

**FACTORS AFFECTING HETEROSEXUAL
HIV-1 TRANSMISSION**

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DECLARATION

I declare that all the work and composition of this thesis was carried out by myself, unless otherwise stated.

Sarah Lockett
September 1998

DEDICATION

I dedicate this thesis to the memory of my grandmother, Doris Adamson, for her love,
generous spirit and belief in me.

Also to my parents, Susan and Douglas Lockett, for their unwavering support and
encouragement in all that I have done.

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ABBREVIATIONS

$\Delta 32$	32 base pair deletion in the <i>CCR-5</i> gene
$^{\circ}\text{C}$	degrees Celsius
^{51}Cr	γ emitting radioactive isotope of chromium
64I	valine to isoleucine amino acid change at position 64 of CCR-2
ADCC	antibody dependant cell cytotoxicity
AGM	African Green Monkey
AIDS	acquired immunodeficiency syndrome
APC	antigen presenting cell
APL	altered peptide ligand
ARI	anal receptive intercourse
AZT	azidodeoxythymidine
BCL	B cell line
BD	Becton Dickinson
bp	base pair
BSA	bovine serum albumin
CAF	CD8 cell antiviral factor
CCR	CC chemokine receptor
cDNA	complementary deoxyribonucleic acid
CI	confidence intervals
CMI	cell mediated immunity
CMV	cytomegalovirus
cpm	counts per minute
CTL	cytotoxic T lymphocyte
CXCR	CXC chemokine receptor
DC	dendritic cell
DCG	DC gay
DEPC	diethyl procarbamine
dH ₂ O	distilled water
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
ds	double stranded
DTH	delayed type hypersensitivity
DTT	dithiothreitol
EBV	Epstein Barr virus
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme linked immunosorbant assay
ER	endoplasmic reticulum
EU	exposed uninfected
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
gp	glycoprotein
GuSCN	guanidinium thiocyanate
HGDS	hemophila growth and development study
HIV	human immunodeficiency virus
HIV ⁺	seropositive for HIV

HLA	human leucocyte antigen
HRP	horseradish peroxidase
HSV	herpes simplex virus
HTLV	human T cell leukaemia virus
ICAM	intracellular adhesion molecule
IDU	injecting drug user
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IP10	interferon inducible protein 10
kb	kilobase
kDa	kiloDalton
LC	langerhans' cell
LFA	leucocyte functional antigen
LPA	lymphoproliferation assay
LTNP	long term non-progressor
m.o.i.	multiplicity of infection
MACS	multicenter AIDS cohort study
MCP	monocyte chemoattractant protein
MHC	major histocompatibility complex
MIG	monokine inducible by interferon gamma
MIP	macrophage inflammatory protein
mRNA	messenger ribonucleic acid
M-tropic	macrophage tropic
NK	natural killer cell
NSI	non-syncytium inducing
NTRI	non-transmitting index
PAF	paraformaldehyde
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCD	programmed cell death
PCP	<i>Pneumocystis carinii</i> pneumonia
PCR	polymerase chain reaction
PE	phycoerythrin
PHA	phytohaemagglutinin
PND	principal neutralising domain
PPD	mycobacterium purified protein derivative
RANTES	reduced upon activation normal T cell expressed and secreted
RFLP	restriction fragment length polymorphisms
rHIV	recombinant HIV cocktail
RNA	ribonucleic acid
RPMI	Rose Park Memorial Institute
RRE	REV response element
RT	reverse transcriptase
rVV	recombinant vaccinia virus
SDF	stromal cell derived factor
SDS	sodium dodecyl sulphate
SI	syncytium inducing
SIV	simian immunodeficiency virus
SNBTS	Scottish National Blood Transfusion Service

ss	single stranded
STDs	sexually transmitted diseases
TAP	transporter associated protein
TAR	transactivation response element
TB	tuberculosis
TCA	trichloroacetic acid
TCID ₅₀	50%-tissue culture infectious dose
TCLA	T cell line adapted
TCR	T cell receptor
T _H	T helper cell
TMB	tetramethylbenzidine
TNF	tumour necrosis factor
TRI	transmitting index
tRNA	transfer ribonucleic acid
TT	tetanus toxoid
T-tropic	T cell tropic
UK	United Kingdom
US	United States
vRNA	viral ribonucleic acid
WT	wild type

ABSTRACT

Heterosexual transmission of human immunodeficiency virus (HIV) is increasing in many Western societies and is the major mode of transmission world-wide. Knowledge of factors which protect individuals who remain uninfected after exposure to HIV (exposed uninfecteds, (EUs)), aid our understanding into the mechanisms of transmission and hence, help prevent further spread. The aim of this thesis was to investigate a broad variety of factors, in a cohort of EU heterosexual partners of HIV⁺ individuals (indexes), which may affect heterosexual transmission.

The immune function of the EUs was assessed and compared to normal controls, by monitoring proliferative responses to mitogen, recall and alloantigens and a combination of recombinant HIV proteins. Cytokine responses to these stimuli were also monitored. The EUs were also confirmed to be uninfected by polymerase chain reaction (PCR). The EUs had similar proliferative responses to controls for both the allogenic and recall antigens and showed a minor difference in the response to the mitogen, phytohaemagglutinin (PHA), which may reflect differences in the kinetics of the response. An increase in the amount of interferon- γ (IFN- γ) produced in response to alloantigen was seen in EUs compared to controls, which could potentially inhibit HIV replication. The proportion of lymphocytes expressing the MHC Class II protein, human leucocyte antigen-DR (HLA-DR), was also elevated in the EUs compared to controls and may reflect an overall increase in the activation status of the EUs' lymphocytes.

Genetic factors which were investigated included the HLA antigens and the recently reported mutations in the CC chemokine receptors (CCR), CCR-2 and CCR-5, utilised by certain strains of HIV as co-receptors for entry. The HLA allele DR5 was elevated in frequency in the EU cohort compared to population controls and to HIV⁺ individuals who were infected by heterosexual exposure. The DR6 allele was decreased in the EUs compared to population controls, but this was not confirmed as a risk factor for heterosexual infection, as no increase in frequency was seen in the HIV⁺ individuals. A significant difference was observed between the degree of HLA mis-match between the index and their partner, with couples discordant for HIV serostatus showing a higher degree of HLA mis-match than concordant couples ($P=0.02$). This suggests allogenic responses may be increased and protective in the EUs. No difference in the frequency of the 32 base pair deletion ($\Delta 32$) in the CCR-5 gene was seen in the EUs compared to heterosexually infected HIV⁺ individuals, or

population controls. In contrast, heterozygosity for a valine to isoleucine mutation at position 64 (64I) of CCR-2 was shown to be acting as a risk factor ($P=0.02$, $RR=1.6$) for HIV infection of females following heterosexual contact, as a higher frequency of 64I heterozygotes were observed in HIV⁺ than EU females. The 64I mutation may be mediating its effect by linkage disequilibrium with other mutations in the CCR genes, including those in the promoter regions of the CCR-5 gene. However, no differences were seen in the frequencies of several polymorphisms in the CCR-5 promoter region between the HIV⁺s and EUs.

In studies of a male index and his four female partners, two HIV⁺ and two EUs, it was investigated whether viral variation in the index could account for the lack of transmission in the later EU contacts. No major differences in the viral variants were seen later in the index's infection which would explain the differences in transmission. The first EU partner, was homozygous for the $\Delta 32$ mutation in the CCR-5 gene and her lymphocytes were shown to be infectible with T-tropic virus, but not M-tropic virus.

These studies of factors influencing susceptibility to heterosexual transmission suggest that the genetic background, immune response, as well as the disease status of the HIV⁺ partner may all influence transmission in a multifactorial way, indicating future studies of cohorts of this kind should incorporate a broad interdisciplinary approach.

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Chapter 1

Introduction

CHAPTER 1 - INTRODUCTION

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1.1. HISTORY

Even before the discovery of the human immunodeficiency virus (HIV), it was apparent that the disease, later named AIDS (acquired immunodeficiency syndrome), was intricately involved with the immune system. A group of previously healthy, homosexual men presented with Kaposi's sarcoma and *pneumocystis carinii* pneumonia (PCP)(Centers for Disease Control (1982a)), diseases previously restricted to the severely immunocompromised such as transplant recipients. Later, reports of these and other such illnesses: persistent cytomegalovirus (CMV) infection, persistent generalised lymphadenopathy and neoplasias, were identified in haemophiliacs, blood transfusion recipients, injecting drug users (IDUs), children born to HIV-infected mothers and heterosexual partners of infected individuals (Centers for Disease Control (1982b); Centers for Disease Control (1982c); Centers for Disease Control (1982d); Centers for Disease Control (1982e); Spira *et al.* (1984)). Such risk groups implied that sexual, vertical and blood borne transmission routes were all possible. As well as these AIDS defining illnesses, patients showed a massive decline in a subset of immune cells, namely the CD4⁺ lymphocytes, from normal adult ranges of 1000-2000 cells/mm³ to levels of <200.

The discovery of the causative agent soon followed (Barré-Sinoussi *et al.* (1983); Gallo *et al.* (1983); Levy *et al.* (1984)) and was shown to infect cells expressing the CD4 receptor, via an interaction with the viral surface glycoprotein, gp120 (Dalgleish *et al.* (1984); Klitzmann *et al.* (1984))(see Section 1.5).

1.2. CLINICAL MANIFESTATIONS

It is estimated that between 50-70% of individuals infected with HIV develop an acute, symptomatic primary HIV infection (reviewed in Tindall & Cooper (1991)). However, the 'mononucleosis like' symptoms are often ignored and ascribed to other causes. Clinical features last from 1 to 2 weeks and include: fever, lethargy, malaise, myalgias, headaches, retro-orbital pain, mucocutaneous ulceration, lymphadenopathy and a maculopapular rash. During this phase, virus and viral antigens are detectable in the bloodstream of the infected individual, viraemia and antigenemia respectively, and finally HIV-specific antibody is detectable. Screening for HIV-specific antibody is the primary test for an individual's infection status and if present is deemed seropositive, or HIV⁺.

Seroconversion is followed by an asymptomatic period, the length of which is variable depending upon the individual. Some subjects can progress rapidly to AIDS, so called rapid

progressors; others have been known to remain disease free for prolonged periods (Lifson *et al.* (1991)) and are termed long term non-progressors (LTNPs).

During progressive HIV infection, the extent and number of diseases suffered by an individual increases and this is used to define the onset of AIDS. Many of the diseases are normally rare in the general population, such as PCP and Kaposi's sarcoma, others are common infections, which rarely cause disease, e.g. CMV infections. A major concern in developing countries is the upsurge of *Mycobacterium tuberculosis* infection (TB) in HIV-infected individuals. Most of the diseases associated with the development of AIDS are caused by secondary infections and malignancies normally controlled by the host immune system and not caused by HIV infection *per se*. One important exception is HIV dementia (Navia, Jordan and Price (1986)) and another is cachexia (weight loss) and diarrhoea (Smith *et al.* (1992)). HIV disease is now staged according to both the diseases suffered and the CD4⁺ cell count of an individual (Centers for Disease Control (1992)).

1.3. TRANSMISSION

Since the start of the global epidemic until July 1996, approximately 28 million people have been infected with HIV (Expert Group of the Joint United Nations Programme of HIV/AIDS (1997)). The major mode of transmission has been unprotected heterosexual intercourse (approximately 70%). The enormity of this proportion is often surprising when one considers that much of the published work in HIV-infected individuals centres around homosexuals. This is a minority risk group globally, but became the first and primary cohorts in Western societies. However, even in the USA and Europe, heterosexual exposure is the most rapidly increasing risk factor for new infections (Balfe (1998)). The contribution of other modes of transmission globally are as follows: vertical transmission (mother-to-child) 8-10%, homosexual intercourse 5-10%, needle sharing amongst IDUs 5-10%, transfusion of blood and blood products 3-5% (Expert Group of the Joint United Nations Programme of HIV/AIDS (1997)).

1.3.1 Transmission by Blood and Blood Products

Prior to the screening of blood for anti-HIV antibodies, transfusion recipients and haemophiliacs were infected with contaminated blood (Centers for Disease Control (1982c); Donegan *et al.* (1990)), or products such as Factor VIII and IX (Bartz, Rogel and Emerman (1996)). Following the introduction of routine screening for blood donors, the risk of

transfusion acquired infection is now extremely rare; heat inactivation of Factor VIII and IX has virtually eliminated the transmission to haemophiliacs.

Needle-stick injuries and other occupational exposures of health care workers also carry a risk of infection with HIV (Marcus and the CDC Cooperative Needlestick Surveillance Group (1988)) and strict guidelines for the prevention of such transmissions have now been implemented (Centers for Disease Control (1989)).

IDUs are a further population at risk from blood borne HIV infection, due to the sharing of needles (Spira *et al.* (1984); Robertson *et al.* (1986)). Increased availability of clean needles and education has reduced the level of transmission in these cohorts; the number of newly acquired IDU infections in Scotland in 1986 was 242/351 (68.9%) compared with 50/165 (30.3%) in 1991 and only 29/173 (16.8%) in 1996 (Scottish Centre for Infection and Environmental Health (1997)).

1.3.2 Vertical Transmission

Transmission of HIV from an infected mother to her child can occur during pregnancy (Backé *et al.* (1993)), at birth (Goedert *et al.* (1991)), or even from breast feeding (van de Perre *et al.* (1991)) and is the predominant form of paediatric HIV infections. Caesarean section, as opposed to vaginal delivery, was shown to reduce the risk of transmission (European Collaborative Study (1992)) and recent trials administering the antiviral drug, azidodeoxythymidine (AZT), has further reduced vertical transmission (Sperling *et al.* (1996)), at least in Western societies. AZT is given during the last trimester of pregnancy and to the child during the first six weeks following birth, although, a short course of AZT has now been shown to be effective in Thailand too (Centers for Disease Control (1998); Morris (1998))

1.3.3 Sexual Transmission

AIDS was first noted in homosexuals (Centers for Disease Control (1982a)), but was also soon identified in heterosexually exposed individuals (Centers for Disease Control (1982b)). The risk of transmission varies from study to study depending upon the criteria of the investigation. In a Swedish cohort of homosexuals and heterosexuals, anal intercourse was found to be twice as infectious as vaginal (Giesecke *et al.* (1992)). This was determined by studying heterosexual and homosexual couples with a HIV⁺ partner and it was found that more homosexual couples were concordant for HIV serostatus, i.e. the sexual contact was

infected, than heterosexual. In general, this same trend is seen in all studies (reviewed in Royce *et al.* (1997)).

HIV is detectable in seminal cells and plasma (Zagury *et al.* (1984); Ho *et al.* (1984); Borzy, Connell and Kiessling (1988)) and HIV deoxyribonucleic acid (DNA) has also been detected in sperm cells (Bagasra *et al.* (1994)). For females, the glandular epithelium in the cervix has been shown to harbour HIV (Nuovo *et al.* (1993)) and both cervical and vaginal swabs yield HIV (Vogt *et al.* (1986); Wofsy *et al.* (1986); Hénin *et al.* (1993); Clemetson *et al.* (1993)); although the latter less readily (Clemetson *et al.* (1993)).

Susceptibility to infection obviously differs for homosexual and heterosexual intercourse and for male-to-female and female-to-male transmission. This is reflected in the risk levels associated with the respective mode of infection. As outlined earlier, sex between men carries a greater risk of infection than heterosexual exposure (Giesecke *et al.* (1992)), but amongst homosexuals, transmission from a seropositive insertive partner is more likely than from a receptive (Moss *et al.* (1987); Detels *et al.* (1989); Giesecke *et al.* (1992)). The same principal applies to heterosexuals: male-to-female transmission is more common than female-to-male (0.19-0.28 versus 0.06-0.12)(Johnson *et al.* (1989); European Study Group (1992); Giesecke *et al.* (1992)).

1.3.4 Epidemiology of Heterosexual HIV Transmission

The epidemiology of HIV and AIDS in the developed world is dramatically different from that of developing world, in particularly in sub-Saharan Africa. The epidemic in the United States (US) and later Europe, initially centred around male homosexuals, IV drug users, their sexual partners, haemophiliacs and small numbers of children. As homosexuals modified risky sexual behaviours, it was feared that the epidemic would then spread to the heterosexual population, including the partners of IDUs. Indeed, the sex ratio of AIDS cases in the US in 1992 was 8:1 male to female compared to 16:1 in the early 1980s (Hunt (1996)).

In direct contrast, the African epidemic has maintained an almost constant sex ratio of 1:1 since the early discovery of AIDS in Africa (Hunt (1996)). Here the major transmission route is heterosexual contact and due to the large numbers of infected women at child-bearing age, a considerable number of children are also HIV⁺ (Chin (1990)).

Many theories exist to try and address the differences in the risk and prevalence of HIV in heterosexuals in the developed versus the developing world. These fall into biological theories, such as that based on the natural history of the infection, and social theories,

encompassing cultural and economic factors (reviewed in (Hunt (1996))). However, no one theory has fully encompassed and accounted for all the known epidemiology. To try and highlight some of the factors involved in the different regions, I will describe three heterosexual epidemics; 1) Uganda, a well established and high prevalence region; 2) a European Collaborative Study, to highlight the factors involved in heterosexual transmission in the developed world; and 3) Thailand, a newly emerging and rapidly spreading heterosexual epidemic.

1.3.4.a Uganda

As already mentioned, heterosexual transmission accounts for a vast majority of HIV infections in Africa and HIV has already been shown to be the major cause of death in many African cities (De Cock *et al.* (1991); Gregson, Garnett and Anderson (1994)). Sexually transmitted diseases (STDs) are particularly important; the presence of both ulcerative (Greenblatt *et al.* (1988)) and non-ulcerative STDs (Laga *et al.* (1993)) have been shown to increase the risk of sexual HIV transmission. STDs are poorly controlled and treated in many African countries and this is thought to have accelerated the rapid spread of HIV. Indeed, in a study in rural Uganda, over 90% of HIV infections were attributed to STDs (Robinson *et al.* (1997)).

Reasons for the high incidence of STDs, later including HIV, may involve cultural factors. Polygamy is more widespread and accepted in African cultures, with often concurrent sexual networks existing both in urban and rural areas (Hudson (1993); Obbo (1993)). A high level of concurrent sexual mixing is perhaps an additional factor for the rapid spread of HIV, a factor also thought to be involved in the infection of highly promiscuous US homosexuals in the 1980s (Jacquez *et al.* (1994)).

It is also common for older men to have sex and form partnerships with younger women, often due to both matters of status and economic gain on the part of the young girl (Konde-Lule, Musagara and Musgrave (1993); Potts, Anderson and Boily (1991); Vos (1994)). It is thought that this is one of the main reasons for the high incidence of HIV infection in young females; women outnumbered men in the 15-24 age band, compared to men outnumbering women in the 30+ age groups in a Ugandan study from 1985-89 (Berkley *et al.* (1990)). Some young girls then form more stable relations with men nearer their own age and hence perpetuate the transmission network. The high incidence of HIV infection in young

women of child bearing age, also means that more children are likely to be subsequently infected through vertical transmission.

Studies in Uganda have shown a gradient of infection from towns, predominately along the trans-African highway, to smaller trading towns and finally to rural villages, with incidences of over 40% to approximately 25% to 8-9% respectively (Nunn *et al.* (1994); Wawer *et al.* (1991); Nunn *et al.* (1996)). It is thought that economic factors, including civil wars, caused the migration of male workers from agriculture to factory work (Hunt (1996)). Along with traders (Pickering *et al.* (1996)) and truck drivers (Carswell, Lloyd and Howells (1989)), it is thought that the migrant workers, away from home for long periods, frequented prostitutes and accounted for the high incidence in the urban areas. The returning migrant worker is then thought to have transferred the virus to more rural areas. However, studies on the prostitutes have highlighted a grading, with the low grade migrant workers frequenting different women from the more wealthy traders and truck drivers (Pickering *et al.* (1997)). This may help to explain the slow spread to the rural areas.

A further possible factor involved in transmission is differences in the infecting virus. Subtype B is known to predominate in Western populations, but a wide degree of subtypes are found in other areas such as Africa (Myers *et al.*(1995)) and may vary in their transmission rates.

Intervention has had mixed results, with some showing effective STD control (Laga *et al.* (1994)) and others reporting little change in behaviour, despite substantial knowledge of AIDS (Wawer *et al.* (1994)). It is clear that health education to reduce the numbers of sexual partners and promote the use of condoms is required to prevent further transmissions and control the epidemic.

1.3.4.b Thailand

The HIV epidemic in Thailand is of recent origin and two main risk groups have been identified: IDUs and female sex workers along with their heterosexual contacts (Weniger *et al.* (1991)). Studies of the molecular epidemiology of HIV in Thailand found distinct genotypes present in these two risk groups, suggesting they were not epidemiologically linked; genotype A predominated in the heterosexuals and genotype B in the IDUs (Ou *et al.* (1993)). Later, genotype A was defined as HIV-1 subtype E and genotype B as HIV-1 subtype B (Kunanusont *et al.* (1995)). The different subtypes in these different risk groups adds further debate to the theory that subtype differences are involved in the vast heterosexual spread of

HIV in Africa, compared to much slower spread in Western countries where subtype B predominates.

Epidemiological studies of the heterosexual contacts of HIV⁺ individuals, revealed that the transmission rate from an infected IDU by sexual contact was significantly lower than that from a heterosexually infected index (48% (27% excluding individuals with an additional IDU-risk) versus 69% respectively) and this was more pronounced when the subtype was considered (52% (26% excluding individuals with an additional IDU-risk) subtype B versus 70% subtype E) (Kunanusont *et al.* (1995)). This suggested that subtype E may be associated with a higher risk of heterosexual transmission. In support of this the estimated risk of female-to-male transmission in the Thai population has been reported to be 31-56 fold greater than estimates for the US population, where subtype B predominates (Mastro *et al.* (1994)).

It is possible that the distinct subtype distribution was one of a founder effect, i.e. the infection of sex workers and their contacts with subtype E, followed by rapid spread such that all HIV-susceptible individuals became infected with this strain and later exposure to other strains was then unimportant. A similar process could then have occurred in the IDUs with subtype B virus, but this whole theory needs further investigation (Mastro *et al.* (1997)). A similar pattern of subtype distinction has also been reported in South Africa, with male homosexuals predominately infected with subtype B virus and heterosexuals with subtype C (Williamson *et al.* (1995)).

A massive public health campaign was initiated in Thailand in 1989, known as the 100% condom campaign, to try and curtail the rapidly growing epidemic, but this only became nation-wide in 1992 (Rojanapithayakorn and Hanenberg (1996)). A decrease in reported visits to sex workers and increased condom usage followed (Rojanapithayakorn and Hanenberg (1996); Nelson *et al.* (1996)), probably associated with the subsequent fall in reports of STDs and the rate of newly acquired HIV infections reported in 1995 (Nelson *et al.* (1996)).

1.3.4.c Europe

The first national survey of sexual attitudes and lifestyles in the United Kingdom (UK) (Johnson *et al.* (1992)), based on a random selection of households, found that young people (16-34), in particular men, were the most likely of all age groups to have had recent multiple heterosexual partners. It was clear therefore that the public health message needed to be focused predominately at this higher risk group. Those with multiple partners were more likely to have attended an STD clinic, suggesting a higher incidence of 'at risk' exposure in

this group. This was reflected in the numbers who reported to have had an HIV test; 1 in 10, of those with 5 or more heterosexual partners in the past 5 years, sought a test for reasons other than blood donation, pregnancy, or insurance purposes. This rose to 1 in 4 among homosexuals.

From this national survey, it seemed that a proportion of individuals were at risk from heterosexually acquired infection and studies into the transmission rates were needed to assess how many of these individuals would subsequently be expected to be, or become HIV⁺. A European Collaborative Study was established from 9 countries across Europe (European Study Group (1992)). 563 stable couples (400 male and 156 female HIV⁺ index patients) were recruited where the contacts only risk factor was sexual contact with the index. A total of 12% of male contacts and 20% of female contacts were HIV⁺, suggesting male-to-female transmission is almost twice as frequent as female-to-male. Factors increasing the risk of male-to-female transmission were stage of disease, anal sex and advanced age (>45) of the female partner. The latter seems to differ from that seen in African populations, where younger females are more likely to be infected, but this may be due to a bias in HIV exposure in the African women (see Section 1.3.4.a). Risk factors for female-to-male transmission were identified as advanced stage of disease in the index and sex during menses.

The vast majority of the index patients had acquired their infection from IV drug use (65% - 66% female; 64% male) and were all probably infected with subtype B virus, highlighting an important difference from that seen in the other two mentioned heterosexual epidemics. However, further evidence would be required to confirm any theories regarding the effect of subtype and viral variation on heterosexual transmission.

1.3.5 Mode of Heterosexual Transmission

Transmission of HIV requires the infection of a susceptible cell, predominately cells expressing the CD4 receptor. These include: the CD4⁺ T lymphocytes, Langerhans' cells (LCs) and macrophages. Studies using monkeys have begun to clarify the complex and unresolved mechanism of sexual transmission. Intravaginal simian immunodeficiency virus (SIV) inoculation, in rhesus macaques, showed that the first cellular targets of infection were in the lamina propria of the cervicovaginal mucosa and appeared to be dendritic in nature (Spira *et al.* (1996)). *In vitro* studies involving dendritic cells (DCs) have yielded conflicting results (Pope *et al.* (1997b); Essex *et al.* (1997)); this may reflect the different modes of

isolation and source of the dendritic cell. Hence, the precise mechanism of infection of these cells is unclear, but a hypothetical model is outlined in Figure 1.1. Essentially, the infected DC migrates to the draining lymph node where it infects CD4⁺ T lymphocytes.

It is important to note that heterosexual and homosexual infection probably occur by different mechanisms. The mode of sexual contact is different, with anal sex carrying an increased risk of tissue damage and exposure to blood than vaginal intercourse. Primate studies also revealed that the oral, cervicovaginal and foreskin epithelia all contained LCs, but they were absent from the rectal and urethral epithelia (Hussain and Lehner (1995)). Furthermore, intestinal epithelial cells form tight junctions unlike that found in vaginal epithelium, which has intercellular gaps sufficient to allow viral transfer (Fantini *et al.* (1997)). Intestinal epithelial cells have also been shown to be susceptible to *in vitro* infection (Fantini *et al.* (1993)).

Factors known to effect the transmission of HIV heterosexually include: circumcision (Royce (1992)), probably due to the removal of the LC-rich foreskin (Hussain and Lehner (1995)); sex during menses (Lazzarin *et al.* (1991); European Study Group (1992)); reproductive tract infections, both non-ulcerative and ulcerative (Laga *et al.* (1993); Greenblatt *et al.* (1988)); and hormonal contraceptives (Clemetson *et al.* (1993)), shown in rhesus macaques to thin the vaginal epithelium and enhance SIV transmission (Marx *et al.* (1996)). The clinical stage of the infecting partner has also been shown to be important, with late stage infection showing an increased risk of transmission (Laga *et al.* (1989)). Models of early epidemics (Ahlgren, Gorny and Stein (1990)) and the high viral loads found during primary infection (Piatak *et al.* (1993)), suggest that acutely infected individuals also are more likely to transmit.

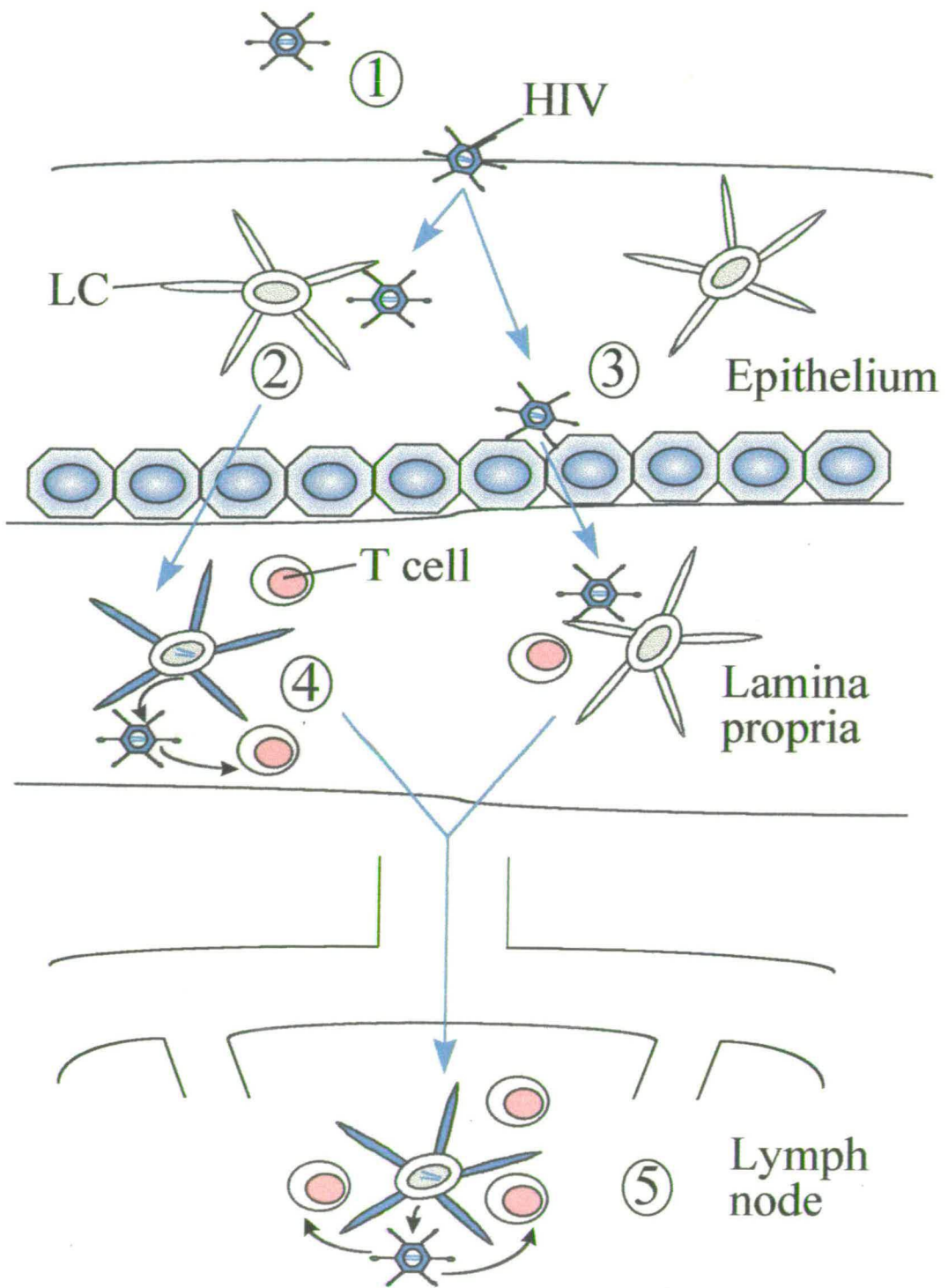
Another factor which may effect transmission is the subtype of the infecting virus and *in vitro* studies of HIV infection of DCs were performed to try and explain the contrasting epidemiology in areas such as Thailand (see Section 1.3.4.b). Soto-Ramirez *et al.* (1996) reported that primary isolates from Thai heterosexuals (subtype E) replicated more readily in DCs than those from US homosexuals (subtype B). Several groups contested this finding (Dittmar *et al.* (1997); Pope *et al.* (1997a); Pope *et al.* (1997b)), but in response Essex *et al.* (1997) suggested that two different mechanisms of DC infection can occur. The first involving DC/T cell conjugates as reported by Pope and colleagues (Dittmar *et al.* (1997); Pope *et al.* (1997b)) and the second involving the productive infection of DC alone (Soto-Ramirez *et al.* (1996); Blauvelt *et al.* (1997); Dittmar *et al.* (1997)). Essex also noted that Dittmar *et al.*

Figure 1.1.

Hypothetical model of HIV infection following heterosexual transmission.

Adapted from Zambruno *et al.* (1995).

1. Invading virus must first penetrate the mucosal epithelium, surviving mechanisms of host resistance (see section 1.6).
2. Virus can then infect Langerhans' cells (LC) present in the epithelium and these activated cells then migrate through the lamina propria.
3. Alternative route: the virus may diffuse through the vaginal epithelium (Fantini *et al.* (1997)) and infect the LC in the lamina propria, as seen in SIV-infected macaques (Spira *et al.* (1996)). It is also theoretically possible that HIV can infect the other CD4⁺ cells present here, namely the T cells and also macrophages, but this has yet to be confirmed.
4. The infected LCs could produce new virus to infect other cells in the lamina propria, such as CD4⁺ T cells. The LCs differentiate and migrate to the draining iliac lymph node.
5. The differentiate LCs, now dendritic cells (DCs), can then produce virus to infect the circulating naïve CD4⁺ T cells. DCs are also antigen presenting cells (APCs); APCs present antigens to T cells to initiate immune responses (see section 1.6.2), hence virus-specific immune responses could also be generated.



(1997) did show a difference in DC infection, with their non-subtype B viruses replicating better in DCs than subtype B ones.

1.4. THE VIRUS

HIV is a member of the Retroviridae, a family of viruses that infect a variety of different hosts. The genome of all retroviruses is composed of single stranded ribonucleic acid (ssRNA) and they share a similar mode of replication, virion structure and genomic organisation. The Retroviridae family is now divided into 7 distinct genera (Coffin (1992)); the Avian Leukosis-Sarcoma virus group (e.g. Rous sarcoma virus), mammalian C-type virus group (e.g. feline leukaemia virus), B-type virus group (e.g. mouse mammary tumour virus), D-type virus group (e.g. Mason-Pfizer monkey virus), spumavirus group (e.g. human foamy virus), lentivirus group (e.g. HIV) and human T cell leukaemia (HTLV)- bovine leukaemia virus group (e.g. HTLV-1).

HIV is currently the only known member of the lentivirus genus to infect man and is sub-divided into two types, HIV-1 and HIV-2, based on serology and sequence analysis (Fauci and Desrosiers (1997)). *Lenti*, meaning slow, denotes the long period between infection and disease, which can be up to 10 years or more with HIV infection (Lifson *et al.* (1991)). Other lentiviruses, particularly SIV, have been used to aid research into their human counterpart, although none exactly mimics it. SIV has been isolated from chimpanzees in the wild (SIV_{CPZ}) and has homology to HIV-1 (Huet *et al.* (1990)), whereas HIV-2 has more homology to other SIV isolates, such as those found in African Green monkeys (SIV_{AGM}) (Hirsch *et al.* (1989)).

HIV-1 can be further divided into 10 subtypes, or clades A-I (Leitner (1996); Kostrikis *et al.* (1995)) and the more diverse subtype O (Gürtler *et al.* (1994)). HIV-2 has so far only been divided into 5 clades, A-E (Gao *et al.* (1994)). This diversity is based predominately on sequence variation in the *env* gene, which encodes for the envelope proteins of the virus (see Section 1.5). Within subtype variation is also seen, reflecting the vast potential for change due to high levels of viral replication and the error-prone reverse transcriptase, a feature of all retroviruses (see Section 1.4.3.c). Recombination, between viruses of different subtypes, is also a means for generating further variation in areas of diverse subtype array such as Central Africa, South America and Southeast Asia (Robertson *et al.* (1995)). HIV-1 subtype B is the predominant subtype in Europe and America and HIV-2 is mainly restricted to West African countries (Clavel *et al.* (1986)).

1.4.1 Genome

HIV contains two identical copies of 9.5 kilobases (kb) long, positive sense ssRNA, i.e. they contain the correct coding sequence for protein translation to occur directly, although this does not occur in retroviruses. As for all retroviruses, the basic genome is composed of three genes *gag-pol-env* and encodes for structural proteins and enzymes (see Figure 1.2). The *gag-pol* transcript is translated to precursor proteins (see Figure 1.3); these are cleaved by virus derived proteases into the structural and regulatory proteins. Smaller accessory proteins are obtained by multiple splicing, occasionally in other open reading frames, maximising the potential number of proteins from a relatively small genome; the herpes viruses contain genomes of approximately 200kb by comparison.

1.4.2 Structure

A schematic representation of the HIV-1 virion is shown in Figure 1.4. The envelope proteins associate as 4 heterodimers to form 'knobs', or 'spikes', of which there are approximately 72 in the 110nm diameter host derived lipid bi-layer. The extracellular gp120 is non-covalently associated to the transmembrane gp41.

The lipid membrane encloses the cone shaped nucleocapsid composed of the capsid protein (p24), which provides the main structural framework for the virion. The matrix (p17), located between the capsid and envelope, also provides structural support and associates with the viral envelope (Höglund *et al.* (1992)). The nucleocapsid proteins, p7/9 and p6, are contained within the capsid too.

The two strands of viral RNA are linked at the 5' end and are associated with a transfer RNA (tRNA) molecule, which acts as a primer for the reverse transcriptase enzyme (RT) to initiate viral replication (see Figure 1.5). The viral genome may also be associated with the nucleocapsid. As well as the RT complex, the virion also contains the integrase and protease enzymes and the regulatory protein, VPR.

1.4.3 The Structural Genes

1.4.3.a *Env*

The envelope glycoproteins, gp120 and gp41, are cleaved from the gp160 precursor (see Figure 1.3) and form a non-covalently linked heterodimer. The extracellular gp120 contains the binding domain for CD4, the principal receptor for entry of the virus, and interacts with the transmembrane gp41. CD4 independent entry has been shown (Harouse *et*

Figure 1.2.

HIV-1 genomic organisation

The proviral genome, highlighting the coding regions for the respective genes.

The 5' and 3' long terminal repeat (LTR) contain identical U3-R-U5 repeats in the integrated form of the genome (see Figure 1.5). The 5' LTR contains the promoter region required for transcription to occur. The following list describes the various proteins encoded in the respective regions:

gag - capsid protein (p24), the matrix protein (p17) and the nucleocapsid proteins (p7 and p9).

pol- viral enzymes (including reverse transcriptase (RT) and integrase).

env - envelope glycoproteins (gp120 and gp41).

vif - Viral Infectivity Factor (VIF), involved in viral infectivity

vpr - VPR, a factor involved in nuclear localisation of the virus and control of cellular gene expression.

vpu - VPU, a factor possibly involved in the extracellular release of the virus and/or CD4 degradation.

tat - Trans-Activator of Transcription (TAT), transcriptional activator of viral gene expression

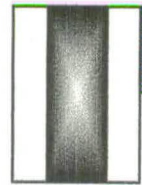
rev - Regulator of Expression of the Virus (REV), involved in the control of the structural genes expression and RNA transport

nef - Negative Effector Function (NEF), involved in the down-regulation of gene regulation, including CD4.

5' LTR

3' LTR

U3 R U5



GAG



POL



VIF



VPR



VPU



ENV



TAT

REV



NEF

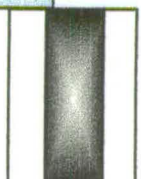


Figure 1.3.

HIV-1 mRNA Transcripts and Expression

- a.) Full length mRNA transcript, complete with the 5' cap, Transactivation Response Element (TAR), the Rev Response Element (RRE) and the 3' poly AAA tail. The regulatory proteins TAT and REV bind to TAR and RRE respectively.
- b.) mRNA transcripts produced during HIV replication, showing the splice sites (dotted lines) and the products following translation (arrow). For the *gag-pol-env* genes the polyprotein precursors are shown, along with the products produced following cleavage with the viral protease.

The first transcripts to be produced during replication are the multiply spliced *tat*, *rev* and *nef*. These are then translated in the cytoplasm and return to the nucleus to exert their effects (see Section 1.4.4 and 1.4.5.d). TAT binds to the TAR, stabilising and increasing the number of full length and singly spliced transcripts. REV binds to the RRE, only present in the full, or singly spliced transcripts and aids transport to the cytoplasm, where translation of the gene products can occur.

In order for production of the *pol* gene products to occur, a -1 frameshift in translation is required which occurs in about 5% of *gag* transcripts.

a.



b.

