GENOTYPIC AND PHENOTYPIC CHARACTERISTICS OF HIV-1 CLADE C RESISTANT VARIANTS SELECTED IN VITRO AGAINST NUCLEOSIDE AND NON-NUCLEOSIDE INHIBITORS OF REVERSE TRANSCRIPTASE

By

Hugues D. Loemba

A thesis submitted to the Faculty of Graduate studies and Research, McGill University, in partial fulfillment of the requirement for the degree of Doctor of Philosophy

Department of Medicine

Division of Experimental Medicine

McGill University, Montreal, Canada

Submitted in August of 2001

ABSTRACT

This thesis project was designed to investigate the phenotypic and genotypic variability of human immunodeficiency virus type 1 (HIV-1) drug-naïve clade C reverse transcriptase (RT) and its potential impact in the development of resistance against inhibitors of RT. Five treatment-naive HIV-1 Ethiopian isolates were classified as subtype C on the basis of *env* gene heteroduplex mobility assays (HMA) profile and phylogenetic analysis of RT sequences. In subtype C RT, a specific KVEQ motif of silent mutations (amino acid 65, 106, 138, 161) at resistance sites was present. Two Ethiopian strains were naturally resistant to non-nucleoside RT inhibitors (NNRTI), as well as to zidovudine (ZDV), based on the natural polymorphisms of G190A and K70R, respectively. The final drug concentration that selected for NNRTI primary resistance mutations in tissue culture assays was significantly higher for clade B than clade C for each of nevirapine (NVP) (10 μM versus 2 or 4 μM), efavirenz (EFV) (1μM versus 0.01μM) and delaviridine (DLV) (10μM versus 1 or 4μM), respectively. In the middle of the selection period with all the NNRTIs, subtype B viruses were harboring a mixture of both wild type and mutated forms, whereas in clade C viruses, primary resistance mutations were fully generated. Thus, we have found that clade C isolates developed more rapidly resistance (8 or 9 weeks with NVP or DLV and 13 weeks with EFV) as compared with clade B controls (at least 15 weeks with NVP or DLV and 30 weeks with EFV). Odd mutations were detected during selection with NNRTIs, such as S98I, and two mutations (A62V and V75E), at sites associated to multi-drug resistance against nucleoside inhibitors (NRTIs). The substitution A62V was initially observed as a drug-naïve silent mutation A62A. NVP and DLV mutants were broadly cross-resistants. Following in vitro selection for drug-resistance with NNRTIs (NVP, DLV and EFV) and NRTIs [lamivudine (3TC) and ZDV], RT immunodominant regions of 14 HIV-1 subtype C treatment-naive isolates, i.e. five from Ethiopia and nine from Botswana, were screened. A significant clustering of baseline and drug-induced polymorphisms have been identified within the CTL and T-helper epitopes of clade C RT, although certain RT immunogenic sequences are

conserved. Peptides harboring such predicted mutations may be used in therapeutic immunization to restrict emergence of drug-resistance in clade C. A global characterization of HIV-1 non-B subtype resistance profile is warranted.

RÉSUMÉ

Cette Thèse a été réalisée pour déterminer les caractéristiques phénotypiques et génotypiques divergentes de la transcriptase inverse (TI) du virus de l'immunodéficience humaine de type 1 (VIH-1) de sous-type C et ainsi que leur impact dans le développement de la résistance aux inhibiteurs de la TI. Cinq isolats cliniques du VIH-1 avaient été prélevés chez des patients d'origine éthiopienne n'ayant jamais reçu d'anti-rétroviraux. Leur appartenance au VIH-1 de sous-type C avait été confirmée par la réaction de mobilité des hétéro-duplex du gène d'enveloppe (*env)* et par l'analyse phylogénétique des séquences de la TI. Un profil spécifique (KVEQ) de mutations silencieuses a été noté à certains sites de résistance de la TI de sous-type C (acides aminés 65, 106, 138 et 133). De plus, deux isolats d'Éthiopie étaient naturellement résistants à certains inhibiteurs non-nucléosidiques de la TI (NNRTIs) et à la zidovudine (ZDV), respectivement à cause des mutations G190A et K70R. Les mutants résistants aux NNRTIs ont été sélectionnés in vitro plus rapidement pour les souches de sous-type C que les sous-types C, avec respectivement 8, 9 et 13 semaines pour avoir des mutants de sous-type C contre la névirapine (NVP), la délavirdine (DLV) et l'Efavirenz (EFV) et par contre 15 semaines (pour la NVP ou la DLV) et 30 semaines (pour l'EFV) en ce qui concerne le sous-type B. Les concentrations des NNRTIs induisant le développement des mutants ont été plus hautes pour les souches de sous-type B que les sous-types C, respectivement (10 μM versus 2 or 4 M de NVP), (10μM versus 1 or 4μM de DLV) et (1μM versus 0.01μM d'EFV). Plusieurs mutations peu communes ont été identifiées chez les mutants de sous-type C sélectionnés avec les NNRTIs, notamment la mutations S98I, ainsi que les deux substitutions (A62V et V75E) associées à des sites de résistance croisée entre plusieurs inhibiteurs nucléosidiques de la TI (NRTIs). La mutation A62V est apparue à un codon ayant été identifié initialement comme représentant une mutation silencieuse A62A. D'autre part, la variabilité des séquences immunogènes de la TI a été analysée chez 14 isolats de sous-type C, obtenus chez 5 patients non-traités aux anti-rétroviraux d'Éthiopie et 9 en provenance du

Botswana après la sélection in vitro pour la résistance aux NNRTIs et aux NRTIs. Un polymorphisme significatif a été noté au niveau de certains épitopes des cellules cytotoxiques et ceux reconnus par les celludes T_4. L'immunisation à l'aide de fragments protéiniques renfermant les mutations préalablement identifiées dans la TI peut potentiellement être utile pour la prévention de l'émergence des mutants résistants. Une caractérisation globale du profil de résistance des sous-types non-B du VIH-1 est de mise.

PREFACE

This Ph.D. thesis was written in accordance to the Guidelines Concerning Thesis Preparation from the Department of Graduates Studies and Research at McGill University. The structure and contents of the thesis conforms to the option which states:

"Candidates have the option, subject to the approval of their department, of including, as part of their thesis, copies of the next of a paper(s) submitted for publication, or the clearly-duplicated text of published papers(s)..... If this option, is chosen, connecting texts, providing bridges between the different papers, are mandatory..... The thesis must include, as separate chapters or sections: (1) a Table of Contents, (2) a general abstract in English and French, (3) and introduction which clearly states the rationale and objectives of the study, (4) a comprehensive general review of the background literature to the subject of the study, when this review is appropriate, (5) a final overall conclusion and/or summary.

In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent; ..... since the task of examiners is made more difficult in these cases, it is the candidate's interest to make perfectly clear the responsibilities of the different authors of co-authored paper."

Original publications presented in chapters 2-5 inclusive are either published or submitted for publication. Chapter 1 includes literature review and a general introduction. Chapter 2 represent a comprehensive background information to the subject of the study. A final general discussion is presented in chapter 6. The author's contribution to the original knowledge and references appear at the end of the thesis.

Four original papers described in chapter 2-5 are presented as followed:

Chapter 2: Loemba H., M.A. Wainberg, and B. Brenner. 2001. Effects of HIV-1 clade diversity on HIV-1 virulence and anti-retroviral drug sensitivity. In Recent Research Developments in Virology. Transworld Research Network Ed. 3: 121-129, ISBN.

Chapter 3: Loemba H., B. Brenner, M.A. Parniak, S. Ma'ayan, B. Spira, D. Moisi, M. Oliveira, M. Detorio, and M.A. Wainberg. 2001. Genetic divergence of HIV-1 Ethiopian clade C reverse transcriptase (RT) and rapid development of resistance against non-nucleoside inhibitors of RT. Submitted to Antimicrobial Agents and Chemotherapy.

Chapter 4: Loemba H., B. Brenner, M.A. Parniak, S. Ma'ayan, B. Spira, D. Moisi, M. Oliveira, M. Detorio, M. Essex and M.A. Wainberg. 2001. Co-receptor use and HIV-1 intra-clade C polymorphisms in the protease and reverse transcriptase genes of HIV-1 isolates from Ethiopia and Botswana. Submitted to Antiviral Therapy.

Chapter 5: Loemba H., B. Brenner, M.A. Parniak, S. Ma'ayan, B. Spira, D. Moisi, M. Oliveira, M. Detorio, M. Essex and M.A. Wainberg. 2001. Polymorphism of cytotoxic T-lymphocyte (CTL) and T-helper epitopes within reverse transcriptase (RT) of HIV-1 subtype C from Ethiopia and Botswana following selection of drug resistance. Submitted to Antiviral Research.

The candidate was responsible for all work described in this thesis, with the exception of the work regarding virus isolation, preparation and stimulation of umbilical cord blood cells, sequencing of *pol* genes, and cell culture of isolates from Botswana. Mervi Detorio and Maureen Oliveira (chapter 3 and 5) contributed to the virus isolation, collection, preparation and stimulation of umbilical cord blood cells. Maureen Oliveira (chapter 4 and 5) also contributed to selection experiments with isolates from Botswana. Daniela Moisi (chapter 3, 4 and 5) helped with the sequencing of HIV-1 isolate *pol* regions. All work was performed in the laboratory of Dr. Mark A. Wainberg.

ACKNOWLEDGEMENTS

I am very grateful to Dr Mark A. Wainberg, my supervisor, for helping me accomplish my Ph.D. studies in its laboratory. He has provided me excellent scientific guidance and supervision as well as research expertise during training in its laboratory. I will never forget what Dr. Wainberg has done for me. Without his help, I may have not been able to finish this thesis.

I would like also to be thankful to Dr. Michael A. Parniak for the financial support throughout the course of my training at McGill University. I am very grateful to Dr. Bluma Brenner for her scientific advice, her help during preparation of manuscripts of chapter 2 and 3, as well as for her high interest in my research work and her criticism. I am also grateful to Bonnie Spira, our lab coordinator, for her substantial help and logistic assistance.

I would like to express special thanks to Dr. G. Price, Dr. Horacio Salomon, Dr. Larry Kleiman, Dr. Ralf Germinario, Dr. Alaoui-Jamali, Dr. Florence Doualla-Bell, Dr. Christos Tsoukas, and Dr. Eric Cohen for their stimulating scientific advice and discussions. I am thanking Dr. Donatien Mavoungou too for providing us for the HIV-1 subtype G sample from Gabon used as control in HMA assays.

I would like to express special thanks to Maureen Oliveira, Daniela Moisi, and Mervi Detorio for their great technical help during my training in Dr. Wainberg Laboratory. I am also grateful to Karidia Diallo for her sardonic remarks, encouragements and scientific discussions. I am also thanking Brian for his technical help and for fixing my computer. I am thankful to all the colleagues and friends in Dr. Wainberg laboratory for their assistance, enriching discussions and their kindness, including: Dr. Matthias Gõtte, Dr. Chen Liang, Dr Yudong, Dr Nathalie Richard, Dr Li Wei, Sam, James Whitney, Rodney Russel, Marco Petrella, Melanie, Bob Xie, Xiaofeng, Ceasar Collasos, Estrella Moyal and Monica.... I am thanking you all whose names could have been forgotten.

Finally, I am very grateful to my wife Yvette, my daughter Sarah, my son Kephane and to all my relatives for their love, encouragement and their support.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

Chapter 1:

Chapter 2:

Chapter 3:

Chapter 4:

Chapter 5:

LIST OF ABBREVIATIONS

AIDS: Acquired immunodeficiency syndrome

ALV: avian leukemia virus

APV: amprenavir

ARV: antiretroviral therapy

ASV: avian sarcoma virus

ATP: adenosine tri-phosphate

AZT: 3'-Azido-3'deoxythymidine

BFV: bovine foamy virus

BLV: bovine leukemia viruses

CA: capsid

CBMCs: umbilical cord blood mononuclear cells

ddC: Zalcitabine

DDDP: DNA-dependent DNA polymerase

ddI: Didanosine

DLV : delavirdine

d4T: Stavudine

EFV: efavirenz

EIAV: equine infectious anemia virus

Env: enveloppe

FDA: Food and Drug Administration

FeLV: feline leukemia virus

FIV: feline immunodeficiency virus

HAART: highly active anti-retroviral therapy

HIV: human immunodeficiency virus

HSRV: human spuma retrovirus

HTLV-1, 2 : human T-cell leukemia virus type 1 and type 2

IC_{50} : Inhibitory concentration 50% (drug concentration inhibiting 50% of viral activity).

IN: integrase

INV: indinavir

LTR: long terminal repeats

MHC: major histocompatibility complex

MLV: murine leukemia virus

MMTV: mouse mammary tumor virus

MP: monophosphate

MPMV: Mason-Pfizer monkey virus

MSV: murine sarcoma virus

NC: nucleocapsid

NFV: nelfinavir

NNRTI: non-nucleoside RT inhibitor

NVP : nevirapine

PBMCs : peripheral blood mononuclear cells

PBS: primer binding site

PCR: polymerase chain reaction

PLV: palinavir

Pol: polymerase

PPi: pyrophosphate

PR: protease

RDDP: RNA-dependent DNA polymerase

RSV: Rous sarcoma virus

RT: reverse transcriptase

RTV : ritonavir

SFV: simian foamy virus

SIV: Simian Immunodeficiency Virus

SQV: saquinavir

SRV: simian retrovirus type 1

SSV: simian sarcoma virus

STLV: simian T-cell leukemia

TPV: tipranavir

TRNA: transfer RNA

TP: tri-phosphate

VIH-1,2: Human immunodeficiency virus type 1 and type 2

ZDV: Zidovudine

3TC: Lamivudine

CHAPTER 1

Literature review and general introduction

1.1. Literature review

1.1.1. General overview of retroviruses

Retroviruses represent a broad group of positive-stranded RNA viruses characterized by a unique replicative strategy which involves the reverse transcription of the viral genomic RNA into a linear double-stranded DNA, followed by the integration of this DNA into the genome of the host cell (Coffin et al., 1997). According to the organization of the genome, there are two major taxonomic divisions among retroviruses: simple and complex. Each of these two categories is divided into several subgroups (Coffin, 1992; Cullen, 1992; Murphy et al., 1994). The category of simple retroviruses contains four subgroups:

- C-type retroviruses of group A, such as Rous sarcoma virus (RSV), avian leukemia virus (ALV), avian sarcoma virus (ASV).

- C-type retroviruses of group B, e.g. murine leukemia virus (MLV), feline leukemia virus (FeLV), murine sarcoma virus (MSV), simian sarcoma virus (SSV).

- B-type retroviruses whose prototype is mouse mammary tumor virus (MMTV).

- D-type retroviruses as represented by Mason-Pfizer monkey virus (MPMV) and simian retrovirus type 1 (SRV-1).

Retroviral type A particles are immature intracellular virions with a hollow structure. Type B particles have an enveloped extracellular profile with an excentrically located condensed isometric core. Type C virions have a core that is assembled simultaneously with the budding process and mature particles have a

concentrically located isometric core. Type D virions are similar to type B particles but with fewer surface projections and an elongated core (Gelderblom et al., 1989; Coffin et al., 1997).

The complex retroviruses are classified into three major subgroups:

- Lentiviruses, to which belong the human immunodeficiency virus type 1 (HIV-1) and 2 (HIV-2), simian immunodeficiency virus (SIV), visna virus, feline immunodeficiency virus (FIV), equine infectious anemia virus (EIAV).

- The subgroup of T- cell leukemia viruses including the human T-cell leukemia virus types 1 (HTLV-1) and 2 (HTLV-2), the simian T-cell leukemia virus (STLV) and bovine leukemia virus (BLV).

- The subgroup of spumaviruses is represented by the human spuma retrovirus (HSRV), the simian foamy virus (SFV) and the bovine foamy virus (BFV).

Lentiviruses bud concomitantly with the assembly of the viral core and their envelopes harbour characteristic knobs of 9-10 nm in length. T-cell leukemia viruses represent an intermediate group with morphological patterns common to both C-type particles and lentiviruses. Spumaviruses have surface projections of 10 nm in length and a preformed core which never fully condenses after virion budding (Gelderblom et al., 1989).

The genome of retroviruses may be considered diploid as they contain two copies of single-stranded RNA molecules that are capped and polyadenylated as observed for mRNA elements. This characteristic structure facilitates genomic recombination and appearance of retroviral genetic diversity (Coffin et al., 1997). In general, retroviruses have three main genetic domains termed *Gag*, *Pol* and

Env, that code respectively for the coat proteins (matrix, capsid, nuleocapsid), the viral enzymes (reverse transcriptase, protease and integrase), and the viral envelope (Coffin, 1992; Cullen, 1992; Coffin et al., 1997). In the integrated DNA provirus, these genes are arranged in the following order: 5'-*Gag-Pol-Env*-3'. Their 5' and 3' extremities are flanked by characteristic long terminal repeat sequences (LTR) that contain enhancer and promotor elements required for efficient transcription of the retroviral genome. Simple retroviruses generally only contain the three main genomic regions *Gag-Pol-Env*, whereas complex retroviruses have a series of additional domains that encode accessory and transcriptional or post-transcriptional regulatory proteins (Cullen, 1992; Coffin et al., 1997).

During the replication of retroviruses, viral gene products are employed for the structural and enzymatic functions of viral particles, whereas regulation of viral gene expression at the transcriptional and post-transcriptional levels is controlled exclusively by *cis*-acting viral DNA or RNA sequences with *trans*-acting host cell transcriptional factors (Cullen, 1992). However, unlike their simple counterparts that encode only singly-spliced (*Env*) and unspliced (*Gag-Pol*) mRNA, complex retroviruses encode at least two other multiply spliced viral mRNA species that encode a LTR-driven trans-activator (the early regulatory protein *TAT* in the case of HIV-1 and *TAX* for HTLV-1), and a protein required for expression of the late structural mRNA products (the regulatory protein Rev for HIV-1 or Rex for HTLV-1) (Cullen, 1992). Thus, complex retroviral replication is divided into two temporal phases: an early regulatory phase and a

later structural phase. Although complex retroviruses have a similar profile of viral gene regulation, the in vivo pathogenetic consequences of this shared replication strategy are not fully understood. In addition, complex retroviruses appear to induce chronic infections, long-term wasting diseases, neurological disorders and immunodeficiencies characterized by a high level of latency (Cullen, 1992; Coffin et al., 1997). According to the type of transmission, retroviruses are classified into exogenous and endogenous. Exogenous retroviruses are transmitted horizontally, from one individual to another. In contrast, endogenous retrovirus transmission is achieved vertically in an inheritable manner from parent to offspring in the form of proviruses integrated into the parental chromosome (Coffin, 1992; Coffin et al., 1997).

1.1.2. Biological properties of HIV-1.

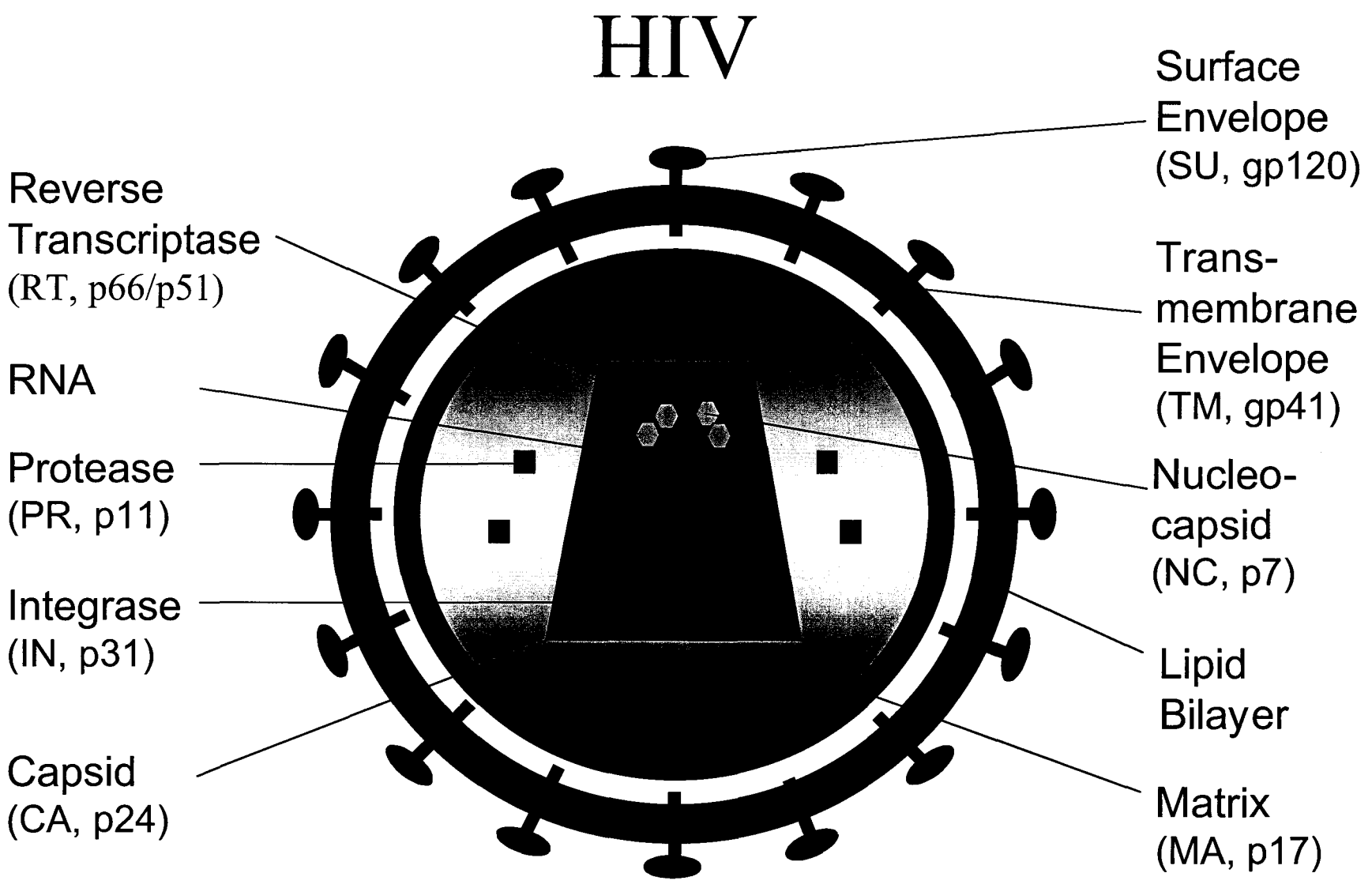
1.1.2.1. Morphological properties of HIV-1.

As shown in Figure 1, HIV-1 is an enveloped virus, which has an icosahedral structure containing 72 external spikes (Gelderblom et al., 1987). Theses knobs are formed by the two major proteins of the viral envelope, the surface glycoprotein gp120 and the transmembrane glycoprotein gp41. The gp120 subunit comprises the external portion of the viral envelope, whereas the gp41 portion spans the membrane and anchors the glycoprotein complex to the surface of the viral particle. The lipid envelope bilayer is also studded with a series of host cell proteins, including β2 microglobulin, ubiquitin, and class I and class II major histocompatibility complex (MHC) antigens acquired during virion budding (Arthur et al., 1992). Various other cellular proteins have been identified inside

the HIV-1 virions: the double-stranded RNA-binding protein staufen, a virion-associated nuclear shuttling protein, and actin-binding proteins such as the translation elongation factor 1-alpha, glyceraldehydes-3-phosphate dehydrogenase, HS-1, Pin1, the C-terminal tail of CD43 (Cimarelli and Luban, 1999; Mouland et al., 2000; Gupta et al., 2000; Ott et al., 2000). The overall diameter of the spherical viral particle is approximately 100 nm (Gelderblom et al., 1987; Gelderblom et al., 1990).

Figure 1. The schematic representation of HIV morphology.

HIV

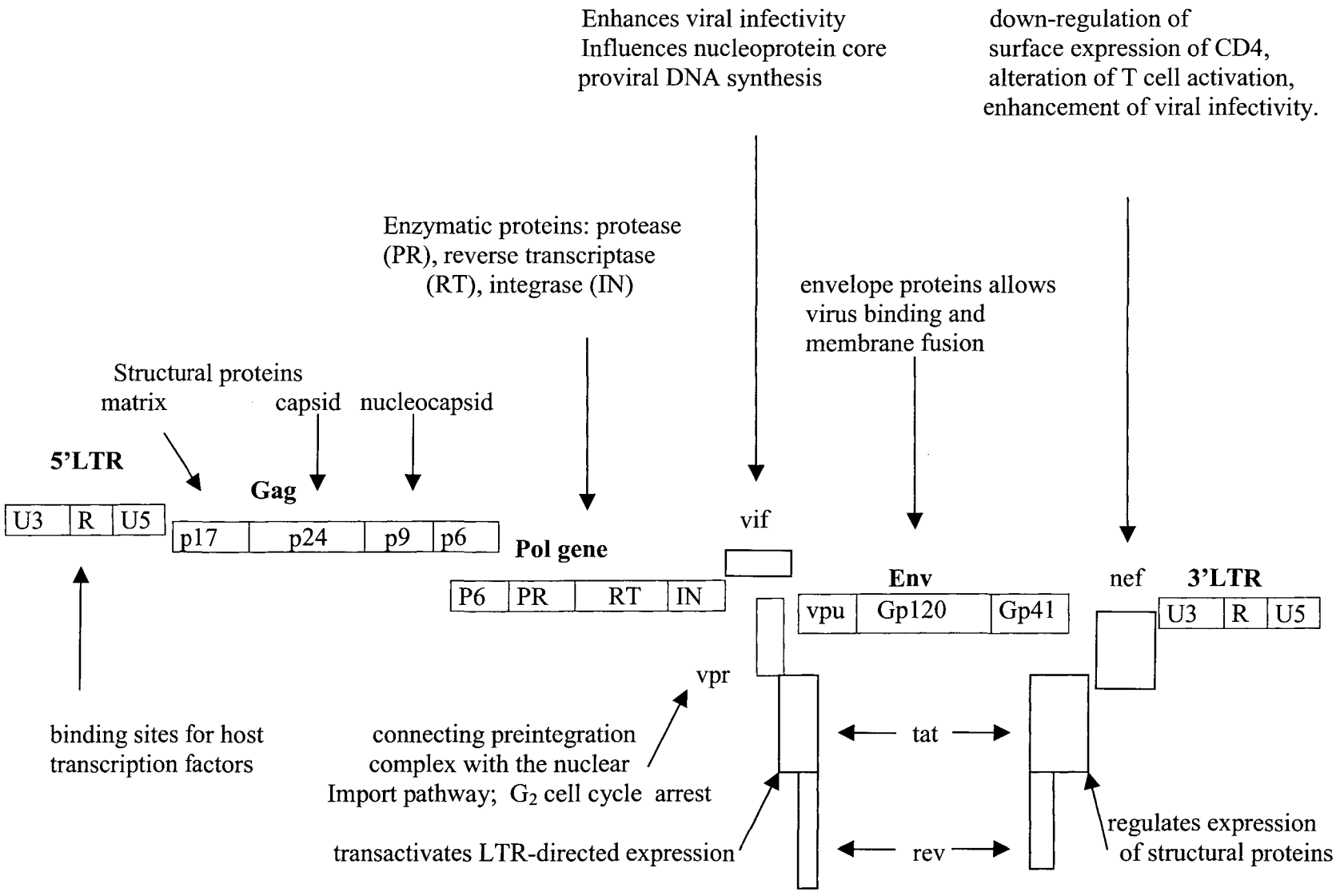

Surface Envelope (SU, gp120)
Trans-membrane Envelope (TM, gp41)
Nucleo-capsid (NC, p7)
Lipid Bilayer
Matrix (MA, p17)
Reverse Transcriptase (RT, p66/p51)
RNA
Protease (PR, p11)
Integrase (IN, p31)
Capsid (CA, p24)

Below the envelope, there is a matrix protein p17 (MA), which is associated with the inner surface of the lipid bilayer and interacts with the later via a myristic acid moiety. The mature HIV-1 virion also contains an inner capsid core whose cone-shaped structure is formed by the capsid protein p24 (CA). Inside the capsid core are located the dimeric viral genomic RNA, the accessory protein vpr, the prolin-rich protein (p9), the viral enzymes reverse transcriptase (p51/p66), protease (p11) and integrase (p31), and approximately 2000 molecules of the processed nucleocapsid (NC) p7 (Chen et al., 1980; Dickson et al., 1985; Lu et al., 1993; Darlix et al., 1995). The proteins p17, p24, p7, and p9 are products of the proteolytic cleavage of the 55-kD Gag precurseur by HIV-1 protease (Gelderblom, 1991; Henderson et al., 1992). The two copies of single-stranded HIV-1 RNA molecules located inside the core are of about 9.2 kilobases each. In general, a single HIV-1 virion is estimated to contain 1200 CA, roughly 80 RT and up to 280 molecules of gp120 (Layne S.P. et al., 1992; Hahn et al., 1994).

1.1.2.2. Molecular organization of HIV-1.

The genomic organization of HIV-1 is illustrated in Figure 2. Two identical elements, the *long terminal repeats* (LTRs), are generated at each end of the provirus during the course of reverse transcription of the viral RNA. These LTRs contain promoters and transcriptional enhancer sequences, such as the TATA promoter, polyadenylation signal sequences, cis-acting elements, negative regulatory elements and NF-kB binding regions that are all necessary for efficient viral replication. Various genes encoding structural proteins and viral enzymes, and genes coding for a series of regulatory and accessory proteins are flanked by the two LTRs.

Figure 2: The genomic organisation of HIV


Enhances viral infectivity
Influences nucleoprotein core
proviral DNA synthesis
down-regulation of
surface expression of CD4,
alteration of T cell activation,
enhancement of viral infectivity.
Enzymatic proteins: protease
(PR), reverse transcriptase
(RT), integrase (IN)
envelope proteins allows
virus binding and
membrane fusion
Structural proteins
matrix
capsid
nucleocapsid
5'LTR
Gag
U3
R
U5
p17
p24
p9
p6
Pol gene
P6
PR
RT
IN
vif
Env
vpu
Gp120
Gp41
nef
3'LTR
U3
R
U5
vpr
tat
rev
binding sites for host
transcription factors
connecting preintegration
complex with the nuclear
Import pathway; G2 cell cycle arrest
transactivates LTR-directed expression
regulates expression
of structural proteins

Roughly, HIV-1 exibits three essential regions located between the 5'-and the 3'LTRs:

- the *Gag* gene that is situated at the 5' end of the genome and that codes for a Pr55 polyprotein, that is the precursor of four smaller virion structural proteins;
- the *Pol* gene that encodes the virion-associated enzymes reverse transcriptase (RT), protease (PR) and integrase (IN);
- and the *Env* gene that encodes the envelope glycoprotein (Varmus et al., 1989).

Like *Gag*, the *Pol* gene is expressed from an unspliced full-length mRNA. Initially, the *Gag* and *Pol* regions are translated by the host cell ribosomes into large polyprotein precursors that are later processed (Jacks et al., 1988; Debouck, 1992). More specifically, the HIV-1 Gag open reading frame is translated as a 55 kD precursor, and the HIV-1 Pol open reading frame is translated as a Gag-Pol fusion polyprotein, $Pr160^{gag\text{-}pol}$, that results from a ribosomal frameshift (approximately 10% frequency) from the Gag to the Pol frames at specific site positioned before the Gag termination codon (Jacks et al., 1988; Debouck, 1992). The proteolytic maturation of $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ is crucial for the successful completion of the retroviral life cycle and the production of infectious virions. This polyprotein processing is not carried out by host cell proteases, but only by the unique protease that is encoded by HIV-1 (Kohl et al., 1988; Debouck, 1992).

In addition to the prototypic *Gag*, *Pol* and *Env* coding sequences, HIV-1 contains at least six other genes coding for auxiliary proteins: *Tat, Rev, Vif, Vpr, Vpu* and *Nef* (Cullen, 1992; Subbramanian and Cohen, 1994). These auxiliary proteins are usually classified in two groups depending on the time of their regulatory expression during HIV replication. *Tat*, *Rev* and *Nef* represent proteins synthesized from Rev-independent multiply spliced mRNA that are coded early in the viral life cycle, whereas *Vif*, *Vpr* and *Vpu* are proteins expressed from Rev-dependent singly spliced mRNAs at late stages of HIV-1 replication (Cullen, 1992; Subbramanian and Cohen, 1994).

The *Tat* gene is a major transactivator of the LTR, whereas *Rev* acts post-transcriptionnally to ensure the switch from the early to late phase of HIV-1 gene expression (Cullen, 1992; Subbramanian and Cohen, 1994). In concert with several host cell factors, Rev facilitates the export of unspliced and singly spliced viral mRNAs into the cytoplasm (Malim et al., 1991; Liu et al., 1994). The shuttling translocation of Rev between nucleus and cytoplasm is based on its capacity to bind to a complex stem-loop structure, the *Rev* Responsive region (RRE) that is present in all Rev-responsive viral mRNAs but is excluded thereafter through the process of splicing (Heaphy et al., 1990). Therefore, *Tat* and *Rev* are required for viral replication unlike the other auxilary gene products that are thought to be accessory, i.e. non-essential for in vitro replication; although accessory proteins play important roles in HIV pathogenesis and disease progression (Cohen et al., 1990; Subbramanian et al., 1994; Trono, 1995).

The gene coding for the *Nef* protein is located at the extreme 3'end of the HIV genome (Ratner et al., 1985; Li et al., 1997). Despite the fact of being expressed early during viral replication like *Tat* and *Rev*, the *Nef* protein belongs to the group of HIV accessory proteins, because *Nef* presence is not essential for HIV replication *in vitro* (Subbramanian and Cohen, 1994). However, *Nef* is required for *in vivo* HIV replication and pathogenicity (Jamieson et al., 1994; Lindemann et al., 1994). The present of *Nef* has been reported to be important for maintaining high viral load values and for the progression of AIDS disease (Kesler et al., 1991). In addition, is has been established that *Nef* specifically down regulates the CD4 surface expression by mediating endocytosis and lysosomal degradation of CD4 (Garcia et al., 1993; Aiken et al., 1994). This Nef-induced process requires interactions with a critical dileucine motif in the proximal portion of the CD4 cytoplasmic domain (Garcia et al., 1993; Aiken et al., 1994).

The *Vif* protein is also called viral infectivity factor and is produced from a singly spliced mRNA that accumulates late in HIV life cycle (Garret et al., 1991). *Vif* acts during assembly by allowing the formation of particles competent to initiate new rounds of infection (von Schwendler et al., 1993; Li et al., 1997). The *Vif* genes exit in several Lentiviruses, including HIV-1, HIV-2 and SIVmac; they are crucial for the infectivity of these viruses in primary cells such as peripheral blood T lymphocytes and monocytes/macrophages whose permissivity to Vif-deficient viruses is highly restricted, suggesting that *in vivo Vif* may be required in these cells (Michaels et al., 1993; Gabuzda et al., 1994; Park et al., 1994;

Subbramanian and Cohen, 1994). During HIV infection in different cell lines, there are varying requirements for *Vif* protein that may be due to the presence or absence of cell line factors having vif-like functions (Fan and Peden, 1992; Sakai et al., 1993; Gabuzda et al., 1994). These cell line factors may also be missing in primary cells (Gabuzda et al., 1994; Subbramanian and Cohen, 1994). According to several reports, *Vif* does not appear to be incorporated in HIV virion (von Schwendler et al., 1993; Sova and Volsky, 1993).

Vpu accessory protein present in HIV-1 is unique because no similar proteins have been found in HIV-2 or in the majority of the SIVs (Cohen et al., 1988; Huet et al., 1990; Subbramanian and Cohen, 1994). The *Vpu* gene overlaps the 5'end of the *Env* gene and the encoded *Vpu* protein shares the same singly spliced mRNA precursor with the envelop glycoprotein (Strebel et al., 1988; Schwartz et al., 1990). *Vpu* has been reported to increase the levels of virions that are released from infected cells but the mechanism of this *Vpu* function is not yet defined (Strebel et al., 1988; Subbramanian and Cohen, 1994; Bour et al., 1995; Li et al., 1997). *Vpu* also plays an important role in CD4 degradation; while *Nef* mediates endocytosis and lysosomal degradation of CD4, *Vpu* induces an efficient and selective degradation of the CD4 molecule in the endoplasmic reticulum, thereby affecting the transport of CD4 to the cell surface (Crise et al., 1990; Willey et al., 1992; Bour et al., 1995). It has been reported that the transmembrane domain of CD4 appears necessary for its Vpu-mediated degradation (Buonocore et al., 1994; Raja et al., 1994).

Unlike the other accessory protein, *Vpr* is packaged into into mature viral particles and this incorporation is probably due to *Vpr* interactions with the Gag polyprotein precursor p55 and particularly with the p6 protein (Cohen et al., 1990; Subbramanian and Cohen, 1994; Lavallée et al., 1994; Kondo et al., 1995). *Vpr* plays an important role during the transport of the preintegration reverse transcription complex from the cytoplasm into the nucleus in non-dividing target cells (Heinzinger et al., 1994; Miller and Sarver, 1997). The proteins Vpr and Matrix independently permit import of the preintegration complex to the nucleus via distinct nuclear localization signal (NLS) sequences (Heinzinger et al., 1994; Miller and Sarver, 1997). *Vpr* has also the ability to alter the cell cycle and proliferation status of the infected host cell (Levy et al, 1993; Subbramanian and Cohen, 1994). Member of the HIV-2/SIV_{SM} group viruses encode, in addition to *Vpr*, a closely related protein called *Vpx*. Although, *Vpr* and *Vpx* share a high sequence homology, there are some distinctions in their functions; the *Vpx* protein is both necessary and sufficient for the nuclear import of the viral reverse transcription complex, while *Vpr* provide just additive nuclear import functions. But unlike *Vpx*, *Vpr* has also the capacity to inhibit the progression of infected host cells from the G_2 to the M phase of the cell cycle. (Levy et al, 1993; Fletcher et al., 1996; Poon et al., 1997). All of these *Vpr* effects have suspected to be mediated by its interactions with cellular proteins. Such interactions with *Vpr* have been reported in the case of the uracil DNA glycolase DNA repair enzyme (Bouhamdan et al., 1996).

1.1.2.3.HIV-1 life cycle.

1.1.2.3.1. Virus binding, fusion and entry.

Infection is initiated by the binding of a virus particle to the HIV-1 specific receptor, the CD4 molecule, located on the surface of the target cell. The interaction between the HIV-1 envelope and the CD4 is mediated by gp120. In addition to the CD4 receptor, other cell surface co-receptors are required for mediating HIV-1 entry, such as CXCR4 (fusin), CCR1, CCR3, CCR5 and CCR2b (Alkhatib et al., 1996; Bjõrndal et al., 1997). Entry occurs via a pH-independent fusion of the viral and cellular membranes. This process is triggered by a conformational change within the envelope gp120 subunit and is activated by a highly hydrophobic region, i.e. a fusogenic domain, located at the N-terminus of gp41. After internalization, the viral particle is partially uncoated in the host cell cytoplasm in preparation for the synthesis of proviral DNA by RT, although cell-free virions may contain a limited amount of partial reverse transcription products (Bedinger et al., 1988; Trono, 1992; Moore et al., 1993)

1.1.2.3.2. Reverse transcription.

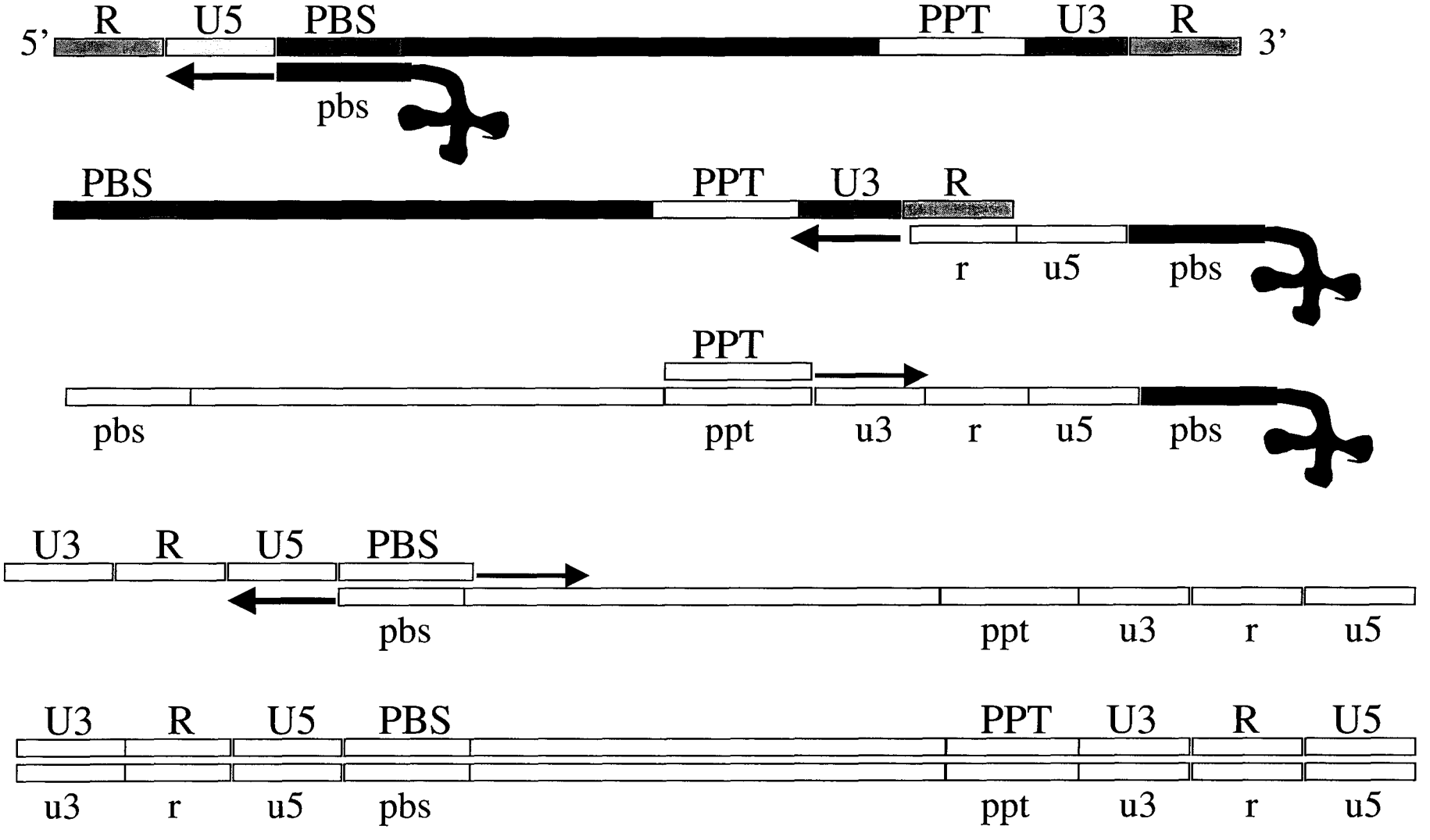
After entry, the viral genomic RNA is reverse transcribed into complementary DNA by the viral RT. The host cell derived $tRNA^{lys3}$ packaged into HIV-1 virions serves to prime the synthesis of minus-strand DNA during the first phase of reverse transcription (Figure 3) (Weiss et al., 1992; Hottiger and Hubscher, 1996). A portion of this $tRNA^{lys3}$ is complementary to sequence situated near the 5' end of the viral genomic RNA that is called the primer binding site (PBS) (Lori, 1992; Trono, 1992; Kohlstaedt et al., 1992b). In

addition, it has been established that RT preferentially bind to $tRNA^{lys3}$ even in the presence of a 100-fold excess of other tRNAs (Barat et al., 1989; Weiss et al., 1992). RT elongates the nascent DNA from the $tRNA^{lys3}$ primer to the 5'end of the genomic RNA to form a short DNA intermediate called minus-strand strong-stop DNA. During DNA synthesis, the RNAse H activity of RT removes the RNA from the RNA/DNA complex. Following removal of the 5'end of viral RNA, the minus-strand strong-stop DNA translocates or jumps to the 3'end of viral genome. This translocation is called the first template switch; it is due to the fact that identical R region sequences exist at the 5' and 3' ends of the viral genome, and fact that sequence of (-) strand strong-stop DNA is complementary to the R region at the 3'end of the genomic RNA (Zack et al., 1990).

