ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

THE BIOLOGICAL PHENOTYPES(NSI/SI) OF HIV-1 ISOLATES OBTAINED FROM ETHIOPIAN AIDS PATIENTS VISITING ST. PAUL`S HOSPITAL IN ADDIS ABABA".

DEREJE DEMISSIE

AUGUST, 1998

Abstract

The aim of this study was to assess the frequencies of syncytium-inducing and non-syncytium-inducing HIV-1 subtype C isolates obtained from Ethiopian AIDS patients. Forty-eight hospitalized AIDS patients ($CD4^+$ T cell counts < 200 cells/μl) with WHO revised conditions of staging system criteria for HIV-1 infection and disease listed in stage III and IV were used. The biological phenotypes of the viruses were determined by MT-2 cocultivation assay. Lymphocyte subsets were enumerated using coulter counter and FACScan analysis. Viral load determination was done by nucleic acid sequence based amplification assay (NASBA). HIV-1 isolates with syncytium-inducing phenotype were detected only in 3/48(6%) of AIDS patients. Lower distribution of absolute $CD4^+$ cell counts and CD4/CD8 ratio were determined in SI carriers as compared to NSI carriers (P=0.04 and 0.03, respectively). However, no difference in viral load was observed between SI and NSI patients (P>0.05). SI patients had higher distribution of immune-complexed dissociated HIV-1 p24 levels than NSI patients (P=0.02). Both patients with SI and NSI variants had comparable $CD8^+$ T cell counts (P=0.72). This finding suggests that the prevalence of SI viruses among Ethiopian C-subtype AIDS patients was significantly lower than among AIDS patients infected with other subtypes.

Acknowledgements

This study has been done in collaboration with the Department of Biology, Addis Ababa University; Ethiopian Health and Nutrition Research Institute (EHNRI), Ethiopian-Netherlands AIDS Research Project (ENARP) and St. Paul's Hospital.

I remain intellectually indebted for Dr. Tobias Rinke de Wit for his continuous guidance, valuable criticisms, unlimited support and inexhaustible suggestions. I find myself at a loss to identify the words that would properly describe the beneficial effects of this model of scholarship, character and intelligence. Unfortunately, not many are similarly blessed.

I would like to extend my thanks to Dr. Araud Fontant and Dr. Elias Kassa to create smooth working conditions with St. Paul's Hospital Physicians in organising samples to be collected and in filling CDC clinical staging questionnaire about the clinical manifestations of patients. My special thanks also go to my most influential teacher and advisor, Dr. Beyene Petros, who through the years has continued to provide sage counsel, much support, constructive criticisms and valuable suggestions.

I would like to give a special note of appreciation to St. Paul's Hospital Physicians who managed in their busy schedules to clinically diagnose and fill the clinical data of patients.

I am especially grateful to Ermias Hailu for his skilled technical assistance, kindness and warm friendship and I also wish to thank and express my deepest appreciation to:

---Tesfaye Tilahun, Asfaw Beyene, Solomon Abebe and Mulu Girma for their skilled technical assistance, Kindness and good companionship.

---Ronan Doorely for programming and technical assistance in data reduction as well as computer-related problems

---all members of the ENARP for their generous help at the time of need

---my colleagues Solomon Hailu, Derebe Hirpo, Lijam Z/Michael, Berhanu Ayele and all others for their encouragement, support, all the good times we have passed together and moral support at the time of need

Finally my deepest gratitude and appreciation goes to my parents for encouraging me all along, for their caring and support.

This work was financially supported by The Netherlands Ministry of Development Cooperation through the Ethiopian Netherlands AIDS research Project (ENARP).

Table of contents

Abbreviations

AIDS	Acquired imuunodeficiency syndrome
Ag	antigen
CCR-5	chemokine receptor-5
CCS	cell culture supernatants
CXCR-4	CXC- chemokine receptor-4
EHNRI	Ethiopian Health and Nutrition Research Institute
ELISA	Enyzme Linked Immunosorbent Assay
ENARP	Ethiopian-Netherlands AIDS Research Project
env	envelope
gp	glycoprotein
HIV-1	Human Immunodeficiency Virus Type 1
ICD-p24Ag	-Immune complex dissociated p24 antigen
M-tropic	Macrophage tropic
MT-2	human T-lymphotropic-I-transformed CD4+ cell line
NSI	non-syncytium-inducing
PBMC	Peripheral Blood Mononuclear Cells
ng	nanogram
RNA	Ribonucleic Acid
SI	syncytium-inducing
SDF-1	stromal-derived factor 1
T-tropic	T-cell tropic

Chemical composition of reagents used in this study.

Reagents	Chemical Composition
A. Reagents for HIV-SPOT	
1. Non-reactive control	1[+].Lyophilized normal human serum non-reactive for hepatitis B surface antigen (HbsAg) as well as for HIV-1 antibodies 2[*]. Preservative: 0.05% Thimerosal
2. Weak-reactive control	1. 1[+] and 2[*]
3. Strong-reactive control	1. 1[+]and 2[*]
4. Lyophilized buffer	1. Tween 20, 2. Bovine serum albumin, 3. heat-treated normal goat serum, and 4. 0.1% sodium azide and 2[*]
6. Conjugate	1. Lyophilized protein A-gold reagent
B. Reagents for ELISA antibody assay	
1. Negative control	1. Human serum non-reactive for anti-HIV-1 Abs 2. Preservatives: a) 0.1 g/l gentamicin sulphate b) 0.2 ml/l cinnamaldehyde
2. Anti-HIV-1 positive control	1. Human serum containing human anti-HIV-1 2. Preservatives: a) 0.1 g/l gentamicin sulphate b) 0.2 ml/l cinnamaldehyde
3. Specimen diluent	1. Stabilizing protein 2. Detergent 3. Preservatives: a) 0.1 g/l gentamicin sulphate b) 0.2 ml/l cinnamaldehyde
5. TMB solution	1. Tetramethylbenzidine in citric acid
6. Urea peroxide solution	1. Urea peroxide 2. Preservative: 1 g/l 2-chloroacetamide
C. Reagents for HIV-1 p24 antigen ELISA on CCS	
1[a]. Negative control	1. Human serum non-reactive for HIV-1 Abs, HBsAg and HCV Ab 2. Preservatives: a) 0.01% gentamicin b) 0.02 cinnamaldehyde
2[b]. Positive control	1. Human HIV-1 Ag: 160 pg/ml p24 core antigen (inactivated) 2. Preservatives: a) 0.01% gentamicin b) 0.02 cinnamaldehyde
3[c]. Anti-HIV-1 conjugate	1. Horseradish-peroxidase-labeled anti-HIV-1 2. Preservatives: a) 0.01% gentamicin b) 0.02 cinnamaldehyde
4. Disruption buffer	1. FD and C red dye no. 2 as coloring agent 2. Preservatives: a) 0.01% gentamicin b) 0.02 cinnamaldehyde
5[d]. TMB solution	1. Citric acid diluent 2. 0.03% tetramethylbenzidine.2HCl
6[e]. Peroxide solution	1. Citric acid-citrate buffer 2. 0.04% urea peroxide

Continued

7[f]. Stop solution	1. 2N sulfuric acid
D. Reagents for Vironostika ICD-HIV-1 p24 antigen	
1. Base dissociation buffer	1. Sodium hydroxide saline and preservatives
2. Negative control	3. Human serum non-reactive for HIV-1 Abs, HBsAg and HCV Ab 4. Preservatives: a) 0.01% gentamicin b) 0.02 cinnamaldehyde
3. Positive control	3. Human HIV-1 Ag: 160 pg/ml p24 core antigen (inactivated) 4. Preservatives: a) 0.01% gentamicin b) 0.02 cinnamaldehyde
4. Anti-HIV-1 conjugate	3. Horseradish-peroxidase-labeled anti-HIV-1 4. Preservatives: a) 0.01% gentamicin b) 0.02 cinnamaldehyde
5. TMB solution	3. Citric acid diluent 4. 0.03% tetramethylbenzidine.2HCl
6. Peroxide solution	3. Citric acid-citrate buffer 4. 0.04% urea peroxide
7. Stop solution	1. 2N sulfuric acid

I. *INTRODUCTION*

1. General

AIDS is the clinical stage of human immunodeficiency virus type 1 or type 2 (HIV-1 or HIV-2) infection (1). It is an immunosuppressive disorder characterized by the depletion of the CD4 helper/inducer lymphocyte sub-population, which is accompanied by a broad variety of clinical manifestations related to impaired immunity. AIDS was first reported in the USA among homosexuals. The oldest confirmed HIV viruses were isolated from serum samples collected in Central Africa in 1959 (2). The first Ethiopian HIV-positive case was reported from serum sample collected in 1984 (3) while the first AIDS case was reported in 1986 (4) in Addis Ababa. Since then the AIDS epidemic is a rapidly growing problem in Addis Ababa and elsewhere in the country (5). AIDS has become one of the leading causes of morbidity and mortality in the world (6,7).

HIV-1 virus isolates are phenotypically diverse varying in cytopathicity, replication rate and cell tropism (8). The biological phenotype of HIV-1 isolates can be divided into two groups, rapid/high, SI; and slow/low, NSI, based on the ability to infect MT-2 cells ($CD4^+$ T cell lines) and induce syncytium formation (9). These differences in the biological phenotype of HIV-1 isolates are largely determined by the charge of two specific amino acids at positions 11 and/or 28 in the V3 loop of the envelope protein gp120 for HIV-1 subtypes A, B, D and E (10,11). HIV-1 typically evolves from an M-tropic, noncytopathic, NSI virus at early asymptomatic stages of infection to a T-cell-tropic, cytopathic and SI virus population as patients progress to AIDS in subtype A, B and D infections (1,4,8-10) in 50-70% of cases. The presence of SI HIV-1 isolates in infected individuals is associated with a rapid decline of $CD4^+$ T-cells (8-10,11-27), an increased virus load (8,10,22-24,28-30), rapid disease progression (21-23,30-33),

reduced survival time after AIDS diagnosis (17,23,31-34), P24 antigen positivity (12,23,25-27,35), higher proportion of infected $CD4^+$ T-cells (7,9,11,15,16,29), AIDS-defining clinical illnesses (14,16,36) as well as with higher cytopathicity (14-18,27,30). The SI HIV-1 isolate have high cytopathicity and broader tropism for human cells/tissues than the M-tropic NSI HIV-1 isolate. Moreover, the biological phenotype of HIV-1 isolates is associated with viral transmission and persistence. The efficacies of antiviral therapy and virus-receptor interactions have also been shown to be influenced by the biological phenotypes of the HIV-1 isolates. Finally, the biological phenotypes can be used for *in vitro* classification of HIV-1 isolates (9,37).

