Molecular-genetic Investigation into Host Susceptibility and Variability to HIV/AIDS in the South African Population

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Declaration:

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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Date :

SUMMARY

The risk of human immunodeficiency virus type-1 (HIV-1) infection and rate of progression towards development of the acquired immunodeficiency syndrome (AIDS) is determined by a combination of viral characteristics, immune function and host genetic variation. Although mutations of the chemokine and chemokine co-receptor genes and allelic variation of the major histocompatibility complex (MHC) have been studied extensively, variation in these host genetic factors does not explain the differences in HIV/AIDS susceptibility in all cases. This study represents the first analysis of new candidate genes implicated in iron metabolism and immune function in relation to HIV-1 disease in the African context. Both case-control association studies and genotype-phenotype correlations were performed to determine the potential functional significance of genetic variants that may be involved, either directly or indirectly, in susceptibility to HIV-1 disease in the South African population.

Genotyping was performed to identify potentially important polymorphisms in the solute carrier family 11 member 1 (*SLC11A1*), haemochromatosis (*HFE*) and protein-tyrosine phosphatase receptor-type C (*PTPRC/CD45*) genes in HIV-seropositive versus HIV-seronegative individuals. This was followed by HLA-B27 genotyping in HIV-1 infected individuals with known disease status to determine the potential impact of combined genotypes for different mutations identified in the same study cohort. Preferential association with any of the mutations screened for in the *CCR5*, *SLC11A1*, *HFE* or *CD45* genes were not detected in HLA-B27 positive individuals identified. These findings were in accordance with the independent protective role of HLA-B27 in relation to disease progression in HIV-1 infected individuals.

Although differences in allelic distribution were not significant between the study groups, an apparently African-specific mutation 32A→G, identified in an exonic splicing silencer element (ESS-1) of the *CD45* gene, appeared to predominate in HIV-1 infected subjects with WHO Class I disease status and slow progression to AIDS. This mutation was present in 35.7% (5/14) of HIV-seropositive individuals with WHO Class I disease status, whilst absent in 22 HIV-seropositive patients with rapid disease progression. This finding may be related to differences in proportions of both CD4+ and CD8+ subsets observed following flow cytometry (FACs) analysis in two HIV-seropositive individuals with mutation 32A→G, compared with an HIV-seropositive individual without this mutation.

Analysis of the iron-related *SLC11A1* and *HFE* genes did not reveal significant associations with modified risk of HIV-1 infection or progression to AIDS in our predominantly African study population. However, the effect of the virus on iron metabolism was demonstrated for the first time at the DNA level. Haemoglobin levels were significantly reduced in both HIV-seropositive (P=0.004) and HIV-seronegative (P=0.02) Black Africans with mutation IVS3-48c→g in the *HFE* gene, compared with mutation-negative individuals in both groups. Since this effect was more pronounced in HIV-infected individuals compared with controls, presence of the *HFE* mutation seems to result in an even stronger effect on haemoglobin levels, which may be related to the acute phase response following virus infection. This effect possibly results from genetic variation in a nearby gene involved in innate immunity, most likely in the HLA region on chromosome 6. It therefore seems possible that genetic variation in any of the host molecules involved in response to infection could contribute to clinical outcome.

The significance of the multitude of host genetic factors investigated in this study, or previously implicated in susceptibility to HIV-1 infection and disease progression, revealed a complex interrelationship between the host and HIV-1. In some instances the disease process following HIV-1 infection depends on combined effects of different mutations occurring in the same individual, while independent effects of specific genes in conjunction with environmental influences may explain diverse clinical outcomes in others.

OPSOMMING

Die risiko vir menslike immuniteitsgebrek virus tipe-1 (MIV-1) infeksie en die progressie-tempo vir ontwikkeling van die verworwe immuniteits gebrek sindroom (VIGS) word hoofsaaklik deur 'n kombinasie van virale eienskappe, immuunfunksie en gasheer genetiese variasie bepaal. Alhoewel mutasies van die chemokien en chemokien ko-reseptor gene en alleliese variasie van die major weefsel-verenigbaarheidskompleks (MWVK) reeds omvattend bestudeer is, verklaar variasie van hierdie gasheer genetiese faktore nie noodwendig verskille in vatbaarheid vir MIV/VIGS in alle gevalle nie. Hierdie studie verteenwoordig die eerste analise van nuwe kandidaatgene, geïmpliseer in yster metabolisme en immuunfunksie in die konteks van MIV-1 siekte in Swart bevolkingsgroepe. Beide gevalle-kontrole assosiasie-studies en genotipe-fenotipe korrelasies is uitgevoer om moontlik betekenisvolle verwantskappe met genetiese variante te bepaal, wat moontlik direk of indirek betrokke mag wees in vatbaarheid vir MIV-1 siekte in die Suid Afrikaanse populasie.

Genotipering van die solute draer familie 11 lid 1 (*SLC11A1*), hemochromatose (*HFE*) en protein-tirosien fosfatase reseptor-tipe C (*PTFRC/CD45*) gene is uitgevoer in beide MIV-seropositiewe en MIV-seronegatiewe individue. Daaropvolgend is genotipering van die menslike leukosien antigeen-B27 (MLA-B27) uitgevoer in MIV-1 geïnfekteerde individue met bekende siekte-status, om die potensiële impak van gekombineerde genotipes te bepaal vir verskillende mutasies wat in dieselfde studie populasie geïdentifiseer is. Voorkeur-assosiasie is nie waargeneem vir enige van die mutasies waarvoor geanaliseer is in die *CCR5*, *SLC11A1*, *HFE* of *CD45* gene nie. Hierdie bevinding is in ooreenstemming met die onafhanklike rol van MLA-B27 in verwantskap met siekte progressie in MIV-1 geïnfekteerde individue.

Alhoewel die alleelverspreiding van 'n Afrika-spesifieke mutasie 32A→G, wat in 'n eksoniese splytingsdemper-element (ESS-1) van die CD45 geen geïdentifiseer is, nie statisties betekenisvolle verskille getoon het tussen studiegroepe nie, is die mutasie oorheersend waargeneem in MIV-1 geïnfekteerde individue met WGO Klas I siekte-status en stadige progressie na VIGS. Hierdie mutasie was teenwoordig in 35.7% (5/14) van HIV-seropositiewe individue met WGO Klas I siekte-status, terwyl dit afwesig was in 22 HIV-seropositiewe pasiënte met vinnige siekteprogressie.

Hierdie bevinding mag moontlik verband hou met verskille in verhoudings van beide die CD4+ en CD8+ substelle, soos waargeneem gedurende vloei sitometriese (VAS, FACs) analise in twee HIV-seropositiewe individue met mutasie 32A→G, in vergelyking met 'n HIV-seropositiewe individu sonder hierdie mutasie.

Analise van die yster-verwante *SLC11A1* en *HFE* gene het nie betekenisvolle assosiasies opgelewer met gemodifiseerde risiko vir MIV-1 siekte of progressie na VIGS in die hoofsaaklik Swart studie-populasie nie. Die effek van die virus op ystermetabolisme is wel vir die eerste keer op DNS vlak gedemonstreer. Hemoglobien vlakke was betekenisvol verlaag in beide MIV-seropositiewe (P=0.004) en MIV-seronegatiewe (P=0.02) Swart individue met die *HFE* geen IVS3-48C→G mutasie, in vergelyking met mutasie-negatiewe individue in beide groepe. Aangesien hierdie effek meer uitgesproke was in MIV-geïnfekteerde individue as in kontroles, blyk dit dat die teenwoordigheid van die *HFE* mutasie die hemoglobienvlakke tot 'n groter mate beïnvloed weens die akute fase respons wat verband hou met die virusinfeksie. Hierdie effek kan moontlik toegeskryf word aan genetiese variasie in 'n naasliggende geen wat in aangebore immuniteit betrokke is, heel moontlik in die MLA gebied van chromosoom 6. Dit wil dus voorkom asof genetiese variasie in enige van die gasheer molekules betrokke by respons op infeksie kan bydra tot die kliniese uitkoms.

Die belangrike rol van die veelvuldige gasheer genetiese faktore wat in hierdie studie bestudeer is, of wat voorheen geïmpliseer is in vatbaarheid vir MIV-1 infeksie en siekte progressie, het 'n komplekse inter-verwantskap tussen gasheer en MIV-1 geopenbaar. In sommige gevalle is die siekte-proses na MIV-1 infeksie afhanklik van gekombineerde effekte van verskillende mutasies in dieselfde individu, terwyl onafhanklike effekte van spesifieke gene tesame met omgewings-invloede uiteenlopende kliniese uitkomste in ander mag verklaar.

In loving memory to my mother and grandmother

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Contents

ABBREVIATIONS

32 bp	thirty-two base pair
7TM	seven transmembrane receptors
Aci I	restriction endonuclease enzyme, source: *Arthrobacter citreus*, incubation 37 ºC 16-18 hours recognition site: 5'...CCGC...3' 3'...GGCG...5'
AIDS	acquired immunodeficiency syndrome
bcg	murine chromosome 1 locus, controls multiplication of *Mycobacterium bovis* in mice
bp	base pair
Bsa JI	restriction endonuclease enzyme, Source: *Bacillus stearo-thermophilus*, incubation: 55 ºC 2-3 hours recognition site: 5'...CCNNGG...3' 3'...GGNNCC...5'
Bsp MI	restriction endonuclease enzyme, source: An *E. coli* strain that carries the cloned *Bsp* MI gene from *Bacillus* species M, incubation: 37 ºC 16-18 hours recognition site: 5'...ACCTGC$(N)_4$ ...3' 3'...TGGACG$(N)_8$ ...5'
β-chemokines	beta chemokines
C2, C4B and BF	HLA class III complement system
CAAT motif	5' nucleotide sequence in conserved DNA region (promoter); eukaryotic transcription start point; specific transcription factors associate with it; usually found at -75 bp with the consensus sequence: GG(T/C)CAATCT
CCR1 or CC CKR1	first receptor for the CC chemokines
CCR2 or CCR2b	second receptor for the CC chemokines (or b)
CCR3	third receptor for the CC chemokines
CCR4 or CXCR4	fourth receptor for the CC chemokines
CCR5 or CXCR5	fifth receptor for the CC chemokines
CCR6	sixth receptor for the CC chemokines
CCR8	eighth receptor for the CC chemokines
CCR9	ninth receptor for the CC chemokines
CCR5Δ32	CCR5 32 bp deletion
CD4+	MHC class II cell receptor
CD4+ cells	MHC class II restricted immune cells

CD8+	MHC class I cell receptor
CD8+ cells	MHC class I restricted immune cells
CD45	alias for PTPRC
CD45RA, CD45RB CD45RAB, CD45RO	isoforms of CD45
cDNA	complementary DNA (or copy DNA)
CF	cystic fibrosis
$\chi 2$	Chi-square
Clq	complement factor Iq
cm	centimetre
CSW or CSWs	commercial sex worker(s)
CTL or CTLs	cytotoxic T-lymphocyte(s)
C-terminal	carboxy terminal
C1-C5	conserved regions of HIV envelope
DCYTB	duodenal cytochrome b ferric reductase
Δ	deletion (delta, *Gr.*)
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
dNTP	deoxy nucleotide triphosphate
dH_2O	deionised water
df	degrees of freedom
DMT1 (see also NRAMP2)	divalent metal transporter protein 1
EDTA	ethylene diamine tetra acetic acid (disodium salt, dihydrate), *chemical formula*: $Na_2C_{10}H_{14}O_8 \cdot 2H_2O$, *molecular weight*: 372.20 g/l; *synonyms*: ethylenediamine etraacetic acid, edetic acid, ethylenedinitrilotetracetatic acid, celon A, gluma cleanser, sequestrene AA, titriplex, nullapon B acid, trilon BS, warkeelate acid, YD 30, versene acid, N,N'-1,2-ethanediylbis(N-(carboxymethyl)glycine)
Env	envelope surface protein of HIV
ER	endoplasmic reticulum
Fe^{2+}	ferric iron, divalent cation
Fe^{3+}	ferrous iron, trivalent cation
g/dl	gram per decilitre

g/l	gram per litre
GP15/BOB	chemokine receptor-like orphan
gp120	HIV glyco protein 120
gp41	glyco protein 41
HCl	hydrochloric acid
HEPS	highly exposed persistently negative
Hex	heteroduplex
Hex-SSCP	heteroduplex single stranded conformation polymorphism
HFE or *HFE*	human haemochromatosis protein or *gene*
HH	hereditary haemochromatosis
HIV or HIV-1	human immunodeficiency virus (or type-1)
HIV-2	human immunodeficiency virus (or type-2)
HLA	human leukocyte antigen
HLA-A, B or C	human leukocyte antigen, class I type A, B or C
HLA-DP, DR or DQ	human leukocyte antigen, class II type DP, DR or DQ
Hp1-1, Hp1-2, Hp 2-2, Hp0	haptoglobin phenotypes
HSV-8	herpesvirus-8
H_2O	water
H_2O_2	hydrogen peroxide
Ig	immunoglobulin
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
IL-8	interleukin-8
IRP1, IRP2	iron-responsive protein 1 and 2
Ity	murine chromosome 1 locus, controls multiplication of *Salmonella typhimurium* in mice
kb	kilo bases
kDa	kilo Dalton
KIR(s)	killer immunoglobulin-like receptor(s)
Ish	murine chromosome 1 locus, controls early multiplication of *Leishmania donovani* in mice
LPS	lipopolysaccharides
LTNP or LTNPs	long-term non-progressor(s)
M	molar, moles per litre
MBL or *MBL*	mannose-binding lectin protein or gene

MCP	monocyte chemotactic protein
MIP-1α and MIP-1β	macrophage inflammatory protein 1 alpha and 1 beta
MHC	major histocompatibility complex
µg/l	microgram per litre
µl	microlitre
µmol/l	micromole per litre
µM	micro molar
mg	milligram
ml	millilitre
mm	millimetre
mM	milli molar
Mn^{2+}	manganese ion, divalent cation
Mr	molecular weight
MS	multiple sclerosis
mRNA	messenger ribonucleic acid
M-tropic	macrophage-tropic HIV strains
Nef or nef	surface protein of HIV
ND or n.d.	not determined
ng	nanogram
NH_4Cl	ammonium chloride
Nramp1 or *Nramp1*	murine natural resistance-associated macrophage protein-1 (protein or *gene*)
NRAMP1 or *NRAMP1* (see also SLC11A1)	human natural resistance-associated macrophage protein-1 (protein or *gene*)
NRAMP2 (see also DMT1)	human natural resistance-associated macrophage protein-2
NSI	non-syncythium inducing HIV strains
N-glycosylation	amino-glycosylation
nt.	nucleotide
N-terminal	amino terminal
OH^-	hydroxy
$OH^•$	hydroxyl radical (toxic)
% C	per cent crosslink
PAA	polyacrylamide
PBS	phosphate-buffered saline
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction

PCT	porphyria cutanea tarda
pH	measurement of acidity/alkalinity
pmol	pico mole
PTPRC or *PTPRC*	protein-tyrosine phosphatase, receptor-type C (protein or *gene*)
RANTES	regulated on activation, normal t expressed and secreted
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
Rsa I	restriction endonuclease enzyme, source: *Rhodopseudomonas sphaeroides* (1), incubation: 37 ºC 16-18 hours recognition site: 5'...CTAC...3' 3'...GATG...5'
RT-PCR	reverse transcriptase PCR
R5	HIV virus strains utilising CCR5 as entry mechanism
R5X4	HIV virus strains utilising CCR4/CCR5 as entry mechanism
SDF-1 (or α and β)	stromal cell-derived factor-1 (or alpha and beta)
SDS	sodium dodecyl sulphate, *chemical formula*: $CH_3(CH_2)_{10}CH_2OSO_3Na$
SI	syncythium-inducing HIV strains
SIV	simian immunodeficiency virus
SLC11A1 or *SLC11A1* (see also NRAMP1)	solute carrier family 11 member 1 protein or *gene*
SNP	single nucleotide polymorphism
SSCP	single stranded conformation polymorphism
SSP-PCR	sequence-specific primer polymerase chain reaction
STRL33	seven transmembrane-domain receptor from lymphocyte clone 33 (a chemokine receptor-like orphan)
TATA motif	highly conserved DNA sequence about 25 bp 5' from gene transcription start site, usually flanked by GC-rich sequences; binding site of transcription factors but not RNA polymerase (*also*: Homeobox, Hogness or Pribnow box)
TE buffer	Tris-EDTA buffer
TEMED	tetramethyl ethylene diamine, *chemical formula*: C6H16N2, *molecular weight*: 116.21 g/l; *synonyms*: N,N,N',N'-tetramethylethylenediamine, N,N,N',N'-Di(dimethyl-amino) ethane, N,N,N',N'-Tetramethyl-1-,2-diaminomethane, 1,2-di-(dimethylamino) ethane, propamine D, tetrameen, TMEDA
TFR	transferrin receptor

T_H1 or T_H2	T helper-1 or 2 cells
TNF (or α and β)	tumour necrosis factor (or alpha and beta)
Tris-Cl	Tris-chloride
T-tropic	T-cell line tropic HIV strains
U	unit or units
US28	human CC chemokine co-receptor, encoded by human cytomegalovirus
UTR	untranslated region
V1-V5	variable regions of HIV envelope
w/v	weight per volume
Z-DNA	form of DNA; left-handed helix, overall appearance elongated and slim
Zn^{2+}	zinc ion, divalent cation

CHAPTER I

INTRODUCTION

Chapter 1 INTRODUCTION

1. HIV/AIDS on the African continent

The human immunodeficiency virus (HIV) epidemic has recently passed its twentieth anniversary, whilst investigation of the complexity of the virus, the mode of infection and pathology of disease progression is continuing. Acquired immunodeficiency syndrome (AIDS) is amongst the primary causes of mortality on the African continent (http://www.who.int/infectious-disease-report/pages/ch1text.html). More than fifty million people worldwide are presently infected with HIV, and more than fifteen thousand new infections occur daily. The majority of individuals are from developing countries, with more than thirty million HIV-infected people living in sub-Saharan Africa. Data from a survey in 2000 and annual data available since the beginning of the epidemic in South Africa, indicated South African HIV prevalence rates rising from 0.7% in 1990 to 24.5% in 2000 (SA Dept. of Health 2001). The continuously increasing rates of HIV infection and prevalence of AIDS in Sub-Saharan Africa are causing a rapid decline in life expectancy - from seventy years of age to less than forty years of age. Although recent government data indicated a stabilising HIV/AIDS prevalence rate in South Africa based on 2001 infection data, the most concerning is the projected drop in life expectancy to about 30 years of age for the continent by 2010.

The increased vulnerability to HIV and sexually transmitted infections has been proposed to be directly associated with poverty, mainly due to urbanisation, modernisation and privatisation. The situation is further exacerbated by factors such as poor nutritional status, lack of health and social services, inadequate education, slow economic growth, and devastating drought conditions. A direct consequence is the risk of tuberculosis infection, causing about one-third of all AIDS-related deaths in developing countries. Activation of latent tuberculosis infection is believed to be due to a weakened immune system, as the risk of initial infection with tuberculosis is amplified by HIV-1 infection.

Whilst more than fifteen antiretroviral therapies have been accessible to most westernised populations, the cost may still be too high for many in developing countries. The continuously rising resistance rates of the virus towards treatment are also a major concern, highlighting the importance of novel strategies in combating the disease.

Up to one in seven reported cases of HIV infection (14%) during 1999 and 2000 revealed HIV strains with tenfold reduction in susceptibility to at least one class of antiretroviral drug. This is compared to 3.5% of individuals infected with HIV strains with comparable resistance levels between 1995 and 1998. The rising rates of therapeutic resistance would result in a narrowing of the range of available therapeutic options in the continuous battle with HIV infection. These resistance rates have been suggested to be due to the rapid mutation potential of HIV, resulting in new quassispecies of HIV.

In addition to the relative inaccessibility of treatment to developing populations, the rapidly rising rate of resistance to antiretroviral therapy, the absence of a preventative vaccine, and the lack of proper education targeting behaviour appear to be major contributing factors in the spread of HIV. Therefore, a possible intervention in the pandemic would be prevention efforts targeting education and behaviours that put individuals at risk for infection. Some African countries have maintained relatively low rates of infection because of early preventative efforts in educating people about the disease and reducing the risk of infection via low-cost interventions. These include voluntary testing to determine HIV status and counselling.

Since the beginning of the HIV/AIDS epidemic, fresh insights have been provided regarding the variation in risk towards infection, as well as development of resistance by the virus to current therapies and development of complications by the host to the existing therapies. This has been substantiated by studies involving pharmacogenetic approaches, the molecular functioning of the virus, as well as case-control studies investigating the risk towards susceptibility to HIV-infection and progression to full-blown AIDS. It is of great importance to investigate novel approaches to HIV/AIDS, especially determining the risk of susceptibility to HIV and the progression to full-blown AIDS. Application of such information for development of new means or therapies to combat infection, or at least to prolong life expectancy, has become a priority.

The basic mechanisms of disease pathology will be discussed, with particular focus on relevant cells and molecules involved in the pathogenesis of HIV/AIDS. These molecules in their respective ways are directly or indirectly involved in susceptibility and/or resistance patterns to disease, and all form part of the complex pathology. It has further been established that various genetic components involved in certain of these pathways are crucial in resistance or susceptibility to infection and/or progression of disease.

This chapter serves as a background to the consecutive discussions investigating involvement of certain host genetic components in HIV/AIDS disease pathology in South African ethnic populations.

2. Human Immune Virus type 1 (HIV-1) Pathogenesis and Human Immune Function – An Overview

The pathogenesis of human immune virus type 1 (HIV-1) infection is highly variable, and factors that influence susceptibility to infection and rate of disease progression involve a combination of viral, host, and environmental factors (Fauci 1996). The multifactorial nature of HIV disease pathogenesis is reflected by the highly variable rates of disease progression observed in HIV-infected individuals.

Whilst most individuals infected with HIV develop AIDS symptoms within 10 years, about 1-5% remains relatively healthy for 15 years or more (long-term non-progressors, LTNP), and others progress to AIDS within the first 2-3 years after infection (fast or rapid progressors). Resistance to infection have been observed in a limited number of individuals, and some appear to even eliminate the virus.

Host factors that play an important role during modulation of the rates of disease progression facilitate widely varying clinical outcomes, even in individuals who were apparently infected from a common source (Liu *et al.* 1997). The complexity of host genetic effects is characterised by the involvement of variants with very subtle, although significant consequences on gene expression or protein function. Although significant advancement had been made in terms of the proper understanding of the roles of potential host factors involved in disease acquisition and progression, this represents only a limited understanding of the complexity of the disease and progression thereof.

Currently no standard practice exists that takes into account the genetic profile of individuals (i.e. genotypes at loci known to be associated with rate of disease progression). Assessment of such factors should be a priority to properly understand disease mechanisms in light of the pandemic. Prediction of disease outcome on an individual basis will only be possible by a better understanding of the gene-environment interactions in HIV disease.

2.1. HIV Infection and Host Cellular Response

Initial cellular target cells of HIV-1 infection are the Langerhans cells, which are tissue dendritic cells found in the lamina propia subjacent to the cervicovaginal epithelium (Spira *et al.* 1996) or the nasopharyngeal tonsil and adenoid tissues (Goldberg *et al.* 1998), rich in cells of dendritic origin. These cells fuse with cells involved in the cellular immune response, and spread to deeper tissues. Viral particles can be detected from draining the iliac lymph nodes within two days after infection.

2.1.1. *HIV Fusion and Cell Entry: Mediated by its own Envelope Structure and Host Receptor Molecules*

Fusion and entry of HIV-1 into target cells (primarily T-cells and macrophages) is mediated by both determinants of the viral envelope (reviewed by Wyatt and Sodroski 1998) and host proteins expressed on the surface of CD4+ lymphocytes or macrophages attracted to areas of inflammation caused by HIV-1 infection. It has been established that primarily CD4+ and chemokine co-receptors are involved in this mode of entry. Infection by HIV-1 takes place by direct fusion of virus cell surface molecules and CD4+ molecules expressed on the target cell membranes (Dalgleish *et al.* 1984, Klatzmann *et al.* 1984, Maddon *et al.* 1986).

The HIV-1 viral envelope (Env) consists of two non-covalently associated subunits after cleavage of a gp160 precursor protein: a) a heavily glycosylated external gp120 subunit, derived from the N-terminal portion of gp120 and containing the CD4 binding site, and b) the membrane-spanning gp41 subunit, derived from the C-terminal portion of the precursor with an N-terminal hydrophobic fusion peptide which is directly involved in membrane fusion (Wyatt and Sodroski 1998). Native Env expressed on the surface of the virion or the infected cell is a trimeric structure containing three gp120/gp41 complexes associated noncovalently via gp41.

Associations between the co-receptor and CD4+ are enhanced in the presence of gp120, although they occur to some extent in the absence of gp120. Furthermore, soluble gp120 binds weakly to co-receptors expressed on cells, with greatly increased affinity upon CD4 binding. This mechanism is referred to as CD4-dependent entry.

The gp120 molecule consists of five hypervariable loops, V1-V5, interspersed with five relatively conserved regions, C1-C5 (Starcich *et al.* 1986). Fusion specificity is primarily determined by the V3 loop (Chesebro *et al.* 1991, Hwang *et al.* 1992, Shioda *et al.* 1992), and dependent on the tropism of the infecting HIV strains. Interaction with CD4+ takes place via relatively conserved amino acid residues in the V3 loop, which is the outermost principal neutralising determinant of the envelope. This binding is in concert with V1, V2, V4 and C4 causing the Env complex to undergo pH-independent CD4-induced conformational changes (Sattentau *et al.* 1993), thereby creating high affinity binding with the CD4+ molecule by exposure of the previously hidden co-receptor binding site. The hypervariable loops protrude over the conserved residues (Moore *et al.* 1996), thus sheltering this presumably highly immunogenic region from host defences. This probably accounts partially for the ability of HIV to evade an effective immunological response, and permitting entry of the retroviral genome into the cytoplasm for viral replication to commence.

Although cell hybrid studies conducted on HIV-entry supports this proposed mechanism, binding of the HIV envelope to the target cell requires the presence of a co-receptor on the surface of the target cell (usually either CCR5 and/or CCR4, dependent on stage of infection), in addition to the CD4+ molecule. Interaction with chemokine co-receptors CCR5 and CCR4 follows the steric changes (conformation or charge) of the conserved receptor-binding site via the V3 loop (Moore *et al.* 1998), although the binding of gp120 has been shown to be unrelated to physiological (sensitisation) state of the receptor. This observation suggested that the underlying transduction pathways are relatively unimportant in the HIV entry mechanism (Aramori *et al.* 1997).

After infection with HIV-1 and replication, cells expressing the gp120 proteins attract and bind other CD4+ expressing primary T-cells, creating a syncythium. A syncythium (*plural* = syncythia) is the clumping together of T-cells expressing the viral Env protein, encoded by the HIV viral RNA after integration into the human genome, and being multinucleated (also referred to as “giant cells”).

Although the CD4-dependent mechanism is the major route for HIV-1 fusion and infection, Env/co-receptor interactions have however been reported in the absence of CD4+ (referred to as the CD4-independent mechanism). The CD4-independent entry pathways appear to be relatively inefficient.

Env's capable of mediating CD4-independent interaction with co-receptor retain the ability to bind CD4+, which markedly enhances functional interaction between gp120 and co-receptor. However, the significance of CD4-independent Env/co-receptor interactions *in vivo* is questionable.

An intriguing hypothesis deduced from these findings is that the evolutionary predecessor of HIV-1 strictly used the chemokines for entry, and that the CD4+ requirement evolved later as a means of conferring greater target cell specificity, as well as protecting the co-receptor binding region from the humoral immune response system.

2.1.2. *HIV-1 tropism and Viral Entry: Specificity of Infection of Different Cell Types via Specific Receptors*

Nomenclature to distinguish between different HIV strains was originally based only on phenotypic properties, as represented by two classification systems.

As depicted by figure 1, some isolates of HIV-1 show efficient infectivity for continuous CD4+ T-cell lines, but poor infectivity for macrophages. These isolates are designated T-cell line tropic (T-tropic), and are generally syncythium-inducing (SI) strains. Some HIV-1 strains show the opposite with preferential infection of macrophages, and are referred to as macrophage-tropic (M-tropic), which are usually non-syncythium inducing (NSI) strains.

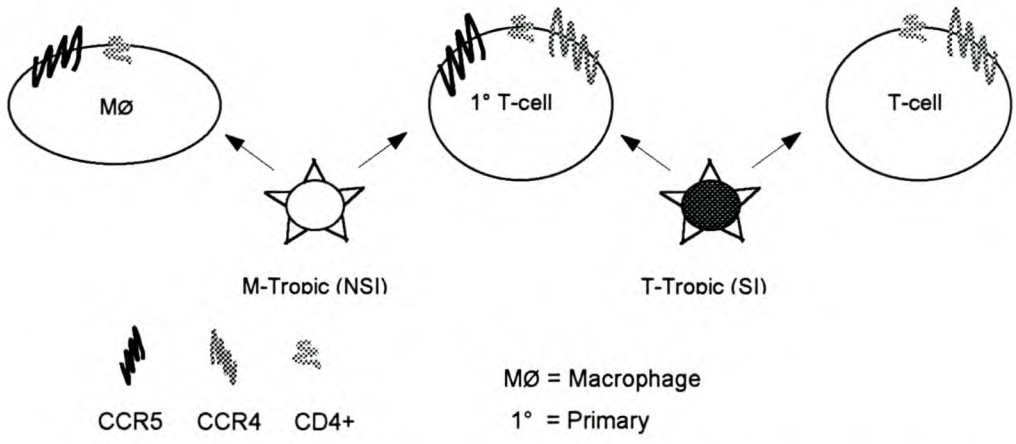

Figure 1. Proposed model for chemokine co-receptor usage and viral entry. M-tropic (NSI) viruses use the CCR5 receptor, expressed in macrophages (MØ) and primary (1°) T-cells. T-tropic (SI) viruses use the CCR4 receptor, expressed on primary T-cells. Viruses enter after binding of both CD4+ receptor and chemokine receptor by fusion with the cell membrane. Cells with the 32 base pair deletion (CCR5Δ32) do not express CCR5, and although M-tropic viruses can bind CD4+, viral entry is not possible.

Isolates that have either preference (i.e. replicate equally efficient in both target cell types) are designated dual-tropic. Viral isolates from peripheral blood, isolated shortly after infection and during the asymptomatic period, are predominantly M-tropic.

Some shortcomings of these classification systems have however been pointed out. Primary infections with T-tropic (SI) strains, among others, and failure to confirm any distinctive tropism for HIV-1 isolates *in vitro* have been reported (Stent *et al.* 1997). The ability of activated T-cells to express both CCR5 as well as CCR4 and allow M-tropic strains to replicate (Trkola *et al.* 1997) further complicates the concept of tropism. Also, macrophages or monocytes show expression of CCR4 in addition to CCR5, although they do not support replication of T-tropic (SI) strains (Yanjie *et al.* 1998). T-tropic (SI) strains typically display higher cytopathic effects *in vitro*, suggesting that they may have a particularly important role in the decline of CD4 T-cells *in vivo*, which is the hallmark of AIDS. Thus, as the infection progresses towards AIDS, predominantly T-tropic (SI) viruses can be isolated from patients.

Further investigation of these phenotypic properties indicated close correlation with the use of specific co-receptors for productive HIV infection. Although all HIV-1 strains infect and replicate in activated primary CD4+ T lymphocytes, various co-receptors are required for entry into T-cells and macrophages/monocytes. Transmission studies indicated that initial infection was almost invariably established by M-tropic (NSI) strains, using the CCR5 co-receptor to gain entry in macrophage/monocyte cell lines (Simmons *et al.* 1996). However, strains relying on a broader range of co-receptors may develop during the course of the infection (Connor *et al.* 1997). Therefore, highly pathogenic SI strains with a preference for the CCR4 receptor on the surface of T-cells, can be isolated prior to the development of AIDS in approximately 50% of all cases (Karlsson *et al.* 1994). Although the co-receptor binding residues are highly conserved, the extreme mutation rate of HIV in combination with the duration of infection allows for strains with other tropisms to develop, further contribute to the complexity HIV disease pathogenesis.

Supported by these observations, construction of a simplified model of molecular events during HIV entry was proposed to yield a more simplified HIV-1 nomenclature system (Berger *et al.* 1998). This facilitated revision of the HIV-1 phenotype to indicate co-receptor usage, rather than the less biochemically defined characteristics of target cell tropism or syncythium-inducing properties (Adopted at the Gordon Research Conference on Chemotactic Cytokines, June 23-28, 1996).

According to this newly adapted nomenclature system, variants using CCR5 as co-receptor and corresponding to M-tropism (NSI), is referred to as R5 variants. For HIV-1 variants specific for CCR4 (or CXCR4) co-receptor binding and corresponding to being T-tropic (SI), reference is made to as X4 variants. Similarly, variants using both CCR4 and CCR5 as co-receptors (thus with dual-tropism), are referred to as R5X4 variants. Development of both the X4 and R5X4 variants is potentially due to the extreme mutation rate of HIV, in concert with the duration of infection. The latter is responsible in the late stage of disease, yielding a rapid decline of CD4+ T-cells and the subsequent breakdown of the immune system, which is indicative of the onset of AIDS.

2.2. Host Mediators of HIV Infection: The Chemokines and Chemokine Receptors

Discovery of chemokines and their receptors have greatly expanded the knowledge of host factors that form part of the pathogenesis of HIV-1 and the progression to AIDS. Clues that chemokine-related events are important in HIV-pathogenesis initially came from work showing that high levels of chemokines could inhibit HIV replication *in vitro* (Cocchi *et al.* 1995), which proposed that chemokines might prevent HIV infection by binding to the elusive HIV cofactor. The general mechanisms of viral infection and pathogenesis involving these cofactors will be described, to further the understanding of basic mechanisms of action of these chemokines and their receptors.

Chemokines (*chemo*tactic cyto*kines*) are small serum protein molecules with molecular weights ranging between 7 and 15 kDa, and ~68-120 amino acids in length. The chemokines are all related in amino acid sequence. They are produced by a variety of cells and their function is important in the inflammatory process (Baggiolini *et al.* 1997), where they induce migration of leukocytes. Chemokines function primarily as chemoattractants for leukocytes, recruiting monocytes, neutrophils and other effector cells from the blood to sites of infection. These biological molecules are released by many different cell types, and serve to guide cells involved in innate immunity, including lymphocytes in adaptive immunity. Functioning primarily during inflammation, the chemokines direct cellular movement in the body from the blood into tissues and to the appropriate location within each tissue. Activation of circulating cells is facilitated by the chemokines, causing them to bind to the endothelium and initiating leukocyte migration across the endothelium. Once a cell has crossed the endothelium, it is capable of responding to a new set of chemokines, which directs its migration through the tissues.

Three major functions exist for the chemokines: they 1) act as biological messengers, recruiting leukocytes to injured or diseased tissue by chemotactic activity along a chemokine concentration gradient and activation of leukocytes, 2) play a regulatory role in leukocyte maturation and development of lymphoid tissues, and 3) have a role in infectious disease susceptibility or pathogenesis.

Chemokines form part of the seven transmembrane G-protein-coupled receptor super-family (Murphy 1996), which is the largest receptor superfamily in the human genome and are intracellularly linked to G-proteins, consist of a major group and a minor group (reviewed by Rollins 1997), and are sub-divided into four classes, based on cysteine motifs near the N-terminus (Table 1). Although not certain for all classes, respective signalling functions are as indicated.

Table 1. Chemokines are divided into two groups and four classes, based on cysteine-motifs near the N-terminus (adapted from Rollins 1997).

Group & Class	***Type***	***Characteristics***	***Signalling Function***
Major Group			
Class I	'α-' or CXC chemokines	two cysteines, separated by a single residue	mainly neutrophils
Class II	'β-' or CC chemokines	two adjacent cysteines	signalling other leukocytes
Minor Group			
Class III	C chemokines	one single cysteine	
Class IV	CX_3C chemokines	first two cysteines, separated by three residues	

Leukocyte populations have specific receptors for appropriate chemokines. Chemokine receptors are all integral membrane proteins containing seven membrane-spanning helices. Most chemokines act on more than one receptor, and most receptors will respond to several chemokines. The receptors are selectively expressed on particular populations of leukocytes, and this determines which cells can respond to signals coming from the tissues. The profile of chemokine receptors on a cell depends on its type and state of differentiation. For example, all T cells express CCR3, whereas T helper-2 (T_H2) cells preferentially express CCR3, and T helper-1 (T_H1) cells CCR5 and CXCR3.

Further, chemokine receptors are functional during microbial infection, and are referred to as chemokine co-receptors as it functions in concert with other molecules involved in microbial entry, for example CD4+.

Why do viruses need co-receptors?

Viral entry into the target cells is critically dependent on the interaction between the surface proteins of the virus and those on the cell membrane (Feng *et al.* 1996a, Samson *et al.* 1996a). As described earlier, initial adherence of the virus is passive to a receptor protein on the cell surface, followed by conformational changes to improve binding, and finally penetration. Entry of the viral genome into the cytoplasm takes place for viral replication to commence. Inhibition of HIV-entry may however be facilitated by chemokine receptor blockade, desensitisation, sequestration, or internalisation: either through alterations in receptor affinity; or by inhibiting postbinding steps (Oravecz *et al.* 1996), such as phosphorylation through G-coupled mechanisms. Before the link between HIV and chemokine receptors had been established, these molecules were known as seven transmembrane-spanning cell-surface proteins or seven transmembrane (7TM) receptors. Figure 2 shows the basic chemokine receptor (co-receptor) structure (reviewed in Broder and Collman 1997).

