Studies on multiply exposed but persistently HIV-1 seronegative sex workers from KwaZulu/Natal, South Africa.

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CONTENTS

ABSTRACT

The overall aim of this study was to determine whether host genetic factors are associated with resistance to HIV-1 infection in a group of highly exposed persistently seronegative sex workers from KwaZulu/Natal, South Africa.

A cohort of 17 African highly exposed but persistently seronegative (HEPS) commercial sex workers (CSW) were identified who had been in sex work for more than four years (range between 4-26 years). The women had been followed monthly for at least four years as part of HIV-1 prevention programmes (Ramjee, *et al.*, 1998). The overall aim of this study was to identify the frequency of polymorphisms and mutations in chemokine genes, chemokine receptors and chemokine receptor promoter region which may be associated with HIV-1 resistance and prolonged disease progression. Secondly, to determine if the chemokine receptors on CD4 T-cells are sufficiently expressed and functional to enable infection. This information will shed light on correlates of immunity as influenced by these polymorphisms and this knowledge will help in the bigger objective of determining factors influencing disease progression as well as the development of an effective HIV-1 vaccine in South Africa.

The DNA of these women was isolated and the chemokine (SDF1-3'A) and chemokine receptor (CCR5Δ32, CCR5m303 and CCR2b-64I) polymorphisms detected by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). To investigate the effect of polymorphisms in the non-coding region of the CCR5 gene, five CCR5 promoter polymorphisms were screened for in the study namely, CCR5 59029G/A, 59353T/C, 59356C/T, 59402G/A and 59653C/T. Fluorescent hybridization probe method was developed to identify these mutations. Peripheral blood mononuclear cells (PBMC) were isolated from these women and the proportion of CD4 T-cells positive for CCR5 and CXCR4 and relative level of expression was determined by flow cytometry.

Lastly, to ascertain whether the chemokine receptors expressed on PBMC surface were functional, *in vitro* infection assays were performed. PBMC from seven HEPS women were exposed to six HIV-1 viral strains of different subtypes and coreceptor usage, included were three R5, one X4 and two dual tropic (R5/X4) viral isolates.

No mutations were detected in the CCR5Δ32 and CCR5m303 variants among the 17 HEPS samples tested. Two of the 17 samples were heterozygous for the SDF1-3'A, resulting in an allelic frequency of 5.8%. This figure was higher than the background population figure of 1% among South Africans (Williamson, *et al.*, 2000). The allelic frequency for the CCR2b-64I variant was found to be 15% among HEPS which was comparable to the 13% found in the background population (Williamson, *et al.*, 2000). Two CCR5 promoter polymorphisms (CCR5-59353T/C and CCR5-59029G/A) associated with disease progression, were found at an elevated frequency among the HEPS women, and were always linked.

There was no difference in the proportions of CD4 T-cells positive for CCR5 and CXCR4 receptors between the HEPS women a control group (n = 5) of seronegative low risk women. The mean for CD4 T-cells positive for CCR5 was 10.7% and 1.7% for HEPS and controls respectively, and for CXCR4 the mean was 89.3% and 83.8% in HEPS and controls. The difference in CCR5 and CXCR4 expressed on the surface of cells between HEPS and controls was not statistically significant (p=0.117 and p=0.964 respectively) indicating normal expression levels among the HEPS population.

All viral strains irrespective of subtype or coreceptor usage replicated in the PBMC of all HEPS samples tested (n=7), indicating that the chemokine receptors were sufficiently expressed on PBMC and that they were functional.

In conclusion, none of the well characterised mutations in the CCR5 gene associated with resistance were identified in this study population.

In addition, CCR5 and CXCR4 expression on the surface of cells was demonstrated to be sufficient and functional as cells from all HEPS samples tested were found to be susceptible to infection with all viral strains tested. An elevated frequency of the SDF1-3'A variant and certain CCR5 promoter polymorphisms was observed. However, the mechanism of how this variant may confer resistance is puzzling as sexually transmitted HIV-1 strains utilize the CCR5 receptor and not CXCR4.

Further work needs to be done to ascertain the background frequencies of the SDF-1 and promoter polymorphisms in South Africa, and their role in HIV-1 pathogenesis. The fluorescent hybridization probe method developed to screen for CCR5 promoter polymorphisms was found to be reliable, when compared to other conventional methods used for point mutation detection such as PCR-RFLP and sequencing. This method was also found to be easier and faster.

This is the first study on HEPS in South Africa, it is hoped that correlates of immunity as influenced by the genetic variants studied here will shed light with regard to the goal of designing and monitoring vaccines for South Africa and Africa.

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ABBREVIATIONS

A	adenine
AIDS	acquired immunodeficiency syndrome
bp	base pair
BSA	bovine serum albumin
C	cytosine
CAT	chloramphenicol acetlytransferase
CTL	cytotoxic T lymphocytes
CSW	commercial sex workers
DNA	deoxyribosenucleic acid
dNTP	deoxyribonucleic acid
°C	degrees centigrade
ELISA	enzyme-linked immunosorbent assay
FCS	foetal calf serum
FITC	fluorescein isothyocyanate
g	gravitational acceleration
G	guanine
h	hours
HEPS	highly exposed but persistently seronegative
HIV-1	human immunodeficiency virus
HLA	human leukocyte antigen
INF	interferon
IL	interleukin
LTR	long terminal repeat
Mab	mononuclear antibody
mg	milligrams
MHC	major histocompatibility complex
MRC	medical research council
µl	micro-litre
min	minutes
MIP-1	macrophage inhibitory protein
MRC	medical research council
ml	millilitre
mM	millimolar
Mol	mole
ND	not done
ng	nanogram
NIV	National Institute for Virology
NSI	non-syncitium inducing
OD	optical density
ORF	open reading frame
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHA	phytohemagglutinin
RANTES	regulated upon activation normal T-cell expressed and secreted
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
SDF-1	stromal derived factor
SI	syncitium inducing
SIV	simian immunodeficiency virus
STIs	sexually transmitted infections

T	thymidine
TAE	Tris-acetate
TBS	tris-buffered saline
Tm	melting temperature
U	unit
UCT	University of Cape Town
UTR	untranslated terminal repeat
UNAIDS	Joint United Nations Programme on HIV/AIDS
USA	United States of America
UV	ultraviolet
v/v	volume per volume

CHAPTER 1

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INTRODUCTION

1.1

Literature review

1.1.1 General introduction

One of the major obstacles in the development of an HIV-1 vaccine is a lack of understanding regarding the mechanisms of natural protection against HIV-1 infection. One approach to identify factors related to protection, has involved the intensive study of cohorts of high-risk individuals who remain uninfected despite repeated exposure to HIV-1, referred to as "nature's experiment" (Shearer, *et al.*, 1996). Cohorts studied include commercial sex workers (Rowland-Jones, *et al.*, 1995), sex partners of HIV-1 infected individuals (Mazzoli, *et al.*, 1997) and, infants born to HIV-1 infected mothers (Clerici, *et al.*, 1993). Of these different groups, commercial sex workers (CSW) represent a particularly compelling population in which to study mechanisms of resistance to HIV-1 infection. Unlike new borns of infected mothers, or HIV-1 discordant couples, who sustain multiple exposures to the same viral variant, commercial sex workers are likely to experience a variety of high-risk exposures to a multitude of HIV-1 variants over a prolonged period of time (Beyer, *et al.*, 1999). Studying these HIV-1 resistant cohorts will shed light on which host genetic factors and immunological parameters are implicated in preventing HIV-1 infection.

It is likely that highly exposed but persistently seronegative (HEPS) individuals are protected from HIV-1 infection by a number of factors. Host genetic factors like polymorphisms in the chemokine receptor genes or their ligands play a role in resistance. An association between human leukocyte antigen (HLA) and resistance to infection and to disease has also been described. Immunological factors like cytotoxic T-lymphocyte (CTL) responses, antibody responses are also thought to confer resistance to HIV-1 infection or delay disease progression.

In most cases, resistance to HIV-1 infection is probably a consequence of a combination of these and more, as yet undiscovered factors. There is currently no data on factors conferring resistance to infection in South Africa populations. This review will focus on host genetic mutations, which contribute to resistance to infection or disease. Immunological correlates of immunity will be covered briefly.

1.1.2 Chemokine receptors as HIV-1 coreceptors

The entry of HIV-1 into human cells has been demonstrated to depend on CD4 receptor molecules on the cell surface (Dalgleish, *et al.*, 1984). However, the CD4 molecule alone is not sufficient to render cells susceptible to HIV-1 infection (Clapham, *et al.*, 1991 and Dragic, *et al.*, 1996) and a coreceptor is required by the virus. Two major HIV-1 coreceptors have been identified, CCR5 and CXCR4, both of which are chemokine receptors. These receptors are members of the seven transmembrane G-protein coupled family (Raport, *et al.*, 1996) and are important components of the immune response to inflammation and infection.

Transformed T-cell lines, such as MT-2 cells, have proven very useful in phenotyping viruses as these cells express high levels of CXCR4, but do not express CCR5. Viruses that fail to form syncytia when grown in MT-2 cells have been referred to as non-syncytium inducing (NSI) and because of their ability to infect macrophages have also been referred to as macrophage tropic (M-tropic) or slow-low in reference to their replication kinetics in culture (Feng, *et al.*, 1996). CCR5 is a coreceptor for non-syncytium inducing (NSI) HIV-1 strains *in vitro*, also classified as R5 viral strains (Berger, *et al.*, 1998). Whereas, viruses able to infect T-cell lines have been referred to as T-tropic, syncytium-inducing (SI), or rapid-high (RH) (Fenyo, *et al.*, 1996). The CXCR4 chemokine receptor is used by syncytium inducing (SI) HIV-1 viral strains *in vitro* (Deng, *et al.*, 1996), these viral strains are also classified as X4 (Berger, *et al.*, 1998). HIV-1 infected patients harbour predominantly R5 HIV-1 isolates during early stages of infection, but accumulate increasing amounts of X4 viral strains just before accelerated T-cell depletion and progression to AIDS (Smith, *et al.*, 1997). This tropic transition indicates that viral adaptation from CCR5 to CXCR4 receptor use may be a key in progression to AIDS (Deng, *et al.*, 1996). However this switch does not occur in all patients.

HIV-1 subtype-C HIV-1 viral isolates, the predominant subtype in South Africa, for reasons that are still unclear predominantly use CCR5 coreceptor with rare reported usage of CXCR4 (Cecilia, *et al.*, 2000).

Chemokines (soluble chemical messengers that attract white blood cells to inflamed areas) and their receptors are critical components of HIV-1 infection. Chemokines are 70-90 amino acids in length and can be subdivided into two groups, the α-chemokines, which contain a single amino acid between the first and second cysteine residues, whose function is to activate neutrophils (D'Souza, *et al.*, 1996). Stromal derived factor (SDF-1) is an example of an α-chemokine and is also a ligand to the CXCR4 α-chemokine receptor.

The β-chemokines have adjacent cysteine residues, and they generally activate monocytes, lymphocytes, basophils and eosinophils. RANTES (regulated upon activation normal T-cell expressed and secreted), macrophage inflammatory protein (MIP-1α and MIP-1β) are β-chemokines and act as ligands to the β-chemokine receptor CCR5 (D'Souza, *et al.*, 1996). Various cytokines produced by CD8 T-cells can suppress HIV-1 replication (Barker, *et al.*, 1998). The β-chemokines RANTES, MIP-1α and MIP-1β can block R5 tropic viruses from replicating in peripheral blood mononuclear cells (PBMC) and CD4 cells (Cocchi, *et al.*, 1995).

In addition to CCR5 and CXCR4, nine other chemokine receptors have been shown to support the cellular entry of one or more virus strains *in vitro*. These include, CCR2b, CCR3, CCR8, GPR1, GPR15 (Bob), STRL33 (Bonzo/TYMSTR), US38, V28 (CX3CR1) and ChemR23 (Choe, *et al.*, 1996, Doranz, *et al.*, 1996, Samsom, *et al.*, 1997 and Deng, *et al.*, 1997). CCR5 is also used by HIV-2 and SIV isolates although CXCR4 is only used by HIV-1. BOB and Bonzo are used by SIV and HIV-2 isolates but only rarely by HIV-1 isolates. The *in vitro* significance of these minor coreceptors in HIV-1 infection still remains uncertain (Zhang and Moore, 1999). Table 1 depicts chemokine receptors and their natural ligands, their expression patterns and viral usage.

Table 1 : Chemokine receptor family members' known function as receptors in HIV and SIV entry

Receptor	Ligand	Expression Pattern	Viral usage
Major Receptors			
CCR5	MIP-1α, MIP-1β, RANTES	Monocytes, T-cells	HIV-1, HIV-2, SIV
CXCR4	SDF-1	Lymphocytes, Macrophages	HIV-1
CCR3	MCP-3, MCP-4, RANTES	Eosinophils, Microglia	HIV-1
BOB/GPR15	?	T-cells, Colon	SIV, HIV-2
Bonzo/STRL33	?	T-cells, Monocytes	SIV, HIV-2
Minor receptors			
CCR2	MCP-1	Monocytes, T-cells	HIV-1, HIV-2
CCR8	I-309	Monocytes, Thymocytes	HIV-1, SIV
GPR1	?	Macrophages	SIV

Littman, 1998.

1.1.3 Ligands and chemokine receptor polymorphisms associated with HIV-1 resistance to infection

The recent discovery of several mutations and polymorphisms in the genes of the HIV-1 chemokine coreceptors (D'Souza, *et al.*, 1996) has led to a greater understanding of the host genetic factors that play an important role in susceptibility to HIV-1 infection and in the rate of disease progression (Dean, *et al.*, 1996). The most studied among the HIV/AIDS resistance mutations is the 32bp nucleotide deletion in the CCR5 gene. The mutation results in truncation of the CCR5 protein and abrogation of its HIV-1 coreceptor function (Liu, *et al.*, 1996 and Samsom, *et al.*, 1996), including its chemokine signallng function. CD4 T-cells from these individuals were highly resistant *in vitro* to the entry of primary M-tropic (R5) HIV-1 isolates but readily infectable with viruses adapted to grow in T-cell lines (Paxton, *et al.*, 1996). The homozygosity for CCR5Δ32 is highly protective against HIV-1 infection, though the protection is not absolute (O'Brien, *et al.*, 1997). Individuals who are heterozygous for the CCR5Δ32 allele have been shown to progress more slowly to AIDS than wild-type homozygous individuals (Husman, *et al.*, 1999), suggesting that CCR5 expression levels may be altered in these individuals, and that this affects HIV-1 replication *in vivo*.

Another CCR5 gene variant was identified which results from a single base substitution. A (T-to-A) substitution at position 303 in the open reading frame (ORF) of the CCR5 gene, introduces a premature stop codon (Quillent, *et al.*, 1998).

This rare variant, CCR5m303 leads to the lack of expression of a functional CCR5 protein and abrogation of its HIV-1 coreceptor function. This variant, was identified in less than 1% Caucasians (Quillent, *et al.*, 1998) and has never been identified in Africans (Williamson, *et al.*, 2000). Individuals with CCR5m303/Δ32 genotype are resistant to infection and it is assumed that CCR5m303 has similar effects to CCR5Δ32 on delaying disease progression.

The CCR5 gene is organised into four exons and two introns. Exons 2 and 3 are not interrupted by an intron. Exon 4 and portions of f exon 3 are shared by all isoforms. Exon 4 contains the open reading frame (Fig 1.1).

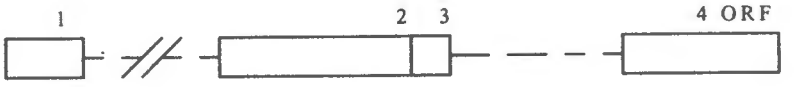

Fig. 1.1 : A schematic representation of the genomic organisation of the CCR5 (Mummidi *et al.*, 1998). Open boxes represents exons or open reading frames (ORF), dashed lines represents connecting introns. Exons are numbered at the upper left of corresponding box.

Stromal derived factor (SDF-1) is the principal ligand for CXCR4, a coreceptor with CD4 for T-tropic (X4) cell line HIV-1 isolates. The SDF1-3'A variant is a result of base substitution in the 3'-untranslated region (UTR) of SDF-1 gene and this G-to-A substitution has been shown to be associated with delayed disease progression in HIV-1 infection (Winkler, *et al.*, 1998). The protective effect of the SDF1-3'A variant is recessive as it is observed only in homozygotes (SDF1-3'A/3'A) allele carriers (Winkler, *et al,*. 1998), no difference is apparent in this respect between wild type (SDF-1) and heterozygotous (SDF1/3'A) individuals (Voevodin, *et al.*, 1999). However, other studies have shown contradictory effects of the SDF1-3'A variant. The SDF1-3'A variant has been shown to accelerate disease progression among seroconverters (Brambilla, *et al.*, 2000). The SDF1-3'A variant is wide spread in all populations, the SDF1-3'A allelic frequency in Caucasians is 21.1% compared to 5.7% in African Americans (Winkler, *et al.*, 1998).

A G-to-A nucleotide substitution has been identified at position 190 of CCR2 that substitutes the CCR2 wild type amino acid residue valine at position 64 with isoleucine. This change is located within the first transmembrane domain of the CCR2 receptor and results in a CCR2b-64I variant (Smith, *et al.*, 1997). This single base substitution was associated with postponed development of AIDS by 2-4 years, but the gene variant was not associated with primary protection against HIV-1 infection (Smith, *et al.*, 1997).

The CCR2b-64I variant is located in a transmembrane domain of the CCR2 protein, which is not a part of the HIV-1 binding site. The protection mechanism is thought to be indirect, since most HIV-1 isolates do not use CCR2 as their coreceptor. In this respect, an interesting hypothesis has been suggested that CCR2b-64I is tracking another polymorphism in the promoter region (Samson, *et al.*, 1996).

Kostrikis *et al.*, (1998) identified a C-to-T base substitution at position 59653, (GenBank Accession Number U95626), in the CCR5 promoter region that was in linkage disequilibrium with CCR2b-64I. The human CCR5 and CCR2 chemokine receptors, are tightly linked on chromsome 3p21-22 (Dean *et al.*, 1996). The CCR2b-64I allelic frequency of 15.1% in African Americans is higher than 9.8% observed in Caucasians (Kostrikis, *et al.*, 1998).

1.1.4 CCR5 promoter polymorphisms and their role in HIV-1 pathogenesis

Recognition of the heterogeneity existing in the regulatory region of the CCR5 gene (Mummidi, *et al.*, 1998) has lead to speculation about the influence of regulatory region polymorphisms on CCR5 receptor expression and consequently on the rate of HIV-1 disease progression (Smith, *et al.*, 1997). Polymorphisms in the regulatory region of the CCR5 gene has been shown to influence HIV-1 vertical transmission, Kostrikis, *et al.*, (1999) identified a base substitution in the promoter region of the CCR5 gene (CCR5-59356-T) that was prevalent in the African American population 20.6% compared to 3.4% in Caucasians and Hispanics (5.6%).

Six hundred and sixty seven (667) infants born to HIV-1 infected mothers not treated with AZT (zidovudine) during pregnancy were studied, three populations were investigated Hispanics (n=65) Caucasians (n=492) and African Americans (n=691). In all population groups the CCR5-59356T variant was found to be associated with increased HIV-1 transmission from mother to child. This is the only study showing an effect on transmission due to a promoter polymorphism.

A CCR5 promoter polymorphism, a G-to-A substitution at position 59029 (GenBank Accession Number U95626) has been associated with altered rates of HIV-1 disease progression (Biti, *et al.*, 1997). The CCR5-59029G allele, which is associated with slower disease progression in HIV-1 positive homozygotes compared with CCR5-59029A homozygotes (McDermott, *et al.*, 1998), is more common than all other variants found to date. Unlike CCR5Δ32 which is found mainly in Caucasians, CCR5-59029G is present in high frequencies in all racial groups (McDermott, *et al.*, 1998). Thus, it is likely that this polymorphism affects HIV-1 pathogenesis worldwide. HIV-1 seroconverters who were homozygous for CCR5-59029A and lacking CCR5Δ32 and CCR2b-64I mutations were found to progress to AIDS on average 3.8 years more rapidly than those who were homozygous for CCR5-59029G allele (McDermott, *et al.*, 1998).

A C-to-T polymorphism at position 59353 was also identified, with the CCR5-59353T allele associated with delayed disease progression. The CCR5-59353C variant occurring with the CCR5-59029A variant in six out of six patient samples sequenced by McDermott, *et al.*, (1998). CCR5-59353C as well as CCR5-59029A were under represented in HIV-1 infected long term non-progressors (Clegg, *et al.*, 2000). This indicates that there is a delay in disease progression in HIV-1 infected individuals carrying CCR5-59353T alleles.

A differential effect on promoter activity between CCR5-59029A/CCR5-59353C and CCR5-59029G/CCR5-59353T has been demonstrated *in vitro* acetlytransferase (CAT) reporter gene constructs containing the CCR5-59029A/CCR5-59353C polymorphisms resulted in 45% more activation than constructs containing the CCR5-59029G/CCR5-59353T polymorphisms (Clegg, *et al.*, 1999).

Increased promoter activity such as that associated with CCR5-59029A/CCR5-59353C could result in increased levels of CCR5 mRNA production and cell surface expression of the CCR5 receptor (Clegg, *et al.*, 1999), and thus affect disease progression. CCR5-59402G/A variant has been identified though its effect of HIV-1 and disease progression it not known (Mummidi, *et al.*, 1998).

Table 2 depicts a summary of all chemokine, chemokine receptor mutations and polymorphisms in the non-coding region of CCR5 and their effects of HIV-1 pathogenesis.

Table 2: Host genetic variants and their role in HIV-1 pathogenesis

Gene variant	Effect on HIV-1 pathogenesis	Reference
Chemokine		
SDF1-3'A	Delays disease progression or Accelerates disease progression	Winkler, *et al.*, 1998 Brambila, *et al.*, 2000
RANTES	Delays disease progression	McDermott, *et al.*, 2000
Chemokine receptors		
CCR5Δ32	Protects against HIV-1 infection (homozygote) and delays disease progression (heterozygote)	Liu, *et al.*, 1996
CCR5m303	Delays disease progression and protects against HIV-1 transmission when linked with CCR5Δ32	Quillent, *et al.*, 1998
CCR2b-64I	Slows down disease progression	Smith, *et al.*, 1997
Promoter polymorphisms		
CCR5-59029A/A	Accelerates disease progression	Mummidi, *et al.*, 1998
CCR5-59353T/T	Delays disease progression	McDermott, *et al.*, 1998
CCR5-59356T/T	Protects against HIV-1vertical transmission	Kostrikis, *et al.*, 1999
CCR5-59402G/G	Function unknown	Mummidi, *et al.*, 1998
CCR5-59653T/T	Delays disease progression when linked with CCR2b-64I	Samson, *et al.*, 1997

1.1.5 Chemokine receptor expression levels and CD4 T-cell susceptibility

The two major HIV-1 coreceptors, CCR5 and CXCR4, have been shown to be differentially expressed in different sub-populations of CD4 T-cells. CCR5 is mainly expressed on memory and activated T-cells as marked by their high expression of CD45RO and low expression of CD45RA, in contrast, CXCR4 is expressed highly by naïve and resting cells as marked by their high expression on CD45 (Ostrowski, *et al.*, 1997). Because naïve cells are resting cells, it is therefore conceivable that most CD4 T-cells expressing CXCR4 are resting cells and are not permissive for the complete viral life cycle and active viral replication. The availability of target cells for viral replication, i.e. activated cells with appropriate viral receptors, may play a crucial role in viral dynamics and pathogenesis of AIDS.

