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Intrapatient evolution of HIV-1 in the context of coreceptor usage

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Som med vederbörligt tillstånd av Medicinska fakulteten vid Lunds universitet för avläggande av doktorsexamen i medicinsk vetenskap kommer att offentligen försvaras i seminarierummet, BMC D15, Sölvegatan 19, Lund, torsdagen den 14 juni 2007, kl 9.00

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To my family

CONTENTS

LIST OF PAPERS

This thesis is based on the papers listed below, which will be referred to in the text by their roman numerals (I-IV):

I **Mattias Mild, Åsa Björndal, Patrik Medstrand, and Eva Maria Fenyö.** Isolation of human immunodeficiency virus-type 1 (HIV-1) clones with biological and molecular properties of the primary isolate. *Virology 350 (2006) 58-66*[1].

II **Mattias Mild, Joakim Esbjörnsson, Eva Maria Fenyö, and Patrik Medstrand.** Frequent intrapatient recombination between HIV-1 R5 and X4 envelopes: Implications for coreceptor switch. *Journal of Virology. 2007 Apr;81(7):3369-76*[2].

III **Mattias Mild, Anders Kvist, Joakim Esbjörnsson, Ingrid Karlsson, Eva Maria Fenyö, and Patrik Medstrand.** Differences in molecular evolution between switch and non-switch HIV-1 populations. *Submitted.*

IV **Mattias Mild, Anders Kvist, Eva Maria Fenyö, and Patrik Medstrand.** Divergence rates and selective pressure in switch and non-switch HIV-1 populations: the influence of R5 and X4 subpopulations. *Submitted.*

ABBREVIATIONS

2',3'-dideoxy-3'-thiacytidine	3TC
Acquired immunodeficiency syndrome	AIDS
Amino acid	aa
Amino-terminal	N-terminal
Analysis of variance	ANOVA
Base pair	bp
CC chemokine receptor	CCR
CD4 binding site	CD4bs
Central memory T-cells	T_{CM}
Circulating recombinant form	CRF
Constant region 1 to 5	C1-C5
CXC chemokine receptor	CXCR
Corboxy-terminus	C-terminal
Cystein	C
Cytotoxic T lymphocyte	CTL
Deoxyribonucleic acid	DNA
Dendritic cell	DC
DC-specific ICAM-3 grabbing nonintegrins	DC-SIGN
Double stranded	ds
Effector memory T-cells	T_{EM}
Enfuvirtide	ENF
Envelope (gene)	*env*
Envelope (protein)	Env
Glycoprotein	gp
Group antigen (gene)	*gag*
Group antigen (protein)	Gag
Gut-associated lymphoid tissue	GALT
Hemagglutinin	HA
Highly active antiretroviral therapy	HAART
Human T-cell lymphotropic virus 3	HTLV-3
Human immunodeficiency virus	HIV
Human immunodeficiency virus type-1	HIV-1
Human immunodeficiency virus type-2	HIV-2
Integrase	IN
Interferon	IFN
Lymphoadenopathy associated virus	LAV
Long terminal repeat	LTR
Long term progressors	LTPs
Low degree of syncytia formation	LDSF
Major group	M-group

Major histocompatibility complex	MHC
Macrophage inflammatory protein	MIP
Maximum-likelihood	ML
Monoclonal antibody	MAb
Months post infection	MPI
Mucosal-associated lymphoid tissue	MALT
Naïve T-cells	T_N
Natural killer cell	NK-cell
Negative regulatory factor gene	*nef*
Novel group	N-group
Nucleoside reverse transcriptase inhibitor	NRTI
Non-nucleoside reverse transcriptase inhibitor	NNRTI
Non-switch populations	nSP
Non-syncytium inducing	NSI
Outlier group	O-group
Peripheral blood mononuclear cells	PBMC
Positive single stranded	+ss
Potential N-linked glycosylation site	PNGS
Polymerase chain reaction	PCR
Polymerase (gene)	*pol*
Pre-integration complex	PIC
Protease	PR
Protease inhibitors	PI
Regulated on activation, normal T-cell expressed, and secreted	RANTES
Regulator of virion protein gene	*rev*
Reverse transcriptase	RT
Rev responsive element	RRE
Ribonucleic acid	RNA
Stromal cell derived factor 1	SDF-1
Simian immunodeficiency virus	SIV
Switch populations	SP
Syncytium inducing	SI
Transactivator gene	*tat*
Transactivation-responsive RNA	TAR
Variable region 1 to 5	V1-V5
Virion infectivity factor gene	*vif*
Viral protein R gene	*vpr*
Viral protein U gene	*vpu*
Zidovudine	AZT

AIMS OF THIS THESIS

The overall aim of this thesis was to investigate the molecular evolution of HIV-1 in the context of coreceptor usage within patients. The studies focused on recombination and its consequences, glycosylation patterns, evolutionary divergence rates and selective pressures in the HIV-1 envelope protein.

Paper I: To establish a biological cloning system for HIV-1 by using cell lines expressing different coreceptors.

Paper II: To investigate intrapatient HIV-1 recombination in patients with coexisting R5 and X4 variants. The consequences of recombination on coreceptor usage were also elucidated.

Paper III: To compare the evolution of potential N-linked glycosylation sites, V2 length and evolution of V3 charge between switch and non-switch virus populations.

Paper IV: To compare the divergence rates and selective pressures in HIV-1 switch and non-switch virus populations, as well as in coexisting R5 and X4 subpopulations.

SUMMARY

The variable region 1 to 3 (V1-V3) of the HIV-1 envelope plays an important role in coreceptor usage. Early in infection HIV- 1 is using CCR5 as coreceptor to enter target cells (R5 viruses) whereas viruses using CXCR4 as coreceptor (X4 viruses) may appear later in infection. This broadening or switch in coreceptor usage is associated with progression to AIDS. In my thesis work, we have compared the molecular evolution of V1-V3 between virus populations that maintained CCR5 coreceptor usage (non-switch populations, nSP) with virus populations that used CXCR4 as coreceptor (switch populations, SP). We also developed a novel biological cloning system.

We found an increase in the number of potential N-linked glycosylation sites in V1 -V3 over time in nSP, while SP showed no change. Since glycans are an important defense against neutralizing antibodies, we hypothesize that the antibody response differed between patients with SP and nSP. We found that V2 length and evolution of V3 charge differed between R5 viruses from SP and nSP, already before coreceptor switch in SP. Therefore, these molecular properties could prove important for understanding, and maybe even for predicting, the evolution of coreceptor usage in HIV-populations. Due to the presence of the X4 subpopulations, SP evolved faster compared to nSP. In addition, R5 and X4 from SP were subjected to different selective pressures. We showed that R5 and X4 viruses recombine frequently. We hypothesize that such rearrangements may affect antibody recognition of X4 and allow for antibody escape and expansion of X4 subpopulations.

INTRODUCTION

The epidemic

In the early 1980s, previously healthy homosexual men were diagnosed with opportunistic diseases associated with immunodeficiency such as Pneumocystis Pneumonia and Kaposi's Sarcoma (83, 105). These were the first reported cases of what became known as acquired immunodeficiency syndrome (AIDS). In 1983, a French group isolated a virus from a patient diagnosed with lymphoadenopathy (17). Within a few months, an American group isolated viruses from immunodeficient patients (89). The newly discovered virus were first called lymphoadenopathy associated virus (LAV) or Human T-cell lymphotropic virus 3 (HTLV-3). However, in 1986, the virus was shown to belong to the lentivirus family and was renamed human immunodeficiency virus (HIV). Later the same year, a closely related virus, named HIV-2, was discovered (45).

Comparison of the UNAIDS/WHO AIDS epidemic reports from 2006 and 2004 reveals that the global HIV epidemic continues to increase (UNAIDS-WHO report 2006, www.unaids.org). Today, approximately 40 million people are infected with HIV worldwide, which is 2.6 million more than in 2004. In 2006, more than 4.3 million people became infected and 2.9 million people died of AIDS. That corresponds to an increase of 400 000 and 200 000 people, respectively. In many regions of the world, a high number of new infections were reported among young people (15-24 years). Young people accounted for 40% of the new infections among people that were 15 years or older. Sub-Saharan Africa continues to bear the burden of the global epidemic with 25 million people infected, which is equivalent to 63% of the total number of cases. In addition, 72% of the people that have died of AIDS lived in Sub-Saharan countries. The highest increase in infected cases (21%) was reported in East Asia, Eastern Europe, and Central Asia. The majority of new cases in Eastern Europe and Central Asia were transmitted via contaminated needles. Globally, and in every region, more adult women than ever before are infected with HIV. The number of women infected increased with over one million compared to 2004. One positive aspect is that the access to treatment and care has increased and it has been estimated that two million life years were gained since 2002 in low- and middle-income countries.

In Sweden, approximately 7500 people have been reported to be HIV positive to date (www.smittsyddsinstitutet.se). The number of newly infected cases were 390 in 2006, which is a decrease compared to the last two years. Only 12 cases were reported among people between 15 and 29 years of age.

HIV-1 genome and structure

HIV belongs to the family of *Retroviridae* (retroviruses), which can be further subdivided into seven generas: *Alpha-*, *Beta-*, *Gamma-*, *Delta-*, *Epsilon-*, *Lenti-*, and *Spumaretroviridae*. All retroviruses are enveloped viruses with an RNA genome and replicate through a DNA intermediate using reverse transcriptase. The viral DNA can be integrated as an obligate parasite into the host cell genome using a viral-encoded integrase. HIV is a complex retrovirus since it has additional regulatory and accessory genes and is classified as a lentivirus.

The HIV-1 genome is approximately 9.7 kilobases and consists of two copies of positive single stranded (+ss) RNA. When integrated as a double stranded DNA (provirus), it is flanked by long terminal repeats (LTR) generated during reverse transcription. The genome contains three open reading frames and like other retro-

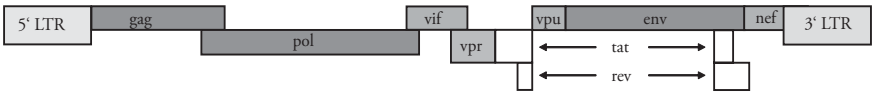

Figure 1. The genomic organization of HIV-1. The genome is flanked by long terminal repeats (LTR). The main genes *gag*, *pol* and *env* encodes the structural proteins (p24, p17 and p7), the viral enzymes (RT, PR and IN) and envelope protein (gp120 and gp41), respectively. Regulatory proteins are encodes by *tat* and *rev*. Finally, *nef*, *vif*, *vpr* and *vpu* encode the accessory proteins.

viruses the main genes are *gag*, *pol* and *env* (Figure 1). The *gag* gene encodes the Gag polyprotein precursor p55 that is cleaved into capsid (p24), matrix (p17), nucleocapsid (p7) and Vpr-binding (p6) proteins, by the viral protease. The *pol* gene encodes the viral enzymes reverse transcriptase (RT), protease (PR) and integrase (IN) that are produced as a Gag-Pol precursor and processed by the viral protease. The polyprotein precursor gp160 is encoded by the *env* gene and is proteolytically cleaved into the transmembrane protein gp41 and the surface protein gp120. Finally, the genome also comprises genes encoding regulatory (Tat and Rev) and accessory (Nef, Vif, Vpr, and Vpu) proteins, which are important regulators of the viral replication, transcription and assembly (Table 1).

HIV is a spherical enveloped virus, approximately 100nm in size (Figure 2). In a cone shaped structure the p24 capsid protein enclose the diploid +ssRNA genome that is coated with nucleocapsid proteins, p7. The viral enzymes and the accessory proteins are found inside the capsid. The matrix proteins p17 line the envelope, and are responsible for maintaining virion structure but it also contains a nuclear localization signal important for the nuclear import of the HIV-1 preintegration complex. The HIV-1 envelope is a host cell derived lipid bilayer. Embedded in the

Table 1. Functions of HIV-1 regulatory and accessory proteins.

Protein	Function
Regulatory	
Tat	Stimulation of transcription; binds TAR to facilitate initiation and elogation of viral transcription
Rev	Regulation of viral mRNA production; bind RRE and facilitates nuclear export of unspliced or singly spliced RNAs
Accessory	
Nef	Can increase and decrease viral replication; reduces expression of CD4 and MHC class I; affects T-cell activation; enhances virion infectivity
Vif	Increases virus infectivity; Affects virion assembly
Vpr	Causes G2 cell cycle arrest; facilitates nuclear entry of PIC
Vpu	Affects virus release; disrupts Env-CD4 complexes; CD4 degradation

envelope is gp41 which anchors the gp120 trimers that protrudes from the envelope. In addition, numerous host cell proteins can be found embedded in the envelope.

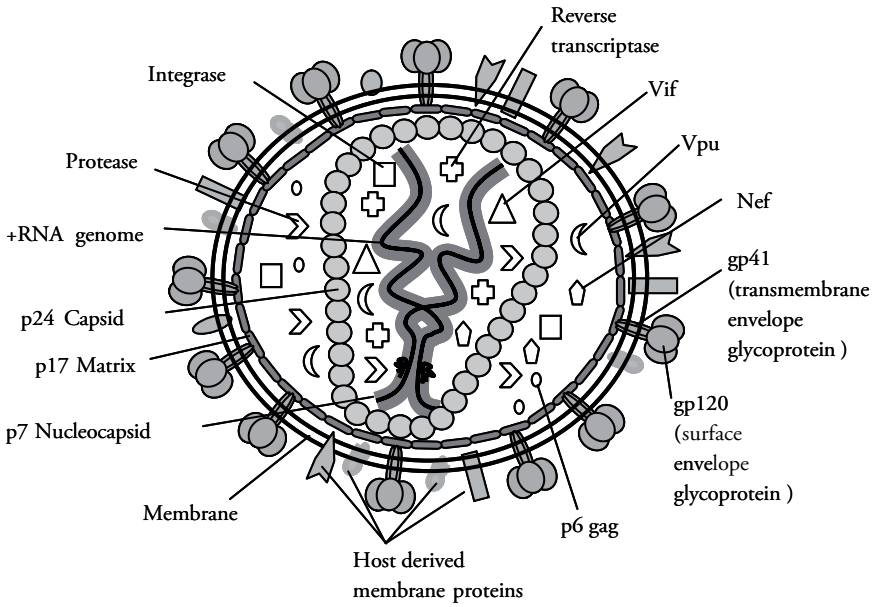

Figure 2. Structure of the HIV-1 particle. Kindly provided by Anna Laurén.