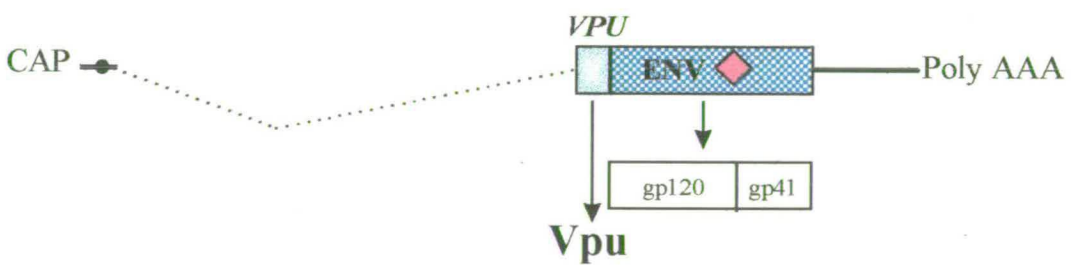
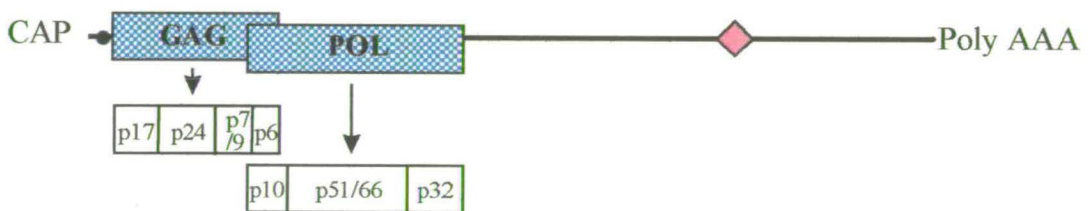
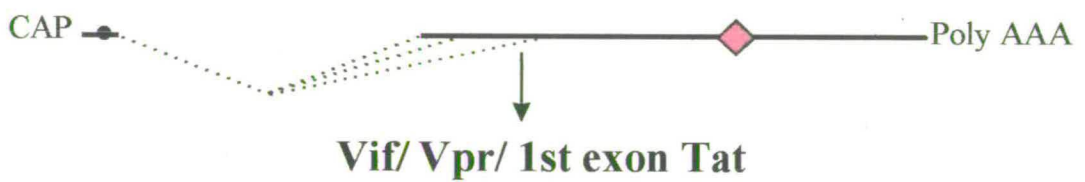
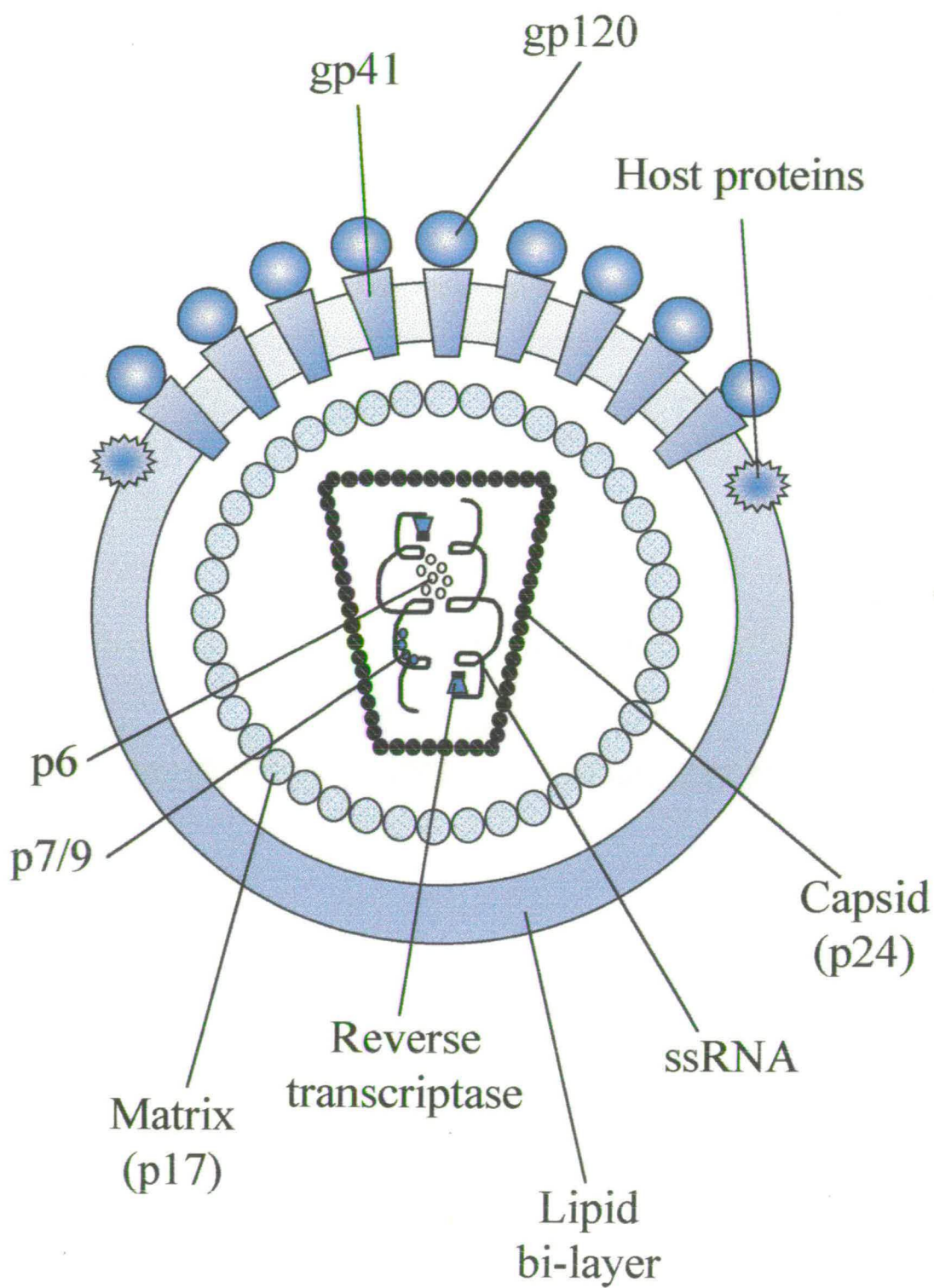


Figure 1.4.

The HIV-1 Virion

A diagrammatical representation of the structure of the HIV-1 virion (not to scale)

The host proteins shown included the Major Histocompatibility Complex (MHC) Class I and Class II proteins and the Class I associated β -2 microglobulin (Arthur *et al.* (1992)).



al. (1989); Tateno, Gonzalez-Scarano and Levy (1989); Harouse *et al.* (1991)), but its importance *in vivo* is unclear. Gp41 is involved in membrane fusion, allowing entry of the virus once CD4-gp120 binding has occurred. The envelope protein and its role in infection will be discussed in more detail later (see Section 1.5)

HIV-1 Env is encoded from a bicistronic (*vpu* and *env*) singly spliced transcript found later than the multiply spliced messenger RNAs (mRNAs) (see Figure 1.3) and its production is dependent on the regulatory proteins TAT and REV (see 1.4.4.b). The resulting Env protein is extensively glycosylated and cleaved to form gp120 and gp41. The highly glycosylated gp120 protein is the hydrophilic external protein and the relatively hydrophobic gp41 is a type 1 integral transmembrane protein.

1.4.3.b Gag

The *gag* gene encodes for the structural components of the HIV virion. The pr53 precursor protein is derived from the unspliced *gag-pol* mRNA transcript (see Figure 1.3). The polyprotein is then cleaved into p24 (capsid), p17 (matrix), p15 (nucleocapsid), the latter of which is further cleaved into p7/9 (nucleocapsid protein) and p6.

As well as providing structural support, the GAG proteins have other suggested functions. The capsid, p24, is known to bind cyclophilins A/B (Luban *et al.* (1993)), which function to mediate the correct assembly of other proteins and hence may be involved in assembly of the virion. The basic, hydrophilic nucleocapsid protein, binds genomic viral RNA (vRNA) and may condense it during packaging (Sakaguch *et al.* (1993)).

1.4.3.c Pol

The virally encoded enzymes are derived from the *gag-pol* transcript by a -1 ribosomal frameshift, occurring at 5% of translations (Jacks *et al.* (1988)). The resulting pr160 precursor protein is then cleaved by the viral protease into: p51/66 (RT complex), p10 (protease) and p32 (integrase) (see Figure 1.3).

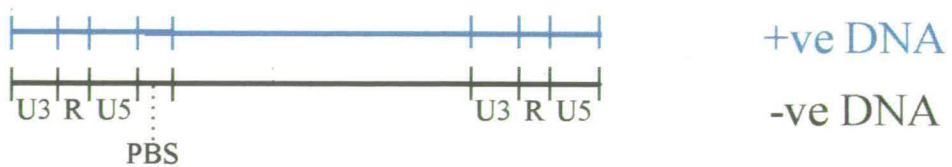
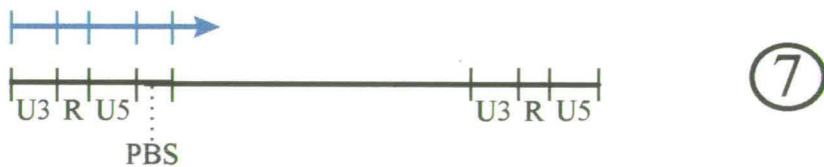
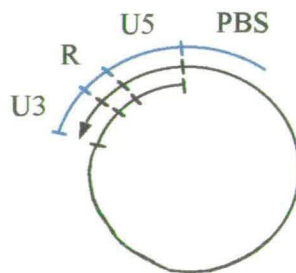
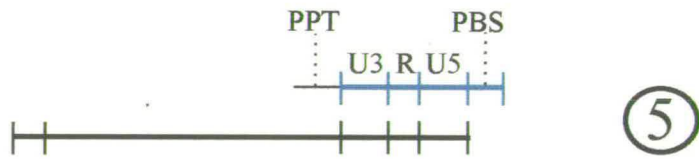
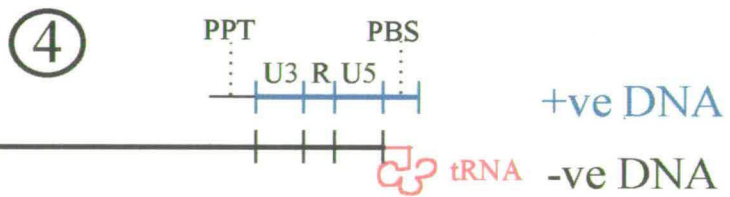
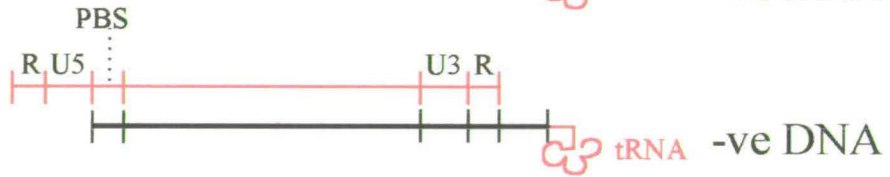
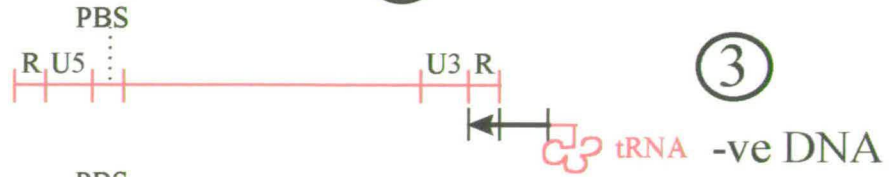
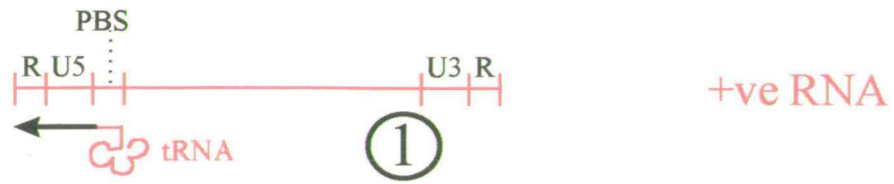
The RT complex is a heterodimer of the RNA dependent DNA polymerase enzyme, encoded by the p51 protein and the RNase H enzyme. RNase H degrades the RNA moiety of the RNA/DNA complexes formed during replication (see Figure 1.5) and generates oligonucleotide primers for the RT enzyme to initiate transcription. The RT of retroviral polymerases lack the 3'-5' exonucleotide proof-reading activity found in other DNA polymerases. This and the high error rate of the enzyme are the main accountable factors for

Figure 1.5.

A schematic representation of reverse transcription.

Adapted from Boucher (1993).

1. The tRNA primer associates with the primer binding site (PBS) just downstream of the U5 region of the 5' LTR, allowing a -ve strand DNA copy to be made, using the reverse transcriptase enzyme (RT).
2. As reverse transcription occurs RNase H, part of the RT complex, degrades the RNA template.
3. The short intermediate ('strong stop' -ve strand) jumps to 3' end of the second copy of RNA, facilitated by the identical repeat sections (R) at the 5' and 3' LTR (Coffin and Haseltine (1977); Resnick, Omer and Faras (1984)). Elongation of the strong stop -ve DNA strand then continues to the PBS.
4. A primer is generated by the RNase H activity of the RT enzyme, which is called Polypurine Track (PPT) (Panganiban and Fiore (1988)). This allows +ve strand DNA synthesis to occur until the tRNA molecule.
5. The tRNA is then removed by RNase H.
6. In order for full double stranded DNA (dsDNA) to be formed, the -ve strand DNA strand circularises, or a second strand jump occurs (Resnick, Omer and Faras (1984)). The -ve strand then contains a U3/R/U5 motif at both the 5' and 3' ends.
7. The strong stop +ve strand is then completed and a ds linear DNA genome formed.



the high degree of diversity seen in the retrovirus family. Approximately 1 inaccurate base/1700-4000 incorporated nucleotides occurs (Preston, Poiez and Loeb (1988); Roberts, Bebenek and Kunkel (1988)), relating to a possible 1-3 mis-incorporations/ replication cycle for the 9.5kb genome. However, the *in vivo* error rate has been reported to be up to 20-fold less than that of these purified cell-free studies (Mansky and Temin (1995)).

The protease enzyme is responsible for the cleavage of the precursor proteins and is related to cellular aspartyl proteases, with which it shares homology and hence, its active form is most likely to be a dimer (Loeb *et al.* (1989)).

The integrase enzyme possesses DNA cleavage and joining activities and its function is the covalent linkage of double stranded viral DNA (ds vDNA) into the host genome, a key feature of retroviral replication (see Figure 1.6). The central domain of the integrase enzyme is conserved across retroviruses, retrotransposons and the transposons of bacterial transposable elements. Further understanding of this and all the viral enzymes will improve the discovery of inhibitors and potential therapeutic agents (Pommier *et al.* (1997)).

1.4.4 The Regulatory Genes

The regulatory genes, *tat* and *rev* are both essential for HIV replication and are derived from multiply spliced transcripts early in infection (see Figure 1.3).

1.4.4.a *Tat*

TAT, or the *trans-activator of transcription*, is a 15kiloDalton (kDa) protein. TAT mediates its effect by binding to a *cis-acting* RNA element, TransActivation Response element (TAR), located at the start of all viral transcripts (Berkhout, Silverman and Jeang (1989)). The binding of TAT to TAR has a positive effect on the elongation of transcription, allowing the formation of full length transcripts required for the production of the structural proteins (Feinberg, Baltimore and Frankel (1991)).

1.4.4.b *Rev*

In the absence of any protective mechanism, all viral transcripts would be spliced by the spliceosome recognising the 5-6 splice sites present in the genome. The function of REV, or the *regulator of expression of the virion*, is to control this.

REV is a 13kDa protein, which binds to the REV Response Element (RRE) (Daly *et al.* (1989)). RRE is another *cis-acting* RNA element, located in the *env* exon, 3' to the

junction between gp120 and gp41, and hence is only expressed on unspliced and singly spliced transcripts (see Figure 1.3). REV binding to RRE allows the transport of these unspliced and singly spliced transcripts from the nucleus to the cytoplasm (Felber *et al.* (1989)). Once in the cytoplasm, translation can occur and the relevant proteins produced. REV contains a nuclear export signal sequence and interacts with cellular nucleoporins to allow transport (Fischer *et al.* (1995)).

1.4.5 The Accessory Genes

The accessory genes (*vif*, *vpr*, *vpu* and *nef*) are encoded from spliced mRNA in different open reading frames (see Figure 1.3). Early *in vitro* tissue culture studies deemed these proteins non-essential for replication, although more recent reports have highlighted some critical functions. The precise role *in vivo* of these proteins is still not clearly resolved.

1.4.5.a *Vif*

Encoded downstream of the *pol* gene, *vif* is found in all lentiviruses with the exception of ELAV. The 23kDa protein accumulates in the cytosol and cytoplasmic membrane of infected cells and early studies revealed a lack of VIF resulted in reduced infectivity (Goncalves, Jallepalli and Gabuzda (1994)). Hence, the protein became known as viral infectivity factor, or VIF. Two proposed functions for VIF are to transport the infecting virus to the nucleus (Karczewski and Strebel (1996)) and stabilise newly synthesised vDNA (Simon and Malim (1996)).

1.4.5.b *Vpr*

VPR is a 15kDa protein translated from singly spliced REV dependent mRNA and associates with the nucleocapsid of mature virions. *Vpr* is encoded by HIV-1 and some SIV strains; other SIV strains along with HIV-2 also encode a homologous gene *vpx*. *Vpx* is thought to be a repeat of *vpr* in HIV-2 and the SIV strains expressing both (Tristem *et al.* (1990)), although a recent report suggests that a latter recombination event may have occurred (Sharp *et al.* (1996)). Sharp *et al.* (1996) suggest that the two genes in HIV-2/SIV may have divergent functions (see below for VPR functions), providing a potential selective advantage.

A mutant virus (HIV-1) lacking both functional VPR and matrix protein, showed a block in nuclear localisation of the pre-integration complex, suggesting both are redundantly involved in nuclear localisation in non-dividing cells (Heinzinger *et al.* (1994)). Therefore, in

non-dividing cells, such as macrophages, VPR is required for efficient infection and integration of the virus.

In dividing cells, such as CD4⁺ T cells, VPR has been implicated as a regulator of viral and cellular gene expression. By maintaining cell cycle in G2 arrest, VPR allows enhanced virus production (Bartz, Rogel and Emerman (1996)).

1.4.5.c Vpu

Vpu encodes a 16kDa protein from a singly spliced REV dependent mRNA. The N-terminal amino acids of VPU are hydrophobic in nature and the rest are hydrophilic and its structure suggests it to be an amphipathic integral membrane protein (type 1) (Maldarelli *et al.* (1993)). In infected cells, VPU is seen in the perinuclear region of the cell, associated with the endoplasmic reticulum (ER) and Golgi. Suggested functions for the protein are the degradation of CD4 in the ER (Willey *et al.* (1992); Bour, Schubert and Strebel (1995)) and enhanced release of virions from infected cells (Göttlinger *et al.* (1993))

1.4.5.d Nef

NEF is translated from two multiply spliced early transcripts and produces a 27kDa protein. It is postrationally modified by the addition of myristic acid, required for attachment to the cell membrane. The functions of NEF are multiple, with pleiotropic effects. Early *in vitro* studies suggested the misnomer that *nef* mutant viruses replicated to higher levels than wild type viruses, hence the name 'negative effector function'. Conversely, NEF-mediated enhancement of viral infectivity has been seen (Miller *et al.* (1994); Spina *et al.* (1994)) and supported by findings of *nef* mutants that appear attenuated and less pathogenic (Deacon *et al.* (1995); Kestler *et al.* (1991)). NEF has also been shown to reduce surface CD4 levels (Garcia, Alfano and Miller (1993)), which is presumed to occur early in replication to prevent superinfection.

1.4.6 Life Cycle

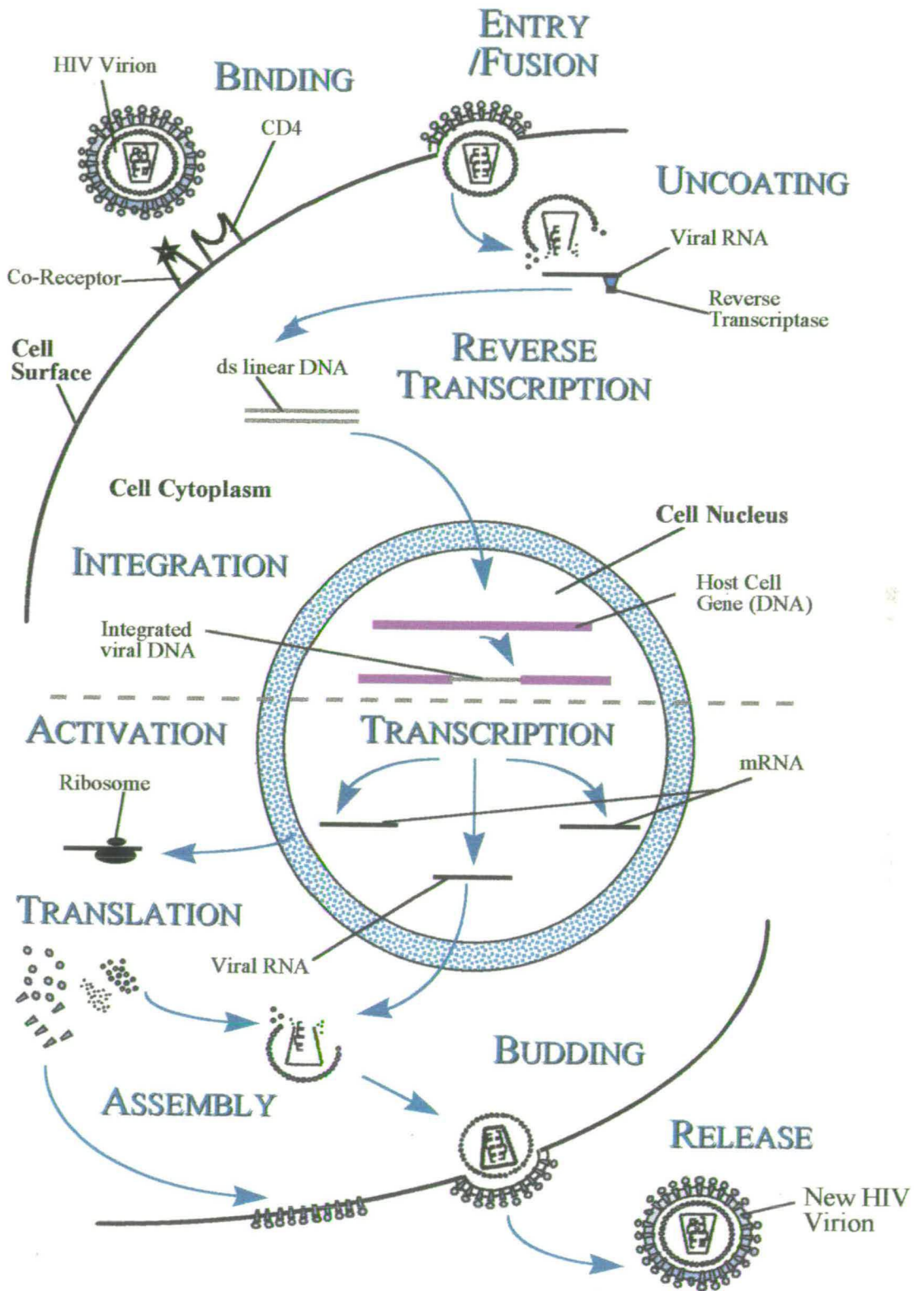
The replication cycle of HIV-1 is summarised in Figure 1.6, along with a brief explanation of the various stages. The production of dsDNA from the ssRNA genome is described in more detail in Figure 1.5.

Figure 1.6.

The replication cycle of HIV-1.

A schematic representation of the replication cycle of HIV-1. The various stages are described in more detail below, with the order indicated by arrows (→).

- a.) **BINDING** - The virion attaches to the susceptible cell, via the external glycoprotein, gp120, binding to the cell surface receptor, CD4 (Dalglish *et al.* (1984); Klatzmann *et al.* (1984)). It has recently been shown that entry also requires interaction with a co-receptor and this will be described in more detail later (see Section 1.5).
- b.) **ENTRY/ FUSION** - The binding of gp120 to the host cell causes conformational changes in the gp120/gp41 proteins, exposes the fusion peptide of gp41. This causes the viral envelope to fuse with the host cell membrane and release the nucleocapsid into the cytoplasm (Brasseur *et al.* (1988)).
- c.) **UNCOATING** - Reverse transcription begins before the RNA genome is released from the nucleocapsid and at some point during this process the capsid is broken down and the pre-integration complex transported to the nucleus. The precise components of this complex are not yet known, but include: the integrase enzyme, the matrix protein and the dsDNA, produced following reverse transcription.
- d.) **REVERSE TRANSCRIPTION** - A more detailed description of this procedure is shown in Figure 1.5. However, the basic process is the production of dsDNA from the ssRNA genome, by the viral reverse transcriptase enzyme.
- e.) **INTEGRATION** - The virally encoded integrase enzyme nicks the viral dsDNA and host cell genome forming 'blunt ends' (Engleman, Mizuuchi and Craigie (1991)) These are overhangs of oligonucleotide bases, which allow insertion of the viral dsDNA into the cell genome. Host cell enzymes are presumed to fill in any gaps and ligate the ends.
- f.) **TRANSCRIPTION/ TRANSLATION** - Following activation, usually cell activation, cellular transcription factors, e.g. NF- κ B, Sp1, bind to the promoter region located in the U3 region of the viral 5' LTR (see Figure 1.5) and initiate transcription. Full length transcripts are multiply spliced by host machinery in the nucleus, producing the early transcripts, *tat*, *rev* and *nef* (see Figure 1.3). These are translated in the cytoplasm by cellular processes and then return to the nucleus to aid production of the late transcripts. These late transcripts encode for the structural proteins and viral enzymes (see Figure 1.3). Some of the full length RNA transcripts act as new RNA genomes.
- g.) **ASSEMBLY** - The newly formed proteins are assembled into virions and the RNA genome and enzymes packaged inside. The glycoproteins gp120 and gp41 insert into the cell membrane and the completed capsid is then targeted to this area by an unknown mechanism.
- h.) **BUDDING/ RELEASE** - The nucleocapsid then buds out of the cell membrane, acquiring a glycoprotein studded lipid bi-layer envelope. The virus is then able to infect further susceptible cells by binding to the cell receptor CD4.



1.5. THE ENVELOPE GLYCOPROTEIN

As described earlier (see Section 1.4.1), the envelope proteins are encoded from the *env* gene of the HIV genome. A schematic representation of the secondary structures of the envelope proteins is shown in Figure 1.7.

The gp120 protein contains six conserved regions (C1-6) interspersed with five hypervariable regions (V1-5) (see Figure 1.7.a)(Starcich *et al.* (1986); Willey *et al.* (1986); Modrow *et al.* (1987)). Intrachain disulphide bonds have been postulated to be important in the structure and function of gp120, with the finding of conserved cysteine residues across diverse HIV-1/2 and SIV strains (Leonard *et al.* (1990); Hoxie (1991)). The regions involved in the gp120-CD4 interaction are found within the conserved regions and form a discontinuous binding region (Olshevsky *et al.* (1990)) (see Figure 1.7.a).

The diversity seen in the V1-5 regions is a major factor involved in distinguishing the viral subtypes (Leitner (1996)) and strains (Hahn *et al.* (1985)); differences in the viral population of an infected individual (inpatient variation), termed a quasispecies, are also seen in these hypervariable regions (Hahn *et al.* (1986); Simmonds *et al.* (1991); Holmes *et al.* (1992)). The heterogeneity seen in an individual patient is most likely caused by the selective pressures of the host immune response and adaptation of the virus to infect different cell types. The host immune response to the virus will be addressed in more detail later (see Section 1.6), but involves escape from neutralising antibodies, a large proportion of which are directed against the envelope proteins. The principal neutralising domain (PND) for tissue culture adapted strains of HIV, is found within the third hypervariable region, V3 (Javaherian *et al.* (1989); Carrow *et al.* (1991)), although other domains are also involved (Chanh *et al.* (1986)).

1.5.1 The V3 Loop

This domain was first shown to be important with the discovery that antibodies directed against this region prevented infection (Javaherian *et al.* (1989); Carrow *et al.* (1991); Emini *et al.* (1992)) and was later shown to play a crucial role in cell tropism, cytopathicity and fusogenicity (Cann *et al.* (1992); Cheng-Mayer *et al.* (1990)). The region contains 35 amino acids arranged in a loop from a disulphide bond (see Figure 1.7.b). The crown of the loop contains a highly conserved GPGRF motif (subtype B) flanked by variable regions either side, with the sequences towards the base of the loop becoming more

Figure 1.7.

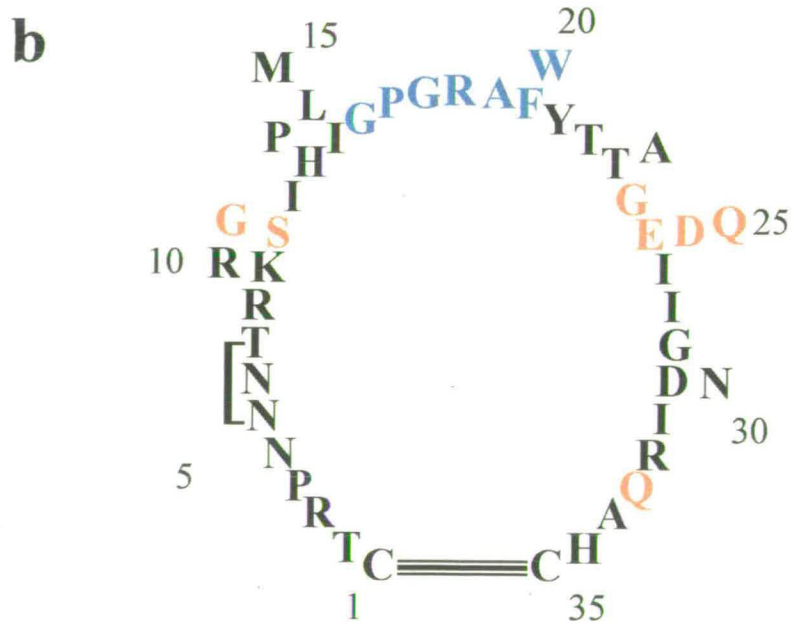
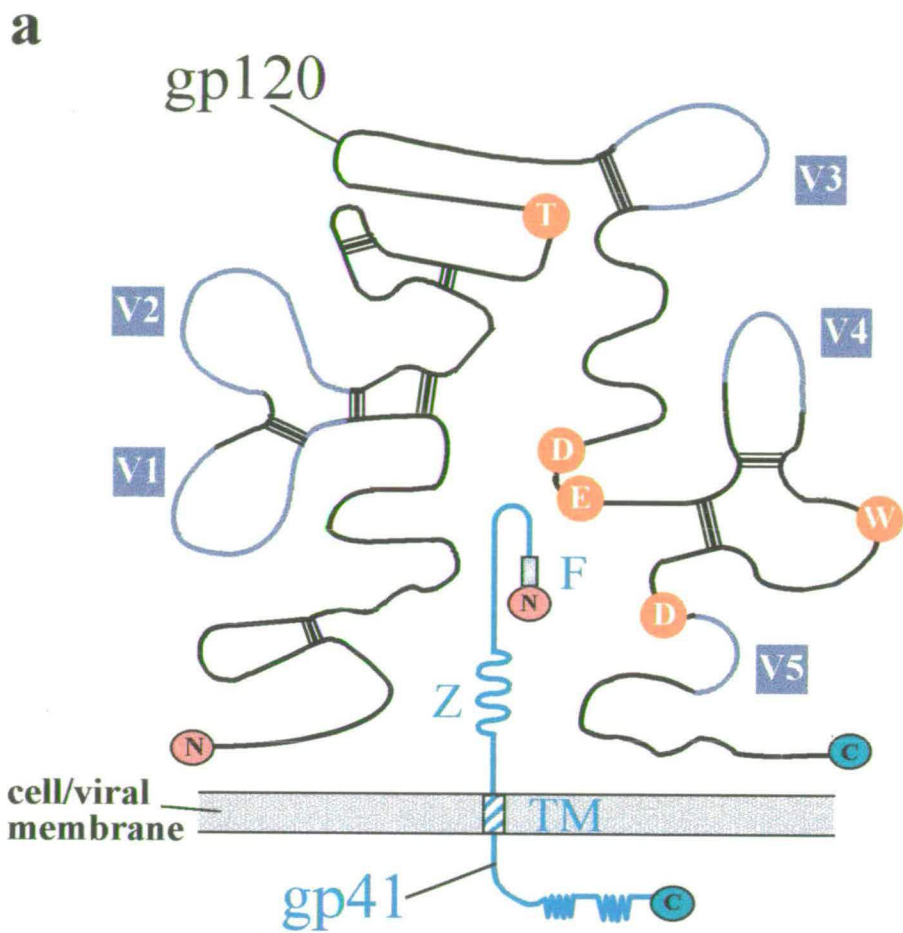
Diagrammatic Representation of HIV-1 Env

a.) The predicted folding pattern of gp120 and gp41 adapted from Luciw (1996)

Gp120 is shown in black, with the hypervariable domains V1-5 shown in blue. The disulphide bonds are represented as three lines and the amino acids involved in binding CD4 are shown in red. The amino and carboxyl termini are labelled as (N) and (C) respectively. Gp41 is shown in light blue, with the fusion peptide, F, represented as a box and the leucine zipperlike region, Z shown as a helix. The transmembrane portion, TM is indicated by a hatched box. The diagram does not consider any interactions of gp120 with gp41, which may affect the precise structure from that shown here.

b.) The V3 loop of HIV-1.

The amino acid sequence shown in the centre is the subtype B consensus sequence, with common alternatives shown outside (>10 sequences of 1078; (Dighe, Korber and Foley (1997))). The conserved crown motif GPGRF is shown in blue. The potential glycosylation signal NNT is highlighted with a bracket, the disulphide bridge linking the two cysteine (C) residues is shown with a stripe and the prominent amino acids involved in cell tropism are shown in red.



conserved again (Dighe, Korber and Foley (1997)). The variation seen between viruses is not completely random and involve amino acid substitutions with similar chemical properties.

1.5.1.a V3 and Cell Tropism

Early definitions of viral phenotypes were dependent upon the ability to induce syncytia, or formation of large multinucleate cells in CD4⁺ T cells lines, such as MT-2 (Lifson *et al.* (1986a); Lifson *et al.* (1986b); Koot (1992)). Viruses able to form syncytia in *in vitro* culture were termed syncytium-inducing, or SI, and those unable to were called non-syncytium inducing, or NSI (Tersmette *et al.* (1988); Koot (1992)). SI viruses grew faster and to higher titres in both T cell lines and primary peripheral blood mononuclear cell (PBMC) cultures and were thought to be more virulent. The presence of a basic amino in one or more of the following positions in the V3 loop: 11, 24, 25 and 32 (see Figure 1.7.b), confers an SI phenotype and if uncharged, or acidic amino acids are present an NSI phenotype is seen (de Jong *et al.* (1992b); de Jong *et al.* (1992a); Fouchier *et al.* (1992); Milich, Margolin and Swanstrom (1993)). The overall charge of the V3 loop is also used to predict the viral phenotype (Fouchier *et al.* (1992); Milich, Margolin and Swanstrom (1993); Donaldson *et al.* (1994)), as is the degree of variability (Chesebro *et al.* (1992); Milich, Margolin and Swanstrom (1993); Donaldson *et al.* (1994)); SI isolates have a higher overall charge and are more heterogeneous than NSI isolates. A combination of these two properties, charge and variability, was used by Donaldson and colleagues (1994) to predict the phenotype of different isolates. Variations in V1 and V2 have also been shown to be involved in tropism to a lesser extent (Boyd *et al.* (1993); Groenink *et al.* (1993); Sullivan *et al.* (1993)).

Chimeric viruses, constructed between different viruses, found that the V3 region was involved in the distinction between macrophage tropism, or M-tropism, and T cell line tropism, or T-tropism (O'Brien *et al.* (1990); Westervelt, Gendelman and Ratner (1991); Cann *et al.* (1992)). Generally, the terms are interchangeable with the NSI/SI phenotype; NSI viruses generally are M-tropic and SI viruses are T-tropic. However, care should be taken as an isolate deemed NSI, due to its lack of ability to induce fusion in T cell lines, is not automatically a M-tropic virus. The terms M and T-tropic are also confusing as many M-tropic and T-tropic isolates will grow in primary PBMC cultures, composed primarily of CD4⁺ T cells, i.e. the term M-tropic means an isolate will not replicate in continuous T cell lines. Another term for T-tropic cell isolates is T Cell Line Adapted (TCLA), which is slightly less ambiguous.

The V3 loop does not interact with CD4 on the host cell, as seen by the fact that antibodies against V3 (Linsley *et al.* (1988)) and mutations in V3 (Page, Stearns and Littman (1992); Grimaila *et al.* (1992)) do not affect gp120-CD4 binding, but do prevent entry and infection. It was proposed many years ago that the V3 region of gp120 may interact with other cell surface molecules, or a co-receptor (Hunter (1997)) and may even be cleaved by a cellular proteinase (Hattori *et al.* (1989); Clements *et al.* (1991)). Such an interaction may play a crucial role in conformational changes required to expose the fusion peptide in gp41 and hence allow viral entry. A potential co-receptor was proposed by Callebaut and colleagues (Callebaut *et al.* (1993)) as the dipeptidyl peptidase IV, CD26. They reported that co-transfection of murine NIH 3T3 cells with CD4 and CD26 rendered them permissive to HIV infection, but this failed to be reproduced by others (Broder *et al.* (1994); Patience *et al.* (1994); Camerini, Planelles and Chen (1994); Alizon and Dragic (1994); Lazaro *et al.* (1994)). It has long been known that CD4 alone is insufficient for HIV infection (Maddon *et al.* (1986)) and that some other factor(s) expressed on human cells is(are) required. This was verified by the fusion of the uninfected murine NIH 3T3 cells co-transfected with human CD4 and non CD4⁺ human cells, as the heterokaryons formed were permissive to HIV infection (Dragic *et al.* (1992)).

Other suggested co-receptors include the adhesion molecules, leukocyte functional antigen-1 (LFA-1) and CD44, but although a role in viral adhesion and fusion was identified (Pantaleo *et al.* (1991); Dukes *et al.* (1995)), a direct role in infection was not seen.

The recent identification of a role for a group of G-protein linked receptors, involved in inflammation, in HIV infection has had a substantial impact on HIV research. The receptors normally bind a group of cytokines called chemokines, but have also been shown to act as co-receptors for entry of HIV.

1.5.2 Chemokines and Their Receptors

These *chemotactic cytokines*, or chemokines are a group of small polypeptides, which chemotactically attract different cells involved in inflammation (Baggiolini, Dewald and Moser (1997)). They are divided into two distinct subgroups based on the positions of the first two highly conserved cysteine residues: the CC chemokines, where the residues are continuous and the CXC chemokines, where they are separated by another amino acid. The chemokines induce the adherence and migration of various white blood cells, or leucocytes to a site of inflammation. In humans, the CXC chemokines are encoded on chromosome 4 and primarily

activate neutrophils and the CC chemokines are localised to chromosome 17 and generally activate monocytes, lymphocytes, basophils and eosinophils.

The chemokines mediate their function by binding to specific receptors found on the susceptible cells. The chemokine receptors belong to a family of G-protein coupled seven transmembrane receptors. Five CC chemokine receptors (CCR) are known to date, CCR-1-5 and four CXC chemokine receptors, CXCR-1-4 (Baggiolini, Dewald and Moser (1997)). The known ligands are summarised in Table 1.1 and the seven transmembrane structure is shown in Figure 1.8.

1.5.2.a Chemokine Receptors and HIV

The recent discovery, by Berger's group, of a co-receptor for entry of HIV (Feng *et al.* (1996)) injected new energy into HIV research. The receptor termed 'fusin' by Berger was identified by construction of a complementary DNA (cDNA) library from the mRNA of the HeLa cell line, a known permissive cell once transfected with CD4. After extensive screening, a single plasmid clone was identified which was capable of allowing the normally non-permissive CD4-NIH 3T3 cells undergo to fusion with NIH 3T3 cells expressing *env* and a *lacZ* gene under a T7 promoter. Fusion was identified by substrate hydrolysis of β -galactosidase, which in the presence of both the T7 polymerase (CD4-NIH 3T3 cells) and the *lacZ* gene under the T7 promoter (ENV-NIH 3T3 cells) is utilised and a change in absorbance recorded. The clone was sequenced and found to encode a protein with homology to a family of G protein linked seven transmembrane receptors.

Later classified as CXC chemokine receptor-4 (CXCR-4), fusin was shown to act as a co-receptor for entry of TCLA strains of HIV. Non-human cells transfected with CD4 and CXCR-4 were permissive for HIV binding and fused with cells expressing TCLA gp120 proteins. Later, its ligand was identified as stromal cell derived factor-1 (SDF-1) (Bleul *et al.* (1996); Oberlin *et al.* (1996)) and it was shown to block entry of TCLA strains of virus, further proof of the receptor's role as a co-receptor. A monoclonal antibody against CXCR-4 (McKnight *et al.* (1997); Strizki *et al.* (1997)) was also shown to inhibit entry of some, but not all TCLA strains, suggesting that different strains of virus may interact in varying ways with the receptor.

It was discovered many years ago, that CD8⁺ cells release a factor which inhibits HIV replication (see Section 1.6.2.g). Much controversy surrounds the identity of this 'non-lytic' suppressive factor, but it was suggested that the CC chemokines, macrophage inflammatory

Table 1.1

Chemokine Receptors and Their Ligands

Group	Receptor	Ligand
CC Receptors	CCR-1	MIP-1 α / RANTES/MCP-3
	CCR-2	MCP-1/ 2/ 3
	CCR-3	Eotaxin/ RANTES/ MCP-3/4
	CCR-4	RANTES/ MIP-1 α
	CCR-5	RANTES/ MIP-1 α / β
CXC Receptors	CXCR-1 (IL-8RA)	IL-8
	CXCR-2 (IL-8RB)	ELR-CXC chemokines
	CXCR-3	IP10/ MIG
	CXCR-4	SDF-1

MIP - Monocyte Inflammatory Protein

RANTES - Reduced upon Activation Normal T cell Expressed and Secreted

MCP - Monocyte Chemoattractant Protein

IL - InterLeukin

ELR-CXC chemokines - majority of CXC chemokines with the amino acid motif ELRCXC

IP10 - Interferon-inducible Protein 10

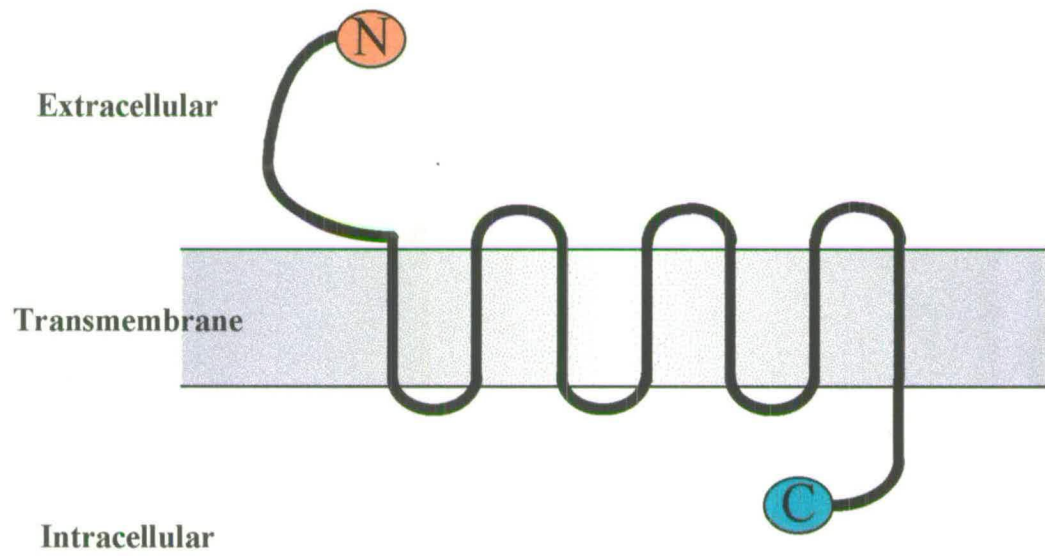
MIG - Monokine Inducible by interferon-Gamma

SDF - Stromal cell Derived Factor

Figure 1.8.