After translocation, RT continues to copy the RNA genome until the complete synthesis of minus-strand DNA, resulting in the formation of an RNA/DNA duplex. The RNA strand is degraded by the RNAse H activity of RT, but this degradation is not complete; a polypurine tract located near the 3'end of the viral RNA remains resistant (Charneau and Chavel, 1991). This short ribonucleotide sequence serves as primer for the synthesis of the second DNA strand called plus-strand DNA (Resnik et al., 1984; Luo et al., 1990; Charneau et al., 1992). Synthesis of plus-strand strong-stop DNA is terminated when extension results in coping of the tRNA still attached to the 5'end of the template (Figure 3). Then, the tRNA primer is removed by the RNAse H activity of RT, allowing the second template switch (Hottiger and Hubscher, 1996).

Figure 3: The reverse transcription

Reverse transcription



The complementarity of 3'end sequences between the minus- and plus- strand PBS facilitates this second template transfer. Both of the 3'end sequences serve as primers for synthesis of the remaining DNA sequences. This results in the production of a double-stranded DNA copy of the viral genome that contains complete LTR elements at the 5' and 3' ends (Resnik et al., 1984; Luo et al., 1990).

1.1.2.3.3. Nuclear localization and integration.

After reverse transcription has taken place in the cell-free virus and mainly in the host cell cytoplasm, the viral double stranded-DNA remains associated with reverse transcriptase and integrase in the context of a preintegration complex that is translocated into the nucleus of the host cell (Roth et al., 1989; Bukrinsky at al., 1992; Fletcher et al., 1996). The preintegration complex of HIV-1 includes the reverse transcribed viral DNA, the integrase, the reverse transcriptase, the proteins Vpr, nucleocapsid and the phosphorilated matrix (p17) (Bukrinsky et al., 1992 and 1993; Heinzinger et al., 1994; Gallay et al., 1995). This nuclear translocation is a process that requires ATP and is independent of cell division (Bukrinsky at al., 1992; Gallay et al., 1995). The linear copy of the HIV genome is subsequently inserted into chromosomal DNA with the aid of the endonuclease activity of viral integrase to form a stable provirus (Vink and Plasterk, 1993). Proviral integration sites are random but may be influenced by chromatin structure. HIV-integrated DNA contains highly conserved di-nucleotide sequences (5' TG-CA 3'), and there is a duplication of a short stretch of cellular DNA at the site of integration (Roth et al., 1989; Vink and Plasterk, 1993).

Because the HIV provirus is covalently integrated into the host cell genetic material, viral DNA stays permanently associated with the host cell chromosome for the remaining life-time period of the cell.

1.1.2.3.4. Expression and regulation of viral genes.

1.1.2.3.4.1. Early expression of HIV-1 regulatory genes.

The integrated viral genome of HIV and other retroviruses contains at both ends a terminally redundant region called long terminal repeat (LTR) that does not encode proteins but is crucial for the regulation of viral replication (Varmus and Brown, 1989; Cullen, 1992). The 5'LTR plays an essential role in the regulation of viral gene expression whereas the 3'LTR is important for transcriptional termination (Gaynor, 1992; Cullen, 1992). There are three different regions that have been determined within the LTRs:

- a 5' unique region (U5) that contains signals mediating polyadenylation of viral messages,
- a terminal redundancy (R) sequence; during the reverse transcription it facilitates DNA strand transfer, and during viral gene expression it marks the beginning of all viral transcripts. In general in HIV and SIV viruses, the sequence R contains the Tat-binding region called Trans-Activation Responsive (TAR) element that is represented by a stable stemloop structure.
- a 3' unique region (U3); it contains enhancer and promotor elements (Goff, 1990; Cullen, 1991; Cullen, 1992; Gaynor, 1992).

Several sites for binding of inducible and constitutive host cell transcription factors have been defined within the LTRs (Gaynor R., 1992). These trans-activation factors stimulate a low but important level of expression of HIV-1 genes. Viral gene expression is divided into two temporal phases, i.e. an early, regulatory phase and a later, structural phase (Cullen, 1991). The early expressed mRNA molecules encode two nuclear regulatory proteins. The first of these proteins is the sequence-specific trans-activator *Tat*. The second regulatory protein *Rev* is required to activate the expression of the late, structural mRNA species. The early phase of expression is characterized by cytoplasmic expression of the multiply spliced 2.0-kilobase viral mRNAs that encode the two regulatory proteins *Tat* and *Rev*, and the accessory protein *Nef* (Cullen, 1991; Cullen, 1992; Trono 1995).

1.1.2.3.4.2. Late expression of HIV-1 structural and enzymatic genes.

Accumulation of the *Rev* protein causes a switch from the early to the late phase of viral gene expression. During the late phase, the production of unspliced and incompletely spliced RNA transcripts as well as the synthesis of structural proteins are observed. *Rev* protein appears to exert its regulatory activity at a posttranscriptional level by activating the cytoplasmic expression of the unspliced (9-kB) and singly spliced (4-kB) transcripts that encode the structural proteins Gag, Pol, and Env as well as the accessory proteins *Vif*, *Vpr*, and *Vpu* (Cullen, 1992). Rev interacts with a cis-acting sequence, called the Rev Responsive Element (RRE). The binding of *Rev* to RRE promotes the stability, nuclear export, and translocation of RRE-containing mRNA and is thus essential for the

expression of gag, gag-pol and env as well as for the expression of the late regulatory and accessory proteins *Vif*, *Vpr*, and *Vpu* (Cohen et al., 1990; Cullen, 1991; Felber et al., 1993; Subbramanian et al., 1994; Trono, 1995).

1.1.2.3.4.3. Morphogenesis of the HIV-1 virion.

The assembly of new virions occurs at the cell surface. HIV-1 *gag* and *pol* gene products are transported to the cell membrane via the cytoplasmic pathway, whereas envelope glycoproteins use a secretory pathway (Hunter et al., 1990; Wills et al., 1991). *Gag* and *pol* products form the core of the mature HIV-1 virion, while *env* products constitute the main exterior coat proteins. The Gag proteins play an essential role in facilitating the recruitment of both viral components and host-cell derived elements into budding viral particles (Luban et al., 1993; Dorfman et al., 1994; Li et al., 1997). Gag polyprotein interacts with the Gag/Pol precursor molecules, the accessory protein Vpr, and the genomic RNA, thereby facilitating their incorporation into nascent virions. The RNA-binding nucleocapsid (NC) protein is required for viral RNA encapsidation through its interactions with the nucleic acid packaging signal located at the 5'end of the viral genome (Lever et al., 1989). The N-terminal myristylation of Gag and Gag/Pol is required for the attachment of these proteins to the plasma membrane (Luban et al., 1993; Dorfman et al., 1994; Li et al., 1997). The overall process of assembly is initiated by p17 (Berg, 1986; Will et al., 1991). Once assembled, the viral core buds through the plasma membrane, where it acquires parts of cell lipid membrane. After release of the virion from the surface, gag and gag/pol

polyproteins are fully processed within the virion by the viral protease, leading to maturation of the viral particle (Kohl et al., 1988; Yu et al., 1992).

1.1.3. HIV-1 reverse transcriptase.

1.1.3.1. Functional properties of HIV-1 RT.

RT is the enzyme that allows HIV-1 to replicate by converting the single-stranded viral RNA genome into double-stranded DNA. HIV-1 RT exhibits three enzymatic activities: an RNA-dependent DNA polymerase (RDDP), a Ribonuclease H (RNAse H) function and a DNA-dependent DNA polymerase (DDDP) activity (Temin et al., 1970; Baltimore D., 1970; Katz et al., 1994). The RDDP function allows RT to synthesise the minus strand of the proviral DNA by copying the genomic RNA, whereas DDDP allows RT to catalyze the synthesis of the plus strand DNA. The RNAse H activity degrades the RNA primer used for the synthesis of the plus DNA strand and removes the host-encoded $tRNA^{Lys3}$ primer used to initiate minus strand DNA synthesis (Coffin, 1979; Varmus et al., 1989; Coffin et al., 1997). During reverse transcription of the viral genome, HIV-1 RT also catalyzes strand transfer and strand displacement leading to DNA synthesis (Huber et al., 1989; Luo et al., 1990; Peliska et al., 1992; Peliska et al., 1994). It has been assumed that the same RT polymerase active site is used for both RNA- and DNA-dependent synthesis. Many properties of HIV-1 RT are similar to those of other DNA polymerases. This includes the absolute requirement for a primer to initiate DNA synthesis, metal requirements, template preferences, processivity, pH, triphosphates, physiological salts and temperature

(Skalka, 1993; Katz et al., 1994). The catalytic properties of HIV-1 RT require a divalent cations, with a preference for Mg2+ over Mn2+ (Le Grice et al., 1990).

1.1.3.2. Structural characteristics of HIV-1 RT.

The HIV-1 RT is initially processed from the Pol polyprotein as a 66-kDa polypeptide that has both polymerase (N-terminus) and RNAse H domains (C-terminus). This polypeptide dimerizes and forms a p66/p66 homodimer. Subsequent proteolytic cleavage removes the RNAse H domain from one of the subunits, generating a heterodimer composed of a 66-kDa subunit (p66) and 51-kDa subunit (p51) (Farmerie et al., 1987; Mous et al., 1988; diMarzo Veronese et al., 1986; McHenry, 1989). The role of each subunit within the heterodimer has been investigated and it appears that mutations in the p51 subunit have little effect, whereas mutations of the p66 subunit can inactivate the heterodimer (Le Grice et al., 1991; Hostomsky et al., 1992; Boyer et al., 1994). Thus, the catalytic site residues present in p51 do not seem to contribute directly to activity and p66 appears to be the subunit that is catalytically active (Howard et al., 1991).

The polymerase domains of the p66 and p51 subunits are divided into four sub-domains termed 'finger', 'palm', 'thumb' and 'connection' (Arnold et al., 1992; Kohlstaedt et al., 1992a; Wang et al., 1994). These subdomains are arranged differently in each subunit and thus the heterodimer is asymmetric. The major contacts between the p66 and p51 subunits occur within the connection domains, whereas RT-DNA interactions involve elements derived primarily from the thumb, fingers and palm sub-domains of the p66 subunit. In the HIV-1 RT-DNA complex, the polymerase and RNAse H active sites are separated by

approximately 17 to 18 nucleotides (Wõhrl et al, 1990; Fufine et al., 1991; Jacobo-Molina et al., 1993). The double-stranded DNA bound to HIV-1 RT has the majority of its 18 base pair duplex region presented in 'B'-form, whereas 6 to 7 base pairs of DNA in the vicinity of the polymerase active site are in the 'A' conformation. In p66, the finger, palm, and thumb sub-domains form a cleft, or ''hand'', presumed to grasp the template-primer, with the polymerase active-site residues positioned in the ''palm'' sub-domain. The amino acid residues at positions 110-117, 160-161, 183-186 and 219-221 are involved in forming the topology of the nucleotide-binding site that is located in the palm subdomain of the p66 subunit of HIV-1 RT. In this region, the amino acid residues Tyr-Met-Asp-Asp (183-186) are the most conserved among lentiviruses, form a YMDD motif, and are essential for polymerase activity. Furthermore, the three aspartic acid residues D110, D185 and D186 appear to play a critical role in catalysis and in coordinating the presence of the required metal ion (Kohlstaedt et al, 1992a; Jacobo-Molina et al., 1993).

1.1.3.3. HIV-1 RT Processivity, fidelity and generation of viral quasispecies.

It is estimated that there are approximately 50-100 RT molecules per virion, and it is unclear whether one or more than one enzyme molecule contributes to the synthesis of a single DNA copy. During replication of the retroviral RNA genome, HIV-1 RT must polymerize about 20,000 nucleotides, 50% of which are RNA-templated, in order to accomplish the synthesis of the proviral double-stranded DNA (Coffin, 1990). Processivity is defined as the average number of bases incorporated during a single round of primer extension.

As with all DNA polymerases, HIV-1 RT is a processive enzyme, although its processivity is highly dependent on template sequence. HIV-1 RT processivity on the best template, poly(rA), is greater than 300 nucleotides, at an elongation rate of 10-15 nucleotides per second. Processivity on natural templates and homopolymers other than poly(rA) is low compared with other replicative DNA polymerases (Huber et al., 1989; Huber et al., 1990).

RT-mediated polymerization is a complex reaction (Kati et al., 1992; Reardon, 1992; Hsieh et al., 1993). A four step general mechanism has been determined for a single nucleotide addition during DNA synthesis:

1- HIV-1 RT binds with its template-primer,

2- binding of the appropriate dNTP to the RT-nucleic acid complex,

3- a nucleophilic attack occurs generating a phosphodiester bond,

4- the pyrophosphate is released.

It has been admitted that HIV-1 RT can complete these four steps approximately 20 times per second and the rate limiting step seems to be either phosphodiester bond formation or the putative RT conformational change preceding nucleotide incorporation.

HIV-1 RT lacks proofreading function and is more highly error-prone than other RTs (Coffin, 1990). HIV-1 RT has a frequency of misincorporation estimated at approximately 1:1700 to 1:4000 (Preston et al., 1988; Roberts et al., 1988; Preston and Garvey, 1992). Since the length of the viral genome is about 9.2 kb, this implies that one mutation will happen during each virus replication cycle. The high error rate of HIV-1 RT may be due to the loose association of RT

with the template, which is necessary for the strand transfer to occur (Pathak et al., 1990; Boyer et al., 1992). It has been determined that fidelity of RT is several fold higher with RNA than with DNA, suggesting that most mutations occur during synthesis of the second DNA strand. The error rate for frameshifts is higher at homopolymeric than at other sequences.

In vitro, RT errors include misinsertions and rearrangements, depending on the template sequence and the composition of the mismatch. In vivo, there are many cofactors, including the pressure exerted by the immune system and antiviral drugs that may influence RT mutation frequencies to be much lower than that is predicted from in vitro assays with purified HIV-1 RT (Mansky et al., 1995). The high turnover rate of HIV-1 replication, the high frequency of defective particles, and the high spontaneous variability of HIV-1 in vivo generate distinct viral genetic variants called quasispecies (Hahn et al., 1986; Boulerice et al., 1990; Ho et al., 1995; Wei et al., 1995). This rapid genetic change of the virus in any individual host may contribute to the prolonged and progressive nature of HIV-1 infection by allowing the virus to escape immune destruction.

1.1.4. HIV-1 Protease.

1.1.4.1. General structural features.

The HIV-1 protease belongs to the aspartyl protease class of enzymes that includes several well-characterized cell-derived enzymes such as the human enzymes renin, pepsin, cathepsin D and E, chymosin, and several other fungal proteases (Katz and Skalka, 1994; Molla et al., 1998). HIV-1 protease has a symmetric structure consisting of two identical subunits. Each of the two

homodimers are 99 amino acid-long, with amino and carboxyl termini interacting intimately at the dimmer interface (Wlodawer et al., 1989; Weber et al., 1989). The active site of the homodimeric HIV-1 protease includes six amino acids (one triad of Asp-Thr-Gly in each monomer) found at positions 25 to 27 (Mager, 2001). The conserved active site motifs are located in loops that approach the center of the dimmer (Boden and Markowitz, 1998).

1.1.4.2. Functional roles and substrates.

HIV protease processes Gag (p55) and Gag-Pol (p160) polyprotein products into functional core proteins and viral enzymes (Boden and Markowitz, 1998). During viral morphogenesis, the polyproteins are cleaved by protease at nine different cleavage sites to yield the structural proteins (p17, p24, p7, and p6) as well as the viral enzymes RT, PR and IN (Boden and Markowitz, 1998). This process is essential for normal viral maturation and the generation of infective viral particles. Inhibition of this cleavage process by either specific inhibitors or by mutation of the active site aspartic acid residue leads to the accumulation of non-infectious, immature virus particles and impairs the spread of virus infection in cell culture (Dubouck, 1992; Molla et al., 1998). The minimal peptide length required for recognition and cleavage by HIV-1 protease was shown to be seven amino acids (Katz and Skalka, 1994). The substrate binding pocket of protease is formed in region along the central axis of the symmetric homodimer. Eight individual subsites are formed along the length of the enzyme surface and are designed to accommodate substrate amino acid side chains. Substrate binding in the subsites is governed mostly by van der Waal's forces that generate stable

interactions between both substrate and enzyme amino acid side chains lining the subsites (Ridky and Leis, 1995). Protease amino acid residues in the substrate binding cleft can be mutated both singly and in combination to produce mutant proteases with altered substrate specificity. Many of these mutants have been shown to retain a reduced ability of processing the wild-type substrate sequences (Ridky and Leis, 1995).

1.1.5. Antiviral therapy.

1.1.5.1. Inhibitors of HIV-1 RT.

Since the beginning of the HIV-1 pandemic, RT has been the most extensively studied chemotherapeutic target of HIV-1. RT inhibitors can be classified into two major groups based on structural considerations: nucleoside analogs and non-nucleoside RT inhibitors NNRTIs.

1.1.5.1.1. Nucleoside analogs.

Nucleoside RT inhibitors represent 2',3'-dideoxy derivatives (ddNTP) of the natural substrates of DNA polymerases. These deoxynucleoside analogs are taken up by cells, and are converted to 5'-triphosphorylated forms that inhibit DNA synthesis by acting as chain terminators, while being incorporated into elongating DNA strands. Chain termination is the result of the lack of a 3'-OH group on ddNTPs, blocking the formation of a potential 5'-3'phosphodiester bond with incoming dNTPs (Furman et al., 1992; Yarchoan et al., 1989; Hart et al., 1992). Chain-termination makes further nucleotide incorporation impossible. However, at higher concentrations than those required to block RT activity, these dideoxynucleoside analogs can also competitively inhibit cellular DNA

polymerases, thereby inducing side effects or toxicity, such as mitochondrial myopathy and cardiomyopathy that are associated to long-term AZT therapy (Fischl et al., 1987; Hart et al., 1992; Herskowitz et al., 1992).

Nucleoside analogs can be classified into different groups:

- simple 2',3'- dideoxyderivatives, e.g. 2',3'-dideoxycytidine (ddC),
- unsaturated 2',3'-didehydro-2',3'-dideoxy derivatives, e.g. 2',3'-didehydro-2',3'-dideoxythymidine (d4T),
- drugs in which the 3'OH group is replaced by other atoms, e.g. 3'-azido-3'-deoxythymidine (AZT),
- drugs in which either a 2'- or 3'- carbon atom is replaced by other atoms, e.g. 2',3'-dideoxy-3'-thiacytidine (3TC), in which the carbon has been replaced by a sulphor,
- acyclic nucleoside phosphates, e.g. 9-(2-phosphonylmethoxyethyl)adenine (PMEA).

The principal nucleoside analogs currently in use in clinical practice are (Sande and Volberding, 1995; Hoetelmans, 1999):

- Zidovudine (AZT or ZDV, 3'-azido-2',3'-dideoxythymidine): it was the first anti-retroviral drug to be approved by the American Food and Drug Administration (FDA) in 1987. AZT represents a thymidine analog; its major toxic effects are anemia, neutropenia, nausea, headache and myopathy. The manufacturer is Glaxo-Smith-Kline.

- Didanosine (ddI, 2',3'-dideoxyinosine): ddI is an adenosine analog and its major toxicities are peripheral neuropathy, pancreatitis, nausea, hepatitis and diarrhea. The manufacturer is Bristol-Myers Squibb.
- Zalcitabine (ddC, 2',3'-dideoxycytidine): ddC is a cytosine analog and its major toxic effects are peripheral neuropathy, aphthous ulcers, pancreatitis and rash. The manufacturer is Roche.
- Stavudine (d4T, 2',3'-didehydro-2'-3'-dideoxythymidine): Like zidovudine, d4T is deoxythymidine derivative. Its major toxic effects are peripheral neuropathy and hepatitis. The manufacturer is Bristol-Myers Squibb.
- Lamivudine (3TC, 2'-deoxy-3'-thiacytidine): Like zalcitabine, 3TC is a deoxycytidine derivative. Its main toxic effects are anemia, nausea and hair loss. The manufacturer is Glaxo-Smith-Kline.
- Abacavir: this drug has a distinct intracellular mechanism of anabolism during what abacavir is stepwise converted to carbovir triphosphate, its pharmacologically active form that represents a deoxyguanosine analogue. The manufacturer is Glaxo-Smith-Kline.

1.1.5.1.2. Non-nucleoside RT inhibitors.

NNRTIs belong to the second class of antiretroviral drugs that has been introduced into the clinical practice. Unlike the NRTIs, NNRTIs are non-competitive inhibitors of RT. Moreover, NNRTIs specifically inhibit HIV-1 RT RNA-and DNA-dependent polymerase activities and do not affect the other human and animal polymerases (Merluzzi et al., 1990; De Clercq, 1992). They interact directly with p66 subunit of RT, and don't bind to the p51 subunit due to

its confirmational differences (Kohlstaedt et al., 1992a; Smerdon et al., 1994). By binding to the hydrophobic region of p66 called the NNRTI binding pocket, located near the catalytic site of RT, they affect the rate of the chemical reaction catalyzed by the RT enzyme and simultaneously inhibit RT conformational flexibility (Rittinger et al., 1995; Spence et al., 1995). While NNRTIs interact with RT, they interfer with mobility of the thumb subdomain and disrupt the orientation of conserved aspartic acid side chains that are essential for RT catalytic activity. In addition, binding of NNRTIs on RT has been shown to induce pausing sites, where DNA polymerization reaction temporarily stops (Gotte et al., 1999). During pausing sites, there is an increased RT enzyme dissociation from the binary complex, composed of the template and the extended primer.

The NNRTI binding pocket involves a series of residues L100, K101, K103, V106, V108, E138, V179, Y181, Y188, E233, L234, and P236 (Smerdon et al., 1994; Arnold et al., 1996). Other amino acids, surrounding these residues in the vicinity of the NNRTI binding pocket, may also interact with these compounds. NNRTIs have no alteration effect on the dNTP substrate binding region of RT, neither on the template/primer binding site. In contrast to NRTIs, NNRTIs don't need metabolic activation and they have a relatively lower cytotoxicity (Hoetelmans, 1999).

NNRTIs include several compounds from diverse class of inhibitors (Coffin et al., 1997). There are:

- Thiobenzimidazolone (TIBO) derivatives (R82150, R82913),

- Dipyridinone derivatives (L-697, 661, L-696, 229),
- Bisheteroarylpiperazine (BHAP) derivatives U-90152 (Delavirdine) and U-87201 (Atevirdine),
- α-anilinophenyl acetamide (α-APA) derivatives (Loviride) 1-[(2-hydroxy-ethoxy)methyl]-6-phenylthiothymidine and derivatives (HEPT, E-EPU, I-EBU, MKC-442),
- [N-(2-phenyl ethyl)-N'-(2-thiazolyl) thiourea (PETT) derivatives (Trovirdine),
- 1,4-dihydro-2H-3,1-benzoxazin-2-ones (L-743, 726, DMP-266),
- TSAO analogs,
- Quinoxaline derivatives (S-2720),
- Calanolides,
- Quinolines derivatives (U-78036),
- Thiazolo-iso-indolinones,
- Diarylsulfone derivatives,
- Inophyllums,
- Substitute naphthalenones,
- Pyrroles,
- Benzothiadiazine derivatives,
- Nitrophenyl phenyl sulfone (NPPS),
- Oxathiin carboxanilide (UC-38) (Coffin et al., 1997).

The most commonly used NNRTIs in AIDS clinics are:

- Nevirapine (11-cyclopropyl-5, 11-dihydro-4-methyl-6H-dipyrido-(3,2-b:2',3'-e)(1,4)diazepin-6-one): it belongs to dipyridodiazepinone class of compounds. Nevirapine can be ingested with or without food. It inhibits the cytochrome P450 enzymes, and this effect gives rise to a number of drug-drug interactions, particularly with some protease inhibitors of HIV-1 and with ketoconazole, methadone, rifampin and rifabutin (Coffin et al., 1997).
- Delavirdine [(1-(3-(isopropylamino)-2-pyridyl)-4-((5-methane-sulfonaminodo indol-2-yl) carbonyl) piperazine]: Delavirdine is a bisheteroarylpiperazine (BHAP) derivative. It can be taken with or without food, but for its solubility, it requires to be administered when there is an acidic pH of the stomach. Delavirdine inhibits the cytochrome P450 and thereby induces several drug-drug interactions particularly with Terfenadine, benzodiazepines, ketoconazole, clarithromycin, fluoxetine, ritonavir, rifampin, rifabutin, carbamazepine, Phenobarbital and phenytoin (Coffin et al., 1997).
- Efavirenz (DMP-266, (S)-6-chloro-4-(cyclopropylethynyl)-1,4-dihydro-4-(trifluoromethyl)-2H-3,1-benzoxazin-2-one): Efavirenz can be ingested with or without food. It also inhibits the cytochrome P450 enzymes and may interacts with several drugs, including protease inhibitors of HIV-1, rifampin, benzodiazepine and ergot derivatives (Coffin et al., 1997).

1.1.5.2. HIV-1 protease inhibitors and related drug resistance.

To date, several protease inhibitors have been used in the clinical management of HIV-1 infected patients. The currently licensed protease inhibitors include saquinavir (Ro 31-8959, Roche), ritonavir (ABT-538, Abbott), indinavir

(MK-639, Merck), and more recently nelfinavir (AG 1343, Agouron) (Molla et al., 1998). A few other compounds, such as amprenavir (141 W94 or VX-478, Glaxo-Smith-Kline), ABT-378 (Abbott), DMP-450 (Triangle), and DMP-851 (DuPont Merck) are in advanced clinical development (Molla et al., 1998). Most of these drugs are substrate-based inhibitors of protease (Molla et al., 1998).

The genotypic and phenotypic patterns that emerge in HIV-1 infected patients treated with protease inhibitors are complex, and cross-resistance between structurally different compounds occurs frequently (Boden and Markowitz, 1998). The majority of protease inhibitors possess a high genetic barrier to emergence of primary resistance. Most reported mutations are clustered around the substrate binding site of the enzyme, although some mutations in the two anti-parallel hairpin flaps have also been described (Moyle, 1995); these may probably represent compensatory mutations that enable the protease to maintain ability of processing substrates in the presence of resistance conferring mutations (Moyle, 1995; Boden and Markowitz, 1998). The majority of the drug-resistant protease mutations that have been identified are relatively conservative changes involving the gain or loss of a methylene group. For exemple, I84V, V82I, V82A, I47V, and V32I. All of these mutations have a significant effect on inhibitor binding (Ridky and Leis, 1995). Many other protease mutations represent amino acid changes that probably do not cause significant changes in protease specificity. The only exception of this latter group of mutations is M46F that is a critical mutation for enzyme specificity (Ridky and Leis, 1995). The key single mutations conferring high level of resistance are located at sites 48, 84, 90 (for saquinavir), 82, 84 (for indinavir), 82, 84 (for ritonavir), 30, 84, 90 (for

nelfinavir), 50 and 84 (for amprenavir) (Ridky and Leis, 1995; Moyle, 1995; Boden and Markowitz, 1998; Molla et al., 1998).

1.1.6. HIV-1 resistance to reverse transcriptase inhibitors.

The benefits of antiretroviral therapy are usually compromised by the emergence of drug resistant viral variants, harbouring RT or PR mutations. Those amino acid substitutions are primarily due to the high error-prone nature of HIV RT. A number of resistance mutations may pre-exist in RT of a minority of viral particles inside the entire viral populations usually called quasispecies (Mohri et al., 1993; Najera et al., 1994; Najera et al., 1995). However, the mutant variants usually are underrepresented in the quasiespecies compared to the wild type that initially predominates due to its competitive advantage in replication. During antiviral therapy, selective drug pressure provides the pre-existing resistant mutants with a replicative advantage over the wild type, thereby becoming the most predominant genotype in the quasispecies (Kellam et al., 1994; Frost et al., 1994; Wainberg, 1999). Selected mutations may affect the shape, the size or the charge of RT active site, the substrate binding site or surrounding regions that may hinder the potential incoming ddNTP substrate.

High level of resistance to certain drugs may develop after a short period of antiviral therapy in the presence of a single mutation, indicating that the particular antiretroviral drug has a low genetic barrier against the development of resistance; this is the case of 3TC, nevirapine and the other NNRTIs, as all of them possess a low genetic resistance barrier (Wainberg and Cameron, 1998; Geretti and Easterbrook, 2001). In contrast some antiretroviral drugs require

accumulation of several mutations that usually are selected during relatively long periods of therapy (from months to several years); this is the case of AZT or abacavir (Larder and Kamp, 1989; Wainberg and Cameron, 1998; Geretti and Easterbrook, 2001).

HIV-1 drug resistance can be generated in cell tissue culture by incubating HIV-1 infected cells with increasing concentrations of antiviral drugs for several weeks or months. Viral resistance occurs relatively quicker in vitro than in vivo (Gao et al., 1994). With a few exceptions, in general, the same mutations that are selected in cell culture are also noted in viral samples from patients receiving the corresponding antiretroviral drug (Richman et al., 1988; Richman et al., 1994; Wainberg, 1999). Tissue culture selection of viral resistant mutants represents a relatively cheap and faster way of identifying mutations conferring resistance to antiviral drugs, therefore it is a great tool of studying drug resistance phenomenon prior to the usage of new medications in clinical trials.

1.1.6.1. Molecular mechanisms of HIV-1 resistance to nucleoside analogs.

Except for ZDV, resistance against 3TC, ddI, ddC and d4T is mostly conferred by a single amino acid substitution (Wainberg, 1999). In addition, resistance against the majority of NRTIs have been associated with several mutations that mostly occur in the vicinity of the dNTP substrate binding pocket of RT and in surrounding regions. Some of these amino acid substitutions induce cross-resistance among various NRTIs and certain resistance mutations are associated with increase susceptibility to other drugs. Normally during the reverse transcription reaction, NRTIs induce a chain-termination (Wainberg, 1999). But

pyrophosphorolysis, the reverse reaction of DNA polymerization, is thereafter induced in the presence of pyrophosphate and nucleoside-triphosphates, resulting in the removal of the incorporated chain-terminated ddNTP and ending up with the rescue of the polymerization reaction (Gotte and Wainberg, 2000). Both decreased rates of incorporation of the chain-terminator nucleoside analog and increased rates of pyrophosphorolysis can reduce the susceptibility of RT to a given NRTI.

- Resistance to AZT (ZDV):

ZDV possesses a high genetic barrier. A high level of resistance against ZDV is observed after accumulation of a set of mutations: the K70R mutation usually appears first, and the continued therapy selects subsequently the mutation T215Y or T215F. Additional mutations may appear later, such as M41L, followed by D67N, then K219Q. These five mutations generated with ZDV therapy may confer different level of resistance, depending on they potential additive or synergistic actions (Larder and Kemp, 1989). Phenotypic resistance is usually noted with the viral variants harbouring the following genotypes: M41L + T215Y/F or D67N +K70R +T215Y/F (Mayer, 1996). The mechanism of resistance to ZDV seems to be unique, and it doesn't appear to be explained by the decreased incorporation of ZDV. Recently, it has been shown that phenotypic resistance against ZDV appears to be a result of increased polymerization processivity of the mutant viral RT and also an increased rate of the Chain-terminated AZT removal by pyrophosphorolysis (Caliendo et al., 1996; Arion et al., 1998; Gotte and Wainberg, 2000). In fact, the mechanism involving

pyrophosphorrolysis to enhance the removal of ZDV from the blocked primer has been demonstrated for the ZDV resistance mutations, located within the β2, β3 and β4 loop region of RT finger subdomain, i.e. D67N and K70R, whose presence affects the RT-AZTTP/dNTP interactions (Huang et al., 1998). In contrast, the mechanism of resistance against ZDV for mutations M41L and T215Y/F is still less clear. Recent crystallographic studies performed on ZDV-mutant RT with both mutations M41L and T215Y/F have shown that conformational changes do not appear to be implicated in resistance mechanism for M41L and T215Y/F (Stammers et al., 2001). Nonetheless, biochemical data have confirmed that the mutations T215Y and K219Q do increase the processivity of viral DNA synthesis, but the rates of chain-terminated AZT-MP removal appeared similar to thoses of the wild type enzyme (Caliendo et al., 1996; Arion et al., 1998). Moreover, it has been reported that the ZDV mutant RT displays increased rates of nucleotide-dependent primer unblocking (Meyer et al., 1999). This effect is likely more attributed to the D67N and K70R mutations. Cross-resistance of ZDV-mutants to other nucleoside analogs containing a 3'-azido group, such as 3'-azido-2',3'-dideoxyguanosine (AZG), has been reported (Larder and Kemp, 1989). An AZT-mediated cross-resistance to nucleoside analogs was particularly noted in therapeutic combinations including ddI plus AZT or ddC plus AZT in the presence of the T215Y and/or M41L mutations (Arts et al., 1998). Nonetheless, most of the ZDV-mutants are often susceptible to other nucleoside analogs and to NNRTIs (Larder and Kemp, 1989; Wainberg, 1999).

- Resistance to 3TC:

3TC possesses a low genetic barrier. High level of resistance (about 1000-fold increased IC_{50}) to 3TC is observed when the amino acid substitution M184V is generated (Wainberg, 1999). This mutation appears in the middle of a highly conserved YMDD motif (amino acid 183-186) of RT), harbouring two of the three critical residues that participate in the formation of RT polymerization active site (D110, D185, and D186) (Patel et al., 1995). A second mutation M184I, inducing high level of resistance to 3TC (about 1000-fold), has been noted at the same resistance site. In fact, the M184I substitution is observed in individuals treated with 3TC, prior to the appearance of the M184V genotype. The transient observance of the M184I mutation is explained by the fact that mutant viruses harbouring the mutation M184V appear to have a better fitness that allow them to have a replication advantage over viral strains that contain the M184I substitution (Back et al., 1996). The molecular mechanism of resistance against 3TC is still not fully understood. However, based on crystallographic studies, the M184 residue appears to be located adjacent to the 3'end of the primer as well as to the sugar ring of the incoming dNTP (Huang et al., 1998). By steric hindrance, the presence of the M184V/I substitution in RT affects positively the discrimination of the dCTP by the mutant RT to the detriment of 3TC-TP, resulting in diminished rates of incorporation of 3TC-MP (Sarafianos et al., 1990; Gõtte et al., 1999; Gõtte and Wainberg, 2000). Whilst for ZDV-resistant RT an increase of primer-unblocking events have been observed, the M184V RT mutant appears to lack the ability to remove 3'-terminal 3TC-MP in the presence of PPi

or ATP, showing that there are reduced rates of primer unblocking with M184V (Gõtte et al., 1999; Gõtte and Wainberg, 2000). The M184V mutation induces low level of cross-resistance to both ddC and ddI, (4- to 8-fold decrease in susceptibility). No cross-resistance has been reported with ZDV, d4T, nevirapine or delavirdine (Wainberg et al., 1996; Wainberg, 1999). On the contrary, the M184V substitution has been associated to induction of a significant resensitization to ZDV of viral resistant strains harbouring both ZDV resistance mutations T215Y/F and K219Q. However, this resensitization to ZDV appears to be incomplete in the presence of the 4 mutations D67N, K70R, T215Y/F and K219Q, all inducing resistance to ZDV. The susceptibility reversal of ZDV resistant mutants to ZDV, induced by the M184V substitution, may be explained by the fact that double-mutant RT is unable to unblock both AZT- and 3TC-terminated primers (Gõtte et al., 1999; Gõtte and Wainberg, 2000). In a recent report, it has been shown that the prolonged incubation of a 3TC resistant mutant strain with ZDV may accelerate the disappearence of the M184V mutation before generation of ZDV resistance mutations, thereby improving the M184V-associated impairement of the chain-terminated ZDV removal (Gõtte et al., 2001). In treated patients, the combination of ZDV and 3TC has been reported to delay the development of resistance to ZDV (Larder et al., 1995; Douglas, 1996; Wainberg, 1999).

- Resistance to ddI and to ddC:

Resistance to ddI is mediated mainly through the amino acid substitution L74V. It produces around 6- to 26-fold reduction of sensitivity to ddI, but may

partially restore susceptibility to ZDV by antagonizing the resistance effect of the T215Y/F mutation. The mutation L74V also induces 8- to 15-fold cross-resistance to ddC (Craig and Moyle, 1997). However, it does not show cross-resistance to unnatural enantiomers, e.g. b-L-ddC, (-)-2',3'-dideoxy-5-fluoro-3'-thiacytidine (FTC) or (-)-b-L-2',3'-dideoxy-5-fluorocytidine (5-F-b-L-ddC) (Van Draanen et al., 1994) (Abrams et al., 1994). Five mutations have been reported to induce resistance against ddC: K65R, Y69D, L74V, Q151F and M184V/I (Ftzgibbon et al., 1992; Craig and Moyle, 1997). All of these mutations are located within or in the vicinity of the RT polymerase active site. The K65R residue substitution is associated with a 5- to 10-fold resistance against ddC as well as a 3- to 5-fold cross-resistance against ddI and a 20-fold cross-resistance against 3TC (Gu et al., 1994a). The mutation K65R has been implicated in altering recognition of dNTPs/ddNTPs by the mutant RT, in affecting interactions between RT and template, and in diminishing frequencies of chain termination (Gu at al., 1994a; Wainberg, 1999). The mutation Y69D has been, so far, the most frequent mutation selected by ddC in treated patients. It induces a 5-fold reduced sensitivity to ddC, but does not lead to cross-resistance to other nucleoside analogues (Sylvester et al., 1995; Schooley et al., 1996). The mutation Q151F confers at least 22-fold resistance to ddC, a 10-fold reduction to ZDV and a 5-fold resistance to ddI. The M184V substitution, involved in high level of 3TC resistance, induces also low level of cross-resistance (4- to 8-fold) to both ddI and ddC (Gu et al., 1992; Gu et al., 1994b; Craig and Moyle, 1997; Wainberg, 1999).

- Resistance to d4T (stavudine):

The mutation V75T emerges during d4T treatment and confers a 7-fold increase in d4T IC_{50} and as well as a cross-resistance to both ddI and ddC, and to other NRTIs (Lacey and Larder, 1994). However, this V75T substitution is generated mostly during d4T resistance selection in cell culture but rarely seen in patients failing d4T treatment (Lacey and Larder, 1994; Hirsch et al., 1998). The mutation I50T induces a 30-fold resistance against only d4T, without cross-resistance with other NRTIs (Gu et al., 1994b).

- Resistance against abacavir:

Various amino acid substitutions at several sites have been implicated in resistance against abacavir: there are mutations K65R, L74V, Y115F, and M184V. Although the full impact of these mutations during abacavir treatment remain to be investigated, it has been determined that the K65R, L74V and Y115F appear to induce a secondary resistance, whereas the M184V substitution represents a primary resistance mutation to abacavir (Hirsch et al., 1998; Birk and Sonnerborg, 1998).

1.1.6.2. Molecular mechanisms of HIV-1 resistance to non-nucleoside analogs.

Resistance mutations against NNRTIs usually appear relatively quickly both in patients and during cell culture resistance selection experiments, often within a few weeks of initiating monotherapy (Numberg et al., 1991; Richman et al., 1991; Richman et al., 1994; Wainberg, 1999). Single-point mutations conferring resistance are usually implicated, and in many cases with high level of

cross-resistance to other NNRTIs. The majority of NNRTI resistance mutations are generated in the regions 100-108 and 181-190, related to the β-sheets adjacent to the catalytic site of RT (Kohlstaedt, 1992a; Smerdon et al., 1994).

- Resistance to nevirapine (NVP):

Several amino acid substitutions conferring resistance to NVP has been reported, including A98G, L1001, K103N, V106A, V108I, Y181C or Y181I, Y188C and G190A (Richman et al., 1994; Wainberg, 1999; Bacheler et al., 2001). Except the mutations A98G and L1001 inducing low level of resistance (6.2- and 5.3-fold, respectively), all the other substitutions confer primary resistance against NVP (Richman et al., 1991; Richman et al., 1994; Bacheler et al., 2001). Moreover, many NVP resistance mutations also confer significant cross-resistance against several NNRTIs. The Y181C substitution appears the most frequent mutations to be noted during NVP monotherapy. It induces about 100-fold resistance against NVP and while conferring cross-resistance to delavirdine (DLV), to dipyridinone derivatives (L-697, 661, L-696, 229) and to TSAO (Richman et al., 1994). Aromatic stacking of Y181 has been suggested to play an important role for the inhibitory activity of NNRTIs, since the amino acid Y181 is situated on the bottom part of the NNRTI binding pocket, and the substitutions Y181W or Y181F have no resistance effect, unlike the mutations Y181S or Y181H (Sardana et al., 1992; Balzarini et al., 1993; Smerdon et al., 1994). In addition, amino acid substitution at the codon 181 has been associated with an antagonistic effect against ZDV resistance induced by the presence of mutations at the codon 41 and 215, suggesting a potential beneficial effect during a

combination therapy with ZDV and NNRTIs (Demeter et al, 1993; Zhang et al., 1994). There is another mutation Y188C that induces resistance against NVP, TIBO and pyridinone derivatives, while the other substitution Y188H at the same site confers resistance against TIBO, pyridinone derivatives and HEPT but not against NVP (Richman et al., 1991; Sardana et al., 1992; Balzarini et al., 1993). The K103N mutation, encoding resistance against NVP, confers also cross-resistance against all NNRTIs, except HEPT (Nunberg et al., 1991; Sardana et al., 1992; Balzarini et al., 1993). The amino acid substitution K103N alters biochemical interactions occurring between the NNRTIs and RT, since the residue K103 is located right at the entrance of the NNRTI binding pocket (Nunberg et al., 1991; Kohlstaedt et al., 1992a; Smerdon et al., 1994; Arnold et al., 1996). The mutation V106A has been reported to induce about 120-fold resistance against NVP as well as about 3.8-fold and 13-fold resistance against efavirenz (EFV) and DLV, respectively (Bacheler et al., 2001). In contrast, the site-directed mutagenesis created V106I substitution confered only 1.9-, 1.3-, and 1.1-fold resistance against NVP, DLV and EFV, respectively. The mutation V108I has been reported to generate a 2.8-, 0.9-, and 1.6-fold decrease in susceptibility to NVP, DLV and EFV, respectively (Bacheler et al., 2001). The G190A mutation induces high level of resistance (41-fold) against NVP, but low cross-resistance against EFV (4.6-fold) and apparently no cross- resistance against NVP, while the mutation G190S seems to generate primary resistance (around 290-fold) against NVP, and EFV (97-fold), without cross-resistance against DLV (Bacheler et al., 2001).

- Resistance against delavirdine:

The P236L mutation confers high level of resistance against DLV, without significant cross-resistance to the other NNRTIs, including NVP (just about 1.7-fold) and EFV (0.60-fold) (Dueweke et al., 1993; Bacheler et al., 2001). In addition, the P236L mutation has been reported to antagonize the resistance effects induced by the presence of the Y181C substitution against NVP, TIBO and pyridinones. Apparently, the enlargment of the NNRTI binding pocket, due to the appearance of the Y181C substitution, seems to be reduced by the presence of the P236L mutation (Smerdon et al., 1994). Unfortunately, like noted for the d4T resistance mutation V75T mostly generated in cell culture, the P236L substitution represents a resistance mutation that is generally selected only in vitro by DLV, but rarely identified in samples collected from patient failing DLV therapy (Dueweke et al., 1993; Hirsch et al., 1998). Yet, other primary resistance mutations, such as K103N and Y181C, have been observed during monotherapy with DLV, but unlike the P236L substitution, their resistance effect appears to be synergistic against DLV and the other NNRTIs. Recently, in a cohort study on the development of resistance against DLV, a frequency of 30% for simultaneous appearance of K103N and Y181C mutations in isolates from patients was reported, whereas the occurrence of the other mutations was: 48% for K103N alone, 10% for Y181C alone, and 3% for each of the P236L, V106A, and K103N+P236L mutations (Demeter et al., 2000). In another recent study, the concommitant emergence of the K103N and Y181C substitutions during DLV

treatment was reported to induce high level of cross-resistance to all NNRTIs, including NVP (more than 1,600-fold), and EFV (32-fold) (Bacheler et al., 2001).