The viral life cycle

Viral entry

HIV-1 most efficiently infect cells of the T-cell and macrophage lineages (51, 79, 94, 152) and enters target cells through a series of interactions between the viral glyco-

proteins, the cellular receptor CD4 and a coreceptor, most often CCR5 or CXCR4 (Figure 3). The surface protein gp120 consists of five constant regions (C1-C5) and

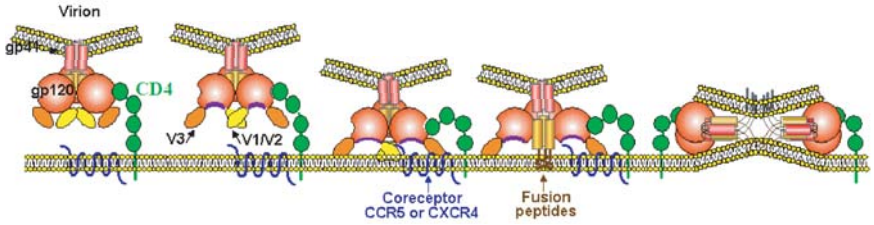

Figure 3. Schematic picture of HIV-1 entry. Gp120 binds to CD4, which induces conformational changes in gp120 and exposure of the coreceptor binding site. Coreceptor binding induces further conformational changes in gp41, which allows insertion of the fusion peptide into the host cell membrane. The final step is formation of the six-helix bundle, which brings the viral and cell membranes together and allows fusion. (Kindly provided by Dr. R.W Doms).

five variable regions (V1-V5) and both constant and variable regions are involved in the sophisticated entry process of HIV-1 (Figure 4). First the CD4 binding do-

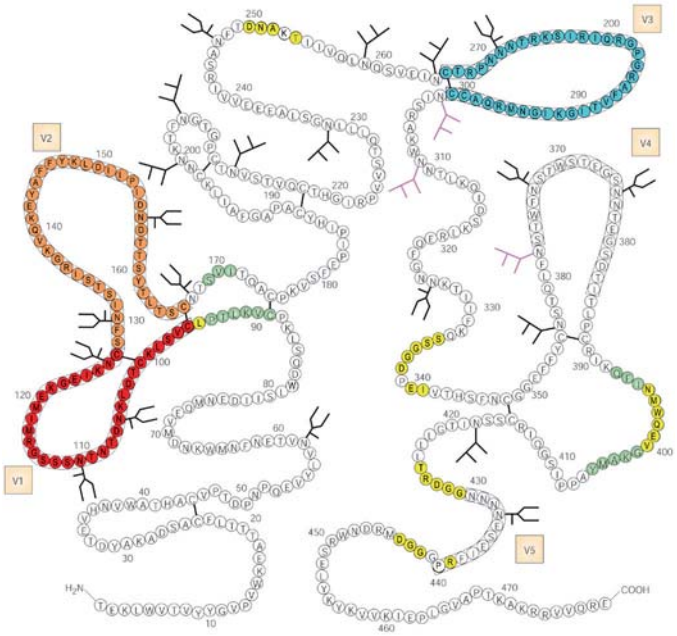

Figure 4. The gp120 molecule. Location of the variable regions are marked in boxes (V1–V5). Variable regions under study are highlighted in colour: V1: red, V2: orange, V3: blue. Residues in contact with CD4 are highlighted in yellow (161). The CD4-induced epitope is highlighted in green (161). The glycosylation sites containing high mannose-type and/or hybrid-type oligosaccharide structures are indicated by the branched structures, and glycosylation sites containing complex-type oligosaccharide structures are indicated by the U-shaped branches. Glycosylations that induce neutralizing antibodies are highlighted in purple. Adopted from (313).

main, key epitopes in V1/V2 stem, C2, C3, C4, V5 and C5 of gp120 interacts with CD4 (161) (Figure 3 and 4). This interaction promotes a conformational change in gp120, which results in exposure of the bridging sheet, composed of four anti-parallel ß-strands from the V1/V2 and C4 regions of gp120 (297). The bridging sheet and the V3 region interact with the coreceptor, leading to a conformational change in gp41. Consequently, a fusion peptide in gp41 is inserted in the host cell membrane. (183). The final step is the formation of a six-helix bundle of gp41 that brings the viral and cellular membranes together and allows fusion to occur (194). Dendritic cells (DC) also play an important part in HIV infection by transferring the virus to susceptible CD4-expressing cells in *trans* (97). HIV-1 gp120 can bind C-type lectins, for example DC-specific ICAM-3 grabbing nonintegrins (DC-SIGN), on the surface of DC. The virus is internalized and can retain infectivity up to 4 days (97). The transfer of HIV-1 from DC to T-cells occurs via the formation of virological synapses, that concentrate viruses and receptors at the site of contact between the two cells (136, 190, 281). It has been suggested that virological synapses protect the virus from recognition by neutralizing antibodies (90).

Transcription and translation

After fusion, the capsid is delivered to the cytoplasm (Figure 5). The +ssRNA is reverse transcribed into double stranded (ds) DNA by RT, inside the semi-dissolved

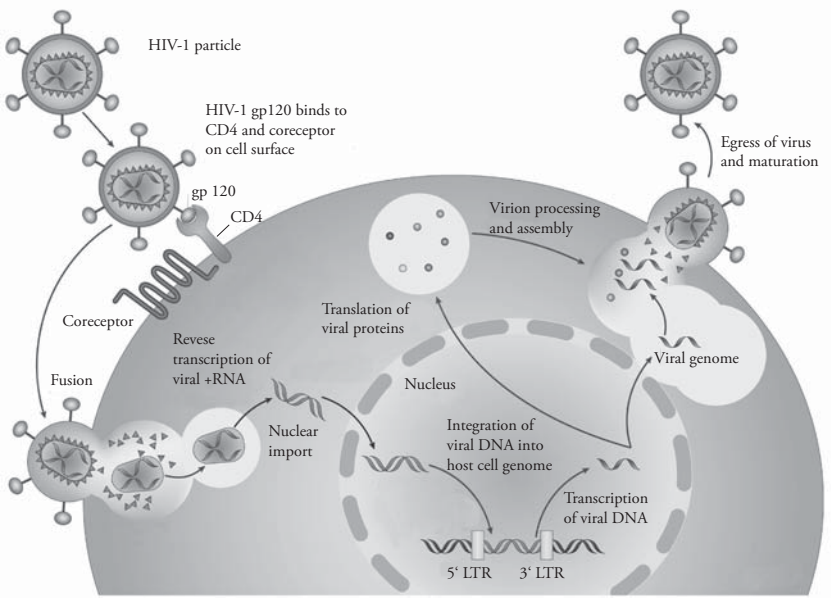

Figure 5. HIV-1 life cycle. HIV-1 enters target cell through interactions with CD4 and a coreceptor. The viral RNA is reverse transcribed and inserted into the host cell genome. Transcription and translation is performed by the cellular machinery and new particles are assembled at the plasma membrane. After virus budding, the viral proteases cleave ploy protein precursors, which generate the mature virus particle. Adopted from (226).

capsid. The viral proteins Vpr, the matrix protein p17 and integrase binds to the dsDNA and forms the pre-integration complex (PIC). A nuclear localization signal within p17 directs the PIC into the nucleus (30) where the provirus is integrated into the host cell genome. The viral integrase catalyzes the integration process and it has been shown that the proviral DNA preferentially integrates within gene regions (254). Once the proviral DNA is integrated, the virus fully relies on the host cell transcription machinery, since the virus lacks RNA polymerase II, required for transcription of dsDNA.

HIV-1 gene expression is regulated by both cellular and viral proteins. The transcription is initiated by binding of cellular transcription factors, including NF-κB, to the enhancer region in the LTR. (204). The early transcripts are mainly short transcripts (140). These are multiply spliced mRNAs and encode the Tat, Rev and Nef proteins. Tat binds to the transactivation-responsive RNA (TAR) element positioned downstream from the enhancer element in the LTR, and facilitate efficient elongation. Therefore, the transcription terminates prematurely in the absence of Tat (145). Consequently, at early time points a high level of Tat, Rev and Nef is observed. Accumulation of Rev results in a shift from early to late transcripts. In addition, Rev binds to the rev responsive element (RRE) in the *env* region and activates the transport of unspliced mRNA to the cytoplasm. There are two types of late transcripts. The first encodes the structural Gag proteins and the viral enzymes. The second type of transcripts consist of 5 singly spliced mRNA that encodes the gp160 Env precursor protein and the accessory proteins Vif, Vpr and Vpu.

All mRNAs are translated in the cytoplasm or in close proximity to the endoplasmatic reticulum. The Env proteins are heavily glycosylated and cleaved by cellular proteinases into gp41 and gp120 in the Golgi apparatus, before being inserted into the membrane. The viral particle is assembled at the plasma membrane and buds off from the cell, thereby acquiring the cell derived envelope. The final maturation step occurs after budding and is performed by the viral protease that cleaves the Gag-Pol polyprotein into smaller proteins. The generation time of HIV has been estimated to 1.2-2.6 days (86, 214, 235).

HIV receptors

In 1984, the CD4 molecule was identified as a receptor for HIV-1 (51, 152, 153). It soon became evident that CD4 was not enough to confer HIV-1 entry. The discoveries in late 1995 that the CC-chemokines could inhibit HIV-1 replication (47), and in early 1996 that the CXC-chemokine receptor CXCR4 was a coreceptor for some HIV-1 strains (71) had an extraordinary impact on the HIV research field. Within a year several groups identified a second coreceptor for HIV-1, CCR5 (41, 56, 61, 62) and it was discovered that a 32-base pair (bp) deletion in the coding region

of the CCR5 gene (CCR5-Δ32) was associated with resistance to HIV-1 infection (54, 175, 243, 311). Since the finding that CCR5 and CXCR4 are the major co-receptors for HIV-1 more than a dozen minor coreceptors have been identified for HIV-1, such as CCR2b (61), CCR3 (41), CCR8 (238), CXCR6 (57), gpr1 (263), gpr15 (57) , RDC1 (264) and APJ (40, 64). However, when these minor receptors are used *in vitro* it is always in combination with CCR5 and/or CXCR4 and the *in vivo* role of these minor receptors is unclear. The chemokine receptors belong to the seven transmembrane spanning G-protein-coupled receptors that are involved in signal transduction (14, 202, 236). The amino-terminus (N-terminal) part of the receptor is located outside the cell and the carboxy-terminus (C-terminal) part is coupled to a signal transducing G-protein and is located inside the cell. The N- and C-terminal are separated by three extracellular loops and three intracellular loops. Disulfide bonds between the first and second extracellular loops bring the extracellular domains together, resulting in a stable barrel-like structure. Chemokines receptors can de divided into four families: C, CC, CXC and CX_3C, chemokines, based on the arrangement of conserved cystein (C) residues (312). Chemokines are small peptides (92-125 amino acids (aa)) involved in leukocyte trafficking (reviewed in (14)). The chemokines that bind to CCR5 are RANTES (Regulated on activation, normal T-cell expressed, and secreted), Macrophage inflammatory protein (MIP) -1α and MIP-1β and the natural ligand for CXCR4 is stromal cell-derived factor 1 (SDF-1). They exhibit suppressive effect of HIV-1 by down regulating coreceptor expression and through competitive binding (3, 9, 207). This has been employed in the development of HIV-1 inhibitors (see Introduction, HIV evolution during antiretroviral therapy).

It has been shown that different parts of CCR5 and CXCR4 are involved in HIV-1 binding. The N-terminal and the first extracellular loop seem to be most important for HIV-1 binding to CCR5 (60, 167), while the first and second extracellular loops are the key epitopes in CXCR4 (25, 26, 177). The binding of HIV-1 to CCR5 and CXCR4 results in a signal cascade that affects the gene expression of the infected cell. The major group of genes affected is involved in the cell cycle and the cell is forced into an active state (43). This promotes transcription of HIV-1 genes and results in increased viral production. In addition, viruses that bind to CCR5 and CXCR4, respectively, seem to induce distinct gene expression in peripheral blood mononuclear cells (PBMC) (43).

HIV-2 and simian immunodeficiency virus (SIV) have been reported to enter cells in a CD4-independent manner (44, 65, 69, 228). In addition, some laboratory adapted HIV-1 strains have been shown to exhibit the same properties (63, 123, 162) while primary CD4-independent HIV-1 has not been isolated until recently (306). The discrepancy in the ability of CD4 independent infections between HIV-1 and HIV-2 and SIV could be a consequence of differences in the conformational changes

needed during the entry process. It is possible that HIV-2 and SIV envelopes have a more open conformation compared to HIV-1 and therefore the initial binding step to CD4 is not essential for exposure of the coreceptor binding site (123). However, the more open structure could also make the virus more vulnerable to neutralizing antibodies, a correlation that has not been fully investigated (67, 278).

Biological phenotype

Before the coreceptors were identified, HIV-1 was defined as being fast or slow replicating in peripheral PBMC (13, 72) or as having the capacity to induce syncytia in PBMC or MT-2 cells (syncytium inducing (SI) and non-syncytium inducing (NSI) respectively) (39, 256, 275, 276). Detection of SI strains in infected individuals was an indicator of poor prognosis since it was associated with accelerated loss of $CD4^+$ T-cells and a more rapid progression to AIDS (142, 232, 255). The discovery that SI-viruses mainly used CXCR4 and NSI-viruses mainly used CCR5 resulted in a new nomenclature for HIV-1 (18). Viruses that used CCR5 were designated R5 viruses, CXCR4-using viruses, X4, and viruses able to utilize both receptors, R5X4. This nomenclature will be used throughout the thesis.

Determinants of coreceptor usage

Numerous studies have shown that the V3 region of gp120 is the principal determinant of coreceptor usage (reviewed in (115)). V3 is a 35 aa long loop structure held together by disulphide bonds between the cysteins in position 1 and 35 (Figure 4). A few amino acid changes in V3 can change the coreceptor usage from CCR5 to CXCR4 (53, 262, 265). High V3 charge and presence of basic aa in position 11 and 25 has been associated with CXCR4 usage (53, 80, 81), whereas negative or uncharged aa in positions 11, 25, or 28/29, resulting in a low V3 charge, or presence of a glycine-proline-glycine motif in position 15-17 has been associated with CCR5 usage (53, 81, 126, 265, 299). In addition, methods for predicting coreceptor usage from V3 amino acid sequences have been developed and are used with good result (132, 133, 230). Although the V3 region is the principal determinant, other regions have also been shown to impact coreceptor utilization such as V1/V2, V4 and C4 (34, 131, 164, 203, 208, 221). In addition, a recent study suggests that the V1/V2 region can compensate for loss of fitness mutations in V3 (212).