Diagrammatic Representation of a Chemokine Receptor

The seven transmembrane G-protein linked receptors all have similar structures. The amino and carboxyl termini are labelled as N and C respectively and the length of these segments varies in each receptor.



proteins (MIP)-1 α/β and RANTES (Reduced upon Activation, Normal T cell Expressed and Secreted) were responsible for the suppressive effect of the supernatant from a CD8⁺ cell line (Cocchi *et al.* (1995)). HIV replication was inhibited in the CD4⁺ cell line, PM1, a continuous line infectible with both T and M-tropic strains of HIV (Lusso *et al.* (1995)). A cocktail of the three chemokines was able to reproduce the suppression caused by the cell supernatant. This inhibition was not effective against the TCLA strain HIV_{IMB}, but was highly effective against the non-TCLA and M-tropic strain HIV_{BAL}. These three chemokines all utilise CCR-5 as a receptor (see Table 1.1) and with the discovery of the CXCR-4 as TCLA virus' co-receptor, the logical progression was soon confirmed that CCR-5 acted as a co-receptor for non-TCLA/M-tropic strains of virus (Alkhatib *et al.* (1996); Choe *et al.* (1996); Deng *et al.* (1996); Doranz *et al.* (1996); Dragic *et al.* (1996)). CCR-2 and CCR-3 were also shown to be utilised by a few strains of virus (Choe *et al.* (1996); Doranz *et al.* (1996); Frade *et al.* (1997)). One important involvement of CCR-3 *in vivo* could be in the infection of microglia in the brain, which have been shown to express both CCR-3 and CCR-5 (He *et al.* (1997)).

Further analysis of a broad range of HIV isolates has shown that M-tropic strains of virus use CCR-5 as the major co-receptor, but dual tropic isolates, many primary isolates and TCLA isolates can use a more varied range of co-receptors (Doranz *et al.* (1996); Björndal *et al.* (1997); Cheng-Mayer *et al.* (1997); Speck *et al.* (1997); Dittmar *et al.* (1997); Zhang *et al.* (1996)), including CCR-5. CCR-5 usage does not therefore completely correlate with the ability to infect macrophages (Dittmar *et al.* (1997); Cheng-Mayer *et al.* (1997); Björndal *et al.* (1997); Speck *et al.* (1997)).

1.5.2.b Chemokine Receptors and Gp120

Sensitivity to CC chemokine mediated suppression, shown to inhibit M-tropic, but not TCLA virus replication, was mapped to *env* and more specifically the V3 loop (Cocchi *et al.* (1996)). The ability to utilise CCR-5 and CCR-3 was found to be dependent upon the sequence of the V3 loop (Choe *et al.* (1996)) and antibodies against V3 blocked the interaction of gp120 with CCR-5 (Trkola *et al.* (1996a); Wu *et al.* (1996); Hill *et al.* (1997)). It might seem therefore that the long reported association of V3 with viral phenotype and tropism may reflect chemokine receptor binding. However, although an association of gp120/CD4 and CCR-5/CXCR-4 has been seen (Lapham *et al.* (1996); Trkola *et al.* (1996a); Wu *et al.* (1996)), no direct interaction of V3 with either co-receptor has yet been reported. It may be that other regions of gp120 are involved in actually binding to the co-receptor and some

conformational requirement, influenced by the V3 structure and/or charge, is needed. This is supported by the fact that diverse subtypes and other lentiviruses, with differing V3 sequences, use CCR-5 and CXCR-4 as co-receptors for entry (Zhang *et al.* (1996); Hill *et al.* (1997); Cheng-Mayer *et al.* (1997); Bron *et al.* (1997); Sol *et al.* (1997)). Therefore, the idea that a more conserved region is actually involved in physically binding to the co-receptor seems more feasible. It is interesting to note that, using chimeras of CCR-5 and other receptors, differing regions of CCR-5 have been found to be involved in binding M-tropic from dual tropic strains (Rucker *et al.* (1996); Doranz *et al.* (1997)). The use of CCR-5 by dual tropic strains may therefore differ from that of M-tropic ones. A similar prediction was seen for CXCR-4, as TCLA and dual tropic viruses showed varying susceptibility to a monoclonal antibody to CXCR-4 (McKnight *et al.* (1997); Strizki *et al.* (1997)), suggesting that different regions of CXCR-4 are perhaps involved in binding with different strains of virus.

Hence, while the discovery of the co-receptors has clarified some earlier questions, it has not provided a simple answer, or complete explanation of the questions of tropism and the role of V3, but has added many more. It has also added a further classification system based on co-receptor usage; R5 isolates using CCR-5, X4 using CXCR-4 and R5X4 using both (Berger *et al.* (1998)).

1.6. THE IMMUNE RESPONSE

Primary infection with HIV is characterised by a transient viraemia (see Section 1.2), which declines concordant with the onset of a specific immune response; most notably seroconversion, the production of HIV-specific antibody. A long infection then ensues before the onset of AIDS. The length of this could be determined either by the effectiveness of the control of infection by the host immune system, or latent infection, via the integration of the virus into the host genome (see Section 1.4.6). In fact it was recently shown that, in contrast to that previously believed, rapid viral dynamics of infection, replication and cell turnover during the asymptomatic phase of infection did in fact occur (Ho *et al.* (1995); Wei *et al.* (1995)).

The human immune system is primarily divided into non-specific, innate defences and specific, acquired ones. Innate responses, such as phagocytic cells and the skin, which imposes a physical barrier, do appear to be involved in protection from HIV infection. For example, at mucosal surfaces, such as the vagina, the risk of infection appears to be enhanced by the presence of ulcerative lesions that disrupt the epithelial barrier (Laga *et al.* (1993)). Also *in*

vitro, activated neutrophils have been shown to have virucidal actions on cell-free virus, via the toxic effects of myeloperoxidase and hydrogen peroxide (Klebanoff and Coombs (1992)). Hence, if stimulated at mucosal surfaces, or inflammatory sites, neutrophils may provide some protection from cell-free virus. If the innate barriers are overwhelmed, specific defences are then relied upon. Historically, the acquired immune response is divided into the humoral and cell-mediated arms and they will be considered separately here.

1.6.1 Humoral Immunity

Humoral immunity involves the activation and differentiation of B lymphocytes into plasma cells. These produce glycoproteins known as antibodies, that are specific for the invading pathogen and act in a variety of ways including: neutralisation of molecules that allow cell entry, or damage; opsonisation, which involves the clumping of antigenic substances and enhancing ingestion by phagocytic cells like macrophages; complement activation, which leads to the formation of membrane attack complexes and the destruction of the antigenic cell, or organism and antibody dependent cell cytotoxicity (ADCC), which involves the lysis of antigenic cells, or organisms via non-specific mechanisms, e.g. natural killer (NK) cells, using antibody for specific recognition (see Section 1.6.2.b).

For a viral infection, the principal role of antibody is neutralisation preventing cell entry. With most viral infections that are acute and self limiting, initial infection 'primes' the induction of antigen-specific antibody, therefore an effective antibody-mediated control is delayed until antibody production commences, some 4-6 days following antigenic stimulation. For a secondary infection, antigen specific B cells are already primed; antibody production is therefore more rapid and more effective at controlling infection and can often occur before symptoms are established. However, following primary infection, HIV induces a life-long persistent infection despite the presence of a strong, measurable antibody response (Brun-Vézinet *et al.* (1984); Cheingsong-Popov *et al.* (1984); Safai *et al.* (1984)).

1.6.1.a Humoral Immunity and HIV

Early research on the immune response to HIV infection concentrated on the role of humoral immunity, due to the strong, easily measurable antibody response seen in infected individuals. The PND for TCLA strains is encoded by a highly variable region of the *env* gene, V3 (Javaherian *et al.* (1989); Zwart *et al.* (1991); Moore and Ho (1993)) (see Section 1.5.1), with V1/V2 (McKeating *et al.* (1993); Moore *et al.* (1993)), C4 (Lasky *et al.* (1987))

and the CD4 binding site (Moore and Ho (1993); Trkola *et al.* (1996b); Lasky *et al.* (1987)) also acting as targets for neutralisation in gp120. Several epitopes in gp41 have also been described (Chanh *et al.* (1986); Muster *et al.* (1993)). Many of these epitopes were identified using TCLA strains of virus and antibodies directed against such regions have been shown to be ineffective at neutralising primary isolates. A rare CD4 binding site epitope (Burton *et al.* (1994)), a conformational epitope denoted by the antibody, 2G12 (Trkola *et al.* (1996b)) and the main gp41 epitope, which binds the antibody, 2F5 (Muster *et al.* (1993)), are the three main epitopes which have been shown to be broadly reactive across a range of primary isolates.

It has frequently been proposed that the presence of specific antibody provides a strong selective pressure for 'escape' mutants to arise, which avoid neutralisation and keep the virus one step ahead of the antibody response (Nara *et al.* (1990)). The virus has also been shown to spread directly from cell to cell, hence avoiding neutralisation (Sato *et al.* (1992)). A further theory, questioning the role of HIV-specific antibody as an effective control mechanism, is the 'original antigenic sin' hypothesis (reviewed in Kohler, Muller & Nara (1994)), whereby initial infection drives a vigorous, yet inappropriate immune response to later viral variants. HIV has also been postulated to induce 'enhancing antibodies', which allow increased viral entry into cells expressing Fc receptors (Robinson, Montefiori and Mitchell (1988)). These receptors bind the constant region (Fc) of antibody and trigger endocytosis of the antigen:antibody complex following binding.

The strong antibody response detected as seroconversion closely follows the decline in primary viraemia. When the nature of the response was analysed in two seroconverters, no antibody capable of neutralising the virus was present (Ariyoshi *et al.* (1992)). This and the other potential problems associated with antibody responses and the persistence of the virus, despite the presence of a strong anti-HIV antibody response, questions the protection offered by humoral immunity against HIV infection.

1.6.2 Cell Mediated Immunity (CMI)

Classically, cell mediated immunity is the principal controlling/ eliminating force against intracellular pathogens (McMichael *et al.* (1983); Moss, Rickinson and Pope (1978); Rickinson *et al.* (1981); Zinkernagel and Welsh (1976)). CMI is composed of four main facets: delayed type hypersensitivity (DTH) reactions; ADCC; non-antigen specific

cytotoxicity, via NK cells; antigen specific cell cytotoxicity, mediated by cytotoxic T lymphocytes (CTLs).

DTH reactions involve the activation of macrophages, by the production of molecules called cytokines from antigen specific T cells. This activation enhances the macrophage's phagocytic and bacteriocidal functions. The protective role this would play in HIV infection is questionable and shall not be discussed further.

1.6.2.a Natural Killer (NK) Cells

NK cells represent a discrete lymphocyte subset defined by CD16/56 expression. They can be induced to proliferate at high concentrations of the cytokines, interleukin-2 (IL-2) and IL-12 (Chehimi *et al.* (1992)). NK cell lysis of tumour and virally infected cells is mediated in an major histocompatibility complex (MHC)-unrestricted and as yet undefined manner (see Section 1.6.2.c/d). Lysis occurs essentially as CTL mediated lysis (see Section 1.6.2.f), i.e. perforin release, etc. The lytic activity is enhanced by the following cytokines: type 1 interferon (IFN), IFN- γ , tumour necrosis factor (TNF), IL-2 and IL-12 and when activated they produce the type 1 cytokines, TNF and IFN- γ (see Section 1.6.2.e). NK cells are an important initial control of viral infections, shown by an individual who, despite normal B and T cells, lacked NK cells and suffered severe viral infections (Biron, Byron and Sullivan (1989)).

1.6.2.a.i NK Cells and HIV

NK cells have been shown to be effective in killing HIV-infected cells (Weinhold *et al.* (1988); Malkovsky *et al.* (1988)). However, in early phase HIV infection, there appears to be a decrease in NK cell function, which decreases further with progression to AIDS (Brenner *et al.* (1989); Cai *et al.* (1990)). The defect appears to be in the lytic ability and can be partially restored *in vitro* by the addition of IL-2 (Brenner *et al.* (1989); Bonavida, Katz and Gottlieb (1986)), or IL-12 (Chehimi *et al.* (1992)). Such findings are consistent with the decrease in IL-12 production by macrophages following HIV infection (Yoo *et al.* (1996)), the impaired production of IL-12 from PBMCs of infected individuals (Chehimi *et al.* (1994)) and with Clerici and Shearers' switching hypothesis (see Section 1.6.2.e). NK cells are also important mediators of ADCC (see Section 1.6.2.b).

1.6.2.b ADCC

Many cells capable of cytotoxicity, express membrane receptors for the Fc portion of antibody including: neutrophils, eosinophils, NK cells and monocytes / macrophages. This allows non-specific cells to specifically lyse infected cells, via the release of lytic components. Macrophages and NK cells can also induce apoptosis, or programmed cell death (PCD) of an infected cell by the release of TNF.

1.6.2.b.i ADCC and HIV

ADCC has been shown to be important in the control of viral infections, including retroviral infection in cats (De Noronha *et al.* (1978)). Both NK and neutrophil mediated ADCC has been shown in HIV infection (Lyerly *et al.* (1987); Tyler *et al.* (1989); Szalc *et al.* (1992)). The antibody involved, cytophilic antibody, is generally specific for more conserved epitopes than neutralising antibody (Lyerly *et al.* (1987); Tyler *et al.* (1989)), with important implications for vaccine design. However, the dominant epitope remains controversial, as does the level of ADCC during disease progression (Lyerly *et al.* (1987); Ljunggren *et al.* (1987); Rook *et al.* (1987); Szalc *et al.* (1992)).

1.6.2.c T Lymphocytes

T cells 'see' antigen, in association with the MHC proteins, via their T cell receptor (TCR). MHC proteins are encoded by highly polymorphic genes, hence every individual possesses an almost unique 'fingerprint-like' pattern of MHC proteins (see Section 1.7.3.b). T cells are selected in the thymus early in development, for their ability to recognise the specific MHC pattern of the individual. This 'thymic education' ensures that self reactive T cells are not present, which would otherwise destroy host tissues in a similar manner to that which causes graft rejection in transplant recipients.

Two classes of MHC exist, Class I and II. Class I is encoded for by the A, B and C genes and Class II the DP, DQ, DR genes. Other MHC genes exist, but their function extends beyond the scope of this thesis.

Class I is expressed on the majority of cells and is recognised by T cells expressing the CD8 molecule. In contrast, Class II expression is restricted to B cells, dendritic cells, macrophages, monocytes, epithelial cells and activated T cells and is recognised by CD4⁺ T cells.

1.6.2.d Antigen Presentation

CD4⁺ T cells predominately respond to exogenous antigens presented in conjunction with MHC Class II on the surface of specialised antigen presenting cells (APCs). Class II antigen presentation involves the uptake of exogenous antigen, enzymatic degradation into peptides, which then binds to Class II and is presented on the cell surface. For efficient antigen presentation, co-stimulatory molecules must also interact, such as intracellular adhesion molecule (ICAM-1) and LFA-1. Without these co-stimuli, antigen specific anergy, or loss of responsiveness, can be induced.

Class I presentation involves endogenous antigen within the cell. These are processed into peptides by the cellular proteasome, which are then transported to the endoplasmic reticulum by transporter associated proteins (TAP) and then bind MHC Class I and an additional protein, β 2 microglobulin, before presentation on the surface.

1.6.2.e T helper (TH) cells

CD4⁺ cells interact with MHC Class II and peptide and the relevant co-stimulatory molecule, resulting in the activation of the cell. The activation induces the production of cytokines (low molecular weight messenger peptides), which play an important role in the activation of B cells, CTLs, macrophages, as well as facilitating their own proliferation and maturation. Activated cells clonally expand to produce more effector cells and memory cells; memory cells persist and respond more readily upon re-encounter with the same antigen.






Studies in mice have identified two distinct subsets of T_H cell: T_{H1} and T_{H2} (Mosmann and Coffman (1987)). The subsets differ in their cytokine profiles and the subsequent immune responses they aid, as shown in Figure 1.9. T_{H1} cells produce IL-2, IFN- γ and are associated with cell mediated responses; T_{H2} cells produce IL-4, IL-5, IL-6, IL-10 and IL-13 and are associated with humoral responses. Controversy still surrounds their existence in humans, with T_{H1} and T_{H2} possibly exhibited a chronically stimulated population (Mosmann and Moore (1991)). Under normal stimulation, cells perhaps produce an increased level of one, or more T_{H1}, or T_{H2} cytokines, giving T_{H1}-like and T_{H2}-like profiles (Mosmann and Moore (1991)).

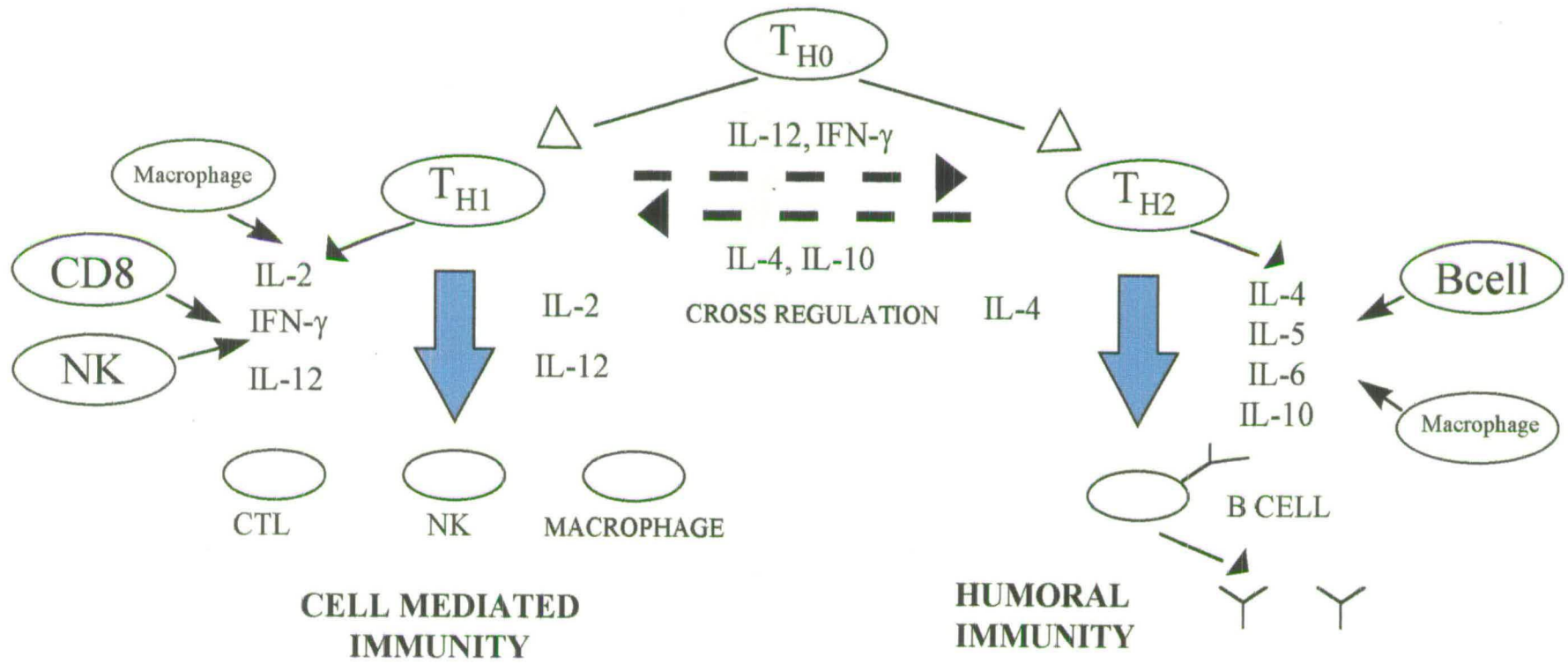
A recent modification to the theory, to incorporate the role of cytokines produced from other cells such as NK cells and macrophages, resulted in the terms 'Type 1' and 'Type 2' responses (Clerici and Shearer (1994)). This nomenclature mostly encompasses the previously described T_{H1} and T_{H2} cytokines, but takes into consideration that they are often

Figure 1.9.

Type 1 and Type 2 Responses

The two subsets of $CD4^+$ cells, T_{HI} and T_{H2} , have a suggested common progenitor, T_{H0} and downregulate each other, via the production of cytokines. The Type 1 cytokines, IL-2 and IL-12, activate and augment the actions of the cells involved in cell mediated immunity (CMI). Type 2 cytokines activate B cells, which then differentiate into plasma cells and produce specific antibody.

Differentiation is indicated by ; secretion is indicated by ;   indicates downregulation; and activation is indicated by .



also produced by other cell types too. A notable exception is IL-12 which is a Type 1 cytokine, not produced by T cells, but produced predominately by monocyte/ macrophages (Chehimi and Trinchieri (1994)).

1.6.2.e.i TH Responses and HIV

It was shown in macaques, using recombinant HIV proteins, that the virus was immunogenic for T cells (Zarling *et al.* (1986)) and this was supported with the finding of HIV-specific T cell responses in HIV uninfected vaccine recipients (Clerici *et al.* (1991); Kovacs *et al.* (1993)). HIV-specific T cell responses in HIV-infected individuals were shown to whole virus, viral antigens and peptides (Kelker *et al.* (1992); Ranki *et al.* (1989); Pontesilli *et al.* (1995); Clerici *et al.* (1993b); Borkowsky *et al.* (1990); Clerici *et al.* (1989a)). However, it has been reported that even before the loss of CD4⁺ T cells in HIV infection, T cell dysfunction occurs (Pontesilli *et al.* (1995); Ranki *et al.* (1989); Clerici *et al.* (1989b); Miedema *et al.* (1988); Teeuwesen *et al.* (1990)). Clerici and co-workers (Clerici *et al.* (1989b)) classified the loss of reactivity, via IL-2 production and proliferation studies, to recall antigens (Flu antigen, tetanus toxoid antigen and later HIV (Clerici *et al.* (1989a))), alloantigens and mitogens (e.g. phytohaemagglutinin, (PHA)). They noted a sequential loss of reactivity *in vitro* first to recall antigens (-/+/+), indicating a loss of MHC self restricted CD4⁺ cell activity; then to alloantigens (-/-/+); and finally to mitogens (-/-/-). Some of the *in vitro* reactivity to recall antigens has been shown to be recoverable in cells from HIV⁺ individuals, by the addition of the cytokine IL-12 and anti-IL-12 antibodies suppressed the responses seen in normal individuals (Clerici *et al.* (1993a)), suggesting a limitation of this cytokine in HIV⁺ individuals.

The cytokine pattern induced in response to infection can greatly influence the disease outcome, as typified by the parasitic infection of leprosy; a strong T_{H1} response is associated with the resistant tuberculoid form and a T_{H2} response characteristic of susceptible lepromatous leprosy (Yamamura *et al.* (1991)). Clerici and Shearer proposed the hypothesis of a T_{H1} → T_{H2} switch occurring during HIV infection, supported by their findings of a loss of type 1 cytokines and an increase in type 2 cytokines concurrent with disease progression (Clerici and Shearer (1993); Clerici and Shearer (1994)). These observations have been verified by some (Diaz-Mitoma *et al.* (1995); Barcellini *et al.* (1994)) and questioned by others (Graziosi *et al.* (1994); Maggi *et al.* (1994)), possibly due to the use of different

protocols (reviewed in Clerici and Shearer (1994)). An alternative hypothesis of a $T_H \rightarrow T_{HD}$ switch was proposed (Maggi *et al.* (1994)). The lack of a predominant T_H response seems to be the consistent factor and is supported with the finding of cell mediated (T_H) responses in potentially protected HIV exposed uninfected individuals (EUs)(see Section 1.7.2).

1.6.2.f Cytotoxic T Lymphocyte cells (CTLs)

CD8⁺ cells recognise peptides in association with MHC Class I and, in order to be activated, also require 'help' in the form of IL-2 from activated T_H cells. Once activated, the CTL lyses the infected cell, via one of several suggested mechanisms (Isaaz *et al.* (1995)). The first involves the release of intracellular granules from the CTL to the target; these contain proteoglycans, various cytokines (e.g. TNF- β), a pore forming protein (perforin) and a family of seven esterases (granzymes A-G). The alternative mechanism is the induction of PCD, or apoptosis, occurring either via signal transduction, or the release of mediators like TNF- β .

1.6.2.f.i CTLs and HIV

HIV-specific CTLs were first identified in the PBMCs of seropositive individuals using recombinant vaccinia viruses (rVV) expressing HIV proteins in autologous Epstein Barr virus (EBV)-transformed B cell lines (BCLs) (Walker *et al.* (1987)) and a chromium (⁵¹Cr) release assay. Reactivity to *env* (Koenig *et al.* (1988); Walker *et al.* (1987); Koenig *et al.* (1988); Koup *et al.* (1989); Lamhamedi-Cherradi *et al.* (1992)), *gag* (Walker *et al.* (1987); Nixon *et al.* (1988); Koup *et al.* (1989); McFarland *et al.* (1993); Lamhamedi-Cherradi *et al.* (1992)) and *pol* (Walker *et al.* (1988); Koenig *et al.* (1988); McFarland *et al.* (1993); Lamhamedi-Cherradi *et al.* (1992)), as well as the regulatory proteins such as *vif*, *nef* and *tat* (Borrow *et al.* (1994); Lamhamedi-Cherradi *et al.* (1992)) have been reported. Hence, CTLs to HIV show a wide degree of reactivity to external and internal proteins, of both a structural and regulatory nature.

CTLs have been detected in peripheral blood, cerebral spinal fluid (Sethi, Näher and Stroehmann (1988)), broncho-alveolar lavage (Plata *et al.* (1987); Autran *et al.* (1995)) and even in the cervical mucosa (Musey *et al.* (1997)). The CTLs exhibit the classic CTL phenotype, CD3⁺, CD8⁺ and MHC Class I restricted. CD4⁺ CTLs have also been reported (Orentas *et al.* (1990)), but their role *in vivo* is uncertain; although they have been reported in other viral infections, such as herpes simplex virus (HSV) (Yasukawa and Zarlign (1984)).

The number of HIV-specific CTLs is often surprisingly high, with the CTLs in PBMCs responding directly *in vitro* without the need for prior stimulation (Hoffenbach *et al.* (1989)).

CTL activity has been monitored in monkeys infected with SIV and the presence and the level of HIV-specific CTL activity has been shown to confer a favourable prognosis (Bourgault *et al.* (1993); Miller *et al.* (1990)). A direct role in containing HIV viraemia was assessed by Castro *et al.* (1992), who *in vivo* depleted the CD8⁺ cell population in two HIV-infected chimps. Following the depletion, virus isolation from the chimps was achieved, an event that had only occurred once in the previous four years. However, HIV/ SIV infections in monkeys, although similar to HIV infection in humans, do have their differences.

The presence of HIV-specific CTLs early in infection have also been shown in humans and correlate to a rapid control of viraemia (Borrow *et al.* (1994); Koup *et al.* (1994)). Generally, HIV-specific CTLs have been interpreted as causing a rapid control of primary infection, slower disease progression and they appear to decline with the loss of CD4⁺ cells and progression to AIDS (Joly *et al.* (1989); Pantaleo *et al.* (1990); Carmichael *et al.* (1993); Ferbas *et al.* (1995); Klein *et al.* (1995)). A direct association of CTL activity with control of viraemia was always difficult to quantify and involved limit dilution approaches, but a novel method was recently reported by Ogg *et al.* (1998). Using tagged MHC proteins binding peptide, they were able to analyse the numbers of antigen specific CTLs using a fluorescent activated cell sorter and confirmed the previously held belief of the inverse correlation of the HIV-specific CTL frequency with plasma viral load. Others have failed to see a correlation of CTL with a lack of progression (Rinaldo *et al.* (1995); Froebel *et al.* (1997)) and a model of HIV dynamics has shown that the control of primary viraemia may not be caused by immune control (Phillips (1996)). However, the detection of HIV-specific CTLs in EUs has added further support to their potential as a protective control mechanism (see Section 1.7.2).

It has been argued that CTLs may have a deleterious effect in HIV individuals, with the detection of CTLs in the cerebral spinal fluid (Sethi, Näher and Stroehmann (1988)) and alveolitis (Autran *et al.* (1995)), thought to be participating in the inflammatory reactions found there. It has also been suggested that CTLs contribute to the decline in CD4⁺ T cells (Grant, Smail and Rosenthal (1994)), but this remains to be clarified.

As with humoral responses, the virus has been shown to mutate to evade the CTL response. These mutants result in a lack of lysis, either by the loss of the critical peptide

sequence, or changes which may effect antigen processing (Phillips *et al.* (1991); Rowland-Jones *et al.* (1992); Couillin *et al.* (1994)). Naturally occurring 'altered peptide ligands' (APLs), which antagonise the normal peptide by competing for MHC binding and can also trigger ineffectual responses, have also been seen in HIV-infected individuals (Klenerman *et al.* (1994); Klenerman *et al.* (1995)). The fact the virus attempts to evade the CTL response suggests it does provide a strong selective pressure against the virus, but the presence of escape mutants does have important implications for vaccine design. A broad, multideterminant response would reduce the risk of escape mutants arising (Borrow *et al.* (1997); Goulder *et al.* (1997)). This is supported by the findings of an HIV⁺ individual who was adoptively transferred a single expanded CTL clone, directed against the NEF protein, and unexpectedly showed a rapid deterioration (Koenig *et al.* (1995)). However, a broader CD8⁺ cell adoptive transfer is being evaluated (Torpey *et al.* (1993)).

1.6.2.g Non-Lytic Suppression

An antiviral effect by CD8⁺ cells was first noted by Walker *et al.* (1986), who demonstrated that the removal of CD8⁺ cells from PBMC cultures allowed the recovery of previously undetectable virus. The factor was soluble, but more efficient inhibition was achieved when direct cell contact was permitted (Walker and Levy (1989)), although this was not MHC restricted (Walker *et al.* (1991)). The effect did not alter CD4⁺ cell activation, but seemed to block HIV RNA expression (Mackewicz and Levy (1992)). The level of activity seems to vary between individuals and its presence was shown to correlate with a slower rate of progression (Mackewicz, Ortega and Levy (1991)). The activity is broadly cross reactive to a variety of strains, including SIV (Mackewicz, Ortega and Levy (1991); Mackewicz and Levy (1992); Walker *et al.* (1991)). African green monkeys (AGM) naturally infected with SIV_{AGM}, which does not lead to an AIDS related illness, have been shown to produce a suppressive factor from CD8⁺ cells, which also inhibits HIV replication (Ennen *et al.* (1994)).

Much controversy surrounds the identity of the factor, but many suggestions have been proposed. IL-16, which is secreted by CD8⁺ cells (Laberge *et al.* (1995)) and thought to bind to CD4 (Cruickshank *et al.* (1994)), was shown to inhibit HIV_{SF2} infection in the CD4⁺ cell line, MT4 (Baler *et al.* (1995)). IL-16 from AGMs was shown to be more effective than

human IL-16. The mechanism of inhibition was unclear, but may function like a neutralising antibody directed against CD4.

The β -chemokines, RANTES, MIP-1 α and β , were isolated from a CD8⁺ cell supernatant, shown to have inhibitory properties on HIV infection in CD4⁺ T cells (Cocchi *et al.* (1995)). Recombinant proteins were shown to induce a dose dependent inhibition and monoclonal antibodies against the three proteins reduced the inhibitory effect of the CD8⁺ cell supernatant. Only M-tropic strains were inhibited and this was explained later by the discovery of the M-tropic virus co-receptor, CCR-5 (see Section 1.5.2), the natural receptor for these ligands. The β -chemokines mediate their inhibition by blocking the co-receptor, CCR-5. The natural ligand for the TCLA co-receptor CXCR-4, SDF-1, blocks infection of TCLA strains of HIV in a similar manner (Bleul *et al.* (1996); Oberlin *et al.* (1996)).

The inhibitory effect of the β -chemokines on infection of CD4⁺ T cells has been confirmed by several groups (Schmidtayerova, Sherry and Bukrinsky (1996); Verani *et al.* (1997)), but their effect on inhibiting infection of macrophages has been seen by some (Verani *et al.* (1997)), but not others (Moriuchi *et al.* (1996); Schmidtayerova, Sherry and Bukrinsky (1996)). One group even showed an enhanced infection in the presence of the β -chemokines (Schmidtayerova, Sherry and Bukrinsky (1996)). The differences may reflect differences in the macrophage cultures, or the viruses used.

It is claimed that the original CD8⁺ T-cell antiviral factor (CAF) is not any known cytokine, including those suggested above (Levy, Mackewicz and Barker (1996)). It is reported to mediate suppression of M and T-tropic strains of virus and inhibit infection of both T cells and macrophages (Levy, Mackewicz and Barker (1996); Moriuchi *et al.* (1996)), unlike the β chemokines (Moriuchi *et al.* (1996); Schmidtayerova, Sherry and Bukrinsky (1996)). The factor, although soluble, also mediates a contact mediated effect, which may differ to that from the soluble factor (Levy, Mackewicz and Barker (1996)).

A precise factor may yet to be elucidated, but the non-cytotoxic nature of this suppression and the discovery of its potential role in protecting EUs (Paxton *et al.* (1996))(see Section 1.7.2), hold great potential as a therapeutic strategy.

1.7. EXPOSED UNINFECTED INDIVIDUALS (EUS)

1.7.1 'Silent Infection' ?

Not everyone exposed to HIV, even those with high risk exposures, becomes infected (Burger *et al.* (1986)), as reflected in the risk values (see Section 1.3.2). Due to the known time lapse that occurs between infection and seroconversion, it was initially feared that many exposed individuals, although seronegative, could be harbouring a 'silent infection' and that initial estimates of transmission may be an underestimate. As the polymerase chain reaction (PCR) first came into use for diagnosis, reports began to appear of 'at risk' individuals who were seronegative, but producing virus in culture and were PCR positive for HIV DNA (Imagawa *et al.* (1989); Pezzella *et al.* (1989); Ensoli *et al.* (1991); Coutlée *et al.* (1994)). Later reports showed this to be extremely rare, with the few exceptions eventually seroconverting (Gibbons *et al.* (1990); Lee *et al.* (1991); Pan *et al.* (1991); Brettler *et al.* (1992); Coutlée *et al.* (1994); MacGregor *et al.* (1995)). Although one report showed a high level of persistently seronegative individuals (Imagawa *et al.* (1989)) and others have reported only transient seropositivity in exposed individuals (Farzadegan *et al.* (1988); Brettler *et al.* (1992)). However, a recent study by Frenkel *et al.* (1998), on reported transient HIV infection in children born to HIV⁺ mothers, highlighted that most were mistaken samples, or contamination, casting doubt on many of the other reports which mostly occurred many years ago (Farzadegan *et al.* (1988); Imagawa *et al.* (1989); Pezzella *et al.* (1989); Ensoli *et al.* (1991); Brettler *et al.* (1992); Coutlée *et al.* (1994)).

The exposed seronegative state may be a transient, or stable state, but if the reports of virus is indeed true, suggests that these individuals may harbour a low level latent infection. It is not however apparent from such individuals if viral clearance is possible.

1.7.2 Immune Responses

The discovery of HIV-specific T cell responses in high risk EUs raised the possibility of exposure and subsequent clearance of HIV infection. Responses to whole virus, viral proteins and viral peptides have been reported by proliferation and IL-2 production in a variety of risk groups: sexual partners of HIV⁺ individuals (Ranki *et al.* (1989); Clerici *et al.* (1992); Kelker *et al.* (1992); Mazzoli *et al.* (1997)); children born to HIV⁺ mothers (Borkowsky *et al.* (1990); Clerici *et al.* (1993b)); and health care workers occupationally exposed to contaminated blood (Clerici *et al.* (1994b)). However, T_H responses can be

generated to viral peptides and proteins and may not reflect actual infection, but merely exposure to viral antigens. Whether these responses will then provide protection is difficult to determine.

Recently, HIV-specific immunoglobulin (Ig)-A was detected in the urine and vaginal washes of heterosexually exposed uninfected females (Mazzoli *et al.* (1997)), suggesting that local mucosal immunity may also play a role in protection from infection with HIV.

CTLs are generated to endogenous antigens and the presence of antigen specific CTLs generally involves at least one round of viral replication. So the discovery of HIV-specific CTLs in a child born to a HIV⁺ mother finally provided some suggestive evidence of infection and clearance of HIV (Rowland-Jones *et al.* (1993)). However, the reactivity was only transient, possibly reflecting the loss of persistent antigenic stimulation. Further reports soon followed of HIV-specific CTLs in exposed children (de Maria, Cirillo and Moretta (1994)), heterosexually exposed individuals (Langlade-Demoyen *et al.* (1994); Rowland-Jones *et al.* (1995)) and even in occupationally exposed health care workers (Pinto *et al.* (1995)).

Protection from infection by HIV-specific immune responses also supports Clerici and Shearers' type 1 → type 2 switching hypothesis (Clerici and Shearer (1993); Clerici and Shearer (1994)) (see Section 1.6.2.e), with EUs potentially retaining a protective type 1 response to HIV and controlling the infection. A study of uninfected high risk IDUs, who appeared to show increased levels of *in vitro* stimulated type 1 cytokines and a decrease of type 2 compared to low risk controls (Barcellini *et al.* (1995)), supports this theory.

It is possible that such EUs were exposed to a defective, or replicatively impaired virus. However, in a group of individuals exposed to a defective virus, with a deletion in the *nef* gene, they still are infected, although they are long term non-progressors (Deacon *et al.* (1995)). The persistently exposed prostitutes (Rowland-Jones *et al.* (1995)) will have undoubtedly been exposed to a variety of isolates, not all of which could have been defective. Also, the recent reports of the clearance of infection in previously infected children born to HIV⁺ mothers suggests that this is possible, although cell mediated responses were not determined in these studies (Bryson *et al.* (1995); Roques *et al.* (1995)).

The protective role of T cell responses is further supported by a study in macaques exposed to a subinfectious dose of SIV (Clerici *et al.* (1994a)). The exposure resulted in virus specific proliferative responses and subsequent protection from lethal challenge. It would seem that the low dose exposure, although not sufficient to induce infection, or seroconversion, was sufficient to induce protective cell mediated responses. Models of infections inferred from

mouse studies, suggest that once a low dose exposure has primed immune responses, infection is unlikely to occur, even with repeated exposure (Salk *et al.* (1993)).

In another macaque study, where a low dose intrarectal exposure to SIV also protected from a high dose challenge, the protection correlated with a CD8-dependent antiviral factor (Salvato *et al.* (1994)). This may be similar to the non-lytic suppressive factor produced by CD8⁺ cells of AGMs following natural infection with SIV_{AGM} and the CAF factor reported by several groups to prevent HIV infection in culture (see Section 1.6.2.g). CD8⁺ cell antiviral activity was found to be elevated in a group of highly exposed homosexual men (Paxton *et al.* (1996)) and found to relate to the levels of the β -chemokines, RANTES, MIP-1 α and β . CD4⁺ T cells and macrophages of two such individuals, EU2 and EU3 (referred to below), were found to resist macrophage tropic virus infection (Connor *et al.* (1996)).

1.7.3 Genetic Factors

1.7.3.a Chemokine Receptor Polymorphisms

1.7.3.a.i CCR-5

EU2 and EU3, along with other EUs were later found to be homozygotes for a 32 base pair (bp) deletion within the coding region of the *CCR-5* gene (Liu *et al.* (1996)). The deletion ($\Delta 32$), in the region corresponding to the second extracellular loop of the receptor, causes a frameshift which results in premature termination of translation just downstream of the deletion site (see Figure 1.10.a). Homozygotes for the mutation do not express the CCR-5 receptor (Liu *et al.* (1996)).

Population studies have shown the mutant allele to occur at a high frequency in Caucasians, but it is absent from all other ethnic backgrounds studied (Liu *et al.* (1996); Samson *et al.* (1996b); Huang *et al.* (1996)). Earlier reports of an allele frequency of 9.2-9.8% in Continental Europeans (Liu *et al.* (1996); Samson *et al.* (1996b)) and 8% in Caucasian Americans (Huang *et al.* (1996)) showed the mutation to be highly prevalent in these populations.

Homozygotes for the deletion ($\Delta 32/\Delta 32$) were found to be highly protected in studies of homosexuals and haemophiliacs (Liu *et al.* (1996); Dean *et al.* (1996); Huang *et al.* (1996)) and have been thought to be uninfected with M-tropic strains of HIV. Table 1.2.a summarises the results of two reports by Dean *et al.* (1996) and Huang *et al.* (1996). The study by Dean *et al.* (1996) is composed of several homosexual and haemophiliac cohorts (see

Figure 1.10.

Diagrammatical Representation (not to scale) of the Chemokine Receptor Mutations.

a.) 32bp Deletion in CCR-5 ($\Delta 32$) - Adapted from Liu *et al.* (1996) and Samson *et al.* (1996)

The diagram shows the nucleic acid sequence (black) of the region flanking the deletion site (blue) along with the corresponding amino acid sequence (pink). The deletion (nucleotide 794 to 825) causes a frameshift during translation (codon 185) which results in a premature termination downstream (* denotes stop codon). The nucleic acid sequence underlined is a 10bp direct repeat thought to be responsible for the deletion.

b.) Valine to Isoleucine Mutation in CCR-2 (64I)

The diagram shows the nucleic acid sequence (black) flanking the G→A (blue) mutation (nucleotide position 190). This results in a valine to isoleucine (V→I) mutation in the amino acid sequence (pink) at position 64 (64I, blue).

