitted is its cellular tropism (Milman G *et al.,* 1994).

Concentration of HIV-1 virions on the surface of follicular dendritic cells (FDCs) in the germinsal centers of lymphoid tissue occurs during the translation from the acute to the chronic phase of HIV infection. Formation of immune complexes (i.e. HIV bound with immunoglobulin and complement) and their seeding in the FDC network are physiological mechanisms generally devoted to the clearance of the pathogen within the reticuloendothelial (lymphoid/macrophage) system, and to the generation and maintenance of effective immune responses, respectively. However, in HIV infection these mechanisms lead to the formation of a stable reservoir of infectious virions that form a continuous source for the infection of CD4+ T-cells, which ultimately result in the destruction of the lymphoid tissue. FDCs may transmit infection to these cells as they migrate through lymphoid follicles. Latently infected lymphocytes and macrophages constitute an intracellular reservoir large enough ultimately to contribute to much of the immune depletion in AIDS (Embretson *et al.,* 1993). B cells also traffic through follicle centres but it is not yet clear as to whether these cells pose any real threat to neighbouring T-cells. Although CD4 expressing B cell lines supported *in vitro* infection of HIV-1, conclusive evidence for the *in vivo*

infection of B cells has yet to be presented (Von Laer *et al.*, 1990; Davis *et al.*, 1991).

Langerhans cells (LCs) constitute 1-3 per cent of the intra-epidermal cell population (Caughman *et al.*, 1986), but also diffusely populate non-cutaneous stratified epithelia, including that of the female genital tract (Breathnach *et al.*, 1988). Several investigators have demonstrated the presence of HIV virions (Ludewig *et al.*, 1995; Rappersburger *et al.*, 1988; Stingl *et al.*, 1990), proviral DNA (Zambruno et al, 1991) and RNA transcripts (Giannetti *et al.*, 1993) within LCs. As part of the physiological process of antigen processing and presentation, LCs carrying HIV migrate towards the T-cell areas of lymphoid tissue.

In the thymus, both lymphocytes and epithelial cells were found to be susceptible to HIV-1 infection (Numazaki *et al.*, 1989). The virus can also infect T-cells of the CNS, thereby causing much of the dementia associated with AIDS. The major target T-cells for HIV-1 in the CNS are the microglia, although the astrocytes and, possibly, brain capillary endothelial cells can also be infected (Moses *et al.*, 1996).

5.3 LOCALISATION OF INTRACELLULAR ANTIGENS

Antibody will not penetrate into cells if applied directly to living cultures. For the localisation of intracellular antigens, therefore, the first consideration is to find a suitable method for rendering cell membranes permeable to the antibodies, thus allowing access to the antigen. The 'universal' fixative tends to be formalin, which causes cross-linking of proteins with fixed tissue. Formalin removes the lipids from cells and severely distorts the teritary structure of proteins, causing protein

precipitation (Poulter *et al.*, 1983). It is important to wash the fixed cells thoroughly between this treatment and the application of the first antibody.

However, the routine methods of fixation for human post-mortem tissues and cells are considered less than optimal when immunocytochemistry is to be used as a research technique. Many methods have been used in an attempt to recover immunoreactivity within formalin-fixed tissues and cultured PBMCs. These include trypsinisation, proteolytic enzymes (Denk *et al.*, 1977; Finley *et al.*, 1982), alkaline hydrolysis in NaOH solutions (Shi *et al.*, 1992), treatment with detergents (Meehan *et al.*, 1989) and the use of formic acid to unmask antigenic sites (Kitamoto *et al.*, 1987). To improve the reactivity of epitopes that are masked by formalin fixation two practical approaches are used. Firstly, protease digestion of formalin-fixed sections to 'unmask' antigenic sites and secondly microwave heating of slides enables efficient antigen retrieval prior to immunohistochemical staining. Microwave antigen retrieval methodology is a powerful tool for obtaining optimal immunohistochemical results in formalin-fixed, paraffin-embedded tissue sections (McQuaid *et al.*, 1995). Cattoretti and Suurmeijer suggested that heat and hydrolysis may both denature and break the tissue proteins at or near the links made by the formalin between adjacent aminso acids and thought it conceivable that self-assembly of unfolded protein chains with subsequent restoration of antigenic sites occurs when the retrieval solution is allowed to cool (Cattoretti *et al.*, Suurmeijer *et al.*, 1995).

If techniques such as antigen retrieval could be used to visualise antigens that were otherwise undetectable, the range of useful immunohistochemical methods would be greatly expanded. A simplified method for antigen retrieval could reduce the

incidence of false-negative immunostaining results. In clinical applications this may translate into increased diagnostic accuracy and improved patient care.

5.4 STAINING METHODS

There are many immuno-enzymatic staining methods which can be used to localise antigens. The choice is based on the individual needs of each laboratory, such as the type of specimen being investigated, the degree of sensitivity required, and the processing time and cost requirements. Immuno-cytochemical techniques are used to identify antigens in specific cell types and to co-localise multiple antigens in individual cells. To detect sequentially two antigens in single sections, co-localisation protocols typically use primary antibodies raised in two different species (van der Loos *et al.*, 1995, Hermiston *et al.*, 1992). In detecting two antisera raised in the same species, however, recognition of both primary antibodies by the secondary antibodies is a major concern. This problem can be overcome by a number of methods. Blocking the antigenicity of the first primary antibody before application of the second primary antibody can be achieved by either eluting the antibody itself (Tramu *et al.*, 1978) or by masking the antibody completely by depositing a permanent reaction product such as silver granules (Roth *et al.*, 1990. Alternatively, primary antibodies can be directly conjugated to fluorophores (Stengl and Hildebrand *et al.*, 1990), enzymes (Bootsma *et al.*, 1984), or other molecules (Hartig *et al.*, 1995; Wurden and Homberg *et al.*, 1993) so that the conjugate itself can then be detected instead of detecting the immunoglobulin portion of the primary antibody.

5.4.1 Direct method

In this technique, an enzyme-labelled primary antibody reacts with the antigen in the tissue. Subsequent use of substrate and chromogen concludes the reaction sequence. Because this method utilises only one antibody, it can be completed quickly, and non-specific reactions are limited. However, since staining involves only one labelled antibody, little signal amplification is achieved. This method is used only rarely for detection of viruses.

5.4.2 Indirect method

In this method, an unconjugated primary antibody binds to the antigen. An enzyme-labelled secondary antibody directed against the primary antibody (now the antigen) is then applied, followed by the substrate-chromogen solution. If the primary antibody is made in rabbit or mouse, the secondary antibody must be directed against rabbit or mouse immunoglobulins, respectively. This method is more versatile than the direct method because a variety of primary antibodies from the same species can be used with the same labelled secondary antibody. The procedure is also several times more sensitive than the direct method because several secondary antibodies are likely to react with different epitopes on the primary antibody.

5.4.3 Avidin-Biotin procedure

As with intracellular antigens, the immunohistochemical localisation of cell surface antigens provides clear-cut answers to questions about antigen localisation. To do this, immunocytochemical techniques such as the Avidin-Biotin Complex procedure using Fluorescein and Diaminsobenzidine were used in the detection of

infected cell types. The most relevant advantages of the Avidin-Biotin methods are their sensitivity and versatility. The sensitivity of Avidin-Biotin methods makes them particularly suitable for immunolocalisation of antigens by means of monoclonal antibodies (MABs). Avidin has on its surface four hydrophobic pockets that behave as specific binding sites for four Biotin residues. However due to the molecular orientation of the Biotin-binding sites, fewer than four molecules of Biotin will bind. Currently two Avidin-Biotin methods are in frequent use – the ABC method and the labelled Avidin-Biotin (LAB) technique. Both methods require a Biotinylated antibody as a link antibody. Biotinylation is a mild process, whereby Biotin is covalently attached to the antibody. Open sites on Avidin from the Avidin-Biotin complex or enzyme-labelled Avidin bind to the Biotin on the link antibody. Avidin can be labelled with markers, such as fluorochrome, an enzyme peroxidase or phosphastase, ferritin and gold particles (Poulter *et al.*, 1983).

A means of enhancing the sensitivity of Avidin-Biotin systems in solid-phase assays was reported by Bobrow *et al* (1989). The method employs horseradish peroxidase (HRP) to catalyse the deposition of Biotinylated tyramide onto proteins attached to the substrate. The binding of the tyramide to the proteins at or near the site of HRP activity is believed to be due to the production of free radicals by the oxygen that is liberated by the HRP. The Biotin sites on the bound tyramide are subsequently used for attachment of Avidin, which is conjugated to HRP. The HRP is then used to catalyse a colour reaction (Bobrow *et al.*, 1989). Adams *et al* (1992) showed that the Biotin amplification procedure has great utility for a variety of applications in neuro-anatomic experiments, lectin histochemistry, and immunohistochemistry because it provides greater sensitivity than standard

procedures. One of the attractive features of this method, aside from the increased sensitivity that it offers, is that it greatly increases the versatility of all procedures to which it can be applied. A potential problem of the increased sensitivity afforded by the amplified Biotin method is the possibility of increasing non-specific staining due to endogenous Biotin.

5.5 MONOCLONAL ANTIBODIES AS IMMUNOCYTOCHEMICAL REAGENTS.

MAbs have proven to be useful in the diagnosis of infectious disease. In particular, they have enhanced existing techniques for the rapid detection of microbial antigens. MAbs differ from polyclonal antibodies in that MAbs are usually raised in mice while polyclonal antibodies are usually raised in the rabbit. MAbs to HIV-1 Gag p24 or Env gp41 have been used in several studies on paraffin embedded brain tissue with various forms of enzymatic pre-treatment (Ranki *et al.*, 1995; Esiri *et al.*, 1991; Davis *et al.*, 1992). The DuPont HIV MAb to p24 core protein has been shown to produce excellent HIV labelling compared to 11 other MAbs tested (Cartun *et al.*, 1988). Peripheral blood lymphocytes experimentally infected with HIV and subsequently fixed with formalin have demonstrated immunocytochemical labelling with the HIV MAb at the light microscopy level as well as at the ultrastructural level. In addition, HIV p24 immunoreactivity in dendritic reticulum cells and interfollicular histocytes in the lymph nodes of AIDS-related-complex patients has been demonstrated with this procedure. The DuPont anti-HIV-1-MAb p24 has also produced excellent labelling of mononuclear and multinucleated perivascular

inflammatory cells in autopsy brain specimens from patients with AIDS encephalitis (Strappe *et al.*, 1997).

Rapid virus diagnosis relies heavily on indirect immunofluoresence to detect viral antigens (Fox *et al.*, 1990). This technique involves testing cells from clinical specimens or tissue culture, for the presence of specific virus antigens by incubating them with, for example, rabbit-anti-virus antibodies. The immune complex is thoroughly washed and bound antibody is detected, following incubation with fluorescein isothiocyanate conjugated to anti-rabbit antibody, by ultraviolet light microscopy.

The development of MAbs for use as immunofluorescent reagents to detect microbial antigens in clinical specimens is not without difficulties. Success depends on the selection of MAbs that possess the necessary affinity, range of reactivity and physiochemical stability. Many MAbs have been shown to react with unrelated proteins, with the result that uninfected cells may display non-specific apparent positivity. Other MAbs have had difficulties in linking to the fluorescein isothiocyanate (Tiffin *et al.*, 1987). In this study the DuPont anti-HIV-1-p24 MAb was used with the routine ABC technique to detect the p24 antigen, since this technique has been proven reliable for sensitive HIV detection (Donaldson *et al.*, 1994; Strappe *et al.*, 1997).

5.6 IN-SITU PCR AMPLIFICATION

For years, the gold standard for viral diagnosis was to isolate virus in cell culture. This technique took several days or even weeks to produce results and only detected viable virus. The PCR was discovered in 1985 and is the most widely used

technique that allows the direct detection of both RNA and DNA viruses in clinical specimens.

Methods to demonstrate the presence of productive HIV-1 infection in PM lymphoid tissue include immunohistochemistry for viral antigen (Donaldson *et al.*, 1994) and in-situ hybridisation for specific mRNA (Ranki *et al.*, 1995). Standard *in situ* hybridisation methods for detecting viral DNA can be highly specific but are not as sensitive in detecting HIV-1 proviral DNA, hence the need for HIV-1 *in situ* gene amplification. Haase *et al* 1990 first demonstrated the cellular localisation of single copies of lentiviral DNA in sheep choroid plexus cells by PCR *in situ* hybridisation (PCR-ISH) where the amplified DNA was detected by hybridisation of an internal oligonucleotide probe. Direct *in situ* PCR (IS-PCR) allows the detection of cellular genes and viral sequences in non-disrupted cells and tissue sections and involves the incorporation during the PCR process of a labelled nucleotide (e.g. Biotin digoxigenin) which can be detected by immunological methods. Previous studies using PCR-ISH have demonstrated the presence of HIV-1 DNA in both formalin-fixed and frozen brain tissue (Takashi *et al.*, 1996, Bagasra *et al.*, 1996) and also in lymphoid tissue (Embretson *et al*; Pantaleo *et al.*, 1993), cervical tissue (Nuovo *et al.*, 1994) and fixed cells (Bagasra *et al.*, 1992, 1993). IS-PCR is potentially a more sensitive technique used for the detection of single copy genes by the incorporation of more labelled nucleotides compared to the hybridisation of a labelled probe. However false-positive signals can occur due to filling in of gaps or nicks in the genomic DNA by the *Taq* polymerase using a primer independent DNA repair mechanism (Long *et al.*, 1993; Nuovo *et al.*, 1994). Microwave pretreatment of the tissue sections combined with hot-start PCR with the appropriate controls can alleviate primer

independent polymerisation and increase the sensitivity of the reaction. Inclusion of positive and negative controls for every experiment is also a vital additional safeguard.

5.7 RESULTS

5.7.1 Detection of CD4 and CD8 expression on PBMCs infected with synctium and non-syncytium inducing isolates of HIV-1.

PBMCs were infected with either a non-syncytium inducing isolate (NSI) or a syncytium inducing isolate (SI) of HIV-1. After cultivation, the cells were washed with PBS, and immersed in ice-cold acetone. The cells were then suspended in formalin which permeabilised the cell membranes for antibody interaction with intracellular antigens. Single label staining reactions were carried out on these cells to detect HIV and cell surface antigens using various immunocytochemical techniques and colour substrates. The monoclonal avidin-biotin complex (ABC) procedure was utilised to detect two cell surface markers CD4 and CD8 both individually and combined on a single well. CD4+ and CD8+ T-cells were both individually stained with fast red and fast blue using a biotinylated rabbit anti-mouse conjugate.

CD4+ T-cells were detected with an *in vitro* conjugated complex of mouse primary antibody, biotinylated rabbit anti-mouse and normal rabbit serum complex, and developed by the avidin-biotin/alkaline-phosphatase (ABC-AP) technique with naphthol-AS-MX-phosphate/Fast Blue BB (figure 5.1). Although not apparent from some low-power images, T-cells appeared as small round cells. CD8+ T-cells were detected by another single label reaction and stained with the fast red substrate using the same methodology as described above (figure 5.2). Cells that were stained using the fast red complex as antibody label revealed a dark red colour, with some paler pink coloured cells in the background. This background shade could be accounted for extracellular debris or dead cells that may also have linked to the fast red substrate.

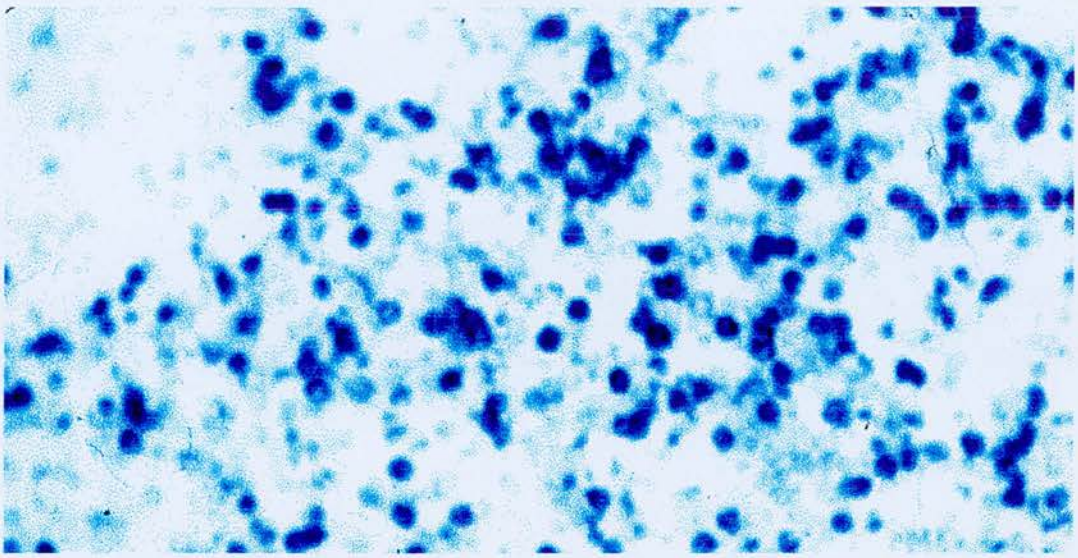


Figure 5.1. Single label. Detection of CD4+ T-cells using the Avidin-Biotin/Alkaline Phosphatase (ABC/AP) technique with naphthol-AS-MX-phosphate/Fast Blue BB. Magnification x400. NSI isolate.

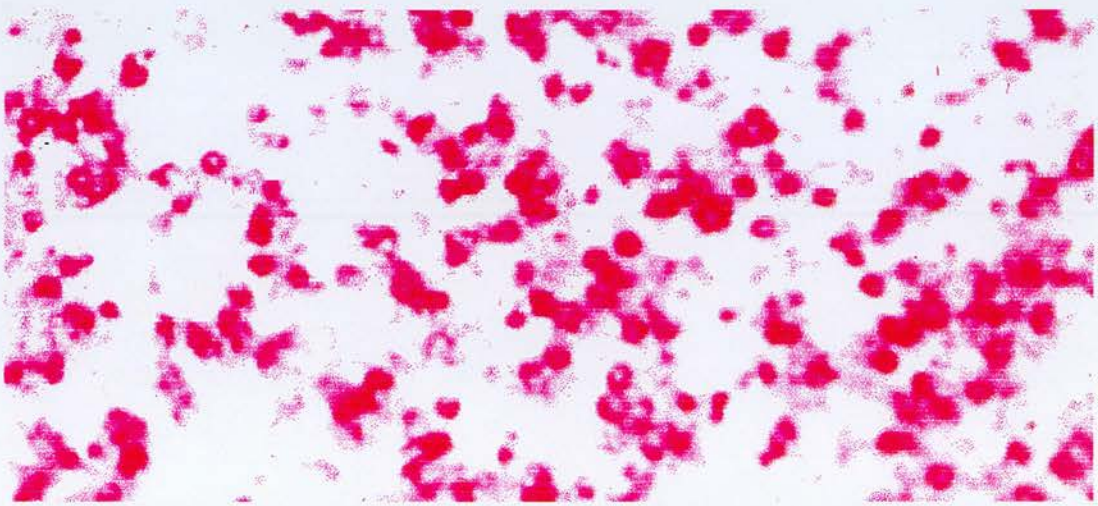


Figure 5.2. Single label. Detection of CD8+ T-cells using the Avidin-Biotin/Alkaline Phosphatase)ABC-AP) technique with naphthol-AS-MX-phosphate/Fast Red. Magnification x400. SI isolate.