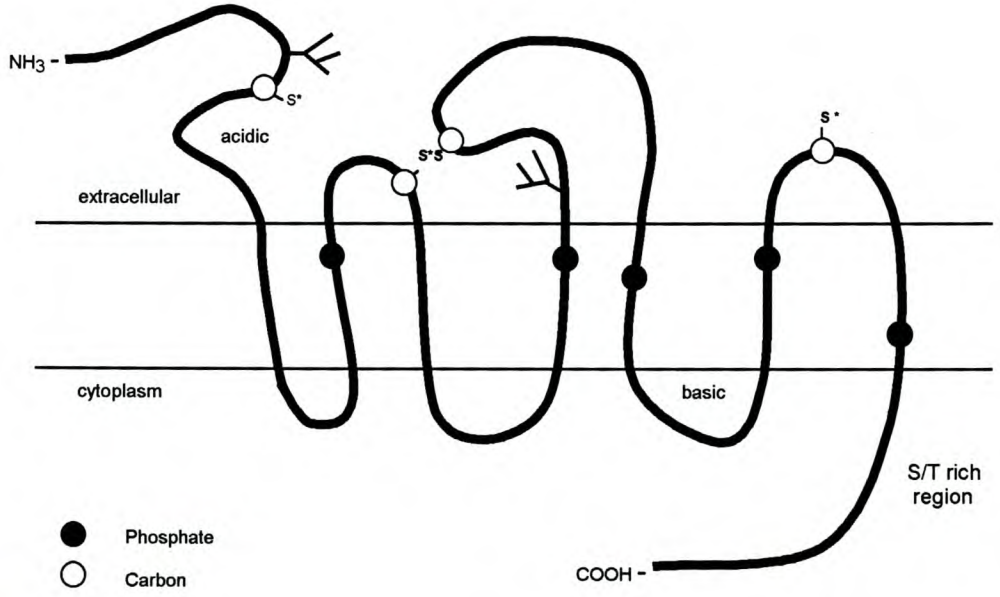

Figure 2. Structure of the seven-transmembrane chemokine receptor (co-receptor) proteins, facilitating viral entry into CD4+ target cells (modified from Broder and Collman 1997).

Receptors for chemokines comprise a subfamily within the G-protein-coupled receptor super-family. The receptor is topologically arranged with seven characteristic serpentine-like transmembrane segments, the N-terminus and three extracellular loops, and the C-terminus and three intracellular loops (Murphy 1994a,b, 1996, Strader *et al.* 1994). The negatively charged acidic extracellular N-terminal domain is proposed to be involved in ligand binding, while the basic C-terminal domain may be a target for phosphorylation by G-protein coupled receptor kinases following ligand binding. The membrane-spanning domain consists of seven α-helices containing three intracellular loops and three extracellular loops composed of hydrophilic amino acids. Disulfide bonds are formed by highly conserved cysteine residues between the first and second extracellular loops, and between the N-terminal domain and third extracellular loop. These bonds are thought to confer a barrel shape, bringing the extracellular domains into close proximity.

In the assumption that Env probably makes initial contact with the extracellular domains, most focus has been on these regions (Alkhatib *et al.* 1997, Doranz *et al.* 1997, Dragic *et al.* 1998, Reeves *et al.* 1998, Wang *et al.* 1998 as examples). Each extracellular region has been implicated in co-receptor function, with the N-terminal segment playing a particularly important role in ligand binding and leukocyte signalling. The transmembrane and/or intracellular domains however also critically influence activity, perhaps by affecting display of the extracellular regions in addition to activation of intracellular signalling pathways. Although the two major chemokine receptors, CCR5 and CCR4, share only about 30% amino acid homology (including conserved amino acid sequences), they probably share similar membrane topology.

Identification of the chemokines and their receptors was preceded by the identification of soluble factors from CD8+ T-cells that control HIV infection *in vitro* (Walker *et al.* 1986). Although further receptors have been identified, the principal chemokine receptors for either T-tropic or M-tropic strains of HIV-1, are CCR4 (Feng *et al.* 1996a) and CCR5 (Alkhatib *et al.* 1996, Choe *et al.* 1996, Deng *et al.* 1996, Doranz *et al.* 1996, Dragic *et al.* 1996) respectively. It was recently shown in the HIV-1 subtype C strains, predominating in South Africa, that some of these strains could use both CCR5 and CCR4 or alternative receptors (Cilliers *et al.* 2003). These observations are relevant in understanding the rapid spread of HIV-1 subtype C in the developing world and for the design of intervention and treatment strategies.

CCR5

CCR5 (*fifth receptor for the CC chemokines*) was identified as a receptor for three β-chemokines (or CC chemokines) RANTES (*r*egulated on *a*ctivation, *n*ormal *t* *e*xpressed and *s*ecreted), MIP-1α and MIP-1β (*m*acrophage *i*nflammatory *p*rotein 1α and 1β, respectively) (Combadiere et al. 1996a, Deng *et al.* 1996, Raport *et al.* 1996, Samson *et al.* 1996a). As it appears that the co-receptors merely serve as anchors for HIV on the cell surface, the suppressive effect in primary T-cells by RANTES, MIP-1α and MIP-1β on HIV replication stems from their ability to occupy and/or internalise the receptor rather than from their physiological effect on the cell.

CCR5 is utilised as a co-receptor by M-tropic (NSI) R5 variants of HIV-1 early in the course of infection. In addition to the involvement of the mentioned β-chemokines, investigation of the possible involvement of other chemokine receptors in the entry mechanism of HIV into cells demonstrated the involvement of CCR3 and CCR2b (Doranz *et al.* 1996). These β-chemokines were the first chemokines shown to act as soluble inhibitors of HIV *in vitro*, are released by CD8+ lymphocytes (Cocchi *et al.* 1995), and bind to and block HIV-1 infection by binding putatively to CCR5. Individuals resisting HIV infection or progression to AIDS had abnormally high levels of these chemokines.

CCR4

CCR4 (*fourth receptor for the CC chemokines*, CXCR4, or 'fusin' as it was referred to originally) was shown to be a co-receptor for T-tropic (SI) X4 and R5X4 variants of HIV-1, which predominate in later stages of infection (Feng *et al.* 1996a). This phenomenon was supported by the observation that co-expression with CD4+ rendered human cells permissive for Env-mediated cell fusion and infection of primary CD4+ T-cells. The CCR4 co-receptor was further shown to be specific for the functional chemokines SDF-1α and SDF-1β, formed by alternative splicing (Bleul et al. 1996, Oberlin et al. 1996), because of the ability of SDF-1 (stromal derived factor-1) to inhibit T-tropic (SI) X4 HIV-1 strains selectively.

The exact mechanisms for the CCR4 and CCR5 chemokines as co-receptors for HIV-1 entry have not been clearly defined together with CD4 binding (Wain-Hobson 1996). However, it appears to involve interactions of the viral V3 loop and other parts of the outer envelope protein gp120 with extracellular domains of CCR5 or CCR2b.

This may involve multistep interactions with CD4+, the chemokine receptor and other cell surface components (Lapham *et al.* 1996).

CCR3 and CCR2

Subsequent to several studies reporting on CCR3 being a minor co-receptor and with a limited number of HIV-1 isolates that can function with CCR3, compared to CCR5, it was indicated that CCR3 has comparable activity with CCR5 and/or CCR4 and function with a broad range of isolates (Rucker *et al.* 1997, Barzan *et al.* 1998). It has further been shown that some HIV isolates may even use both CCR5 and CCR4 co-receptors, in addition to CCR3 or CCR2b (Doranz *et al.* 1996).

CCR3 was originally reported to be specific to eosinophils (Combadiere *et al.* 1995), although it has been reported that brain microglia express CCR3 along with CCR5. This suggested that either molecule could mediate HIV-1 entry into microglia (He *et al.* 1997) with development of neurological disease (HIV encephalopathy), resulting from HIV infection in the brain. In contrast, CCR2 is highly expressed on blood monocytes and macrophages, and exists in two isoforms, CCR2a and CCR2b (Combadiere *et al.* 1995). Further studies suggested that alternate pathways could be available for HIV entry by some strains in primary macrophages (Rana *et al.* 1997), indicating possible inclusion of the involvement of CCR2b.

Other major chemokine receptors

Although the relative importance and contribution to HIV replication and cellular pathology is not fully clear, a wide array of chemokine receptors acting as co-receptors have been identified. Table 2 shows a summary of these cofactors, along with a brief description of certain co-receptors that have been identified to play an active role in the inflammatory process of HIV infection.

From several studies involving chemokine co-receptors, absence of activity in HIV infection has been noted for some, for example CCR6 and CXC chemokine receptors other than CXCR4, and several other chemokine receptor-like orphans. Additional to the mentioned human proteins, certain other HIV co-receptors have been identified with activity in HIV-1 infection. These include CCR2b, CCR3, CCR8, CCR9, CX3CR1; and the chemokine receptor-like orphans STRL33/Bonzo, GP15/BOB and Apj.

Another CC chemokine co-receptor (US28), encoded by human cytomegalovirus (Rucker *et al.* 1997, Pleskoff *et al.* 1997) has been indicated in active HIV infection. These observations suggest the possibility of other as yet unidentified, but related seven transmembrane molecules supporting HIV entry, because of the ability of some isolates to promiscuously utilise a broad range of co-receptors. Thus, identification of strategies to block infection via CCR5 or CCR4 may result in selection for variants with enhanced ability to enter by alternative co-receptors.

Table 2. Summary of chemokine receptors and their ligands: tissue distribution and pathogens (adapted from McNicholl *et al.* 1997, with modification)

Chemokine receptors (Old names) [a]	Ligands	Tissue distribution	Pathogens [b]
CC Receptors			
CCR1 (CC CKR1)	RANTES, MIP-1α, MCP-2, MCP-3	Monocytes, T-cells	HIV-1
CCR2A (MCP-1Ra)	MCP-1, MCP-3	T-cells, Basophils, Monocytes	
CCR2B (MCP-1Rb)	MCP-1, MCP-2, MCP-3, MCP-4		HIV-1(NSI)
CCR3 (CKR3)	Eotaxin, Eotaxin-2, RANTES, MCP-2, MCP-3, MCP-4	Eosinophils, Basophils, Microglial Cells, and possibly Monocytes; little expression in Peripheral Blood T-lymphocytes or Dendritic cells	HIV-1(NSI)
CCR4 (CXCR4)	TARC, RANTES, SDF-1 MIP-1α, MCP-1, MDC	Basophils, T-cells	HIV-1(SI)
CCR5 [c] (CC CKR5)	RANTES, MIP-1α, MIP-1β	Monocytes, Dendritic Cells Microglial Cells, T-cells	HIV-1 (SI,NSI) HIV-2, SIV
CCR-8	I-309, MIP-β	Monocytes, Thymocytes	HIV-1
CCR9	Unknown	PBMC	HIV-1
CXC Receptors			
CXCR1 (IL-8 RA)	IL-8	Neutrophils, NK cells	
CXCR2 (IL-8 RB)	IL-8, MGSA, *gro*-, NAP-2, IP-10, ENA-78, Mig		
CXCR3	IP-10, Mig	Activated T-cells	
CXCR4 (Fusin, LESTR, HUMSTR)	SDF-1	*Wide*: $CD4^+$ and $CD4^-$ cells, Monocytes, Macrophages, Dendritic cells, B cells; other tissues, e.g. brain, lung, spleen	HIV-1(SI), HIV-2
CC/CXC Receptor			
DARC (Duffy Antigen)	RANTES, MCP-1, TARC, IL-8, MGSA, *gro*-, etc.	Endothelial Cells, Erythrocytes	*Plasmodium vivax*

...../(cont..)

Table 2 (cont..)

Chemokine receptors (Old names) [a]	Ligands	Tissue distribution	Pathogens [b]
Others [d]			
STRL33	ND	Lymphoid Tissues and activated T-cells	HIV-1
HCMV-US28	MIP-1, RANTES	Fibroblasts infected with CMV	HIV-1
ChemR1	ND	T-lymphocytes, Polymorphonuclear cells	
Chem R23	Unknown	Monocytes, Macrophages	
CMKBRL1	ND	Neutrophils, Monocytes, Brain, Liver, Lung, Skeletal Muscles	
*TER*1	ND	Thymus, Spleen	
V28	ND	Neural and Lymphoid Tissue	
D2S201E	ND	*Wide*: incl. cells of hemopoietic origin	
BLR1	ND	B-lymphocytes	
EBI1	ND	B-lymphocytes	
GPR1,2,5	ND	ND	
BONZO/ STRL33	Unknown	T-cells, monocytes, placental cells	HIV-1(SI), HIV-2, SIV
BOB/GPR-15	Lymphotaxin	T-lymphocytes, colon	
Apj	Unknown	PBMC, brain	

[a] New nomenclature for CC and CXC chemokine receptors was adopted at the Gordon Research Conference on Chemotactic Cytokines, June 23-28, 1996.

[b] Pathogens using this receptor for infection.

[c] The 32 bp deleted allele of CCR5 has been referred to as CCR5-2, or CCR5Δ32.

[d] Chemokine receptor-like genes whose predicted proteins have 7 transmembrane domains.

Abbreviations: BLR1, Burkitt's lymphoma receptor-1; CMKBRL1, Chemokine receptor like-1; DARC, duffy anti gen/receptor for chemokines; EBI1, Epstein-Barr virus-induced receptor; ENA78, epithelial-derived neutrophil-activating peptide-78; GPR, G protein coupled receptor; *gro*, growth related gene product; HCMV, human cytomegalovirus; HUMSTR, human serum transmembrane segment receptor; IL, interleukin; IP-10, interferon-gamma inducible 10kD protein; LESTR, leukocyte-expressed seven-transmembrane-domain receptor; MCP, monocyte chemotactic protein; MDC, macrophage-derived chemokine ; Mig, monokine induced by interferon gamma; MIP, macrophage inflammatory protein; NSI, non-syncythium inducing; N/A, not applicable; NAP-2, neutrophil- activating protein-2; ND, not determined; RANTES, regulated on activation, normal T-cell expressed and secreted; PBMC, peripheral blood mononuclear cells; SDF-1, stromal cell-derived factor-1; STRL33, seven transmembrane-domain receptor from lymphocyte clone 33; TARC, thymus and activation regulated chemokine.

3. Host genetics and resistance or susceptibility to microbial infection

A rapid transition in the understanding of the involvement of genetics and genomics in infectious disease pathology has been achieved over the past decade. Most human populations are affected by microbial infections, which is especially true for developing countries where some infections may predominate. Varying clinical outcomes are often seen between individuals or populations, ranging from asymptomatic to severe disease pathogenesis. Some pathological disorders may become apparent within days or weeks, whereas others are detected only after a number of years of infection. For the majority of infections, only a proportion of individuals exposed to the pathogen become infected with consecutive development of clinically evident disease.

It has been suggested that the variation between individuals is mainly due to combination effects of host genes and proteins, controlling the quality and possibly quantity of host-parasite interaction and human immune responses. Environmental factors, density and distribution of the infecting organism, as well as differing susceptibilities amongst different ethnic groups due to genetic differences, may all contribute significantly to the disease outcome. The phenotypic and/or genetic characteristics of the infecting organism itself however also play an important role in disease pathogenesis.

Assessment of the importance of host genetics in disease development has been complicated by the multiplicity of environmental factors. For example, nutritional requirements can be modulated by genetic mechanisms that might further affect microbial transmission. Discovery of genetic mechanisms of both host and pathogen associated with infectious disease pathology, and elucidating the involvement of environmental factors in the genetic mechanisms that underlie acquisition of infectious disease, may therefore aid in development of novel therapeutic strategies.

Speculation of polygenic control of infectious disease susceptibility has been confirmed recently by identification of several genetic regions containing genes that determine susceptibility to *Leishmania major*, the agent for cutaneous leishmaniasis in humans (Roberts *et al.* 1997a).

Further, infectious diseases can act as strong selective influences in moulding human evolution and population structure. It was established that the heterozygous advantage of mutant alleles compensate for the deleterious effect of homozygotes in the face of malaria challenge, associated with sickle cell anaemia and thalassemia (Allison 1969, Flint *et al.* 1986, Hill *et al.* 1987, Weatherall *et al.* 1987, Clegg and Weatherall 1999). The high prevalence of mutated alleles of the *β-globin* gene in areas of high malaria endemicity confirmed that this gene provided protection against severe malaria. Using similar strategies, it was demonstrated that certain HLA haplotypes and *TNFα* alleles (McGuire *et al.* 1994, Kroeger *et al.* 1997, Abraham and Kroeger 1999) could also be responsible for modified host susceptibility to malaria.

At present no vaccines exist towards therapy of many of these pathogenic diseases. Preventative therapies in general are often inadequate, mainly because of economic considerations and toxicity of drugs used in the treatment regimens. The fact that evolution of the infecting organism also plays an important role in the pathogenesis suggests proper assessment of the potential host genetic mechanisms that could contribute to increased susceptibility or resistance to microbial infection.

As with most infections, no standard practice presently exists for HIV/AIDS with regard to the prediction of disease outcome on an individual basis, taking into account the genetic profile (i.e. genotypes at loci associated with rate of disease progression) of individuals subjected to functional studies for immune responsiveness to HIV-1. This would only be possible with a better understanding of the various effects on HIV disease. Such an approach was proposed recently by Carrington and co-workers (Carrington *et al.* 2001), which may clarify the critical steps required in the disease pathogenesis, thereby providing novel targets for vaccines or chemotherapy.

Various host genetic mechanisms, which could potentially form integral parts in novel treatment strategies towards HIV infection and/or disease progression to AIDS, will be discussed.

3.1. Host genes and HIV-1 infection and progression to AIDS

Significant differences in the rate of disease progression have been observed in longitudinally followed HIV-infected persons since the beginning of the HIV epidemic. Identification of persons who were persistently exposed to HIV but remained uninfected facilitated studies of the effect of host genes on susceptibility to HIV infection (Rowland-Jones *et al.* 1995a,b, Fowke *et al.* 1996, Huang *et al.* 1996, Liu *et al.* 1996, Paxton *et al.* 1996, Stephens *et al.* 1996).

Prior to the discovery of the role of chemokine receptor gene polymorphisms in HIV infection, only genes of the HLA system were thought to protect against HIV infection. However, as with many infectious diseases, it was suggested that polygenic control might contribute to the individual risk to susceptibility to HIV-infection. For example, certain distributions of HLA class I alleles were observed in uninfected female commercial sex workers (CSWs) in Africa and Thailand, who had been highly exposed to HIV (Fowke *et al.* 1993, Plummer *et al.* 1993, Stephens *et al.* 1996). Additional class I and II alleles have been identified, that may be associated with those remaining uninfected (Malkovsky 1996). Mechanisms possibly related to cytotoxic T-cell function, have been suggested to explain these findings (Rowland-Jones *et al.* 1995, Rowland-Jones and McMichael 1995a, Fowke *et al.* 1996, Stephens *et al.* 1996).

Several non-HLA genetic factors appear to influence susceptibility to HIV infection and the course of HIV disease, for example the presence of certain alleles of the CCR5 co-receptor gene that provide protection against HIV infection (Dean *et al.* 1996). This has led to speculation of evolutionary development of a selective force by maintaining one such allele to control the virus. The major mechanisms providing resistance or susceptibility to HIV-1 infection and disease progression are discussed.

3.1.1. *The HLA (human leukocyte antigen) genotype*

The human leukocyte antigen (HLA) gene complex forms part of the major histocompatibility complex (MHC) agglomerate (or antigenic cluster), and is encoded by a 3800 kilo base (kb) segment located on the short arm of chromosome 6, locus 6p23-p21 (Dunham *et al.* 1987). Other immune-related genes (for example the *TNF*α and *TNF*β genes) are also encoded by this region, as was shown by molecular mapping of the MHC containing the HLA complex (Spies *et al.* 1986). Expression of the MHC gene products (HLA genes) plays a crucial role in activation of the immune response to infectious agents. This primary function is fulfilled by the binding and presentation of antigens to class I-restricted CD8+ cytotoxic T-lymphocytes (CTLs), thereby recruiting other cells involved in the immune response.

The MHC consists of three classes, which form part of a large multigene family within the MHC. Class I contains the HLA-A, B and C genes, which encode a protein that forms a heteroduplex with β_2-microglobulin and is expressed on most body tissues. MHC class I molecules play an indispensable role in the immune defence against intracellular infections, including HIV-1 infection (Walker *et al.* 1987, Nixon *et al.* 1998). Regulation of the immune system by these molecules takes place by presentation of antigenic peptide epitopes to CTL, generated as a result of immune stimulation. Initiation of specific immune responses is activated through this mechanism in order to control clearance of foreign material (Shiga *et al.* 1996, Tomiyama *et al.* 1997, Weinhold *et al.* 1998, Rowland-Jones *et al.* 1999). Class II molecules, on the other hand, encoded by the HLA-DR, DQ and DP genes, are essential for initiation and control of elements of the immune system. Located on the surface of immune cells (for example cells of the macrophage lineage, B cells and activated T-cells), the class II molecules are responsible for presentation of antigens in the form of peptides by antigen presenting cells including dendritic cells, macrophages and B cells, to CD4+ T helper cells (Collins *et al.* 1986). Although very little is known about class III molecules, these include the molecules involved in the complement system C2, C4B and BF and tumour necrosis factor (TNF).

The HLA system is the most polymorphic biological system known in humans, and large racial and geographical variations of specific alleles have been shown. Further, alleles identified for the respective classes and types vary in number, and no gender-associated variation has been observed.

For example, at the peptide level for class I the HLA-A locus contains approximately 27 allelic variants, 60 for the B locus and 10 for the C locus. In class II, 60 variants for HLA-DR, 32 for HLA-DQ and 40 for HLA-DP have been identified. At the molecular (DNA) level however, many more variants exist for both classes. These variants are suggested to have evolved in response to a variety of pathogenic confrontations. They have been maintained specifically within populations in order to elicit immune responses to given pathogens, thus yielding protective mechanisms in different populations (Parham and Ohuta 1996, Hughes and Yeager 1998). Accurate assigning of HLA loci to specific infections has been complicated by the extremely polymorphic nature of the MHC, and relatively even distribution of HLA alleles characterising the HLA loci.

Due to the polymorphic nature, the possibility of linkage disequilibrium patterns within the MHC further complicates the assignment of causative loci to specific diseases and/or disorders. Because of the complexity of the multiple effects of the host genetic composition on pathogens, multiple HLA loci may therefore be required to elicit an effective immune response. Although substantial associations have been made, the effects of multiple variants in complex gene systems clearly need to be investigated in considerably large populations and by organized comprehensive approaches.

Current methods for accurate detection of specific HLA loci associated with diseases are limited. Until recently, serological detection methods were applied almost exclusively. These detection methods only allowed for the basic subtypes (for example HLA-A, B and C) to be identified, and were very time consuming. However, since the amino acid sequences of all but the most rare variants of HLA-A, B, Cw and DR have been elucidated, molecular typing techniques are being developed. These methods include polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) typing (Moribe *et al.* 1997, Nieto *et al.* 1997), sequence-specific primer polymerase chain reaction (SSP-PCR) (Geisthorpe *et al.* 1999), real-time PCR (Bon *et al.* 2000), flow cytometric analysis (Chou *et al.* 1997), amongst others. The advantage of these techniques over the more traditional tissue typing techniques is predominated by the robustness of molecular techniques, as well as relatively high-throughput made possible by such techniques. Although more typing methods are being evaluated, routine application of these methods will only be possible after a number of years due to the complexity of allelic variation in the HLA region.

To understand the potential mechanisms of the HLA system providing protection from HIV-1 infection or yielding susceptibility to HIV/AIDS, a review of the complex interaction of the virus with the host in the context of the immune system is necessary. The following section provides background to susceptibility and/or resistance mechanisms that could be ascribed to genetic variation of the host HLA system.

CTL-response and HIV-1 escape from the immune system

Cytotoxic T-lymphocytes (CTLs) play an indispensable role in control and clearance of infections. Variation in the epitopes expressed by cells involved in the primary immune response is crucial for effective immune responses to take place. Such variation in epitopes recognised by CTL however frequently offers potential escape mechanisms for infecting organisms, as seen in the case of HIV-1 infection.

The polymorphic nature of HLA determines which cytotoxic T-lymphocyte (CTL) epitopes are expressed on the surface of infected cells. The breadth of the immunological response may therefore be a factor of the genotype of the host, and not necessarily due to variation in the infecting organism, although phenotypic variation of viruses can also elicit specific CTL-responses. However, evasion from the CTL response often takes place for survival of the virus, possibly due to extreme mutation rates known to occur especially in viruses. A number of examples exist for viral infections other than HIV, in which the virus can escape from control by the CTL by means of mutation (Pircher *et al.* 1990, De Campos Lima *et al.* 1993,1994, Bertoletti *et al.* 1994a,b, Niewiesk *et al.* 1995, Weiner *et al.* 1995).

For HIV-infection, the combination of immunodominant T-cell responses, the integration of single copies of DNA in infected cells, together with high HIV-1 turnover and accumulation of mutants during persistent infection, makes it almost inevitable that HIV-1 escapes CTL. Several reports support the hypothesis of escape (Phillips *et al.* 1991, Johnson *et al.* 1993, Couillin *et al.* 1994,1995, Harrer *et al.* 1994, Koup 1994a, Nietfield *et al.* 1995), whilst others argue against it (Meyerhans *et al.* 1991, Chen *et al.* 1992). Although many examples exist for mutations in the provirus, which could affect recognition by CTL from the same patient, sufficient evidence that escape has occurred has not been provided (Johnson *et al.* 1992, Zhang *et al.* 1994, Safrit *et al.* 1994, Meier *et al.* 1995, Nietfield *et al.* 1995, Nowak *et al.* 1995, Wolinsky *et al.* 1996).

The CTL response is capable of reducing viral levels by approximately 100-fold, but no further in the case of HIV-infection (Koup *et al.* 1994b, Safrit and Koup 1995). In contrast with other viral infections, T-cell control ultimately fails in HIV-1 pathogenesis, with viral escape due to the rapid HIV-1 replication rate. Estimates based on decline in HIV-1 virion levels observed after administration of anti-retroviral drugs, have indicated a daily generation of 10^9-10^{10} virions (Ho *et al.* 1995, Wei *et al.* 1995). Further, with the HIV mutation potential of 10^{-5} per base (bp) per generation (and genome size of 10^4 bp), on average 10^8 mutant viruses should be generated daily (Coffin 1995). Although the immune response is challenged with this extreme mutation rate, only a minute portion of the mutant virions yield viable viruses and most are defective with a slower replication rate. Inefficient competition with wild type virus thus normally allows for mainly wild type virus to elicit necessary CTL responses. Best-adapted viruses however predominate distinctly in different geographical regions, referred to as HIV-1 quassispecies, subtypes or clades (Louwagie *et al.* 1993). These are based on strong conservation of consensus sequences of the virus clades. The tendency to revert to the "mean", with little initial selective pressure from the immune system at each transmission event, therefore allows eventual escape of the virus from the immune response due to evolution within the host.

HLA and susceptibility to HIV/AIDS

Numerous HLA loci from both classes I and II have been assigned in susceptibility to HIV-infection and disease progression patterns between individuals, and also with differing distributions and effects between populations. The role of the HLA system in determining the course of infection has been examined by using measures such as the CD4+ cell count or the length of time between HIV-1 infection and AIDS (Steel *et al.* 1988, Hill 1996, Kaslow *et al.* 1996, Malkovsky 1996, McNeil *et al.* 1996). From these and other studies, it had been established that small contributions of multiple interactive HLA marker combinations (primarily in class I) could significantly modulate the course of HIV-1 infection. However, similar to the 32 bp deletion mutation occurring in the *CCR5* gene (CCR5Δ32), it has been suggested that these alleles contribute equally, although independently to disease acquisition or progression status. Together, these observations strongly support a genetic component in HIV-1 disease pathogenesis.

Although many associations have been made between alleles of the HLA complex and HIV disease acquisition or progression, inconsistent results have however not facilitated delineation of a clear pattern on how HLA influences disease progression (Hendel *et al.* 1996, Hill 1996, Malkovsky 1996, Roger 1998).

Studies on HIV/AIDS susceptibility indicated that the class I HLA-A26, HLA-B27 and HLA-B57 are associated with slow progression (Kaslow *et al.* 1996), whereas the HLA-B35 allele which almost always neighbours HLA-Cw*04, has been consistently associated with rapid progression (Scorza Smeraldi *et al.* 1986, Itescu *et al.* 1992, Sahmoud *et al.* 1993, Just 1995, Carrington *et al.* 1999). The accelerating function of HLA-B35,Cw*04 has been observed only in Caucasian individuals and not in Africans, suggesting this allele may be operating immunologically or may be associated with another locus accelerating progression to AIDS (Carrington *et al.* 1999). It was further shown by these investigators that HIV-seropositive Caucasian individuals lacking the heterozygous class I AIDS-associated loci HLA-B*35 and Cw*04, avoided AIDS for 10 or more years. Subtypes of the HLA-B*35 allele, possibly influencing the rapid progression to AIDS, were assessed by Gao and co-workers (2001). Progression was shown to be attributable to the HLA-B*35-Px alleles, and it was also concluded by these authors that the previously observed association of HLA-Cw*04 with progression to AIDS (Carrington *et al.* 1999) was due to linkage disequilibrium with HLA-B*35-Px alleles. The association with B*35-Px was observed in Caucasians, and this allele was absent from Africans. The only difference between B*35-Px and B*35-Py was one amino acid substitution, which probably facilitated association with disease progression. In African individuals from the population investigated by these authors, the HLA-B*5301 allele was associated with susceptibility to disease progression. This study supported the hypothesis that HLA-B alleles exert an effect on the immune response to HIV-1 infection, which appear to differ between populations.

Strong association for a number of alleles associated with non-progression (such as B14, B27, B57, C8 and C14, DR11) have been shown (Hendel *et al.* 1999), whereas others favoured rapid progression (A29, B22, B35, C16). These observations confirmed previous associations of the B27, B57, B35 and DR11 alleles with slow HIV disease progression (Scorza Smeraldi *et al.* 1986, Cruse *et al.* 1991, Hendel *et al.* 1996, Kaslow *et al.* 1996 Malkovsky 1996, McNeil *et al.* 1996, Roger 1998).

It was recently established that homozygosity of B alleles sharing the HLA-Bw4 allele, is markedly associated with suppression of HIV-1 viremia (Flores-Villanueva *et al.* 2001). This association was independent from the CCR5Δ32 mutant in protection and also from other HLA alleles that could confound the association. This study further showed independent association of HLA-B44 and control of viremia. Furthermore, it was shown that specificity for the HLA-Bw6 allele is associated with a worse disease outcome. The hypothesis that the ability to control disease progression is multifactorial, and that additional genetic advantages remain to be discovered, was supported by these results.

Some HLA alleles are in linkage disequilibrium and commonly occur on the same haplotype. For the study conducted by Hendel and co-workers (1999), such linkage was observed for A29-C16, B8-C7, B14-C8, B27-C1, B27-C2, B35-C4, B51-C14, B57-C6, B57-DR7 and A1-B8-C7-DR3, explaining similar association for some A, B and C alleles. These authors further showed that the DR11 allele is protective and it was present in two patients with the protective CCR5Δ32 mutation, supporting the hypothesis of polygenic control of susceptibility patterns to HIV/AIDS.

Extended haplotype analysis with disease progression established association between a number of HLA haplotypes and HIV-1 disease progression. In Caucasian individuals, the haplotypes HLA A1-B8-DR3 (Kaslow *et al.* 1990, McNeil *et al.* 1996, Steel *et al.* 1988), HLA A1-Cw7-B8 and Cw4-B35 (Steel *et al.* 1988, Kaslow *et al.* 1990, Carrington 1999, Keet *et al.* 1999) appear to be associated with rapid progression. HLA A1-Cw7-B8-DR3-DQ2 and HLA A11-Cw4-B35-DR1-DQ1 were also found to be associated with fast progression to AIDS.

In some studies two subtypes identified from the same allele (for both class I and II) have shown differences in susceptibility patterns between ethnically different populations. Costello and co-workers (1999) showed that in the Rwandan population the HLA-B5703 subtype predominated in slow progressors, whereas this allele was not present in Caucasians. Instead, the HLA-B5701 subtype was present almost exclusively in Caucasians.

Haplotypes identified predominantly in the Rwandan population included HLA B57-Cw04, whereas the HLA B57-Cw06 haplotype predominated in Caucasian individuals, although neither of the C-antigens alone suggested any progression association (Costello *et al.* 1999). Similarly, it was shown that HIV-1 infection is affected by differing HLA genotypes between African Americans and Caucasians (Roe *et al.* 2000). The HLA-DQB1*0603 subtype was positively associated with HIV-infection, and the HLA-DQB1*03032 subtype was associated with protection from HIV-1 infection in Caucasians, but not in African Americans. Presence of HLA-DQB1*0201 to be associated with HIV-1 infection in African Americans was further demonstrated by these authors, as it occurred more frequently in HIV-seropositive individuals, whereas HLA-DRB1*04 was positively associated with HIV-1 infection in Caucasians from the study population.

The influence of HLA types on susceptibility to HIV-1 infection in highly exposed individuals was assessed in a population of CSWs in Kenya (MacDonald *et al.* 2000). MHC class I HLA-A2, HLA-A28, and HLA-B18 were associated with decreased risk of HIV-1 infection in this population, while HLA-A23 was associated with increased risk. Subtyping of class I alleles identified a supertype, consisting of the HLA-A2 subtypes HLA-A*0202, -A*0205, and -A*0214 and an HLA-A28 subtype, HLA-A*6802, which appeared to be associated with a significantly decreased rate of HIV-1 seroconversion. A significantly decreased risk of HIV-1 seroconversion associated with the HLA-DRB1*0102 allele of the HLA-DRB1*01 determinant was further observed. Resistance to HIV-1 infection in this cohort was associated with immunological responses to HIV-1, and not with protective chemokine receptor polymorphisms (for example CCR2). The HLA-A2/6802 supertype and the DRB1*01 determinant were proposed to mediate protection through presentation and restriction of conserved epitopes, although these alleles are neither completely necessary nor sufficient for resistance.

Other alleles in association with alleles from the HLA complex have been shown to contribute to progression to AIDS. It was reported that the activating KIR allele KIR3DS1, in combination with HLA-B alleles (HLA-B Bw4-80Ile), is associated with delayed progression to AIDS (Martin *et al.* 2002). In the absence of KIR3DS1, the HLA-B Bw4-80Ile allele was not associated with any of the AIDS outcomes. By contrast, in the absence of HLA-B Bw4-80Ile alleles, KIR3DS1 was significantly associated with more rapid progression to AIDS.

The KIRs encode the killer immunoglobulin-like receptors on natural killer (NK) cells and it was shown to regulate the inhibition and activation of NK cell responses through recognition of HLA class I molecules on target cells.

Combined, these observations confirm that disease progression appears to be influenced by several HLA genes or haplotypes. The effects of the associations are however complex, and may depend on interactions with other host genes. The observation by Martin and co-workers (2002) strongly suggested a model involving an epistatic interaction between 2 loci. Although polygenic control involving the HLA system has been proposed, it should be noted that the effect of HLA genotypes on survival or disease progression are independent from other host genes that from part of the pathogenesis of, and protection from HIV infection (Kaslow *et al.* 1997).

In the context of polygenic control of susceptibility to HIV/AIDS, some recent studies investigated the possible contribution of variations in the HLA region with those present in chemokines. Certain HLA alleles, for example HLA-A32 and A25, were present at higher frequencies, whereas others (HLA-B8) were present at reduced frequencies in HIV-infected individuals in progression groups compared with HIV-seronegative individuals (Geczy *et al.* 2000). These differing allele frequencies were not accompanied by HIV-disease associated variants in either the *CCR5* or *CCR2* genes in long-term survival groups. Confounding effects of viral mechanisms (for example in the case of nef-defective HIV-1) could however be a possible explanation for the association between certain HLA alleles and long-term survival in HIV-1 infection.

Furthermore, data presented by Magierowska and co-workers (1999) have shown that the combined host genetic background strongly influences the evolution of HIV-1 disease. long-term non-progressors (LTNPs) studied shared a particular genotype for both chemokine receptor and HLA loci, which independently influenced the outcome of their disease. The odds of subjects heterozygous for the CCR5 32-bp deletion and homozygous for SDF-1 wild-type of being LTNPs, were increased by 16-fold; by 47-fold when an HLA-B27 allele is present with HLA-DR6 absent, and by 47-fold also if at least three of the following alleles are present: HLA-A3, HLA-B14, HLA-B17, HLA-DR7. This model facilitated the accurate classification of 70% of LTNPs and 18% of progressors, suggesting that other, as yet undefined host susceptibility factors remain to be investigated.

3.1.2. *The chemokines and chemokine receptors*

The chemokines, also known as chemo-attractant cytokines, are a family of proteins that play a crucial role in inflammatory and immune reactions and viral infections (reviewed in Luster 1998). As described earlier, several chemokine receptors have been characterised and many have multiple ligand specificities. These receptors play a major role in lymphocyte function, recruiting these molecules to sites of infection or inflammation. In the case of HIV-1 infection, various chemokines are involved in the inflammatory response.

Supported by epidemiological data, some mechanisms have been shown to be crucial in susceptibility patterns to HIV-infection and disease progression. This section will emphasise some of these mechanisms involving chemokine receptors and co-receptors with host genetic variation to susceptibility for HIV-disease.

CCR5 and *CCR2*

The main focus in the investigation of chemokine receptors and their influence on HIV-1 disease status has been on the human chemokine *CCR5* and *CCR2* genes, both located on the short arm of chromosome 3p22-p21. *CCR5* is amongst a group of genes that encode multiple chemokine receptors, including the *CCR2* gene (Liu *et al.* 1996, Samson *et al.* 1996a). These two genes are tightly linked, and separated by less than 20 kb, suggesting a single promoter(s) may regulate expression of both genes. The respective coding regions of the two genes share 75% nucleic acid and amino acid identity. Both gene products can serve as co-receptors with CD4+ for HIV as indicated by several studies. However, common allelic variants in both genes (which may occur in linkage disequilibrium) are associated with HIV-1 disease progression and these mechanisms will be discussed.

CCR5

The *CCR5* gene consists of 4 exons spanning 6 kb, and two introns (Mummidi *et al.* 1997). Exons 2 and 3 are continuous as no intron is present between the two exons, and the open reading frame and complete 3' untranslated region (UTR) are present in exon 4. A region upstream of exon 1 was identified with strong sequence homology (~89%) with sequences in the 3'-flanking region of *CCR5*.

Two distinct AT-rich promoters have been identified around exon 1 (one upstream and one downstream), both lacking TATA and CAAT motifs. P_U, a weak promoter which resides proximal to exon 1, and P_D, a stronger promoter which is located upstream of exon 3. It is conceivable that regions further upstream of exon 1, or constructs shorter than those tested, may support strong promoter activity for P_U. A strong silencer element (nt. –244 to –80) and enhancer element (nt. –486 to –244) have been located, suggesting the gene region between nt. –486 and –1 may be regulating the expression of CCR5 in monocytes/macrophages and T-lymphocytes (Guignard *et al.* 1998). Multiple CCR5 transcripts have been identified, which are due to a complex alternative splicing pattern in the 5' UTR and exon 4. Intact, *CCR5* encodes a 352 amino acid protein CCR5, with a molecular mass of 40,600 kilo Dalton (kDa) and contains a potential N-glycosylation site (Samson *et al.* 1996a). The regulatory sequences and non-coding exons have however been shown to be polymorphic, whereas the amino acid coding sequence appears to be more conserved.