A

CCR-5 WT F P Y S Q Y Q F W K N F Q T L K I V I L G L V L P
-TTTCCATACAGTTCAGTATCAATTCTGGAAGAATTTCCAGACATTAAGATAGTCATCTTGGGGCTGGTCCTGCCG-
CCR-5 Δ32 F P Y I K D S H L G A G P A

Δ32 Deletion

CCR-5 WT L L V M V I C Y S G I L K T L L R C R N E K K R
-CTGCTTGTCATGGTCATCTGCTACTCGGGAATCCTAAAACTCTGCTTCGGTGTGCGAAATGAGAAGAAGACG-
CCR-5 Δ32 A A C H G H L L L G N P K N S A S V S K * Stop Codon

B

F V G N M L V V L CCR-2 WT
-TTGTGGGCAACATGCTGGTTCGTCCT-
↑
190
↓
-TTGTGGGCAACATGCTGGTTCATCCT- CCR-2 64I
F V G N M L V I L

Table 1.2.**Genotypes for CCR-5 in Exposed Uninfected (EUs) and HIV⁺ cohorts****A. $\Delta 32/\Delta 32$ Homozygotes in Cohort Studies**

	$\Delta 32/\Delta 32^*$ (%)	Total	$\Delta 32$ Allele Frequency (%)
Dean Paper^{#†}			
EUs	3.0	573	10.6
HIV ⁺ s	0.0	877	10.6
Huang Paper[†]			
EUs	3.6	446	12.8
HIV ⁺ s	0.0	461	10.1
Controls	1.4	637	8.1

B. Effect of Increased Risk in The MACS StudyDistribution of CCR-5 Genotype According to Risk (%)[†]

Genotype [*]	All Caucasian EUs			Followed > 8 years	
	Any Risk	>3 ARI [§]	>6 ARI	>3ARI	>6ARI
WT/WT	78.0	71.7	63.3	67.4	50.0
WT/ $\Delta 32$	18.4	18.2	16.7	20.9	16.7
$\Delta 32/\Delta 32$	3.6	10.1	20.0	11.6	33.3
Total	446	99	30	43	12

* Genotype for the 32bp deletion in the *CCR-5* gene ($\Delta 32$); homozygous wild-type (WT/WT), homozygous mutant ($\Delta 32/\Delta 32$) and heterozygote (WT/ $\Delta 32$).

Dean *et al.* (1996)

† Combined data from the DC Gay cohort (DCG), Multicenter AIDS Cohort Study (MACS, homosexuals), San Francisco City Clinic (SFCC) Cohort (homosexuals), Hemophilia Growth and Development Study (HGDS), Multicenter Hemophilia Cohort Study (MHCS)

† Huang *et al.* (1996)

§ Reported number of partners of whom had anal receptive intercourse (ARI) in preceding 6 months

Table 1.2.a, excluding ALIVE Study data, as predominately non-Caucasians), some of which did not show an effect when considered separately (DC Gay (DCG), Hemophila Growth and Development Study (HGDS)). However, the combined effect, of individuals exposed to HIV who remained uninfected compared to those who became HIV⁺, revealed a substantial difference ($P=5.8 \times 10^{-8}$) in the distribution of genotypes for the $\Delta 32$ mutation when Caucasians only were considered.

The study by Huang *et al.* (1996) on the Chicago Multicenter AIDS Cohort Study (MACS) of homosexuals (Kaslow *et al.* (1987)) was more in depth. It involved more extensive knowledge of the actual exposure of the individuals concerned, in terms of the number of partners with whom they had anal receptive intercourse (ARI) in the preceding six months. With increasing numbers of partners came an increased risk of infection and when graded in this way revealed an even greater outcome of the protective effect of homozygosity for the $\Delta 32$ mutation in CCR-5 (see Table 1.2.b, $P=6.0 \times 10^{-7}$ for those with >6 ARI in last 6 months who had remained seronegative for > 8 years compared to all other seronegative cases).

A few reports of $\Delta 32/\Delta 32$ HIV⁺ individuals have now been seen, although they are extremely rare (Biti *et al.* (1997); Balotta *et al.* (1997); O'Brien *et al.* (1997); Theodorou *et al.* (1997)). It is not clear if these individuals were infected with a CXCR-4 dependent strain, hence removing the requirement for CCR-5, or if the $\Delta 32/\Delta 32$ state is not 100% protective.

HIV⁺ individuals heterozygous (wild type (WT)/ $\Delta 32$) for the CCR-5 mutation have been found to progress to AIDS at a slower rate than WT/WT individuals (Huang *et al.* (1996); Dean *et al.* (1996); Stewart *et al.* (1997); Eugen-Olsen *et al.* (1997); Meyer *et al.* (1997)). Heterozygotes have been shown to express less CCR-5 on the surface of their cells (Wu *et al.* (1997b)), which may result in reduced rate of viral replication. If this is the case it would explain the effect of heterozygosity on progression to AIDS, as early viral load has been shown to be related to the rate of progression (Ruiz *et al.* (1996)). An early report of a decrease number of heterozygotes in the HIV⁺ group, suggesting a mild protective effect from infection (Samson *et al.* (1996b)), was not confirmed by others (Dean *et al.* (1996); Huang *et al.* (1996)), who reported heterozygosity for $\Delta 32$ was not protective following homosexual contact.

1.7.3.a.ii CCR-2

A mutation in the *CCR-2* gene has also been identified (see Figure 1.10.b) (Smith *et al.* (1997)). This is a single point mutation (G→A position 190), which causes a valine to isoleucine amino acid substitution (64I) in the second transmembrane loop region of the receptor (Smith *et al.* (1997)). This mutation results in this domain becoming identical to the corresponding region of *CCR-5*, with which it shares most homology (76%; Figure 1.11). Much of the homology is shared in the transmembrane regions and when these regions are considered alone, the homology increases to 92% (Samson *et al.* (1996a)). The least homology is shared at the amino terminal of the receptors and is reflected in the different ligands which bind the two receptors (see Table 1.1).

CCR-2 occurs as two RNA-splicing variants, *CCR-2a* and *CCR-2b*, which differ in their COOH-terminal regions and may effect signal transduction, but not ligand binding (Steinberg, Crumacker and Chatis (1991); Folks *et al.* (1988)). Envelope chemokine receptor interactions appear not to require the signalling functions of the CCRs (Atchison *et al.* (1996); Rucker *et al.* (1996); Doranz *et al.* (1997)), therefore this difference will not effect HIV binding. *CCR-2* only functions as a co-receptor for a restricted number of viral isolates, in selected *in vitro* systems (Doranz *et al.* (1996); Frade *et al.* (1997)).

The *CCR-2* and *CCR-5* genes are both encoded in chromosome 3 (Baggiolini, Dewald and Moser (1997)) and are very closely located (~17.5bp apart). This perhaps explains why mutations resulting in the $\Delta 32$ and 64I changes are in linkage disequilibrium and are never found together on the same chromosome (Smith *et al.* (1997)). In order that mutations may become freely distributed amongst the population, recombination between two chromosomes needs to occur and as the *CCR-2* and *CCR-5* genes are so closely located, the chance of a recombination event occurring within such a small area is almost impossible. Hence, individuals homozygous for both mutations are never seen and heterozygotes for both (WT/ $\Delta 32$ and 64I/WT) were rarer than if the mutations were randomly associated. Unlike the *CCR-5* $\Delta 32$, the 64I mutation is also present in a variety of ethnic backgrounds (Smith *et al.* (1997)), suggesting that it is a more ancient mutation in the human population (Mummididi *et al.* (1998)).

The amino acid change (V→I) is a conserved mutation and has not shown to effect expression, ligand binding, or usage as a co-receptor (Kostrikis *et al.* (1998)). With all this in mind, it is surprising that an effect of the mutation was seen on progression in a homosexual cohort (Smith *et al.* (1997)), with individuals expressing the 64I mutant form of the receptor



Figure 1.11.

Amino Acid Sequence Alignment for CCR-5/2a and 2b Genes

Adapted from Raport *et al.* (1996) and Baggiolini, Dewald & Moser (1997)

The sequences are aligned with to CCR-5, with identical amino acids denoted with a dot. Dashes signify gaps placed to align the sequences correctly. The putative transmembrane regions (TM1-7) are indicated with a line above the sequence. The valine (V) which is mutated to give isoleucine (I) in some forms of CCR-2 is at position 64 and makes this TM1 region identical to that of CCR-5. CCR-2a and 2b are mRNA splice variants, which are identical until the carboxy terminal end (371 end) at amino acid 319 onwards. The numbering given is essentially for CCR-2a as the CCR-5 and CCR-2 are shorter (CCR-2a: 374 amino acids; CCR-2b: 360 amino acids; CCR-5: 352 amino acids). The frame shift which results from the 32 base pair deletion occurs after the FPY at position 195-7 on diagram (position 182-4 of CCR-5 amino acid sequence) (see Figure 1.10).


```

      1      11      21      31      41      51      61      71      80
      |      |      |      |      |      |      |      |
CCR-5 -----MDYQVSSPIYDINYTSEPCQKINVKQIAARLLPPLYSLVFI FGFVGNMLVILILINCKRLKSMTDI
CCR-2B MLSTSRSRFIRNTNEESGEEVTTFFD.DYGA..H.FD....G.Q.....V.....K..CL...
CCR-2A MLSTSRSRFIRNTNEESGEEVTTFFD.DYGA..H.FD....G.Q.....V.....K..CL...

      81      91      101      111      121      131      141      151      160
      |      |      |      |      |      |      |      |
CCR-5 YLLNLAISDLFFLLTVPFWAHYAAAQWDFGNTMCQLLTGLYFIGFFSGIFFIILLTIDRYLAVVHAVFALKARTVTFGVV
CCR-2B .....L..I.L.L...S..NE.V...A..K.F...H..Y.G.....I.....
CCR-2A .....L..I.L.L...S..NE.V...A..K.F...H..Y.G.....I.....

      161      171      181      191      201      211      221      231      240
      |      |      |      |      |      |      |      |
CCR-5 TSVITWVVAVFASLPGIIFTRSQKEGLHYTCSSHPYSQYQFWKNFQTLKIVILGLVPLLVMVICYSGILKTLRLCRNE
CCR-2B .....L.....V.....KC...DSV.V.GPY..R---G.N..H.IMRN.....I.....
CCR-2A .....L.....V.....KC...DSV.V.GPY..R---G.N..H.IMRN.....I.....

      241      251      261      271      281      291      301      311      320
      |      |      |      |      |      |      |      |
CCR-5 KKRHRAVRLIFTIMIVYFLFWAPYNIVLLNTFQEFFGLNCCSSNRLDQAMQVTETLGMTHCCINPIIYAFVGEKFRNY
CCR-2B .....V.....T.....I.....S..E.TSQ...T.....R.
CCR-2A .....V.....T.....I.....S..E.TSQ...T.....SI

      321      331      341      351      361      371
      |      |      |      |      |      |
CCR-5 LLVFFQKHIA---KRFC-----KCCSIFQOEAPERASSVYT---RSTGEQEISVGL
CCR-2B .S...R...T.....Q.PV.YR.TVDGVT.TN...P.....V.A..
CCR-2A FHIALGCR..PLQ.PV.GGPGVRPG.NVKVTT.GLLDGRGKKSIGRAPEASLQDKE.A

```

showing a slower progression to AIDS. The effect was questioned by another study (Michael *et al.* (1997)) and it now seems that the mutation shows a protective effect only in cohorts where the date of seroconversion is known ('seroconverter cohorts')(Rizzardi *et al.* (1998); Kostrikis *et al.* (1998)). No protective effect against infection has been reported, with WT/64I heterozygotes and 64I/64I homozygous individuals occurring in EU and HIV⁺ homosexuals (Michael *et al.* (1997); Smith *et al.* (1997); Kostrikis *et al.* (1998)).

A possible explanation for how the mutation may effect progression was later revealed by Kostrikis *et al.* (1998), who showed that the 64I mutation was in 100% linkage disequilibrium with a mutation in a putative promoter region of *CCR-5* (C→T at nucleotide 59653 for whole of chromosome 3, also referred to as nucleotide 927 of *CCR-5*; Figure 1.12) (Mummidi *et al.* (1997); Guignard *et al.* (1998)). The mutation is in fact in a putative intron, but it is possible that the *CCR-2* 64I mutation is merely acting as a marker for this, or other mutations, which may effect the level and distribution of expression of *CCR-5*. Several polymorphisms have now been reported in the newly described promoter region for the *CCR-5* gene (Figure 1.12), but their affects on the expression of *CCR-5* and on HIV infection / disease have yet to be truly clarified (Samson *et al.* (1996a); Kostrikis *et al.* (1998); Mummidi *et al.* (1998)).

1.7.3.b HLA

As described earlier, the genes encoding the MHC are highly polymorphic and are critical to acquired cell mediated immune responses (see Section 1.6.2.c). The discovery and definition of the human leucocyte antigens (HLA) came from observations of multiparous women and individuals who had received several blood transfusions. Such individuals harboured antibodies against the 'foreign' antigens which they were exposed to, in a similar manner to that of the ABO blood group antigens. With widespread international collaboration of both knowledge and reagents, the HLA groups were established and have help revolutionise transplantation surgery, previously restricted by massive graft rejection. To date 164 serological and cellular HLA specificities have been defined, including: 28-A, 61-B, 10-Cw, 26-Dw, 24-DR, 9-DQ and 6-DP (Bodmer *et al.* (1997)).

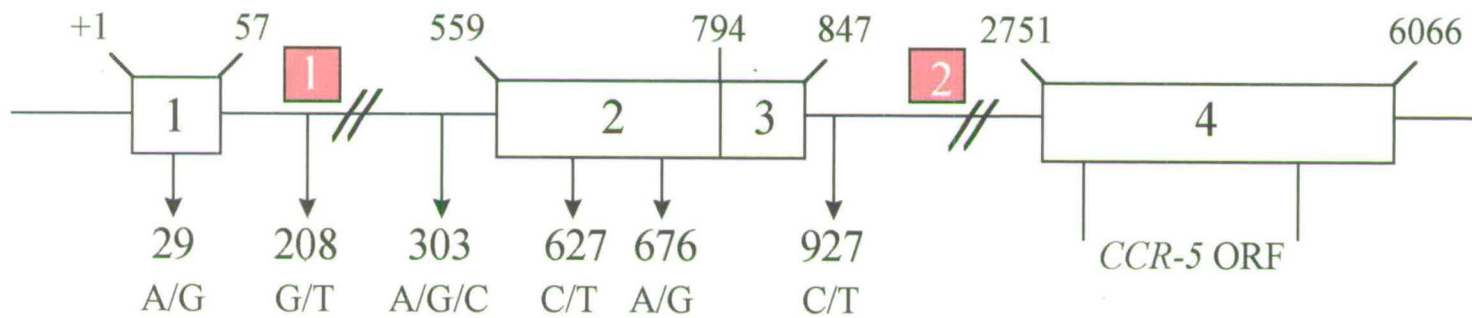
The majority of typing has been performed using serological techniques involving antigen specific anti-sera and complement mediated cell cytotoxicity (Klein (1986a)). If the cells of the individual tested express the antigen to which the antisera is specific for, the antibody will bind the antigen and will activate the complement cascade to form the membrane

Figure 1.12.

Diagrammatical Representation (not to scale) of the *CCR-5* Gene and Promoter.

Adapted from Mummidi *et al.* (1998).

The diagram shows the *CCR-5* locus on chromosome 3. The four exons (open boxes) and two introns (pink boxes) are shown and numbered. Arrows indicate the polymorphisms identified in the promoter region, contained within the region from exon 1 to the end of exon 3. It is unknown if the 927 C→T mutation, out with this region, has any functional role. The *CCR-5* open reading frame (*CCR-5* ORF) is marked in exon 4.



attack complex, which will then lyse the cell. This can be monitored in several ways, but often involves the ^{51}Cr release assay described earlier (see Section 1.6.2.f.i). As the methodology became more advanced, several 'splits' of broader serologically defined HLA types were identified (Hurley *et al.* (1997)). The advent of molecular techniques has seen the use PCR based technologies to further define more and more precise antigens, which will probably replace serological techniques in a few years (Bidwell (1994)).

Every individual possesses two copies of each chromosome, one from each of their parents. Hence, the exact combination of HLA antigens which an individual encodes for in the MHC loci, is defined as the genotype. The genes encoded for by each chromosome, are therefore defined as a haplotype, or half genotype. Several alleles at different loci tend to occur on the same chromosome at a higher than expected frequency due to linkage disequilibrium (see Section 1.7.3.a.ii).

1.7.3.a.i HLA and HIV Progression

Several infectious diseases are known to have HLA associations, either in a protective, or susceptible role and include: hepatitis B and C (Czaja *et al.* (1993); Carbonara *et al.* (1983)), malaria (Hill *et al.* (1991)) and the mycobacterial infections tuberculosis and leprosy (Mehra (1990)). The ethnic and geographical diversity of HLA antigens (Klein (1986b)), was undoubtedly shaped by exposure to the variety of environment exposures including infectious diseases. Individual variation seen in HIV infection and AIDS, lead to investigations of the influence of HLA on HIV infection and disease (reviewed in (Just (1995))).

One of the earliest reports of an HLA haplotype association came from a study of Edinburgh haemophiliacs exposed to Factor VIII contaminated with HIV. Steel and colleagues (1988) noted a strong association of the haplotype A1B8DR3 with rapid progression to AIDS and AIDS related illnesses. Additional studies have also seen associations of A1B8DR3 with rapid progression (Fabio *et al.* (1990); Kaplan *et al.* (1990); Kaslow *et al.* (1990); Mallal *et al.* (1990); McNeil *et al.* (1996)). Other associations have been reported, such as B27 with a slow progression (McNeil *et al.* (1996)) and DR5 with Kaposi's sarcoma (Pollack, Safai and Dupont (1983); Friedman-Kien *et al.* (1982)), but are rarely widely reported due to limited sample sizes, the large number of HLA antigens and ethnic variations which can occur depending on the population studied (Just (1995)).

Kaslow *et al.* (1996) attempted to devise a system by which a variety of genes could be assessed and the combined effects noted. They identified 6 HLA types with an associated decreased time to AIDS (A25, A32, B18, B27, B51 and B57) and 5 and association with a shorter time to AIDS (A23, B37, B49 and the TAP alleles 1400-0101-0503 and 1300-0102-0604 (see Section 1.6.2.d)). These associations were confirmed in a second cohort and proved that HLA haplotype can effect the course of HIV infection and disease.

1.7.3.b.ii HLA and HIV Infection

A1B8DR3 was weakly associated with an increased risk of seroconversion in the Edinburgh haemophiliacs (Steel *et al.* (1988)) and several HLA types, including: B52, B44, Cw4, DR4 and DR6 have been shown to be associated with protection from HIV infection, although none were seen in more than one study (Fabio *et al.* (1990); Fabio *et al.* (1992); Cruse *et al.* (1991)). The lack of a clear association of HLA with protection from infection probably results from the often restricted numbers in EU cohorts and the large degree of potential HLA alleles. HLA discordance between mother and child was also seen to be protective, presumably reflecting an allogeneic infant anti-maternal MHC immune response (MacDonald *et al.* (1998)). The TAP proteins involved in antigen presentation (see Section 1.6.2.d) have also been reported to affect susceptibility, with the variants TAP1.4 and TAP1.4 and 2.3 being increased in exposed seronegative individuals (Detels *et al.* (1996)).

1.8. THE EDINBURGH HETEROSEXUAL COHORT

1.8.1 The Edinburgh Epidemic

Due to a hepatitis B outbreak in heroin users, in 1982, routine blood sampling of IDUs was being carried out in the early 1980s in Edinburgh. With the discovery of a high incidence of HIV seropositivity in Edinburgh IDUs (Peutherer *et al.* (1985)), stored samples were retrospectively tested for HIV antibodies. An alarming 51% of 164 heroin users were HIV⁺, higher than that reported elsewhere in the UK, and the first cases were traced back to 1983 (Robertson *et al.* (1986)). The major factor involved in the rapid dissemination of the virus in this population was thought to be needle sharing, caused by the lack of availability of 'clean' needles at that time (Robertson *et al.* (1986); Brettle *et al.* (1987)).

Later, phylogenetic studies revealed that the majority of individuals were infected with a closely related subtype B virus (Holmes *et al.* (1995); Leigh Brown *et al.* (1997)), presumably reflecting needle sharing networks thought to involve many individuals (Robertson

et al. (1986)). The virus was genetically divergent from that which infected the Edinburgh haemophiliacs, implying different sources of infection (Holmes *et al.* (1995); Leigh Brown *et al.* (1997)). However, virus from heterosexually infected individuals clustered with the IDUs (Holmes *et al.* (1995)), suggesting that they were the source of the heterosexually acquired infection.

1.8.2 The Heterosexual Partner Study Group

The Edinburgh Heterosexual Partner Study was established to monitor factors associated with heterosexual transmission. As the index cases were infected with genetically related viruses (Holmes *et al.* (1995); Leigh Brown *et al.* (1997)), this provided a study group which did not have the added factor of variations (e.g. subtype differences) in the infecting virus, which may also influence transmission as suggested by studies in Thailand (see Section 1.3.4.b).

Individuals were recruited to the study by partner tracing through a general practice, an infectious diseases out patient department and in the home as required. Couples comprised of an index, who was a known infection risk for HIV, i.e. was the HIV⁺ potentially transmitting partner and allowed interview contact with their heterosexual partner (contact). All interviews and recruitment were performed by a qualified research nurse, who was also able to take blood samples when required. This allowed home visits and a level of trust to be obtained which was fundamental to this study, involving often very non-compliant individuals. Interviews with the contact were arranged, or the contact traced by the research nurse, where detailed knowledge of their past sexual behaviour with the index was obtained for the 5 years preceding the interview. An example of the interview used is given in Appendix 1.1 and involved the interviewer asking and completing the form, which ensured continuity.

The sexual behaviour was recorded in yearly intervals and periods of abstinence and condom usage were assessed to deduce the proportion of the year at which unprotected intercourse was occurring. Abstinence included periods of time spent apart (often in prison), or short break-ups of the relationship. An HIV test was offered along with counselling and advice on safe sex and 'at risk' activities was also provided. If discordant for HIV serostatus after testing (contact was HIV-negative), the contact was followed up, by interview, at approximately 6 monthly intervals. During follow-up, the sexual behaviour was re-assessed and an HIV test offered as previously. If the relationship terminated, the contact was also encouraged to attend a final follow-up interview and an HIV test offered several months later.

A detailed analysis of the cohort was performed from October 1987 to April 1993 by Fielding *et al.* (1995). This analysis was a longitudinal approach where the risk of seroconversion over time was modelled and a behavioural and biological profile constructed over yearly blocks. An example of the information considered is given in Figure 1.13 for several couples. The basic information concluded for the study over this time is given in Table 1.3 and includes the number of concordant couples with respect to sex and the length of relationship. A total of 125 couples were assessed at this time and 18 were excluded for either a lack of HIV testing (8 couples), or consistent condom use/ abstinence during their relationship (8 couples). This left a remaining 109 couples which were used in the analysis.

During cross-sectional analysis of the 109 couples, only 'high risk' sexual practises and the length of the relationship were significant risk factors for seroconversion ($P=0.0009$ and $P=0.02$ respectively). 'High risk' sexual practises were ever engaged in anal sex, or experience post coital bleeding for female contacts and sex during menstruation for male contacts, all previously defined risk factors (Royce *et al.* (1997)). In multifactorial cross sectional analysis only 'high risk' sexual practices was significant ($P=0.0009$). Longitudinal analysis attempted to control for the periods and extent of sexual practises and behaviour over the study period, therefore factors such as the age of the contact were not affected, but unprotected intercourse then became a significant factor ($P=0.040$). This is presumably because the periods where the contact was most at risk (immediately following seroconversion of the index) and the frequency of exposure were considered in this form of analysis. The square root of the CD4 count of the index, was also a significant risk factor for seroconversion of the contact ($P=0.033$) and suggests a more advanced stage of disease to be a significant risk factor. The effect of a low CD4 count remained in the multifactorial longitudinal analysis ($P=0.009$). 'High risk' sexual practises also remained a significant factor in both the single factor and multifactorial analysis ($P=0.001$ and $P=0.013$ respectively).

The final recruitment data for the whole study was reported by Robertson *et al.* (1998) and is summarised in Table 1.4. Following exclusion for IDU risk, only 120 discordant contacts remained and some of these individuals had no known 'at risk' exposure, i.e. no periods of unprotected intercourse. Others did not have a sample available for research purposes; either stored, or provided. The precise numbers studied in this thesis vary depending upon the availability of samples. Some of the study required fresh blood samples and was therefore limited to individuals still involved in the study and willing to provided a sample at

Figure 1.13.

Information Determined by Interview from Couples in Heterosexual Partner Study

Adapted from Fielding *et al.* (1995)

Information established from interview and HIV testing on several couples enrolled in the Heterosexual Partner study.

Line indicates the length of the relationship. [indicates the last negative HIV test of the index,] indicates the first positive test for the index and ● indicates the index knowledge of their seropositivity, often after first positive due to retrospective testing of stored samples. { indicates the last negative of the contact and } indicates the first positive of the contact. ▲ marks the initial interview of the contact and ▼ indicates the last follow-up, which sometimes occurred after the relationship had ended.

Couple Number

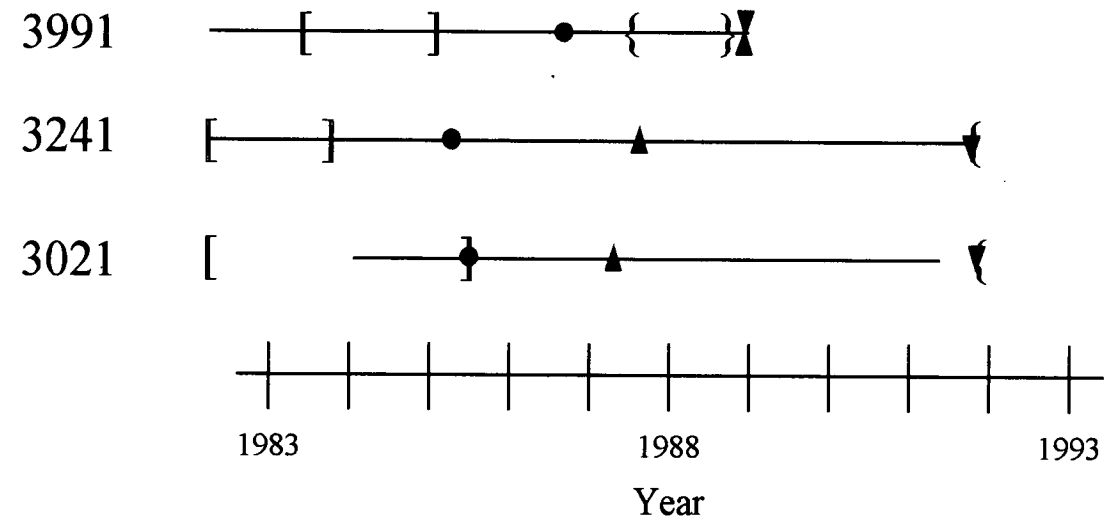


Table 1.3.

Summary of Heterosexual Partner Study Group Data 1987-1993*

	Male	Female
Number of Indexes[‡]	82/105	23/105
Number of Contacts	23/109	86/109
Number of Contacts Concordant[†]		
At recruitment	3/23 (13.0%)	21/86 (24.4%)
Follow-up	1	1
Mean Age of Contact at Recruitment[§] (years(range))	32 (22-51)	26 (17-58)
Median Length of Relationship[§] (months (range))	41.1 (9.4-109.6)	44 (1.8-304.9)
	Concordant	Discordant
Median Length of Relationship[§] (months (range))	47.5 (2.9-133.8)	41.1 (1.8-304.9)

* as published in Fielding *et al.* (1995) for period 1987-1993

‡ Two contacts with the same index were considered as two independent couples

† Contact was concordant for HIV serostatus, i.e. HIV⁺, having acquired the infection from heterosexual contact with the HIV⁺ index.

§ No statistical difference between the two group (Mann-Whitney test)

Table 1.4.**Summary of Heterosexual Partner Study Group Data 1987-1996***

	Male	Female
Number of Indexes[‡]	145/202	57/202
Number of Contacts	66/246	180/246
IDU risk [†]	32/94	62/94
Non-IDU risk	34/152	118/152
Number of Contacts Concordant[#]		
At recruitment	14/66 (21%)	45/180 (25%)
IDU risk	7/32 (22%)	20/62 (32%)
Non IDU risk	7/34(21%)	25/118 (21%)
Follow-up (non-IDU risk)	5 (1)	1 (1)
Mean Age of Contact at Recruitment[§] (years(range))	30 (\pm 6)	29 (\pm 5)
Median Length of Relationship[§] (months (Max))	42 (172)	52 (313)

* as published in Robertson *et al.* (1998) for period 1987-1996

[‡] Two contacts with the same index were considered as two independent couples

[†] Known risk for injecting drug use

[#] Contact was concordant for HIV serostatus, i.e. HIV⁺, having acquired the infection from heterosexual contact with the HIV⁺ index.

[§] No statistical difference whether contact also had IDU risk.

that time. Other sections relied on identifying retrospectively stored samples and was limited by the availability relative to potential clinical uses, which obviously took precedence.

Some of the discordant contacts had high levels of unprotected intercourse, over 1000 'at risk' exposures in some cases, yet they remained uninfected. With the ever increasing reports of factors present in such exposed uninfected individuals, individuals were recruited into a study of host factors of resistance to heterosexual transmission.

1.9. WORK PRESENTED IN THIS THESIS

The aim of this thesis was to investigate a wide variety of factors in the Edinburgh Heterosexual Partner Study Group which may affect heterosexual transmission. Most studies focus on one aspect of immunity, or a particular genetic factor, so it was intended to ascertain the effect of several different aspects in the one cohort. Immune functions investigated included proliferative responses to mitogen, recall and alloantigens, as well as HIV-specific proliferative responses. The cytokine responses to these antigens was also analysed to see if the responses to different antigens differed in EUs compared to that of normal controls. It was also hoped to investigate the presence of HIV-specific CTL responses in the EUs, using naturally infected cells to enable the stimulation of a broad range of responses and not restricting to known CTL epitopes, as if responses were present in the EUs they may differ from those of HIV⁺ individuals.

The effect of HLA type of the risk of transmission was investigated by comparing the antigen frequencies in the EU group to that of HIV⁺ individuals who acquired their infection heterosexually and a group of population controls. The HLA frequencies of the indexes were also compared to assess their effect on transmission, by comparing the indexes who transmitted to their heterosexual partner (Transmitting Index (TRI)) to those who did not transmit (non-TRIs). The level of HLA mismatch between the index and contact was also compared to ascertain if a higher degree of HLA discordance may reduce the risk of heterosexual HIV transmission. This was achieved by comparing the mean level of HLA mismatch in concordant compared to discordant couples with respect to HIV serostatus.

The frequency of the recently described mutations in the chemokine receptors, CCR-2 and CCR-5 (see Section 1.7.3.a), were compared in the EUs to that of heterosexually infected HIV⁺s and population controls, to assess if these mutations had any affect upon heterosexual HIV transmission, in contrast to the known associations in homosexual cohorts (see Section

1.7.3.a). The frequency of these mutations in the TRI and NTRIs was also compared to assess if they influenced transmission too. Recently described polymorphisms in the *CCR-5* promoter region were also investigated to see if they differed in frequency in EUs compared to HIV⁺ and population controls.

Finally, an index and his four heterosexual partners were studied to see if changes in the viral population present in the index may account for differences in transmission, as the first two partners were HIV⁺ and the final two were EUs. It was hoped to infect cells of the EU contacts to ascertain if virus isolated from the index was capable of infecting these and other EUs, or if they were intrinsically protected from HIV infection. The co-receptor usage, SI/NSI phenotype of the index's virus was investigated over time and the degree of relatedness of these isolates deduced by sequence analysis and phylogenetic reconstructions.

The importance of all different aspects with the findings were discussed in the context of heterosexual HIV transmission and related to that of differed populations. Also how contrasting findings from that of homosexual cohorts may be explained by differences in the mode of transmission, highlighting the importance of investigating different risk groups and different populations.

Chapter 2

Materials and Methods

CHAPTER 2 - MATERIALS AND METHODS

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2.1. GENERAL EQUIPMENT AND REAGENTS

2.1.1 Areas of Work

All DNA/RNA extractions, tissue and viral culture were performed in a BioMat Class II Microbiological Safety cabinet (Medical Air Technology, Manchester, UK) under Category II or III safety conditions as appropriate (Butler (1995)).

cDNA synthesis and PCR reactions were performed in a separate area from extractions, with secondary, nested PCRs and gel electrophoresis done in two further separate areas. These procedures were to reduce the risk of nucleic acid contamination (Krogstad and Zack (1995)).

2.1.2 General Supplies

Unless otherwise stated all chemicals were obtained from Sigma (Sigma-Aldrich Company Ltd, Poole, Dorset, UK) and all plasticware was obtained from Costar (Corning Costar, High Wycombe, Bucks, UK) at tissue culture grade. The following list of supplies and equipment were common to most procedures.

Gilsons (P20, P200, P1000, Gilson Medical Electronics, Villiers-le-Bel, France)

Multichannel pipette (25-200 μ l, Anachem Ltd, Luton, Beds, UK)

Pipetboy acu (Integra BioSciences, Letchworth, Herts, UK)

Gilson Tips (1-10 μ l, 10-200 μ l, 20-1000 μ l, sterilised, Alpha Laboratories Ltd, Eastleigh, Hampshire, UK)

Pastettes (1ml sterile, Alpha)

Stripettes (5ml, 10ml, 25ml sterile)

1.5ml screw capped tubes (flat and conical, sterilised, Alpha)

Small and large Treff tubes (500 μ l and 1.5ml, sterilised, Treff Lab[®] from Scotlab Ltd, Coatbridge, Scotland, UK)

Cryotube[®] (1.8ml internal thread sterile, Nunc from Life Technologies, Paisley, Scotland, UK)

Polystyrene, round bottomed tubes (Falcon tubes, 12 x 75mm, Falcon[®], Becton Dickinson (BD), Oxford, UK)

7ml Bijoux (sterile, Bibby Sterilin, Stone, Staffordshire, UK)

100 x 16mm conical tubes (sterile, Bibby Sterilin)

30ml Universals (sterile, Bibby Sterilin)

50ml Centrifuge Tubes (sterile, Corning Costar)

96 well plates (U, V and flat bottomed sterile)

Tissue culture plates (48 well, 24 well and 6 well sterile)

Tissue culture flasks (25, 75, 175 cm² sterile)

Cell Counting Chambers (Bio-Stat Diagnostics, Manchester, UK)

Rose Park Memorial Institute 1640 medium (RPMI, Hyclone, Cramlington, UK and later Sigma)

Dulbecco's Modified Eagle's Medium (DMEM, Hyclone and later Sigma)

Heat inactivated foetal calf serum (FCS, Advanced Protein Products Ltd, Brockmoor, Brierley Hill, West Midlands, UK and later Sigma)

Heat inactivated human AB serum (Scottish National Blood Transfusion Service (SNBTS), Edinburgh, Scotland, UK)

10% RPMI (RPMI medium supplemented with 10% (v/v) FCS, 20µM L-glutamine (HyClone), 50IU/ml penicillin and 50µg/ml streptomycin (GibcoBRL, Life Technologies) (pen/strep/glutamine))

10% DMEM (DMEM medium supplemented with 10% (v/v) FCS, pen/strep/glutamine)

5% AB RPMI (RPMI medium supplemented with 5% (v/v) AB serum, pen/strep/glutamine)

1X phosphate buffered saline (PBS, (10mM phosphate buffer, 2.7mM KCl, 137mM NaCl), Oxoid, Ltd, Basingstoke, Hampshire, UK)

0.1% (w/v) diethyl procarbamine in distilled water (dH₂O) and sterilised (DEPC-treated dH₂O)

Bench-top centrifuge (Centra 3C, IEC from Life Sciences International, Basingstoke, Hampshire, UK)

Microfuge (Biofuge 15, Heraeus, Brentwood, Essex, UK)

Thermal Cycler (GeneE, Techne, Cambridge, UK)

HEPA filtered, CO₂ incubator (37°C, IR incubator, Forma Scientific from Life Sciences International)

Vortex (Vortex Genie, Scientific Industries, NY, US)

Hot Block (BT1 Block Thermostat, Grant Instruments, Cambridge, UK)

Autoclave (Double Entry Herald, Rodwell Scientific Instruments, Bentalls, Basildon, Essex, UK).

2.2. INDIVIDUALS STUDIED

2.2.1 Edinburgh Heterosexual Partner Study

The Edinburgh Heterosexual Partner Study was established to look at factors associated with heterosexual HIV-1 transmission. Further details of the cohort can be found in Section 1.8.

Index patients were assigned a study number prefixed with the letter I, e.g. I3881, and the contact, irrespective of serostatus was assigned the same number prefixed with the letter C, e.g. C3881. Any further contacts of the same index were increased by one, e.g. C3882.

2.2.1.a Exposed Uninfected (EUs) Contacts

Heterosexually exposed individuals were selected for their continued seronegative status, despite high risk exposure to HIV-1. The level of exposure was derived from initial interview data. The number of sexual episodes was deduced from the frequency of 'at risk' sexual activity reported for the 5 years preceding the interview, or the duration of the relationship if shorter than this. Any periods of abstinence during this time were subtracted, as were the number of 'protected' sexual episodes, estimated from the reported frequency of condom usage. Follow up data were analysed similarly and added to the value obtained for the initial interview. This provided an estimated level of exposure in these EU individuals. The number of individuals studied, varied in the different aspects of this thesis and details are given at the relevant sections.

2.2.1.a.i HIV-specific Antibody Testing

Individuals were tested for the presence of HIV-specific antibody using the Abbott IMX HIV-1/2 Third Generation kit (Abbott Diagnostics, Chicago, IL, USA), with positive, or indeterminate results confirmed using the Abbott Biostat HIV-1/2 third generation plus EIA kit. Positive, or indeterminate results from the second kit were confirmed by western blot using Cambridge Biotech Western Blot kit (from Ortho Diagnostics, Raritan, NJ, US). All tests were performed in the Department of Medical Microbiology, University of Edinburgh.

2.2.1.b Concordant Contacts

Contacts (n= 24, 4 male, 20 female) who became HIV⁺ following heterosexual contact were part of the HIV⁺ control cohort, used to assess the effect of genotypic variation in CCR-2 and CCR-5 on heterosexual infection.

2.2.1.c Index Patients

HIV⁺ index patients were divided into those who transmitted virus heterosexually (Transmitting Indexes (TRIs), n=19, 19 male, 3 female) and HIV⁺ partners of the EU individuals (Non-TRIs (NTRIs), n=38, 28 male and 10 female). These individuals were analysed to ascertain if mutations in the CCR-2 and CCR-5 gene had any effect upon the transmission of HIV during heterosexual exposure. One patient (I3151), who had transmitted to his first two partners (C3151, C3152), but not his later two (C3153, C3154), was assessed further to see if variation in the circulating virus had any effect upon the transmission of HIV.

2.2.2 MRC Molecular Epidemiology Repository, Edinburgh

The Repository was established to provide an archive store of patients infected with HIV. Samples were collected from all over Scotland, the North of England and Ireland and PBMCs, serum and plasma stored. Seroconversion dates, if known, were recorded along with details concerning the risk group, country of infection, antiviral therapy history and a recent CD4 count.

Caucasian patients were selected (n=62, 17 male and 45 female) who were infected heterosexually from the Central Scotland area and were used in the HIV⁺ control cohort, used to assess the effect of genotypic variation in CCR-2 and CCR-5 on heterosexual infection. Samples from many of the index patients analysed from the Heterosexual Partner Study were also obtained from the Repository, including Sample 2 and 3 from I3151 studied in Chapter 5 (see Section 5.2.1).

2.2.3 Low Risk Controls

DNA samples (n=50) were kindly provided by Dr. Alan Wright (MRC Human Genetics Unit, Edinburgh, Scotland, UK) from individuals selected for a study of polycystic kidney disease without regard for risk of HIV infection. All were Caucasian in origin and 95% reside in Central Scotland. These individuals were used as a low risk control group in studies of CCR-2 and CCR-5 genotyping.

Samples for infectivity assays, controls for proliferation assays and PBMCs for making phytohaemagglutinin (PHA)-blasts for HIV culture work were obtained from either fellow laboratory workers, not involved in Category III HIV culture work, or from blood donors, kindly provided from the SNBTS. The blood from the SNBTS was either in the form

of blood packs (buffy coats), used for viral culture work, or small samples from plasmapheresis donors for controls in proliferation assays.

2.2.4 Blood Samples

All blood samples were collected in tubes containing anti-clotting agents for collection of PBMCs and plasma. For functional T cell studies, samples were collected in heparin and all remaining samples were collected in ethylenediamine tetraacetic acid (EDTA), with the exception of buffy coats from the SNBTS which were collected with sodium citrate. Serum was obtained from clotted blood. All samples were processed within 12 hours of being taken and PBMCs isolated by density gradient centrifugation.

2.3. ISOLATION OF PBMCs

PBMCs were isolated by density gradient centrifugation (Lymphoprep, Nycomed, Birmingham, UK). 8-15ml of heparinised/EDTA treated blood was carefully layered over an equal volume of warmed Lymphoprep ($\approx 37^{\circ}\text{C}$) and centrifuged at 2 100rpm for 30 minutes. After this time, a discrete band of PBMCs was visible at the interface, which was carefully removed using a pastette and placed into a clean tube. The cells were then washed twice in 15-20ml of 1X PBS by resuspending the cells, centrifuging at 1 500rpm for 10 minutes and discarding the supernatant. Cells were then used for further analysis, or cryopreserved in liquid nitrogen.