Most of the data demonstrating the biological phenotypes of HIV-1 isolates and their biological consequences are based on studies of subtypes A, B, D and E HIV-1 isolates. Studies on Ethiopian HIV-1 isolates have shown the presence of subtype C (11,38). However, data on the biological phenotypes (SI/NSI) are not available for subtype C HIV-1 isolates circulating in Ethiopia.

2.0 THE VIRUS

The human immunodeficiency virus is a retrovirus that belongs to the lentivirus subfamily. HIV-1 variants have been genetically classified into the major (M) and the outlier (O) groups. The M group is further divided into 10 genetically distinct subtypes (A- J) based on sequence diversity of the gene encoding the envelope (env) protein, gp120, where members of the same subtype differ by less than 10%, and those of different subtypes by 15% or more (39,40). The O group contains several very heterogeneous viruses (40).

Figure 1 illustrates the genomic and biological characteristics of an HIV-1 virion. Like other retroviruses, HIV virions contain at least three reading frames (gag, *pol* and *env* genes) encoding three structural proteins (41-43) (Figure 2). *Gag* encodes the $P55^{gag}$ precursor protein that is processed into the $p24^{gag}$ (capsid), $p17^{gag}$ (matrix), $p7^{gag}$ (nucleocapsid) and $P6^{gag}$ mature proteins (43,44). The *pol* gene encodes enzymes necessary for replication in the host cell, $P51/P66^{pol}$ (reverse transcriptase/RNase H), $P34^{pol}$ (integrase) and $P11^{pol}$ (protëase). *Env* encodes the envelope precursor protein, $gp160^{env}$ and it is processed by a cellular protease into the two envelope proteins, $gp120^{env}$ and $gp41^{env}$ (45).

The outer envelope protein, gp120, facilitates viral entry into the host cell by binding to CD4, the main cellular receptor for all primate lentiviruses (46,47) whereas the transmembrane protein, gp41, is involved in the fusion of viral envelope with the cellular membrane (48). In addition to the structural genes, the HIV-1 genome contains several genes (*tat, nef, rev, vif, vpu and vpr*) encoding regulatory and accessory proteins (49-56) (Figure 2). $P14^{tat}$ and $p19^{rev}$ are involved in regulation of mRNA expression (50-53). $P23^{vif}$ and $P16^{vpu}$ are thought to have function in virion assembly and release. Although, the exact function of $P15^{vpr}$ and $P27^{nef}$ is controversial, they have been described to be important for replication in primary lymphocytes and monocytes/ macrophages (49). The proviral genome is flanked by long terminal repeats (LTR) which contain sequences necessary for initiation and regulation of transcription and polyadenylation of mRNA (43,54).

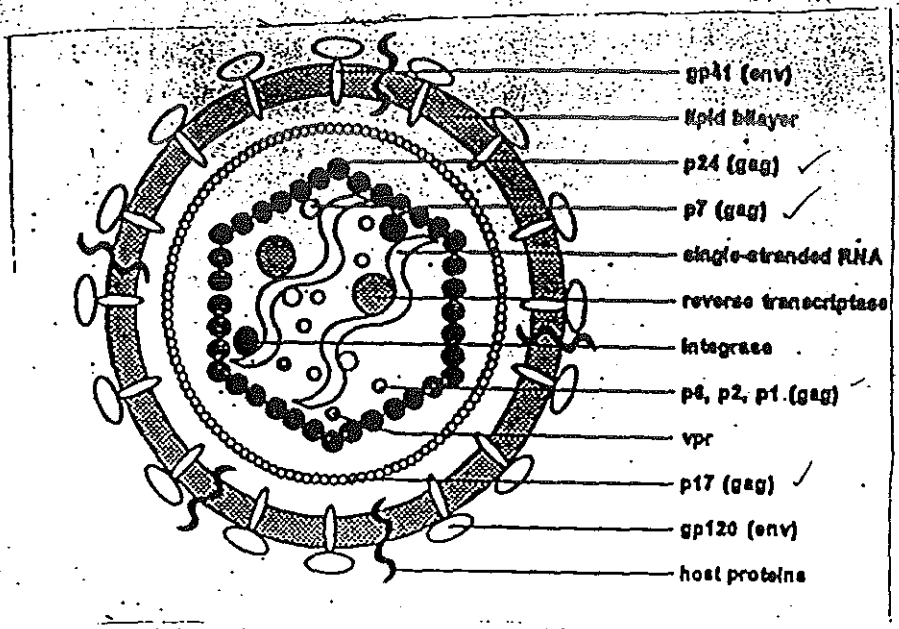


Figure 1. Schematic representation of an HIV virion.

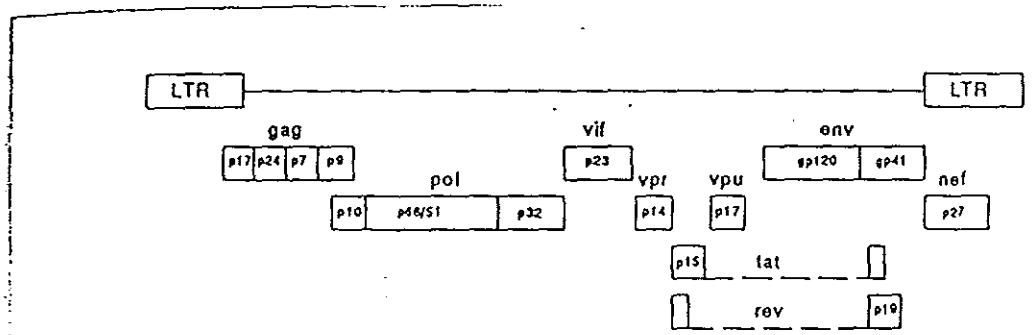


Figure 2. Genomic organization of HIV-1 and the proteins encoded by its genes.

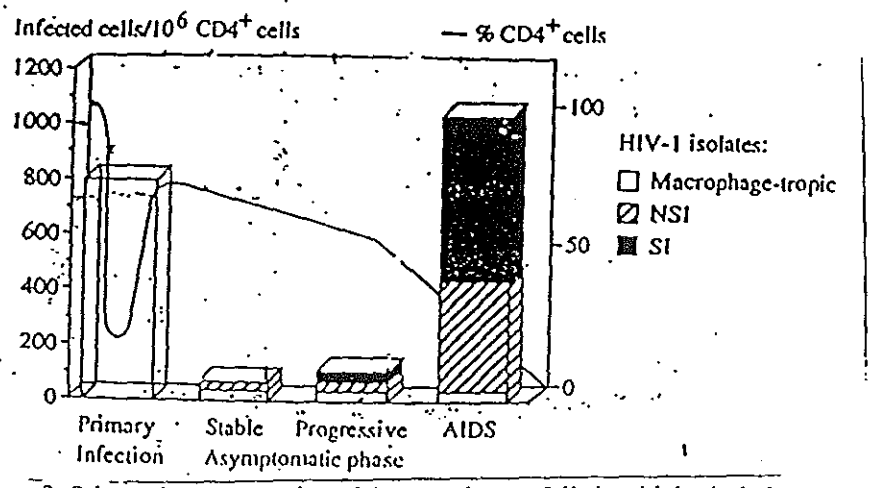


Figure 3. Schematic representation of the prevalence of distinct biological phenotypes of HIV-1 variants in the course of HIV-1 infection (adopted from Schuitemaker, H. 1994. J. Leukoc. Biol., 56:218-25).

1.1 Biological Phenotypes (SI/NSI) of HIV-1 Isolates

In vitro primary HIV-1 isolates can be subdivided into two distinct groups according to their biological phenotypes. The two distinct groups of primary HIV-1 isolates were classified as rapid/high or syncytium-inducing (SI) and slow/low or non-syncytium-inducing (NSI) isolates based on virus isolates replication characteristics and ability to form syncytia in MT-2 cells, respectively (9, 14, 32, 57). HIV-1 strains isolated from recently infected individuals are predominantly slow replicating, macrophage-tropic (M-tropic) and NSI *in vitro* (32, 33) and they use CCR5 coreceptor as entry ports in combination with CD4 molecule (58-67). Over the course of HIV-1 infection, viral phenotype and coreceptor uses broaden to include the appearance of T-cell tropic (T-tropic) (14, 16, 29, 34). T-tropic strains appear in about 50% to 70% of individuals infected with various HIV-1 subtypes as patients progress to AIDS (14, 23, 27, 32, 33). T-tropic strains induce the formation of syncytia in CD4 cell lines *in vitro*, infect peripheral blood mononuclear cells (PBMCs) faster, and replicate more aggressively than do the early M-tropic isolates (27-29). T-tropic, SI HIV-1 isolates enter target cells by means of CD4 and CXCR4 as a coreceptor (63,68, 69). The early classification of biological phenotypes of HIV-1 isolates based on their *in vitro* ability to form syncytia in MT-2 cells is imprecise and sometime misleading (70). Therefore, it is gradually replaced by a new classification system which is based on coreceptor use. According to this classification scheme virus isolates that use CCR5 but not CXCR4 were termed R5 viruses, isolates using CXCR4 but not CCR5 were designated X4 viruses and isolates that are able to use both coreceptors with comparable efficiency were called R5X4. In addition, whether an X4 or R5X4 virus is a cell-line-adapted isolate should be specified.

The occurrence of T-tropic, SI strains has been reported to be a predictive marker for increased progression towards AIDS (22-25) and disease progression to AIDS is faster in SI patients as compared to NSI patients (42, 72). The prevalence of distinct biological phenotypic variants is reported to be dependent on the stage of infection and is an important determinant in the variable clinical course of the infection (33, 71) [Figure 3]. NSI isolates can be detected throughout HIV-1 infection, SI isolates generally only develop in the course of HIV-1 infection and tend to precede the development of AIDS in 50% to 70% of patients. Reports from several authors have shown that the majority of the viruses isolated from asymptomatically infected individuals are M-tropic and of the NSI phenotype (40,42,44-46). *In vitro* studies suggest that HIV-1 isolates obtained from patients with advanced immunodeficiency in about 50% of individuals infected with subtypes A, B, D and E are SI phenotype (39, 40, 44-46). The risk of a rapid $CD4^+$ cell decline and onset of AIDS rises significantly with the appearance of SI variants in the peripheral blood of asymptomatic seropositive individuals (44, 72-74) [Fig.3]. SI variants in general correlate with faster disease progression to AIDS. SI arise after the initial loss of $CD4^+$ T cells but precede an accelerated $CD4^+$ T cell decline. The more efficient replication and cytopathicity of SI viruses as compared to NSI variants result in an increased viral load, thus leading to accelerated $CD4^+$ T cell killing. Mean $CD4^+$ T-cell count in patients developing AIDS with B subtype SI strains is lower than those developing AIDS with NSI HIV-1 variants (33,99). The blood of AIDS patients with Subtype B SI strains has been shown to contain more HIV-1 RNA copies in the plasma than the blood of AIDS subjects with NSI strains (1, 21, 75).