- Resistance against efavirenz:

The mutation K103N seems to be the most frequent amino acid substitution reported from patients failing EFV therapy (Bacheler et al., 2000). K103N substitution appears to confer between 19- to 36-fold decrease of susceptibility to EFV. However, various other mutations (L100I, V106I, V108I, E138K, V179D, Y181C, Y188H, P225H and F227L), conferring secondary resistance against EFV as single mutation, have been noted. The multiple associations of these secondary mutations may induce high-level resistance to EFV (more than 100-fold) (Young et al., 1995; Winslow et al., 1996; Birk and Sonnerborg, 1998; Bacheler et al., 2000). Moreover, these secondary mutations may enhance the level of primary resistance to EFV and NVP when noted simultaneously with the K103N mutation in RT. Amino acid substitutions that have been identified in viral isolates, harbouring at the same time the K103N mutation, are notably the L100I, K101E/Q, V108I, G190A or –S, P225H, and K238T substitutions (Bacheler et al., 2000; Bacheler et al., 2001). The double mutant, harbouring the synergistic K103N/Y181C substitutions with regard to resistance against various NNRTIs, seems to be rare in samples from individuals failing EFV therapy, while being often observed in patients failing NVP or DLV treatment (Bacheler et al., 2000). As stated previously, the mutation G190S generates a much higher level of resistance against EFV (97-fold) and than the G190A (4.6-fold), although both mutations confer primary resistance against NVP (290-fold and 41-fold,

respectively) (Bacheler et al., 2000; Bacheler et al., 2001). The mutation E138K, conferring secondary resistance to EFV (1.1-fold), to TSAO and to some TIBO derivatives, represents the only amino acid change, conferring resistance against NNRTIs, that is harbored by the p51 subunit of RT, since the p51 E138 residue is situated in a region participating in the formation of the NNRTI binding pocket (Balzarini et al., 1993, Jonckheere et al., 1994; Wainberg, 1999; Bacheler et al., 2001). In addition, it has been shown that the EFV selected mutations, E138K in the p51 and V179D in the p66, may generate resistance against two distinct TIBO derivatives but with a quasi-identical structure, since the amino acid V179 and is situated in the vicinity of the p51 E138 residue (Nanni et al., 1993; Boyer et al., 1994).

1.1.6. Combination therapy and multi-drug resistance.

Current anti-retroviral agents provide relative clinical benefit of variable and often limited durability when used in monotherapy (Collier et al., 1993; Yarchoan et al., 1994). In contrast, combination antiviral therapy has been shown to suppress more effectively HIV-1 replication and in some cases to reduce stronger the emergence of drug resistant strains than does single drug treatment (Dornsife et al., 1991; Hammer et al., 1994). Several in vitro studies as well as clinical trials have suggested that the most successful treatment of HIV disease must comprise the combination of two or more of the available nucleoside and non-nucleoside reverse transcriptase inhibitors with protease inhibitors (Dornsife et al., 1991; Craig et al., 1993; Robins et al., 1993; Hammer et al., 1994).

Selection of a single primary mutation, that induces cross-resistance to several other closely related drugs, is particularly significant for NNRTIs, and these mutations are selected relatively quickly, often within a few weeks of treatment. This is the case of NNRTI primary mutations K103N or the Y181C, although it is frequent to observe association of several emerging NNRTI secondary substitutions that may generate substantial cross-resistance to other NNRTIs (Richman et al., 1994; Birk and Sonnerborg, 1998; Wainberg, 1999; Bacheler et al., 2000; Bacheler et al., 2001).

In contrast, most of NRTIs, except 3TC, possess a high genetic barrier to resistance. However, the emergence of cross-resistance and multi-nucleoside resistance, mediated by inhibitor-specific mutations or secondary less specific mutations, is also a preoccupation in long-term combination treatment. This is particularly, the case for cross-resistance against ddI, ddC and abacavir, which may select for similar amino acid substitutions at the codon K65R, L74V and M184V/I. Nonetheless, the mutation M184V/I, known to induce high level of resistance to 3TC and low-level of resistance to ddI, ddC and abacavir, is unique since the presence of the M184V substitution may increase the fidelity of the 3TC-mutant RT, thereby decreasing the genetic diversification of viral quasispecies (Wainberg et al., 1996). Furthermore, the presence of M184V may be useful during combination therapy because its emergence induces a resensitization of formely AZT-resistant viruses (Gõtte and Wainberg, 2000). Considering that the presence of the ZDV-resistant mutations, in a regimen comprising ddI plus AZT or ddC plus AZT, may generate multi-nucleoside cross-

resistance, all of these findings must be considered in therapeutic combinations involving ZDV and other nucleoside analogs, in the presence of the genotype including M184V or T215Y and/or M41L (Mayers, 1996; Arts et al., 1998; Gõtte and Wainberg, 2000; Spira et al., 2001). Although the majority of HIV-1 infected individuals treated with ZDV plus ddC and ddI may often generate AZT-resistance mutations T215Y and or M41L, a small number of those patients may also develop the primary mutation Q151M that mediates high-level of multiple dideoxynucleoside resistance (Wainberg, 1999; Garcia-Lerma et al., 2000). In addition, several other mutations, such as A62V, V75I, F77L, and F116Y, inducing resistance to all used NRTIs including ZDV, ddI, ddC, 3TC, d4T and abacavir, have been reported in approximately 3 to 16% of individuals treated with ZDV plus ddC and/or ddI, and in some cases multi-drug resistance viruses are vertically or horizontally transmitted (Kavlick et al., 1998; Schmit et al., 1998; wainberg, 1999; Little, 2000; Routy et al., 2001). Appearance of those mutations may severely diminish alternative antiretroviral therapy options.

1.1.7. Clinical impact of HIV-1 drug resistance.

The clinical benefit of variable drug regimens have been documented but the development of drug resistance and the overlapping toxicity of different antiviral medications has triggered the necessity of continuous development of alternative drug associations to achieve effective suppression of HIV (Richman et al., 1987; Hochster et al., 1990; Shafer et al., 1993; Larder et al., 1993). The goal of multiple-drug therapy is to target different stages of the HIV life cycle in order to have a better viral suppressive efficacy than obtained with single-drug

regimens, and additionally to delay the emergence of resistance as much as possible. However, the highly active anti-retroviral therapy (HAART) does not inhibit completely HIV replication, although it usually reduces substantially HIV RNA levels below clinically detectable limits (Pomerantz, 2001). In the absence of possible viral eradication in an HIV-1 infected patient, treated with current antiretroviral drugs, the persistent and residual viral replication allows accumulation of mutations that are generated during each viral life cycle, since RT lacks proofreading activity. Moreover, the penetration of most antiretroviral drugs into certain tissues, such as nervous system, lymph nodes, testes and eyes, is very limited (Pomerantz et al., 1987; Pantaleo, et al., 1993; Zhang et al., 1998; Gunthard et al., 2001). Despite HAART, these compartments progressively become sanctuary reservoirs where HIV escapes and persists in long-term (Furtado et al., 1999).

The genotypic and phenotypic changes, generated under drug-pressure during HAART, decrease the viral susceptibility to one or more antiretroviral drugs (Geretti and Easterbrook, 2001). In addition, resistance mutations usually cause structural and functional alterations in RT and protease that reduce mutant viral fitness compared to wild type. However, the accumulation of additional compensatory mutations along HAART, enable the mutant variants, initially existing as minority quasispecies, to prevail in the viral populations (Nijhuis et al., 1999).

Along with the emergence of resistance during HAART, there is often a progressive narrowing of therapeutic options, especially in the absence of new

strategies and more potent HIV inhibitors. Considering the importance of drug resistance in the outcome of the antiviral treatment, the relative long-term successfulness of an anti-HIV chemotherapy has become a real challenge (Shafer et al., 1992; Sande and Volberding, 1995; Wainberg and Cameron, 1998). Several factors, that may play a critical role during HAART in precipitating occurrence of resistance, must be taken in account in the therapeutic regimen. These elements that must be considered include the knowledge of the initial genotypic and phenotypic resistance profiles of the patient, the choice of the initial drug regimen, the timing of the therapy changes, the occurrence of cross-resistance events and the availability of salvage therapy, the tolerance and adherence of the patient to the treatment, the potential drug toxicity and side effects, and finally the progressive restoration of the immune system (Shafer et al., 1992; Sande and Volberding, 1995; Wainberg and Cameron, 1998).

1.2. General introduction.

Group M viruses are responsible for the vast majority of HIV cases worldwide and this group has been subdivided into at least 11 distinct subtypes (A to K). Although there are overall similarities in genomic arrangement among HIV-1 clades, a marked inter-strain sequence divergence has been noted, with the highest variation seen in *Env*, then in *Gag* genes, but much less in *pol* regions (Korber et al., 1993; Dighe et al., 1997; Haesevel et al., 1997; Cornelissen et al., 1997).

To date, the majority of HIV-1 RT and PR variability analyses have been performed with HIV-1 subtype B, although clade B cases of HIV infection occur

mostly in western countries (Gao et al., 1994; Shaafer et al., 1998; Schinazi et al., 1996; Birk and A. Sonnerborg, 1998; Hirsch et al., 1998). In contrast, cases caused by non-B subtypes are expanding in the developing world, and subtype C is now thought to be responsible for approximately half of all new cases (Van Harmelen et al., 1997; Soto-Ramirez et al., 1996; Janssens et al., 1997; Essex, 1998). Characterization of potential genotypic divergence of *pol* sequences between different HIV-1 subtypes is now being investigated. The RT and PR regions are also of interest, since the enzymes encoded by these genes have been the main targets of current anti-retroviral drugs (Haesevel et al., 1994; Cornelissen et al., 1997; Shaafer et al., 1998; Schinazi et al., 1996; Palmer et al., 1998; Apetrei et al., 1998; Sato et al., 2000).

Several studies have phylogenetically classified viral isolates based on variability in *env* regions, but relatively few have examined genetic diversity of RTs from different clades; this is, in part, because *pol* is considered to be the most conserved structural gene of HIV (Selbert et al., 1995; Quinones-Mateu et al., 1996). However, diversity in RT and PR regions can impact on viral replication, drug susceptibility, and evolution of drug resistance. A recent report on 20 HIV-1 clade C infected patients treated in Israel suggested that baseline mutations associated with resistance to protease inhibitors (PI) can affect the likelihood of developing phenotypic resistance against these agents (Grossman et al., 2001). Given that the potential impact of HIV-1 subtype diversity on the long-term outcome of the anti-retroviral treatment is not fully understood, this study was

undertaken to investigate that issue and the main objectives of this research work were the following:

- To study the genotypic and phenotypic characteristics of HIV-1 clade C drug-naïve isolates and screen for potential divergence from wild type clade B controls.
- To study the potential impact of HIV-1 clade C genetic diversity on emergence of drug resistance in tissue culture assays.
- To identify the polymorphisms and drug selected resistance mutations occurring in the conserved regions of clade C RT corresponding to known clade B RT immunogenic epitopes.

CHAPTER 2

Effects of HIV-1 clade diversity on HIV-1 virulence and anti-retroviral drug sensitivity

Loemba H., M.A. Wainberg, and B. Brenner.

Preface to Chapter 2:

In chapter 1, we have described a general introduction into biological and morphological properties of HIV as well as antiviral therapy and drug resistance. As several HIV-1 subtypes have been characterized, it is of interest to overview the diversity of HIV-1 clades in the context of genomic variation, pathogenesis and drug susceptibility.

2.1. Abstract.

The growing HIV/AIDS pandemic has had a devastating global impact, eroding the public health, social, and economic infrastructures of entire communities, nations, and continents. The vast majority (95%) of people with HIV/AIDS live in Africa and Asia. Given HIV genotypic diversity, the global significance of non-B viral subtypes (particularly C and E) has markedly increased. Nevertheless, our knowledge of HIV pathogenesis is based on our experience with clade B strains, with little comparative information available on other viral subtypes. Non-B clades show marked genotypic diversity in regions of the HIV-1 reverse transcriptase and protease. This review summarizes current knowledge of the impact of HIV-1 clade genetypic diversity on cellular tropism, HIV-1 virulence and differential viral sensitivity to the three classes of ARVs (antiretroviral drugs), i.e nucleoside and non-nucleoside reverse transcriptase inhibitors (NRTIs and NNRTIs), as well as protease inhibitors (PIs). Adaptation of antiviral strategies to prevent and manage new waves of non-B clade HIV infections will require a detailed understanding of the unique biological and molecular properties of Asian and African viral strains.

2.2. Introduction.

Human immunodeficiency virus (HIV) is a major challenge to medical science, and, despite some success in western countries, AIDS remains out of control in the main HIV epidemic zones of the world. Currently, at least 33.6 million persons are infected by HIV worldwide, of whom 23.3 million live in Sub-Saharan Africa. HIV-1 disease continues to expand in Africa, in Latin

American countries, in Eastern Europe, and in South-Eastern Asia, including India and China (European commission workshop report and UNAIDS, 1997; UNAIDS, 2000).

To date, two distinct types of HIV viruses have been characterized, HIV-1 and HIV-2. HIV-2 prevalence rates remain relatively low and HIV-2 infection is endemic mostly in West African Countries, although a limited number of cases have been observed elsewhere (Janssens et al., 1994, 1997; Toure-Kane et al., 2000). In contrast, HIV-1 viruses are responsible for most of the world's HIV pandemic (Janssens et al., 1997; Essex, 1998; UNAIDS, 2000).

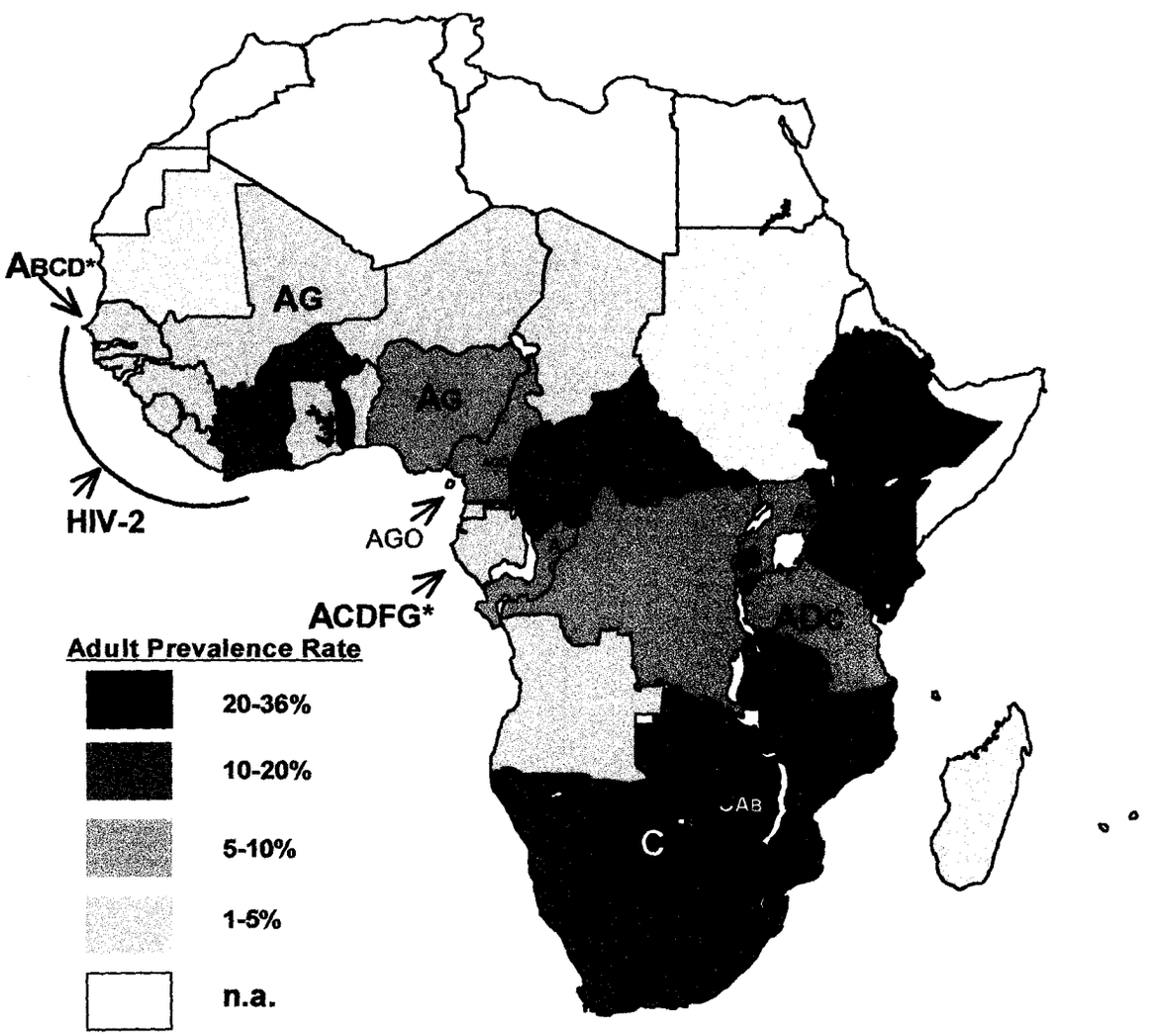
Our knowledge of HIV-1 diversity in the context of pathogenesis and treatment efficacy is still limited (Janssens et al., 1997; Quinones-Mateu and Arts, 1999; Hu et al., 1999). In this review, we focus on HIV-1 subtypes, genetic and biological polymorphism, and on clade differences in susceptibility to anti-retroviral drugs. The potential impact of clade diversity on HIV disease pathogenesis, treatment, and prevention strategies will be discussed.

2.3. Classification and distribution of HIV-1 subtypes

HIV-1 displays a high genetic variation and the different HIV-1 strains circulating in the world have been classified into three distinct phylogenetic groups, named M (major), O (outlier), and N (new) (Carr et al., 1998; Simon et al., 1998). The group O viruses represent a highly divergent minority among HIV-1 strains and have been mainly reported from Central Africa (Delaporte et al., 1996; Janssens et al., 1997; Takehisa et al., 1998). Recently, a new group designated N has been discovered in Cameroon (Simon et al., 1998). Based on

HIV envelope gene (env) sequence divergence, HIV-1 group M viruses have been subdivided into at least 11 different subtypes (A to K) and are responsible for over 90% of reported AIDS cases (Janssens et al., 1997; Carr et al., 1998; Simon et al., 1998 Quinones-Mateu et Arts, 1999; Triques et al., 2000). The majority of HIV-1 subtypes have been detected in Africa, with the highest clade diversity in the Central African area (Delaporte et al., 1996; Janssens et al., 1997; Takehisa et al., 1998; Bikandou et al., 2000). The Figure 1 shows a view of HIV subtype diversity in different regions of Africa. The global geographic distribution of various HIV-1 subtypes and HIV-1 inter-subtype recombinants is heterogeneous, but the predominant clades are A, B, and C. Clade A predominates in West and Central Africa; clade B is more prevalent in Europe and in North and South America; clade C is largely predominant in Southern Africa, in Eastern Africa, in India and in Nepal (Figure 1). The recent explosive spread of HIV-1 subtype C in Southern African countries such as Botswana, Zimbabwe, Malawi, Zambia, Namibia, Lesotho and South Africa, as well as in the Indian sub-continent, Nepal and China, has rendered these African and Asian regions the current epicentre of epidemic (Weniger et al., 1994; Janssens et al., 1997; Essex, 1998; Quinones-Mateu et Arts, 1999; Novitski et al., 1999; Oelrichs et al., 2000). Presently, HIV-1 subtype C is thought to be responsible for about half of new cases of HIV infections worldwide and for approximately half of infection in Sub-Saharan Africa (Weniger et al., 1994; Janssens et al., 1997; Renjifo et al., 1998; Essex, 1998; Novitski et al., 1999; Downing et al., 2000; Oelrichs et al., 2000).

Figure 1: A view of HIV subtype diversity in Africa


ABCD*
AG
AG
HIV-2
AGO
ACDFG*
ACD
CAB
C
Adult Prevalence Rate
20-36%
10-20%
5-10%
1-5%
n.a.

Clade D is mostly present in East and Central Africa, although a few subtype D cases have been detected in South and West Africa (Janssens et al., 1994, 1997; Delaporte et al., 1996; European commission wokshop report and UNAIDS, 1997; Triques et al., 1999; Toure-Kane et al., 2000; Bikandou et al., 2000). Most cases involving Clade E appear to be A/E mosaics, mostly observed in Asian countries as Thailand, the Philippines, China and in Central African area; however, cases have been reported from Eastern Europe and the American continent (Artenstein et al., 1995; Kalish et al., 1995; Janssens et al., 1997; Quinones-Mateu et Arts, 1999). Clade F has been detected in Central Africa, South America and Eastern Europe (Delaporte et al., 1996; Janssens et al., 1997; Takehisa et al., 1998; Quinones-Mateu and Arts, 1999; Triques et al., 1999; Triques et al., 2000). Subtype G and various recombinant A/G viruses have been reported in West African countries, in Central Africa and Eastern Europe, whereas subtype H is so far localised in Central Africa (Delaporte et al., 1996; Janssens et al., 1997; Takehisa et al., 1998; Quinones-Mateu et Arts, 1999; Bikandou et al., 2000). Subtype I has been detected once in Cyprus; in contrast subtype J has been reported exclusively from Central Africa (European commission wokshop report and UNAIDS, 1997; Bikandou et al., 2000). Recently, a new HIV-1 subtype designated K has been identified in the Democratic Republic of Congo and in Cameroon (Triques et al., 2000). It appears that the list of HIV-1 subtypes is far from being exhausted, and more HIV-1 subtypes will be discovered in the future. In addition, the dynamic phylogenetic evolution of different HIV subtypes and the constant migration of populations

into different areas may contribute to substantial changes in the geographic distribution of various HIV-1 subtypes with time.

2.4. Genomic diversity among HIV-1 subtypes.

The sequence diversity among HIV viruses of different phylogenetic lineages is appreciable particularly in the env protein. In general, group N virus strains appear to be equidistant from groups M and O, but sequence diversity in the env protein between the groups M and O is approximately 50 % (Gurtler et al., 1994; Gao F. et al., 1994; Carr et al., 1998; Simon et al., 1998). In contrast, the average divergence in env amino acid sequence between different group M subtypes ranges from 20 to 30%, whereas intrasubtype env diversity can vary from 5 to 15% (Korber et al., 1993; Myers et al., 1995). The gag nucleotide sequence of group O can vary from other HIV-1 clades by 30%, whereas gag gene sequence variation between HIV-1 group M subtypes ranges from approximately 10 to 15% (Gao F. et al., 1994, 1998).

Little is known about the pol sequence divergence among HIV-1 subtypes, as compared to env and gag. Roughly, there is a two- to three-fold less variability in the Pol gene than in env genes of group M isolates. Group O strain variation for the pol gene ranges from 3.3 to 12.2% (Quinone-Mateu et al., 1998), but the average value for Pol gene nucleotide divergence among HIV-1 group M clades is 10% (Gao F. et al., 1998). About 10% nucleotide divergence between RT sequences of subtypes E and B also exists, and RT amino acid sequences variation is about 7% (Korber et al., 1997; Sato et al., 2000). Pol nucleotide sequence diversity between clade C isolates from India was about 3 to 5% (Soto-Ramirez et

al., 1996). In our studies, RT nucleotide sequences of five Ethiopian clade C isolate revealed an average divergence of 6.8 to 10% compared with three different clade B reference strains, whereas RT sequence variation ranged from 3.5 to 5.8% the five Ethiopian clinical isolates and four clade C reference strains from Ethiopia, Southern Africa, India and Southern America (Table No...).

2.5. HIV-1 subtype variations in HIV-1 transcriptional promoters.

Significant differences may exist among HIV-1 clades in the long terminal repeat (LTR) genomic region that encode the transcriptional promoters of HIV (Montano et al., 1997; Zacharova et al., 1997; Jeeninga et al., 2000). Despite relatively conserved elements such as the Sp1 sites, the TATA box and the Trans-Activation Responsive (TAR) element, there are subtype-specific variants in copy numbers and exact nucleotide sequences of enhancer and promoter structures (Montano et al., 1997; Naghavi et al., 1999; Jeeninga et al., 2000). Subtype C virus LTRs were found to contain three to four NF-kB binding sites, whereas subtype B isolate LTRs contain two NF-kB sites and subtype E LTRs have just one. A typical deletion of one T nucleotide was observed in the subtype E NF-kB binding site (Jeeninga et al., 2000). In other LTR transcriptional activation regions, located upstream of the NF-kB binding sites, some variations specific to certain HIV-1 subtypes were seen. A region, which overlapped with Nef and was specific for binding of the transactivation factor USF, was only found in subtype B strains (Jeeninga et al., 2000). In contrast, potential binding sites for transciptional factors AP-1, located in the upstream U3 region of the LTR, were observed in a variety of HIV-1 subtypes but not clades B and D. One AP-1 motif

was found in subtypes C, E, and G, but two AP-1 sites were observed in clades A and F (Jeeninga et al., 2000). Furthermore, a specific motif for the transcription factor NF-IL6, located in the –170 region of U3 of all subtype B isolates, was entirely absent from the LTRs of subtypes A, C, D, and O (Zacharova et al., 1997). This factor NF-IL6, also termed C/EBP-B, is known to transactivate HIV-1 LTR in cell lines of monocytic origin (Henderson et al., 1995; Zacharova et al., 1997). There were also subtype discrepancies in the negative regulatory element (NRE) sequence; clades C, D, and E seem to contain a subtype-specific NRE sequence different from that of clade B (Naghavi et al., 1999).

Depending on the trans-activation system and on the cell line used, differential responses to various transcriptional factors were found among different HIV-1 clades. Transcriptional activation was stronger in subtype C LTR than in subtypes B and E in responsiveness to either the NF-kB binding factor Rel-p65 or to both purified recombinant human NF-kB and nuclear HeLa extract (Montano et al., 1997; Naghavi et al., 1999). In another investigation, LTR stimulation with TNF-α was more significant for clade C LTR than for subtypes A, B, D, F, and G LTRs; the lowest degree of TNF-α stimulation was seen with clade E. Although clades A and C LTRs were shown to have relatively high basal activity compared to clade B, the strongest basal activity was found in clade C LTR (Jeeninga et al., 2000). Given the structural and functional differences found in the transcriptional regulation of HIV-1 subtype LTRs, one can speculate on the potential impact of LTR diversity on HIV-1 gene expression and replication kinetics. In addition, the LTR has been reported to play a functionally critical role

as a determinant of cell tropism in murine retroviruses (Rosen et al., 1985). Further studies need to be conducted to evaluate the subtype and cell-type specific kinetics of HIV-1 transcription and replication depending on LTR variation.

2.6. Regulatory and accessory protein variation among HIV-1 clades.

It has been reported that important genetic polymorphism exists among various HIV-1 subtypes in regard to Nef sequences (Jubier-Maurin et al., 1999). Variation of Nef sequences between subtypes ranges from 14.4 to 23.8%; the lowest nef divergence is between subtypes B and D. Within the same subtype, Nef gene variation is from 9.6% to 12.1% (Jubier-Maurin et al., 1999). Although the overall structure of major nef functional domains is conserved among HIV-1 clades, some subtype specific patterns have been noticed; the myristylation signal is highly divergent among subtype C strains but not among other HIV-1 clades; in the majority of HIV-1 subtypes (A to H) but not clade B, a methionine serving as an internal initiation site for a truncated nef protein was mutated to an isoleucine at position 20, and a highly conserved acidic region required for the enhancement of viral replication was less conserved in subtype G. The biological impact of nef sequence divergence between subtypes in viral pathogenicity is unknown; nonetheless, the recent observation of specific nef sequence variations, associated with different stages of HIV disease in subtype B infected patients, points to the need to investigate whether important nef genetic variation among HIV-1 clades can have divergent functional implications for HIV replication and disease pathogenesis (Kawano et al., 1997; Kirchhoff et al., 1999). Finally, the presence of a truncated rev protein and an enlarged Vpu protein in subtype C strains, as

well as the detection of a deletion in the Tat protein C-terminus of clade D viruses, imply that other regulatory and accessory genes of HIV-1 may play a significant role in HIV-1 subtype genetic and biological diversity (Gao F. et al., 1998).

2.7. Clade diversity in co-receptor usage, cell tropism and syncytium formation.

HIV-1 subtype B strains can been distinguished by their in vitro replicative and cytopathic properties, and can be classified into rapid/high or syncytium-inducing (SI) and slow/low, i.e. non-syncytium-inducing (NSI) viruses (Fenyo et al., 1988; Tersmette et al., 1988; Worgall et al., 1999). In addition, it has been shown that the main coreceptor for non-T cell adapted primary isolates, also called macrophage-tropic NSI viruses, is the β-chemokine receptor CCR5, although some NSI strains can use either CCR3 or CCR5 (Choe et al., 1996). The major coreceptor for T-cell line-adapted viruses is the α-chemokine receptor CXCR4 (Cocchi et al., 1995; Deng et al., 1996; Doranz et al., 1996). A correlation between the biological phenotype of virus isolates and the immunological status or clinical stage of HIV-1 disease has been established for subtype B; the CCR5-positive phenotype predominates during primary infection and clade B CXCR4-positive strains prevail at the latest stages of the disease, when there is a lower CD4 cell count and a worsened clinical status (Keet et al., 1993; Nielsen et al., 1993; Cornelissen et al., 1995). In addition to subtype B, this correlation status appear to be similar for the majority of other HIV-1 subtypes; however, this may not be necessary be the case for clade C, A and D viruses. In fact, CXCR4-positive/SI variants are rare among subtype C strains, even at terminal stages of

disease (Zhang et al., 1996; Tscherning et al., 1998; Abebe et al., 1999; Peeters et al., 1999; Chen et al., 2000). On the other hand, subtype D viruses are more likely to display dual tropism for CCR5 and CXCR4 and clade A strains tend to be more CCR5 prone (Zhang et al., 1996; Tscherning et al., 1998). There is no definitive explanation for the CXCR4 phenotype rarity among subtype C viruses. Persistent immune activation observed in subjects in African countries, as a consequence of high infectious disease prevalence triggering over-expression of CCR5 on activated and memory T-cells, has been suggested as an explanation (Bleul et al., 1997; Abebe et al., 1999; Bentwich et al., 1998, 2000). However, HIV-1 clade C is the world's most predominant HIV-1 subtype and is widespread in several countries with different epidemiological backgrounds.

HIV-1 viruses of all subtypes, when isolated at the beginning of primary infection, i.e. after sexual transmission, are NSI or CCR5 viruses (Keet et al., 1993; Nielsen et al., 1993; Cornelissen et al., 1995). Preliminary studies have reported the existence of divergence among subtypes in ability to infect Langerhans cells with a correlation between dendritic cell tropism and heterosexual transmission; however, subsequent investigations did not support those observations (Soto-Ramirez et al., 1996; Dittmar et al., 1997; Essex, 1998; Hu et al., 1999). In fact, various considerations can influence the transmission of HIV, including epidemiological and environmental factors as well as host and viral factors. Variations in rates of mother-to-child vertical transmission in different regions may be due to variations in methodology and the presence of confounding factors. The latter includes HIV-1 clade diversity, cellular tropism,

coreceptor usage phenotype, and specific amino acid variations in the V3 loop, especially the distribution of charged amino acids at positions 11 and 25 (Hwang et al., 1991; de jong et al., 1992; Kato et al., 1999). There are more positively charged amino acids in the V3 loop of SI HIV-1 strains than in NSI isolates. For instance, specific amino acid changes in the V3 loop of subtype E have been reported to be critical for coreceptor usage, infectivity and cell tropism (Kato et al., 1999). Subtype D strains display a highly variable pattern of V3 loop amino acids compared to the other HIV-1 group M clades. A correlation exits between the extent of positive charge in V3 and define biological and phenotypic characterisitics. Subtype C viruses also have divergent V3 loop patterns. In contrast to other HIV-1 subtypes, clade C strains have less variation and fewer positive charges in the V3 loop than other subtypes (Dighe et al., 1997; Peeters et al., 1999; Shiino et al., 2000). Furthermore, clade C viruses do not contain the highly conserved potential N-linked glycosylation site commonly found in the V3 loop of all HIV-1 subtypes (Orloff et al., 1993).There is a controversy on the role of this glycosylation site, as it has been associated with maternal-infant transmission, this remains to be confirmed (Wolinsky et al., 1992; Orloff et al., 1993).

Divergence of HIV-1 subtypes in regard to preferential and predominant route of transmission has been reported (Soto-Ramirez et al., 1996; van Harmelen et al., 1997; Essex, 1998; Janssens et al., 1997; Novitski et al., 1999; Hu et al., 1999). HIV-1 clade B is more prevalent in regions in which heterosexual transmission is responsible for only a minority of cases and where the major

transmission methods are intravenous drug use and anal sex. Subtypes A, C, D and E predominate in countries and regions where heterosexual transmission is dominant. Subtype B is more prevalent among injection drug users in Thailand and is more prevalent among homosexual subjects in South Africa. On the other hand, subtype E is associated with HIV-1 heterosexual transmission in Thailand, and clade C viruses are the leading cause of heterosexual spread of HIV in South Africa and surrounding countries (Soto-Ramirez et al., 1996; van Harmelen et al., 1997; Janssens et al., 1997; Novitski et al., 1999; Hu et al., 1999). Other host co-factors that could differentially transactivate HIV-1 subtypes are also potentially involved (John et al., 1988; Novitski et al., 1999; Neilson et al., 1999).

A limited number of reports indicate that subjects infected with different HIV-1 subtypes differ in rates of disease progression (Hu et al., 1999; Kanki et al., 1999; Neilson et al., 1999). In a recently published prospective study, subtypes A and G appear to be associated with longer AIDS-free survival, whereas the rate of disease progression in clade C infected subjects may be significantly faster, while Clade D cases may be intermediate in this regard (Kanki et al., 1999). Furthermore, a correlation between HIV-1 subtypes with different markers of disease has been observed in a cross-sectional study (Neilson et al., 1999). Subjects infected with subtype C strains appear to have the highest median viral load and a lower median CD4 cell count as well as faster progression to AIDS than those infected with either subtypes A or D. Additional longitudinal studies with various HIV-1 subtypes need to be conducted to better understand the

biological characteristics of each subtype and to design better strategies for the control of specific subtypes.

2.8. Effect of reverse transcriptase and protease sequence polymorphism.

Genotypic divergence of pol gene sequences between different HIV-1 subtypes is now being investigated, although the encoded reverse transcriptase (RT) and protease enzymes are the main targets of anti-retroviral therapy (Gao Q. et al., 1994; Haesevel et al., 1994; Cornelissen et al., 1997; Shaafer et al., 1998). HIV-1 group O viral isolates were shown to be naturally resistant to non-nucleoside RT inhibitors (NNRTIs), while showing similar sensitivity to nucleoside RT inhibitors (NRTIs) and protease inhibitors (PIs) as clade B viruses (Descamps et al., 1995, 1997). Group O viruses carry the natural Y181C polymorphism similar to the Y181I sequence divergence seen in HIV-2 viruses which are also intrinsically refractory to NNRTIs (Tantillo et al., 1994; Descamps et al., 1995, 1997). Clade F isolates, showing approximately 11% nucleotide sequence variation from clade B and from the other HIV-1 group M viruses, have also been reported to have reduced sensitivity to specific NNRTI drugs, such as TIBO, while demonstrating similar phenotypic susceptibility to other inhibitors such as Nevirapine, Delavirdine, NRTIs and PIs (Apetrei et al., 1998). In contrast, the phenotypic sensitivity of clade C isolates from five drug-naive infected Zimbabweans to NRTIs and NNRTIs was reported to be similar to that of clade B isolates (Shafer et al., 1997). In a recent study conducted with a panel of Ethiopian clade C clinical isolates, we have also seen similar drug susceptibility of subtype C as compared to subtype B. However, the relatively high frequency of

resistance mutations, that has been observed among drug-naïve subjects carrying certain non-B subtypes strains, such as the NRTI and NNRTI mutations detected in some Ethiopian clade C isolates, may jeopardize the outcome of anti-retroviral treatment. This may warrant a global screening program for the genotypic and phenotypic resistance characteristics of non-B subtypes.

2.9. Conclusion

This overview summarizes current knowledge of specific genetic characteristics of HIV-1 subtypes, their epidemiological distribution and divergence in pathogenicity and susceptibility to anti-retroviral drugs. It is difficult to evaluate the implication of a single specific genetic feature of a given HIV-1 subtype that could determine by itself a possible increase in transmissibility, virulence, cytopathogenicity and disease progression. Perhaps, it is better to consider the biological impact of those HIV-1 subtypes genetic specificities in the context of diverse interacting forces, such as environmental, epidemiological, demographic, immunological and virological factors. Clade variation in natural susceptibility to antiviral drug represents a major concern regarding the potential impact in the efficacy and outcome of antiviral treatment in the epidemic areas where different HIV-1 subtypes and inter-subtype recombinants are circulating.

CHAPTER 3

Genetic divergence of HIV-1 Ethiopian clade C reverse transcriptase (RT) and rapid development of resistance against non-nucleoside inhibitors of RT

Loemba H., B. Brenner, M.A. Parniak, S. Ma'ayan, B. Spira, D. Moisi, M. Oliveira, M. Detorio, and M.A. Wainberg.

Preface to Chapter 3:

As stated in Chapter 2, HIV-1 has displayed high genomic and biological variations. Clade C represents one of the most predominant HIV-1 subtypes and is currently responsible of half of new HIV-1 cases worldwide. Given the natural polymorphisms of different HIV-1 subtypes described in chapter 2, it is pertinent to investigate the genotypic and phenotypic characteristics of HIV-1 subtype C and emergence of drug resistance.

ABSTRACT

We have sequenced and phylogenetically analysed the reverse transcriptase (RT) region of five human immunodeficiency virus type 1 (HIV-1) isolates from treatment-naïve Ethiopian émigrés to Israel, which were classified as subtype C on the basis of *env* gene structure. Heteroduplex mobility assays were performed to confirm the clade C status of RT genomic regions. The RT sequences showed that the strains clustered phylogenetically with clade C isolates, and a KVEQ specific motif of silent mutations (amino acids 65, 106, 138, 161 respectively) at resistance sites was present in the polymerase region of all studied Ethiopian isolates and subtype C reference strains. In addition, many other silent mutations were observed in the clade C viruses at various resistance sites. In general, the Ethiopian isolates were more closely related genotypically to a clade C reference strain from Botswana (Southern Africa) than to previously sequenced Ethiopian reference strains. Phenotypic analysis revealed two Ethiopian strains that were shown to be naturally resistant to non-nucleoside RT inhibitors (NNRTI), including nevirapine (NVP), delavirdine (DLV) and efavirenz (EFV), as well as to zidovudine (ZDV), based on the natural polymorphisms G190A and K70R. Moreover, variants resistant to NVP, DLV and EFV were more rapidly selected in tissue culture with clade C than clade B wild-type (WT) isolates. In subtype C RT, various amino acid changes were observed after selection with NVP (K103N, V106A, V108I and Y181C), including a novel mutation S98I. After selection with DLV, a drug-naïve silent mutation A62A initially observed in the Ethiopian isolate 4762, mutated to A62V, a secondary substitution associated with multi-

drug resistance against nucleoside RT inhibitors (NRTI). Phenotypic analysis of clade C mutants selected against NVP, DLV and EFV revealed a broad cross-resistance, particularly between NVP and DLV variants. These findings suggest that RT genotypic diversity may influence the efficacy of drug response, as well as the emergence of drug resistance. As such, global programs for evaluation of phenotypic and genotypic characteristics of different HIV-1 subtypes in regard to drug resistance is probably warranted.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) has taken on distinct viral forms globally. Viruses have been stratified into three major phylogenetic groups, namely M (Major), O (outlier) and N (new) (Myers et al., 1995; Janssens et al., 1997; Simon et al., 1998). Group M viruses can be subclassified into at least 10 different subtypes, designated clades A-J (Burke and McCutchan, 1997; Janssens et al., 1997; Simon et al., 1998). In North America and Europe, subtype B is predominant, and in other regions of the world various HIV-1 subtypes are endemic, with the greatest diversity found in Central Africa (Louwagie et al., 1995; Myers et al., 1995; Burke and McCutchan, 1997). Global epidemics with group M (non-B, A through J) and O clades are expanding with at least 33.6 million infected persons worldwide and 5.6 million new cases in 1999. Sub-Saharan Africa (clades C, A, D, E, F, G, H, J, O) and Southeast Asia (clades C, E) represent the epicentre of HIV-1 infection with 69% and 19% respectively of the total of HIV-1 infected persons in the world (Myers et al., 1994; 1995; Louwagie et al., 1995; Janssens et al., 1997). In densely populated regions of southern Africa and India, clade C virus may be responsible for almost 50% of new HIV-1 infections (Becker et al., 1995; UNAIDS and WHO, 1999; Novitski et al., 1999). Clade C virus may become the most commonly transmitted HIV-1 subtype worldwide, given the exponentially growing number of infected persons in India and southern Africa (Botswana, South Africa, Malawi, Zambia, Mozambique, Namibia) (Workshop report from the European Commission and UNAIDS, 1997; Novitski et al., 1999; Lole et al., 1999).

Although there are overall similarities in genomic arrangement among HIV-1 clades, there is marked inter-strain divergence with variations of 30% to 40% in *env* amino acid sequences, whereas intra-strain heterogeneity ranges from 5 to 20% (Becker et al., 1995; Jeeninga et al., 2000). Characterization of the genotypic divergence of *pol* sequences among different HIV-1 subtypes is not yet complete, although the reverse transcriptase (RT) and protease (PR) enzymes are the major targets of antiretroviral therapy (Haesevel et al., 1994; Gao Q. et al., 1994; Cornelissen et al., 1997; Shaafer et al., 1998). The *in vitro* and *in vivo* evolution of RT polymorphism and the appearance of resistance mutations have been extensively documented for subtype B viruses (Wainberg et al., 1993; Schinazi et al., 1996; Cornelissen et al., 1997; Birk and Sonnerborg, 1998 Wainberg, 1999; Sato et al., 2000). Little information is available on the impact of viral subtype diversity on natural susceptibility to antiretroviral drugs. Moreover, it is not known whether preexisting polymorphisms of RT and PR can influence the development of drug resistance patterns through various sequence evolution pathways and impact on the outcome of anti-retroviral therapy (Cornelissen et al., 1997; Shafer et al., 1997; Birk and Sonnerborg, 1998; Apetrei et al., 1998; Telenti et al., 1999; Becker-Pergola et al., 2000).

In this report, we have analyzed RT sequences from five drug-naive Ethiopian émigrés to Israel infected with clade C HIV-1. These sequences were compared to RT sequences of subtype B and to subtype C reference strains from various regions of the world, as well as to reference strains of other clades. The phenotypic susceptibility of these strains was compared to clade B clinical

isolates. We have characterized phenotypic and genotypic drug resistance patterns in clade C Ethiopian clinical isolates grown in increasing concentrations of NVP, DLV and EFV.

MATERIALS AND METHODS:

Study subjects and virus isolates. Five treatment-naive HIV-1 infected patients from Ethiopia were included in the study. The patients were identified as HIV-seropositive in 1994-1995, shortly after emigrating to Israel. HIV-1 was isolated from blood samples using umbilical cord blood mononuclear cells (CBMC), pre-stimulated for 3 days with phytohaemagglutinin (PHA) and cultured in RPMI-1640 supplemented with interleukin-2 (IL-2) (Boehringer-Mannheim, Inc, Montreal, Canada) as described previously (Salomon et al., 1994; Boulerice et al., 1990). Incubation was carried out at 37^0C under 5 per cent CO_2 in a volume of 5 ml of RPMI-1640 medium, supplemented with 10% fetal calf serum, 2 mM glutamine, 200 U/ml penicillin, 200 μg/ml streptomycin. At regular intervals, culture fluids were evaluated for the presence of p24 antigen (Abbott Laboratories, North Chicago, Illinois, USA) and RT activity as previously described (Salomon et al., 1994; Boulerice et al., 1990). Fresh donor CBMC were added at seven days intervals, and cultures were considered positive if greater than 20 pg/ml of p24 antigen was detected in each of two consecutive samples and if the second reading was at least three times higher than the first. DNA was extracted from 10^6 CBMC for viral genotyping.