Independent studies identified the CCR5 protein as a coreceptor for HIV-1 (Deng *et al.* 1996, Dragic *et al.* 1996). Some individuals however resisted infection despite repeated exposure to HIV-1, and a possible mechanism of resistance to HIV-1 infection involving a homozygous 32 bp deletion in the *CCR5* gene (two copies CCR5Δ32/CCR5Δ32) was identified (Liu *et al.* 1996, Samson *et al.* 1996a, Biti *et al.* 1997).

CCR5Δ32 involves a deletion of the nucleotide sequence between bases 794-825 of the *CCR5* gene, at a site of a repeat motif consisting of 10 bases (Figure 3). The deletion results in a non-functional truncated protein due to a frame-shift after amino acid 174, inclusion of 7 novel amino acids and premature a stop-codon at codon 182 (Liu *et al.* 1996). Loss of three of the seven transmembrane domains (two of the three outer loops, and the intracellular domain) results from the deletion, and the truncated protein is not expressed on the surface of immune cells. The predicted structures of the wild-type protein and the truncated form containing the 32 bp deletion are depicted in Figure 4. The deletion does not appear to impair immune function, but reduces the risk for HIV-1 infection. This occurs because of the inability of the virus utilising this protein as co-receptor for attachment to and integration into CD4+ cells.

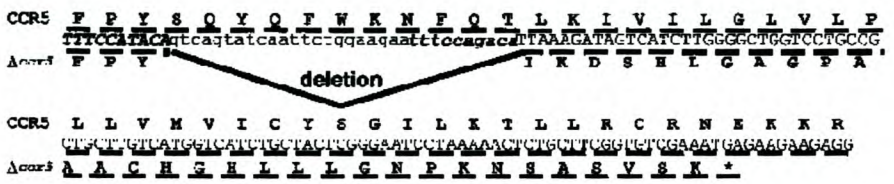

Figure 3. Partial *CCR5* gene and amino acid sequence with 32 bp deletion (CCR5Δ32). Nucleotide sequence of the CCR5 gene surrounding the deleted region, and translation into the normal receptor (top lines) or the truncated mutant (CCR5Δ32, bottom lines). The 10 bp direct repeat is represented in bold italics and the deleted nucleotides are non-capitalized (adapted from Zhang *et al.* 1996).

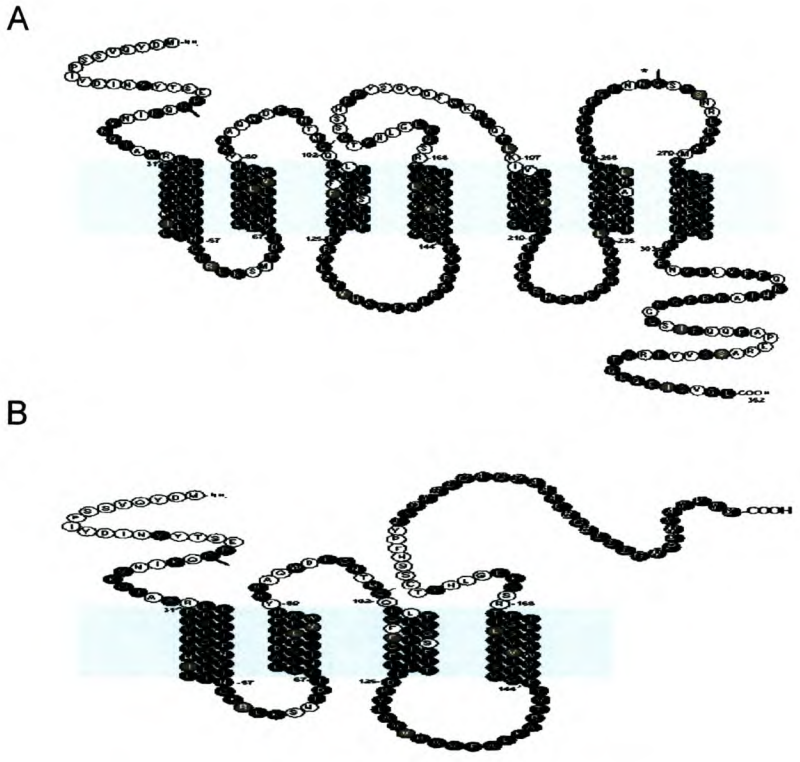

Figure 4. Predicted structures of the *CCR5* chemokine co-receptor (Doranz et al.1996). A. Wild-type CCR5 protein. The typical serpentine structure is depicted with three extracellular (top) and three intracellular (bottom) loops and seven transmembrane domains. B. The predicted CCR5Δ32 protein structure. The mutant protein lacks the last three transmembrane segments of CCR5, as well as regions involved in G-protein coupling. The transmembrane organization is given by analogy with the predicted structure of the wild-type CCR5 protein (adapted from McNicholl *et al.* 1997).

In contrast with the protective effect of the homozygous genotype for the deletion mutant, heterozygosity for the 32 bp deletion (one copy, *CCR5* wild type/CCR5Δ32) did not show protection against HIV-1 infection (Samson *et al.* 1996). Rather, retardation of progression to AIDS as the result of heterozygosity for CCR5Δ32 had been noticed predominantly in Caucasian populations (Dean *et al.* 1996, Garred 1996, Eugen-Olsen *et al.* 1997, Meyer *et al.* 1997, Michael *et al.* 1997). Furthermore, prolonged AIDS-free survival has been associated with a slower decline in CD4+ T-lymphocytes and a lower viral load (Katzenstein *et al.* 1997). At the onset of AIDS however, heterozygosity does not offer protective advantage over the wild type (Garred *et al.* 1997a, Michael *et al.* 1997a,b, Smith *et al.* 1997a,b). The effect in heterozygotes appear to be a suppression of wild-type CCR5 co-receptor function due to an intracellular association of these defective and normal gene products, which is retained in the endoplasmic reticulum (ER) (Benkirane *et al.* 1997). Retention in the ER was shown to result in decreased surface expression of CCR5 and degradation of the truncated CCR5 protein.

However, although the protective effect of this deletion against HIV-1 infection has been confirmed, some isolated individuals with the homozygous deletion subsequently became infected with HIV-1 (Balotta *et al.* 1997, O'Brien *et al.* 1997, Theodorou *et al.* 1997). This genotype therefore does not confer absolute resistance against HIV infection, and infection can infrequently be established by T-tropic HIV strains (NSI) which use the CCR4 receptor for infection, as well as strains which can enter via alternative receptors (Balotta *et al.* 1997, O'Brien *et al.* 1997, Rana *et al.* 1997, Simmons *et al.* 1997, Theodorou *et al.* 1997).

Population studies have shown that homozygosity for CCR5Δ32 occurs at a frequency of approximately 1% amongst Northern American or European Caucasians, whereas the homozygous genotype was absent in Africans (Dean *et al.* 1996, Michael *et al.* 1997a, McNicholl *et al.* 1997). Conversely, these and other studies showed that heterozygosity occurs in 10-20% of Caucasian populations from North America and the European continent. In non-Caucasian populations however, heterozygosity has been found in 1.7-6% of African Americans, 7% of Hispanics, 13% of Native Americans and less than 1% of Asians.

Further investigations on non-Caucasian populations, including persons from parts of Africa (Zaire, Burkina Faso, Cameroon, Senegal, Benin, Uganda, Rwanda, Kenya, Malawi, Tanzania, Sierra Leone), Haiti, parts of Asia (Thailand, India, China, Korea, Japan, the Philippines) and Venezuela have not found CCR5Δ32 among HIV-infected or HIV-uninfected individuals, including individuals from African ethnic origin (Huang *et al.* 1996, Liu *et al.* 1996, Samson *et al.* 1996b, Zimmerman *et al.* 1997). Recent investigations of *CCR5* in the South African Black population showed the 32 bp deletion was virtually absent, whereas it was present in approximately 10% of Caucasians investigated (Williamson *et al.* 2000, Petersen *et al.* 2001).

A north-south gradient (ranging from less than 1% to 16%) was observed in the CCR5Δ32 allelic distribution in Europe, and indicated a frequency of 11-16% of the mutant in Scandinavian countries, 9-11% in France and Belgium, 8-10% in central European countries, 4-9% in Mediterranean countries and as low as 0.9% in Corsicans (Libert *et al.* 1998, Lucotte and Mercier 1998, Stephens *et al.* 1998). Furthermore, heterozygosity was observed to follow a similar pattern in these populations, and homozygosity was less frequent. These studies did not show presence of CCR5Δ32 in Middle Eastern, African, East Asian and American Indian populations, although in Central Asian populations (Uzbeks and Kazaks, amongst others) the mutant was less frequent. It was concluded from these observations that the CCR5Δ32 deletion had a recent origin in North-eastern Europe.

Several other point mutations in *CCR5* have been identified (Dean *et al.* 1996, Ansari-Lari *et al.* 1997, Carrington *et al.* 1997), either resulting in amino acid transitions or not, and some of these mutations appear to be associated with HIV-disease. Others are population-specific occurring in either Caucasian or non-Caucasian populations, or both. It was pointed out by Carrington and co-workers (1997) that the majority of missense mutations identified in Caucasians occurred in more conserved regions of the *CCR5* gene, whereas those occurring in African Americans occurred in more variable regions. This further supported the more recent origin of population-specific mutations in the *CCR5* gene that may be protective in Caucasian individuals. It has also been suggested that some of these mutations may be in linkage disequilibrium with other as yet unknown mutations that might affect disease acquisition or progression specifically in different populations.

In the South African population investigated by Petersen and co-workers (2001), novel mutations were identified, including an African-specific single nucleotide polymorphism (SNP) at codon 35, P35. A significant difference in allelic distribution was observed between HIV-seropositive and HIV-seronegative individuals in the Cape Coloured population of mixed ancestry. This observation raised the possibility that this sequence change occurs in linkage disequilibrium with another polymorphism either in the regulatory promoter region of *CCR5*, or in the *CCR2* gene as these genes are in close proximity on chromosome 3. A further significant association in this study population was observed for the A335V SNP with slow disease progression in individuals of African ethnic origin (Hayes *et al.* 2002). This is however in contrast with the observation by Carrington and co-workers (1997), where this SNP was not associated with disease progression in either Caucasian or African populations. Furthermore, a recent study in which this polymorphism, amongst others, was included for investigation indicated no significant association with HIV/AIDS in the Ugandan population (Ramaley *et al.* 2002a,b).

Another point mutation in the *CCR5* gene that appears to be of significance in HIV-infection is the m303 mutation causing a T→A substitution at position 303. This results in a premature stop codon, implicating a loss of functional expression of CCR5 (Quillent *et al.* 1998). When present together with the heterozygous CCR5Δ32 mutation, it was shown to render resistance to HIV-1 infection. The CCR5Δ32/m303 genotype was shown to be functionally indistinguishable from CCR5Δ32 homozygosity. Although present in the general population described by these authors, the inheritance of the m303 mutation was studied further in a family of Western European origin. It was present in association with the heterozygous CCR5Δ32 mutation in one individual from this family at high risk for HIV-1 infection, and whom remained HIV-seronegative. The South African study by Williamson and co-workers (2000) indicated a frequency of 0.9% for m303 in Caucasians, whereas it was absent in Africans.

Further, mutations in the regulatory region of *CCR5* have also been shown to be associated with rate of disease progression (Martin *et al.* 1998). A significantly higher presence of the -59353T/C promoter polymorphism (C-allele) was shown to occur in nonprogressors, where the -59029G/A promoter polymorphism (G-allele) was associated with disease retardation.

The effects of mutations in the *CCR5* regulatory region were shown to be independent of the protective effects provided by the CCR5Δ32 and CCR2-64I polymorphisms. Also, a promoter haplotype was found to be associated with rapid disease progression. This observation involved the -59353 (allele C) and -50402 (allele A) polymorphisms, but not the –59029 polymorphism (Martin et al. 1998). Different frequencies were reported for these alleles, which could possibly be explained by ethnic admixture in the respective populations investigated. Also, in a recent study in CSWs in Thailand, highly exposed persistent seronegative (HEPS) women had higher frequencies of the *CCR5* –59402 promoter mutation (homozygous for the G allele), compared with controls (Sriwanthana *et al.* 2001). This suggested a potential reduction in disease progression amongst these high-risk individuals.

CCR2

It was recently shown that a mutant allele of the *CCR2* gene V64I (CCR2b-64I), which is in strong linkage disequilibrium with CCR5Δ32, is more frequently found in individuals whose progression to AIDS was postponed, compared with fast progressors (Smith *et al.* 1997a, Anzala *et al.* 1998, Kostrikis *et al.* 1998, Mummidi *et al.* 1998, Rizzardi *et al.* 1998). It was shown that this point mutation is present in a 1.9 kb intron, which may be dispensable for optimal promoter activity (Guignard *et al.* 1998, Mummidi *et al.* 1998). However, as both genes are potentially regulated by the same promoter, some studies have confirmed that CCR5 expression is not influenced in CCR2-64I heterozygotes (Kostrikis *et al.* 1998, Rizzardi *et al.* 1998). Although not associated with HIV-1 infection per se, CCR2-64I has been shown to contribute to prolonged progression to AIDS (2 to 4 years longer than individuals without the mutation). When in combination with the heterozygous CCR5Δ32 genotype, it was however protective against progression for more than 16 years.

Compared with CCR5Δ32, the CCR2-64I point mutation was shown to occur in different ethnic populations and was not only confined to Caucasians. This polymorphism was also reported recently to be relatively frequent in South African Black individuals (Williamson *et al.* 2000). In the population studied by these investigators, CCR2-64I was present in a significantly higher frequency (13.1%) in Black Africans, compared with 7.2% of Caucasians, confirming the higher incidence of this mutation in Africans (Mummidi *et al.* 1998).

A subsequent study investigating South African populations (Petersen *et al.* 2002) indicated a similar distribution of the CCR2-64I polymorphism in African individuals to that previously reported by Williamson and co-workers (2000). It was further shown by these authors that this polymorphism was present more frequently in HIV-seronegative Black African and Coloured individuals of mixed ancestry (16.9% and 17.4%, respectively) than in HIV-seronegative Caucasian individuals (9.5%). Further, in HIV-seropositive Black African individuals from the cohort investigated, the frequency was similar (13.0%) to that of population-matched healthy individuals (16.9%). The polymorphism however differed significantly in HIV-seropositive individuals of mixed ancestry compared with healthy population-matched individuals, whereas it was absent in the low number of HIV-seropositive Caucasians included in the study cohort. A number of novel mutations and SNPs in this gene was reported in the population investigated, some with conservative amino acid transitions, although no association with HIV-infection or disease progression was found. This study also indicated that for the silent *CCR2* C→T mutation N260, described by Clark and co-workers (2001), the mutant T allele was present at lower frequencies in the HIV-seropositive individuals compared with healthy individuals, but this did not reach statistical significance for the individual populations investigated.

Furthermore, as shown by Anzala and co-workers (1998) in a cohort of CSWs from Nairobi, Kenya (lacking the CCR5Δ32 mutation), the CCR2b-64I was present in 33% of HIV-1 infected individuals asymptomatic for more than 12 years, whereas it was present in 11% in those whose infection progressed to AIDS within 4 years (rapid progressors). These investigators concluded that this mutation provided a two-fold protection in African individuals compared with Caucasians towards protection from HIV-disease progression. Interestingly, a recent investigation indicated a significant association of CCR2b-64I with mother-to-child transmission of HIV-1, as well as a delay in disease progression (Mangano *et al.* 2000). Protection was not confounded by either the SDF1-3'A or CCR5Δ32 mutations in this study.

Other chemokine receptors

Other chemokine receptor polymorphisms include two alleles (coding for a serine or threonine at amino acid position 276) in the *CCR3* gene (Combadiere *et al.* 1996, Daugherty *et al.* 1996, Ponath *et al.* 1996). The population prevalence of this polymorphism and role in HIV infection or disease progression have however not been reported. Furthermore, a recent study showed an association with more rapid disease progression for a haplotype identified in the CX3CR1 chemokine receptor (Faure *et al.* 2000). HIV-infected Caucasian individuals homozygous for the CX3CR1 I249-M280 haplotype affecting two amino acids (isoleucine-249 and methionine-280) progressed more rapidly than those with other haplotypes. In a consecutive study, no associations with disease progression were found (Kwa *et al.* 2003). No polymorphisms in the *CCR4* gene encoding CCR4 (the entry co-factor for T-tropic HIV virus strains) have been reported to date.

SDF-1

The *SDF-1* gene was additionally identified as a potential marker for HIV-disease progression. *SDF-1* is located on chromosome 10q and encodes the ligand for the CXCR4 chemokine receptor, SDF-1 (Shirozu *et al.* 1995).

SDF-1 forms part of the family of integrins mainly encoded by genes located on chromosomes 4q or 17q. The integrins are a class of proteins that link the outside of cells with their interior, thus integrating a response, for example the mediation of adhesion of neutrophils to endothelial cells. Strong evolutionary conservation and unique chromosomal localization of *SDF-1* suggested that SDF-1 might have important functions, distinct from those of other members of the integrin family. Two isoforms due to alternative splicing, SDF-1α and SDF-1β, were identified. These proteins share 92% homology, and consist of 89 and 93 amino acids respectively. The *SDF-1* gene encodes both isoforms, and the isoforms are encoded by 3 and 4 exons respectively.

Homozygosity for the chemokine 3'UTR (untranslated region) *SDF-1* gene variant SDF1-3'A (wild type, GG), was shown to confer a recessive protective effect in long-term nonprogressors (LTNPs) (Winkler *et al.* 1998). HIV-1 infected individuals showed a remarkably slower disease progression compared to wild type individuals.

It was furthermore shown by these authors that in association with either the CCR5Δ32 deletion or CCR2b-64I mutations, HIV-seropositive individuals progressed even slower with the SDF1-3'A polymorphism.

Subsequent to the observation by Winkler and co-workers (1998), it was shown that the homozygous SDF1-3'A genotype (mutant, AA) had an accelerating effect on progression to disease although it was shown to yield a subsequent prolonged survival after AIDS diagnosis (van Rij *et al.* 1998, Brambilla *et al.* 2000). This polymorphism is present in 1% of Africans from South Africa, compared with 19.8% of Caucasian individuals, as shown by Williamson and co-workers (2000). This was in line with previous investigations (O'Brien 1998), indicating that the mutation was present at a much lower frequency in African individuals. It was proposed by O'Brien (1998) that SDF1-3'A can up-regulate biosynthesis of SDF-1, making the protein highly available to compete with HIV for binding to CCR4 and thereby blocking the emergence of T-tropic HIV isolates.

In the study by Sriwanthana and co-workers (2001) in CSWs in Thailand, highly exposed persistent seronegative (HEPS) women had higher frequencies of the SDF-1 3'A mutation, in addition to the *CCR5* promoter -59402GG genotype known to influence HIV transmission or course of disease. This appeared to potentially yield a reduced risk for disease progression in these individuals.

Interestingly, it was shown that the maternal heterozygote genotype (wild type/SDF1 3'A) is associated with perinatal transmission of HIV-1 (John *et al.* 2000), and particularly transmission through breastmilk. The infant genotype however, had no effect on mother-to-infant transmission.

3.1.3. *Mannose-binding lectin, MBL*

The mannose binding lectin (MBL), previously known as mannose-binding or mannan-binding protein, is the most extensively studied human collectin, and is recognised as a versatile macromolecule with many functional characteristics of the immunoglobulins (IgM, IgG) and complement factor, Clq. MBL plays an important role in immune defence, and appears to have functional significance in paediatric infection (Ross and Densen 1984).

It has been suggested that MBL is particularly important in first line host defence acting before establishment of an adaptive (protective) immune response by T- and B-cells (Sastry and Ezekowitz 1993). The ability of MBL to bind a wide spectrum of oligosaccharides through multiple lectin domains in the presence of calcium, suggested its role in immune function upon microbial challenge. Furthermore, a range of mannose-rich organisms appears to activate complement during the immune response, supporting the functional importance of MBL during infection.

The *MBL* gene encoding the MBL protein has been mapped to chromosome 10q11.2-q21 (Sastry *et al.* 1989, Schuffenecker *et al.* 1991). Three functional alleles were identified in exon 1 of the *MBL* gene, present in codons 52, 54, and 57 respectively, and are referred to as the B, C and D alleles, whereas the normal allele is referred to as A (Madsen et al. 1994), and designation of any of the variant alleles is O. Allele B results in a glycine to glutamic acid transition (Symiya *et al.* 1991), C in a glycine to glutamic acid transition (Lipscombe *et al.* 1992), and D in an arginine to cysteine transition (Madsen *et al.* 1994). Since these mutations are present in the collagen region of the gene, formation of the collagenous triple helix of the 96 kDa MBL subunits has been suggested to be disrupted by the mutations. The disrupted subunits are more vulnerable to degradation thereby reducing the amount of functional MBL subunits in heterozygous individuals approximately 5-8 times (Symiya *et al.* 1991, Garred *et al.* 1992, Madsen *et al.* 1994).

Population studies have shown that the known mutations vary between populations. For example, the B allele has been shown to be present at higher frequencies in Caucasian and Asian individuals (17% and 11% respectively), whereas the mutation was very rare in the Gambians (0.3%). Furthermore, the C allele was observed at a higher frequency in West Africans, Gambians (23-29%). Earlier studies indicated the D allele occurs at much reduced frequency in both African and Caucasian individuals (5%), and is absent from Eskimos (Madsen *et al.* 1994). A recent population study investigating the origin of the infectious disease-associated B-allele, suggested that absence of the mutation from the founder gene pool of Australian indigenous individuals possibly contributed to the vulnerability to intracellular infections such as tuberculosis in this population (Turner *et al.* 2000).

The mutant alleles were further shown to contribute significantly to a number of infections. Low serum MBL concentrations or an increased frequency of mutations have been observed in individuals with suspected immunodeficiencies and those with frequent unexplained infections (Turner 1998). It was suggested that the protein serum levels might be profoundly reduced in individuals both homozygous and heterozygous for the mutant alleles. This was confirmed to activate complement through the mannose-binding protein-initiated classical pathway (Lipscombe *et al.* 1992). Homozygosity for the B allele was subsequently identified in individuals with recurrent infections (Summerfield *et al.* 1995), confirming the association with infectious diseases.

Studies investigating susceptibility to meningococcal disease showed that homozygosity or compound heterozygosity for the three alleles occurred at significantly higher frequencies in subjects with meningococcal disease compared to healthy control subjects (Hibberd *et al.* 1999, Jack *et al.* 1997). Furthermore, investigation of the functional role of genetic variants of the *MBL* gene in lung infections frequently observed in cystic fibrosis (CF), which are a primary cause of morbidity and mortality of this immune disorder, suggested *MBL* variant alleles are potential risk factors for susceptibility to these infections (Garred *et al.* 1999). Variant alleles were especially confined to patients with chronic *Pseudomonas aeruginosa* infection, and heterozygotes for the variants were significantly more at risk to acquire *Burkholderia cepacia* infection compared with homozygotes. Follow-up studies confirmed this observation, and showed complement activation upon binding of MBL to *B. cepacia*, which was not the case for the more common colonizing organism in CF, *Ps. aeruginosa* (Davies *et al.* 2000). It was suggested that CF patients with MBL deficiency would be at a particularly high risk of *B. cepacia* colonization. The study by Garred and co-workers (1999) also indicated that the predicted survival age was significantly reduced in heterozygous carriers, compared with individuals with wild-type alleles. Furthermore, the lack of binding of MBL to *P. aeruginosa* suggested the effect of this organism on lung function in patients with MBL-deficient CF, reflecting a role for MBL possibly involved in inter-current infections with other organisms or in the inflammatory process.

MBL and HIV-infection

Homozygously inherited *MBL* genetic variants are associated with a greater risk for immunodeficiency (Garred *et al.* 1995, Summerfield *et al.* 1995). Further, association studies of HIV-infected individuals showed that homozygosity for any *MBL* variant allele was significantly increased compared to high-risk uninfected control individuals or healthy individuals (Garred *et al.* 1997b). Disease progression in HIV-infection was however more rapid when mutations are present in the *MBL* gene (Maas *et al.* 1998), accompanied by reduced serum MBL. The study conducted by Garred and co-workers (1997) also showed that individuals heterozygous for the variant alleles had significantly shorter survival times compared to the homozygous wild type genotype. These observations supported previous data by Nielsen and co-workers (1995), although differing from a study by Senaldi and co-workers (1995), which may possibly be explained due to random events or by differences in population-distribution of the variant alleles.

Although the exact mechanism for variant *MBL* alleles facilitating susceptibility to HIV-infection has not been elucidated, it has been proposed to have a direct role in HIV infection due to the ability of MBL to selectively bind HIV-infected cells, thereby inhibiting infection of CD4+ cell lines (Ezekowitz *et al.* 1989). This takes place by binding of MBL to oligosaccharides present on the HIV-1 gp120 glycoprotein, which are ligands for MBL (Larkin *et al.* 1989), followed by compliment activation (Haurum *et al.* 1993). Therefore, low serum MBL concentrations and compliment activation may impair sequestration and contribute to an increased viral load in HIV-1 infection.

3.1.4. *Iron status*

Iron is of vital importance for most living organisms. It is involved in many metabolic processes including oxygen transport, DNA synthesis and electron transport. However, regulation of iron concentrations in body tissues is essential, as excessive iron often causes tissue damage as a result of free radicals. Iron metabolic disorders are amongst the most common human diseases, with diverse clinical manifestations including iron overload and iron deficiency or anaemia. Numerous environmental determinants affect the clinical outcome of both iron overload and iron deficiency. These factors include age, gender and nutrition, in addition to genetic associations. Therefore, even where an iron-loading allele is highly prevalent, high iron status is not necessarily affected if dietary iron intake is low and iron deficiency is common in a population.

Proper understanding of iron regulation, or homeostasis, is therefore important to provide proper diagnosis and treatment in severe cases. Various studies have assisted in understanding the regulation and molecular mechanisms of iron homeostasis from the perspectives of molecular biology, biochemistry and genetics.

Iron and opportunistic infection

Although iron overload has been proposed not to be directly involved in susceptibility to microbial infection, compared to the mechanisms involved in more central systems, a number of infections have been reported in individuals with iron overload (Brennan *et al.* 1983, Capron *et al.* 1984, Bullen *et al.* 1991). Some investigations have shown that iron chelation therapy could be important in controlling infectious diseases related to iron overload (Boelaert *et al.* 1988, Rex 1988, Daly *et al.* 1989), whereas others have indicated that iron chelators are often more harmful in stimulating microbial growth and enhancing the risk of severe infection (Robins-Browne and Prpic 1985). The decision to commence iron chelation therapy should therefore be carefully individualised. The benefits of therapy to prevent iron-induced organ damage however mostly outweigh the risk of infectious complications.

Iron overload has been associated with opportunistic infections in HIV-1 disease. It has been reported that higher levels of iron is associated with both *Mycobacterium tuberculosis* and *Pneumocystis carinii* infections, two of the most common opportunistic infections associated with HIV (Weinberg 1994, Lounis *et al.* 2001). These studies showed that iron chelation therapy inhibits growth of both organisms, suggesting that excess iron may enhance microbial propagation in humans, thereby worsening the clinical outcome. Furthermore, the association of excess iron with Kaposi's sarcoma as a result of herpesvirus-8 (HSV-8) infection was shown in HIV-infected individuals in Uganda (Ziegler *et al.* 2001). These investigators pointed out that exposure to wet clay soils in Africa may be an important risk factor for accumulating iron through skin uptake, contributing to the pathogenesis of Kaposi's sarcoma caused by an opportunistic infection associated with HIV/AIDS.

African Iron overload: a potential risk factor for HIV infection and disease progression

Dietary iron overload is a common condition in sub-Saharan Africa, affecting up to 10% of individuals in rural populations (Gordeuk 1992a,b). It is characterised by heavy iron deposits in both parenchymal cells and in macrophages, and these are important in the defence against many microbial infections including *Mycobacterium tuberculosis* (Bothwell *et al.* 1965, Learmonth *et al.* 1991, Gordeuk *et al.* 1996, Lounis *et al.* 2001).

Iron loading reduces the function of macrophages and other cells of the immune system, increasing the vulnerability to infection. In Africans, iron overload can be attributed to the accumulation of iron due to high amounts of bioavailable iron. The principle source of excessive iron in these populations is home-brewed beer fermented in steel drums (Bothwell *et al.* 1964, Gordeuk *et al.* 1992a,b). In addition to the potential increased risk for infectious diseases, various other clinical outcomes, including immune disorders for example *Diabetes mellitus*, are often associated with iron overload in these populations (Bothwell and Bradlow 1960, Isaacson *et al.* 1961, Seftel *et al.* 1961, Bothwell *et al.* 1984). It has further been suggested that Africans may be genetically predisposed to iron loading, possibly as a result of a gene that differs from the HLA-linked haemochromatosis (*HFE*) gene in Caucasians (Gordeuk *et al.* 1992a,b). It would therefore be of interest to investigate genes related to iron-metabolism in populations of African descent, to establish whether genetic mechanisms exist that could facilitate susceptibility to HIV-disease in these populations.

Iron and HIV infection

Iron metabolic pathways and the immune system both play an important role in homeostasis. These systems regulate one another, and infectious agents like HIV can easily break the regulation. The discovery of the HLA genes involved in haemochromatosis (Simon *et al.* 1976, Feder *et al.* 1996) and the haptoglobin phenotype involved in iron metabolism (Delanghe *et al.* 1998) supported this mechanism.

The genetic origin of iron overload was initially described for haemochromatosis, or iron loading disease by possible association of the MHC system (Simon *et al.* 1976). The human leukocyte antigen loci HLA-A3 and HLA-B14 were shown to be associated with haemochromatosis. It was subsequently shown that the underlying genetic mechanism of haemochromatosis however involved the *HFE* gene (Feder *et al.* 1996).

Identification of disease-associated mutations in this gene facilitated interest in studies of gene-environment interactions affecting the individual risk of iron overload. Both these HLA-A3 and HLA-B14 alleles are implicated in slow progression to AIDS (Magierowska *et al.* 1999). Further, haptoglobin has been shown to be important in regulating iron levels by preventing the formation of free radicals (Gutteridge 1995a,b). An increase in iron accumulation due to the haptoglobin Hp2-2 phenotype was observed, yielding less efficient haemoglobin-binding capacity and weaker antioxidant function (Delanghe *et al.* 1998).

Based on theoretical and experimental considerations that excessive iron stores may have an adverse effect on immunity, it has been suggested that high iron levels may be of concern in individuals with HIV-infection. As reviewed by Gordeuk and co-workers (2001), sufficient evidence has been provided to suspect that high iron may adversely affect the outcome of HIV-1 infection. Optimum HIV replication requires chelatable iron (Cinatl *et al.* 1997), and the progression of HIV-1 infection towards AIDS is accompanied by increasing iron stores (Boelaert *et al.* 1996a,b). Furthermore, excess iron has the potential to enhance oxidative stress, thereby impairing immune defence mechanisms and directly promoting the growth of microbial cells (van Asbeck *et al.* 2001). Iron accumulation occurs in several tissues as a result of increasing ferritin levels during HIV disease progression (reviewed by Savarino *et al.* 1999). Shorter survival in HIV disease therefore appears to be directly associated with excess iron due to iron-mediated oxidative stress.

Further, many of the activities of the target cells of HIV are iron dependent or modulate iron metabolism (Bierer and Nathan 1990, Pattanapanyasat *et al.* 1992, Whitley *et al.* 1993, Brock 1994). Macrophages are particularly important as they play a central role in iron metabolism (Brittenham 1994), and these cells form an integral part in primary immune function. A number of genes involved in iron metabolism express their products to localise in macrophages. Investigation of the possible effects of mutations in these genes in HIV pathogenesis is therefore important.

Iron deficiency and anaemia in HIV infection

Iron deficiency occurs frequently among HIV-seropositive individuals, contributing significantly to morbidity and mortality (Forsyth *et al.* 1996). A further complication that accounts for increased morbidity and mortality in HIV disease is anaemia, often as a result of iron deficiency.

The overall incidence of anaemia among HIV-seropositive individuals ranges from 10% in asymptomatic patients to 92% in individuals with full-blown AIDS (Zon *et al.* 1987, Mir and Krantz 1992). The multifactorial nature of anaemia however complicates differential diagnosis and adequate treatment in HIV-infection.

Intestinal malabsorption was shown to be a direct cause of anaemia (Castaldo *et al.* 1996). Poor nutritional status and even malnutrition often predominate in HIV-infection, contributing to reduced iron intake. This in turn correlates with poor prognosis in HIV-infected individuals (Brettler *et al.* 1990). The typical pre-terminal stage of HIV disease, cachexia (also referred to as 'wasting'), is related to malnutrition due to intestinal malabsorption. Moreover, iron deficiency contributes to immune and neurologic impairment, both common features in HIV-infected children (Falloon *et al.* 1989). Assessment of iron status with clinical correlation could therefore provide a better understanding of the effect of iron in disease pathogenesis.

Genetic determinants of iron-related disorders in HIV-disease outcome

Genetic mechanisms associated with iron metabolism have been proposed to contribute to the clinical outcome of HIV disease, and the genes involved in HIV pathogenesis are discussed in the context of association with HIV-infection and disease progression.

The Haptoglobin gene, *Hp*

Haptoglobin (Hp) is a polymorphic acute phase haemoglobin-binding plasma glycoprotein with haemoglobin-binding capacity (Langlois and Delanghe 1996), and is present in all vertebrates (Bowman and Kurowsky 1982). Hp is part of a group of human serum α2-globulins, so called due to the ability to complex with haemoglobin. Although the function of the Hp protein is not completely understood, it may function in limiting iron loss during erythrocyte turnover and haemolysis. The main physiological function of haptoglobin is the clearance of free haemoglobin from the plasma to facilitate lower free radical formation. Hp-haemoglobin complexes are formed following haemolysis, which are rapidly taken up by hepatocytes (Kino *et al.* 1980). Hp has been proposed to have an antioxidant function by preventing haem-iron mediated generation of free radicals (Gutteridge 1987, 1995a,b).

The haptoglobin (*Hp*) gene is located on chromosome 16q22.1 and consists of 7 exons. Almost immediately adjacent to the *Hp* gene is the haptoglobin-related (*Hpr*) gene, consisting of 5 exons, sharing high nucleotide sequence homology with the *Hp* gene (Maeda *et al.* 1984, Bensi *et al.* 1985, Maeda 1985). The two common alleles of the *Hp* gene, Hp^1 and Hp^2, have been reported to result in three phenotypes, Hp1-1, Hp2-1 and Hp2-2 (Smithies *et al.* 1962, Langlois and Delanghe 1996). Several functional differences have been reported between these phenotypes (Langlois and Delanghe 1996, Langlois *et al.* 1997a,b). For example, Hp2-2 has been reported to have less efficient haemoglobin-binding capacity and hence a weaker function than Hp1-1 and Hp2-1. The Hp2-2 phenotype may result in retention of iron, resulting in oxidative stress induced by excess iron. Also, vitamin C (an antioxidant) is depleted in individuals with Hp2-2, and was shown to contribute to increased oxidative stress (Sadrzadeh and Eaton 1988, Gordeuk *et al.* 1994, Livrea *et al.* 1996, Langlois *et al.* 1997a,b). Furthermore, Hp polymorphisms may influence the course of certain infections, for example viral hepatitis, and the Hp2-2 variant has been shown to also have an adverse effect in tuberculosis infection in Black Africans (Kasvosve *et al.* 2000). Earlier studies identified another major phenotype, Hp0, predominantly in West African individuals (Allison *et al.* 1958). This phenotype is causative of an absence of Hp, resulting primarily in anhaptoglobinaemia (or anti-haptoglobin antibody), often characteristic of anaphylactic reactions in blood transfusion individuals. Hp0 was shown to be due to a deletion in the *Hp* cluster (Koda *et al.* 1998), involving complete deletion of both the *Hp* and *Hpr* genes. Hp^{del} was shown to be approximately 28 kb in size, occurring from 5.2 kb upstream of the *Hp* gene to the 3' region of intron 4 of the *Hpr* gene (Koda *et al.* 2000).

Haptoglobin and HIV-infection

Recently it was shown that the clinical outcome in HIV-infected individuals differs greatly according to various haptoglobin phenotypes. Determination of haptoglobin phenotypes in the serum of HIV-infected subjects has demonstrated that HIV-seropositive patients carrying Hp2-2 show a worse prognosis, compared to the Hp1-1 and Hp1-2 phenotypes (Delanghe *et al.* 1998). This was reflected by a more rapid rate of viral replication in the absence of anti-retroviral treatment. Sufficient evidence was provided by this study, that the highest HIV-1 viral load was found in Hp2-2 carriers compared with those with other Hp phenotypes, suggesting a more rapid viral replication rate in Hp2-2 carriers.

The lowest haptoglobin concentrations were further observed in the Hp 2-2 carriers. As a consequence, quantitative binding of haemoglobin is lower in Hp2-2 plasma than in Hp1-1 or Hp1-2 plasma, suggesting a less efficient removal of free haemoglobin from the plasma and ultimately a degree of haem iron accumulation. These patients accumulate more iron and oxidise more vitamin C, suggesting that less efficient protection against haemoglobin/iron-driven oxidative stress may be a direct mechanism for stimulating viral replication. Excess iron induces an iron-driven peroxidation of vitamin C (Langlois *et al.* 1997a, Stadtman 1991).

Although vitamin C is a powerful antioxidant (free radical 'scavenger'), in the presence of excess Fe^{3+} iron it has pro-oxidant activity (Herbert *et al.* 1996). This facilitates the conversion of Fe^{3+} trapped within ferritin to Fe^{2+}, and vitamin C itself becoming oxidized (Gordeuk *et al.* 1994). Fe^{2+} then leaks out of the ferritin protein, generating free radicals by means of the Fenton reaction (Gutteridge 1995a,b).

Fenton Reaction: $H_2O_2 \xrightarrow[Fe^{2+}\ Fe^{3+}]{} OH^{\bullet} + OH^{-}$

Oxidative stress caused for example in this manner due to reactive oxygen radicals (for example $OH^{\bullet}$), stimulates HIV replication through activation of the nuclear transcription factor NF-κB, thereby contributing to development of cell damage and immunodeficiency (Boelaert *et al.* 1996, Wong *et al.* 1991, Schreck *et al.* 1991). It has been suggested that oxidative stress is increased in HIV infection due to iron excess, which may potentially activate viral replication (Boelaert 1996a,b). Vitamin C on the other hand suppresses HIV reverse transcriptase activity and viral replication in chronically HIV-infected cells (Harakeh *et al.* 1990). A combination of higher iron-driven oxidative stress and vitamin C depletion, may therefore contribute to the increased HIV replication rate as observed in Hp2-2 individuals.