Investigations on V2 length and the impact on the coreceptor repertoire and disease progression have generated contradictive results. Studies have suggested that an elongated V2 is associated with switch to CXCR4 usage (131) or from NSI to SI phenotype (108) whereas others have claimed, without statistical support, that an elongated V2 is associated with slow disease progression (266) and maintained CCR5 usage (185).

The coreceptor switch - facts and hypotheses

Early in infection, the viral population uses CCR5 whereas coreceptor usage may switch, or broaden, to include CXCR4 usage later in infection. The broadening of coreceptor usage to include CXCR4 is associated with accelerated loss of $CD4^+$ T-cells and faster progression to AIDS (250). After the appearance of X4 viruses, the R5 and X4 populations most often coexist in the host (73, 283) and **Paper II**). Despite intense efforts, the cellular and molecular mechanisms responsible for HIV-1 coreceptor switch remain unclear, although several hypotheses have been presented (Reviewed in (229)). The transmission-mutation hypothesis, the target-cell-based hypothesis and the immune-based hypothesis are considered the main hypotheses.

The transmission-mutation hypothesis suggests that R5 viruses are preferentially transmitted and are gradually mutated into X4 viruses. The main argument for the transmission-mutation hypothesis is that people that are homozygous for a 32 bp deletion in the CCR5 gene, and consequently do not express CCR5 on cell surfaces, are resistant to HIV-1 infection (175, 289). In addition, a stochastic model of HIV-1 showed that the virus would switch coreceptor usage in approximately 50% of individuals which is in agreement with experimental data (154). The model assumed that only a few amino acid changes are required for switch from CCR5 to CXCR4 and that the fitness for the intermediate mutants was lower than the initial R5 variant (229). Finally, a recent study suggested that the X4 subpopulation identified in two children evolved from their own R5 subpopulation and was not caused by transmission of X4 variants (46).

The target-cell-based hypothesis emphasizes that a gradual shift in availability of CCR5- and CXCR4-expressing cell populations is responsible for the appearance of X4 viruses (52). The hypothesis presented by Davenport *et al.* was based on the differential expression of CCR5 and CXCR4 on naïve and memory $CD4^+$ T-cells as well as the dynamics of these cell populations during the course of infection (23, 168). CXCR4 is mainly expressed on naïve $CD4^+$ T-cells, whereas memory $CD4^+$ T-cells mainly express CCR5. During the course of infection the proliferation rate of the naïve cells increases which would act as a selective pressure in favor of X4 viruses (116). A mathematic model based on division rates of naïve and memory $CD4^+$ T-cells and the ability of R5 and X4 viruses to bind these cells presented by the same research group support this hypothesis (231). However, investigation of CXCR4 and CCR5 expression during HIV-1 infection presents contradictive data (284). Van Rij et al. showed that the percentage of CXCR4-expressing $CD4^+$ T-cells was not associated with development of X4 variants. Instead, they found that early viral load and $CD4^+$ T-cell count was associated with the switch (284). Even if we do not understand the details of HIV-1 susceptible cell dynamics during the course of infection it is reasonable to assume that such factors influence HIV-1 pathogenesis and coreceptor switch.

Finally, the immune-based hypothesis suggests that X4 viruses are better recognized by the immune system and are subsequently suppressed early in infection. X4 populations may emerge as a consequence of gradual dysfunction of the immune system. Several experimental data support this hypothesis (24, 31, 114, 300). First, studies in macaques have shown that dual-infections with R5 and X4 results in dominance of R5 variants. However, monoclonal antibody depletion of the $CD8^+$ T-cells in the macaques prior to infection resulted in dominance of X4 variants (114). Second, genetic analysis of the *env* gene has shown that X4 subpopulations are under stronger selective pressure, possibly from the immune system, than R5 subpopulations (24, 300). Third, a recent study showed that X4 clones are more sensitive to neutralizing antibodies than the coexisting R5 clones (31). Finally, it has also been shown that X4 viruses only appeared after the CD4-count had dropped below 400 cells/µl (155).
In summary, the coreceptor switch from CCR5 to CXCR4 is very complex. None of the studies that favor either hypothesis argue against the remaining two and all three hypotheses are supported by several observations. It is therefore likely that mechanisms presented in favor for each hypothesis are all involved in the coreceptor switch.

It should be emphasized that individuals with viral populations that do not undergo coreceptor switch also progress to AIDS. Such viral populations alter the mode of CCR5 usage (143). Early R5 isolates seem to be dependent on the N-terminal part of CCR5 for cell entry, whereas late R5 isolates may acquire the ability to use other parts of CCR5 (144).

HIV-1 Pathogenesis

The main routes for HIV-1 transmission are sexual contacts, contaminated needles, blood transfusions and mother-to-child transmissions. The rate of disease progression varies between HIV patients and the time from infection to development of AIDS may vary from one to 25 years. HIV-1 infection can be divided into two distinct phases: Acute infection and chronic infection (59). During acute infection some individuals experience flu like symptoms while others remain asymptomatic. The acute infection is associated with massive viral replication in the mucosal-associated lymphoid tissue (MALT) and especially in the gut-associated lymphoid tissue (GALT) which results in depletion of $CD4^+CCR5^+$ effector memory T-cells (T_{EM}) (27, 111, 174, 188, 193, 218, 286) and establishment of a chronic hyperactive immune system (27, 109). Recent studies have shown that within 10 days after infection the majority of extra-lymphoid tissue-based T_{EM} have either been infected or undergone apoptosis as a result of receptor interactions with the virus (174, 188). Within 10-21 days the viremia peaks with viral loads of 10^7 to over 10^8 million copies/ml, which is associated with a dramatic, and sometimes complete, depletion of $CD4^+$ T-cells in extra-lymphoid effector sites (27, 174, 188, 193, 218, 286). Since $CCR5^+$

T_{EM} cells only are minor components of the $CD4^+$ T-cell population in blood and lymph nodes ($CCR5^-$ naïve T-cells (T_N) and $CCR5^-$ central memory T-cells (T_{CM}) dominate), the $CD4^+$ T-cell populations at these sites are not as dramatically affected during acute infection. In addition, the importance of coreceptor usage of the infecting virus has been shown in macaques. CXCR4 is expressed at high levels on T_N, and infection with an X4 virus results in depletion of this T-cell population while the $CCR5^+$ T_{EM} population remains relatively preserved (122, 206, 218).

The massive depletion of $CCR5^+$ T_{EM} cells leaves a scar on the overall immune system function and the remaining infection is a battle for recovery for the immune system. Another consequence of the massive viral replication is the establishment of a persistent, hyperactive state of the immune system. The surviving $CD4^+$ memory T-cells display an increased proliferative activity and new short lived $CCR5^+$ T_{EM} are produced. This stabilizes the depletion in extra-lymphoid tissue sites that occurred during acute infection and a complete immune collapse and death is temporarily avoided. However, the newly regenerated T-cell pool also provides the viral population with new infectable targets needed for a chronic infection (109, 174, 218). The regeneration of T-cell pools is thought to be associated with the decline in peak viral replication and the establishment of the viral set-point of chronic infection. The viral set-point has been shown to be correlated to disease progression and can be used as a prognostic marker (195). The activation of $CD4^+$ memory T-cells is only one component of a more generalized immune activation that involves $CD4^+$ and $CD8^+$ memory T-cells, NK-cells, B-cells and accessory cells (217). At start, this activation is not fundamentally different from the innate immune response. Such response wanes in other infection but in the case of HIV-1 it persists indefinitely, resulting in chronic infection. The average duration of the chronic phase is approximately 10 years (211) and it is characterized by low but persistent viral replication and a slow, continuous loss of $CD4^+$ T-cells and high $CD8^+$ anti-HIV responses. Most individuals remain relatively healthy during the chronic phase but some suffer from clinical symptoms such as diarrhea, chronic fever or weight loss. Eventually the number of effector T-cells needed to mount an adequate immune response can no longer be maintained. The failure is most likely a consequence of irreversible loss of regenerative capacity. Such loss could result from a decrease in a specific immune cell population, e.g. T_{CM} or T_N, antigen presenting cells or virus specific lymphocytes. But it could also be a consequence of failure to coordinate these depleted and excessively stimulated cell populations (109). As a result of the non-functional immune system the infected individual gets susceptible to opportunistic diseases such as Pneumocystis Pneumonia, tuberculosis, toxoplasmosis and herpes virus infections. The appearance of opportunistic diseases marks the final AIDS stage and without treatment the patient usually dies within a few years.

The origin of HIV

The evolutionary history of HIV-1 and HIV-2 has been reconstructed by inferring phylogenetic trees from primate lentiviruses (92, 93, 112, 147, 156, 240, 244, 260, 305). Results from these studies showed that HIV-1 is most closely related to SIV from chimpanzee (Figure 6) which is found in the chimpanzee sub-species *Pan troglodytes troglodytes* and *Pan troglodytes schweinfurthii* (92, 147, 244). The habitats of *P.t. troglodytes* include the region in Africa (Cameroon, Gabon and Congo) with the greatest HIV-1 diversity and it has therefore been suggested that this was where

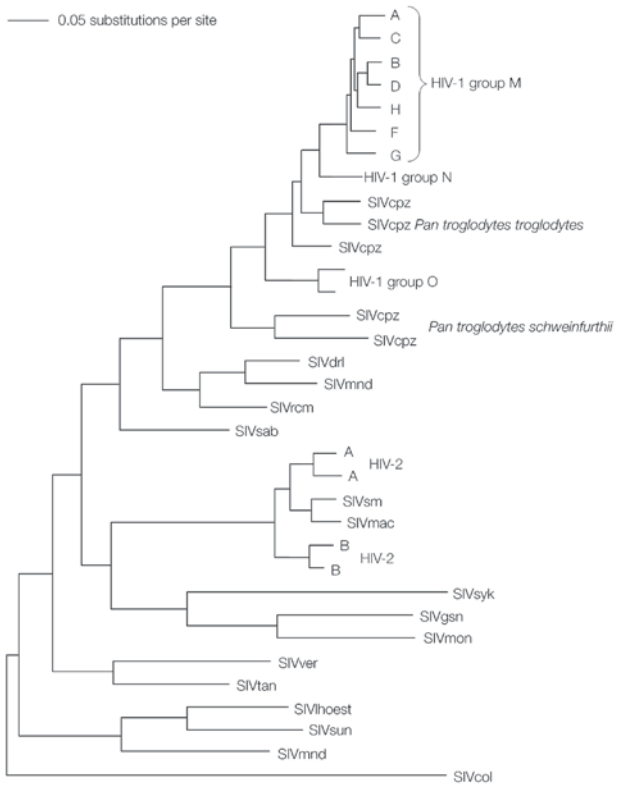

Figure 6. Maximum likelihood tree reconstructed using on alignment of 34 nucleotide sequences of the *pol* gene. Since HIV-1 and HIV-2 cluster with SIV from different primate species, they represent independent cross-species events. Three transmissions from chimpanzees to humans have resulted in the major (M), novel (N) and outlier (O) groups of HIV-1. Adopted from (226).

HIV-1 first emerged (226). HIV-2 shares the closest genetical relationship with SIV from sooty mangabey monkeys (*Cercocebus atys*) that is most frequently found in West Africa (Figure 6). Therefore, it is likely that the first cross-species transmission of HIV-2 to humans occurred in West Africa (93).

It has been difficult to determine the exact number of cross-species transmission of HIV from monkeys to humans but at least four transmissions are likely for HIV-2 (112), whereas three transmissions from chimpanzees to humans have been suggested for HIV-1. These transmissions have resulted in the three groups of HIV-1: The major (M), novel (N) and outlier (O) group (92, 260) (Figure 6).

It has been estimated that HIV-1 group M originated in the 1930s with a range of ± 10 years (156, 241, 305). Although most estimates for the time of origin for HIV-1 are consistent, recombination events have probably biased these estimates since recombination increases apparent variation in rates among nucleotide sites and reduces the genetic distances between sequences (251, 296). Therefore, in order to retrieve a reliable estimate of the time of origin for HIV-1 M group, archive sequences have been analyzed. The oldest sequence of HIV-1 M originates from 1959 (308) and it fits well with the extrapolation of the timescale obtained when the 1930s were calculated as the origin of HIV-1 group M (156). The introduction of HIV-2 in the human population has been estimated to around 1940-1950 (170). More accurate estimation of the origin of HIV-1 and HIV-2 would require analysis of more archive sequences.

HIV-1 genetic subtypes

Multiple introductions of HIV-1 to the human population and extensive genetic variation have resulted in several genetic lineages that can be divided into groups according to their phylogenetic relationship. The HIV-1 group M is the largest and most diverse group and is found worldwide, whereas group N and O are mainly found in West Africa. The M group can be further divided into subtypes A1, A2, B, C, D, F1, F2, G, H, J and K (169) (Figure 6). The subtypes differ by up to 30% of amino acids in Env and by up to 15% in Gag. In addition to the subtypes, 34 circulating recombinant forms (CRFs) have been identified (http://www.hiv.lanl.gov/content/index) (169). CRFs have emerged as a result of recombination events between viruses from different subtypes and have become circulating strains in the HIV-1 epidemic. In some areas in Central Africa CRFs have become the most prevalent circulating strains (118, 210), which further supports that Central Africa represents the epicenter of HIV-1 origin. The number of identified CRFs will probably increase over the years since better techniques for sequencing and detection of recombinants are constantly being developed.

HIV-1 variation

HIV-1 is the fastest evolving human pathogen and the worldwide variation is enormous. The accelerated evolution of HIV-1 is a consequence of several factors. First, the substitution rate is very high due to the error-prone viral reverse transcriptase (180). Second, the viral turnover is very high with as many as 10^{10} virus particles

produced daily (121, 216). Third, the immune system exerts a high selective pressure on the viral population (4, 85) and finally, recombination events between viruses from different subtypes, within subtypes and within subpopulations of an individual result in evolutionary leaps (36, 151, 157, 196, 215, 285). The best way to illustrate genetic variability is to construct phylogenetic trees. The genetic variability in the influenza virus hemagglutinin (HA) gene of the worldwide influenza virus epidemic in 1996 is comparable to the variation in HIV-1 V2-C5 of *env* within a single individual 73 months post infection (Figure. 7A and B). When HIV-1 phylogenies from cohorts like the Amsterdam cohort (23 HIV-1 infected individuals) or demographic areas like Democratic Republic of the Congo are constructed the degree of variation becomes enormous and it becomes evident that HIV-1 variation is the main obstacle when developing antiviral drugs and in vaccine design (Figure 7 C and D).