Heteroduplex mobility assay. Subtype determination of the different clinical isolates was performed by heteroduplex mobility assays (HMA), using protocol

and reagents obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. DNA was extracted from CBMCs using the QIAmp DNA purification kit (Qiagen, USA). A region of the *env* gene spanning the C2-V5 sequence was amplified using two rounds of PCR. A gamma-^{32}P labeled 3'primer was used during the second round of PCR to generate 0.7 kb labeled fragments. A 5-µl sample from the nested PCR product of each uncharacterized HIV-1 isolate was mixed with 5-µl of corresponding DNA fragments, amplified from plasmids containing *env* genes from reference strains representing different HIV-1 subtypes A2, B1, B2, B3, C1, C2, C3 and E1. 1.1 µl of HMA annealing buffer (100mM Nacl, 10 mM tris, pH 7.2, 2 mM EDTA) was added to each sample mixture. Homo- and heteroduplexes were formed between sample and reference strains by thermal denaturation at 94^{0}C for 2 minutes, and reannealing after cooling rapidly on wet ice. The duplexes were mixed with loading dye, then loaded onto a 5% acrylamide gel and separated by electrophoresis on a 160 X 160 X 1.5 mm gel apparatus (Protean II cell; Bio-Rad, Hercules, Ca), using a constant voltage of 150 V for 4 hours. The mobility of heteroduplexes was visualized by autoradiography.

HIV-1 RT sequencing. Viral RNA was isolated from culture supernatants of infected cells using the QIAamp viral extraction kit (Qiagen Inc., Chatsworth, California). The TruGene HIV-1 Assay Gene Kit was used in conjunction with the Open Gene automated DNA sequencing system (Visible Genetics Inc. Atlanta, GA) to sequence the PR and RT regions of HIV-1 cDNA. Testing involved simultaneous clip sequencing of PR and codons 35-244 of RT from

amplified cDNA in both the 3' and 5' directions. Sequences were aligned and compared to a LAV-1 consensus sequence using Visible Genetics Gene Librarian software (Visible Genetics Inc., Toronto, Ontario, Canada).

Phylogenetic analysis. A multiple alignment of five drug-naive Ethiopian clade C isolates was performed with 4 reference clade C strains, i.e. ETH2220 (from Ethiopia), 92BR025.8 (from Brazil), IN21068 (from India), and 96BW05.02 (from Botswana). In addition, genotypic variations of the Ethiopian clinical isolates were compared to reference isolates of subtype A (U455 and 92UG037.1 from Uganda, Q2317 from Kenya), subtype D (NDK from Zaire/DRC), subtype E (CM240 from Thailand, 90CF402.1 from Central African Republic, 93TH253.3 from Thailand), subtype F (93BR020.1 from Brazil), subtype G (SE61165 from both Sweden and Zaire/DRC), subtype H (90CF056.1 from Central African Republic), subtype J (SE9280.9 from Sweden), subtype O (MVP5180 from Cameroon), Subtype CPZ (CPZGAB from Gabon) and subtype B (LAV from France, JRFL from USA). The sequences of all subtype reference strains were obtained from the HIV sequence database at the " http://hiv-web.lanl.gov " web site. All alignments were gap-stripped and a total of 25 sequences, each having 397 base pairs in length corresponding to the same polymerase region (mainly the fingers and palm sub-domains) of reverse transcriptase, were generated using *Genetool* and *peptool* software (from BIOTOOLS Incorporated, Edmonton, Canada). A multiple alignment pairwise matrix, based on percent identity of sequences, was performed. Phylogenetic trees based on distances between sequences were constructed by the neighbor joining method, using the

phylogenetic programs Dnadist, Neighbor and Drawtree/Drawgram (HIV-WEB Treemaker interface at http//hiv-web.lanl.gov).

Phenotypic drug susceptibility assay. Drug susceptibility was measured by determining the extent to which ARVs inhibit *in vitro* HIV replication (Boulerice et al., 1990; Gao Q. et al., 1992; Salomon et al., 1994). Clade C and B isolates were amplified and quantified by RT enzyme assays in order to generate defined and titrated clinical isolates with a minimum of inter-inoculum effects (Boulerice et al., 1990). CBMCs infected with patient isolates were then plated in 96 well plates both in the absence and presence of a variety of ARV concentrations. After 7 days, RT enzyme assays were used to determine the 50% drug inhibitory concentration (IC_{50}) (Wainberg et al., 1993; Schinazi et al., 1996; Wainberg, 1999). The observed IC_{50} values of patient viral isolates were then compared to the known IC_{50} values of treatment-naïve clade B isolates and clinical isolates known to possess resistance to select ARVs as drug-resistant controls (Salomon et al., 2000).

Selection for NNRTI Resistance. Using procedures previously described in our laboratory, we selected for resistance to the NNRTIs, NVP and DLV, by growing cells in the presence of increasing concentrations of drugs. In these experiments, clade B and C isolates were grown in parallel, by repeated passage of wild-type clinical isolates in PBMCs in the presence of increasing concentrations of NNRTIs over 8 weeks using initial drug doses of 0.01 μM to final doses of 4 μM. RT assays were performed weekly to assess viral replication. At times of RT peaks, genotyping was performed to identify changes associated with drug-

resistance. Time to development of resistance and genotypic profiles were compared in clade B and C isolates.

RESULTS

Phylogenetic analysis of *env* regions. HIV-1 clinical isolates were obtained from antiviral naïve Ethiopian émigrés to Israel who entered the country between 1994-1995. Previous phylogenetic screening of *env* regions performed in Israel indicated that this immigrant population, in general, harbored clade C infections. Heteroduplex mobility assays were performed to confirm the phylogenetic classifications of amplified viral isolates from five individuals. Subtype determination was based on evaluation of the mobility of heteroduplexes formed by DNA fragments amplified from the viral clinical samples and the corresponding PCR product from the *env* gene of various reference strains. As shown in Fig. 1A, clinical isolates 4742, 4761, 4762 and 4766 showed electrophoretic mobilities consistent with clade C viral reference strains. In contrast, isolate 4743 envelope appeared to be a clade B/C mosaic and displayed a heterogeneous heteroduplex mobility with evolutionary relationships to both subtype B and C reference viruses. The North-American clinical isolate, 4246, was used as a control for subtype B viruses.

Several clones harboring *env* genes of certain subtype reference strains were used in the HMA assay. The subtype C reference strains C1, C2 and C3 correspond to the HIV-1 clones pCMA959 (Thailand), HIV-1 pZM18 (Zimbabwe) and HIV-1 pIN868 (India), respectively. For the other subtypes, A2 represents the subtype A reference strain clone IC144 (Ivory Cost), E1 designates

subtype E pTH22 (Thailand), whereas B1, B2 and B3 represent subtype B pBR20 (Brazil), pTH14 (Thailand) and pSF162 (USA), respectively. With the exception of recombinant strain 4743, all the Ethiopian clade C isolates in this HMA assay harbored an envelope gene that was phylogenetically closer to subtype C strain C3 from India and C2 from Zimbabwe than to the reference virus C1 from Thailand (Fig.1A).

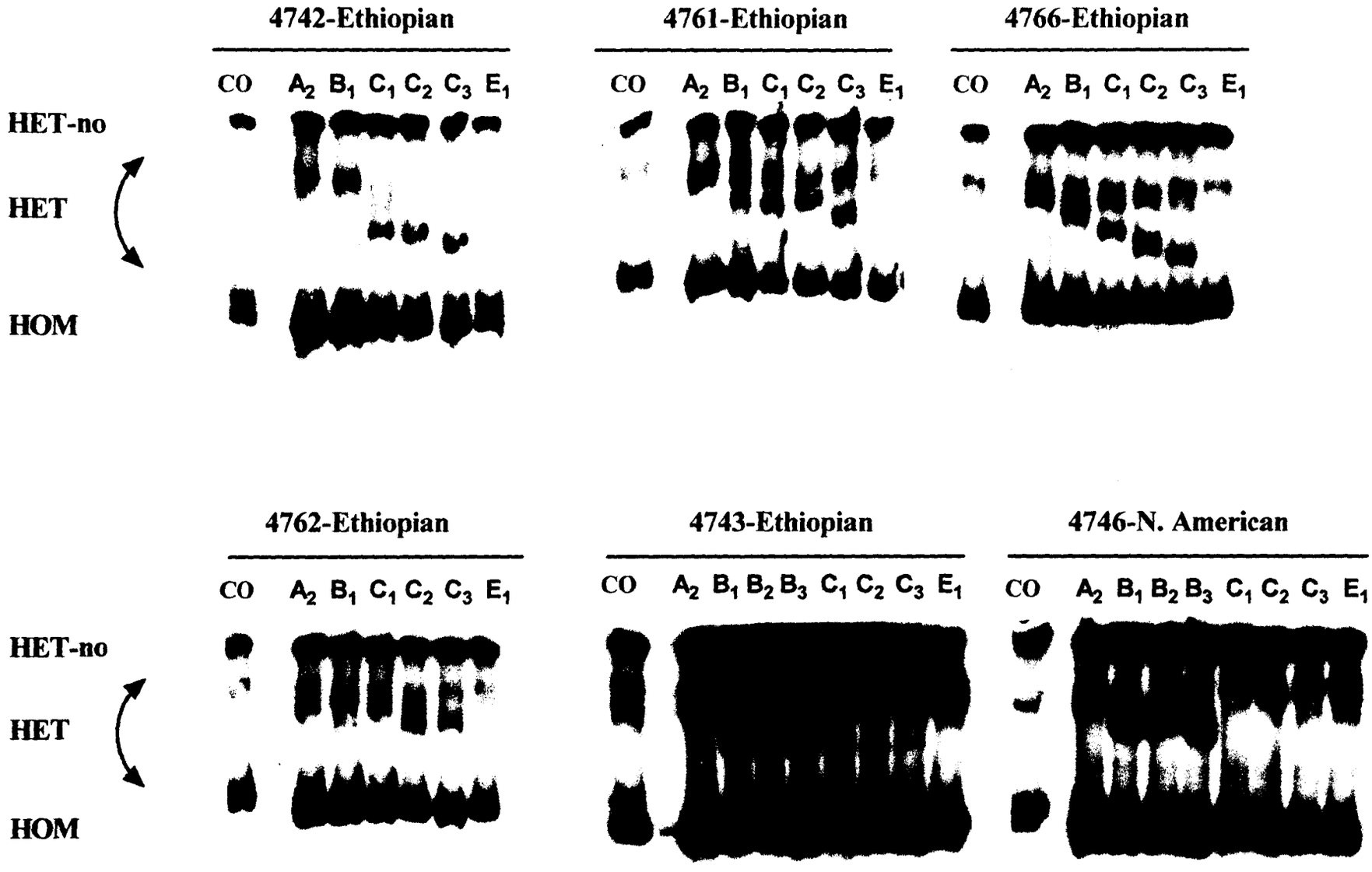
Figure 1. Phylogenetic profiles of clinical isolates from five drug-naïve Ethiopian individuals.

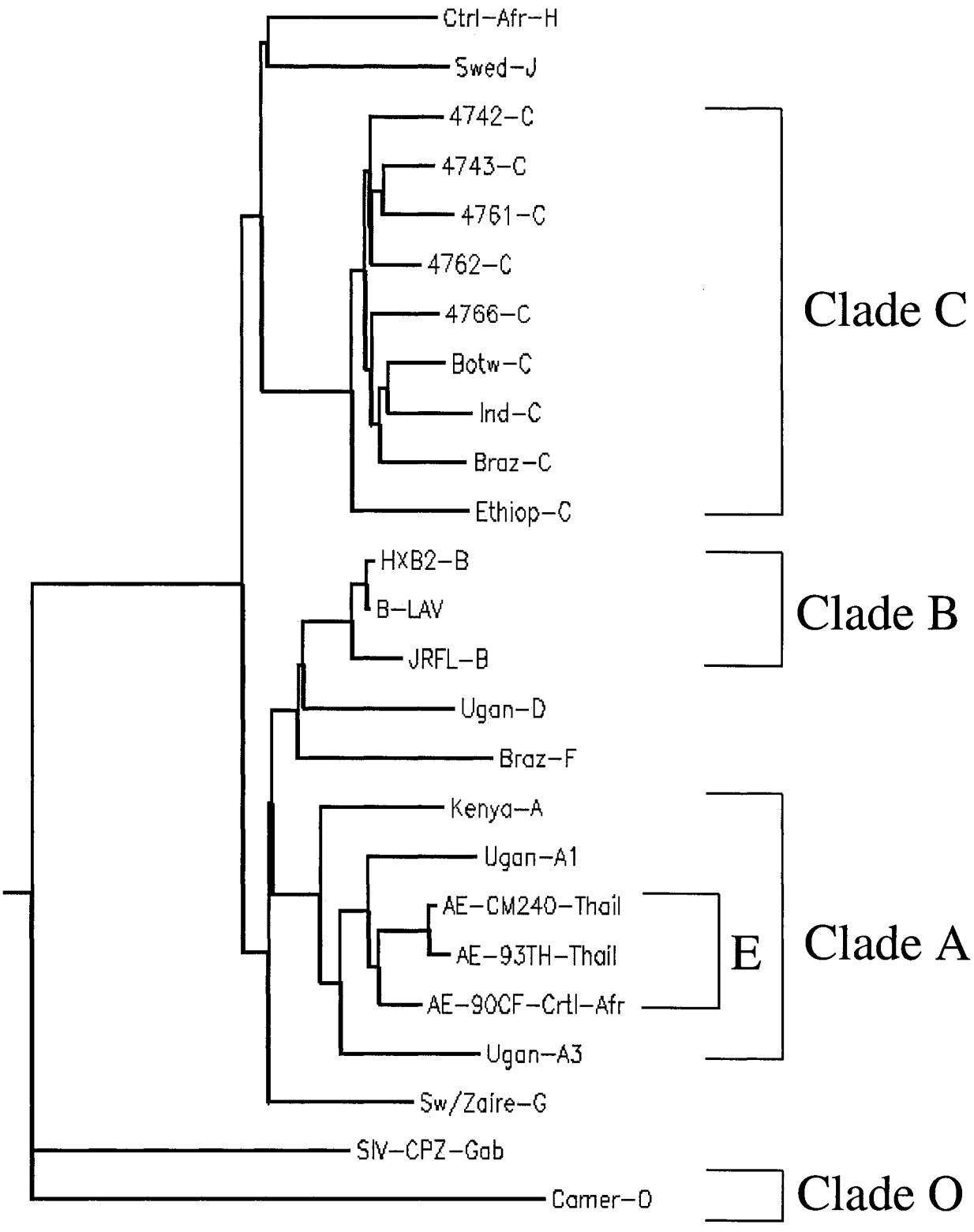
(A). Heteroduplex mobility analysis of isolates from five Ethiopian drug-naïve patients.

Heteromobility duplex assays were performed as described in Materials and Methods. Heterocomplexes were formed by mixing the amplified DNA from the viral isolates with the PCR-amplified *env* sequences of reference strains. A more rapid migration on acrylamide gels indicates relative degree of similarity between the unknown isolate and the reference strain sequences. For comparative purposes, a wild-type (WT) clade B (4746) is presented. Lanes CO show the control for each of the Ethiopian samples; lanes A_2, B_1, B_2, B_3, C_1, C_2, C_3 and E_1 show respectively the reference strains of different HIV-1 subtypes A, B, C and E. HOM, homoduplex; HET, intra-subtype heteroduplex; HET-no, inter-subtype heteroduplex.

(B). Phylogenetic analysis of reverse transcriptase sequences from HIV-1 Ethiopian isolates.

Phylogenetic analysis comparing the RT regions of HIV-1 pol genes from five Ethiopian clinical isolates and 20 different reference strains. Tree topology was inferred by neighbour-joining method and was based on an alignment of 397 nucleotides from which columns containing gaps were deleted. The subtype O prototype isolate was treated as an outgroup.


4742-Ethiopian
4761-Ethiopian
4766-Ethiopian
CO A2 B1 C1 C2 C3 E1
HET-no
HET
HOM
4762-Ethiopian
4743-Ethiopian
4746-N. American
CO A2 B1 B2 B3 C1 C2 C3 E1


Ctrl-Afr-H
Swed-J
4742-C
4743-C
4761-C
4762-C
4766-C
Botw-C
Ind-C
Braz-C
Ethiop-C
HXB2-B
B-LAV
JRFL-B
Ugan-D
Braz-F
Kenya-A
Ugan-A1
AE-CM240-Thail
AE-93TH-Thail
AE-90CF-Crtl-Afr
Ugan-A3
Sw/Zaire-G
SIV-CPZ-Gab
Camer-O
Clade C
Clade B
E
Clade A
Clade O
Distance 0.1

Reverse transcriptase genotypic analysis. Direct sequencing of the RT region was performed using Visible Genetics technology. The RT sequences of five Ethiopian isolates were aligned with a panel of reference strain RT sequences from different geographic regions. The Ethiopian isolate RT sequences had an average divergence of 6.8 to 10% from the different subtype B reference strains, whereas RT sequence variation was just 3.5 to 5.8 % between the Ethiopian viruses and the clade C reference strains.

The inter- and intra-species diversity of RT is depicted on the phylogenetic tree in Fig.1B. The neighbor-joining tree was constructed using multiple alignment of 25 RT sequences of the Ethiopian viruses and a broad panel of reference strains. As shown, the RT sequences of the Ethiopian clinical isolates obtained from the Israeli immigrants were more closely related to the clade C Botswana, Southern African reference strain, than to the Ethiopian reference strain 2220 (North-Eastern Africa). The RT regions of the clade C viruses clustered together apart from clade B and from the majority of other non-subtype B viruses.

Amino acid diversity at codons associated with drug resistance for the five Israeli Ethiopian isolates, as compared to clade C isolates from four other geographic locations, is summarized in Table 1. As shown, natural polymorphisms were present in some of subtype C isolates at key codon sites associated with resistance to NNRTIs and to ZDV [14,17]. Amino acid substitutions at positions 98, 138, 139 and 190, that have been associated with primary (G190A) or secondary (low-level) resistance to NNRTIs, were also

Table 1

Genotypic Diversity in RT nucleotide sequences of 5 treatment-naïve clade C HIV-1 Ethiopian Isolates versus RT sequences of clade C reference strains from Botswana, Ethiopia, and India.

amino acid site	HIV-1 clade B wild type codon	HIV-1 clade B resistance codon	Drug	HIV-1 Ethiopian clade C Isolates 4743	4742	4761	4762	4766	HIV-1 clade C Prototypes BOTSW	ETHIOPIA	INDIA
A. Resistance Mutations											
K70R	AAA	AGG	ZDV			**R(AGG)**					
A98S	GCA	TCA	NNRTI		**S(TCA)**		**S(TCA)**				
E138K	GAG	AAG	NNRTI		**A(GCA)**						
T139I	ACA	GCA	NNRTI		**A(GCA)**						
G190A	GGA	GCA	Nev	**A(GCA)**							
L214F	CTT	TTT	ZDV	**F(TTT)**	**F(TTC)**	**F(TTC)**	**F(TTC)**	**F(TTC)**	F(TTC)	F(TTC)	F(TTC)
B. Silent Mutations at Resistant Sites											
A62V	GCC	GTA	NRTI				A(GCT)				
K65R	AAA	AGA	ddI/ddC/ Aba	K(AAG)	K(AAG)	K(AAG)	K(AAG)	K(AAG)	K(AAG)	K(AAG)	K(AAG)
K70R	AAA	AGG	ZDV	K(AAG)	K(AAG)	**R(AGG)**		K(AAG)	K(AAG)	K(AAG)	K(AAG)
F77L	TTC	CTA	NRTI	F(TTT)							
V106A	GTA	GCA	Nev	V(GTG)	V(GTG)	V(GTG)	V(GTG)	V(GTG	V(GTG)	V(GTG)	V(GTG)
F116Y	TTT	TAT	NRTI	F(TTC)	F(TTC)	F(TTC)	F(TTC)	F(TTC)		F(TTC)	
E138K	GAG	AAG	NNRTI	E(GAA)	**A(GCA)**	E(GAA)	E(GAA)	E(GAA)	E(GAA)	E(GAA)	E(GAA)
Q161L	CAA	CTA	Foscar	Q(CAG)	Q(CAG)	Q(CAG)	Q(CAG)	Q(CAG)	Q(CAG)	Q(CAG)	Q(CAG)
Y181 C/I	TAT	TGT/ ATT	NNRTI			Y(TAC)					
K219 Q/E	AAA	CAA/ GAA	ZDV		K(AAG)		K(AAG)	K(AAG)		K(AAG)	K(AAG)
C. Silent Mutations in the YMDD Motif											
Y183	TAC			Y(TAT)	Y(TAT)	Y(TAT)	Y(TAT)	Y(TAT)	Y(TAT)	Y(TAT)	Y(TAT)
D186N	GAT	AAC		D(GAC)	D(GAC)	D(GAC)	D(GAC)	D(GAC)	D(GAC)	D(GAC)	D(GAC)

Viral RT sequences are the same as the clade B consensus sequence unless otherwise noted. RT Regions of drug-naïve Ethiopian clade C viruses were sequenced and compared to a panel of clade C reference strain RT sequences obtained from the HIV Los Alamos Database. Series of resistance mutations were noted as well as silent mutations at resistant sites and in the conserved YMDD motif.

observed. In addition, amino acid substitutions were observed at positions K70R and L214F, associated with resistance to ZDV (Haesevel et al., 1994; Schinazi et al., 1996; Cornelissen et al., 1997; Lole et al., 1999).

Specific silent mutations were observed in all clade C clinical isolates and all subtype C reference strains at sites encoding resistance to certain anti-retroviral drugs. In this regard, a KVEQ specific motif of silent mutations (amino acids 65, 106, 138, 161) at resistant sites has been observed in the polymerase region of all clade C strains studied, including all subtype C reference strains. An additional silent mutation at amino acid 116, a site encoding cross-resistance to nucleoside analogues, was specifically noted in the Ethiopian clade C isolates and the Ethiopian subtype C reference strain (Table 1).

Phenotypic drug susceptibility. The sensitivities of three drug-naive Ethiopian isolates to a panel of NRTIs (ZDV, 3TC) and NNRTIs (NVP, DLV, and EFV) were investigated in cell culture as indicated in Material and Methods. The presence of the primary G190A resistance mutation in the clinical isolate 4743 resulted in ~100-fold resistance to NVP (Table 5). This isolate remained relatively sensitive to the other NNRTIs, i.e. DLV and EFV. The overall susceptibility of the Ethiopian clade C isolates, compared to the Clade B control, as judged by IC_{50} values, was relatively low with respect to NVP, and was higher for EFV, for DLV and the NRTIs (Table 5). The presence of the K70R and L214F polymorphisms did not reduce phenotypic susceptibility to ZDV.

Selection of Drug Resistant Variants. Selection of resistance to NVP, EFV and DLV was performed to identify genotypic variations that may arise in clade C

versus clade B isolates. As shown in Tables 2, 3 and 4, some of the mutations that arose in clade C viruses selected for resistance against NNRTIs were the same as those seen in subtype B, although a few clade C mutations may have appeared through either synonymous or non-synonymous codon change. As indicated, concentrations of NVP, EFV and DLV that generated primary resistant mutations were respectively 10 μM, 1 μM and 10 μM for the subtype B controls and 2 - 4 μM, 0.01 μM and 4 μM, respectively, for the Ethiopian subtype C isolates. The number of weekly passages needed to generate mutations associated with primary resistance to NVP, EFV and DLV were 15, 30 and 15 weeks, respectively, for the clade B isolates and 9, 13 and 8 weeks for the Ethiopian subtype C viruses. At the mid-point of the selection period, the clade B viruses still contained a mixture of wild type and mutated types, while the clade C isolates harbored already primary mutations. A few amino acid changes not previously reported were noted in the clade C isolates, e.g. A98I in the 4742 C NVP resistant virus and V106M in the EFV resistant mutant (Tables 2 and 4), a mutation usually detected in clade B viruses as V106A against NVP. During DLV selection, a codon change from GCC to GTA was generated at position 62 as previously noted in the Ethiopian isolate 4762 C as the silent mutation A62A (Table 1). This, in turn, yielded a secondary mutation, A62V, associated with multi-nucleoside resistance (Table 3). Another mutation V75E was detected in the Ethiopian isolate 4743 after selection with DLV (Table 3). This substitution is also associated with multi-drug resistance against NRTIs in clade B strains (V75T).

Table 2.

Selection of viral mutants resistant against nevirapine (NVP)

	4746 clade B		**4742 clade C**		**4761 clade C**		**4762 clade C**	
Passage number (week)	NVP (μM)	Mutations observed	NVP (μM)	Mutations observed	NVP (μM)	Mutations observed	NVP (μM)	Mutations observed
0	----	no mutation	----	A98S * E138A * T139A * L214F	----	K70R L214F	----	A98S * L214F
1	0.01		0.01		0.001		0.001	
2	0.05		0.05		0.01		0.01	
3	0.1		0.1		0.01		0.01	
5	0.4		0.4		0.02		0.02	
6	1.0		1.0		0.05		0.05	A98S *
7	2.0		2.0		0.1		**0.1**	**V108I ←** L214F
8	**4.0**	**V106A/V <** **Y181Y/C <**	**4.0**	**S98I ⊕** E138A * T139A * L214F	1.0		1.0	A98S * **V108I ←**
9	10.0				**2.0**	K70R **Y181C ←** L214F	**2.0**	**K103N ←** L214F
10	10.0							
11	10.0							
13	10.0							
15	**10.0**	**V106A/V <** **Y181C ←** **V108I/V <**						

Cell culture selection of resistance-associated mutations in clade B and clade C isolates against NVP. As indicated in Material and Methods, infected CBMC were cultured in the presence of increasing concentrations of drugs. First appearance of drug resistance mutations was monitored by direct sequencing of RT regions. * secondary resistance mutation; < not fully selected primary mutation (mixture); ← fully selected primary resistance mutation; ⊕ odd mutation. Amino acid substitutions generated during selection as well as corresponding passage number and drug concentration are indicated in bold.

Table 3

Selection of viral mutants resistant against delavirdine

Passage number (week)	4246 subtype B		4742 subtype C		4743 subtype C		4762 subtype C	
	Del (μM)	Genotype	Del (μM)	Genotype	Del (μM)	Genotype	Del (μM)	Genotype
0	----	no mutations	----	A98S E138A * T139A * L214F	---	G190A	----	A98S L214F
4	0.2		0.2		0.4		0.4	A98S **V108I** *
8	**4.0**	**Y181Y/C <** **P236L ***	**4.0**	A98S E138A * T139A * L214F **P236L ***	0.8		**1.0**	**Y181C←** L214F
9					**2.0**	**V75E ⊕** **K103T ←** G190A		A98S **V108I**
10	10.0				**10.0**	**V75E ⊕**	**2.0**	**Y181C←** L214F
11						**K103T ←** G190A		**A62A/V ⊕** A98S
15	**10.0**	**Y181C ←** **P236L ***					**10.0**	**V108I *** **Y181C ←**
16								L214F

Cell culture selection of resistance associated mutations in clade B and clade C isolates against DLV. As indicated in Material and Methods, infected CBMC were cultured in the presence of increasing concentrations of drugs. First appearance of drug resistance mutations was monitored by direct sequencing of RT regions. * secondary resistance mutation; ← fully selected primary resistance mutation; < not fully selected primary mutation (mixture); ⊕ odd mutation.

Table 4

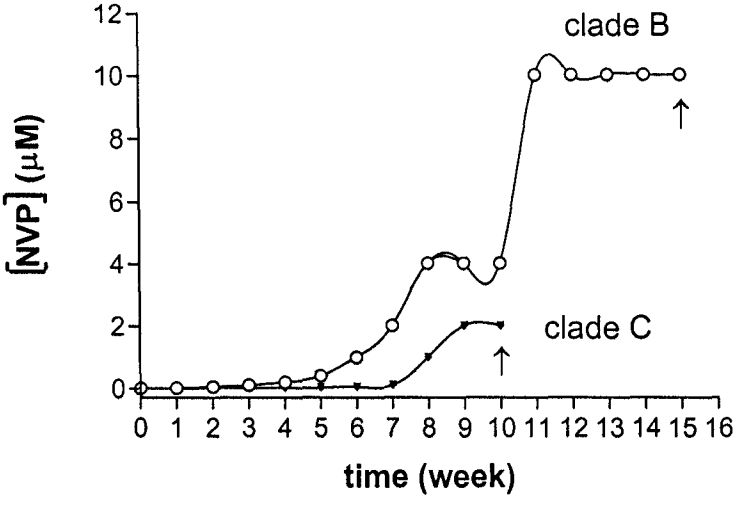
Selection of viral mutants resistant against efavirenz (EFV)

Passage number (week)	5346 subtype B		4742 subtype C	
	EFV (μM)	genotype	EFV (μM)	genotype
0	---	no mutations	---	A98S E138A T139A
1	0.001		0.001	L214F
6	**0.004**	**L100L/I** * **K101K/E** *	0.004	A98S E138A T139A
13	0.004		**0.01**	L214F **K103E** ←
19	0.02		**0.2**	A98S E138A T139A **Y188Y/C** < **G190G/A** * L214F
20	**0.04**	**L100L/I** *	1.0	
30	**1.0**	**K103N** ←	**1.0**	A98S **V106M** ←, ∇ E138A T139A **Y188Y/C** < **G190G/A** * L214F

Cell culture selection of resistance associated mutations in clade B and clade C isolates against EFV. As indicated in Material and Methods, infected CBMC were cultured in the presence of increasing concentrations of drugs. First appearance of drug resistance mutations was monitored by direct sequencing of RT regions. * secondary resistance mutation; ← fully selected primary resistance mutation; ∇ resistance site not previously reported with EFV; < not fully selected primary mutation (mixture); ⊕ odd mutation.

Fig. 2. Progression to resistance against nevirapine (NVP) in cell culture. Ethiopian clade C and clade B control viruses were selected for resistance against NVP, by growing cells in the presence of increasing concentrations of drugs. Concentrations of NVP selecting for primary resistance mutations and amount of time required (in weeks) are shown.

Appearance of primary resistance mutations during selection with NVP



Emergence of resistance to NNRTIs in Clade B versus clade C. Figure 2 illustrates the main tendencies of progression to resistance against NVP for the clade B and Ethiopian clade C isolates as described in Table 2. Similar trends were being observed for EFV and DLV. Clade C Ethiopian isolates generally developed more rapidly primary resistance against NNRTIs and lower concentrations of NNRTIs were needed than observed with clade B isolates. Susceptibility to NRTIs and NNRTIs among the Ethiopian clade C resistant mutants as well as cross-resistance profiles were investigated (Table 6). NVP and DLV resistant isolates were broadly cross-resistant and the majority of EFV resistant mutants did not show significant cross-resistance with the isolates selected against NVP and DLV (data not shown).

Table 5

Phenotypic drug sensitivity (IC_{50} values) of Ethiopian clinical isolates to nucleoside and non-nucleoside reverse transcriptase inhibitors

Drug	Ethiopian Isolate			Clade B Control
	4742	4743	4761	5346
	A98S,E138A,T139A L214F	G190A,L214F	K70R,L214F	WT
ZDV	0. 00044	0.00132	0.00105	0.00172
3TC	0.00341	0.00341	0.00673	0.00830
DLV	0.00966	0.00509	0.01445	0.00633
EFV	0.00091	0.00322	0.00048	0.00016
NVP	0.06018	1.343*	0.02147	0.01957

CBMCs were infected with Ethiopian HIV-1 clade C strains and clade B isolate and incubated in microtiter plates for 7 days in the presence of different NNRTIs and NRTIs. p24 production in each well was measured and the IC_{50} values for each antiviral drug was calculated. In all cases, r^2 were higher than 0.8. Pre-existing genotypic polymorphism in each isolate was noted.

Table 6

Phenotypic profiles of HIV-1 Ethiopian clade C drug resistant mutants selected in vitro

		4742		4743	4761		4762		4762 control
Non-nucleoside inhibitor		**NVP**	**DLV**	**DLV**	**NVP**	**DLV**	**NVP**	**DLV**	No drug
Final Genotype		S98I Y181C	S98X/L P236L	V75E K103T G190A	Y181C K70R	L100I K70R	V108I K103N	Y181C V108I A62A/V A98S	wild type
Drug sensitivity	NVP	> 10 μM	> 2 μM	> 10 μM	> 10 μM	> 2 μM	> 10 μM	> 2 μM	.40 ± 0.55 μM
	DLV	> 10 μM	> 10 μM	> 10 μM	> 10 μM	> 10 μM	> 10 μM	> 10 μM	.064 ± 0.08 μM
	EFV	.916 nM	.591 nM	7.74 nM	17.4 nM	55 nM	1.94 nM	> 0.4 μM	.07 μM
	ZDV	----	----	.0006 μM	.0014 μM	0.021 μM	----	> 2 μM	.0024 μM

CBMCs were infected with Ethiopian HIV-1 clade C strains and clade B isolate and incubated in microtiter plates for 7 days in the presence of different NNRTIs and NRTIs. p24 production in each well was measured and the IC_{50} values for each antiviral drug was calculated. In all cases, r^2 values were higher than 0.8. Final viral mutant genotypes are indicated.

DISCUSSION

Although numerous studies have phylogenetically classified viral isolates based on variability in *env* regions, few studies have charted the effects of genetic diversity of RTs among different clades (Najera et al., 1995; Cornelissen et al., 1997; Novitski et al., 1999). However, diversity in this region may have enormous ramifications and impact on viral replication, drug susceptibility and evolution of drug resistance. In this study, we evaluated the genotypic diversity of Ethiopian clade C strains, in conjunction with phenotypic drug susceptibility and emergence of RT mutations conferring drug resistance.

Our findings show that five Ethiopian clinical isolates studied were of clade C origin. Of interest, these isolates were more closely related to Botswana clade C variants than to the Ethiopian strains previously described (Salminen et al., 1996). Although the predominant subtype in Ethiopia is clade C, phylogenetic divergence of some Ethiopian isolates from other HIV-1 strains has previously been observed (Kefenie et al., 1989; Ayenie et al., 1991; Salminen et al., 1996). Similarly, HIV-1 strains from India were reported to be highly divergent from prototypic African and US/European strains but linked to the South African reference strain (Dietrich et al., 1993).

Heteroduplex mobility assays confirmed that the Ethiopian isolates were of C subtype. Interestingly, the envelope of isolate 4743 appeared to be a clade B/C mosaic, but RT region sequencing revealed homology to other Ethiopian isolates and to the different subtype C reference strain RTs. These studies argue for extensive RT screening and drug resistance surveillance for non-subtype B

viruses. Recombination has been reported to be a common feature among retroviruses and particularly among various HIV-1 strains (Miguel and Arts, 1999). In addition, mutation and recombination may both contribute to rescuing high-fitness HIV-1 variants that harbor phenotypicaly-relevant genetic alterations. The recent identification of individuals infected with HIV-1 isolates of two subtypes and inter-subtype recombinants suggests that this phenomenon may be common among viruses co-circulating in specific regions such as parts of Africa or Asia (Xin et al., 1995; Janini et al., 1998; Miguel and Arts, 1999). Considering that various non-subtype B strains are currently being reported to carry resistance mutations, inter-subtype mosaics may pose problems for the application of antiviral therapies in populations where the predominant HIV-1 subtypes are non-clade B (Descamps et al., 1995; Cornelissen et al., 1997; Shafer et al., 1997; Apetrei et al., 1998).

Numerous resistance mutations, polymorphisms, and silent mutations in RT have been linked to resistance to NNRTIs and NRTIs. Phenotypic drug testing revealed resistance to NVP in isolate 4743, that carryied the G190A primary mutation. As confirmed in our studies, this mutation is not associated with primary resistance to DLV, and the IC_{50} for EFV was only slightly higher than that of the clade B control. It is interesting to note that the Ethiopian clade C isolates 4742 and 4762 initially harbored an A98S secondary mutation associated with resistance to NVP. After cell culture selection for resistant variants with NVP, A98I mutation appeared in isolate 4742. In subtype B strains, the NVP selected mutation at this position was reported to be A98G and has been observed

in vivo (Richman et al., 1994). We have now demonstrated that this mutation can also be selected by NVP in cell culture.

The final drug concentration that selected for primary resistance mutations was significantly higher for the clade B than clade C viruses for each of NVP (10 versus 2μM), EFV (1 versus 0.01μM) and Del (10 versus 1μM), respectively. Furthermore, resistant variants were fully selected more rapidly in the clade C isolates (8 or 9 weeks with NVP or DLV and 13 weeks with EFV) as compared with the clade B control (at least 15 weeks with NVP or DLV and 30 weeks with EFV).

In the middle interval of the selection period, the subtype B virus harbored a mixture of both wild-type and mutated forms in regard to all the NNRTIs (Tables 2, 3 and 4). These findings suggest that clade C viruses can be rapidly selected for resistance to NNRTIs. Recently, it was reported that non-subtype B HIV-1 strains were likely to be less susceptible to highly active anti-retroviral therapy (HAART) (Caride et al., 2000). In addition, Non-B sequences were statistically associated with rapid progression to resistance after HAART, and had different mutational patterns than B isolates (Caride et al., 2000). Another recent study has shown some evidence of HIV-1 subtype impact on the development of NNRTI resistance mutations; there was an increased prevalence of specific mutations and polymorphisms among non-clade B viruses that may have predisposed to NNRTI treatment failure (Pillay et al., 2000). These *in vivo* reports correlate with our cell culture observations and the development of

NNRTI resistance mutations not previously observed *in vitro* for HIV-1 subtype C.

It has been established that HIV-2 RT, that shows 60% sequence homology to HIV-1 RT, is not inhibited by any of the NNRTIs in current use (Tantillo et al., 1994). This has been mechanistically linked to differences in the NNRTI binding pocket of HIV-2 RT, that harbors the natural Y181I polymorphism. In other studies, ten Cameroonian group O HIV-1 isolates were shown to be naturally resistant to NNRTIs (NVP, DLV, R82913) while showing similar sensitivity to NRTIs (ZDV, ddI, ddC, 3TC) and PIs (Saquinavir, Ritonavir) as compared to clade B (Descamps et al., 1995, 1997). Group O viruses carry the natural Y181C polymorphism similar to the Y181I divergence seen in HIV-2. In addition, four Romanian clade F isolates, showing 7.4% genotypic variation from clade B isolates, have been reported to have reduced sensitivity to certain NNRTIs, e.g. TIBO, while demonstrating similar phenotypic susceptibility to NVP, DLV, NRTIs and PIs (Apetrei et al., 1998). In contrast, the phenotypic sensitivity of clade C isolates from five drug-naive infected Zimbabweans to NRTIs and NNRTIs was reported to be similar to that of clade B isolates (Shafer et al., 1997).

In our study, the presence of certain secondary mutations associated with resistance to NNRTIs and to ZDV did not significantly decrease the susceptibility of Ethiopian clade C strains to RT inhibitors, except for strain 4743 that harbored a NVP resistance primary mutation. The natural genotypic diversity of HIV RT among different subtypes, variations in drug susceptibility and, the development

of resistance to certain drugs, all indicate a need for global genotypic and phenotypic surveillance of non-subtype B strains. The emergence of recombinant viruses in areas endemic for various HIV-1 subtypes may potentially accelerate the selection of highly resistant mosaics and may represent another challenge for the treatment of HIV disease (Gu et al., 1995; Moutouh et al., 1996).

CHAPTER 4

Co-receptor use and HIV-1 intra-clade C polymorphisms in the protease and reverse transcriptase genes of HIV-1 isolates from Ethiopia and Botswana.

Loemba H., B. Brenner, M.A. Parniak, S. Ma'ayan, B. Spira, D. Moisi, M. Oliveira, M. Detorio, M. Essex and M.A. Wainberg.

Preface to Chapter 4:

In chapter 3, we have described the genotypic and phenotypic divergence of HIV-1 clade C RT from clade B. With subtype C isolates, a rapid development of resistance against non-nucleoside inhibitors has been noted. Since several silent and resistance mutations have been detected at resistance loci in the Ethiopian clade C RT, it is of interest to investigate the intra-clade C variations and frequency in baseline mutation polymorphisms within clade RT and PR of clade C isolates from two different areas.

ABSTRACT.

Knowledge of baseline variations in drug-naïve HIV isolates that arise at key positions in the protease (PR) and reverse transcriptase (RT) genes of non-B viruses may yield information that is important in our understanding of drug resistance. We have analyzed *Pol* sequence variations within HIV-1 clade C strains isolated from fourteen treatment-naïve patients from Ethiopia and Botswana. PR and RT sequences were compared to consensus sequences of subtype B. Polymorphisms within RT were found to be more common among clade C strains from Ethiopia than Botswana, although the L214F, R211K, and E138K substitutions were detected in both groups of isolates. In contrast, the PR of the clade C isolates from Botswana were more variable at drug resistance loci than those from Ethiopia, and the only PR mutation shared among almost all isolates from both regions was M36I. Analysis of co-receptor phenotypes of the clade C isolates from Ethiopia and Botswana showed preferential usage of CCR5 and none of the viruses studied used the CXCR4 co-receptor exclusively.

INTRODUCTION.

HIV-1 protease (PR) and reverse transcriptase (RT) are two critical enzymes necessary for the completion of the HIV-1 biological life cycle and both remain the chief targets of HIV-1 antiretroviral therapy (Schinazi et al., 1996; Telenti et al., 1999). The discovery of several HIV-1 subtypes, including the major group M (from A through K), the highly divergent group O (outlier) and the new group N, has revealed similarities in overall baseline genomic

organization (Myers et al., 1995; Cornelissen et al., 1997; Janssens et al., 1997). While important genetic divergence has been detected mostly in the Env and Gag genes, RT and PR are among the most conserved regions among HIV-1 subtypes. Among all circulating clades of HIV-1, subtype C is considered responsible for approximately half of new infections worldwide and half of new HIV-1 cases in Sub-Saharan Africa (Janssens et al., 1997; Essex, 1998).

The existence of genetic variations and the presence of baseline mutation polymorphisms within HIV-1 clade C *pol* sequences may impact on the development of drug resistance and long-term outcome of antiretroviral treatment. Knowledge in regard to inter and intra-clade diversity of HIV-1, and the drug resistance polymorphisms within PR and RT, is limited (Sato et al., 1996; Cornelissen et al., 1997; Shaafer et al., 1998; Quinones-Mateu et al., 1998; Apetrei et al., 1998).

In this study, we have analyzed *pol* sequence variations within two distinct groups of drug-naïve HIV-1 clade C isolates originating from Ethiopia and Botswana. RT and PR sequences have been screened for inter and intra-clade C diversity and the frequency of naturally occurring drug resistance-associated mutations. Similarities and distinctive genotypic patterns have been revealed within the RT and PR genes of clade C isolates from Ethiopia and Botswana. Co-receptor usage analysis has shown either an exclusive or preferential CCR5 usage among the clade C strains from Ethiopia and Botswana.

MATERIALS AND METHODS.

Cells and viruses. Blood samples collected from fourteen HIV-1 antiviral drug-naïve infected patients were included in the study. Five individuals were from Ethiopia (north-eastern Africa) and their HIV-seropositivity was primarily diagnosed in 1994-1995, shortly after emigrating to Israel. Nine other HIV-1 infected patients were from Botswana (southern Africa). Isolation of HIV-1 strains from blood samples was performed by co-culture of peripheral blood mononuclear cells (PBMCs) from Ethiopian and Botswana patients with umbilical cord blood mononuclear cells (CBMCs) obtained from seronegative donors after Ficoll-paque centrifugation (Salomon et al., 1994). Prior to co-culture, donor CBMCs were stimulated with interleukin-2 (IL-2) (Boehringer-Manneim, Inc., Montreal, Canada) and phytohemagglutinin (PHA) for 3 days in RPMI-1640 tissue culture medium as described previously (Salomon et al., 1994). The co-culture medium was supplemented with 10% fetal calf serum, 2mM glutamine, 200 U/ml penicillin and 200 μM/ml streptomycin, and the cells were incubated at 37^0C in the presence of 5% CO2. Production of HIV-1 p24 antigen (Abbott Laboratories, North Chicago, Illinois, USA) and RT activity in cell supernatants was monitored at least once weekly as described (Boulerice et al., 1990; Salomon et al., 1994). Proviral DNA was extracted from CBMC lysates, using the QIAmp DNA purification kit (Qiagen, USA), and was subsequently amplified by PCR for the sequencing of RT regions. The isolates from Ethiopia were designated 4742, 4743, 4761, 4762 and 4766, whereas the HIV-1 strains

from Botswana were termed BG05, BG15, HSTmok, Mol01, Mol03, Mol13, Mol14, Mol18 and Mol36.