In general, variation in the biological phenotype of HIV-1 isolates plays an important role in classifying HIV-1 isolates *in vitro* (14, 15, 29, 37, 76), in monitoring the efficacy antiviral therapy (37), in understanding the mechanisms of AIDS immunopathogenesis

(18, 75, 77-84), in prognosis of HIV-1 infection (9, 21-26, 48, 71) and in viral persistence and transmission (74, 85-92).

1.2 Molecular Basis of the Variation in the Biological Phenotypes (Cytotropism) of HIV-1 Isolates:

The course of HIV-1 disease progression is characterized by the presence of closely related diverse genetic variants or "quasispecies" of viruses in HIV-1 infected individual (37). These HIV-1 variants display variation in biological properties such as replication rate, cell tropism and SI capacity (14, 16, 32). The viral determinants for cytotropism reside mainly in the envelope (Env) V3 region of glycoprotein gp120 (10, 93). Sequence analysis of the V3 regions of HIV-1 SI isolates from subtypes A, B, C, D, E and O has revealed positively charged amino acids at positions 11 and 28 to be associated with SI phenotype (94-101). However, this association has not been confirmed for HIV-1 subtype C. Elongation of V2 domain with added or relocated N-linked glycosylation sites (102-106), the charge of amino acids of the V1V2 regions (73, 107-111) and changes in the amino acid in V1 loop (112) were also postulated to herald an NSI to SI phenotype switch in HIV-1 subtype B isolates. But, the role of these regions in determining the biological phenotype of HIV-1 subtype C variants is not documented.

Recent studies indicated that a major molecular determinant of HIV-1 tropism (phenotype) lies at the level of virus entry into target cells, which in turn is governed by the expression of coreceptors in conjunction with CD4 (14, 27, 113); either CCR5 or CXCR4 or both. Therefore, CXCR4 use is a defining feature of viruses that form syncytia in T-cell lines; use of CCR5 is a property of NSI, M-tropic viruses; and many T-tropic isolates can use both coreceptors (60, 70, 113). This suggests that the co-receptor used would thus provide a precise molecular designation of a given isolate that largely explains its phenotype.

3.0 OBJECTIVES OF THE STUDY

There are no data on the SI/NSI phenotype of HIV-1 isolates obtained from Ethiopian HIV-1 infected individuals and despite some in depth studies (ENARP, unpublished data) there are no MT-2 positive results reported in HIV^+ Ethiopians. Thus, the general objective of this study was to establish the biological phenotypes (the presence of SI/NSI phenotype) and the frequency of SI and NSI variants of HIV-1 strains obtained from Ethiopian HIV-1 infected AIDS patients. AIDS patients were chosen for this study, because according to literature, this could maximize the chance of finding SI viruses.

Specific objectives of the thesis are to determine the correlation between SI/NSI biological phenotypes and

(1) clinical staging of the disease,

(2) immune status as determined by $CD4^+/CD8^+$ T-cell counts, and

(3) Viral load as determined by p24 or NASBA technologies.

II. MATERIALS AND METHODS

1. The study subjects and blood collection

In this study 48 hospitalized Ethiopian AIDS patients ($CD4^+$ T cell counts < 200 cells/μl of whole blood) from a nearby central referral hospital of Addis Ababa, Saint Paul Hospital were studied. Seven patients were clinically diagnosed as III patients and 41 subjects as stage IV patients (114) according to the revised WHO criteria for staging of HIV-1 infection and disease. Whole blood was collected using coded EDTA vacutainer tubes (Becton and Dickinson) between 2 A.M. and 5 A.M. This was done three days per week from November 1996 to February 1997. Samples were transported at ambient temperature (20-25°C) to EHNRI/ENARP laboratory. In addition, coded HIV results were reported to St. Paul's Hospital within 24-48 hours of sample collection.

2. Laboratory Methods

2.1 HIV serology

Screening for plasma antibodies (Abs) against HIV-1 was performed by HIV-SPOT test using HIV-SPOT kits (Genelabs Diagnostics, CA, USA). In brief, a drop of plasma sample was put on HIV-SPOT kit that was previously wetted with three drops of reconstituted liquid buffer. Then two drops of liquid buffer and two drops of wash buffer solution were added. This was followed by two drops of reconstituted conjugate and three drops of wash buffer solution. Appearance of a distinct red spot at the centre of the kit was interpreted to be positive for antibodies to HIV-1, whereas clear membrane after the procedure (or overall pink color) was interpreted to be negative for antibodies to HIV-1.

Plasma samples were retested for HIV-1 Abs by Enzyme linked immunosorbent assay (ELISA) using Organon Vironostika kits (Organon Teknika, BV, the Netherlands) according to the recommendations of the manufacturer. That is, 100 µl specimen diluent was pipetted into wells of 96-well plates. Fifty µl plasma samples and controls were pipetted into assigned wells. Two controls were included on each plate. After plates were incubated at 37°C for 60 minutes, each well was washed six times with phosphate buffer. Then, 100 µl tetramethylbenzidine (TMB) substrate was pipetted and plates were incubated at room temperature for 30 minutes. The reaction was stopped by adding 100 µl of 2N sulfuric acid to each well. The absorbance of the solution in each well was read at 450nm using an automatic ELISA reader. Test sample was considered reactive when absorbance value of plasma was greater than or equal to the cutoff value. A test sample was interpreted non-reactive when the absorbance value of the plasma sample was less than the cutoff value.

2.2 WBC count and flow cytometric analysis

To determine WBC, 20µl EDTA whole blood was mixed with 10ml Isoton and 3 drops of Isoton/Triton-100X. WBC count was determined in triplicate by Coulter counter (Coulter Electronics LTD., U. K.), according to the standard procedure. Absolute WBC count was done inorder to calculate the absolute count of CD4 cells in the peripheral blood of the patient.

Lymphocyte subsets and leukocyte three part differential (percent granulocyte, monocyte and lymphocyte) were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) using monoclonal antibodies combinations

CD45/CD14, IgG1/IgG2 isotype control, CD3/CD19, CD3/CD4, CD3/CD8 andCD3/CD16+56 with SimulSET software according to the manufacturers recommendations (36) (see Appendix 1). In brief, 100 µl EDTA whole blood was mixed with 10 µl of each combination of mAbs in 6 separate tubes and incubated at room temperature for 15 to 20 minutes in the dark. Red blood cells were lyzed by adding 2 ml of lysing solution per tube (FACSlyse, Becton Dickinson, CA, USA). After vortexing, tubes were incubated for 10 minutes at room temperature in the dark. The cells were centrifuged at 300g for 5 minutes and then washed twice with Isoton (Becton Dickinson, San Jose, CA, USA). The cell pellet was resuspended in 500 µl Isoton and analyzed using the SimulSET software of the FACScan on the same day. A minimum of 2000 events was acquired for analysis. The FACScan was calibrated with fluorescent beads (CaliBrite) and the AutoComp software weekly.

2.3 Biological phenotyping of HIV-1 isolates and cell culture supernatant (CCS) p24 ELISA

To assess the presence of SI and NSI HIV-1 variants, patients' PBMC were cocultured with MT-2 cells as described by Koot *et al.* (9). Briefly, when $CD4^+$ T cell counts of a given patient were less than 50 cells/µl of whole blood, five million PBMC were cocultured with one million MT-2 cells. When $CD4^+$ T cell counts of a given patient were greater than 50 cells/µl of whole blood, one million PBMC were cocultured with one million MT-2 cells. Ten µl SI isolates were cocultured with one million MT-2 cells as positive control and one million MT-2 cells were cultured alone as a negative control. Controls were included with every batch of MT-2 assay. The cultures were maintained at 37°C for three weeks and refreshed twice a week Cultures were checked for the

presence of syncytia twice a week under an inverted microscope (4X magnification). Virus replication was measured by ELISA for HIV-1 p24 antigen production using Vironostika kits (Organon Teknika, BV, the Netherlands) on CCS at syncytium formation or after a maximum of three weeks of coculturing. CCS with absorbance values less than the cutoff value were considered negative for HIV-1 antigen. Whereas, coculture supernatants with potical densities (OD) greater than or equal to the cutoff value were considered positive for HIV-1 antigen.

2.4 Detection of plasma p24 antigen

Immune-complexed dissociated HIV-1 p24 antigen (ICD-HIV-P24 AG) in plasma was measured semiquantitatively by a solid-phase, sandwich-type enzyme immunoassay (EIA, Abbott Laboratories, North Chicago, IL) according to a standard procedure. That is, 25 µl of base dissociation reagent was pipetted into all specimen and control wells. After 10 minutes, 100 µl of each test specimen and controls were added into the assigned wells. On each plate three negative controls and 6 positive controls, that is, five serial 2-fold dilutions of positive control (PC) (160-5ng/ml) were included. Strips were incubated at 37°C for 60 minutes. After each well was washed and soaked 4 times with diluted phosphate buffer, 100 µl of anti-HIV-1 conjugate (horseradish peroxidase-labeled anti HIV-1 antibody) was pipetted into each well and then incubated at 37^{O}C for 60 minutes. Then, 100 µl of solution containing 0.03 %TMB and 0.04 % urea peroxide was pipetted into each well. Strips were incubated at room temperature for 30 minutes. The reaction was stopped by adding 2N sulfuric acid to each well. The absorbance of the solution in each well was read at 450 nm using ELISA reader. Plasma samples with OD values less than the cutoff value were considered negative for ICD-HIV-1 p24 antigen.

Whereas, plasma samples with OD values greater than or equal to the cutoff value were considered positive for ICD-HIV-1 p24 antigen.

2.5 Viral load determination

HIV-1 RNA copy numbers in 100 µl plasma samples were quantitated by using a nucleic acid sequence based amplification assay (NASBA) as described by Kievit et al., (115). In brief, RNA was extracted from 100 µl plasma using an established guanidinium isothiocyanate based method (116) and isothermally amplified in the presence of RNA standards using NASBA kits (Organon Teknika, the Netherlands) (see Appendix 2 for detail).