2.4. FREEZING AND THAWING OF CELLS

2.4.1 Freezing cells

Viable cells were stored in liquid nitrogen, by resuspending 5×10^6 - 2×10^7 cells in freezing medium (10% (v/v) dimethylsulphoxide (DMSO), 40% (v/v) RPMI medium and 50% (v/v) FCS). The cell solution was quickly placed in internal threaded cryovials (Nunc) and into a freezing box (Nalgene, Hereford, UK). The freezing box is designed to lower the temperature gradually, when placed at -70°C overnight, to increase later viability. Finally, the cells were stored in liquid nitrogen in the vapour phase.

2.4.2 Thawing Cells

Cells were removed from liquid nitrogen and allowed to thaw. When almost melted, an equal volume of 1X PBS was added, left for 15-30 seconds and mixed to dilute the DMSO,

which is toxic to the cells at room temperature. The diluted mixture was then added drop-wise, using a pastette, to 10-20ml of 1X PBS. This was centrifuged at 1 200rpm for 5 minutes to form a pellet and the cells resuspended in the relevant media.

2.5. ESTABLISHMENT OF B CELL LINES (BCLS)

2.5.1 Transformation of PBMCs

PBMCs were resuspended in 2ml of 10% RPMI containing 0.1µg/ml Cyclosporin A (Sandoz Chemicals Ltd (UK), Horsforth, Leeds, West Yorkshire) to inhibit T cell growth and EBV-containing supernatant (see Section 2.5.2) (1:10 dilution). The cells were left for a week at 37°C, 5% CO₂ and then fed twice weekly with fresh media. The cells were fed by removing 1ml of the existing media and adding 1ml of fresh 10% RPMI media. This procedure was repeated until transformation had occurred, usually within 1-2 months. The cell line was then expanded and aliquots frozen (see Section 2.4.1).

2.5.2 Production of EBV supernatant

EBV containing supernatant was obtained from the EBV infected Marmoset cell line, B95-8 (Miller and Lipman (1973)). The cell line was cultured to confluence and starved (i.e. no fresh medium added) for 7 days. The supernatant was then harvested by removal of any cells by centrifugation and the resulting cell-free solution filtered through a 0.45µm filter (Sartorius, Epsom, Surrey, UK) and stored in liquid nitrogen.

2.5.3 Titration of EBV containing supernatant

PBMCs were obtained as described previously (see Section 2.3), from cord blood and incubated at 37°C for 1 hour, in various dilutions of EBV containing supernatant. 6×10^6 cells were incubated in 1.2ml of either neat, 1:10, 1:100, 1:1000 supernatant diluted in 10% RPMI. The cells were then placed in a 96 well U-bottomed microtitre plate in 6 x 200µl aliquots/ dilution, i.e. 1×10^6 cells/ well and left for a week at 37°C. The cells were then fed twice weekly with fresh media for 4 weeks. A 1:10 dilution of supernatant yielded 6/6 transformed wells and was therefore used for future transformations to ensure success.

2.6. CELL LINES AND CELL CULTURE

2.6.1 EBV Transformed BCLs

EBV transformed BCLs were obtained as outlined in Section 2.5. Once transformed, cells were cultured in 10% RPMI at approximately 5×10^5 cells/ml and were split (1:5-10) and fed with fresh media twice weekly, or as required if bulking up cell numbers. Cells were incubated in a moist environment at 37°C in 5% CO₂.

2.6.2 Continuous CD4⁺ Cell Lines

T cell tropic viruses were propagated in continuous CD4⁺ T cell lines. Early viral stocks were made in the cell line C8166 (Lee *et al.* (1984)), which was obtained from the AIDS Reagent Project (NIBSC, South Mimms, Hertfordshire, UK donated by Dr. G. Farrar, CAMR, Porton Down, Salisbury, Wiltshire, UK). C8166 cells are a CD4⁺ human T-lymphoblastoid cell line.

Later, following the discovery of the cell line PM1 (Lusso *et al.* (1995)), which can support the growth of T cell and some macrophage tropic isolates, viral stocks were propagated in this cell line. PM1 cells are a clonal derivative of the HUT-78 cell line, a CD4⁺ human cutaneous T-cell lymphoma and was also obtained from the AIDS Reagent Project (kindly donated by Dr. M. Reitz, Institute of Human Virology, MD, USA).

Both C8166 and PM1 are non-adherent and are cultured in 10% RPMI as for BCLs (see Section 2.6.1). The BCL Preiss (Hurley *et al.* (1982)) was also cultured in the same way.

The promonocytic cell line, U1 (Folks *et al.* (1987)) was obtained from the AIDS Reagent Project (kindly donated by Dr. T. Folks, CDC, Atlanta, USA) to perform sensitivity studies of HIV PCR methods. The U1 cell line is a sub-clone of U937, a human monocyte-like cell from a histiocytic lymphoma, infected with a single copy of integrated HIV DNA per cell and hence was used to determine the end point level of methods used (see Section 2.12).

The promonocytic cell line, U87.MG, transfected with CD4 and also the chemokine receptors, CCR-1/2b/3/5 and CXCR-4 were obtained from the AIDS Reagent Project (see Section 2.6.6).

2.6.3 Primary T cell Lines (PHA blasts)

PBMCs were established as outlined in Section 2.3 and cultured in 10% RPMI with 10µg/ml of the mitogen PHA for 24-48 hours at 37°C, 5% CO₂. After this time, the media

was replaced with 10% RPMI supplemented with 5µg/ml PHA and 10U/ml recombinant IL-2 (AIDS Reagent Project), or 5% (v/v) Lymphocult-T® (LC, Biotest, Shirley, West Midlands, UK). Cells were cultured at approximately 5 x 10⁵ cells/ml, fed twice weekly and split as required. PHA blasts were maintained in this way for 2-3 weeks before being discarded.

2.6.4 CD8⁺ Cell Depletion

CD8⁺ cells were removed from PHA blasts for HIV infectivity studies (see Section 2.9.6) by magnetic depletion. 2 x 10⁷ cells washed twice in 1X PBS, resuspended in 500µl of 1X PBS and placed in a Falcon tube. To this 50µl of washed (1% (v/v) FCS in 1X PBS) anti-CD8 coated Dynabeads (Dyna, Bromborough, Merseyside, UK) were added, mixed and incubated on ice for 30 minutes with mixing halfway. 4-5ml of 1X PBS was added, half of the cell suspension placed in another tube and both tubes topped up. The tubes were then placed on a magnet (Immunotech, Marseilles, France) for 5 minutes and the supernatant carefully removed with a pastette and placed into another tube on the magnet for a further 5 minutes. The supernatant was again carefully removed and the cells pelleted by centrifugation, then washed twice in 1X PBS. The cells were then resuspended in the respective culture supernatant and used accordingly (see Section 2.9.6).

2.6.5 Primary Macrophage Cultures.

PBMCs were separated as described previously (see Section 2.3). Macrophages were isolated by plastic adherence by culturing 5 x 10⁶ PBMCs/ml of serum free Iscove's Modified Dulbecco's media (Gibco BRL) in a tissue culture flask laid flat for 90 minutes. Non-adherent cells were removed and pelleted by centrifugation. Macrophages were then cultured in Iscove's with 5% human serum. For macrophage tropic virus culture, the non-adherent cells were added back with 10µg/ml PHA for two days prior to infection (see Section 2.9.3).

2.6.6 U87-CD4 Cells + Chemokine Receptors

U87.MG-CD4 (U87-CD4) cells are human glioma cells transduced with an amphotrophic retrovirus, expressing CD4 and a neomycin selectable marker (Clapham, Blanc and Weiss (1991)) kindly donated by Dr. P. Clapham. CD4 expression was selected for by geneticin (G418, GibcoBRL) and hence the adherent cell line was cultured in 10% DMEM supplemented with 250µg/ml G418.

The U87-CD4 cell line was then transduced with amphotropic retroviruses encoding the following chemokine receptors, CCR-1/2b/3/5 and CXCR-4, under puromycin selection (Björndal *et al.* (1997)). The chemokine receptor expressing cells were also obtained from the AIDS Reagent Project and were kindly donated by Dr. D. Littman (New York University Medical Center, NY, USA). Chemokine receptor expression was selected for by puromycin resistance, thus, these cells were grown in 10% DMEM supplemented with 250µg/ml G418 and 0.5µg/ml puromycin.

The cells were cultured at 37°C, 5% CO₂ until approximately 80-90% confluent and then split (1:4 for maintenance, less for bulking up) as necessary. The cells were split by removing the media and washing with 1X PBS to remove all traces of serum and then incubated with enough 1X trypsin (0.25% (w/v) GibcoBRL) to cover the cells and left at 37°C for approximately 5 minutes. The trypsin was 'quenched' with excess 1X PBS, the cells pelleted by centrifugation and resuspended in 10% DMEM. The cells were fed 2-3 times a week with fresh media and trypsinised at least every 7-10 days.

2.7. FUNCTIONAL T CELL STUDIES

2.7.1 Lymphoproliferation Assays

Freshly isolated PBMCs were incubated in 5% AB RPMI (1 x 10⁵ cells/well) in a 96 well U-bottomed microtitre plate in the presence of various antigens : the mitogen PHA (5/1µg/ml), mycobacterium purified protein derivative (PPD, 100U/ml, Evans Medical Ltd, Leatherhead, UK), tetanus toxoid (TT, 2.5/1.25µg/ml, Calbiochem-Novobiochem Ltd, Nottingham, UK), a recombinant HIV protein 'cocktail' (1.25/0.625/0.125µg/ml, see Section 2.7.1.a) and Mitomycin C fixed allo-reactive BCLs (allo, 1:1 ratio, see Section 2.7.1.b).

Plates were incubated at 37°C, 5% CO₂ for 7 days, with the addition of 0.1mCi/ml ³H-thymidine (85Ci/mmol stock specific activity, Amersham International plc, Little Chalfont, Bucks, UK) 18 hours prior to harvesting using a Skatron cell harvester (Skatron, Lier, Norway) and 1205/401 filtermats (Wallac UK, Milton Keynes, UK). Prior to harvesting 100-150µl of supernatant was transferred to a new plate and stored at -20°C for later cytokine analysis (see Section 2.7.2). Thymidine incorporation was measured using a Wallac Beta plate counter (1205 Beta Liquid Scintillation Counter) and Stimulation Indices (SI) determined by mean value/ mean background (no antigen added). A value was deemed significant if an SI>2 was achieved. Each antigen was performed in triplicate to quintuplicate (most in the latter) and the geometric mean deduced from these (see Section 2.19)

2.7.1.a Recombinant HIV Cocktail

A cocktail of recombinant HIV proteins was made from the following proteins, all obtained from the AIDS Reagent Project: gp120 from the MN strain derived in Baculovirus, gp120 from the SF2 strain derived in CHO cells kindly provided by Dr. K. Steimer (Chiron Corporation, CA, USA), p24 derived in Baculovirus kindly provided by Dr. I. Jones (Institute of Virology, Oxford, UK), tat, nef and reverse transcriptase (p66) all derived in *E.Coli* (tat - kindly provided by Dr. J. Raina, Agmed Corps, Bedford, MA, USA; nef - kindly provided by Dr. V. Erfle, GSF, Munich, Germany; RT - kindly provided by Dr. D. Stammers, Glaxo Wellcome, Beckenham, Kent, UK).

2.7.1.b Preparation of Allo-Reactive BCLs

The allo-reactive BCLs were a mixture of two cell lines, the Preiss cell line and a line made from an individual of oriental descent, hence less likely to share MHC homology with the Caucasian cohort studied. 5×10^6 cells of each cell line (10^7 cells) was incubated with $100\mu\text{g/ml}$ of Mitomycin C (1ml) in 1X PBS at 37°C for 1-2 hours. The 'fixed' cells were then washed three times in 1X PBS and resuspended to 10^6 cells/ml in 5% AB RPMI.

2.7.2 Cytokine ELISA

Supernatant from proliferation assays was stored at -20°C and then thawed and pooled with other replicates to allow enough volume to assay for the presence of cytokines by enzyme linked immunosorbant assay (ELISA). Genzyme DuoSet IL-4 and IFN- γ ELISAs (Genzyme Diagnostics, West Malling, Kent, UK) were performed essentially as per the manufacturer's instructions. Initial checkerboard plates were performed using recombinant standard cytokines to check the recommended reagent concentrations were suitable.

2.7.2.a IL-4

Plates were coated (Immuno™ plate, Nunc) overnight at 4°C with $100\mu\text{l/well}$ of capture mouse anti-human IL-4 monoclonal antibody ($2\mu\text{g/ml}$; coating buffer - 0.1M NaHCO_3 (pH 9.5)). The following day the plates were washed 6 x $200\mu\text{l/well}$ with wash buffer (1X PBS, 0.05% Tween 20) using a plate washer (Handiwash, Dynatech Laboratories Ltd, Billingshurst, West Sussex, UK), then blocked with $250\mu\text{l/well}$ of blocking buffer (1X PBS, 4% bovine serum albumin (BSA) for 2 hours at 37°C .

The blocking buffer was removed and 100µl/well of sample was added, including diluted standards (8 x 1:2 dilutions from 1500pg/ml rIL-4), and incubated for 1 hour at 37°C. Plates were again washed and 100µl/well of biotinylated polyclonal sheep anti-human IL-4 detection antibody added for 1 hour at 37°C (IL-2 - 1.25µg/ml; IL-4 - 1µg/ml; diluted in 1X PBS, 0.05% Tween 20, 1% BSA). The excess antibody was removed by washing and 100µl/well of horseradish peroxidase (HRP) conjugated to streptavidin (1:1000 in 1X PBS, 0.05% Tween 20, 1% BSA) for 15 minutes at 37°C.

Excess detection reagent was removed by washing and 100µl/well of tetramethylbenzidine (TMB) added for 10 minutes at room temperature. After this time the reaction was stopped with 100µl/well of stop solution (1M H₂SO₄) and the absorbance read at 450nm on a Multiskan Biochromatic spectrophotometer (Labsystems from Life Sciences International). The concentration in each sample was determined by comparison to the standards using Genesis (Genesis, Version 2.12, Life Sciences (UK) Ltd).

2.7.2.b IFN-γ

Plates were coated as for IL-4 (IFN-γ - 1:800). The following day the plates were washed and blocked as before, then samples and standards added (8 x 1:2 dilutions from 1000pg/ml rIFN-γ) and incubated for 2 hours at 37°C. Plates were again washed and 100µl/well of anti-human IFN-γ conjugated to HRP (2µg/ml diluted in 1X PBS, 0.05% Tween 20, 1% BSA) for 30 minutes at 37°C. The excess antibody was removed by washing and 100µl/well of TMB substrate added for 10 minutes at room temperature. The reaction was then stopped with 100µl/well of stop solution and the absorbance read at 450nm of a spectrophotometer. The results were processed as outlined for IL-4.

2.8. FLOW CYTOMETRY AND IMMUNOFLUORESCENT MICROSCOPY

2.8.1 FACScan™ Analysis

All flow cytometry work was performed by the use of a FACScan™ (BD) using Lysis™ II software (BD). Stained cells were resuspended in 300-500µl of 1% (w/v) paraformaldehyde (PAF) and stored in the dark at 4°C for not more than 24 hours after staining.

All staining was performed using directly conjugated monoclonal antibodies (BD, unless otherwise stated) with either fluorescein isothiocyanate (FITC) for single staining, or

FITC and phycoerythrin (PE) for double staining. Live cells were gated for analysis as determined by forward and side scatter sizes.

Approximately 2×10^5 cells added to a Falcon tube. The cells were washed twice in 1X PBS by centrifugation and the pellet resuspended in 100 μ l of 1X PBS. To this 3 μ l of the respective antibodies were added, including the control (anti IgG2-FITC; anti IgG1-PE) and incubated at room temperature for 15 minutes in the dark. Excess antibody was removed by washing three times in 4-5ml of 1X PBS and finally the pellet resuspended in 300-500 μ l of 1% PAF.

Monoclonal antibodies used for staining were against CD3-FITC: CD4-PE, CD3-FITC: CD8-PE, CD45-FITC:CD14-PE (leucogate control), HLA-DR-FITC, CD45RO-PE (BD).

2.8.2 Immunofluorescent Staining of HIV-infected Cells

To determine HIV infection in endpoint dilution experiments (see Section 2.9.5) the FITC conjugated monoclonal antibody, KC57 (Coulter, Luton, Beds, UK), was used on permeabilised fixed cells and visualised under an UV immunomicroscope (Nikon phase contrast 1.25UV photomicroscope). KC57 reacts to the core proteins of HIV, including p24. Cells were scored as positive, or negative, with the degree of staining being irrelevant and the uninfected cell were always co-stained to ensure no non-specific staining had occurred.

2.8.2.a Fixing and Permeabilisation

Cells were removed from the culture supernatant by centrifugation and washed twice in 1X PBS. The cells were rendered permeable by resuspending the cell pellet in 50 μ l of PBS:dH₂O (4:6) and 15 μ l of the resulting cell suspension was spotted onto a PTFE coated multispot microscope slide (Hendley, Loughton, Essex, UK). The slide was then dried in a Class I/II hood for 45-60 minutes. The cells were then fixed in methanol: acetone (1:1) for 10 minutes at room temperature. Slides were then either stored at -20°C, or stained immediately. If stored at -20°C, slides were warmed to room temperature prior to staining. Fixed cells could also then be removed from the Category III.

2.8.2.b KC57 Staining

15µl per well of diluted KC57:FITC (1:150 in 1X PBS) was spotted onto the slides and incubated in the dark for 30 minutes at room temperature. The antibody was then rinsed off with 1X PBS and washed for 30 minutes in 1X PBS in a staining rack. Slides were then blotted dry and coverslips added (22 x 64mm, BDH, Lutterworth, Leicestershire, UK) after the addition of Mowiol (see Section 2.8.2.c). After storing overnight at 4°C in the dark, the slides were examined under a Nikon phase contrast 1.25UV photomicroscope with a filter to monitor green fluorescence and using a 50X water immersion UV lens. Photographs were taken with a Nikon FX 3A camera using 1600 ASA colour negative film.

2.8.2.c Preparation of Mounting Medium

2.4g of Mowiol-4-88 was mixed in 6ml of glycerol, then 6ml of dH₂O for 2 hours at room temperature. Finally, 12ml of 0.2M Tris/HCl (pH 8.5, ICN, Biomedicals Ltd, Thame, Oxfordshire, UK) was added, mixed and incubated at 50°C until dissolved. The solution was then stored at -20°C until use, where 2-3 grains of 1,4 diazabicyclo(2,2,2)octane was added as an anti-fade agent.

2.9. HIV VIRUS CULTURE

All HIV culture work was performed under Category III conditions in a Class II Hood (see Section 2.1). Viral cultures were assayed to be positive by either p24 ELISA or reverse transcriptase activity (see Section 2.10). High titre viral stocks were then made by successive culturing and quantified by 50%-tissue culture infectious dose (TCID₅₀), i.e. the dose of virus that will infect 50% of cells in the PM1 cell line, which allows both infection of T cell line adapted strains and some macrophage tropic strains.

2.9.1 HIV Virus Strains Used

The strains of virus used were two TCLA, SI strains: HIV_{C98}, HIV_{IBB} and two M-tropic, NSI strains: HIV_{BaL} and HIV_{139.6b}. HIV_{IBB} (Antoni, Stein and Rabson (1994)) was obtained from the AIDS Reagent Project (kindly donated by Dr. R. Gallo, National Cancer Institute, MD, USA and Dr. M. Popovic Institute of Human Virology, MD, USA) and was the original isolate of HIV-1. HIV_{C98} is an isolate derived from an Edinburgh haemophiliac patient and is now available from the AIDS Reagent Program (called HIV_{EHC98}).

HIV_{BaL} was also obtained from the AIDS Reagent Project (donated from the NIH AIDS Research and Reference Reagent Program, USA) and was originally derived from human infant lung tissue (Gartner *et al.* (1986)).

HIV_{139.6b} cloned virus, with a patient derived *env* gene cloned in HIV_{BXB2} background. The *env* gene was derived from an Edinburgh haemophiliac patient and was made by Dr. Sarah Ashelford (Ashelford (1996) PhD thesis).

2.9.2 Primary Isolations

Several attempts, by different methods, were tried to isolate virus from the index, I3151, studied in Chapter 5 (see Section 5.2.1)

2.9.2.a PHA blast co-culture

PHA blasts were produced from donor cells (see Section 2.6.3) and then co-cultured with PBMCs from an HIV⁺ individual in 10% RPMI with 5µg/ml PHA and 10U/ml rIL-2, at an approximate ratio of 1:2 HIV-infected PBMCs: PHA blast. The culture was fed twice weekly with fresh 10% RPMI with IL-2 and samples of culture supernatant stored at -70°C and tested for p24 antigen (see Section 2.10.2) and RT activity (see Section 2.10.3). Fresh PHA blasts were added as required, approximately once a week.

2.9.2.b PM1 cell co-culture

PBMCs from an HIV⁺ individual were co-cultured with the cell line PM1 (see Section 2.6.2) at an approximate ratio of 3:1 PBMC:PM1 in 10% RPMI with 5µg/ml PHA and 10U/ml IL-2 and incubated at 37°C and 5% CO₂. The culture was fed twice weekly with fresh 10% RPMI and split as required. Samples of culture supernatant were taken and stored outlined above (see Section 2.9.2.a).

2.9.2.c Isolations from Plasma

5 x 10⁶ CD8⁺ depleted PHA blasts (see Section 2.6.4), or 1 x 10⁶ PM1 cells (see Section 2.6.2), were incubated for 1 hour at 37°C with 0.5 ml of plasma from an HIV⁺ individual. Then the volume was adjusted to 4-5ml with 10% RPMI with 5µg/ml PHA and 10U/ml rIL-2, transferred to a 6 well plate and incubated at 37°C and 5% CO₂. Fresh media was added twice weekly by removal of 1ml and addition of fresh and further CD8⁺ depleted

cells were added after a week and samples of culture supernatant stored as outlined above. PM1 cultures were split as required.

2.9.2.d U87-CD4-CCR-5 Cells

U87-CD4-CCR-5 cells were seeded in 6 well plate (see Section 2.6.6) in 10% DMEM and until approximately 60-70% confluent, then either 1ml of, or $2-3 \times 10^6$ PBMCs, or plasma from an HIV⁺ individual were added in 10% DMEM with 5µg/ml and 10U/ml rIL-2. The plasma was left for 1 hour at 37°C, then the final volume adjusted to 5ml; 10% DMEM for plasma culture and 10% DMEM with 5µg/ml PHA and 10U/ml rIL-2 for PBMC culture. Cultures were fed twice weekly with fresh media and samples stored and tested as outlined above (see Section 2.9.2.a).

2.9.3 HIV_{BaL} Viral Stocks

HIV_{BaL} stocks were made in primary macrophage cells (see Section 2.6.5). Macrophages were isolated by plastic adherence and non-adherent cells were removed then added back with 10µg/ml PHA. After 2 days, the culture supernatant was removed and 2-3mls of virus containing supernatant was added to the macrophages and incubated at 37°C for 1 hour. The original supernatant, containing the PBMCs, was then added back and incubated for 7 days. At 7 days post infection, half the volume of supernatant was removed and replaced with fresh 5% normal human serum supplemented Iscove's and cultured for a further 7 days. At 14 days post infection, the supernatant was removed and clarified by centrifugation at 2000rpm for 10 minutes. Aliquots of the virus containing supernatant were stored at -70°C and tested for the presence of p24 (see Section 2.10.2) and later the TCID₅₀ (see Section 2.9.5) obtained.

2.9.4 Other Viral Stocks

All the other viral stocks for use in an infectivity assay (see Section 2.9.7) were produced by co-culture of virus containing culture supernatant with the PM1 cell line (see Section 2.6.2). The TCLA strains HIV_{C98} and HIV_{MB} were previously cultured in C8166 cells to obtain a high titre stock. The method is analogous to that described for PM1 cells.

Approximately 5×10^6 cells were pelleted and resuspended in 0.5-1ml of virus containing supernatant for 1 hour at 37°C. After this time the cells were washed in 1X PBS and resuspended in 10% RPMI and incubated at 37°C, 5% CO₂ for 2-3 days. The cells were

then fed with fresh 10% RPMI and a sample of the discarded media stored for and RT testing (see Section 2.10.2 and 2.10.3). This was repeated 2-3 days later, until visual signs of syncytia were seen for the TCLA strains and for approximately 14 days for NSI strains. The cells were then co-cultured with fresh cells at a ratio of approximately 1:3-5 cells from the viral culture: uninfected cells and treated as above. After 2-3 co-cultures, the culture supernatant was harvested as outlined for the HIV_{BaL} stocks. When high p24 counts were obtained, the TCID₅₀ of the stock was determined (see Section 2.9.5).

2.9.5 TCID₅₀ Quantitation in PM1 Cells

Due to the variability of infection in PHA blasts from different donors and the inability to infect many CD4⁺ T cell lines with macrophage tropic viruses, the cell line PM1 was chosen to perform repeatable quantitation studies and could be used for all viral isolates used. The method used was based on that by Johnson and Byington (1990).

PM1 cells were prepared at 4×10^5 cells/ml in 10% RPMI. 133 μ l of 10% RPMI was added to the first six wells of a 96 well U-bottomed microtitre plate (only the central 60 wells were used and one plate per isolate) and 150 μ l to the rest. 67 μ l of virus containing supernatant was added to the first six wells (1:3 dilution, sextuplicate) and mixed using a multichannel pipette. 50 μ l of this was transferred to the next row of wells and the processes repeated, until the final row where 50 μ l was discarded. 50 μ l of the PM1 cell suspension was added (2×10^4 cell/well) making a 1:4 dilution of the viral stock in row 1, 1:16 in row 2, etc. The plate was then incubated in a humidified environment at 37°C, 5% CO₂ for 4 days,

On day 4, the wells were mixed and 100 μ l removed and replaced with fresh 10% RPMI. The plate was fed with fresh media in this way twice weekly. On day 14 the cells were fixed and stained for p24 by immunofluorescent microscopy as outlined in Section 2.8. Wells were scored as either '+' or '-', with the degree of staining considered irrelevant. The TCID₅₀ was calculated by the method of Reed and Muench (Dulbecco (1988)).

2.9.6 Co-receptor usage of viral stocks

To determine the co-receptor usage by the viral stocks used for the infectivity assay (see Section 2.9.7), the U87-CD4 cells transfected with the various chemokine receptors (see Section 2.6.6) were infected at a multiplicity of infection (m.o.i) of 0.01. The m.o.i relates to TCID₅₀ units (see Section 2.9.5) / cell, hence a m.o.i of 1, is 1 TCID₅₀ unit/ 1 cell. Cells were seeded overnight in 6 well plates (3×10^5 cells/ well) in 3 ml of 10% DMEM + 250 μ g/ml

G418 and 0.5µg/ml puromycin (except CD4 only cells which were without puromycin). The next day the media was removed and 1ml of diluted virus stock added (in 10% DMEM + G418), resulting in a m.o.i. of 0.01 and incubated at 37°C for 2 hours and then a further 1ml of media added and incubated overnight. The following day the supernatant was removed and the cells washed with 1X PBS to remove any unbound virus and 3ml of fresh media added and incubated at 37°C, 5% CO₂. Supernatant was removed at day 5 and 8 of infection and replaced with fresh media. The cultures were terminated at day 8 of infection. P24 production was monitored by ELISA (see Section 2.10.2) and each sample tested in duplicate and the final value represented by the average of these two values and the average of the two experiments, as each experiment was performed in duplicate.

2.9.6 Infectivity Assay of PHA blasts

Cyropreserved PBMC samples were thawed (see Section 2.4.2) and cultured in 10% RPMI with 10µg/ml PHA for 48 hours at 37°C and 5% CO₂ at approximately 3-5 x 10⁶ cells/ml. The cells were then pelleted by centrifugation and half the media replaced with fresh 10% RPMI with 5µg/ml PHA and LC added to a final concentration of 5% and incubated. Following a further 48 hours, fresh media was added by removal of half of the supernatant and addition of fresh 10% RPMI + 5µg/ml PHA + 5% LC. The volume was increased if the culture growing well to expand the cell yield further.

After a total of 8 days of culture (6 days after the addition of LC), the cells were counted and CD8⁺ depleted by magnetic depletion (see Section 2.6.4). Following depletion the cells were resuspended in media (10% RPMI + PHA + LC) at 6 x 10⁵ cells/ml and left at 37°C for an hour to recover. The cells were then seeded at 3 x 10⁵ cells/ well (500µl) in a 48 well plate and 500µl of the appropriate virus dilution added. Viruses were diluted to obtain a m.o.i. of 0.001, with HIV_{BaL} at both 0.01 and 0.001. The viruses used were HIV_{IIIb}, HIV_{C98}, HIV_{BaL} and HIV_{139.6b} (see Section 2.9.1).

At day 3, 6, 9, 12 and 15 post infection, 300µl of supernatant was removed and stored at -70°C for p24 analysis (see Section 2.10.2). 300µl of fresh 10% RPMI + PHA + LC was added at day 3 and 9 and 350µl at day 6 and 9 (to account for evaporation); the cultures were terminated at day 15 post infection.

2.10. HIV DETECTION ASSAYS

2.10.1 Immunofluorescent Staining

HIV infection in the PM1 TCID₅₀ assay (see Section 2.9.5) was determined by immunofluorescent microscopy using an anti-p24 monoclonal antibody as outlined in Section 2.8.2.

2.10.2 p24 ELISA

Based on an assay developed by Dr. W. James (Sir William Dunn School of Pathology, University of Oxford, UK) as provided by the AIDS Reagent Project and quantifies the amount of the core gag protein, p24, in the supernatant of HIV-infected cells relative to a standard curve of recombinant p24 protein.

2.10.2.a Coating of Plates

MicroELISA strip plate-8 immunosorbant strips were coated with 100µl/well affinity purified sheep anti-HIV-1 p24 (D7320, 1:100 in 150mM NaHCO₃ (pH 9.0), Aalto BioReagents Ltd, Dublin, Eire) and left overnight. The plate was washed 6 x 200µl/well with a 1X TBS (0.144M NaCl, 25mM Tris-HCl (pH 7.5)) using a plate washer. Once the final wash had been done, excess liquid was removed by banging the plate upside down on tissue.

The wells were then blocked by the addition of 200µl/well of a milk solution (2% (w/v) Marvel in 1X TBS) for 30 minutes at room temperature. This was then removed and the plates dried in at 37°C. The plates were then sealed into plastic bags with silica gel sachets and stored at -20°C until use.

2.10.2.b ELISA

The anti-p24 coated strips were removed from the -20°C freezer and allowed to thaw to room temperature. Then the blocking protein was removed by washing six times in 1X TBS.

Whilst the plate was thawing, the virus in the samples was lysed to release the internal p24 protein and inactivated by incubation at 56°C for 30 minutes. This was done by the addition of 1% (w/v) Empigen solution in 10% RPMI at a 1:10 dilution, i.e. final concentration of 0.1% Empigen in 10% RPMI, or the relevant culture medium. The samples were diluted, if necessary in 0.1% Empigen in 10% RPMI and placed 100µl/well in the washed plates. p24 standards (recombinant p24 obtained from the AIDS Reagent Project, see

Section 2.7.1.a) were also added in $\frac{1}{2}$ log dilutions (1000, 316, 100, 31.6, 10, 3.16, 1 and 0 ng/ml), and one well was left empty as a blank. The plate was sealed using a plate sealer and incubated overnight at room temperature.

Next day, the plate was washed six times as before, but this time in 0.05% (v/v) Tween 20 in 1X TBS and blotted dry. Then 100 μ l/well of biotinylated mouse anti-p24 monoclonal antibody (1:1000 in 1X TBS, 20% (v/v) FCS, 0.05% (v/v) Tween 20, AIDS Reagent Project, kindly donated by Dr. W. James, Sir William Dunn School of Pathology, Oxford) to all wells except the blank. The plate was sealed and incubated for 2 hours at room temperature, then washed six times in 0.05% (v/v) Tween 20 in 1X TBS.

100 μ l/ well of Extravidin-alkaline phosphatase (1:4000 in 1X TBS, 0.05% Tween 20) was then added to all but the blank and incubated at room temperature for an hour. The plate was then washed as before in 0.05% Tween 20 in 1X TBS and 100 μ l/well of TMB substrate added to all wells, including the blank. After 15 minutes the reaction was stopped with 50 μ l/well of 2N sulphuric acid and the optical density read at 450nm on a spectrophotometer (see Section 2.7.2.a).

2.10.3 Reverse Transcriptase (RT) Assay

The presence of p24 protein does not prove the presence of virus, therefore to confirm this an RT assay was performed. Reverse transcriptase is a virally encoded enzyme that converts ssRNA to dsDNA. The following assay provides all the materials to perform this reaction, apart from the enzyme. If HIV RT was present dsDNA would be formed including radioactively labelled ^3H -Thymidine (^3H -TTP), which can be then monitored on a beta plate counter.

A 5X RT mix (750mM KCl, 50mM MgCl₂, 50mM Tris-HCl (pH 8.0) (ICN), 2.5mM ethylene glycol tetraacetic acid, 0.5% (v/v) Triton X-100, 125 μ g/ml BSA, 10% (v/v) ethane diol) was made and stored in aliquots at -20°C until required. A 2.5X RT mix was then made by the addition of 10mM dithiothreitol (DTT), 75 μ g/ml polyA.oligo dT (12-18, Pharmacia, St Albans, Herts, UK), 0.1 μ Ci/ μ l of ^3H -TTP and DEPC-treated dH₂O. 10 μ l of 2.5X RT mix was then added to a 96 well V-bottomed microtitre plate with 15 μ l of culture supernatant, yielding a 1X RT solution. The plate was sealed and incubated at 37°C for 48 hours.

Any DNA produced was then precipitated by the addition of 150 μ l/well of 10% (w/v) trichloroacetic acid (TCA) with 50 μ g/ml yeast RNA and incubated on ice for 15 minutes. The DNA was then harvested onto DEAE impregnated mats (1205/405, Wallac) using a Skatron

cell harvestor. The first wash was 5% (w/v) TCA, then 3% (w/v) sodium pyrophosphate and finally 70% (v/v) ethanol. The filter mat was dried in the microwave and placed in a bag with liquid scintillant (Betaplate Scint, Wallac) and sealed. ³H activity was then measured using a beta plate counter in counts per minute (cpm). Samples were performed in triplicate or quintuplicate and the mean and standard deviation determined. A sample was deemed RT positive and hence virus positive if the mean was at least twice the value of the negative control (HIV free cell culture supernatant).

2.11. EXTRACTION

2.11.1 Phenol/ Chloroform DNA Extraction

DNA was extracted from PBMCs, or BCLs essentially as outlined previously (Simmonds *et al.* (1990a)). Cells were pelleted (approximately 5×10^5 - 1×10^7 cells) and incubated in 400µl of TNE buffer (0.1M NaCl, 50mM Tris-HCl (pH 8.0), 1mM EDTA (pH 8.0)) with 0.5% (w/v) sodium dodecyl sulphate (SDS), 1mg/ml proteinase K (Boehringer Mannheim Ltd, Lewes, East Sussex, UK) and 40µg/ml poly A (Boehringer Mannheim) for 2 hours at 37°C. The resulting viscous solution was pipetted repeatedly through a fine tip, helping to shear the DNA. 450µl of TE (10mM Tris-HCl (pH 8.0), 1mM EDTA)-saturated phenol was added, vortexed three times extensively and centrifuged for 15 minutes at 13 000rpm.

The upper aqueous layer was transferred to a clean tube containing 450µl of phenol/ chloroform (1:1) and 75µl of TNE buffer with 0.1% (w/v) SDS, taking care not to disturb the interface. This was vortexed vigorously and centrifuged at 13 000rpm for 10 minutes. The upper aqueous layer was again removed and transferred to a fresh tube containing 450µl chloroform/ isoamylalcohol (50:1), vortexed and centrifuged at 13 000rpm for 10 minutes. Following removal of the aqueous layer to a clean tube, containing 40µl of 3M sodium acetate (pH 5.2), 800µl of chilled ethanol (-20°C) was added, mixed and incubated overnight at -20°C, or at -70°C for 30 minutes.

Nucleic acid was collected by centrifugation at 13 000rpm for 30 minutes and the resulting supernatant discarded. The pellet was washed in chilled 80% (v/v) ethanol (-20°C) and then dried on a hot block at 40°C for 15-20 minutes. Finally, the nucleic acid was resuspended in 30-50µl of DEPC-treated dH₂O. To ensure thorough mixing of the DNA, this was then heated at 68°C for 10 minutes and then pipetted up and down several times to shear the DNA.

2.11.2 Estimating DNA Concentration

5 μ l of the resulting DNA solution was added to 700 μ l of DEPC-treated dH₂O and the absorbance recorded on a spectrophotometer (CE 594, Cecil from Jencons (Scientific) Ltd, Leighton Buzzard, Bedfordshire, UK) at 260 and 280nm. The ratio of the absorbance at 260:280 determines the purity of the DNA and should be greater than 1.5. The absorbance value obtained at 260nm can then be used, with the following formula, to estimate the concentration of DNA in the sample.

Concentration of DNA in sample (μ g/ μ l) = Absorbance 260nm x dilution factor (e.g. 140) x 50 (1 OD₂₆₀ unit \equiv 50 μ g/ml of DNA) \div 1000 (to convert to μ g DNA / μ l).

Therefore for an absorbance value of 0.100 at 260nm, the concentration of DNA = 0.100 x 140 x 50 \div 1000 = 0.7 μ g of DNA / μ l. The concentration of samples was then adjusted so that they were approximately the same and divided into several smaller aliquots before storage at -70°C.

2.11.3 Plasma DNA Extraction

The low concentration of DNA in plasma and the lack of other available samples, required an alternative, more sensitive method of extraction. The protocol used is essentially as described by Boom *et al.* (1990) and relies on the ability of silica to bind nucleic acid.

200 μ l of plasma was incubated with 900 μ l of warmed Lysis buffer (10M Guanidinium thiocyanate (GuSCN, Fluka Chemicals, Gillingham, Dorset, UK), 0.1M Tris-HCl (pH 6.4), 35mM EDTA, 0.02% (v/v) Triton X-100) and 100 μ l of silica coarse (60g silica oxide/ 500ml dH₂O, pH 2.0, vortexed prior to use), mixed and left at room temperature for 10 minutes, mixing every two. The silica-DNA complex was then collected by centrifugation at 1 500rpm for 2 minutes and the supernatant discarded. The pellet was washed twice in Wash buffer (1ml 10M GuSCN, 0.1M Tris-HCl (pH 6.4)) by vortexing, centrifuging at 10 000rpm for 15 seconds and then discarding the supernatant. Further washes were performed in the same manner, but twice with 70% (v/v) ethanol and once with acetone.

The pellet was then dried at 56°C for 10 minutes and the nucleic acid eluted from the silica using 10-20 μ l of DEPC-treated dH₂O, followed by incubation at 56°C for a further 10 minutes. The silica was then removed by centrifugation at 10 000rpm for 2 minutes and the DNA/RNA containing supernatant transferred to a clean tube and stored at -70°C until use.

2.11.4 Plasma Viral RNA Extraction

Viral RNA was extracted from plasma by a different GuSCN based extraction method modified from the Stratagene Micro RNA isolation kit (Stratagene, Cambridge, UK). 250µl of plasma was added to an equal volume of denaturing solution (6M GuSCN, 0.04M sodium citrate, 1% (w/v) sarcosyl, 1.44% (v/v) β-mercaptoethanol) on ice. 50µl of 2M sodium acetate (pH 4.0) and 500µl of water saturated phenol were added, followed by 100µl of chloroform:isoamylalcohol. The mixture was vortexed vigorously, incubated on ice for 15 minutes, then centrifuged at 13 000rpm for 5 minutes.

Two phases were then formed and the upper phase transferred to a clean tube. 500µl of isopropanol was added with 1µl of glycogen as a carrier and the solutions mixed. This was then incubated at -20°C for 30 minutes and centrifuged at 13 000rpm for 30 minutes to pellet the RNA. After careful removal of the supernatant, the pellet was washed with 75% (v/v) ethanol and air dried at room temperature. The pellet was then resuspended in 10µl of DEPC-treated dH₂O. Two identical extractions were performed for each sample and like samples were pooled and stored at -70°C until use.

2.12. HIV PCR

2.12.1 Sensitivity Testing

To define the level of sensitivity of the HIV PCR method by which to say the EUs are HIV PCR negative, the cell line U1 (see Section 2.6), which contains a single copy of HIV DNA per cell, was mixed with uninfected Preiss cells (see Section 2.6). U1 cells were mixed at a level of 0/10⁶, 1/10⁶, 2.5/10⁶, 5/10⁶, 10/10⁶, 50/10⁶, 100/10⁶, 1000/10⁶, 10⁶/0 uninfected cells and 5 x 10⁶ cells of the mixture was pelleted and extracted by phenol: chloroform extraction (see Section 2.12.2).

As all the EUs were expected to be HIV PCR negative, the same DNA was amplified with primers specific for the MHC locus HLA-DQα, primers 26 and 27 (see Section 2.20). The reaction was set up and performed at the same time as the HIV PCR (see Section 2.12.4), but with the HLA specific primers. Identical reaction conditions were used, but only a single round PCR was necessary.

2.12.2 Extraction

DNA was isolated from infected cells by phenol:chloroform extraction, as outlined in Section 2.11.1. Where plasma derived HIV RNA was assayed, a GuSCN based extraction method was used (see Section 2.11.4).

2.12.3 cDNA Synthesis

When HIV RNA was assayed, primer specific cDNA was made using the outer antisense primers, 534 for *gag* (see Section 2.20) and 633 for V3 *env* (see Section 2.20) and Expand RT (Boehringer Mannheim) essentially as outlined by the manufacturer.