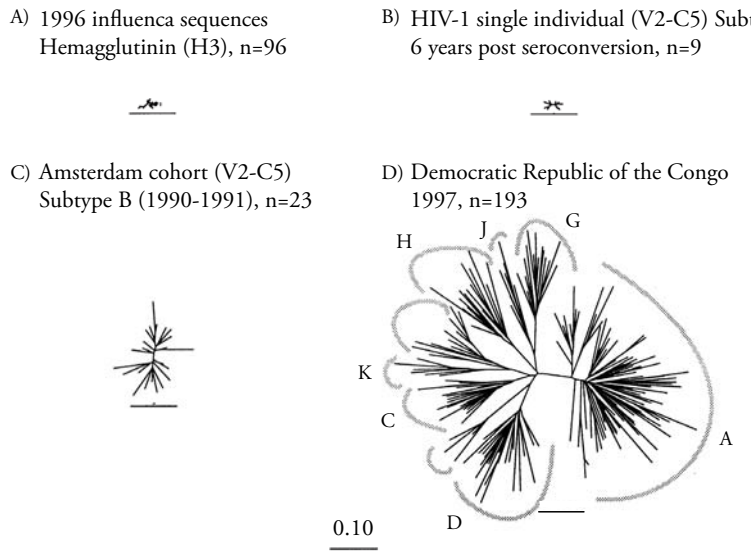

Figure 7. Comparisons of genetic variation of influenza hemagglutinin gene and HIV-1 C2-V5 *env* sequences through phylogenetic analysis. A) Tree based on 96 HA sequences encoding the HA1 domain of human influenza H3N2 viruses from 1996. B) Tree based on 9 HIV-1 subtype B C2-V5 *env* sequences from an infected individual 73 months post seroconversion. C) Tree based on HIV-1 subtype B C2-V5 *env* sequences from 23 individuals residing in Amsterdam 1990-1991. D) Tree based on HIV-1 C2-V5 *env* sequences sampled from 193 individuals residing in the Democratic Republic of the Congo in 1997. Adopted from (156).

HIV-1 recombination

Recombination is a shared feature among retroviruses. It occurs by template switching of the reverse transcriptase between the two RNA templates in the diploid virion, generating a daughter DNA provirus that is a mosaic between the two parental genomes (127) (Figure 8). Recombination events may lead to major genome rear-

rangements, and therefore exceed the impact of nucleotide substitution on HIV-1 variation. This makes recombination the most important contributor in the generation and diversification of HIV-1 subpopulations (226).

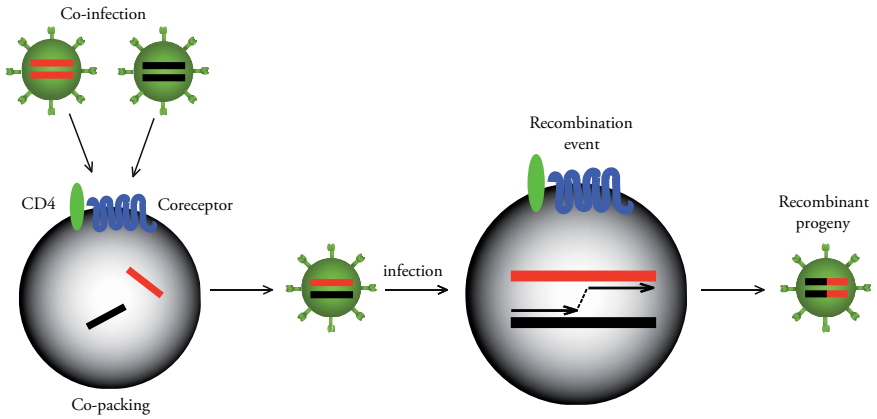

Figure 8. HIV-1 recombination. Two genetically distinct (red and black) virus particles infect the same cell. During assembly both genomes are packed in the same particle. Strand transfer event during reverse transcription in the second cell results in a mosaic genome and consequently a recombinant progeny.

It has been found that splenocytes of HIV-1 infected patients contained on the average three or four proviruses per cell (138), which indicate that cells frequently become coinfected which is a prerequisite for a recombination event to take place. From single cycle experiments, the recombination rate for HIV-1 has been estimated to range from two to 9 events per round of replication (134, 172, 310) and as much as 30 crossover events have been detected in single cycle experiments in macrophages (172). The strand transfer does not seem to occur at random sites in the genome and a hot-spot for recombination has been identified in the C2 region (between V2 and V3) of *env* (88, 200) (Figure 4). Reshuffling of the V2 with respect to V3 could have consequences for virus immune escape since the conformational arrangements in V2 and V3 with the respect to CD4 binding is recognized by neutralizing antibodies (303).

Recombination has been found repeatedly in studies of HIV-1 evolution. Recombination events between viruses of different subtypes of the M-group have resulted in 34 CRFs (169). Extensive recombination has been reported in intrapatient studies (37, 285) and (**Paper II**) and recombination between viruses derived from different anatomical sites within the same individual has been reported (149, 215). Establishment of recombinant viruses within the infected individual may lead to serious consequences for the patient due to rapid spread of drug resistance in the virus population (148) and accelerated progression towards AIDS (176).

Intrapatient HIV-1 evolution

Early in infection, the HIV-1 population is relatively homogenous (181, 182, 192, 259, 294, 300, 307, 309). With time the viral population diversifies and a complex population develops. However, 99% of the diversity in *gag* and *env* is lost during transmission (66). The consequences of such bottleneck depend on the mode of selection during transmission. If transmission is a neutral process the effect of genetic drift will be strong, which will have a negative effect on viral fitness. However, natural selection during transmission could alleviate the deleterious effects of genetic drift (66). In addition, it has been suggested that HIV-1 *env* evolves toward ancestral states during transmission. This would mean that HIV-1 recovers certain ancestral features when infecting a new host (117). The most variable part of the HIV-1 genome is the envelope gene and the V3 region can vary up to 15% on the amino acid level within the viral population of a patient. Due to its high variability and important role in coreceptor usage and neutralizing antibody recognition, *env* is the most extensively studied gene. Shankarappa *et al.* (259) described a consistent pattern in the evolution of the C2-V5 region of *env*: An early phase with linear increase (~ 1% per year) in both divergence (intra time-point genetic distance) and diversity (inter time-point genetic distance); an intermediate phase characterized by a continued increase in divergence but with stabilization or decline in diversity; and a late phase characterized by a slowdown or stabilization in divergence and a continued stabilization or decline in diversity. X4 viruses emerged around the time of early- to intermediate-phase transition and began a decline around the transition between the intermediate and late phase. The peak in diversity was supported statistically, while the stabilization in divergence was not. In order to evaluate the model for HIV-1 evolution proposed by Shankarappa *et al.*, this data set have been reanalyzed with more robust methods (189, 291). The increase in diversity followed by a decrease has been confirmed using regression modeling (189). However, the stabilization in divergence was not confirmed using the same model (189). Williamson *et al.* (291) used a set of statistical procedures to analyze the proposed stabilization in the data set from Shankarappa *et al.*. They evaluated the contribution of non-synonymous mutations (mutations that result in amino acid change) and synonymous mutations (mutations that do not result in amino acid change) separately. First, when the data were not combined across patients they lacked the power to reject a constant rate of evolution. However, in the analysis of combined data, they found evidence for divergence stabilization at non-synonymous, but not at synonymous sites (291). Thus, the pattern of change in divergence is not completely resolved and additional methods that can characterize changes in diversity and divergence needs to be developed.

A number of studies have investigated HIV-1 genetic evolution in the context of disease progression. Diverse populations were found in asymptomatic adults and children while limited viral heterogeneity was detected in rapid progressors (15, 55, 91,

191, 294). Analyses of the relationship between disease progression and divergence have resulted in contradictive results. Some studies reported a positive relationship between divergence rate and disease progression (181, 273) while others have suggested the opposite (15, 55, 191). A possible explanation for the contradictive results could be that relative small sample sizes have been studied.

Few studies have analyzed the molecular evolution of coexisting R5 and X4 subpopulations separately (46, 73, 283). It has been suggested that R5 subpopulations are more homogeneous compared to coexisting X4 subpopulations and compared to R5 populations from patients that do not have X4 variants (73). However, no reports of divergence rates of coexisting R5 and X4 subpopulations have, to my knowledge been presented.

HIV-1 intrapatient evolution is complex and is mainly influenced by selective pressure (171). The nature of the selective pressure can be analyzed by computing the ratio of the number of non-synonymous mutations per potential non-synonymous site and the number of synonymous mutations per potential synonymous sites (*dN/dS*). A *dN/dS* ratio above one indicates positive selection and *dN/dS* ratio below one indicates negative selection. Accordingly, the *env* gene of HIV-1 appears to be under positive selective pressures and it has been shown that divergence rates are correlated to selective pressures (15, 91, 181, 294). In these studies the selective pressure was analyzed for the whole *env* gene in relatively small data sets. In an attempt to characterize the pattern and rate of adapted evolution of the *env* gene within patients Williamson (290) analyzed a number of longitudinal data sets from previously published reports (91, 124, 181, 259, 272, 293). The data sets covered the V1-V5 region of *env* and viral populations from 50 patients were studied in total. The results showed that one adaptive substitution occurred every 3.3 months. He also found that one adaptive mutation is driven to a high frequency (>50%) every 2.5 months. Taken together, the adaptive evolution rate in HIV-1 *env* is the fastest ever recorded for a protein-coding gene. He also found that virus populations from slow progressors had a higher adaptive rate (290). This was in line with a previous report that made a detailed analysis of selective pressure on individual sites in *env* (237). Results from this study showed that viruses from slow progressors had more sites under positive selective pressure compared to viruses from patients with fast progression (237).

HIV evolution during antiretroviral therapy

Four classes of anti-HIV drugs are available today. Two classes are acting against the viral reverse transcriptase. Nucleoside reverse transcriptase inhibitors (NRTIs) are nucleoside analogues that terminate the transcription when incorporated into DNA. Non-nucleoside reverse transcription inhibitors (NNRTIs) inhibit the function of reverse transcription by binding to the enzyme. The third class, protease inhibitors (PI), prevents cleavage of the polyproteins to mature proteins during HIV-1

budding, which results in non-infectious particles. The fourth class is fusion inhibitors, which prevent the six helix bundle formation of gp41.

Initial treatment with the nucleoside analogue zidovudine (AZT) was successful with decreased mortality among AIDS patient in the late 1980s (77). However, it soon became evident that treatment with mono- and dual therapy generated drug escape variants resulting in treatment failure (165, 166). The introduction of combination therapy, usually referred to as highly active antiretroviral therapy (HAART) include at least three different antiretroviral drugs where at least one is a NNRTI or PI. HAART has decreased HIV-1 disease progression and the number of deaths caused by AIDS (209) and a mathematical model predicted that HAART would eradicate HIV-1 within 2-3 years (213). However, the model failed to take two important HIV aspects into account: viral reservoirs and evolution. Viral reservoirs have been found in a variety of cell types including $CD4^+$ T-cells (76), DC (270) and macrophages (129) and virus from these reservoirs can replenish the pool of replicating viruses. Soon after the introduction of HAART HIV-1 latent reservoirs were identified in patients receiving HAART (76). The evolution of HIV-1 has also proved to be more complicated under HAART than first anticipated. It was believed that the virus was not acquiring drug resistant mutations in patients with undetectable levels of viral load (76, 295). However, the virus continue to evolve even during successful treatment and eventually drug resistant mutations would appear (184). It has also been shown that antiviral treatment increase the mutation rate of HIV-1. Single cycle replication experiments have shown that RT inhibitors AZT and 2',3'-dideoxy-3'-thiacytidine (3TC) increased the mutation rate by 7.6 and 3.4 fold respectively (178). It has also been shown that the mutation rate is further increased when a drug-resistant virus replicates in the presence of drug. The mutation rate of AZT-resistant HIV-1 increased 24-fold compared to the wild type when replicating in the presence of AZT (179). In addition, replication of 3TC/AZT dual-resistant HIV in the presence of AZT and 3TC increased the mutation rate 22.5-fold. Newly administrated drugs can also increase the mutation rate of drug-resistant virus. For example, 3TC increased the mutation frequency of AZT-resistant virus 13.6 fold (179). Finally, drug resistant HIV-1 variants isolated from primary isolates have also been shown to have higher replication rate and increased infectivity (268).

Another complicating factor is HIV's ability to recombine. Resistance to drugs has been shown to spread vertically via recombination in both experimental systems (110, 201) and in vivo (148). In addition, computer simulations have suggested that recombination can both create (35) and break up drug-resistant mutations (28) and it is likely that the effective population size affects the outcome of recombination on drug-resistance, where a large population size would increase the generation of drug-resistance (8). Finally, it has also been shown that drug-resistant mutations in RT can increase template switching (205).

In 2003, the first entry inhibitor enfuvirtide (ENF) was licensed by the food and drug administration in the US and today several entry inhibitors are in clinical trial. ENF binds to gp41 and interferes with the formation of the fusogenic six-helix bundle. As with other antiretroviral drugs, resistance to ENF has been observed (163, 227, 269, 287).

It is apparent from the above discussion that the development of new drugs is important. One class of drugs that has been under development for years is CCR5 inhibitors and recently the first approved CCR5 inhibitor (maraviroc) was released on the market. Such inhibitors have been debated since it has been suggested that such treatment could select for viruses able to use CXCR4 and consequently result in faster disease progression.

Immune responses to HIV

Innate immunity

The first line of defense that all pathogens encounter is the innate immunity which acts within hours of a pathogen's appearance in the body. The response is non-specific and does not require antigen stimulation via major histocompatibility complex (MHC) molecules for activation and consequently does not confer long-lasting protective memory. The activation is mediated by non-specific binding of pathogens to cell surface receptors such as pattern recognition receptors and Toll-like receptors (75, 130). The defense involves a number of cell populations such as neutrophils, macrophages, DC, natural killer cells (NK-cells) and γδ-T-cells and has several important functions (173). Macrophages and DC activate the adaptive immune response by presenting antigens on MHC molecules. Interferon (IFN) α and β are produced by HIV-1 infected cells as a response to abnormal large amounts of double stranded RNA and induces an anti-viral state in neighboring cells. Upon HIV-1 infection such cells undergo apoptosis and limits spread of the virus. Furthermore, IFN stimulates upregulation of MHC I and therefore increased presentation of viral peptides to cytotoxic $CD8^+$ T-lymphocytes (CTL). NK-cells and γδ-T-cells kill infected cells through cytolytic mechanisms (21, 257). Infected cells can also be rapidly killed via activation of the classical pathway of the complement system through the formation of membrane attack complexes. Finally, the phagocytes (neutrophils and macrophages) engulf pathogens and clear foreign material.