2.6 Statistical data analysis

The distribution of immunological and virological data ($CD4^+$ and $CD8^+$T cell counts, CD4/CD8 ratio, ICD-p24 antigen concentration and viral load) was compared between patients with SI and NSI phenotypes using the non-parametric Wilcoxon ranksum test (Mann-Whitney U test). Proportions were compared using two-sided Fisher's exact test. Mean, median and standard deviations were calculated for each viro-immunological data for patients harboring SI and NSI virus phenotype.

III. RESULTS

3. Clinical characteristics of the study subjects

A total of 48 hospitalized Ethiopian AIDS patients aged 22-54 years participated in this study. All patients were at clinical stages III or IV of the WHO staging system of HIV-1 infection and disease (114). The mean ages were 36 (females, n= 20) and 35 (males, n=28)years. The most common manifestation of the WHO clinical staging observed was HIV wasting syndrome (n=39), followed by oral candidiasis (n=28) and pulmonary tuberculosis (n=23).

3. Viro-immunological characteristics of the study subjects

The mean and median of the viro-immunological data measured on 48 AIDS patients grouped according to the biological phenotypes of HIV-1 isolates in MT-2 assay are presented in Table 1.

All patients were selected to have absolute $CD4^+$ cell counts < 200 cells/μl whole blood (see Table 2). The absolute $CD4^+$ T cell counts ranged from 2 (PHD.092) to 194 cell/μl (PHD.049) with median value of 54 cells/μl (see Table 2). The absolute $CD8^+$ T cell varied between 20 cells/μl whole blood (PHD.092) and 1989 cells/μl whole blood (PHD.072); CD4/CD8 ratios ranged between 0.0 and 0.8 with median value of 0.1. All patients had HIV-1 RNA copy numbers, ranging from 4.26 $\log_{10}$ (PHD.030) and 7.08 $\log_{10}$ per ml of whole blood(PHD.009) with median of 5.59 $\log_{10}$ RNA copies/ml of plasma (see Table 2).

Table 1. Mean (SD✠) and median of viro-immunological data from 48 Ethiopian AIDS patients harboring SI and NSI strains.

	SI	Patients(n=3)	NSI	Patients(n=45)	
Parameter	Mean(SD✠)	Median	Mean(SD)	Median	P value
CD4 count	23(23)	16	79(56)	62	0.04
CD8 count	589(320)	568	611(555)	395	0.72
CD4/CD8 ratio	0.03(0.06)	0.00	0.21(0.21)	0.10	0.03
ICD-p24Ag conc.	163(18)	160	58(70) (n=43)	23 (n=43)	0.02
RNA load	5.30(0.54)	5.78	5.72(0.08)	5.89	0.50

✠SD = standard deviation

HIV-1 ICD-p24 antigen was detected in 30/46 (65.2%) of the plasma samples assayed. The concentration of ICD-p24 antigen ranged from 3 to 190 ng/ml of plasma with median of 18 ng/ml.

Of the 48 AIDS patients studied, 45/48 (94%) had NSI viruses and 3 (6%) harbored SI viruses as determined in the MT-2 assay (patients: PHD.030, PHD.074, PHD.079) (see Table 2). The distributions of $CD4^+$ count of SI AIDS patients was lower (median=16 cells/μl) compared to that of the NSI AIDS patients (median=568 cells/μl), P=0.04 (see Table1). Also, the distributions of CD4/CD8 were lower in SI patients (median=0.00) as compared to NSI patients (median=0.10), P=0.03 (Table1). Both NSI and SI patients had comparable RNA copy numbers in the plasma; 5.89 $\log_{10}$ RNA copies/ml for NSI versus 5.57 $\log_{10}$ RNA copies/ml for SI, P>0.05. In addition, they had comparable distribution of $CD8^+$ T cell counts; median value were 568 and 395 for SI patients and NSI patients, respectively (P=0.72). However, the distribution of ICD-p24 antigen levels in SI patients was higher (median=160) as compared to mean of the NSI patients (median=23), P=0.02.

Table 2. Immunological and virological data obtained from 48 Ethiopian AIDS patients.

LABID	AGE	SEX	$CD4^+$ Count	$CD8^+$ Count	CD4/CD8 Ratio	VIRUS PHENO TYPE	STAGE	LOAD†	P24 (ng/ml)
PHD005	32	M	22	307	0.10	NSI	IV	5.46	ND
PHD006	45	M	110	918	0.10	NSI	IV	5.72	ND
PHD009	40	M	61	745	0.10	NSI	IV	7.08	99
PHD019	43	M	20	452	0.00	NSI	IV	6.46	175
PHD020	25	M	83	1082	0.10	NSI	IV	6.38	17
PHD024	33	M	75	98	0.80	NSI	III	4.86	5
PHD028	30	M	8	410	0.20	NSI	IV	5.51	73
PHD029	25	F	117	395	0.30	NSI	IV	5.76	13
PHD030	30	M	5	281	0.00	SI	III	4.26	160
PHD032	50	F	155	295	0.50	NSI	IV	5.00	0
PHD033	48	F	53	1064	0.10	NSI	IV	5.38	103
PHD034	24	M	190	248	0.80	NSI	IV	6.08	163
PHD035	50	M	27	257	0.10	NSI	IV	6.53	179
PHD036	35	M	48	338	0.10	NSI	IV	5.49	0
PHD037	30	M	61	174	0.40	NSI	IV	5.79	0
PHD039	30	M	58	431	0.10	NSI	IV	6.15	0
PHD042	31	F	122	346	0.40	NSI	IV	6.04	0
PHD043	54	F	39	69	0.60	NSI	IV	5.69	0
PHD044	26	F	55	291	0.20	NSI	III	4.63	51
PHD045	35	F	62	83	0.80	NSI	IV	6.43	142
PHD046	38	F	25	332	0.10	NSI	IV	4.72	0
PHD049	23	F	194	1940	0.10	NSI	IV	5.15	18
PHD050	35	M	191	1319	0.10	NSI	IV	5.32	0
PHD051	45	F	35	475	0.10	NSI	IV	5.23	154
PHD054	30	F	186	647	0.30	NSI	III	5.26	175
PHD055	26	F	17	210	0.10	NSI	IV	5.20	0
PHD069	52	M	83	704	0.10	NSI	IV	5.38	0
PHD070	35	M	137	2218	0.10	NSI	IV	4.58	99
PHD072	30	F	155	1989	0.10	NSI	IV	6.08	0
PHD074	45	F	49	568	0.10	SI	IV	5.57	146
PHD076	45	M	149	1663	0.10	NSI	IV	5.79	156
PHD077	45	F	158	1760	0.10	NSI	IV	5.20	0
PHD079	30	M	16	919	0.00	SI	IV	6.08	182
PHD081	53	M	54	158	0.30	NSI	IV	5.97	0
PHD086	45	M	92	990	0.10	NSI	IV	5.59	77
PHD088	35	M	114	212	0.50	NSI	IV	6.72	190
PHD089	23	F	12	184	0.10	NSI	III	5.96	0
PHD091	25	F	25	143	0.20	NSI	IV	6.39	175
PHD092	38	M	2	60	0.00	NSI	IV	4.79	0
PHD094	32	M	103	393	0.30	NSI	IV	5.91	8
PHD099	38	F	63	599	0.10	NSI	IV	6.28	91
PHD100	35	M	126	632	0.20	NSI	IV	6.00	3
PHD101	50	F	9	305	0.00	NSI	IV	5.83	62
PHD102	22	M	29	572	0.10	NSI	IV	5.78	0
PHD104	25	M	62	341	0.20	NSI	III	5.45	0
PHD106	26	M	53	830	0.10	NSI	III	5.85	0
PHD108	28	F	26	672	0.00	NSI	IV	6.39	153
PHD115	33	M	20	136	0.10	NSI	IV	6.08	116

†: in $\log_{10}$ copies of HIV-1RNA/ml, ND: not done

IV. DISCUSSION

HIV-1 subtype C is the predominant HIV-1 variant in Addis Ababa (11, 117, 118). However, the prevalence of SI strains of HIV-1 isolates among the Ethiopian AIDS patients is not documented. This study was done to assess the prevalence of SI viruses among Ethiopian AIDS patients who were shown to be infected by HIV-1 C subtype by an independent study (Almaz Abebe *et al.*, personal communication). Only 3/48 (6%) of the patients had SI viruses. Low prevalence of the SI viruses among subjects infected with HIV-1 subtype C has also been reported from other countries, like Dijoubti, India, China, Brazil and South Africa (111, 117-123). The reasons why the frequency of SI strains is low among HIV-1 subtype C variants remain to be elucidated.

A low sensitivity of MT-2 assay for the determination of the biological phenotypes of HIV-1 viruses is a possible explanation for the low frequency of SI strains detected in this study. In line with this explanation, studies have demonstrated that coculture supernatant assay is more sensitive than MT-2 assay for the determination of NSI and SI phenotypes (129). Researchers have also indicated that HIV-1 isolates with NSI phenotype in MT-2 assay were found to be of SI phenotype in $CD4^+$ cell clones and other cell lines (37, 70, 131). In addition, it should be noted that an MT-2 assay may give false negative results unless the coreceptor(s) used is included in the classification of HIV-1 isolates as SI and NSI phenotypes (70). On the other hand, MT-2 assay was confirmed positively by p24, increased PBMC input, and V3 loop sequence on the clonal level (Almaz Abebe et al., personal communication). Finally, it should be realized that the *in vitro* result of a MT-2 assay does not necessarily reflect the *in vivo* situation. Subjects with NSI variants by MT-2 assay have been reported to harbor $CD4^+$ syncytia

as determined by high resolution microscopy and immunofluoresent staining (Stapleton et al., Abstract 21111, Geneva AIDS conference, 1998).

It could also be argued that lower expression of CXCR4, possible CXCR4 gene defects or increased CCR5 expression on cells of HIV-1 subtype C infected subjects which would provide less selective advantage for the NSI viruses to evolve into SI strains. The HIV-1 coreceptors CCR5 and CXCR4 are reported to be expressed differentially on activated and memory T cells, and on naïve T cells, respectively (68). HIV-positive Ethiopian subjects have been demonstrated to possess increased numbers of activated T cells and (in AIDS stage) decreased numbers of naïve T cells (Tsehaynesh Messele et al., personal communication). Furthermore, it was recently reported that subjects with delta32/wt CCR5 genotype carried more often SI virus than subjects with wt/wt genotype (124).