The RNA and primer (40 pmoles) were first denatured at 65°C for 10 minutes, then immediately placed on ice. The reaction was performed after the addition of the following: Expand RT Buffer (1X final, Boehringer Mannheim), DTT, (10mM final), deoxynucleotide triphosphates (dNTPs, 1mM final of each nucleotide, deoxy adenosine triphosphate (dATP), deoxycytidine triphosphosphate (dCTP), deoxyguanosine triphosphate (dGTP), thymidine triphosphate (dTTP), Promega, Southampton, UK), RNase Inhibitor (20U final, Promega), Expand RT (50 units). The total reaction (20µl) was then incubated at 42°C for 1 hour.

2.12.4 PCR

PBMCs from EUs were screened by PCR for the presence of HIV p17 *gag* using nested PCR. Plasma derived HIV RNA from HIV-infected individuals was also amplified by nested PCR in both p17 *gag* and V3 *env*. Primer specific cDNA was limit diluted to ensure amplification from a single molecule for sequencing. Positive primary PCR reactions were then reamplified with biotinylated primers for T7 sequencing (see Section 2.13).

All reactions were performed in essentially the same way. For the screening of the EUs 1-1.5µg of DNA was added and for cDNA amplification 1µl of the respective cDNA dilution was used. Primary PCR amplifications were performed in a 20µl reaction mixture containing 1X Storage Buffer B (Promega), 33µM dNTPs, 0.25µM of each primer, 0.05 units/µl of Taq DNA polymerase (Promega), or later with the use of Taq Supreme and 1X Reaction buffer PC2 (Helena BioSciences, Sunderland, Tyne and Wear, UK). For p17 *gag* outer primers used were 531 and 534 (see Section 2.20) and for V3 *env*, 634 and 332 (see Section 2.20). The reaction was overlaid with paraffin and subject to 30 cycles of 94°C for 25 seconds, 55°C for 35 seconds and 68°C for 2.5 minutes, followed by a final extension at 68°C for 7 minutes.

1µl of the primary reaction was then transferred to the secondary reaction mix which was identical to the primary mixture, but for the use of inner, nested primers, 532/533 for p17 *gag* (see Section 2.20) generating a 390bp fragment and 306/634 for V3 *env* (see Section 2.20) generating a 436bp fragment. The mixture was again overlaid with paraffin and subject to a further 30 cycles of the same conditions as for the primary amplification.

The product of the secondary reaction was then visualised on a 1% TBE agarose gel (see Section 2.17.1). For HIV sequencing, any positive, secondary reactions occurring in ≤ 20% of reactions is deemed to be derived from a single molecule of HIV RNA (Leigh Brown and Simmonds (1995)). The corresponding primary PCR product was then reamplified with biotinylated primers (see Section 2.13).

2.13. HIV SEQUENCING

Sequences were generated using a direct solid phase automated sequencing approach (Leigh Brown and Simmonds (1995)) for both the sense and antisense strands of the *gag* and *env* generated products. Once biotinylated strands were formed by the use of labelled primers, the products were bound to streptavidin coated magnetic beads, then single stranded DNA magnetically purified. Sequencing was performed by the use of an Applied Biosystems PRISM Sequenase Terminator Single Stranded DNA Sequencing Kit (PE Applied Biosystems, Warrington, Cheshire, UK) and an Applied Biosystems 373A automated DNA sequencer.

2.13.1 Production of Biotinylated PCR Product

3µl of primary PCR products (see Section 2.12.4) were amplified in a 120µl secondary reaction using internal primers, one biotinylated primer and one normal primer (532/533 for *gag*, 306/634 for *env*), therefore two reactions per sample. The conditions were identical to that outlined in Section 2.12.4. 10-15µl were run on a 1.5% TBE agarose gel (see Section 2.17.1) and only samples where both sense and antisense reactions were positive were continued.

2.13.2 Purification of Single Stranded DNA and Dynabead Complex

Streptavidin coated magnetic beads (200µg/PCR product (20µl), Dynal Dynabeads M280) were washed in an equal volume of binding and washing buffer (B & W; 10mM Tris-HCl (pH 7.5), 1mM EDTA, 3.5M NaCl) and resuspended to twice their initial volume in B &

W buffer. 40µl of the resulting bead solution was added to 100µl of biotinylated secondary amplification product and incubated for 30 minutes at 48°C. The dynabead/PCR product complex was then placed on a magnetic separator (MPC, Dynal), supernatant removed and the complex washed with 40µl of B & W buffer.

The DNA strands were separated in 0.1M NaOH at room temperature for 10 minutes and the supernatant and non-biotinylated strand removed using the MPC as before. The single biotinylated strand/ dynabead complex was washed once with B & W buffer (50µl) and once with TE buffer (50µl). Finally, the complex was resuspended in 14µl of DEPC-treated dH₂O.

2.13.3 T7 Dye Terminator Sequencing

8 pmoles (1µl) of the complementary non-biotinylated primer was incubated with the single biotinylated strand/ dynabead complex (14µl) in 1X SS MOPS Buffer (5µl of 5X buffer, equal volume of MOPS and Mn²⁺ Isocitrate) at 65°C for 2 minutes, then slowly cooled to 30°C. 4µl of T7 dye terminator mix was added and incubated at 37°C for 2 minutes, then the extension performed at 37°C for a further 10 minutes after the addition of 1µl of T7 DNA polymerase (1.5U). The resulting products were washed twice in Tris/ Tween (50µl, 0.01M Tris-HCl (pH 8.0), 0.1% (v/v) Tween 20) and once with TE buffer (50µl). Finally, the beads were resuspended in 3-4µl of FE, denatured at 90°C for 2 minutes and placed on ice until loaded on a 6% (w/v) acrylamide sequencing gel (see Section 2.18).

2.14. CCR-5 GENOTYPING PCR

2.14.1 Extraction

Genomic DNA was extracted from either EBV transformed BCLs (see Section 2.5), derived from individuals, or archive cryopreserved PBMCs by phenol/chloroform extraction (see Section 2.11.1). Where only plasma samples were available, a more sensitive extraction method was required based on binding of nucleic acid to silica particles (see Section 2.11.3).

2.14.2 Pilot Approach

2.14.2a PCR Amplification of the Whole CCR-5 Gene

A 1.2kb fragment containing the entire coding region of the CCR-5 gene was amplified using primers 28 and 29 (see Section 2.20), in total volume of 50µl containing 1X Reaction Buffer PC2 (50mM Tris-HCl, pH 9.1, 16mM Ammonium sulphate, 3.5 mM MgCl₂,

150µg/ml BSA, Helena BioSciences), 33µM dNTPs, 0.25µM of each primer, 0.125U/µl of Taq Supreme (Helena BioSciences), 3.0ng/µl of DNA and overlaid with paraffin. Amplification conditions were 1 cycle of 94°C for 5 minutes, 60°C for 5 minutes, followed by 30 cycles of 72°C for 2 minutes, 94°C for 1 minute, 60°C for 1.5 minutes and a final extension at 72°C for 10 minutes. The PCR product was purified using a Wizard™ PCR Preps DNA Purification System (Promega) as described below.

2.14.2.b Purification of PCR Product

Amplified PCR product was purified prior to restriction digest using a Wizard™ PCR Preps DNA Purification System (Promega) essentially as outlined by the manufacturer.

The lower aqueous phase of the PCR reaction was transferred to a clean tube and 100µl of Direct Purification Buffer added and vortexed briefly to mix. To this 1ml of warmed ($\approx 37^{\circ}\text{C}$) resin was added and vortexed three times over a one minute period. The resultant resin/PCR mix was then pipetted into a 3ml syringe barrel with a Wizard™ Minicolumn attached. Via insertion of the syringe plunger, the mix was gently pushed into the Minicolumn. With the syringe detached from the column prior to removal of the barrel, the column was then washed with 2ml 80% (v/v) isopropanol and then dried by insertion of the Minicolumn into a 1.5ml microcentrifuge tube and centrifugation for 2 minutes at 13 000rpm. The minicolumn was then transferred to a clean tube and 50µl of DEPC-treated dH₂O added and left for 1 minute at room temperature. The DNA was eluted from the column by centrifugation at 13 000rpm for 20 seconds. 5µl of the cleaned-up product was run on a 1% TBE agarose gel (see Section 2.17.1) to confirm the reaction and clean-up had worked. The remaining 45µl was then digested with the restriction enzyme Asp700 (Boehringer Mannheim) as outlined below.

2.14.2.c Restriction Digest

The purified whole CCR-5 gene was digested with the restriction enzyme Asp700 which for a wild type gene should have generated products of 56, 201, 328 and 527bp and for a $\Delta 32$ deleted gene 225, 328 and 527bp. 45µl of purified PCR product was digested with 20U of Asp700 in Buffer B (Boehringer Mannheim) for 3 hours at 37°C in a total volume of 60µl. The resulting products were then precipitated with 150µl of chilled absolute alcohol (-20°C) and 6µl of 3M sodium acetate (pH 5.2) and incubated at -70°C for 30 minutes. The precipitate was collected by centrifugation at 13 000rpm for 30 minutes, washed in chilled

70% (v/v) alcohol and dried at 40°C for about 15-20 minutes. Next, the pellet was resuspended in 16µl of DEPC-treated dH₂O and the fragments resolved on a 4% Metaphor gel (see Section 2.17.2).

Due to the inability to completely digest the whole product, despite lots of enzyme and a long incubation with Asp700, a combination of two restriction enzymes, EcoRI and BglII was also developed. The same PCR, purification and digest was used as outlined for Asp700, but the digest was performed in Buffer D (Boehringer Mannheim) with 20U of EcoRI (Boehringer Mannheim) and 10U of BglII (Boehringer Mannheim). Fragments of 282, 319 and 511bp were generated for wild type and 250, 319 and 511bp for Δ32 gene.

With the advice of Dr. Linqi Zhang (Aaron Diamond Institute, NY, USA) a more direct and less laborious method was used to screen all the cohorts, as outlined below.

2.14.3 PCR

Genotypes were determined by PCR amplification of the region of the CCR-5 gene where the deletion site is located, using the following primers, C and D (see Section 2.20)(Huang *et al.* (1996)). PCR amplifications were performed in a 20µl reaction mixture containing 1X Storage Buffer B (50mM KCl, 10mM Tris-HCl (pH 9.0 at 25°C), 1.5mM MgCl₂, 0.1% (v/v) Triton X-100)(Promega), 33µM dNTPs, 0.25µM of each primer, 0.04 units/µl of Taq DNA polymerase (Promega), 1.5ng/µl of DNA (except for plasma derived DNA, where the DNA concentration was unknown) and overlaid with paraffin. Later samples were amplified with Taq Supreme (Helena BioSciences) under identical conditions apart from the use of 1X Reaction Buffer PC2 (Helena BioSciences) instead of Storage Buffer B. This was then subject to 5 cycles of 94°C 1 minute denaturation, 55°C 1 minute annealing and extension at 72°C for 1.5 minutes, then a further 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 45 seconds and a final extension at 72°C for 10 minutes. The resulting 189bp (wild type, WT/WT), 157bp (mutant Δ32/Δ32), or 157 and 189bp (heterozygotes, WT/Δ32) fragments were resolved on a 3% Metaphor gel (see Section 2.17.2).

2.14.4 Confirmation PCR and Digest

To confirm the results obtained in the above PCR reaction, random samples were selected and amplified with the sense primer 29 (See Section 2.20) and the antisense primer D from the CCR-5 genotyping PCR, under identical conditions. The resulting 706bp product was digested with 10U of BglII in storage Buffer D (Boehringer Mannheim) for 2 hours at

37°C, resulting in fragments of 195, 511bp for WT/WT, 163, 511bp for $\Delta 32/\Delta 32$ and 163, 195, 511bp for WT/ $\Delta 32$. These products were also resolved on a 3% Metaphor gel (see Section 2.17.2).

2.15. CCR-5 SEQUENCING

2.15.1 PCR Amplification of the Whole CCR-5 Gene

A 1.2kb fragment containing the entire coding region of the CCR-5 gene was amplified using primers 28 and 29 (see Section 2.20), in total volume of 200 μ l (2 x 100 μ l) as described in Section 2.14.2.a. 5 μ l of the final product was run on a 1% TBE agarose gel (see Section 2.17.1) to confirm the reaction had worked and the remaining product was purified using a Wizard™ PCR Preps DNA Purification System (Promega) as described in Section 2.14.2.b, with the two reactions pooled. 5 μ l of the purified product was also run on a 1% TBE agarose gel (see Section 2.17.1) to confirm success prior to sequencing.

2.15.2 Sequencing of CCR-5 Gene

The purified products were then sequenced using an ABI Prism™ Dye Terminator Cycle Sequencing Ready Reaction Kit, with AmpliTaq® DNA Polymerase, FS (PE Applied Biosystems), as outlined by the manufacturer and using an Applied Biosystems 373A automated DNA sequencer. All four of the CCR-5 primers (29, 28, C, D (see Section 2.20)) were used for sequencing each sample to form overlapping segments, spanning the whole of the amplified gene.

A reaction mix containing 8 μ l of Terminator Ready Reaction Mix (A-dye Terminator, C-dye Terminator, G-dye Terminator, T-dye Terminator, dTTP (deoxyinosine triphosphate), dATP, dCTP, dTTP, Tris-HCl (pH 9.0), MgCl₂, thermal stable pyrophosphate and AmpliTaq DNA Polymerase, FS), 3.2pmoles of primer, 8 μ l of PCR template and the volume adjusted to 20 μ l with DEPC-treated dH₂O, was overlaid with mineral oil and thermal cycling performed. A total of 25 cycles of 96°C for 30 seconds denaturation, 50°C for 15 seconds annealing and 60°C for 4 minutes extension were performed. Due to the light sensitive nature of the Terminators, the tubes were covered with foil during the cycles.

The excess Terminators were then removed by ethanol precipitation. The products from thermal cycling were placed in a clean tube, taking care to avoid carry over of mineral oil and precipitated by the addition of 50 μ l of 95% (v/v) ethanol and 2 μ l of 3M sodium acetate (pH 5.2) and left on ice for approximately 30 minutes. The precipitate was collected

by centrifugation at 13 000rpm for 30 minutes, washed in 75% (v/v) ethanol and dried at 40°C for 15-20 minutes. The sample was then kept on ice in the dark until ready to load in the gel, when it was resuspended in 4-5µl of formamide/EDTA (FE, deionised formamide and 25mM EDTA (pH 8.0)), vortexed, spun, denatured at 90°C for 2 minutes and placed on ice until loaded (see Section 2.18 for details of gel and sequencer preparation).

2.16. CCR-2 GENOTYPING PCR

2.16.1 Extraction

Genomic DNA was obtained as for CCR-5 genotyping (see Section 2.11.1)

2.16.2 PCR

A 128bp fragment of the CCR-2 gene was amplified using primers A and Z (see Section 2.20). Primer A contains a mis-match base (C→A position 184), which in the presence of a mutation in the CCR-2 gene (G→A position 190, causes a V→I amino acid substitution at a-a 64)) generates a restriction site for the enzyme BsaBI in the amplified product.

A 25µl reaction containing 1X Reaction Buffer PC2, 33µM dNTPS, 0.1µM of each primer, 0.05 units/µl of Taq Supreme (Helena BioSciences), 1.5ng/µl of DNA (except for plasma derived DNA, where the DNA concentration was unknown) was mixed and overlaid with paraffin. This was then subject to 5 cycles of 94°C 1 minute denaturation, 60°C 1 minute annealing and extension at 72°C for 1.5 minutes, then a further 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 45 seconds and a final extension at 72°C for 10 minutes. 5µl of the PCR product was run on a 2% TBE agarose gel (see Section 2.17.1) to confirm success.

2.16.3 Precipitation of PCR Product.

The remaining 20µl was transferred to a clean tube and the nucleic acids precipitated by the addition of 50µl of chilled absolute alcohol (-20°C) and 2µl of 3M sodium acetate (pH 5.2) and incubated at -20°C overnight. The precipitate was collected by centrifugation at 13 000rpm for 30 minutes, washed in chilled 70% (v/v) alcohol and dried at 40°C for about 15-20 minutes. Next, 8µl of DEPC-treated dH₂O was added, heated to 60°C for a few minutes to ensure resuspension of the DNA and spun.

2.16.4 Restriction Digest.

The precipitated product was digested with 5U of BsaBI (New England BioLabs, Hitchin, Herts, UK), by diluting the enzyme to 5U/ μ l in 1X NEBuffer 2 (50mM NaCl, 10mM Tris-HCl, 10mM MgCl₂, 1mM DTT (pH 7.9 at 25°C) and adding 1 μ l 10X NEBuffer 2 and 1 μ l of diluted enzyme to the 8 μ l product. The digestion was performed at 60°C for 2 hours. The samples were then stored at -20°C until they could be run on a 3% Metaphor gel (see Section 2.17.2).

DNA of individuals encoding the mutation in the CCR-2 gene, the mis-match base in primer A allows digestion of the 128bp PCR product to 18 and 110bp fragments. Individuals homozygous wild-type (WT/WT) will only show the uncut 128bp product, homozygous mutant individuals (64I/64I) show only the digested 110bp fragment, as the 18bp fragment was too small to resolve and heterozygous individuals (WT/64I) will show both the 110 and 128bp fragments.

2.17. AGAROSE GEL ELECTROPHORESIS

2.17.1 TBE Agarose Gels

Varying % gels (w/v, i.e. 1g/100ml \equiv 1% gel) were made by the addition of routine electrophoresis grade agarose (Flowgen, Litchfield, Staffordshire, UK) to 1X Tris-Borate (TBE), made from a 10X stock (890mM Tris-borate, 20mM EDTA (pH 8.0)) and dH₂O, which were then microwaved for 5 minutes to melt the agarose and stirred for 5-10 minutes to cool. Once cooled, ethidium bromide was added at a final concentration of 0.3 μ g/ml and the gel cast and allowed to set.

Typically 300ml gels were cast using four 22-tooth combs/ gel. Samples were loaded and gels ran for 40-90 minutes at 100-150V depending upon the resolution required and % of the gel. Gels were run with 1X TBE as running buffer and electrophoresis equipment used was either a GNA 200 electrophoresis tank (Pharmacia) and a BioRad Model 250/2.5 power pack (BioRad Laboratories, Hemel Hempstead, Herts, UK), or a Hybraid Maxi Gel electrophoresis tank and a Microgel PSU power pack (Hybraid, Teddington, Middlesex, UK). DNA bands were visualised using a UV transilluminator (Vilber Lourmat, Marne la Vallee, France).

2.17.2 Metaphor Gels

To resolve the smaller products and to distinguish between the small differences generated by the CCR-5 and CCR-2 genotyping PCRs (see Sections 2.14 and 2.16), Metaphor agarose (FMC Bioproducts supplied from Flowgen) gels were used. Typically, 3% gels (w/v) were made by sprinkling 9g of Metaphor agarose into 300ml of chilled 1X TBE in a beaker of at least 3 x this volume (1000ml), and left for approximately 10 minutes for the agarose to hydrate. This was then weighed and microwaved until the mixture began to boil and carefully swirled to cool. Care was taken when the gel was swirled as 'hot spots' can cause the gel to boil over. This process was repeated until the agarose was completely dissolved and the gel adjusted back to its original weight with the addition of warmed dH₂O. Any bubbles were removed by placing the beaker in warm water for a few minutes. After allowing the gel to cool for a few minutes, it was then cast. For the genotyping assays, due to the large numbers screened at once, 2 x 30-tooth combs were used/ gel, rather than the normal 22-tooth comb, to allow more samples/ gel.

Samples were loaded with 6X Loading buffer (15% (w/v) Ficoll (type 400) in dH₂O with 0.25% (w/v) Bromophenol blue and 0.25% (w/v) Xylene cyanol) in a final volume of 10-15µl. 300ml gels were run for 1.5-2 hours at 80-100V in 1X TBE for the CCR-5 genotyping PCRs and if smaller gels were ran the voltage was adjusted accordingly. Due to the small difference in fragment size for the CCR-2 genotyping PCRs, a 300 ml gel was ran at 90V for 3-4 hours at approximately 10°C, with fresh 1X TBE added halfway.

As the gels were run for a long time and ethidium bromide is positively charged so eventually runs out of the gel, the Metaphor gels were stained after electrophoresis. This was done by placing the gel in approximately 300ml of 1X TBE containing 0.5µg/ml of ethidium bromide and gently swirled on a shaker (Gyrotory[®] model G2, New Brunswick Scientific Co, Inc, Edison, NJ, USA) for about 20-30 minutes. The gel was then 'destained' in dH₂O for a further 10 minutes, to reduce the background staining, and the DNA visualised on a UV transilluminator.

2.17.3 Photography of Gels

Gels were photographed as a long term record of results using a Polaroid CU-5 land camera with the gel on the transilluminator. Black and white Polaroid ISO 3000/ 30° film was used and where a more permanent record was required positive and negative film was used and a clearer, enlarged picture taken from the negative.

2.18. ACRYLAMIDE SEQUENCING GEL ELECTROPHORESIS

Both T7 and Taq sequencing products were run on an Applied Biosystems 373A DNA sequencer using 6% (w/v) acrylamide gels.

Prior to assembly, glass plates, spacers and the comb were washed thoroughly with Alconox™ (Alcono, Inc, NY, USA), rinsed with hot water, then dH₂O and allowed to air dry. Plates were then assembled and sealed with electrical tape.

Meanwhile, the gel was made by dissolving 30g of Urea in 9ml of 40% (w/v) acrylamide/bis (19:1) solution (BioRad), 20ml of dH₂O and 0.5g of Amberlite™ MB-150 resin (Supelco, Bellefonte, PA, USA) for approximately one hour. 6ml of filtered 10X TBE was added and the volume adjusted to 60ml. The solution was then filtered through a 0.2µm cellulose acetate filter unit under vacuum and degassed for a few minutes.

300µl of freshly made 10% (w/v) ammonium persulphate and 33µl of TEMED (IBI, Ltd, A Kodak Company, Cambridge, UK) were added to the acrylamide solution and gently mixed. This was poured immediately, using a 50ml syringe, up to 3-5cm from the top of the plates. The casting comb was then added and secured with clamps. The gel was left to set horizontally for at least 1.5 hours, after which time the clamps and tape were removed and any excess acrylamide removed by thorough washing in tap water and then dH₂O. The plates were again left to air dry.

The sequencing machine was then prepared for running the samples. Firstly, a sample sheet was set up on the Macintosh. Next, the lower buffer chamber and the gel were placed in the machine. The filter set was selected from the configuration menu on the sequencing machine ('Main Menu', then 'Calibration', then 'Configuration', then 'more' until 'Filter Set', where appropriate filter set was selected); filter set A was used for Taq sequencing and filter set B for T7 sequencing. To ensure the plates were sufficiently clean, a plate check run was performed ('Main Menu', then 'Start Pre Run', then 'Plate Check', then 'Full Scan'). Once a flat base-line was established after any necessary plate cleaning, the PMT setting was checked on the Macintosh. This should be between 800-1000 and adjusted on the sequencing machine if not.

The rest of the gel equipment was then assembled, the 24 sharks-tooth comb inserted and the gel firmly clamped in place. The upper and lower buffer chambers were next filled with 1X TBE and the wells rinsed of any build up of urea with a syringe and buffer. The gel was then checked again after plugging in both the positive and negative electrodes ('Main

Menu', then 'Pre Run Gel', then 'Start Scan'). Once a flat trace was again obtained, the samples were placed in FE loading buffer.

The wells were rinsed again with 1X TBE and odd samples loaded and run for 5 minutes ('Main Menu', then 'Choose Run', then 'Sequence Run', then 'Full Scan', then 'Start Run'). After 5 minutes, 'Interrupt Run' was selected, wells washed out once more, even samples loaded and 'Resume Run'. The data collection was then commenced by selecting 'Collect' on the Macintosh and the machine left to run overnight. Sequences were then processed as outlined in Section 2.19.

2.19. SEQUENCE ANALYSIS

Sequences were transferred from the Macintosh to a Sun SPARC station using Columbia Apple Talk Package (CAP). Raw nucleotide sequences were preliminarily edited (ends cut) via TED (part of the STADEN computer package (Staden (1993))) using the SEQPROCESS script (written by Dr. C. Wade, Centre for HIV Research, Edinburgh). Further editing and assembly of sequences was then performed using the Xbap software (STADEN package). Sequences were then aligned and translated using version 2.2 of the Genetic Data Environment (GDE) package (Smith *et al.* (1994)).

Phylogenetic analysis of the nucleotide sequence data was carried out using the neighbour-joining method (NEIGHBOR taken from version 3.52c of the Phylogeny Interface Package (PHYLIP)). Bootstrap resampling was performed to assign support with 100 replicates using SEQBOOT and CONSENSE (Felsenstein (1985))). Distances were estimated for each pairwise nucleotide sequence comparison using the method of Kimura (1980).

2.20 STATISTICS

For lymphoproliferative data (Chapter 3) the geometric mean was calculated and 95% Confidence Intervals deduced using Microsoft Excel. These data were then used to calculate the Stimulation Indexes given (see Section 3.2.4).

Parametric comparisons between data (Chapter 3 and 4) were performed using the t test (two-tailed) for normally distributed unpaired data using SPSS (SPSS for Windows, version 6.0, Chicago, Illinois, USA). Data were normalised by either taking the square root of the values, or log transformed values where required.

The non-parametric test, Mann-Whitney U/Wilcoxon Rank Sum W Test (SPSS) was used to assess the HLA mismatch scores (see Section 4.3.2), to confirm the t test data, due to the small data set which therefore meant the normal distribution was less significant.

95% Binomial CI for allele frequencies (Chapter 4) were interpolated from Table W of Rohlf and Sokal (Rohlf and Sokal(1969)) and the difference between the groups obtained from the standardised normal deviate (z).

2x2 contingency tables were assembled to assess for differences of a particular property, between two groups (Chapter 3 and 4). These were tested by χ^2 with Yate's correction for contingency and also by Fisher's Exact test. The Exact test was performed manually and involved lengthy calculation, therefore only when the actual 2x2 table gave a probability (P) of less than 0.10, were more extreme tables constructed. Therefore, the Exact test values when $P > 0.10$ were an underestimate of the precise significance and hence the use of χ^2 . However, the Exact test has greater accuracy for low values, which were often seen for the data analysed here, so was performed when significance was approached. Other contingency tables were assessed by χ^2 .

2.21. PRIMER SEQUENCES

2.21.1 HIV *gag* (p17) Primers

2.21.1.a Outer

531 sense : 5'-GCGAGAGCGTCAGTATTAAGCGG-3'
(795-817*)

534 antisense : 5'-TCTGATAATGCTGAAAACATGGG-3'
(1296-1318*)

2.21.1.b Inner

532 sense : 5'-GGGAAAAAATTCGGTTAAGGCC-3'
(833-856*)

533 antisense : 5'-CTTCTACTACTTTTACCCATGC-3'
(1247-1270*)

2.21.2 HIV *env* (V3) Primers

2.21.2.a Outer

332 sense : 5'-TACAATGTACACATGGAATT-3'

(6957-6976*)

633 antisense : 5'-GGAGGGGCATACATTGC-3'
(7520-7537*)

2.21.2.b Inner

306 sense : 5'-TGGCAGTCTAGCAGAAGAAG-3'
(7009-7028*)

634 antisense : 5'-ATTCTGCATGGGAGTGTG-3'
(7465-7482*)

* position of primer on HIV-HXB2 genome

2.21.3 HLA Primers

26 sense : 5'-GTGCTGCAGGTGTAAACTTGTACCAG-3'

27 antisense : 5'-CACGGATCCGGTAGCAGCGGTAGAGTT-3'

Obtained from the AIDS Reagent Project.

2.21.4 CCR-5 Primers

2.21.4.a Genotyping

C sense : 5'-CAAAAAGAAGGTCTTCATTACACC-3'
(747-770[#])

D antisense : 5'-CCTGTGCCTCTTCTTCTCATTTCG-3'
(912-935[#])

2.21.5.b Whole Gene

29 sense : 5'-CTCGGATCCIGGTGGAACAAGATGGATTAT-3'
(229-248^{£#})

28 antisense : 5'-CTCGTCGAICATGTGCACAACCTCTGACTG-3'
(1324-1343^{£#})

[#] position of primer on CCR-5 consensus sequence (Genbank accession number: X91492).

[£] counting from line onwards as cloning primers with additional sequence.

2.21.6 CCR-2 Primers

A sense : 5'-TTGTGGGCAACATGATGG-3'
(209-226^s)

Z antisense : 5'-GAGCCCACAATGGGAGAGTA-3'
(318-337^s)

^s position of primer on CCR-2 consensus sequence (Genbank accession number: U80924).

Chapter 3

Immunological Factors

CHAPTER 3 - IMMUNOLOGICAL FACTORS

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3.1. INTRODUCTION

Heterosexual contact with an HIV⁺ individual was recognised as a risk factor for acquiring HIV infection in 1982 (Centers for Disease Control (1982b)). Following this discovery, it was feared that a world-wide heterosexual epidemic of HIV would occur. Epidemiological studies were established to monitor the prevalence of and factors associated with heterosexual HIV transmission. These studies included a European Collaborative Study (European Study Group (1992)) (see Section 1.3.4.c), of which the Edinburgh Heterosexual Partner Study was part (see 1.8.2). Important risk factors were established from such cohort studies, including: anal sex, sex during menses, ulcerative STDs and it was also discovered that barrier methods of contraception dramatically reduced the risk of infection (see Section 1.3.4). It became apparent that while in some Western cohorts the incidence of heterosexual transmission was low (see Section 1.3.4), heterosexual transmission is an immense and growing problem in developing countries, particularly in Africa (van de Perre (1995)) (see Section 1.3.4.a). An increased understanding of heterosexual transmission is therefore paramount for controlling HIV infection on a global scale.

From epidemiological studies it was also apparent that some individuals remained uninfected despite high risk exposure. If the factors which protected these individuals could be determined, it may help in the struggle towards finding a vaccine. It was initially thought that perhaps a proportion of EUs were merely harbouring a low level infection and would eventually produce detectable levels of virus and virus specific antibody, known as seroconversion. Individuals who are seronegative, but show evidence of HIV infection, either by PCR, or virus culture have been reported (Imagawa *et al.* (1989); Pezzella *et al.* (1989); Ensoli *et al.* (1991); Coutlée *et al.* (1994)) and a few have been shown to persist in this state without seroconversion (Imagawa *et al.* (1989)).

Many other viral infections are acute and self limiting and control is often due to specific immune responses to the virus in question (Zinkernagel and Welsh (1976); Moss, Rickinson and Pope (1978); Rickinson *et al.* (1981); McMichael *et al.* (1983)). Immune responses in HIV⁺ individuals have been detected, with the presence of HIV-specific CMI correlating with a more favourable prognosis (see Section 1.6.2). A progressive loss of CD4⁺ T cell reactivity, first to recall antigens and later even mitogenic responses has been seen to correlate with progression to AIDS (Miedema *et al.* (1988); Clerici *et al.* (1989b); Ranki *et al.* (1989); Teeuwesen *et al.* (1990); Pontesilli *et al.* (1995)). Also, the presence of HIV-

specific CTLs has been shown to relate to increased AIDS-free survival (Bourgault *et al.* (1993); Miller *et al.* (1990)).

The correlation of CMI with disease progression lead to a hypothesis by Clerici and Shearer (Clerici and Shearer (1993)) (see Section 1.6.2.e). They proposed that progression to AIDS related to a switch from a predominately T_H response, inducing CMI, to a T_H response, associated with antibody production. The theory was later modified to encompass other cells and cytokines and became termed a type 1 to a type 2 switch (Clerici and Shearer (1994)).

Clerici and Shearer also postulated that EUs may remain uninfected by retaining a protective type 1 response. This was supported by the finding of HIV-specific proliferative responses (Ranki *et al.* (1989); Borkowsky *et al.* (1990); Clerici *et al.* (1992); Kelker *et al.* (1992); Clerici *et al.* (1993b); Clerici *et al.* (1994b); Mazzoli *et al.* (1997)) and later with the discovery of HIV-specific CTLs in a few EUs (de Maria, Cirillo and Moretta (1994); Langlade-Demoyen *et al.* (1994); Pinto *et al.* (1995); Rowland-Jones *et al.* (1995)).

This study was established to determine if the EUs present in the Edinburgh Heterosexual Study showed any immunological responses, including those against HIV, which might explain their apparent lack of infection, despite continued high risk exposure. It was also necessary to ensure that these individuals were truly uninfected and not harbouring a persistent low level infection, whilst remaining seronegative.

3.2. MATERIAL AND METHODS

3.2.1 Subjects

Details regarding the Edinburgh Heterosexual Partner study, including interview data and recruitment criteria are given in Section 1.8.2. DNA samples for PCR testing were obtained from cryopreserved PBMCs (see Sections 2.4.2 and 2.11). Fresh blood samples were required for lymphoproliferation assays (see Section 3.2.4) and were collected with informed consent specifically for research purposes by the research nurse (see Section 2.2.1.a and 2.2.4).

A total of 15 samples from 15 EUs were obtained over the period January to November 1996, at which time further funding was not forthcoming and the Heterosexual Study came to a close. One of the EU individuals (C5571) was subsequently shown to be HIV⁺ at the time of sampling and was excluded from further analysis. A sample was also obtained from an individual known to be undergoing acute seroconversion (C5751).

Control samples from 18 low risk controls (Donors) were obtained from the Blood Transfusion Service where they were plasmapheresis donors (see Section 2.2.3). Details of the EUs and Donors analysed are summarised in Table 3.1.

3.2.2 Antibody and PCR Testing

HIV antibody testing was performed by ELISA and confirmed by western blotting (see Section 2.2.1.a.i). PCR testing was performed on PBMC derived DNA (see Section 2.11) with HIV-specific primers to the *gag* gene by nested PCR (see Section 2.12). Nested PCR allows the detection of genes to a copy number as low as one per PCR reaction (Simmonds *et al.* (1990b)).

To define the detection level of the PCR reaction under the conditions used, serial dilutions of the U1 cell line, which contains a single HIV genome per cell, were performed with HIV-negative Preiss cells (see Section 2.12.1). DNA was then extracted and the PCR performed (see Section 2.12.4). Samples from EUs were assessed in an identical manner. As the EUs were expected to yield a negative result, a positive control PCR reaction was performed in parallel to confirm the presence of DNA. This was achieved using primers specific for the HLA gene, *HLA-DQ α* gene (see Section 2.12.1).

3.2.3 Lymphocyte Subset Analysis

The percentage of lymphocytes was determined by two colour flow cytometry using two fluorochromes: FITC and PE (see Section 2.8.1). Lymphocytes were gated, as determined by size and granularity (forward and side scatter) and confirmed using a leucocyte marker, CD45:FITC and CD14:PE. Only CD45 is present on the lymphocytes, thus distinguishing them from the CD45⁺ CD14⁺ monocytes.

3.2.4 Lymphoproliferation Assays (LPA)

PBMCs were isolated from heparinised blood samples (see Section 2.2.4) and incubated for 7 days with a variety of antigens (see Section 2.7.1). The antigens used included the mitogen PHA which was used as a positive control to determine reactivity in the assay.

The recall antigens were included to assess whether normal recall immune responses were present in the EUs. As most adults have been vaccinated against mycobacterium and *Clostridium tetani*, the PPD from mycobacterium and the toxin from *Clostridium tetani* (TT) were chosen to monitor the recall responses of the cohort.

Table 3.1.
Details of Individuals Studied

EUs	DOB*	Age*	Sex	All Donors	DOB	Age	Sex	HIV+s	DOB	Age	Sex
C3153	08.04.62	34	F	D1 ^{+s}	21.03.57	39	F	C5571	24.05.68	27	M
C3154	09.06.78	17	F	D2 ^{+s}	06.02.56	40	M	C5751	29.08.69	27	M
C3942	15.11.65	30	F	D3 ^{+s}	23.02.52	44	M				
C4051	01.01.59	37	M	D4 ⁺	04.12.35	60	M				
C4371	24.08.62	33	F	D5 ⁺	30.11.61	34	M				
C4401	02.06.60	28	F	D6 ^{+s}	02.08.64	31	M				
C4712	21.07.66	29	F	D7 ^{+s}	04.02.69	27	F				
C5191	25.01.62	34	F	D8 ^{+s}	14.09.59	36	M				
C5411	21.01.61	35	F	D9 ^{+s}	06.02.56	40	M				
C5451	19.12.67	28	M	D10 ⁺	22.12.60	35	M				
C5621	03.02.51	45	F	D11 ⁺	21.08.56	39	M				
C5711	28.08.67	28	F	D12 ⁺	27.06.71	25	M				
C5721	25.01.71	25	F	D13 ⁺	13.03.49	47	F				
C5731	14.09.64	31	M	D14 ^s	02.08.64	31	M				
				D15 ^s	12.07.49	46	F				
				D16 ^s	02.11.75	20	F				
				D17 ^s	09.05.44	51	F				
				D18 ^s	11.06.66	29	M				
Mean Age		31.0				All 37.4	Subset ⁺ 38.2	LPA ^s 36.2			
Range		17-45				20-60	25-60	20-51			
Male:Female Ratio		3:11				12:6	10:3	7:5			

* Date of Birth

* Age at time of Sampling

+ Donors used in Subset Analysis (Subset)

^s Donors used in Lymphoproliferation Assays (LPA)

An alloantigen (Allo) was made from a mixture of BCLs and included to investigate the presence and extent of allo reactivity in the EUs. A strong response to mismatched MHC proteins, expressed on an invading infected cell, or virus, has been suggested as a potential protective mechanism from HIV infection (see Section 1.7.3.b).

Finally, to assess if any of the EUs had proliferative responses to HIV, a cocktail of recombinant proteins was made which included all the major viral proteins: ENV derived from two different strains (gp120, HIV_{MN} and HIV_{SF2}), GAG (p24), NEF, reverse transcriptase (p66, RT) and TAT (see Section 2.7.1.a)

Proliferations were considered significant if a stimulation index (SI) of greater than 2 was achieved as determined by the geometric mean value counts per minute (cpm)/ geometric mean background value (cells with no antigen added) (see Section 2.7.1).

3.2.5 Cytokine ELISA

Prior to harvesting, 100µl/well of supernatant was removed from all the wells of the proliferation assay and stored at -20°C. This was subsequently thawed and the wells for each particular antigen were pooled to give a sufficient volume of supernatant to assay. The cytokine content was analysed by Genzyme's DuoSet ELISAs for IL-4 and IFN-γ essentially as outlined by the manufacturer, with checkerboard assays performed previously to optimise the reagent concentrations. All samples were analysed in duplicate undiluted, except for the mitogen, PPD and allo assays, which were also assessed at both 1:25 and 1:50 dilutions.

3.2.6 CTL Proposal

A strategy was also established for assaying CTL activity in follow up samples. It was planned to infect stored autologous CD8 depleted PHA blasts with HIV, which were then to be used as stimulators to expand HIV-specific CTLs. It was decided that this method would be more likely to detect HIV-specific responses in the EUs than polyclonal stimulation with PHA, as the numbers of HIV-specific CTL circulating in the EUs may be much lower than that of HIV⁺ individuals, who would have wide scale *in vivo* stimulation. Peptide specific stimulation (Rowland-Jones *et al.* (1995)) would have required detailed knowledge of the reactive peptides in these individuals which may differ from those of HIV⁺ individuals. A broader stimulation was also thought to be more likely to detect responses in a wide variety of individuals, rather than being limited to those of certain HLA types.

Following specific stimulation, it was hoped to monitor CTL activity in a standard chromium release assay using either recombinant vaccinia virus (rVV) infected autologous BCLs, expressing specific HIV proteins, or HIV-infected autologous PHA blasts. Again it was hoped that a broader target may detect a wider degree of reactivity than peptide based detection methods.

3.3. RESULTS

3.3.1 Confirmation of Exposed Uninfected (EU) State

3.3.1.a Antibody Testing

All individuals recruited into the Heterosexual Partner study were offered a HIV test. These tests were performed by staff in the Department of Medical Microbiology, University of Edinburgh. Unless otherwise stated, consistent negative results were obtained for the EUs within the cohort.

3.3.1.b Polymerase Chain Reaction (PCR) Testing

To investigate the possibility of a 'silent' latent infection in the seronegative contacts, PCR analysis of the *gag* gene of HIV was performed on PBMC derived DNA. Dr. David Yirrell and Pamela Robertson, Centre for HIV Research, analysed 41 seronegative contacts by PCR and found them all to be negative. However, the sensitivity of the reaction and the input of nucleic acid was not controlled for, so 22 contacts, for whom PBMC derived DNA was available, were re-assessed.

The sensitivity of the reaction was determined using the U1 cell line, which contains a single copy of the HIV per cell. The cell line was mixed with the uninfected B cell line, Preiss, at ratio of : 0/10⁶; 1/10⁶; 2.5/10⁶; 5/10⁶; 10/10⁶; 50/10⁶; 100/10⁶; 1000/10⁶; 10⁶/0 U1 cells/uninfected Preiss cells.

HLA amplifications were routinely positive (see Figure 3.1). The HIV DNA was repeatedly detected to a level of 1 copy/μg of DNA, equivalent to approximately 1 copy/ 10⁵ cells (see Figure 3.2.a). At some of the higher concentrations of U1 cells and hence, HIV DNA, the larger 480bp primary PCR product was also detected, seen as a second band above the main 390bp one (see Figure 3.2.a). Occasionally, lower concentrations were detected (as low as 1 copy/ 10⁶ cells, lane 18 in Figure 3.2.a), but not routinely. None of the EU individuals tested gave a positive PCR amplification for HIV (see Figure 3.2.b) with

Figure 3.1.