Ostrowski, *et al.*, (1997) found that the heightened state of cellular activation in HIV-1 infected individuals is associated with CCR5 up-regulation and CXCR4 down-regulation on CD4 T-cells, and that this milieu may favour the propagation of macrophage-tropic (R5) viruses. Coreceptor expression levels were found to correlate with the levels of cellular activation *in vivo*. Lower expression levels of CCR5 and higher expression of CXCR4 on CD4 T-cells was found to correlate with early disease state, while, lower expression levels of CXCR4 and higher expression of CCR5 on CD4 T-cells correlated with advanced disease stage (Ostrowski, *et al.*, 1997).

Expression levels are highly variable on cells from individuals with the wild type CCR5 genotype (Moore, *et al.*, 1997), and there seems to be a correlation between the level of expression and *in vitro* infectability with R5 tropic HIV-1 strains (Reynes, *et al.*, 2000). Individuals carrying the CCR5Δ32 allele and HEPS we found to have CD4 T-cells with reduced infectability by R5 HIV-1 strains. This reduction in *in vitro* infection by CD4 T-cells was attributed to low CCR5 expression levels and may be associated with protection against sexual transmission of HIV-1 (Paxton, *et al.*, 1998).

For both genotypic groups (CCR5 and CCR5Δ32), the percentages of CCR5-expressing cells were higher in the HIV-1 infected group than in the uninfected group. CCR5 expression was shown to increase with progression of disease, possibly as a consequence of continuous immune activation associated with HIV-1 infection (Husman, *et al.*, 1999).

1.1.6 Human leukocyte antigens (HLA) associated with HIV-1 resistance.

Certain HLA phenotypes have been found to be prevalent in HEPS CSW. An increased frequency of the HLA-18 and HLA-A11 phenotype was found in HEPS in Thailand (Beyrer, *et al.*, 1999). Interestingly HLA-18 was also the class I allele associated with resistance to HIV-1 infection in the East African HEPS (Plummer, *et al.*, 1993). In addition, HLA-B18 has been reported to be associated with slow or non-progression to AIDS in HIV-1 infected Caucasians (Kaslow, *et al.* 1996).

In a study done in Gambia, the most prevalent HLA phenotype among the HEPS population was HLA-B35, which was found in higher frequencies in HEPS compared to HIV-1 infected individuals (Rowland-Jones, *et al.*, 1995). The HLA-A6082 and HLA-B18 phenotypes were found to have the strongest protective activity in HEPS individuals in Nairobi, Kenya (Rowland-Jones, *et al.*, 1998). The HLA system does play a significant role in HIV-1 resistance as indicated in these diverse studies done around the world. In a preliminary study done on the HEPS women from the sex worker cohort in KwaZulu/Natal showed a clustering of HLA-A24 compared to background frequencies in women from the same region of KwaZulu/Natal (Dr. A.J Puren, XIII International AIDS, 2000). Whether A24 is a true resistance marker remains to be determined.

1.1.7 Immunological responses associated with HIV-1 resistance

Globally, over 72% of HIV-1 infections occur in the developing world, with heterosexual transmission accounting for most infections in these regions (UNAIDS, December 2000). As most HIV-1 transmission is sexual, immune responses in the genital mucosa may be important in mediating protection against HIV-1 infection

Husman, *et al.*, (1999) found that HIV-1 infected individuals carrying the CCR5 allele had higher percentages of CCR5-expressing CD4 T-cells as compared with HIV-1 infected individuals carrying the (CCR5Δ32) heterozygote allele.

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Certain HLA phenotypes have been found to be prevalent in HEPS CSW. An increased frequency of the HLA-18 and HLA-A11 phenotype was found in HEPS in Thailand (Beyrer, *et al.*, 1999). Interestingly HLA-18 was also the class I allele associated with resistance to HIV-1 infection in the East African HEPS (Plummer, *et al.*, 1993). In addition, HLA-B18 has been reported to be associated with slow or non-progression to AIDS in HIV-1 infected Caucasians (Kaslow, *et al.* 1996).

In a study done in Gambia, the most prevalent HLA phenotype among the HEPS population was HLA-B35, which was found in higher frequencies in HEPS compared to HIV-1 infected individuals (Rowland-Jones, *et al.*, 1995). The HLA-A6082 and HLA-B18 phenotypes were found to have the strongest protective activity in HEPS individuals in Nairobi, Kenya (Rowland-Jones, *et al.*, 1998). The HLA system does play a significant role in HIV-1 resistance as indicated in these diverse studies done around the world. In a preliminary study done on the HEPS women from the sex worker cohort in KwaZulu/Natal showed a clustering of HLA-A24 compared to background frequencies in women from the same region of KwaZulu/Natal (Dr. A.J Puren, XIII International AIDS, 2000). Whether A24 is a true resistance marker remains to be determined.

(Kaul, *et al.*, 1999). Several HIV-1 specific immune responses have been associated with resistance to infection, including cytotoxic T-lymphocyte responses (CTL), T-helper responses and mucosal IgA responses. Major histocompatibility complex (MHC) alleles determine the molecular targets of the cellular immune response in a given host, and immunological resistance in these cohorts was therefore also linked to increased frequency of certain HLA allele (as described in 1.1.6).

1.1.7 a) Cellular immune responses

There have been two African cohorts which have been well characterised with respect to their immune responses to HIV-1 infection, one in Gambia and one in Kenya. In both cohorts systemic and local CTL responses have been demonstrated. In a study done in Gambia on HEPS commercial sex workers (CSW) with HLA-B35, three out of six HEPS women generated CTL responses to most of the specific HIV-1 peptides tested (nef, pol, p24 and p17) compared to none of the 12 seronegative individuals (Rowland-Jones, *et al.*, 1995). The strongest response was generated towards the HIV-1 pol peptide, a peptide located near to the active site of reverse transcriptase, and to a highly conserved peptide in nef which is conserved between HIV-1 and HIV-2. The HEPS in Kenya recognize many CTL epitopes presumably because, these women are exposed to multiple subtypes of HIV-1 (Rowland-Jones, *et al.*, 1998).

HIV-1 specific cytotoxic T-lymphocytes are present in the genital tract of most HIV-1 infected women (Musey, *et al.*, 1997). HEPS CSW in a study done in Kenya had 69% HIV-1 specific CD8 T-cell response in their cervix and no CTL responses were detected in HIV-1 uninfected controls (Kaul, *et al.*, 2000).

Detection of HIV-1 specific CTL in HEPS suggest that one of the mechanisms of resistance is through the induction of specific CTL response, by the class I-MHC (Goh, *et al.*, 1999). Of interest is that Kenyan sex workers who left sex work for a period of time, became infected once they resumed sex work (Dr. Sarah Roland-Jones, XIII International AIDS, 2000). This raises questions on why these women were no longer resistant, and implies non-host related factors conferring resistance.

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In addition to CTL responses, T-helper responses have also been demonstrated in exposed uninfected individuals. Systemic T-helper lymphocyte responses to HIV-1 envelope peptides were present in 11 out of 20 (55%) exposed uninfected sex workers, 4 out of 18 (22%) infected women, and one out of 25 (4%) lower risk women in a study done in Kenya (Kaul, *et al.*, 1999).

1.1.7 b) Antibody response

HIV-1 infection in humans leads to an antibody response directed against most of the viral structural antigens, and of particular interest are neutralising antibodies directed against HIV-1 envelope antigens, namely, the precursor gp-160 and its cleavage products, gp-120 and gp-41 (Brown, *et al.*, 1999). The major class of immunologlobulin is IgG which circulates in blood and is also present in mucosal exudates. The secretory form is IgA which is found in mucosa. HIV-specific IgA is made by immunocytes in the cervical mucosa and fallopian tubes and represents another possible mechanism of immune resistance to HIV-1 infection.

HEPS are consistently HIV-1/2 IgG seronegative. However a number of studies have shown that IgA antibodies against HIV proteins can be detected. Mazzoli, *et al.*, (1997) observed HIV-1 specific IgA responses, but not IgG, in serum, urine and vaginal washes of the 15 HIV-1 uninfected heterosexual partners of HIV-1 infected individuals. In a study done in Kenya, IgA was detected in higher levels in HEPS than in HIV-1 infected women and HIV-1 uninfected women (Kaul, *et al.*, 1999). The same results were obtained from a study on HEPS CSW in Thailand where anti gp 160 IgA was detected in six of 13 women tested (Beyrer, *et al.*, 1999).

However, no IgA response was detected to gp 120. This data indicates the presence of HIV-1 envelope specific mucosal IgA responses in HEPS, consistent with the finding in a discordant couple cohort (Mazzoli, *et al.*, 1997).

The role of IgA in HIV-1 resistance is demonstrated by the prevalence of these antibodies in HEPS cohorts. There were higher levels of IgA (76%) detected in HEPS than in HIV-1 infected women (26%) and HIV-1 uninfected women (11%) tested in Kenya, which indicates the protective role played by the IgA antibodies in the genital tract of HEPS individuals (Kaul, *et al.*, 1999). HIV-1 specific IgA with neutralizing activity were detected in higher frequency (73%) in plasma and vaginal fluid (79%) of HEPS (Devito, *et al.*, 2000). The prevalence of IgG detected in sera was in direct opposite to the prevalence of IgA detected in vaginal washings. None of the 21 HEPS tested had IgG and all of the 19 HIV-1 infected women had IgG antibodies in their sera (Kaul, *et al.*, 1999).

HIV-1 specific IgA but not IgG was detected in vaginal wash samples from HIV-1 exposed seronegative individuals, whereas both IgA and IgG were observed in HIV-1 seropositive individuals (Rowland-Jones, *et al.*, 1998). The prevalence of HIV-1 specific IgA was found to be higher (82%) in HEPS than in HIV-1 infected individuals (1%), whereas in HIV-1 infected individuals the levels of IgG were higher than the levels of IgA (Rowland-Jones, *et al.*, 1998). This combined data suggest that HIV-1 specific IgA plays a crucial role in HIV-1 resistance.

1.1.8 Project motivation

This is the first study to investigate host factors which may confer immunity to infection with HIV-1 in a South African population of highly exposed but persistently seronegative (HEPS) commercial sex workers. The overall aim of this study is to identify the frequency of polymorphisms and mutations in chemokine genes, chemokine receptors and chemokine receptor promoter region which may be associated with HIV-1 resistance and prolonged disease progression. Secondly, to determine if the chemokine receptors on CD4 T-cells are sufficiently expressed and functional.

This information will shed light on correlates of immunity as influenced by these polymorphisms and this knowledge will help in the bigger objective of determining factors influencing disease progression as well as the development of an effective HIV-1 vaccine in South Africa.

This study was done on 17 HEPS commercial sex workers from a closely monitored cohort based in KwaZulu/Natal (G.Ramjee, MRC). This group of women have been participating in a prevention programme and were screened monthly for HIV-1 for four years. Commercial sex workers make a compelling group for such a study as they are multiply exposed to HIV-1 and are also exposed to more than one HIV-1 strain. However, the limitation in looking at this group is that results obtained can not be accurately extrapolated to the normal population for the very reasons given above. It is possible that multiple exposures to multiple partners prime the immune system differently compared to individuals with exposure to a restricted number of partners.

Resistance is almost certainly multifactorial, and associated with both host and immune factors. In this project only the host genetic factors associated with HIV-1 resistance were investigated.

Polymorphisms in the chemokine (SDF1-3'A) and chemokine receptors (CCR5Δ32, CCR5m303 and CCR2b-64I) are known to be associated with decreased disease progression. Only the loss of function of CCR5 due to Δ32 and m303 have been associated with resistance. However, in addition to investigating the frequency of mutations known to result in abrogation of CCR5, it is relevant to investigate frequency of polymorphisms which affect disease progression as the mechanism governing resistance to disease may also decrease the susceptibility to infection. In addition, it has been shown that individuals with low levels of chemokine receptors on their T-cells may be less susceptible to infection.

In order to address these questions we determined the proportion of CD4 T-cells positive for chemokine receptors (CCR5 and CXCR4) and the expression levels of these receptors on CD T-cells by flowcytometry.

We also tested peripheral blood mononuclear cells (PBMC) susceptibility to infection using different HIV-1 sub-types *in vitro*. Lastly, a number of studies have shown that polymorphisms in the non-coding region of the CCR5 gene affect levels of expression and therefore, possibly, susceptibility to infection. In this study, HEPS samples were screened for the presence of four (CCR5-59029G/A, CCR5-59353T/C, CCR5-59402G/A and CCR5-59653C/T) variants. To do this, a novel method was developed using fluorescent hybridization probes on the LightCycler (Roche), results obtained were compared to known methods of genotyping like PCR-RFLP and sequencing.

These polymorphisms have never been investigated in South African HEPS, hence their role in HIV-1 resistance if any has never been determined in this country.

This study is part of a multicentre initiative between the (MRC, S.A) in Durban and other research institutions within South Africa. Dr. G. Ramjee and Prof. SS Abdool Karim (MRC) established and run the sex work cohort. Dr. Adrian Puren is investigating the relationship between the Human Leukocyte Antigen (HLA) and resistance and Dr. Clive Gray is studying the immunology of the sex work cohort, both are from the National Institute for Virology (NIV, SA).

CHAPTER 2

DETECTION OF CHEMOKINE AND CHEMOKINE RECEPTOR POLYMORPHISMS IMPLICATED IN RESISTANCE TO HIV-1 INFECTION OR DISEASE.

2.1) INTRODUCTION

The role played by chemokines and their receptors in the pathogenesis of AIDS is a rapidly expanding field. One of the most interesting findings in this field has been the discovery of mutations in genes coding for chemokine receptors, and their ligands, which confer resistance to HIV-1 infection and AIDS (Voevodin, *et al.*, 1999). In addition, non-coding polymorphisms in the CCR5 promoter region have also been found to influence transmission and disease progression (Chapter 4).

Since 1995, chemokines and their receptors have been shown to be determinants in the infection process inasmuch as they represent competitive inhibitors and coreceptors for HIV-1 infection, respectively (DeSouza, *et al.*, 1996). The chemokine receptors CCR5, and CCR2b, and SDF-1, the ligand of CXCR4 influence HIV-1 disease progression (Samsom, *et al.*, 1996).

The most studied among HIV/AIDS resistance mutations is a 32bp deletion in the CCR5 gene, referred to as CCR5Δ32. This mutation results in truncation of the CCR5 protein and abrogation of its HIV-1 coreceptor function (Liu, *et al.*, 1996). The defective CCR5 gene (CCR5Δ32) affords near total protection against HIV-1 infection in individuals homozygous for the deletion (Biti, *et al.*, 1997), and delays the development of AIDS in HIV-1 infected individuals who are heterozygous for the deletion (Dean, *et al.*, 1996). The CCR5Δ32 mutation is found in the Caucasian population at an allelic frequency of 10-20% and is extremely rare in people of African or Asian ancestry (Williamson, *et al.*, 2000 and Martison, *et al.*, 2000).

A single base substitution (T-to-A) at position 303 of the CCR5 gene open reading frame (ORF), referred to as CCR5-m303 (Quillent, *et al.*, 1998) introduces a premature stop codon in a coding sequence of the first extracellular loop of CCR5 protein. This rare variant leads to the lack of expression of a functional CCR5 protein and is thus likely to delay development of AIDS, as has been shown for the CCR5Δ32 mutation (Dean, *et al.*, 1996).

This mutation was identified at a frequency of less than 1% in the South African Caucasian population and has never been identified in people of African ancestry (Williamson, *et al.*, 2000).

Stromal derived factor (SDF-1) is the principal ligand for CXCR4, a coreceptor for X4 HIV-1 strains. The SDF1-3'A variant is a result of a single base substitution (G-to-A) in the 3'untranslated region (UTR) of SDF-1 gene. SDF1-3'A variant affects the rate of progression to AIDS in individuals who are homozygous (SDF1-3'A/3'A) for this variant (Winkler, *et al.*, 1998). The proposed hypothesis for SDF1-3'A/3'A mechanism of action, is that it up-regulates the quantity of SDF-1 protein available to bind CXCR4 and thus stems the appearance of late stage X4 HIV-1 strains (Winkler, *et al.*, 1998). However recent studies have shown that there is a correlation between SDF1-3'A/3'A genotype and accelerated disease progression after onset of AIDS (Brambilla, *et al.*, 2000).

The SDF1-3'A variant is wide spread and has being identified in all the populations in the world. This variant exists at different allelic frequencies in the different parts of the world, influenced by ethnic and racial background of the population. In South Africa the allelic frequency of SDF1-3'A is 1.0% among the African population and 20% among the Caucasian population (Williamson, *et al.*, 2000), and in North America the allelic frequency is 39% among the Caucasian population and 15% among the African American population (Huang, *et al.*, 1996).

The CCR2b-64I variant has a G-to-A substitution at position 190 in the CCR2 gene that substitutes the CCR2 wild type amino acid residue valine at position 64 with isoleucine, a conservative change located within the first transmembrane domain of the CCR2 (Smith, *et al.*, 1997). The amino acid substitution is located in a transmembrane domain of the CCR2b receptor which is not a part of the HIV-1 binding site, suggesting that the effect of this polymorphism is probably by some indirect mechanism (Smith, *et al.*, 1997). The CCR2b-64I polymorphism does not result in protection from HIV-1 infection, but, has been reported to significantly prolong HIV-1 disease progression to AIDS in CCR2b-64I carriers (Smith, *et al.*, 1997).

The CCR2b-64I variant is found in higher allelic frequencies in people of African ancestry, with an allelic frequency of 13.3% in Africans compared to 7.2% of Caucasians in South Africa (Williamson, *et al.*, 2000). The allelic frequency in African Americans is 27.7% compared to 17% of their Caucasian counterparts (Mummidi, *et al.*, 1998).

Table : 2.1.1 : The frequencies of chemokine and chemokine receptor variants in different populations.

Population	Gene Variants and Allelic Frequency (%)			
	CCR5Δ32	*CCR2b-64I*	*SDF1-3'A*	*CCR5m303*
Europe				
[1]Paris	28.6 (n=145)	18.9 (n=145)	39.3 (n=110)	1.0 (n=209)[6]
[2]Greece	1.9 (n=135)	15.5 (n=135)	ND	ND
North America				
[3,4]Caucasian	22 (n=446)	17.7 (n=479)	38.8 (n=179)	ND
[3,4]African American	0 (n=137)	27.7 (n=288)	15.1 (n=58)	ND
South Africa				
[5]Caucasian	9.4 (n=144)	7.2 (n=146)	20.3 (n=145)	0.7 (n=145)
[5]African	0.1 (n=1247)	13.3 (n=180)	1.0 (n=198)	0.0 (n=687)
[2]***Kuwait***	ND	11.9 (n=123)	25.9 (n=108)	0.0 (n=200)

ND = Not Done
[1]Hendel, *et al.*, 1998.
[2]Martison, *et al.*, 2000.
[3]Huang, *et al.*, 1996.
[4]Mummidi, *et al.*, 1998.
[5]Williamson, *et al.*, 2000.
[6]Quillent, *et al.*, 1998.

Most studies investigating host genes associated with resistance to infection have focused on Caucasian cohorts. Studies analysing the chemokine receptors and their ligands have shown that only mutations in the CCR5 gene confer resistance to infection. However, mutations affecting levels of expression of the receptors or their ligands have been shown to play a role in the rate of disease progression. As the role of these genetic variants may influence susceptibility to infection and therefore the dynamics of HIV-1 transmission, it is useful to screen for these mutations in South African highly exposed but persistently seronegative (HEPS) population, where their role in HIV-1 transmission has never been investigated. In this study four of these genetic polymorphisms, namely CCR5Δ32, CCR5m303, CCR2b-64I and SDF1-3'A were screened for in a population of 17 HEPS sex workers to ascertain their frequency and role, if any, in HIV-1 resistance.

2.2) MATERIALS AND METHODS

2.2.1 Study Population

The study was done on samples from 17 African (black) HEPS commercial sex workers from KwaZulu/Natal who, despite multiple sexual exposures for over four years, have resisted HIV-1 infection. See Table 2.2.1 for the characteristics of the study population. Samples were obtained from G. Ramjee (MRC, S.A) from an established sex worker cohort (Ramjee, *et al.*, 1998).

This cohort includes women from five truck-stops in the KwaZulu/Natal midlands area of South Africa who were recruited to participate in a number of prevention programmes including literacy and condom usage programmes and a Phase III microbicide trial (Ramjee, *et al.*, 2000). Informed consent was obtained from all participants and ethical approval was acquired from all participating institutions (Universities of Natal, Witswatersrand and Cape Town).

Women participating in the microbicide trial were screened monthly for sexually transmitted infections (STIs) including HIV-1. STIs screened for include *Trichomonas vaginalis*, *Neisseria gonorrheae*, *Chlamydia trachomatis*, *Haemophilus ducreyi* and *Treponema pallidunam.* Antibody to HIV-1 was detected using HIV-1-s ELISA (Abbott, Chicago), and second confirmatory ELISA was performed using the Vironostika HIV-1 Uniform II micro-ELISA 4 system (Omnimed, UK). For details of STI results from this cohort please refer to Ramjee *et al.*, (1998).

Table 2.2.1: Characteristics of the study population*

Sample ID	#Age	Partners per/wk	Condom use (%)	Anal sex	CD4 count	CD4:CD8	**Years in sex work
Du4	39	10	50-75	Yes	939	2.139	4.10
Du6	24	16	25-50	No	921	1.393	6.5
Du7	24	15	<25	No	1523	1.765	13.5
Du20	38	10	25-50	No	739	1.711	26.1
Du53	27	21	25-50	Yes	1250	1.553	5.5
Du85	30	10	25-50	Yes	1429	1.100	5.1
Du96	25	27	50-75	Yes	960	2.172	6.5
Du117	31	16	25-50	Yes	1172	3.417	7.5
Du126	38	24	25-50	Yes	879	2.021	8.5
Du154	18	9	25-50	No	1191	0.855	4.8
Du175	38	20	25-50	No	875	1.519	7.10
Du188	36	40	25-50	Yes	621	2.416	5.11
Du205	27	10	25-50	No	739	1.334	8.0
Du250	25	10	25-50	No	780	1.889	8.5
Du270	38	11	25-50	Yes	773	2.438	9.1
Du272	30	12	25-50	No	1113	2.553	14.1
Du366	30	21	25-50	Yes	960	1.282	7.5

*Information provided by Dr. G. Ramjee, (MRC-Durban)
#Age at time of recruitment (Sep. 1996 - Oct.1997)
**Years in sex work at time of sample collection (Nov. 1999)

Table 2.2.2 depicts all the sexually transmitted infections (STIs) tested for in the sex work cohort at the time of recruitment.