It is also possible that probable oversecretion of SDF-1 chemokine could help prevent the emergence of SI variants by binding to CXCR4 receptor making this receptor inaccessible for SI uses. This is consistent with previous *in vitro* observations in subtype B viruses (69, 125). In addition, recent experiments have shown that SDF-1α is capable of down regulating CXCR4 on cells by induction of endocytosis and effectively blocking infection by T-tropic (SI) strains (62, 69, 125). The relevance of this explanation, however, needs to be validated by further independent studies.

Compared to the above speculated host related immunlogical factors the virus itself is the most likely reason for the low prevalence of SI strains among individuals infected with C subtype HIV-1 variants. This has been repeatedly reported among subjects

infected with subtype C viruses in Dijoubti, South Africa India, China and Brazil (111, 117-123). It is unlikely for subjects from such diverse genetic and environmental backgrounds to have identical immunological factors as described above. This suggests that it is unlikely for the host to account for the low prevalence of SI strains in this study. Therefore, it is the virus itself that is most likely resulted in the absence of NSI to SI switch among C subtype HIV-1 infections.

It is also possible that restriction by the V3 regions of Env gp120 may be involved in preventing frequent NSI to SI shift in HIV-1 subtype C infections. It was demonstrated that the V3 regions of Ethiopian C subtype SI viruses was different from those of other subtype A, B, D, E and O HIV-1 SI viruses (10, 94, 96-98). However, in HIV-1 subtype B, use of CXCR4 as a coreceptor has been shown to be associated with the presence of positively (basic) charged amino acids at positions 11 and 28 in V3 region (69, 125). This suggests that the absence of this association among Ethiopian C subtype viruses may restrict the use of CXCR4 as a coreceptor and thus a selective evolution of SI strains. Therefore, structural features of the V3 loop as well as other Env regions may play a role in preventing the development of SI capacity of subtype C HIV-1 viruses.

Another observation in this study is that there was an association between SI strains and low CD4 cell counts. Clearly this indicates that SI strains of HIV-1 isolates contributed to a significant depletion of $CD4^+$ T cells. There is still an on going argument regarding the immunological and virological factors contribute to low $CD4^+$ cell counts in NSI patients. The following factors are suggested play a role in the depletion of CD4 cells in

B subtype HIV-1 infection: (1) natural CD4 count in HIV-negative individuals, (2) direct cytopathicity, (3) apoptosis/high turnover, and (4) immune exhausition.

Although the appearance of SI variants of subtype B HIV-1 isolates was strongly associated with an increased rate of depletion of $CD4^+$ T cells and more rapid development of AIDS (9, 19, 34), faster disease progression towards AIDS and low absolute $CD4^+$ T cell counts in Africa, particularly in the majority of Ethiopian who were infected with NSI C subtype HIV-1 viruses are not documented. It could be speculated that immune activation because of persistent co-infection with environmental pathogens and the naturally low $CD4^+$ T cell counts in healthy Ethiopians which will be further decreased upon HIV-1 infections (Aster Tsegaye *et al.*, personal communication) may explain faster disease progression to AIDS and low $CD4^+$ T cell counts in C subtype HIV-1 infections.

The present finding clearly indicate that M-tropic, NSI and potentially transmitting HIV-1 subtype C variants are the predominant variants in the majority of Ethiopians. The biological consequence of higher proportion of M-tropic NSI variants is that they could effectively initiate HIV-1 infection after sexual transmission (89) even in AIDS patients. This might be the reason for an argument that supposes a higher transmission rate in C subtype epidemics. The role of SI and NSI HIV-1 subtype C strains, however, in virus transmission, disease progression towards AIDS and $CD4^+$ T cell depletion among HIV-1 infected Ethiopian individuals remain to be elucidated.

In contrast to the low prevalence of p24 antigen positivity (4, 35, 131), in this study base-dissociated ICD-HIV-1 p24 antigen was detected in 67.2%(30/46) of individuals. This finding is contrary to the previous reports that argue racial origin is the reason for

differences in p24 antigen positivity (131, 134). This is consistent with a recent report that the frequencies of p24 antigen positivity are independent of racial difference (130). The likely explanation for the earlier low prevalence of p24 antigen positivity among Ethiopian AIDS patients (4, 35) could be due to variation in the sensitivity of the method employed in detecting p24 antigen. High amount of HIV-1 immune-complexed and dissociated p24 antigen concentration was significantly correlated with the SI HIV-1 variants. This result is in agreement with the previously reported findings of the association of plasma p24 antigen positivity with the emergence of SI strains in North America and Europe (132).

There is a good correlation between viral RNA copy numbers and p24 antigen amounts in the plasma ($P = 0.02$). Similar results were reported for subtype B HIV-1 isolates obtained from North America and Europe (35, 48, 133, 134). This suggests that the quantitative analysis of HIV-1 viral RNA by Q-NASBA will be a valuable method in monitoring HIV-1 RNA levels in AIDS subjects and may serve as a predictive marker for disease progression.

V. CONCLUSION AND RECOMMENDATIONS

The biological phenotypes of the majority of (96%) HIV-1 isolates obtained from the study subjects were of NSI phenotype regardless of the clinical status and immunological and virological characteristics of the patients. Therefore, the prevalence of SI variants among Ethiopian subtype C HIV-1 isolates is remarkably low. Further studies are necessary to elucidate the likely immunological and virological factors that attributed to the lack of NSI→SI switch in subtype C HIV-1 infections.

VI. REFERENCES

1. **Miedema, F. L., Meyaard, M., Koot, M. R., *et al.*, 1994.** Changing virus-host interactions in the course of HIV-1 infection. *Immunol. Reviews*, 140:35-72.
2. **Zhu, T., Korber, B. T., Nahmias, A. J., *et al.*, 1998.** An African HIV-1 sequence from 1959 and implications for the origin of the epidemic. *Nature*, 391:594-7.
3. **Lester, F. T., Seyoum Ayehunie and Debrework Zewdie 1988.** Acquired immunodeficiency syndrome: seven cases in an Addis Ababa hospital. Ethiop. Med. J., 26:139-45.
4. **Seyoum Ayehunie, Sonnerborg, A., Belta Desta, *et al.*, 1992.** Relationship between cell-free viraemia, antigenaemia and antibody levels in HIV-1 infected Ethiopian patients. *AIDS*, 6: 651-657.
5. **Hailegnaw Eshete and Tefera Sahlu 1996.** The progression of HIV/AIDS in Ethiopia: Review article. *Ethiop. J. Health Dev.*, 10:179-90.
6. **Nkowane, B. 1991.** Prevalence and incidence of HIV infection in Africa: a review of data published in 1990. *AIDS*, 5(Suppl 1): S7-S14.
7. **Who, 1997.**
8. **Fouchier, R. A., Meyaard, L., Brouwer, M., *et al.*, 1996.** Broader tropism and higher cytopathicity for $CD4^+$ T-cells of a syncytium-inducing compared to a non-syncytium-inducing HIV-1 isolate as a mechanism for accelerated $CD4^+$ T-cell decline in vivo. *Virol.*, 219: 87-95.
9. **Koot, M. Vos, A. H., Keet, R. P., *et al.*, 1992.** HIV-1 biological phenotype in long-term infected individuals evaluated with an MT-2 cocultivation assay. *AIDS*, 6: 49-54.
10. **Fouchier, R. A. M., Groenink, M., Kootstra, N., *et al.* 1992.** Phenotype-associated sequence variation in the third variable domain of the HIV-1 gp120 molecule. *J. Virol.*, 66: 3183-3187.
11. **Almaz Abebe, Kuiken, C. L., Goudsmit, J., *et al.*, 1997.** HIV-1 subtype C in Addis Ababa, Ethiopia. *AIDS Res. Hum. Retrovirus*, 13:1071-5.
12. **Cao, Y., Qin, L., Zhang, L., *et al.*, 1995b.** Virologic and immunologic characterization of long-term survivors of HIV-1 infection. *NEJM*, 332: 201-8.
13. **Ho, D. D., Neumann, A. U. and Perelson, A. S. 1995.** Rapid turnover of plasmid virions and CD4 lymphocytes in HIV-1 infection. *Nature*, 373: 123-6.

14. **Schuitemaker, H., Koot, M., Kootstra, N. A., *et al.* 1992.** Biological phenotype of HIV-1 clones at different stages of infection: progression of disease is associated with a shift from monocytotropic to T-cell-tropic virus population. *J. Virol.*, **66**: 1354-60.

15. **Tersmette, M., Gruters, R. A., De Wolf, F., et al., 1989a.** Evidence for a role of virulent HIV variants in the pathogenesis of AIDS: studies on sequential HIV isolates. *J. Virol.*, **63**: 2118-26.

16. **Asjo, B., Morfeldt-Manson, L., Albert, J., *et al.*, 1986.** Replicative properties of HIV from patients with varying severity of HIV infection. *Lancet,* **ii**: 660-2.

17. **Connor, R. I., Mohri, H., Cao, Y. and Ho, D. D. 1993.** Increased viral burden and cytopathicity correlate temporally with CD4 T-lymphocyte decline and clinical progression in HIV-1 infected individuals. *J. Virol.*, **67**: 1772-7.

18. **Miedema, F. L., Meyaard, M., Ros, S. A., *et al.*, 1993.** Immunological and virological mechanisms of $CD4^+$ cell depletion. In New Concepts of AIDS Pathogenesis, Montagnier, L. and Gougeon, M. L. (eds.), Dekker Inc., pp. 169-81.

19. **Schellekens, P. T., Tersmette, M., Roos, M. T., *et al.*, 1992.** Biphasic rate of $CD4^+$ cell count decline during progression to AIDS correlates with HIV-1 phenotype. *AIDS*; **6**: 665-9.

20. **Munoz, A., Kirby, A. J., He, Y. D., et al., 1995.** Long-term survivors with HIV-1 infection: incubation period and longitudinal patterns of $CD4^+$ lymphocytes. *J. AIDS Hum. Retrovirol.*, **8**:496-505.

21. **Tsoukas, C. M. and Bernard, N. F. 1994.** Markers predicting progression of human immunodeficiency virus-related disease. *Clin. Microbiol., Rev.* **7**: 14-28.

22. **Jurriaans, S., Gemen, B. V., Weverling, G. J., *et al.* 1994.** The natural history of HIV-1 infection: virus load and virus phenotype independent determinants of clinical course? *Virol.*, **204**: 223-233.

23. **Lafeuillade, A., Tamalet, C., Pellegrino, P., *et al.*, 1994.** Correlation between surrogate markers, viral load and disease progression in HIV-1 infection. *J. Acquir. Immune Syndr.*, **7**: 1028-33.

24. **Verhofstede, C., Reniers, S., Van Waanzeele, F. and Plum, J. 1994.** Evaluation of proviral copy number and plasma RNA level as early indicators of progression in HIV-1 infection: correlation with virological and immunological markers of disease. *AIDS,* **14**:21-7.