The innate immunity fights the viral infection until the specific adaptive immune responses are activated. The adaptive immunity includes B-cells and T-cells and can be divided into the cellular and humoral response. The cellular response mainly includes CTL and the humoral response involves the production of antibodies by B-cells. A central role in both the cellular and humoral responses is played by the

$CD4^+$ T-cells that act as helper T-cells. By cell to cell contact and production of cytokines $CD4^+$ T-cells activate both CTL and B-cells and is therefore crucial for a functional adaptive immune response and control of HIV infection.

Cellular response to HIV

In vitro studies indicate that CTL can both effectively kill HIV-1 infected cells and inhibit viral replication through cytolytic and non-cytolytic mechanisms (301, 302). Studies of SIV in rhesus macaques have shown that depletion of CTL during primary infection led to loss of initial control of viral replication and to marked increase in viremia during chronic infection (252). In addition, rhesus macaques vaccinated to elicit CTL responses and then infected with SIV or SHIV (SIV with an HIV-1 envelope) had lower viral loads, better-preserved $CD4^+$ T-cell populations and survived longer than unvaccinated monkeys (10, 16, 258, 267). This clearly shows the importance of an effective CTL response in the control of SIV.

CTL responses are detectable in most study subjects and responses to all 9 HIV-1 proteins have been reported (1, 33, 82). Early in infection the CTL response is intimately linked to viral load and the number of HIV-1 specific CTL peak when the viral load levels off (292). In most viral infections, the CTL response is able to clear or control the pathogen to a level that is harmless for the host. Since HIV-1 infects cells of the immune system the response is impaired which results in loss of HIV-1 control. Another problem is the extensive genetic diversity generated in HIV-1, which results in mutants that escape the immune system. HIV-1 mutants that escape HIV-1 specific CTL responses have been detected in both early (96, 137, 224) and chronic infection (5, 6, 96, 107). In addition, a recent report suggested that escape from CTL responses is a major driving force in HIV-1 evolution (4). Together these findings clearly show that the cellular immune response acts as a strong selective force on HIV-1 evolution. However, a recent report taking the founder effect of the virus population into account challenges previous findings on CTL response driven HIV-1 evolution (19). They showed that 60 of 80 of the associations between human leukocyte antigen (HLA) and HIV-1 mutations could be explained by demographic and geographic structure of the HIV-1 epidemic rather than immune pressure (19). They suggest that the previously reported selective pressure from CTL is overestimated. This study clearly shows the importance of analyzing human and viral genetics in parallel and that the epidemiological linkage should be taken into account. This issue needs to be further investigated in order to obtain a uniform picture of the selective force exerted by CTL on HIV-1.

Humoral response to HIV-1

The general opinion on the role of antibodies in controlling HIV-1 infection has varied widely over the last 20 years from highly important in the 1980s to negligible in the mid 1990s. However, immunization experiments have repeatedly shown the

importance of antibodies in controlling HIV-1 infection (68, 95, 187, 225, 261). In addition, passive immunization experiments have shown that antibodies decrease the infectious inoculum and induce more effective cellular and humoral immune responses to the replicating viruses (113). In addition, depletion of the B-cell pool in rhesus macaques resulted in lower neutralizing antibody titers, less effective immunological control and higher viral loads compared to monkeys with intact B-cell repertoire (135, 253). These results clearly demonstrate the importance of potent neutralizing antibody for an effective HIV-1 control and the common view in HIV-1 research today is that antibodies have an important role in suppressing HIV-1 infection.

The neutralizing antibody response seems to be the major antibody action mechanism in HIV-1 infection (234). Neutralizing antibodies are mainly directed towards gp120 which prevent viral interaction with host cell receptors (99, 100, 199, 245, 247). However, non-neutralizing antibodies are also involved in post-attachment mechanisms, e.g. by interfering with the formation of the gp41 coiled-coil and thereby preventing fusion with the host cell (20, 98, 104). In addition, non-neutralizing antibodies have been suggested to be involved in activation of the classical pathway of the complement system (128, 271) and killing of infected cells via antibody-dependent cell mediated cytotoxicity (7, 158, 282).

A significant neutralizing antibody response towards autologous virus is generated early in infection, whereas responses to heterologous viruses is generally lower and delayed (2, 233, 288). However, due to the high mutation rate HIV-1 rapidly evolves to escape the neutralizing antibody response (2, 12, 85, 197, 233), which suggests that neutralizing antibodies exert a selective pressure and drive the evolution of HIV-1 envelope diversification. As a consequence neutralizing antibodies are more likely to recognize earlier autologous virus than contemporaneous ones. The mechanism for neutralizing antibody escape has been shown to include evolution of potential N-linked glycosylation sites (PNGS) in gp120, a mechanism that has been called the evolving "glycan shield" (288). The evolving glycan shield hypothesis has been supported by a recent study of HIV-1 subtype A (239) and an earlier report of SIV in monkeys (36). However, the acquisition of additional glycans is probably accompanied by a fitness cost since the virus that established the new infection had a more compact envelope structure and reduced glycosylation density compared to the transmitting pool of viruses after heterosexual transmission of HIV-1 subtype A and C (42, 58). In the case of subtype C, this was accompanied with a more neutralization sensitive phenotype (58). It is not clear if the evolution of PNGS is uniform for all HIV-1 subtypes since investigation of HIV-1 subtype B have failed to confirm that the viral population in the recipient have a more compact gp120 with fewer PNGS compared to the source virus (84). In addition, a recent study of HIV-1 subtype B found no correlation between the rate of neutralizing antibody escape and the rate of evolution of PNGS. Instead, this study reported continuous amino acid

changes over the entire envelope suggesting a continuous ongoing escape of HIV-1 from neutralizing antibody (85). Other mechanisms have also been proposed to be involved in HIV-1 neutralizing antibody escape: (i) structural domains of Env are sterically hindered (32) or only transiently exposed (38), and (ii) the high level of intrinsic entropy of Env-receptor binding makes the envelope a moving target for neutralizing antibodies (160).

Mapping neutralizing epitopes and vaccine design

Human monoclonal antibodies (MAbs) have been isolated from infected individuals or by using phage display techniques. MAbs have been useful tools for identification of neutralizing epitopes of gp120 and gp41 (50, 74, 99, 100, 102, 119, 120, 146, 199, 247, 277, 279, 298), which is important in order to produce an effective HIV-1 vaccine.

Epitopes identified in gp41 are exposed during a short time during infection which probably limits the effect of antibodies elicited towards these epitopes. Consequently, these epitopes are poorly immunogenic and antibodies towards these epitopes are detected infrequently in HIV-1-infected individuals (48).

The main neutralizing epitopes targeted by MAbs in gp120 are the CD4 binding site (CD4bs), CD4-induced epitope, carbohydrate residues and the variable loops V1/V2 and V3 (Figure 4). The CD4bs is composed of several parts of gp120 and all but one isolated MAb directed towards the CD4bs (IgG1b12) (150) has poor neutralizing capacity on primary HIV-1 isolates. The complex structure of the CD4bs makes the production of potent neutralizing antibodies towards this epitope a challenging task. The structure that is induced after interaction of gp120 and CD4 has been defined as the CD4-induced epitope and a number of MAbs specific for this structure have been isolated (199, 277, 298). Unfortunately, as for the Mabs directed towards CD4bs, these MAbs have shown poor neutralizing capacity to primary HIV-1 isolates (298).

The monoclonal antibody 2G12 targets carbohydrate residues of gp120 (Figure 4) and has broad neutralizing capacity (247, 279). However, the carbohydrates targeted are poorly immunogenic and the dynamics of the carbohydrates in gp120 are very complex which raises questions regarding the importance of such epitopes in an HIV-1 vaccine design.

The V2 and V3 have been shown to induce potent neutralizing antibodies (87, 103, 125, 304). However, neutralizing antibodies against the V2 region are isolate specific and therefore the V2 is improbable as a target in HIV-1 vaccine design. Early induced antibodies towards the V3 region are also isolate specific (106, 186) but later in infection more broadly specific V3 antibodies are induced as a result of so-

matic hypermutations in the immunoglobulin gene (11). Such antibodies have been shown to neutralize subtype A, B and F (49, 101-103, 159).

It is apparent from the above reports that eliciting broad neutralizing antibodies is very challenging, yet it is of great importance to continue the search for such antibodies. It is possible that gp120 have additional potent neutralizing epitopes that have not been identified during the selective procedure used to screen for monoclonal antibodies. Such epitopes could be of importance for future vaccine design. As described earlier, CTL responses are important for controlling HIV-1 infection and the general opinion today is that future vaccines should induce both a strong CTL response and an effective neutralizing antibody response in order to give a protective immunity against HIV-1 infection or disease.

MATERIALS AND METHODS

Virus isolates used in the study

In **Paper I** HIV-1 isolates 29 and 31 were obtained from patients with progressive HIV-1 infection. The isolates have previously been shown to use CCR3, CCR5 and CXCR4 as coreceptors (22).

The HIV-1 isolates under study in **Paper II-IV** were collected from 9 patients. The patients were selected from a larger cohort of 53 homosexual or bisexual men living in Sweden that have been described previously (141). The patients were selected on the basis of the coreceptor evolution of their virus populations. Five patients had virus populations that switched coreceptor usage to CXCR4 (switch populations, SP) and four had viral populations that maintained CCR5 usage throughout the infection (non-switch populations, nSP) (143). All isolates were classified as HIV-1 subtype B. Patients with SP and one with nSP were under intermittent retroviral therapy (see Figure 1, **Paper III**).

The first samples from patients with nSP were generally obtained later in infection (average: 49.5 months post infection (MPI), range: 41-58 months) compared to the first sample from patients with SP (average 23.4 MPI, range 10-49 months). The coreceptor switch for SP occurred on average 65.6 months post infection (range 47-76 months), which corresponds to 16.1 months after first isolation time-point for patients with nSP. In addition, the CD4-count was generally higher for patients with nSP compared to patients with SP, which is expected since emergence of X4 is intimately linked to low CD4-count.

Virus isolation

Viruses were isolated from PBMC using a standard protocol (249). Viral stocks were produced in phytohemagglutinin P-stimulated human PBMC from two healthy donors as described in (**Paper I**). Virus stocks were not passaged more than twice after primary isolation to minimize in vitro manipulation of the viral population.

Determination of coreceptor usage

Coreceptor usage of the viral isolates and V1-V3 chimeric viruses (see below) was determined using the U87.CD4 indicator cell line system. U87 is a glioma cell line stably transfected to express CD4 and either of the coreceptors CCR3, CCR5 or CXCR4 (56). The infected cultures were analyzed for syncytia formation and either reverse transcriptase activity (**Paper I**) or HIV-1 p24 antigen production (**Paper II-IV**).

Molecular cloning system

The genomic region under study in **Paper II-IV** was gp120 V1-V3 region. To be able to correlate the coreceptor usage of viral clones from the HIV-1 isolates with DNA sequence a cloning system was set up using the protocol by Trouplin *et al.* (280), with some modifications (Figure 9). Viral RNA was isolated from HIV-1 isolates and

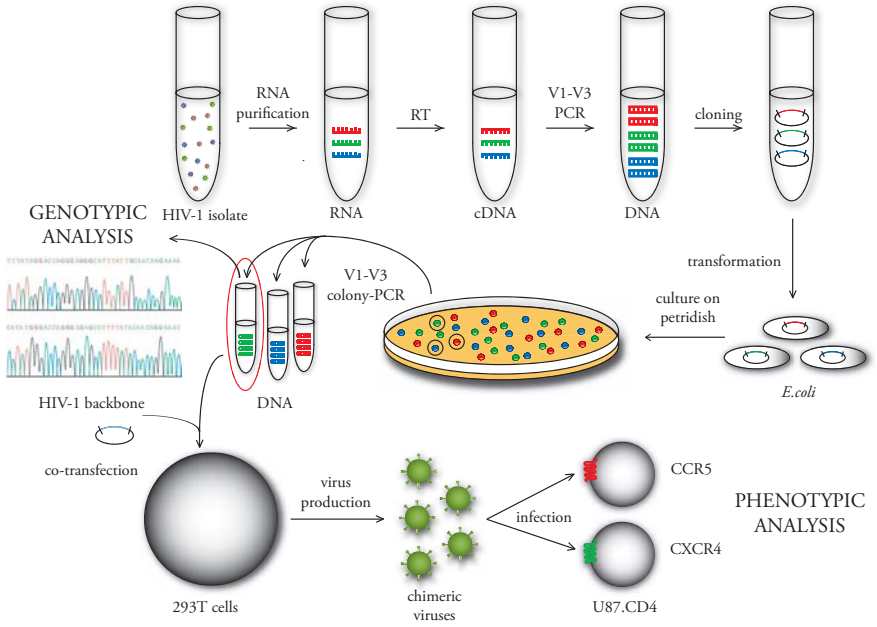

Figure 9. Experimental design. Viral RNA was extracted and purified from primary HIV-1 isolates. After reverse transcription the V1-V3 region of *env* was amplified and cloned into chemically competent *E.coli*. Clones were collected and the V1-V3 region was amplified and sequenced for genotypic analysis. In addition, the V1-V3 region and an HIV-1 backbone was used to construct chimeric viruses. The coreceptor usage of the chimeric viruses was determined by infection of U87.CD4 cells expressing CCR5 and CXCR4.

reverse transcribed using standard protocol. Next, the V1-V3 region from the viral population was amplified and cloned using TOPO-TA cloning. The cloned product was transformed and to obtain a fair representation of the viral population, 10 to 12 V1-V3 clones from each transformation were used for further analysis. To determine the coreceptor usage of the V1-V3 clones, chimeric viruses were constructed using an HIV-1 backbone with a deleted V1-V3 region. During transfection a homologous recombination occurs and the V1-V3 fragment under analysis is inserted into the backbone. The plasmid is transcribed in the transfected cells and progeny virus will be produced. The coreceptor usage of the chimeric viruses was determined by infecting U87.CD4 cells expressing CCR5 or CXCR4. In addition, the nucleotide

sequence of the V1-V3 fragment was determined. This approach allowed us to correlate the coreceptor usage with the nucleotide sequence of the V1-V3 region.