The details of the basic double immunolabelling protocol used are outlined in figure 5.3 and results examined from cells infected with an SI isolate of HIV-1 stained red (CD4 cells) and blue (CD8 cells) (figure 5.3 and figure 5.3.1). Both antibodies used in this technique demonstrated precise localisation of antigen-antibody complexes with minimal non-specific background staining. Morever, to investigate the reproducibility of staining results, these experiments were repeated several times on different days with the same working conditions.

5.7.2 Immunocytochemical identification of the p24 antigen (alone) in CD4 and CD8 cells after infection with HIV-1.

Labelling of the intracellular p24 antigen with diaminobenzidine (DAB) was achieved using the monoclonal ABC procedure. Uninfected cells were exposed to exactly the same procedures as HIV-1 positive cells to serve as negative controls. In addition, infected cells with an omission of the primary antibody were also included as a negative control. The attachment of monoclonal antibodies to the infected cell was indicated by a reddish-brown precipitate formed by the action of the peroxidase enzyme in association with hydrogen peroxide and the diaminobenzidine substrate (DAB). With DAB, the p24 antigen-positive cells could be easily identified on cells infected with either the SI and NSI isolate. Positive cells stained reddish-brown while negative cells remained colourless since no counterstain was utilised (figure 5.4; figure 5.4.1; figure 5.4.2. Negative controls, in which the primary antibody was omitted, and the normal, HIV-1 negative controls, showed no positive p24 signal with the ABC technique. This confirmed the specificity of the p24 mAb (DuPont) with the

Figure 5.3 and Fig 5.3.1. Double label reaction. Detection of CD4+ and CD8+ T – cells using the Avidin-Biotin/Alkaline Phosphatase (ABC-AP) technique on an SI isolate of HIV-1. CD4+ cells were detected using Fast Red and CD8+ cells detected with fast blue substrate.

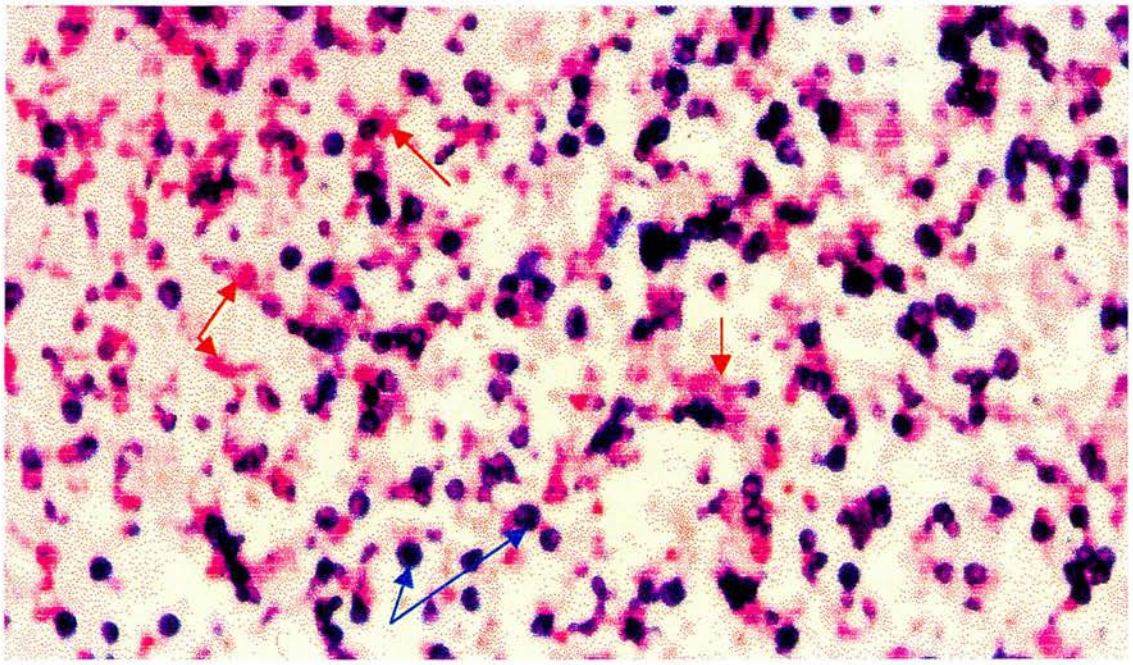


Figure 5.3. Magnification x400
CD4+ cells- Fast Red (RED ARROWS). CD8+ cells – Fast Blue (BLUE ARROWS). SI isolate of HIV-1.

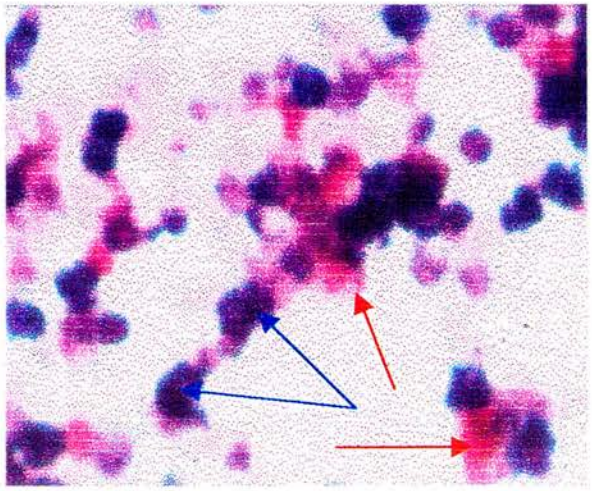


Fig 5.3.1. Magnification x100.
CD4+ cells- Fast Red (RED ARROWS). CD8+ cells – Fast Blue (BLUE ARROWS). SI isolate of HIV-1.

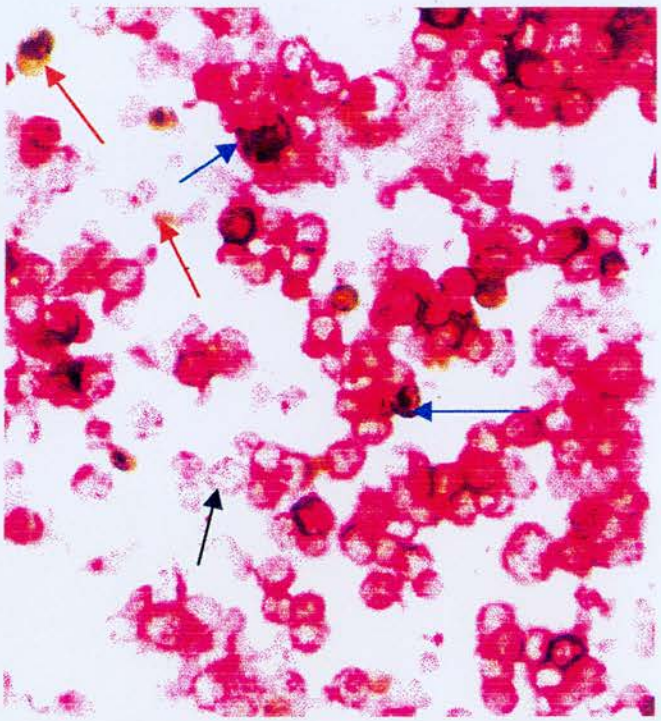


Figure 5.4. Double label reaction. Detection of CD4+ T-cells using the ABC-AP technique on an NSI isolate of HIV-1. CD4+ cells were detected using the Fast Red substrate while the p24 antigen was detected using the DAB substrate (brown precipitate – red arrows). p24+ CD4+ cells stained a red-brown colour (blue arrow). Cells negative for CD4 and p24 (black arrows). Magnification x 200.

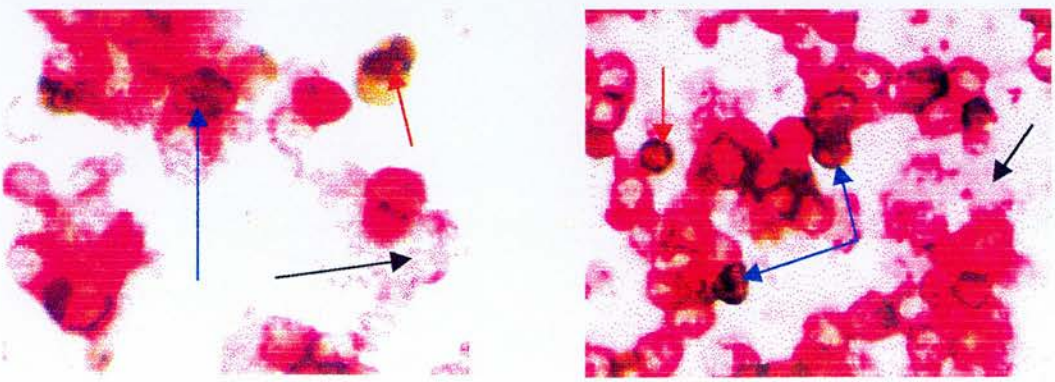


Figure 5.4.1 (left) and Figure 5.4.2 (right). Double immunolabelling staining for p24+CD4+ cells on an NSI isolate of HIV-1. Typical p24 staining (brown precipitate) was observed (red arrow). A portion of CD4+ cells on these slides were double-stained with HIV-1 p24 MAb (blue arrows), although some of them remained negative (black arrows). Magnification x100.

ABC technique and showed that non-specific signals were not amplified. These results were duplicated on different days utilising the same reaction conditions.

Immunofluoresence has been reported as a useful tool in multiple labelling for demonstrating the cellular localisation of viral proteins. In this study, detecting the p24 antigen with the fluorescent substrate, FITC, gave inconclusive results and limited the application of immunofluorescence with formalin-fixed cells. Following the procedure outlined in 2.8.1 and using fluorescein avidin D to detect p24 positive cells it was surprising to find some uninfected cells giving off the same bright green fluorescence as the infected cells (data not shown). In addition to this, background fluorescence was prominent making it quite difficult to determine which cells were positive for the HIV-1 p24 antigen. To try to reduce the background fluorescence the slides were firstly incubated in hydrogen peroxide in methanol for five minutes but this method proved inconclusive as it was difficult to visualise individual cells.

Due to the inconclusive results, an alternative secondary antibody, FITC rabbit anti-mouse was tried instead on the CD4 and CD8 cells on individual slides (no avidin-biotin reaction) and the CD4+ and CD8+ T-cells were clearly identified by bright green fluorescence. The p24 monoclonal and synctia were evidently fluorescent but not as intensely as on the CD8 and CD4 monoclonal (figure 5.5).

A double label reaction was carried out on the p24 antigen with FITC and CD4 with Fast Red. Under the light microscope, approximately 85-90% of CD4+ cells stained a clear red. FITC labelled rabbit anti-mouse was used to detect p24 antigen on the same cells and when the slide was viewed under the UV light, it became apparent that the fast red substrate was also giving a fluorescent red colour. Positive cells were indicated by the presence of a bright green fluorescence amongst

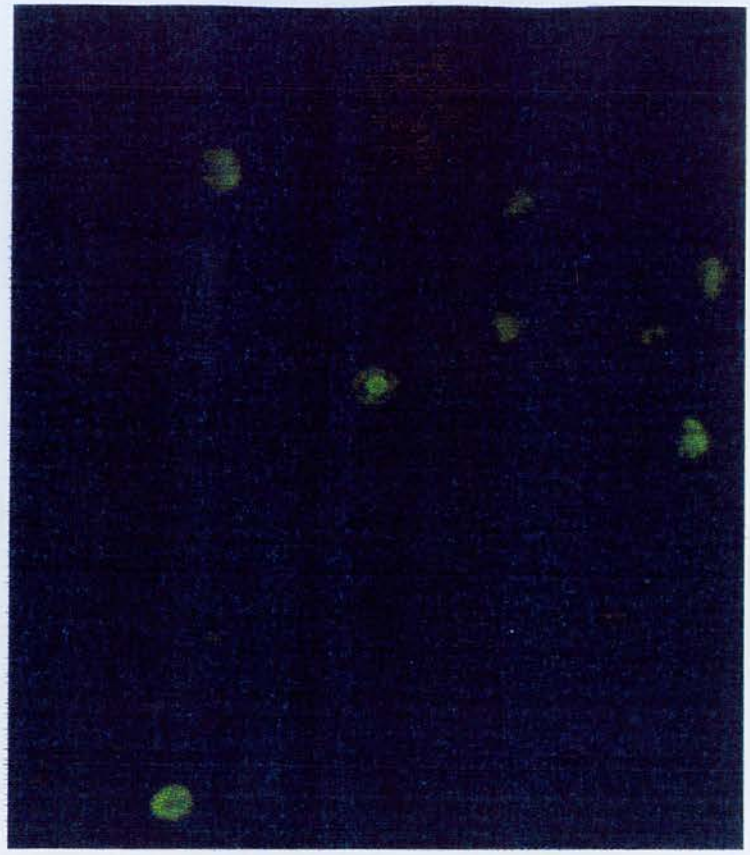


Figure 5.5. Immunoflourescent staining of CD8+ T lymphocytes. p24 immunofluorescent staining was evidently fluorescent although not as intensely as the fluorescene observed with CD8+ T cells. Magnification x100.

the red cells but this was rarely achieved, most T-cells usually giving the fluorescent red colour. One probable cause of the non-specific staining in this type of immunofluorescence procedure was the fact that perhaps the FITC-labelled rabbit anti-mouse secondary antibody was also acting with primary CD4 mouse monoclonal from the first incubation. Inconsistent results revealed that the procedure was extremely delicate and other factors including the concentration of primary antibody, biotinylated secondary antibody, avidin, the duration of incubation times, even the diluents might also contribute to non-specific staining. Therefore, to eliminate the problem of non-specific staining is a major task for further optimisation of the ABC technique with immunofluoresence.

5.7.3 Double immunocytochemical labelling of the CD4 and CD8 cells infected with HIV-1.

To evaluate the sensitivity of immunocytochemistry in detecting CD4 and CD8 infected cells, HIV-1 DNA in individual CD4+ or CD8+ T-cells was detected using the fast red or fast blue complex in conjunction with diaminobenzidine (DAB) for the p24 antigen. CD4 positive cells were identified from both SI and NSI cultures, respectively (figures 5.6 and 5.6.1). Staining of CD8+ T-cells and the p24 antigen allowed direct visualisation of infected CD8+ T-cells. PBMCs from an NSI culture were fixed and the CD8+ T-cells were stained with fast blue and counterstained for the HIV-1 p24 antigen. The occurrence of rare p24+ individual CD8+ T-cells indicated that HIV could indeed infect some of these cells (figure 5.7). The p24 antigen was observed in other unstained cells as well as CD8+ T-cells (see arrows;

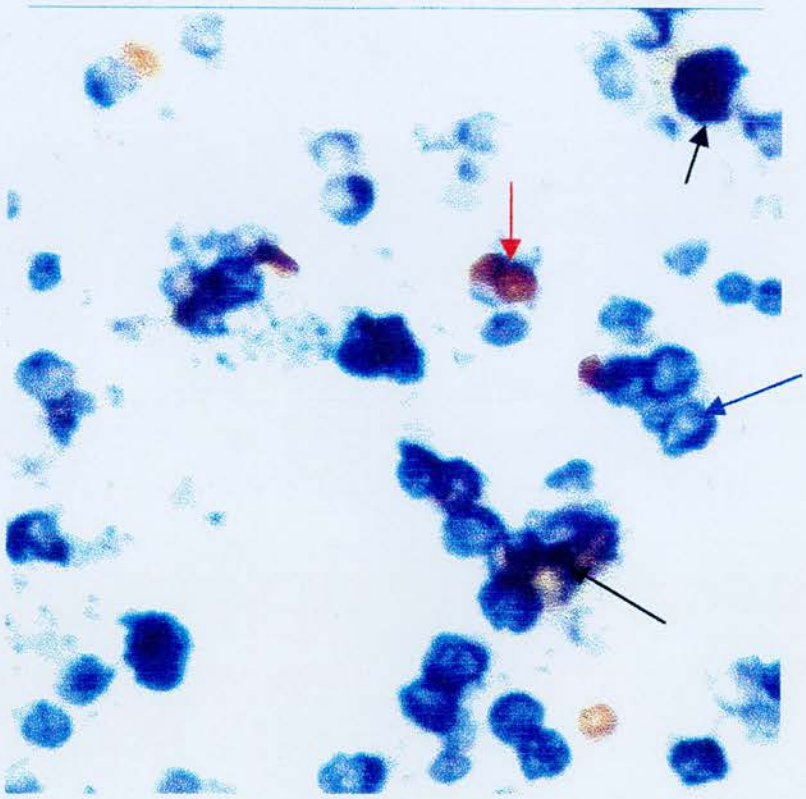


Figure 5.6. Double label reaction. Detection of CD4+ T-cells from an SI isolate of HIV-1 using the ABC-AP technique. CD4+ cells were detected using the Fast Blue substrate (blue arrow) while the p24 antigen was detected using the DAB substrate (brown precipitate, red arrow). p24+ CD4+ cells stained a red-brown colour (as indicated by black arrows. Magnification x100.