Further, a recent study investigating the contribution of the deletion of the haptoglobin gene due to Hp^{del} in HIV disease, suggested a significant association with fewer HIV-infected patients and with limited destruction of CD4+ cells, which favours an improved prognosis (Quaye *et al.* 2000).

The importance of host receptor molecules in the infection mode of HIV (via for example CCR5, CCR4, CCR3, and CCR2b) was highlighted by this observation. As haptoglobin has been shown to be a ligand for some HIV-1 cellular adhesion molecules (Sanchez-Madrid *et al.* 1983, Valentin *et al.* 1990), these investigators postulated that limited destruction of CD4+ cells in the Hp0 phenotype might serve as a cofactor in HIV transmission from cell to cell. Therefore, although not preventing spreading of the virus, the absence of haptoglobin might limit virus transmission between cells. It was pointed out via this study that CD4+ cells were diminished in HIV-seropositive individuals with Hp2-2, and that HIV transmission might be higher in this group of individuals. This could possibly be ascribed to the weak binding capacity of Hp2-2 to haemoglobin, making unbound iron available for HIV-1 replication and transmission.

Other genes potentially involved in HIV-pathogenesis due to their role in iron homeostasis

A number of other genes have been proposed to be associated with iron-related disorders, including genes encoding transferrin, transferrin receptor 1, transferrin receptor 2, ferritin-L, ferritin-H, IRP1, IRP2, β2-microglobulin, mobilferrin/calreticulin, ceruloplasmin, ferroportin, NRAMP2 (DMT1), heme oxygenase-1, heme oxygenase-2, hepcidin, USF2, ZIRTL, duodenal cytochrome b ferric reductase (DCYTB), TNFα, keratin 8, and keratin 18 (Lee *et al.* 2002). The roles of genes involved in iron metabolic pathways therefore need further investigation to fully understand their possible role in HIV-pathogenesis.

Genes involved in iron metabolism and/or immune function selected for further investigation in the present study, are discussed in more detail in the context of HIV disease.

a. The *SLC11A1* (*NRAMP1*) gene

The *NRAMP1* (natural resistance-associated macrophage protein 1) gene, recently renamed the *SLC11A1*, solute carrier family 11 (proton-coupled divalent ion transporter), member 1 gene is located on chromosome 2, locus 2q35 (Cellier *et al.* 1994, Liu *et al.* 1995). It consists of 15 exons, 14 introns, an upstream 1 kb promoter, and an alternatively spliced exon encoded by an Alu element within intron 4. Furthermore, the *SLC11A1* gene is located on a fragment of 220 kb in close proximity to the *VIL* gene, encoding villin with the interleukin-8 (IL-8) genes in between (White *et al.* 1994).

Prior to the discovery of the *SLC11A1* gene, its murine homologue *Nramp1*, was mapped to mouse chromosome 1 (Vidal *et al.* 1993), which is located close to the murine *bcg* (*ity*, *lsh*) gene. The *Bcg* gene was shown to be important in natural resistance to infection with unrelated parasites such as Mycobacteria, Salmonella, and Leishmania (Vidal 1995). *Nramp1* encodes a macrophage-specific polypeptide with predicted features characteristic of an integral membrane protein. It was suggested that *Nramp1* activity may control its own expression by negative autoregulation, which may be important in iron homeostasis and maintenance of low cytoplasmic redox active levels in macrophages (Baker *et al.* 2000).

Exonic polymorphisms of *SLC11A1* appear to be rare, possibly due to the functional nature of the gene. However, some mutations have been identified primarily in the promoter region of the *SLC11A1* gene. Nine sequence variants associated with the *SLC11A1* gene were identified by Liu and co-workers (1995), in addition to a dinucleotide repeat in the 3'UTR of the gene, that was deleted in some cases. This mutation was suggested to be involved in regulating gene expression (Liu *et al.* 1995, Buu *et al.* 1995).

Analysis of the promoter region of *SLC11A1* indicated a polymorphism encoding a Z-DNA forming dinucleotide repeat 5'$[GT]_n$, containing various alleles (Searle and Blackwell 1999, Graham *et al.* 2000, Kojima *et al.* 2001). It was established that the two most commonly occurring alleles, alleles 2 and 3, occurred at gene frequencies ranging from 0.20-0.25 and 0.75-0.80 respectively in most populations (Table 3). Alleles 1 and 4 were however less frequent (0.001), and alleles 5 and 6 are very rare.

Table 3. *SLC11A1* 5'$[GT]_n$ DNA repeat alleles, nucleotide sequences and allele frequencies (Blackwell *et al.* 1995, Graham *et al.* 2000, Kojima *et al.* 2001)

Allele	Nucleotide sequence	Allele frequency
1	$t(gt)_5ac(gt)_5ac(gt)_{11}ggcaga(g)_6$	0.001
2	$t(gt)_5ac(gt)_5ac(gt)_{10}ggcaga(g)_6$	0.20-0.25
3	$t(gt)_5ac(gt)_5ac(gt)_9ggcaga(g)_6$	0.75-0.80
4	$t(gt)_5ac(gt)_9g$ ggcaga$(g)_6$	0.001
5	$t(gt)_4ac(gt)_5ac(gt)_{10}ggcaga(g)_6$	rare
6	$t(gt)_5ac(gt)_5ac(gt)_4at(gt)_4ggcaga(g)_7$	rare

Although the ability to drive gene expression differed for all four alleles, alleles 1, 2, and 4 were shown to have poor promoter activity in the absence of exogenous stimuli, whereas allele 3 was responsible for increased gene expression. It was suggested that chronic hyperactivation of macrophages associated with allele 3 was functionally linked to autoimmune disease susceptibility, while the poor level of *SLC11A1* expression promoted by allele 2 contributed to infectious disease susceptibility (Blackwell and Searle 1999). This hypothesis was confirmed by the observation that bacterial lipopolysaccharides (LPS) significantly reduced expression due to allele 2, although expression was enhanced by allele 3. Conversely, it was shown that allele 3 protects against infectious disease and allele 2 against autoimmune disease.

From these observations it was speculated that alleles that are detrimental in relation to autoimmune disease susceptibility, might be maintained in the population because of improved survival to reproductive age following infectious disease challenge (Searle and Blackwell 1999). This observation was recently supported by Kotze and co-workers (2001) in demonstrating a significantly increased frequency of allele 3 in elderly subjects, compared with younger individuals.

Susceptibility to Infectious Diseases

Various studies showed associations between the *SLC11A1* gene and infectious diseases. Polymorphisms in *SLC11A1* were typed in a case-control study of tuberculosis in the Gambia, West Africa (Bellamy *et al.* 1998), and four polymorphisms were significantly associated with tuberculosis infection. Heterozygosity for 2 *SLC11A1* polymorphisms in intron 4 and the 3'UTR were significantly associated with tuberculosis infection in this population, compared with the most common genotype 3,3. This observation was confirmed in a study conducted in Guinea-Conakry (Cervino *et al.* 2000). It has further been suggested that susceptibility to leprosy might also have a genetic component involving *SLC11A1* and additional genetic loci (Abel *et al.* 1998). Susceptibility studies conducted in different geographical regions indicated clear association with tuberculosis infection with the *SLC11A1* gene (Greenwood *et al.* 2000, Marquet et al. 1999). Other associations with infectious diseases, including viral (HIV), bacterial (tuberculosis, meningococcal meningitis) and protozoan pathogens have been described (Shaw *et al.* 1997, Marquet *et al.* 1999, Gao *et al.* 2000, Ryu *et al.* 2000).

The study by Marquet and co-workers (1999) indicated that four variants in the *SLC11A1* gene (5'$[GT]_n$, 274C/T, 469+14G/T and 823C/T) are associated with modified risk to HIV infection. It was pointed out that three of these markers (5'$[GT]_n$, 274C/T, 469+14G/T) were in linkage disequilibrium. These three markers were furthermore associated with a reduced risk to infection, whereas the fourth independent marker (823C/T) was associated with an increased risk to HIV infection. These observations strongly suggested a risk modifying effect involving the *SLC11A1* gene.

Localization and functional mechanism of the SLC11A1 *protein*

The human *SLC11A1* gene encodes a membrane protein of 550 amino acids, with 85% homology to the mouse homologue (Blackwell *et al.* 1995). The SLC11A1 protein contains 10-12 putative transmembrane domains, 2 N-linked glycosylation sites (Vidal *et al.* 1993), and an evolutionarily conserved consensus transport motif (Cellier *et al.* 1994, Kishi 1994). An N-terminal SH3-binding domain has further been characterised (Feng *et al.* 1996a). Since discovery of the human *SLC11A1* gene, several investigations identified homologues in other organisms and mammals (Cellier *et al.* 1995, Hu *et al.* 1997).

SLC11A1 is believed to function in iron homeostasis involving the recycling of iron from effete macrophages (Atkinson and Barton 1998), possibly through lysosomal iron exocytosis. SLC11A1 localizes to membranes of late endosomes and lysosomes in macrophages (Gruenheid *et al.* 1997, Searle *et al.* 1998), but not to early endosomes. It was shown that SLC11A1 targets phagosomes (Searle *et al.* 1998), which are activated by cytokines after infection and engulfment of the pathogen. As the function of SLC11A1 is pH-dependent, this mechanism creates an acidic environment for proper functioning of SLC11A1.

Although the exact mechanistic function of SLC11A1 has not been clarified, it is believed to be a divalent cation (Fe^{2+}, Zn^{2+} and Mn^{2+}) transporter (Goswami *et al.* 2001). In contrast with the symporter SLC11A2/DMT1 (Gunshin *et al.* 1997), SLC11A1 is an antiporter that can flux divalent cations in either direction against a proton gradient. In late endosomes, i.e. after internalisation of pathogens and maturation of endosomes, SLC11A1 delivers divalent cations from the cytosol into the acidic compartment. Ferrous iron is utilised in the Fenton reaction (refer to page 45) in the acidic environment to generate toxic hydroxyl ($OH^{\bullet}$) radicals (Zwilling *et al.* 1999) for destruction of the pathogen.

b. The haemochromatosis gene, *HFE*

Haemochromatosis, or iron loading disease (often referred to as iron storage disease), is one of the most common autosomal recessive disorders in individuals of European descent. Progressive accumulation of dietary iron by major body organs is a major clinical feature of haemochromatosis, which has varied consequences for major organs, including the skin where milder clinical outcomes have been observed. Haemochromatosis can result in a wide range of clinical complications, including hepatic cirrhosis, diabetes mellitus, hypermelanotic skin pigmentation, hypergonadism, arthritis and cardiomyopathy (Bothwell *et al.* 1995, Adams and Valberg 1996, Niederau *et al.* 1996). Regulating the metabolic levels of iron may therefore have significant outcomes where overload is diagnosed, and this is effectively achieved by periodic phlebotomy (or bloodletting).

The haemochromatosis protein, HFE, has been shown to have a direct link with iron loading disease, and is often associated with the inability to down-regulate iron absorption. Although various types of iron overload exist, the term hereditary haemochromatosis (HH) should however be reserved for the HLA-linked inherited disease, determined by inappropriately high absorption of iron by the gastrointestinal mucosa.

The *HFE* gene encoding the HFE protein is located on chromosome 6p21.3. It was previously named the HLA-H gene and is related to the MHC class I family (Feder *et al.* 1996, Bodmer *et al.* 1997, Mercier *et al.* 1997). HLA-H was renamed to *HFE*, as HLA-H was also used for a presumed pseudogene in the HLA class I region. The gene was identified to be involved in hereditary haemochromatosis (HH), after the identification of the MHC class I alleles HLA-A3 and HLA-B14 (both located in the MHC region on chromosome 6) was linked to iron overload (Simon *et al.* 1976, 1977a,b,c, Ritter *et al.* 1984). The gene was suggested to be located on chromosome 6 (Stevens *et al.* 1977), possibly in linkage disequilibrium with the HLA-A locus. Some investigations suggested this gene to be located between the HLA-A and HLA-B loci (Edwards *et al.* 1985, 1986). It was further suggested that the locus for the haemochromatosis gene was in close proximity to two ferritin heavy chain genes, one being a pseudogene (Dugast *et al.* 1990). However, Calandro and co-workers (1995) established that the gene involved in HH was telomeric to the 5' end of the HLA-F locus. Subsequent studies confirmed the locus was in fact 4.6 megabases (Mb) from the HLA-A locus (Lonjou *et al.* 1998) and co-localised with the preferred marker D6S105, although this molecular marker is 2 Mb from the *HFE* gene.

The *HFE* gene consists of seven exons covering 12 kb of DNA (Feder *et al.* 1996), with the exon/intron structure identical to other MHC class I genes (Malissen *et al.* 1982). It comprises an open reading frame of 1 kb encoding 343 amino acids, and encodes an MHC class I-like molecule that requires β2-microglobulin association for normal presentation on the cell surface. Unlike other MHC class I genes where promoters are relatively close to translation start-sites, no promoter or *cis*-acting elements have been identified to promote transcription of the *HFE* gene. It has been suggested that an intron and/or an additional 5' untranslated exon exists separating the first known exon and actual transcription start-site, which may be involved in transcriptional activity (Feder 1999a,b).

HFE is expressed in most tissues as a 4.0 kb major transcript (Feder *et al.* 1996), although two minor transcripts have been identified at 5.7 and 2.4 kb. It is unknown whether this derivation may be due to alternative splicing amongst other possible mechanisms. The only tissue where HFE mRNA has not been identified is the brain. This expression pattern is suggestive of the involvement of HFE in most if not all tissues involved in iron homeostasis. HFE is however most notably expressed in the liver and small intestine, suggesting its role in cellular uptake of iron, although perhaps not an exclusive role in the regulation of iron absorption from the gut. Further, because HFE forms part of the MHC class I molecules it connects haemochromatosis with the immune system, although the mechanism has not yet been elucidated. Specifically T-cells may have a direct role in iron loading, as certain HLA alleles and haplotypes (A3 or A3B7) and CD4-CD8 ratios (CD4:CD8 >3) have been associated with a greater degree of iron overload (De Sousa 1989).

Various molecules related to HFE have been identified to utilise splice variants to regulate metabolic pathways. For HLA-G, a non-typical MHC class I protein with significant homology to HFE, it was shown that inclusion of a premature stop codon in intron 4 yields an alternatively spliced molecule (Moreau *et al.* 1995). Similarly, a truncated form of HFE that lacks the transmembrane domain as a result of alternative splicing was recently identified (Jeffrey *et al.* 1999). It was suggested by further studies that the *HFE* gene does indeed undergo alternative splicing, as different mRNAs have been identified (Rhodes and Trowsdale 1999, Thenie *et al.* 2000). A number of these molecules may have biological significance.

Furthermore, other metal transport proteins also use alternative splicing pathways to produce different protein isoforms. These include the iron regulatory element (IRE) and non-IRE containing mRNA isoforms of the divalent metal transporter 1 (DMT1/SLC11A2), found to be differentially expressed in different body tissues (Lee *et al.* 1998). It was therefore postulated that this mechanism might allow for regulated uptake of iron by the enterocyte.

Localization and functional mechanism of the HFE *protein*

Although HFE has structural homology to other class I MHC molecules, comparison of the HFE sequence with other MHC class I molecules suggested that it is unlikely that HFE plays a role in peptide presentation to cells of the immune system due to the structure of its putative peptide-binding groove (Feder *et al.* 1996, Lebron *et al.* 1998). MHC class I molecules are integral membrane molecules with three extracellular loops (α1, α2 and α3), a transmembrane region and a short cytoplasmic tail. Similarly, the HFE protein consists of a peptide binding domain, a single transmembrane region and a short cytoplasmic tail (Figure 5).

Two functional missense genetic alterations C282Y and H63D (figure 5), were identified by Feder and co-workers (1996). The C282Y (cysteine to tyrosine) mutation resulted from a G to A transition at nucleotide (nt.) 845, and the H63D (histidine to aspartic acid) mutation resulted from a C to G substitution at nt. 187. C282Y was predicted to disrupt a critical disulphide bond in the α3 loop of the HFE protein (the most highly conserved domain within class I MHC molecules), and to abrogate binding of the mutant HFE protein to β2-microglobulin and its transport to and presentation on the cell surface. Formation of a heterodimer between HFE and β2-microglobulin was however shown to be essential for correct intracellular trafficking and transport of HFE to the cell membrane, confirming the prediction of disruption by C282Y (Feder *et al.* 1997).

Various investigations confirmed that both C282Y and H63D were associated with haemochromatosis in different populations. Approximately 85% (60%-100% in different populations) of patients with haemochromatosis are homozygous for the C282Y mutation (Beutler *et al.* 1996, Jouanolle *et al.* 1996, Merryweather-Clarke *et al.* 1997, Borot *et al.* 1997, Feder *et al.* 1998, Rochette *et al.* 1999). In some studies the mutation was associated homozygously in up to 100% of HH patients (Jazwinska *et al.* 1995).

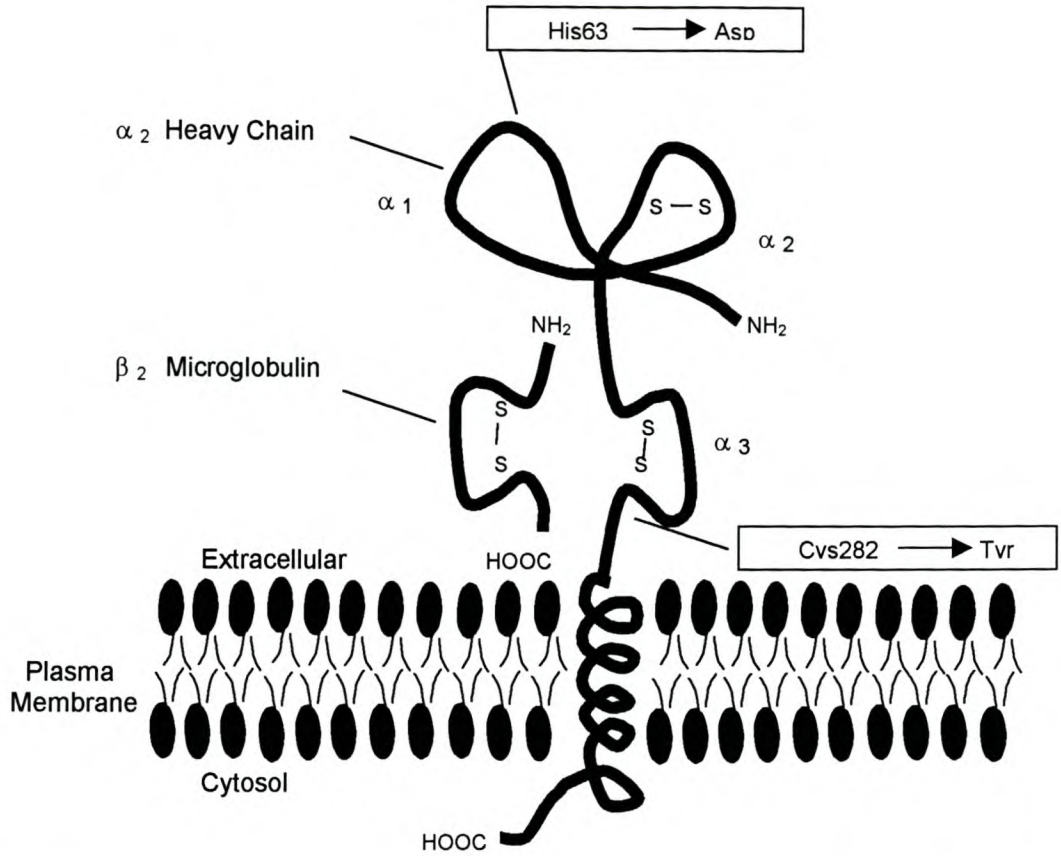

Figure 5. The predicted structure of HFE, including hereditary haemochromatosis-associated mutations (modified from Feder *et al.* 1996).

Beutler and co-workers (1996) reported that the mutations at nt. 845 and 187 where the respective genetic variations exist were in complete linkage disequilibrium: nt. 187 was a C on all chromosomes with the 845A mutation. This accounted for both the homozygously or heterozygously inherited mutations. The H63D mutation was thought to occur in a site that might cause disruption of ligand-receptor interaction in the protein. It was further shown that H63D, unlike C282Y, had no effect on β2-microglobulin-association. The frequency of H63D was found to be approximately 17% in the general population, and occurred homozygously in 2.8% of the population. In individuals with HH however, this mutation was found heterozygously in ~89% (8 out of 9) of HH patients who were compound heterozygotes for the C282Y mutation (Feder *et al.* 1996). Thus, on its own H63D did not appear to cause HH, whereas in combination with C282Y haemochromatosis could result (Feder *et al.* 1996, Beutler 1997a,b,c, Risch 1997).

Although studies indicated that both the C282Y and H63D mutations were present at low frequencies in Asian populations, where haemochromatosis is not a major health concern, it was concluded that C282Y is almost always associated with the Caucasian haplotype (Chang *et al.* 1997, Merryweather-Clarke *et al.* 1997, Cullen *et al.* 1998). This suggested its presence in these populations to be due to genetic admixture. Conversely, the H63D mutation has been found on many haplotypes.

Some studies have however shown that approximately 15% of individuals with HH do not have any mutation of the *HFE* gene (Feder *et al.* 1996, Carella *et al.* 1997, Beutler *et al.* 1997). In addition, some of those who were heterozygous for C282Y, but not C282Y/H63D compound-heterozygotes failed to show any consistent haplotype on the non-C282Y chromosome. This suggested that many different mutations in *HFE* should be present, each with a unique haplotype. However, this has not been supported by most investigations, suggesting that additional disease-causing *HFE* mutations are probably rare. Alternatively, for the majority of individuals with iron overload symptoms and no *HFE* mutations, this could possibly be due to causes other than mutations of the *HFE* gene.

The presumed role of the *HFE* gene product in the regulation of iron homeostasis suggested that varying iron levels might regulate the gene itself (Feder *et al.* 1999a,b). It was demonstrated that both wild type and H63D HFE proteins form stable complexes with the transferrin receptor (TFR), possibly contributing to its function in iron homeostasis (Feder *et al.* 1998, Parkkila *et al.* 1997). However, the C282Y mutation almost completely prevented association of the mutant HFE protein with TFR. Overexpression of the wild type HFE protein could further decrease the affinity of TFR for transferrin. The overexpressed H63D protein however did not have this effect, providing direct evidence for a functional consequence of the H63D mutation. These observations established a molecular link between HFE and a key protein involved in iron transport, and raised the possibility that alterations in this regulatory mechanism may play a role in the pathogenesis of HH.

A molecular model for the function of HFE protein and mechanism by which mutations in *HFE* may lead to HH has recently been proposed (Townsend and Drakesmith 2002). It was suggested that HFE has two mutually exclusive activities in cells: inhibition of uptake or inhibition of release of iron.

The balance between serum transferrin saturation and serum transferrin-receptor concentrations determines which of these functions predominates. HFE enables the intestinal crypt cells and reticuloendothelial system to interpret the body's iron requirements and regulate iron absorption and distribution. It was further proposed that *HFE* mutations might result in overabsorption of dietary iron with consecutive tissue iron deposition.

HFE and HIV/AIDS disease progression

A possible link between HFE and HIV-disease progression was recently suggested by the identification of a HIV-seropositive individual with relatively high iron loading due to HH (Nielsen *et al.* 1999). It was shown that HH in this individual was positively linked to homozygous inheritance of the C282Y mutation. Long-term survival (more than 10 years) was further achieved after regular phlebotomy. Although this was the only case reported to date with HH, this observation suggested a potential important role of iron in HIV/AIDS disease progression. It was further suggested that HIV-infection in this individual might have been protective against iron loading damage, as no clinical symptoms for HH were observed. However, as iron loading could be disadvantageous in HIV-infection, one might expect that HIV-seropositive individuals with HH may present with a worse clinical outcome due to iron-mediated acceleration of HIV infection (Dhople *et al.* 1996). The significant observation described by this report, further supported by preliminary data from polytransfused individuals, suggested that iron chelation therapy might assist in preventing (or reversing) negative consequences normally associated with both iron overload and HIV infection.

Does C282Y and H63D affect African iron overload?

From various population studies, an association for the C282Y substitution in the *HFE* gene of African individuals and iron overload have not been found, whereas it is highly prevalent in populations of European ancestry (Merryweather-Clarke *et al.* 1997, Roth *et al.* 1997, Barton and Acton 2001). This supports the previously suggested Celtic origin of haemochromatosis (Jazwinska *et al.* 1995). In contrast however, the H63D substitution is not restricted to European populations, as it has been detected in some African populations, suggesting the H63D substitution may have occurred earlier than the C282Y substitution.

Furthermore, the H63D and C282Y mutations have not been detected in Southern African individuals diagnosed with African iron overload (McNamara *et al.* 1998). It has been suggested that this condition may have a genetic cause, in addition to being directly related to excess dietary iron intake (Gordeuk *et al.* 1992b). However, it does not appear as though the known *HFE* mutations have an effect on this condition. It was also shown that African iron overload was not linked to the HLA locus on the short arm of chromosome 6 (Gordeuk *et al.* 1992b), suggesting the involvement of another gene(s) in this condition in Africans. Therefore, if African iron overload had a genetic cause, it appears as though this differs from that found in the European condition. Alternatively, different *HFE* mutations may be responsible for this condition in African individuals.

It has been suggested that individuals with iron overload who do not have the known H63D or C282Y causative mutations, may have mutations in other genes contributing to iron loading (McNamara *et al.* 1998). This suggestion can be supported by the fact that excess iron accumulates in the macrophages of individuals with African iron overload, whereas it is not the case in Caucasian individuals (Bothwell and MacPhail 1998). It is therefore possible that unidentified mutations in other genes may contribute to the condition of African iron overload.

Other HFE mutations

A number of other mutations have been identified in *HFE*. Of these, the S65C mutation (serine to cysteine) is the most commonly occurring mutation affecting the *HFE* gene, and resulting in a mild form of HH (Fagan and Payne 1999, Mura *et al.* 1999). Also, rare *HFE* mutations were recently reported by Bradbury and co-workers (1999). These mutations, E277K and V212, were observed in two isolated individuals (in an Asian and a Caucasian individual, respectively) with diabetes, but no signs of iron overload.

Recently, a study on South African populations (de Villiers *et al.* 1999) identified novel missense and silent mutations in the *HFE* gene. An exon 2 mutation resulting in a V53M amino acid substitution was identified in South African Black and Bushman (Khoi-San) populations, and a second exon 2 mutation resulting in a V59M substitution was identified in a South African Caucasian. An exon 5 mutation resulting in a R330M substitution was also identified in South African Caucasian individuals from this study.

Of note, a mutation occurring in exon 3 (Q127H) was identified, which may disrupt a critical disulphide-binding area of the HFE protein. This may resemble the reported situation for the C282Y mutation, which disrupts a critical disulphide bond in the α3 extracellular loop of HFE (Parkkila *et al.* 1997). Some of these and other mutations described by these investigators indicated a possible association with porphyria cutanea tarda (PCT), often associated with hepatic siderosis and iron overload (Roberts *et al.* 1997b, Bonkovsky *et al.* 1998, Sampietro *et al.* 1998). This relationship has recently been confirmed in South African individuals with PCT (Elder and Dorwood 1998, Hift *et al.* 1997).

A previously described intron 2 mutation IVS2+4T was recently identified in 70% of PCT patients from Denmark (Christiansen *et al.* 1999, Douabain *et al.* 1999), which almost always occurred heterozygously or homozygously with the known H63D or S65C mutants. Further, a splice-site mutation in intron 3 (IVS3+1G/T) in compound heterozygosity with C282Y was identified in two Caucasian siblings with iron overload (Wallace *et al.* 1999). Although extremely rare, it was shown that this mutation functionally alters RNA splicing causing obligate skipping of exon 3. The functionality of this mutation highlighted the possibility of other rare *HFE* mutations, possibly in compound heterozygosity with C282Y, causing high iron overload.

African-specific HFE polymorphism

A common polymorphism in intron 3 (IVS3-48C→G) was identified recently, occurring almost exclusively in African American individuals (Beutler and Gelbart 2000). This polymorphism was detected after several investigators noted that the binding site for PCR-amplification primers used in routine diagnosis of HH (described by Feder and co-workers in 1996) was hampered by a mutation in intron 4 (de Villiers *et al.* 1999, Beutler *et al.* 2000, Merryweather-Clarke *et al.* 1997). It was indicated that presence of both the intron 3 and intron 4 polymorphisms may lead to misdiagnosis of C282Y.

The IVS3-48 C→G polymorphism occurs in a site that closely resembles the splice site also occurring in intron 3 (Beutler and Gelbart 2000), and this mutation had a greater effect than one in intron 4, which prevent amplification of the preferred C282Y allele.

Although this mutation is located in a potential alternative splice site, no evidence has been reported on alternative splicing of HFE or iron homeostasis. The elevated frequency in Africans (12.8% compared with 0.6% of Caucasians) is however a notable observation. Alternatively, this intronic mutation may be causative of alternative splicing in tissues other than leukocytes, which may be involved in iron homeostasis. This study furthermore indicated that serum iron parameters were not altered in individuals with the mutation, and it was concluded that it only affects accurate diagnosis of the C282Y mutation. It might however be important to investigate the possible significance of this polymorphism in different population groups, including HIV-infected individuals where viral infection may affect iron homeostasis.

c. Immune Function: The *CD45* (*PTPRC*) gene

In addition to non-MHC genes that may determine susceptibility to HIV, for example *CCR5* and *SLC11A1*, the modulation of T-cell responses to particular antigens determines disease severity. T-cell differentiation depends on the availability of iron, and iron metabolism appears to be regulated by these molecules, possibly through interactions with the MHC class I-like HFE protein (reviewed by Bowlus 2002). Alterations in iron metabolism have a potentially hazardous impact on immune-related disorders, including immunodeficiency. Both iron deficiency and overload have been shown to contribute to T-cell abnormalities. The role of iron in the pathogenesis of HIV/AIDS can be supported by the association of the iron transporter gene *SLC11A1* with risk to HIV infection (Marquet *et al.* 1999) and several other infectious and autoimmune disorders. Additionally, iron can catalyse the production of cryptic epitopes of several autoantigens, supporting the role in immunity. It is further possible that the polygenic nature of HIV/AIDS susceptibility could be attributed by the involvement of molecules involved in immune activation, for example CD45 (Tchilian *et al.* 2001a).

The *CD45* (or *PTPRC*, protein tyrosine phosphatase receptor type C) gene is located on chromosome 1q31-32, and contains 35 exons encoding the CD45 (PTPRC) molecule, a member of the protein tyrosine phosphatase (PTPase) family (Akao 1987, Ralph *et al.* 1987, Seldin 1988). It has been estimated to be more than 120 kb in size (Fernandez-Luna *et al.* 1991, Hall *et al.* 1998), and co-localises with marker D1S413 on chromosome 1 (Goff *et al.* 1999). Further, other genes of immunologic interest have been reported to cluster in this chromosomal region (Ravetch *et al.* 1986).

Although control elements in the CD45 gene has not been detected, analysis of the 5' flanking region of the human CD45 gene indicated a region with strong promoter activity in the highly conserved first intron of the gene (Timon and Beverley 2001). The promoter activity is not tissue-restricted, and is strongest at the 3' end of intron 1, although the nucleotide sequence lacks similarities with known promoters and initiators. An alternative exon 1, designated 1a, was further identified which can be spliced to exon 2. Also, due to the unusually large intron sequence between exons 2 and 3, which appears to be very conserved, the possibility of control sequences being located within intronic sequences of the CD45 gene was recently suggested (Virtis and Raschke 2001).

CD45, also known as PTPRC (or T200 glycoprotein or leukocyte common antigen, Lca) is an integral transmembrane tyrosine phosphatase that is expressed on nucleated haemopoietic cells where it comprises up to 10% of the cell surface (Charbonneau *et al.* 1988, Tonks *et al.* 1988, 1990, Trowbridge and Thomas 1994).

CD45 functions as a tyrosine phosphatase in leukocyte signalling, regulating both T-cell receptor-associated kinases and Janus kinases, and is involved in signal transduction from cytokine receptors. T-cell receptor-mediated activation of B- as well as T-lymphocytes is facilitated by CD45 after antigen stimulation by dephosphorylation of an inhibitory site on the Src-family of tyrosine kinases, yielding an enhanced kinase ability of the substrate essential for initiation of the signalling event (Thomas and Brown 1999). Efficient lymphocyte antigen receptor signal transduction is dependent on the phosphatase activity of CD45 (Kishihara *et al.* 1993, Byth *et al.* 1996, Kung *et al.* 2000, Tchilian *et al.* 2001b). A lack of CD45 has been shown to be responsible for severe immunodeficiency, due to a lack of T-cells in the periphery and impaired T and B cell responses. CD45 can further function as a Janus kinase (Jak) phosphatase, negatively regulating cytokine receptor activation involved in differentiation, proliferation and anti-viral immunity of haemopoietic cells (Irie-Sasaki *et al.* 2001).

CD45 is a type I transmembrane protein with a large heavily glycosylated extracellular domain (consisting of 391-552 amino acids), and an extensive cytoplasmic tail (consisting of 700 amino acids) that contains two domains with phosphatase homology. The proximal cytoplasmic domain of CD45 is responsible for phosphatase activity, whereas the second domain is required for proper folding and substrate recruitment.

Substrates for CD45 include various members of the Src family of tyrosine kinases, which are operated by CD45 either as a positive or negative regulator (Ostergaard *et al.* 1989, Shiroo *et al.* 1992). Although the cytoplasmic domain is highly conserved, low sequence similarities have been observed between CD45 extracellular domains of different species (Okumura *et al.* 1996, Diaz Del Pozo *et al.* 2000). This supports the essential role of CD45 as a phosphatase for normal antigen-induced signal transduction.

At least eight different isoforms have been identified for CD45, which are generated by complex alternative splicing. The alternative spliced isoforms range in molecular weights from 180 to 220 kDa (Alexander 1997). Alternative splicing of exons 4, 5 and 6 at the N-terminus of the extracellular domain of the molecule is responsible for the different isoforms (Saga *et al.* 1986, Streuli *et al.* 1987). Exons 4, 5 and 6 are also termed A, B and C respectively. Thus, CD45RABC refers to an isoform containing all three variable exons 4, 5 and 6, while the CD45R0 isoform refers to the null isoform in which exons 4, 5 and 6 have been spliced out.

Expression of the different CD45 isoforms depends on the state of activation and differentiation of haemopoietic cells (Akbar *et al.* 1988, Hathcock *et al.* 1992, Craig *et al.* 1994), and plays a distinct role in cellular signalling (Chui *et al.* 1994, Leitenberg *et al.* 1996). In humans and other mammals differential expression of CD45 isoforms on the cell surface has been used as a measure to distinguish between subsets of T-cells with distinct functional properties (Akbar *et al.* 1988, Lee *et al.* 1990, Mackay *et al.* 1990, Powrie *et al.* 1990).

B-lymphocytes express the high molecular weight isoform of 220 kDa, CD45RABC (also called B220). Immature CD4+CD8+ thymocytes express mainly low molecular weight isoforms, whereas mature CD4+ and CD8+ thymocytes and peripheral T-cells can express multiple isoforms. Expression of different isoforms also changes during T-cell activation. Two major subsets of T-lymphocytes have been identified in humans expressing high molecular weight isoforms containing the A exon (CD45RA cells) or the l80 kDa isoform (CD45R0 cells), also termed naïve and memory cells respectively. The two subsets however have distinct properties (Michie *et al.* 1992, Pilling *et al.* 1996, Hamann *et al.* 1997, Young *et al.* 1997, Maini *et al.* 1999, 2000). It is further known that HIV preferentially infects CD45RO cells (memory cells), which are lost earlier in HIV infection than their CD45RA counterparts (Helbert *et al.* 1997).

Various mutations in the *CD45* gene have been linked to the function of CD45 in immune-related disorders. The phenotypic changes caused by these mutations could alter the function of CD45 in immune function in humans, suggesting that CD45 is a potential candidate target for immune intervention, and that its modulation may affect autoimmune-related disorders, amongst others. Alterations in the respective mutation carriers may lead to changes in ligand binding, homodimerization of CD45, and altered immune responses, suggesting the involvement of natural selection in controlling the carrier frequencies of the mutations.

Recently, a homozygous 6 bp deletion was identified in exon 11 of the *CD45* gene, which results in a lack of surface CD45 expression and severe combined immunodeficiency (SCID) (Tchilian *et al.* 2001c). The deletion resulted in a loss of two essential amino acids of the extracellular domain of CD45. Another study also reported lack of CD45 expression associated with SCID (Kung *et al.* 2000).

Additional to the association of SCID, further abnormalities of CD45 splicing have been reported in humans yielding lack of CD45 surface expression. A C to G transversion in the fourth exon of CD45 (exon A, C77G) was shown to disrupt normal splicing of the N-terminal region (Thude *et al.* 1995, Zilch *et al.* 1998). Although this silent mutation does not change the amino acid sequence, it prevents splicing by disrupting a strong exonic silencer (Lynch *et al.* 2001). Both high and low molecular weight CD45 isoforms are expressed by activated or memory T-lymphocytes in affected individuals, in contrast to the normal pattern of low molecular weight isoform expression. Development of multiple sclerosis (MS) was recently associated with expression of the C77G polymorphism (Jacobsen *et al.* 2000). This association could not be confirmed in subsequent studies (Barcellos *et al.* 2001, Vorechovsky *et al.* 2001). Furthermore, an increased frequency of the C77G variant G-allele was observed in HIV-1 seropositive individuals, compared to healthy individuals (Tchilian *et al.* 2001a). Further associations involving alternative splicing of CD45 due to C77G have also been described (Bujan *et al.* 1993, Wagner *et al.* 1995). Combined, these studies suggested an association of abnormal CD45 splicing with impaired viral clearance. It appears that expression of different isoforms of CD45 alters T-cell related (TCR)-mediated signal transduction, although the exact functions of individual isoforms are unknown. It is thus likely that abnormal CD45 splicing may be associated with immunodeficiency, autoimmunity or impaired anti-viral responses.

Population studies have indicated that the frequency of the C77G transversion varies considerably in the general population. Reported frequencies were 0.16% in Germany and 0.85% in the United Kingdom, and ranged between 1.4 and 1.8% in North America and Sweden (Jacobsen *et al.* 2000, Vorechovsky *et al.* 2001). Striking differences have been observed in different African and Asian populations, where it could be as high as 6.7% in Pamiris from Central Asia (Tchilian *et al.* 2002). The observation of the highest frequency in central Asian populations, suggested that the variant might have arisen in Central Asia. However, the mutation appears to be absent from African individuals (Tchilian *et al.* 2002).