Several notes should be made regarding the molecular cloning system. First, it would have been favorable to analyze the complete gp120 protein since some studies have found additional motifs outside the V1-V3 region that affect coreceptor usage (34, 131, 164, 203, 208, 221). Initially, attempts were made to clone the complete *env* gene and produce gp120 pseudotype viruses. However, due to technical problems the cloning efficiency was too low to be used on a large material. Therefore, since the V1-V3 region has been shown to be the principal determinant of coreceptor usage for HIV-1, we chose this region for our analysis. Second, we chose to analyze 10-12 clones as representative of the viral population. However, this approach only generates a rough estimate of the complete population and additional HIV-1 genotypes would probably have been identified if a larger number of clones were analyzed. Due to resource and time limits this was not possible. Third, the primers used for polymerase chain reaction (PCR) could have reduced binding affinity for certain *env* gene variants present in the population. Such variants could have been negatively selected during PCR and consequently remained undetected. Fourth, the HIV-1 backbone used for production of chimeric V1-V3 clones was derived from a virus with X4 phenotype. Again, since additional motifs outside V1-V3 have been shown to affect coreceptor usage it would have been optimal to confirm the coreceptor usage of our clones with chimeric viruses produced with an R5 backbone. However, the coreceptor usage predicted by position-specific scoring matrices (PSSM) (132) confirmed the results from infections with chimeric viruses (95% concordance). In addition, our phylogenetic analyses (see results) showed that the determined R5 and X4 viruses were significantly separated in the trees. Therefore, we are confident that the coreceptor usage determined using the X4 backbone is correct. Fifth, we can not rule out that recombination could have occurred during PCR. The optimal approach would have been to perform single genome PCR instead of using a molecular cloning system. Finally, although the enzyme used for PCR exhibit proof reading capacity, we can not rule out that misincorporations have occurred during PCR.

Reconstruction of phylogeny

In many publications inappropriate models of nucleotide substitution are used without testing which model that fit the data set best. This may result in inaccurate phylogenies and conclusions from such phylogenies could be misleading. A program for identifying the appropriate nucleotide substitution model for dataset is available (Modeltest (223)).

In **Paper II, III** and **IV** Modeltest was used to identify the appropriate nucleotide substitution model. All models tested belong to a general class of models known as time-homogenous time-continuous stationary Markov models and when applied to

model nucleotide substitutions they all share the following assumptions:

(i) At any given site, the rate of change from base i to base j is independent of the base that occupied that site prior to i (Markov property). This corrects for multiple substitutions that occur at the same site.
(ii) Substitution rates do not change over time (homogeneity).
(iii) The relative frequencies of A, C, T and G are at equilibrium (stationarity).

All nucleotide substitution models use the Q-matrix, which specifies the relative rate of change of each nucleotide along the sequence.

Phylogenetic trees were constructed using maximum-likelihood (ML) estimates. ML evaluates a hypothesis about evolutionary history in terms of the probability that the proposed model and the hypothesized history would give rise to the observed data set. The model searches for the phylogenetic tree with highest likelihood from a wide range of trees where the branches have been repositioned. Statistical support for internal branches in the trees was obtained by 100 bootstrap replicates and ML-based zero-branch-length test (274). A branch was considered significantly separated if the bootstrap value was above 80% and the p-value < 0.001 in the ML-based zero-branch-length test.

Identification of recombinant clones

Identification of recombinant clones was done in a multi-step fashion (Figure 10). Since recombinants have acquired genetic material from at least two sources, their evolutionary history should also be a mixture. This can be visualized by using phylogenetic methods if the two parental sequences have low degree of similarity. First, the data set for each patient was split into two regions: V1/V2 and V3. The phylogenies were constructed for each sub-region and clones that showed significant discordant clustering in the two trees were considered recombinants. Putative parental sequences were identified as the sequences most similar to the recombinant in the respective tree. Second, BootScan analysis (242) was performed to confirm recombinants and to identify recombination breakpoints. BootScan uses a sliding window approach. As it slides over the gene, it constructs and bootstraps phylogenetic trees. The bootstrap values are used to identify recombinant and parental sequences. The two putative parental sequences were considered true parental sequences if they clustered together with the recombinant in more than 90% of the permuted trees. If the recombination breakpoint was identified, the dataset was split at that position and two trees were generated and inspected for discordant clustering of the recombinant as a second confirmation. Finally, the recombinants and their parental sequences were inspected manually.

The methodology applied to identify recombinants has some limitations. It requires

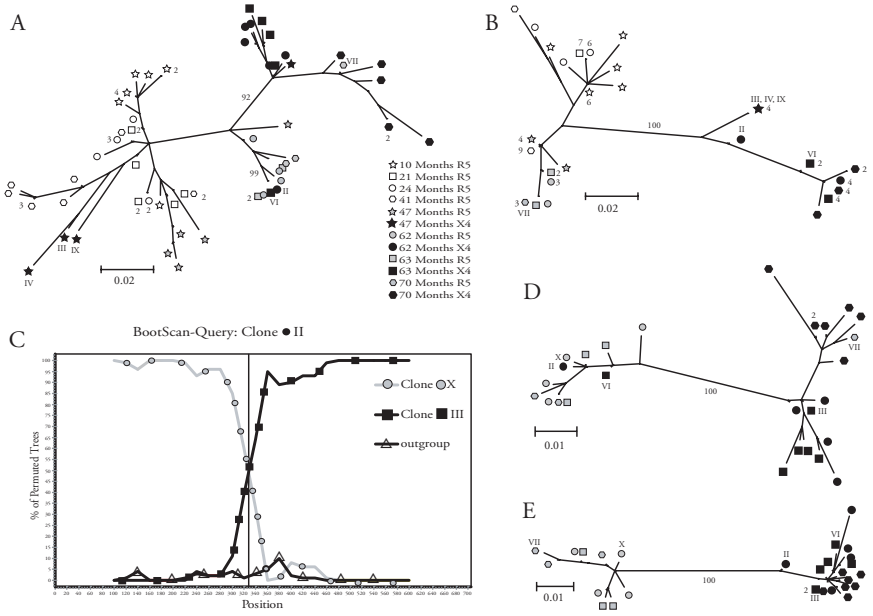

Figure 10. Identification of recombinant clones. Phylogenetic trees were constructed for the A) V1/V2 and the B) V3 region. Clones that showed significant discordant clustering were considered recombinants. C) Identified recombinants were analyzed with Bootscan for identification of recombination breakpoints. D-E) The data set was split at the identified breakpoint and two trees were generated and recombinants were confirmed by discordant clustering. Open symbols show clones from R5 isolates, grey symbols indicate phenotypically characterized R5 clones from R5X4 isolates and black symbols represent phenotypically characterized X4 clones from R5X4 isolates. Recombinants (II, VI and VII) and parentals (III and X) clones are indicated with roman numerals. Sequences that differed by 3 nucleotides or less are represented by one terminal branch and the number of clones that are represented at a branch is indicated. Bootstrap values that separated groups and were used for identification of recombinants are indicated.

that the sequences analyzed show enough dissimilarity to be significantly separated in phylogenetic trees. Hence, it is possible that recombination events that involve small parts of the sequence analyzed and recombination events between similar sequences are missed. However, the impact of such recombination events will be small on analyses using these V1-V3 sequences in comparison to recombination events that involve large pieces of genetic material with low similarity.

Statistical analysis

For the statistical analysis in **Paper III** and **IV** we used linear mixed effects models (29). In observational studies it is difficult to obtain measures of each subject at each given time or state. Therefore, such studies often suffer from an unbalanced design. In addition, repeated measurements within each subject are not independent, which

is a necessary assumption for many statistical tests. The mixed model appropriately handles repeated-measurements data in an unbalanced design.
Mixed models are similar to models used in conventional analysis of variance (ANOVA) but allow the inclusion of random effects in addition to fixed effects. A fixed effect represents a fixed set of categories, all of which are included in the study, e.g. SP and nSP. Usually we want to compare the mean of the dependent variable between these categories, e.g. V2 length, potential N-linked glycosylation sites, V3 charge, divergence or *dN/dS*. A random effect represents a population of possible categories from which a random sample is collected for the study, e.g. patients selected from a cohort of infected patients. Usually we want to control for the variance in the dependent variable accounted for by the random effect, rather than comparing specific categories. In our case we want to control for random differences between virus populations from different patients in order to perform appropriate tests for the fixed effects. Thus, while ANOVA only include fixed effects, the mixed model procedure includes random effects. Therefore, mixed models handle the dependence between repeated measures in each patient and allow us to generalize our statistical inferences to the population from which our patients were sampled. Model estimates were obtained using a restricted maximum-likelihood approach. This approach yields the same results as conventional ANOVA in balanced designs, but avoids the bias introduced in unbalanced designs (29)

For detailed information of the specific models and model parameters used see the material and methods sections in **Paper III** and **IV**.

RESULTS AND DISCUSSION

Biological cloning

Isolation of HIV-1 biological clones has routinely been carried out by limiting dilutions in PBMC (255). However, the uneven distribution of coreceptors expressed on PBMC (23, 168) could select for viruses using CXCR4. In an attempt to avoid such selection we established a cloning system using U87.CD4 indicator cell lines expressing CCR3, CCR5 or CXCR4 (Figure 11).

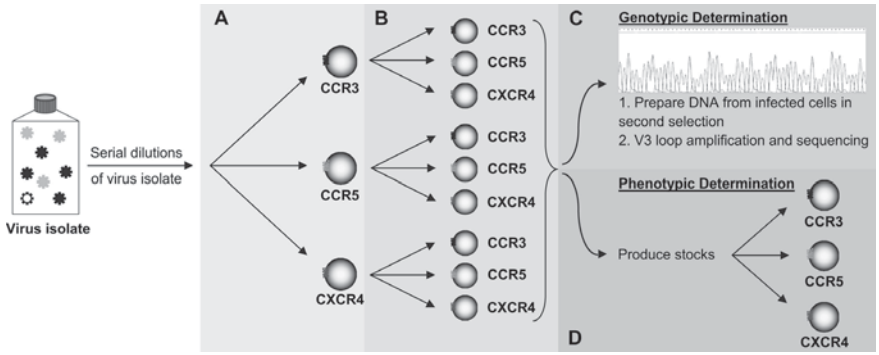

Figure 11. Biological cloning system. A) First selection: Viral isolates were diluted and passaged 1 to 3 times on U87.CD4-CCR3, -CCR5, and -CXCR4. B) Second selection: U87.CD4-CCR3, -CCR5, and -CXCR4 were infected with supernatants from first selection. C) Genotypic determination: clonality were confirmed by V3 sequencing. D) Phenotpic determination. Biological clones were grown on PBMC to produce viral stocks and the coreceptor usage was determined on U87.CD4 cells.

Two multitropic R3R5X4 isolates were serially diluted before infection of the three U87.CD4 coreceptor expressing cells lines (first selection). Cultures were analyzed for syncytia formation and RT activity. Cultures showing five or less syncytia per well were defined as having low degree of syncytia formation (LDSF). Virus supernatants from first selection were subjected to a second selection step by infection of the three U87.CD4 cell lines. The V3 and flanking regions (≈300 bp) obtained from proviral DNA of cells infected in the second selection was amplified and sequenced. A correlation was observed between dilution in the first selection and homogenous V3 sequence. All supernatants obtained from LDSF-cultures resulted in biological clones with homogenous V3 sequences after the second selection independent of coreceptor expression of the cells used in first selection.

We also found that the coreceptor usage of the biological clones reflected that of the primary isolates. However, no R5 clones were isolated. We therefore subjected both multitropic isolates to serial passages on CCR5-expressing cells using diluted virus

supernatant at each passage. Using this approach we isolated R5 clones from one of the isolates with identical homogenous V3 sequence, which differed from the genotypes of the previously isolated clones. The results were confirmed by sequence analysis of 20 molecular V3 clones from the primary isolates. All genotypes identified in the primary isolates were represented by biological clones. However, the genotypes of clones obtained after serial passages was not represented by the molecular clones from the primary isolates, which suggest that these biological clones represented a minority population.

The cloning system developed was sensitive and specific and we suggest it to be used as an alternative to the existing PBMC-based method. By using cell lines we can avoid the selection for CXCR4-using variants which is apparent in the PBMC-method. Instead, the method takes the biological properties of each virus variant into account. Therefore, the probability to isolate variants other than CXCR4-using viruses will be higher. We also found that our method is fast and non-labor intensive. The cell lines are easy to work with and are used routinely in the lab. In contrast, preparation of PBMC requires fresh blood and involves several purification steps. In addition, differences between PBMC preparations can be significant and may affect the result of the cloning.

Intrapatient recombination

Recombination is the major contributor to the enormous variation observed in HIV-1 (226) and recombination has been observed repeatedly in studies of HIV-1 evolution and genetics. However, only a few studies have addressed recombination between HIV-1 subpopulations within patients (37, 149, 215, 285) The extent of intrapatient recombination was analyzed using 253 V1-V3 *env* clones from 27 sequential HIV-1 subtype B isolates from four patients with virus populations that switched coreceptor usage to CXCR4. The coreceptor usage of clones from dual-tropic R5X4 isolates was characterized experimentally, which enabled us to dissect the impact of recombination on coreceptor usage. Recombinant clones were identified by discordant clustering of clones in the V1/V2 region and the V3 region (240) and by Bootscan analysis (242) (Figure 10). The results showed that 11 of 125 (9%) clones from CXCR4-using isolates had originated by recombination events between R5 and X4 viruses. For six of the 11 recombinants, both parental sequences were identified and recombination breakpoint could be identified. The majority of breakpoints were positioned in constant region 2 (Figure 12), which is in line with recent reports (88, 200). Identification of recombination breakpoints between V1/V2 and V3 allowed us to analyze the impact on coreceptor usage of these two regions. Manual inspection of amino acid sequence alignments of the recombinants and parental sequences showed that all recombinants had the same phenotype as the parental sequences that donated the 3' part (including V3) of the recombinant sequences (Figure 12). Our results therefore suggest that intrapatient recombination between

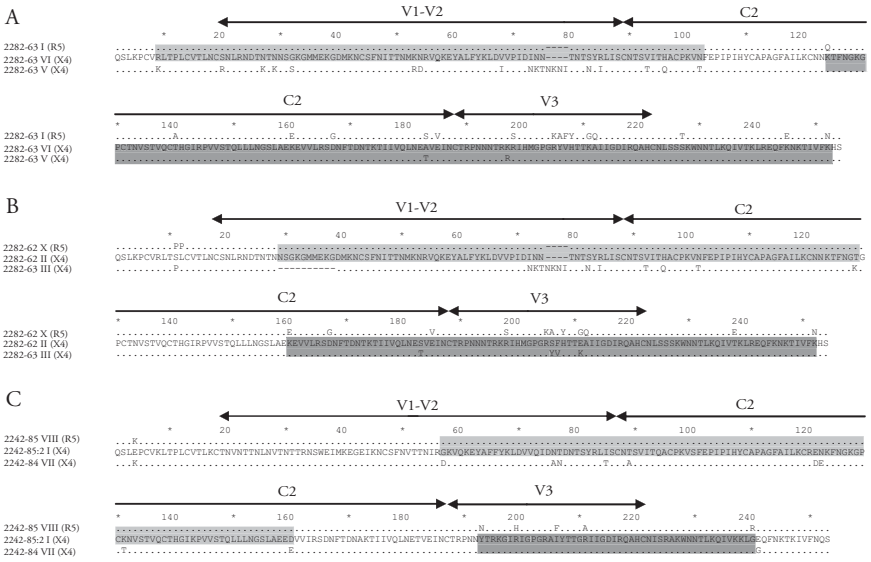

Figure 12. Amino acid sequences of recombinant clones. (A) X4 clone 2282-63 VI, (B) X4 clone 2282-62 II X4 , (C) X4 clone 2242-85:2 I. Recombinant sequences are shown in the middle of each alignment, and the parental sequences are shown above and below each recombinant sequence. The coreceptor usage is indicated in parentheses. Shaded regions indicate where the recombinants are most similar to one of the parental sequences. Regions shaded in light grey indicate similarity between the recombinant and the R5 parental sequence, and regions highlighted in dark grey show regions of similarity between the recombinant and the X4 parent. Dots represent identical amino acids between the recombinant and parental sequences.

viruses from different subpopulations occur frequently and that the V3 region determined coreceptor usage for the identified recombinant clones.