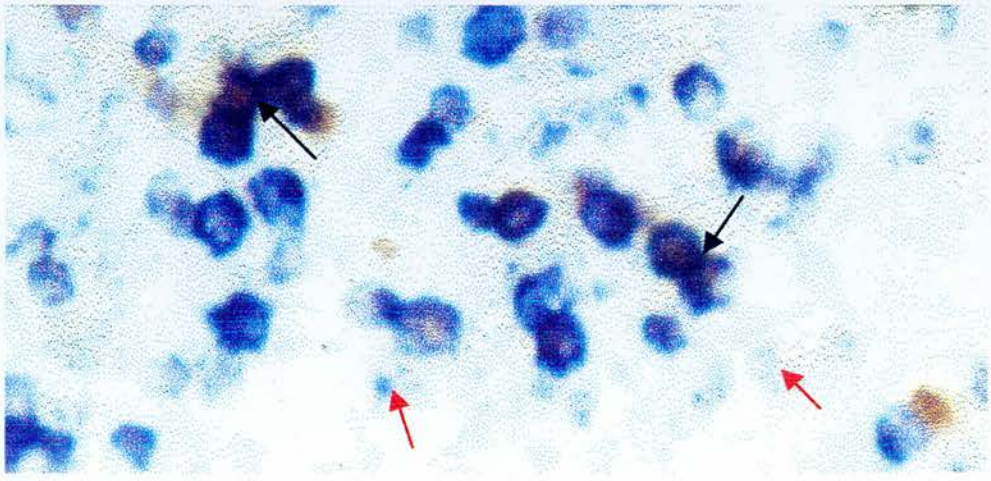


Figure 5.6.1. Double immuno-labelling staining for p24/CD4 cells from an NSI isolate of HIV-1. Typical p24 staining was observed. Most of the p24 positive cells were double-stained with Fast Blue (as indicated by black arrows), but some CD4+ T-cells remained p24 negative (red arrows). Magnification x100.

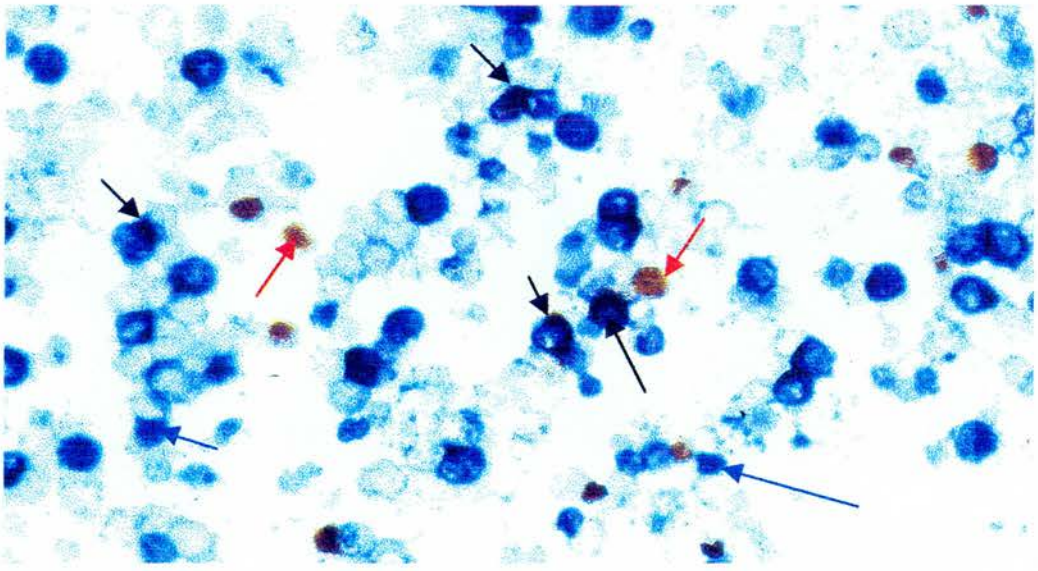


Figure 5.7. Double label reaction. Detection of CD8+ T cells using the ABC-AP technique from an SI isolate of HIV-1. CD8+ cells were detected using the Fast Blue substrate (blue arrow) while the p24 antigen was detected using the DAB substrate (brown precipitate- red arrow). p24+ CD8+ cells stained a blue-brown colour (black arrows). Magnification x200.

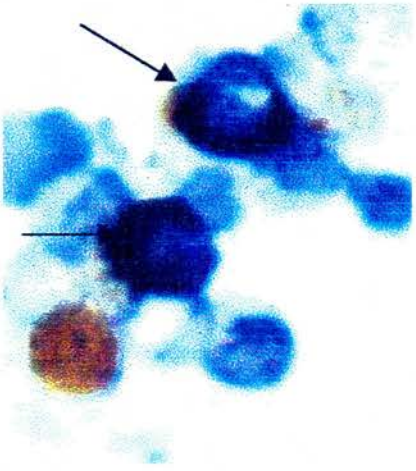


Figure 5.7.1

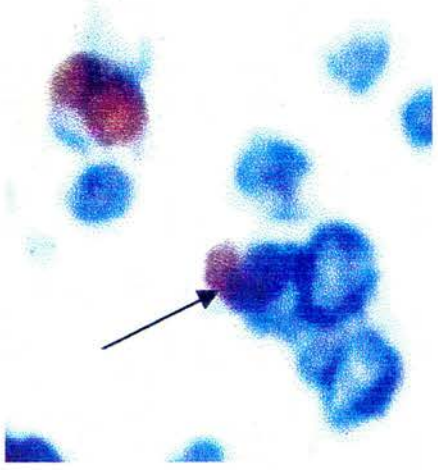


Figure 5.7.2

Double immuno-labelling staining of p24 (DAB) and CD8 (Fast Blue) demonstrating that some of these p24 positive cells are CD8 positive (as indicated by arrows). Magnification x100. SI isolate.

figure 5.7). The proportion of the CD8+ T-cells co-expressing the p24 antigen was lower than those observed with the CD4+ T-cells (figure 5.7.1 and figure 5.7.2). Similar micrographs also showed CD8+ T-cells stained with fast red and p24 with DAB (figure 5.7.3 and figure 5.7.4). The red arrow indicated a cell which is both p24 negative and CD4 negative, the blue arrow indicates a positive signal for p24 antigen expression and the black arrows indicate both CD4 and p24 antigen expression (figure 5.7.3 & 5.7.4). These findings confirm the presence of HIV-1 in the CD4+ and CD8+ T-cells of PBMCs cultured with isolates of HIV-1.

5.7.4 Detection of CD4, CD8 and p24 antigen expression by triple label reactions.

Immunocytochemical identification of the p24 antigen in CD4+ and CD8+ T-cells on the same slide can be achieved by a triple labelling reaction. This technique involves labelling three monoclonals, CD4, CD8 and p24 using fast blue, fast red and fast yellow respectively. On individual control wells using one MAb and the respective stain, it was evident that all three cell surface and internal markers existed, but a triple label caused a mass of colour mixture on the well which made it difficult to interpret HIV-infected cells (data not shown). The staining pattern was blurred and background staining also increased.

In the next series of experiments a clearer staining pattern was observed. Replacement of the fast yellow complex with the DAB substrate for detecting the p24 antigen gave much clearer results. This triple label reaction included CD4 stained with fast red, CD8 with fast blue and p24 with DAB (figure 5.8). Closer analysis was performed and revealed some positive CD4+ and CD8+ T-cells respectively (see

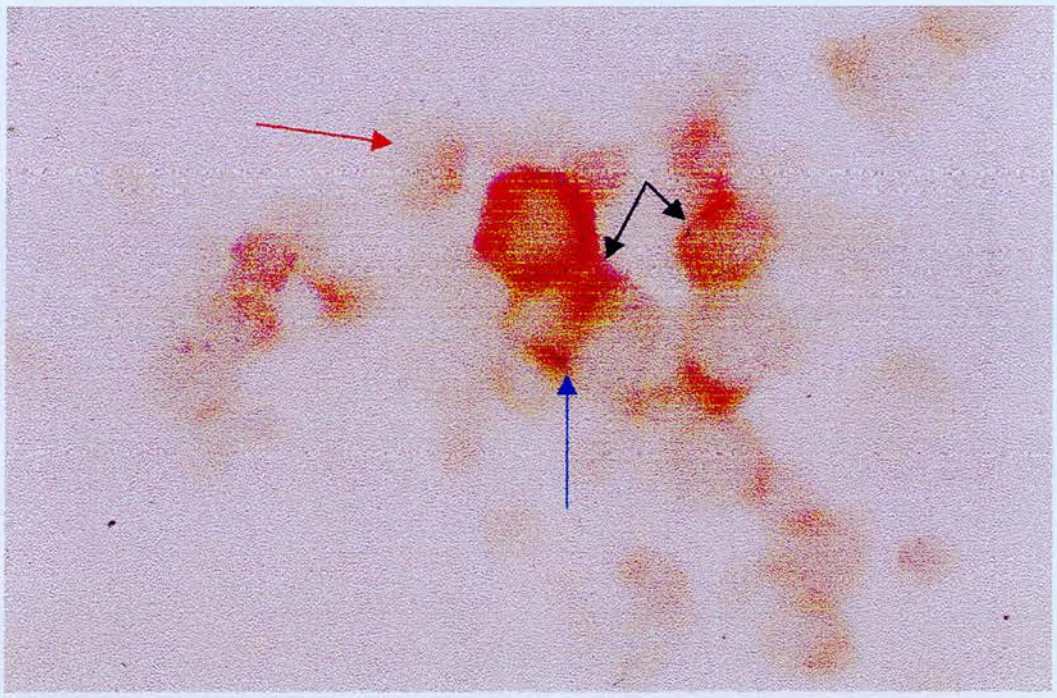


Figure 5.7.3.

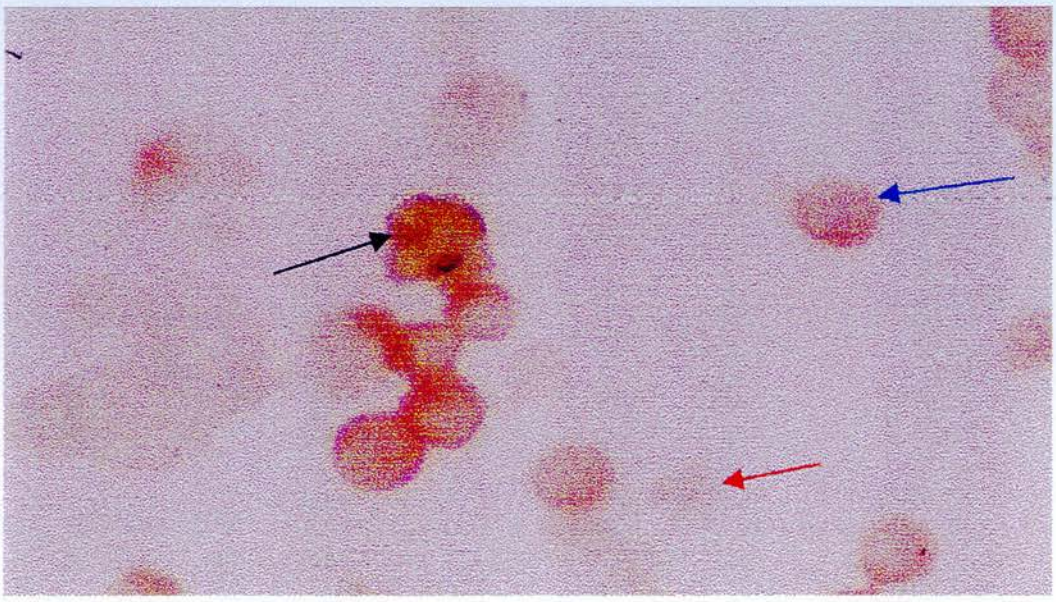


Figure 5.7.4.

Figure 5.7.3 & 5.7.4. Double immuno-labelling staining of p24 (DAB) and CD8 (Fast Red) demonstrating that some p24 positive cells are CD8 positive (as indicated by arrows). Magnification x100. NSI isolates.

Red Arrow indicates a negative signal for both p24 antigen and CD8+ T-cells.
Blue Arrow indicates a positive signal for the p24 antigen alone.
Black arrow indicates a positive signal for both p24 and CD8 T-cells.

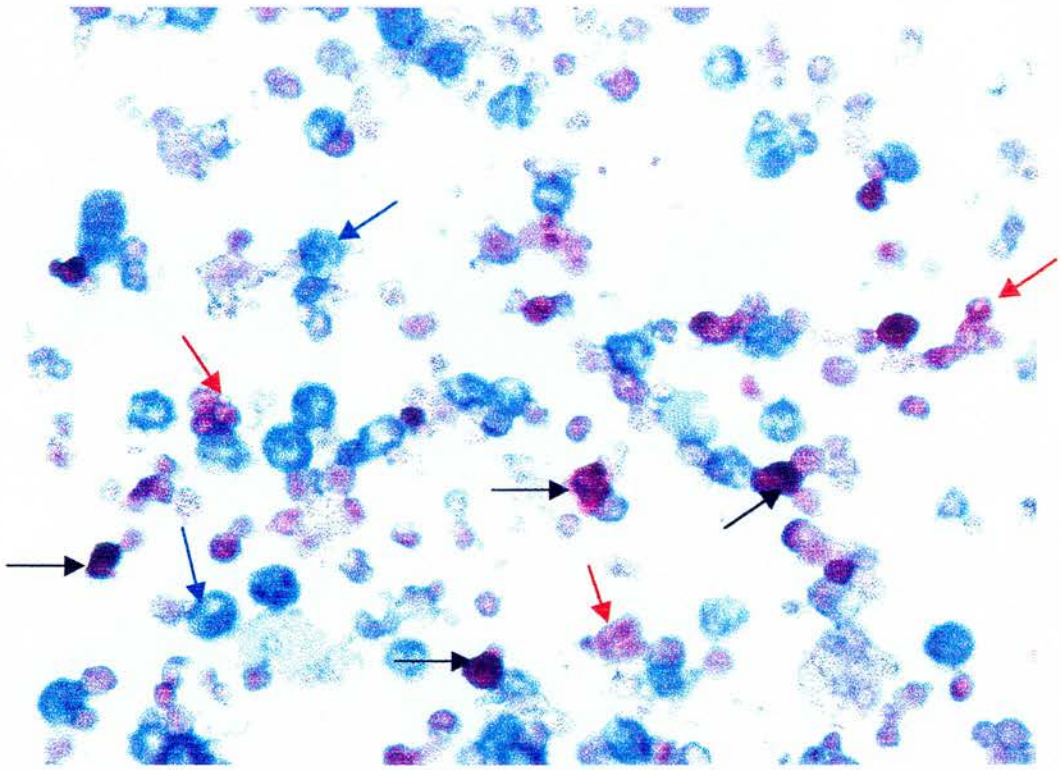


Figure 5.8. Triple label reaction. Detection of CD4 and CD8+ T cells using the ABC-AP technique from an NSI isolate of HIV-1. CD8+ cells were detected using the Fast Blue substrate (blue arrow). CD4+ cells were detected using the Fast red substrate (red arrow) and the p24+ antigen was detected using the DAB substrate (brown precipitate). p24+ CD8+ cells stained a blue-brown colour (black arrows). Magnification x200.

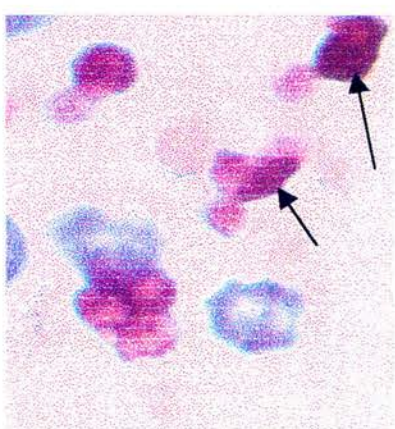


Figure 5.8.1

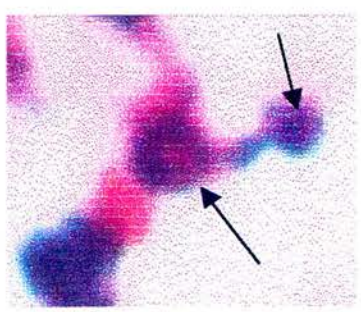


Figure 5.8.2

Triple immuno-labelling staining of p24 (DAB), CD4(Fast Red), and CD8 (Fast Blue). p24 (brown) stained with both CD4+ T-cells and CD8+ T-cells (see arrows). Magnification x40.

arrows in figure 5.8.1 and figure 5.8.2). This procedure allowed accurate analysis of the three markers which were clearly distinct from each other, indicating that our procedure precluded any cross-reactivities between the three different stains. All of the staining techniques were repeated several times on different days with the same working conditions and reproducible immunocytochemical staining results were obtained. More multiple staining reactions are required to show some of the viral characterisitics of HIV-1 *in vivo*.

5.7.5 Detection of HIV-1 p24-positive cells by IS-PCR

In this study, we demonstrated the increased sensitivity of direct in situ PCR for HIV-1 DNA in formalin-fixed, paraffin-embedded lymph node and spleen tissue sections by biotin amplification using the tyramide signal amplification detection system. Lymph node (LN) and spleen tissue sections from HIV-1 seropositive individuals and donor controls were subjected to in-situ PCR using gag-specific primers. All of the tissue sections analysed were positive for the HIV-1 provirus (figure 5.9). Uninfected tissue sections subjected to the same procedure did not demonstrate any positive provirus (data not shown). Most tissue sections survive *the in situ* PCR procedure. Equivocal results are rarely noted with positive cells demonstrating dense nuclear staining. Detection of incorporated biotin using the tyramide signal amplification produced a strong dark nuclear staining with the DAB substrate after 30 cycles of the two step PCR. In addition to the strong staining intensity, these microwave irradiated sections showed good cellular structure and morphology.

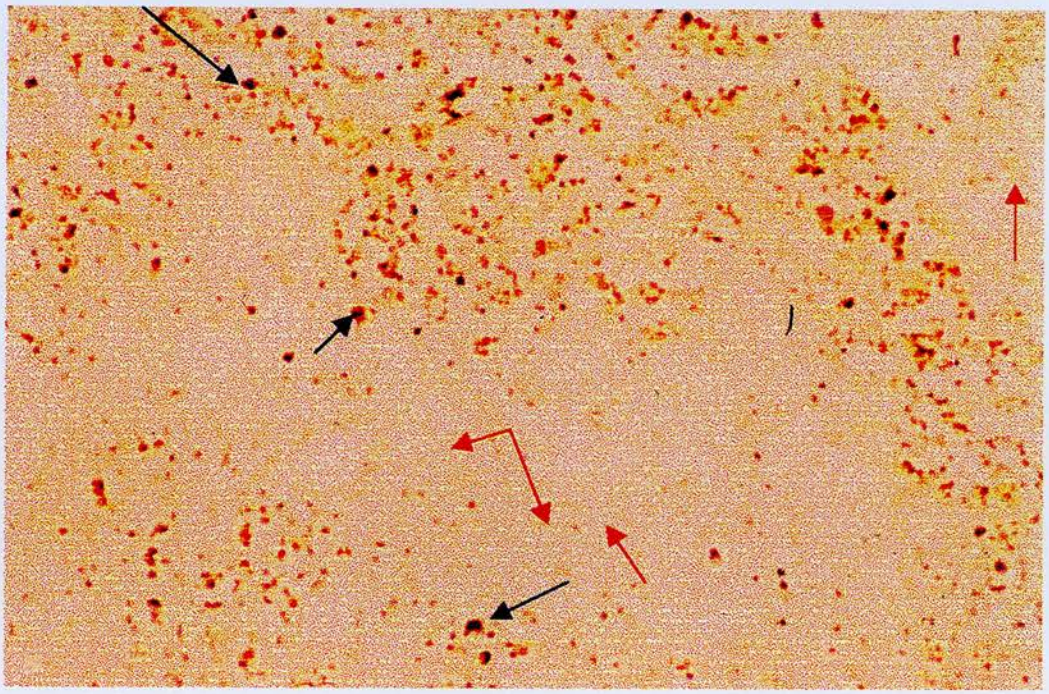


Figure 5.9. Detection of p24 positive cells in the lymph node tissue sections from a seropositive individual using *in situ* PCR. Proviral positive cells were demonstrated by a dark nuclear stain (black arrows) compared to that of uninfected cells (red arrows). Magnification x 400.