Altered isoform expression could further be due to a second mutation in exon 4 (C59A) causing interference with splicing in MS patients (Jacobsen *et al.* 2002). In addition to a disruption of the stronger of two *CD45* exonic splicing silencer elements, ESS-1, C59A causes a non-conservative amino acid exchange (H19Q, histidine to glutamine) in the CD45A antigen, suggesting this mutation may be causative of a structurally altered protein.

Most recently, a polymorphism in exon 6 (A138G) was detected in the *CD45* gene and it was also shown to interfere with alternative splicing (Stanton *et al.* 2003). The polymorphism results in a T47A (threonine to alanine) amino acid substitution, a potential O- and N-linked glycosylation site. The A138G variant is present at a frequency of 23.7% in the Japanese population, although absent in Caucasians. Peripheral blood T-cells from individuals carrying A138G showed significant reduction in the proportion of cells expressing the A, B, and C isoforms of CD45 and a high frequency of CD45R0+ cells.

The CD45 locus has furthermore been suggested to be a site of strong natural selection in other vertebrates (Raschke *et al.* 1995, Symons 2000, Ballingall *et al.* 2001), which appears to be pathogen driven.

As CD45 has an important role in immune function, with association in various immune-related disorders including HIV-1 infection, predominantly due to alternatively spliced isoforms expressed in different cell types of the immune system, it is therefore important to investigate the association of possible genetic variation in the *CD45* gene with HIV/AIDS in the diverse South African population.

OBJECTIVES AND AIMS

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The complexity of interactions between host cells and microorganisms at the molecular level suggests that genetic variants of any of the host molecules involved in the host response to infection might alter the fate of the infectious agent within the host. Differing susceptibility patterns between individuals and between different ethnic populations furthermore contribute to the complexity of infectious diseases. Most host genetic investigations have been conducted in Western and Asian populations. The importance of investigating African populations is therefore clear, as the majority of HIV-infections occur in developing countries, specifically from sub-Saharan Africa.

SPECIFIC OBJECTIVES

1) To contribute to a better understanding of the genetic background of HIV-1-infected subjects in order to eventually increase the predictive ratio of disease evolution.

2) To identify genetic variants involved in HIV/AIDS susceptibility in the South African population and investigate possible phenotypic effects of relevant mutations studied.

3) To gain knowledge from studying South African subjects at the molecular level which may ultimately improve treatment strategies and may serve, in addition to viral load and CD4 cell counts, as decision-making tools for clinical management of HIV-1 infected subjects.

Even if the promise of preventive and therapeutic intervention based on HIV/AIDS resistance genes is not fulfilled, discovery of relevant genetic factors potentially contributing to susceptibility to HIV infection or progression to AIDS may have prognostic value. This may therefore offer an opportunity to refine ongoing evaluation of other promising therapeutic or preventive measures.

ETHICAL APPROVAL

Ethical approval for the project was obtained from the Research Committee of Stellenbosch University (project number 2000/C068). All the patient material has been obtained with informed consent from collaborating clinicians who are responsible for patient management.

CHAPTER 2

RESULTS AND DISCUSSION

A. The *SLC11A1* gene and modified risk to human immunodeficiency virus infection susceptibility in South Africa

Objectives: To investigate the likelihood that variation in the *SLC11A1* gene, encoding the iron modulating solute carrier family 11 member 1 protein, modifies the risk of HIV infection and/or progression to AIDS in South African individuals of predominantly African origin. The ultimate aim is to gain insight into the mechanisms underlying host susceptibility to disease progression.

Design: The functional 5'[GT]n repeat polymorphism in the promoter region of the *SLC11A1* gene and a potentially detrimental 9-bp deletion in exon 2, were analysed in 374 HIV-seropositive individuals and 410 HIV-seronegative population-matched controls.

Methods: Mutation detection was performed using polymerase chain reaction (PCR)-based methods, including restriction enzyme analysis and combined heteroduplex-single strand conformation polymorphism (Hex-SSCP) analysis, followed by direct sequencing where appropriate.

Results: Genotype distribution and allele frequencies did not differ significantly between HIV-seropositive and HIV-seronegative individuals, although genotype 2,3 of the *SLC11A1* 5'[GT]n repeat polymorphism appears to predominate in patients attending the Infectious Diseases Clinic at Tygerberg Hospital compared with population-matched controls.

Conclusions: The data obtained in this study largely exclude the likelihood that the studied *SLC11A1* polymorphisms play a major role in HIV/AIDS susceptibility in our predominantly African population group. Since polymorphic variants may become clinically relevant only in certain environments or genetic backgrounds, we conclude that *SLC11A1* host factors involved in modified risk of HIV infection may be population-specific.

Introduction

Host genetic factors have been shown to influence susceptibility to human immunodeficiency virus type 1 (HIV-1) infection and progression to AIDS (Klein and Miedema 1995, Bollinger *et al.* 1996, Haynes *et al.* 1996), in addition to immunological factors and viral characteristics. Variation in a number of genes have been found to completely confer resistance or at least protect against infectious agents, such as mutations in chemokine co-receptors and the presence of certain alleles of the major histocompatibility complex (MHC) (Liu *et al.* 1996, Dean *et al.* 1996, Laumbacher and Wank 1998). Magierowska and co-workers (1999) have shown that combined genotypes of CC chemokine receptor-2 (*CCR2*) and -5 (*CCR5*), the chemokine gene *SDF-1*, encoding the ligand for the CXCR4 chemokine receptor, together with certain alleles of both MHC Class I and II, may strongly influence HIV/AIDS disease progression. Accurate classification of approximately 70% of long-term non-progressors (LTNPs) and 18% of progressors was possible by applying this proposed model, suggesting that other as yet unidentified host factors that may provide protection or increase susceptibility to HIV-infection in some individuals remain to be identified.

Although various susceptibility mechanisms have been proposed for HIV/AIDS, most models involving host genetics are based on studies performed in Caucasians and Asian populations, with limited data from African populations. Also, genes included were mainly those related to chemokines and the histocompatibility antigen (human leukocyte antigen, HLA). Recent HIV-association studies of chemokine-related genes in South Africa (Williamson *et al.* 2000, Petersen *et al.* 2001) and elsewhere (Ramaley *et al.* 2002a) have shown that except for the CCR2b-64I genetic variant, protective genetic variation is relatively rare in Africans. Since HLA studies have indicated that different alleles are implicated in HIV/AIDS susceptibility in Africans and Caucasians (Rohowsky-Kochan *et al.* 1998, Roe *et al.* 2000), further studies of new candidate genes are warranted in the African context.

Genetic variation in genes encoding proteins involved in immune defence, expressed in cells of the monocyte/macrophage lineage, may modify the risk of HIV-1 infection and progression to AIDS.

Interest broadened in the family of proton-coupled divalent metal ion-transporter proteins, specifically the solute carrier family 11 member 1 (SLC11A1), formerly known as the natural resistance-associated macrophage protein 1 (NRAMP1), following evidence that monocytes and macrophages are important in the early phase of HIV infection (Gill *et al.* 1996). Association of the *SLC11A1* gene encoding this protein with modified risk to HIV infection (Marquet *et al.* 1999, Ramaley *et al.* 2000) was in accordance with the proposed role of SLC11A1 in infectious disease susceptibility (Skamene *et al.* 1998, Buschman *et al.* 1997, Blackwell *et al.* 1996). Studies performed by Searle and Blackwell (1999) demonstrated that the poor level of expression of allele 2 [$(t(gt)_5ac(gt)_5ac(gt)_{10}g$] of the 5'[GT]n polymorphism in the promoter region of the gene is associated with infectious disease susceptibility. Furthermore, chronic hyperactivation of macrophages associated with allele 3 [$(t(gt)_5ac(gt)_5ac(gt)_9g$] is functionally linked to autoimmune disease susceptibility. Whilst this differential effect was mediated by bacterial lipopolysaccharides *in vitro*, an extension of these studies including ferric ammonium citrate as an external stimulus suggested the importance of cellular iron status in differential regulation of macrophage activation (Kotze *et al.* 2003). The finding that allele 3 may protect against infectious disease and allele 2 against autoimmune disease (Searle and Blackwell 1999), raised the possibility that this phenomenon is related to low expression of allele 2 in the presence of high cellular iron, thereby exerting protection against autoimmune disease. Conversely, high expression of allele 3 upon iron loading may be related to the body's defence mechanism of withholding iron from invading pathogens. It seems likely that such complex gene-environment interaction might explain the paradoxical observations that infectious agents can both induce and suppress autoimmune disorders (Bach 2002). Availability of cellular iron related to dietary iron intake and subsequent biological processes involved in iron homeostasis could possibly influence the outcome of case-control studies in diverse populations exposed to different environments. Further, infrequent occurring alleles of 5'[GT]n have previously been described, and occur at much reduced (0.1% respectively) frequencies (Graham *et al.* 2000). These alleles have been associated with other immune-related disorders, but no association with infection has been observed.

Marquet and co-workers (1999) studied six different polymorphisms in the *SLC11A1* gene and reported independent association of two gene regions with HIV susceptibility in Columbians.

Their data indicated significant association between HIV/AIDS susceptibility and the functional 5'[GT]n polymorphism, which appeared in strong linkage disequilibrium with two downstream polymorphisms, 274C/T and 469+14G/T. In the present study the promoter polymorphism and a potentially detrimental 9 base pair (bp) deletion in exon 2 encoding the putative SH3 binding domain of SLC11A1, were analysed in 374 HIV-seropositive and 410 HIV-seronegative population-matched individuals from predominantly African origin from South Africa. The 9-bp deletion removes three amino acids from an SH3-binding domain of the protein and has been associated with susceptibility to HIV-1 infection in the Ugandan population (Ramaley *et al.* 2000). In the mentioned study by Marquet *et al.* (1999) allele 2 of the 5'[GT]n promoter polymorphism, occurring at a frequency of 20-25% in the general population (allele 3, 75-80%) (Searle and Blackwell 1999), was reported to be protective towards HIV infection in Columbians.

Materials and Methods

Study Population

The study cohort consisted of HIV-seropositive individuals recruited in the Western Cape, Northwest and Gauteng Provinces of South Africa, and HIV-seronegative population-matched control individuals (Table 1). Venous blood samples were obtained with informed consent from these study participants and the Ethics Review Committees of the Universities of Stellenbosch and Potchefstroom approved the study.

HIV-seropositive study participants from the Western Cape Province (n=219) attended the Infectious Diseases Clinic at Tygerberg Hospital in Cape Town and included 125 subjects of African origin (mainly Xhosa) and 94 of mixed ancestry (Cape Coloured population of San, Khoi, African, Madagascar, Javanese and European origin). Estimated date of infection and HIV-1 disease-associated complication data were recorded for the majority of these individuals, and ages were calculated based on date of blood sampling. Data on disease status were denoted according to WHO clinical disease classification (classes I-IV, http://who.int), which was available for 171 individuals. HIV-seropositive individuals were further classified as normal progressors when progressive loss in CD4 cell counts were associated with development of AIDS-related symptoms within 10 years after HIV-1 infection, as fast progressors when they progressed to full-blown AIDS within 2-3 years from seroconversion, and as slow progressors when they remained unaffected after 8-10 years from seroconversion.

Table 1. Study Cohort

Study Population	HIV-seropositive			HIV-seronegative		
Western Cape Province	Male	Female	Total	Male	Female	Total
African	37	88	125	26	38	64
Age range (years)	21-59	19-56	19-59	19-47	17-49	17-49
Mean Age	33.19	30.61	31.43	27.5	28.6	27.94
Mixed Ancestry	25	69	94	56	10	83 *
Age range (years)	21-56	22-73	21-73	17-52	26-45	17-52
Mean Age	36.20	34.15	34.71	30.14	34.38	30.67
Northwest Province	Male	Female	Total	Male	Female	Total
African	55	91	146	52	99	151
Age range (years)	15-81	17-69	15-81	7-71	17-77	7-77
Mean Age	35.00	33.76	34.28	42.24	36.23	38.08
Gauteng Province	Male	Female	Total	Male	Female	Total
African	8	1	9	54	58	112
Age range (years)	32-48	44	32-48	22-62	21-59	21-62
Mean Age	38.50	44	39.11	34.19	30.83	32.38
TOTAL	125	249	374	188	205	410

*Ages and gender available for 66 HIV-seronegative individuals of mixed ancestry

Progression status according to these criteria was available for only 72 of the HIV-seropositive individuals. HIV-seronegative population-matched individuals for the African individuals from the Cape Province (n=64) were selected from volunteers with no previous history of HIV-related infectious disease or immune disorders. For the study participants of mixed ancestry (n=83), HIV-seronegative population-matched individuals were recruited from healthy individuals attending blood donation clinics in the Western Cape.

For the population recruited from the Gauteng Province blood samples were obtained anonymously from predominantly healthy individuals, and the population consisted exclusively of African individuals (9 HIV-seropositive and 112 HIV-seronegative). The African population from the Northwest Province formed part of a cross-sectional study previously performed by James *et al.* (2000), and consisted of 146 HIV-seropositive and 151 HIV-seronegative individuals.

Since these individuals were recruited from a random population sample, HIV status was unknown at the time of entry and was assessed upon inclusion into the study. These individuals originate from a more rural setting compared with the study cohort recruited in the Cape Province and Gauteng Province, and disease progression status was asymptomatic for the majority of the HIV-seropositive individuals.

DNA Extraction and Amplification

Genomic DNA was extracted from whole blood using a standard method (Miller *et al.* 1988). Primer sets used for polymerase chain reaction (PCR)-based DNA analysis of the *SLC11A1* genomic regions of interest were as follows: 5'-CCCCAGAACCTTGAGGTCTA-3' (forward) and 5'-CCCACACCAGTACCCCAT-3' (reverse) for analysis of the 5'[GT]n promoter polymorphism; 5'-AGGAGGGAAAGGATCAGG-3' (forward) and 5'-GGATCCTGTCAATCTTGCAAGC-3' (reverse) for analysis of the 9-bp deletion polymorphism in exon 2. DNA amplification was performed in 25 μl reaction volumes containing ~20 ng of genomic DNA, 5 mM each deoxyribonucleoside triphosphates (dNTPs), 15 pmol of each PCR primer, Mg^{2+}-containing reaction buffer and 0.375 units of DNA *Taq* polymerase (Roche Diagnostics, Switzerland). A step-down PCR amplification procedure was applied with one cycle initial denaturation for 3 minutes at 95°C, ten cycles of denaturation at 95°C for 45 seconds, annealing at 65°C for 30 seconds and extension at 72°C for 45 seconds, followed by twenty-five cycles of denaturation at 95 °C for 45 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 45 seconds, and final extension at 72°C for 10 minutes. PCR amplification products were visualised in 2% agarose (Seakem) by ethidium bromide staining and ultraviolet transillumination.

DNA analysis

A standard combined heteroduplex single-strand conformation polymorphism (HEX-SSCP) procedure (Kotze *et al.* 1995) was applied for detection of the 9-bp deletion in exon 2. Polyacrylamide gels were supplemented with 7.5% urea and electrophoresis was performed at 270 V for 17 hours (overnight) at 4 °C. Sequencing analysis confirmed the 9-bp deletion (not shown). Alleles of the *SLC11A1* 5'[GT]n promoter polymorphism were distinguished by *Rsa* I (Promega Inc., UK) restriction enzyme analysis (0.5 units per reaction) as previously described (Graham *et al.* 2000). Digested PCR products were electrophoresed at room temperature in 5% cross-linked polyacrylamide gels at 300 V for 2 hours.

DNA fragments in the respective polyacrylamide gels were visualised by ethidium bromide staining and ultraviolet transillumination. Genetic variants were confirmed by automated sequence analysis, where appropriate.

Statistical Analysis

Allele frequencies were determined by genotype count, and the exact test of the Hardy Weinberg proportion was applied using the Markov Chain algorithm (Guo and Thompson 1992, available from: http://www2.biology.ualberta.ca/brzusto.hwenj.html). Genotype distribution and allele frequencies were compared among HIV-seropositive and HIV-seronegative individuals within the same populations and between populations. Where no differences in genotype distribution were observed, populations were combined to improve statistical power. Testing for heterogeneity in mutation frequency was based on the Fischer exact test for 2-sided probability, using GraphPad InStat® Version 3.05 for Win 95/NT, GraphPad Software, San Diego California USA (www.graphpad.com), and a 2-tailed *P*-value <0.05 was considered significant. Odds ratios and 95%CI (with Woolf approximation) were determined for combined homozygous mutant and heterozygous genotypes as a measure of relative risk for affected genotypes and alleles of interest and HIV-status. For populations with differing genotypic distribution between HIV-seropositive and HIV-seronegative individuals, theoretical sample size for significant power ($P<0.05$) was calculated according to observed proportions and resulting odds ratios.

Results

Genomic DNA samples of 374 HIV-seropositive and 410 HIV-seronegative South African individuals (Table 1) were screened for variation in two functionally important regions of the *SLC11A1* gene. Figure 1 illustrates ethidium bromide stained gels showing the 5'[GT]n repeat using *Rsa* I digestion in the *SLC11A1* gene (A) and a 9 bp deletion polymorphism detected by HEX-SSCP analysis (B), respectively. The 32 bp deletion of the *CCR5* gene (CCR5Δ32), which was also screened for in all study participants in an attempt to exclude possible confounding effects as a consequence of the known protective effect in relation to HIV/AIDS, could be readily detected by polyacrylamide gel electrophoresis (Figure 1C). The deletion mutation was detected in three Cape Coloured males whilst absent in Black Africans included in the study, and co-existence with allelic variants of the *SLC11A1* gene did not influence the outcome of the present study.

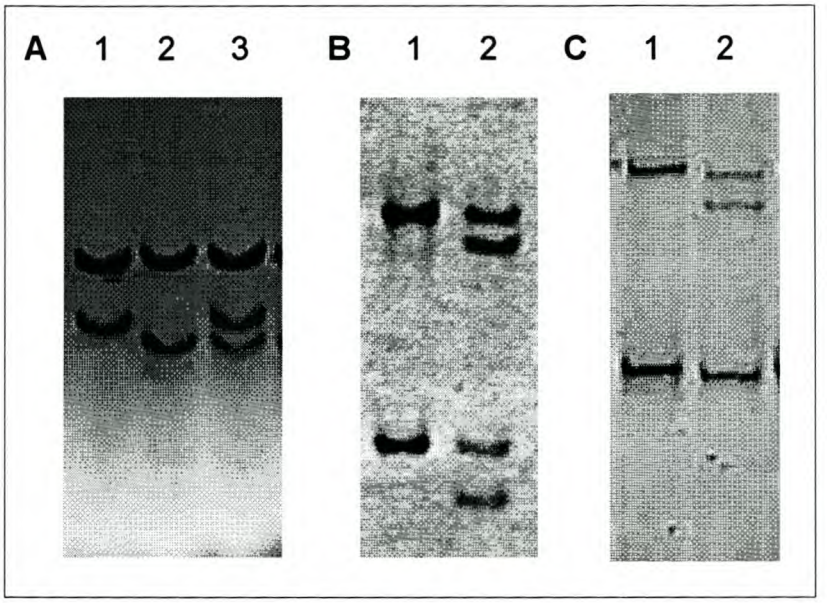

Figure 1. Analysis of the *SLC11A1* and *CCR5* genes using PCR-based mutation detection methods and gel electrophoresis. A. *Rsa* I restriction enzyme analysis of the *SLC11A1* 5'[GT]n promoter polymorphism showing genotype 2,2 (lane 1), genotype 3,3 (lane 2) and genotype 2,3 (lane 3). B. Detection of the 9-bp deletion in exon 2 of the *SLC11A1* gene. The wild type allele is shown in lane 1 and a heterozygote for the 9 bp deletion in lane 2. C. Detection of the *CCR5* 32 bp deletion. The wild type allele is shown in lane 1 and a heterozygote for mutation *CCR5*Δ32 in lane 2. Sequence analysis confirmed the 9-bp deletion (not shown).

The 9-bp deletion was present in 1.3% of HIV-seropositive (1/81) and 2.5% of HIV-seronegative (1/40) Africans in the Western Cape, and in 2.1% of African HIV-seropositive (2/95) and 3.3% of HIV-seronegative (4/122) individuals from the Northwest Province. A single HIV-seronegative individual of mixed ancestry (1.7%, n=60) presented with the deletion mutation, but this sequence change was not detected in the Cape Coloured HIV-seropositive group. The allelic distribution of the 5'[GT]n repeat polymorphism was also similar in HIV-seropositive Africans from different geographical regions in South Africa, as well as in HIV-seronegative Africans recruited as controls in the same areas. Since the genotype distribution and allele frequencies in these groups did not differ significantly from that observed in the Cape Coloured population of South Africa (data not shown), all patient and control groups were pooled for comparative analysis.

The alleles of the 5'[GT]n *SLC11A1* polymorphism were in Hardy-Weinberg equilibrium in the different population groups studied. A comparison of genotype distribution and allele frequencies of the *SLC11A1* 5'[GT]n promoter polymorphism in HIV-seropositive individuals compared with HIV-seronegative population-matched control individuals, did not reveal any significant allelic associations (table 2).

Table 2. Genotype distribution and allele frequencies of the *SLC11A1* 5'[GT])n repeat polymorphism in HIV-seropositive individuals compared with HIV-seronegative population-matched controls

Population		HIV+	HIV-	OR	95%CI	*P*
	Genotype	n=280	n=326	1.081	0.7697-1.518	0.6657
	2,2	5 (0.018)	10 (0.031)			
Africans	2,3	90 (0.321)	95 (0.291)			
n=606	3,3	185 (0.661)	221 (0.678)			
	Allele	n=560	n=652	0.9935	0.7395-1.335	1.000
	2	100 (0.179)	115 (0.176)			
	3	470 (0.821)	537 (0.823)			
	Genotype	n=94	n=83	1.668	0.8897-3.128	0.1174
	2,2	2 (0.021)	3 (0.036)			
Mixed Ancestry	2,3	36 (0.383)	21 (0.253)			
n=177	3,3	56 (0.596)	59 (0.711)			
	Allele	n=188	n=166	1.391	0.8105-2.389	0.2769
	2	40 (0.212)	27 (0.163)			
	3	148 (0.787)	139 (0.837)			
	Genotype	n=374	n=409	1.198	0.8898-1.613	0.2555
	2,2	7 (0.019)	13 (0.032)			
Total	2,3	126 (0.337)	116 (0.284)			
n=783	3,3	241 (0.644)	280 (0.684)			
	Allele	n=748	n=818	1.078	0.8334-1.395	0.5989
	2	140 (0.174)	142 (0.174)			
	3	618 (0.826)	676 (0.826)			

In the Cape Coloured population, the homozygous 2,2 genotype was observed in 3.6% of HIV-seronegative (3/83) and 2.1% of HIV-seropositive individuals (2/94), and the 2,3 genotype in 38.3% of HIV-seropositive (36/94) compared with 25.3% of HIV-seronegative individuals (21/83).

An increased risk of 66.8% was calculated in association with genotype 2,3 in the Cape Coloured population (OR=1.668, 95% CI 0.8897-3.128, *P*=0.1174), but statistical significance was not reached. The sample size required to enable detection of significant differences was calculated at 188 for HIV-seropositive (2-fold increase) and 108 for HIV-seronegative (1.3 fold increase) coloured individuals (OR=1.685, 95% CI 1.013-2.803, *P*=0.0454). In a similar assessment of the total group of African HIV-seropositive and HIV-seronegative individuals, no increased risk in association with genotype 2,3 was detected, although a predominance of this genotype was detected in African HIV-seropositive patients attending the infectious Diseases Clinic at Tygerberg Hospital (data not shown). One HIV-seropositive individual (0.3%) from this population presented with the 1,2 genotype while it was absent from HIV-seronegative individuals, and this observation was not included for statistical analysis (results not shown).

In African individuals from the Northwest and Gauteng Provinces, the homozygous 2,2 genotype was present at similar frequencies in both HIV-seropositive (3.2%, 5/155) and HIV-seronegative (2.7%, 7/263) individuals. Genotype 2,2 was not detected in African HIV-seropositive individuals attending the Infectious Diseases Clinic at Tygerberg Hospital, whilst present in 4.8% (3/63) of HIV-seronegative individuals.

Possible association of the *SLC11A1* functional 5'[GT]n promoter variant with progression to AIDS was investigated in HIV-seropositive individuals from the Western Cape Province, divided into clinical subgroups according to the WHO clinical disease classification (Table 3) and according to disease progression (Table 4). No statistically significant associations were observed. The 2,2 genotype was present in individuals with a class III classification (4.3%, 2/46), and absent from individuals in classes I, II and IV. The 2,3 genotype distribution was similar in classes I, II and III (37.0%-42.6%), and much reduced in class IV (21.7%). Similarly, allele 2 was present at higher frequency in classes I, II and III (20.6%-22.8%) and reduced in class IV individuals (10.9%). The homozygous 2,2 genotype was detected in 6.1% of normal progressors (6/24), in 3.4% of asymptomatic individuals (5/146), and absent in both slow and fast progressors.

Table 3. Genotype distribution and allele frequencies of the *SLC11A1* 5'[GT]n-repeat promoter polymorphism in 171 HIV-seropositive patients attending the Infectious Diseases Clinic at Tygerberg Hospital, Western Cape, subdivided according to WHO clinical disease classification (Classes I, II, III, IV), compared with asymptomatic HIV-seropositive individuals.

Population		Progressors (n=171), IDC				Asymptomatic, NWP
		Class I	Class II	Class III	Class IV	
		A (n=39) M (n=29)	A (n=19) M (n=15)	A (n=19) M (n=27)	A (n=14) M (n=9)	A (n=146)
	Genotype	n=68 [a]	n=34	n=46	n=23 [a]	n=146
A & M	2,2	0	0	2 (0.043)	0	5 (0.034)
n=317	2,3	29 (0.426)	14 (0.412)	17 (0.370)	5 (0.217)	39 (0.267)
	3,3	39 (0.574)	20 (0.588)	27 (0.587)	18 (0.783)	102 (0.699)
	OR	1.724	1.623	1.631	0.6439	
	95%CI	0.9491-3.131	0.7519-3.502	0.8221-3.237	0.2248-1.844	
	P	0.0886	0.2268	0.2071	0.4695	
	Allele	n=136	n=68	n=92	n=46	n=292
	2	29 (0.213)	14 (0.206)	21 (0.228)	5 (0.109)	49 (0.168)
	3	107 (0.787)	54 (0.794)	71 (0.772)	41 (0.891)	243 (0.832)
	OR	1.344	1.286	1.467	0.6048	
	95%CI	0.8051-2.244	0.6623-2.496	0.8248-2.608	0.2274-1.608	
	P	0.2826	0.4795	0.2155	0.3900	

IDC, Infectious Diseases Clinic; NWP, Northwest Province; A, African; M, mixed ancestry

Genotypes : [a] class I vs. class IV: OR=2.677, 95% CI 0.8897-8.054, *P*=0.0857

Table 4. Genotype distribution and allele frequencies of the *SLC11A1* 5'[GT]n promoter variant in 72 HIV-seropositive patients attending the Infectious Diseases Clinic at Tygerberg Hospital, Western Cape, subdivided according to disease progression (normal, slow and fast), compared with asymptomatic HIV-seropositive individuals.

Population		Progressors, IDC			Asymptomatic, NWP
		Normal	Slow	Fast	
		A (n=16) M (n=17)	A (n=6) M (n=9)	A (n=15) M (n=9)	A (146)
	Genotype	n=33	n=15	n=24	n=146
A & M	2,2	2 (0.061)	0	0	5 (0.034)
n=218	2,3	13 (0.394)	6 (0.400)	6 (0.250)	39 (0.267)
	3,3	18 (0.545)	9 (0.600)	18 (0.750)	102 (0.699)
	OR	1.932	1.545	0.7727	
	95%CI	0.8934-4.177	0.5185-4.606	0.2873-2.079	
	P	0.1034	0.5583	0.8094	
	Alleles	n=66	n=30	n=48	n=292
	2	17 (0.258)	6 (0.200)	6 (0.125)	49 (0.168)
	3	49 (0.742)	24 (0.800)	42 (0.875)	243 (0.832)
	OR	1.721	1.240	0.7085	
	95%CI	0.9150-3.235	0.4814-3.193	0.2854-1.758	
	P	0.1125	0.6154	0.5322	

IDC, Infectious Diseases Clinic; NWP, Northwest Province; A, African; M, mixed ancestry

Distribution of the 2,3 genotype was similar between normal (39.4%, 13/33) and slow (40.0%, 6/15) progressors, and also between fast (25.0%, 6/25) progressors and asymptomatic (26.7%, 39/146) individuals. Further, distribution of allele 2 between the respective progression groups was similar compared to the WHO classification. Allele 2 was present at elevated frequencies in normal and slow progressors (25.8% and 20% respectively) and reduced in individuals with fast disease progression (12.5%), although not significant.

Failure to demonstrate an association between polymorphisms in the *SLC11A1* gene and HIV/AIDS susceptibility could not be ascribed to antiretroviral treatment of patients attending the Infectious Diseases Clinic, since only 22% of these individuals received antiviral treatment. No gender-related differences in allelic distribution were furthermore observed in the South African populations studied (data not shown).

Discussion

The human *SLC11A1* gene studied in 374 HIV-seropositive and 410 HIV-seronegative South African individuals in relation to susceptibility to HIV-1 infection and progression to AIDS, has previously been associated with both autoimmune and infectious diseases (Searle and Blackwell 1999). Chronic hyperactivation of macrophages associated with allele 3 is functionally linked to autoimmune disease susceptibility, while the poor level of expression of the *SLC11A1* allele 2 contributes to infectious disease susceptibility. The likelihood that allele 3 would conversely protect against infectious disease and allele 2 against autoimmune disease, was supported by data from Kotze *et al.* (2001), by comparison of allele frequencies of the promoter variant between different age groups. It appeared that *SLC11A1* alleles considered to be detrimental in relation to autoimmune disease susceptibility might be maintained in the population as a consequence of improved survival to reproductive age following infectious disease challenge. These studies demonstrated the complex interplay between genetic and environmental factors related to multifactorial conditions, and provided the background for analysis of the *SLC11A1* gene in the study populations included in the present study.

The frequency of the 9-bp deletion in exon 2 of the *SLC11A1* gene, previously associated with susceptibility to HIV-1 infection in sub-Saharan Africans (Ramaley *et al.* 2000), was very low in our study population. It does however not appear to be associated with modified risk to HIV-1 infection in South African individuals, since allelic distribution was similar in the patient and control samples.

We therefore focused on the *SLC11A1* 5'[GT]n promoter variant in an attempt to elucidate the role of allele 2 as a modifying factor of HIV infection and/or progression to AIDS. While homozygotes for allele 2 were protected from HIV-1 infection in an earlier study (Marquet *et al.* 1999), this genotype was detected at similar frequencies in the South African patient groups compared with population-matched HIV-seronegative individuals. Our findings, therefore does not provide evidence that homozygosity for allele 2 is protective in Africans.

This may be explained in part by differences in ethnic background, which may include co-existence of other important *SLC11A1* allelic variants or mutations in other genes occurring in linkage disequilibrium with specific alleles in different populations. The possible role of the *CCR5* 32-bp deletion as a confounding factor was excluded in our study population. Further studies are however warranted to investigate the significance of the *SLC11A1* promoter polymorphism -237C/T in this context. *In vitro* studies performed by M Zaahl (PhD study, submitted) have demonstrated that the presence of the T-allele in association with allele 3 results in an opposite effect *in vitro* upon iron loading, similar to that of allele 2.

We hypothesise that the availability of cellular iron related to dietary iron intake, and subsequent biological processes involved in iron homeostasis, may have had an influence on the outcome of the case-control comparisons in this study. Iron homeostasis is of vital importance for proper cellular function, specifically cells of the phagocytic lineage involved in modulation of immunological responses to infection (for example macrophages). Iron dysregulation has been shown to be associated with various human diseases, including some infectious diseases and autoimmune disorders (Dhople *et al.* 1996). Recent studies have shown that the SLC11A1 protein plays a significant role in iron homeostasis in macrophages (Blackwell *et al.* 2000), and is closely associated with another structurally related divalent cation transporter, DMT1 (Fleming *et al.* 1997, Gunshin *et al.* 1997). This model facilitated explanation of the multiple pleiotropic effects of *SLC11A1* and its complex roles in infectious and autoimmune disorders.

Analysis of the *SLC11A1* gene in our study cohort has contributed to a better understanding of the complexity of host genetic factors implicated in modified risk to HIV-1 infection. The proposed effect of the functional *SLC11A1* 5'[GT]n promoter polymorphism on HIV-related infectious disease (Marquet *et al.* 1999) may be mediated though a complex gene-environment interaction within the context of the genetic background of an individual. Multiple low-penetrance host alleles contribute to susceptibility to HIV-1 infection and progression to AIDS, and a combination of these factors together with viral load and CD4+ counts, could provide a valuable decision-making tool in the management of HIV-infected individuals in a population-specific manner. We conclude that *SLC11A1* host factors involved in modified risk of HIV infection are population-specific.

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B. The African-specific *HFE* gene polymorphism IVS3-48c→g correlates significantly with reduced haemoglobin levels in HIV-infected South African individuals

Background: Human immunodeficiency virus (HIV)-infected individuals experience a range of haematological complications, including anaemia, representing a prognostic marker for future disease progression or death independent of CD4+ counts or viral load.

Objective: Given the ability of HIV to alter iron metabolism, we aimed to identify host genetic factors associated with altered iron parameters that may be of prognostic value.

Subjects and Methods: DNA samples of 111 HIV-seropositive and 145 HIV-seronegative Africans were amplified by the polymerase chain reaction (PCR) method and screened for an African-specific polymorphism IVS3-48c→g in intron 3 of the haemochromatosis (*HFE*) gene. Iron parameters were compared between individuals with and without this point mutation.

Results: Significantly reduced haemoglobin levels were detected in both HIV-seropositive (P=0.004) and HIV-seronegative (P=0.02) Africans with the intronic polymorphism IVS3-48c→g in the *HFE* gene, in comparison with individuals in these respective study groups without this point mutation.

Conclusions: Although the g-allele of the IVS3-48c→g polymorphism may occur in linkage disequilibrium with a functional mutation in the HLA region of chromosome 6, possibly implicated in HIV infection, our data identified the *HFE* gene polymorphism as a potentially useful genetic marker of poor clinical outcome in HIV-infected individuals.

Introduction

Progression of human immunodeficiency virus (HIV) infection towards advanced disease is accompanied by alterations in iron metabolism and increased body iron stores (Boelaert *et al.* 1996a,b). In the absence of antiviral treatment elevated iron levels cause shorter survival times (De Monye *et al.* 1999), promote growth of AIDS-opportunistic pathogens (Boelaert *et al.* 1996a,b) and HIV-1 replication (Sappey *et al.* 1995), and contribute to an increased risk for cancer, for example Kaposi's Sarcoma (Simonart *et al.* 1998). Impairment of immune defence mechanisms is enhanced by oxidative stress due to excess iron, directly promoting growth of microbial cells (Dhople *et al.* 1996). Recently it was demonstrated that HIV-1 infected individuals carrying the haptoglobin Hp2-2 phenotype show a worse prognosis (Delanghe *et al.* 1998). An increased oxidization of vitamin C due to iron accumulation in affected individuals suggests a less efficient protection mechanism against haemoglobin/iron-driven oxidative stress, which may stimulate viral replication directly. Iron deficiency or anaemia conversely represents a prognostic marker of poor clinical outcome in HIV-infected individuals, independent of CD4+ counts and viral load (Means 1997, Moyle 2002). However, while many of the activities of HIV target cells (for example polymorphonuclear granulocytes and macrophages) are iron-dependent and/or modulate iron metabolism (Cantinieaux *et al.* 1992, van Asbeck *et al.* 1982), the exact mechanical basis of the associated deleterious effects is not fully understood.

In addition to the complex interaction between infectious agents and host cells, variation in genes involved in host response to infection can be expected to alter the clinical outcome in HIV-seropositive subjects. Reduced haemoglobin levels in HIV-infected patients are associated with enhanced cellular immune activation, as seen by release of certain cytokines and changes of iron metabolism (Fuchs *et al.* 1993). Furthermore, significantly lower haemoglobin levels in HIV-seropositive compared with HIV-seronegative individuals most likely result from virus infection (Spada *et al.* 1998).

Risk of HIV-1 infection and progression to AIDS has been shown to be modified due to genetic variation in genes encoding chemokine receptors and their ligands, and the major histocompatibility complex (Liu *et al.* 1996, Magierowska *et al.* 1999, Laumbacher and Wank 1998, Winkler *et al.* 1998).

The functional linkage between immune function and iron absorption became evident with the identification of the major histocompatibility complex (MHC)-encoded HFE protein, underlying the common iron overload condition hereditary haemochromatosis (Feder *et al.* 1996, 1998). Evidence that MHC-encoded class I molecules might play a role in iron metabolism (Salter-Cid *et al.* 2000), led to targeting of the human haemochromatosis gene (*HFE)* as a potential candidate gene involved in iron dysregulation related to HIV infectivity. Prolonged progression status has been reported in an HIV-seropositive patient treated for iron overload as a consequence of hereditary haemochromatosis caused by homozygosity for the C282Y mutation in the *HFE* gene (Nielsen *et al.* 1999).

In the present investigation the *HFE* gene (Feder *et al.* 1996) was studied in 111 HIV-seropositive and 145 HIV-seronegative Black South African individuals with known iron status. The previously-documented *HFE* gene mutations, V53M, V59M, H63, H63D, S65C and C282Y were present at a relatively low frequency in both HIV-seropositive and HIV-seronegative Africans, or were not detected at all in this study population (D Pretorius, unpublished data). This finding was in accordance with the previously reported low frequencies for these mutations occurring among these populations (de Villiers et al. 1999). The African-specific *HFE* polymorphism IVS3-48c→g that does not appear to affect RNA splicing, nor does it influence serum iron parameters (Beutler and Gelbart 2000), was therefore investigated by mutation analysis. We investigated the likelihood that haemoglobin levels and serum iron parameters are differentially affected upon HIV-infection, in relation to the IVS3-48c→g polymorphism.

Materials and Methods

The Ethics Review Committees of the Universities of Stellenbosch and Potchefstroom approved the study.

Study Population

Blood samples were obtained with informed consent from 111 HIV-seropositive (42 males, 69 females) and 145 HIV-seronegative (59 males, 86 females) Black South African individuals recruited from the Northwest Province of South Africa.

HIV status and iron parameters of all study participants were determined and previously described (James *et al.* 2000), as part of a cross-sectional study performed by these authors. The study population represents a random sample of individuals unaware of their HIV status at the time of entry.