HLA-DQ- α Amplification

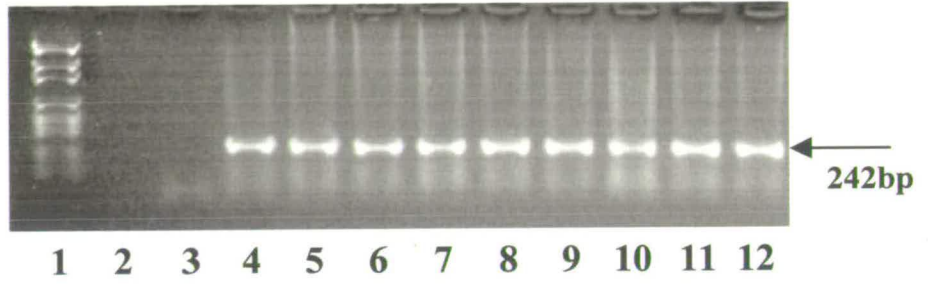
Typical results obtained from a *HLA-DQ α* PCR (see Section 2.12.1), showing the 242bp fragment marked with an arrow, run on a 1.3% Agarose gel, 80-100V, for approximately 1 hour.

a.) Results from the U1/Preiss titrated DNA. Lane 1 contains pGEM markers; Lane 2 is a no primer control; Lane 3 is a no DNA control; Lanes 4-12 contain U1/Preiss titrated DNA.

Lanes	U1/Preiss cell ratio
4	$10^6/0$
5	$1000/10^6$
6	$100/10^6$
7	$50/10^6$
8	$10/10^6$
9	$5/10^6$
10	$2.5/10^6$
11	$1/10^6$
12	$0/10^6$

b.) Typical results obtained from EU derived DNA. Lane 1 contains pGEM markers; Lane 2-11 EU DNA.

A



B



Figure 3.2.

HIV gag Amplification

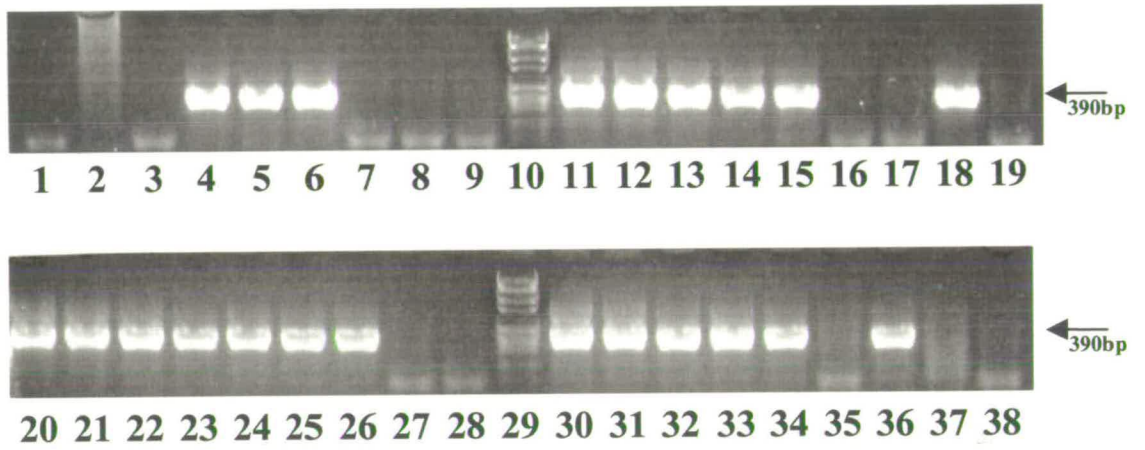
Typical results obtained after a nested HIV *gag* PCR (see Section 2.12), showing the 390bp fragment marked with an arrow, run on a 1.3% Agarose gel, 80-100V, for approximately 1 hour.

a.) Results from the U1/Preiss titrated DNA. Lanes 10 & 29 contain pGEM markers; Lane 1 & 3 contain no DNA controls (1 for 1° reaction; 3 for 2° reaction); Lane 2 contains a no primer control (1° & 2° reaction). Lanes 11-19/20-28/ 30-38 are direct repeats of the U1/Preiss titrated DNA, with 4-9 lacking the highest three concentrations of U1 cells.

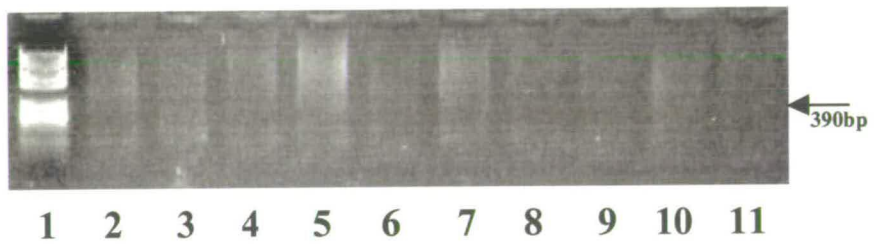
Lanes	U1/Preiss cell ratio
11/ 20/ 30	10 ⁶ /0
12/ 21/ 31	1000/10 ⁶
13/ 22/ 32	100/10 ⁶
4/ 14/ 23/ 33	50/10 ⁶
5/ 15/ 24/ 34	10/10 ⁶
6/ 16/ 25/ 35	5/10 ⁶
7/ 17/ 26/ 36	2.5/10 ⁶
8/ 18/ 27/ 37	1/10 ⁶
9/ 19/ 28/ 38	0/10 ⁶

b.) Typical negative results obtained from EU derived DNA. The positive control amplification for *HLA-DQ-α* for these individuals is shown in Figure 3.1.b. Lane 1 contains pGEM markers; Lanes 2-11 EU DNA.

A



B



duplicates for each individual, although all were positive for *HLA-DQ α* (see Figure 3.1.b). The much smaller band seen in some of the lanes is the unused primer, or 'primer front'.

The EUs tested were therefore confirmed to be both HIV-negative in terms of HIV-specific antibody and for the presence of virus as assessed by PCR to at least a level of below 1 copy of HIV/ 10^5 cells.

3.3.1.d Lymphocyte Subsets

Blood samples were obtained from 13 of the 14 individuals assessed for proliferative responses and were also analysed for lymphocyte subsets. The remaining sample was not analysed due to mechanical problems with the FACscan. Thirteen normal donors were also assessed, along with the known seroconverter, C5751.

A typical scatter can be seen in Figure 3.3.a., with the region marked R1 indicating the gated region of lymphocytes. Using irrelevantly conjugated controls, the unstained cells were selected (see Figure 3.3.b). This allowed the percentage of populations expressing the antigen in question to be determined. The percentage of $CD3^+ CD4^+$ and $CD3^+ CD8^+$ were obtained in this manner (see Figure 3.3.c and d). Typically, >90% of the gated cells were lymphocytes (93-100%) (see Figure 3.3.e), as determined by the leucogate marker (CD14/CD45).

For 9/13 EU samples, 9/13 donor samples and the seroconverter sample, the percentage of lymphocytes expressing HLA-DR and CD45-RO were also assessed (see Figure 3.3.f). The total percentage of cells expressing one of the markers was the sum of cells expressing the molecule alone and those expressing both.

The mean percentages of CD4 and CD8 expressing cells for the EUs did not differ from that seen in normal donors, as assessed by a t-test of mean values following log transformation (see Table 3.2 and Appendix 3.1 for actual values). Although a few EUs showed increased percentages of $CD8^+$ cells (C4051, C4401, C5191, C5451, C5731) (see Figure 3.4.a), this was also seen in some donors (D6, D10, D11) (see Figure 3.4.b) and was much less marked than in the seroconverter (C5751), whose lymphocytes consisted of over 70% $CD3^+ CD8^+$ cells (see Figure 3.4.a). Although, C4051 did show a marked decrease in $CD4^+$ (17%) and elevated $CD8^+$ cells (53%), he remained seronegative.

The mean percentage of cells expressing the memory marker, CD45-RO, did not differ in the two groups, as assessed by a t-test of mean values following log transformation

Figure 3.3.

Flow Cytometry of Lymphocyte Subsets

a.) A typical scatterplot obtained from PBMCs using Lysis II software (see Section 2.8.1). The x-axis shows side scatter and is dependant on the granularity of cells. The y-axis shows forward scatter, dependent on size. The lymphocytes are gated (R1) and subsequent analysis is performed on the gated region of cells. The population of cells above the R1 region are activated, dividing cells and the population of cells below the R1 region are dead cells and debris.

The remaining plots show the staining pattern obtained at 660nm (FITC on FL1) and 560nm (PE on FL2). The flurochromes are directly conjugated to monoclonal antibodies specific for specific cell surface molecules. The % values are calculated as the % of the total number contained within that quadrant.

- b.) The gates are set with two irrelevant conjugated antibodies (see Section 2.8.1).
- c.) CD3:FITC and CD4:PE.
- d.) CD3:FITC and CD8:PE
- e.) CD45:FITC and CD14:PE (Leucogate marker).
- f.) HLA-DR:FITC and CD45RO:PE.

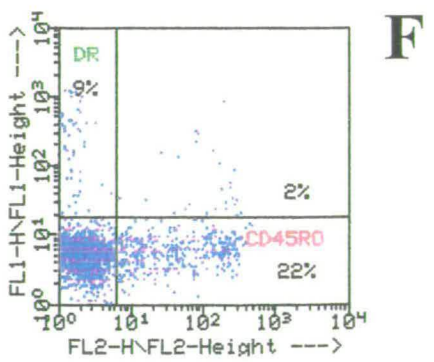
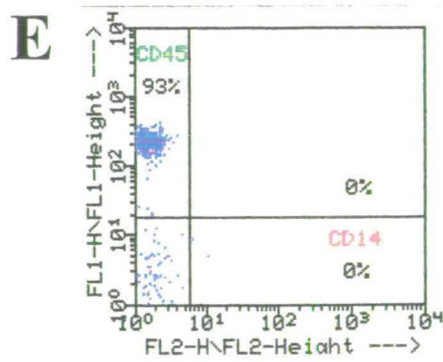
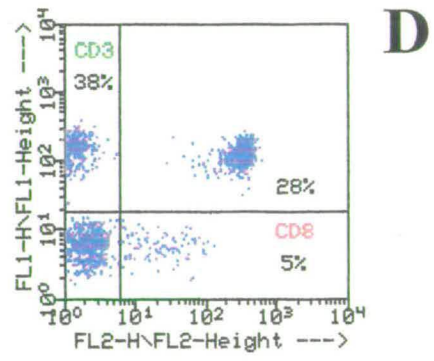
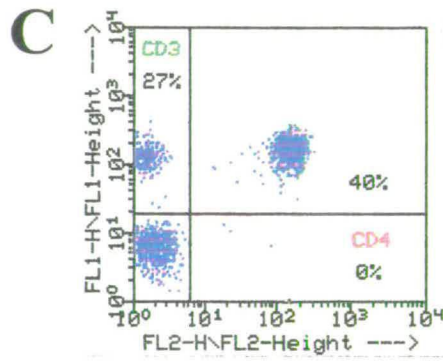
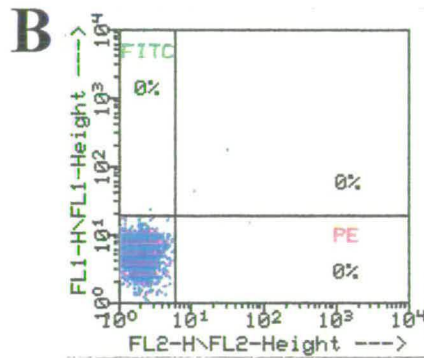
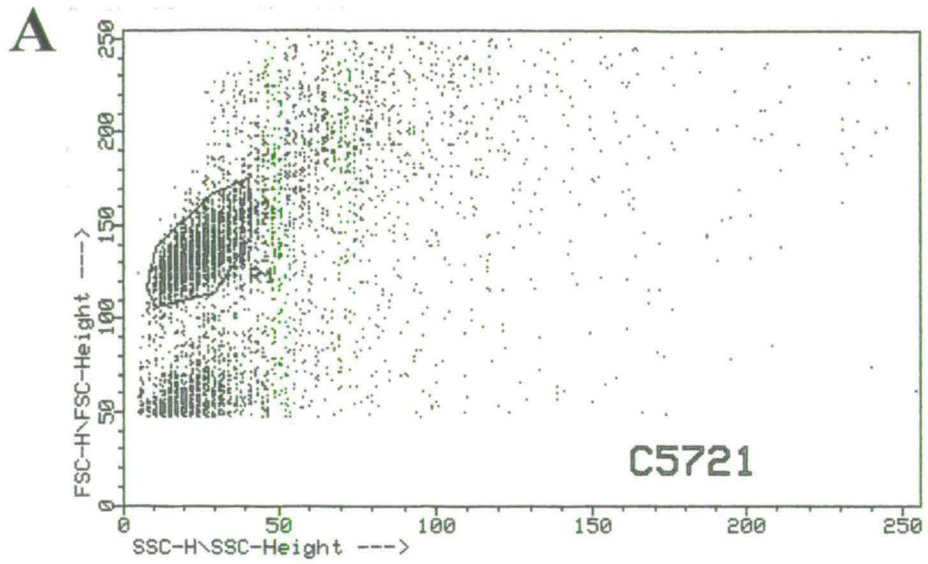


Table 3.2.

Lymphocyte Subsets of EUs and Controls and T-test Results

% of Lymphocytes	EUs			Controls			t*	P [#]
	Mean	Standard Deviation	Number	Mean	Standard Deviation	Number		
CD3 ⁺ :CD4 ⁺	38	12	13	40	9	13	0.66	0.52
CD3 ⁺ :CD8 ⁺	28	11	13	24	7	13	0.97	0.34
DR ⁺	20	8	9	14	4	9	2.33	0.03
CD45RO ⁺	35	11	9	32	10	9	0.45	0.66
DR ⁺ :RO ⁺	4	2	9	4	2	9	0.17	0.87
CD4:CD8 Ratio	1.49	1.0	13	1.98	0.7	9		

* t-test performed on log transformed values

[#] Probability (P) value of difference between log transformed mean values.

Figure 3.4.

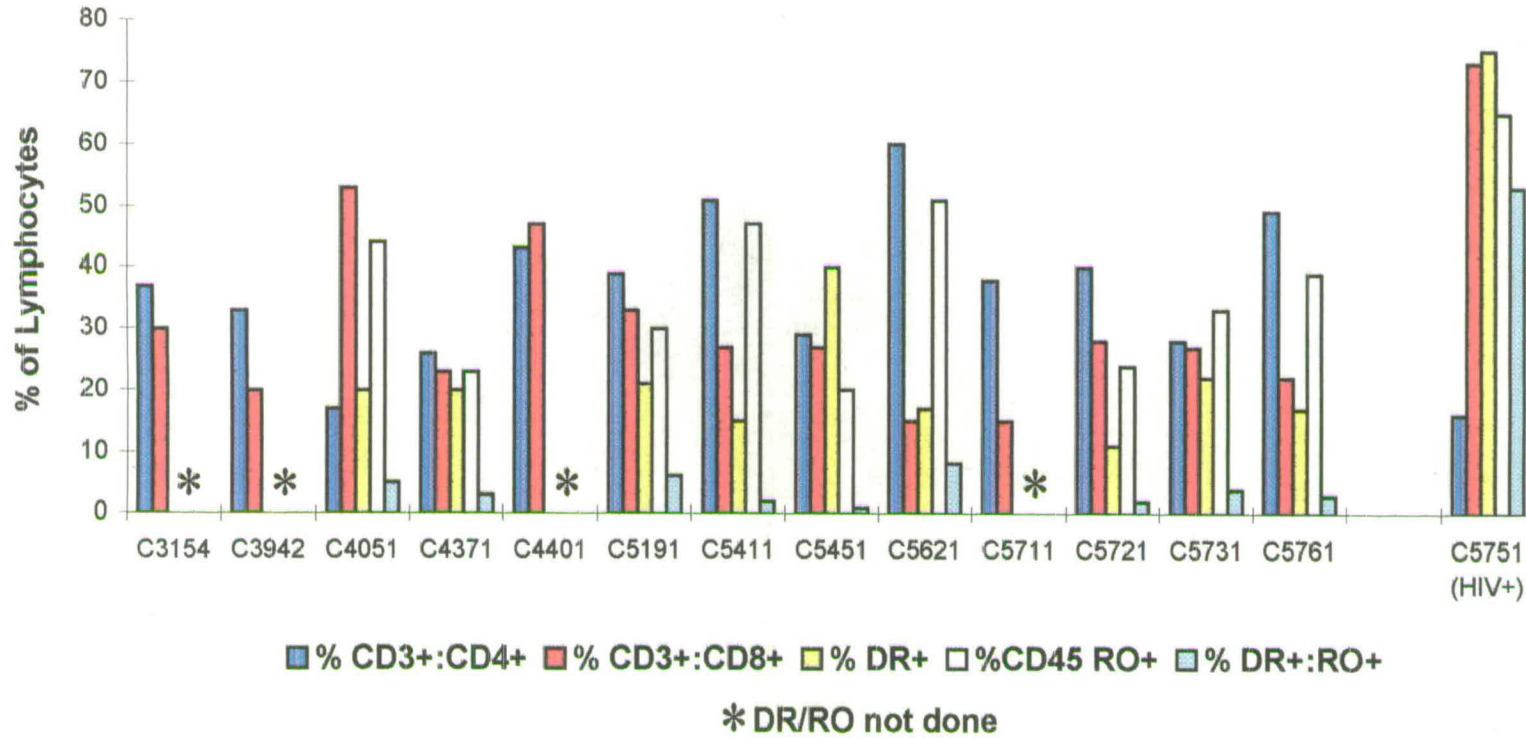
Lymphocyte Subsets

The following graphs show the % composition of lymphocytes, as determined by co-staining (FITC and PE) and flow cytometry, using Lysis II software (see 2.8.1).

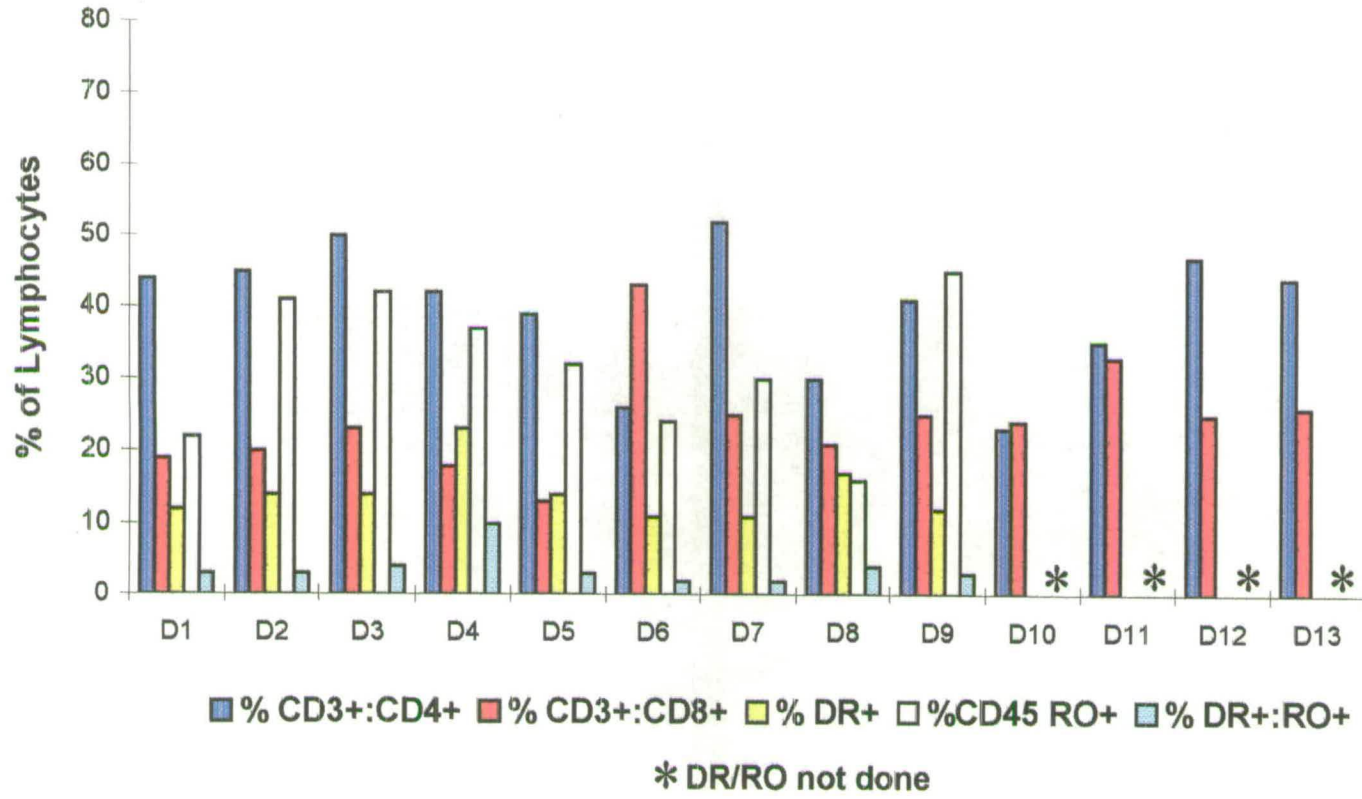
a.) EUs and Seroconverter (C5751)

b.) Low risk donor controls

A



B



(see Table 3.2 and actual data presented in Figure 3.4.a and b and Appendix 3.1). In contrast, the seroconverting patient, C5751, showed an expansion of HLA-DR⁺ (75%) and CD45-RO⁺ cells (65%), as well as HLA-DR⁺ CD45-RO⁺ cells (53%) (see Figure 3.4.a). The mean percentage of cells expressing the HLA-DR marker was increased significantly in the EUs compared to donors (see Table 3.2; EUs: 20%; Donors: 14%; $t=2.33$, $P=0.03$).

The mean ratio of CD4:CD8 cells was also compared (see Table 3.2 and Appendix 3.1 for actual data). The ratio of CD4:CD8 cells in the HIV seroconverting individual was completely reversed at 0.22 compared to an average of 1.66 seen in the normal donors. The average in the EUs (1.36), did not differ significantly from the normal donors. C4051, who showed the large increase in CD8⁺ cells, had a ratio of 0.32, but did not show the abnormal levels of HLA-DR⁺, or CD45-RO⁺ cells seen in the seroconverting patient.

3.3.2 Lymphoproliferation Assays

14 EUs were assessed for proliferative responses to mitogen, alloantigens, recall antigens and HIV antigens. 12 normal donors were also analysed. The full SI data are presented in Appendix 3.2 and the range of actual geometric mean values obtained for each antigen are given in Appendix 3.3 with the 95% Confidence Intervals.

3.3.2.a Mitogen Responses

Activity to the mitogen PHA was monitored as a positive control, to assess if cells were reactive in the assay. Mitogenic responses peak at 3-4 days at an optimal PHA concentration of 10µg/ml. However, recall responses peak later (6-7 days) and in order that the assay could be harvested as a whole, suboptimal concentrations of the mitogen were used (5µg/ml and 1µg/ml) and left for the full 7 days.

All but one EU (C5721, 13/14) and all of the donors (12/12) had significant responses to both concentrations of PHA, i.e. a SI greater than 2 and most had SI values over 100 and 25 for the 5µg/ml and 1µg/ml concentrations respectively (see Figure 3.5.a and b). C5721 had a poor response to the higher concentration of PHA (SI=0.53), but at the time of harvesting few cells were seen and the media had turned yellow, reflective of exhaustion rather than unresponsiveness. In support of this, measurable levels of cytokine were detected from this assay (see Section 3.2.3). The range of responses varied between individuals, but the pattern of variation did not differ between the two groups (see Figure 3.5.a and b).

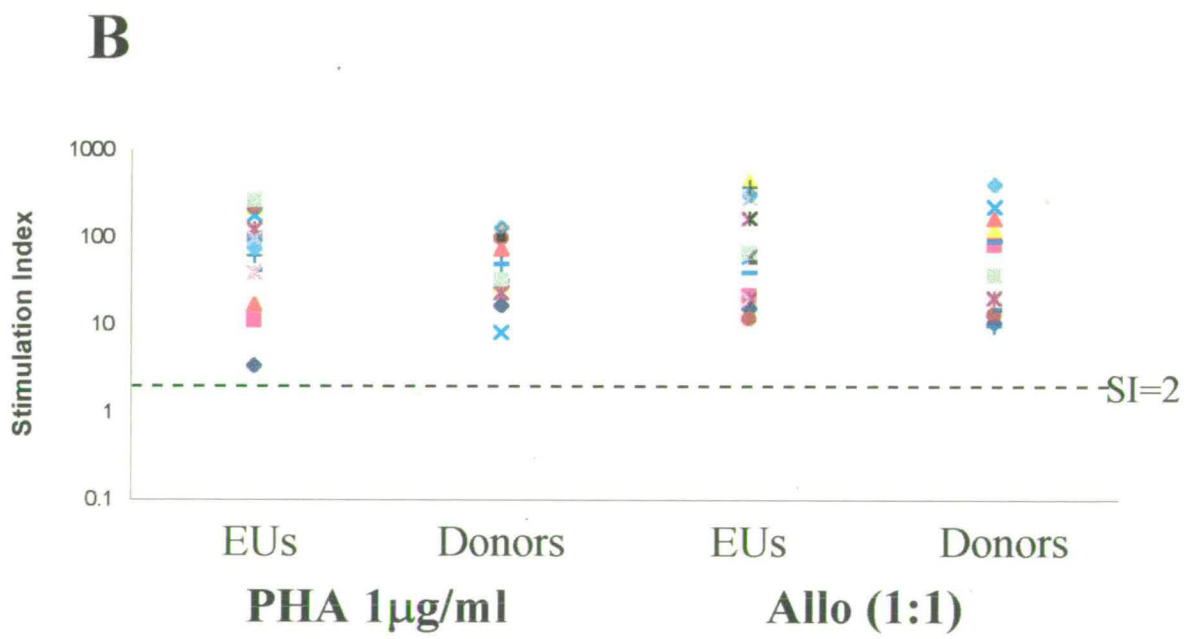
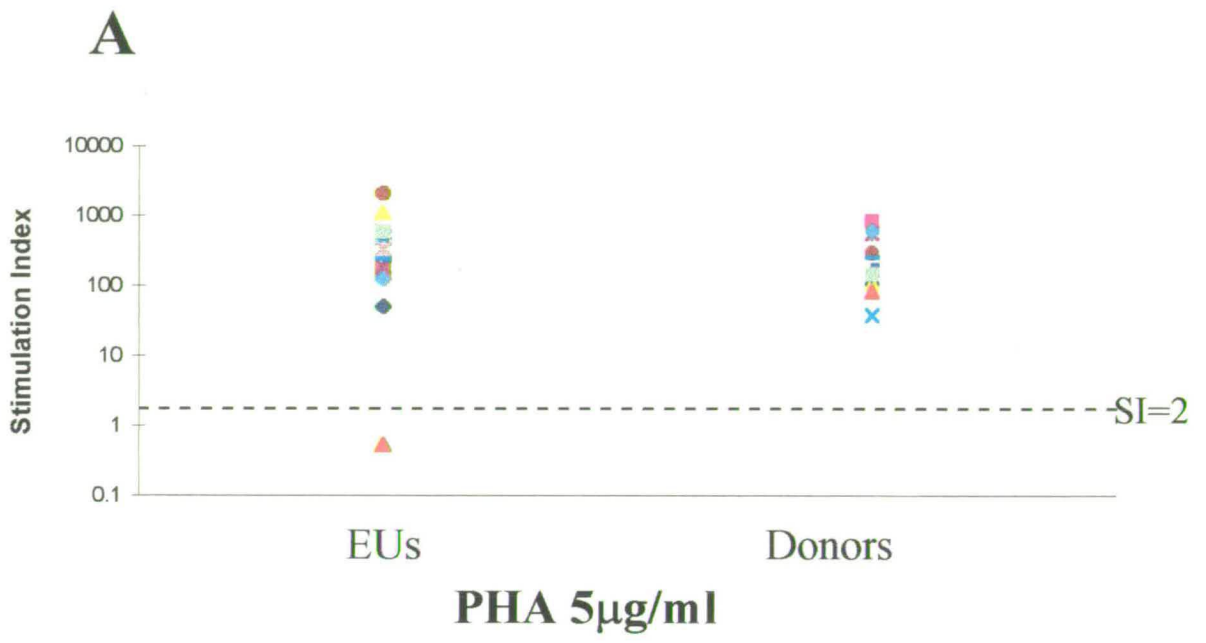
Figure 3.5.

Lymphoproliferations – Mitogen and Allogenic Responses

The following graphs show the stimulation indices (SI) plotted on a log scale obtained from culturing PBMCs with various antigenic stimuli (see Section 2.7.1). Values shown are those obtained from EUs and low risk donor controls (Donors). SI was determined by the following equation:

$$SI = \frac{\text{CPM obtained from cells alone (medium only)}}{\text{CPM obtained cells with antigen}}$$

- a.) Values obtained with the mitogen, PHA, at a concentration of 5µg/ml.
- b.) Values obtained with the mitogen, PHA, at a concentration of 1µg/ml and a mix of mitomycin C inactivated BCLs at a ratio of 1:1 BCLs: PBMCs (Allo).



Two of the EUs had SI>1000 (C4051: 1085; C4712: 2065) not seen in any of the donors and to determine if this was due to an earlier peak of responses in the donors compared to the EUs, some of the later samples were assessed over a time course (see Figure 3.6). Of the donors analysed, all but one had a decline in responses from day 4 through to 7. The remaining donor (Donor 13) had a small increase in SI at day 7, but the actual values at each time point were much closer than that for the other donors and the increase was in fact due to a decline in the background proliferation, rather than an increase in activity. Only two EUs were able to be assessed (see Section 3.2.1); C4401 did show a later peak than the donors (day 5), but by day 7, the value dropped considerably and was not above that seen for donors. C5761 had relatively similar values at days 4 and 5 and this had declined by day 7 in a similar manner to the donors (see Figure 3.6).

No difference was seen between the mean SI obtained in EUs compared to donors for either concentration of mitogen, assessed by a t-test following log transformation (see Table 3.3; 5µg/ml - EUs: 453; Donors: 283; $t=0.03$, $P=0.98$; 1µg/ml - EUs: 103; Donors: 47; $t=1.25$, $P=0.23$). Using cut off values of SI>2, 10 or 100 and analysed by Fisher's Exact test, there were also no differences between the proportion of responders versus non-responders (see Table 3.4).

3.3.2.b Allogeneic Responses

More of the EUs showed high responses to allo (SI - C4051: 448; C5191: 379; C5621: 306; C5731; Donor 7: 441; Donor 17: 227)(see Figure 3.5.b), no significant difference in the proportion of non-responders versus responders was observed between EUs and donors, even with a cut off of SI>100 (see Table 3.4). The range of SI values was similar for both groups (EUs: 12-448; Donors: 10-411) and there was no difference in the mean SI obtained for each group, assessed by a t-test following log transformation (See Table 3.3; EUs: 144; Donors: 100; $t=0.73$, $P=0.47$).

3.3.2.c Recall Responses

A PPD concentration of 100U/ml was found to be optimal and induced cell proliferation in several control individuals. Reactivity to TT was found to be more variable and hence two concentrations of this antigen were used, 2.5µg/ml and 1.25µg/ml.

Generally, more individuals were reactive to PPD than TT (SI>2, PPD: 22/26; TT 2.5µg.ml:15/26 ; TT 1.25µg/ml: 16/26) (see Figure 3.7.a and Table 3.4). The responses to

Figure 3.6.

Lymphoproliferations - Mitogen Time Course

The following graph shows the stimulation indices (SI) obtained from culturing PBMCs with the mitogen, PHA, at 5µg/ml (see Section 2.7.1). Values shown are those acquired, from named EUs and low risk donor controls (Donors), after harvesting the cultures at day 4, 5 and 7. SI was determined by the following equation:

$$SI = \frac{\text{CPM obtained from cells alone (medium only)}}{\text{CPM obtained cells with antigen}}$$

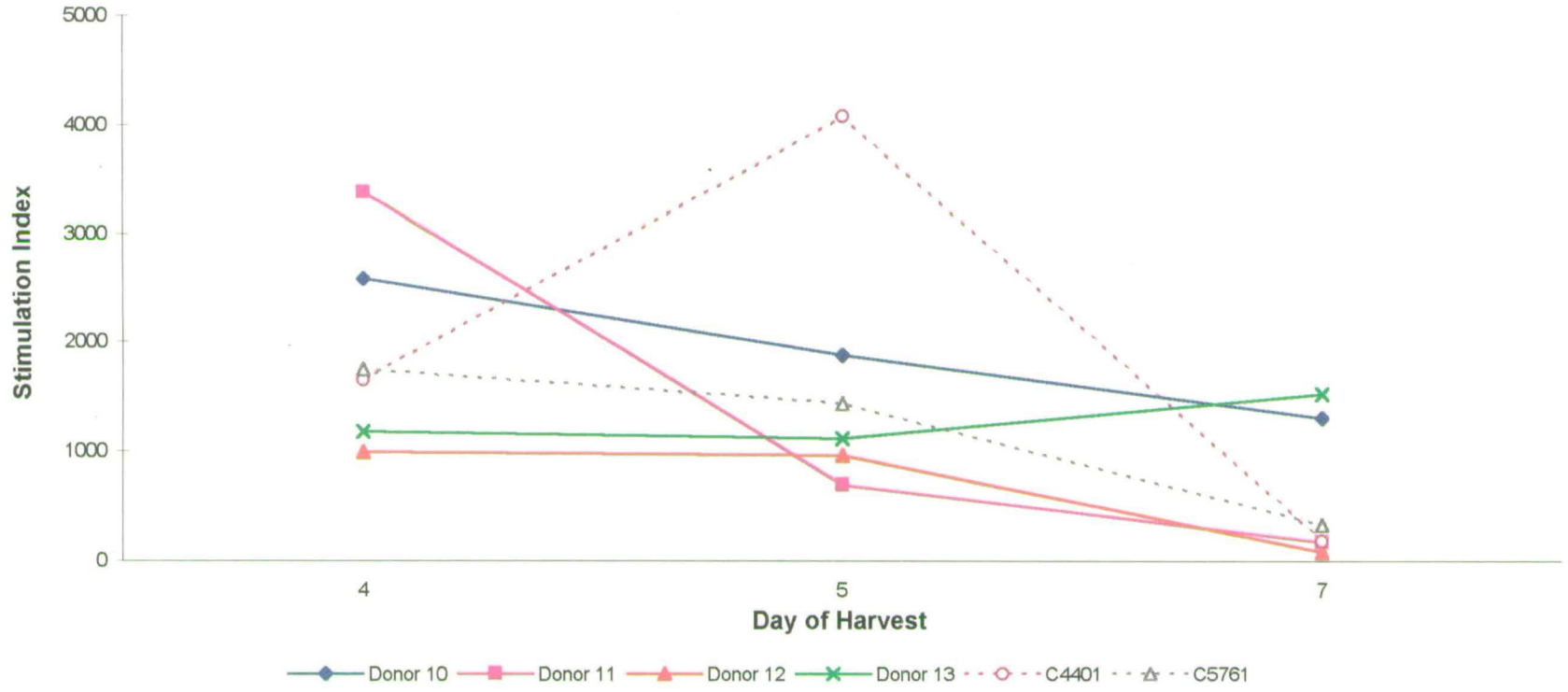


Table 3.3.**Mean Stimulation Indices for EUs and Donors and T-Test Results.**

	E Us				Don ors				<i>t</i> ^{\$}	P [£]
	n [*]	Mean	SD [#]	Range	n	Mean	SD	Range		
PHA (5µg/ml)	14	452.73	538.46	1-2065	12	283.43	253.29	38-837	0.03	0.98
PHA (1µg/ml)	14	103.06	86.10	3-262	12	47.06	35.15	8-127	1.25	0.23
Allo (1:1)	13	143.55	155.64	12-448	12	100.34	120.40	10-411	0.73	0.47
PPD (100/ml)	14	98.98	117.97	1-440	12	311.37	459.95	2-1427	0.86	0.40
TT (2.5µg/ml)	14	37.00	54.01	1-182	12	34.19	69.20	1-226	1.20	0.24
TT (1.25µg/ml)	14	51.16	77.87	1-273	12	21.50	34.33	1-110	1.08	0.29
rHIV (1.25µg/ml)	12	2.68	2.34	0-8	12	2.96	1.67	1-7	1.14	0.27
rHIV (0.625µg/ml)	12	3.91	3.80	1-13	12	3.06	1.97	1-7	0.10	0.92
rHIV (0.125µg/ml)	7	5.53	5.07	1-14	12	10.15	9.13	2-26	1.36	0.19

* Number of Individuals in Group

Standard Deviation

\$ T-test value (*t*) for difference between means of log transformed values, obtained using SPSS (see Section 2.20)

£ Probability (P) for difference between means of log transformed values

Table 3.4.**Proportion of Responders versus Non-responders in Proliferation Assays for EUs and Donors.**

	SI>2*			SI>10*			SI>100*		
	EUs	Donors	P [#]	EUs	Donors	P [#]	EUs	Donors	P [#]
PHA (5µg/ml)	13/14	12/12	0.54	13/14	12/12	0.54	12/14	11/12	0.40
PHA (1µg/ml)	14/14	12/12	1.00	13/14	11/12	0.52	5/14	1/12	0.10
Allo (1:1)	13/13	12/12	1.00	13/13	11/12	0.48	5/13	4/12	0.31
PPD (100/ml)	11/14	11/12	0.29	10/14	8/12	0.32	6/14	6/12	0.29
TT (2.5µg/ml)	10/14	5/12	0.10	7/14	3/12	0.14	2/14	2/12	0.40
TT (1.25µg/ml)	9/14	7/12	0.30	9/14	4/12	0.095 [§]	3/14	1/12	0.29
rHIV (1.25µg/ml)	5/12	8/12	0.16	0/12	0/12	1.00	[£] NT	NT	NT
rHIV (0.625µg/ml)	6/12	8/12	0.23	1/12	0/12	0.50	NT	NT	NT
rHIV (0.125µg/ml)	5/7	10/12	0.36	2/12	5/12	0.33	NT	NT	NT

* Number of Individuals with a Stimulation Index (SI)>2/10/100 compared to total numbers of Individuals

[#] Probability of difference between the two groups assessed by Fisher's Exact test. Exact test for 2x2 table only.[§] Sum of more extreme tables than initial 2x2 P=0.119[£] Not Tested

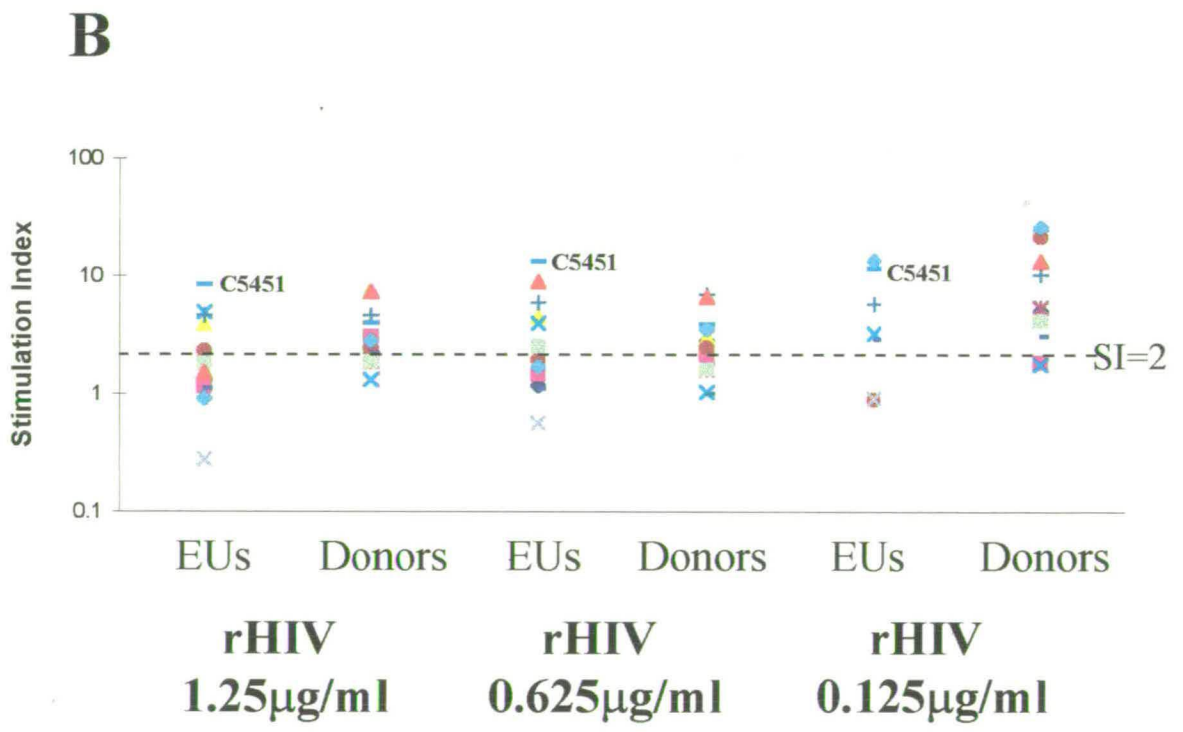
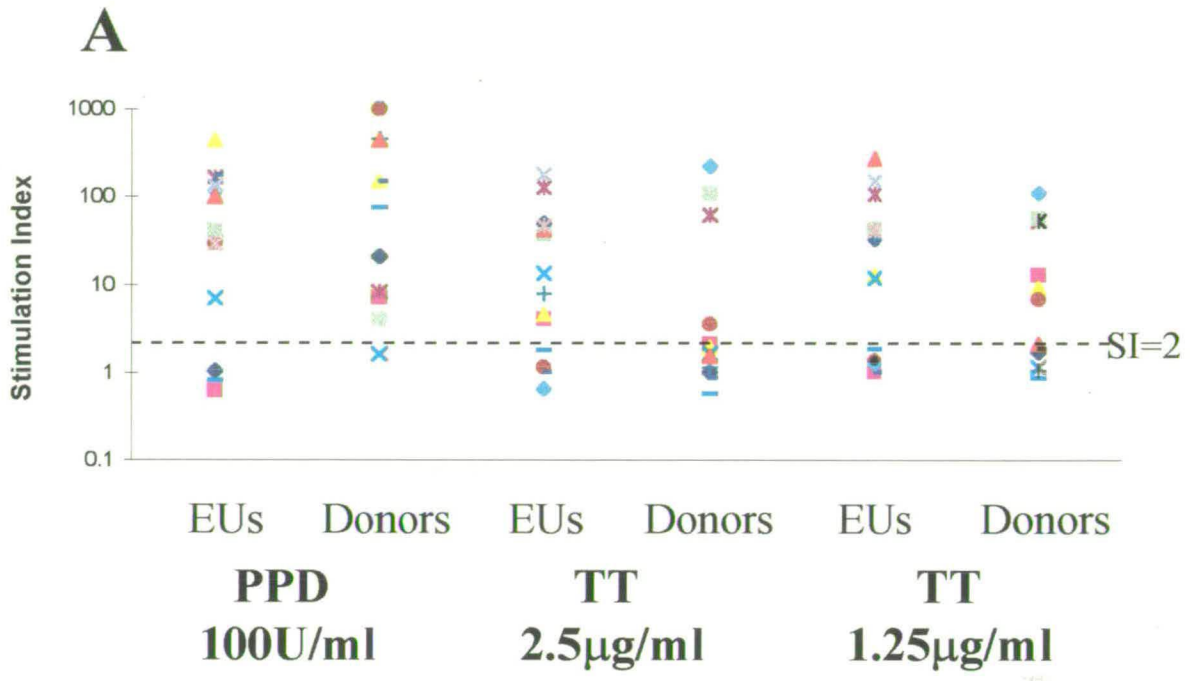
Figure 3.7.