Determination of subtype by heteroduplex mobility assays (HMA). Subtype determination of the different clinical isolates was performed by HMA of *env* regions. For this purpose, protocol and reagents obtained through the AIDS Research and Reference Reagent Program of the NIH (Division of AIDS, NIAID, NIH, USA) were used as previously described (Delwart et al., 1993). Briefly, the C2-V5 *env* region i.e. approximately 0.7 kb of each viral isolate, was amplified by two rounds of PCR, using a gamma-^{32}P labeled 3'primer in a nested amplification reaction. Parallel PCRs were performed to amplify similar fragments from plasmids harboring *env* genes of a panel of HIV-1 reference strains, representing HIV-1 subtypes from different parts of the world. 5-µl of each of the unknown sample and each reference PCR product were mixed in a sample tube containing 1.1µl of HMA annealing buffer (100mM NaCl, 10mM tris, pH 7.2, 2mM EDTA). Homo-and hetero-duplex DNA fragments, that were generated by denaturation at 94^{0}C for 2 min and reannealing on ice, were first separated under non-denaturating conditions by electrophoresis in a 5% acrylamide gel at 150 V for 4 hours, and then visualized by autoradiography.

Phylogenetic analyses of PR and RT sequences. Subtype status was confirmed by analysis of RT and PR gene sequences. Viral *Pol* regions, amplified by PCR, were sequenced using the automated DNA sequencing system (Visible Genetics Inc. Atlanta, GA) as previously reported (Durant et al., 1999). Ethiopian and Botswana isolate RT sequences were aligned and compared to corresponding RT

regions of various reference strains representing different HIV-1 subtypes, obtained from the Los Alamos HIV sequence database (http://hiv-web.lanl.gov). These included the strains ETH2220 (subtype C from Ethiopia), 92BR025.8 (subtype C from Brazil), IN21068 (subtype C from India), 96BW05.02 (subtype C from Botswana), U455 and 92UG037.1 (subtype A from Uganda), and Q2317 (subtype A from Kenya), NDK (subtype D from Zaire/Congo), CM240 (subtype E from Thailand), 90CF402.1 (subtype E from Central African Republic), 93TH253.3 (subtype E from Thailand), 93BR020.1 (subtype F from Brazil), SE61165 (subtype G from both Sweden and Zaire/Congo), 90CF056.1 (subtype H from Central African Republic), SE9280.9 (subtype J from Sweden), MVP5180 (subtype O from Cameroon), CPZGAB (Subtype CPZ from Gabon) and subtype B strains (LAV from France, JRFL from USA). Overall, 34 sequences representing RT polymerase domain were analyzed, using *Genetool* and *peptool* software (from BIOTOOLS Incorporated, Edmonton, Canada). Each sequence was 397 base pairs in length and the alignments were gap-stripped. Percent homology between selected pairs of RT sequences were determined.

Chemokine receptor usage: Co-receptor usage was tested in HOS-CD4 cell lines engineered to stably express the CCR5 and CXCR4 (fusin) chemokine receptors. The HOS-co-receptor transfected cell lines were obtained through the "Research and Reference Reagent Program", Division of AIDS, NIAID, NIH, USA. The HOS-CD4 cells were maintained in complete Dubelcco's modified Eagle's medium (DMEM) (GIBCO/BRL) supplemented with 10% fetal bovine serum. Co-receptor usage was assessed by co-culture of CBMCs with HOS-CD4 cells

that stably expressed CCR5 and CXCR4, as previously described (Bjorndal et al., 1997; Abebe et al., 1999). Briefly, CBMCs were first infected with different HIV-1 strains from Ethiopia and Botswana, using viral samples containing at least 1ng/ml p24 Ag to yield infection of 4 million CBMCs. The clade B isolate 5346 and a mock-infected sample containing sterile culture medium were used as controls. After 2 hours of incubation with gentle shaking, unbound virus was washed out by two centrifugations in phosphate-buffered saline (PBS) and once with fresh medium. The CBMCs were subsequently plated and maintained in a 37^0 C tissue culture incubator containing 5% of CO2, and assessment of RT activity as well as p24 production in cell supernatant was performed at day 7 post-infection. At day 5 after CBMC infection, the HOS-CD4 transfectant cell lines were plated in 24 well plates at a concentration of $5X10^5$ cells per well in 2 ml of medium and maintained for 2-3 days until the cells reached half confluence. The pre-infected CBMCs were washed at day 7 post-infection with PBS, and numbers of infected CBMCs for each viral sample were counted. For each virus isolate, $0.5x10^6$ infected CBMCs were added in duplicate to the different co-receptor expressing HOS-CD4 cell lines that had been plated into 24 well plates. The co-culture was maintained in 2 ml medium per well for 48 hours. The CBMCs were removed by washing each well twice with sterile PBS, and 2 ml fresh DMEM culture medium was added. The cells were monitored for 3 weeks for a progressive increase in p24 production in HOS-CD4 cell supernatants collected at day 2 (just before the co-culture with CBMCs), and at days 4, 6, 10, and 14.

RESULTS.

Phylogenetic analysis of Pol regions of Ethiopian and Botswanian isolates. Sequencing of the RT region was performed using Visible Genetics technology as described in Materials and Methods. The RT sequences of the five Ethiopian isolates and nine Botswanian isolates were aligned with corresponding regions of various HIV-1 subtype reference strains from different geographic regions. As shown in Table 1, the Ethiopian isolate RT sequences had an average divergence of 6.8 to 10% from the different subtype B reference strains, and just 3.5 to 5.8 % sequence variation is noted among them. While having the highest variation of 20% with the group O prototype strain RT, Ethiopian isolate RTs have an average divergence of 8.5 to 11.6% from RTs of other group M non-B viruses. Similar inter and intra clade RT sequence variation values are seen with the Botswanian HIV-1 isolates (Table 1).

Table 1

RT sequence homology among clade C Ethiopian and Botswana isolates as well as HIV-1 clade prototype strains from different countries (expressed in percentage %)

Subtype reference strains	Ethiopian clinical isolates (n=5)					Botswana clinical isolates (n=9)								
	4742	4743	4761	4762	4766	BG 05	BG 15	Mol 01	Mol 03	Mol 13	Mol 14	Mol 18	Mol 36	HST mok
A-Uganda U455	89.5	91.0	89.5	90.5	89.2	89.5	89.5	88.4	90.0	89.7	90.0	89.5	90.0	89.5
A-Uganda 92UG037.1	89.2	89.5	89.2	90.2	89.5	88.9	90.2	88.7	89.5	90.2	89.7	89.7	89.5	89.7
A-Kenya Q2317	90.0	90.7	89.7	90.7	89.7	90.0	91.0	89.5	90.5	90.2	89.7	90.2	91.7	89.7
B-LAV	91.0	91.0	91.2	93.0	92.7	92.0	93.2	92.0	92.5	92.7	92.0	91.5	92.7	91.5
B-HXB2	90.7	90.7	91.2	93.2	92.5	91.7	93.0	91.7	92.2	92.5	91.7	91.2	92.5	91.2
B-JRFL	90.2	90.2	90.0	91.7	92.0	91.2	92.5	90.2	91.7	92.0	90.7	90.2	92.0	90.7
C-Botswana 96BW05.02	95.2	96.0	95.0	96.0	96.5	97.5	97.5	95.5	96.2	96.5	96.0	95.5	96.5	95.7
C-Brazil C-92BR025.8	94.5	95.0	95.5	95.0	95.5	94.7	96.2	94.5	94.7	95.2	94.5	94.7	95.2	94.0
C-Ethiopia ETH2220	94.2	94.7	94.2	94.7	94.5	94.7	94.5	94.0	94.0	94.2	94.2	93.7	94.7	93.0
C-India IN21068	94.5	94.7	94.2	95.2	95.2	95.5	96.7	94.7	95.5	95.7	95.0	95.2	96.2	95.0
D-Zaire NDK	88.4	89.7	88.7	90.0	89.7	90.0	90.7	88.7	89.5	89.2	88.9	89.5	90.2	88.4
E- Thailand (1)	90.5	90.7	90.7	90.7	90.0	90.2	90.2	89.5	90.2	90.5	91.0	90.2	90.7	90.2
E-Central African Rep.	90.5	90.5	90.2	91.0	90.2	91.0	91.0	90.0	91.0	91.2	91.2	90.5	91.5	90.7
E-Thailand (2)	90.2	90.5	90.5	90.7	90.0	90.0	90.0	89.2	90.0	90.2	90.7	90.5	90.5	90.0
F-Brazil 93BR020.1	88.7	88.9	87.9	89.7	89.2	89.7	90.0	90.0	88.9	90.2	90.0	89.2	90.5	88.7
G-Sweden/Zaire SE 61165	90.0	90.0	90.2	90.7	90.2	90.5	91.2	90.2	91.0	92.0	90.0	90.7	91.7	90.5
H-Central Africa 90cf056.1	90.0	90.5	90.5	91.5	90.7	91.0	92.0	90.2	90.7	92.0	90.5	90.7	91.7	89.2
J-Sweden SE9280.9	89.7	90.5	90.2	91.0	91.0	91.5	91.5	91.0	91.2	92.7	91.0	90.7	92.2	89.2
SIV-CPZGAB	83.2	83.4	83.4	84.4	84.9	83.4	84.4	83.9	84.2	83.7	83.7	82.9	84.4	83.2
O-Cameroon MPV5180	80.7	80.2	80.4	80.4	79.7	80.2	80.4	80.9	80.7	80.4	79.7	80.2	81.2	79.7
4742	---	95.2	95.0	95.7	95.2	94.5	95.2	94.0	95.0	94.7	95.0	95.2	95.2	94.2
4743	95.2	---	96.2	96.0	95.5	95.0	95.5	94.5	94.7	95.2	95.2	95.0	95.0	94.0
4761	95.0	96.2	---	96.2	95.5	94.2	95.7	94.5	94.5	95.0	94.5	94.2	94.7	93.7
4762	95.7	96.0	96.2	---	96.5	95.0	96.0	95.2	95.5	96.2	94.7	94.5	95.5	94.0
4766	95.2	95.5	95.5	96.5	---	95.7	97.0	95.2	96.0	96.0	95.5	94.5	95.5	94.5

RT nucleotide sequences of Ethiopian and Botswana isolates were aligned and compared to the corresponding sequences of different subtype reference strains obtained from the HIV Los Alamos Database. For each virus, a 397 bp region of RT polymerase domain was used in the alignment. Percent sequence homology is indicated. The percent matrix identity values were obtained using the software Genetool (Biotools Inc., Edmonton, Canada.

As described in Chapters 3 and 5 (phylogenetic analysis performed using the heteroduplex mobility assay profiles of amplified env genes and the alignment of RT sequences), all the HIV-1 isolates from Ethiopia and Botswana were classified as subtype C, with the exception of the Ethiopian isolate 4743 which has a B/C recombinant envelope membrane while harboring a subtype C *pol* region.

HIV-1 clade C intra-subtype polymorphisms of PR and RT sequences at resistance sites: RT and PR sequences were analysed for the presence of resistance-associated mutations by comparing them to the consensus subtype B RT and PR sequences obtained from the Los Alamos HIV sequence database. The diversity and frequency of naturally occurring drug resistance mutations was evaluated. As shown in Table 2, the RT of Ethiopian clade C isolates had more polymorphisms than the RT of clade C isolates from Botswana, although the substitutions L214F, R211K, and E138K were detected in both groups. The mutations L214F was the most conserved drug resistance mutation among clade C isolates, but almost all the strains from Botswana appeared to also have the R211K resistance mutation. In contrast, the PR of clade C isolates from Botswana had a higher number of polymorphisms related to drug resistance, whereas the only PR resistance mutation shared among almost all isolates from Ethiopia and Botswana was M36I. The latter is conserved among all Ethiopian isolates as well as in the vast majority of clade C strains from Bostwana (Table 2). In addition, a Botswanian isolate harbored a new substitution, M36V. Most of the drug-naïve

Table 2

***Pol* protein sequence polymorphisms at resistance sites in HIV-1 clade C drug-naïve isolates from Ethiopia and Botswana**

HIV-1 enzyme	Resistance Site (position)	Clade C isolates from Ethiopia (n = 5)	Clade C isolates from Botswana (n = 9)	Resistance mutations reported for HIV-1 clade B	Antiviral drugs
Protease	20	----	R (1/9)	K20R/M	INV, RTV,APV
	23	F (1/5)	----	L23I	palinavir group
	36	I (5/5)	V (1/9), I (6/9)	M36I	RTV, NFV
	60	----	E (1/9)	D60E	INV, NFV
	63	----	P (4/9),T (1/9),V (1/9)	L63P	INV, NFV,PLV
	71	----	I (1/9), T (1/9)	A71T/V	INV, RTV NFV, PLV
	77	----	I (1/9)	V77I	NFV
	82	----	I (1/9)	V82A/F/I/S/T	INV, RTV, SQN, APV,TPV
RT	**70**	R (1/5)	----	K70R	ZDV
	98	S (2/5)	----	A98G	NVP, EFV
	138	A (1/5)	A (1/9)	E138K	EFV, TIBO, TSAO, UC-82
	139	A (1/5)	----	T139I	Calanolide A
	190	A (1/5)	----	G190A/E/Q/T	NVP, EFV
	211	K (1/5)	K (7/9)	R211K	ZDV, NRTIs
	214	F (5/5)	F (8/9)	L214F	ZDV, NRTIs

RT and protease amino acid sequences of treatment-naïve HIV-1 clade C isolates from Ethiopia and Botswana were analysed for the presence of amino acid substitutions at sites associated to antiretroviral drug resistance. The amino acid positions within RT or protease are shown and primary resistance sites are written in bold. The frequency of each mutations among clade C isolates from Ethiopia and Botswana is indicated in parentheses. Previously characterized drug-resistance mutations in HIV-1 clade B are noted.

amino acid changes detected in RT and PR of the Ethiopian and Botswanian strains were located at secondary resistance sites, although a few mutations were noted at primary resistance sites.

Co-receptors: The usage of the two major co-receptors CCR5 and CXCR4 by the clade C isolates from Ethiopia and Botswana were assessed using the HOS-CD4 stably transfected cell lines co-cultured with CBMCs pre-infected with the different viral strains. All HIV-1 clade C isolates from Botswana and Ethiopia were strongly CCR5 positive and none of them was exclusively CXCR4 positive. However, half of them could additionally induce less efficient productive infection of CXCR4 expressing cells as compared to CCR5 cells (Table 3). The clade B control isolate was exclusively CXCR4 positive and no viral p24 antigen production was detected in the mock-infected HOS-CD4 cells.

Table 3

Increase in p24 production over time in HOS-CD4 cell lines expressing chemokine receptors

HIV-1 clade isolates		CCR5	CXCR4
from Ethiopia	4742	+++	++
	4743	+++	---
	4761	++	---
	4762	++	---
from Botswana	BG05	+++	++
	BG15	+++	++
	Mol 01	+++	+++
	Mol 03	+++	++
	Mol 13	+++	++
	Mol 14	+++	++
	Mol 18	+++	+
	Mol 36	+++	---
	HSTmok	++	---
Controls	Clade B isolate 5346	---	+++
	Mock-infected (medium)	---	---

CBMCs were pre-infected for 7 days with 3-4 ml containing at least 1ng/ml of p24 antigen of different HIV-1 isolates and then co-cultured with HOS-CD4 cell lines (chemokine receptor transfectants) expressing the co-receptor CCR5 and CXCR4 (fusin). Cells were monitored for 3 weeks and p24 production was assessed in the supernatants collected at different time points. p24 positive cultures were considered indicative of the virus using the corresponding co-receptor for cell infection.

DISCUSSION.

We have investigated HIV-1 intra-clade C *Pol* sequence variation within strains isolated from fourteen treatment-naïve patients originating from Ethiopia and Botswana as well as their co-receptor usage. Genotypic divergence of *pol* sequences between different HIV-1 subtypes has not been extensively studied, although the RT and PR are the main targets of anti-retroviral therapy (Sato et al., 1996; Cornelissen et al., 1997; Shaafer et al., 1998). We have detected a series of mutations at sites associated with drug resistance in clade C RT and PR. A few resistance mutations, such as substitutions L214F in RT and M36I in PR, were highly conserved within both the Ethiopian and Botswanian clade C isolates. In addition, the R211K mutation in RT was also highly conserved among the isolates from Botswana and less among the Ethiopian strains. Various amino acid substitutions were also detected at the resistance site 63 in PR, with L63P being the most frequently noted. However, no mutation was identified at position 63 in the PR of the five clade C isolates from Ethiopia. Recent reports indicate that HIV-1 clade C infected patients, who failed treatment with protease inhibitors in Israel, had baseline resistance mutations in the PR gene (Grossman et al., 2001). Drug-naïve individuals possessed only a M36I substitution or combinations of either M36I + K20R or M36I + K20R + L23P (Grossman et al., 2001). Patients failing HAART therapy showed strong correlation between infection with a non-B subtype and the prevalence of resistance mutations in RT and PR (Herman, 2001).

Our preliminary study on intra-clade *pol* variation shows that polymorphisms in RT are more common among clade C strains from Ethiopia than Botswana. In contrast, the PR of clade C isolates from Botswana has higher diversity than Ethiopia in regard to resistance-associated polymorphisms. Larger studies must be performed to assess overall baseline variability among clade C and other HIV-1 subtype viruses from different regions.

Our analysis of co-receptor usage of clade C isolates from Ethiopia and Botswana supports the known preferential usage of CCR5. No Ethiopian or Botswanian isolates exclusively used the CXCR4 co-receptor as can occur with clade B viruses.

CHAPTER 5

Polymorphisms of cytotoxic T-lymphocyte (CTL) and T-helper epitopes within reverse transcriptase (RT) of HIV-1 subtype C from Ethiopia and Botswana following selection of antiretroviral drug resistance

Loemba H., B. Brenner, M.A. Parniak, S. Ma'ayan, B. Spira, D. Moisi, M. Oliveira, M. Detorio, M. Essex and M.A. Wainberg.

Preface to Chapter 5:

In Chapter 4, an intra-clade C *Pol* sequence variations have been detected. Given the presence of several baseline resistance mutations noted in RT of clade C isolates from Ethiopia and Botswana, genotypic divergence may also be observed within immunogenic regions of RT. RT represents a target for the immune system. Therefore, it is important to study the polymorphisms of CTL and T-helper epitopes within clade C RT and screen the resistance mutations that may allow viral escape.

ABSTRACT.

Immunodominant regions characterized in HIV-1 clade B RT and peptides harboring predicted drug selected mutations in each epitopes may be of interest in therapeutic immunization protocols to restrict antiviral drug resistance. However, little is known about potential divergence in other HIV-1 subtype RTs. Five isolates from Ethiopia (north-eastern Africa) and nine from Botswana (southern Africa), characterized as subtype C, were included in this study. A clustering of genotypic epitope variability was observed, with a few epitopes remaining relatively conserved. Additional mutations within the immunogenic regions of clade C RT were generated through tissue culture selection of drug resistant variants, using increasing concentrations of non-nucleoside RT inhibitors (NNRTIs) [i.e. nevirapine (NVP), delavirdine (DLV), and efavirenz (EFV)] and nucleoside RT inhibitors (NRTIs), [i.e. lamivudine (3TC) and zidovudine (ZDV)]. At sites associated with drug resistance, the mutations (S98I, Y181C, V108I and K103N), (A62V, V75E, L100I, K103T, V108I, Y181C, L210M), (K103E, V106M, V179D, Y188C, Y188H, G190A), (M184I, M184V), and (K70R) were noted during selection with NVP, DLV, EFV, 3TC and ZDV, respectively. New amino acid substitutions in CTL and T-helper epitopes, but non-related to known drug-resistant sites, were observed during selection with NVP (D186N), DLV (V90I, D113I, I132L, K154E, F171L, A173V, I178M), 3TC (T39K and A173V) and ZDV (I135M). A global characterization of CTL and T-helper anchor motifs and of predicted drug induced mutations in RT from HIV-1 non-B subtype

viruses, may lead to optimal immuno-therapeutic strategies to control that may control the emergence of drug resistant strains.

INTRODUCTION.

The management of highly active antiretroviral therapy (HAART) in high-income countries has contributed to a significant reduction of HIV-1 associated mortality and morbidity over the past few years. However, emergence of drug resistant mutants during treatment may results in a need to change a drug regimen to more drug regimen for more potent and expensive drug cocktails (Schinazi et al., 1996; Wainberg and Cameron, 1998; Wainberg, 1999). Although an effective vaccine is the ultimate goal of the field, other preventive measures and the management of antiretroviral drugs (ARVs) may limit the devastating impacts of HIV infection (Nabel, 2001; Richman, 2001). Immune-based measures may also be important and, if successful, may be a cheaper alternative to use than some ARVs (Pantaleo, 1997; Richman, 2001). Among therapeutic immunization strategies that could be considered is the targeting of HIV-1 *Pol* immunogenic sequences that harbor predicted drug resistance mutations, before the emergence of viral mutants (Myers et al., 2001). This may improve the long-term efficacy of existing antiretroviral drugs and significantly delay the onset of resistant variants.

Each of the HIV *Env*, *Gag, Pol, Nef, Rev, Vif* and *Tat* gene products can be immunogenic (Buseyne et al., 1993; Culmann-Penciolelli et al., 1994; Dupuis et al., 1995; Haas et al., 1998; Rowland-Jones et al., 1998; Brander et al., 2000). A panel of HIV-1 RT sequences has also been identified as strong inducers of cytotoxic T lymphocyte (CTL) immune responses. Many of these RT epitopes are

HLA class 1 and class 2 restricted, and generally, they have been characterized using HIV-1 subtype B (Walker et al., 1988; van der Burg et al., 1995; Haas et al., 1998; Samri et al., 2000). Epidemics involving HIV group M (non-B, A through J) and O clades are expanding in the developing world, and 50% of new HIV-1 infections in southern Africa and in heavily infected regions of Asia are attributable to clade C strains (Dietrich et al., 1993; Burke and McCutchan, 1997; Essex, 1999; UNAIDS, 2000). Although overall similarities exist among HIV-1 subtypes, there is a need to investigate potential divergence within immunogenic *pol* sequences among different HIV-1 clades, in order to determine the conserved epitopes that could be used in a candidate therapeutic vaccine.

This study was undertaken to screen the diversity of immunodominant regions of HIV-1 subtype C RT and identify potential drug selected mutations that may affect recognition of these epitopes by cellular immune response. A total of 14 HIV-1 clade C treatment-naive isolates were studied, comprising samples collected from five patients from Ethiopia and nine from Botswana. Our results demonstrate a significant inter and intra-clade C natural polymorphism in many epitopes within the RT polymerase domain, although a portion of RT immunogenic sequences appear to be relatively conserved between clades B and C. Additional mutations within the CTL and T-helper epitopes were selected in vitro in the presence of increasing concentrations of different non-nucleoside RT inhibitors (NNRTIs) [nevirapine (NVP), delavirdine (DLV) and efavirenz (EFV)] as well as nucleoside RT inhibitors (NRTIs) [lamivudine (3TC) and zidovudine (ZDV)].

MATERIALS AND METHODS.

Subjects and HIV-1 isolation. Blood samples collected from fourteen HIV-1 antiviral drug-naïve infected patients were included in the study. Five individuals were from Ethiopia (north-eastern Africa) and their HIV-seropositivity was primarily diagnosed in 1994-1995, shortly after emigrating to Israel. Nine other HIV-1 infected patients were from Botswana (southern Africa). Isolation of HIV-1 strains from blood samples was performed by co-culture of peripheral blood mononuclear cells (PBMCs) from Ethiopian and Botswana patients and umbilical cord blood mononuclear cells (CBMCs) obtained from seronegative donors after Ficoll-paque centrifugation (Salomon et al., 1994). Prior to the co-culture, donor CBMCs were stimulated with interleukin-2 (IL-2) (Boehringer-Manneim, Inc., Montreal, Canada) and phytoheamagglutinin (PHA) for 3 days in RPMI-1640 tissue culture medium as described previously (Salomon et al., 1994). The co-culture medium was supplemented with 10% fetal calf serum, 2mM glutamine, 200 U/ml penicillin and 200 μM/ml streptomycin, and the cells were incubated at 37^0C in the presence of 5% CO2. Production of HIV-1 p24 antigen (Abbott Laboratories, North Chicago, Illinois, USA) and RT activity in cell supernatant were monitored at least once weekly as described (Boulerice et al., 1990; Salomon et al., 1994). Proviral DNA was extracted from CBMC lysates, using the QIAmp DNA purification kit (Qiagen, USA), and was subsequently amplified by PCR for sequencing RT regions. The isolates from Ethiopia were designated 4742, 4743, 4761, 4762 and 4766, whereas the HIV-1 strains from

Botswana were BG05, BG15, HSTmok, Mol01, Mol03, Mol13, Mol14, Mol18 and Mol36.

Subtype assessment by hetero-duplex mobility assays (HMA). Subtype determination of the different clinical isolates was performed by HMA of *env* regions. For this purpose, protocol and reagents obtained through the AIDS Research and Reference Reagent Program of the NIH (Division of AIDS, NIAID, NIH, USA) were used as previously described (Delwart et al., 1993). Briefly, the C2-V5 *env* region of approximately 0.7 kb of each viral isolate was amplified by two PCR rounds, using a gamma-^{32}P labeled 3'primer in a nested amplification reaction. Parallel PCRs were performed to amplify similar fragments from plasmids harboring *env* genes of a panel of HIV-1 reference strains, representing different HIV-1 subtypes originating from various parts of the world. An equal amount of 5-µl of both the unknown sample and each reference PCR product were mixed in a sample tube containing 1.1µl of HMA annealing buffer (100mM NaCl, 10mM tris, pH 7.2, 2mM EDTA). Homo-and hetero-duplex DNA fragments, that were generated by denaturation at 94^{0}C for 2 min and reannealing on ice, were first separated under non-denaturating conditions by electrophoresis in a 5% acrylamide gel at 150 V for 4 hours, and then visualized by autoradiography.

Phylogenetic analyses of RT sequences. Subtype status was confirmed by analysis of RT and protease gene sequences. Viral *Pol* regions, amplified by PCR, were sequenced using the automated DNA sequencing system (Visible Genetics Inc. Atlanta, GA) as previously reported (Durant et al., 1999). Ethiopian

and Botswana isolate RT sequences were aligned and compared to corresponding RT regions of various reference strains representing different HIV-1 subtypes, obtained from the Los Alamos HIV sequence database (http://hiv-web.lanl.gov). This included the strains ETH2220 (subtype C from Ethiopia), 92BR025.8 (subtype C from Brazil), IN21068 (subtype C from India), 96BW05.02 (subtype C from Botswana), U455 and 92UG037.1 (subtype A from Uganda), and Q2317 (subtype A from Kenya), NDK (subtype D from Zaire/Congo), CM240 (subtype E from Thailand), 90CF402.1 (subtype E from Central African Republic), 93TH253.3 (subtype E from Thailand), 93BR020.1 (subtype F from Brazil), SE61165 (subtype G from both Sweden and Zaire/Congo), 90CF056.1 (subtype H from Central African Republic), SE9280.9 (subtype J from Sweden), MVP5180 (subtype O from Cameroon), CPZGAB (Subtype CPZ from Gabon) and subtype B strains (LAV from France, JRFL from USA). Overall, 34 sequences representing RT polymerase domain were analyzed, using *Genetool* and *peptool* software (from BIOTOOLS Incorporated, Edmonton, Canada). Each sequence was 397 base pairs in length and the alignments were gap-stripped. Percent homology between selected pairs of RT sequences was determined. Phylogenetic neighbor joining trees were generated, based on distances between sequences, using the phylogenetic computed programs Dnadist, Neighbor and Drawtree/Drawgram.

Selection of drug-resistant viral mutants. Resistance to the NNRTIs (NVP, DLV and EFV) as well as to the NRTIs (3TC and ZDV) was selected by growing infected CBMCs in the presence of increasing concentrations of these

drugs as previously described (Gao Q. et al., 1992). HIV-1 clinical isolates were grown by repeated weekly passages in CBMCs over 8 to 30 weeks for NNRTI selections, 8 to 12 weeks for 3TC, and up to 11 months for AZT. Sub-optimal drug concentrations were used, beginning with 0.01 μM for NVP, DLV, and 3TC, or 0.001 μM for EFV and ZDV, then ending at 1 to 10 μM. RT assays were performed weekly to monitor viral replication. When RT activity in cell supernatant peaked, genotyping was performed to detect emergence of drug-selected mutations. All amino acid substitutions that appeared within the RT CTL and T-helper epitope sequences of the clinical isolates from Ethiopia and Botswana were screened.

Epitope prediction in HIV-1 clade C RT. RT protein sequences of the fourteen HIV-1 antiviral drug-naïve isolates from Ethiopia and Botswana were analyzed and screened for potential CTL or T-helper restricted anchor residue motifs within RT, using the epitope recognition computed program «MultiMotifScanner», that predicts HIV-1 best-suitable sequences for binding to CTL and CD4+ T-helper cells (http://phage.lanl.gov/cgi-bin/EPI_PREDICT/MultiMotifScanner.pl). Predicted epitope sequences, that were conserved among HIV-1 clade C strains from Ethiopia and from Botswana, were analyzed.

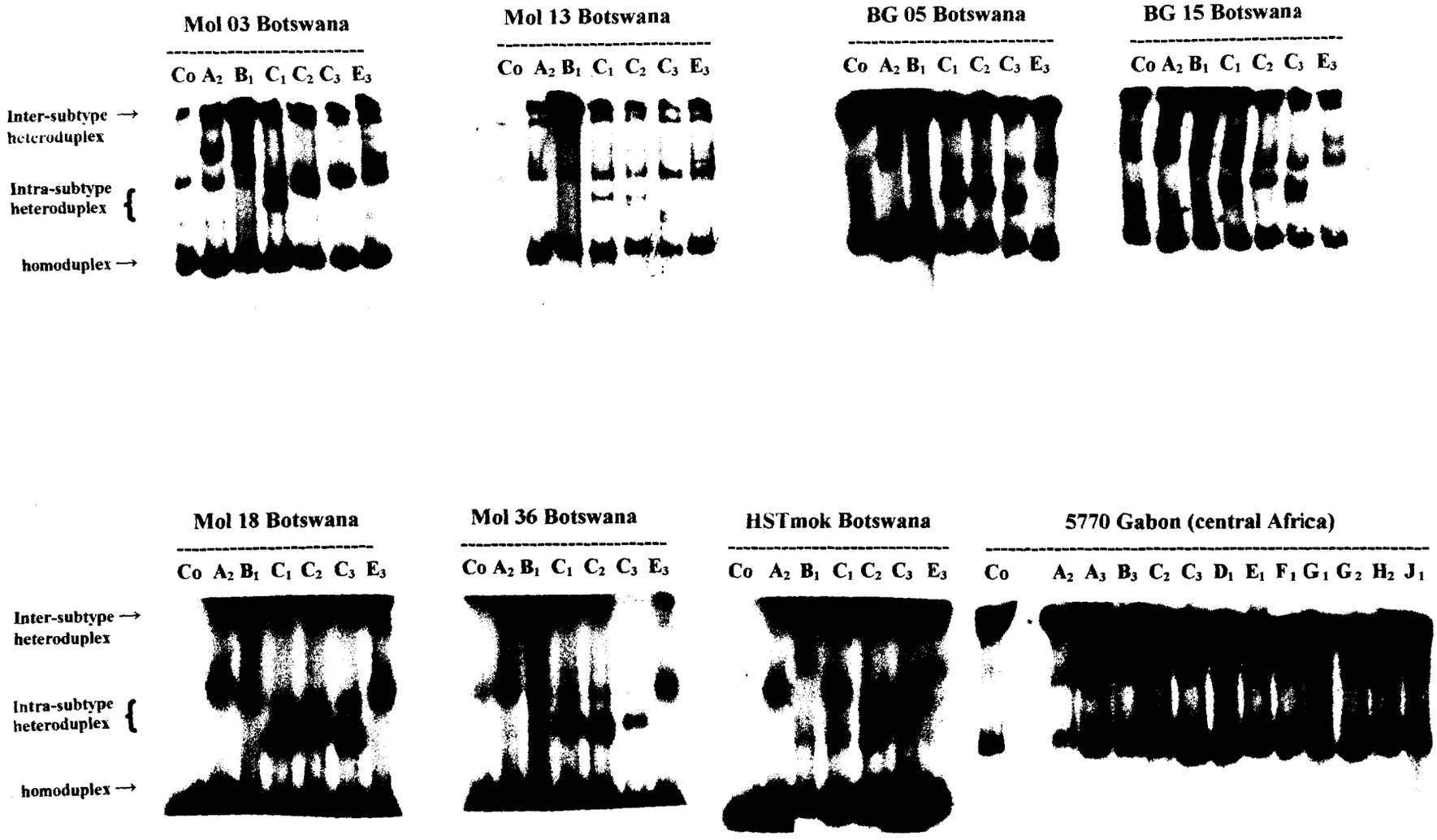
RESULTS.

Subtype characterization of isolate env genes. The phylogenetic analysis of Ethiopian and Botswana isolate *env* regions was performed by HMA. Designation to a defined HIV-1 subtype was based on the comparison of the

HMA mobility profile of a given sample duplexes formed with different subtype reference strains. The *env* regions of the nine samples from Botswana all belonged to subtype C, as faster moving hetero-duplexes were observed in appropriate lanes representing duplexes generated with clade C reference strains. Figure 1 shows an overall view of the HMA profiles of seven Botswanian samples. The clinical isolate 5770 from Gabon, used in the HMA as a control, was determined to be a subtype G strain. The subtype C status of our Ethiopian isolates has been described previously (Loemba et al., 2001). The set of reference strains used in the HMA reactions is defined as follows: clade C viruses C1, C2, C3 representing the HIV-1 strains MA959 (accession number U08453), ZM18 (accession number L22954) and IN868 (accession number U07103), respectively. Clade A viruses A2 and A3 as well as clade E strain E1 represent reference strains IC144 (accession number unknown), SF170 (accession number M66533), and TH22 (accession number U09131), respectively. For clade B viruses, B1 and B3 represent strains BR20 (accession number U08797) and SF162 (accession number M65024), respectively. The samples D1, F1, G1, G2, H2 and J1 designate the strains UG21 (accession number U08804), BZ162 (accession number L22084), RU131 (accession number U08364), LBV21-7 (accession number U09664), and V1557 (accession number U09666), respectively.

Figure.1. Heteroduplex mobility analysis of isolates from Botswana drug-naïve patients.

Heteromobility duplex assays were performed as described in Materials and Methods. Hetero-complexes were formed by mixing the amplified DNA from the viral isolates with the PCR-amplified C2-V5 *env* sequences of reference strains mixed with the same amount of amplified C2-V5 *env* fragments from the Botswana isolates have generated Hetero-complexes. A more rapid migration on acrylamide gels of hetero-duplex DNA indicates relative degree of similarity between the unknown isolate and the reference strain sequences. For comparative purposes, a wild-type (WT) HIV-1 clade G strain (5770) from Gabon was used. Lanes CO show the control for each of the samples from Botswana; lanes A_2, A_3, B_1, C_1, C_2, C_3, D_1, E_1, F_1, G_1, G_2, H_2, and J_1 show respectively the reference strains of different HIV-1 subtypes A, B, C, D, E, F, G, H and J.

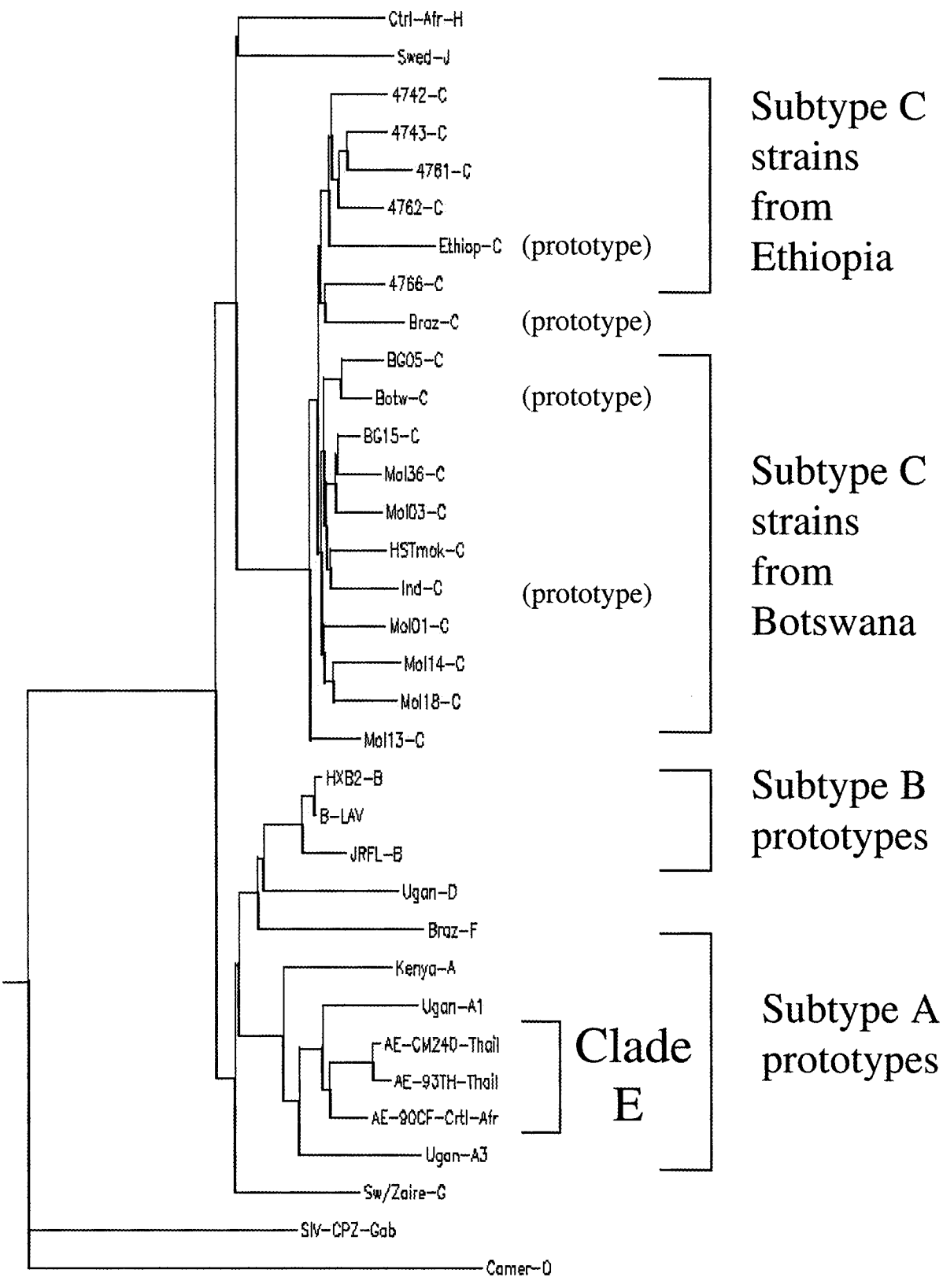

Mol 03 Botswana
Co A2 B1 C1 C2 C3 E3
Mol 13 Botswana
Co A2 B1 C1 C2 C3 E3
BG 05 Botswana
Co A2 B1 C1 C2 C3 E3
BG 15 Botswana
Co A2 B1 C1 C2 C3 E3
Inter-subtype heteroduplex
Intra-subtype heteroduplex
homoduplex
Mol 18 Botswana
Co A2 B1 C1 C2 C3 E3
Mol 36 Botswana
Co A2 B1 C1 C2 C3 E3
HSTmok Botswana
Co A2 B1 C1 C2 C3 E3
5770 Gabon (central Africa)
Co A2 A3 B3 C2 C3 D1 E1 F1 G1 G2 H2 J1
Inter-subtype heteroduplex
Intra-subtype heteroduplex
homoduplex

Phylogenetic analysis of RT sequences. A segment encompassing the fingers and palm sub-domains of Ethiopian and Botswana isolate RT was obtained, using automated "Visible Genetics" DNA sequencing technology. RT nucleotide sequences were aligned with corresponding regions of a set of various reference strains representing different HIV-1 subtypes from diverse regions of the world. RT regions of Ethiopian and Botswana isolates shared 94.2 to 97.5% sequence homology with each of the four different clade C reference strains, between 90 and 92.5% homology with three other subtype B reference strains, and between 88.9 and 91.7% homology with the subtype A reference strains. The lowest degree of RT sequence homology of Ethiopian and Botswana viruses was seen with the clade O reference strain, i.e. 80%.

A phylogenetic tree was constructed by the neighbor-joining method, using the alignment of 34 corresponding RT sequences of the Ethiopian and Botswanian isolates and different HIV-1 subtype prototype strains (Fig. 2). Eleven major branches are seen in the tree, harboring sequences of eleven different subtypes (A, B, C, D, E, F, G, H, J, CPZ, and O). All five isolates from Ethiopia and nine isolates from Botswana clustered with the four different HIV-1 clade C prototype strains from Ethiopia, Brazil, India, and Botswana. Two different sub-clusters were observed in the tree: one representing clade B prototype strains and the other for clade A and the closely related E reference strains.

Figure 2. Phylogenetic analysis of reverse transcriptase sequences of HIV-1 isolates from Ethiopia and Botswana.

Phylogenetic analysis comparing the RT regions of HIV-1 *pol* genes from five Ethiopian clinical isolates, nine HIV-1 isolates from Botswana and twenty different prototype strains representing various HIV-1 subtypes. Tree topology was inferred by neighbour-joining method and was based on an alignment of 397 nucleotides from which columns containing gaps were deleted. The subtype O prototype isolate was treated as an outgroup.


Distance 0.1
Ctrl-Afr-H
Swed-J
4742-C
4743-C
4761-C
4762-C
Ethiop-C
(prototype)
4766-C
Braz-C
(prototype)
BG05-C
Botw-C
(prototype)
BG15-C
Mol36-C
Mol03-C
HSTmok-C
Ind-C
(prototype)
Mol01-C
Mol14-C
Mol18-C
Mol13-C
HXB2-B
B-LAV
JRFL-B
Ugan-D
Braz-F
Kenya-A
Ugan-A1
AE-CM240-Thail
AE-93TH-Thail
AE-90CF-Crtl-Afr
Ugan-A3
Sw/Zaire-C
SIv-CPZ-Gab
Camer-O
Subtype C strains from Ethiopia
Subtype C strains from Botswana
Subtype B prototypes
Clade E
Subtype A prototypes

Analysis of genetic variability of RT, CTL, and T-helper epitope sequences. A set of known HIV-1 RT antigenic regions, corresponding to RT CTL and T-helper epitopes of subtype B, was obtained from the LANL Molecular Immunology Database. The epitope amino acid sequences characterized for the HIV-1 subtype B fingers and palm sub-domains of RT were aligned with corresponding RT sequences of clade C isolates from Ethiopia and Botswana. Sequences were screened for potential genotypic polymorphisms that may exist in specific RT CTL and T-helper epitopes of HIV-1 clade C. Table 1 and Table 2 illustrate the polymorphisms within the sequences of Ethiopian and Botswana clade C isolates. Major genotypic differences were observed in many epitope sequences of clade C viruses, corresponding to the characterized CTL epitope regions of clade B RT (Table 1). A clustering of sequence divergence was also detected within the RT of Ethiopian clade C isolates and the RT regions of Botswana clade C isolates. However, a few RT CTL epitopes were conserved between the subtype B and subtype C strains from Ethiopia and Botswana (CTL epitopes 103-118, 108-123, 118-128, 128-136, 180-190 and 192-201) (Table 1). The polymorphism of T helper recognition motifs, existing within RT regions of clade C isolates from Ethiopia and Botswana, was concentrated mainly in the N-terminus portion of the RT fingers sub-domain (CD4 T-cell epitopes 36-53, 39-53 and 48-53) and in the C-terminus portion of the RT palm sub-domain (T-helper epitopes 171-191, 195-210 and 196-216) (Table 2). The C-terminus of the RT fingers region and the N-terminus portion of the palm sub-domain of HIV-1 RT appear to be relatively conserved between HIV-1 clade B and the Ethiopian and

Botswana clade C strains (CD4 T-cell epitopes 62-78, 88-100, 133-148 and 144-159).