DNA Analysis

Genomic DNA was extracted from whole blood following a standard procedure (Miller *et al.* 1988). The IVS3–48c→g polymorphism in intron 3 of the *HFE* gene was analysed following restriction enzyme analysis of polymerase chain reaction (PCR)-amplified DNA (Beutler and Gelbart 2000). One PCR cycle of 3 minutes at 95 °C was followed by 35 cycles of 95 °C for 45 sec, 55 °C for 30 sec and at 72 °C for 45 sec, and a final cycle at 72 °C for 10 min. PCR products (10-15 μl) were digested with 0.75 units *Bsa J1* restriction enzyme (New England Biolabs, Beverley, MA, USA) for 2 hours at 60 °C, and compared with DNA from an individual heterozygous for the polymorphism as positive control (Beutler and Gelbart 2000). Digested products were electrophoresed at room temperature in a 3.4% cross-linked polyacrylamide gel for two hours and 300 V. DNA fragments were visualised by ultraviolet transillumination following ethidium bromide staining.

Statistical Analysis

Allele frequencies were determined by genotype count, and the exact test of the Hardy Weinberg proportion was applied using the Markov Chain algorithm (Guo and Thompson 1992, http://www2.biology.ualberta.ca/jbrzusto/hwenj.html). Genotype distribution and allele frequencies were compared between HIV-seropositive and HIV-seronegative individuals within the same population group. Chi-square (χ^2) statistical analysis was performed using Simcalc V1.0 (Provalis Research, USA) to test for heterogeneity in mutation frequencies, with *P*-values <0.05 considered as significant. For continuous variables, groups were compared using unpaired *t* tests. Statistical comparisons of iron parameters were done after correction for age and gender, where appropriate. Groups were compared by multivariate analysis, adjusting for age using the GLM (General linear model) with LSD (least significant differences) procedure. The total means were also adjusted for gender. Mutation data was provided blindly to the statistician for correlation with biochemical parameters of iron status.

Results

Analysis of the IVS3-48c→g polymorphism in intron 3 of the HFE gene is shown in Figure 1, in an individual homozygous for the wild type c-allele (lane 1), a heterozygote with genotype cg (lane 2), and a homozygote for the mutant g-allele (lane 3).

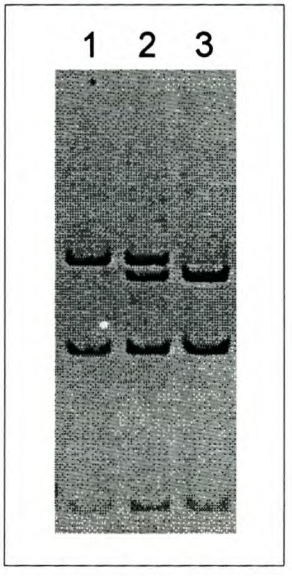

Figure 1. Detection of the *HFE* intron 3 polymorphism, IVS3-48c→g, using *Bsa* J1 restriction enzyme digestion. Lane 1: homozygous wild type genotype cc, lane 2: heterozygous genotype cg, lane 3: homozygous mutant genotype gg.

The genotype distribution and allele frequencies determined in 111 HIV-seropositive and 145 HIV-seronegative South African individuals with known iron status are indicated in Table 1. Both alleles were in Hardy-Weinberg equilibrium in the respective HIV-seropositive and HIV-seronegative study groups. Although mutant allele frequencies were similar in HIV-seropositive individuals (12.6%) compared with those previously reported (12.8%) for African individuals (Beutler and Gelbart 2000) and lower in HIV-seronegative individuals (9.6%), the allelic distribution of the IVS3-48c→g polymorphism did not differ significantly between these groups. Theoretical estimates indicated that a sample size of 444 HIV-seropositive and 580 HIV-seronegative individuals is required for sufficient statistical power to confirm or exclude association between HIV infection and the *HFE* polymorphism.

Table 1. Comparison of genotype distribution and allele frequencies of the *HFE* IVS3-48c→g polymorphism in HIV-seropositive and HIV-seronegative Africans.

	HIV-seropositive			*HIV-seronegative*		
	Males n=42	**Females n=69**	**Total n=111**	**Males n=59**	**Females n=86**	**Total n=145**
Genotype						
cc	33 (0.786)	52 (0.754)	85 (0.766)	47 (0.797)	70 (0.814)	117 (0.807)
cg	9 (0.214)	15 (0.217)	24 (0.216)	12 (0.203)	16 (0.186)	28 (0.193)
gg	0	2 (0.029)	2 (0.018)	0	0	0
Allele						
c	75 (0.893)	119 (0.862)	194 (0.874)	106 (0.898)	156 (0.907)	262 (0.903)
g	9 (0.107)	19 (0.138)	28 (0.126)	12 (0.102)	16 (0.093)	28 (0.097)

Comparison of iron parameters in relation to the IVS3-48c→g polymorphism revealed significantly lower haemoglobin levels in individuals with the g-allele ($P<0.05$) (Table 2). This association was observed between mutation groups in both the HIV-seropositive ($P=0.004$) and HIV-seronegative ($P=0.021$) individuals. Although haemoglobin levels were significantly lower in HIV-seropositive compared with HIV-seronegative individuals as reported previously (HH Vorster *et al.*, unpublished data), it was consistently lower in the presence of the IVS3-48c→g polymorphism.

When males and females were considered separately, the most striking observation was the significantly reduced haemoglobin levels in HIV-seropositive females (10.95 g/dl) with the g-allele compared with HIV-seronegative females (13.10 g/dl) without the g-allele ($P=0.01$). The two HIV-seropositive individuals from this population homozygous for the g-allele were both females, and their haemoglobin levels were reported to be 10.70 g/dl and 11.50 g/dl, respectively. Although the numbers are small, the mean haemoglobin value for these two homozygous female individuals (11.10 g/dl) was below the normal reference value of 11.5-16.5 g/dl, while that of females heterozygous for the polymorphism (12.47 g/dl) was within the normal range (12-15 g/dl for females; Mahan and Escott-Stump 2000).

In an attempt to determine whether the IVS3-48c→g polymorphism affects a branch site in intron 3 that may possibly affect gene splicing in an exonic splice element (ESE), the web-based ESEfinder release 2.0 computer program (Cartegni *et al.* 2003, http://exon.cshl.edu/ESE) was used. It was however indicated that IVS3-48c→g appears not to cause a disruption that may result in a splicing defect.

Table 2. Comparison of mean values for different iron parameters measured in Black African HIV-seropositive and HIV-seronegative individuals recruited from the Northwest Province of South Africa in relation to the presence of the *HFE* IVS3-48c→g polymorphism.

HIV seropositive **Parameter**	**Genotype**	**Total HIV+[a] (n=111)**	**95% CI Lower**	**Upper**	**Males (n=42)**	**95% CI Lower**	**Upper**	**Females (n=69)**	**95% CI Lower**	**Upper**
Hb (g/dl)	cc	12.77[b]	12.25	13.28	13.67[c]	12.85	14.50	12.20[d]	11.53	12.88
	cg/gg	11.21	10.30	12.13	11.38	9.52	13.24	10.95	9.89	12.01
SI (µmol/l)	cc	15.11	13.53	16.70	17.86	15.36	20.35	13.44	11.35	15.53
	cg/gg	15.40	12.58	18.23	16.99	11.35	22.63	14.15	10.86	17.43
TIBC	cc	67.58	64.27	70.89	62.59	59.12	66.06	70.64	65.58	75.69
	cg/gg	64.44	58.55	70.34	59.47	51.64	67.30	67.43	59.48	75.37
TS (%)	cc	23.92	21.27	26.57	29.57	24.97	34.18	20.48	17.22	23.75
	cg/gg	24.56	19.84	29.29	28.79	18.40	39.18	21.62	16.49	26.75
Fe (µg/l)	cc	112.75	53.82	172.09	195.06	42.45	347.66	61.92	48.90	74.94
	cg/gg	64.75	-40.70	170.21	76.72	-267.81	421.24	41.92	21.44	62.39
HIV seronegative **Parameter**	**Genotype**	**Total HIV+[a] (n=145)**	**95% CI Lower**	**Upper**	**Males (n=59)**	**95% CI Lower**	**Upper**	**Females (n=86)**	**95% CI Lower**	**Upper**
Hb (g/dl)	cc	13.43[e]	13.07	13.80	13.89	13.37	14.40	13.10[f]	12.57	13.63
	cg/gg	12.23	11.31	13.16	12.86	10.54	15.18	11.91	10.88	12.94
SI (µmol/l)	cc	15.98	14.58	17.38	18.55	16.51	20.60	13.99	12.10	15.89
	cg/gg	18.56	15.05	22.08	11.13	1.92	20.34	18.67	14.98	22.37
TIBC	cc	63.32	61.23	65.40	58.51	55.79	61.24	66.99	63.89	70.10
	cg/gg	66.22	60.97	71.47	69.72	57.47	81.96	67.76	61.71	73.81
TS (%)	cc	26.19	23.85	28.53	31.90	28.40	35.40	21.90	18.76	25.05
	cg/gg	29.14	23.25	35.04	19.22	3.47	34.96	27.94	21.81	34.07
Fe (µg/l)	cc	113.82	80.88	146.47	196.33	126.59	266.08	56.21	36.09	76.33
	cg/gg	157.56	74.66	240.47	400.83	87.13	714.52	64.35	25.15	103.55

Hb, haemoglobin: SI, serum iron; TIBC, total iron binding capacity; TS, transferrin saturation; Fe, ferritin

[a] Means adjusted for age and gender
[b] HIV-seropositive with (cg/gg) and without (cc) HFE IVS3-48c/g: P=0.004
[c] *Males*: HIV-seropositive with (cg/gg) and without (cc) HFE IVS3-48c/g: P=0.029
[d] *Females*: HIV-seropositive with (cg/gg) and without (cc) HFE IVS3-48c/g: P=0.051
[e] HIV-seronegative with (cg/gg) and without (cc) HFE IVS3-48c/g: P=0.021
[f] *Females*: HIV-seronegative with (cg/gg) and without (cc) HFE IVS3-48c/g: P=0.033

Although almost identical to the downstream splice-site nucleotide sequence (ttcctg**tc**aag) of the same intron, this finding is in accordance with the previously reported data indicating that the IVS3-48c→g polymorphism (ttcctgg**g**aag, wild type ttcctgg**c**aag) does not affect alternative splicing (Beutler and Gelbart 2000).

Discussion

The present investigation was prompted by a previous finding of significantly lower haemoglobin levels in South African HIV-seropositive individuals compared with population-matched HIV-seronegative individuals (HH Vorster *et al.*, unpublished data). It however was unclear whether variation in genes involved in host response to infection would influence the acute response upon infection, and possibly alter clinical outcome in HIV-infected individuals. Since HIV has the ability to affect iron metabolism, we aimed to identify host genetic factors that may be of prognostic value and could possibly define potential targets for future therapeutic intervention. HFE is expressed on the cell surface and it has been speculated that this protein may be a receptor for some infectious agents (Rochette *et al.* 1999).

The finding that the presence of the IVS3-48c→g polymorphism correlates significantly with a reduction in haemoglobin levels, irrespective of HIV status, largely excluded the possibility that this apparent phenotypic effect is related only to an acute phase response due to HIV infection. The g-allele shown to possibly be related to this effect most likely occurs in linkage disequilibrium with a functional sequence variant in a gene in close proximity to the *HFE* gene, since only the haemoglobin levels were significantly affected and not serum iron parameters, for example serum ferritin and transferrin saturation that are typically altered by deleterious mutations in the *HFE* gene (Feder *et al.* 1996). These findings are in accordance with the genotype-phenotype correlation studies performed by Beutler and Gelbart (2000), failing to demonstrate any effect of the IVS3-48c→g polymorphism on serum iron parameters or RNA integrity.

The HLA-A3 and HLA-B14 loci, associated with slow disease progression in HIV-infected individuals (Magierowska *et al.* 1999) were shown to occur in strong linkage disequilibrium with the *HFE* gene. It therefore seems likely that our findings may be explained by the occurrence of other unidentified MHC class I molecules (also encoded on chromosome 6) in close proximity to *HFE* that may affect iron status, as speculated by Salter-Cid *et al.* (2002).

A limitation of the present study is the fact that disease status was unknown in the study population, and due to ethical constraints follow-up studies could not be performed for comparison of clinical outcome between individuals with and without the IVS3-48c→g polymorphism. Comparison of allelic distribution between the 111 HIV-seropositive and 145 HIV-seronegative individuals did not reveal any significant differences, even after increasing the sample size to include 233 HIV-infected and 274 population-matched control individuals (data not shown). The additional samples were however from populations recruited from the Western Cape and Gauteng Provinces, and from individuals originating from different ethnic backgrounds.

It is further possible that the polymorphism may have an impact on disease progression once an individual is infected with the virus, but does not play a role in HIV infection. Preliminary data from analysis of 165 HIV-infected individuals with disease status classified according to the WHO criteria (classes I-IV, http://www.who.int) did however not reveal any significant association with disease status or rate of disease progression. These HIV-infected subjects with known progression status were recruited from the population attending the Infectious Diseases Clinic at Tygerberg Hospital. Homozygosity for the g-allele of the IVS3-48c→g polymorphism was not detected in any of these HIV-seropositive individuals. A predominance of heterozygotes with a single copy of the g-allele was noted in HIV-seropositive individuals compared with HIV-seronegative individuals, but the sample size was too small to obtain conclusive results (data not shown).

Detection of significantly reduced haemoglobin levels in association with the IVS3-48c→g polymorphism is an important consideration with respect to nutritional status, since previous studies have indicated that anaemia independently predicts poor clinical outcome and contributes to morbidity in HIV-infected children and adults (Tovo *et al.* 1992, Forsyth *et al.* 1996, Sullivan *et al.* 1998). Recovery from anaemia reduces the risk of disease progression to approximately the same level as observed in patients who are not anaemic. Administration of iron supplementation or blood transfusion to correct this condition may activate HIV expression and probably worsen immunosuppression. It therefore seems likely that our findings may be explained by the close proximity of other unidentified MHC-encoded class I molecules that affect iron status as speculated (Salter-Cid *et al.* 2002). Thus, it is important to identify factors contributing to iron deficiency, particularly since recombinant human erythropoietin may provide an effective means of improving haemoglobin and reducing transfusion requirements in patients who have low endogenous erythropoietin levels (Moyle 2002).

We conclude that a significant association was detected between the IVS3-48c→g polymorphism and haemoglobin levels, irrespective of HIV status. This effect was more pronounced in HIV-seropositive than in HIV-seronegative individuals, which may point to an added effect with HIV infection. Iron metabolic pathways and the immune system are regulated by one another, however HIV infection can break this inter-relationship resulting in disruption of cellular homeostasis. Since a reduction in haemoglobin levels is associated with poor nutritional status and consequently poor clinical outcome in HIV-infected individuals, it seems possible that detection of the IVS3-48c→g polymorphism in HIV-seropositive individuals may have prognostic value.

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C. Sequence variation in the exonic splicing silencer element (ESS1) of the *CD45* gene: a possible role in HIV-disease

Background: The 77C→G polymorphism in the protein tyrosine phosphatase receptor type C (*CD45/PTPRC)* gene has previously been associated with increased risk of HIV-infection in the Caucasian population, but this point mutation is virtually absent in Africans.

Objective: The aim of this study was to identify possible functional mutations in the exonic splicing silencer element (ESS-1) and promoter region of the *CD45* gene that may be related to increased risk of HIV-1 infection or progression to AIDS.

Design: DNA analysis was performed in 300 HIV-seropositive individuals and 354 HIV-seronegative population-matched control individuals, followed by functional studies to evaluate the possible significance of the mutations identified.

Methods: Polymerase chain reaction (PCR)-based mutation detection methods were applied, which included combined heteroduplex-single stranded conformation polymorphism (HEX-SSCP) analysis, restriction enzyme analysis and direct sequencing. The potential significance of the mutations identified was assessed using the web-based ESEfinder (ESE - Exonic Splice Element) program and flow cytometry (FACs) analysis.

Results: Although modified risk to HIV infection was not demonstrated for two novel mutations 32A→G and 54A→G identified in the ESS-1 situated in exon 4 of the *CD45* gene, FACs analysis indicated a significantly reduced number of CD45RA- CD45R0+ cells in the CD8+ subset (9.8% and 16.6% versus 40.6% of CD8+ cells) in two HIV-seropositive individuals with the 32A→G mutation. Application of the ESEfinder program furthermore indicated that the missense mutation, T18A (54A →G), potentially results in alternative splicing due to disruption of important splicing elements.

Conclusions: The 32A→G mutation in the *CD45* gene appears to be predominantly associated with slow disease progression in the HIV-seropositive individuals studied.

This finding may be related to differences in proportions of both CD4+ and CD8+ subsets observed following FACs analysis in HIV-seropositive individuals with mutation 32A→G, compared with a control without this mutation. Further studies are however warranted to exclude the possibility of false positive results due to small sample size and the effect of HIV disease status on CD45 expression.

Introduction

The protein tyrosine phosphatase receptor type C (*PTPRC*) gene, also known as *CD45*, encodes an abundantly expressed transmembrane protein that is expressed on all hematopoietic cells (Trowbridge and Thomas 1994). CD45 appears at one of the earliest stages of hematopoietic development, suggesting a role in the generation of a functional immune system. Structural heterogeneity of CD45 is facilitated by alternative splicing of exons 4,5 and 6, giving rise to multiple isoforms (Saga *et al.* 1986, Streuli *et al.* 1987). T-cell receptor (TCR)-mediated activation of both B- and T- lymphocytes is facilitated by CD45 after antigen stimulation by dephosphorylation of an inhibitory site on the Src-family of tyrosine kinases, yielding an enhanced kinase ability of the substrate essential for initiation of the signalling event (Thomas and Brown 1999). This molecule further functions as a negative regulator of cytokine receptor signalling, due to its function as a hematopoietic Janus kinase (Jak) phosphatase (Irie-Sasaki *et al.* 2001).

Recently it was shown that an exon 6 deletion causes a lack of CD45 expression and appears to be associated with severe combined immunodeficiency (SCID) in humans (Tchilian *et al.* 2001c). Loss of CD45 expression with consecutive development of SCID has also been reported in a child bearing a large deletion in one *CD45* allele and a point mutation in the other (Kung *et al.* 2000). It therefore became clear that CD45 is essential for normal immunological functioning. This was further exemplified by the development of autoimmune disease due to an inactivating point mutation in the *CD45* gene in a murine model (Majeti *et al.* 2000).

The role of CD45 in autoimmune diseases was highlighted by detection of two point mutations, 59C→A and 77C→G, in patients with multiple sclerosis (MS) (Jacobson *et al.* 2000, 2002). The 77C→G mutation may also be associated with an increased risk of HIV-1 infection (Tchilian *et al.* 2001a), and appears to be absent in Africans (Tchilian *et al.* 2002). Both mutations 59C→A and 77C→G cause disruption of the exonic splicing silencer element-1 (ESS-1) in exon 4 of the *CD45* gene, resulting in altered isoform expression due to interference with exon-intron splicing (Thude *et al.* 1995, Tchilian *et al.* 2001b, Jacobson *et al.* 2002). ESS-1 usually suppresses splicing at a splice site located at the 5' end of exon 4 (Lynch and Weiss 2001), resulting in continuous expression of CD45RA isoforms on activated T-lymphocytes (Zilch *et al.* 1998). The mutation at nucleotide position 59 causes a non-conservative amino acid change (H19Q) in the CD45 antigen, while 77C→G represents a silent mutation.

Since the CD45 molecule has been implicated in various immune-related disorders, it seems likely that the CD45 gene may be involved in HIV-1 infection, as reported by Tchilian and co-workers (2001a). In the present study an attempt was made to identify functional mutations in the ESS-1 element and promoter regions of the *CD45* gene in non-Caucasian South Africans, which may affect risk for HIV/AIDS in the South African population. Preliminary data revealed two novel mutations in the CD45 gene, 32A→G and 54A→G, in our predominantly African study population (Jooste *et al.* 2002). We have subsequently screened a total of 300 HIV-seropositive individuals and 354 HIV-seronegative population-matched control individuals for these mutations, to determine their possible involvement in HIV/AIDS susceptibility. Mutations 32A→G and 54A→G were furthermore assessed using the web-based ESEfinder release 2.0 computer program (Cartegni *et al.* 2003) to theoretically determine whether these mutations would yield alternative splicing patterns. Functional studies using flow cytometry (FACs) analysis were additionally performed to investigate the possible functional significance of mutation 32A→G, occurring most frequently in the local population. This investigation was deemed important, since the identification of functional *CD45* gene variations may provide molecular targets for immune intervention.

Methods

Study population

Informed consent was obtained from all study participants following approval of the study by the Ethics Review Committee of the University of Stellenbosch. The study population consisted of 300 HIV-seropositive and 354 HIV-seronegative individuals (table 1).

The HIV-seropositive population consisted predominantly of Black African (Xhosa-speaking) individuals (n=124, 39 males and 85 females) and non-Caucasian individuals of Mixed Ancestry (n=96, 27 males and 45 females). The estimated date of infection and HIV-1 disease-associated complication data were recorded for the majority of individuals, and clinical monitoring was performed for at least 5 years in most individuals. Ages were calculated based on the date of blood sampling.

Table 1. Study Cohort

Study Population	**HIV-seropositive**			**HIV-seronegative**		
Western Cape Province	Male	Female	Total	Male	Female	Total
African	39	85	124	27	45	72
Age range (years)	21-59	22-59	21-59	19-47	17-49	17-49
Mean Age	33.76	31.28	32.76	28.04	26.66	27.18
Mixed Ancestry	27	69	96	52	10	62
Age range (years)	21-55	22-73	21-73	17-52	26-44	17-52
Mean Age	36.30	34.16	34.77	30.38	35.20	31.16
Northwest Province	Male	Female	Total	Male	Female	Total
African	20	50	70	36	59	95
Age range (years)	21-81	17-69	17-81	7-71	17-77	7-77
Mean Age	39.06	33.55	35.39	42.47	37.45	39.16
Gauteng Province	Male	Female	Total	Male	Female	Total
African	7	3	10	61	64	125
Age range (years)	32-47	30-44	32-48	22-62	21-59	21-62
Mean Age	37.14	37	39.11	33.96	31.61	32.66
TOTAL	93	207	300	176	178	354

Disease status was denoted according to the WHO clinical disease classification (http://www.who.int), and was available for the majority (n=178) of HIV-seropositive individuals attending the clinic. HIV-seropositive individuals were further classified as normal progressors when progressive loss in CD4 cell counts was associated with development of AIDS-related symptoms within 10 years after HIV-1 infection, as fast progressors when they progressed to full-blown AIDS within 2-3 years from seroconversion, and as slow progressors when they remained unaffected after 8-10 years from seroconversion. Progression status according to this classification was unknown for the majority of individuals and in those with known progression status, approximately 20% were receiving antiretroviral treatment. Xhosa-speaking HIV-seronegative control individuals (n=72, 27 males and 45 females) were recruited from healthy individuals without a previous history of tuberculosis or immune disorders. Healthy blood donors (n=62, 52 males and 10 females) were included as controls for HIV-seropositive individuals of Mixed Ancestry.

Additionally, 70 African HIV-seropositive individuals (20 males and 50 females) and 95 HIV-seronegative population-matched individuals (36 males and 59 females) originating from the North West Province in South Africa were included in the study. This population formed part of a cross-sectional study previously performed by James *et al.* (2000), and disease progression status was unknown for HIV-seropositive individuals. An anonymous, predominantly HIV-seronegative population of African origin from the Gauteng Province of South Africa was also included in the study, consisting of 10 HIV-seropositive (7 males and 3 females) and 125 HIV-seronegative (61 males and 64 females) individuals.

In this study African (or Black African) refers to individuals of central African descent, and individuals of Mixed Ancestry (also referred to as Cape Coloured, or Coloured) are of San, Khoi, African, Madagascan, Javanese as well as European origin.

Mutation Analysis

EDTA blood samples of the study population were collected for genomic DNA extraction and mutation screening of exon 4 and the proposed promoter region of the *CD45* gene, described by Timon and Beverley (2001). DNA was isolated from peripheral blood using a standard procedure (Miller *et al.* 1988), and amplified by the polymerase chain reaction (PCR) using *CD45*-specific primers following a step-down PCR amplification procedure. Primers used for PCR amplification (Table 2) of exon 4 and the promoter region of the *CD45* gene were designed according to the Genbank gene sequence for *CD45* (Accession Numbers AF492459 and M23461).

Table 2. PCR amplification primers for exon 4 and the proposed promoter region of the *CD4* gene

Gene fragment		PCR primer nucleotide sequence (5'→3')	Fragment size (bp)
Exon 4	forward	ATTTATTTTGTCCTTCTCCCA	260
	reverse	GTTAACAACTTTTGTGTGTGCC	
Promoter	forward	CCGAATCTGACATCATCACC	324
	reverse	CCTTGTACTTACCTGTCACAA	

PCR amplification conditions consisted of initial denaturation at 95 °C for 3 min, followed by 10 cycles of 95 °C for 45 sec, 60 °C for 30 sec, 72 °C for 30 sec; and 30 cycles of 95 °C for 45 sec, 55 °C for 30 sec, 72 °C for 45 sec; with final extension at 72 °C for 10 min. Amplification products were visualized in 2% agarose (Seakem) by ultraviolet transillumination and ethidium bromide staining. Mutation screening was performed using a combined heteroduplex single strand conformation polymorphism (Hex-SSCP) procedure (Kotze *et al.* 1995). Heat-denatured PCR amplification products were electrophoresed in 10% cross-linked polyacrylamide gels supplemented with urea as a mild denaturing agent, for 17 hours at 270V and 4°C, and visualised by ultraviolet transillumination and ethidium bromide staining. Aberrant fragments detected with Hex-SSCP analysis were subjected to automated sequencing on an ABI Prism 310 Genetic Analyser (Applied Biosystems, Foster City, CA, USA).

Lymphocyte Isolation and FACscan cellular assay

For functional analysis of the 32A→G mutation, cellular lymphocyte isolates were prepared following a standard isolation procedure. Venous blood was collected in heparinised syringes, diluted in an equal volume of sterile phosphate buffered saline (PBS) and slowly layered onto Ficoll Paque (Amersham Pharmacia, UK) in 50 ml Falcon tubes. Blood was centrifuged at 500xg for 30 minutes at room temperature (RT). Cells were removed from the interface and washed twice in sterile PBS at 350-400xg, and resuspended in 90% foetal calf serum (FCS), 10% dimethyl sulphoxide (DMSO) (Merck, UK) before cryopreservation at –80°C.

Cell viability was tested using the trypan blue exclusion method. Viable cells were counted using a haemocytometer (improved Neubauer chamber) and light microscopy (Leica), after dilution in an equal volume of trypan blue solution (Sigma, UK), and by exclusion of trypan blue. The number of cells in suspension was calculated by the formula: number of viable cells in 25 squares x 2 (dilution factor) x 10^4 = number of cells/ml.

Flow cytometry (FACS) analysis of CD45 variant splicing was performed as previously described (Tchilian *et al.* 2001). Briefly, 2×10^5 PBMC were stained with either APC-conjugated CD4 (S3.3, Caltag, Silverstone, UK) or CD8-APC (clone RPA/T8, Pharmingen, San Diego, CA) along with FITC-conjugated CD45RA (clone HI10, Pharmingen) and PE-conjugated CD45R0 (clone UCHL1, Pharmingen) monoclonal antibodies (mAbs) in a single step at 4°C for 20 minutes and washed with phosphate-buffered saline (PBS), containing 0.2% BSA. Isotype-matched mAbs were used as controls. Ten thousand events per sample were collected on a FACSCalibur (Becton Dickinson, Mountain View, CA), and analysed using Win MDI software.

Statistical analysis

Allele frequencies were determined by genotype count, and the exact test of the Hardy Weinberg proportion was applied using the Markov Chain algorithm (Guo and Thompson 1992, available from: http://www2.biology.ualberta.ca/jbrzusto/hwenj.html).

Data was analysed for significance of associations tested using Simcalc V1.0 for Windows (Provalis Research). Chi-square (χ^2) analysis was performed, and a two-tailed *P*-value <0.05 was considered significant.

Results

Mutation Analysis

No mutations were identified in the proposed promoter region of the *CD45* gene, which is in accordance with the fact that the nucleotide sequence of this gene fragment appears to be relatively conserved (Timon and Beverley 2001). Aberrant bands detected in exon 4 by Hex-SSCP were analysed by direct sequence analysis to characterise the gene variations. Among individuals attending the Infectious Diseases Clinic in the Western Cape, only one Coloured HIV-seropositive individual (1.04%, n=96) was heterozygous for the 77C→G polymorphism, known to be more common in individuals of Caucasian origin but absent in Black Africans (Tchilian *et al.* 2002). This female individual was classified with class IV disease status according to the WHO criteria. Although progression status for this individual was unknown, she was diagnosed with both cryptococcal meningitis and tuberculosis, and no antiretroviral therapy has been provided.

Mutation 32A→G was identified heterozygously in 6 of 123 HIV-seropositive Black Africans (4.9%) and 1 of 95 Coloured HIV-seropositive individuals (1.1%) (Table 3). The mutation was however absent in the respective HIV-seronegative control groups from this population, including 72 African and 62 Coloured individuals. This apparent association between the 32A→G mutation and HIV-1 infection was marginally significant (*P*=0.057) in the African individuals. The likelihood that this mutation is associated with increased risk of HIV-1 infection was however largely excluded by analysis of African study cohorts including individuals unaware of their HIV status at the time of entry into the study. The 32A→G mutation was present at a lower frequency in HIV-seropositive (1.4%, 1/70) compared with HIV-seronegative (3.2%, 3/94) African individuals recruited from the North West Province.

Furthermore, 5 of 120 (4.2%) of African HIV-seronegative individuals from the Gauteng Province population presented with the 32A→G polymorphism, compared with none of the 10 HIV-seropositive individuals from this population.

Table 3. Allelic distribution of the 32A→G mutation in exon 4 of the *CD45* gene in South African populations of African ethnicity and Mixed Ancestry per geographical region

		HIV + n=298	HIV - n=348	P
Western Cape Province				
African	Genotype	n=123	n=72	
n=195	aa	117 (0.951)	72 (1.00)	0.057
	ag	6 (0.049)	0	
	Alleles	n=246	n=144	
	a	240 (0.976)	144 (1.00)	0.059
	g	6 (0.024)	0	
Mixed Ancestry	Genotype	n=95	n=62	
n=157	aa	94 (0.989)	62 (1.00)	0.418
	ag	1 (0.011)	0	
	Alleles	n=190	n=124	
	a	189 (0.995)	124 (1.0)	0.418
	g	1 (0.005)	0	
North West Province				
African	Genotype	n=70	n=94	
n=164	aa	69 (0.986)	91 (0.968)	0.469
	ag	1 (0.014)	3 (0.032)	
	Alleles	n=140	n=188	
	a	139 (0.993)	185 (0.984)	0.472
	g	1 (0.007)	3 (0.016)	
Gauteng Province				
African	Genotype	n=10	n=120	
n=130	aa	10 (1.00)	115 (0.958)	0.510
	ag	0	5 (0.042)	
	Alleles	n=20	n=240	
	a	20 (1.00)	235 (0.979)	0.514
	g	0	5 (0.021)	

When genotypes for all African individuals were combined to improve the statistical power, neither the genotype distribution (*P*=0.680) or allele frequencies (*P*=0.683) differed significantly between HIV-seropositive individuals (n=203) compared with HIV-seronegative individuals (n=286).

In order to determine a possible relationship between the 32A→G mutation and disease classification, allele frequencies were determined among patients subdivided according to the WHO HIV-1 disease classification. No statistically significant differences were observed between groups, although the are numbers very small and therefore findings were considered inconclusive (data not shown). The same accounted for comparisons between rapid and slow progressors. It is however noteworthy that the 5 individuals with this mutation for whom disease status were known were all classified according to the WHO criteria as class I. Detection of mutation 32A→G in 35.7% (5/14) of HIV-seropositive individuals classified as WHO class I and slow progression compared with its absence in 22 HIV-seropositive patients with rapid disease progression therefore suggests a positive link with slower disease progression. Table 4 shows a summary of clinical data of the 5 HIV-seropositive individuals with the 32A→G mutation with known disease status according to the WHO criteria. Two were classified as LTNPs, two remained asymptomatic after several years of infection, and progression status was unknown in the remaining individual.

Table 4. Clinical characteristics of HIV-seropositive individuals heterozygous for the *CD45* 32A→G mutation

No.	Popul./ Gender	Age [a] (years)	Est. infection length (years)	WHO class.	Disease Progress	CD4+ ($x10^6$ cells/l)	ARV	HIV-1 disease-assoc. symptoms
HIV118	BF	45	unknown	I	unknown	293	yes	unknown
HIV125	CF	43	> 4	I	normal	980	no	asymptomatic
HIV24/262	BF	46	>10	I	LTNP	350, 325	no	lymphadenopathy
HIV54	BF	31	~ 9	I	slow	585	no	asymptomatic
HIV245	BF	19	~ 11	I	LTNP	unknown	no	HIV-associated polyadenitis, PTB

[a] Age at blood sampling; Popul., population; ARV, antiretroviral therapy; BF, Black female; CF, Coloured female; LTNP, long-term non-progressor; PTB, pulmonary tuberculosis

The second novel mutation identified in the South African population, 54A→G, causing a non-conservative amino acid change T18A, was identified in only one African HIV-seropositive individual (1/118, 0.8%) from the Western Cape whilst absent in the HIV-seronegative individuals (n=72) from this study cohort (Table 5). Disease progression was unknown for the Black African female with mutation 54A→G, classified with class I disease stage after approximately one year of HIV-infection. This missense mutation was not detected in the Coloured study cohort including 94 HIV-seropositive and 62 HIV-seronegative individuals. Further, 54A→G was detected in only one of 92 (1.1%) African HIV-seronegative individuals from the North West Province, and absent in 69 HIV-seropositive individuals from this population. Similarly, mutation 54A→G was absent in 10 Black African HIV-seropositive individuals recruited from the Gauteng Province and present in 5 of 120 (4.2%) HIV-seronegative individuals recruited at the same time in this region. Statistical analysis did not reveal any significant associations between HIV infection and the 54A→G mutation. Combined genotypes for all African individuals to increase the statistical power also failed to show any significant association for either genotype distribution (P=0.148) or allele frequencies (P=0.168) for HIV-seropositive individuals (n=197), compared with HIV-seronegative individuals (n=284).

Application of the ESEfinder program

The possible significance of the mutations identified in the South African population was further assessed by using the web-based ESEfinder release 2.0 (ESE – Exonic Splice Element) computer program (Cartegni *et al.* 2003). Figure 1 shows differences observed with regard to disruption or creation of binding sites for the four splice factors analysed by this program (SF2ASF, SC35, SRp40 and SRp55), based on theoretical threshold values. The previously identified mutations 77C→G and 59C→A, with a well-established effect on CD45 expression due to alternative splicing (Jacobson *et al.* 2000, 2002), was first tested to determine the usefulness of theoretical predictions using this program. In both cases, presence of the mutant alleles abolished known binding sites for the splicing factors as shown in Table 6. The results obtained for the 54A→G missense mutation suggest that binding sites for SRp40 and SRp55 would be abolished in the presence of the mutated g-allele, with no new binding sites created for any other splice factors.

Table 5. Distribution of the *CD45* (*PTPRC*) exon 4 54A→G SNP in South African populations of African ethnicity and Mixed Ancestry, per geographical region

		HIV + n=291	HIV - n=346	P
Western Cape Province				
African	Genotype	n=118	n=72	
n=190	aa	117 (0.992)	72 (1.00)	0.434
	ag	1 (0.008)	0	
	Alleles	n=236	n=144	
	a	235 (0.996)	144 (1.00)	0.434
	g	1 (0.004)	0	
Mixed Ancestry	Genotype	n=94	n=62	
n=156	aa	94 (1.00)	62 (1.00)	-
	ag	0	0	
	Alleles	n=190	n=124	
	a	188 (1.00)	124 (1.0)	-
	g	0	0	
North West Province				
African	Genotype	n=69	n=92	
n=161	aa	69 (1.000)	91 (0.989)	0.385
	ag	0	1 (0.011)	
	Alleles	n=138	n=184	
	a	138 (1.00)	183 (0.995)	0.386
	g	0	1 (0.005)	
Gauteng Province				
African	Genotype	n=10	n=120	
n=130	aa	10 (1.00)	115 (0.958)	0.510
	ag	0	5 (0.042)	
	Alleles	n=20	n=240	
	a	20 (1.00)	235 (0.979)	0.514
	g	0	5 (0.021)	

This may generate aberrant mRNAs that are either unstable or code for defective or deleterious protein isoforms. In the case of mutation 32A→G, no evidence of disruption of any of the binding sites analysed were obtained.

Flow Cytometry (FACs) analysis

Since assessment of the 32A→G mutation revealed no apparent splicing effect utilising the ESEfinder program, FACs analysis was performed to determine whether this mutation (observed predominantly in slow progressors compared to fast progressors) affects CD45 expression. The results obtained, as represented by Figure 2, suggested that this silent mutation might potentially affect expression of *CD45*. Figure 2A shows FACs profiles from two individuals with the 32A→G mutation, and a control without this mutation. All three individuals were HIV-seropositive. Peripheral blood mononuclear cells (PBMCs) were gated on CD4+ helper or CD8+ cytotoxic T-cells and CD45RA and CD45R0 expression was analysed. Striking differences in the proportions of CD4+ subsets in 32A→G individuals were observed, however these could possibly be due to differences in the stage of disease of the three individuals since HIV is known to infect CD4+ T-cells. The two individuals with the 32A→G mutation showed a significantly reduced number of CD45RA- CD45R0+ cells in the CD8+ subset (9.8% and 16.6% versus 40.6% of CD8+ cells), which might indicate altered splicing in CD8+ T-cells. Interestingly, the effect of the well-characterised 77C→G mutation on splicing (Figure 2B) appears to be more pronounced in CD8+ than CD4+ cells. Differences in proportions of both CD4+ and CD8+ subsets were obvious in the case of the 32A→G mutation.

The likelihood that the differences observed with FACs analysis (as seen in Figure 2) could be due to differences in the stage of disease or viral load in the individuals studied has to be considered, since viral infections generally affect the phenotype of CD8+ T-cells (Appay *et al.* 2002).