In this experiment, a combination of microwave irradiation of tissue sections and the use of a 30 cycle PCR showed that no primer-independent false-positive signals were detected. The negative controls included amplification reactions with the omission of one primer, both primers, *Taq* polymerase and biotin-dUTP. 30 cycles of IS-PCR were also performed on HIV-1 negative tissue with no positive signal being detected after incorporating the avidin-biotin-alkaline-phosphatase method.

5.8 DISCUSSION

Immunocytochemistry has been reported as a useful multi-labelling tool in detecting and demonstrating the cellular localisation of viral proteins. The exact interaction between HIV-1 and different T-cell types is poorly understood. It was believed that direct and productive infection of these cells *in-vivo* might be responsible for many of the clinical abnormalities found in HIV-1 infected individuals. Study of infected blood cells has focused chiefly on CD4 lymphocytes in the past. This part of the study was aimed to investigate further the cellular distribution of HIV-1 infection in PBMCs by visually demonstrating HIV-1 infecting $CD8^+$ T lymphocytes *in-vitro*. The CD8 T-cell function is essential in maintaining the possible homeostasis of the immune system. Because it is still held widely that lentiviruses do not infect $CD8^+$ T-cells, the disruption of this cytotoxic function has been neglected as a potential indirect mechanism of lentivirus-induced immune dysfunction.

5.8.1 Single, double and triple label expression of CD4, CD8 and HIV-1 p24 antigen.

The preliminary strategy in this study was to complete firstly a single stain with one primary antibody using a Biotinylated secondary antibody and a colour substrate to give a positive label. $CD4^+$ and $CD8^+$ T lymphocytes were characterised in this way. A relatively new aspect in the field of immunocytochemical staining is the application of an amplification method for double immunostainings with primary antibodies from the same species. Double immunolabellings are an easy and

convenient method to use in immunocytochemistry, but not usually for co-localisation, where one colour product tends to swamp the other. We demonstrated here the efficiency and the reliability of this method, using antibody pairs that are known to label easily distinguishable cell types

Double immunolabellings involved using a second primary antibody and secondary Biotinylated antibody before detection using the ABC-AP technique with either fast blue or fast red as chromogen. Although one label was developed to completion before commencing with the second, the cells retain their antigenicity (a beneficial feature of this method). Double immunostaining techniques were also used to detect intracytoplasmic viral antigen and the cell surface that is being infected. The purpose of this was not only viral detection but also the identification of the cellular host of HIV-1. With optimal conditions, the immunocytochemical technique demonstrated a clear population of p24 positive $CD8^+$ and $CD4^+$ cells which were simultaneously identified using the fast red, fast blue and DAB substrate as internal and cell surface markers respectively. Results were validated by a number of independent experts comprised of a neuropathologist and a collaborating post-doctoral researcher working in the immunocytochemistry field.

5.8.2 Immunocytochemical identification of the HIV-1 p24 antigen.

We found that immunocytochemical markers of the p24 antigen, employed here under carefully optimised conditions, labelled numerous HIV positive $CD8^+$ and CD4+ T-cells *in vitro*. By identifying the presence of detectable virus in the $CD8^+$ T-cells, we can confirm yet another significant factor correlating with the severity of lymphoid damage observed with HIV-1 infection during disease progression.

Although immunofluoresence methods have considerable advantages in revealing several co-localised antigens, the double immunofluorescent technique used here to detect p24 antigen using an FITC-labelled secondary antibody could not detect antigen in cells that were positive by ABC-AP detection. Some stains revealed high background fluorescence which made it difficult to distinguish particular cell types. Several technical problems occurred while applying this technique, such as different pre-treatments required for the two working antibodies, cross-reaction because the monoclonal antibodies were raised in the same species, and also in balancing the intensity of the two positive signals. We had also had little success using fluorophore (FITC)-tagged secondary antibodies or Avidin-fluorophores as the second label to detect the *in-vitro* conjugated secondary/primary antibody complex calling into question the sensitivity of these fluorescent methods. In our experiments, even routine single immunofluorescent methods rendered unsatisfactory results with CD4 and CD8 MAbs. There was considerable cross-recombination of colours at the end-point which resulted in confusion when trying to read the results. Therefore, enzymatic labelling took precedence in this study, because end-results were more easily interpreted, and permanant. Furthermore, the colour intensity of each stain may be developed to achieve the optimal contrast for the single and double-label profiles.

The combination of the ABC-AP and Fast Red/Fast Blue/DAB seems to be the optimal method presently available for double and triple immuno-labelling and co-localisation studies given the low sensitivity and high background of antibodies and methodology available for immunofluoresence. These results have, however, not been confirmed by other investigators. The optimising investigations described above suggest that further refinement of the immunocytochemical technique could assist in

enhancing the staining sensitivity. Perhaps when more sensitive antibodies are available, which can be used at high titres, fluorescent labelling may be preferred since different fluorophores can be differentiated by appropriate filter combinations and are suitable for confocal microscopy. The ABC-AP method used in this study offers an alternative technique that can be used instead of existing techniques, irrespective of the source of antibodies.

At present, the immunofluoresence (IF) detection sensitivity of HIV antigens has seriously limited its application and it is still used mainly as a research tool. Further improvements are required within this field before IF can be used extensively for HIV in clinical diagnostics. The techniques discussed above therefore had their potential problems and limitations. For immunofluorescence, the use of multiple amplification steps in connection with the sensitivity of detection systems such as the ABC-IF did not provide better detection sensitivity. Instead, the complicated staining protocols and the achievement of full optimisation with each of the new reagents in each protocol restricted the application of these methods in this study. The problems of non-specific staining of background debris and inconsistent results remain the most significant obstacles in an endeavour to improve and promote immunofluoresence for future use in relation to HIV in the clinical diagnostic and medical research fields.

The tyramide signal amplification (TSA) method was not used in this study, but does represent a possible way forward since it is a promising tool when conventional IF methods fail to detect specific signals (Dr Ting-Huei Wang, personal communication). Confocal microscopy is an essential method in characterising the exacT-cellular localisation of antigens using multiple IF labelling in the same tissue section. Since in many circumstances the availability of primary antibodies is limited,

the TSA method can be critical for double IF labelling with unconjugated primary antibodies raised in the same host species. The benefit of using TSA-IF also provides an opportunity for the development and improvement of double-labelling immunofluoresence (Wang *et al.,* 1999). The tyramide signal amplification (TSA) has been shown to not only increase the intensity of immunohistochemical staining but also numerically detect more HIV-1 antigen expressing cells in brain tissue (Strappe *et al.,* 1997).

5.8.3 Detection of HIV-1 positive cells by in-situ PCR.

Primary infection with HIV-1 is generally followed by a burst of viremia with or without clinical symptoms. This is in turn followed by a prolonged period of clinical latency. During this period there is little if any detectable viremia, the numbers of infected cells in the blood are low, and it is extremely difficult to demonstrate virus expression in these cells. In recent years, a new approach, in-situ PCR, has been used to amplify specific genetic elements within intacT-cells. In comparison to standard PCR techniques, in-situ PCR has demonstrated high levels of proviral harbouring PBMCs in HIV-1 infected individuals. It is claimed that the results are extremely sensitive because the cell itself acts as the amplification vessel and one copy of target sequence can be demonstrated in individual cells (Bagasra *et al.,* 1993; Nuovo *et al.,* 1992; Chiu *et al.,* 1992). In this part of the study we looked at the distribution of HIV in the PBMC's of lymph node and spleen sections and these results indicate a major localisation of HIV-infected cells in these lymphoid organs. Of note however, high levels of HIV expression have been observed in regions other than the lymph nodes,

such as adenoids and tonsils (Pantaleo *et al.*, 1993). It is also likely that HIV disease is active in the lymphoid tissue even throughout the period of clinical latency.

In order to have effective clearance of HIV, it is important that virus-specific CTLs are concentrated mainly in lymphoid tissue during the early phases of infection. Naïve lymphocytes traffic between secondary lymphoid organs, probably until they die or are activated by a specific antigen. Memory cells on the other hand, display a migratory capacity to a broad range of tertiary lymphoid organs such as the skin or intestinal laminsa propria and also to sites of inflammation. Rapid spreading of HIV infection within the naïve and memory cells can lead to high levels of virus replication which may occur in lymphoid organs.

The very close proximity between reactive $CD4^+$ T-cells and $CD8^+$ T-cells seen in HIV-1 infected lymph nodes strongly suggest that passage of virus could occur by cell-cell transfer. If infection can occur by cell-cell transfer then the immune response to HIV infection may result in a greater transmission to uninfected cells. Follicular dendritic cells were shown to trap the virus in the lymph node by carrying virus on their surface, which would facilitate infection of adhering T-cells (Cameron *et al.*, 1992). A previous study comparing the different T-cell subsets that exist within the lymph nodes and blood has shown that B cells and CD4+ T-cells predominsate in the lymph nodes (Emilie *et al.*, 1990). As CD4+ T-cells are susceptible to latent infection early in the course of disease, they are potentially able to pass virus to closely adjacent T-cells such as the $CD8^+$ naïve cell lineage within the lymph nodes. HIV-1 p24 protein is a viral structural protein, which is only expressed during productive infection. HIV-1 is able to infect several different T-cell lines non-productively and cells *in vivo* such as astrocytes without expression of structural

proteins. In addition, HAART has suppressed viral replication in many of these cellular targets *in vivo*. Under these conditions it may be extremely difficult to detect virus antigen expression in infected cells. A different technique will be required to investigate the frequency and distribution of latently infected cells, which highlights the potential value of *in situ* techniques. As immunocytochemical p24 detection only reveals productively infected cells it is important to use techniques such as *in-situ* PCR to detect how many cells are latently infected, particularly in individuals where HAART suppresses productive infection.

Besides lymphocytes, cells of the macrophage lineage are major target T-cells for HIV-1 (Gartner *et al.*, 1986; Nicholson *et al.*, 1986; Von Briesen *et al.*, 1990). HIV-1 infected macrophages persist in tissues for extended periods of time containing latent proviral DNA or large numbers of infectious particles within cytoplasmic vacuoles (Meltzer *et al.*, 1990). Furthermore monocytes/macrophages may be important as vehicles for viral disseminsation throughout the body (Levy *et al.*, 1993; Weiss *et al.*, 1993). Carriage of HIV-1 by dendritic cells may facilitate the lysis and loss of antigen specific T lymphocytes in AIDS (Cameron *et al.*, 1992).

For *in-situ* PCR however, improvements can also be made. Protocols with better specificity, as well as new methods to prevent the spread of the PCR amplicons need to be developed. Future developments should involve establishing reproducible protocols with good sensitivity and specificity. Meanwhile, the techniques used in this study are current research tools and the results suggest that further investment in optimisation is worth pursuing.

5.8.4 Summary

In summary, this study confirms the results of the earlier PCR experiments by demonstrating that HIV-1 can infect cytotoxic T lymphocytes *in vivo*. It is known that these cells decline in number in untreated AIDS patients and it may be that loss of these cells and their function in AIDS is a more significant factor in the advancing immune incompetence than the loss of T-helper cell function, as previously mentioned (refer to section 1.8.2.1). The ABC-AP technique is a useful tool in the developmental progression of immunocytochemistry, especially in detection of HIV-1 p24 antigen with $CD4^+$ and $CD8^+$ T lymphocytes. The sensitivity of detection was greatly enhanced, and the ABC-AP technique was considered to be another beneficial tool for the p24 investigations in the cells analysed. Our experiments also demonstrated that *in situ* PCR can be accomplished in cell populations known to carry the HIV-1 provirus. Further development and validation of *in-situ* PCR in localising and quantifying HIV infected cells in vivo could be combined with a laser capture micro-dissection microscope providing a powerful method to detect, separate, genetically characterise and clone sequences from HIV infected cell types from different tissues.

CHAPTER SIX: GENERAL DISCUSSION

Implications of HIV infection for the immune system

The pathogenesis of an infectious disease involves interactions between the infectious agent and the host's immunological response to the challenge. In HIV-1 infection, these interactions appear to have fundamental consequences for many different aspects of the disease. Disruption of the T-cell network and suppression of the cellular immune response in HIV disease result in opportunistic infections and neoplasms that contribute to AIDS-related morbidity and mortality. Despite the recent advances observed with antiretroviral therapy, there is still no cure for AIDS or HIV infection. Drug therapy, although promising, remains problematic because of crippling side effects, vast expense and poor compliance. In addition, only 10% of people infected with HIV live in countries where an adequate supply of HIV drugs is available, while limited resources make sophisticated HIV therapy beyond the reach of the other 90% (UNAIDS/WHO, 1999).

The understanding of disease caused by HIV-1 has been hampered by the complexity of the interactions of this virus with the human immune system. HIV infection *in vivo* is a dynamic process involving continuous rounds of infection, replication and cell death. The immunological hallmark of successful infection with HIV-1 is a progressive decline in CD4 T-cell count which ultimately results in the appearance of infections characteristic of AIDS. As well as predominsantly infecting CD4+ cells, HIV also infects cells of the monocytic/macrophage lineage, which express low level CD4+ and MHC class II. The turnover of CD4+ T-cells during HIV infection is thought to be rapid, with the entire population of peripheral CD4+ T-cells

estimated to be replaced on average every 15 days (Ho *et al.*, 1995). While the great majority of infected individuals do not present clinical signs of disease for extended periods, virus replication remains high, especially in reservoirs such as the lymph nodes and spleen (Donaldson *et al.*, 1994). In patients who die of AIDS related illnesses, infection is extensive, targeting brain, lung, colon and liver cells as well as lymphoid tissue (Donaldson *et al.*, 1994). Persistent and high levels of virus replication result in the loss and destruction of normal lymphoid architecture. As the immune system chronically deteriorates, virus and virus-infected cells are less efficiently removed by the host.

Persistence of virus in the host in the presence of ineffective immune system clearance results in the state of chronic immune system activation. Activation of cells in the course of the immune response further favours the spread and establishment of HIV in new target CD4+ T-cells and macrophages. Progression of HIV infection is characterised by complex dysregulation of T-helper cell maturation and development which can involve the T-cells themselves and/or antigen presenting cells (reviewed in Copeland *et al.*, 1996).

The analysis of the number and type of cells that HIV infects in the lymphoid system, and the discovery of widespread covert infection in macrophages and CD4+ T-cells at early stages brings a fresh perspective to understanding the pathogenesis of disease and an increased appreciation of difficulties in treating and preventing infection. The potential for understanding the mechanism of immune dysregulation and cell targetting by HIV in general was recently enhanced by the discovery of secondary co-receptors for HIV-1 entry into target T-cells (Feng *et al.*, 1996; Bleul *et al.*, 1996, Alkhatib *et al.*, 1996; Choe *et al.*, 1996; Berger *et al.*, 1997; Oberlin *et al.*,

1996; Dragic *et al.*, 1996). These discoveries not only provide insight into the mechanism of viral entry, but also explain the mechanism by which early viremia progresses to immunodeficiency. Acquistion of the ability to use additional co-receptors may broaden viral tropism and influence disease pathogenesis.

The 'tap and drain' hypothesis

HIV infection is seen as a Titanic struggle between the virus and the immune system. 3.5 x 10^6 $CD4^+$ T-cells are lost daily in HIV infected individuals and since in most normal individuals the decline in total $CD4^+$ T-cell numbers over time is very gradual, the conclusion is that an almost equivalent number must be regenerated to reconstitute the peripheral pool. A number of studies have recently described an analogy where the HIV seropositive patient can be compared to a bathtub, in which a drain (HIV) causes a steady loss of water (the T-lymphocytes), while a tap (the thymus) steadily strives to maintain the water level. The putative gene controlling the CD4:CD8 ratio operates at a pre-thymic level, and the undifferentiated precursor, all of the CD4/CD8 lineage, is genetically geared to preferentially give rise to either $CD4^+$ or $CD8^+$ T-cells. During HIV infection, an increased loss of CD4+ T-cells results in an increased production of CD3+ T-cells which are maintained at a constant level in high-ratio individuals who produce T-cells with a balance favouring CD4+ T-cells over $CD8^+$ T-cells. The decline in this production is slower and less pronounced, compared with low-ratio seropositive individuals, who preferentially produce CD8+ T-cells in response to a CD3+ T-cell depleting event, and hence undergo more marked CD8+T-cell lymphocytosis. The evidence suggests that seropositive individuals genetically predisposed to a high CD4:CD8 ratio would be more efficient

in replenishing $CD4^+$ T-cell losses than those predisposed to a low CD4:CD8 ratio. Of course, this hypothesis assumes that the 'drain' would be equal in different patients, and that different exogenous factors such as therapeutic interventions would not interfere with the 'tap and drain' balance (Ho *et al.*, 1995; Heeney *et al.*, 1995; Amadori *et al.*, 1996).

However, HIV may also impair T-cell regeneration by targeting and damaging the thymus (tap) itself. The architecture of the thymus is disrupted in AIDS patients (McCune *et al.*, 1997; Ho *et al.*, 1995) although the extent to which thymus dysfunction contributes to the development of AIDS remains unclear. It is known however, that any alteration of thymopoiesis would limit the production of naïve T-lymphocytes (water) which is consistent with the preferential depletion of these cells in HIV-infected individuals (Roederer *et al.*, 1997). Histopathologic lesions in the thymus of AIDS patients have been observed which suggests some degree of thymus dysfuntion (Haynes *et al.*, 1998; McCune *et al.*, 1997) which may result from the emergence of X4 viruses that target thymic progenitors (Pedroza-Martins *et al.*, 1998). Severe congenital thymus defects have also been observed in HIV-infected infants that progress rapidly to disease (Kourtis *et al.*, 1996; Nahmias *et al.*, 1998), suggesting that the mechanisms of T-cell depletion is in part thymus dependent. Thymopoiesis is critical to the restoration of normal T-cell numbers after profound T-cell depletion, as is seen following bone-marrow transplantation. Thus, HIV-induced thymic damage may impair the regeneration of CD4 and CD8 T-cells in patients with severely depleted numbers of T-cells.