Table 1

HIV-1 RT CTL epitopes (Clade B polymerase domain)		Polymorphism of corresponding HIV-1 subtype C RT CTL epitope sequences		Additional mutations generated within the clade C RT CTL epitopes after cell tissue culture selection of drug resistant isolates				
	Amino acid	HIV-1 Clade C drug naïve sequence		NVP	DLV	EFV	3TC	AZT
Location	sequence	5 Ethiopian isolates	9 Botswana isolates	7-9 weeks	8-15 weeks	13–30 weeks	12 weeks	44 weeks
33 - 44	ALVEICTEMEKE	V35T (4/5), E36A (3/5) T39E (5/5)E40D (1/5), E40K (1/5)K43N (1/5), K43R(1/5)	T39E (9/9)					
42 - 53	EKEGKISKIGPE	K43N (1/5), K43R (1/5) I47F (1/5), S48T (2/5)	S48T (8/9), S48E (1/9)					
103-118	KKSVTVLDVGDA YFSV	--	K104R (1/9)	**K103N**	**K103T, V108I,** D113I	**K103E, V106M**		
108-123	VLDVGDAYFSVP LDED	D123S (3/5), D123G (2/5)	D123S (2/9), D123G (3/9) D123N (1/9)	**V108I**	**V108I**			
118-128	VPLDEDFRKYT	D123S (3/5), D123G (2/5)	D123S (2/9), D123G (3/9) D123N (1/9)					
128-136	TAFTIPSIN	I135T (1/5)	I135T (1/9)		I132L			I135M
157-179	PAIFQSSMTKILE PFRKQNPDI	A158S (2/5), Q174K (3/5) K173A (5/5), D177E (4/5) D177G (1/5)	S162A (1/9), S162C (1/9) T165I (3/9), K166R (2/9) E169K (1/9), K173T (1/9) K173E (1/9), K173A (7/9) Q174R (1/9), Q174K (2/9) D177E (8/9) I178M (2/9)		F171L A173V I178M	**V179D**	A173V	
175-184	NPDIVIYQYM	D177E (4/5), D177G (1/5)	D177E (8/9), I178M (2/9)	**Y181C**	**Y181C** I178M	**V179D**	**M184I M184V**	
180-190	IYQYMDDLYYG	**G190A** (1/5)	---	**Y181C** D186N	**Y181C**	**Y188C,Y188H G190A**	**M184V M184I**	
192-201	DLEIGQHLLR	T200A (4/5)	T200A (9/9)					
201-210	KIEELRQHLL	Q207E (4/5), Q207D (1/5)	I202V (1/9), E204K (1/9), Q207E (7/9), Q207K (1/9) Q207R (1/9)		**L210M**			
209-220	LLRWGLTTPDKK	**R211K** (1/5), **L214F** (5/5)	**R211K** (6/9), **R211Q** (1/9) **L214F** (9/9)		**L210M**			

Protein sequences of the known CTL epitopes in HIV-1 subtype B RT were analyzed for natural divergence in corresponding sequences of clade C RT of Ethiopian and Botswana isolates. The frequency of each drug-naïve mutation is noted in parentheses. Additional mutations were selected in clade C RT, using increasing concentrations of antiviral drugs in tissue culture. Average number of weeks needed for generating viral mutants in indicated. Mutations noted at drug resistance sites in RT are written in bold.

Table 2

HIV-1 RT T-Helper epitopes (Clade B polymerase domain)		Polymorphism of corresponding HIV-1 subtype C RT T-Helper epitope sequences		Additional mutations generated within the putative clade C RT T-Helper epitopes after in vitro selection of drug resistant isolates				
Location	Amino acid sequence	HIV-1 Clade C drug naïve sequence		Nev (7-9 weeks)	Del (8-15 weeks)	Efv (13-30 weeks)	3TC (12 weeks)	AZT (44 Weeks)
		5 Ethiopian isolates	9 Botswana isolates					
36 - 53	EICTEMEKEGKIS KIGPE	E36A (3/5), T39E (5/5) E40D (1/5), E40K (1/5) K43N (1/5), K43R(1/5)	T39E (9/9), S48T (8/9), S48E (1/9)				T39K	
39 - 54	TEMEKEGKISKIG PEN	T39E (5/5), E40D (1/5), E40K (1/5)K43N (1/5), K43R(1/5), I47F (1/5) S48T (2/5)	T39E (9/9), S48T (8/9),S48E (1/9)					
48 - 73	SKIGPENPYNTPV FAI	S48T (2/5), N57K(1/5) D67G (1/5), T69P (1/5) **K70R** (1/5)	S48T (8/9), S48E (1/9)		**A62V**			**K70R**
62 - 78	AIKKKDSTKWRK LVDFR	D67G (1/5), T69P (1/5)	--		**V75E**			
88 - 100	WEVQLGIPHPAGL	**A98S** (2/5)	--	**S98I**	V90I, **L100I**			
133-148	PSINNETPGIRYQY NV	I135T (1/5), **E138A** (1/5) **T139A** (1/5)	I135T (1/9), E138A (1/9) I142V (1/9)					I135M
144-159	YQYNVLPQGWKG SPAI	A158S (2/5)	A158S (1/9)		K154E			
171-191	FRKQNPDIVIYQY MDDLYVGS	K173A (5/5), Q174K (3/5) D177E (4/5), D177G (1/5) **G190A** (1/5)	K173T (1/9), K173E (1/9), K173A (7/9), Q174R (1/9), Q174K (2/9), D177E (8/9) I178M (2/9)	**Y181C**	F171L, I178M **Y181C**	**V179D, Y188C Y188H, G190A**	**M184V M184I**	
195-210	IGQHRTKIEELRQ HLL	I195L (1/5), G196R (1/5) T200A (4/5), K201N (1/5) Q207E (4/5), Q207D (1/5)	T200A (9/9), I202V (1/9), E204K (1/9), Q207E (7/9), Q207K (1/9), Q207R (1/9)		**L210M**			
196-216	GQHRTKIEELRQ HLLRWGLT	G196R (1/5), T200A (4/5), K201N (1/5), Q207E (4/5), Q207D (1/5), **R211K** (1/5) **L214F** (5/5)	T200A (9/9), I202V (1/9), E204K (1/9), Q207E (7/9), Q207K (1/9), Q207R (1/9) **R211K** (6/9), **R211Q** (1/9) **L214F** (9/9)		**L210M**			

Protein sequences of the known T-Helper epitopes in HIV-1 subtype B RT were analyzed for natural divergence in corresponding sequences of clade C RT of Ethiopian and Botswana isolates. The frequency of each drug-naive mutation in clade C RT is noted in parentheses. Additional mutations were generated in clade C RT under drug pressure in tissue culture. Average number of weeks needed for generating viral mutant isolates are indicated. Mutations noted at drug resistance sites are written in bold.

Selection of HIV-1 clade C mutants with antiretroviral drugs in cell culture. In order to predict mutations that may appear within RT epitope sequences, Ethiopian and Botswana strains were grown in the presence of increasing concentrations of NNRTIs (NVP, DLV, and EFV) and NRTIs (3TC and ZDV), using weekly passages in primary cells. Tables 1 and 2 reveal specific-drug resistance mutations that were generated within clade C RT immunogenic regions, as well as other amino acid substitutions, related to clade C polymorphism at sites non-associated with drug resistance but overlapping the epitopes. The mutations S98I, Y181C, V108I and K103N were generated during selection with NVP. Incubation with DLV led to previously characterized mutations at sites associated with resistance to DLV (K103T, Y181C) and other NNRTIs (L100I, V108I), as well as ZDV (L210M) and multi-drug secondary resistance to NRTIs (A62V, V75E). Selection with EFV led to the EFV-related mutations K103E, V179D, Y188C, Y188H, G190A and a mutation at a site associated with resistance to NVP (V106M). Cell culture selection with 3TC and ZDV generated the mutations M184I, M184V and K70R associated with primary resistance against 3TC and ZDV, respectively.

However, multiple other amino acid substitutions, outside of known drug-resistance sites, were observed in HIV-1 clade C RT regions overlapping CTL and T-helper epitope sequences. A D186N mutation was noted during selection with NVP in the CTL epitope (RT region 180-190). 3TC and ZDV led to emergence of mutations T39K (T-helper epitope 36-53), A173V (CTL epitope 157-179) and I135M (T-helper epitope 133-148) (Table 1 and Table 2). Other

amino acid substitutions at sites not related to drug resistance were generated during selection with DLV, i.e. V90I, K154E, and F171L, I178M within the CD4 T-cell epitopes (88-100), (144-159), and (171-191), respectively. The mutations D113I, I132L, and F171L, A173V, I178M were seen in the CTL epitopes (103-118), (128-136) and (157-179), respectively. No amino acid changes, outside of known antiviral resistance sites, were noted during selections with EFV.

Clade C RT sequence screening for potential immunogenic motifs. The most conserved RT epitope sequences among all Ethiopian isolates and those conserved among all nine isolates from Botswana were analyzed (Table 3). In the case of Ethiopian strains, complete sequence identity was observed with the Ethiopian HIV-1 clade C reference strain ETH2220 in 14 of the predicted RT epitope sequences. Similarly, Botswana viruses shared 18 highly conserved sequences with the Botswana HIV-1 clade C reference strain 96BW0502. The clade C prototype strains ETH2220 and 96BW0502 were obtained from the Los Alamos HIV sequence database. Among all predicted immunogenic regions of RT, just three areas, located in the N-terminal portion of the fingers (amino acids 145-154, 146-154 and 147-154), were completely conserved among all clade C strains from Ethiopia and Botswana (Table 3).

Table 3

Predicted HLA class 1 and class 2 conserved epitopes within RT of HIV-1 clade C isolates from Ethiopia and Botswana

Conserved epitopes among 100% of clade C isolates from Ethiopia		Conserved epitopes among 100% of clade C isolates from Botswana		Conserved epitopes shared among 100% of all clade C isolates from Ethiopia and Botswana		Conserved epitopes shared among 60 – 80% of all clade C isolates from Ethiopia and Botswana	
Location	Sequence	Location	Sequence	Location	Sequence	Location	Sequence
56 - 64	YNTPVFAIK	38 - 46	CEEMEKEGK	145-154	QYNVLPQGWK	38 - 46	CEEMEKEGK
56 - 65	YNTPVFAIKK	39 - 46	EEMEKEGK	146-154	YNVLPQGWK	39 - 46	EEMEKEGK
57 - 64	NTPVFAIK	61 - 70	FAIKKKDSTK	147-154	NVLPQGWK	56 - 64	YNTPVFAIK
57 - 65	NTPVFAIKK	62 - 70	AIKKKDSTK			56 - 65	YNTPVFAIKK
57 - 66	NTPVFAIKKK	63 - 70	IKKKDSTK			57 - 64	NTPVFAIK
58 - 65	TPVFAIKK	64 - 73	KKKDSTKWRK			57 - 65	NTPVFAIKK
58 - 66	TPVFAIKKK	65 - 73	KKDSTKWRK			57 - 66	NTPVFAIKKK
59 - 66	PVFAIKKK	92 - 101	LGIPHPAGLK			58 - 65	TPVFAIKK
73 - 82	KLVDFRELNK	93 - 101	GIPHPAGLK			58 - 66	TPVFAIKKK
74 - 82	LVDFRELNK	93 - 102	GIPHPAGLKK			59 - 66	PVFAIKKK
75 - 82	VDFRELNK	94 - 101	IPHPAGLK			63 - 70	IKKKDSTK
145-154	QYNVLPQGWK	94 - 102	IPHPAGLKK			64 - 73	KKKDSTKWRK
146-154	YNVLPQGWK	94 - 103	IPHPAGLKKK			66 - 73	KDSTKWRK
147-154	NVLPQGWK	95 - 102	PHPAGLKK			75 - 82	VDFRELNK
		95 - 103	PHPAGLKKK			145-154	QYNVLPQGWK
		96 - 103	HPAGLKKK			146-154	YNVLPQGWK
		146-154	YNVLPQGWK			147-154	NVLPQGWK
		147-154	NVLPQGWK				

RT protein sequences of 5 clade C isolates from Ethiopia and 9 from Botswana were scanned for identification of putative HLA anchor residue motifs using the computer-based epitope search program «MultiMotifScanner», at the web site http://phage.lanl.gov/cgibin/EPI_PREDICT/MultiMotifScanner.pl. The non-conserved motifs have been excluded from the table.

DISCUSSION.

We have described natural variations and drug-selected mutations in RT immunogenic regions of fourteen HIV-1 clade C Ethiopian and Botswanian isolates. Those RT regions correspond to known specific CTL and CD4 T-cell anchor sequences in HIV-1 clade B RT. The existence of polymorphisms in the CTL and T-helper epitopes may impact on immunological recognition of HIV-1 RT regions and immunlogical cross-reactivity. We have mainly investigated the fingers and palm sub-domains of RT, as the majority of drug resistance mutations are generated within these regions. The phylogenetic characterization of the *Env* gene of Ethiopian and Botswana isolates by HMA and phylogenetic analysis of their RT sequences have confirmed that they are related to HIV-1 subtype C strains.

The immunogenic properties of HIV-1 RT have been documented by numerous studies (Walker et al., 1988; van der Burg et al., 1995; Haas et al., 1998; Samri et al., 2000; Day et al., 2001). As HIV-1 *pol* is one of the most conserved genes among different HIV-1 subtype strains, reverse transcriptase represents a potential target for induction of immune cross-recognition among different HIV-1 subtypes. In our study, an average sequence homology of about 90 to 92.5% was found between Ethiopian and Botswanian clade C isolates and the other HIV-1 group M prototype strains. Despite relatively little sequence variation, large immunological differences may exist among RTs from different HIV-1 subtypes. The various HIV-1 subtype natural polymorphisms that exist at amino acid anchor positions may alter antigen processing and presentation,

allowing immune escape of HIV-1 subtype variants (Del et al., 1991; Ossendorp et al., 1996; Goulder et al., 1997).

At early stages of HIV-1 infection, the immune system, e.g. CD8+ cells, may help to suppress viral replication (Koup et al., 1994; McMichael and Rowland-Jones, 2001). Strong CTL activity directed against multiple conserved HIV-1 epitopes may be protective against HIV-1 infection (Rowland-Jones et al., 1998; McMichael and Rowland-Jones, 2001). However, selection of variants harboring new mutations in CTL epitopes may enable HIV-1 to evade the cellular immune response. Knowledge of such mutations in RT and elsewhere including CTL epitopes, among different HIV clades is important. Clustering of genotypic divergence has also been reported in Gag-specific CTL epitopes (Buseyne et al., 1993), and may be involved in HIV-1 escape from HLA-B27-restricted CTL responses (Kelleher et al., 2001). To date, there has been no report on the genotypic divergence of CTL epitope sequences in clade C RT from different regions.

T-helper lymphocytes (CD4+ cells) are important in induction of CTL responses, maintaining CD8+ T-cell memory, and maturation of CD8+ T-cell functions (Ridge et al., 1998; McMichael and Rowland-Jones, 2001). We have shown that Ethiopian and Botswanian isolates have clustered polymorphisms within certain CD4+ T cell epitopes, mainly the N-terminal part of the RT fingers and the C-terminal region of RT palm subdomain. T-helper epitope diversity in clade C RT may contribute to cross-reactivity of RT regions.

In vivo CTL recognition of RT epitopes can be affected by drug resistance mutations (Dalod et al., 1998; Wainberg and Cameron, 1998; Gray et al., 1999; Wainberg, 1999; Samri et al., 2000). Cell culture selection experiments, with NNRTIs and NRTIs, using Ethiopian and Botswana subtype C strains, has revealed common mutations at sites associated with drug resistance, as well as number of other amino acid changes. A62V, V75E, S98I, K103E, V106M and L210M mutations were observed with particularly with NNRTIs. Mutations were mainly noted within two relatively conserved immunogenic regions of RT that overlap CTL epitopes 103-118, 108-123, 175-184, and 180-190. In the case of T-helper epitopes, half of drug resistance mutations arose in a relatively conserved region 48-73, 62-78, 88-100 and the other half in highly polymorphic epitopes 171-191, 195-210, and 196-216. Many other mutations, unrelated to drug resistance, were also noted within regions that overlap the CTL and T-helper epitopes of HIV-1 clade C RT. Some mutations in clade B RT may also increase immunogenicity (Samri et al., 2000).

We used an epitope prediction approach, effective for defining optimal MHC ligands and peptide motifs. Interestingly, predicted HLA class 1 and class 2 epitopes, conserved among subtype C strains from Ethiopia and Botswana, were located in the regions of RT (amino acids 63-82 and 145-154), that span the C-terminus portion of the RT fingers sub-domain and the N-terminus of palm region. These regions are relatively conserved among clade B and clade C strains, with regard to previously characterized T-helper epitopes. Given the susceptibility of CD4+ and CD8+ T cells to epitope sequence variations, inter- and intra-clade

variability may have important impact on vaccine development. CTL activity against various clade B antigens (Gag, RT, Env, and Nef) in patients with non-subtype B viruses was relatively high in clade A and G infected subjects and low in a clade C infected individual (Cao et al., 1997). The clade B RT antigens in the latter individual yielded only 5% lysis of target cells compared with controls, while the same antigens yielded between 17 - 60% lysis when tests were performed with most clade A and G infected patients (Cao et al., 1997).

Antiretroviral therapy during acute stages of HIV-1 infection may be beneficial for regeneration of both CD8+ and CD4+ T cells and restoration of immune functions (Richman, 2001; McMichael and Rowland-Jones, 2001). The knowlegde of the mutations emerging within the conserved epitopes of HIV-1 clade B and clade C RTs may be useful for the prevention of RT drug resistance. The pre-immunization of patients during the acute phase of HIV infection using peptides harbouring sequence variations and expected drug resistance mutations may yield specific immune responses against mutated segments of RT of different HIV-1 subtypes.

CHAPTER 6

General discussion

We have evaluated the genotypic diversity of 5 HIV-1 isolates from treatment-naïve Ethiopian émigrés to Israel, as well as their phenotypic drug susceptibility and the relationship between the emergence of RT mutations conferring drug resistance in HIV-1 clade C strains versus clade B controls. This has involved phylogenetic analysis of *Env* regions and sequencing the RT of those viruses. We showed that the Ethiopian clinical isolates were of clade C origin on the basis of *Env* gene HMA profiles and phylogenetic analysis of RT sequences. Of interest, these isolates were more closely related to a Botswana clade C prototype than to previously described Ethiopian strains (Salminen et al., 1996). Although the predominant subtype in Ethiopia is clade C, the establishment of new HIV-1 epidemics has resulted sequence diversity among circulating viruses in that area. A phylogenetic divergence of some Ethiopian isolates from other HIV-1 close strains was previously observed (Kefenie et al., 1989; Ayenie et al., 1991; Dietrich et al., 1993; Salminen et al., 1996). Similarly, HIV-1 strains from India were reported to be highly divergent from prototypic African and US/European strains, but linked to the South African reference strain (Dietrich et al., 1993). Interestingly, the envelope of isolate 4743 appeared to be a clade B/C mosaic; but RT region sequencing revealed homology to other Ethiopian isolates and to different subtype C reference strain RTs. These results indicate that the *Env* and RT regions may be distinct phylogenetically, warranting extensive RT sequence screening and drug resistance mutational surveillance for non-subtype B.

Recombination has been reported to be a common feature among retroviruses and, as well, between various HIV-1 strains (Robertson et al., 1995; Miguel and Arts, 1999). Moreover, mutation and recombination may both contribute to rescuing high-fitness HIV-1 variants that harbor phenotypically relevant genetic alterations. Recent identification of individuals infected with HIV-1 isolates from two subtypes and inter-subtype species suggests that this may happen often among HIV-1 viruses co-circulating in specific geographic regions (Zhu et al., 1995; Xin et al., 1995; Janini et al. 1998; Takehisa et al., 1998; Miguel and Arts. 1999). Recently, a study in Ivory Cost showed that almost all HIV-1 patients were infected with non-B subtypes, with a predominance of recombinant A/G viruses (Adje et al., 2001). In addition, a high prevalence of 57.4% of genotypic and phenotypic HIV-1 drug-resistant strains were reported among 68 patients who were treated with NNRTIs, NRTIs and protease inhibitors between 1998 and 1999 (Adje et al., 2001). A comparably high prevalence of 30%-50% viral variants harboring drug resistance mutations has been previously reported in some developing countries, among HIV individuals receiving antiretroviral therapy (Fatkenheuer et al., 1997; Deeks et al., 1999). Since various non-subtype B strains can carry resistance mutations, inter-subtype recombinants may pose problems for the application of antiviral therapies in populations where the predominant HIV-1 subtypes are non-clade B. This is increasingly relevant as the use of antiretroviral drugs is becoming increasingly prevalent in developing countries (Cornelissen et al., 1997; Janssens et al., 1997; Descamps et al., 1997; Davis et al., 1999).

The analysis of Ethiopian isolate RT sequences showed that these strains clustered phylogenetically with clade C RTs, with a KVEQ specific motif of silent mutations (amino acid 65, 106, 138, 161 respectively) at resistance sites in the polymerase region of all studied Ethiopian isolates and subtype C reference strains. In addition, there were numerous resistance mutations, polymorphisms, and silent mutations at RT sites linked to NNRTIs and NRTIs (AZT, multi-nucleoside resistance). We have also noted some intra-clade C variation among drug-naïve resistance mutations within RT and PR. Polymorphisms in RT seem more accentuated among clade C strains from Ethiopia than in RT of Botswana isolates, although the substitutions L214F, R211K, and E138K were detected in both groups of isolates. In contrast, the PR of clade C isolates from Botswana had more polymorphisms in regard to drug resistance mutations. The only PR resistance polymorphism shared among all clade C samples from Ethiopia and Botswana was M36I.

Our evaluation of Ethiopian isolate drug susceptibility showed that most viruses displayed similar naïve sensitivities, confirming observations reported for clade C strains from Zimbabwe (Shafer et al., 1997). However, intrinsic resistance to NVP was seen with isolate 4743, carrying the G190A primary mutation. As confirmed in our studies, this mutation is not associated with primary resistance to DLV, and the IC_{50} for EFV was only slightly higher than that of the clade B control. This result corroborates data on the resistance impact of the G190A substitution in clade B (Bachelier et al., 2001).

The final drug concentration that selected for primary resistance mutations was significantly higher for clade B than clade C viruses for each of NVP (10 versus 2μM), EFV (1 versus 0.01μM) and Del (10 versus 1μM), respectively. Furthermore, resistant variants were fully selected more rapidly with the clade C isolates (8 or 9 weeks with NVP or DLV and 13 weeks with EFV) as compared with the clade B control (at least 15 weeks with NVP or DLV and 30 weeks with EFV). In general, at the middle interval of the selection period, the subtype B viruses harbored a mixture of wild type and mutated forms in regard to all the NNRTIs (NVP, DLV or EFV). These findings suggest that clade C viruses can more rapidly select for resistance to NNRTIs. Recently, it was reported that non-subtype B HIV-1 strains were likely to be less susceptible to highly active anti-retroviral therapy (HAART) (Caride et al., 2000). In addition, Non-B sequences were statistically associated with rapid progression to resistance after HAART, and had different mutational patterns than B isolates (Caride et al., 2000). Another recent study showed some evidence of HIV-1 subtype impact on the development of NNRTI resistance mutations; there was an increased prevalence of specific mutations and polymorphisms among non-clade B viruses that may have predisposed to NNRTI treatment failure (Pillay et al., 2000). These *in vivo* reports correlate with our observations in cell culture; the discrepancies we have seen in the development of NNRTI resistant mutations have not been previously noted *in vitro* for subtype C RT.

It is interesting to note that the Ethiopian clade C isolates 4742 and 4762 initially harbored a A98S secondary mutation associated with resistance to NVP.

After cell culture selection with NVP, a new S98I mutation appeared in isolate 4742. In subtype B HIV-1 strains, the mutation at this position has been reported to be A98G and has been observed in vivo (Richman et al., 1994). This is the first report on the presence of the S98I mutation in RT *in vitro* selected by NVP. In clade C resistant variants selected with NVP, several other amino acid changes were also generated, including A98I, A98S, K103N, V106A, V108I and Y181C. Except for A98I and A98S, all codon changes at positions 103, 106, 108 and 181 were generally noted in subtype B infected patients failing NVP therapy (Hirsch et al., 1998; Wainberg, 1999). Yet, the emergence of some NVP resistance mutations may be more accelerated in certain HIV-1 non-B subtypes and more facilitated by pre-existing genetic polymorphisms. In a recent clinical trial conducted in Uganda for prevention of mother-to-child transmission of HIV-1 with NVP, the K103N mutation was generated in 20% of treated women by 6 weeks after receiving a single dose of NVP at the onset of the labor (Jackson et al., 2000). This mutation not detected in samples at 33 months after delivery, showing that the accelerated emergence of resistance to NVP may not affect the prevention of HIV-1 transmission during the intrapartum period, but is a concern for the post-partum prevention of transmission via breastfeeding (Jackson et al., 2000).

After selection with DLV, a silent mutation, A62A, initially observed in Ethiopian isolate 4762, became A62V, a secondary mutation previously associated with multi-drug resistance against NRTIs (Hirsch et al., 1998). This shows that silent mutations at sites related to drug resistance in clade C RT have

potential impact in facilitating codon changes for emergence of resistance. A different secondary mutation at a site associated with cross-resistance among multiple NRTIs, i.e. substitution V75E, was generated in the Ethiopian clade C isolate 4743 during selection with DLV (Hirsch et al., 1998). Once again, this suggests that clade C RT may have specific patterns of drug resistance that need to be considered. In addition, these findings demonstrate that clade C viruses may progress rapidly to resistance after treatment with NNRTIs. Additional prospective studies need to be conducted in order to assess the incidence of drug resistance-related mutations in populations infected with subtype C strains and undergoing drug therapy.

It has been established that HIV-2 RT, showing 60% sequence homology with HIV-1 RT, is not inhibited by any of the known NNRTIs (Tantillo R et al. 1994). This has been mechanistically linked to differences in the NNRTI binding pocket of HIV-2 harboring the natural Y181I polymorphism. In other studies, ten Cameroonian group O viral isolates were shown to be naturally resistant to NNRTIs (Nev, Del, R82913) while showing sensitivity to NRTIs (AZT, ddI, ddC, 3TC) and PIs (Saq, Rit) (Descamps et al. 1997.Descamps et al. 1995). Group O viruses carry the natural Y181C polymorphism similar to the Y181I divergence seen in HIV-2. Four Romanian clade F isolates, showing 7.4% genotypic variation from clade B isolates, were also shown to have reduced sensitivity to TIBO, while demonstrating phenotypic susceptibility to other NNRTIs such as NVP, DLV as well as to NRTIs and PIs (Apetrei et al. 1998). In contrast, the phenotypic sensitivity of clade C isolates from five drug-naive infected

Zimbabweans to NRTIs and NNRTIs was reported to be similar to that of clade B isolates (Shafer et al. 1997).

In our study, the presence of certain secondary mutations associated with resistance to NNRTIs and to ZDV did not significantly decrease the susceptibility of Ethiopian clade C strains to RT inhibitors, except for 4743 that harbored a NVP resistance primary mutation.

Several immunodominant regions have been characterized in HIV-1 clade B RT. Therefore, the peptides harboring drug selected mutations that appear in these epitopes may be of interest in therapeutic immunization protocols to restrict emergence of antiviral drug resistance. However, little is known about potential divergence among RTs of different HIV-1 subtypes. We hypothesized that diversity in the *pol* regions of HIV-1 clade C, corresponding to known CTL and T-helper epitopes within clade B RT, could be important and confound immuno-therapeutic strategies that target RT immunogenic regions.

A total of 14 clade C antiviral treatment-naive isolates were included in our study, comprising the five previously charaterized clade C isolates (4742; 4743; 4761; 4762 and 4766) from Ethiopia and nine other HIV-1 isolates obtained from nine drug-naïve individuals originating from Botswana. Phylogenetic analysis of RT sequences and HMA patterns of *Env* genes confirmed the clade C status of the nine Botswana isolates. We have screened the diversity of immunodominant regions of HIV-1 subtype C RT and identified amino acid substitutions that may affect recognition of these epitopes by cellular immune response effectors.

Surprisingly, we found a polymorphism that clustered within certain CTL epitopes of clade C isolates from Ethiopia and Botswana. Such clustering has been reported in Gag-specific CTL epitopes in HIV-1 infected individuals (Buseyne et al., 1993). These clustered mutations in Gag may be required for HIV-1 escape from HLA-B27-restricted CTL responses (Kelleher et al., 2001). To date, there has been no previous report on the genotypic divergence of CTL epitope sequences in clade C RTs from different regions. Such inter- and intra-clade C variations may affect RT immunogenicity and CTL cross-reactivity for different strains of HIV-1, allowing viral escape from immune control.

In addition, our analysis of T-helper epitopes in clade C RT of Ethiopian and Botswana isolates has also shown that clustered polymorphisms were present in certain CD4+ T cell epitopes, mainly in the N-terminal part of the RT fingers and the C-terminal region of the RT palm subdomain. T-helper epitope diversity observed in clade C RT is another factor that may potentially contribute to a divergent immune cross-reactivity of RT regions. The role of CD4+ T cells in priming immune responses against HIV has been widely documented. T-Helper lymphocytes have been reported to be critical for the induction of CTL responses, as well as for maintaining CD8+ T-cell memory and for the maturation of CD8+ T-cell function (Ridge et al., 1998; McMichael and Rowland-Jones, 2001). The polymorphism found in clade C RT sequences, corresponding to known clade B regions that trigger immunodominant T-helper responses, emphasizes the need for global screening of distinct immunogenetic patterns among HIV-1 subtypes. This

may reveal immune correlates for a broadly cross-reactive immuno-therapeutic approach to prevent the destruction of CD4+ T cells by HIV.

Despite the natural polymorphisms among different HIV-1 subtypes, the recognition of RT epitopes by CTLs and T-helper cells has been reported to be affected by antiviral drug resistance mutations (Dalod et al., 1998; Gray et al., 1999; Samri et al., 2000). A series of mutations selected by NNRTIs and NRTIs can lead to viral drug resistance (Wainberg and Cameron, 1998; Wainberg, 1999). Mutations generated during HAART could also decrease immune responsiveness to RT, in the case of amino acid substitutions within epitopes that are normally recognized by CTLs and T-helper cells.

To identify drug resistance mutations that might be generated within immunogenic motifs of clade C RT, we used increasing concentrations of different NNRTIs (NVP, DLV and EFV) as well as NRTIs (3TC and ZDV). Experiments performed with Ethiopian and Botswana subtype C strains revealed a panel of common mutations at sites associated with drug resistance as well as odd amino acid changes, A62V, V75E, S98I, K103E, V106M and L210M seen particularly with NNRTIs. In the case of RT CTL recognition motifs, mutations at drug resistance sites were mainly noted within two relatively conserved immunogenic regions of RT overlapping CTL epitopes 103-118, 108-123, 175-184, and 180-190. In the case of RT T-helper epitopes, half of the drug resistance mutations arose in relatively conserved regions 48-73, 62-78, 88-100 and half arose in highly polymorphic epitopes, i.e. 171-191, 195-210, and 196-216. Many other mutations, unrelated to drug resistance sites, were also noted within regions

overlapping the CTL and T-helper epitopes of HIV-1 clade C RT. The impact of these residues, including the drug resistance mutations and amino acid substitutions due to clade C polymorphism, on the specific immunogenicity of clade C RT is unknown. Some antiviral drug mutations in clade B RT have been reported to increase the immunogenicity of previously poor immunogenic regions of RT (Samri et al., 2000).

We have mainly investigated the fingers and palm sub-domain of RT, as the majority of drug resistance mutations are generated within these areas of RT (Tantillo et al., 1994; Hirsch et al., 1998; Wainberg, 1999). We have noted the existence of an important polymorphism in the CTL and T-helper epitopes in clade C RT. An average sequence homology of 88.4 to 91.5% was found among Ethiopian and/or Botswana clade C isolates and other HIV-1 group M subtype prototype strains. These observations suggest that large immunological differences may exist among RTs.

The immunogenic properties of HIV-1 RT have been documented in several studies (Walker et al., 1988; van der Burg et al., 1995; Haas et al., 1998; Samri et al., 2000; Day et al., 2001). Thus, HIV-1 subtype natural polymorphisms that exist at critical amino acids may anchor positions within epitopes that may alter mechanisms of HIV-1 RT fragment processing and presentation, allowing immune escape of HIV-1 subtype variants (Del Val et al., 1991; Ossendorp et al., 1996; Goulder et al., 1997). Substantial evidence shows that the immune system may control and significantly suppress viral replication during early stages of HIV-1 infection (Koup et al., 1994; McMichael and Rowland-Jones, 2001).

Genotypic divergence noted in clade C RT suggests that a global characterization of CTL and T-helper anchor motifs and predicted drug selected mutations in HIV-1 non-B subtype RT is warranted. Knowledge of frequently arising baseline polymorphisms and drug-related mutations in the context of immune responsiveness to RT, may aid our understanding of the immuno-therapeutic control of HIV infection.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

The work presented herein was adapted from articles published or prepared for submission to refereed scientific journals. The following is a brief summary of my original work and contributions to the scientific research community under the supervision of Dr. M.A. Wainberg and in collaboration with several colleagues listed in the title page of each chapter.

Chapter 2. Despite relative similarities between various HIV-1 subtypes, HIV has displayed a great degree of genetic and biological variations. Since HIV-1 clade diversity represents a major challenge in regard to the HIV chemotherapy, drug resistance, and HIV vaccine development, we reviewed relevant data in the following areas: the classification and distribution of HIV-1 subtypes, diversity among viral genomes and structural genes, structural and functional variations in transcriptional promoters, variations within regulatory and accessory genes, diversity in co-receptor usage, cell tropism and syncytium-inducing phenotype, and effects of RT and PR genotypic polymorphism on drug susceptibility of different HIV-1 subtypes.

Chapter 3: We have investigated the genotypic diversity of Ethiopian clade C strains, in conjunction with phenotypic drug susceptibility and emergence of RT mutations conferring drug resistance. No previous *in vivo / in vitro* study investigated clade C patterns of antiretroviral drug resistance against inhibitors of RT. We found a KVEQ specific motif of silent mutations (amino acid 65, 106, 138, 161, respectively) at resistance sites present in the RT regions of all studied

Ethiopian isolates and all subtype C reference strains. In addition, we identified numerous resistance mutations, polymorphisms, and silent mutations at RT sites linked to resistance to NNRTIs and NRTIs (ZDV, multi-nucleoside resistance sites). We also showed that the final drug concentrations that selected for primary resistance mutations was significantly higher for clade B than clade C viruses for each of NVP (10 versus 2μM), EFV (1 versus 0.01μM) and Del (10 versus 1μM), respectively. In addition, resistant variants were fully selected more rapidly in the clade C isolates (8 or 9 weeks with NVP or DLV and 13 weeks with EFV) as compared with the clade B control (at least 15 weeks with NVP or DLV and 30 weeks with EFV). During cell culture experiments for selection of clade C variants resistant to NVP, we detected a new S98I substitution in RT that had not been previously reported. In addition, two odd mutations were detected during selection of DLV resistant mutants, i.e. A62V, previously noted as a silent mutation, A62A, and V75E mutations; both the latter are associated with secondary resistance against multiple NRTIs. This is the first report on the impact of baseline genotypic polymorphisms in clade C RT on emergence of drug resistance against NNRTIs and NRTIs.

Chapter 4: The issue of intra-clade C variation of resistance mutational frequency has not been studied previously. We have detected a series of baseline drug-naïve mutations at sites associated with drug resistance in clade C RT and PR. Certain distinct patterns were noted between clade C isolates from Ethiopia and Botswana in our study. A few resistance mutations, e.g. L214F in RT and

M36I in PR, were highly conserved within both Ethiopian and Botswanian clade C isolates. In addition, the R211K mutation in RT was highly conserved among isolates from Botswana and less so among Ethiopian strains. We also detected substitutions at resistance site 63 in PR, with L63P being noted most frequently. However, no mutation was identified at position 63 in the PRs of the five clade C isolates from Ethiopia.

Chapter 5: We examined the diversity of immunogenic regions of subtype C RT, identified baseline amino acid polymorphisms, and studied the frequencies of their occurrence among different clade C strains of two distinct African origins. We identified a sequence polymorphism that clustered among certain CTL epitopes of clade C isolates from Ethiopia and Botswana and we showed that these RT CTL epitopes were relatively conserved among subtype B and C strains (CTL epitopes 103-118, 108-123, 118-128, 128-136, 180-190 and 192-201). We also showed the existence of a clustered polymorphism in some CD4+ T cell epitopes, mainly in the N-terminal region of the RT fingers and the C-terminal domain of the RT palm. Within the CTL and T-helper epitope sequences of clade C RT, we used cell culture assays to generate a series of mutations at sites associated with NNRTI and NRTI resistance, as well as odd amino acid changes, e.g. A62V, V75E, S98I, K103E, V106M and L210M, seen with NNRTIs. In addition, we identified several other mutations, unrelated to drug resistance, within regions overlapping the CTL and T-helper epitopes of HIV-1 clade C RT. This is the first investigation of frequently arising amino acid substitutions as well

as drug-induced mutations that may affect recognition of these epitopes by the immune system and allow HIV-1 clade C viral escape.

REFERENCES.

Abebe A., D. Demissie, J. Goudsmit, M. Brouwer, C.L. Kuiken, G. Pollakis, H. Schuitemaker, A.L. Fontanet, and T.F. Rinke de Wit. 1999. HIV-1 subtype C syncytium and non-syncytium-inducing phenotypes and coreceptor usage among Ethiopian patients with AIDS. AIDS 13: 1305-1311.

Abrams D.I., A.I. Goldman, C. Launer et al. 1994. A comparative trial of didanosine or Zalcitabine after treatment with zidovudine in patients with human immunodeficiency virus infection. N. Engl. J. Med. 330: 657-662.

Adje C., R. Cheingsong, T.H. Roels, C. Maurice, G. Djomand, W. Verbiest, K. Hertogs, B. Larder, B. Monga, M. Peeters, S. Eholie, E. Bissagene, M. Coulibaly, R. Respess, S.Z. Wiktor, T. Chorba, and J.N. Nkengasong. 2001. High prevalence of genotypic HIV-1 drug-resistant strains among patients receiving antiretroviral therapy in Abidjan, Côte d'Ivoire. J. AIDS 26: 501-506.

Aiken C., J. Konner, N.R. Landau, M.E. Lenburg, and D. Trono. 1994. Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain. Cell 76: 853-864.

Alkhatib G., C. Combadiere, C.C. Broder, Y. Feng, P.E. Kennedy, P.M. Murphy, and E.A. Berger. 1996. CC-CKR5: A RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. Science 272: 1955-1958.

Apetrei C., D. Descamps, G. Collin, I. Loussert-Ajaka, F. Damond, M. Duca, F. Simon and F. Brun-Vezinet. 1998. Human Immunodeficiency Virus type 1 subtype F reverse transcriptase sequence and drug susceptibility. J. Virol. 72: 3534-3538.

Arion D., N. Kaushik, S. McCormic et al. 1998. Phenotypic mechanism of HIV-1 resistance to 3'-azido-3' deoxythymidine (AZT): increased polymerization processivity and enhanced sensitivity to pyrophosphate of the mutant viral reverse transcriptase. Biochemistry 37: 15908-15917.

Arnold E., A. Jacobo-Molina, R.G. Nanni, R.L. Williams, X. Lu, J. Ding, A.D.Jr. Clark, Zhang A., A.L. Feris, P. Clark, A. Hizi, and S.H. Hughes 1992. Structure of HIV-1 reverse transcriptase/DNA complex at 7 A resolution showing active site locations. Nature 357: 85-89.

Arnold E., K. Das, J. Ding, P.N.S. Yadav, Y. Hsiou, P.L. Boyer, and S.H. Hughes. 1996. Targeting HIV-1 reverse transcriptase for anti-AIDS drug design: structural and biological considerations for chemotherapeutic strategies. Drug Des. Disc. 13: 29-47.

Artenstein A.W., J. Coppola, A.E. Brown, J.K. Carr, E. Sanders-Buell, E. Galbarini, J.R. Mascola, T.C. VanCott, P. Schonbrood, and F.E. McCutchan.

1995. Multiple introductions of HIV-1 subtype E into the western hemisphere. The Lancet 346: 1197-1199.

Arthur et L.O., J.W. Jr. Bess, R.C. Sowder, et al. 1992. Cellular proteins bound to immunodeficiency viruses: implications for pathogenesis and vaccines. Sciences (Wash. DC) 258: 1935-1938.

Arts E., M.E. Quinones-Mateu, J.L. Albright, J.P. Marois, C. Hough, Z. Gu, and M.A. Wainberg. 1998. 3'-azido-3'-deoxythymidine (AZT) mediates cross-resistance to nucleoside analogs in the case of AZT-resistant human immunodeficiency virus type 1 variants. J. Virol. 72: 4858-4865.

Ayenie S., B.O. Johansson, M.O. Salminen, P. Leinikki, A. Sonnerborg, D. Work-Zewdie, S. Britton and O. Strannegard. 1991. HIV-1 in Ethiopia; Phylogenetic divergence from other HIV-1 strains. Virus Genes 5: 359-366.

Bacheler L., E.D. Anton, P. Kudish, D. Baker, J. Bunville, K. Krakowski, L. Bolling, M. Aujay, X.V. Wang, D. Ellis, M.F. Becker, A.L. Lasut, H.J. George, D.R. Spalding, G. Hollis, and K. Abremski. 2000. Human immunodeficiency virus type 1 mutations selected in patients failing efavirenz combination therapy. Antimicrobial Agents and Chemotherapy 44: 2475-2484.

Bacheler L., S. Jeffrey, G. Hanna, R. D'Aquila, L. Wallace, K. Logue, B. Cordova, K. Hertogs, B. Larder, R. Buckery, D. Baker, K. Gallagher, H. Scarnati, R. Tritch, and C. Rizzo. 2001. Genotypic correlates of phenotypic resistance to efavirenz in virus isolates from patients failing

Back N.K., M. Nijhuis, W. Keulen et al. 1996. Reduced replication of 3TC-resistant HIV-1 variants in primary cells due to a processivity defect of reverse transcriptase enzyme. EMBO J. 15: 4040-4049.

Baltimore D. RNA-dependent DNA polymerase in virions of RNA tumour viruses. 1970. Nature 226: 1209-1211.

Balzarini J., A. Karlsson, M.J. Perez-Perez et al. 1993. HIV-1 specific reverse transcriptase inhibitors show differential activity against HIV-1 mutant strains containing different amino acid substitutions in the reverse transcriptase. Virology 192: 246-253.

Barat C., V. Lullien, O. Schatz, G. Keith, M.T. Nugeyre, F. Gruninger-Leitch, F. Barre-Sinoussi, S.F. Le Grice, and J.L. Darlix. 1989. HIV-1 reverse transcriptase specifically interacts with the anticodon domain of its cognate primer tRNA. EMBO J. 8: 3279-3285.

Becker M.L.B., G. de Jager and W.B. Becker. 1995. Analysis of partial gag and env gene sequences of HIV type I strains from Southern Africa - AIDS Res. Human. Retroviruses 11: 1265-1267.

Becker-Pergola G., P. Kataaha, L. Johnston-Dow, S. Fung, J.B. Jackson and S.H. Eshleman. 2000. Analysis of HIV type 1 Protease and reverse transcriptase in anti-retroviral drug-naïve Ugandan adults. AIDS Res. Hum. Retroviruses 8: 807-813.

Bedinger P., A. Monarty, R.C. von Borstel. et al. 1988. Internalization of the human immunodeficiency virus does not require the cytoplasmic domain of CD4. Nature 334: 162-165.

Bentwich Z., A. Kalinkovich, Z. Weisman, and Z. Grossman. 1998. Immune activation in the context of HIV infection. Clin. Exp. Immunol. 111: 1-2.

Bentwich Z., G. Maartens, D. Torten, A.A. Lal and R.B. Lal. 2000. Concurrent infections and HIV pathogenesis. AIDS, 14: 2071-2081.

Berg J. M. 1986. Potential metal-binding domains in nucleic acid binding proteins. Science 232: 485-487.

Bikandou B., J. Takehisa, I. Mboudjeka, E. Ido, T. Kuwata, Y. Miyazaki, H. Moriyama, Y. Harada, Y. Taniguchi, H. Ichimura, M. Ikeda, P.J. Ndolo, M.Y. Nzoukoudi, R. M'Vouenze, M. M'Pandi, H.J. Parra, P. M'Pele, and M. Hayami. 2000. Genetic subtypes of HIV type 1 in Republic of Congo. AIDS Research and Retroviruses 16: 613-619.

Birk B. and A. Sonnerborg. 1998. Variations in HIV-1 *pol* gene associated with reduced sensitivity to anti-retroviral drugs in treatment-naïve patients. AIDS, 12: 2369-2375.

Bjõrndal A., H. Deng, M. Jansson, J.R. Fiore, C. Colognesi, A. Karlsson, J. Albert, G. Scarlatti, D.R. Littman, and E.M. Fenyõ. 1997. Co-receptor usage of primary human immunodeficiency virus type 1 isolates varies according to biological phenotype. J. Virol. 71: 7478-7487.