Table 2.2.2 : Prevalence of sexually transmitted infections in the sex work cohort*

Sample ID	Condom use (%)	Incidence of STIs at screening					
		Trichomonas vaginalis	*Candida. albicans*	*.Neisseria. gonorrheae*	*Haemophilus ducreyi*	*.Chlamydia trachomatis*	*Treponema pallidunam*
Du4	50-75	Neg.	Neg.	Pos.	Neg.	Pos.	Neg.
Du6	25-50	Neg.	Pos.	Neg.	Neg.	Pos.	Neg.
Du7	<25	Neg.	Pos.	Pos.	Neg.	Neg.	Neg.
Du20	25-50	Neg.	Neg.	Neg.	Neg.	Neg.	Pos.
Du53	25-50	Neg.	Pos.	Neg.	Neg.	Neg.	Pos.
Du85	50-75	Pos.	Neg.	Neg.	Neg.	Neg.	Neg.
Du96	25-50	Neg.	Pos.	Neg.	Neg.	Neg.	Neg.
Du117	25-50	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.
Du126	25-50	Neg.	Neg.	Neg.	Neg.	Neg.	Pos.
Du154	25-50	Pos.	Pos.	Neg.	ND	Pos.	Pos.
Du175	25-50	Neg.	Neg.	Neg.	ND	Neg.	Neg.
Du188	25-50	Neg.	Neg.	Neg.	ND	Neg.	Neg.
Du205	25-50	Pos.	Pos.	Neg.	ND	Neg.	Pos.
Du250	25-50	Neg.	Neg.	Neg.	ND	Neg.	Neg..
Du270	25-50	Neg.	Neg.	Neg.	ND	Neg.	Neg.
Du272	25-50	ND	ND	ND	ND	ND	ND
Du366	25-50	Pos.	Pos.	ND	ND	ND	Pos.

*Informatiom provided by DR. G. Ramjee (MRC, S.A) Durban.
Neg. = Negative
Pos. = Positive
ND = Not done

2.2.2 Detection of genetic polymorphisms in chemokines and chemokine receptors

The chemokine receptor mutation (CCR5Δ32) was detected by polymerase chain reaction (PCR) and CCR5m303, CCR2b-64I and SDF1-3'A polymorphisms were detected by PCR followed restriction fragment length polymorphism (RFLP). Genomic DNA was isolated from peripheral blood mononuclear cells (PBMC) of 17 HEPS individuals using the Pure PCR Template Preparation Kit (Boerhinger Mannheim, Germany) according to the manufacturer's instructions. DNA was amplified using the Perkin Elmer 9600 thermocycler following the PCR cycling protocols shown in Table 2.2.4.

Table 2.2.4 : PCR protocols and $MgCl_2$ concentration for different chemokine receptor genes

Coreceptor Variant	$MgCl_2$ (mM)	PCR Cycling Protocol (30 cycles)
CCR5Δ32	2.5	95°C, 60°C and 72°C for 30s at each temperature
CCR5-m303	2.0	94°C, 58°C and 72°C for 20s at each temperature
CCR2b-64I	2.5	94°C, 63°C and 72°C for 20s at each temperature
SDF1-3'A	2.0	94°C, 65°C and 72°C for 30s at each temperature

The following were used for the PCR master mixes ; standard PCR buffer (10mM Tris-HCL, pH 8.3; 50mM KCL), 10mM of deoxynucleotide triphosphates (dNTP), 25pmol/µl of each primer, 0.125µl (5U/µl) Super Therm (Taq) polymerase (ICN, Biochemicals, USA) in a total volume of 20µl.

a) CCR5Δ32 Detection

The CCR5 primers, CCR5 (F) and CCR5 (R) (Table 2.2.5) amplified a 225bp fragment of the CCR5 gene including the CCR5Δ32 mutation site. The CCR5 wild type allele generated a 225bp fragment and the mutant allele (CCR5Δ32) generated a 193bp DNA fragment, 32bp shorter than the fragment generated by the wild type allele. All PCR master mixes were made up to 20µl, each containing 2.5µl of the 10X buffer, 2µl of 10mM dNTPs, 1.0µl of each primer (25pMol/µl), 5.0µl of DNA template, $MgCl_2$ and made up to 20µl with distilled water. DNA amplification cycling and the concentration of $MgCl_2$ used in each PCR programme are shown in Table 2.2.4. The PCR products were visualized on 2.5% agarose gel containing ethidium bromide (10ng/ml) after gel electrophoresis.

Table 2.2.5 : PCR primers for detection of chemokine and chemokine receptor polymorphisms

Name	Gene variant	Sequence	References
CCR5 (F)	CCR5Δ32	5'-TAATACGACTCACTATAGGG-'3	Michael, *et al.*, (1997) **A**
CCR5 (R)	CCR5Δ32	5'-GTTTTCCCAGTCACGACG-'3	Michael, *et al.*, (1997) **A**
CCR5-m303 (F)	CCR5m303	5'-GCTGCAGGTGTAATGAAGAC-'3	Williamson, et al., 2000
CCR5-m303 (R)	CCR5m303	5'-CTGAAGAGCATGACTGACAT-'3	Williamson, et al., 2000
CCR2b (F)	CCR2b-64I	5'-TTGTGGGCAACATGATGG-'3	Michael, *et al.*, (1997) **B**
CCR2b (R)	CCR2b-64I	5'-TGTGAATAATTTGCACATTG-'3	Michael, *et al.*, (1997) **B**
SDF-1 (F)	SDF1-3'A	5'-CAGTCAACCTGGGCAAAGC-'3	Winkler, et al., (1998)
SDF-1 (R)	SDF1-3'A	5'-AAGCTTTGGTCCTGAGAGTC-'3	Winkler, et al., (1998)

b) CCR5m303 Detection

For the detection of the CCR5-m303 polymorphism, the primers used CCR5m303 (F) and CCR5m303 (R) (Table 2.2.5) amplified a 350bp fragment of the CCR5 gene that included the CCR5m303 polymorphic site.

DNA amplification cycling was carried out as shown in Table 2.2.4. Amplified DNA fragments were analysed by restriction fragment length polymorphism (RFLP) using the restriction endonuclease, *Hinc II* (Boerhinger Mannheim, Germany). All the restriction digests were carried out as described by Sambrook, *et al.*, (1989). A single digestion reaction (5µl) contained 2.5µl of buffer, 0.5µl of *Hinc II* (500U/ml) and 2µl of distilled water, was added to the 20µl PCR product and incubated for 4 hours at 37°C.

The CCR5m303 wild type allele has a restriction site for *Hinc II* and the mutant allele loses this restriction site. Two fragments of 230 and 120bp were generated for the CCR5 wild type allele, and a 350bp fragment was generated for the homozygote mutant allele. The PCR products analysed by gel electrophoresis and were visualized on 2.5% agarose gel containing ethidium bromide (10ng/ml).

c) CCR2b-64I Detection

The primers CCR2 (F) and CCR2 (R) (Table 2.2.5) were used for the detection of CCR2b-64I polymorphism. DNA amplification cycling was carried out as shown in Table 2.2.4. The PCR product generated a 183bp fragment that included the site of a single nucleotide substitution in the CCR2 gene (Smith, *et al.*, 1997). Amplified DNA fragments were analysed by restriction fragment length polymorphism (RFLP) using *BsaB I* (ICN Biomedicals, USA) endonuclease.

A 5µl restriction digest mix containing 2.5µl of buffer, 0.5µl of *BsaB I* (1,000U/ml) and 2.0µl of distilled water, was added to the 20µl PCR product and incubated for an hour at 60°C. The CCR2b-64I allele has a restriction site for *BsaB I* and the wild type allele (CCR2b) loses its restriction site. Two fragments of 165 and 18bp were generated for the CCR2b-64I homozygous allele. The wild type allele generated a fragment of 183bp.

d) SDF1-3'A Detection

Primers SDF-1(F) and SDF-1(R) (Table 2.2.5) were used to amplify a 303bp DNA fragment of the SDF-1 gene, including the SDF1-3'A polymorphic site (Winkler, *et al.*, 1998). DNA amplification cycling was carried out as shown in Table 2.2.4. Amplified DNA fragments were analysed by restriction fragment length polymorphism (RFLP) using *Msp I* (Boerhinger Mannheim, Germany) endonuclease. A 5µl restriction digest mix containing 2.5µl of buffer, 0.5µl of *Msp I* (1,000U/ml) and 2µl of distilled water, was added to the 20µl PCR product and incubated for 4 hours at 37°C. The SDF-1 wild type allele has a restriction site for *Msp I* and the mutant (SDF1-3'A) allele loses its restriction site. Two fragments of 179 and 124bp were generated for the SDF-1 wild type allele, and a 303bp fragment was generated for the homozygote mutant allele.

2.2.3 Sequencing

A 303bp DNA fragment of the SDF-1 gene that included the SDF1-3'A polymorphic site (Winkler, *et al.*, 1998) was sequenced. Sequencing was performed by Mr Peter Owira of the Department of Medical Microbiology University of Cape Town using the ABI Prism 310 Genetic Analyzer (Perkin Elmer). The forward and the reverse strands were aligned and compared to a published sequence, polymorphisms were identified by analysis of aligned sequences using the Auto Assembler 2.1 package of the Prism 310 Genetic Analyzer.

2.3) RESULTS

Seventeen women were identified who had been in sex work for greater than four years, while remaining HIV-1 negative. Four years was selected based on data generated from the sex-worker cohort in Nairobi which showed that women were most likely to become infected in the first two years of sex work and the risk of infection decreases with every year in sex-work thereafter (Fowke, *et al.*, 1996).
A similar analysis is not yet available on the KwaZulu/Natal cohort, and the inclusion of these women into the HEPS cohort will have to be reviewed once the analysis is complete.

In general, these women had proven exposure to HIV-1 as they had a low reported frequency of condom usage, which was reflected in the high prevalence of sexually transmitted infections (STIs) detected at entry to the study (Table 2.2.2). In addition, the overall HIV-1 incidence in this cohort is 18% (G. Ramjee personal communication). The time in sex work ranged from four years eight months to 26 years with an average of nine years. The average number of partners per week was 17 with the reported condom usage in 16 of the 17 women being below 50%. The prevalence of HIV-1 among the truck drivers who are clients to these sex workers is reported to be 56% (G. Ramjee personal communication).

Women were screened for mutations or genetic polymorphisms in three chemokine genes namely, CCR5Δ32, CCR5m303 and CCR2b-64I, and the untranslated terminal repeat (UTR) of one chemokine ligand, SDF1-3'A. The frequencies of these variants in the HEPS were compared to previously published frequencies from South African populations.

The CCR5Δ32 mutation is characterised by a 32bp deletion. The CCR5 wild type allele produces a 225bp DNA fragment and the mutant allele produces a fragment of 193bp. The CCR5m303 variant is characterised by an elimination of a *Hinc II* restriction site. The wild type allele produces two DNA fragments of 230 and 120bp, the mutant allele produces a 350bp fragment. CCR2b-64I variant has a *BsaB I* restriction site that is lost by the wild type CCR2b allele. The wild type allele produces a 183bp fragment and the mutant allele produces two fragments 165 and 18bp long.

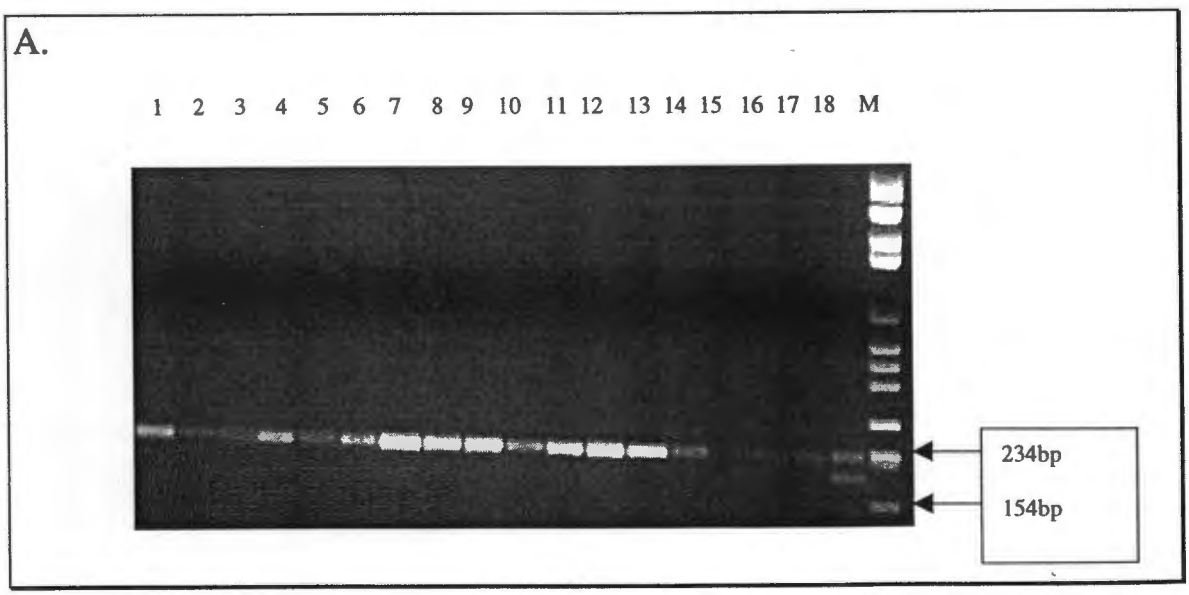
The SDF-1 ligand has a *Msp I* restriction site which is lost by the mutant allele (SDF1-3'A). The wild type allele produces a fragment of 179 and 124bp and the mutant allele produces a fragment of 303bp. In Table 2.3.1 band sizes characteristic for each chemokine and chemokine receptor variants are presented.

Table 2.3.1 : Sizes of bands characteristic for different genotypes

Gene variant	Genotype		
	Wild type (wt/wt)	*Heterozygous* (wt/vt)	*Homozygous* (vt/vt)
CCR5Δ32	225bp	225 and 193bp	193bp
CCR2b-64I	183bp	183, 165 and 18bp*	165 and 18bp*
SDF1-3'A	179 and 124bp	303, 179 and 124bp	303bp
CCR5m303	230 and 120bp	350, 230 and 120bp	350bp

*Band not visible in regular agarose gel.
wt/wt = wild type
wt/vt = heterozygote (wild type/variant)
vt/vt = homozygote (variant/variant)

Among the 17 HEPS individuals tested for the CCR5m303 and CCR5Δ32 mutations by RFLP and PCR respectively, no mutant alleles were detected (Table 2.3.3). These two mutations have previously been shown to be rare among the African population (Williamson, *et al.*, 2000). The typical gel electrophoresis results of CCR5Δ32 and CCR5m303 genotyping are presented in figure 2.3.1 a and b.



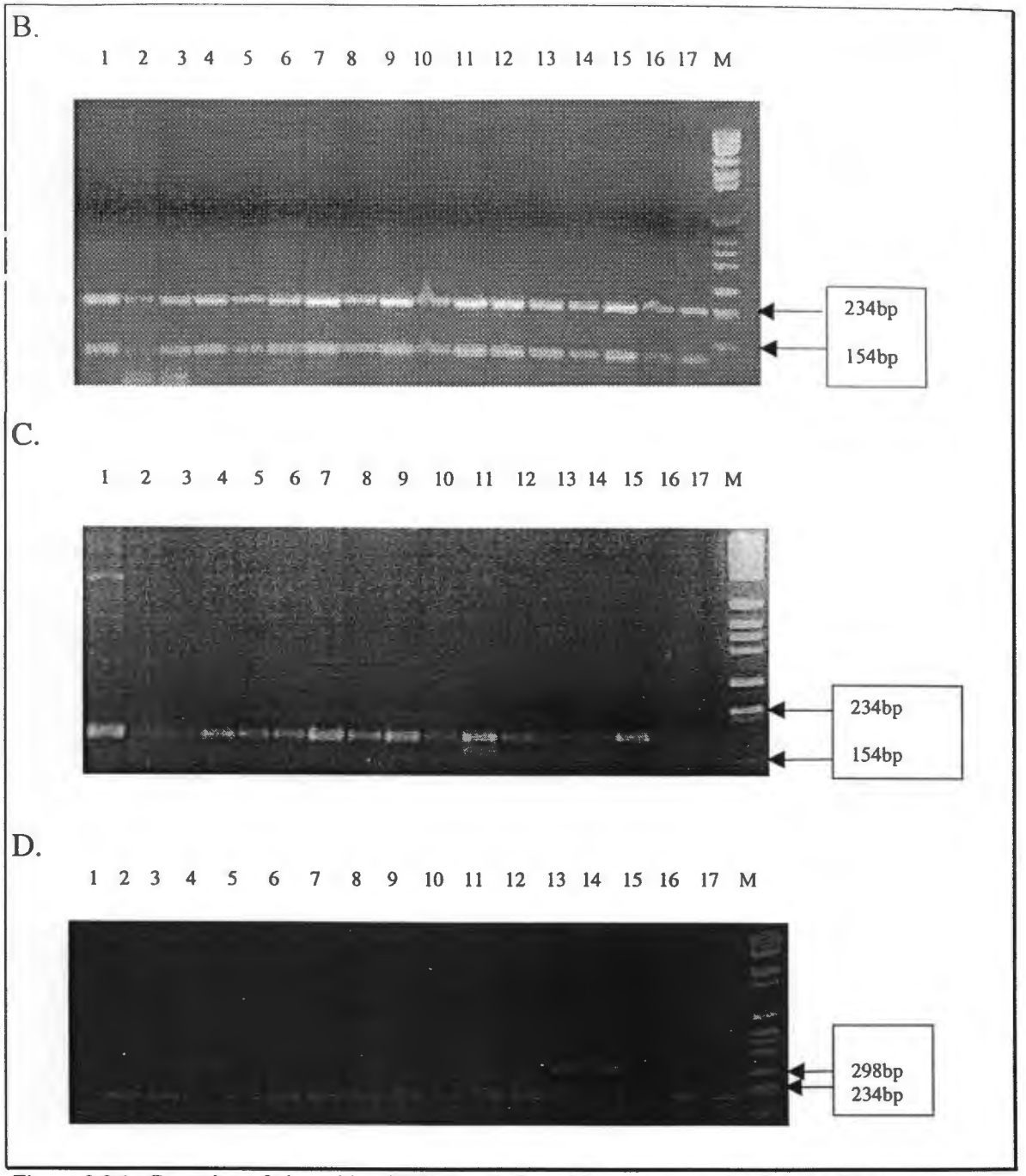


Figure 2.3.1 : Detection of chemokine (SDF-1) and chemokine receptor (CCR2b and CCR5) variants in a population of HEPS by PCR and PCR-RFLP analysis. **(a)** CCR5Δ32 mutation detection, all samples (lanes 1-17) were CCR5 wild type (w/w) band size 225bp. Lane 18 positive control known heterozygote (w/Δ32) band sizes 225 and 193bp. **(b)** Detection of the CCR5m303 variant, all samples (lanes 1-17) were wild type band sizes 230 and 120bp. **(c)** Detection of CCR2b-64I variant, CCR2b-64I heterozygous (w/64I) lanes (2, 11 and 15) band sizes 165, 147 and 18bp (not visible). CCR2b wild type (w/w) lanes (1,3,4,5,6,7,8,9,10,12,13,14, and 16) band size 165bp. CCR2b-64I homozygotes (64I/64I) lane (17) band sizes 147 and 18bp (not visible). **(d)** SDF1-3'A detection, SDF-1 wild type (w/w) lanes (1,2,3,5,6,7,8,9,10,11,12,15,16,17) band sizes 179 and 124bp. SDF1-3'A heterozygotes (w/3'A) lanes (4,13 and 14) band sizes 303, 179 and 124bp. All bands visualised on 2.5% agarose gel containing Ethidium bromide (10ng/ml).

Seventeen HEPS individuals were genotyped for CCR2b-64I polymorphism by PCR-RFLP (Figure 2.3.1c). Among the HEPS population an allelic frequency of 15% (Table 2.3.3) was determined. This compared with a frequency of 13 % in the South African population (Williamson, *et al.*, 2000).

Highly exposed but persistently seronegative (HEPS) women were genotyped for SDF1-3'A variant by PCR-RFLP. Among the HEPS population three individuals were identified as being heterozygous for this variant. A typical gel electrophoresis result of the SDF-1 genotyping is presented in figure 2.3.1d. However, only two out of three of these variants were confirmed by sequencing, indicating that the enzyme was not digesting to completion. Figure 2.3.2 depicts the polymorphisms in the HEPS samples as compared to a wild type sequence. Sequence analysis did not reveal any other variant in this region. The confirmed allelic frequency of this variant was therefore 5.9%. This compared with a frequency of only 1% in the South African population (Williamson, *et al.*, 2000), and the difference between HEPS and the background frequency in the South African population was markedly significant.

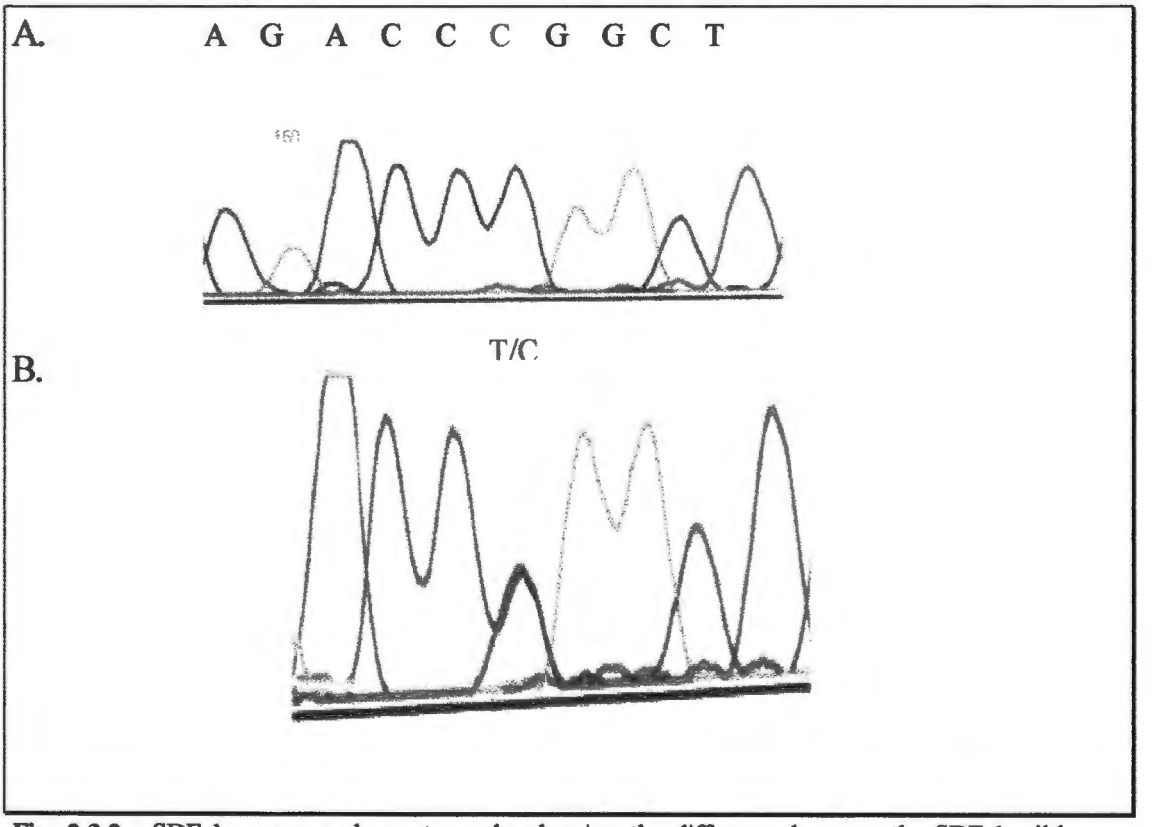


Fig. 2.3.2 : SDF-1 sequence chromatographs showing the difference between the SDF-1 wild type, **(A)** and heterozygote **(B)** variants.