25. **Schellekens, P. T., Koot, M., Roos, M. T., *et al.*, 1995** Immunologic and virologic markers determining progression to AIDS. *J. Acquir. Immune Defic. Syndr. Hum. Retrovirol.*, **10** (suppl 2) : S62-6.

26. **Hogervorst, E., Jurriaans, S., De Wolf, F., *et al.*, 1995.** Predictors for non- and slow progression in HIV-1 infection: low viral RNA copy numbers in serum and maintenance of high HIV-1 p24-specific but not V3-specific antibody levels. *J. Infect. Dis.*, 171: 811-21.

27. **Fenyo, E. M., Morfeldt-Manson, L., Chiodi, F., *et al.*, 1988.** Distinct replicative and cytopathic characteristics of HIV isolates. *J. Virol.*, 62: 4414-9.

28. **De Wolf, F., Roos, M., Lange, J. M. A., *et al.*, 1988.** Decline in $CD4^+$ cell numbers reflects increases in HIV-1 replication. *AIDS Res. Hum. Retroviruses*, 4: 433-40.

29. **Connor, R. I. and Ho, D. D. 1994.** HIV-1 variants with increased replicative capacity develop during the asymptomatic stage before disease progression. *J. Virol.*, 68: 4400-8.

30. **Wei, X., Ghosh, S. K., Taylor, M. E., *et al.*, 1995.** Viral dynamics of HIV-1 infection. *Nature*, 373: 117-22.

31. **Karlsson, A., Parsmyr, K., Sandstorm, E., *et al.*, 1994.** MT-2 cell tropism as a prognostic marker for disease progression in HIV-1 infection. *J. Clin. Microbiol.*, 32: 364-70.

32. **Tersmette, M., de Goede, R. E. Y., Al, B. J. M., *et al.*, 1988.** Differential syncytium-inducing capacity of HIV isolates: frequent detection of SI isolates in patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex. *J. Virol.*, 62:2026-32.

33. **Koot, M., Keet, I. P., Vos, A. H., *et al.* 1993.** Prognostic value of HIV-1 syncytium inducing phenotype for rate of $CD4^+$ cell depletion and progression to AIDS. *Ann. Intern. Med.*, 118: 681-688.

34. **Tersmette, M., Lange, J. M. A., de Goede, R. E. Y., *et al.*, 1989b.** Association between biological properties of HIV-1 variants and risk for AIDS and AIDS mortality. *Lancet*, I:983-6.

35. **Lange, J., Paul, D., Huisman, H., *et al.*, 1986.** Persistent HIV antigenaemia and decline of HIV core antibodies associated with transition to AIDS. *BMJ.*, 293: 1459-62.

36. **CDC 1994.** 1994 revised guidelines for the performance of $CD4^+$ T-cell determinations in persons with human immunodeficiency virus (HIV) infection. *MMWR*, 43(No. RR-3): 4-21.

37. **Mckeating, J. A. 1996.** 1995 Fleming Lecture. Biological consequences of HIV-1 envelope polymorphism: does variation matter? *J. Gen. Virol.*, 77: 2805-2904.

38. **Seyoum Ayehunie, Johansson, B., Sonnerborg, A., *et al.*, 1993.** Sequence analysis of selected regions of the env(V3 loop and gp41) and gag(p7) reading frames of Ethiopian HIV-1 strains. *Arch. Virol.*, **128**:229-39.

39. **Louwagie, J., Janssens, W., Mascola, J., *et al.*, 1993.** Genetic diversity of the envelope glycoprotein from different HIV-1 isolates of African origin. *J. Virol.*, **69**:263-71.

40. **Myers, G. 1994.** HIV: between past and future. *AIDS Res. Hum. Retroviruses*, **10**: 1317-24.

41. **Ratner, L., Haseltine, W. A., Patarca, R., *et al.*, 1985.** Complete nucleotide sequence of AIDS virus, HTLV-III. *Nature*, **313**:277-84.

42. **Sanchez-Pescador, R., Power, M. D., Barr, P. J., *et al.*, 1985.** Nucleotide sequence and expression of an AIDS-associated retrovirus (ARV-2). *Science*, **227**:484-92.

43. **Wain-Hobson, S., Sonigo, P., Danos, O., *et al.*, 1985.** Nucleotide sequence of the AIDS virus, LAV. *Cell*, **40**:9-17.

44. **Connor, R. I. And Ho, D. D. 1994.** Biology and molecular biology of HIV. In: *Pediatric AIDS: The Challenge of HIV Infection in Infants, Children and Adolescents*; Pizzo, P. A. and Wilfert, C. M.(eds.). Williams and Wilkins, Baltimore, PP.97-113.

45. **Barrie-Sinoussi, F. 1996.** HIV as the cause of AIDS. *Lancet*, **348**:31-5.

46. **Dalgleish, A. G., Beverly, P. C. L., Clapham, P. R., *et al.*, 1984.** The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature*, **312**:763-70.

47. **Klatzmann, D., Champagne, E., Chamaret, S., *et al.*, 1984.** T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. *Nature*, **312**: 767-8.

48. **Mellors, J. W., Rinaldo, C. R. Jr., Gupta, P., *et al.*, 1996.** Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. *Science*, **272**:1167-70.

49. **Subbramanian, R. A. and Cohen, E. A. 1994.** Molecular biology of the HIV accessory proteins. *J. Virol.*, **68**:6831-5.

50. **Coffin, J. M. 1992.** Structure and classification of retroviruses. In: *The Retroviridae*, Vol. I; Levy, J. A. (ed.). New York: Plenum, PP.19-20.

51. **Levy, J. A. 1993.** Pathogenesis of HIV infection. *Microbiol. Rev.*, **57**:183-9.

52. **Sodroski, J., Goh, W. C., Rosen., *et al.*, 1986.** Role of the HTLV-III/ LAV envelope in syncytium formation and cytopathicity. *Nature*, **322**: 470-4.

53. **Strebel, K., Kimkait, T. and Martin, M. A. 1988.** A novel gene of HIV-1, vpu and its 16-kilodalton product. *Science*, **241**:1221-3.

54. **Cullen, B. R. and Green, W. C. 1990.** Functions of the auxiliary gene products of HIV-1. *Virol.*, 178:1-5.

55. **Kestler, H. W., Ringler, D. J., Mori, K., et al., 1991.** Importance of the nef gene for maintenance of high virus loads and for the development of AIDS. *Cell*, 65:651-62.

56. **Strebel, K., Daugherty, D., Clouse, K., et al., 1987.** The HIV a (SOR) gene-product is essential for virus infectivity. *Nature*, 328:728-30.

57. **von Briesen, H., Andresen, R. and Rubsamen-Waigmann 1990.** Systematic classification of HIV biological subtypes on lymphocytes and monocytes/macrophages. *Virol.*, 178:597-602.

58. **Alkhatib, G., Combadiere, C., Broder, C. C., *et al.*, 1996.** CC CKR-R: A RANTES, MIP-1, MIP-1 receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science*, 272: 1955-8.

59. **Choe, H., Farzan, M., Sun, Y., *et al.*, 1996.** The β chemokine receptors CCR-3 and CCR-5 facilitate infection by primary HIV-1 isolates. *Cell*, 85: 1135-48.

60. **Doranz, B. J., Rucker, J., Yi, Y., *et al.*, 1996.** A dual-tropic primary HIV-1 isolate that uses fusin and β-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell*, 85: 1149-58.

61. **Dragic, T., Litwin, V., Allaway, G. P., *et al.*, 1996.** HIV-1 entry into $CD4^+$ cells is mediated by the chemokine receptor CC-CKR-5. *Nature*, 381:667-73.

62. **Feng, Y., Broder, C.C., Kennedy, P. E. and Berger, E. A. 1996.** HIV entry factor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science*, 272: 872-7.

63. **Bates, P. 1996.** Chemokine receptors and HIV-1: an attractive pair? *Cell*, 86: 1-3.

64. **Broder, C. C., Earl, P. L., Long, D., *et al.*, 1994.** Antigenic implications of HIV-1 envelope quaternary structure: oligomere-specific and -sensitive monoclonal antibodies. *Proc. Natl. Acad. Sci. USA*, 91: 11699-703.

65. **Wu, L., Gerard, N. P., Wyatt, R., *et al.*, 1996.** $CD4^+$-induced interaction of primary HIV-1 gp120 glycoproteins with chemokine receptor CCR-5. *Nature*, 384: 179-83.

66. **Trkola, A., Dragic, T., Arthes, J., *et al.*, 1996.** CD4-dependent, antibody-sensitive interactions between HIV-1 and its coreceptor CCR-5. *Nature*, 384: 184-7.

67. **Zhang, L., Huanq, Y., He, T., Cao, Y. and Ho, D. D. 1996.** HIV-1 subtypes and second coreceptor use. *Nature*, 383: 768-70.

68. **Bleul, C. C., Wu, L., Hoxie, J. A., *et al.*, 1997.** The HIV coreceptors CXCR-4 and CCR5 are differentially expressed and regulated on human T lymphocytes. *Proc. Natl. Acad. Sci. USA*, 94:1925-30.

69. **Oberlin, E., Amara, A., Bachelrie, F., *et al.*, 1996.** The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line adapted HIV-1 . *Nature*, **382**:833-5.

70. **Berger, E. A., Doms, R.W., Fenyo, E.- M., *et al* 1998.** A new classification for HIV-1. *Nature*, 12:729-36.

71. **Meyaard, L. 1995.** Mechanisms of T cell dysfunction in HIV infection. PHD disseration; University of Amsterdam, pp. 11.

72. **Koeing, S., Gendelam, H., Orenstein, J., *et al.*, 1986.** Detection of AIDS virus in macrophages in brain tissue from AIDS patients with encephalopathy. *Science*, **233**:1089-92.

73. **Groenink, M., Fouchier, R. A., Broersen, S., *et al.*, 1993.** Relation of phenotype evolution of HIV-1 envelope V2 configuration. *Science*, **260**: 1513-6.

74. **Zhu, T., Mo, H., Wang, N., *et al.*, 1993.** Genotypic and phenotypic characterization of HIV-1 patients with primary infection. *Science*, **261**: 1179-81.

75. **Schuitemaker, H., Groenink, M., Meyaard, L., *et al.*, 1993.** Early replication steps but not cell-type specific signaling of the viral LTR determine HIV-1 monocytotropism. *AIDS Res. Hum. Retrovirus*, 9:669-75.