The *env* V1-V3 region havs repeatedly been shown to be important for coreceptor usage (53, 78, 131, 132, 203) and as target for neutralizing antibodies (74, 99, 100, 102, 103, 119, 146, 277, 279, 298). It has also been shown that the V1/V2 region influence neutralization by soluble CD4 and by MAbs directed towards V3 and CD4 induced epitope (70, 139, 198, 219, 246). The V1/V2 region has even been suggested to be the global regulator of the sensitivity of primary HIV-1 isolates to neutralizing antibodies (220). It has also been demonstrated that conformational arrangement of V2 and V3 with respect to the CD4 binding region of gp120 appeared to be critical for the recognition by neutralizing antibodies in a SHIV model (303). Thus, recombination events in the C2 region of *env* could affect coreceptor usage and the neutralization sensitivity of the progeny. Accordingly, assuming that the immune-based hypothesis (see Introduction, Biological phenotype, The coreceptor switch - facts and hypotheses) is correct, recombination could play a critical role in the coreceptor switch. A recombination event between an immune resistant R5 and

an X4 virus could potentially generate an X4 offspring with potential to evade the immune response. The broadening in cell tropism of the viral population to include CXCR4-expressing cells would result in increased CD4+ cell death and further impair the immune system which would allow the suppressed X4 population to expand (Figure 13).

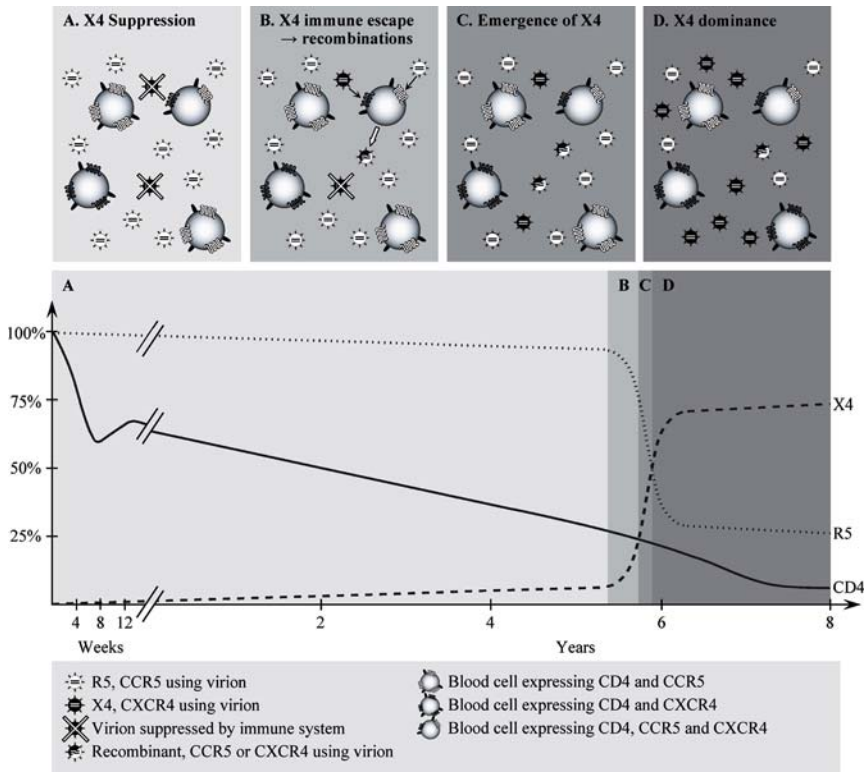

Figure 13. Possible involvement of R5/X4 recombinants in coreceptor switch. A) Early in infection X4 viruses are suppressed according to the immune based hypothesis. B) Because of immune system impairment a small fraction of X4 viruses are allowed to replicate, which allows for superinfection of U87.CD4 CCR5$^+$ CXCR4$^+$ cells and recombination between R5 and X4 viruses. Rearrangements in the V1-V3 region results in antibody escape of the recombinants. C-D) Broadening in cell tropism further impair the immune system which allows the suppressed X4 population to expand and become the dominate population.

Evolution of potential N-linked glycosylation sites

Glycosylations have many important functions in HIV-1. They protect the virus particle from recognition by neutralizing antibodies, they can bind to DC-SIGN and allow trans infection and they also affect coreceptor specificity. Here, we analyzed potential N-linked glycosylation sites (PNGS) in the context of coreceptor evolution of the vial population.

Mixed model statistics was used to analyze the evolution of PNGS in switch (SP) and non-switch (nSP) populations (**Paper III**). The number of PNGS increased significantly with time since infection in nSP (t=2.64, p=0.037). The pattern was similar in all nSP and the average increase was 0.30 (range 0.24 to 0.37) PNGS per year. In contrast, the number of PNGS did not increase significantly with time in SP (t=0.52, p=0.62). The range in PNGS varied from -0.50 to 0.23 PNGS/year. Despite the large variation between the four SP, the difference in PNGS over time between nSP and SP was near significant (F=5.02, p=0.066). Therefore we suggest that the evolution of PNGS differs between nSP and SP. The number of PNGS increased with time in nSP while no uniform pattern was observed for SP.

Evolution of the HIV-1 "glycan shield" has been shown to be important for neutralization escape (288) and neutralizing antibodies have been shown to drive the evolution in HIV-1 *env* in subtype B (85). Evolution towards a denser glycosylated envelope have also been observed in subtype A and C (58, 239). However, late in infection the number of glycosylations may decrease, probably as a response to an impaired antibody response (Repits et al, personal communication). This indicates that the glycan shield is a prerequisite for survival in an immune competent environment but that it is also associated with a fitness cost. Based on these findings, it is possible that the differences observed in PNGS evolution could be a consequence of differences in antibody response directed towards nSP and SP. We therefore hypothesize that a stronger antibody response is elicited towards nSP than SP which results in positive selection of PNGS in nSP. The stronger antibody response could be a consequence of a more functional immune response in patients having nSP, which is indicated by the generally higher $CD4^+$ T-cell count in patients with nSP compared to patients with SP. Our hypothesis is supported by the finding that patients harboring NSI viruses had a stronger antibody response than patients having SI viruses (248). In addition, a recent study showed that long-term infected progressors (LTPs) have more sites under positive selection than short-term infected progressors, which suggest a more functional immune response in LTPs (237).

Correlation analyses between the number of PNGS and V1/V2 length showed a significant positive correlation within all eight patients' virus populations (Pearson correlation, p<0.031) (**Paper III**). This indicates that the mechanism for variability of the number of PNGS in the V1-V3 region could be insertions/deletions in the V1/V2 region. Thus, our results suggest that the evolution of PNGS was associated with coreceptor usage evolution and that the number of PNGS in the V1-V3 region was correlated to V1/V2 length. This adds evidence to the idea that PNGS evolution is associated with length variation of the variable regions (58, 239).

An large variation was observed in glycosylation profiles both between and within virus populations from the eight patients (**Paper III**). No general pattern that dis-

tinguished viruses from SP and nSP or R5 from X4 was identified. The only general trend was that more sites showed an increase in PNGS with time in nSP than in SP. These results clearly demonstrate the complexity of the HIV-1 glycan shield and highlight the difficulties associated with constructing an effective HIV-1 vaccine. In addition, a recent report demonstrated that PNGS in HIV-1 envelope frequently interact, which adds yet another level of complexity (222).

Evolution of V2 length

The evolution of V2 length has been proposed to influence evolution of coreceptor usage and disease progression although results have been contradictive (108, 131, 185, 266). Statistical support has been presented in studies suggesting that an extended V2 is associated with an upcoming switch from R5 to X4 (131), or from NSI to SI phenotype (108). In contrast, studies where it is implied that an extended V2 is associated with maintained CCR5 usage (185) or slower disease progression (266), lack statistical analysis.

A mixed model was used to analyze differences in V2 length between SP and nSP (**Paper III**). No significant interaction was found between time since infection and viral population group ($F=0.137$, $p=0.724$), which means that V2 length evolved in a similar manner in both nSP and SP. A reduced mixed model, assuming that V2 evolution did not differ between SP and nSP showed that V2 length increased significantly with time ($F=7.60$, $p=0.028$). Results using this model also showed that V2 was longer in SP than in nSP at comparable time-point over the course of infection ($F=7.3$, $p=0.035$). The average difference in V2 length between SP and nSP estimated from the model was 4.2 amino acids (SE=1.56).

Our results contradict previous results that an extended V2 is associated with maintained CCR5 usage (185), and we suggest that V2 extension is not associated with evolution in coreceptor usage. In contrast, our results support previous findings that R5 from SP isolated just prior to coreceptor switch have a longer V2 than R5 viruses from nSP isolated at corresponding time since infection (131). In fact, our results showed that R5 clones from SP had a longer V2 throughout the course of infection. Thus, the V2 was longer for SP than for nSP already at the first isolation time-point for nSP. The first isolation time-point for nSP was on average 16.1 months before the observed coreceptor switch in SP. First isolation occurred later for nSP compared to SP and therefore we cannot address the relationship between coreceptor switch and V2 length early in infection. It is possible that the V2 length differed already early in infection between SP and nSP. However, this assumes that the pattern of change in V2 that we observed can be extrapolated to earlier stages of infection.

Evolution of V3 charge

The gp120 V3 region is without doubt the most studied part of HIV-1. Since the early 1990's, V3 has been associated with differences in cell tropism for HIV-1 and later it was shown to be the principal determinant for coreceptor usage. A high V3 charge and amino acid signatures have been associated with CXCR4 usage (53, 80, 81) and models for predicting coreceptor usage have been presented and are used with good results (132, 133, 230). However, no method for predicting an upcoming switch using molecular data has been presented.

We used mixed model statistics to analyze the differences in V3 charge evolution between R5 viruses from nSP and SP (**Paper III**). Results showed that the evolution of V3 charge differed significantly between SP and nSP ($F=12.4$, $p=0.011$). The V3 charge of R5 clones from SP increased significantly ($t=2.81$, $p=0.025$) with 0.15 units of charge per year, whereas a non significant decrease ($t=2.16$, $p=0.074$) with 0.12 units of charge per year was observed for nSP. The difference in V3 charge evolution was even more evident when comparing R5 clones from nSP with R5 clones from SP obtained before the coreceptor switch ($t=4.53$, $p=0.004$).

In nSP a charge of ≥ +4 was found in 15% (27 of 176) of the clones. The corresponding number for R5 clones before switch in SP was 80% (154 of 192 clones). We also analyzed R5 clones before switch from four SP from a study by Van Rij and colleagues (283) and found that 80% (20 of 25) of the clones had a V3 charge of ≥ +4. Taken together, R5 populations from nSP and SP showed different evolution of V3 charge. Our results also suggest that a V3 charge ≥ +4 is more common among R5 viruses isolated before switch from SP compared to R5 viruses from nSP.

In conclusion, the differences in V3 charge and V2 length were present in R5 viruses already before the switch in coreceptor usage. Therefore, these molecular properties could be of importance for understanding if and when a coreceptor switch occurs. Since switch in coreceptor usage is intimately linked to dramatic decline in $CD4^+$ T-cell count and progression to AIDS this could also be of importance in a future clinical setting.

Evolutionary divergence rates of R5 and X4 subpopulations

Previous studies have estimated the divergence rate of HIV-1 *env* to range from 0.3-1.0 % per year (181, 259) and a consistent pattern for HIV-1 *env* evolution has been proposed (259) (see Introduction, Intrapatients HIV-1 evolution). In addition, several attempts have been made to correlate the divergence rate in *env* to progression of disease (15, 55, 181, 191, 273, 294). Despite this, the relationship between *env* divergence and disease progression remains unsolved. R5 and X4 subpopulations preferentially infect different cell subsets (23, 168) allowing for independent evolu-

tion under different conditions. Few studies have analyzed the molecular evolution of coexisting R5 and X4 subpopulations separately (46, 73, 283) and comparisons of divergence rates between coexisting subpopulations with different coreceptor usage evolution have not been presented.

The average pairwise distances were computed between V1-V3 *env* clones isolated at the first time point and all clones from subsequent time-points, to obtain intra time-point distances (divergence). Mixed model, taking patient-specific effects into account, was used to analyze differences in divergence rates between SP and nSP (**Paper IV**). The divergence rate was significantly higher for SP compared to nSP (F=6.03, p=0.049) (Figure 14). nSP diverged on average 0.65% per year and SP diverged on average 1.12% per year. The residual divergence was higher for X4 (+0.52) clones than for R5 (-0.6) clones within SP. Since X4 subpopulations were generally present late in infection, the positive residuals would tend to increase the

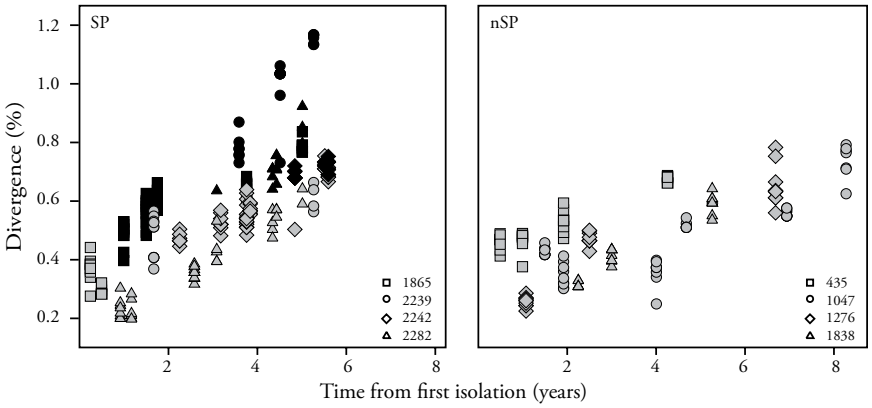

Figure 14. Divergence of the HIV-1 gp120 V1-V3 region in switch- and non-switch populations. Grey symbols represent R5 clones and black symbols represent X4 clones. Clones from different patients are represented by different symbols.

estimated divergence rate for the SP. We therefore removed the X4 clones from the SP dataset and compared the divergence rates of R5 subpopulations from SP with R5 nSP. The results showed no significant difference in divergence between R5 SP and nSP (F=0.006, p=0.943) and the divergence rates of the two population groups were similar. This suggests that the R5 subpopulations from SP diverged at similar rates as R5 nSP and that the inflated divergence rate in SP was caused by X4 subpopulation.