In summary, the allelic frequencies of the CCR5Δ32, CCR5m303 and CCR2b-64I variants were comparable between the HEPS and the general South African population, whereas the HEPS had an elevated frequency of the SDF1-3'A variant compared to the South African population.

Table 2.3.3 : Genotype and allelic frequencies found in the study population versus allelic frequencies found in the South African population (SAP)*

Gene Variant	**Genotype**						**Allelic Frequency (%)**		
	Wild type (wt/wt)		*Heterozygotes* (wt/vt)		*Homozygotes* (vt/vt)				
	HEPS	SAP*	HEPS	SAP*	HEPS	SAP*	HEPS	vs	SAP*
CCR5Δ32	17	1245	0	2	0	0	0	vs	0.1
CCR5-m303	17	687	0	0	0	0	0	vs	0
CCR2b-64I	12	134	3	44	1	2	15	vs	13
SDF1-3'A	14	194	3	4	0	0	5.9	vs	1

*Williamson, *et al.*, (2000). Study done on South African Population.
wt/wt = wild type
wt/vt = heterozygote (wild type/variant)
vt/vt = homozygote (variant/variant)

2.4) DISCUSSION

Global, regional and ethnic distribution of frequencies of HIV/AIDS protective mutations can vary significantly (Table 2.4.1). The CCR5Δ32 mutation is extremely rare among Africans (Huang, *et al.*, 1996) and its frequency is different even among the Caucasian population with higher frequencies in Europeans from Northern Europe (20%) compared to those in Southern Europe (2.0%) (Martison, *et al.*, 2000). CCR2b-64I variant exist at a higher frequency (13.3%) in the African and the African American (27.7%) populations than in Caucasians (17.7 -27.7%) (Williamson, *et al.*, 2000 and Mummidi, *et al.*, 1998). The SDF1-3'A variant is more frequent in Caucasians (39%), compared to African Americans (15.1%) (Huang, *et al.*, 1996 and Mummidi, *et al.*, 1998). In South Africa Caucasians have a frequency of 20.3% compared to only 1% in Africans (Williamson, *et al.*, 2000).

Table 2.4.1 : Global distribution of chemokine and chemokine receptor variants associated with HIV-1 pathogenesis

Population	Gene Variant and Allelic Frequency (%)			
	CCR5Δ32	*CCR2b-64I*	*SDF1-3'A*	*CCR5m303*
Europe				
[1]Paris	28.6 (n=145)	18.9 (n=145)	39.3 (n=110)	1.0 (n=209)[6]
[2]Greece	1.9 (n=135)	15.5 (n=135)	ND	ND
North America				
[3,4]Caucasian	22 (n=446)	17.7 (n=479)	38.8 (n=179)	ND
[3,4]African American	0 (n=137)	27.7 (n=288)	15.1 (n=58)	ND
South Africa				
[5]Caucasian	9.4 (n=144)	7.2 (n=146)	20.3 (n=145)	0.7 (n=145)
[5]African	0.1 (n=1247)	13.3 (n=180)	1.0 (n=198)	0.0 (n=687)
HEPS	0 (n=17)	15 (n=17)	6 (n=17)	0 (n=17)
[2]***Kuwait***	ND	11.9 (n123)	25.9 (n=108)	0.0 (n=200)

[1]Hendel *et al.*, 1998.
[2]Voevodin *et al.*, 1999.
[3]Huang *et al.*, 1996.
[4]Mummidi *et al.*, 1998.
[5]Williamson *et al.*, 2000.
[6]Quillent, *et al.*, 1998.

Mutations that result in non-functional CCR5, such as CCR5Δ32 and CCR5-m303, confer resistance to HIV-1 infection (O'Brien, *et al.*,1997 and Quillent, *et al.*, 1998), however, these mutations were not responsible for resistance in the women participating in this study. This is not surprising as these mutations are extremely rare in individuals of African origin.

In a recent study by Petersen, *et al.,* (2001) several novel mutation in the CCR5 gene were found which had no effect on HIV-1 resistance, included in that study were seven HEPS women from this cohort.

One out of the 17 HEPS samples genotyped for CCR2b-64I was homozygous (64I/64I) and three of the 17 samples were heterozygous (wt/64I), giving the allelic frequency of 15% for this study population. This value compares well with the allelic frequency of 13.1% reported for South African population (Williamson, *et al.*, 2000). This mutation is not associated with protection from HIV-1 infection but was found to delay disease progression in HIV-1 infected individuals (Smith *et al.*, 1997).

The role of the SDF1-3'A variant is controversial. The SDF1-3'A variant has been reported to both delay the rate of progression to AIDS in individuals who are homozygous (SDF1-3'A/3'A) for this variant (Winkler, *et al.*, 1998), while other researchers have found homozygosity results in more rapid progression after diagnosis of AIDS (Brambila, *et al* ., 2000). Interestingly, Winkler *et al.*, (1998) found that heterozygosity had a protective effect against HIV-1 infection. In the study by Winkler *et al.*, (1998) a group of high-risk exposed uninfected individuals showed a highly significant elevation in SDF1-3'A variant wherein more than half (50.6%) were heterozygous (wt/3'A) for the SDF-1 allele compared to 31.1% among infected individuals. In the current study HEPS women were found to have an allelic frequency of 5.8% (two out of 17 were heterozygote) compared with only 1% in the background population (4 out of 198) individuals were heterozygous (Williamson, *et al.*, 2000).

In conclusion, none of the well characterised mutations in the CCR5 gene associated with resistance were identified in this study population. However, an elevated frequency of the SDF1-3'A variant was observed. The mechanism of how this variant confer resistance is puzzling as sexually transmitted HIV-1 strains generally utilize the CCR5 receptor and not CXCR4. Further work needs to be done to ascertain the role of this variant in HIV-1 pathogenesis.

CHAPTER 3

INVESTIGATION OF LYMPHOCYTE SUSCEPTIBILITY TO HIV-1 INFECTION AND DETERMINATION OF EXPRESSION LEVELS OF CHEMOKINE RECEPTORS.

3.1) INTRODUCTION

Some individuals remain uninfected by HIV-1, despite multiple sexual exposures to the virus. Peripheral blood mononuclear cells (PBMC) of such individuals are resistant to *in vitro* infection by R5 viral strains, but not X4 viral strains (Connor, *et al.*, 1997 and Paxton, *et al.*, 1996). This resistance is associated with a homozygous 32bp deletion (Δ32) in the CCR5 gene (Samson, *et al.*, 1996 and Liu, *et al.*, 1996) that renders the receptor non-functional. Resistance of PBMC to R5 HIV-1 viral strains was found to be a result of a blockage at the level of viral entry (Dragic, *et al.*, 1996). Individuals heterozygous for the CCR5 deletion (CCR5Δ32) are susceptible to infection (Dean, *et al.*, 1997) and their PBMC are permissive for CCR5 dependent HIV-1 strains (Samson, *et al.*, 1996). However, these individuals have delayed disease progression possibly as a consequence of low levels of CCR5 expression (Husman, *et al.*, 1999).

The role of β-chemokines, natural ligands for CCR5, in the pathogenesis of HIV-1 has been studied extensively *in vivo* and *in vitro*. Several studies have shown that β-chemokines can inhibit replication of R5 viruses *in vitro* (Alkhatib, *et al.*, 1996 and Dragic, *et al.*, 1996). Paxton, *et al* (1996) reported that highly exposed but uninfected individuals produced high amounts of β-chemokines RANTES, MIP-1α and MIP-1β (Paxton, *et al.*, 1996) which may contribute to their natural resistance to HIV-1 infection.

Primary HIV-1 isolates in early disease stage use CCR5 as a coreceptor, these viruses are classified as R5 or non syncytium-inducing (NSI) based on the cytopathic effect they have on the MT-2 cell line. In HIV-1 subtype-B, as disease progresses there is an emergence of syncytium-inducing (SI) viruses which utilise the CXCR4 receptor (R5X4 or X4 viruses). The emergence of these variants results in a decline in CD4 T-cells and rapid disease progression (Samson, *et al.*, 1996).

The expression levels of CCR5 are crucial in the *in vitro* cell infectability by HIV-1 R5 isolates (Reynes, *et al.*, 2000). The two chemokine receptors (CCR5 and CXCR4) are differentially expressed in different CD4 T-cell subpopulations. CCR5 is mainly expressed in memory and activated T-cells and CXCR4 is expressed in naive T-cells (Bleul, *et al.*, 1997). HIV-1 infected individuals heterozygous for the Δ32 deletion have some advantage over CCR5 homozygote wild-type individuals in relation to disease progression (Zimmerman, *et al.*, 1997). The mechanism by which heterozygous individuals are protected against disease progression is not fully understood. It has been demonstrated that *in vitro* infectability correlates with CCR5 expression levels at the cell surface, which in turn is influenced by the genotype of the CCR5 gene (Wu, *et al.*, 1997). Ostrowski, *et al.*, (1998) demonstrated that CXCR4 on CD4 T-cells is significantly down regulated, and CCR5 expression is up-regulated in HIV-1 infected individuals compared with uninfected controls (Ostrowski, *et al.*, 1998).

In addition, differential functioning of these receptors may be a factor in HIV-1 resistance. Individuals who are infected with HIV-1 and carrying the wild type CCR5 allele were found to have higher levels (28%) of CD4 T-cells expressing CCR5 compared to HIV-1 infected individuals who are heterozygous (21%) for the CCR5Δ32 mutation (Husman *et al.*, 1999). Lower expression levels of the CCR5 receptor correlates to reduced infectability of CD4 T-cells with R5 HIV-1 isolates *in vitro* (Wu, *et al.*, 1997). HIV-1 infected individuals who progress rapidly to AIDS have higher levels of CD4 T-cells expressing CCR5 than slow progressors (Husman, *et al.*, 1999). There is a significant correlation between disease stage and chemokine receptor expression levels, with CXCR4 expression levels higher than the CCR5 levels in the early stages of the disease. In advanced disease stage the expression levels of CCR5 are higher than the expression levels of CXCR4 (Ostrowski, *et al.*, 1998).

To determine the influence of chemokine receptors in HIV-1 pathogenesis in HEPS individuals, the levels of CCR5 and CXCR4 were quantified on unstimulated PBMC from 17 HEPS and a control group of five HIV-1 seronegative women.

In addition, we examined whether resistance to HIV-1 infection in HEPS is associated with differences in the susceptibility of their CD4 T-cells to infection with phenotypically distinct primary isolates of HIV-1 including R5, R5X4 and X4 of different subtypes.

3.2) MATERIALS AND METHODS

a) Blood samples

Blood samples were obtained from 17 sex workers who had previously tested HIV-1 antibody negative despite repeated exposure to HIV-1 (HEPS). These samples were re-tested for antibodies to HIV using an ELISA assay (Genscreen HIV-1/2 version 2, Sanofi Pasteur, France) and for CD4 T-cell numbers by flow cytometry (Becton Dicknson) at the National Institute for Virology. A group of five HIV-1 seronegative laboratory co-workers considered to be at low risk of HIV-1 infection were used as controls.

b) Isolation of PBMC

PBMC were isolated from approximately 20ml of whole blood by Ficoll hypaque density gradient centrifugation. Whole blood was centrifuged at 1,500rpm for 15 minutes and the plasma collected and stored. The buffy coat was removed and mixed with 5ml of phosphate buffered saline (PBS). This cell suspension was carefully layered onto 5ml Ficoll (Pharmacia) in a 15ml conical tube and centrifuged at 2,000 rpm for 30 minutes at room temperature with brake off.

The mononuclear cells which sedimented on top of the Ficoll layer were carefully removed and washed twice in PBS at 1,000 rpm to remove platelets. Viable cells were counted using trypan blue exclusion criteria under a hemocytometer.

c) Quantification of chemokine receptor expression levels by flow cytometry.

The proportion CD4 T-cells positive for CCR5 and CXCR4 was measured on unstimulated CD4 T-cells from HEPS and controls by three colour flow cytometry. This part of the work was done under the guidance of Ms Stephina Nyoka at the National Institute for Virology.

PBMC (2 x $10^{5)}$ were labelled with anti-human CD3-APC (SK7) and anti-human CD4-PerCP (Becton Dickinson) to delineate CD4 positive T-cells. To this was added either anti-human CCR5-FITC (2D7), or anti-human CXCR4-PE (12G5) (BD Pharmingen). All antibodies were diluted 1:10 in PBS in a total volume of 50µl. IGg2a-FITC and IGg2a-PE (Becton Dickinson) were used as isotype controls. Cells were then washed with PBS containing 0.5% bovine serum albumin (BSA) and fixed in 200µl of PBS containing 2% BSA and 5% formalin for 10 minutes. Acquisition and surface staining analysis was performed using Cell Quest software program 3.2.1 (BD Facsort, San Jose, CA). Statistical analysis was done using the Mann Whitney student t-test performed on Prism 2.0 program.

d) Cell culture

Isolated PBMC were stimulated with 5µg/ml phytohemagglutinin (PHA) (Sigma, USA) for 72 hours in RPMI 1640 medium supplemented with 20% fetal calf serum plus 100 U/ml of streptomycin and penicillin (complete medium) at a density of 2 x 10^{6}/ml in a 15 ml flask at 37°C. After two-three days cells were washed and counted and used as targets for HIV-1 infection assays.

e) HIV-1 isolates used in the study

PBMC from both HEPS and controls, were exposed to three R5 (Du151, Du422, and SM2), one X4 (Acute6) and two R5X4 (Du179 and CM9) HIV-1 tropic viral strains (Table 3.1). Four of the HIV-1 viral strains were HIV-1 subtype-C (Du151, Du422, Du179 and CM9), one was HIV-1 subtype-B (SM2) and one was an HIV-1 subtype-D (Acute 6). The three isolates with the Du prefix were collected from women in the same geographic region as the women in the HEPS cohort. Three of the isolates caused syncitia (SI) in the MT-2 cell line and this correlated with their ability to use the CXCR4 coreceptor. Two of these isolates also used CCR5 and were classified as dual-tropic. Three isolates were non-syncitium inducing (NSI) and used the CCR5 coreceptor only.

The MT-2 and coreceptor assays were performed by Mr Tonie Cilliers at the National Institute for Virology.

Table 3.1: Genotypic and phenotypic characteristics of HIV-1 isolates used in the study

Isolate	Subtype gag/env	MT-2 assay	Co-receptor used
Du151	C/C	NSI	CCR5
Du422	C/C	NSI	CCR5
Du179	C/C	SI	CCR5/CXCR4
CM9	C/C	SI	CCR5/CXCR4
SM2	-/B	NSI	CCR5
Acute 6	D/-	SI	CXCR4

f) *In vitro* infectivity assay

Mitogen-stimulated PBMC from HEPS and control subjects were plated at a density of 2 x 10^5 cells per well in 96-well plates, in complete RPMI medium containing IL-2 (5%) in a volume of 100µl. Cultures were inoculated with approximately 1,000 $TCID_{50}$ of various HIV-1 isolates in a final volume of 200µl. Virus replication was assessed at regular intervals (days 4, 7, 10, and 14) over a two week period by measuring the production HIV-1 p24 antigen in the culture supernatants by an ELISA assay. Half of the supernatant from the cultures was removed for the p24 assay, and replaced with fresh media containing IL-2.

g) p24 antigen ELISA assay

The levels of p24 antigen that accumulated in tissue culture supernatants were measured using an in-house p24 antigen ELISA (Appendix B4). For this, each well of a high binding 96 well plate was coated with 100µl of anti-HIV-1 p24 antibody (D7320, Aalto Bio, Ireland) and incubated overnight at room temperature. Plates were washed twice with Tris Buffered Saline (TBS) followed by 100µl of 2% milk in TBS (to block non-specific binding sites on the plate) and incubated for 1 hour at room temperature.

Plates were washed once with TBS after which 100µl of culture supernatant plus 100µl of 1% Empigen (to inactivate the virus) was added to each well. Plates were incubated for 2 hours at 37 C, after which they were washed thoroughly with TBS.

To each well 100µl of anti-HIV1-p24 antibody conjugated to alkaline phosphatase (EH12AP, Aalto Bio, Ireland) in 2% sheep serum (Sigma) and 0.05% Tween in TBS was added and incubated for 1 hour at 37 C. Plates were washed 4x with AMPAK buffer (Dako) using an automated washer, followed by the addition of 50µl AMPAK substrate (Dako) and incubated for an hour at room temperature. After this 50µl of AMPAK amplifier (Dako) was added for 3 minutes at room temperature and then 50µl of stop solution (0.5 M HCL) was added. The optical density (OD) was read using an automatic plate reader at 492nm. Positive and negative controls were included in all ELISA'S.

3.3) RESULTS

All the samples from the HEPS women used in this study were confirmed as HIV-1 seronegative by ELISA. The CD4 T-cell count of all the samples was within the normal range of 500-1,200cell/µl indicating that these women had no evidence of immunosuppession (Table 3.3.1).

Table 3.3.1 : Depicting the HIV-1 status of the HEPS women

Sample ID	HIV-1/2 antibody	CD4 T-cell count (cells/µl)
Du4	Negative	939
Du6	Negative	921
Du7	Negative	1523
Du20	Negative	739
Du53	Negative	1250
Du85	Negative	1429
Du96	Negative	773
Du117	Negative	1172
Du126	Negative	879
Du154	Negative	1191
Du175	Negative	875
Du188	Negative	621
Du205	Negative	739
Du250	Negative	780
Du270	Negative	759
Du272	Negative	1113
Du366	Negative	960
Average		**980**

a) Chemokine receptor expression levels

The percentage of CD4 T-cells positive for CCR5 and CXCR4 of 17 HEPS and four controls was determined by three-color flow cytometry. The mean percentage of CD4 T-cells positive for CCR5 was 10.7% (STD deviation 10.6%) for HEPS and 1.7% (STD deviation 6.8%) for the control group (Table 3.3.2). The difference in CCR5 levels between the two groups was not statistically significant (p=0.117). Similarly, the difference in CXCR4 positive CD4 T-cells in the HEPS and the control group was not statistically significant (p=0.964). The mean percentage of CD4 T-cells positive for CXCR4 for HEPS was 89.3% (STD deviation 9.4%) and for the control group 88.3%

(STD deviation 3.0%) (Table 3.3.2). The proportion of CD4 T-cells expressing CXCR4 was higher than for CCR5 in both groups.

In order to determine whether the levels of CCR5 and CXCR4 receptor expression on CD4 T-cells differed between HEPS and controls we analyzed the mean fluorescence intensity (MFI) (Table 3.3.2). For CCR5 the MFI on HEPS and controls was 16.2 and 11.1 respectively. For CXCR4 it was 151.7 (HEPS) and 138 (controls). The difference in expression levels of both chemokine receptors was not statistically significant for CCR5 (p=0.433) or CXCR4 (p=2.13) between the two groups.

Collectively these data indicate that both HEPS women and controls had similar proportions on CD4 T-cells positive for CCR5 and CXCR4, and that the levels of expression of both chemokine receptors was similar in both groups.

Table 3.3.2 : CCR5 and CXCR4 expression levels on CD4 T-cells of HEPS women and controls

Sample ID	Mean fluorescence intensity (MFI)		% positive CD4 T-cells	
	CCR5	*CXCR4*	*CCR5*	*CXCR4*
Du4	9.6	126.9	1.0	97.6
Du6	14.3	47.4	24.8	78.1
Du7	17.7	272.2	0.5	98.2
Du20	40.6	153.8	37.3	95.9
Du53	10.0	58.2	15.0	79.1
Du85	19.2	114.8	11.8	93.1
Du96	10.5	236.5	2.0	98.9
Du117	9.8	234.2	2.3	99.5
Du126	9.7	147.7	1.3	98.0
Du154	11.6	49.5	19.9	67.4
Du175	10.1	150.9	23.8	77.0
Du188	48.9	335.7	7.6	84.8
Du205	11.9	187.6	3.6	98.2
Du250	16.5	158.7	7.0	83.1
Du270	8.9	61.2	2.3	92.5
Du272	8.7	160.2	1.0	92.5
Du366	16.0	84.3	9.7	92.1
Mean	**16.2**	**151.7**	**10.7**	**89.3**
Control 1	9.7	161.2	1.4	95.2
Control 2	11.0	139.0	2.7	87.1
Control 3	9.2	173.7	1.0	93.8
Control 4	11.5	115.0	1.2	88.3
Control 5	13.8	101.4	2.3	77.3
Mean	**11.1**	**138.0**	**1.7**	**88.3**

There was considerable variability between individuals within the HEPS group in the expression of CCR5 on CD4 T-cells as observed by others (Moore, *et al.*, 1997). This is shown graphically in Figure 3.3.1. A few HEPS women had higher levels of CCR5 than controls despite the fact that as a group there was no significant difference between them. Representative scatter plots of flow cytometric analysis of PBMC from three HEPS and three controls are shown in Fig. 3.3.2.