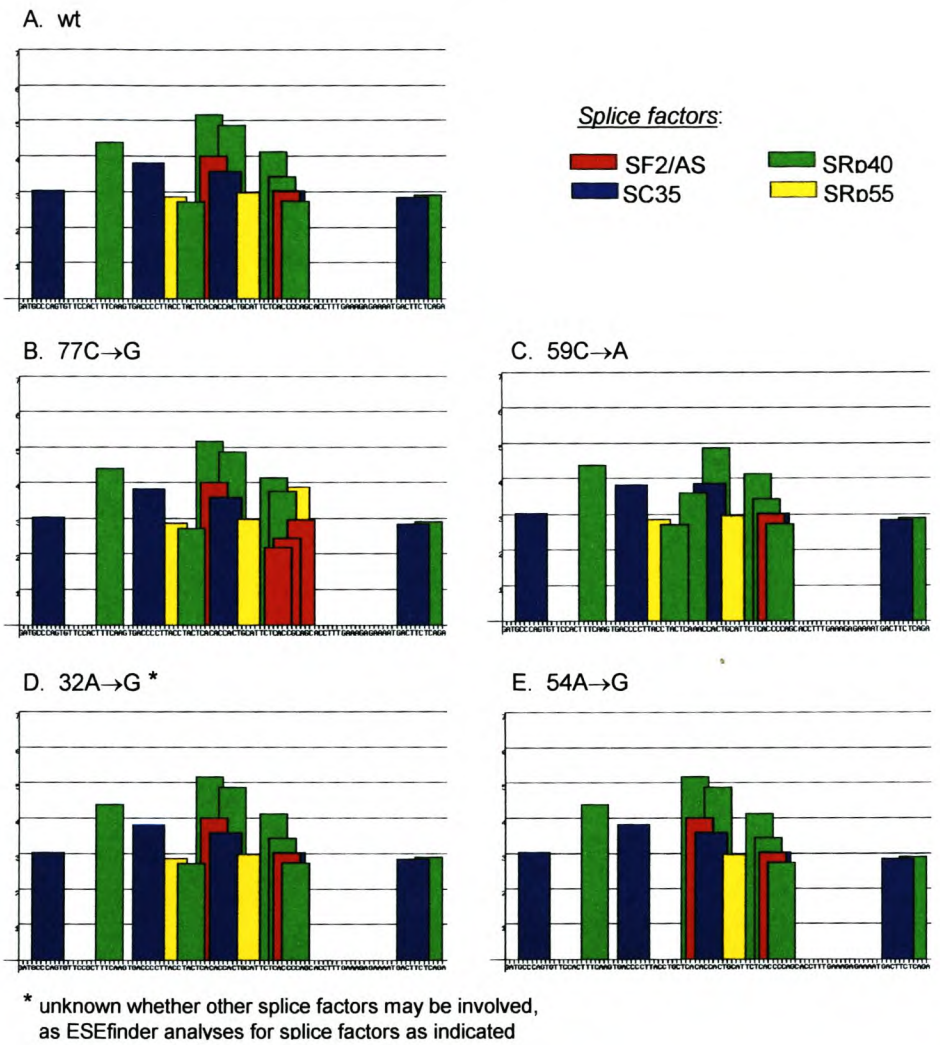

Figure 1. Theoretical predictions for creation or abolishment of binding sites for splice factors SF2ASF, SC35, SRp40 and SRp55 in the exonic splicing silencer element 1 (ESS-1) of the CD45 gene, according to the ESEfinder program. Panel A shows the wild type (wt) nucleotide sequence. Panels B and C show the known mutations 77C→G and 59C→G respectively, previously associated with alternative splicing. Panels D and E show the novel mutations 32A→G and 54A→G, respectively. Splice factors are as indicated by different colours.

Table 6. ESEfinder results obtained for abolishing binding or creation of new binding sites for splicing factors due to nucleotide sequence alterations in the *CD45* ESS-1 gene sequence. Nucleotide sequences for recognition sites of respective splicing factors are as indicated for the wild type (wt.) and mutant (mut.) sequences (bold and underlined). Nucleotide positions (nt.) are based on the nucleotide sequence as indicated in the footnote, used in determination of binding sites for splicing factors, and representing a portion of the PCR-fragment used in mutation analysis for *CD45* exon 4.

Mutation	Binding site abolished			New binding site created		
	Splice factor(s) for wt. sequence	Recognition nt. sequence (wt)	Nucleotide (nt.) position #	Splice factor(s) for mut. sequence	Recognition nt. sequence (wt)	Nucleotide (nt.) position #
77 C→G [a]	SF2/ASF	cacc**C**cag	57 - 64	SF2/ASF	ctcacc**G**	55 -61
				SF2/ASF	cacc**G**c	57-62
				SF2/ASF	c**G**cagca	60-66
	SC35	cacc**C**cagc	57 - 65	SRp55	c**G**cagc	60-65
	SRp40	cc**C**cagc	59 - 65	SRp40	tcacc**G**ca	56-63
59 C→A [a]	SRp40	tca**C**acc	40 - 46	SRp40	tca**A**acc	40-46
	SF2/ASF	ca**C**acca	41 - 47	SC35	**A**accactg	43-50
	SC35	**C**accact	43 - 49			
54 A→G [b]	SRp40	tacct**A**	33 - 39	none	-	-
	SRp55	ct**A**ctca	36 - 42	none	-	-
32 A→G [b]	none *	-	-	none *	-	-

[a] known mutations causing alternative splicing of *CD45* [b] novel mutations identified by the present study

* unknown whether other splice factors may b e involved, as ESEfinder analyses for four splice factors only: SF2/ASF, SC35, SRp40, SRp55

Nucleotide sequence for ESEfinder analysis (wild type): GATGCCCAGT GTTCC**A**CTTT CAAGTGACCC CTTACCT**A**CT CA**C**ACCACTG CATTCTCACC **C**CAGCACCTT TGAAAGAGAA AATGACTTCT CAGA (94 bases, nt. 1-94)

Disease progression status for the two HIV-seropositive individuals with mutation 32A→G was reported to be asymptomatic for HIV125 (Coloured female) and unknown for HIV118 (Black African female), while both were of class I WHO disease classification. Further, HIV118 has not received any antiretroviral therapy with a CD4+ count of 283 x 10^6 cells/l, whereas HIV125 received antiretroviral therapy for at least three years and maintained a CD4+ count of 980 x 10^6 cells/l.

HIV disease progression was uncertain in the HIV-seropositive control individual (HIV087/100) without mutation 32A→G who was included for comparison. However, WHO classification was reported to be class I at initial blood sampling, whereas follow-up clinical data approximately two years later reported class II disease classification, and CD4+ counts of 251 x 10^6 cells/l with no antiretroviral therapy.

Discussion

Analysis of exon 4 and the proposed promoter area (located between exons 1 and 2) of the *CD45* gene in South African populations from different ethnic backgrounds were performed in this study, with the aid of PCR-based mutation detection methods. The data obtained in this study confirmed the absence of the 77C→G mutation in exon 4 of the *CD45* gene in Africans, as previously reported (Tchilian *et al.* 2002). This mutation was however observed in one individual of Mixed Ancestry in our study population, suggesting inheritance due to Caucasian admixture. This mutation has been identified in individuals of Caucasian origin, but appears to be absent in Africans (Jacobson *et al.* 2000, Tchilian *et al.* 2001b, 2002). The frequency of 77C→G varies considerably between different ethnic groups and allele frequencies in healthy control groups have been reported to be 1.8% in individuals from the USA (Jacobson *et al.* 2000), whereas it could be as high as 6.7% in Pamiris from Central Asia (Tchilian *et al.* 2002). While this polymorphism was virtually absent in the populations investigated in our study, association of this mutation with HIV-infection or disease progression (Tchilian *et al.* 2001a) needs to be confirmed in different populations of large sample size.

Mutations 32A→G and 54A→G identified in exon 4 of the *CD45* gene have not previously been detected in the Caucasian population of South Africa (JNP de Villiers, unpublished data) and are therefore considered to be African-specific polymorphisms. Both mutations are present in the ESS-1 element of exon 4, located between nucleotides 29 and 88 of *CD45* (Lynch and Weiss 2001).

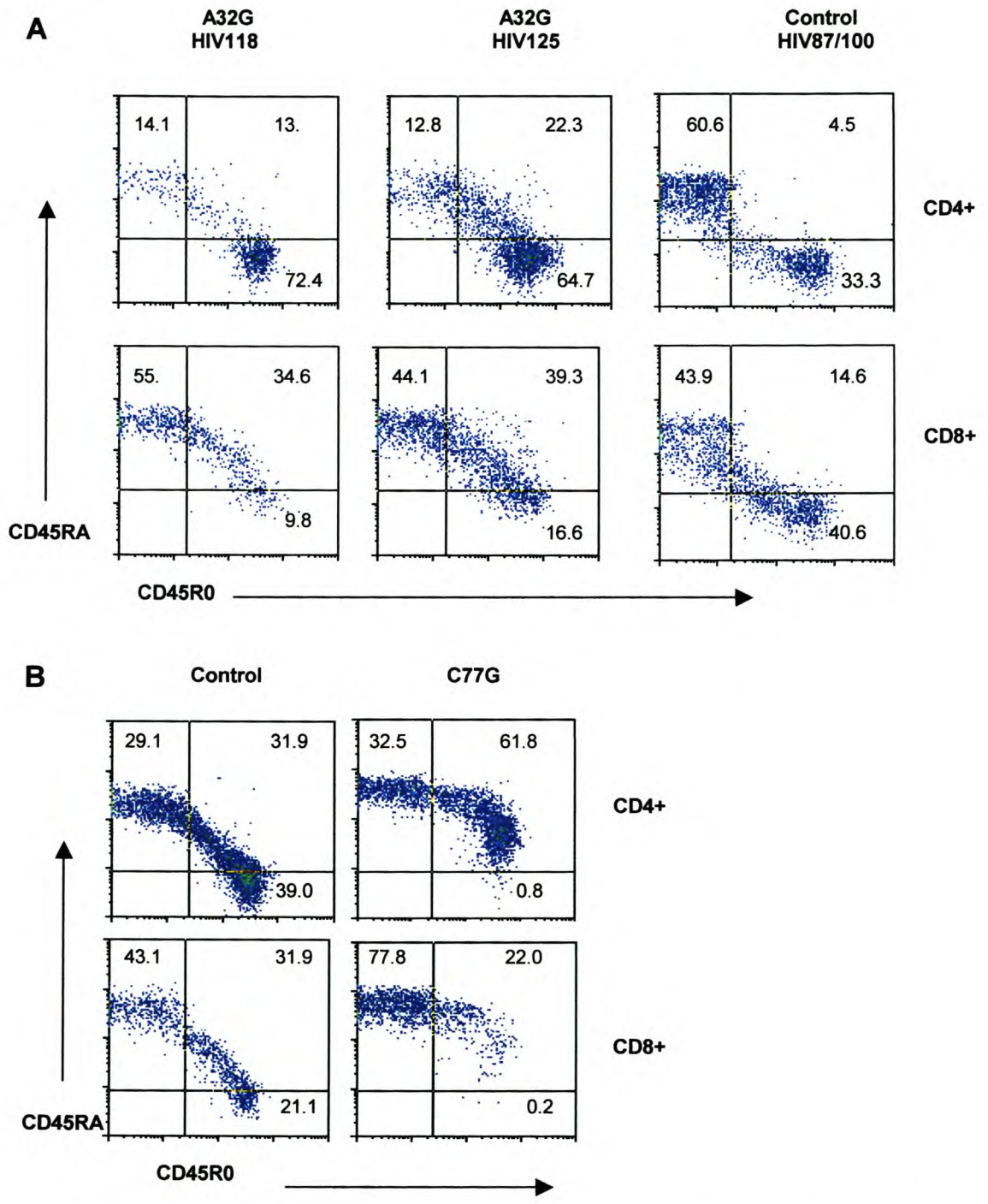

Figure 2. Variant expression of the CD45 protein in relation to the 32 A→G mutation. FACs analysis profiles of 32A →G, indicating markedly reduced CD8+ cells (figure 2A), compared with a wild type control, 32A. Isolates presenting with the 32A→G polymorphism were compared to both a non-HIV control isolate and an isolate presenting with the 77C→G mutation (figure 2B).

Mutations in ESS1 have been associated with alternatively spliced isoforms of *CD45* (Jacobsen *et al.* 2000, Lynch and Weiss 2001). Similar to the previously documented 77C→G and 59C→A substitutions, both the 32A→G and 54A→G transitions is therefore likely to contribute to alternative splicing.

Application of the ESEfinder program predicted alternative splicing in the presence of mutation 54A→G, due to abolishment of known binding sites for factors that are essential for normal gene splicing. The creation of new binding sites were not indicated by this program, as was the case for two well-characterised mutations in the *CD45* gene, 77C→G and 59C→A where known binding sites were abolished and new ones were predicted. Further, in addition to being situated within the ESS1, the 54A→G mutation causes an amino acid substitution, T18A, which may alter the CD45 protein structure. In the case of mutation 32A→G, no indication was obtained that this sequence change is likely to abolish or create binding sites for the splicing factors analysed. It is however possible that this mutation has a favourable effect on immune function, or occurs in linkage disequilibrium with another functional mutation, which may be associated with differential expression.

Based on the results obtained using FACs analysis, the likelihood that mutation 32A→G affects splicing of the CD45 protein cannot be excluded, since clear differences in proportions of both CD4+ and CD8+ subsets were observed in individuals with this mutation compared with the control individual. However, since HIV is known to infect CD4+ T-cells, these differences might also be due to differences in the stage of disease. The disease status of the two HIV-seropositive 32A→G heterozygotes included for FACS analysis was both classified as WHO class I. However, the HIV-seropositive control individual without the 32A→G mutation had much reduced CD4+ counts. Further, according to clinical data this individual progressed from class I to II within two years from seroconversion, and could be classified as a rapid progressor. The individual with mutation 32A→G for whom clinical data was available has remained asymptomatic for more than four years since HIV-1 disease diagnosis.

Analysis of clinical data of three more individuals with mutation 32A→G (all class I disease status and either normal or long-term non-progressors, LTNP), raise the possibility that this mutation is associated with protection against progression to AIDS following HIV infection, especially since none of the 22 rapid progressors subjected to mutation screening tested positive for this mutation.

This was exemplified by the fact that none of the slow progressors or LTNPs with mutation 32A→G and with known progression status received antiretroviral therapy. Rather, they remained relatively healthy and maintained relatively stable or high CD4+ counts for extended periods of 9-11 years since seroconversion.

Since our data does not provide conclusive proof that the 32A→G mutation affects CD45 splicing, FACs analysis on healthy HIV-seronegative individuals with the mutation is required to clarify the possible phenotypic effect of this mutation. Differences in the stage of disease or viral load in the HIV-seropositive patients analysed may be responsible for the differences observed between patients and controls, since the phenotype of CD8+ T-cells are affected due to viral infections (Appay *et al.* 2002). RNA studies or analysis of possible splicing effects using minigenes (Zilch *et al.* 1998) containing the mutation could clarify the potential role of mutation 32A→G on expression of the *CD45* gene.

In this study we have provided evidence that the apparently African-specific polymorphisms, 32A→G and 54A→G identified in the *CD45* gene, may affect protein expression. Final elucidation of the functional significance of these mutations would contribute to our understanding of the involvement of CD45 in immune disorders and modification of risk for HIV/AIDS in the African context. Increased understanding of the role of CD45 in host immunity may provide potential targets for intervention strategies aimed at slowing disease progression in affected individuals.

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D. Assessment of HLA-B27 genotypes in South African HIV-seropositive individuals with slow versus rapid progression to AIDS in relation to predetermined genotypes of the *CCR5, SLC11A1, HFE* and *CD45* genes

Objectives: The potential protective effect of HLA type B27 was investigated in relation to combined genotypes determined for several candidate susceptibility genes in HIV-seropositive individuals. The objective of this study was to identify genetic markers that, alone or in combination with other mutations, might explain differences in rate of disease progression from stage I to IV according to WHO criteria in South African patients.

Study Design: Thirty-six HIV-seropositive individuals classified according to disease status were selected for HLA-B27 genotyping. These individuals have previously been screened for genetic polymorphisms in the *CCR5, SLC11A1, HFE* and *CD45* genes, and formed part of a cohort attending an infectious diseases clinic in the Western Cape Province of South Africa.

Methods: The study population included 15 Xhosa, 17 Coloured and 4 Caucasian HIV-seropositive individuals. HLA-B27 genotyping was performed by using a real-time polymerase chain reaction (PCR)-based mutation detection method.

Results: Two individuals tested positive for HLA-B27, and remained to be classified as WHO stage I after more than 5 years of infection. No predominant co-existence with any of the mutations screened for in the *CCR5, SLC11A1, HFE* and *CD45* genes were detected in these individuals.

Conclusions: Although only two of 36 individuals analysed tested positive for HLA-B27, the clinical features of the HIV-seropositive individuals with this allele is in accordance with the protective effect previously ascribed to this allele occurring in individuals of Caucasian origin. Our findings provide support for a multifactorial basis for the development of AIDS in HIV-1 infected individuals.

Introduction

The multifactorial nature of HIV disease pathogenesis is reflected by the highly variable rates of disease progression in HIV-infected individuals and between populations. Significant differences in the rate of HIV-1 disease progression have been observed in longitudinally followed HIV-infected persons since the beginning of the HIV epidemic. Although most infected individuals develop AIDS symptoms within ten years, a small number remains relatively healthy for periods longer than fifteen years, whereas others progress to full-blown disease within the first three years after infection (Phair *et a*l. 1992, Muñoz *et al.* 1997). Some individuals however resist infection or have the ability to eliminate the virus.

Various studies have indicated that susceptibility to many infectious diseases, including HIV/AIDS and tuberculosis, may be under host genetic control (Bellamy *et al.* 1998, Liu *et al.* 1996, Samson *et al.* 1996a,b,c). Modulation of the rate of disease progression can be influenced by host factors that can be either intrinsic or acquired, and most host genetic mechanisms contributing to HIV/AIDS susceptibility have been shown to influence the immune defence system. Genetic variants contributing to resistance to infection have been identified in persons who were persistently exposed to HIV-1 but remained uninfected (Rowland-Jones and McMichael 1995, Liu *et al.* 1996, Paxton *et al.* 1996). Although a 32-bp deletion of the *CCR5* gene, CCR5Δ32, was shown to be associated with resistance to HIV-1 infection in several studies (Liu *et al.* 1996, Samson *et al.* 1996a,b,c, Biti *et al.* 1997), this mutation is not responsible for a protective effect in all cases (Balotta *et al.* 1997, O'Brien *et al.* 1997, Theodorou *et al.* 1997). This also appears to be true in the Black South African population (Williamson *et al.* 2000, Petersen *et al.* 2001), since the deletion-mutation is virtually absent in Africans. Several reports have indicated that the mechanisms underlying HIV/AIDS susceptibility differ amongst most risk groups and between populations (Costello *et al.* 1999, Geczy *et al.* 2000, Roe *et al.* 2000, Gao *et al.* 2001).

Resistance or susceptibility to HIV infection and disease progression has been ascribed largely to mutations in chemokine co-receptors and the presence of certain alleles of the major histocompatibility complex (MHC) (Liu *et al.* 1996, Laumbacher and Wank 1998, Magierowska *et al.* 1999).

Speculation of polygenic control of infectious disease susceptibility has been confirmed by identification of several genetic regions containing genes that determine susceptibility to infection (Roberts *et al.* 1997a). For HIV-1 disease, it was shown that combinations of genetic variants might contribute significantly to susceptibility patterns and disease progression (Magierowska *et al.* 1999, Geczy *et al.* 2000). Strong association for a number of alleles from the HLA system, including HLA-B27, have furthermore been associated with slow HIV disease progression and even non-progression, whereas others favoured rapid progression (Hendel *et al.* 1996, 1999; Malkovsky 1996, Kaslow *et al.* 1996, McNeil *et al.* 1996, Roger 1998, Scorza Smeraldi *et al.* 1986, Cruse et al. 1991). Although alleles of the HLA system were shown to function independently from other host susceptibility genetic variants (Flores-Villanueva *et al.* 2001), a combination of protective alleles from the HLA system and chemokines was suggested to be responsible for slow disease progression in a large proportion of individuals studied (Magierowska *et al.* 1999).

In addition to immunological factors and host genetic variants implicated in susceptibility to HIV/AIDS, environmental and viral factors contribute significantly to the complexity of disease pathogenesis (Klein and Miedema 1995, Bollinger *et al.* 1996, Haynes *et al.* 1996). Assessment of the importance of host genetic involvement in disease development has however been complicated by the complexity of environmental factors and nutritional requirements that can be modulated by genetic mechanisms, which might further affect microbial transmission. Furthermore, the variation of disease outcome between individuals has been speculated to be due to combination effects of host genes and proteins controlling the quality and possibly quantity of host-parasite interaction and human immune responses. Genetic variants often have subtle but significant consequences on gene expression or protein function. A delicate balance among these factors most likely determines the net rate of viral replication in HIV-infected individuals.

Recently, Carrington and co-workers (2001) suggested an approach that may assist to clarify the influence of genetic mechanisms involved in microbial disease pathogenesis. However, due to the complexity of host-pathogen interaction no standard practice exists for prediction of the disease outcome based in part on the genetic profile of HIV-seropositive individuals.

In this study we investigated the potential protective effect of HLA type B27 in relation to combined genotypes determined for several candidate susceptibility genes in HIV-seropositive individuals, including the *CCR5*, *SLC11A1*, *HFE* and *CD45* (*PTPRC*) genes. The objective was to identify genetic markers that, alone or in combination with other mutations, might explain differences in rate of disease progression from WHO stage I to IV in South African patients. Discovery of host genetic mechanisms associated with infectious disease pathology, and defining the involvement of environmental factors interacting with genetic mechanisms underlying acquisition of infectious disease, may aid in development of novel targets for vaccines or chemotherapy.

Materials and Methods

Study Population

Ethical approval for the study was obtained from the Ethics Review Committee of the University of Stellenbosch. Venous blood samples were obtained with informed consent from all the study participants and the DNA was extracted using standard methods. Thirty six HIV-seropositive individuals with known disease progression status and WHO classification (Table 1) were selected for HLA-B27 genotyping from a cohort of HIV-seropositive individuals attending the Infectious Diseases Clinic at Tygerberg Hospital in Cape Town, South Africa. This study group has previously been subjected to mutation analysis of the *CCR5, SLC11A1, HFE* and *CD45* (*PTPRC*) genes (see sections A, B and C of this chapter), and included 15 Black Africans (12 female, 3 male) of Xhosa origin, 17 Coloured individuals of mixed ancestry (13 females, 4 males) and 4 Caucasian males. The Coloured individuals of mixed ancestry are of San, Khoi, African, Madagascar, Javanese and European origin. Estimated date of infection and HIV-1 disease-associated complication data were recorded for the majority of individuals included in the study, and ages of all study participants were calculated based on date of blood sampling. Data on HIV disease status was denoted according to WHO clinical disease classification (classes I-IV, http://who.int), and HIV-seropositive individuals were classified as normal progressors when progressive loss in CD4 cell counts were associated with development of AIDS-related symptoms within 10 years after HIV-1 infection, as rapid progressors when progression to full-blown AIDS occurred within 2-3 years from seroconversion, and as slow progressors when they remained unaffected after 8-10 years from seroconversion.

Table 1. Clinical data of 36 HIV-seropositive South African individuals in relation to genotypes determined for HLA-B27 and the *SLC11A1*, *HFE*, *CD45* and *CCR5* genes.

	Clinical data						Genetic Data					
HIV No.	Classification Progr.	WHO	Age (y.o.) & Gender	CD4+ count X10^6 cells/ml	Clinical Symptoms	ARV	*SLC11A1* 5'[GT]n	*HFE* IVS3-48c/g	*HLA* -B27	*CD45* Exon2	*CCR5* P35	*CCR5* Δ32
Black African Population **(n=11)**												
HIV54	S,9y	I	31 F	585	As	+	3,3	-	-	32A→G	-	-
HIV116	N,6y	II	52 F	216	As	+	3,3	+	-	-	-	-
HIV118	*unk.*	I	45 F	293	*unk.*	+	3,3	-	-	32A→G	-	-
HIV122	N,5y	II	26 F	672, 874	Seb	+	3,3	-	-	-	-	-
HIV155	R	IV	28 F	47	TB	-	3,3	-	-	-	-	-
HIV200	R	II	20 F	207	Seb, Hb10	*unk.*	2,3	-	-	-	-	-
H IV202	R	IV	43 M	*unk.*	PTB,O thr	*unk.*	3,3	-	-	-	+	-
HIV209	R,6y	III	35 F	113	TB	+	3,3	+	-	-	-	-
HIV215	LTNP,2y	I	28 M	1106, >1000	As	*unk.*	3,3	-	-	-	-	-
HIV245	LTNP,11y	I	19 F	*unk.*	HIV-as poliade, PTB	-	3,3	-	-	32A→G	-	-
HIV257	N	IV	34 M	<50	OcTB,O thr,Per Neu	+	3,3	+	-	-	-	-
HIV262/24	LTNP,>10y	I	46 F	325, 350	Lymphadenopathy	-	2,3	+	-	32A→G	-	-
HIV267	*unk.*	I	26 F	*unk.*	*unk.*	-	3,3	-	-	54A→G	-	-
HIV272	S	I	28 F	727	As	*unk.*	2,3	-	-	-	-	-
HIV273	S,6y	II	37 F	239	Foll	-	3,3	-	-	-	-	-
Coloured Population **(n=16)**												
H IV93	N,9y	II	32 F	*unk.*	Seb	+	3,3	-	-	-	-	-
HIV98	Asymp,>3y	I	49 F	400, 175	As	-	2,3	-	-	-	-	-
HIV111	R,>4y	II	27 F	250, 291, 319	Seb	+	3,3	-	-	-	-	-
H IV113	N	*unk.*	46 M	120, 658	*unk.*	+	3,3	-	-	-	NR	32 bp
HIV125	N,>4y	I	43 F	980	As	+	2,3	-	-	32A→G	-	-
HIV137	N,>5y	I	39 M	305	Dia Mel	+	2,3	-	+	-	-	-

...../cont.

Table 1 *cont.*

	Clinical data						Genetic Data					
HIV No.	Classification Progr.	WHO	Age (y.o.) & Gender	CD4+ count X10^6 cells/ml	Clinical Symptoms	ARV	*SLC11A1* 5'[GT]n	*HFE* IVS3-48c/g	*HLA* -B27	*CD45* Exon2	*CCR5* P35	*CCR5* Δ32
HIV142	N	III	25 F	*unk.*	PTB	-	3,3	-	-	-	-	-
HIV163	N	III	49 F	*unk.*	*unk*	*unk.*	3,3	-	-	-	-	-
HIV171	R	IV	25 F	20	Per TB, HSV	+	3,3	-	-	-	-	-
HIV174	S	IV	29 F	804, 909	TB	*unk.*	3,3	-	-	-	-	-
HIV180	S	II	32 F	609	Seb, Fe30	*unk.*	2,3	+	-	-	-	-
HIV195	*unk.*	IV	41 F	*unk.*	Crypt men, TB	+	3,3	-	-	77C→G	-	-
HIV206	N	IV	44 M	748	TB	*unk.*	2,3	-	-	-	-	-
HIV231	N,>6y	II	37 F	444	Seb	*unk.*	2,3	+	-	-	-	-
HIV232	LTNP	II	29 F	288, 415	Seb	*unk.*	3,3	NR	-	-	-	-
HIV252	R,4y	II	35 M	244, 112, 55	p24	*unk.*	2,3	-	-	-	-	-
HIV255	R,>2y	IV	29 F	128	PTB	-	3,3	-	-	-	-	-
***Caucasian population* (n=4)**												
HIV109 (Homo)	N,6y	I	33 M	*unk.*	*unk.*	+	3,3	-	+	-	-	-
HIV183	LTNP,4y	I	36 M	650,658,473	As	*unk.*	3,3	NR	-	77C→G	NR	-
HIV266	R	I	32 M	21	*unk.*	*unk.*	2,3	-	-	-	-	-
HIV277 (Homo)	R,2y	IV	26 M	*unk.*	Oes Ca,Can	*unk.*	2,3	NR	-	-	-	-

***Abbreviations*:** y.o. = years old, Δ32 = 32 bp deletion, Progr. = Progression, ARV = anti-retroviral therapy, N = normal, R = rapid, S = slow, LTNP = long term non-progressor, As = asymptomatic, Seb = seborrhoea, TB = tuberculosis, Hb = haemoglobin, Foll = folliculitis, PTB, pulmonary tuberculosis, O thr = oral thrush, Oc TB, occult tuberculosis, Per Neu = pericardial neuropathy, HIV-as poliade = HIV-associated polyadenitis, HSV = herpes simplex virus, Crypt men = cryptococcal meningitis, Fe = ferritin, Dia Mel = Diabetes mellitus, p24 = persistent HIV p24 antigen, Homo = homosexual, Oes Ca = oesophageal cancer, Can = candida, *unk.* = unknown, NR = no result

HLA-B27 *genotyping*

HLA-B27 genotyping was initially performed using the commercially available HLA-B27 Gene Detection Assay (ViennaLab, Austria). The assay procedure was followed as specified by the manufacturers, which is based on a reverse-hybridisation procedure with horseradish peroxidase staining. Briefly, HLA-B gene exon 2 sequences were PCR-amplified and terminally labelled with a reporter molecule (included in the assay procedure) before hybridisation to group-specific oligonucleotide probes (either HLA-B27 or control, CTL). Horseradish peroxidase-labelled antibody was added to the reporter molecule and colour substrates. CTL was included as an indicator of successful PCR amplification. Positive samples were observed visually, although a micro plate reader (not available) is recommended for visualisation of HLA-B27 positive samples. Confirmation of positive signals obtained with the HLA-B27 Gene Detection Assay was performed by real-time PCR on a Roche LightCycler as described by Tiemann *et al.* (2001). β-Globin-specific PCR oligonucleotide primers were used as an internal control for this procedure. The oligonucleotide primers, PCR conditions and mutation detection methods previously used for mutation detection in the study population are summarised in Table 2, together with that applied for HLA-B27 genotyping performed additionally in this study.

Results and Discussion

Clinical and mutation data of 36 HIV-seropositive individuals selected for this study based on disease stage classified according to the WHO criteria, is summarised in Table 1. The mutation detection methods previously used for genotyping of polymorphisms in the *CCR5, SLC11A1, HFE* and *CD45* genes in these individuals are given in Table 2. Figure 1 illustrates genotype results obtained for HLA-B27, using the commercially available HLA-B27 Gene Detection Assay (A), followed by confirmation of positive signals using real-time PCR (B). All samples observed to be positive with the HLA-B27 Gene Detection Assay (both light blue and strong dark blue staining wells) were subjected to real-time PCR using LightCycler technology.

Only the two strongest positive signals representing the DNA samples of individuals HIV137 and HIV109 (wells A7/A8 and A11/A12, respectively), were confirmed as HLA-B27 positive following analysis by real-time PCR. According to disease progression status and WHO disease classification, both individuals HIV137 and HIV109 had normal progression status and were of WHO class I status.

Table 2. Oligonucleotide primers and condition used for PCR-based mutation detection in the South African study population.

Gene & variation	PCR Primer nucleotide sequences (5'→3')	$T_{A1/A2}$ (°C)	Size (bp)	DNA Analysis
SLC11A1 (NRAMP1)				
5'[GT]n promoter variant	F CCCCAGAACCTTGAGGTCTA R CCCACACCAGTACCCCAT	65/60	116	*Rsa* I RE digestion
HFE [a]				
IVS3–48c→g	F AGTGAAAGTTCCAGTCTTCC R CTCACTGCCATAATTACCTC	55	202	*Bsa* J1 RE digestion
CD45 (*PTPRC*)				
Exon 2	F CCGAATCTGACATCATCACC R CCTTGTACTTACCTGTCACAA	60/55	324	Hex-SSCP analysis
CCR5 [b]				
P35	F ATTATACATCGGAGCCCTGC R AGCATAGTGAGCCCAGAAGG	60/55	280	Hex-SSCP analysis
*CCR5*Δ32	F CTGGCCATCTCTGACCTGTT R GATGATTCCTGGGAGAGACG	65/60	332	12% PAGE (3.4% C)
HLA-B [c]				
HLA-B27	F GGGTCTCACACCCTCCAGAAT R CGGCGGTCCAGGAGCT	65/60	250	Real-time PCR (with β-globin primers[#] as internal standard

T_A annealing temperature, bp base pair, F forward, R reverse, RE restriction enzyme, Hex-SSCP heteroduplex single-stranded conformation polymorphism

[#] β-globin specific primers BG1: CAACTTCATCCACGTTCACC, BG2: GAAGAGCCAAGGACAGGTAC

[a] Beutler *et al.* 2000, [b] Petersen *et al.* 2001, [c] Tiemann et al. 2001. These references refer to the first description of the mutation analysed, or the method of mutation detection used.

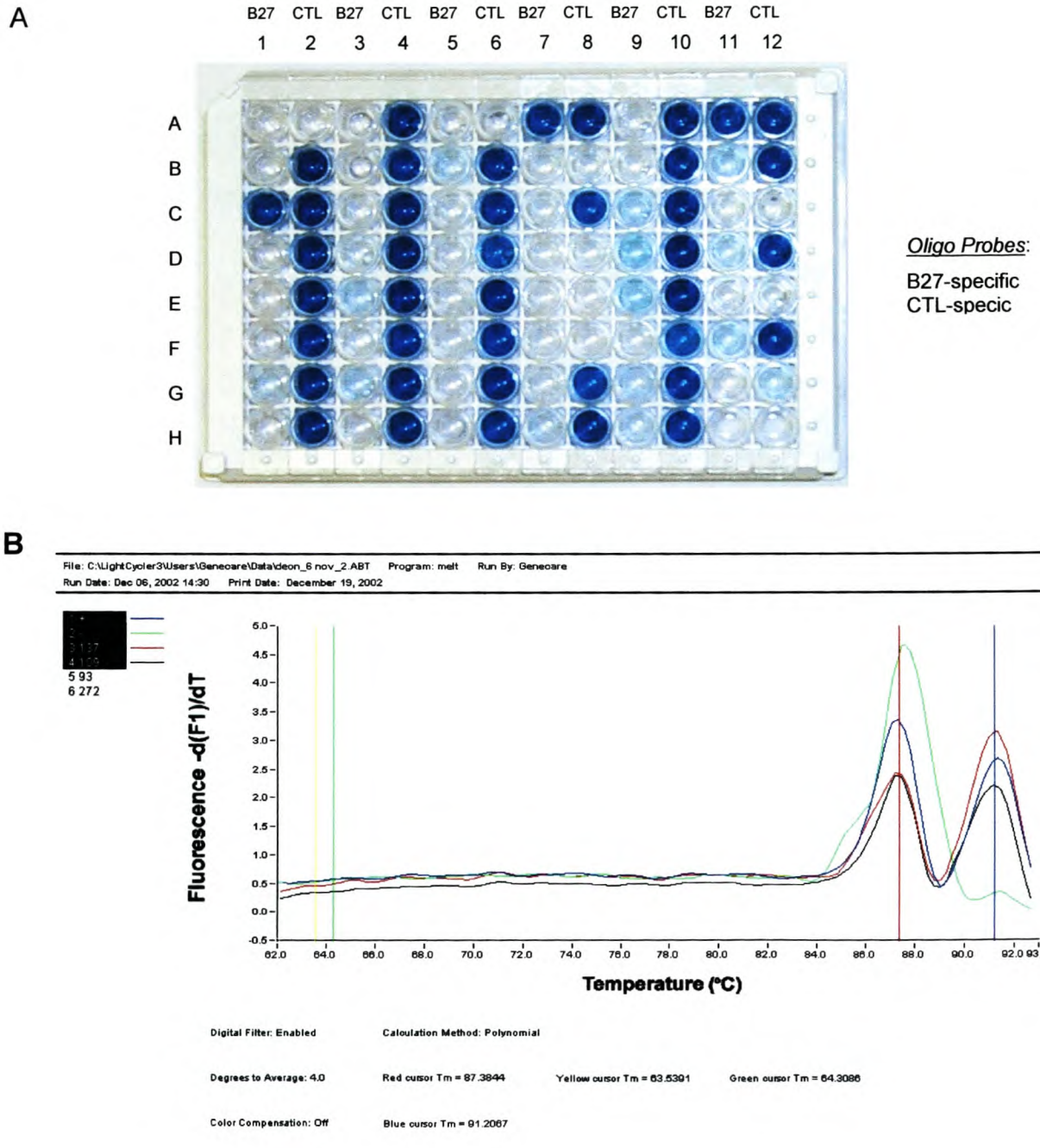

Figure 1. HLA-B27 genotyping using the commercially available HLA-B27 Gene Detection Assay (A) and real-time PCR using the Roche LightCycler (B). A. Exon 2 of the HLA-B gene was PCR-amplified and terminally labelled with a reporter molecule before hybridisation to a group-specific HLA-B27 and generic control (CTL) oligonucleotide probes in alternating cavities of a microwell plate. Bound sequences are detected by addition of horse-radish peroxidase-labelled antibody to the reporter molecule(s) and colour substrates. Positive signals are indicated in wells A7/A8 (HIV137) and A11/A12 (HIV139), respectively. B. The two strongest signals observed by using the HLA-B27 Gene Detection Assay were confirmed as being positive by real-time PCR on the Roche LightCycler. Sample numbers H137 and H109 is indicated in red and black respectively, while the positive and negative controls are indicated by blue and green respectively.

HIV137, a Coloured male individual, has been HIV-seropositive for more than 5 years with no clinical symptoms related to HIV-1 infection. This individual also had a diagnosis of Diabetes Mellitus. HIV109, a Caucasian male, seroconverted 6 years prior to entry into the study and was classified as stage I disease at the time. No additional clinical data was available for this individual. Although only a limited number of gene mutations were analysed in the study population, none of these individuals showed any predominant association with a combination of gene variations in the *CCR5, CD45, HFE* and *SLC11A1* genes. It therefore seems likely that the presence of the HLA-B27 allele could contribute to the health status of the two mutation-positive individuals considered to be relatively good. Although this could also be ascribed to antiretroviral therapy recorded for both individuals, our findings are in accordance with a protective role of HLA-B27 against progression to AIDS, seeing that none of the rapid progressors had this allele. Although HIV137 also tested positive for allele 2 of the *SLC11A1* 5'[GT]n polymorphism, previously implicated as a protective modifying factor in HIV-infected patients (Marquet *et al.* 1999), our earlier studies (see section A of this chapter) could not confirm this association in the South African population.