76. **Cheng-Mayer, C., Seto, D., Tateno, M. and Levy, J. A. 1988.** Biologic features of HIV-1 that correlate with virulence in the host. *Science* , **240**: 80-2.

77. **Giorgi, J., Nishanian, P., Schmid, I., *et al.*, 1987.** Selective alterations in immunoregulatory lymphocyte subsets in early HIV (human T-lymphotropic virus type III/lymphadenopathy-associated) virus infection. *J. Clin. Immunol.*, 7: 140-50.

78. **Rosenberg, Z. F. and Fauci, A. S. 1994.** Immunopathology and pathogenesis of HIV infection. In: *Pediatric AIDS: The Challenge of HIV Infection in Infants, Children and Adolescents*; Pizzo, P. A. and Wilfert, C. M. (eds.). Williams and Wilkins, Baltimore, PP. 115-128.

79. **Lane, H. C. and Fauci, A. S. 1985.** Immunologic abnormalities in the acquired immunodeficiency syndrome. *Ann. Rev. Immunol.*, **3**: 477-500.

80. **Meyaard, L. and Miedema, F. 1994.** Programmed death of T-cells in HIV infection: result of immune activation? *Current Topics Microbiol. Immunol.*, (In Press).

81. **Meyaard, L., Schuitemaker, H. and Miedema, F. L. 1993.** T-cell dysfunction in HIV infection: anergy due to defective antigen-presenting cell function? *Immunol. Today,* 14:161-4.

82. **Meyaard, L., Otto, S. A., Jonker, R. R., *et al.*, 1992.** Programmed death of T cells in HIV-1 infection. *Science*, **257**:217-9.

83. **Lifson, J. D., Reyes, G. R., McGrath, M. S., *et al.*, 1986.** AIDS retrovirus induced cytopathicity; giant cell formation and involvement of CD4 antigen. *Science*, **232**: 1123-7.

84. **Goudsmit, J. 1995.** The role of viral diversity in HIV pathogenesis. *J. AIDS Hum. Retrovirol.*, 10(Suppl 1): S15-9.

85. **Soto-Ramierez, L. E., Renjifo, B., McLane, M. F., *et al.*, 1995.** Differential growth of the HIV-1 subtypes in Langerhan's cells: Implications for vaccine development. *Dixieme Colloque des Cent Gardes*, 127-30.

86. **Spira, A. I., Marx, P. A., Paterson, B. K., *et al.*, 1996.** Cellular targets of infection and route of viral dissemination after an intravaginal inoculation of SIV virus into rhesus macaques. *J. Exptal. Med.*, **183**: 215-25.

87. **Fiore, J. R., Bjorndal, A., Peipke, K. A., *et al.*, 1994.** The biological phenotype of HIV-1 is usually retained during and after sexual transmission. *Virol.*, **204**: 297-303.

88. **Scarlatti, G., Hodara, V., Rossi, P., *et al.*, 1993.** Transmission of HIV-1 from mother to child correlates with viral phenotype. *Virol.*, **197**: 624-9.

89. **Leitner, T., Escanilla, D., Marquina, S., *et al.*, 1995.** Biological and molecular characterization of subtype D, G, and A/D recombinant HIV-1 transmission in Sweden. *Virol.*, **209**: 136-46.

90. **Cornelissen, M., Mulder Kampingna, G., Veenstra, J., et al., 1995.** Syncytium-inducing phenotype suppression at seroconversion after intramuscular inoculation of a non-syncytium-inducing/SI phenotypically mixed HIV population. *J. Virol.*, **69**:6777-80.

91. **Michael, N., Chang, G., Louie, L. G., *et al.*, 1997.** The role of viral phenotype and CCR-5 gene defects in HIV transmission and disease progression. *Nature Med.*, **3**: 338-40.

92. **Zhang, L. Q., MacKenzie, P., Cleland, A., *et al.*, 1993.** Selection for specific sequences in the external envelope protein of HIV-1 upon primary infection. *J. Virol.*, **67**: 3345-56.

93. **Andeweg, A. C., Leeflang, P., Osterhaus, A. D. and Bosch, M. L. 1993.** Both the V2 and V3 region of HIV-1 surface glycoprotein functionally interact with other envelope regions in syncytium formation. *J. Virol.*, 67: 3232-9.

94. **Yu, X., McLane, M. F., Ratner, L., *et al.*, 1994.** Killing of primary $CD4^+$ T-cells by non-syncytium-inducing macrophage-tropic HIV-1. *Proc. Natl. Acad. Sci. U.S.A.*, 91: 10237-41.

95. **De Wolf, F., Hogervorst, E., Goudsmit, J., *et al.*, 1994.** Syncytium-inducing and non-syncytium-inducing capacity of HIV-1 subtypes other than B: phenotypic and genotypic characteristics. *AIDS Res. Hum. Retroviruses,* **10**: 1387-1400.

96. **Zhong, p., Peeters, M., Janssens, W., *et al.*, 1995.** Correlation between genetic and biological properties of biologically cloned HIV-1 viruses representing subtypes A, B, and D. *AIDS Res. Hum. Retroviruses*, 11:239-48.

97. **De Jong, J. J., Simon, F., van der Groen, G., et al., 1996.** V3 loop sequence analysis of seven HIV-1 subtype 1 group O isolates phenotyped in PBMCs and MT-2 cells. *AIDS Res. Hum retroviruses*, **12**:1503-7.

98. **Hwang, S. S., Boyle, T. J., Lyerly, H. K. and Cullen, B. R. 1991.** Identification of the envelope V3 loop as the primary determinant of cell tropism in HIV-1. Science, 253: 71-4.

99. **Shioda, T., Oka, S., Ida, S., *et al.*, 1994.** A naturally occurring single basic amino acid substitution in the V3 region of HIV-1 env protein alters the cellular host range and antigenic structure of the virus. J. Virol., 68: 7689-96.

100. **Fouchier, R. A., Brouwer, M., Broersen, S. M. and Schuitemaker, H. 1995.** Simple determination of HIV-1 syncytium-inducing V3 genotype by PCR. *J. Clin. Microbiol.*, **33**: 906-11.

101. **Harrawe, G. and Cheng-Mayer, C. 1995.** Amino acid substitutions in the V3 loop are responsible for adaptation to growth in transformed T-cell lines of a primary HIV-1 infection. *Virol.*, **210**: 490-494.

102. **Fouchier, R. A., Broersen, S. M., Brouwer, M., *et al.*, 1995.** Temporal relationship between elongation of HIV-1 glycoprotein 120 V2 domain and the conversion toward a syncytium-inducing phenotype. *AIDS Res. Hum. Retroviruses,* **11**: 1473-78.

103. **Bosch, M. L., Andeweg, A. C., Schipper, R., *et al.*, 1994.** Insertion of N-linked glycosylation sites in the variable regions of the HIV-1 surface glycoprotein through AAT triplet reiteration. *J. Virol.*, **68**: 7566-9.

104.**Cornelissen, M., Hogervorst, E., Zorgdrager, F., et al., 1995.** Maintenance of SI phenotype of HIV-1 is associated with positively charged residues in the HIV-1 gp120 V2 domain without fixed positions, elongation or relocated N-linked glycosylation sites. *AIDS Res. Hum. Retroviruses*, 11:1169-75.

105.**Chesebro, B., Wehrly, K., Nishio, J. and Perryman, S. 1992.** M-tropic HIV isolates from different patients exhibit unusual V3 envelope sequence homogeneity in comparison with T-cell tropic isolates: definition of critical amino acids involved in cell tropism. *J. Virol.*, 66: 6547-54.

106. **De Jong, J. J., de Ronda, A., Keulen, W., et al., 1992.** Minimal requirements for the HIV-1 V3 domain to support the SI phenotype: analysis by single amino acid substitution. *J. Virol.*, 66:6777-80.

107.**Koito, A., Harrowe, G., Levy, J. A. and Cheng-Mayer, C. 1994.** Functional role of the V1V2 region of HIV-1 envelope gp120 in infection of primary macrophages and soluble CD4 neutralization. *Virol.*, **206**: 878-84.

108.**Wang, W. K., Essex, M. and Lee, T. H. 1996.** Single amino acid substitution in constant regions one or four of gp120 causes the phenotype of a HIV-1 variant with mutations in V1 and V2 to revert. J. Virol., 70: 607-11.

109.**Sullivan, N., Thali, M., Furman, C., *et al.*, 1993.** Effect of amino acid changes in the V1V2 region of HIV-1 gp120 glycoprotein on subunit association syncytium formation and recognition by a neutralizing antibody. *J. Virol.*, 67: 3674-9.

110.**Wu, Z., Kayman, S. C., Honnen, W., *et al.*, 1995.** Characterization of neutralization epitopes in the V2 region of HIV-1 gp120: role of glycosylation in the correct folding of the V1V2 domain. *J. Virol.*, 69: 2271-8.

111.**Fox, D. G., Balfe, P., Palmer, C. P., *et al.*, 1996.** Length polymorphism within the V2 region of the HIV-1 gp120 affects accessibility of the receptor binding site. *J. Virol.* (in press).

112.**Boyd, M. T., Simpson, G. R., Cann, A. J., *et al.*, 1993.** A single amino acid substitution in the V1 loop of HIV-1 gp120 alters cellular tropism. *J. Virol.*, 67: 3649-52.

113. **Berger, E. A. 1997.** HIV entry and tropism: the chemokine receptor connection. *AIDS*, **11**(suppl.):S3-S16.

114.**CDC 1993.** 1992 revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. *MMWR*, **41**(No. RR17): 1-35.

115. **Kievits, T., van Gemen, B., van Striip, D., et al., 1991.** NASBA isothermal enzymatic in vitro nucleic acid amplification optimized for the diagnosis of HIV-1 infection. *J. Virol. Methods*, 35:273-86.

116. **Boom, R., Sol, C. J. A., Salimans, M. M. M., *et al.*, 1990.** A rapid and simple method for purification of nucleic acids. J. Clin. Microbiol., **28**:495-503.

117. **Seyoum Ayehunie, Johansson, B., Sonnerberg, A., *et al.*, 1993.** Sequence analysis of selected regions of the env(V3 loop and gp41) and gag (p7) reading frames of Ethiopian strains. *Arch. Virol.*, **128**:229-39.

118. **Sherefa, K., Sonnerborg, A., Steinbergs and Sallberg, M. 1994.** Rapid grouping of HIV-1 infection in subtypes A to E by V3 peptide serotyping and its relation to sequence analysis. *Biochem. Biophys. Res. Communications*, **205**:1658-64.