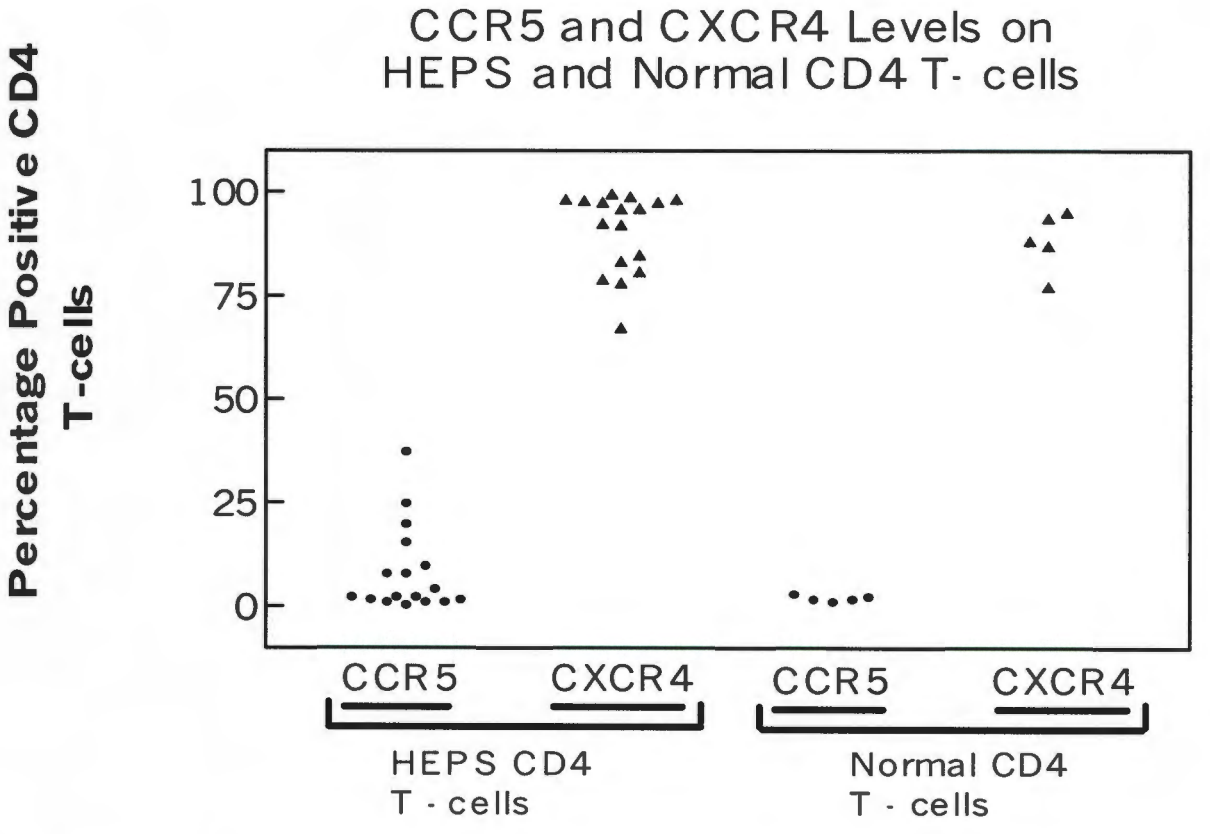


Fig 3.3.1 : CCR5 and CXCR4 expression levels on CD4 positive T-cells, of HEPS and HIV-1 seronegative control individuals.

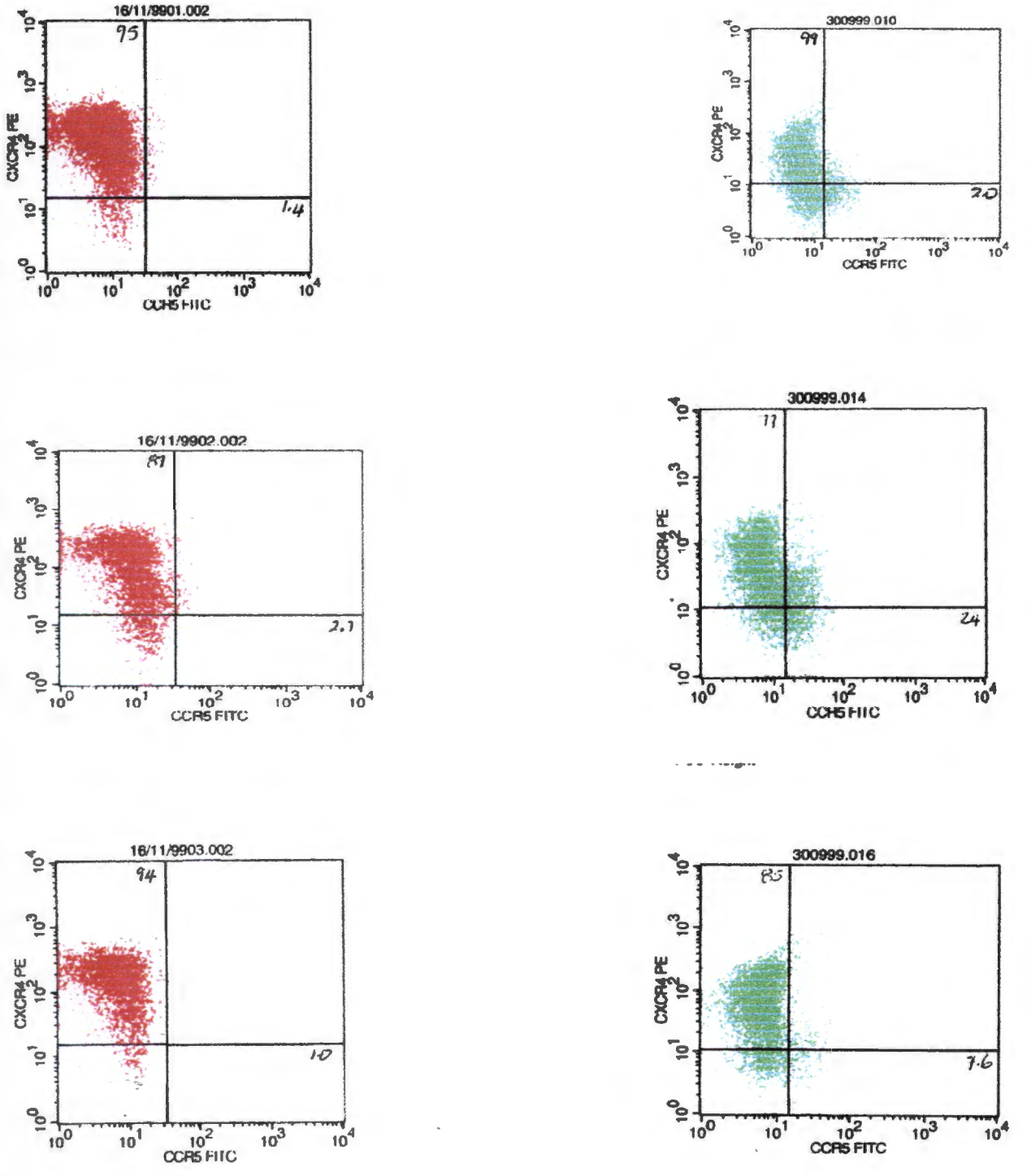


Fig. 3.3.3 : Representative scatter plots of flow cytometric analysis of CD4 T-cells from (A) Controls (1, 2 and 3) and (B) HEPS (Du96, Du179 and Du188). A three-color staining protocol was used to asses the expression of CCR5 (x-axis) and CXCR4 (y-axis in all plots). Quadrants were set according to the staining of control mABs (IgG2a-FITC and IgG2a-PE). The percentage of stained cells is shown in each quadrant.

b) PBMC *in vitro* infectivity assays

PBMC from seven HEPS women and two controls were infected with HIV-1 and monitored at regular intervals for HIV-1 p24 antigen production. The isolates used included three R5, two R5/X4 and one X4 isolate (Table 3.1). All viral isolates showed evidence of replication in cells from both HEPS and controls, except CM9 which replicated in only three of the seven HEPS samples (Table 3.3.2). The levels of replication of each viral isolate varied between PBMC samples as did the ability of each PBMC sample to support the replication of different viral isolates (Table 3.3.2).

Table 3.3.2 : Replication of R5 and X4 tropic viral strains in PBMC from HEPS and control group.

	HIV-1 Isolates used in the study					
	Du151	Du422	SM2	Acute6	Du179	CM9
Sample ID						
Du4	++++	++++	++++	+++	++++	++++
Du7	++	+	++	+	+++	-
Du85	+++	+	++++	+++	+	-
Du117	+++	+++	+++	++	+++	+++
Du126	++++	++++	++++	++++	+++	++++
Du250	+	+	+++	++	+	-
Du272	+++	+++	++++	++	++++	-
Control 1	+++	++	++++	+++	++++	++
Control 2	++++	++	+++	++++	++++	++

< 250 p/ml = negative
+ = 0.5 - 1 ng/ml
++ = 1 - 2 ng/ml
+++ = 2 - 4 ng/ml
++++ = 4 - 8 ng/ml

The replication kinetics of all six isolates in two HEPS and one control PBMC sample is shown in Figure 3.3.3. Du179, an (R5/X4) virus showed the fastest replication kinetics with maximum p24 antigen levels by day four in both HEPS and control PBMC. This was clearly not the result of its ability to use both HIV-1 coreceptors as the other dual-tropic virus, CM9 had the lowest replication capacity of all the viruses tested. In this case, p24 antigen was only observed after 11 days of infection in Du272 PBMC although the kinetics were slightly faster on control PBMC (Fig. 3.3.3).

Acute 6, the only X4 viral strain used established infection in all HEPS PBMC, but replicated poorly as indicated by the low HIV-1 p24 antigen production in Du7 and Du272 (Fig. 3.3.3). In both cases Acute 6, failed to reach maximum infection levels even after 18 days of infection.

The three R5 isolates tested (Du151, SM2, and Du422) infected all HEPS PBMC, although the replication of Du422 was slightly delayed compared with that of SM2 (Fig. 3.3.3). Du151 established the fastest infection with highest p24 antigen production among the three R5 HIV-1 isolates. There were no apparent differences in the capacity to infect PBMC from HEPS and the control group by the three R5 viral strains tested.

Du7 PBMC supported viral replication of all viral isolates tested, except CM9 (Figure 3.3.3). In these cells Du179 replicated to the highest level followed by Du151 and SM2 while, Du422 and Acute 6 showed lower replication kinetics. A similar pattern was observed for Du272 PBMC although in this case CM9 did establish an infection in these cells, although replication rates were low. PBMC from the control sample supported replication of all viral isolates tested with Du179 and Du151 producing maximum p24 antigen, followed by and CM9, with Acute 6 producing minimal p24 antigen.

In summary, PBMC from HEPS women and the control sample showed no significant difference in their ability to support viral replication. For viral strains that failed to replicate (CM9) or those that replicated poorly (SM2 and Acute 6), it is clear that the failure of these isolates to replicate is a phenomena of the isolates themselves and not resistance of PBMC.

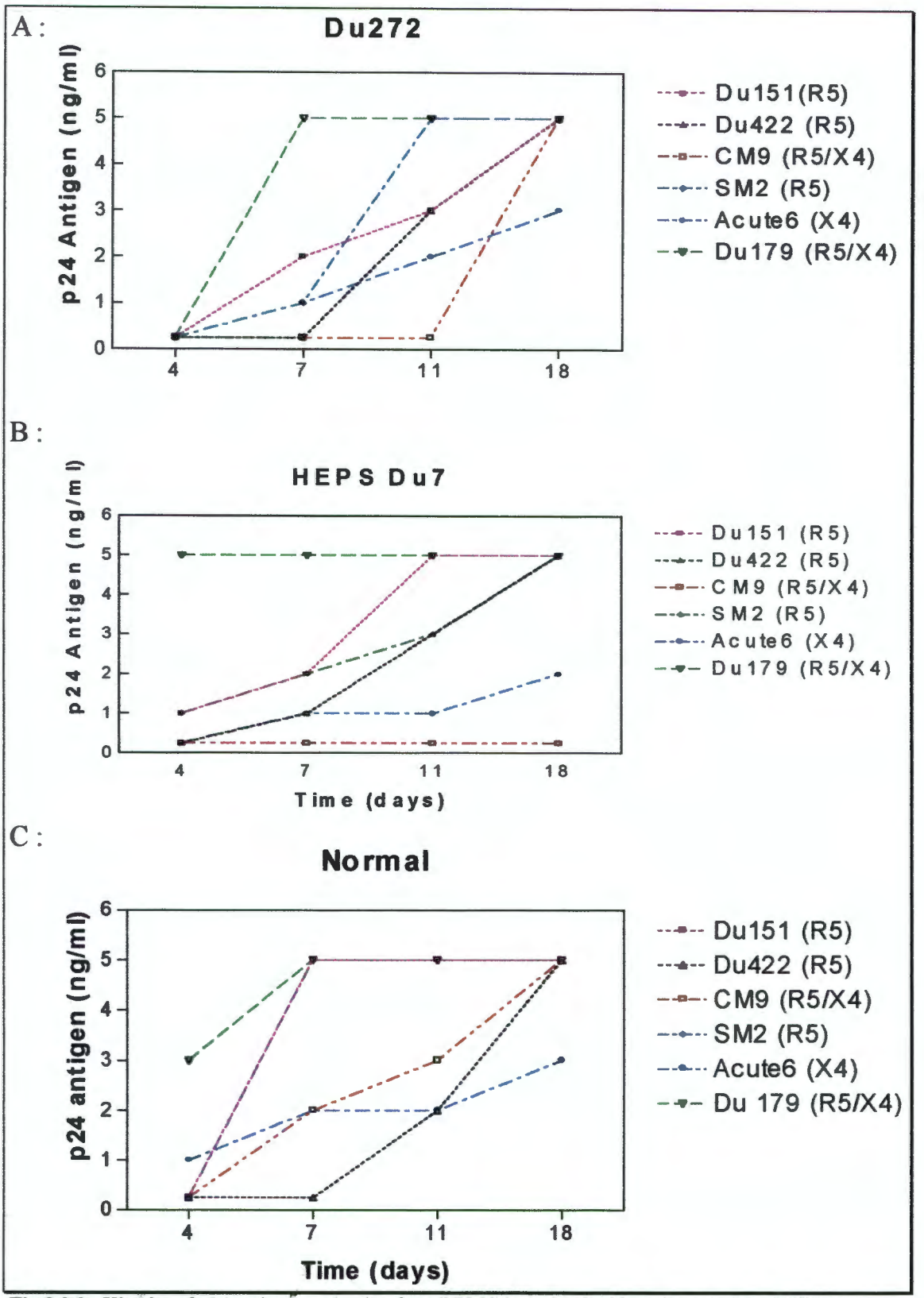


Fig 3.3.3 : Kinetics of p24 antigen production from PBMC inoculated with various strains of HIV-1 on **A)** HEPS Du272, **B)** HEPS Du7 and **C)** Control sample.

3.4) DISCUSSION

PBMC from all the HEPS sex workers and the control group were infected by most of the HIV-1 viral strains used. The capacity to infect PBMC was shown not to be tropism specific, in that R5, X4 and R5/X4 viral phenotypes could initiate a productive infection. The above results also demonstrate that sufficient HIV-1 coreceptors are expressed on the cell surface of HEPS PBMC to facilitate infection. The mechanism underlying resistance to HIV-1 infection in these women is by some means other than cells being resistant to viral entry.

Previous studies (Paxton, *et al.*, 1996 and Connor, *et al.*, 1996) have demonstrated that PBMC from HEPS individuals were resistant to infection by R5 viral strains due to a defective CCR5 protein (Paxton, *et al.*, 1996). In this study we showed that blood cells from HIV-1 seronegative sex workers from KwaZulu/Natal could be infected with R5, R5X4 and X4 viral strains indicating that both the CCR5 and CXCR4 coreceptors were functional. This supports studies reported in Chapter 2 showing that all HEPS women had the wild-type CCR5 gene with no evidence for the delta 32 mutation.

The levels of CCR5 expression have also been shown to correlate with susceptibility to infection with R5 strains, *in vivo* (Husman, *et al.*, 1999). In our study there was no difference in the expression levels of both CCR5 and CXCR4 between the HEPS and the control group. There was however, considerable individual variability in the expression levels of CCR5 on PBMC. The variability observed, could be attributed to polymorphisms on the promoter region of the CCR5 gene. The influence of the CCR5 promoter polymorphisms in HIV-1 pathogenesis is investigated in Chapter 4. A similar high level of variability has been reported by other authors and could be due to cytokine production, such as IL-2, IFNγ, and IL-10 which upregulate CCR5 on the cell surface (Loetscher, *et al.*, 1996 and Bleul, *et al.*, 1997). It was reported that CCR5 levels are higher in cells of HIV-1 infected and uninfected Africans compared to their Italian counterparts, possibly as a result of a higher level of immune activation (Clerici, *et al.*, 2000).

Thus resistance among these women is likely due to other factors, such as acquired anti-HIV immunity (Rowland-Jones, *et al.*, 1998) or other host factors operating in the context of the *in vivo* mileu of the mucosal interface.

The levels of CCR5 on CD4 T-cells measured in this study were significantly lower than has previously been reported in the literature (Paxton, *et al.*, 1998). It is possible that due to the fact that we used an anti-CCR5 monoclonal antibody (Mab) conjugated to FITC, which is a fairly weak fluorochrome. In order to test this, we conducted a control experiment using three samples stained with an anti-CCR5 antibody conjugated to either FITC or PE (done by Stephina Nyoka at NIV). The CCR5-PE antibody labeled approximately 30% more cells than CCR5-FITC antibody used in the study (Table 3.4.1). Thus it is likely that our data are underrepresented by a similar amount. Unfortunately we were not able to get fresh blood samples from HEPS sex workers to repeat this experiment. Nevertheless, since both HEPS and the control samples were stained with the same fluorochrome using the same protocol, our data remain accurate, i.e. there is no difference in the numbers of CCR5 positive cells between HEPS and control samples.

Table 3.4.1 : Percentage of CD4 T-cell positive for CCR5 comparison of CCR5-PE and CCR5-FITC Stephina Nyoka (NIV).

Sample	CCR5-PE	CCR5-FITC	Ratio (%)
Sample 1	48.8	9.1	18.6
Sample 2	24.2	5.4	22.3
Sample 3	32.8	10.3	31.6
Median	**32.8**	**9.1**	**27.7**

HEPS samples used in this study were collected in Durban and shipped overnight to Johannesburg. In order to simulate this, control samples that were collected locally were left overnight before staining. Previous studies showed that CXCR4 expression is up-regulated in stored blood samples (Wu, *et al.*, 1997). It is likely that our reported levels of CXCR4 are therefore an over-estimate of the true levels. Nevertheless our finding that there was no difference between HEPS and control samples in terms of CXCR4 expression is most likely accurate as all samples were treated similarly.

The β-chemokines RANTES, MIP-1α and MIP-1β have been shown to inhibit replication of primary R5 isolates, but not X4 strains of HIV-1 (Cocchi, *et al.*, 1995). It would therefore be of interest to quantify β-chemokines produced by the HEPS PBMC compared to amounts produced by a group of PBMC. HEPS PBMC could be protected from infection *in vivo* by high levels of β-chemokines that could block the binding of HIV-1 to the CCR5 coreceptor.

The infectivity of CD4 T-cells *in vitro* correlates with the risk of acquiring HIV-1 infection through sexual exposure. It would thus be expected that a large proportion of individuals who are resistant despite multiple sexual exposures would have relatively resistant CD4 T-cells. This was not the case among the group of HEPS individuals in this study. Cells from these individuals showed a high degree of susceptibility to HIV-1 infection *in vitro*. Clearly other mechanisms are in operation to protect these women from infection. Studies on similar cohorts have shown that HIV-specific immune responses, including mucosal IgA, are present in many of these individuals (Devito, *et al.*, 2000). Further studies are needed, particularly at the sites of infection, to determine if strong immune responses at the mucosal surface are responsible for protecting these women from HIV-1 infection.

CHAPTER 4

DETECTION OF CCR5 PROMOTER POLYMORPHISMS

4.1) INTRODUCTION

In addition to mutations within the CCR5 gene that abrogate coreceptor function, a number of non-coding polymorphisms in the CCR5 promoter region have been identified that that may affect the transmission of HIV-1 or the rate of disease progression (McDermott, *et al.*, 1998). Some of these polymorphisms in the CCR5 promoter region are thought to influence the expression levels of HIV-1 coreceptors, which may indirectly influence the susceptibility of PBMCs to HIV-1 viral entry. At least ten single nucleotide polymorphisms have been described of which five are most commonly described. These CCR5 promoter polymorphisms identified are, CCR5-59029G/A, CCR5-59353T/C, CCR5-59356C/T, CCR5-59402G/A and CCR5-59653C/T where the first letter indicates the wild type allele and the second the variant.

In a cohort of HIV-1 seroconvertors lacking both CCR5Δ32 and CCR2b-64I, individuals carrying the CCR5-59029G/G allele progressed to AIDS 3.8 years slower than individuals carrying the CCR5-59029 A/A allele (McDermot, *et al.*, 1998). When *in vitro* promoter activity was measured using a chloramphenicol acetyltransferase (CAT) reporter system, the CCR5-59029G had a lower activity than the CCR5-59029A variant. This implies that individuals with the CCR5-59029G allele would have a lower production of mRNA and thus lower levels of CCR5 expression. The CCR5-59029G allele was prevalent in all racial groups screened, with an allelic frequency of 43% in whites (n=473), 57% in blacks (n=41) and 53% in Asians (n=97) (McDermott, *et al.*, 1998).

A T-to-C polymorphism at position 59353 was also identified, which was associated with prolonged disease progression among HIV-1 infected individuals (Mummidi, *et al.*, 1998). The CCR5-59353C variant always occurred with CCR5-59029A. Individuals with CCR5-59353T, and not CCR5-59353C had prolonged disease progression either independently or in association with CCR5-5929G (Mummidi, *et al.*, 1998). The promoter activity between CCR5-59029A/59353C and CCR5-59029G/59353T have been compared *in vitro* utilising the CAT reporter system (McDermott *et al.*, 1998).

The constructs containing the CCR5 59029A/59353C polymorphisms resulted in 45% more activation than constructs containing the CCR5-59029G/59353T variants. Another

The constructs containing the CCR5-59029A/59353C polymorphisms resulted in 45% more activation than constructs containing the CCR5-59029G/59353T variants. Another polymorphism in the promoter region, where A substitutes G (CCR5-59402G/A) is thought to influence HIV-1 disease progression (Mummidi, *et al.*, 1997), however its functional significance has not been confirmed.

A polymorphism CCR5-59653C/T was identified by Kostrikis *et al.*, (1999) with the CCR5-59653T allele which was 100% associated with CCR2b-64I variant. However, in another study by Mummidi *et al.*, (1998) the link between CCR2b-64I and CCR5-59653T polymorphisms was identified, but in contrast with the report by Kostrikis *et al.*, (1999) the association between the two polymorphisms was not absolute. HIV-1 infected individuals with a CCR5-59653T allele progressed to AIDS or death slower than individuals carrying the CCR5-59653C allele (Mummidi, *et al.*, 1998).

Kostrikis, *et al.*, (1999) identified a polymorphic site in the CCR5 promoter region that influences the rate of perinatal HIV-1 transmission among infants born to HIV-1 infected mothers. To determine the effect of the CCR5-59356T variant in perinatal transmission, infants born to AZT (zidovudine) treated and untreated mothers were compared. Among the African American infants in the untreated group, there was a highly significant increase in HIV-1 transmission to infants with CCR5-59356T/T alleles (47.6%), compared to with those carrying none or one mutant allele (13.4 % and 14.1 %). The CCR5-59356T variant was found to be common among African Americans 20.6% (n=552), whereas it was rare among Caucasians 3.4% (n=174) and Hispanics 5.6% (n=113).

As polymorphisms in the CCR5 promoter region can have a direct or indirect influence in HIV-1 resistance, in this study we screened for the prevalence of five CCR5 promoter polymorphisms in a group of HEPS commercial sex workers (n=17). The summary of the five CCR5 promoter variants and their effects on HIV-1 pathogenesis is presented in Table 4.1.

A novel method was developed to screen for point mutations, using fluorescent hybridization probes on the LightCycler instrument (Roche, Germany). The reliability of this method of detection was ascertained by comparing results to those obtained using other conventional methods of genotyping such as PCR-RFLP and sequencing.