HIV infected CD8+ T lymphocytes *in vivo*.

In vivo infection of CD8 lymphocytes has been described for HIV-1 (Huang *et al.*, 1999; Mercure *et al.*, 1993; Livingstone *et al.*, 1996; Yang *et al.*, 1998; Flamand *et al.*, 1998), for simian immunodeficiency viruses in sooty mangabeys (SIVmac) (Dean *et al.*, 1996) and African green monkeys (SIVagm) (Muruayama *et al.*, 1999) and for lentiviruses infecting other mammals, such as feline immunodeficiency virus (English *et al.*, 1993; Dean *et al.*, 1996). In this study, frequencies of infection, proviral contaminsation and genetic relationships between HIV-1 variants infecting naïve and memory CD4 and CD8 lymphocytes were investigated. Significant infection of the naïve subset of CD8 T lymphocytes was detected in 8 study subjects at frequencies ranging from 31-1444 proviral copies/10^6 cells, more frequently than in CD3+, CD8+ lymphocytes expressing the RO isoform of CD45 (n=3, 20-259 copies/10^6 cells). In agreement with previous studies there was no evidence for a similar preferential infection of CD4 naive lymphocytes.

The propensity of T lymphocytes to die, as evidenced by spontaneous apoptosis, is increased in HIV-1 infected patients and in SIV-infected macaques (Ameisen *et al.*, 1991; Estaquier *et al.*, 1994; Gougeon *et al.*, 1993, Meyaard *et al.*, 1994). Evidence has also been presented suggesting that the SIVmac may target the thymus of experimentally infected macaques, and infect immature lymphocytes expressing both CD4 and CD8 during thymopoiesis (Dean *et al.*, 1996). Similarly, infection of CD4+, CD8+ thymocytes in human thymic implants in SCID mice leads to the subsequent appearance of HIV-infected CD8+, CD4- lymphocytes in the peripheral circulation (Kitchen *et al.*, 1997; Lee *et al.*, 1997). The work carried out in this thesis provides supporting evidence for the contribution of intrathymic infection

in the pre-activated, antigen naïve population of CD8 lymphocytes *in vivo*. HIV-1 infection of CD4+, CD8+ immature thymocytes destined to become CD8 lymphocytes during thymic maturation would provide both a plausible mechanistic explanation for their infection, and would also explain the presence of proviral sequences in the naïve subsets of both CD4 and CD8 lymphocytes in peripheral blood.

Decline in total lymphocyte numbers has been attributed to a failure of T lymphocyte homeostasis, although the observation of a delayed although substantial decline in CD8 frequencies suggests that their production is also impaired or that their destruction in the periphery occurs at a similar intensity to that of CD4 lymphocytes. Contributory information on the influence of HIV on CD8-lymphocyte mediated immunity is provided by several studies investigating the dynamics of production of CD8 lymphocytes in HIV-infected individuals, and the effect of HAART on turnover. Investigation of T-cell turnover by expression of the Ki-67 antigen or by labelling of cells with [2H]-labelled deuterium or by BrdU in experimentally infected macaques with SIV indicated increased turnover of both CD4 and CD8 cells upon disease progression (Rodriguezalfageme *et al.*, 1998; Hellerstein *et al.*, 1999; Sachsenberg *et al.*,1998; Rosenweig *et al.*, 1998; Mohri *et al.*, 1998) and specifically of CD8 lymphocytes by investigations of telomere length. (Wolthers *et al.*, 1996).

Combination monoclonal antibody cell separation techniques and in-situ PCR for proviral sequences established that HIV infects a wide range of cell types in the PBMC population, including CD8 lymphocytes. Ultimately, it is important to identify whether these infected CD8 T lymphocytes are productively or latently infected. Clearly, immunostaining to identify viral protein is insufficient and much work needs to be carried out to improve the limitations of this technique (as discussed in Chapter

3). Evidence of HIV distribution within the lymphoid areas such as the lymph nodes and spleen was also presented in this thesis project. Lymphocyte depletion is the major abnormality seen in the spleen with HIV-1 infected-individuals (Pantaleo *et al.*, 1994; Levy *et al.*, 1998). Lymph nodes are commonly sites for opportunistic infection in AIDS, usually resulting in lymphadenopathy. Generally infections are multiple and are sometimes found in association with neoplasms. It could be speculated that CD8 lymphocytes themselves could represent a reservoir for HIV-1 which may persist for long periods at low levels in many treated individuals despite the use of maximally suppressive antiretroviral regimens. CD8 T lymphocytes are known to traffic within the CNS and they may also represent a target for infection during AIDS alongside other targeted cells within the CNS such as microglial cells, multinucleated giant T-cells (MGCs) and brain macrophages (reviewed in Bell, 1998). Infected cells release toxic cellular factors leading to tissue damage and apoptosis (Esser *et al.*, 1998). Therefore, HIV-1 is likely to have its damaging effect indirectly in tissue of the CNS. Common problems occurring after infection of these cell types include HIV-1 specific neuropathology and many opportunistic infections (Gray *et al.*, 1996; Price *et al.*, 1996; Bell *et al.*, 1998; Esser *et al.*, 1998).

Recent work undertaken post thesis completion

Recently, it has been suggested that there is a possibility that CD8 lymphocytes purified by negative selection may contain null (CD4-,CD8-) cells. In this thesis project it was not possible to positively select for CD4 and CD8 T lymphocytes prior to the separation of naïve and memory cells since positive selection can potentially alter the activation status of isolated cells and therefore the isoform of

CD45 expressed on the cell surface. However, after completing all the work detailed in this thesis, further experiments were undertaken to address this issue. In the attached manuscript T-cells from the PBMCs of 7 new individuals were positively selected for CD4 and CD8 lymphocytes. Purified CD8 lymphocytes prepared in this way showed a range of proviral loads (Table 3, McBreen *et al.*, 2000, manuscript attached) similar to those found in CD8 lymphocytes isolated by the negative selection. In addition, examinsation of the HIV proviral sequences detected in CD8-positive lymphocytes supports the conclusion that the proviral sequences obtained from the negative selection procedure used in this thesis project were not likely to have originated from null cells. This provides support for the validity of results in the main project.

Evidence that the positive and negative selection procedures used to separate CD4 and CD8 T lymphocytes are highly effective at removing monocytes and other non-T lymphocytes from purified cells has also been shown in the attached manuscript (McBreen *et al.*, 2000). Previous studies have documented a relatively low frequency of infection of monocytes in PBMCs *in vivo* which makes it highly unlikely that monocytes could contribute significantly to the proviral loads detected in CD4 and CD8 T lymphocytes in this study (Hsia *et al.*, 1995; Schnittman *et al.*, 1989; Livingstone *et al.*, 1996).

Further work was also carried out to examinse the quiescent nature of HIV-1 infection in CD8 naïve lymphocytes. A method was designed to quantify complete proviral sequences (i.e. that span the primer binding site [PBS} and U5 region; C-LTR-primers) and initial transcripts (primers amplifying the region immediately downstream from the PBS (pan-LTR). Within the expected accuracy of the limiting

dilution method used for proviral quantitation, no observations were made between the two sets of primers for an excess of incomplete proviral sequences indicating that the sequences detected in both CD4 and CD8 lymphocytes were complete. Their stable integration in the cellular genome has been suggested by previous studies investigating the half-lives of proviral DNA in PBMCs *in vivo* and the observations in this thesis document the stability of proviral load in CD8 lymphocytes in individuals receiving antiretroviral therapy. From this data it can be concluded that there is no evidence for the existence of significant numbers of incomplete transcripts which might be associated with abortively infected cells.

Future Investigations

At present it remains unclear whether HIV-infected CD8 lymphocytes *in vivo* represent recent thymic emigrants or recently activated naïve CD8 lymphocytes. Identifying the maturation stage of infected CD8 lymphocytes will give a broader insight into the mechanism and effect of their infection on immune function. Future experiments involve separating CD3+ lymphocytes into CD4-CD8- and CD8+CD4+ subsets using MACS beads and subsequently sorting cells with cell surface markers CD69 and CD1 to differentiate between immature thymic emigrants and recently activated naïve CD8 lymphocytes. Limiting dilution PCR will be carried out on the sorted cell subsets to analyse for HIV proviral sequences. The frequency of productively infected cells will be assayed by p24 antigen staining of fixed immobilised cells.

Although HIV sequences can be detected in CD8 lymphocytes present in PBMCs, there is currently no information on whether the cells are actively infected

with HIV, or whether this can be induced by mitogenic or antigenic stimulation. It would be interesting to investigate whether CD8 lymphocytes have the potential to produce infectious virus. This could be achieved by developing a fully quantitative infectivity assay for separated lymphocytes. Simple bulk culture of purified CD8 lymphocytes is evidently insufficient to carry out this analysis. A quantitative method is crucial in order to calculate the contribution of contaminsating CD4 lymphocytes within the isolated CD8 lymphocytes.

Further development and validation of the methods used in this study to localise and quantify HIV-infected cells *in vivo* such as p24 antigen immmunocytochemistry and *in-situ* PCR would prove very useful in not only allowing precise localisation but also in defining the genetic characterisation of variants infecting particular cell types *in vivo*. New studies involving cell isolation using a laser capture microdissection microscope would provide a powerful method to detect, separate, genetically characterise and clone sequences from HIV-1 infected cell types in different tissues. These methods could measure the frequency and viral expression of HIV infecting cells in the lymphoid tissue and infiltrates by co-localising with cell surface markers such as CD3, CD4, CD8 and CD14 which would allow identification of infected cell types.

Forthcominsg studies should also focus on phenotypically characterising variants infecting the two cell types by applying a pseudotyping method to characterise the properties of entire gp120 sequences amplified by PCR from purified CD4 and CD8 lymphocytes. This would not only provide further information on co-receptor usage but would also indicate whether CD4-independent entry of HIV-1 can

occur by avoiding the potential selection effects observed when carrying out *in-vitro* culture.

In summary, this study provides extensive evidence for the infection of CD4 and CD8 naïve and memory cells *in vivo*, signifying that HIV has a broader tropism for cell types *in vivo* than previously thought. The primary outcome of many viral infections is immune containment rather than the eradication of infection. In order to develop an effective vaccine, HIV-specific Th cell responses as well as virus-specific CTLs should be induced. The stronger the response the broader its direction, resulting in increased opportunity for variant virus strains could be controlled efficiently. Exactly how to elicit these responses still remains to be established. The most effective antigen delivery vehicle and routes of adminsistration have also yet to be defined.

Although many questions remained unanswered, in particular concerning the process underlying CD4 lymphocyte decline as well as the mechanisms of CD8+ T-cell infection, much is understood about how to treat HIV effectively. The recent development and use of HAART has provided optimism that T-cell immune competence can be restored in patients with chronic infection and preserved in those with acute infection. The aim is to improve the capacity for T-cells to regenerate effectively with a reduction in T-cell activation which may prevent anergy and apoptosis which together may ameliorate the CD4 and CD8 T-cell deficiencies and dysfunction noted in this thesis project. The hope must be that the increased understanding of HIV infection which new therapies have provided and new reliable assays which quantify HIV levels lead us to a greater understanding of the therapeutic constraints on HIV replication and eventually to a prophylactic vaccine.

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Infection of the CD45RA$^+$ (Naive) Subset of Peripheral CD8$^+$ Lymphocytes by Human Immunodeficiency Virus Type 1 In Vivo

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To investigate the mechanism and functional significance of infection of CD8$^+$ lymphocytes by human immunodeficiency virus type 1 (HIV-1) in vivo, we determined frequencies of infection, proviral conformation, and genetic relationships between HIV-1 variants infecting naive (CD45RA$^+$) and memory (CD45RO$^+$) peripheral blood CD4$^+$ and CD8$^+$ lymphocytes. Infection of CD3$^+$ CD8$^+$ CD45RA$^+$ cells was detected in 9 of 16 study subjects at frequencies ranging from 30 to 1,400 proviral copies/10^6 cells, more frequently than CD3$^+$ CD8$^+$ lymphocytes expressing the RO isoform of CD45 (n = 2, 70 and 260 copies /10^6 cells). In agreement with previous studies, there was no evidence for a similar preferential infection of CD4$^+$ naive lymphocytes. Proviral sequences in both CD4$^+$ and CD8$^+$ lymphocyte subsets were complete, as assessed by quantitation using primers from the long terminal repeat region spanning the tRNA primer binding site. In six of the seven study subjects investigated, variants infecting CD8$^+$ lymphocytes were partially or completely genetically distinct in the V3 region from those recovered from CD4$^+$ lymphocytes and showed a greater degree of compartmentalization than observed between naive and memory subsets of CD4$^+$ lymphocytes. In two study subjects, arginine substitutions at position 306, associated with use of the chemokine coreceptor CXCR4, were preferentially found in CD4 lymphocytes. These population differences may have originated through different times of infection rather than necessarily indicating a difference in their biological properties. The preferential distribution of HIV-1 in naive CD8$^+$ lymphocytes indeed suggests that infection occurred early in T-lymphocyte ontogeny, such as during maturation in the thymus. Destruction of cells destined to become CD8$^+$ lymphocytes may be a major factor in the decline in CD8$^+$ lymphocyte frequencies and function on disease progression and may contribute directly to the observed immunodeficiency in AIDS.

CD4$^+$ lymphocytes are considered to be the principal target of human immunodeficiency virus type 1 (HIV-1), but more recently a number of studies have shown the in vivo infection of other lymphoid cell types including CD8$^+$ T lymphocytes (12, 21, 31, 33, 51). CD8$^+$ T cells are a major immunological defence against HIV-1 infection. In most individuals, HIV mediates a strong specific cytotoxic activity that eliminates productively infected cells. This response also blocks intracellular viral replication in CD4 cells by production and secretion of a number of soluble inhibitory factors such as macrophage inflammatory proteins 1α and 1β and RANTES (regulated upon activation, normal T-cell expressed and secreted), interleukin-16, and other, as yet unidentified factors (17, 32). During the course of infection, there is a gradual loss of CD4 cells; after an initial increase in the number of cytotoxic T cells, the CD8 cell number similarly falls during disease progression (29). The observed decline in both the number of CD8 cells and specific HIV cytotoxic activity coincides with an increase in viral load and may ultimately be a contributory factor to the eventual collapse of the immune system and the development of AIDS.

The mechanisms leading to CD8 T-cell dysfunction and depletion are still unclear. CD8 T-cell function may be the indirectly influenced by a defective HIV-1-specific CD4 T helper response that is necessary for the maturation and function of cytotoxic T cells. It has also been demonstrated after antigenic stimulation some CD8 T cells develop a state of unresponsiveness and eventual death is mediated via apoptosis (19, 30). However, it has been observed that productive HIV-1 infection of cytotoxic T lymphocytes in vitro and in vivo in animal models can occur (47). The peripheral interaction between CD4 and CD8 cells occurring in vivo as part of the immune response may allow direct transmission of infection to the CD8 lymphocytes. Alternatively, it is possible that damage to or deficiency of the thymus may also confer direct infection. HIV-1 may infect and destroy both intrathymic T progenitor cells (CD3$^-$ CD4$^+$ CD8$^-$), double-positive thymocytes (CD3$^+$ CD4$^+$ CD8$^+$) (4, 49) and mature CD3^{hi} CD8$^+$ thymocytes (28). Recent studies alternatively suggest that infection of CD8 lymphocytes may occur by a conventional CD4-dependent mechanism, as CD4 expression is up-regulated following activation through the T-cell receptor complex (12, 49, 51).

Information on the mechanisms of CD8 lymphocyte infection can be obtained by analysis of relative frequencies of infection of CD8 lymphocytes expressing the CD45RA (naive) and CD45RO (memory/effector) isoforms, as has been previously described for CD4 lymphocyte infection (36). Abortive or noncytopathic infection of double-positive (CD4$^+$ CD8$^+$)

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TABLE 1. Clinical profile and frequencies of infection of naive and memory subsets of CD4 and CD8 T lymphocytes.

Study subject	Sex[a]	Risk group[b]	Viral load (genome eq/ml)	CD4 count (μl)	Proviral copies/10^6 cells					
					$CD4^+$ lymphocytes		$CD8^+$ $CD45RA^+$ (naive) cells		$CD8^+$ $CD45RO^+$ (memory) cells	
					$CD45RA^+$	$CD45RO^+$	Proviral load	CD4 contamination[c]	Proviral load	CD4 contamination
p1	F	IDU	14,000	10	98	834	<10	0.9	<10	11
p2	M	MH	514,500	29	1,106	587	107	10 (9.4)	<10	8.0
p3	M	MH	116,000	74	133	278	1,444	1.2 (0.1)	<10	3.8
p4	M	MH	18,000	130	596	4,272	<10	5.4	<10	58
p5	M	MH	<400	232	686	457	422	6.2 (1.5)	67	6.2 (17)
p6	F	Het	52,000	265	866	1,566	<10	7.9	<10	21
p7	M	IDU/Het	48,000	267	95	520	<10	0.9	<10	7.1
p8	M	IDU	460,000	280	4,307	2,137	22	39 (180)	259	29 (21)
p9	M	MH	>750,000	282	575	2,321	40	5.2 (13)	<10	32
p10	M	MH	81,540	301	1,307	464	622	12 (1.9)	<10	6.3
p11	M	MH	661,000	311	834	3,916	<10	7.6	20	53 (270)
p12	F	IDU	64,680	329	55	115	<10	0.5	<10	1.6
p13	M	MH	67,980	354	115	222	71	1.0 (1.5)	<10	3.0
p14	F	Het	<513	360	533	980	119	4.9 (4.1)	<10	13
p15	M	MH	60,589	748	653	Negative	31	5.9 (19)	<10	<0.1
p16	M	MH	1,680	850	143	25	<10	1.3	<10	0.3

[a] F, female; M, male.
[b] Abbreviations: IDU, injecting drug user; MH, male homosexual; Het, infection acquired through heterosexual contact.
[c] Estimated contribution of contaminating CD4 cells to proviral load (percent of total) detected in CD8 lymphocytes, using mean purity data from controls (Table 4).

thymocytes during maturation in the thymus would produce differentiated, naive $CD8^+$ lymphocytes in the circulation that contained stably integrated HIV proviral sequences. However, if infection occurred during the phase of CD4 expression after antigenic stimulation of CD8 lymphocytes, then proviral sequences would be preferentially distributed in the memory/effector population, as the activation process would result in a change of phenotype from $CD45RA^+$ to $CD45RO^+$. In this study, we investigated the frequency of HIV infection in CD4 and CD8 naive and memory cell populations separated from peripheral blood mononuclear cells (PBMCs) of HIV-seropositive individuals by quantitative PCR for HIV-1 proviral sequences. We also investigated possible genetic differences between variants of HIV-1 isolated from each of the lymphocyte subsets that might relate to time of infection or determine differences in cellular tropism.