119. **Lasky, M., Perret, J. L., Peeters, M., *et al.*, 1997.** Presence of multiple non-B subtypes and divergent subtype B strains of HIV-1 in individuals infected after overseas deployment. *AIDS*, **11**:43-51.

120. **Jameel, S., Zafrullah, M., Ahmed, M., Kapoor S.G. and Sehgal, S. 1995.** A genetic analysis of HIV-1 from Punjab, India reveals the presence of multiple variants. *AIDS*, 9:685-690.

121. **Chi-Chang Luo, Tian, C., Hu, D.J., *et al.*, 1995.** HIV-1 subtype C in China. *Lancet*, **345**:1051-1052.

122. **Claudio Csillag 1994.** HIV-1 subtype C in Brazil. Lancet, **344**:1354.

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

124. **Bratt, G., Leandersson, A. C., Albert, J., *et al.*, 1998.** MT-2 tropism and CCR5 genotype strongly influence disease progression in HIV-1 infected individuals. *AIDS*, **12**:729-36.

125. **Bleul, C. C., Farzan, M., Choe, H., *et al.*, 1996.** The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. *Nature*, **382**:829-32.

126. **Liesnard, C., Delforge, M. L., Tchetcheroff, M., *et al.*, 1997.**Importance of method in the determination of Si phenotype of HIV-1 clinical isolates. *J. Virol. Methods,* **64**:137-145.

127. **Todd, B. J., Kedar, P. and Pope, J. H. 1995.** Syncytium induction in primary $CD4^+$ T-cell lines from normal donors by HIV-1 isolates with non-syncytium-inducing genotype and phenotype in MT-2 cells. *J. Virol.*, **69**: 7099-105.

128. **Perez Alvarez, L., Verdejo, J., Gonzalez Lahoz, J., *et al.*, 1998.** Expression of different HIV-1 phenotypic subpopulations through the natural history of the infection. *Med.Clin.*, **110** :441-5.

129. **Collman, R., Balliet, J. W., Gregory, *et al.*, 1992.** An infectious molecular clone of an unusual macrophage-tropic and highly cytopathic strain of HIV-1. *J. Virol.*, **66**: 7517-21.

130. Easterbrook, P. J., Farzadegan, H., Hoover, D. R., *et al.*, 1996. Racial differences in rate of CD4 decline in HIV-1-infected homosexual men. *AIDS,* **10**: 1147-55.

131. **Fiscus, S. A., Wallmark, E. B., Folds, J. D., *et al.*, 1991.** Detection of infectious immune complexes in HIV-1 infections: correlation with plasma viremia and CD4 cell counts. *JID.*, **164**: 765-69.

132. **Yu, X. F., Wang, Z., Beyrer, C., *et al.*, 1995.** Phenotypic and genotypic characteristics of HIV-1 from patients with AIDS in northern Thailand. *J. Virol.*, **69**: 4649-55.

133. **Leal, M., Torres, Y., Medrano, F. J., *et al.*, 1996.** HIV-1 viraemia changes in patients with and without SI phenotype treated with nucleoside analogues: a case-control study. *Europ. J. Clinic. Investig.*, **26**: 923-8.

134. **Prazuck, T., Troisvallets, D., Vallantin, X., et al., 1996.** Lack of predictive value for progression of dissociated circulating p24 antigen in HIV-1 infected black patients. *J. Med. Virol.*, **50**:181-7.

ENARP

SimulSET Lab Report

Director: TOBIAS RINKE DE WIT
Operator: DEREJE

Cytometer: FACScan
Software: SimulSET v 3.1

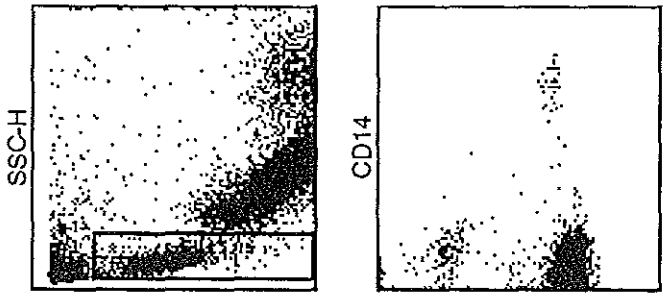
Patient ID: F
Patient Name: PHD 076

Date Acquired: 12-Feb-97 13:10:14
Date Analyzed: Fri, 16 Oct 1998 10:48 am
Panel Name: IMK-Lymphocyte

F04.01 **LeucoGATE**

Events Acquired: 15000 **Data Set:**[1]
Gated Events: 5454

	FSC	SSC
Mean	119	24
Gate	53	52
	255	52
	255	9
	53	9



Preparation:
Lysed Whole Blood

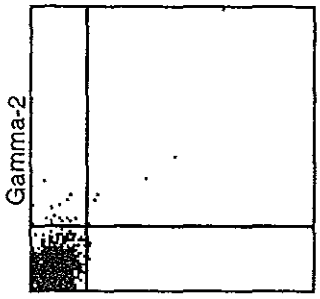
	Lymphs	Monos	Grans	Debris	
Gate Composition (%):	95	1	1	3	100% of all lymphocytes are in the gate
Calculated 3 Part Diff.	41	7	52	-	

Percent Lymph Conversion On

F04.02

Events acquired:6000 **Data set:**[1]
Gated Events:2200

	FSC	SSC
Means:	119	24
	FL1	FL2
Marker	48	56

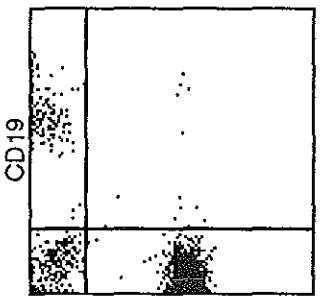


	Control IgG1/IgG2	Conv
Q	Cell Type	%L
Q1	NSS PE	1
Q2	NSS ++	0
Q3	Unstained	99
Q4	NSS FITC	0

F04.03

Events acquired:6000 **Data set:**[1]
Gated Events:2254

	FSC	SSC
Means:	119	24
	FL1	FL2
Marker	48	56

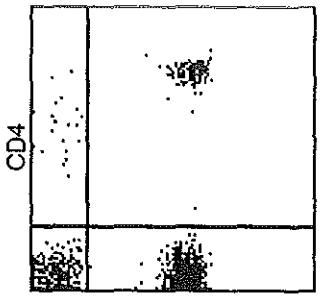


	CD3/CD19	Conv	
Q	Cell Type	%L	
Q1	CD3- CD19+	7	
Q2	CD3+ CD19+	1	
Q3	CD3- CD19-	5	
Q4	CD3+ CD19-	87	
	Subset Name	Conv %L	
	Total T (CD3+) Lymphocytes	87	>>
	Total B (CD19+) Lymphocytes	7	OK

Patient ID: F (Continued)
Patient Name: PHD 076

F04.04
Events acquired:6000 Data set:[1]
Gated Events:2261

	FSC	SSC
Means:	118	24
	FL1	FL2
Marker	48	56



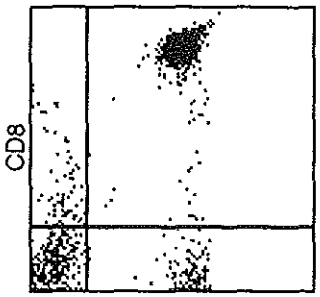
CD3/CD4

Q	Cell Type	Conv %L
Q1	CD3- CD4+	1
Q2	CD3+ CD4+	8
Q3	CD3- CD4-	12
Q4	CD3+ CD4-	79

Subset Name	Conv %L	
Total T (CD3+) Lymphocytes	87	>>
T Helper (CD3+, CD4+) Lymphocytes	8	<<

F04.05
Events acquired:6000 Data set:[1]
Gated Events:2187

	FSC	SSC
Means:	120	24
	FL1	FL2
Marker	48	56



CD3/CD8

Q	Cell Type	Conv %L
Q1	CD3- CD8+	4
Q2	CD3+ CD8+	79
Q3	CD3- CD8-	9
Q4	CD3+ CD8-	9

Subset Name	Conv %L	
Total T (CD3+) Lymphocytes	88	>>
T Suppressor (CD3+,CD8+) Lymphs	79	>>

F04.06
Events acquired:6000 Data set:[1]
Gated Events:2203

	FSC	SSC
Means:	120	23
	FL1	FL2
Marker	48	56

CD3/CD16+56

Q	Cell Type	Conv %L
Q1	CD3- CD16+/CD56+	6
Q2	CD3+ CD16+/CD56+	4
Q3	CD3- CD16-/CD56-	4
Q4	CD3+ CD16-/CD56-	86

Subset Name	Conv %L	
Total T (CD3+) Lymphocytes	90	>>
Total NK (CD16+/CD56+)Lymphocytes	6	OK

Tube Name/ Consistency Ck	Subset Name/ Ck Name	Conv. Percent Lymphs	
Average CD3	Total T (CD3+) Lymphocytes	88	>>
Sum of Cells	T + B + NK	101	OK
Ratio	T Lymph H/S CD3,CD4/CD3,CD8 Ratio	0.10	<<

Appendix 2. Viral Load Determination

HIV-1 RNA copy numbers in 100 µl plasma samples were quantitated by using a nucleic acid sequence based co-amplification assay (NASBA) using NASBA kits (M. G. and S. J., Organon Teknika, N. V., Belgium), as described by Kievit et al. (115). In brief, RNA was extracted from100 µl of plasma stored at -80oC using guanidinium isothiocyanate (GuSCN) as described by Boom et al (116). That is, plasma samples were lyzed in 5.25 M GuSCN, 50 mM Tris/HCl, pH 6.4, 20 mM EDTA and 13% w/v Triton X-100. Nucleic acid was bound by 50 µl activated silica (1 mg/ml pore size). Silica particles were washed twice with5.25 M GuSCN, 50 mM Tris/HCl, pH 6.4, twice with 70% ethanol and once with acetone. Nucleic acid was eluted in 50 µl distilled water and aliquoted in 5 µl portions. Ten-fold serial dilutions ranging from 10 2 to10 6 molecules of Q-RNA were made and mixed with the 5 µl aliquoted nucleic acid isolated from plasma samples. Ten µl of diluted primers was added to the reaction mixture and then incubated at 65oC for 5 minutes to allow primer annealing and subsequently cooled down to 41oC for 5 minutes. Amplification was started by adding 5 µl enzyme mixture. Reactions were incubated for 90 minutes at 41oC in a total volume of 25 µl. Amplification was performed isothermally in the presence of RNA standards. Detection of amplified RNA was performed on a NASBA reader (Micro SLT 510, Organon Teknika, Belgium).