Table 4.1 : CCR5 promoter variants and their effects on HIV-1 pathogenesis

Polymorphisms	Effect on HIV-1 pathogenesis	Referecnce
CCR5-59029A/A	Accelerates disease progression	Mummidi, *et al.*, 1998
CCR5-59353T/T	Slows down disease progression	McDermott, *et al.*, 1998
CCR5-59356T/T	Protects against HIV-1 transmission	Kostrikis, *et al.*, 1999
CCR5-59402G/G	Function unknown	Mummidi, *et al.*, 1998
CCR5-59653T/T	Slows down disease progression when linked with CCR2b-64I	Samson, *et al.*, 1997

4.2) METHODS

The prevalence of these polymorphisms in 17 HEPS commercial sex workers were compared to a group of HIV-1 infected women (n=13) from the same cohort. Genomic DNA was isolated from PBMC using Pure PCR Template Preparation Kit (Boehringer Mannheim, Germany) according to the manufacture's instructions (see Appendix A1, for details).

Characteristics of HIV-1 seropositive individuals (n=13), from the same sex workers cohort are depicted in Table 2.2.3. These women had seroconverted in the six months prior to sample collection. These samples were used to compare the prevalence of the CCR5 promoter polymorphisms, SDF1-3'A and the CCR2b-64I variants to the HEPS cohort.

Table 4.2.1: Characteristics of the acute seroconvertors cohort*

Sample ID	#Age	Partners per/wk	Condom use (%)	Anal sex	**Years in sex work
Du11	23	7	>75	No	5.5
Du21	21	11	25-50	No	6.1
Du23	19	35	25-50	No	4.1
Du104	23	25	25-50	Yes	6.5
Du156	35	20	25-50	No	5.6
Du172	22	10	25-50	Yes	5.5
Du174	28	18	25-50	No	7.5
Du179	36	30	25-50	Yes	4.11
Du258	22	20	25-50	Yes	3.10
Du368	18	20	25-50	Yes	7.5
Du422	44	20	50-75	Yes	9.11
Du457	33	10	25-50	No	5.5
Du467	26	25	25-50	No	5.11

*Information provided by Dr. G. Ramjee, (MRC-Durban)
#Age at time of recruitment (Sep. 1996 - Oct.1997)
**Years in sex at the time of samples aquisition (Nov. 1999)

4.2.1 Detection of CCR5 promoter polymorphisms by PCR-RFLP.

a) Detection of CCR5-59029G/A

The screening for CCR5-59029G/A variant was performed by PCR-RFLP as described by McDermott *et al.*, (1998) and compared to fluorescent hybridization probes utilising the LightCycler method (4.2.3).

Essentially, a *Bsp* I restriction site is present in the CCR5-59029G allele but absent in the CCR5-59029A allele. After PCR using CCR5-59029G/A primer pair (Table 4.2.1), the amplicon is digested using *Bsp* I (New England Biotechnology) endonuclease. The predicted fragment size for the CCR5-59029A/A was a 275bp fragment, for CCR5-59029G/G allele 183 and 92bp and for CCR5-59029G/A was 275, 183 and 92bp.

Table 4.2.1 : Primers used in PCR for the detection of point mutations

Gene Variant	Primer Sequence
CCR5 59029G/A	5'-TTCCGTTTACAGAGAACAATA-'3 (Forward) 5'-GTGGGCTTTTGACTAGAT-'3 (Reverse)
CCR5 59353T/C	5'-ATATTTTCTAACAGATTCTG-'3 (Forward) 5'-CCGTTCCCCTACAAGAAACTCT-'3 (Reverse)
CCR5 59402G/A	5'-ATATTTTCTAACAGATTCTG-'3 (Forward) 5'-CCGTTCCCCTACAAGAAACTCT-'3 (Reverse)
CCR5 59653C/T	5'-TCATCTGGCCAGAAGAGCTG-'3 (Forward) 5'-ACGGTTATAACATCAAAGAT-'3 (Reverse)

Samples were amplified with 1.3U Taq polymerase (ICN Biochemicals, USA) in a total volume of 20µl. PCR mixes contained 1.0µl (1.25mM) $MgCl_2$, 2.0µl 10X buffer, 2.0µl dNTP (10mM), 0.5µl of each primer at 25pmol/µl, 5.0µl DNA and made up to 20.0µl with distilled water. Conditions of PCR amplification comprised 2 min denaturation at 94°C, 35 cycles of 30s at 94°C, 58°C and 72°C, and 1 min final extension at 72°C for 5 min in a Perkin Elmer 9600 thermocycler. PCR products were digested with *Bsp* I (New England Biotechnology) for 5 hours at 37°C and analysed on a 1.5 % agarose gel, electrophoresed in 1X TAE (Appendix C3) running buffer and stained with ethidium bromide (10ng/ml) at a concentration (v/v) of 1µl/100ml.

4.2.2 The LightCycler

a) Principle

The LightCycler is a microvolume fluorometer integrated with a thermal cycler that combines rapid-cycle DNA amplification with real-time fluorescence monitoring. Fluorescence monitoring of amplification is based on the concept that a fluorescence

between two adjacent fluorescently labeled sequence-specific hybridization probes (Wittwer, *et al.*, 1997).

The two probes are designed to hybridize to the same strand between two unlabeled primers. One of the probes is labeled at the 3' end with fluorescein and the other probe is labeled at the 5' end with a LightCycler red fluorophore (LC-Red 640). The anchor probe was labeled with LC-Red-640 on the 5' end and phosphorylated on the 3' end to prevent probe elongation. The sensor probe was labeled with fluorescein on the 3' end. After hybridization, the two probes are in close proximity, which allows fluorescent energy transfer between these fluorophores. The donor fluorophore, fluorescent (FLU) is excited by the light source of the instrument, and part of the excitation energy is transferred to LightCycler Red 640 (LC-Red 640), an acceptor fluorophore. The emitted fluorescence is measured and is proportional to the amount of specific target sequences in the reaction mixture. With an appended melting curve analysis of the obtained amplification products, the method is suitable for mutation detection (Mangasser-Stephan, *et al.*, 1999).

4.2.3) Detection of CCR5 Promoter Polymorphisms by fluorescent hybridization probes on the LightCycler

Screening for CCR5-59353T/C, 59402G/A and 59653C/T variant was done using the fluorescent hybridization probe method. Forward and reverse primers (Table 4.2.1) were used to amplify DNA together with hybridization probes to detect point mutations (Table 4.2.2). PCR and point mutation detections were performed in 20µl volumes in glass capillaries (Boehringer Mannheim, Germany). For all the point mutation detections the following protocol was followed : 6.4µl H_2O, 2.0µl $MgCl_2$ (2.5mM), 1.0µl Primer at 25pMol/µl each, 1.3µl (0.006µM) of each hybridization probe (TIB MOLBIOL, Germany) 2.0µl of DNA-Master Hybridization Probes (Roche, Germany) containing Taq DNA polymerase, reaction buffer, dNTP mixture and 10mM $MgCl_2$ as a 10X concentrate. Five micro litres of genomic DNA (20-200ng) was used as template. Fluorescently labelled hybridization probes were synthesized by TIB MOLBIOL (Germany).

Five micro litres of genomic DNA (20-200ng) was used as template. Fluorescently labelled hybridization probes were synthesized by TIB MOLBIOL (Germany).

Cycling conditions were as follows; 95°C for 10min (activation), 45 cycles at 95°C for 10 seconds, 55°C for 10 seconds and 72°C for 12 seconds with a ramping time of 20°C/s. After amplification, melting curves were generated following denaturation of the PCR product. The melting curves protocol followed was denaturation at 95°C for 10 seconds, annealing at 40°C for one minute and then slowly heating the samples to 95°C with a ramp rate of 0.2°C/s and simultaneous monitoring fluorescence decline. Melting curves were converted to melting peaks by plotting the negative derivative of the fluorescence with respect to temperature (-dF/dT) against time. The wild type allele produced specific peaks which differed in melting temperature (Tm) to those of the mutant allele.

a) Detection of CCR5 59029G/A

In addition to the PCR-RFLP method, CCR5-59029G/A was also genotyped using fluorescent labelled hybridization probes. CCR5-59029G/A primers (Table 4.2.1) were used to amplify DNA, and two hybridization probes, the anchor probe CCR5-029(A) and the sensor probe CCR5-029(S) were included (Table 4.2.2).

Table 4.2.2 : Hybridization probes used in the detection of point mutations.

Name	Probe Sequence
CCR5-029(A)	5'-LC-Red 640-CACTCTCCCCGTATCCCCTATCCCAC-P
CCR5-029(S)	5'-TTAACCCTGTGCCCCCTTTTTCT-'3FLU
CCR5-353(A)	5'-LCRed640-GATCTATTCTCTAGCTTATTTTAAGCTCAAC-P
CCR5-353(S)	5'-CCGTAAATAAACTTTCAGACCA-'3
CCR5-653(A)	5'-LC-Red 640-CAAACACCAAGTGCTCATACAATTATC-3'-P
CCR5-653(S)	5'-GGAAACCCATAGAAGACATTTG-'3-FLU

(A) = Anchor probe
(S) = Sensor probe

b) Detection of CCR5-59353 T/C

The CCR5-59353T/C variant was genotyped using fluorescent hybridization probes, and the results were ascertained by sequencing. CCR5-59353T/C primers (Table 4.2.1) were used to amplify DNA, hybridization probes CCR5-353(A) and CCR5-353(S) (Table 4.2.2) were used for genotyping for the CCR5-59353T/C variant.

c) Detection of CCR5 59402 G/A

The CCR5-59402G/A variant was genotyped using fluorescent hybridization probes, results were ascertained by sequencing using CCR5-59402G/A primers (Table 4.2.1).

d) Detection of CCR5-59653C/T

The CCR5-59653C/T variant was genotyped using fluorescent hybridization probes method. CCR5-59653C?T primers (Table 4.2.1) were used together with the CCR5-653(A) and CCR5-653(S) hybridization probes (Table 4.2.2).

4.2.4 Sequencing

A 297bp fragment between 59272 and 59569 bases of the CCR5 promoter region as numbered in the GenBank (U95626) was sequenced. The fragment included three known polymorphic sites, CCR5-59353T/C, CCR5-59356C/T and CCR5-59402G/A. Sequencing was performed by Mr Peter Owira of the Department of Medical Microbiology U.C.T using the ABI Prism 310 Genetic Analyzer (Perkin Elmer). The forward and the reverse strands were aligned and compared to a published sequence. Polymorphisms were identified by analysis of aligned sequences using the Auto Assembler 2.1 package of the Prism 310 Genetic Analyzer.

c) Detection of CCR5 59402 G/A

The CCR5-59402G/A variant was genotyped using fluorescent hybridization probes, results were ascertained by sequencing using CCR5-59402G/A primers (Table 4.2.1).

d) Detection of CCR5-59653C/T

The CCR5-59653C/T variant was genotyped using fluorescent hybridization probes method. CCR5-59653C/T primers (Table 4.2.1) were used together with the CCR5-653(A) and CCR5-653(S) hybridization probes (Table 4.2.2).

4.2.4 Sequencing

A 297bp fragment between 59272 and 59569 bases of the CCR5 promoter region as numbered in the GenBank (U95626) was sequenced. The fragment included three known polymorphic sites, CCR5-59353T/C, CCR5-59356C/T and CCR5-59402G/A. Sequencing was performed by Mr Peter Owira of the Department of Medical Microbiology U.C.T using the ABI Prism 310 Genetic Analyzer (Perkin Elmer). The forward and the reverse strands were aligned and compared to a published sequence. Polymorphisms were identified by analysis of aligned sequences using the Auto Assembler 2.1 package of the Prism 310 Genetic Analyzer.

Figure 4.2.1 is the schematic representation of the CCR5 gene including the promoter region (Kostrikis, *et al.*, 1999). The promoter region lies upstream of the 5' end of the exon four of the CCR5 gene. Appendix A5 depicts the actual CCR5 promoter sequence, the position of the primers and the position of the probes used to screen the CCR5 promoter polymorphisms in the study.

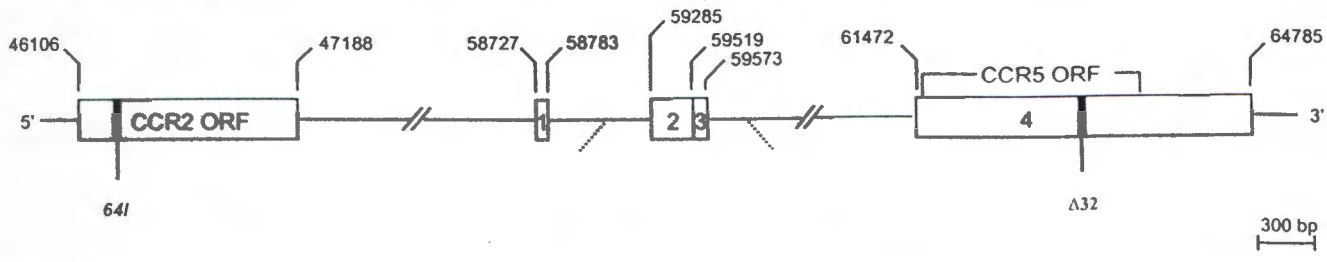


Fig. 4.2.1 : Schematic representation of the genomic organization of the CCR2 and CCR5 genes on chromosome 3 and locations of polymorphic sites in the regulatory region of CCR5 (59029-G/A, 59353-T/C, 59356-C/T, and 59402-A/G) and in the coding regions of CCR5 (Δ32) and CCR2 (641) genes. Open boxes indicate noncoding exons and open reading frames (ORF); lines signify introns. Exons and mutations are numbered based on the nucleotide position of the unpublished sequence with GenBank accession no. U95626. For each CCR5 polymorphism, the first letter indicates the wild-type nucleotide and the second indicates the mutant nucleotide.

4.3) RESULTS

The fluorescent hybridization probe method was developed to screen single base pair nucleic acid changes in the CCR5 promoter region. This method was compared to sequencing to detect the CCR5-59353T/C variants. The hybridization probes had different melting temperatures (Tm) depending on the sequence (Fig. 4.3.1). For samples homozygous for T variant had one peak with a Tm of 57°C and homozygotes C had one peak at a Tm of 52°C clearly differentiating the two alleles. Heterozygotes had two peaks at Tm of 57°C and 52°C. These results were confirmed by sequencing analysis where homozygotes yielded one peak on the chromatogram and heterozygotes two peaks at the same position.

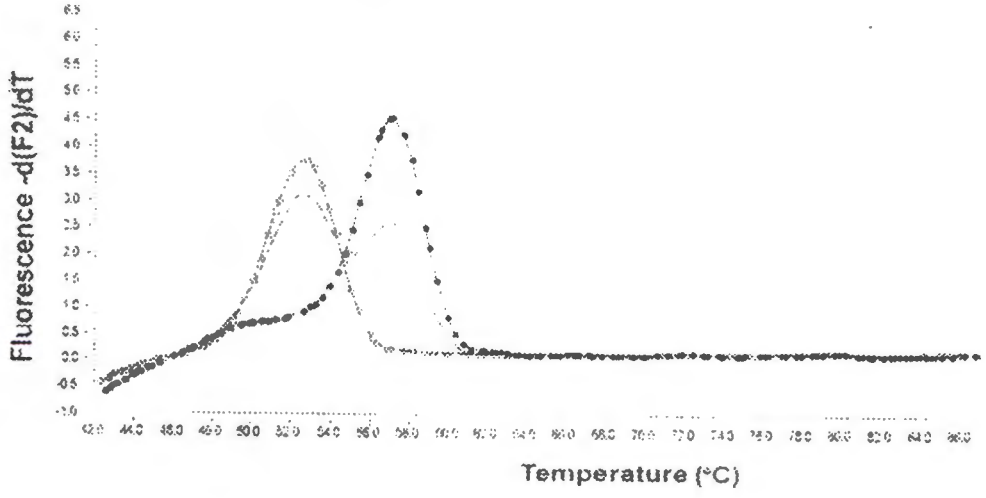


Fig. 4.3.1 : A representative graph illustrating the shift in melting curves due to point mutation in CCR5-59353T/C. The melting temperature (Tm) of the sensor probes are shown. Tm for 59353 T/T is 57°C, Tm for 59353 C/C is 52°C and Tm for 59353 T/C is 57°C and 52°C.

This correlation, along with large differences in melting temperature between the two probes, indicated that fluorescent hybridization probes were a reliable method for detection of point mutations (Table 4.3.1). Using this method six samples were found to carry the CCR5-59353T/T variant, eight carried the CCR5-59353T/C and three had the CCR5-59353C/C variants, resulting in an allelic frequency of 59% among the 17 HEPS women.

Among a group of HIV-1 infected women (n=13) used as a comparative group, three samples carried the T/T variant, five had the T/C and seven the C/C variants resulting in an allelic frequency of 39%.

Table 4.3.1 : Different methods used to detect point mutations in the CCR5 promoter region

Gene Variant	Method of detection		
	PCR-RFLP	*Hybridization*	*Sequencing*
CCR5-59029			
G/G	6	7	ND
G/A	9	8	ND
A/A	2	2	ND
CCR5-59353			
T/T	ND	6	6
T/C	ND	8	8
C/A	ND	3	3
CCR5-59402			
G/G	ND	ND	6
G/A	ND	ND	8
A/A	ND	ND	3
CCR5-59653			
C/C	ND	12	ND
C/T	ND	4	ND
T/T	ND	1	ND

However, when this method was compared with PCR-RFLP for detection of the CCR5-59029G/A variant, there was one discrepancy. RFLP detected a G to A base change through the elimination of a *Bsp* I restriction site in CCR5-59029A/A allele. The CCR5-59029A/A produced a band of 275bp, the CCR5-59029G/A produced bands of 275bp, 183bp and 92bp, and the CCR5-59029G/G produced bands of 183bp and 92bp. A typical gel electrophoresis result of this variant is depicted in Fig. 4.3.2. Among the HEPS population six samples were found to carry the CCR5-59029G/G variant, nine carried the CCR5-59029G/A variant and two had the CCR5-59029A/A variant (Table 4.3.1). The discrepancy could be attributed to a partial digest by the restriction enzyme resulting in nine instead of eight 59029G/A alleles detected by PCR-RFLP.

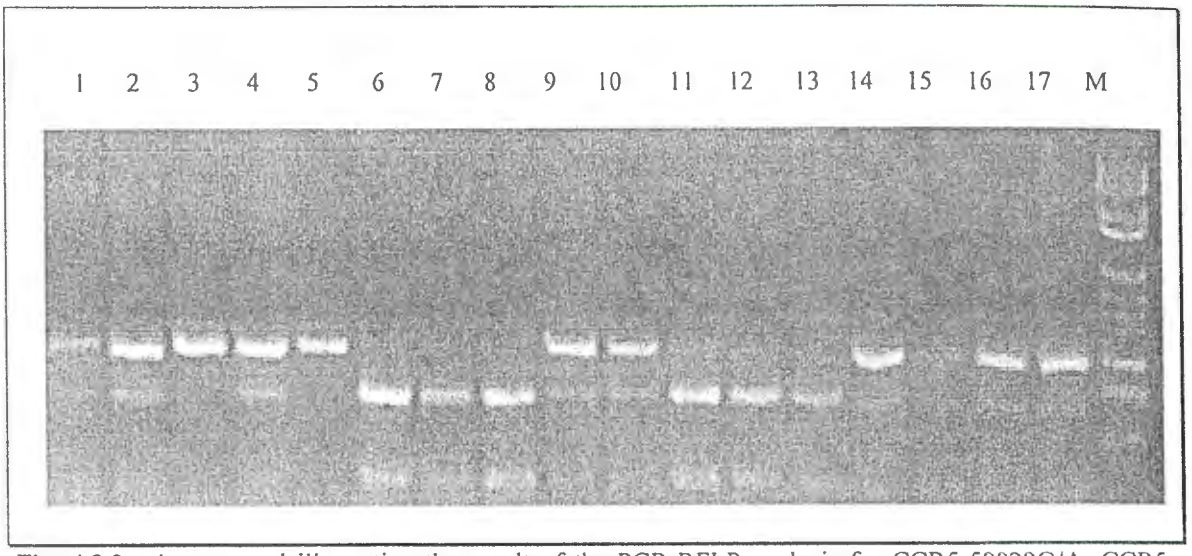


Fig. 4.3.2 : Agarose gel illustrating the result of the PCR-RFLP analysis for CCR5-59029G/A. CCR5-59029G/G illustrated in (lanes 6,7,8,11,12,13), CCR5-59029G/A (lanes 1,2,4,9,10,14,15,16,17) and CCR5-59029A/A (3 and 5).

Given the clear differentiation of melting curves (Fig. 4.3.3), samples homozygous for CCR5-59402G variant had one peak with a Tm of 68°C and homozygotes CCR5-59402A had one peak at a Tm of 61°C and CCR5-59402G/A (heterozygotes) had two peaks at Tm of 68°C and 61°C. The PCR-RFLP method has previously been shown to produce false positive (Chapter 2), the fluorescent hybridization probe method has been shown to be a more reliable method of detection and it was therefore applied to detect the CCR5-59653C/T.

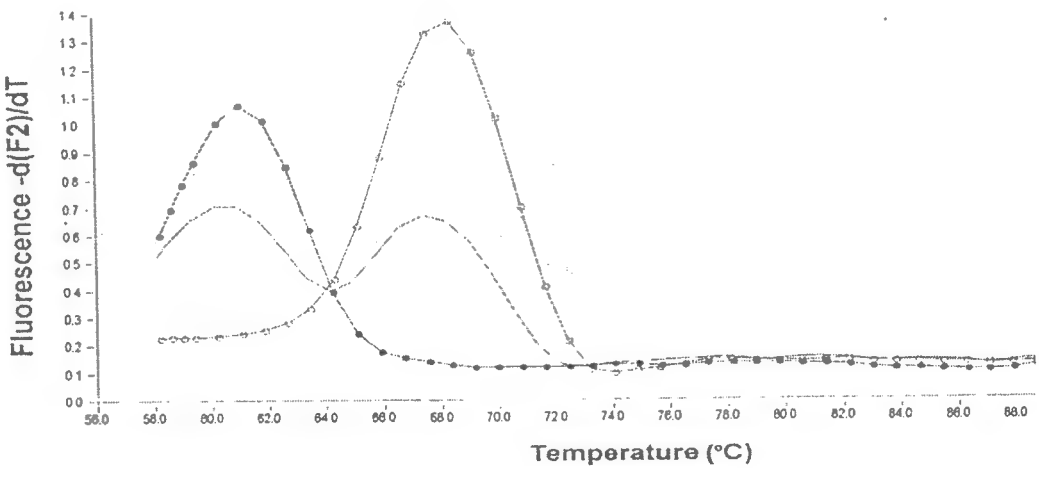


Fig. 4.3.3: A representative graph illustrating the shift in melting curves due to point mutation in CCR5-59029G/A. The melting temperature (Tm) of the sensor probes are shown. Tm for 59029G/G is 68°C, Tm for 59029A/A is 60.8°C and Tm for 59029G/A is 68°C and 60.8°C.