MATERIALS AND METHODS

Samples and clinical details of study patients. Samples (20 to 30 ml) of whole blood anticoagulated with EDTA were collected from seropositive individuals attending the genitourinary medicine clinic or the infectious disease unit in Edinburgh. CD4 counts, viral load information, and risk groups from the patient group in whom distribution of HIV in naive and memory subsets of CD4 and CD8 lymphocytes were analyzed (Table 1). Plasma virus loads were determined by a commercially available PCR (Roche Monitor, Lewes, East Sussex, United Kingdom). Further samples were collected from five healthy, HIV-seronegative control for cell purity measurements.

Cell separations. Blood samples were diluted with an equal volume of phosphate-buffered saline and PBMCs were isolated by density centrifugation over a Ficoll-Hypaque gradient (Lymphoprep; Nycomed). T lymphocytes were purified from PBMCs using a negative selection pan-T isolation kit on an automated MACS cell sorter (Miltenyi Biotec). $CD8^+$ and $CD4^+$ lymphocytes were isolated from the purified T cells by positive selection using CD8- and CD4-conjugated MACS beads. Naive and memory subsets of CD4 and CD8 lymphocytes were separated from PBMCs by initial separation into $CD4^+$ and $CD8^+$ lymphocytes by negative selection (Miltenyi Biotec). Naive and memory subsets of isolated CD4 and CD8 lymphocytes by obtained by positive selection using CD45RA- and CD45RO-conjugated MACS beads.

Analysis of the purity of isolated subsets by flow cytometry. For analyses in this report, the following combinations of labeled monoclonal antibodies were used (10 μl per 10^5 cells; 30 min at 4°C): (i) fluorescein isothiocyanate isomer 1 (FITC)-conjugated CD8, phycoerythrin (PE)-conjugated CD3, and phycoerythrin-Cy5 (Cy5)-conjugated CD4; (ii) FITC-CD45RA and PE-CD45RO; (iii) FITC-CD45RA, PE-CD45RO, and Cy5-CD4; (iv) FITC-CD45RO, PE-CD45RA, and Cy5-CD4; (v) FITC-CD45RA, PE-CD45RO, and Cy5-CD8; and (vi) FITC-CD45RO, PE-CD45RA, and Cy5-CD8 (DAKO, Glostrup, Denmark). Cells were washed twice in phosphate-buffered saline with 2% bovine serum albumin and fixed in 200 μl of 2% formalin. The purity of the isolated $CD4^+$ $CD45RA^+$, $CD4^+$ $CD45RO^+$, $CD8^+$ $CD45RA^+$, and $CD8^+$ $CD45RO^+$ cell populations was assessed on a Coulter Epics Elite after gating on lymphocytes based on their forward and side scatter characteristics. Each analysis was based on a minimum of 5,000 events.

Detection and quantitation of HIV sequences. DNA was extracted from isolated cell subsets as previously described (44). HIV proviral sequences were quantified by limiting-dilution nested PCR (44, 54) using nested sets of highly conserved PCR primers from the long terminal repeat (LTR) region. Pan-LTR primers were 5′-GRAACCCACTGCTTAASSCTCAA-3′ (outer, sense) 5′-TG TTCGGGCGCCACTGCTAGAGA-3′ (outer, antisense), 5′-CTCAATAAAGC TTGCCTTGAG-3′ (inner, sense), and 5′-GAGGGATCTCTAGNYACCAGA GT-3′ (inner, antisense) (5′ base positions 506, 626, 524, and 578, respectively, in the HXB2 genome). Complete LTR (C-LTR) primers were 5′-ACTCTGGT RNCTAGAGATCCCTC-3′ (outer, sense), 5′-GGCGTACTCACCAGTCG CCG-3′ (outer, antisense), 5′-TCTCTAGCAGTGGCGCCCGAAC-3′ (inner, sense), and 5′-TCAGCAAGCCGAGTCCTG-3′ (inner, antisense) (5′ base positions 578, 735, 626, and 692, respectively, in the HXB2 genome). Both primary and secondary PCRs for pan-LTR and C-LTR primers were carried out using the following parameters: 94°C for 18 s, 55°C for 21 s, and 72°C for 1.5 min for 30 cycles, followed by a final extension step of 72°C for 6 min. PCR amplicons were run at 150 V for 30 min on 2% agarose gels containing 0.5 μg of ethidium bromide/ml and visualized under UV light.

Quantification was performed by limiting-dilution PCR as previously described (44). Nucleotide sequences from the V3 region (patient samples p2, p3, p4, p11, p13, p14, and p15) were amplified using previously described primers (24, 40, 43). To serve as negative controls, parallel separations, extractions, and amplifications were carried out with PBMCs isolated from buffy coats leukocytes derived from HIV-negative blood. HIV-1 DNA could be detected in CD4 T lymphocytes and CD8 T lymphocytes from HIV-seropositive individuals but not in any negative controls.

Cloning of the PCR products. Amplified DNA was ligated into a plasmid vector prior to nucleotide sequencing using the pGEM-T vector system (Pro-

TABLE 2. Purity of isolated pan-T and $CD8^+$ lymphocytes

Sample	T lymphocytes		CD8 lymphocytes	
	% $CD4^+$ $CD45^+$	% $CD3^+$ $CD45^+$	% $CD4^+$ $CD8^-$	% $CD3^+$ $CD8^+$
HIV negative				
1	2.6	98.1	0.2	99.0
2	0.5	96.4	0.2	98.2
3	0.1	99.8	0.3	96.0
4	0.1	96.7	0.4	97.3
Mean	0.6	98.2	0.3	97.3
HIV positive				
1	2.5	94.0	0.4	97.2
2	1.3	98.0	0.5	99.0
3	0.7	99.0	0.5	96.4
Mean	1.5	97.0	0.4	97.5

mega, Southampton, United Kingdom). The ligated product was transformed into competent cells (JM109; Promega) and plated on Luria-Bertani plates (200 μg of ampicillin/ml). The plasmid DNA was denatured by incubation with 1/10 volume of 2 M sodium hydroxide–2 mM EDTA at 37°C for 30 min. DNA was precipitated by addition of 1/10 volume of 3 M sodium acetate and 2 volumes of ethanol and incubation at −20°C overnight.

Nucleotide sequencing and analysis. Dideoxynucleotide sequencing was carried out with a U.S. Biochemical Sequenase 2.0 kit (Amersham Life Science, Piscataway, N.J.) with [^{35}S]dATP, and patient plasma samples were sequenced using a Thermosequenase radiolabeled terminator cycle sequencing kit according to the manufacturer's instructions.

Sequences were aligned and distances were estimated using the Simmonic 2000 sequence editor package. Phylogenetic analysis was carried out using the MEGA program (27). The nucleotide sequences from V1/V2 and V3 amplified from each of the study subjects were compared with each other and with a range of standard HIV-1 variants. Each set of sequences from the four study subjects was monophyletic in both genomic regions and distinct from those of the published sequences of subtype B: HIVSF2 (K02007), HIVRF (M17451), HIVOYI (M26727), HIVLAI (K02013), HIVJRFL (M74978), HIVYU2 (M93258), HIVCAM1 (D10112), HIVNY5CG (M38431), HIVHAN (U43131), HIVWMJ22 (M12507), and HIVSFAAA (M65024).

Nucleotide sequence accession numbers. Nucleotide sequences obtained in this study have been submitted to GenBank and assigned accession numbers AF353734 through AF353941.

RESULTS

Detection of HIV proviral sequences in CD8 lymphocytes. T lymphocytes were separated from PBMCs by negative selection using immobilized magnetic beads. Fluorescence-activated cell sorting analysis showed substantial purity of the separated T cells, with ≥94% of cells coexpressing CD3 and CD45 (mean, 97.4%) (Table 2). Monocyte contamination, detected by the expression of CD14 and CD45, was minimal (<3%; mean, 1.1%). Purified $CD3^+$ lymphocytes were subsequently separated by positive selection for CD8 expression and, in a separate step carried on the remaining cells, for CD4. Analysis of the purity of selected CD8 lymphocytes revealed extremely low numbers of contaminating CD4 lymphocytes (Table 2; Fig. 1), in no case exceeding 0.5% of the total population. Knowledge of this frequency combined with quantitation of proviral load in purified CD8 and CD4 lymphocytes allowed calculation in worst-case situations (i.e., 0.5% CD4 contamination) of the contribution of CD4 lymphocytes to the proviral load detected in CD8 lymphocytes (see below).

Having established the effectiveness of the cell separation method, we separated samples from seven HIV-infected individuals into CD4 and CD8 T lymphocytes and assayed them for HIV proviral DNA sequences using highly conserved primers from the LTR. These pan-LTR primers amplify DNA sequences immediately downstream from the tRNA primer binding site and are therefore potentially able to detect incomplete transcripts resulting from abortively infected cells (18, 52). Frequencies of infected cells ranged from 30 to 670 proviral copies/10^6 CD4 lymphocytes and from 8 to 500 /10^6 CD8 lymphocytes. The worst-case scenario, where CD4 lymphocytes comprise 0.5% of the CD8 population (see above), failed to account for the proviral sequences detected in CD8 lymphocytes in at least five of the seven samples (Table 3; Fig. 2A). Maximum contributions from contaminating CD4 lymphocytes ranged from <5% in these four samples and 27 and 33% in the remaining two. These latter samples contained low proviral loads in the CD8 lymphocyte subset (<10 copies/10^6 cells). Similar results were obtained on retesting proviral loads using a different set of primers (C-LTR) (Table 3). In this case, CD4 contamination accounted for ≤1% of proviral load detected in five samples and for 16 and 44% in the remaining two).

Detection of complete proviral transcripts. To determine whether proviral sequences detected in CD4 and CD8 lymphocytes were incomplete or complete transcripts, DNA samples from each subset were quantified using a second set of primers spanning the region on either side of the primer binding site. Quantitation of the plasmid HXB2 demonstrated that pan-LTR and C-LTR primers had equal sensitivities (data not shown), and so the detection of greater proviral loads using the pan-LTR primers in CD4 or CD8 lymphocytes would indicate the presence of incomplete proviral transcripts. Proviral loads using the two sets of primers were comparable in each case, with no evidence for higher virus loads detected with the pan-LTR primers (median ratio of pan-LTR to C-LTR, 0.638; $Z = 0.76$ using Wilcoxon signed rank test; $P = 0.445$ [not significant]).

Distribution of HIV in naive and memory subsets of CD4 and CD8 lymphocytes. To obtain phenotypically unchanged $CD4^+$ and $CD8^+$ T lymphocytes for separation of naive and memory subsets, it was necessary to use negative selection to isolate CD4 and CD8 lymphocytes prior to positive selection with CD45RA and CD45RO monoclonal antibodies. The purity of the isolated $CD4^+$ $CD45RA^+$, $CD4^+$ $CD45RO^+$, $CD8^+$ $CD45RA^+$, and $CD8^+$ $CD45RO^+$ cell populations from five HIV-1-negative individuals and one HIV-1-positive individual was determined (Table 4). $CD4^+$ lymphocytes contained 0.9 to 1.3% contaminating $CD8^+$ cells, similar to that found in the sample from the HIV-positive sample (0.7 to 1.2%). Contamination of the separated $CD8^+$ lymphocytes by $CD4^+$ cells was similarly low (0.8 to 1.7%). As described above for CD4 and CD8 lymphocytes separated by positive selection, the contribution of contaminating $CD4^+$ lymphocytes could not account for the proviral load detected in the majority if CD8 lymphocytes samples (see below).

HIV sequences in DNA from each of the purified lymphocyte subsets of the 16 study subjects was quantified by limiting dilution (Table 1; Fig. 2B and C). HIV-1 proviral DNA was detected in the CD4 naive and CD4 memory subsets of all patients studied (between 55 and 4,307 provirus copies per 10^6 cells) except in the CD4 memory subset of p15 (Table 1). The frequency of infection and proportion of virus load contributed

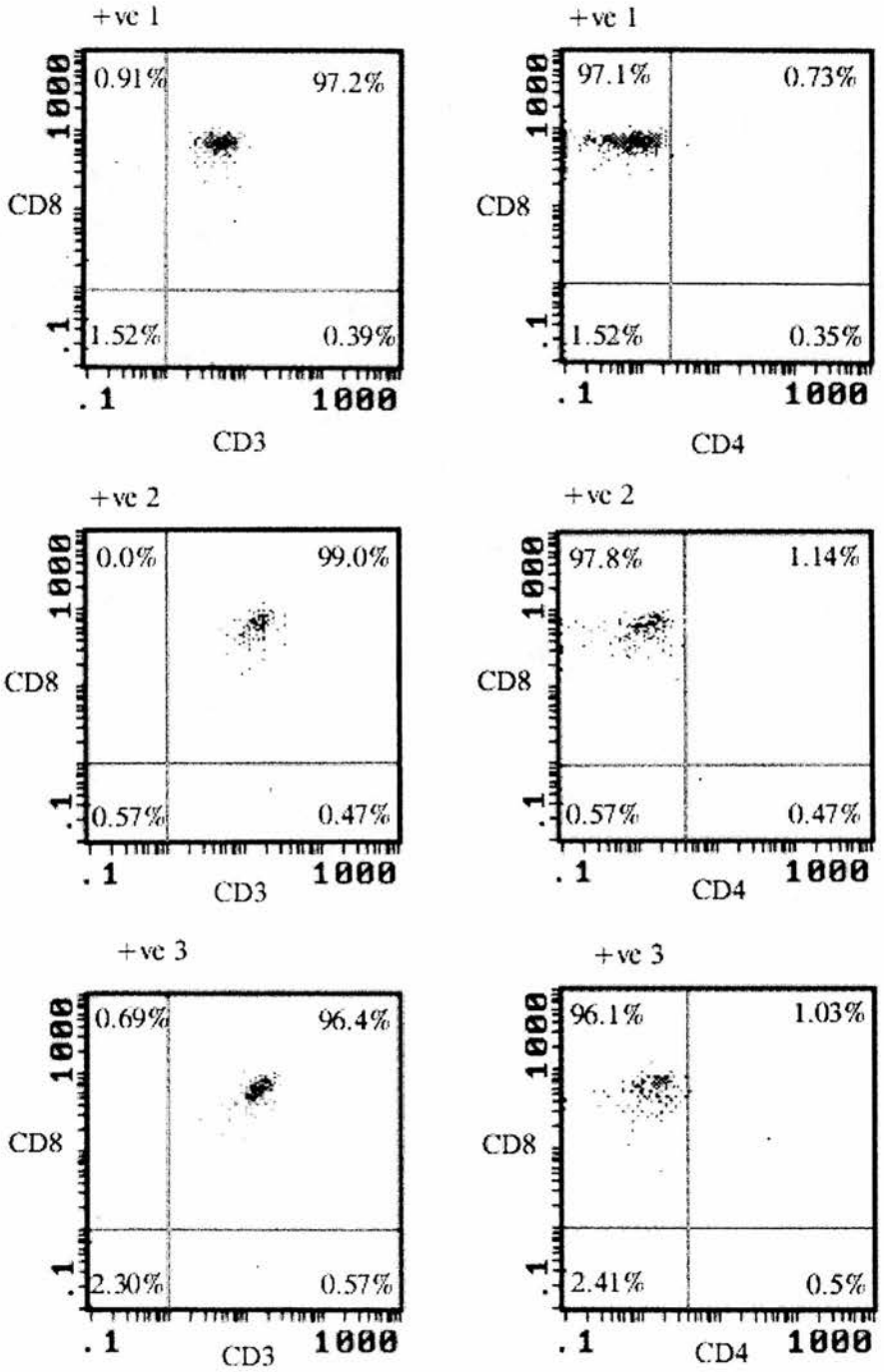


FIG. 1. Analysis by three-color flow cytometry of CD8 lymphocytes purified by positive selection from PBMCs of three HIV-positive (+ve) individuals. Cells were labeled with monoclonal antibodies to CD3 (PE), CD8 (FITC), and CD4 (Cy5).

by naive and memory subsets of CD4 lymphocytes varied with absolute CD4 count. CD4 naive lymphocytes contributed the majority of infected cells in those with high CD4 counts ($r = 0.703$, $P = 0.02$), while memory cells were predominantly infected in individuals with low CD4 counts ($r = -0.450$, $P = 0.08$).

HIV DNA was also detected in nine samples of separated $CD8^+$ $CD45RA^+$ lymphocytes, and in three $CD8^+$ $CD45RO^+$ subsets from 10 of the 16 individuals. The contribution of contaminating CD4 lymphocytes to the measured virus loads was small or insignificant in 10 of these samples (Table 1), and these were considered to represent infection of CD8 lymphocytes. In eight individuals, HIV preferentially infected the naive subset of CD8 T lymphocytes, with proviral frequencies ranging from 25 to and 1,440 provirus copies per 10^6 cells). There was a tendency for higher frequencies of infected $CD8^+$

TABLE 3. Frequencies of infection of CD4 and CD8 lymphocytes and contribution of contaminating CD4 lymphocytes to the proviral load detected in CD8 lymphocytes

Study subject	CD4 (proviral copies/ 10^6 CD4 lymphocytes)	Proviral contamination[a]			CD8 (proviral copies/ 10^6 CD8 lymphocytes)	% Mean (range) CD4 contribution[b]
		Minimum	Mean	Maximum		
Pan-T LTR primers						
p17	215	0.41	0.73	1.01	101	0.72 (0.40–1.00)
p18	309	0.59	1.05	1.45	23	4.55 (2.55–6.31)
p19	672	1.28	2.28	3.16	56	4.07 (2.28–5.64)
p20	579	1.10	1.96	2.72	10	19.6 (11.0–27.2)
p21	30	0.06	0.10	0.14	19	0.53 (0.30–0.74)
p22	563	1.07	1.91	2.65	8	23.8 (13.4–33.1)
p23	551	1.05	1.87	2.59	499	0.37 (0.21–0.52)
C-LTR primers						
p17	216	0.41	0.73	1.01	505	0.14 (0.08–0.20)
p18	514	0.98	1.74	2.42	585	0.30 (0.17–0.41)
p19	52	0.10	0.18	0.24	56	0.31 (0.31–0.44)
p20	901	1.71	3.05	4.23	9.6	31.82 (17.8–44.1)
p21	63	0.12	0.21	0.30	29	0.74 (0.41–1.02)
p22	271	0.51	0.92	1.27	8	11.48 (6.4–15.92)

[a] Contribution of CD4 lymphocytes to proviral load detected in CD8 lymphocyte subset, corresponding to minimum (0.19%), mean (0.34%), and maximum (0.47%) contamination levels observed in control experiments (Table 2), expressed as copies per 10^6 cells.
[b] Percentage of proviral load in CD8 lymphocyte subset attributable to CD4 contamination.