No possible gene-gene interactions were furthermore observed when LTNPs and asymptomatic HIV-seropositive individuals without the HLA-B27 allele were assessed for combinations of any of the other genes analysed in the same study cohort. Predominance of mutation 32A→G in HIV-seropositive individuals classified as WHO class I and slow progression compared with its absence in HIV-seropositive patients with rapid disease progression (see section C of this chapter), suggests a positive link with slower disease progression irrespective of interaction with any of the other genes studied. Two of the five individuals with mutation 32A→G included in this study were classified as LTNPs, two remained asymptomatic after several years of infection, and progression status was unknown in the remaining individual.

The African-specific polymorphism IVS3–48c/g of the *HFE* gene involved in iron overload was identified in the LTNP HIV262/24. This polymorphism occurs at a position in intron 3 of the gene that closely resembles a splice site nucleotide sequence, but further studies did not demonstrate a functional role for this variant (Beutler and Gelbart 2000). Our analysis of this variant (D Pretorius 2003a, section B of this chapter), although not significantly associated with HIV-1 disease, indicated that the *HFE* IVS3–48c/g variant was markedly associated with reduced haemoglobin levels (P=0.004).

This observation appears to be related to linkage disequilibrium with a functional mutation in another gene in close association with the HFE gene. The combination of these two genetic variants, together with *SLC11A1* genotype 2,3 in the same individual, raise the possibility of gene-interaction between the *SLC11A1*, *CD45* and *HFE* genes resulting in the LTNP phenotype. However, co-existence of these mutations in the same individual may also be due to chance, especially since no preferential association of the IVS3-48c→g mutation with disease status or progression was observed in individuals with this *HFE* mutation.

Although one HIV-seropositive Coloured male, HIV113, tested positive for the CCR5Δ32 deletion known to be associated with slow disease progression, this individual was classified with normal disease progression and unknown WHO disease status, while receiving antiretroviral therapy. CD4+ counts improved from 120 to 658 between two clinical follow-up events.

Conclusions

In view of our results within the context of published data, it seems likely that susceptibility to HIV/AIDS following exposure to HIV-1 infection may to a large extent be determined by non-MHC genes such as *CCR5* and *SLC11A1* amongst others, while severity of the condition is determined by modulation of T-cell responses to particular antigens. This can be supported by the proposed role of CD45 (Tchilian *et al.* 2001a,b,c), known to be essential for the activation of T-cells, which is also implicated in this process and probably forms part of the polygenic nature of HIV/AIDS susceptibility. Iron status appears to be central to this process, since sufficient availability of iron is required for development of T-cells, which in turn appears to regulate iron metabolism perhaps through interactions with HFE (Bowlus 2002) encoding a non-classical major histocompatibility gene.

Our results are in accordance with the notion that HIV-1 disease susceptibility and progression are regulated by polygenic control mechanisms, in addition to the influence of environmental factors that are of vital importance in the regulation of cellular activities and the recruitment of cells of the innate immune response. Investigating various host genetic changes in the same population as performed in this study, could be important for the development of a screening procedure to predict clinical outcome of HIV/AIDS.

This may further aid in understanding the involvement of host factors in host immunity, which may provide potential targets for intervention strategies aimed at slowing disease progression in affected individuals.

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CHAPTER 3

CONCLUSIONS AND FUTURE PROSPECTS

Chapter 3 CONCLUSIONS AND FUTURE PROSPECTS

Prior to the initiation of this study very limited genetic analysis had been performed in the South African population with regard to susceptibility to HIV/AIDS. The overall objective was therefore to identify host genetic variants that may contribute to modified risk of HIV-1 infection and/or progression to AIDS, particularly in the non-Caucasian South African population. It was anticipated that possible phenotypic effects of relevant mutations investigated for their potential involvement in HIV-disease might provide an improved understanding of the importance of host genetic mechanisms in the African context. The elucidation of disease mechanisms may ultimately increase the predictive ratio of disease evolution, thereby providing an opportunity to refine the ongoing evaluation of therapeutic or preventive measures, or contribute to the development of novel therapeutic strategies. Host genetic factors found to be involved in HIV/AIDS may have prognostic value and may serve, in addition to CD4+ counts and viral load, as decision-making tools in clinical management of HIV-infected individuals. The observations made in this study were considered in the context of the variety of genetic mechanisms previously implicated in susceptibility to HIV-1 infection and disease progression, in order to evaluate the potential impact of our findings in relation to current knowledge.

It is known from the literature that various host genetic factors may be associated with HIV-1 disease acquisition or progression to full-blown AIDS. The most extensively studied factors associated with HIV-1 disease are summarised in table 1. Susceptibility to HIV-1 disease has mainly been attributed to variation of the chemokine and chemokine co-receptor genes and alleles of the major histocompatibility complex (MHC). For example, various mutations in the chemokine co-receptor genes, *CCR5* and *CCR2*, may protect against HIV-1 infection or are associated with slow disease progression. Alleles of the MHC system are however more varied with regard to their involvement in HIV/AIDS. Some alleles are associated with slow disease progression and others with rapid progression, while the specific alleles involved also differ between Caucasian and African population groups. Alleles of the *MBL* gene appear to be protective to HIV-1 infection, although not against progression to AIDS. Once an individual has been infected, progression to AIDS may occur rapidly irrespective of the *MBL* genotype. The proposed HIV/AIDS susceptibility mechanisms and gene variations vary considerably between individuals, and in some a combination of mutations in chemokine receptors together with specific HLA alleles are responsible for diverse clinical outcomes (Magierowska *et al.* 1999).

Table 1. Genetic mutations (alleles and haplotypes) implicated with modified risk to HIV-1 infection and the rate of disease progression.

		HIV		*HIV-Progression*				
Gene	**Allele/Haplotype**	**Protection**	**Infection**	**LTNP**	**Slow**	**Normal**	**Rapid**	**Reference**
CCR5	CCR5Δ32 (Cau)	✓ (hom)	-	✓ (het)	-	-	-	Liu *et al.* 1996; Samson *et al.* 1996; Garred 1996; Biti *et al.* 1997; Eugen-Olsen *et al.* 1997; Meyer. 1997; Michael *et al.* 1997
	-59353T/C	-	-	✓	-	-	-	Quillent *et al.* 1998
	-59029G/A	-	-	-	✓	-	-	Quillent *et al.* 1998
	-50402A/G (CSW [a])	-	-	-	✓	-	-	Sriwanthana *et al.* 2001
Haplotype	M330/CCR5Δ32 (Cau)	✓	-	-	-	-	-	Quillent *et al.* 1998
	-59353T/C / -50402A/G	-	-	-	✓	-	-	Martin *et al.* 1998
CCR2	V64I (incl. CSW [b])	-	-	-	✓	-	-	Smith *et al.* 1997, Anzala *et al.* 1998 [c] Kostrikis *et al.* 1998, Mummidi *et al.* 1998, Rizzardi *et al.* 1998,Mangano *et al.* 2000
SDF-1	SDF1-3'A	-	-	✓	-	-	-	Winkler *et al.* 1998, van Rij *et al.* 1998, Brambilla *et al.* 2000
MBL	A, B, C (hom)	✓	-	-	-	-	✓	Nielsen *et al.* 1995, Garred *et al.* 1997, Maas *et al.* 1998
SLC11A1	5'[GT]n Allele 2, 274C/T, 469+G/T	✓	-	-	-	-	-	Marquet *et al.* 1999
	823C/T	-	✓	-	-	-	-	Marquet *et al.* 1999
Hp	Hp2-2 (phenotype)	-	✓	-	-	-	✓	Sanchez-Madrid *et al.* 1983, Valentin *et al.* 1990, Delanghe *et al.* 1998
CD45	C77G	-	✓	-	-	-	-	Tchilian *et al.* 2001

...../cont.

Table 1*cont.*

Gene	Allele/Haplotype	*HIV*		*HIV-Progression*				Reference
		Protection	Infection	LTNP	Slow	Normal	Rapid	
HLA	A26, B14, B27 B57, C8, C14, DR11	-	-	-	✓	-	-	Hendel *et al.* 1996, 1999; Kaslow *et al.* 1996, Malkovsky 1995, McNeil *et al.* 1996, Roger *et al.* 1998, Scorza Smeraldi *et al.* 1986
	A2, A28, B18 (Afr, CSW [b])	✓	-	-	-	-	-	MacDonald *et al.* 2000
	A23 (Afr, CSW [b])	-	✓	-	-	-	-	MacDonald *et al.* 2000
	Bw4	-	-	✓	-	-	-	Flores-Villanueva *et al.* 2001
	Bw6, B35	-	-	-	-	-	✓	Flores-Villanueva *et al.* 2001, Scorza Smeraldi *et al.* 1986
	B5703 (Afr)	-	-	-	-	-	✓	Costello *et al.* 1999
	DQB1*0603 (Cau)	-	✓	-	-	-	-	Roe *et al.* 2000
	DQB1*03032 (Cau)	✓	-	-	-	-	-	Roe *et al.* 2000
	DQB1*04 (Cau)	-	✓	-	-	-	-	Roe *et al.* 2000
	DQB1*0201 (Afr)	-	✓	-	-	-	-	Roe *et al.* 2000
	A*0202, A*0205, A*0214, A*6082 DRB1*0102 (Afr, CSW [a])	-	-	✓	-	-	-	MacDonald *et al.* 2000
Haplotype	A1-B8-DR3	-	-	-	-	-	✓	Steel *et al.* 1988, McNeill *et al.* 1996, Kaslow *et al.* 1990
	A1-Cw7-B8, Cw4-B35	-	-	-	-	-	✓	Steel *et al.* 1988, Kaslow *et al.* 1990, Carrington *et al.* 1999, Keet *et al.* 1999
	A1-Cw7-B8-DR3-DQ2	-	-	-	-	-	✓	Steel *et al.* 1988, Kaslow *et al.* 1990, Carrington *et al.* 1999, Keet *et al.* 1999

LTNP, long-term non-progression; hom, homozygous ; het, heterozygous; Afr, African; Cau, Caucasian; CSW, commercial sex workers; [a] Thailand; [b] Kenia

Since mechanisms involving both the immune system and iron-related metabolic pathways have been implicated in impaired antiviral responses, candidate genes considered to be of importance in this context were selected for study. These included the iron-related genes *SLC11A* and *HFE*, as well as the *CD45* (*PTPRC*) gene involved in host immunity. Although these genes have previously been investigated in relation to HIV disease, the findings obtained have not yet been confirmed in follow-up studies. In fact, the *HFE* gene has only been studied in a single HIV-infected individual, whilst the information provided for the two other genes is mainly limited to Caucasian populations. This study represents the first to investigate genetic variations of these candidate genes for their involvement in HIV/AIDS in predominantly Black and Coloured South African populations. The selected genes were studied to determine possible independent effects in relation to HIV disease, and were also evaluated together with HLA-B27 to assess the likelihood that a combination of any of the variants studied could explain diverse clinical outcomes in patient subgroups.

SLC11A1

Variant alleles of the functional 5'[GT]n polymorphism present in the regulatory promoter region of the *SLC11A1* gene, have previously been associated with susceptibility to autoimmune disorders and infectious disease, in addition to regulating gene expression (Blackwell and Searle 1999). Allele 2 [$t(gt)_5ac(gt)_5ac(gt)_{10}ggcaga(g)_6$] is associated with susceptibility to infectious disease (Bellamy *et al.* 1998, Marquet *et al.* 1999), whereas allele 3 [$t(gt)_5ac(gt)_5ac(gt)_9ggcaga(g)_6$] has been associated with susceptibility to autoimmune disorders (Shaw *et al.* 1996, Kotze *et al.* 2001). The SLC11A1 protein is predominantly expressed in immune cells and functions in iron homeostasis (Atkinson and Barton 1998). Differential expression of SLC11A1 due to the functional 5'[GT]n polymorphism may therefore yield altered iron homeostasis, which could contribute to altered susceptibility to immune disorders.

Although allelic variation in the *SC11A1* gene has previously been implicated in HIV/AIDS susceptibility (Marquet *et al.* 1999), genotype and allele frequencies did not differ significantly between HIV-seropositive and HIV-seronegative individuals in the South African population. Analysis of a 9 base-pair deletion of exon 2, previously associated with modified risk to HIV-1 infection in Africans (Ramaley *et al.* 2000) also did not provide evidence for association with HIV-1 disease in our study population. These results suggested that allelic variation of *SLC11A1* could be population-specific.

Furthermore, phenotypic effects of functional *SLC11A1* gene variants in HIV-1 pathogenesis appear to be mediated by complex gene-environment interaction, in the context of the genetic background of an individual.

HFE

It has been speculated that molecules of the major histocompatibility complex (MHC) may play a role in iron metabolism (Salter-Cid *et al.* 2000). The non-classical MHC haemochromatosis (*HFE*) gene, known for its significant role in iron loading (Feder *et al.* 1996), was recently studied in the context of HIV infection. Although only described in an isolated case, the presence of the iron-loading mutation C282Y was reported in relation to HIV-1 disease progression (Nielsen *et al.* 1999). Molecular investigation of the *HFE* gene was therefore deemed important in a population-based study, to investigate the potential involvement of this gene in HIV/AIDS. The mutations identified in exons 2 and 4 of the *HFE* gene (H63D, C282Y, S65C and V53M) were rare in the study population, which was in accordance with a previous finding in Black Africans recruited from the general population (De Villiers *et al.* 1999). However, genotype-phenotype correlation studies indicated a possible direct effect of HIV-1 infection on iron metabolism. The African-specific and apparently non-functional intron 3 polymorphism IVS3-48C→G (Beutler and Gelbart 2000), showed a significant association with reduced haemoglobin levels in HIV-seropositive (P=0.004) and HIV-seronegative (P=0.02) Black Africans. The sample size was similar in both these groups, which further emphasised the more pronounced effect of the mutation on haemoglobin levels upon HIV infection. Although a linkage disequilibrium effect cannot be excluded, it became clear that genetic variation might differentially affect the degree of an acute phase response that could most likely alter disease susceptibility or clinical outcome.

CD45

Molecules directly involved in immune activation, for example CD45, may contribute to the multifactorial nature of HIV/AIDS susceptibility (Tchilian *et al.* 2001a). Activation of lymphocytes is facilitated by CD45 upon antigen stimulation (Byth et al. 1996, Thomas and Brown 1999). However, HIV-1 infected individuals show several immunological alterations, some of which may be attributed to disease progression, including increased susceptibility to cell death and a significant reduction in the number of CD4+ T-lymphocytes (Gougeon and Montagnier 1993, Hellerstein *et al.* 1999).

A lack of CD45 as a consequence of the lack of circulating T-cells and impaired immune responses often results in immunodeficiency. It is also known that CD45 is differentially expressed on different cell types, dependent on the state of activation and differentiation of haemopoietic cells (Hathcock *et al.* 1992, Craig *et al.* 1994). Genetic variation of the *CD45* gene could therefore alter CD45 function, which may contribute to immunodeficiency or impaired anti-viral responses (Tchilian *et al.* 2001a,b). Functional mutations of *CD45*, for example 77C→G, have been identified for their involvement in differential expression of CD45, and may be associated with a modified risk for HIV-1 infection (Jacobsen *et al.* 2000, 2002, Tchilian *et al.* 2001a). The *CD45* mutation 77C→G was however shown to be absent in African populations that were sampled (Tchilian *et al.* 2002). Identification of functional mutations of *CD45* in relation to HIV-1 disease in different populations may therefore be important in understanding the involvement of genetic variation of this molecule in immunodeficiency.

Investigation of the *CD45* gene in this study identified two African-specific polymorphisms, 32A→G and 54A→G, in the exonic splicing silencer element 1 (ESS-1) located in exon 4. Although modified risk to HIV-1 infection was not demonstrated, the silent 32A→G mutation appears to predominate in slow disease progressors in the HIV-seropositive individuals studied, while the missense mutation 54A→G (T18A) was predicted to affect differential expression of CD45. Functional studies of 32A→G by flow cytometry (FACs) analysis showed a reduced number of CD45RA- CD45R0+ cells in the CD8+ subset in two HIV-seropositive individuals (9.8% and 16.6%) selected for further study, compared with a control without the mutation (40.6%), which may be due to differences in disease status. Although numbers were small, assessment of the clinical data of these individuals and HIV-seropositive individuals with WHO Class I disease status and presenting with 32A→G (5/14, 35.7%), suggested a possible protective effect in disease progression, compared with 22 rapid progressing HIV-seropositive individuals without the mutation.

HLA-B27 and combined genotypes

In view of the multitude of environmental factors that contribute to HIV-1 disease outcome, compounded by the differing effects of individual or combinations of genetic variations, alleles of the highly polymorphic MHC system are crucial in immune activation in response to infectious agents. HLA molecules function by binding and presenting antigens to class I-restricted CD8+ cytotoxic T-lymphocytes (CTLs), thereby recruiting other cells involved in the immune response.

The polymorphic nature of HLA determines expression of CTL epitopes on the surface of infected cells, and HLA alleles encoded by this system are known to differ significantly between geographical populations. However, certain alleles have probably been maintained within individual population groups as a consequence of natural selection, and may be responsible for protection against certain infections. Although combinations of certain HLA alleles with other functional disease-associated mutations, for example CCR5Δ32 and CCR2-64I, have been shown to significantly modulate the course of HIV-1 infection, these alleles contribute independently to HIV-1 infection or disease progression. Class I HLA type B27 has also been implicated independently with slow disease progression (Hendel *et al.* 1999), and can also function as part of the protective mechanism when present on the same haplotype as other HLA alleles (Magierowska *et al.* 1999).

The potential protective effect of HLA type B27 was therefore analysed in our study population, in relation to possible combined genotypes determined for the above-mentioned candidate susceptibility genes in HIV-seropositive individuals. This was done to identify genetic markers that might, independently or in combination with other mutations, explain differences in the rate of disease progression in the population investigated. From a selected group of 36 HIV-seropositive individuals with known progression status and attending a clinic for the management of HIV-disease, two individuals who remained relatively healthy after more than 5 years of HIV-1 infection tested positive for HLA-B27. Absence of this allele in the Black Africans studied was in line with the previous association of HLA-B27 with protection from HIV-1 disease progression in individuals of Caucasian origin. Since no predominant co-existence with any of the mutations screened for in the *SLC11A1, HFE*, *CD45* and *CCR5* genes were detected in this study group, it was concluded that HLA-B27 might independently contribute to the clinical features observed in these individuals.

An attempt was made to establish whether gene-gene interactions might have contributed to specific disease outcomes in the study population. Although a limited number of HIV-seropositive individuals presented with combinations of genetic mutations identified during this study, comparison of HIV-seropositive individuals with known progression status did not indicate a distinct possibility for gene-gene interaction with regard to the gene variations studied.

However, in view of the diversity of host genetic mechanisms previously described for their involvement in HIV/AIDS, these mutations may possibly in combination with other mutations, have a significant impact on disease outcome in the South African population.

Future Prospects

In view of the data obtained by this study, the following aspects were identified for further investigation.

Since the findings are not in agreement with the proposed HIV-1 protective role for allele 2 of the *SLC11A1* gene 5'-[GT]n promoter polymorphism, further studies are warranted to clarify the effect of this polymorphism in HIV/AIDS susceptibility. It seems possible that the differential allelic effects of this polymorphism on transcriptional activity observed in the presence and absence of the nearby polymorphism -237C→T (M. Zaahl PhD study, submitted), may affect the outcome of case-control studies. When the –237 t-allele occurs in combination with allele 3 of 5'[GT]n, expression of SLC11A1 is down-regulated, similar to the allelic effect associated with allele 2. Reassessment of allelic associations based on expression profiles as demonstrated by these *in vitro* studies involving different *SLC11A1* promoter constructs, therefore needs to be performed in our study population. It seems likely that the inability to replicate disease associations previously reported in relation to *SLC11A1* in different populations may be due to a linkage disequilibrium effect, and therefore analysis of haplotypes rather than single mutations could be more appropriate.

Indications that the intronic polymorphism IVS3-48c→g in the *HFE* gene, which correlates significantly with altered haemoglobin levels, may occur in linkage disequilibrium with a functional mutation in a nearby gene raised an important question. Is it possible that genetic variation in another gene on chromosome 6 results in differential immune responses upon HIV-infection? The association between the presence of this mutation and reduced haemoglobin levels appears to be more pronounced in HIV-seropositive individuals than in HIV-seronegative individuals. This finding provides support for the notion that genetic variation in any of the host molecules involved in response to infection could influence HIV-infectivity and consequently clinical outcome in affected individuals. A direct role of the *HFE* gene in this process cannot be excluded, since the HFE protein has both structural and potential functional similarities with the immune-associated molecules encoded by the MHC genetic region.

Therefore, haplotype studies in conjunction with mutation studies, may prove useful to investigate the likelihood that genetic variation in a nearby gene may be responsible for the phenotypic effects observed in this study, in relation to the *HFE* mutation IVS3-48c→g.

The genetic alterations identified in the *CD45* gene appear to affect expression of the resulting protein, although further studies are required to confirm these findings. Due to the fact that HIV-1 infection and disease status may influence expression of CD45, mutation 32A→G needs to be screened for in a population of healthy individuals who are contactable for follow-up investigations, once this mutation is detected. Due to ethical constraints, the control individuals testing positive for mutations in the *CD45* gene in this study, could not be contacted to obtain tissue samples for functional studies. Furthermore, screening of additional HIV-seropositive individuals with diverse clinical features for mutation 32A→G, is of great importance to confirm or exclude the possibility that this mutation protects against disease progression following HIV-1 infection. Confirmation of the proposed role of genetic variation in the *CD45* gene with the rate of progression to AIDS may possibly provide a novel molecular target for immune intervention, aimed at slowing disease progression in affected individuals.

Other genes of interest for future studies in the context of HIV/AIDS are those involved in drug response, in light of the clinical impact that pharmacogenetic testing may have on patient management. These include genes encoding drug transporter proteins, which may also alter the risk of immune disorders and infectious disease (Lee *et al.* 2000). Genetic variation of the *MDR1* gene, encoding the multidrug-resistant transporter MDR1/P-glycoprotein, possibly contributes to HIV-1 susceptibility (Lee *et al.* 2000). Over-expression of MDR1 has been associated with reduced HIV-1 viral production, and the *MDR1* genotype was suggested to contribute to this observation. Furthermore, since antiretroviral drugs are substrates for MDR1, it is likely that the therapeutic outcome could also depend on the *MDR1* genotype (reviewed by Sakaeda *et al.* 2002). MDR1 may be fundamental in immune function (Adams *et al.* 1998, Johnstone *et al.* 2000) as it is expressed on cells of the innate immune system (Chong *et al.* 1993, Klimecki *et al.* 1994, Randolph *et al.* 1998). Expression of MDR1 is however affected by genetic variation of the *MDR1* gene. A silent mutation at position 3435 in exon 26, C3435T, appears to be important in both MDR1 expression and pharmacokinetics, and occurs in significantly higher frequencies in Africans compared with other populations (Ameyaw *et al.* 2001, Schaeffeler *et al.* 2001).

Although the function of this mutation has not been clearly defined, linkage with another SNP may possibly affect the function of this molecule. The possibility of treatment and disease diagnosis based on *MDR1* genotyping was highlighted by the observation that MDR1 may be important in susceptibility to for example HIV-1 infection, in addition to being vital in drug delivery. However, since the expression pattern of MDR1 appears to differ between populations and in different cell types, intensive study of this gene is required before any attempts can be made to use genetic information for the design of gene-based therapeutic agents against HIV/AIDS.

Limitations of this study included the relatively small sample size of the study population in relation to the complexity of the condition studied, as well as a lack of clinical information for the majority of individuals subjected to mutation screening. Furthermore, although it was shown that the *HFE* gene IVS3-48c→g mutation is associated with reduced haemoglobin levels, future studies will require assessment of iron status in all individuals included, especially those with known disease status. Other limiting factors that need to be considered are that of cost and time. For HLA genotyping only HLA-B27 was analysed, since commercially available screening kits that can be used for complete genotyping and for haplotype analysis of the HLA region, are very expensive.

During this study it became clear that both host genetic factors and immunological responses might contribute significantly to HIV-1 disease susceptibility. The complexity of disease pathogenesis may furthermore be compounded by nutritional insufficiencies, environmental influences and viral characteristics. Investigation of all these factors in large population samples would therefore be essential to provide conclusive answers to the many questions raised during this study. Given the absence of therapeutic or preventive vaccines at present, findings of relevant genetic differences in or between populations may provide the basis for future development of intervention-based HIV therapeutic or prevention strategies, focussing on the interaction of HIV-1 with host factors.

This study has contributed to a better understanding of the influence of host genetics on susceptibility to HIV-1 disease in the South African population. The effect of HIV-1 on iron metabolism in the context of immune response was demonstrated for the first time at the DNA level, as indicated by the differences in haemoglobin levels observed in relation to the presence of the *HFE* mutation.

Although no direct support was provided for a multifactorial basis for genetic susceptibility to HIV/AIDS, sufficient data in the literature suggests that combined effects of different genes determine the clinical outcome in some individuals, while single genetic factors may be sufficient to produce diverse clinical outcomes in others, in conjunction with relevant environmental factors.

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APPENDICES

APPENDICES: DETAILED EXPERIMENTAL PROCEDURES AND PROTOCOLS

1. *Genomic DNA Extraction*

Long term storage, high yield – long salt-extraction procedure

Reference: SA Miller, DD Dykes, HF Polesky. A simple salting out procedure for extracting DNA from human nucleated cells. 1988 Nucleic Acid Research 16:1215

Day 1:

1. Place EDTA blood ca. 10 ml in 50 ml Falcon tube
2. Add 30 ml cold lysis buffer. Place 15-20 min on ice with gentle agitation every 5 min.
3. Centrifuge 10 min at 1500 rpm
4. Carefully decant supernatant and keep pellet
5. Rinse with 10-15 ml Phosphate-buffered saline (PBS)
6. Centrifuge 10 min at 1500 rpm
7. Carefully decant supernatant and keep pellet
8. Dissolve pellet in: 3 ml Nucleus Lysis Buffer
   30 µl Proteinase K
   300 µl 10% SDS

   Mix well and incubate overnight at 55 °C

Day 2:

1. Add 1ml 6M NaCl and shake vigorously for 1 minute
2. Centrifuge 15 min at 2500 rpm
3. Transfer supernatant to new 50 ml Falcon tube (taking care not to transfer foam and pellet)
4. Add 2 volumes ice cold 100% Ethanol and precipitate DNA
5. Wash DNA in 70% Ethanol (room temp.)
6. Air dry for 20-30 min
7. Dissolve DNA in 200-800 µl sterile dH_2O or TE buffer, depending on pellet size
8. Store tube at 4 °C

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Genomic DNA Extraction (cont.)

Buffer Solutions: DNA Extraction

		Per	1000 ml	5000 ml
1. *Cold Lysis Buffer*				
0.01M Tris-Cl	Mr=121.1		1.211 g	6.055 g
0.4M NaCl	Mr=58.44		23.4 g	117.0 g
0.002M EDTA	Mr=292.2		0.6 g	3.0 g
Set pH8.2 with concentrated HCl				
2. *Nucleus Lysis Buffer*				
0.155M NH_4Cl	Mr=53.49		8.3 g	
0.01M $KHCO_3$	Mr=100.12		1.1 g	
0.001M EDTA	Mr=292.9		0.03 g	

3. *PBS* (phosphate-buffered saline) — Dissolve 10 pellets in 1 litre dH_2O

4. *Proteinase K* — Add 10 ml sterile dH_2O per 10mg vial and dissolve at room temperature. Decant in 1.5 ml Eppendorf tubes and freeze (-20 °C) for long term preservation

5. *10% SDS* — Dissolve 10 g SDS in 100 ml dH_2O

6. *6M NaCl* — Add 350 g NaCl to 1 litre dH_2O and mix well. Allow salt to settle out prior to use

...../(cont.)

Precipitation of DNA

Precipitation:

1. Add 1 volume Phenol-chlorophorm to DNA
2. Centrifuge ~10 min
3. Keep top phase
4. Add. 1 volume chlorophorm
5. Centrifuge ~10 min
6. Keep top phase
7. Add 2 volumes ice-cold 100% Ethanol, stand at room temperature for 30 min
8. Centrifuge 1 minute @ RT
9. Discard supernatant and air-dry pellet
10. Dissolve in dH_2O or TE Buffer

Ethanol Precipitation:

1. Add $^1/_{10}$ volume 3M Sodium Acetate
2. Put in freezer for 20 minutes
4. Centrifuge 10 minutes
5. Discard supernatant
6. Wash with 2 volumes 70% Ethanol (room temperature)
7. Vortex and leave 5 minutes @ RT
8. Centrifuge 1 minute @ RT
9. Discard supernatant and air-dry pellet 5-10 minutes at room temperature
10. Dissolve in dH_2O or TE Buffer (pH7.4)

2. Gel Electrophoresis

PAA (poly-acrylamide) Gel Matrices:

Mix all ingredients, except TEMED. Add TEMED immediately before pouring, mix well and pour immediately (takes about 6 minutes to polymerise at room temperature)

2.1. *Combined Heteroduplex-SSCP (Hex-SSCP) analysis*:

Volumes are 60 ml per gel (Hex-SSCP analysis slab gels), although the required volume is 40 ml per 18cm x 32cm (0.75mm thickness), in case of leakage

A. 10% Urea gel

7.5% Urea	4.5 g
5 x TBE buffer	18 ml
dH_2O	26 ml
1%C (40% stock)	15 ml
10% Ammonium persulphate (APS - freshly prepared)	800 µl
TEMED	80 µl

Use 1.5 x TBE running buffer

B. 10% Glycerol gel

Glycerol	3 ml
5 x TBE buffer	6 ml
dH_2O	36 ml
1%C (40% stock)	15 ml
10% Ammonium persulphate (APS - freshly prepared)	800 µl
TEMED	80 µl

Use 0.5 x TBE running buffer

2.2. *Restriction Enzyme analysis*: Volumes - 20 ml

A. 12% PAA - 5% C gel

5 x TBE buffer	1 ml
dH_2O	4 ml
5%C (40% stock) solution	6 ml
10% Ammonium persulphate (APS - freshly prepared)	160 µl
TEMED	80 µl

Use 1.5 x TBE running buffer

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Restriction Enzyme analysis (cont.)

B. 12% PAA - 3.4% C gel

5 x TBE buffer	4 ml
dH_2O	9 ml
3.4%C (30% stock) solution	7 ml
10% Ammonium persulphate (APS - freshly prepared)	160 µl
TEMED	80 µl

Use 1.5 x TBE running buffer

PAA Gel matrix stock solutions:

1. *Hex-SSCP* :

1% Cross-link (1% C), 40% Stock

	per 100ml
Acrylamide	39.6 g
Bis-acrylamide	0.4 g

Mix well and keep refrigerated until needed

2. *Restriction Enzyme analysis and general*:

A. 3.4% Cross-link (3.4% C), 30% Stock

	per 100ml
Acrylamide	29 g
Bis-acrylamide	1 g

Mix well and refrigerate until needed

B. 5% Crosslink (5% C), 40% stock

	per 100ml
Acrylamide	38 g
Bisacrylamide	2 g

...../(cont.)

Buffers:

Electrophoresis:

5x TBE (Stock): Storage - Room Temperature

	Mr	per 1000 ml
0.445M Tris·HCl	121.1 g/l	54.5 g
0.445M Boric Acid	61.83 g/mol	27.5 g
0.11M EDTA	292.2 g/l	2.9 g

(pH ~8.0)

Gel loading:

Gel Loading Buffer: Storage - Room Temperature

0.25% Bromo Phenol Blue (w/v)
0.25% Xylene Cyanol (w/v)
15% Ficoll (Type 400) (w/v)
in dH_2O

Denaturing Gel Loading Buffer: Storage - Room Temperature

	Mr	per 200 ml
50 mM Tris·HCl	121.1 g/l	121.10 mg
1 mM EDTA	292.2 g/l	7.44 mg
0.1% Bromophenol Blue	w/v	200 mg
0.1% Xylene Cyanol	w/v	200 mg
80% Formamide (deionized)	v/v	160 ml

3. *Flow Cytometry (FACS) Analysis of CD45 mutant cells*:

1. Isolation of peripheral blood mononuclear cells:

Venous blood was collected in heparinised syringes via 19G x 1 inch needles (Terumo Europe, Belgium), diluted in an equal volume of phosphate buffered saline a (PBSa) and slowly layered onto Ficoll Paque (Amersham Pharmacia, UK) in 50ml Falcon tubes. Blood was centrifuged at 500 x g, 30 mins at room temperature (RT) without brake. Cells were removed from the interface and washed twice in RPMI 1640 10% Human serum (R10H) or sterile PBSa at 350-400 x g and resuspended at the required concentration.

2. Cell viability test:

Cell viability was tested using the trypan blue exclusion method. Cells were diluted in an equal volume of trypan blue solution (Sigma, UK) and live cells, determined by exclusion of trypan blue, were counted on a haemocytometer (improved Neubauer chamber) viewed under a light microscope (Leica). The number of cells in the suspension is calculated by the formula:

No. viable cells in 25 squares x 2 (dilution factor) x 10^4 = No. cells per ml

3. Cyropreservation of cells

Excess cells were frozen in 90% foetal calf serum (FCS)(PAA Laboratories, Austria), 10% dimethyl sulphoxide (DMSO) (Sigma, UK) at –80ºC overnight in a polystyrene box then transferred to liquid nitrogen for long term storage.

4. Flow cytometric analysis

Flow cytometric analysis of CD45 variant splicing was performed as previously described (ref). Briefly, 2×10^5 PBMC were stained with either APC-conjugated CD4 (S3.3, Caltag, Silverstone, UK) or CD8-APC (clone RPA/T8, Pharmingen, San Diego, CA) along with FITC-conjugated CD45RA (clone HI10, Pharmingen) and PE-conjugated CD45R0 (clone UCHL1, Pharmingen) mAbs in a single step at 4ºC for 20 minutes and washed with PBS, containing 0.2% BSA. Isotype matched mAbs were used as controls. 10,000 events per sample were collected on a FACSCalibur (Becton Dickinson, Mountain View, CA) and analysed using WinMDI software.

Reference: Tchilian EZ, Wallace DL, Imami N *et al.* The exon A (C77G) mutation is a common cause of abnormal CD45 splicing in humans. 2001 J Immunol 166:6144-148.

4. *Iron Parameters*: Reference values for analyses of volunteer isolates recruited from the Northwest Province

Parameter	Reference value
Serum Iron	10 - 30 μmol/L
TIBC	55 +- 5 μmol/L
TS%	35+- 5%
Ferritin	>8.5 μmol/L
Hb	*females* : 12-15 g/dL *males* : 14-17 g/dL

TIBC, total iron binding capacity; TS, serum transferrin; Hb, haemoglobin

Reference: Mahan KL, Escott-Stump SE. 2000. Krause's Food, nutrition & diet therapy. 10th ed. W.B. Saunders, London p 1194.

5. *Selection criteria for Xhosa Control samples from the University of Transkei (UNITRA)*:

Control subjects were randomly selected from volunteers who responded to the advertisement:

Healthy people, men or women between the ages of 18 - 45 with no history of TB (now or before), diabetes, hypertension and psychiatric disorders.

Informed consent was obtained from all volunteers who participated in the study.

HIV status was assessed first by a screening test and positives were confirmed using the confirmatory test.

1. *Screening* : Primary screening of blood sample was done by Enzymun-Test, HIV combi (Roche Immunodiagnostics cat. No. 1877631)

   Test principle: ELISA/2-step sandwich assay, aided by Streptavidin technology

2. *Confirmation*: Positive samples were confirmed, using IMX HIV-1/HIV-2 111 plus (Abbot Diagnostic division)

   Test principle: Microparticle Enzyme Immuno assay (MEIA) technology

6. *Modified CDC Classification system of HIV infection* (*similar to WHO classification*):

GROUP	CRITERIA
1 Initial infection	Patients in this group may be designated as *symptomatic seroconversion* or *asymptomatic seroconversion*. Symptomatic infection may include a mononucleosis-like syndrome, aseptic meningitis, rash, musculoskeletal complaints, and haematological abnormalities as well as other clinical and laboratory findings. Asymptomatic infection may occur with or without haematological abnormalities.
2 Chronic asymptomatic infection	Patients in this group may be designated as having normal laboratory evaluation, specified laboratory abnormalities, or laboratory evaluation pending or incomplete. Laboratory abnormalities associated with HIV infection include anaemia, leukopenia, lymphopenia, decreased T-helper lymphocyte count, thrombocytopenia, hypergammaglobinemia, and cutaneous anergy.
3 Persistent generalized lymphadenopathy	Patients in this group may be designated on the basis of laboratory evaluation in the same manner as those in Group 2.
4 *Other diseases*:	Medical evaluation must exclude the presence of other intercurrent illnesses that could explain the symptoms.
Subgroup 4 A *Constitutional disease*	Patients in this group may be designated as having more than 1 month involuntary weight loss greater than 10% of baseline body weight, diarrhea lasting more than 1 month, or any combination of these.
Subgroup 4 B *Neurologic disease*	*Category 1*: Central nervous system disorders, including a) dementia, b) cute atypical meningitis (occurring after infection), and c) myelopathy. *Category 2*: Peripheral nervous system disorders, including a) painful sensory neuropathy, and b) inflammatory demyelinating polyneuropathy.
Subgroup 4 C *Secondary infectious diseases*	*Category 1*: Patients in this group may be designated as having one or more of the following: *Pneumocystis carinii* pneumonia, chronic cryptosporidiosis, toxoplasmosis, extraintestinal strongyloidiasis, isosporiasis, candidiasis (esophageal, bronchial or pulmonary), cryptococcosis, disseminated histoplasmosis, mycobacterial infection with *M.* avium complex or *M. kansasii*, disseminated cytomegalovirus infection, chronic mucocutaneous or disseminated herpes simplex virus infection, and progressive multifocal leukoencephalopathy. *Category 2*: Patients in this group may be designated as having one or more of the following: oral hairy leukoplakia, multidermatomal herpes zoster, recurrent *Salmonella* bacteremia, nocardiosis, tuberculosis, or oral candidiasis (thrush).
Subgroup 4 D *Secondary cancers*	Patients in this group may be designated as having one of the following: Kaposi's sarcoma, non-Hodgkin's lymphoma (small, noncleaved lymphoma or immunoblastic sarcoma), or primary lymphoma of the brain.
Subgroup 4 E *Other conditions*	Includes patients with clinical findings or diseases not classifiable above, that may be attributed to HIV infection and/or that may be indicative of a defect in cell-mediated immunity. Patients in this group may be designated on the basis of the types of clinical findings or diseases diagnosed, e.g. chronic lymphoid interstitial pneumonitis.

Reference: Michael NL, Burke DS. Natural Human Immunodeficiency Virus Infection. 1991 Dermatol Clin 9:429-44.