Twelve samples were found to carry the CCR5-59653C/C variant, four carried the CCR5-59653T/C and only one had the CCR5-59653T/T variant, resulting in an allelic frequency of 18% among the 17 HEPS women, a typical graph is depicted in Fig. 4.3.4. The allelic frequency of this variant was higher (27%) among the HIV-1 infected women, seven women had the CCR5-59653T/T variant, five carried CCR5-59653T/C and only one had the CCR5-59653T/T variant. The above data indicate that the fluorescent hybridization probe method, the preferred method for genotyping, once optimised it is more robust, reliable and quicker.

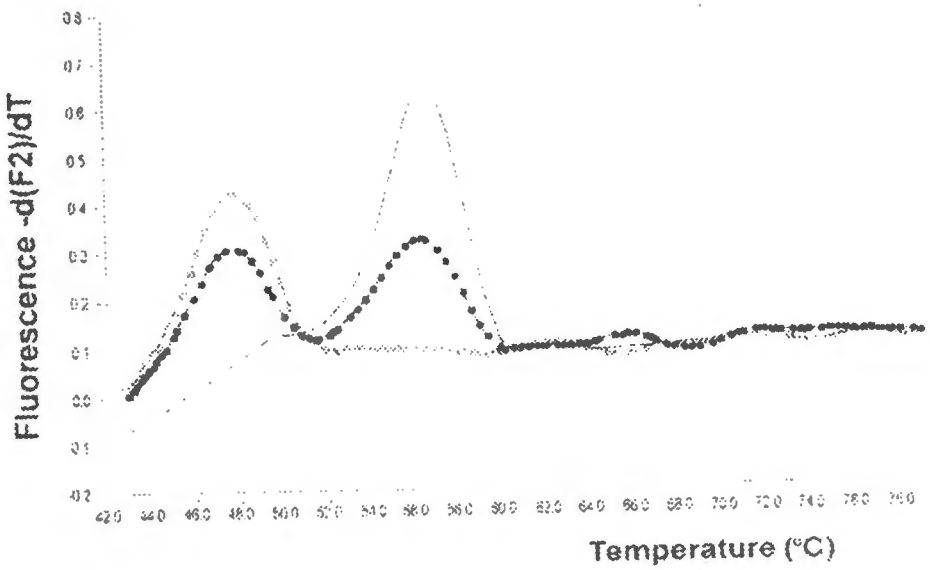


Fig. 4.3.4 : A representative graph illustrating the shift in melting curves due to point mutation in CCR5-59653C/T The Tm of the individual detection probes are shown. Tm for 59653C/C is 56.7°C, Tm for 59653 T/T is 48°C and Tm for 59653 C/T is 56.7°C and 48°C.

However, difficulty was experienced in designing certain probes such as probes to detect the CCR5-59402G/A variant. The CCR5-59402G/A and CCR5-59356C/T variants were both detected by sequencing in the HEPS population. All 17 women carried the wild type allele CCR5-59356C, and for the CCR5-59402G/A, six had the CCR5-59042G/G variant, eight carried the CCR5-59402G/A and three the CCR5-59402A/A variant. Absolute linkage was observed between CCR5-59353T/C and CCR5-59402G/A, with six women carrying both CCR5-59353T/T and CCR5-59402G/G,

eight carrying both CCR5-59353T/C and CCR5-59402G/A and three with CCR5-59353C/C and CCR5-59402A/A both with an allelic frequency of 59% (Fig. 4.3.5).

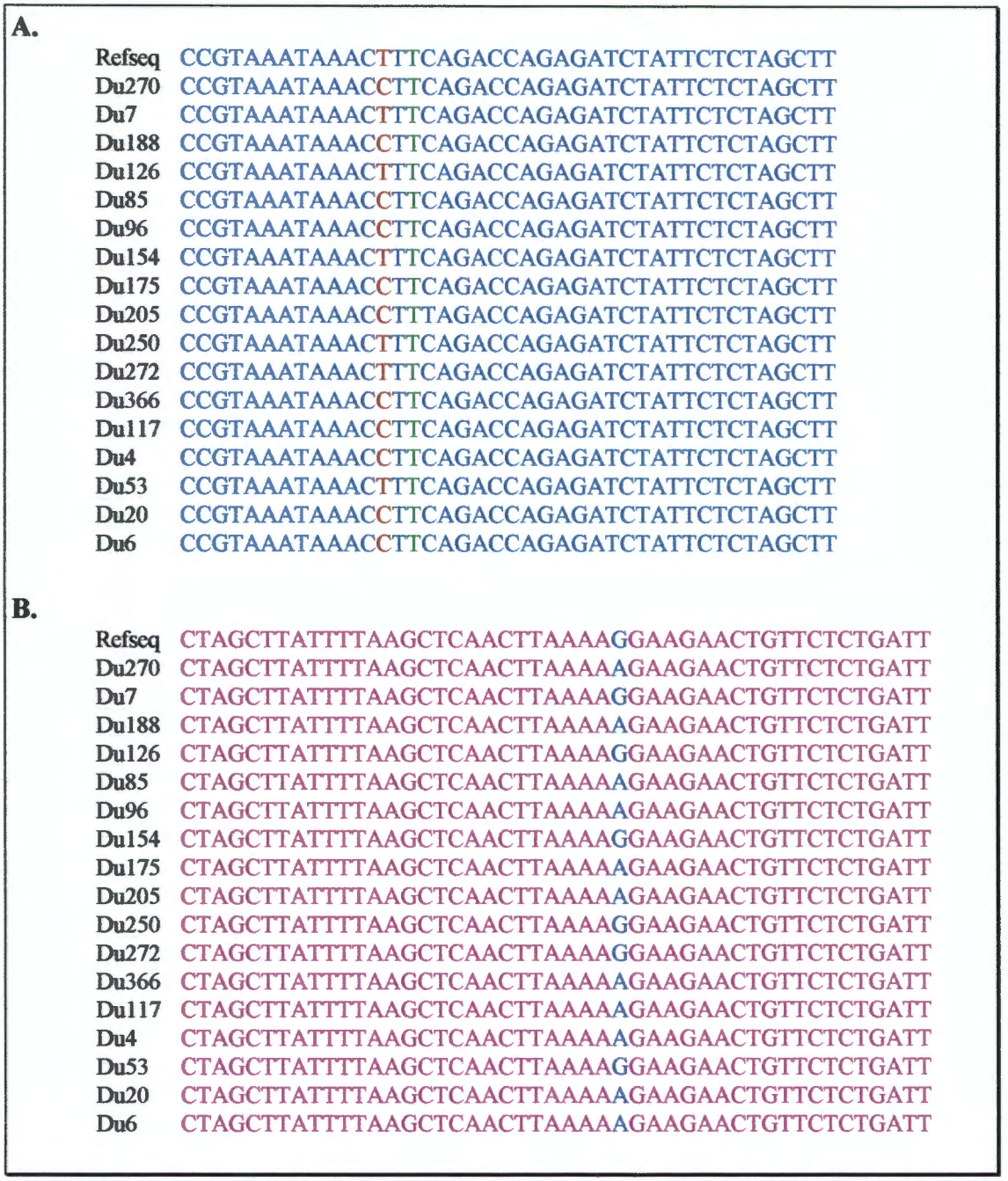


Fig. 4.3.5 : Sequence analysis of the CCR5 promoter region between the bases 59342 and 59381 (GenBank U 95626) including the polymorphic sites 59353T/C (Red) and 59356C/T (Green) **A**, and **B** 59402G/A polymorphic site (Blue) found between the bases 59382-59421.

Of the 17 HEPS samples genotyped by fluorescent hybridization probe method, seven carried the CCR5-59029 G/G variant, two had the CCR5-59029A/A and eight had the CCR5-59029G/A variant. A typical graph showing results of different alleles genotyped by the fluorescent hybridization probe is depicted in Fig. 4.3.3. The allelic frequency of 60% was obtained for the CCR5-59029G variant compared to 62% among HIV-1 infected women (Table 4.3.2).

Table 4.3.2 : Allelic frequencies of CCR5 promoter polymorphisms observed in the study population.

Gene Variant	HEPS (n=17)	HIV-1 (+) (n=13)	*Non-progressors
CCR5 59029			
G/G	7	5	9
G/A	8	6	11
A/A	2	2	7
Allelic frequency (%) **(A)**	**35**	**38**	**46**
CCR5 59353			
T/T	6	3	1
T/C	8	4	15
C/C	3	6	9
Allelic frequency (%) **(T)**	**59**	**39**	**44**
CCR5 59402			
G/G	6	3	5
G/A	8	4	11
A/A	3	6	9
Allelic frequency (%) **(G)**	**59**	**39**	**42**
CCR5 59653			
C/C	12	7	ND
C/T	4	5	ND
T/T	1	1	ND
Allelic frequency (%) **(T)**	**18**	**27**	**ND**

*Easterbrook, *et al.*, (1999). Study done on a population of non-progressors from a cohort in the United Kingdom. (ND = not done).

Table 4.3.2 depicts the allelic frequencies of the four CCR5 promoter polymorphisms studied in HEPS and HIV-1 infected women from the same cohort, compared to a group of non-progressors. Elevated frequencies were observed for the CCR5-59353T and the CCR5-59402G variants (59%) among the HEPS population compared to 39% found in the HIV-1 infected women. The allelic frequency of the CCR5-59029A variant was found to be lower lower (35%) among the HEPS women compared to the HIV-1 infected women (38%). An elevated allelic frequency was observed among the HIV-1 infected women (27%) compared to HEPS women (18%) for the CCR5-59653T variant.

Unlike McDermott (1998) who showed absolute linkage between CCR5-59029G and CCR5-59353T, in this study eight individuals who had the CCR5-59029G/A variant also carried the CCR5-59353T/C variant. However, of seven individuals carrying the CCR5-59029G/G only six had the CCR5-59353T/T variant. Three individuals carried the CCR5-59353C/C and only two carried the CCR5-59029A/A (Table 4.3.3).

There was absolute linkage between the CCR5-59353T/C and CCR5-59402G/A variants. Six individuals who had the CCR5-59353T/T variant also had the CCR5-59402G/G variant, eight individuals had both the CCR5-59353T/C and CCR5-59402G/A variants, finally three individuals had both CCR5-59353C/C and CCR5-59402A/A variants (Table 4.3.3).

Table 4.3.3 : Linkage among CCR5 promoter polymorphisms in the HEPS women.

Sample ID	59029	59353	59353	59402
Du4	G/A	T/C	T/C	G/A
Du6	G/A	T/C	T/C	G/A
Du7	G/G	T/T	T/T	G/G
Du20	G/A	T/C	T/C	G/A
Du53	G/G	T/T	T/T	G/G
Du85	G/A	T/C	T/C	G/A
Du96	G/A	T/C	T/C	G/A
Du117	G/A	T/C	T/C	G/A
Du126	G/G	T/T	T/T	G/G
Du154	G/G	T/T	T/T	G/G
Du175	G/G	T/C	T/C	G/A
Du188	G/A	T/C	T/C	G/A
Du205	G/A	C/C	C/C	A/A
Du250	G/G	T/T	T/T	G/G
Du270	A/A	C/C	C/C	A/A
Du272	G/G	T/T	T/T	G/G
Du366	A/A	C/C	C/C	A/A

The strong linkage between the CCR5-59029G/A and the CCR5-59353T/C variants was also observed among the HIV-1 infected women. Two individuals who carried the CCR5-59029G/A variant also carried the CCR5-59353T/C variant, four individuals who had the CCR5-59029G/G variant also had the CCR5-59353T/T variant and two individuals who carried both the CCR5-59029A/A and CCR5-59353T/T variants (Table 4.3.4).

Table 4.3.4 : Linkage among CCR5 promoter polymorphisms in HVI-1 infected women.

Sample ID	59029	59353
Du11	G/A	T/C
Du21	G/A	T/C
Du23	G/A	C/C
Du104	G/A	C/C
Du156	G/G	T/T
Du172	A/A	C/C
Du174	G/G	T/C
Du179	G/G	T/T
Du258	G/A	C/C
Du368	G/G	T/T
Du422	G/A	C/C
Du457	A/A	C/C
Du467	G/G	T/T

4.4) DISCUSSION

This is the first study looking at the frequency of promoter polymorphisms in South African Populations. The allelic frequency for the CCR5-59029A variant in the HEPS population (35%) was lower compared to the HIV-1 infected group (38%). Although other studies have reported a linkage between the CCR5Δ32 mutation and the CCR5-59029A variant (McDermott, *et al.*, 1998), this was not observed in the current study, as none of the 17 HEPS women had the CCR5Δ32 mutation (Chapter 2). Although the CCR5-59029G variant and the CCR5Δ32 mutations have been associated with delayed disease progression and protection from infection, the protective effect of CCR5-59029G relative to CCR5-59029A was found to be independent of any protection conferred by CCR5Δ32 mutation (McDermott, *et al.*, 1998). This is important especially to Africans who lack the CCR5Δ32 mutation.

An elevated frequency for CCR5-59353T (59%) in the HEPS population was observed compared to HIV-1 infected women (39%). There was a strong linkage between CCR5-59029G and CCR5-59353T variants in this study. But unlike other studies the linkage was not absolute. This study showed absolute linkage between CCR5-59353T and CCR5-59402G which has never been previously reported.

The allelic frequency for CCR5-59653T was higher in the HIV-1 infected group (27%) compared to the HEPS population (18%). The CCR5-59653T variant is associated with delayed disease progression (Clegg, *et al.*, 2000). In this study, two out of the three HEPS women who were heterozygous for CCR2b-64I were also found to carry the CCR5-59653T variant, an association previously identified by Kostrikis, *et al.*, (1998).

This study provides evidence of the polymorphic nature of the CCR5 gene and its possible implications in resistance to infection. This study also highlights the high level of linkage among CCR5 promoter variants. Some of the polymorphisms associated with HIV-1 disease may not be directly responsible for the clinical effects but are associated via linkage with other mutations or polymorphisms.

Two variants CCR5-59353T and CCR5-59402G, which were previously been identified to be associated with increased survival were also found in higher frequencies in the HEPS population compared with the HIV-1 infected group. It would be of interest to extend this study to include a large population to get more accurate data on background frequency.

Although the CCR5-59356T variant has not been reported to affect the transmission of HIV-1 by sexual intercourse, it was linked to increased risk of perinatal transmission. This variant was not identified in this study group and its role if any in HIV-1 transmission by sexual intercourse needs to be investigated.

Polymorphisms in the CCR5 promoter region are hypothesized to mediate the wide variation in CCR5 expression levels thereby influence HIV-1 disease progression. Identification of the functionally active polymorphisms and the mechanisms by which they mediate their effects will be an important step in identifying potential treatment targets, devising new strategies for slowing the progression of HIV-1 to AIDS and will aid in the development of an AIDS vaccine.

CHAPTER 5

CONCLUSIONS

The complexity of interpreting the impact of host polymorphisms on resistance is illustrated by the extent of linkage between genetic changes (Table 5.1), as well as the as well as variation in their frequency within different populations (Figure 5.2). No mutations associated with HIV-1 resistance (CCR5Δ32 and CCR5m303) were detected in the CCR5 coding region in any of the HEPS women screened. However, an elevated frequency in the SDF1-3'A variant was observed among the HEPS compared to the background population (Figure 5.1). The role of SDF1-3'A is uncertain as SDF it is the ligand for CXCR4 receptor, a receptor not associated with transmission. Further work needs to be done to understand the role of this mutation as controversial results have also been obtained in looking at its relationship to disease progression. Studies have shown this variant to delay disease progression (Winkler, *et al.*, 1998), while other studies have shown this variant to be associated with accelerated disease progression (Brambile, *et al.*, 2000). It is possible this mutation is acting as a marker for another biologically relevant mutation. It would be of interest include quantifying the levels of chemokines in the study population, since elevated chemokines have been reported to prevent HIV-1 infection.

For the HIV-1 virus to infect PBMCs it requires both the CD4 marker and a coreceptor. In the current study, all PBMCs tested were infectable by R5, R5X4 and X4 viral strains. This data confirmed with the genotyping results that revealed all samples are carrying the wild type CCR5 variant, furthermore the ability of PBMCs to support viral replication is indicative of functional chemokine receptors. Studies have shown that using low titre of virus, it is possible to identify low levels of resistance to infection (Castillo, *et al.*, 2000). It would be of interest to apply this method to PBMCs from HEPS to determine if there are more subtle levels of resistance that were not being detected when challenging with high titres of viral stocks.

The level of chemokine receptor expression levels are a determining factor in infection, it has been show that individuals who have high levels of expression are easily infected compared to individuals who have lower levels of chemokine receptors. The expression levels of chemokine receptors are also a manifestation of the genotype, individuals carrying the CCR5 allele express more levels of the chemokine receptor than individuals carrying the CCR5Δ32 variant. In this study all HEPS women had the wild type CCR5 allele, however, an apparent increase in frequency of certain promoter polymorphisms were identified. These polymorphisms have been reported to delay disease progression and do not appear to affect HIV-1 infection. However, it is possible that they may contribute to resistance by affecting levels of CCR5 expression. This study needs to be extended to determine the background frequency of promoter polymorphism in the South African population.

In conclusion, the precise mechanisms responsible for resistance to HIV-1 infection remains unclear. It is apparent that resistance is not absolute as there are women in this and other similar cohorts who seroconverted after being many years in sex work. It is possible that there are certain host genetic mutations that give some women the advantage over others. These could be CCR5 promoter polymorphisms which affect levels of expression or other as yet undiscovered polymorphisms. It is also probable that resistance is not due to one factor, but a combination of factors, and that immunological responses will impact on resistance.

Table5.1 : Summary of all genetic polymorphism detected in for each women.

Sample ID	Number of years in sex work	Host Genetic Variants Associated with HIV-1 Pathogenesis								
		Chemokine and Chemokine Receptors variants				*CCR5 promoter vairants*				
		CCR5Δ32	CR5m303	CCR2b-64I	SDF1-3'A	590229	59353	59356	59402	59653
Du4	4.10	wt/wt	wt/wt	wt/wt	wt/vt	G/A	T/C	C/C	G/A	C/T
Du6	6.5	wt/wt	wt/wt	wt/wt	wt/wt	G/A	T/C	C/C	G/A	C/C
Du7	13.5	wt/wt	wt/wt	wt/wt	wt/wt	G/G	T/T	C/C	G/G	C/C
Du20	26.1	wt/wt	wt/wt	wt/wt	wt/vt	G/A	T/C	C/C	G/A	C/C
Du53	5.5	wt/wt	wt/wt	wt/wt	wt/wt	G/G	T/T	C/C	G/G	C/C
Du85	5.1	wt/wt	wt/wt	wt/vt	wt/wt	G/A	T/C	C/C	G/A	C/T
Du96	6.5	wt/wt	wt/wt	wt/vt	wt/wt	G/A	T/C	C/C	G/A	C/T
Du117	7.5	wt/wt	wt/wt	wt/vt	wt/wt	G/A	T/C	C/C	G/A	C/T
Du126	8.5	wt/wt	wt/wt	wt/wt	wt/wt	G/G	T/T	C/C	G/G	T/T
Du154	4.8	wt/wt	wt/wt	wt/wt	wt/wt	G/G	T/T	C/C	G/G	C/C
Du175	7.10	wt/wt	wt/wt	wt/wt	wt/wt	G/G	T/C	C/C	G/A	C/C
Du188	5.11	wt/wt	wt/wt	wt/wt	wt/wt	G/A	T/C	C/C	G/A	C/C
Du205	8.0	wt/wt	wt/wt	wt/wt	wt/wt	G/A	C/C	C/C	A/A	C/C
Du250	8.5	wt/wt	wt/wt	wt/wt	wt/wt	G/G	T/T	C/C	G/G	C/C
Du270	9.1	wt/wt	wt/wt	vt/vt	wt/wt	A/A	C/C	C/C	A/A	C/C
Du272	14.1	wt/wt	wt/wt	wt/wt	wt/wt	G/G	T/T	C/C	G/G	C/C
Du366	7.5	wt/wt	wt/wt	wt/wt	wt/wt	A/A	C/C	C/C	A/A	C/C

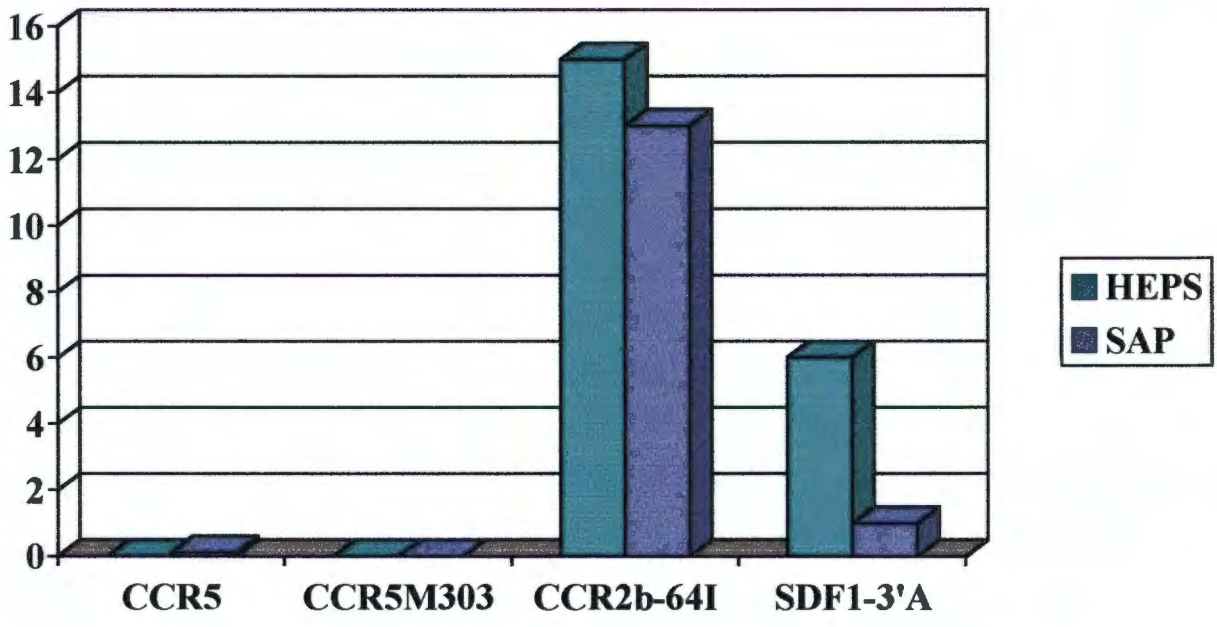


Fig. 5.1 : Chemokine and chemokine receptor allelic frequencies identified in HEPS women and in the back population (South African Population)* Study by Williamson, *et al.*, 2000.