Bleul C.C., L. Wu, J.A. Hoxie, T.A. Springer, and C.R Mackay. 1997. The HIV co-receptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes. Proc. Natl. Acad. Scien. USA 94: 1925-1930.

Boden D. and M. Markowitz. 1998. Resistance to immunodeficiency virus type 1protease inhibitors. Antimicrob.Agents and Chemotherapy 42: 2775-2783.

Bouhamdan M.,S. Benichou, F. Rey, J.M. Navarro, I. Agostini, B. Spire, Camonis J., Slupphaug G., Vigne R., Benarous R., and Sire J. 1996. Human immunodeficiency virus type 1 Vpr protein binds to the uracil DNA glycosylase repair enzyme. J. Virol. 70: 697-704.

Boulerice F., S. Bour, R. Geleziunas et al. 1990. High frequency of isolation of defective human immunodeficiency virus type 1 and heterogeneity of viral gene expression in clones of infected U-937 cells. J. Virol 64: 1745-1755.

Bour S., U. Schubert, and K. Strebel. 1995. The Human immunodeficiency virus type 1 Vpu protein specifically binds to the cytoplasmic domain of CD4: implications for the mechanism of degradation. J. Virol. 69: 1510-1520.

Boyer J.C., K. Bebenek, and T.A. Kunkel. 1992. Unequal human immunodeficiency virus type 1 reverse transcriptase error rates with RNA and DNA templates. Proc. Natl. Acad. Sci. USA 89: 6919-6923.

Boyer P.L., J. Ding, E. Arnold et al. 1994. Subunit specificity of mutations that confer resistance to non-nucleoside inhibitors in human immunodeficiency virus type 1 reverse transcriptase. Antimicrob. Agents Chemother. 38: 1909-1914.

Brander C., and P.J.R Goulder. 2000. The evolving field of HIV CTL epitope mapping: new approaches to the identification of novel epitopes. In C. Brander,

B.T.M. Korber, B.D. Walker, R.A. Koup, J. Moore, B. Haynes, and G. Meyers (ed.), HIV molecular immunology database. Los Alamos National Laboratory, Los Alamos, New Mexico.

Bukrinsky M.I., N. Sharova, M.P. Dempsey et al. 1992. Active nuclear import of human immunodeficiency virus type 1 preintegration complexes. Proc.Natl. Acad. Sci. USA 89: 6580-6584.

Bukrinsky M.I., N. Sharova, T.L. McDonald, T. Pshkarskaya, W.G. Tarpley and M. Stevenson. 1993. Association of integrase, matrix and reverse transcriptase antigens of HIV-1 with viral nucleic acids following acute infection. Proc. Natl. Acad. Sci. USA 90: 6125-6129.

Buonocore L., T.G. Turi, B. Crise, and J.K. Rose. 1994. Stimulation of heterologous protein degradation by the vpu protein of HIV-1 requires the transmembrane and cytoplasmic domains of CD4. Virology 204: 482-486.

Burke D.S., and F.E. McCutchan. 1997. Global distribution of human immunodeficiency virus-1 clades. In: AIDS: Biology, diagnosis, treatment and prevention. (Vincent T, De Vita J, Hellman S, Rosenberg SA, eds). Philadelphia: Lippiancott-Raven: 119-126.

Buseyne F., M. McChesney, F. Porrot, S. Kovarik, B. Guy, and Y. Riviere. 1993. Gag-specific cytotoxic T lymphocytes from human immunodeficiency virus type 1-infected individuals: Gag epitopes are clustered in three regions of the $p24^{gag}$ protein. J. Virol. 67: 694-702.

Caliendo A.M., A. Savara, D. An et al. 1996. Effectsof Zidovudine-selected human immunodeficiency virus type 1 reverse transcriptase amino acid substitutions on processive DNA synthesis and viral replication. J. Virol. 70: 2146-2153.

Cao H., P. Kanki, J.L. Sankale, A. Dieng-Sarr, G.P. Mazzara, S.A. Kalams, B. Korber, S. Mboup, and B.D. Walker. 1997. Cytotoxic T-lymphocytes cross-reactivity among different human immunodeficiency virus type 1 clades: implications for vaccine development. J. Virol. 71: 8615-8623.

Caride E., Hertogs, B. Larder, Dehertogh, Brindeiro, E. Machado, Cam de Sa, W.A. Eyer-Silva, F.S. Sion, L.F.C. Passioni, J.A. Meneses, A.R. Calazans, and A.Tanuri. 2000. Genotyping and phenotyping analysis of B and non-B HIV-1 subtypes from Brazilian patients under HAART. Antiviral Therapy 5 (Supplement 3): 128. Abstract 164.

Carr J.K., T. Laukkanen, M.O. Salminen, J. Albert, A. Alaeus, B. Kim, E. Sanders-Buell, D.L. Birx, and F.C. McCutchan. 1999. Characterization of subtype A HIV-1 from Africa by full genome sequencing. AIDS 13: 1819-1826.

Carr J.K., B.T. Foley, T. Leitner, M. Salminen, B. Korber, and F. McCutchan. 1998. (eds): Human Retrovirus and AIDS-1998: A compilation and analysis of nucleic acid and amino acid sequences. Los Alamos National Laboratory, Los Alamos, New Mexico, III10-III19.

Charneau P., and F. Clavel. 1991. A single-stranded gap in human immunodeficiency virus unintegrated DNA defined by a central copy of the polypurine tract. J. Virol. 65: 2415-2421.

Charneau P., M. Alizon and F. Clavel. 1992. A second origin of DNA plus-strand synthesis is required for optimal human immunodeficiency virus replication. J. Virol. 66: 2814-2820.

Chen M., C. Garon and T. Papas. 1980. Native ribonucleoprotein is an efficient transcriptional complex of avian myeloblastosis virus. Proc. Natl Acad. Sc. USA 77: 1296-1300.

Chen Z., Y. Huang, X. Zhao, E. Skulsky, D. Lin, A. Gettie, and D.D. Ho. 2000. Enhanced infectivity of an R5-tropic simianéhuman immunodeficiency virus

carrying human immunodeficiency virus type 1 subtype C envelope after serial passages in pig-tailed macaques (Macaca nemestrina). J.Virol. 74: 6501.

Choe H., M. Farzan, Y. Sun, N. Sullivan, B. Rollins, P.D. Ponath, L. Wu, C.R. Mackay, G. LaRosa, W. Newman, N. Gerard, C. Gerard, and J. Sodroski. 1996. The β-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. Cell 8: 1135-1148.

Cimarelli A., and Luban J. 1999. Translation elongation factor 1-alpha interacts specifically with the human immunodeficiency virus type 1 Gag polyprotein. J. Virol. 73: 5388-5401.

Cocchi F., A.L. DeVico, A. Garzino-Demo, S.K. Arya, R.C. Gallo, and P. Lusso. 1995. Identification of RANTES, MIP-1α, and MIP-1β as the major HIV-suppressive factors produced by CD8+ T cells. Science 270: 1811-1815.

Coffin J. M. 1979 Structure, replication and recombination of retroviruses genomes : some unifying hypotheses. J. Gen. Virol. 42: 1-26.

Coffin J.M. 1990. Endogenous viruses. In : Virology, 2nd ed., Fields B.N., Knipe D.M., Chanock R.M., Melnick J., Roizman B., and Slope R. ed. (New York: Raven), 1437-1500.

Coffin J.M., S.H. Hughes, and H.E. Varmus. 1997. Retroviruses. Cold Spring Harbor Laboratory Press.

Cohen E. A., E.F. Terwilliger, J.G. Sodroski, and W.A. Haseltine. 1988. Identification of a protein encoded by the vpu gene of HIV-1. Nature 334: 532-534.

Cohen E.A., G. Dehni, J.G. Sodroski and W.A. Haseltine. 1990. Human immunodeficiency virus *vpr* product is a virion-associated regulatory protein. J. Virol. 64: 3097-3099.

Collier A.C., R.W. Coombs, M.A. Fischl et al. 1993. Combination therapy with zidovudine and didanosine compared with zidovudine alone in HIV-1 infection. Ann. Intern. Med. 119: 786-93.

Cornelissen M., G. Mulder-Kampinga, J. Veenstra, F. Zorgdrager, C. Kuiken, S. Hartman, J. Dekker, L. van der Hoek, C. Sol, R. Coutinho, and J. Goudsmit. 1995. Syncytium-inducing (SI) phenotype suppression at seroconversion after intramuscular inoculation of a non-syncytium-inducing/SI phenotypically mixed human immunodeficiency virus population. J. Virol. 69: 1810-1818.

Cornelissen M., R. Van Den Burg, F. Zorgdrager, V. Lukashov and J. Goodsmit. 1997. *Pol* Gene diversity of five Human Immunodeficiency virus type 1 subtypes:

evidence for naturally occurring mutations that contribute to drug resistance, limited recombination patterns, and common ancestry for subtypes B and D. J. Virol. 71: 6348-6358.

Couto-Fernandez J.C., W. Janssens, L. Heyndrickx, J. Motte, K. Fransen, M. Peeters, E. Delaporte, B. Galvao-Castro, P. Piot, and G. van der Groen. 1994. Genetic and antigenic variability of HIV type 1 in Brazil. AIDS Research and Human Retroviruses 10: 1157-1163.

Craig J.C., I.B. Duncan, L. Whittaker, and N.A. Roberts. 1993. Antiviral synergy between inhibitors of HIV proteinase and reverse transcriptase. Antimicrob. Agents Chemother. 4: 161-166.

Criag C., and G. Moyle. 1997. The development of resistance of HIV-1 to Zalcitabine. AIDS 11: 271-279.

Crise B., L. Buonocore and J.K. Rose. 1990. CD4 is retained in the endoplasmic reticulum by the human immunodeficiency virus envelope glycoprotein precursor. J. Virol. 64: 5585-5593.

Cullen B.R. Regulation of Human immunodeficiency virus replication. 1991. Ann. Rev. Microbiol. 45: 219-250.

Cullen B.R. 1992. Mechanism of action of regulatory proteins encoded by complex retroviruses. Microbiological Reviews 56: 375-394.

Culmann-Penciolelli B., S. Lamhamedi-Cherradi, I. Couillin, N. Guegan, J.P. Levy, Guillet J.G., and E. Gomard. 1994. Identification of multi-restricted immunodominant regions recognized by cytolytic T lymphocytes in the human immunodeficiency virus type 1 Nef protein. J. Virol. 68: 7336-7343.

Dalod M., M. Harzic, I. Pellegrin, B. Dumon, B. Hoen, D. Sereni, J.C. Deschemin, J.P. Levy, A. Venet, and E. Gomard. 1998. Evolution of cytotoxic T lymphocytes responses to human immunodeficiency virus type 1 in patients with symptomatic primary infection receiving antiretroviral triple therapy. J.Infect. Dis. 178: 61-69.

Darlix J.L., M. Lapadat-Tapolsky, H. de Rocquigny and B.P. Roques. 1995. First Glimpses at structure-function relationships of the nucleocapsid protein of retroviruses. J. Mol. Biol. 254: 523-537.

Davis F., P. Msellati, N. Meda, et al. 1999. 6 month efficacy, tolerance, and acceptability of a short regimen of oral zidovudine to reduce vertical transmission of HIV in breastfed children in Cote d'Ivoire and Burkina Faso: a double-blind placebo-controlled multicentre trial. Lancet 353: 786-792.

Day C.L., A.K. Shea, M.A. Altfeld, D.P. Olson, S.P. Buchbinder, F.M. Hecht, E.S. Rosenberg, B.D. Walker, and S.A. Kalams. 2001. Relative dominance of epitope-specific cytotoxic T-lymphocyte responses in human immunodeficiency virus type 1-infected persons with shared HLA alleles. J.Virol. 75: 6279-6291.

Debouck C. 1992. The HIV-1 protease as a therapeutic target for AIDS. AIDS Res. Hum. Retroviruses 8: 153-164.

De Clercq E. 1992. HIV inhibitors targeted at the reverse transcriptase. AIDS Res. Hum. Retroviruses 8: 119-134.

Deeks S.G., F.M. Hecht, M. Swanson et al. 1999. HIV-1 RNA and CD4 cell counts response to both initial and salvage therapy. AIDS: 13: F35-43.

Del Val M., H.J. Schlicht, T. Ruppert, M.J. Reddehase, and Koszinowski. 1991. Efficient processing of an antigenic sequence for presentation by MHC class 1 molecules depends on its neighboring residues in the protein. Cell 66: 1145-1153.

de Jong J.J., A. de Ronde, W. Keulen, M. Termestte, and J. Goudsmit. 1992. Minimal requirements for the human immunodeficiency virus type 1 V3 domain to support the syncytium-inducing phenotype: analysis by single amino acid substitution. J. Virol. 66: 6777-6780.

Delaporte E., W. Janssens, M. Peeters, A. Buve, G. Dibanga, J.L. Perret, V. Ditsambou, J.R. Mba, M.C. Georges Courbot, A. Georges, A. Bourgeois, B. Samb, D. Henzel, L. Heyndrickx, K. Fransen, G. van der Groen, and B. Larouze. 1996. Epidemiological and molecular characteristics of HIV infection in Gabon, 1986-1994. AIDS 10: 903-910.

Delwart E.L., E.G. Shpaer, F.E. McCutchan, J. Louwagie, M. Grez, H. Rubsamen-Waigmann, J.I. Mullins. 1993. Genetic relationships determined by DNA heteroduplex mobility assay: analysis of HIV-1 Env genes. Sciences 262: 1257-1261.

Demeter L., L. Resnick, T. Nawaz et al. 1993. Phenotypic and genotypic analysis of atevirdine (ATV) susceptibility of HIV-1 isolates obtained from patients receiving ATV monotherapy in a phase 1 clinical trial (ACTG 187): comparison to patients receiving combination therapy with ATV and zidovudine. Third Workshop on Viral Resistance, Gaithersburg.

Demeter L.M., R.W. Shafer, P.M. Meehan, J. Holden-Wiltse, M.A. Fischl, W.W. Freimuth, M.F. Para, and R.C. Reichman. 2000. Delavirdine susceptibilities and associated reverse transcriptase in human immunodeficiency virus type 1 isolates from patients in a phase I/II trial of delavirdine monotherapy (ACTG 260). Antimicrob. Agents Chemother. 44: 794-797.

Deng H., R. Lui, W. Ellmeier, S. Choe, D. Unutmaz, M. Burkhart, P. DiMarzio, S. Marmon, R.E. Sutton, M. Hill, C.B. Davis, S.C. Pelper, T.J. Schall, D.R. Littman, and N.R. Landou. 1996. Identification of a major co-receptor for primary isolates of HIV-1. Nature 381: 661-666.

Descamps D., G. Collin, F. Letourneur, C. Apetrei, F. Damond, I. Loussert-Ajaka, F. Simon, S. Saragosti, and F. Brun-Vezinet. 1997. Susceptibility of human immunodeficiency virus type 1 group O isolates to antiretroviral agents: in vitro phenotypic and genotypic analysis. J. Virol. 71: 8893-8898.

Descamps D., G. Collin, I. Loussert-Ajaka, S. Saragosti, F. Simon, and F. Brun-Vezinet. 1995. HIV-1 group O sensitivity to antiviral drugs. AIDS 9: 977-978.

Dickson C., R. Eisenman, H. Fan, H. Hunter, and N. Reich. 1985. Protein biosynthesis and assembly. In *RNA Tumor Viruses*, (Weiss R., N. Teich., H. Varmus and J. Coffin eds), part 2, second edition, pp. 513-648. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Dietrich U., M. Grez, H. von Briesen, B. Panhans, M. Geissendorfer, H. Kuhnel, J. Maniar, G. Mahambre, M.L.B. Becker and H. Rubsamen-Waigmann. 1993. HIV-1 strains from India are highly divergent from prototypic African and US/European strains, but are linked to a South African isolate. AIDS 7: 23-27.

Dighe P.K., B. Korber, and B.T. Foley. 1997. Global variation in the HIV-1 V3 region, p.74-206. In B. Korber, B. Hahn, B.T. Foley, J. Mellors, T. Leitner, G. Myers, F. McCutchan, and C. Kuiken (ed), Human retrovirus and AIDS, vol.III. Theoretical biology and Biophysics, Los Alamos National Laboratory, Los Alamos, N. Mex.

Dittmar M.T., G. Simmons, S. Hibbitts, O'Hare, S. Louisirirotchanakul, S. Beddows, J. Weber, P.R. Clapham, and R.A. Weiss. 1997. Langerhans cell tropism of human immunodeficiency virus type 1 subtype A through F isolates derived from different transmission groups. J. Virol. 71: 8008-8013.

Doranz B.J., J. Rucker, Y. Yi, R.J. Smyth, M. Samson, S.C. Pelper, M. Parmentier, R.G. Collman, and R.W. Doms. 1996. A dual- tropic primary HIV-1 isolate that uses fusin and β-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusin cofactors. Cell 85: 1149-1158.

Dorfman T.F., F. Mammano, R.L. Shoeman, and H.G. Gottlinger. 1994. Role of matrix protein in the virion association of the HIV-1 envelope protein. J. Virol. 68: 1689-1696.

Dornsife R.E., M.H. St Clair, A.T. Huang et al. 1991. Anti-human immunodeficiency virus synergism by zidovudine (3'-azidothymidine) and

didanosine (dideoxyinosine) contrasts with their additive inhibition in normal human marrow progenitor cells. Antimicrob. Agents Chemother. 35: 322-8.

Downing R., D. Pieniazek, D.J. Hu, B. Biryahwaho, C. Fridlund, M.A. Rayfield, S.D. Sempala, and R.B. Lal. 2000. Genetic characterization and phylogenetic analysis of HIV-1 subtype C from Uganda. AIDS Research and Retroviruses 16: 815-819.

Dueweke T.J., T. Pushkarskaya, S.M. Poppe et al. 1993. A mutation in reverse transcriptase of bis(hetroary)piperazine-resistant human immunodeficiency virus type 1 that confers increased sensitivity to other non-nucleoside inhibitors. Proc. Natl. Acad. Sci. USA 90: 4713-4717.

Dupuis M., S.K. Kundu, and T.C. Merigan. 1995. Characterization of HLA-A 0201-restricted cytotoxic T cell epitopes in conserved regions of the HIV type 1 gp160 protein. J. Immunol. 155:2232-2239.

Durant J., P. Clevenbergh, P. Halfon, P. Delgiudice, S. Porsin, P. Simonet, N. Montagne, C.A.B. Boucher, J.M. Schapiro, and P. Dellamonica. 1999. Drug resistance genotyping in HIV-1 therapy: the VIRADAPT randomized controlled trial. Lancet 353: 2195-2199.

DiMarzo Veronese F.D., T.D. Copeland, A.L. DeVico, R. Rahman, S. Oroszlan, R.C. Gallo and M.G. Sarngadharan. 1986. Characterization of highly immunogenic p66/p51 as the reverse transcriptase of HTLV-III/LAV. Science 231: 1289-1291.

Essex M. 1998. State of the HIV pandemic. J. Human Virology 1: 427-429.

Essex M. 1999. Human immunodeficiency viruses of the developing world. Adv.Virus Res. 53: 71-88.

Fan L., and K. Peden. 1992. Cell-free transmission of vif mutants of HIV-1. Virology 190: 19-29.

Fatkenheuer G., A. Theisen, J. Rockstroh et al. 1997. Virological treatment failure of protease inhibitor therapy in an unselected cohort of HIV-infected patients. AIDS 11: F113-116.

Farmerie W.G., D.D. Leob, N.C. Casavant, C.A.D. Hutchinson, M.H. Egell, and R. Swanstrom. 1987. Expression and processing of the AIDS virus reverse transcriptase in Escherichia coli. Science 236: 305-308.

Felber B.K. and G.N. Pavlakis. 1993. Molecular biology of HIV-1: positive and negative regulatory elements important for virus expression. AIDS 7: S51-S62.

Fenyo E.M., L. Morfeldt-Manson, F. Chiodi, A. Lind, A. von Gegerfelt, J. Albet, E. Olausson, and B. Asjo. 1988. Distinct replicative and cytopathic characteristics of human immunodeficiency virus isolates. J.Virol. 62: 4414-4419.

Fischl M.A., D.D. Richman, M.H. Grieco et al. 1987. The efficacy of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex: a double-blind placebo-controlled trial. N. Engl. J. Med. 317: 185-191.

Fitzgibbon J.E., R.M. Howell, C.A. Haberzettl, S.J. Sperber, D.J. Gocke, and D.T. Dubin. 1992. Human immunodeficiency virus type 1 pol gene mutations which cause decreased susceptibility to 2',3'-dideoxycytidine. Antimicrob. Agents Chemother. 36: 153-157.

Fletcher T.M., B. Brichacek, Sharova N., M.A. Newman, Stivahtis, P.M. Sharp, M. Emerman, B. H. Hahn and M. Stevenson. 1996. Nuclear import and cell cycle arrest functions of the HIV-1 Vpr protein are encoded by two separate genes in HIV-2/SIV_{SM}. The EMBO Journal 15: 6155-6165.

Frost S.D.W., and A.R. McLean. 1994. Quasispecies dynamics and the emergence of drug resistance during zidovudine therapy of HIV infection. AIDS 8: 323-332.

Furfine E.S., and J.E. Reardon. 1991. Reverse transcriptase: RNAse H from the human immunodeficiency virus. Relationship of the DNA polymerase and RNA hydrolysis activities. J. Biol. Chem. 266: 406-412.

Furman P.A., J.A. Fyfe, M.H. St Clair et al. 1986. Phosphorylation of 3'-azido-3'deoxythymidine and selective interactions of the 5'-triphosphate with human immunodeficiency virus reverse transcriptase. Proc. Natl. Acad. Sci. USA 83: 8333-8337.

Furtado M.R., D.S. Callaway, J.P. Phair et al. 1999. Persistence of HIV-1 transcription in peripheral blood mononuclear cells in patients receiving potent antiretroviral therapy. N. Engl. J. Med. 340:1614-1622.

Gabuzda D.H., H. Li, K. Lawrence, B.S. Vasir, K. Crawford, and E. Langhoff. 1992. Essential role of *vif* in establishing productive HIV-1 infection in peripheral blood T lymphocytes and monocytes/macrophages. J. Acquired Immune Defic. Syndr. 7: 908-915.

Gallay P., S. Swingler, J. Song, F. Bushman, and D. Trono. 1995. HIV nuclear import is governed by the phosphotyrosine-mediated binding of matrix to the core domain of integrase. Cell 83: 569-576.

Garcia J.V., J. Alfano and A.D. Miller. 1993. The negative effect of human immunodeficiency virus type 1 nef on cell surface CD4 expression is not species specific and requires the cytoplasmic domain of CD4. J. Virol. 67: 1511-1516.

Garcia-Lerma J.G., P.J. Gerrish, A.C. Wright, S.H. Qari, and W. Heneine. 2001. Evidence of a role for the Q151L mutation and the viral background in development of multiple dideoxynucleoside-resistant human immunodeficiency virus type 1. J. Virol. 74: 9339-9346.

Garret E., L. Tiley and B. Cullen. 1991. Rev activates expression of the HIV-1 Vif and Vpr gene products. J. Virol. 65: 1653-1657.

Gao F., L. Yue, D.L. Robertson, S.C. Hill, H. Hui, R.J. Biggar, A.E. Neequaye, T.M. Whelan, D. Ho, G.M. Shaw, P.M. Sharp, and B.H. Hahn. 1994. Genetic Diversity of human immunodeficiency virus type 2: evidence for distinct sequence subtypes with differences in virus biology. J.Virol. 68: 7433-7447.

Gao F., D.L. Robertson, C.D. Carruthers, S.G. Morrisson, B. Jian, Y. Chen, F. Barre-Sinoussi, M. Girard, A. Srinivasan, A.G. Abimiku, G.M. Shaw, P.M. Sharp, and B. Hahn. 1998. A comprehensive panel of near-full-lengh clones and reference sequences for non-subtype B isolates of Human immunodeficiency virus type 1. J.Virology 72: 5680-5698.

Gao Q., Z. Gu, M.A. Parniak, X. Li, and M.A. Wainberg. 1992. In vitro selection of variants of human immunodeficiency virus type 1 resistant to 3'-azido-3'-deoxythymidine and 2',3'-dideoxyinosine. J. Virol. 66: 12-19.

Gao Q., Z. Gu, H. Salomon et al. 1994. Generation of multiple drug resistance by sequential in vitro passage of the Human immunodeficiency Virus type 1. Arch. Virol., 36: 111-122.

Gaynor R. 1992. Cellular transcription factors involved in the regulation of HIV-1 gene expression. AIDS 6: 347-363.

Gelderblom H.R., E.H.S. Hausmann, M. Ozel et al. 1987. Fine structure of human immunodeficiency virus (HIV) and immunolocalization of structural proteins. Virology 156: 171-176.

Gelderblom H.R., M. Ozel and G. Pauli. 1989. Morphogenesis and morphology of HIV. Structure-function relations. Arch. Virol. 106: 1-13.

Gelderblom H.R. 1991. Assembly and morphology of HIV: potential effect of structure on viral function. AIDS 5: 617-638.

Geretti A.M. and P. Easterbrook. 2001. Antiretroviral resistance in clinical practice. International Journal of STD & AIDS 12: 145-153.

Goff S.P. 1990. Retroviral reverse transcriptase: synthesis, structure, and function. J. Acq. Immune Def. Syn. 3 : 817-831.

Gõtte M., D. Arion, L. Cellai et al. 1999. Effects of nucleoside and non-nucleoside analogue RT inhibitors on the initiation of plus-strand DNA synthesis. Antiviral Therapy 4: 18-19, abstract 23.

Gõtte M., K. Diallo, B. Brenner, M. Oliviera, D. Moisi, and M.A Wainberg. 2001. AZT pressure accelerates the disappearance of the M184V mutation. Tenth Annual Canadian Conference on HIV/AIDS Research, CAHR, Toronto, Canada. Abstract 123.

Gõtte M. and M.A Wainberg. 2000. Biochemical mechanisms involved in overcoming HIV resistance to nucleoside inhibitors of reverse transcriptase. Drug Resistance Updates 3: 30-38.

Goulder P.J., R.E. Phillips, R.A. Colbert, S. McAdam, G. Ogg, M.A. Nowak, P. Giangrande, G. Luzzi, B. Morgan, A. Edwards, A.J. McMichael, and J.S. Rowland. 1997. Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. Nat.Med. 3: 212-217.

Gray C.M., J. Lawrence, J.M. Schapiro, J.M. Altman, M.A. Winters, M. Crompton, M. Loi, S.K. Kundu, M.M. Davis, and T.C. Merigan. 1999. Frequency of class 1 HLA-restricted anti-HIV CD8+ T cells in individuals receiving highly active antiretroviral therapy (HAART). J.Immunol.162: 1780-1789.

Grossman Z., E. Paxinos, S. Maayan, E. Shahar, F. Mileguir, C. Petropoulos, and J. Schapiro. 2001. The development of protease inhibitor resistance in clade C HIV-1: Role of baseline drug susceptibility. The 1st IAS Conference on HIV Pathogenesis and treatment, July, Buenos Aires, Argentina. Abstract 578.

Gupta K., Ott D., Hope T.J., Siliciano R.F., and Boeke J.D. 2000. A human nuclear shuttling protein that interacts with human immunodeficiency virus type 1 matrix is packaged into virions. J.Virol. 74:11811-11824.

Gurtler L.G., P.H. Hauser, J. Eberle, A. von Brunn, S. Knap, L. Zekeng, J.M. Tsague, and L. Kaptue. 1994. A new subtype of human immunodeficiency virus type 1 (MVP-5180) from Cameroon. J. Virol. 68: 1581-1585.

Gu Z., Q. Gao, X. Li, M.A. Parniak, and M.A. Wainberg. 1992. Novel mutation in the human immunodeficiency virus type 1 reverse transcriptase gene encodes cross-resistance to 2',3'-dideoxyinosine and 2',3'-dideoxycytidine. J. Virol. 66: 7128-7135.

Gu Z., R.S. Fletcher, E.J. Arts et al. 1994a. The K65R mutant reverse transcriptase of HIV-1 cross-resistance to 2',3'-dideoxycytidine, 2',3'-dideoxy-3'-thiacytidine, and 2',3'-dideoxyinosine shows reduced sensitivity to specific dideoxynucleoside triphosphate inhibitors in vitro. J. Biol. Chem. 269: 28118-28122.

Gu Z., Q. Gao, H. Fang et al. 1994b. Identification of novel mutations that confer drug resistance in the human immunodeficiency virus polymerase gene. Leukemia. 8 (Suppl. 1): 5166-5169.

Gundhard H.F., D.V. Havlir, S. Fiscus et al. 2001. Residual human immunodeficiency virus (HIV) type 1 RNA and DNA in lymph nodes and HIV RNA in genital secretions and in cerebrospinal fluid after suppression of viremia for 2 years. J. Infect. Dis. 183: 1318-1327.

Haas G., A. Samri, E. Gomard, A. Hosmalin, J. Duntze, J.M. Bouley, H.G. Ihlenfeldt, C. Katlama, and B. Autran. 1998. Cytotoxic T-cell responses to HIV-1 reverse transcriptase, integrase and protease. AIDS 12: 1427-1436.

Haesevel de M.V., J.L. Decourt, R. J. Deeleys et al. 1994. Genomic cloning and complete sequence of a highly divergent African Human Immunodeficiency Virus isolate. J. Virol. 68:1586-1596.

Hahn B.H., G.M. Shaw, S.K. Arya, et al. 1984. Molecular cloning and characterization of the HTLV-III virus associated with AIDS. Nature 312: 166.

Hahn B.H., M.A. Gonda, G.M. Shaw, M. Popovic, R.C. Gallo, and F. Wong-Staal. 1985. Genomic diversity of the acquired immune deficiency syndrome virus HTLV-III: different viruses exhibit greatest divergence in their envelope genes. Proc. Natl. Acad. Sci. USA 82: 4813-4817.

Hammer S.M., H.A. Kessler, and M.S. Saag 1994. Issues in combination antiretroviral therapy: a review. J.AIDS 7(Suppl. 2): S24-S37.

Hart G.J., D.C. Orr, C.R. Penn et al. 1992. Effects of (-)2'-deoxy-3'-thiacytidine (3TC) 5'-triphosphate on human immunodeficiency virus reverse transcriptase and mammalian DNA polymerases alpha, beta and gamma. Antimicrob. Agents Chemother 37: 918-920.

Heaphy S., C. Dingwall, I. Ernberg, M.J. Gait, S.M. Green, J. Karn, A.D. Lowe, M. Singh and M.A. Skinner. 1990. HIV-1 regulator of virion expression (rev) binds to an RNA stem-loop structure located within the rev-responsive element region. Cell 60: 685-693.

Heinzinger N.K., M.I. Bukrinsky, S.A. Haggerty S.A., A.M. Ragland, V. Kewalramani, M.A. Lee, H.E. Gendelman, L. Ratner, M. Stevenson and M.

Emerman. 1994. The Vpr protein of HIV-1 influences nuclear localization of viral nucleic acids in non-dividing cells. Proc. Natl. Acad. Sci. USA 91: 7311-7315.

Henderson A.J., X. Zou, and K.L. Calame. 1995. C/EBP proteins activate transcription from the human immunodeficiency virus type 1 long terminal repeat in macrophages/monocytes. J. Virol. 69: 5337-5344.

Henderson L.E., M.A. Bower, R.C. Sowder et al. 1992. Gag proteins of the highly replicative MN strain of human immunodeficiency virus type 1: posttranslational modifications, proteolytic processing, and complete amino acid sequences. J. Virol. 66: 1856-1865.

Herman P. 2001. In patients failing HAART, patterns of PI and RT mutations differ in B versus non-B subtypes. The 1st IAS Conference on HIV Pathogenesis and treatment, July, Buenos Aires, Argentina. Abstract 396.

Herskowitz A., S.B. Willoughby, K.L Baughman et al. 1992. Cardiomyopathy associated with antiretroviral therapy in patients with HIV infection: A report of six cases. Ann. Intern. Med. 116: 3111-3113.

Hirsch M.S., B. Conway, R. D'Aquila, V.A. Johnson, F. Brun-Vezinet, B. Clotet, L.M. Demeter, S.M. Hammer, D.M. Jacobsen, D.R. Kuritzkes, C. Loveday, J.W.

Mellors, S. Vella, and D.D. Richman for the International AIDS Society-USA panel. 1998. Antiretroviral drug resistance testing in adults with HIV infection. Implications for clinical management. JAMA 279: 1984-1991.

Ho D.D., A.U. Neumann, A.S Perelson et al. 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Science 273: 123-126.

Hochster H., D. Dieterich, S. Bozzette et al. 1990. Toxicity of combined ganciclovir and zidovudine for cytomegalovirus disease associated with AIDS. Ann. Intern. Med. 113: 111-117.

Hoetelmans R.M.W. 1999. Clinical pharmacokinetics of antiretroviral drugs. AIDS rev. 1: 167-177.

Hoguin A., B. Rodes, U. Dietrich U., and V. Soriano. 1999. Human immunodeficiency viruses type 1 subtypes circulating in Spain 1. J. Medical Virology 59: 189-193.

Hostomsky Z., Z. Hostomska, T.B. Fu, and J. Taylor. 1992. Reverse transcriptase of human immunodeficiency virus type 1: functionality of subunits of the heterodimer in DNA synthesis. J. Virol. 66: 3179-3182.

Hottiger M. and U. Hubscher. 1996. Human immunodeficiency virus type1 reverse transcriptase. Biol. Chem. Hoppe-Seyler, Vol. 377: 97-120.

Howard K.J., K.B. Frank, I.S. Sim, and S.F. Le Grice. 1991. Reconstitution and properties of homologous and chimeric HIV-1/HIV-2 p66/p51 reverse transcriptase. J. Biol. Chem. 266: 23003-23009.

Hsieh J.C., S. Zinnen, and P. Modrich. 1993. Kinetic mechanism of the DNA-dependent DNA polymerase activity of human immunodeficiency virus reverse transcriptase. J. Biol. Chem. 268: 24607-24613.

Hu D.J., A. Buvé, J. Baggs, G. van der Groen, and T.J. Dondero. 1999. What role does HIV-1 subtype play in transmission and pathogenesis? An epidemiological perspective. AIDS 13: 873-881.

Huang H., R. Chopra, G.L. Verdine et al. 1998. Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implication for drug resistance. Science 282: 1669-1675.

Huber H.E., J.M. McCoy, J.S. Seehra, and C.C. Richardson. 1989. Human immunodeficiency virus 1 reverse transcriptase. Template binding, processivity, strand displacement synthesis, and template switching. J. Biol. Chem. 264: 4669-4678.

Huber H.C. and C.C. Richardson. 1990. Processing of the primer for plus-strand DNA synthesis by human immunodeficiency virus type 1 reverse transcriptase. J. Biol. Chem. 265: 10565-10573.

Huet T., R. Cheynier, and A. Meyerhans. 1990. Genetic organization of a chimpanzee lentivirus related to HIV-1. Nature: 345: 356-359.

Human Retrovirus and AIDS-1998. 1998. A compilation and analysis of nucleic acid and amino acid sequences (Carr J.K., B.T. Foley, T. Leitner, M. Salminen, B. Korber, and F. McCutchan, eds): Los Alamos National Laboratory, Los Alamos, New Mexico, III: 10 –19.

Hunter E. and R. Swanstrom. 1990. Retrovirus envelope glycoproteins. In: Vogt, Swanstrom R. eds. Current topics of microbiological immunity. New York: Springer-Verlag: 187-253.

Hwang S.S., T.J. Boyle, H.K. Lyerly, and B.R. Cullen. 1991. Identification of the envelope V3 loop as the primary determinant of cell tropism in HIV-1. Science 253: 71-74.

Irwin K.L., C.P. Pau, D. Lupo, D. Pienazek, C.C. Luo, N. Olivo, M. Rayfield, D.J. Hu, J.T. Weber, R.A. Respess, R. Janssen, P. Minor, J. Ernst, and the Centers for

Disease Control and Prevention-Bronx Lebanon HIV Serosurvey Team. 1997. Presence of Human immunodeficiency virus (HIV) type 1 subtype A infection in a New York community with high HIV prevalence: a sentinel site for monitoring HIV genetic diversity in North America. The J. Infect. Diseases 176: 1629-33.

Jacks T., M.D. Power, F.R. Masiarz, P.A. Luciw, P.J. Barr and H.E. Varmus. 1988. Characterization of ribosomal frameshifting in HIV-1 gag-pol expression. Nature 331: 280-283.

Jackson J.B., G. Becker-Pergola, L.A. Guay, P. Musoke, M. Mracna, M.G. Fowler, L.M. Mofenson, M. Mirochnick, F. Mmiro, and S.H. Eshleman. 2000. Identification of the K103N resistance mutation in Ugandan women receiving nevirapine to prevent HIV-1 vertical transmission. AIDS 14: F111-F115.

Jacobo-Molina A., J. Ding, R.G. Nanni, A.D.Jr. Clark, X. Lu, C. Tantillo, R.L Williams, G. Kamer, A.L Ferris, P. Clark, A. Hizi, S.H. Hughes, and E. Arnold. 1993. Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 A resolution shows bent DNA. Proc. Natl. Acad. Sci. USA 90: 6320-6324.

Jamieson B.D., G.M. Aldrovandi, V. Planelles, J.B.M. Jowett, L. Gao, L.M. Bloch, I.S.Y. Chen, and J.A. Zack. 1994. Requirement of human

immunodeficiency virus type 1 *nef* for in vivo replication and pathogenicity. J. Virol. 68:3478-3485.

Janini L., A.Tanuri, M. Schechter, et al. 1998. Horizontal and vertical transmission of Human Immunodeficiency Virus type 1 dual infections caused by viruses of subtypes B and C. J. Infect. Dis. 177: 227-231.

Janssens W., A. Buve, and J.N. Nkengasong. 1997. The puzzle of HIV-1 subtypes in Africa. AIDS 11: 705-712.

Janssens W., L. Heyndrickx, Y. Van de Peer, A. Bouckaert, K. Fransen, J. Motte, G.M. Gershy-Damet, M. Peeters, P. Piot, and G. Van der Groen. 1994. Molecular phylogeny of part of the env gene of HIV-1 strains isolated in Cote d'Ivoire. AIDS 8: 21-26.

Jeeninga R.E., M. Hoogenkamp, M. Armand-Ugon, M. de Baar, K. Verhoef, and B. Berkhout. 2000. Functional differences between the long terminal repeat transciptional promoters of human immunodeficiency virus type 1 subtypes A through G. J. Virol. 74: 3740-3751.

John G., J. Neilson, P. Lewis, J. Overbaugh, R.W. Nduati, D.A. Mboringacha, and J. Kreiss. 1988. HIV-1 subtype and shedding of cervicovaginal HIV-1 DNA

during pregnancy. Abstract 13117. In Program of the 12th World AIDS Conference, Geneva, Switzerland.

Joint United Nations Programme on HIV/AIDS (UNAIDS). June 2000. AIDS epidemic update.

Jonckheere H., J.M. Taymans, J. Balzarini et al. 1994. Resistance of HIV-1 reverse transcriptase against [2',5'-bis-O-(tert-butyldimethylsilyl)-3'-spiro5''-(4''-amino-1''2''-oxathiole-2'',2''dioxide)] (TIBO) derivatives is determined by the mutation Glu138-Lys on the p51 subunit. J. Biol. Chem. 269: 25255-25258.

Jubier-Maurin V., S. Saragosti, J.L. Perret, E. Mpoudi, E. Esu-Williams, C. Mulanga, F. Liegois, M. Ekwalanga, E. Delaporte, and M. Peeters. 1999. Genetic characterization of the nef Gene from human immunodeficiency virus type 1 group M strains representing genetic subtypes A, B, C, E, F, G, and H. AIDS Research and Human Retroviruses 15: 23-32.

Kalish M.L., A. Baldwin, S. Rakthma, C. Wasi, C.C. Luo, G. Schochetman, T.D. Mastro, N. Young, S. Vanichseni, Rubsamen-Waigmann, H. von Briesen, J.I. Mullins, E. Delwart, B. Herring, J. Esparza, W.L. Heyward, and S. Osmanov. 1995. The evolving molecular epidemiology of HIV-1 envelope subtypes in injecting drug users in Bangkok, Thailand: implications for HIV vaccine trials. AIDS 9: 851-857.

Kanki P.J., D.J. Hamel, J.L. Sankale, C.C. Hsieh, I. Thior, F. Barin, S.A. Woodcock, A. Gueye-Ndiaye, E. Zhang, M. Montano, T. Siby, R. Marlink, I. Ndoye, M.E. Essex, and S. Mboup. 1999. Human immunodeficiency virus type 1 subtypes differ in disease progression. The Journal of Infectious Diseases 179: 68-73.

Kati W.M., K.A. Johnson, L.F. Jerva, and K.S. Anderson. 1992. Mechanism and fidelity of HIV reverse transcriptase. J. Biol. Chem. 267: 25988-25997.

Kato K., H. Sato, and Y. Takebe. 1999. Role of naturally occuring basic amino acid substitutions in the human immunodeficiency virus type 1 subtype E envelope V3 loop on viral coreceptor usage and cell tropism. J. Virol. 73: 5520-5526.

Katz R.A. and A.M. Skalka. 1994. The retroviral enzymes. Ann. Rev. Biochem. 63: 133-173.

Kawano Y., Y. Tanaka, N. Misawa, R. Tanaka, J.I. Kira, T. Kimura, M. Fukushi, K. Sano, T. Goto, M. Nakai, T. Kobayashi, N. Yamamoto, and Y. Koyanagi. 1997. Mutational analysis of human immunodeficiency virus type 1 (HIV-1) accessory genes: requirement of a site in the nef gene for HIV-1 replication in activated CD4+ T cells in vitro and in vivo. J.Virol. 71: 8456.

Kavlick M.F., K. Wyvill, R. Yarchoan, and H. Mitsuya. 1998. Emergence of multi-dideoxynucleoside-resistant human immunodeficiency virus type 1 variants, viral sequence variation, and disease progression in patients receiving antiretroviral chemotherapy. J. Infect. Dis. 98: 1506-1513.

Keet I.P.M, P. Krijnen, M. Koot, M.A. Lange, F. Miedema, J. Goudsmit, and R.A Couthino. 1993. Predictors of rapid progression to AIDS in HIV-1 seroconverters. J. Acquired Immune Defic. Syndr. 7: 51-57.

Kefenie H., S. Butto, B. Desta, P. Virani, F. Titti, L. Sernicola, M. Rapicetta, P. Pasquini and G.B. Rossi. 1989. Serological survey of Human Immunodeficiency Virus in Ethiopia. J. Med.Virol. 28: 21-24.

Kellam P., C.A.B. Boucher, J.M.G.H. Tijnagal, and B.A. Larder. 1994. Zidovudine treatment results in the selection of human immunodeficiency virus type 1 variants whose genotypes confer increasing levels of drug resistance. J. Gen. Virol. 75: 341-351.

Kelleher A.D., C. Long, E.C. Holmes, R.L. Allen, J. Wilson, C. Conlon, C. Workman, S. Shaunak, K. Olson, P. Goulder, C. Brander, G. Ogg, J.S. Sullivan, W. Dyer, I. Jones, A.J. McMichael, S. Rowland-Jones, and R.E. Phillips. 2001.

Clustered mutations in HIV-1 Gag are consistently required for escape from HLA-B27-restricted CTL responses. J. Exp. Med. 193: 375-386.

Kestler H. W., D.J. Ringler, K. Mori, D.L. Panicali, P.K. Sehgal, D. Daniel and R.C. Desrosiers. 1991. Importance of the nef gene for maintenance of high virus loads and for developments of AIDS. Cell 65: 651-662.

Kirchhoff F., P.J. Easterbrook, N. Douglas, M. Troop, T.C. Greenough, J. Weber, S. Carl, J.L. Sullivan, and R.S. Daniels. 1999. Sequence variations in human immunodeficiency virus type 1 Nef are associated with different stages of disease. J. Virol. 73: 5497- 5508.

Kohl N., E. Emini, W. Schleif, et al. 1988. Active human immunodeficiency virus protease is required or viral infectivity. Proc. Natl. Acad. Sci. USA. 85: 4686-4690.