A possible explanation for the difference in V1-V3 *env* divergence rate between SP and nSP could be that the X4 subpopulations were under different selective pressure

than R5 subpopulations. The average *dN/dS* was higher for the X4 subpopulation (1.51, SD=0.14) than for the R5 subpopulation in SP (1.09, SD=1.09) (**Paper IV**). Most importantly, the average *dN/dS* was significantly above 1 for the X4 subpopulation (t=3.55, p=0.01) but not for the R5 subpopulation (t=0.63, p=0.55). Thus, the X4 population was under positive selection, while the R5 population was under neutral selection. Therefore it is possible that the two subpopulations in SP interact with the immune system in different ways. This is in line with previous observation (24, 300) and further supports the immune-based hypothesis as an explanation for the coreceptor switch. However, the difference in divergence rate may also be a consequence of shorter generation time for the X4 subpopulation.

Our phylogenetic analysis (**Paper IV**) showed that X4 subpopulations formed monophyletic groups within each tree (Figure 16). This suggests that the split between R5 and X4 subpopulations occurred only once. The genetic distances between R5 and X4 populations were generally large. One possible explanation is that the split between R5 and X4 occurred long before X4 viruses were sampled in peripheral blood.

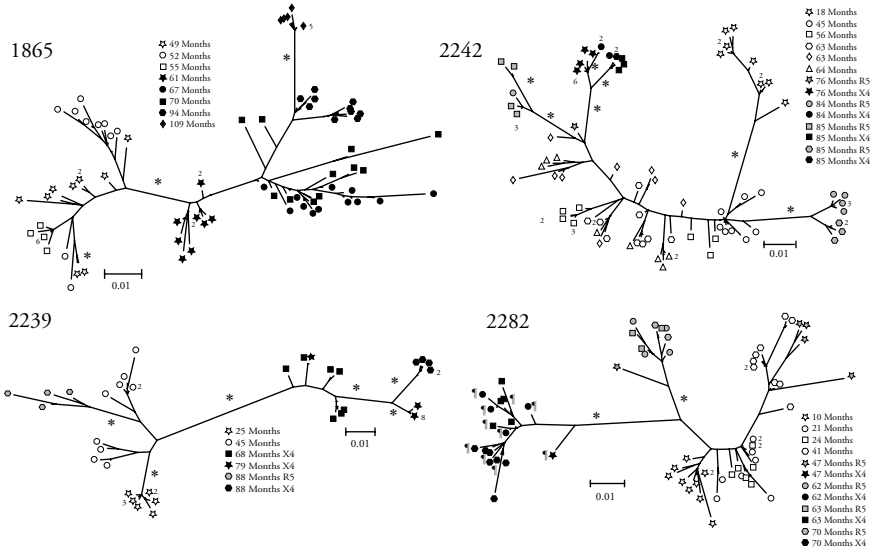

Figure 15. Maximum-likelihood phylogenetic trees for the HIV-1 gp120 V1-V3 region of switch populations from patients 1865, 2242, 2239 and 2282. Internal branches with significant support are indicated with asterisks. Clone sequences from different isolates are represented by different symbols. Open symbols represent R5 clones from monotropic R5 isolates, grey symbols indicate R5 clones isolated from R5X4 isolates and black symbols represent X4 clones. Dualtropic R5X4 clones (patient 2282) are indicated with (¶).

CONCLUSIONS AND FUTURE PERSPECTIVES

A new biological cloning system for HIV-1 was developed using the U87.CD4 cell lines engineered to express CD4 and different coreceptors. Conventional biological cloning in PBMC selects for X4 variants as a consequence of overexpression of CXCR4 on PBMC. Importantly, this is avoided when using our system since viruses with different coreceptor usage are propagated in different cell lines. The cloning system is non-labor intensive, fast and the coreceptor usage of the clones isolated reflects that of the primary isolate. We suggest this system to be used as a complement to the conventional cloning in PBMC. In order to use this method in a high through-put setting we would have to optimize the methodology in a 96-well format. In addition, a better confirmation of clonality would be to sequence a larger part than V3 and flanking regions (≈300 bp).

HIV-1 populations with different coreceptor usage evolution differed in molecular evolution of the V1-V3 region of *env*. The V2 region was longer over the course of infection in populations that switched coreceptor usage to CXCR4 (switch populations, SP) as compared to populations that maintained CCR5 usage throughout the infection (non-switch populations, nSP). In addition, the V3 charge increased over time in the R5 subpopulations from SP, while it decreased or remained unchanged in nSP. These differences might prove useful for understanding the evolution of coreceptor usage and predicting an upcoming coreceptor switch. Since the switch is associated with a more severe disease progression a prediction model could be of importance in a future clinical setting.

HIV-1 nSP evolved toward a more glycan dense gp120 in a stepwise manner, whereas the pattern of change was more irregular in SP. We hypothesize that the difference observed reflect differences in the capacity to elicit an effective antibody response and that patients with nSP mount a stronger antibody response than patients with X4 viruses. This could affect the coreceptor usage evolution of the viral population and counteract a switch in coreceptor usage to CXCR4.

R5 and X4 viruses preferentially infect different T-cell subsets, which allows for independent evolution of R5 and X4 subpopulations. Due to the presence of X4 viruses, SP diverged faster than nSP in the V1-V3 region of *env*. This could be a consequence of the different selective pressures acting on R5 and X4 subpopulations. The differences in selective pressures may reflect differences in immune response elicited towards these subpopulations.

Although R5 and X4 preferentially infect different T-cell subsets their cell tropism overlaps to some extent. This allows for superinfection and recombination events between R5 and X4 variants. We showed that recombination events between R5

and X4 variants occur frequently. The recombination breakpoints in the C2 region resulted in rearrangement of V1/V2 in respect to V3. This could affect the antibody binding to the V3 region since V1/V2 partly shields the V3 region in the trimeric gp120 structure. Consequently, recombination events could be a mechanism for neutralizing antibody escape. If the X4 population is suppressed because it is better recognized by the immune system than R5 viruses, recombination events between R5 and X4 viruses could be involved in the appearance of X4 viruses and thereby contribute to a faster disease progression and AIDS.

Based on the results presented in this thesis, we hypothesize that the antibody response is stronger and more potent throughout the course of infection in patients that maintained exclusive CCR5 usage compared to patients with virus populations that switch coreceptor usage to CXCR4. This may cause the differences in coreceptor usage evolution. We hypothesize that there is a link between human genetics and the evolution of coreceptor usage in HIV-1. In SP the selective pressure, possibly from the immune system, is stronger towards the X4 subpopulation than towards the R5 subpopulation, resulting in suppression of the X4 subpopulation under immune competent conditions. As a result of immune system failure, some X4 variants are allowed to replicate. Recombination events between immune resistant R5 and X4 could generate an X4 virus with potential to evade the immune system. The increased cell tropism results in increased cell pathology and further impairment of the immune system which allows the suppressed X4 population to expand.

In order to test the hypotheses outlined in this thesis we would have to perform neutralization experiments with autologous sera on individual clones. As mentioned in prior sections, such experiments have been performed, albeit with a small number of clones and sera. We would also need to determine the amount and quality of the antibody and CTL response elicited by patients with nSP and SP. Such studies would extend our knowledge of the interactions between the virus components of the quasispecies and the adaptive immune system.

Finally, the intrahost evolution of HIV-1 is complex and far from understood. The number of extensive studies using sequentially collected samples is few and the consensus knowledge from these studies is limited. In order to map HIV-1 evolution, which is pivotal in order to engineer a successful HIV-1 vaccine, more detailed genetic and biological studies of sequentially collected samples should be undertaken. In addition, it's becoming more evident that the evolution of HIV-1 depends on the genetics of the host. Therefore, to increase knowledge further, evolutionary studies of HIV-1 should be brought out in parallel with genetic and functional analysis of the human immune system.

SAMMANFATTNING PÅ SVENSKA

Humant immunbrist virus (HIV) infekterar och dödar celler som utgör en viktig del av immunsystemet. Med tiden försvagas immunsystemet och blir slutligen oförmöget att bekämpa infektioner som under normala betingelser inte drabbar människor. Detta stadium av sjukdomen har definierats som AIDS. Enligt världshälsoorganisationen har mer än 25 miljoner människor dött i AIDS och ytterligare cirka 40 miljoner är idag infekterade med HIV.

HIV är den humana patogen som förändrar sig snabbast. Detta resulterar i att en mängd olika virusvarianter med olika egenskaper samexisterar inom en patient. Den snabba förändringen hos viruset har gjort det komplicerat att utveckla mediciner och vaccin mot HIV. Idag finns varken botemedel eller vaccin mot HIV. Det som finns tillgängligt är bromsmediciner som i de flesta fall kan hålla virusnivåerna låga. För att i framtiden kunna utveckla bättre mediciner och ett vaccin är det viktigt att vi förstår hur HIV förändrar sig under infektionen.

För att infektera en cell binder HIV till receptorer på cellens yta. När HIV infekterar en cell interagerar det med två cellulära receptor. Först CD4 och sedan en coreceptor, oftast CCR5 eller CXCR4. Dessa coreceptorer uttrycks främst på olika celltyper i immunsystemet även om det finns cellpopulationer som uttrycker bägge receptorerna. Virus som binder till CCR5 eller CXCR4 klassificeras som R5- repektive X4-virus. Virusets protein som binder till coreceptorn heter gp120 och vilken coreceptor som används bestäms huvudsakligen av variabel region 1 till 3 (V1-V3) i detta protein. Tidigt i en HIV-infektion detekteras vanligtvis endast R5-virus men i ungefär hälften av HIV-infekterade individerna så förvärvar viruspopulationen egenskapen att använda både CCR5 och CXCR4 senare under infektionen. Detta fenomen kallas ”coreceptor switch” och är starkt kopplat till en försämrad prognos för patienten. Efter coreceptor switch är viruspopulationen oftast sammansatt av både R5- och X4-virus. Det skulle vara av stor vikt att finna mekanismen bakom coreceptor switch och att kunna förutspå om den kommer att ske inom en patient.

I den här avhandlingen har vi utvecklat en metod för att separera olika virusvarianter från en blandning av virus. Vi har även jämfört genetiska förändringar i V1-V3-regionen från HIV-1-populationer som byter coreceptoranvändning till CXCR4 (switch populationer, SP) med viruspopulationer som behåller exklusiv CCR5-användning (non-switch populationer, nSP). Slutligen har vi studerat R5- och X4-varianter inom SP samt om dessa utbyter genetiskt material.

Den metod som vanligast används för att separera virusvarianter använder celler där CXCR4 coreceptorn är överrepresenterad i jämförelse med CCR5. Således kommer detta att selektera för virusvarianter som använder CXCR4. I den metod vi utvecklat

används olika celltyper som vardera bara uttrycker en coreceptor, därmed undviks selektion för X4-varianter. Vårt system var lätt att använda och de separerade varianternas egenskaper stämde bra överrens med egenskaperna hos den blandning av virus varifrån de separerades.

Våra studier av virus från SP och nSP visar att dessa två populationer förändrar sig på olika sätt. Mängden sockermolekyler i V1-V3-regionen ökade i nSP medan den i SP förblev oförändrad. Sockermolekyler är ett sätt för HIV att skydda sig mot immunförsvarets antikroppar, vilka annars skulle inaktivera viruset. Vi föreslår därför att den ökande mängden sockermolekyler hos virus från nSP har uppkommit som ett resultat av ett starkt antikroppssvar hos dessa patienter.

Vidare fann vi att R5-virus från nSP och SP skiljde sig åt. V2-regionen var längre hos R5-virus från SP jämfört med R5-virus från nSP. Dessutom ökade laddningen i V3-regionen hos R5-virus från SP medan den förblev oförändrad hos R5-virus från nSP. Dessa två egenskaper skulle kunna användas för att förutspå om en viruspopulation kommer att byta coreceptoranvändning till CXCR4 senare under infektionen.

Samexisterande R5- och X4-varianter visade sig vara genetiskt olika och de förändrar sig olika snabbt. X4-varianter utsattes även för hårdare selektionstryck, vilket skulle kunna förklara den högre förändringshastigheten. En annan förklaring kan vara att X4-varianterna förökar sig snabbare. Vår slutsats blir därför att R5- och X4-virus förändrar sig oberoende av varandra.

Om två olika viruspartiklar infekterar samma cell så kan det ske en utväxling av genetisk material mellan dem (rekombination). Den resulterande rekombinanten blir en genetisk blandning mellan de två infekterande virusvarianterna. Rekombination är ytterligare en process som bidrar till den enorma genetiska variationen i HIV. Vi fann att R5- och X4-virus rekombinerar relativt ofta i V1-V3-regionen. Närmare analys av rekombinanterna visade att rekombinationen skedde mellan V1/V2 och V3 och att V3 bestämde vilken coreceptor rekombinanten använde. HIV-inaktiverande antikroppar binder ofta till V3 och tidigare studier har visat att förändringar i V1/V2 påverkar dessa antikroppars förmåga att binda till V3. Rekombination skulle därför kunna påverka hur väl viruset känns igen av inaktiverande antikroppar.

HIV är ett mycket komplext virus och vår förståelse om hur viruset förändrar sig är begränsad. Därför är det viktigt att göra fler studier där viruspopulationerna studeras över en längre tid inom en patient. Patientens immunförsvar spelar också en viktig roll i hur viruset förändrar sig. Därför tror vi att det även är av stor vikt att studera immunförsvaret och dess underliggande genetik i detalj hos den infekterade individen.

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