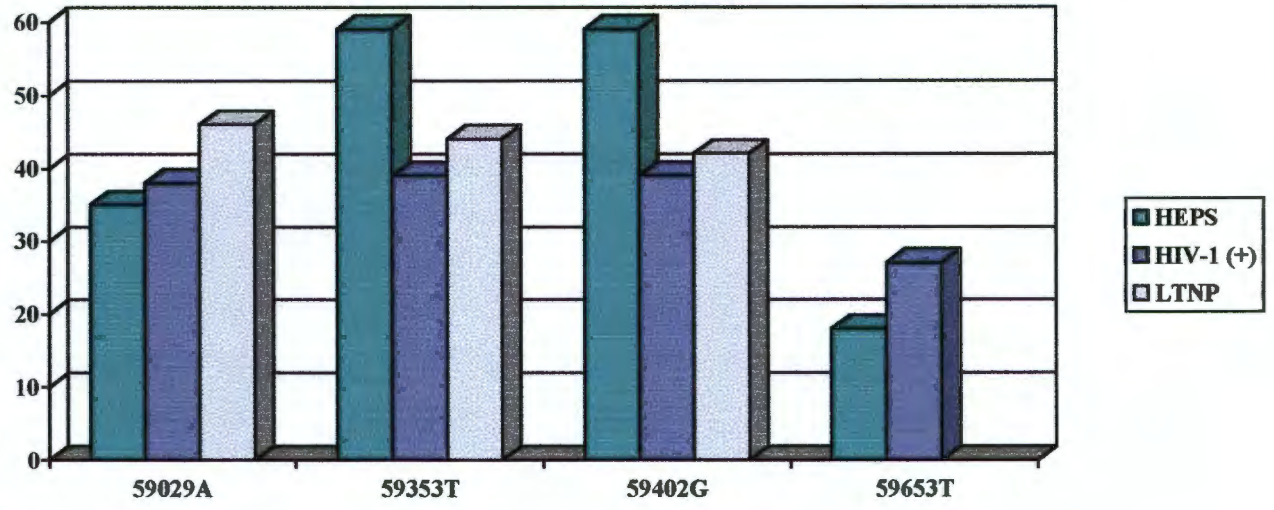


Fig. 5.2 : Frequencies of CCR5 promoter polymorphsms identified in HEPS women, HIV-1 infected women and in long term non-progressors.

APPENDICES

APPENDIX A : DNA TECHNIQUES

A.1 DNA extraction

DNA was extracted from PBMC by Pure PCR Template Preparation Kit (Boehringer Mannheim, Germany) according to the manufacturer's instructions. Blood sample (200µl) was added to equal volume of binding buffer, and 40µl of proteinase K was added and mixed. Incubated for 10 minutes at 72°C. Isopropananol (100µl) was added and the whole mixture added into combined high pure filter tube and centrifuged for a minute at 200g force. The column was washed twice with 500µl of wash buffer. DNA was eluted with 200µl of prewarmed (70°C) elution buffer. DNA aliquats (20µl) were stored at -20°C.

A.2 PCR protocols

a) CCR5Δ32 detection

The CCR5 gene was amplified by CCR5-F and CCR5-R primers (Table 1) in order to detect the CCR5Δ32 polymorphism. A band of 225bp was generated for the CCR5 (w/w) wild type variant. The PCR master mixes (20µl) contained the following :

* 2.5µl 10X PCR buffer
* 2.0µl of 10mM dNTP's
* 2.0mM $MgCl_2$
* 1.0µl of each primer (25pmol/µl)
* 5U/µl of *Taq* DNA polymerase

The reaction mixes were made up to 20ul with sterile water and 5.0µl of sample DNA added. 5µl of sterile water was added instead of sample DNA for the negative control. Amplification conditions in the Perkin-Elmer 9600 thermocycler :

* Initial denaturation at 95°C for 2 minutes
* 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds (30 cycles)
* Final extension at 72°C for 5 minutes

Agorose gel electrophoresis was used for the analysis of the PCR product.

b) CCR5m303 detection

The CCR5-m303 gene was amplified by CCR5-m303(F) and CCR5-m303(R) primers (Table 1) in order to detect the CCR5-m303 polymorphism. A band of 350bp was generated by PCR for RFLP analysis. The CCR5-m303 (w/w) wild type variant generated bands of 230 and 120bp. The PCR master mixes (20µl) contained the following :

* 2.5µl 10X PCR buffer
* 2.0µl of 10mM dNTP's
* 1.5mMl $MgCl_2$
* 1.0µl of each primer (25pmol/µl)
* 5U/µl of *Taq* DNA polymerase

The reaction mixes were made up to 20ul with sterile water and 5.0µl of sample DNA added. 5µl of sterile water was added instead of sample DNA for the negative control. Amplification conditions in the Perkin-Elmer 9600 thermocycler :

* Initial denaturation at 94°C for 2 minutes
* 94°C for 20 seconds, 58°C for 20 seconds and 72°C for 40 seconds (30 cycles)
* Final extension at 72°C for 6 minutes

Restriction endonuclease digestion ,

The restriction digest was carried out as described in Sambrook, *et.al* (1989). The following were added for a single digestion reaction :

* 2.5µl restriction endonuclease buffer (10X) (Boerhinger Manheim, Germany)
* 0.5µl (500U/µl) *Hinc II* (Boerhinger Manheim, Germany)
*2.0µl sterile water

The 5.0µl mix was added to the 20.0µl PCR product and incubated for 4 hours at 37°C. Agorose gel electrophoresis was used for the analysis of the PCR-RFLP product.

c) SDF1-3'A detection

The SDF-1 gene was amplified by SDF-1(F) and SDF-1(R) primers (Table 1) in order to detect the SDF1-3'A polymorphism. A band of 303bp was generated by PCR for RFLP analysis. The SDF-1 (w/w) wild type variant generated bands of 179 and 124bp, the homozygote mutant (SDF1-3'A/ SDF1-3'A) allele produced a band of 303bp and the heterozygote produced bands of 303, 175 and 112bp. The PCR master mixes (20µl) contained the following :

* 2.5µl 10X PCR buffer
* 2.0µl of 10mM dNTP's
* 1.5mM $MgCl_2$
* 1.0µl of each primer (25pmol/µl)
* 5U/µl of *Taq* DNA polymerase

The reaction mixes were made up to 20ul with sterile water and 5.0µl of sample DNA added. 5µl of sterile water was added instead of sample DNA for the negative control. Amplification conditions in the Perkin-Elmer 9600 thermocycler :

* Initial denaturation at 94°C for 2 minutes
* 94°C for 30 seconds, 65°C for 30 seconds and 72°C for 30 seconds (30 cycles)
* Final extension at 72°C for 6 minutes

Restriction endonuclease digestion ,

The restriction digest was carried out as described in Sambrook, *et.al* (1989). The following were added for a single digestion reaction :

* 2.5µl restriction endonuclease buffer (10X) (Boerhinger Mannheim)
* 0.5µl (1,000U/ml) *Msp I* (Boerhinger Mannheim, Germany)
*2.0µl sterile water

The 5.0µl mix was added to the 20.0µl PCR product and incubated for 4 hours at 37°C. Agorose gel electrophoresis was used for the analysis of the PCR-RFLP product

d) CCR2b-64I detection

The CCR2b gene was amplified by CCR2b(F) and CCR52b(R) primers (Table 1) in order to detect the CCR2b-64I polymorphism. A band of 183bp was generated by PCR for RFLP analysis. The CCR2b (w/w) wild type variant generated a band of 183bp, the homozygote mutant (64I/64I) allele produced bands of 165 and 18bp and the heterozygote produced bands of 183, 165 and 18bp. The PCR master mixes (20µl) contained the following :

* 2.5µl 10X PCR buffer
* 2.0µl of 10mM dNTP's
* 2.0mM $MgCl_2$
* 1.0µl of each primer (25pmol/µl)
* 5U/µl of *Taq* DNA polymerase

The reaction mixes were made up to 20ul with sterile water and 5.0µl of sample DNA added. 5µl of sterile water was added instead of sample DNA for the negative control. Amplification conditions in the Perkin-Elmer 9600 thermocycler :

* Initial denaturation at 94°C for 2 minutes
* 94°C for 20 seconds, 53°C for 20 seconds and 72°C for 20 seconds (5 cycles)
* 94°C for 20 seconds, 63°C for 20 seconds and 72°C for 20 seconds (25 cycles)
* Final extension at 72°C for 6 minutes

Restriction endonuclease digestion ,

The restriction digest was carried out as described in Sambrook *et. al* (1989). The following were added for a single digestion reaction :

* 2.5µl restriction endonuclease buffer (10X) (Separations)
* 0.5µl (1,000U/ml) *BSaB I* (Separations)
*2.0µl sterile water

The 5.0µl mix was added to the 20.0µl PCR product and incubated for 1 hour at 60°C. Agorose gel electrophoresis was used for the analysis of the PCR-RFLP product.

A.3 Electrophoresis

Agarose gel electrophoresis was performed as described in Sambrook, *et al.*, (1989). The agarose powder was melted with frequent swirling to prevent clumping in 1X TAE at concentrations of one to 2.5%, depending on the size of the DNA fragments to be separated. Once melted, ethidium bromide (10ng/ml) stain was added to a final concentration of 1ug/100ml of agarose. The agarose gel slabs were allowed to set at room temperature for 30-60 minutes after which the well combs were removed. Samples were mixed with 1/5 their volume of loading dye and added to the 1X TAE submerged wells. The agarose gel was electrophoresed at 85V for an hour until the fragments were separated. The DNA fragments were then visualised using the ultraviolet transilluminator box and photographed with the computerised gel imager by Kodac digital Science1D-camera. Molecular weight marker VI (Boerhinger Mannheim) was used to determine DNA fragment size.

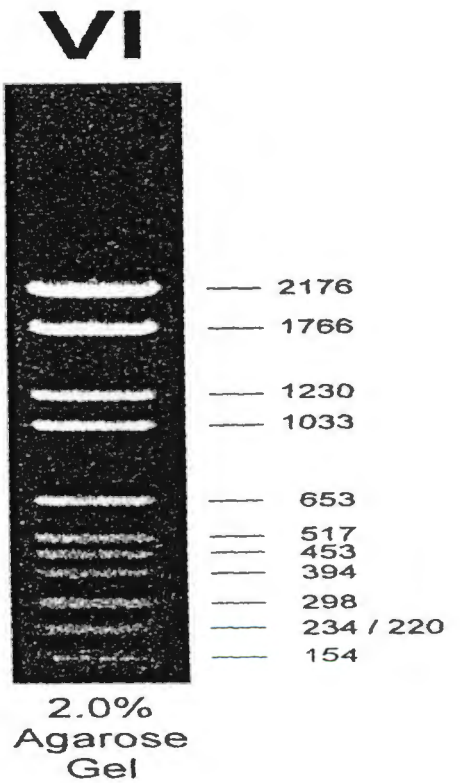


Fig. 1: Marker VI

A.4 LightCycler

a) Product Description

Light Cycler (LC) System is a fast PCR amplification and analysis system. Central to the function of the LC system are the use of air as a medium for heating and cooling and the use of capillaries as reaction vessels for efficient temperature exchange between the air and the reagents in the capillary. The capillaries are made of borosilicate glass. Fluorescence data is recorded by the optical unit of the instrument and are displayed on a computer screen as PCR proceeds and are stored for further analysis.

Fig. 3: Schematic representation of the working components of the LightCycler.

b) Principle

The Hybridization Probes format is a fluorescent format used as a reporter in the LC system. The Hybridization Probes, labelled with two different fluorescent molecules, hybridize next to each other on the target DNA molecule. The first fluorescent dye, the donor dye fluorescein, is excited at 470nm by the light source of the LC instrument. Instead of emitting light at 530nm, the fluorescein can transfer its energy in a non-fluorescent manner to an acceptor dye, LightCycler-Red 640(LC-Red 640) in a process called Fluorescence Resonance Energy Transfer (FRET).

The principle behind point mutation detection, is based on the usage of two distinct hydridization probes (short oligonucleotides). One hydridization probe matches the wild-type sequence with the variable nucleotide in the middle of the sequence, and has LC-Red640 as the fluorophore at its 5' end (sensor probe), a second hybridization probe (anchor probe) is located upstream at a distance of one nucleotide and is labelled with fluorescein at its 3' end. After hybridization, the two probes are in close proximity, which allows fluorescence energy transfer between the fluorophores. The donor fluorophore fluorescein is excited by the light source of the instrument, and part of the excitation energy is transferred to LC-Red 640, an acceptor fluorophore.

Upon completion of PCR, the PCR product is denatured, then the temperature is lowered to 40°C to facilitate binding of the hybridization probes, thus generating maximum fluorescence, and slowly raised to 95°C to permit melting of the detection probe, which is monitored by the decline of the fluorecence. Melting curves are automatically converted to melting peaks, allowing easy distinction of wild type from mutant allele. The distinct peaks are specific to either allele (wild or mutant) and are indicative of the temperature at which the sensor probe is detached from the PCR template.

c) Point mutation detection protocol

The CCR5 promoter polymorphisms were detected by LC and each allele was characterised by a specific melting temperature (Tm).
The PCR master mixes (20µl) contained the following :

* 1.3µl of each hybridization probe (0.006µmol/µl) (Table1)
* 2.0mM $MgCl_2$
* 1.0µl of each primer (25pmol/µl) (Table1)
* 2.0µl of master mix (Taq DNA polymerase, reaction buffer, dNTP mixture and 10mM $MgCl_2$ as a 10X concentrate)

The reaction mixes were made up to 20µl with sterile water and 5.0µl of sample DNA added. 5µl of sterile water was added instead of sample DNA for the negative control.
Amplification conditions were as follows :

* Activation; 95°C for 10 minutes
* PCR; 95°C for 10 seconds, 45°C for 10 seconds and 72°C for 12 seconds (42 cycles)
* Melting; 95°C for 1 minute, 40°C for 1 second and to 95°C at a rate of 0.2°C
* Cooling at 40°C for 2 seconds

The resulting melting curves were converted to melting peaks and used to determine the melting temperatures (Tm), each peak characteristic of a particular allele variant.

The resulting melting curves were converted to melting peaks and used to determine the melting temperatures (Tm), each peak characteristic of a particular allele variant.

Table 1 : Probes and primers used for point mutation detection on the LightCycler

Variant	Primer Pair (25pmol/)	Probe Pair (0.006 lmol/ l)
59029 G/A	5 -GTGGGCTTTTGACTAGAT- 3 5 -TTCCGTTTACAGAGAACAATA- 3	5 -LC-Red 640-CACTCTCCCCGTATCCCCTATCCCAC- 3p 5 -TTAACCCTGTGCCCCCTTTTTCT- 3FLU
59353 T/C	5 -ATATTTTCTAACAGATTCTG- 3 5 -CCGTTCCCCTACAAGAAACTCT- 3	5 -LC-Red -AGATCTATTCTCTAGCTTATTTTAAGCTCA AC-3 p 5 - CCGTAAATAAACTTTCAGACCA- 3-FLU
59402 G/A	5 -CCGTTCCCCTACAAGAAACTCT- 3 5 -ATATTTTCTAACAGATTCTG- 3	5 -AGATCTATTCTCTAGCTTATTTTAAGCTCAAC- 3-FLU 5 -LC-Red 640-TAAAAAGAAGAACTGTTCTCTGATTC - 3 p
59653 C/T	5 -TCATCTGGCCAGAAGAGCTG- 3 5 -ACGGTTATAACATCAAAGAT- 3	5 -LC-Red 640-CAAACACCAAGTGCTCATACAATTATC- 3p 5 -GGAAACCCATAGAAGACATTTG- 3-FLU

Fluorescent labelled hydridization probes were synthesized by TIB MOLBIOL (Germany).

A.5 CCR5 Promoter Sequence

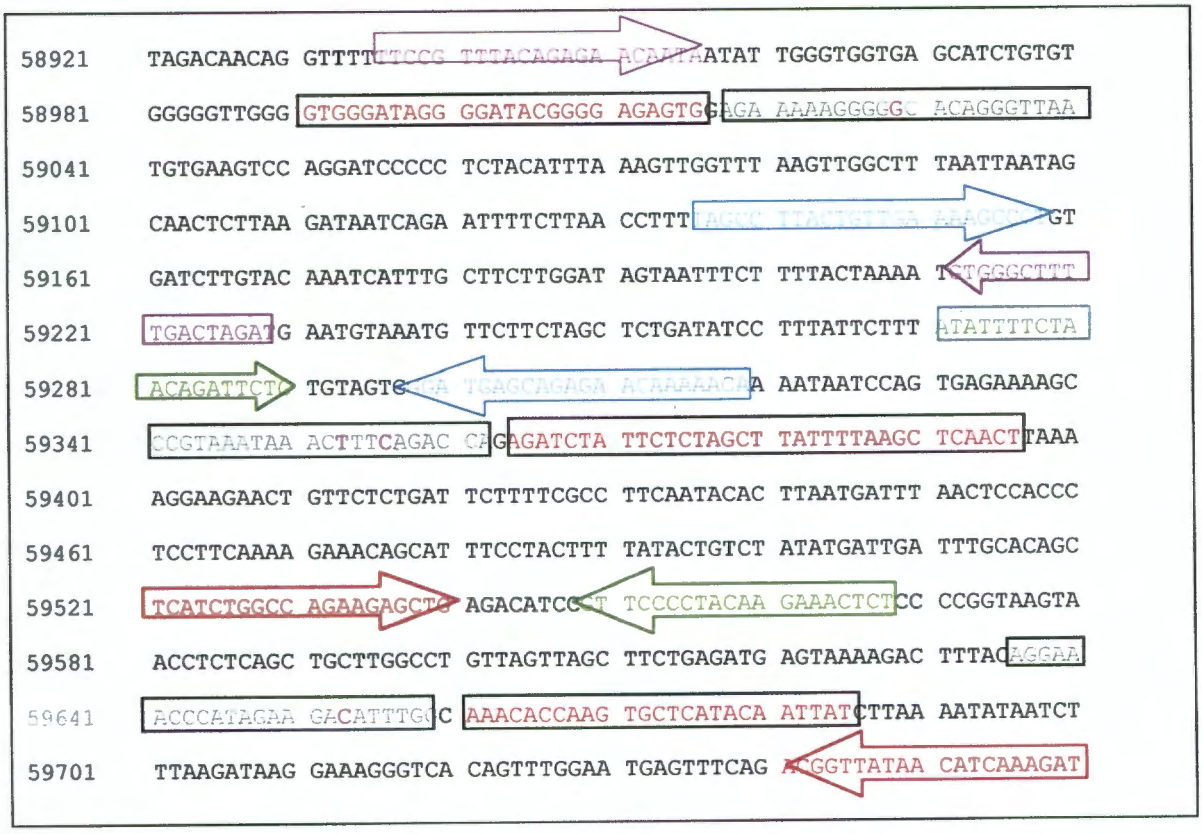


Fig 5 : CCR5 promoter sequence (U95626), arrows indicate the position and direction of the primers. Rectangles represent the position of the probes, in red are the anchor probes and in blue are the sensor probes. Within the sensor probes indicated in purple are the actual polymorphic sites, 59029G/A, 59353T/C, 59356 C/T and 59653C/T.

APPENDIX B: TISSUE CULTURE TECHNIQUES

B.1 Isolation of PBMC

PBMC were isolated from approximately 20ml whole blood by Ficoll hypaque density gradient centrifugation. The tubes were centrifuged at 250g for 15 minutes and the plasma collected and stored. The buffy coat was removed and mixed with 5ml of phosphate buffered saline (PBS). This cell suspension was carefully layered onto 5ml Ficoll in a 15ml conical tube and centrifuged at 400g for 30 minutes.

The mononuclear cells which sedimented on top of the Ficoll layer were carefully removed and washed twice with PBS then resuspended in 1ml medium. Viable cells were counted using trypan blue exclusion criteria under a haemocytometer.

B.2 *In vitro* stimulation

PBMC from 17 HEPS and 5 controls (laboratory co-workers considered to be at low risk for HIV-1 infection), were isolated from whole blood by standard Ficoll-hypaque density centrifugation. The cells were stimulated with phytohemagglutinin (PHA) 5% for 72hours in RPMI 1640 medium supplemented with 20% fetal calf serum - 100 U/ml of streptomycin and penicillin (complete medium) at a density of 1×10^7 in a 15 ml flask at 37°C.

B.3 *In vitro* infection assay

PBMC from HEPS and control subjects were plated at a density of 2×10^5 cells per well in 96-well plates, in complete RPMI medium containing IL-2 (5%) in a volume of 100µl. Cultures were inoculated with HIV-1 supernatants in a final volume of 200µl. Virus replication was assessed at regular intervals (days 4,7, and 14) over a 2 week period by measuring the production HIV-1 p24 antigen in the culture supernatants by an ELISA assay. Half of the supernatant from the cultures was removed for the p24 assay, and replaced with fresh media containing IL-2 (5%).

B.4 p24 ELISA

Each well of a high binding 96 well plate was coated with 100µl of anti-HIV-1 p24 antibody (D7320, Áalto Bio, Ireland) and incubated over night at room temperature. Plates were washed twice with Tris Buffered Saline (TBS) followed by 100µl of 2% milk in TBS and incubated for 1 hour at room temperature.

Plates were washed with TBS and 100µl of culture supernatant plus 100µl of 1% Empigen was added to each well. Plates were incubated for 2 hrs at 37°C, after which they were washed thoroughly with TBS.

To each well 100µl of anti-HIV1 p24 antibody conjugated to alkaline phosphatase (EH12AP, Aalto Bio, Ireland) in 2% sheep serum (Sigma) and 0.05% Tween in TBS was added and incubated for 1 hour at 37°C. Plates were washed 4x with AMPAK buffer (Dako, Denmark) using an automated washer, followed by the addition 50µl substrate (Dako, Denmark) and incubated for an hour at room temperature. After this 50µl of amplifier (Dako, Denmark) was added for 3 minutes at room temperature and then 50µl of stop solution (0.5 M HCL) was added. The optical density (OD) was read using an automatic plate reader at 492nm.

APPENDIX C: MATERIALS

C.1 Loading buffer (10x concentrated)

Ficoll (Type 400, Pharma)	20%
Na_2EDTA	0.1M
SDS	1%
Bromophenol blue	0.25%
Xylene Cyanol	0.25%

The above mentioned ingredients were mixed with distilled water to the required volume. The solution was dispensed into 10ml aliquots and kept at room temperature (Ausebel, *et al.*, 1987).

C.2 Phosphate Buffered Saline (PBS)

NaCl	8.0g
KCL	0.2g
Na_2HPO_4	0.12g

The above ingredients were dissolved in 900ml of distilled water and pH was adjusted to 7.5. Distilled water was added to a final volume of 1 000ml and the solution was dispensed into aliquots of 200ml. PBS was sterilized by autoclaving and stored at room temperature.

C.3 Tris-acetate (TAE) Buffer

50x concentrated:	
TRIS	242g
Glacial acetic acid	57.1ml
EDTA	100ml

The above mentioned ingredients were mixed with distilled water to a final volume of 1 00ml, autoclaved and stored at room temperature (Sambrook, *et al.*, 1989).

C.4 Trypan Blue

Trypan Blue powder	5g
Physiological saline	100ml

The powder was dissolved in the saline solution and filtered through Whatman number1 paper. The dye was sterilized by filtering through a 0.2μm filter, dispensed into 2ml aliquots.

C.5 Ethidium Bromide

10ng	Ethidium bromide

Dissolved in 1ml of distilled water

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