Kohlstaedt L.A., J. Wang, J.M. Friedman, P.A. Rice, and T.A. Steitz. 1992a. Crystal structure at 3.5A resolution of HIV-1 reverse transcriptase complexed with an inhibitor. Science 256: 1783-1790.

Kohlstaedt L.A., and T.A. Steitz. 1992b. Reverse transcriptase of human immunodeficiency virus can use either human tRNA-(3Lys) or Escherichia coli

tRNA(2Gln) as a primer in an in vitro primer-utilization assay. 1992b. Proc. Natl. Acad. Sci. USA 89: 9652-9656.

Kondo E., F. Mammano, E.A. Cohen and H. Gottlinger. The p6 gag domain of HIV-1 is sufficient for the incorporation of Vpr into heterologous particles. J. Virol. 69: 2750-2764.

Korber B., A. Gifford, and G. Myers. 1993. Patterns of variation among international isolates in the highly immunogenic V3 region of the HIV-1 envelope protein. [abstact]. AIDS Res. Hum. Retrovirus 9 (suppl. 1): S 98.

Korber B., B. Hahn, B. Foley, J.W. Mellors, T. Leitner, G. Myers, F. McCutchan, and C. Kuiken. 1997. Human retroviruses and AIDS 1997: a compilation and analysis of nucleic acid and amino acid sequences. Los Alamos National Laboratory, Los Alamos, New.Mexico.

Koup R.A., J.T. Safrit, Y. Cao, C.A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, and D.D. Ho. 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J. Virol. 68:4650-4655.

Lacey S.F., and B.A. Larder. 1994. Novel mutation (V75) in human immunodeficiency virus type 1 reverse transcriptase confers resistance to

2',3'didehydro-2',3'-dideoxythymidine in cell culture. Antimicrob. Agents Chemother. 38: 1428-1432.

Larder B.A. and S.D. Kemp. 1989. Multiple mutations in HIV-1 reverse transcriptase confer high level of resistance to zidovudine (AZT). Science 246: 1155-1158.

Larder B.A., S.D. Kemp, R. Harrigan. 1995. Potential mechanism for sustained antiretroviral efficacy of AZT/lamivudine combination therapy. Science 269: 696-699.

Lavallée C., X.J. Yao, A. Ladha, H. Gottlinger, W.A. Haseltine, and E.A. Cohen. 1994. Requirement of the Pr55gag precursor for incorporation of the Vpr product into human immunodeficiency virus type 1 viral particles. J. Virol. 68: 1926-1934.

Layne S.P., M.J. Merges, M. Dembo et al. 1992. Factors underlying spontaneous inactivation and susceptibility to neutralization of human immunodeficiency virus. Virology 189: 695-714.

Le Grice S.F. and F. Gruninger-Leitch. 1990. Rapid purification of homodimer and heterodimer HIV-1 reverse transcriptase by metal chelate affinity chromatography. Eur. J. Biochem. 187: 307-314.

Le Grice S.F., T. Naas, B. Wohlgensinger, and O. Schatz. 1991. Subunit-selective mutagenesis indicates minimal polymerase activity in heterodimer-associated p51 HIV-1 reverse transcriptase. EMBO J. 10: 3905-3911.

Lever A., H. Gottlinger, W. Haseltine and J. Sodroski. 1989. Identification of a sequence required for efficient packaging of human immunodeficiency virus type 1 RNA into virions. J. Virol. 63: 4085-4087.

Levy D.N., L.S. Fernandes, W.V. Williams, and D.B. Weimer. 1993. Induction of cell differentiation by HIV-1 vpr. Cell 72: 541-550.

Li X., Quan Y., and M.A. Wainberg. 1997. Controlling elements in replication of the human immunodeficiency virus type 1. Cellular and Molecular Biology 43 (3): 443-454.

Lighfoote M.M., J.E. Coligan, T.M. Folks, A.S. Fauci, M.A. Martin, and S. Venkatesan. 1986. Structural characterization of reverse transcriptase and endonuclease polypeptides of the acquired immunodeficiency syndrome retrovirus. J. Virol. 60: 771-775.

Lindemann D., R. Wilhelm, P. Renard, A. Althage, R. Zinkernagel, and J. Mous. 1994. Severe immunodeficiency associated with a human immunodeficiency virus 1 Nef/3' long terminal repeat transgene. J. Exp. Med. 179: 797-807.

Little S.J. 2000. Transmission and prevalence of HIV resistance among treatment-naive subjects. Antiviral Therapy 5: 33-40.

Liu J., C. Woffendin, Z.Y. Yang, and G.J. Nabel. 1994. Regulated expression of a dominant negative form of Rev improves resistance to HIV replication in T cells. Gene Ther. 1: 32-37.

Lole K.S., R.C. Bollinger, R.S. Paranjape, D. Gadkari, S.S. Kulkarni, N.G. Novak, R. Ingersoll, H.W. Scheppard and S.C. Ray. 1999. J. Virol. 73: 152-160.

Loemba H., B. Brenner, M.A. Parniak, S. Ma'ayan, B. Spira, D. Moisi, M. Oliveira, M. Detorio, and M.A. Wainberg. 2001. Divergence in reverse transcriptase sequence and selection for resistance of human immunodeficiency virus type 1 Ethiopian subtype C against non-nucleoside inhibitors. AIDS. Submitted.

Lori F., F. di Marzo Veronese, A.L. De Vico, et al. 1992. Viral DNA carried by human immunodeficiency virus type 1 virions. J. Virol. 66: 5067-5074.

Louwagie J., W. Janssens, J. Mascola., et al. 1995. Genetic diversity of the envelope glycoprotein from human immunodeficiency virus type 1 isolates of African origin. J. Virol. 69: 263-271.

Lu Y.L., P. Spearman and L. Ratner. 1993. Human immunodeficiency virus type 1 viral protein R localization in infected cells and virions. J. Virol. 67: 6542-6550.

Luban J., L. Bossolt, E.K. Franke, G.V. Kalpana and S.P. Goff. 1993. HIV-1 gag protein binds to cyclophilin A an B. Cell 73: 1067-1078.

Luo G.X. and J. Taylor. 1990. Template switching by reverse transcriptase during DNA synthesis. J. Virol. 64: 4321-4328.

Mager P.P. 2001. The active site of HIV-1 protease. Med. Res. Rev. 21: 348-353.

Malim M.H., D.F. McCarn, L.S. Tiley, and B.R. Cullen. 1991. Mutational definition of the human immunodeficiency virus type 1 Rev activation domain. J. Virol. 65: 4248-4254.

Mansky L.M., and H.M. Temin. 1995. Mechanism of resistance of human immunodeficiency virus type 1 that predicted from the fidelity of purified reverse transcriptase. J. Virol. 69: 5087-5094.

Mayers D. 1996. Rational approaches to resistance: nucleoside analogues. AIDS 10 (suppl 1): S9-S13.

McHenry C.S. 1989. Molecular biology of chromosome function. K.Adolph (ed), (New-York; Springer Verlag), pp.97-123.

McMichael A.J. and S.L. Rowland-Jones. 2001. Cellular immune responses to HIV. Nature 410:980-987

Merluzzi V.J., K.D. Hargrave, M. Labadia, K. Grozinger, M. Stoog, J.C. Wu, C-K. Sthih, K. Eckner, S. Hattox, J. Adams, A.S. Rosenthal, R. Faanes, R.J. Eckner, R.A. Koup, and J.L. Sullivan. 1990. Inhibition of HIV-1 replication by a non-nucleoside reverse transcriptase inhibitor. Science 250: 1411-1413.

Meyer P.R., S.E. Matsuura, A.M. Mian et al. 1999. A mechanism of AZT resistance: an increased in nucleotide-dependent primer unblocking by mutant HIV-1 reverse transcriptase. Mol. Cell 43: 35-43.

Michaels F.H., N. Hattori, R.C. Gallo and G. Franchini. 1993. The human immunodeficiency virus type 1 (HIV-1) Vif protein is located in the cytoplasm of infected cells and its effect on viral replication is equivalent in HIV-2. AIDS Res. Hum. Retroviruses 9: 1025-1030.

Miguel E.Q. and E.J. Arts. 1999. Recombination in HIV-1: Update and implications. AIDS Rev. 1: 89-100.

Miller R.H., and N. Sarver. 1997. HIV accessory proteins as therapeutic targets. Nature Medicine 3: 389-394.

Mohri H., M.K. Singh, W.T.W. Ching, D.D. Ho. 1993. Quantitation of Zidovudine-resistant human immunodeficiency virus type 1 in the blood of treated and untreated patients. Proc. Natl. Acad. Sci. USA 90: 25-29.

Molla A., G.R. Granneman, E. Sun, D.J. Kempf. 1998. Recent developments in HIV protease inhibitor therapy. Antiviral Research 39: 1-23.

Montano M.A., V.A. Novitsky, J.T. Blackard, N.L. Cho, D.A. Katzenstein, and M. Essex. 1997. Divergent transcriptional regulation among expanding human immunodeficiency virus type 1 subtypes. J. Virol. 71: 8657-8665.

Moore J.P., B.A. Jameson, R.A. Weiss, and Q.J. Sattentau. 1993. The HIV-cell fusion reaction. In: Bentz J., ed. Viral fusion mechanisms. Boca Raton: CRC Press, 233-289.

Mouland A.J., Mercier J., Luo M., Bernier L., DesGroseillers and E.A. Cohen. 2000. The Double-stranded RNA-binding protein staufen is incorporated in human immunodeficiency virus type 1: evidence for a role in genomic RNA encapsidation. J. Virol. 74: 5441-5451.

Mous J., E.P. Heimer and S.F.J. Le Grice. 1988. Processing protease and reverse transcriptase from human immunodeficiency virus type 1 polyprotein in Escherichia coli. J. Virol. 62: 1433-1436.

Moyle G. 1995. Resistance to antiretroviral compounds: implications for the clinical management of HIV infection. Immunol. Infect. Dis. 5: 170-182.

Murphy F. A., C.M. Fauquet, D.H.L. Bishop, S.A. Ghabrial, A.W. Jarvis, G.P. Martelli, M.A. Mayo, and M.D. Summers. 1994. Virus taxonomy: The classification and nomenclature of viruses, Retroviridae. Springer-Verlag, Vienna.

Muzoke P., L. Guay, D. Bagenda, et al. 1999. A phase I/II study of the safety and pharmacokinetics of nevirapine in HIV-1 infected pregnant Uganda women and their neonates (HIVNET 006). AIDS 13: 479-486.

Myers G., B. Korber, B. Hahn, Human Retroviruses and AIDS 1995. 1995. A compilation and analysis of nucleic acid and amino acid sequences. Los Alamos.

New Mexico: Los Alamos National Laboratory - Theoretical Biology and Biophysics Group.

Myers G., B. Korber, S. Wain-Hobson, K-T. Jeang, L. Henderson, and G.N. Pavlakis. 1994. Human Retroviruses and AIDS, 1994 - Los Alamos. Los Alamos National Laboratory.

Myers G., B. Korber, S. Wain-Hobson, K-T. Jean, L.E. Henderson, and G.N Pavlakis. 1995. Human retroviruses and AIDS 1995. Los Alamos: Los Alamos National Laboratory.

Myers J., J. Gamberg, M.I. Bowmer, and M. Grant. 2001. Immunological recognition of drug resistant HIV. Tenth Annual Canadian Conference on HIV/AIDS Research, CAHR , Toronto, Canada. Abstract 106.

Nabel G.J. 2001. Challenges and opportunities for development of an AIDS vaccine. Nature 410: 1002-1007.

Naghavi M.H., S. Schwartz, A. Sonnerborg, and A. Vahlne. 1999. Long terminal repeat promoter/enhancer activity of different subtypes of HIV type 1. AIDS Research and Retroviruses 15: 1293-1303.

Najera I., A. Holguin, M.E. Quinones-Mateu, M.A. Munoz-Fernandez, Najera R., C. Lopez-Galindez and E. Domingo. 1995. *Pol* Gene Quasispecies of Human Immunodeficiency virus from patients undergoing no drug therapy. J. Virol., 69: 23-31.

Najera I., D.D. Richman, I. Olivares et al. 1994. Natural occurrence of drug resistance mutations in the reverse transcriptase of human immunodeficiency virus type 1 isolates. AIDS Res. Hum. Retroviruses 10: 1479-1488.

Nanni R.G., J. Ding, A. Jacobo-Molina et al. 1993. Review of reverse transcriptase three-dimensional structure: implication for drug design. Perspect. Drug Design 1: 189-196.

Nielsen C., C. Pedersen, J.D. Lundgren et al. 1993. Biological properties of HIV isolates in primary HIV infection: consequences for the subsequent course of infection. AIDS 7: 1035-1040.

Neilson J.R., G.C. John, J.K. Carr, P. Lewis, J.K. Kreiss, S. Jackson, R.W. Nduati, D. Mbori-Ngacha, D.D. Panteleeff, S. Bodrug, C. Giachetti, M.A. Bott, B.A. Richardson, J. Bwayo, J. Ndinya-Achola, and J. Overbaugh. 1999. Subtypes of human immunodeficiency virus type 1 and disease stage among women in Nairobi, Kenya. J. Virol. 73: 4393-4403.

Nijhuis M., R. Schuurma, D. de Jong, J. Erickson, E. Gustchina, J. Albert, P. Schipper, S. Gulnik, and C.A.B. Boucher. 1999. Increased fitness of drug resistant HIV-1 protease as result of acquisition of compensatory mutations during suboptimal therapy. AIDS 13: 2349-2359.

Novitski V.A., M.A. Montano, M.F. McLane, B. Renjifo, F. Vannberg, B.T. Foley, T.P. Ndung'u, M. Rahman, M.J. Mahema, R. Marlink, and M. Essex. 1999. Molecular cloning and phylogenetic analysis of Human immunodeficiency virus type 1 subtype C: a set of 23 full-length clones from Botswana. J. Virology 73: 4427-4432.

Numberg J.H., W.A. Schlief, E.J. Boots et al. 1991. Viral resistance to human immunodeficiency virus type 1-specific pyridinone reverse transcriptase inhibitors. J. Virol. 65: 4887-4892.

Oelrichs R.B., I.L. Shrestha, D.A. Anderson, and N.J. Deacon. 2000. The explosive Human immunodeficiency virus type 1 epidemic among injecting drug users of Kathmandu, Nepal, is caused by a subtype C virus of restricted genetic diversity. J. Virology 74: 1149-1157.

Orloff G. M., M.L. Kalish, J. Chiphangwi, K.E. Potts, C.Y. OU, G. Schochetman, G. Dallabetta, A.I. Saah, and P.G. Miotti. 1993. V3 loops of HIV-1 specimens

from pregnant women in Malawi uniformly lack a potential N-linked glycosylation site. AIDS Research and Human Retroviruses 9: 705-706.

Ossendorp F., M. Eggers, A. Neisig, T. Ruppert, M. Groettrup, A. Sijts, E. Mengede, P.M. Kloetzel, J. Neefjes, U. Koszinowski, and C. Melief. 1996. A single residue exchange within a viral CTL epitope alters proteasome-mediated degradation resulting in lack of antigen presentation. Immunity 5: 115-124.

Ott D.E., Coren L.V., Johnson D.G., Kane B.P., Sowder II R.C., Young D.K., Kim Y.D., Fisher R.J., Zhou X.Z., Lu K.P., and Henderson L.E. 2000. Acting-binding cellular proteins inside human immunodeficiency virus type 1. Virology 266: 42-51.

Paladin F.J., O.T. Monzon, H. Tsuchie, M.R. Aplasca, Jr.G.H. Learn, and T. Kurimura. 1998. Genetic subtypes of HIV-1 in the Philippines. AIDS 12: 291-300.

Pantaleo G. 1997. How immune-based interventions can change HIV therapy. Nature Medicine 3: 483-490.

Pantaleo G., C. Graziosi, J.F. Dernarest et al. 1993. HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. Nature 362: 355-358.

Palmer S., Alaeus, J. Albert and S. Cox. 1998. Drug susceptibility of subtypes A, B, C, D and E Human Immunodeficiency Virus type 1 primary isolates. AIDS Res. Hum. Retroviruses 14: 157-162.

Park I.W., K. Myrick, and J. sodroski. 1994. Effects of *vif* mutations on cell-free infectivity and replication of simian immunodeficiency virus. J. Acquired Immune Defic. Syndr. 7: 1228-1236.

Patel P.H., A. Jacobo-Molina, J.P. Ding, C. Tantillo, A.D. Clark, R. Raag, R.G. Nanni, S.H. Hughes, and E. Arnold. 1995. Insights into DNA polymerization mechanisms from structure and function analysis of HIV-1 reverse trancriptase. Biochemistry 34: 5351-5363.

Pathak V.K., and H.M Temin. 1990. Broad spectrum of in vivo forward mutations, hypermutations, and mutational hotspots in a retroviral shuttle vector after a single replication cycle: substitutions, frameshifts, and hypermutations. Proc. Natl. Acad. Sci. USA 87: 6019-6023.

Peliska J.A., S. Balasubramanian, D.P. Giedroc and S.J. Benkovic. 1994. Recombinant HIV-1 nucleocapsid protein accelerates HIV-1 reverse transcriptase catalyzed DNA strand transfer reactions and modulates RNAse H activity. Biochemistry 33: 13817-13823.

Peliska J.A. and S.J Benkovic. 1992. Mechanism of DNA strand transfer reactions catalyzed by HIV-1 reverse transcriptase. Science 258: 1112-1118.

Peeters M., R. Vincent, J.L. Perret, M. Lasky, D. Patrel, F. Liegeois, V. Courgnaud, R. Seng, T. Matton, S. Molinier, and E. Delaporte. 1999. Evidence for differences in MT2 cell tropism according to genetic subtypes of HIV-1: syncytium-inducing variants seem rare among subtype C HIV-1 viruses. J. Acquir. Immune Deficienc. Syndr. Hum. Retrovirol. 20: 115-121.

Pillay D., K. Sinka, P. Rice, B. Peters, J. Clarke, J. Workman , B. Evans, and P.A. Cane. 2000. Impact of HIV-1 subtype on NNRTI resistance mutations. Antiviral Therapy. 5 (Supplement 3): 128. Abstract 163.

Pomerantz R.J. 2001. Residual HIV-1 infection during antiretroviral therapy: the challenge of viral persistence. AIDS 15: 1201-1211.

Pomerantz R.J., D.R. Kuritzkes, S.M. de la Monte, and M.S. Hirsch. 1987. Infection of the retina by human immunodeficiency virus type 1. N. Engl. J. Med. 317: 1643-1647.

Poon B., J.B.M. Jowett, Stewart S.A., Armstrong R.W., Rishton G.M., and I.S.Y. Chen. 1997. Human immunodeficiency virus type 1 vpr gene induces phenotypic

effects similar to those of the DNA alkylating agent, nitrogen mustard. J. Virol. 71: 3961-3971.

Poss M., and J. Overbaugh. 1999. Variants from the diverse virus population identified at seroconversion of a clade A human immunodeficiency virus type 1-infected woman have distinct biological properties. J. Virol. 73: 5255-5264.

Preston B.D., B.J. Poiesz, and L.A. Loeb. 1988. Fidelity of HIV-1 reverse transcriptase. Science 242: 1168-1171.

Preston B.D. and N. Garvey. 1992. Retroviral mutation and reverse transcriptase fidelity. Pharma. Technol. 16: 34-51.

Quinones-Mateu M.E., E.J. Arts. 1999. Recombination in HIV: Update and implications. AIDS Rev. 1: 89-100.

Quinones-Mateu M., J. Holguin, I. Dopazo, I. Najera, and E. Domingo. 1996. Point mutation frequencies in the pol gene of human immunodeficiency virus type 1 are two- to threefold lower than that of env. AIDS Res. Hum. Retroviruses 12: 1117-1128.

Quinones-Mateu M.E., J.L. Albright, A. Mas, V. Soriano, and E.J. Arts. 1998. Analysis of *Pol* gene heterogeneity, viral quasispecies, and drug resistance in

individuals infected with group O strains of Human immunodeficiency virus type 1. J. Virol. 72: 9002-9015.

Raja N.U., M.J. Vincent, and M.A. Jabbar. 1994. Vpu-mediated proteolysis of gp160/CD4 chimeric envelope glycoproteins in the endoplasmic reticulum: requirement of both the anchor and cytoplasmic domains of CD4. Virology 204: 357.

Ratner L., W. Haseltine, R. Patarca, K.J. Livak, B. Starcich, S.F. Josephs, E.R. Doran, J.A. Rafalski, E.A. Whitehorn, K. Baumeister, L. Ivanoff, Jr. S.R. Petteway, M.L. Pearson, J.A. Lautenberger, T.S. Papas, J. Ghrayeb, N.T. Chang, R.C. Gallo and F. Wong-Staal. 1985. Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature 313: 277-284.

Reardon J.E. 1992. Human immunodeficiency virus reverse transcriptase: steady-state and pre-steady-state kinetics of nucleotide incorporation. Biochemistry 31: 4473-4479.

Renjifo B., B. Chaplin, D. Mwakagile, P. Shah, F. Vannberg, G. Msamanga, D. Hunter, W. Fawzi, and M. Essex. 1998. Epidemic expansion of HIV type 1 subtype C and recombinant genotypes in Tanzania. AIDS Research and Retroviruses 14: 635-638.

Resnick R., C.A. Omer, and A.J. Faras. 1984. Involvement of retrovirus reverse transcriptase-associated RNAse H in the initiation of strand-stop (+) DNA synthesis and the generation of the long terminal repeat. J. Virol. 51: 813-821.

Richman D.D. HIV chemotherapy. 2001. Nature 410: 995-1001.

Richman D.D., C.K. Shih, I. Lowy et al. 1991. Human immunodeficiency virus type 1 mutants resistant to non-nucleoside inhibitors of reverse transcriptase arise in cell culture. Proc. Natl. Acad. Sci. USA. 88: 11241-11245.

Richman D.D., D. Havlir, J. Corbeil et al. 1994. Nevirapine resistance mutations of human immunodeficiency virus type 1 selected during therapy. J. Virol. 68:1660-1666.

Richman D.D., M.A. Fischl, M.H. Grieco et al. 1987. The toxicity of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex: a double-blind, placebo-controlled trial. N. Engl. J. Med. 317: 192-197.

Ridge J.P., F. Di Rosa, and P.A. Matzinger. 1998. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. Nature 393: 474-478.

Ridky T. and Leis J. 1995. Development of drug resistance to HIV-1 protease inhibitors. J. Biological Chemistry 270: 29621-29623.

Rittinger K., G. Divita, and R. Googy. 1995. Human immunodeficiency virus reverse transcriptase substrate-induced conformational changes and the mechanism of inhibition by non-nucleoside inhibitors. Proc. Natl. Acad. Sci. 92: 8046-8049.

Robins T., and J. Plattner. 1993. HIV protease inhibitors: their anti-HIV activity and potential role in treatment (review). J. AIDS 6: 162-70.

Roberts J.D., K. Bebenek, and T.A. Kunkel. 1988. The accuracy of reverse transcriptase from HIV-1. Science 242: 1171-1173.

Robertson D., P. Sharp, F. McCutchan, and B.H. Hahn. 1995. Recombination in HIV-1. Nature 374: 124-126.

Rosen C.A., W.A. Haseltine, J. Lenz, R. Ruprecht, and M.W. Cloyd. 1985. Tissue selectivity of murine leukemia virus infection is determined by long terminal repeat sequences. J. Virol. 55: 862-866.

Roth M.J., P.L. Schwartzberg, and S.P. Goff. 1989. Structure of the Termini of DNA intermediates in the integration of retroviral DNA: Dependence on IN function and terminal DNA sequence. Cell 58: 47-54.

Routy J.P., B Brenner, H. Salomon, B. Spira, P. Junod, R. Sekaly, and M.A. Wainberg. 2001. Viral evolution in untreated patients who acquired drug resistant HIV-1 during primary infection. The 1st IAS Conference on HIV Pathogenesis and Treatment, July. Buenos Aires, Argentina. Abstract No 582.

Rowland-Jones S.L., T. Dong, K.R. Fowke, J. Kimani, P. Krausa, H. Newell, T. Blanchard, K. Ariyoshi, J. Oyugi, E. Ngugi, J. Bwayo, K.S. MacDonald, A.J. McMichael and F.A. Plummer. 1998. Cytotoxic T-cell Responses to multiple conserved HIV epitopes in HIV-resistant prostitutes in Nairobi. J.Clin.Invest. 102: 1758-1765.

Sakai H., R. Shibata, J.I. Sakuragi, S. Sakuragi, M. Kawamura, and A. Adachi. 1993. Cell-dependent requirement of human immunodeficiency virus type 1 Vif protein for maturation of virus particles. J. Virol. 67: 1663-1666.

Salminen M.O., B.O. Johansson, A. Sonnerborg, S. Ayehunie, D. Gotte, P. Leinikki, D.S. Burke and F.E. McCutchan. 1996. Full-lengh sequence of Ethiopian Human Immunodeficiency Virus type 1 (HIV-1) isolate of genetic subtype C. AIDS Research and Human Retroviruses 12: 1329-1339.

Salomon H., A. Belmonte, K. Nguyen, Z. Gu, M. Gelfand, and M.A. Wainberg. 1994. Comparison of cord blood and peripheral blood mononuclear cells as targets for viral isolation and drug sensitivity studies involving human immunodeficiency virus type 1. J Clin Microbiol. 32: 2000-2002.

Samri A., G. Haas, J. Duntze, J.M. Bouley, V. Calvez, C. Katlama, and B. Autran. 2000. Immunogenicity of mutations induced by nucleoside reverse transcriptase inhibitors for human immunodeficiency virus type 1-specific cytotoxic T cells. J. Virol. 74: 9306-9312.

Sande M.A., and P.A. Volberding. 1995. The medical management of AIDS. Fourth edition, WB. Saunders company, Philadelphia, PA.

Sarafianos S.G., K. Das, A.D. Clark et al. 1990. Lamivudine (3TC) resistance in HIV-1 reverse transcriptase involves steric hindrance with beta-branched amino acids. Proc. Nat. Acad. Sc. USA 96: 10027-10032.

Sardana V.V., E.A. Emili, L. Gotlib et al. 1992. Functional analysis of HIV-1 reverse transcriptase amino-acids involved in resistance to multiple non-nucleoside inhibitors. J. Biol. Chem. 267: 17526-17530.

Sato H., Y. Tomita, K. Shibamura, T. Shiino, T. Miyakuni, and Y. Takebe. 2000. Convergent evolution of reverse transcriptase (RT) genes of human imunnodeficiency virus type 1 subtypes E and B following nucleoside analogue RT inhibitor therapies. J.Virol. 74: 5357-5362.

Schinazi R.F, B. A. Larder and J.W. Mellors. 1996. Mutations in retroviral genes associated with drug resistance. Intl. Antiviral News 4: 95-107.

Schmit J.C., K. Van Laethem, L. Ruiz, P. Hermans, S. Sprecher, A. Sonnerborg, Leal M., T. Harrer, B. Clotet, V. Arendt, E. Lissen, M. Witvrouw, J. Desmyter, E. De Clercq, and A.M. Vandamme. 1998. Multiple dideoxynucleoside analogue-resistant (MddNR) HIV-1 strains isolated from patients from different European countries. AIDS 12: 2005-2015.

Schooley R.T., C. Ramtrez-Ronda, J.M.A. Lange et al. 1996. Virological and immunologic benefits of initial combination therapy with zidovudine and zalcitabine or didanosine compared with zidovudine monotherapy. J. Infect. Dis. 173: 1354-1366.

Schwartz S., B.K. Felber, E.M. Fenyo, and G.N. Pavlakis. 1990. Env and Vpu proteins of Human immunodeficiency virus type 1 are produced from multiple bicistronic mRNAs. J. Virol. 64: 5448-5456.

Selbert S.A., C.Y. Howell, M.K. Hughes and A.L. Hughes. 1995. Natural selection on the gag, pol, and env genes of human immunodeficiency virus type 1 (HIV-1). Mol. Biol. Evol. 12: 803-818.

Shaafer R.W., M.A. Winters, S. Palmer, and T.C. Merigan. 1998. Multiple concurrent reverse transcriptase and protease mutations and multi-drug resistance of HIV-1 isolates from heavily treated patients. Ann. Intern. Med. 128: 906-911.

Shafer R.W., J.A. Eisen, T.C. Merigan and D.A. Katzenstein. 1997. Sequence and drug susceptibility of subtype C reverse transcriptase from Human Immunodeficiency Virus type 1 seroconverters in Zimbabwe. J. Virol. 71: 5441-5448.

Shafer R.W., M.J. Kozal, M.A. Winters et al. and the ACTG 143 protocol virologists. 1992. Com bination therapy with ZDV + ddI suppresses virus load but does not prevent the emergence of HIV-1 isolates with ZDV resistance. The IX International Conference on AIDS. Amsterdam, The Netherlands; Abstract MoB 0055.

Shiino T., K. Kato, N. Kodaka, T. Miyakuni, Y. Takebe, and H. Sato. 2000. A group of V3 sequences from human immunodeficiency virus type 1 subtype E non-syncytium-inducing, CCR5-using variants are resistant to positive selection pressure. J.Virol. 74: 1069-1078.

Simon F., P. Mauclere, P. Roques, I. Loussert-Ajaka, M.C. Muller-Trutwin, S. Saragosti, M.C. Georges-Courbot, F. Barre-Sinoussi, and F. Brun-Vezinet. 1998. Identification of a new human immunodeficiency virus type 1 distinct from group M and group O. Nature Medicine 4: 1032-1037.

Skalka A.M., and S.P Goff. 1993. Reverse transcriptase. ed. Cold Spring Harbor, N.Y: Cold Spring Harbor Lab. Press.

Smerdon S.J., J. Jager, J. Wang et al. 1994. Structure of the binding site for non-nucleoside inhibitors of the reverse transcriptase of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA 91: 3911-3915.

Spence R.A., W.M. Kati, K.S. Anderson, and K.A. Johnson. 1995. Mechanism of inhibition of HIV-1 reverse transcriptase by non-nucleoside inhibitors. Science 267: 988-993.

Spira B., B. Brenner, M. Petrella, D. Moisi, J.P. Routy, and M.A. Wainberg. 2001. Prevalence of drug resistant HIV-1 variants in the Montreal PHI cohort. The 1st IAS Conference on HIV Pathogenesis and Treatment, July. Buenos Aires, Argentina. Abstract No 583.

Stammers D.K., J. Ren, C. Nichols, P. Chamberlain, L. Douglas, J. Lennerstrand, B. Larder, and D.I. Stuart. 2001. Crystal structures of HIV-1 reverse transcriptase resistant to zidovudine or lamivudine containing mutations at 41, 184 and 215. Antiviral Therapy 6 (Supplement 1): 54. Abstract 72.

Strebel K., T. Klimkait, M.A. Martin. 1988. A novel gene of HIV-1, vpu, and its 16 kDa product. Science 241: 1221-1223.

Sova P., and D.J. Volsky. 1993. Efficiency of viral DNA synthesis during infection of permissive and nonpermissive cells with vif-negative human immunodeficiency virus type 1. J. Virol. 67: 6322-6326.

Soto-Ramirez L.E., B. Renjifo, M.F. McLane, R. Marlink, C. O'Hara, R. Sutthent, C. Wasi, P. Vithayasai, V. Vithayasai, C. Apichartpiyakul, P. Auewarakul, V.P. Cruz, D.S. Chui, R. Osathanondh, K. Mayer, T.H. Lee, and M. Essex. 1996. HIV-1 Langerhans' cell tropism associated with heterosexual transmission of HIV. Science 271: 1291-1293.

Soto-Ramirez L.E., S. Tripathy, B. Renjifo, and M. Essex. 1996. HIV-1 pol sequences from India fit distinct subtype pattern. J. Acquired Immunodeficiency Syndrome and Human Retroviruses 13: 290-307.

Subbramanian R.A. and E.A. Cohen. 1994. Molecular Biology of the human immunodeficiency virus accessory proteins. J.Virol. 68: 6831-6835.

Sylvester S., A. Caliendo, D. An et al. 1995. HIV-1 resistance mutations and plasma RNA during ZDV and ddC combination therapy. Fourth International Workshop on HIV Drug Resistance. Sardina. Abstract 47.

Takehisa J., L. Zekeng, E. Ido, I. Mboudjeka, H. Moriyama, T. Miura, M. Yamashita, L.G. Gurtler, M. Hayami, and L. Kaptue. 1998. Various types of HIV mixed infections in Cameroon. Virology 245: 1-10.

Tantillo C., A. Ding, R. Jacobs-Molina et al. 1994. Locations of anti-AIDS drug binding sites and resistance mutations in three-dimensional structure of HIV-1 reverse transcriptase. Implications for mechanisms of drug inhibition and resistance. J. Mol. Biol. 243: 369-387.

Telenti A., M. Nunoz, G. Bleiber, B. Ledergerber and D.D. Kaufmann. 1999. Heterogeneity in the response to anti-retroviral therapy. AIDS Rev. 1: 147-155.

Temin H.M. and S. Mizutani. 1970. RNA-dependent DNA polymerase in virions of Rous sarcoma virus. Nature 226: 1211-1213.

Tersmette M., R.E.Y. De Goede, B.J. Al, I. Winkel, B. Gruters, H.T. Cuypers, H.G. Huisman, and F. Miedema. 1988. Differential syncytium-inducing capacity of human immunodeficiency virus isolates: frequent detection of syncytium-inducing isolates in patients with AIDS and AIDS-related complex. J. Virol. 62: 2026-2032.

Toure-Kane C., C. Montavon, M. Awa Faye, P. Mandoumbe Gueye, P.S. Sow, I. Ndoye, A. Gaye-Diallo, E. Delaporte, M. Peeters, and S. Mboup. 2000. Identification of all HIV type 1 group M subtypes in Senegal, a country with low and stable seroprevalence. AIDS Research and Human retroviruses 16: 603-609.

Triques K., A. Bourgeois, N. Vidal, E. Mpoudi-Ngole, C. Mulanga-Kabeya, N. Nzilambi, N. Torimiro, E. Saman, E. Delaporte, and M. Peeters. 2000. Near-full-length genome sequencing of divergent African HIV type 1 subtype F viruses leads to the identification of a new HIV type 1 subtype designated K. AIDS Research and Human Retroviruses 16: 139-151.

Triques K., A. Bourgeois, S. Saragosti, N. Vidal, E. Mpoudi-Ngole, N. Nzilambi, C. Apetrei, M. Ekwalanga, E. Delaporte, and M. Peeters. 1999. High diversity of HIV-1 subtype F strains in Central Africa. Virology 259: 99-109.

Trono D. 1992. Partial reverse transcripts in virions from human immunodeficiency and murine leukemia viruses. J. Virol. 66: 5067-5074.

Trono. D. 1995. HIV accessory proteins: Leading roles for the supporting cast. Cell 82: 189-192.

Tscherning C., A. Alaeus, R. Fredriksson, A. Bjorndal, H. Deng, D.R. Littman, E.M. Fenyo, and J. Albert. 1998 Differences in chemokine coreceptor usage between genetic subtypes of HIV-1. Virology 241: 181-188.

UNAIDS, and WHO - 22 June 1999. Report on the global HIV/AIDS Epidemic - June 1998. UNAIDS and WHO, Geneva, Switzerland.

Vallejo A., A. Heredia, A. Mas, S.F. Lee, J.S. Epstein, V. Soriano, and I.K. Hewlett. 1998. Tropism, coreceptor use, and phylogenetic analysis of both the V3 loop and the protease gene of three novel HIV-1 group O isolates. J. Acquired Immune Deficiency Syndrome and Human Retrovirology 18: 417-425.

van der Burg S.H., M.R. Klein, C.J.H. van de Velde, W.M. Kast, F. Miedema, and J.M. Melief. 1995. Induction of a primary human cytotoxic T-lymphocyte response against a novel conserved epitope in a functional sequence of HIV-1 reverse transcriptase. AIDS 9: 121-127.

van Harmelen J., R. Wood, M. Lambrick, E.P. Rybicki, A.L. Williamson, and C. Williamson. 1997. An association between HIV-1 subtypes and mode of transmission in Cape Town, South Africa. AIDS, 11: 81-87.

van't Wout A.B., H. Blaak, L.J. Ran, M. Brouwer, C. Kuiken, and H. Schuitemaker. 1998. Evolution of syncytium-inducing and non-syncytium-inducing biological virus clones in relation to replication kinetics during the course of human immunodeficiency virus type 1 infection. J. Virol. 72: 5099-5107.

Vandamme A.M., Z. Debyser, R. Pauwels, et al. 1994. Characterization of HIV-1 strains isolated from patients treated with TIBO R82913. AIDS Res. Hum. Retroviruses 10: 39-46.

Vanden Haesevelde M., J-L. Decourt, R.J. De Leys, B. Vanderborght, G. van der Groen, H. Van Heuverswyn, and E. Saman. 1994. Genomic cloning and complete sequence analysis of a highly divergent African human immunodeficiency virus isolate. J. Virol. 68: 1586-1596.

Varmus H. and P. Brown. Retroviruses. 1989. In D.E.Berg and M.M. Howe (ed), Mobile DNA. American Society for Microbiology, Washington, D.C. p.53-108.

Vink C., and R.H.A. Plasterk. 1993. The HIV-1 integrase protein. Trends Genet. 9: 433-437.

Von Schwelder U., J. Song, C Aiken, and D. Trono. 1993. Vif is crucial for HIV-1 proviral DNA synthesis in infected cell. J. Virol. 67: 4945-4955.

Wainberg M.A. 1999. HIV resistance to antagonists of viral reverse transcriptase. In AIDS and the new viruses, second edition, A. Dalgleish and R. Weiss, (eds). Chapter 14. Academic Press, London. pp223-249.

Wainberg M.A. and D.W. Cameron. 1998. HIV resistance to antiviral drugs: public health implications. Drug Resistance updates 1: 104-108.

Wainberg M.A., Z. Gu, Q. Gao, E. Arts, R. Geleziumas, S. Bour., R. Beaulieu, C. Tsoukas, J. Singer and J. Montaner. 1993. Clinical correlates and Molecular Immune Deficiency Syndromes 6: S36-S46.

Wainberg M.A., W.C. Drosopoulos, H. Salomon et al. 1996. Enhanced fidelity of 3TC-selected mutant HIV-1 reverse transcriptase. Science 271: 1282-1284.

Walker B.D., C. Flexner, T.J. Paradis, T.C. Fuller, M.S. Hirsch, R.T. Schooley, and B. Moss. 1988. HIV-1 reverse transcriptase is a target for cytotoxic T lymphocytes in infected individuals. Science 240: 64-66.

Wang J., S.J. Smerdon, J. Jager, L.A. Kohlstaedt, P.A. Rice, J.M. Friedman, and T.A. Steitz. 1994. Structural basis of asymmetry in the human immunodeficiency virus type 1 reverse transcriptase heterodimer. Proc. Natl. Acad. Sci. USA 91: 7242-7246.

Wei X., S.K Ghosh, M.E. Taylor et al. 1995. Viral dynamics in human immunodeficiency virus type 1 infection. Science 273: 117-122.

Weiss S., B. Konig, H.J. Muller, H. Seidel, and R.S. Goody. 1992. Synthetic human tRNA(UUUlys3) and natural bovine tRNA(UUUlys3) interact with HIV-1 reverse transcriptase and serve as specific primers for retroviral cDNA synthesis. Gene 111: 183-197.

Weniger B.G., Y. Takebe, C-Y. Ou, and S. Yamazaki. 1994. The molecular epidemiology of HIV in Asia. AIDS 8 (suppl. 2): S13-28.

Willey R.L., F. Maldarelli, M.A. Martin, and K. Strebel. 1992. Human immunodeficiency virus type 1 Vpu protein regulates the formation of intracellular gp160-CD4 complexes. J. Virol. 66: 226-234.

Wills J.W., R.C. Craven. 1991. Form, function, and the use of retroviral Gag proteins. AIDS 5: 639-654.

Winslow D., S. Garber, C. Reid, H. Scarnati, D. Baker, M. Rayner, and E. Anton. 1996. Selection conditions affect the evolution of specific mutations in the reverse transcriptase gene associated with resistance to DMP 266. AIDS 10: 1205-1209.

Wlodawer A., M. Miller, M. Jaskolski, B.K. Sathyanarayana, E. Baldwin, I.T. Weber, L.M. Selk, L. Clawson, J. Schnei

Wõhrl B.M., and K. Moelling. 1990. Interaction of HIV-1 ribonucleotide H with polypurine tract containing RNA-DNA hybrids. Biochemistry 29: 10141-10147.

Wolinsky S.M., A.M. Wike, B.T.M. Korber, C. Hutto, W.P. Parks, L.L. Rosenblum, K.J. Kunstman, R. Furtado, and J.L. Munoz. 1992. Selective transmission of human immunodeficiency virus type 1 variants from mothers to infants. Science 255: 1134-1137.

Worgall S., R. Connor, R.J. Kaner, E. Fenamore, K. Sheridan, R. Singh, and R.G. Crystal. 1999. Expression and use of human immunodeficiency virus type 1 coreceptors by human alveolar macrophages. J. Virol. 73: 5865-5874.

Workshop report from the European commission (DG XII, INCO-DC) and the Joint United Nations Programme on HIV/AIDS (UNAIDS). 1997. HIV-1 sutypes:

implications for epidemiology, pathogenicity, vaccines and diagnostics. AIDS 11: 17-35.

Workshop report from the European Commission (DG XII, INCO-DC) and the joint United Nations Programme on HIV/AIDS. AIDS 1997, No. 11; UNAIDS 17 - UNAIDS 36.

Xin K., X. Ma, K. Crandall, et al. 1995. Dual infection with HIV-1 Thai subtype B and E. Lancet 346: 1372-1373.

Yarchoan R., H. Mitsuya, C.E. Myers et al. 1989. Clinical pharmacology of 3'-azido-2',3'-dideoxythymidine (zidovudine) and related dideoxynucleosides. N. Engl J. Med 321: 726-738.

Yarchoan R., J.A. Lietzau, B-Y. Nguyen et al. 1994. A randomized pilot study of alternating or simultaneous zidovudine and didanosine therapy in patients with symptomatic human immunodeficiency virus infection. J. Infect. Dis. 169: 9-17.

Young S.D., S.F. Britcher, L.O. Tran, L.S. Payne, W.C. Lumma, T.A. Lyle, J.R. Huff, P.S. Anderson, D.B. Olsen, S.S. Carroll, D.J. Pettibone, J.A. O'Brien, R.G. Ball, S.K. Balani, J.H. Lin, I-W. Chen, W.A. Schleif, V.V. Sardana, W.J. Long, V.W. Byrnes, and E.A. Emini. 1995. L-743, 726 (DMP-266): a novel, highly

potent nonnucleoside inhibitor of the human immunodeficiency virus type 1 reverse transcriptase. Antimicrob. Agents Chemother. 39: 2602-2605.

Yu X., X. Yuan, Z. Matsuda, T-H. Lee, and M. Essex. 1992. The matrix protein of human immunodeficiency virus type 1 is required for incorporation of viral envelope protein into mature virions. J. Virol. 66: 4966-4971.

Zack J.A., S.J. Arrigo, S.R. Weitsman, A.S. Go, A. Haislip and I.S.Y. Chen. 1990. HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. Cell 61: 213-222.

Zacharova V., M.L.B. Becker, V. Zachar, P. Ebbesen, and A.S. Goustin. 1997. DNA sequence analysis of the long terminal repeat of the C subtype of human immunodeficiency virus type 1 from Southern Africa reveals a dichotomy between B subtype and African subtypes on the basis of upstream NF-IL6 motif. AIDS Research and Human Retroviruses 13: 719-724.

Zhang D., A.M. Caliendo, J.J. Eron et al. 1994. Resistance to 2',3'-dideoxycytidine conferred by a mutation in codon 65 of the human immunodeficiency virus type 1 reverse transcriptase. Antimicrob. Agents Chemother. 38: 282-287.

Zhang H., G. Dornadula, M. Beumont et al. 1998. HIV-1 in the semen of men receiving highly active antiretroviral therapy. N. Engl. J. Med. 339: i803-i809.

Zhang L., Y. Huang, T. He, Y. Cao, and D.D. Ho. 1996. HIV-1 subtype and second-receptor use. Nature 383: 768.

Zhu T., N. Wang, A. Carr, S. Wolinsky, and D. Ho. 1995. Evidence for coinfection by multiple strains of Human Immunodeficiency virus type 1 subtype B in an acute seroconvertor. J Virol. 69: 1324-1327.