$CD45RA^+$ cells to be found in individuals with lower CD4 counts, although this difference did not reach statistical significance ($P = 0.286$). Frequencies of HIV-infected CD8 lymphocytes did not correlate with disease status, risk group, antiviral therapy, or total CD4 lymphocyte counts. There was no correlation between frequency of infection of CD8 cells with either CD4 subset.

Effect of antiviral therapy on CD4 and CD8 lymphocyte infection. CD4 and CD8 naive and memory lymphocyte subsets were separated from pretreatment and sequential posttreatment samples collected from five individuals receiving combination antiviral treatment and then analyzed for proviral sequences (Table 5). Treated individuals showed rises in circulating CD4 counts and reductions in circulating viremia. Despite the effectiveness of the antiviral therapy, the distribution and frequency of infected cells in each of the subsets remained relatively stable, in all cases remaining within a ± 1-$\log_{10}$ range of the levels detected in the initial samples. Infection of CD8 lymphocytes remained detectable in the three individuals whose first samples were positive, while the two individuals with negative CD8 lymphocytes in the pretreatment samples remained negative during treatment. There was evidence for a decline in the frequency of infected $CD4^+$ $CD45RA^+$ and $CD4^+$ $CD45RO^+$ lymphocytes over the course of treatment ($r = -0.428$; $P = 0.098$ and $r = 0.545$; $P = 0.029$ respectively). A similar decrease in the frequencies of infected $CD8^+$ $CD45RA^+$ cells was not observed, although fewer observations were made ($r = 0.070$; $P = 0.870$).

Sequence comparison of HIV infecting different lymphocyte subsets. To investigate the possible genetic partitioning and differences in predicted phenotype of HIV infecting CD4 and CD8 lymphocytes, proviral sequences of the V3 hypervariable region of *env* were compared (Fig. 3 and 4). From seven study subjects, approximately 10 clones derived from amplified DNA extracted from the separated lymphocyte subsets were sequenced. The combined set of V3 sequences from each individual were monophyletic and distinct from those of previously published V3 region sequences (data not shown).

Sequence diversity within lymphocyte subsets and the degree of partitioning between them varied considerably between the study subjects. V3 sequences from the naive CD4 and CD8 subsets of p15 grouped together and showed mean diversities of 0.002 to 0.0064, similar to the mean pairwise distance between subsets (0.0071). In marked contrast, sequences from CD8 lymphocytes of p14 were distinct from all other cell types, forming a separate clade supported in 100% of bootstrap resamplings. In this individual, the mean pairwise distance between the CD8 and CD4 lymphocytes ranged from 0.113 ($CD4^+$ $CD45RA^+$) to 0.119 ($CD4^+$ $CD45RO^+$), much greater than the diversity within the CD8 population (0.0076). There was no correlation between sequence diversity in any of the subsets with disease progression, nor was there correlation with proviral load (data not shown).

Among the six study subjects from whom CD8 lymphocyte sequences were obtained, five showed either partial or complete separation from CD4 virus populations. In contrast, only one of six study subjects showed any evidence for separation of CD4 naive and memory subsets (p8) (Fig. 4). Most sequences contained neutral or acidic residues at positions 306 and 320. However, sequences of certain subsets of lymphocytes from p8 ($CD4^+$ $CD45RA^+$) and p14 ($CD4^+$ lymphocytes and plasma) contained arginine residues at position 306 along with several other amino acid changes in V3 and flanking regions. These sequences formed phylogenetically distinct clades that grouped separately from variants found in other subsets.

DISCUSSION

The use of magnetic bead separation provides an effective method to isolate subsets of lymphocytes that can be assayed for HIV infection by conventional PCR. One of the problems with the technique when used to investigate infection of CD8

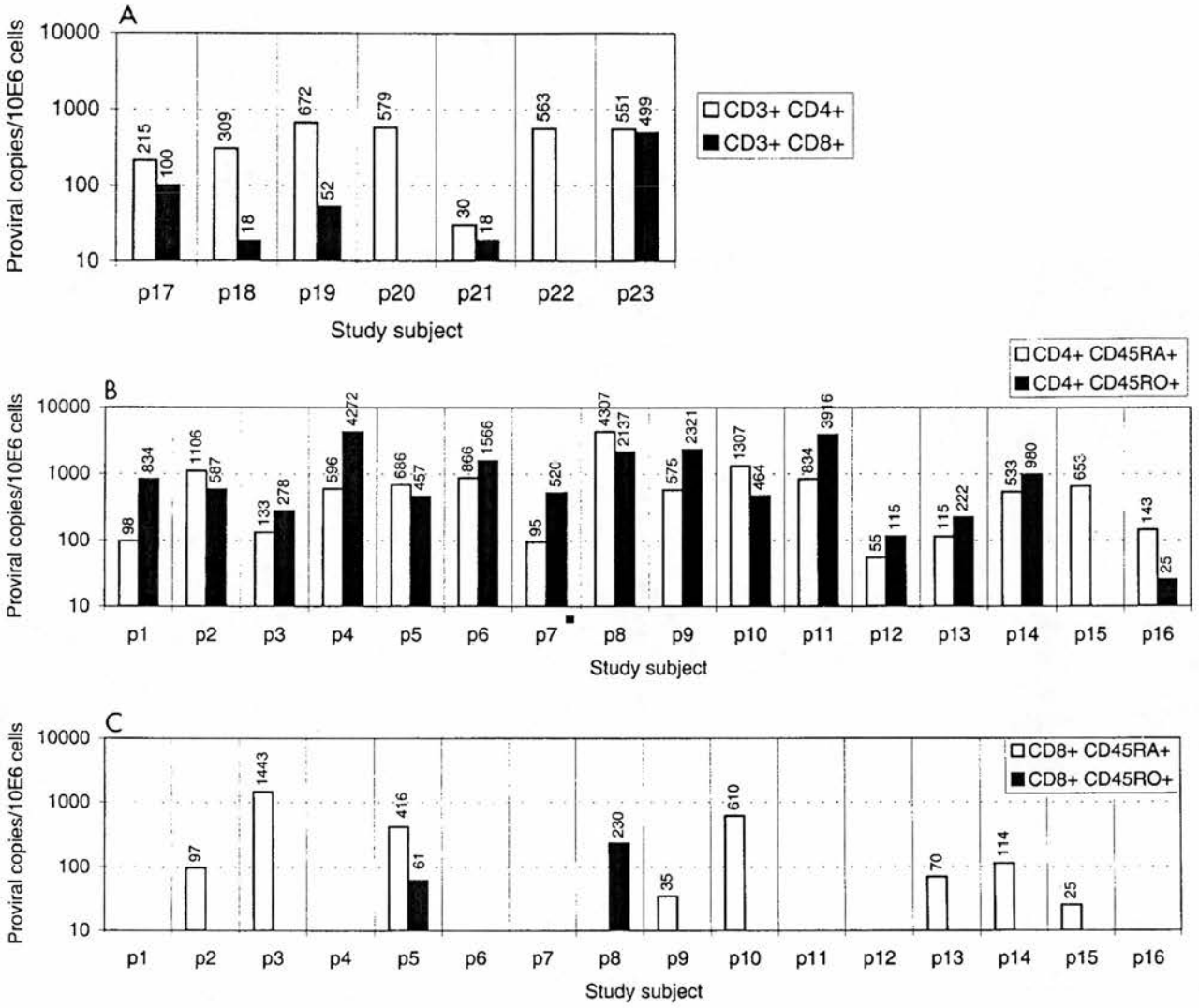


FIG. 2. Comparison of net proviral load in CD4 and CD8 lymphocytes separated by positive selection (A), and in CD4 lymphocytes (B) and CD8 lymphocytes (C) separated by negative selection, followed by positive selection for CD45RA and CD45RO.

lymphocytes is the possibility that even low-level contamination by CD4 lymphocytes may produce false positive results. To verify that CD8 lymphocytes were infected, we combined purity measurements with calculations of the contribution to proviral load of contaminating CD4 lymphocytes. Using positive selection methods to isolate CD4 and CD8 lymphocytes, we demonstrated that CD4 contamination could not account for the proviral load detected in the separated CD8 lympho-

TABLE 4. CD3, CD4, and CD8 expression on isolated $CD4^+$ $CD45RA^+$, $CD4^+$ $CD45RO^+$, $CD8^+$ $CD45RA^+$, and $CD8^+$ $CD45RO^+$ lymphocytes[a]

Sample	CD3 expression (%)				CD4 expression (%)				CD8 expression (%)			
	$CD4^+$ RA^+	$CD4^+$ RO^+	$CD8^+$ RA^+	$CD8^+$ RO^+	$CD4^+$ RA^+	$CD4^+$ RO^+	$CD8^+$ RA^+	$CD8^+$ RO^+	$CD4^+$ RA^+	$CD4^+$ RO^+	$CD8^+$ RA^+	$CD8^+$ RO^+
HIV negative												
1	79.1	98.7	98.7	68.5	73.4	93.9	2.5	2.4	2.3	1.9	93.1	60.9
2	97.2	93.8	99.2	97.6	95.6	90.9	0.4	1.3	0.6	0.3	98.4	97.5
3	98.9	99.9	98.4	91.8	96.1	97.1	0.9	2.6	1.6	1.3	92.6	79.2
4	99.4	99.2	88.4	96.8	95.2	96.5	0.1	0.3	0	0	84.2	91.0
5	99.2	97.6	88.8	50.2	98.1	95.3	0	0	1.7	0	80.9	45.6
Mean	97.6	97.6	94.7	81.0	91.7	86.5	7	1.6	1.3	0.9	83.3	70.1
HIV positive	94.8	97.8	94.5	92.8	95.1	94.7	0.8	1.3	1.2	0.7	89.8	78.8

[a] RA^+ and RO^+, $CD45RA^+$ and $CD45RO^+$.

TABLE 5. Infection of lymphocyte subsets of study subjects receiving combination antiviral therapy

Patient	Therapy[a]	Time (days) since start of treatment	CD4 count/μl	Circulating plasma virus load (genome eq of RNA/ml)[b]	Proviral copies/10^6 cells			
					$CD4^+$		$CD8^+$	
					$CD45RA^+$	$CD45RO^+$	$CD45RA^+$	$CD45RO^+$
p1	Pre	0	10	14,000	98	834	Neg	Neg
	+	27	150	1,700	34	239	Neg	Neg
p9	Pre	0	283	750,000	575	2,321	40	Neg
	+	48		3,000	72	1,807	215	Neg
p10	Pre	0	301	81,540				
	+	49	7	Neg	1,307	464	622	Neg
	+	62	466	Neg	533	533	697	Neg
	+	100		Neg	878	364	1,049	Neg
	+	115		±	667	577	2,764	Neg
	+	154	428	±	1,498	1,390	489	Neg
	Stop	360	349	16,000	734	2,884	435	Neg
p12	Pre	0	329	64,680	55	115	Neg	Neg
	+	14	477	Neg	15	188	Neg	Neg
	+	41	540	Neg	19	119	Neg	Neg
	+	100	442	Neg	9	48	Neg	Neg
	+	340	207	Neg	18	56	Neg	Neg
p14	Pre	0	360	Neg	533	980	119	Neg
	+	27	418	Neg	158	1,307	203	Neg
	+	153	465	Neg	351	183	144	Neg

[a] Combination treatment over study period. Pre, sample collected pretreatment (tested in Table 1); +, sample affected while on treatment; stop, treatment stopped because of side effects.

[b] Neg, <400 genome eq/ml; ±, equivocal result.

cytes from at least five of the seven samples tested (Table 3). A similar conclusion was drawn from comparison of frequencies of infected $CD45RA^+$ and $CD45RO^+$ CD8 lymphocytes with measurements of CD4 contamination (Table 1). The purity of T cells isolated by the pan-T negative selection method and in particular the lack of significant monocyte contamination (Table 2) provided evidence against a significant contribution to proviral load by other cell types in the PBMC population.

Further evidence was provided by lack of a correlation between frequencies of infected CD4 lymphocytes and detection of proviral sequences in either the $CD3^+$, $CD8^+$, $CD8^+$ $CD45RA^+$ or $CD3^+$, $CD8^+$, $CD8^+$ $CD45RO^+$ populations (Tables 1 and 3). For example, the sample with the highest frequency of infected CD4 lymphocytes (p4) contained undetectable frequencies of CD8 infection; similarly, the sample with the highest CD8 proviral load contained relative low frequencies of infection of both subsets of CD4 cells. Third, although there was considerable intersubject variability in the detection of CD8 lymphocyte infection, longitudinal sampling of the same individual over time revealed remarkable stability in proviral loads in each of the lymphocyte subsets. For example, pretreatment samples from p1 and p12 contained undetectable frequencies of infected CD8 lymphocytes, while samples subsequently remained negative. The remaining individuals with infected CD8 lymphocytes pretreatment remained PCR positive subsequently. The consistency of provirus detection in the lymphocyte subset argues against sporadic contamination by CD4 lymphocytes as a reason for our observations. Finally, CD4 contamination would not account for the frequent genetic differences observed in the V3 hypervariable regions between variants recovered from CD8 with those from the $CD45RA^+$ and $CD45RO^+$ subpopulations of CD4 lymphocytes (Fig. 3).

In this study, expression of the RA and RO isoforms of CD45 was used to select between two functionally different subsets of T lymphocytes (naive and memory /effector, respectively) in the peripheral circulation. The separation method had to be modified to avoid potential cellular activation and resulting change in CD45 expression that may occur during the two rounds of positive selection required by the original method (positive selection for CD8 or CD4, followed by bead removal and positive selection by CD45RA or CD45RO monoclonal antibodies). Evidence for the effectiveness of the second method is provided by the similarity in the combined frequencies of infected $CD45RA^+$ and $CD45RO^+$ CD8 and CD4 cells with those measured in CD8 and CD4 lymphocytes separated by positive selection in this (Fig. 2) and previous (31) studies. It is unlikely that CD8 lymphocytes were significantly contaminated with CD4 lymphocytes with Nef-induced down-regulation of CD4 expression. It has been long established that the proportion of provirus-positive PBMCs that are actively infected with HIV is extremely low. PBMCs expressing HIV mRNAs detectable by in situ hybridization were undetectable or present only at very low frequencies (16), frequencies considerably adrift from the numbers of PBMCs containing proviral DNA sequences (42, 44). More recently, quantitative PCR methods to detect multiply spliced mRNA transcripts (from which *nef* is translated) have been developed. They have confirmed that few, if any, cells in the peripheral circulation contain transcriptionally active HIV (2). In a variety of HIV-infected individuals, frequencies of multiply spliced mRNA ranged from 0 to 700 copies per 2×10^5 PBMCs. As a productively infected cell expresses at least 100 to 1,000 spliced transcripts, the expression detected in vivo can be accounted for by a few or even single virus-expressing cells in the sample. These frequencies are considerably adrift from the numbers of provirus-positive cells detected in the CD8 fraction purified by negative selection (Table 1).

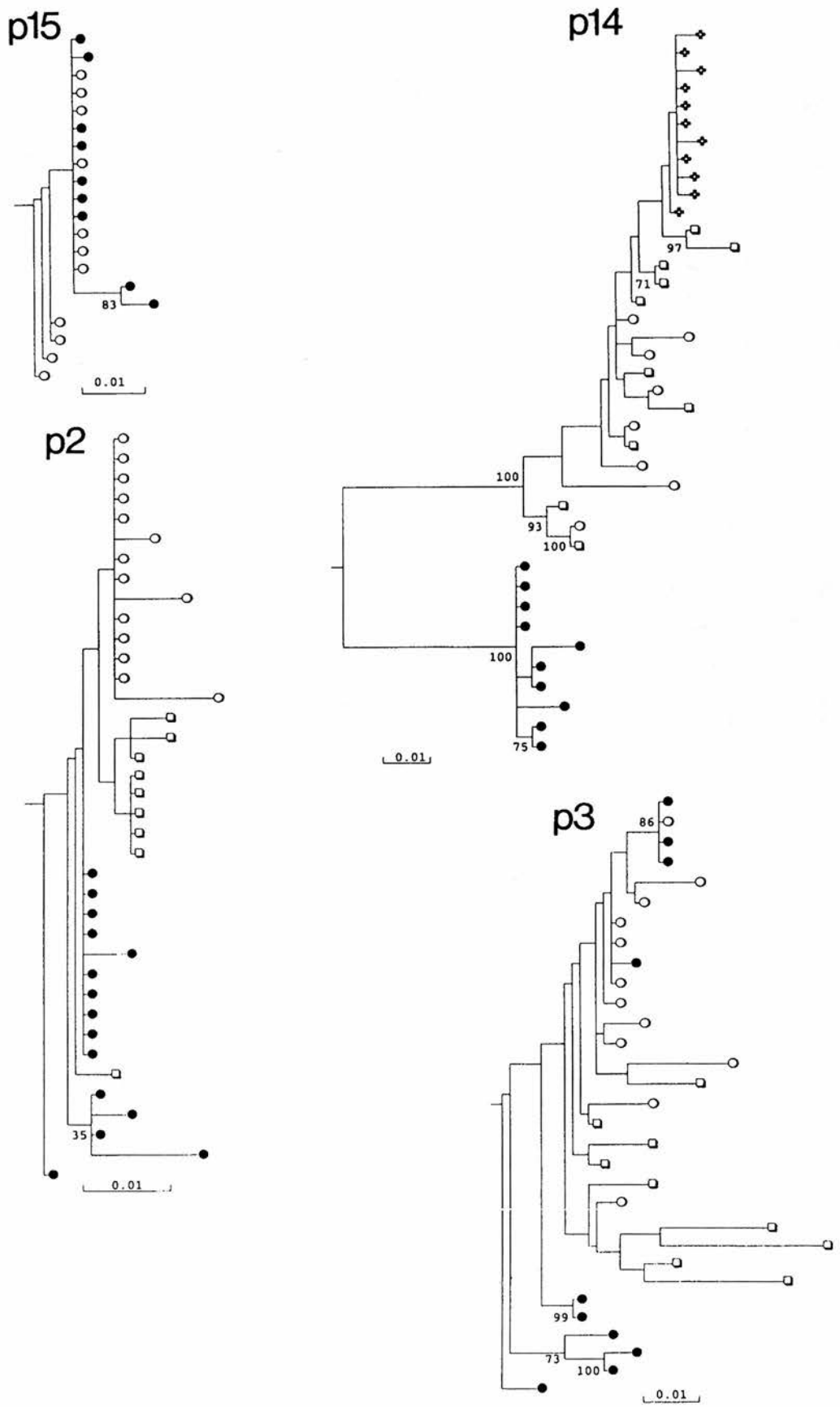


FIG. 3. Phylogenetic analysis of V3 region sequences from different lymphocyte subsets of seven study subjects. Symbols: ●, $CD8^+$ $CD45RA^+$ (naive) cells; ○, $CD4^+$ $CD45RA^+$ cells; ■, $CD8^+$ $CD45RO^+$ cells; □, $CD4^+$ $CD45RO^+$ cells; ✤, cDNA sequences from plasma (p14). Trees were rooted using the $HIV\text{-}1_{MN}$ sequence; a scale bar indicated at the bottom. Clades with >70% bootstrap supported are indicated by numbers on branches.

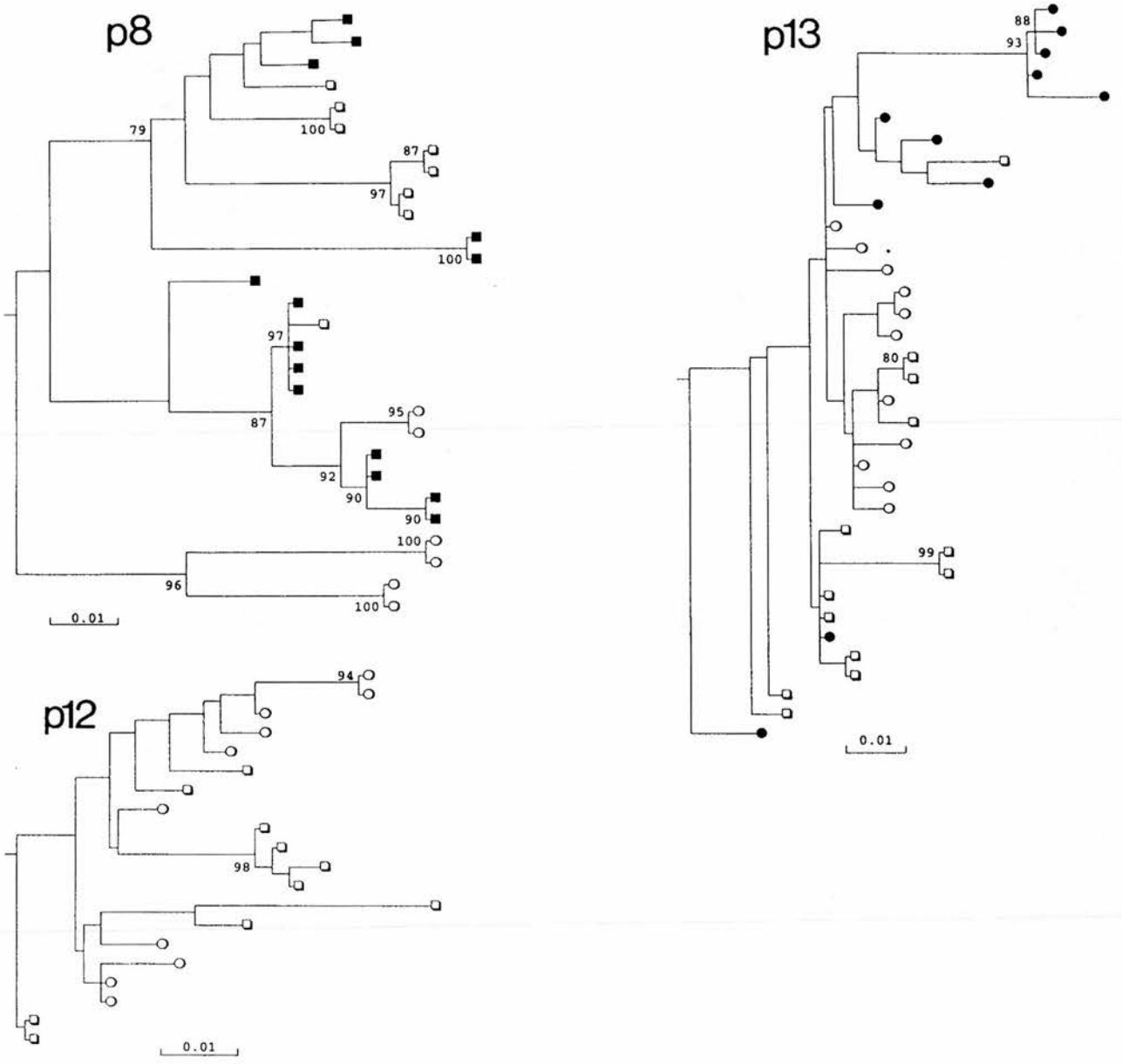
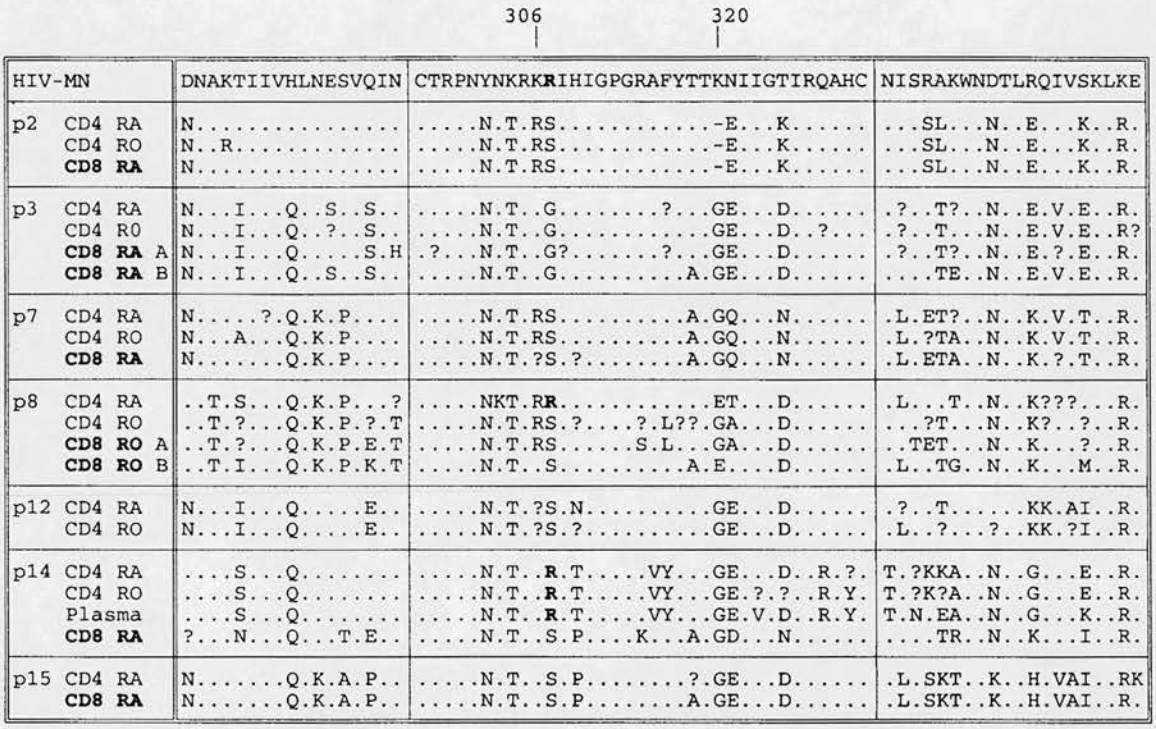


FIG. 3—*Continued.*

Quantitation of proviral sequences in these two subsets indicated that the CD45RA$^+$ (naive) subset was infected with HIV-1 in vivo, in contrast to the distribution of proviral sequences in the RO and RA subsets of CD4$^+$ lymphocytes. Separate identification of HIV-1 infection in naive and memory/effector cells provides information on the time of infection of T cells relative to maturation and activation, which in turn provides indirect information on the likely mechanisms underlying infection of peripheral CD8 lymphocytes. While expression of CD45RA and CD45RO has been the most commonly used method for separating naive and memory/effector cells, expression of other markers in combination with CD45 has been used to more strictly define functionally distinct subsets. Markers of cellular activation such as CD38 expression and down-regulation of CD27 and CD28 have been used to identify effector from memory and naive CD8$^+$ lymphocytes (8, 14, 15, 20, 35). Expression of CD62L in combination with CD45RA has been used to define purer populations of naive CD4 cells, as there is evidence that a proportion of memory CD4 cells of HIV-positive individuals express CD45RA (36, 39). Whether abnormal expression of CD45RA on the CD8 lymphocytes of HIV-positive lymphocytes also occurs has not been determined. Recently it has been reported that the isoform of CD45 expressed on memory cells may ultimately revert to RA, potentially producing a subset of CD45RA$^+$ cells functionally distinct from the naive phenotype (3).

While it is possible that a proportion of the CD45RA$^+$ CD4 and CD8 populations analyzed in our study may have originally been memory cells, the predominance of HIV-1 infection in the CD8 CD45RA$^+$ subset argues empirically that the main



			306	320	
HIV-MN			DNAKTIIVHLNESVQIN	CTRPNYNKRK**R**IHIGPGRAFYTTKNIIGTIRQAHC	NISRAKWNDTLRQIVSKLKE
p2	CD4	RA	N.....	.....N.T.RS.....-E...K.....	...SL...N..E...K..R.
	CD4	RO	N..R.....	.....N.T.RS.....-E...K.....	...SL...N..E...K..R.
	CD8	**RA**	N.....	.....N.T.RS.....-E...K.....	...SL...N..E...K..R.
p3	CD4	RA	N...I...Q..S..S..	.....N.T..G.....?...GE...D.....	.?..T?..N..E.V.E..R.
	CD4	R0	N...I...Q..?..S..	.....N.T..G.....GE...D..?...	.?..T...N..E.V.E..R?
	CD8	**RA** A	N...I...Q.....S.H	.?...N.T..G?.....?...GE...D.....	.?..T?..N..E.?.E..R.
	CD8	**RA** B	N...I...Q..S..S..	.....N.T..G.....A.GE...D.....	....TE..N..E.V.E..R.
p7	CD4	RA	N.....?.Q.K.P....	.....N.T.RS.....A.GQ...N.....	.L.ET?..N..K.V.T..R.
	CD4	RO	N...A...Q.K.P....	.....N.T.RS.....A.GQ...N.....	.L.?TA..N..K.V.T..R.
	CD8	**RA**	N.....Q.K.P....	.....N.T.?S.?.....A.GQ...N.....	.L.ETA..N..K.?.T..R.
p8	CD4	RA	..T.S...Q.K.P...?	.....NKT.R**R**.....ET...D.....	.L...T..N..K???...R.
	CD4	RO	..T.?...Q.K.P.?.T	.....N.T.RS.?....?.L??.GA...D.....	...?T...N..K?..?..R.
	CD8	**RO** A	..T.?...Q.K.P.E.T	.....N.T.RS.....S.L...GA...D.....	..TET...N..K...?..R.
	CD8	**RO** B	..T.I...Q.K.P.K.T	.....N.T..S.....A.E....D.....	.L..TG..N..K...M..R.
p12	CD4	RA	N...I...Q.....E..	.....N.T.?S.N.....GE...D.....	.?..T.....KK.AI..R.
	CD4	RO	N...I...Q.....E..	.....N.T.?S.?.....GE...D.....	.L..?...?..KK.?I..R.
p14	CD4	RA	....S...Q.....	.....N.T..**R**.T.....VY...GE...D..R.?.	T.?KKA..N..G...E..R.
	CD4	RO	....S...Q.....	.....N.T..**R**.T.....VY...GE.?.?..R.Y.	T.?K?A..N..G...E..R.
	Plasma		....S...Q.....	.....N.T..**R**.T.....VY...GE.V.D..R.Y.	T.N.EA..N..G...K..R.
	CD8	**RA**	?...N...Q...T.E..	.....N.T..S.P....K...A.GD...N.....	....TR..N..K...I..R.
p15	CD4	RA	N.....Q.K.A.P..	.....N.T..S.P.....?.GE...D.....	.L.SKT..K..H.VAI..RK
	CD8	**RA**	N.....Q.K.A.P..	.....N.T..S.P.....A.GE...D.....	.L.SKT..K..H.VAI..R.

FIG. 4. Consensus V3 and flanking region sequences of lymphocyte subsets from seven study subjects. Separate consensus sequences were calculated for the two phylogenetic groupings of CD8 sequences from p3 and p8. Amino acid residues are numbered according to HIV-1_{HXB2}; positions 306 and 320 are indicated; basic residues are indicated in bold. Symbols: ., sequence identity with HIV-1_{MN}; ?, variable sites without 75% majority consensus residue.

target CD8 lymphocyte is the naive subpopulation. For example, it is unlikely that the infected CD8 cells detected are memory revertants, as it would be likely that a similar frequency of nonrevertant CD45RO$^+$ lymphocytes would also be infected.

Infection of naive cells has been documented extensively in CD4 lymphocytes (36, 41, 45) and is confirmed in this study, although in contrast to our findings, frequencies of infected CD45RA$^+$ have been reported to be lower than in the CD45RO$^+$ effector/memory population. The latter cells are considered to be the main contributors to the pool of infected lymphocytes in vivo, where cellular activation on antigenic stimulation produces cells susceptible to infection and destruction by HIV-1 (9, 11, 48, 52, 53). Indeed, productive infection of naive CD4 lymphocytes in vitro by laboratory isolates of HIV-1 is demonstrably inefficient, most likely through lack of T-cell activation necessary for efficient reverse transcription and integration of the HIV genome after entry (9, 11, 39, 46, 48, 52, 53), and because they do not express the CCR5 chemokine coreceptor required for the entry of primary (non-syncytium-inducing) variants (5, 34). Hypotheses developed to account for the infection of naive CD4 lymphocytes may also be relevant for the infection of naive CD8 lymphocytes in vivo reported here.

There are several potential mechanisms for the infection of CD8 lymphocytes, although most would not predict the preferential distribution of infection in the naive subset. Peripheral CD8 lymphocytes express low levels of CD4 on mitogenic and antigenic stimulation in vitro, and it has been hypothesized that this allows productive infection of CD8 lymphocytes in vivo to occur (12, 25, 51). This hypothesis is supported by our recent finding that CD4 expression can be detected on the CD69$^+$, activated subpopulation of CD8 lymphocytes in PBMCs of both HIV-negative and HIV-positive individuals (S. Imlach et al., unpublished data), but the requirement for cellular activation suggests that the CD45RO$^+$ population would be the main reservoir of infected CD8 lymphocytes. The absence of any measurable excess of incomplete proviral transcripts in CD8 lymphocytes (Table 3) provides further evidence against active infection of this subset.

It is possible that genetic variants of HIV-1 evolve during persistent infection to infect cells by a non-CD4-dependent mechanism; high levels of virus replication and extensive depletion of the CD4 target population may contribute to this switch. While phenotypic characterization of HIV variants infecting CD4 and CD8 lymphocytes would clearly be of value, and the genetic differences observed in V3 provide evidence for a genetic difference between the CD4 and CD8 populations, CD4-independent infection would likely remain cell cycle-dependent, or perhaps become more so if based on expression of high levels of coreceptors, and again would preferentially target the memory/effector CD8 cell population.

HIV-1 infection of CD4$^+$ CD8$^+$ immature thymocytes destined to become CD8 lymphocytes during thymic maturation (26, 28) would provide both a plausible mechanistic explanation for their infection, and would also explain the presence of proviral sequences in the naive subsets of both CD4 and CD8 lymphocytes in peripheral blood. The thymus represents a ma-

jor target for HIV-1 infection in vivo, and destruction of thymopoietic areas is observed on autopsy examination of AIDS cases. Destruction of CD8 precursor cells would also explain the eventual failure of CD8 homeostasis and decline in circulating numbers of first naive and then memory CD8 lymphocytes on disease progression (37, 38), as well as the recovery in numbers of naive $CD8^+$ (and $CD4^+$) lymphocytes generally observed after commencement of antiviral treatment (1, 6). Without biopsy material, it was not possible to directly observe the replication of HIV within thymocytes. Indeed, proviral sequences in naive cells in peripheral blood can be detected only in circumstances where thymocytes survive infection with HIV and mature into CD4 and CD8 lymphocytes. Survival of infected cells may occur because the infecting virus was defective or because the provirus integrated into a site in the host chromosome that prevented transcription. As documented for naive CD4 lymphocytes in the peripheral circulation, where frequencies of provirus-positive cells were approximately 100-fold greater than infectivity titers (36), it is likely that the vast majority of integrated proviral sequences detected in naive CD8 lymphocytes would also be defective. The presence of culturable virus from this subset therefore provides little information on the degree of CD8 destruction that occurs in the thymus.

Evidence for the quiescent nature of HIV-1 infection in CD8 naive lymphocytes, consistent with thymic infection, is provided by the observation of equivalence in proviral load in CD8 lymphocytes between the pan-LTR and C-LTR primers (Table 3), indicating that HIV proviruses were complete and potentially stably integrated into the cellular genome. Second, we observed a great stability in frequencies of infected CD8 population on antiretroviral therapy; combination treatment achieved a complete clearance of circulating viremia in the majority of study subjects (Table 5), while over the period of virus suppression ranging from 1 to 11 months, there was no consistent reduction in frequencies of infected CD8 lymphocytes. Over the same period, frequencies of infected naive and memory subsets of CD4 lymphocytes showed a modest decline, consistent with previous kinetic studies reporting half-lives of proviral sequences from 21 to 58 weeks (7, 22, 23, 50).

Sequences recovered from the CD8 lymphocytes were frequently distinct from those from CD4 lymphocytes, whereas the $CD45RA^+$ and $CD45RO^+$ subsets of the latter were generally undifferentiated from each other. Although there was great individual variation in the sequence relationships between different cell types, CD8 lymphocytes of p14 retained V3 sequences that lacked positively charged amino acid residues associated with a CXCR4-dependent phenotype (10, 13), when both subsets of CD4 lymphocytes and the plasma population contained variants with arginine residues at these sites and which were likely to have replaced the original population. This indicates a slower turnover of the CD8 population in this individual. Similar differences in rates of cell turnover may therefore underlie the genetic differences between CD4 and CD8 lymphocytes in other study subjects.

In summary, the evidence for infection of the $CD45RA^+$ population of peripheral CD8 lymphocytes provides the basis for testing a number of competing theories for the mechanism CD8 lymphocyte infection in vivo. In the future, phenotypic characterization of variants infecting the two cell types should indicate whether CD4-independent entry of HIV-1 can occur. Further studies on the turnover of HIV-1-infected CD8 lymphocytes in vivo, such as the acquisition of antiviral resistance during treatment relapse, will provide information on the dynamics and consequences of CD8 infection and potentially on the mechanisms underlying the loss of CD8 lymphocytes on disease progression.

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