Lymphoproliferations – Recall and HIV specific Responses

The following graphs show the stimulation indices (SI) plotted on a log scale obtained from culturing PBMCs with various antigenic stimuli (see Section 2.7.1). Values shown are those obtained from EUs and low risk donor controls (Donors). SI was determined by the following equation:

$$SI = \frac{\text{CPM obtained from cells alone (medium only)}}{\text{CPM obtained cells with antigen}}$$

- a.) Values obtained with the recall antigens, purified protein derivative from Mycobacterium (PPD:100U/ml) and tetanus toxoid (TT: 2.5µg/ml; 1.25µg/ml).
- b.) Values obtained with a recombinant HIV protein 'cocktail' (1.25µg/ml; 0.625µg/ml; 0.125µg/ml for each of the constituent proteins).



PPD were also greater in magnitude than those to TT (SI>100, PPD: 12/26; TT: 4/26 both concentrations) (see Figure 3.7. and Table 3.4).

The range of responses to PPD was greater for the donors (SI: 2-1427) than the EUs (SI:1-440) (see Figure 3.7.a), but the mean SI for the two groups (EUs: 99; Donors: 311) did not differ significantly, as assessed by t-test following log transformation (see Table 3.3; $t=0.86$, $P=0.40$). There was also no difference between the proportion of non-responders versus responders using cut off values of SI>2, 10 or 100 and analysed by Fisher's Exact test (see Table 3.4).

The range of responses to TT was similar in both groups (2.5µg/ml - EUs: 1-182; Donors: 1-226; 1.25µg/ml - EUs: 1-273; Donors: 1-110) (see Figure 3.7.a and Table 3.3) and the mean SI did not differ significantly (see Table 3.3; 2.5µg/ml - EUs: 37; Donors: 34; $t=1.20$, $P=0.244$; 1.25µg/ml - EUs: 51; Donors: 22; $t=1.08$, $P=0.29$). The proportion of responders compared to non-responders, at the lower concentration of antigen (1.25µg/ml) and a basal level of SI>10, gave a difference of 9/14 EUs compared to only 4/12 donors, but this was not significant for the limited sample size analysed here (see Table 3.4; $P=0.12$, Exact test).

3.3.2.d HIV-Specific Responses

As control individuals would not be expected to show responses to HIV and HIV⁺ individuals soon lose HIV-specific reactivity, control experiments could not be performed to assess the optimal concentration of antigen. Hence a broad range of concentrations was chosen (1.25µg/ml, 0.625µg/ml and 0.125µg/ml for each of the representative proteins) and preliminary experiments showed no reactivity to the recombinant HIV cocktail (rHIV) in control subjects.

Reactivity was detected in 5/12; 6/12; 5/7 EUs, but also 8/12; 8/12; 10/12 donors to 1.25; 0.625; 0.125µg/ml respectively (see Figure 3.7.b and Table 3.4) with an SI>2. Although, more donors than EUs appeared to react to the cocktail at concentrations of 1.25 and 0.625µg/ml, the range of responses were similar in both groups (see Figure 3.7.b and Table 3.3). The range of responses in donors was greater than that of the EUs for the lowest concentration of antigen (0.125µg/ml) (EUs: 1-14; Donors: 2-26)(see Figure 3.7.b). The proportion of individuals responding to the HIV proteins did not differ between the groups, as assessed by Fisher's Exact test using a cut of value of SI>2, or SI>10, for any concentration (see Table 3.4).

The mean SI for the Donors and EUs, as assessed by a t-test of log transformed values, was not significantly different for any concentration of the HIV cocktail (see Table 3.3; 1.25µg/ml - EUs: 2.7; Donors: 3.0; $t=1.14$, $P=0.27$; 0.625µg/ml - EUs: 3.9; Donors: 3.1; $t=0.10$, $P=0.92$; 0.125µg/ml - EUs: 5.5; Donors: 10.2; $t=1.36$, $P=0.19$)

The high proportion of apparent HIV-specific activity in the donors was unexpected and implied that cross reactivity of some sort may be occurring. Further investigation of the antigens suggested that the cross reactivity was due to responses to NEF, RT and TAT, which were all recombinant, *E.coli*-derived proteins (Table 3.5). No responses were seen to the Baculovirus-derived gp120 (HIV_{MN} ENV) and p24 protein (GAG), or the gp120 (HIV_{SF2} ENV) derived in Chinese hamster ovary cells (CHO) (Table 3.5). Subsequent samples were then assessed for reactivity to the GAG and ENV proteins alone. However, due to the cessation of the Heterosexual Partner Study (see Section 3.2.1), only 2 EU samples were received during this time and neither showed reactivity to this modified cocktail. No further samples were then available for analysis to determine if this modification would have allowed HIV-specific responses to be detected.

Several of the EUs did show responses to the HIV proteins (see Figure 3.7.b). One of the EU individuals (C5451) showed marked responses to all the concentrations of the HIV cocktail (see Figure 3.7.b, SI= 8, 13 and 11 for 1.25, 0.625, 0.125µg/ml concentrations respectively). This was greater than that seen in of any of the other EUs, or the donors (for the lowest two concentrations), but due to the aforementioned problems, it was impossible to determine if this was in fact a true, HIV-specific response, or not.

3.3.3 Cytokine Production

Measurable levels of cytokine were only obtained for IFN-γ and some assays produced over the maximal amount i.e were greater than the range defined by the standard curve (>1500pg/ml if neat and >75000pg/ml if 1:50 dilution). For statistical calculations, values >1500pg/ml and >75000pg/ml were taken as just 1500pg/ml and 75000pg/ml. As this underestimates the value obtained, the values were also assessed as the proportion responding versus not responding using a range of cut of points (Any response, >1500pg/ml, >10000pg/ml and >75000pg/ml). Where duplicate values were inconsistent (>20% difference between samples), the values were ignored as they could not be repeated due to a lack of further culture supernatant for testing. Actual data is presented in Appendix 3.4. Spontaneous IFN-γ production in the wells without antigen were detected in 4/11 EUs and 3/8 Donors (see

Table 3.5.**Donor Stimulation Indices to Recombinant HIV Proteins**

ANTIGEN	DONOR A	DONOR B	DONOR C
PHA 5µg/ml	190	1394	310
gp120 _{MN} 1.25µg/ml	1	1	1
gp120 _{MN} 0.125µg/ml	1	1	1
gp120 _{SF2} 1.25µg/ml	1	1	1
gp120 _{SF2} 0.125µg/ml	1	1	1
p24 1.25µg/ml	1	1	1
p24 0.125µg/ml	1	3	1
nef 1.25µg/ml	8	11	19
nef 0.125µg/ml	3	17	9
RT 1.25µg/ml	2	12	9
RT 0.125µg/ml	2	10	5
tat 1.25µg/ml	3	13	6
tat 0.125µg/ml	1	1	1

Table 3.6) and for individuals with a recorded value of the spontaneous IFN- γ production, the values were recalculated subtracting this background and the statistics re-calculated. The adjusted data are shown in Appendix 3.5.

The amount of IFN- γ produced in response to mitogen was similar for both EUs and donors (see Figure 3.8), with no difference observed between the square root (to normally distribute the data) of the means, assessed by a t-test (see Table 3.7; PHA 5 μ g/ml: $t=0.75$, $P=0.46$; 1 μ g/ml: $t=0.80$, $P=0.43$). However, responses to allo were very different in the EUs compared to donors (see Figure 3.8). The donors either produced a large amount of IFN- γ in response to the allogeneic stimulus, or very little. In contrast, there was a broad range of responses within the EUs, all producing over 1500pg/ml (14/14), which was significantly different to the number of donors, as only 4/10 produced more than this amount (see Table 3.6; $P=0.002$, Exact Test). The number of EUs that produced over 10000pg/ml differed too, although not formally significant (see table 3.6; EUs: 10/14; Donors: 3/10, $P=0.055$, Exact test). The difference between the square root of the mean values for both groups also approached significance (see Table 3.7; EU: 34499; Donors: 20275; $t=1.84$, $P=0.079$).

The range of IFN- γ produced in response to the recall antigens, PPD and TT was similar for both groups (see Figure 3.8 and 3.9.a) and as for the proliferative response, the maximal responses to PPD were higher than that to TT (see Table 3.6; >1500pg/ml IFN- γ - PPD: 10/23; TT (2.5 μ g/ml): 4/20; TT (1.25 μ g/ml): 3/22). No significant difference was seen for the proportion of individuals producing IFN- γ in EUs compared to donors for either antigen (see Table 3.6).

Responses to the higher concentrations of the rHIV cocktail used were generally poor in the donors, consistent with the proliferation results (see Figure 3.9.b), with only 2/7 and 2/10 showing measurable levels of IFN- γ for 1.25 μ g/ml and 0.625 μ g/ml of rHIV respectively. A higher proportion of EUs showed a response at these concentrations (1.25 μ g/ml: 4/9; 0.625 μ g/ml: 3/9) and two recorded values over 1500pg/ml (see Figure 3.9.b), but the difference between the two groups was not significant (see Table 3.6).

Of the EUs responding, C3154, C5411 and C5731 did not show a corresponding proliferative response to these antigens (see Figure 3.9.b) and this was consistent for all the concentrations tested, as all lacked a recordable response to one concentration. Of the donors who produced a measurable IFN- γ response to rHIV, all but one (D3 0.625 μ g/ml rHIV) had a consistent proliferative response, especially at the lowest concentration of antigen, which also gave the highest proliferative responses (see Figure 3.9.b).

Table 3.6.**Proportion of Responders versus Non-responders for IFN- γ Production in EUs and Donors**

	Responders/Total			>1500pg/ml			>10000pg/ml			>75000pg/ml		
	EUs	Donors	P [#]	EUs	Donors	P [#]	EUs	Donors	P [#]	EUs	Donors	P [#]
No Antigen	4/11	3/8	0.37	0/11	0/8	1.00	NT	NT	NT	NT	NT	NT
PHA (5 μ g/ml)	13/13	10/10	1.00	13/13	10/10	1.00	7/13	8/10	0.16	1/13	0/10	0.57
PHA (1 μ g/ml)	13/13	10/10	1.00	11/13	6/10	0.16	6/13	3/10	0.25	0/13	0/10	1.00
Allo (1:1)	14/14	8/10	0.16	14/14	4/10	[£] 0.002	10/14	3/10	[§] 0.055	2/14	1/10	0.45
PPD (100/ml)	13/14	7/9	0.29	6/14	4/9	0.33	3/14	2/9	0.39	0/14	0/9	1.00
TT (2.5 μ g/ml)	7/12	3/8	0.24	3/12	1/8	0.36	NT	NT	NT	NT	NT	NT
TT (1.25 μ g/ml)	8/13	4/9	0.25	3/13	0/9	0.19	NT	NT	NT	NT	NT	NT
rHIV (1.25 μ g/ml)	4/9	2/7	0.24	1/9	0/7	0.56	NT	NT	NT	NT	NT	NT
rHIV (0.625 μ g/ml)	3/9	2/10	0.33	1/9	0/10	0.47	NT	NT	NT	NT	NT	NT
rHIV (0.125 μ g/ml)	5/7	6/6	0.27	1/7	3/6	0.20	NT	NT	NT	NT	NT	NT

* Number of Individuals producing IFN- γ to defined level compared to total number of Individuals

[#] Probability of difference between the two groups assessed by Fisher's Exact test. Exact test for initial 2x2 table only, unless P<0.10, then one tailed test for sum of more extreme tables ([§])

[£] P<0.05

NT - Not Tested

Figure 3.8.

IFN- γ Production in Lymphoproliferation Assays : Mitogen, Allo and PPD

The following graph shows the amount of the cytokine, IFN- γ , produced as monitored by ELISA (see Section 2.7). The results shown are for EUs and low risk donor controls (Donors) for the following antigenic stimuli: PHA: 5/1 μ g/ml; allogenic response (allo) and the recall antigen PPD (100U/ml).

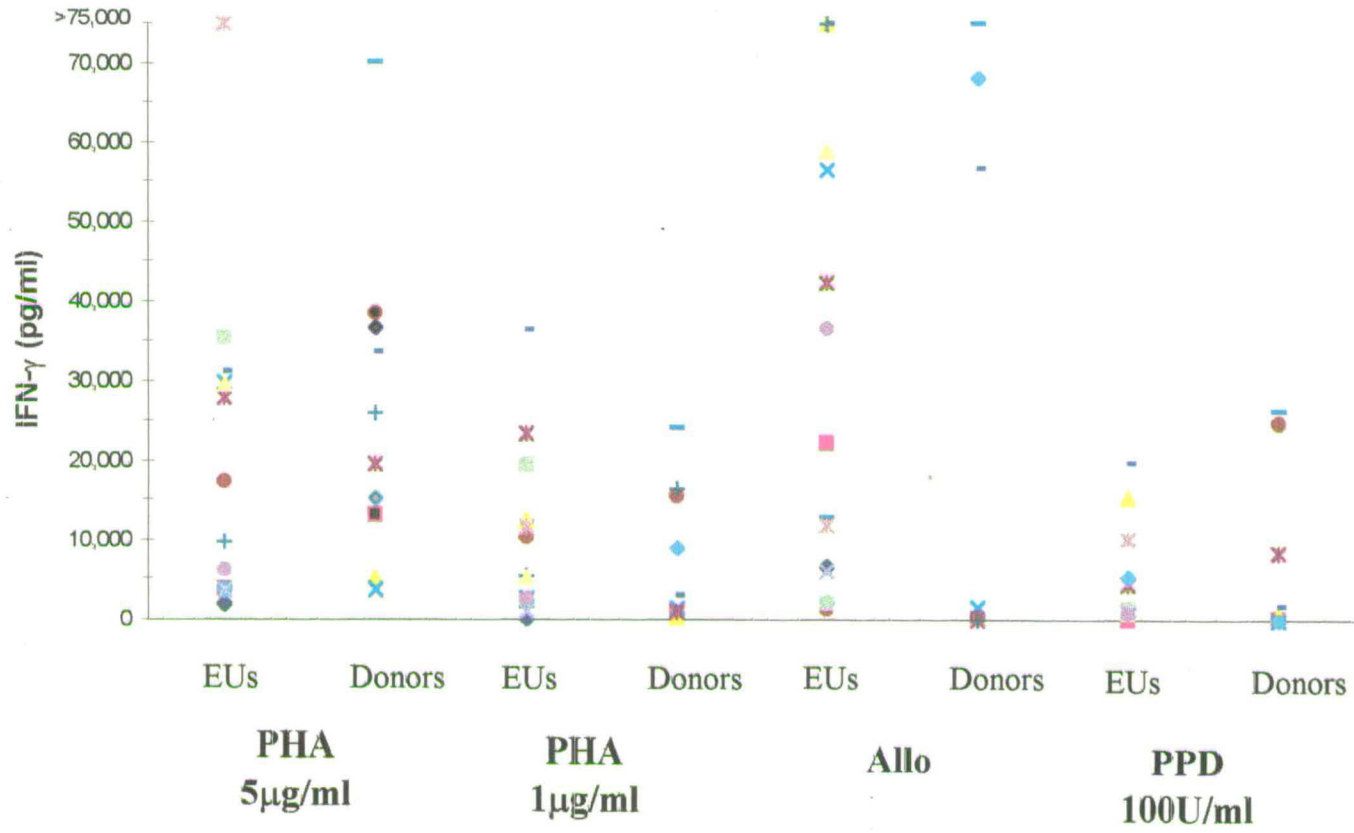


Table 3.7.**Mean IFN- γ (ng/ml) Production for EUs and Donors and T-Test Results.**

	EUs				Donors				t^s	P^{ϵ}
	n*	Mean	SD [#]	Range	n	Mean	SD	Range		
No Antigen	11	218.9	362.4	0-1109	8	136.6	236.4	0-672	0.33	0.75
PHA (5 μ g/ml)	13	21217.7	20475.4	1978->75000	10	26171.5	19806.9	3727-70100	0.75	0.46
PHA (1 μ g/ml)	13	10210.1	10697.9	144-36400	10	7259.3	8576.1	362-24100	0.80	0.43
Allo (1:1)	14	34498.7	28990.1	>1500->75000	10	20275.1	32289.3	0->75000	1.84	0.08
PPD (100/ml)	14	4213.5	6304.1	0-19800	9	6952.2	10902.5	0-26300	0.27	0.79
TT (2.5 μ g/ml)	12	580.7	650.6	0->1500	8	290.0	542.8	0->1500	1.08	0.30
TT (1.25 μ g/ml)	13	599.5	627.5	0->1500	9	220.7	612.4	0-939	1.42	0.17
rHIV (1.25 μ g/ml)	9	297.6	493.1	0->1500	7	73.2	127.3	0-298	1.05	0.31
rHIV (0.625 μ g/ml)	9	323.5	552.8	0->1500	10	37.0	78.0	0-187	1.38	0.18
rHIV (0.125 μ g/ml)	5	917.6	552.9	265->1500	6	904.1	653.3	265->1500	1.04	0.32

* Number of Individuals in Group

⁺ Mean value taking >1500 and >75000 as 1500 and 75000 respectively

[#] Standard Deviation

^s T-test value (t) for difference between square root of means, obtained using SPSS (see Section 2.20)

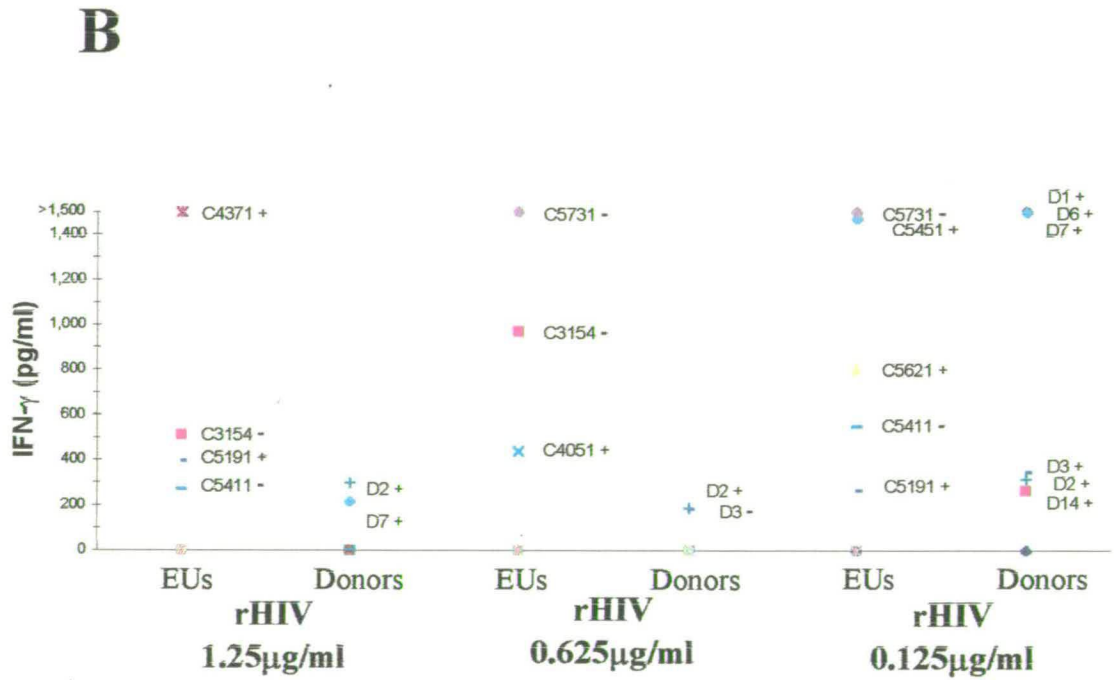
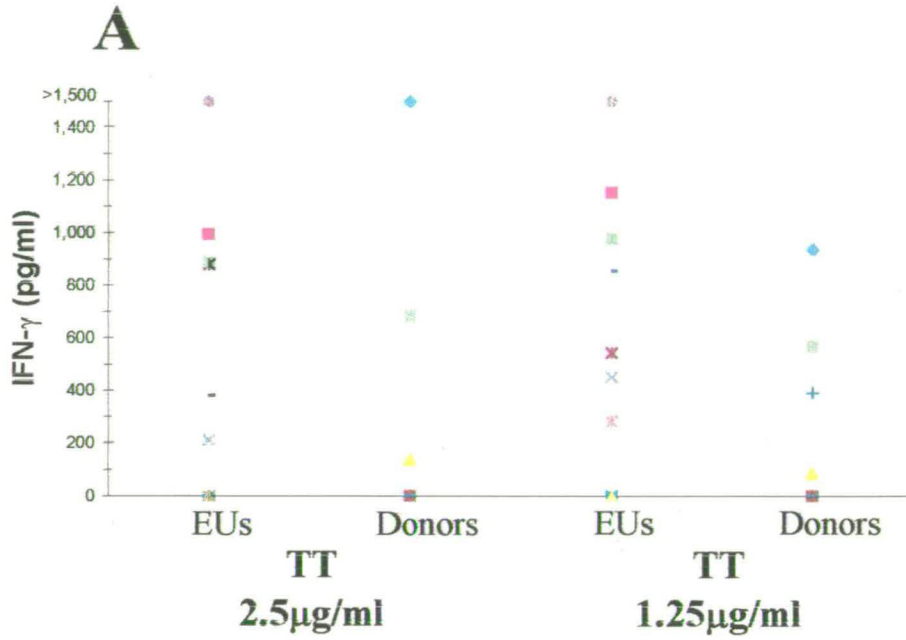
^{\epsilon} Probability (P) for difference between the square root of means

Figure 3.9.

IFN- γ Production in Lymphoproliferation Assays : TT and rHIV

The following graphs show the amount of the cytokine, IFN- γ , produced as monitored by ELISA (see Section 2.7). The results shown are for EUs and low risk donor controls (Donors).

- a.) Results obtained with the recall antigen tetanus toxoid, TT, at 2.5 μ g/ml and 1.25 μ g/ml.
- b.) Results obtained with the recombinant HIV 'cocktail' (rHIV), at 1.25/0.625 and 0.125 μ g/ml for each of the constitutive proteins. Individuals responding have been noted as producing IFN- γ and proliferating in response to rHIV (SI>2), denoted as '+', or producing IFN- γ , but not showing a consistent proliferative response to the cocktail, marked as '-'.



Despite increased mean values for the EUs for the higher concentrations of antigen, the difference was not significant with the small numbers analysed (see Table 3.7). In contrast, for the lowest concentration (0.125µg/ml), the donors had the highest mean, but again this did not differ significantly between the two groups (see Table 3.7).

3.3.3.a Adjusted Values

For individuals who had a recorded result for the spontaneous production of IFN-γ, i.e. 'no antigen' value (EUs n=10, Donors n=8), the values were re-calculated deducting any spontaneous IFN-γ production (see Appendix 3.5). Despite minor fluctuations in the mean values for the two groups, from that obtained in the unadjusted data, no major differences were seen in the adjusted data (see Table 3.8). The proportion of responders versus non-responders was also not greatly altered in these adjusted figures (see Table 3.9). The only real difference to the data in was seen for the IFN-γ production in response to alloantigen. The difference between the means between the EUs and Donors was closer to significance in the adjusted data (EUs: 38669; Donors: 16561; $t=2.07$, $P=0.054$; see Table 3.9). Also the proportion of individuals producing >1500pg/ml, was still significant (EUs 10/10; Donors 2/8; $P<0.002$; see Table 3.9) and the proportion producing >10000pg/ml was significant in the adjusted values (EUs 8/10; Donors 2/8; $P=0.003$; see Table 3.9). Therefore, the level of background IFN-γ production did not greatly affect the outcome of the data and was not altering the apparent increase in IFN-γ production seen in response to alloantigen.

3.3.4 Combined Effects of IFN-γ and Proliferations

Regression plots were calculated for the log transformed SI values and the square root of the IFN-γ production for each of the respective antigens. For all the individuals assessed (donors and EUs pooled together) a significant positive association between the level of proliferation and the amount of IFN-γ produced was seen for both concentrations of mitogen (5 and 1µg/ml $P<0.05$), both recall antigens ($P<0.01$ for all) and for the alloantigen ($P<0.02$). These associations were maintained for the recall antigens when the two cohorts were assessed separately ($P<0.01$ for all, except donors TT 1.25µg/ml; $P<0.02$).

When the donors and EUs were considered separately, there was no significant difference observed, between the two groups, for any antigen. This was particularly pertinent for the allo responses, which had shown a significant difference in the IFN-γ production of the two groups. Although most of the donors produced little, if any, IFN-γ, despite a proliferative

Table 3.8.**Mean IFN- γ (ng/ml) Production for EUs and Donors and T-Test Results - Adjusted Values**

	EUs				Donors				t^s	p^t
	n^*	Mean	SD [#]	Range	n	Mean	SD	Range		
PHA (5 μ g/ml)	9	26061.0	21805.1	3105->75000	8	30211.9	19873.8	5138-69897	1.07	0.30
PHA (1 μ g/ml)	10	12381.7	11148.6	557-36117	8	7628.0	9328.4	362-23897	0.94	0.36
Allo (1:1)	10	38668.9	30519.7	>1500->75000	8	16560.6	30742.6	0-74797	2.07	0.05
PPD (100/ml)	10	5508.3	7016.4	0-19517	7	8878.4	11714.7	63-26097	0.73	0.47
TT (2.5 μ g/ml)	9	551.0	680.6	0->1500	6	216.1	529.4	0-1297	0.91	0.38
TT (1.25 μ g/ml)	9	542.6	649.8	0->1500	7	161.3	292.7	0-736	1.19	0.25
rHIV (1.25 μ g/ml)	6	250.0	612.4	0->1500	7	42.6	112.6	0-298	0.43	0.68
rHIV (0.625 μ g/ml)	5	388.2	650.2	0->1500	8	23.4	66.2	0-187	1.19	0.26
rHIV (0.125 μ g/ml)	5	586.3	586.5	0->1500	6	812.6	691.4	0->1500	1.14	0.28

* Number of Individuals in Group

⁺ Mean value taking >1500 and >75000 as 1500 and 75000 respectively

[#] Standard Deviation

^s T-test value (t) for difference between square root of means, obtained using SPSS (see Section 2.20)

^t Probability (P) for difference between the square root of means

Table 3.9.

Proportion of Responders versus Non-responders for IFN- γ Production in EUs and Donors - Adjusted Values

	Responders/Total			>1500pg/ml			>10000pg/ml		
	EUs	Donors	P [#]	EUs	Donors	P [#]	EUs	Donors	P [#]
PHA (5 μ g/ml)	9/9	8/8	1.00	9/9	8/8	1.00	7/9	7/8	0.42
PHA (1 μ g/ml)	10/10	8/8	1.00	9/10	4/8	^{\$} 0.09	6/10	3/8	0.24
Allo (1:1)	10/10	6/8	0.18	10/10	2/8	[£] 0.002	8/10	2/8	^{£\$} 0.003
PPD (100/ml)	9/10	7/7	0.59	5/10	4/7	0.36	3/10	2/7	0.41
TT (2.5 μ g/ml)	4/9	1/6	0.25	2/9	0/6	0.34	NT	NT	NT
TT (1.25 μ g/ml)	5/9	2/7	0.23	2/9	0/7	0.30	NT	NT	NT
rHIV (1.25 μ g/ml)	1/6	2/7	0.44	1/6	0/7	0.46	NT	NT	NT
rHIV (0.625 μ g/ml)	2/5	1/8	0.28	1/5	0/8	0.38	NT	NT	NT
rHIV (0.125 μ g/ml)	3/5	5/6	0.36	1/5	2/6	0.45	NT	NT	NT

* Number of Individuals producing IFN- γ to defined level compared to total number of Individuals

[#] Probability of difference between the two groups assessed by Fisher's Exact test. Exact test for initial 2x2 table only, unless P<0.10, then one tailed test for sum of more extreme tables (^{\$})

[£] P<0.05

NT - Not Tested

response to allo, three donors did produce IFN- γ and hence, the donor population as a whole gave a similar pattern of responses to the EUs (see Figure 3.10).

3.4. DISCUSSION

3.4.1 Exposed uninfected status

The individuals recruited into this study had been shown to be antibody negative, despite repeated heterosexual exposure to HIV. A subset of individuals were assessed by PCR to be negative for HIV below a level of 1 copy/ 10^5 PBMCs, which is lower than that normally found in asymptomatic patients (Simmonds *et al.* (1990b)). It is unlikely that these individuals were harbouring a very low level infection as most were monitored over a prolonged period and were persistently seronegative. One report claimed to find a high proportion of persistently PCR⁺ seronegative individuals (Imagawa *et al.* (1989)), but this has not been found in other, more recent studies (Gibbons *et al.* (1990); Lee *et al.* (1991); Pan *et al.* (1991); Brettler *et al.* (1992); Coutlée *et al.* (1994); MacGregor *et al.* (1995)); even with the use of a high-input PCR methodology designed to detect low level infection (Lee *et al.* (1991)). Frenkel *et al.* (1998) recently looked extensively at transient infection in children born to HIV⁺ mothers and highlighted that many earlier reports of such cases could in fact be attributed to contamination, or sample mixing. It is thought that this is also possible for many of the early reports of PCR⁺, yet persistently seronegative individuals.

3.4.1.a Lymphocyte Subsets

The lymphocyte subset composition of the PBMCs was determined to further confirm the uninfected status of the EUs. Perturbations in the CD4:CD8 ratios are known to occur before seroconversion and relate to massive expansion of CD8⁺ cells, including cells co-expressing the memory/activation markers, HLA-DR and CD45-RO (Yagi *et al.* (1991); Zaunders *et al.* (1995)).

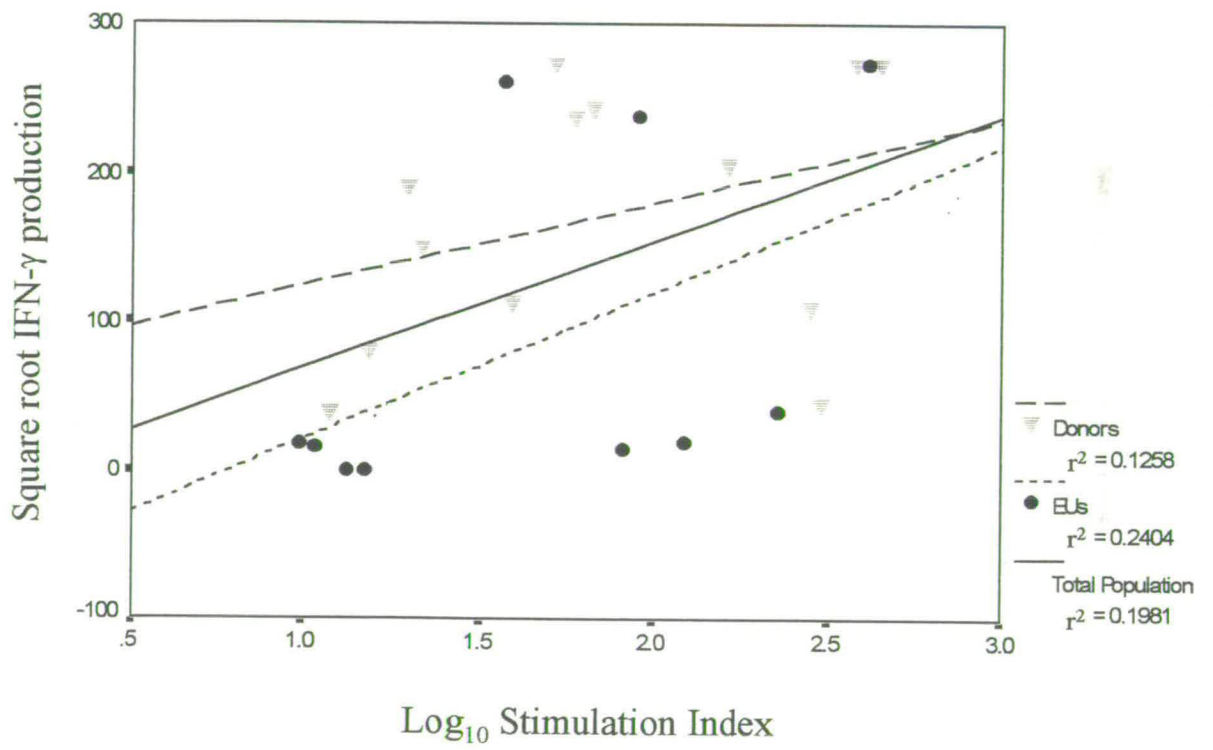
The percentage of CD4⁺ and CD8⁺ cells varied from individual to individual, but did not differ from the range seen in normal donors. In contrast, the level of CD8⁺ cells was markedly increased in an individual known to be undergoing seroconversion (C5751). The same individual showed a concurrent increase in the percentage of lymphocytes expressing HLA-DR and CD45-RO and a large proportion expressing both.

Figure 3.10.

Regression Plot of IFN- γ Production versus Proliferation to Allo Antigen

The following graph shows the regression plot, obtained using SPSS, of the square root of the amount of IFN- γ produced (pg/ml) compared to the \log_{10} of the stimulation index obtained after allo antigen stimulation.

Responses by individual Donors are represented by triangles and EUs by closed circles. The population regression is shown by a dashed line for the Donors, dotted line for the EUs and a solid line for all individuals. The regression value r^2 is shown for these three lines and the significance of the correlation are: Donors and EUs; P= not significant; All individuals: P<0.02.



The percentage of lymphocytes from EUs expressing the memory marker CD45-RO also did not differ from the range seen in normal donors. The seroconverter, in contrast, showed a large percentage of lymphocytes expressing these markers and although the cells were not co-stained for CD8, the fact that over 70% of the lymphocytes are CD8⁺, a large proportion must be CD8⁺ cells. This pattern is in concordance with that observed in other seroconverting (Yagi *et al.* (1991); Zaunders *et al.* (1995)) and HIV⁺ individuals (Prince and Jensen (1991); Ho *et al.* (1993); Watret *et al.* (1993)).

The percentage of cells expressing the activation marker, HLA-DR, was significantly elevated in the EUs compared to donors and may reflect differences in the lifestyles of the two groups (see Section 3.4.2.a).

A perturbed CD4:CD8 ratio was seen in one contact (C4051), although no increase in HLA-DR and CD45-RO was seen in the lymphocytes overall. However, if the CD8⁺ cells had been co-stained with HLA-DR, or CD45-RO, it may have help explain if this was also due to widespread activation. The increased expression of HLA-DR and CD45-RO on CD8⁺ cells is thought to represent an increase in immunologically reactive cells, namely CTLs (Ho *et al.* (1993); Rowland-Jones *et al.* (1993); Watret *et al.* (1993)). Although, similar patterns are seen in other viral infections (Fauci *et al.* (1991); Zaunders *et al.* (1995)), the population of activated CD8⁺ cells tends to decline following recovery from infection. In HIV infection in contrast, these activated cells persist (Prince and Jensen (1991); Zaunders *et al.* (1995)), probably reflecting the persistence of viral antigen.

The activation markers, HLA-DR and CD45-RO, reflect the activation status of lymphocytes, in particular co-expression of HLA-DR and CD45-RO. However, if used in conjunction with CD4 and CD8 staining, this may have been more informative. Co-expression of HLA-DR and CD45-RO was shown in early infection by Prince and Jensen (1991), who reported a decrease in CD8⁺ cells singly expressing HLA-DR, or CD45-RO, but an increase in those expressing both. Dual staining for CD8 and HLA-DR/CD45-RO would not therefore have shown this and triple staining would have been required. A proportion of the HLA-DR⁺ CD45-RO⁺ cells in the seroconverter may also have been CD4⁺, as this subset has also been shown to be elevated during HIV infection (Kestens *et al.* (1994)).

The addition of the marker CD38 with CD8 may also provide further indication of infection. CD38 is a multilineage marker and is thought to be involved in activation and/ or

adhesion (Malavasi *et al.* (1994)). However, although CD38 is elevated on CD8⁺ cells early in infection (Prince and Jensen (1991); Yagi *et al.* (1991); Kestens *et al.* (1992); Giorgi *et al.* (1994); Zaunders *et al.* (1995)), HLA-DR has been shown to be increased before this (Kestens *et al.* (1992)).

Although the infection state of an individual could not have been deduced from such analysis, it would have been a significant indicator and highlighted any potential cases prior to the presence of HIV-specific antibody (Yagi *et al.* (1991); Rowland-Jones *et al.* (1993); Giorgi *et al.* (1994); Zaunders *et al.* (1995)), as can be seen in the seroconverting individual.

3.4.2 Proliferations

3.4.2.a Mitogen Responses

To determine whether the EUs possessed any proliferative capacity which enabled them to remain protected from heterosexual infection, lymphoproliferation assays were performed using various antigens. The mitogen, PHA, which non-specifically activates cells through both the T cell receptor and CD2 (Coligan *et al.*(1996)), was used as a positive control to confirm reactivity in the assay. There was no observed difference between the response in EUs compared to normal donors, although two of the EUs had higher responses. To ascertain if this was due to a difference in the time taken to achieve a maximal response, a time course was assessed. However, despite one of the EUs showing a delay in peak response from day 4 to day 5, too few samples were able to be assessed in this way. If this had been confirmed in larger numbers, it may reflect a difference in the proportion of CD45-RA⁺ to CD45-RO⁺ cells. CD45-RO⁺ cells have been shown to respond more slowly to mitogen than CD45-RA⁺ cells (Merkenschlager *et al.* (1988); Merkenschlager and Beverley (1989); Morimoto *et al.* (1985)) and this could have been confirmed by flow cytometry staining for CD4/8 and CD45-RO/RA.

The CD45-RO isoform is expressed on memory cells (Clement (1992)) and a higher proportion of cells expressing this marker could suggest persistent activation. Persistent activation from exposure to HIV could theoretically lead to a difference in the two groups, although it seems unlikely that the EUs would have a constant level of exposure to HIV, which would induce an overall difference in all EU individuals at all times. No difference in the percentage of CD45-RO⁺ cells was seen in the two groups either. However, the level of HLA-DR expression was elevated in the EUs, suggestive of possible on going activation.

It is possible that there are differences in life-style and socio-economic factors between the EUs and donors. Most of the EUs are recruited from one of the poorer regions of Edinburgh which may lead to differences in nutritional status and hence overall health, allowing increased occurrence of infectious diseases and hence more immune activation. However, socio-economic differences are less likely to lead to such changes in a modern society than they would have in the past, or in a less developed society, such as in Africa. The possibility that such differences occur still exists, but it would require a large scale study just to assess nutritional differences alone and extends well beyond the scope of this study.

A further difference between the groups is the age range. The average age of the EUs is 31.0 (range 17-45) and 37.4 (20-60) for all donors, which is a significant difference ($t=2.11$, $P=0.04$). However, when only the donors used in the LPA were assessed, the mean age was 36.2 (20-51) which was not significantly different from the EUs ($t=1.72$, $P=0.10$). Age-related effects on the immune system include: a decrease in naïve T cells ($CD45-RA^+$), an increase in memory T cells ($CD45-RO^+$) and a decrease in IL-2 production (Miller (1996)). However, studies are often conflicting and are mostly concerned with the more elderly individuals than those studied here (60+)(Miller (1996)). The age difference in the EUs and Donors is not marked and may therefore not be an important issue, but an aged-matched control population would have ruled out the potential age effect if they had been available.

An important difference, which could not be controlled for is the effect of circadian rhythms, the daily fluctuations which occur in lymphocyte recirculation and populations (Levi *et al.* (1985); Malone *et al.* (1990)). The donors were bled in the morning and most of the EUs were bled from lunch-time onwards, although this did vary. The lymphocyte counts and CD4% are lower in the morning, rising to a peak at around 10pm (Malone *et al.* (1990)). The effect of such circadian rhythms was highlighted in a study looking at subset populations in different risk groups (Bofill *et al.* (1992)): increased lymphocyte, CD4 and CD8 counts and percentages were seen in heterosexual (HIV-negative) women compared to heterosexual men and other controls, but when the time of sampling was considered it was found that these women were bled in the afternoon, unlike most of the other individuals who were bled in the morning. In the present study, such differences may have affected the proliferation results, causing the donors to have lower numbers of reactive $CD4^+$ cells, although no differences were seen between the percentage of $CD4^+$ cells in the two groups. However, the circadian rhythms may explain the difference in HLA-DR expression between the two groups.

To gain a clearer insight into the immune function of the EUs an ideal control cohort would have been sex, aged and lifestyle matched, sexually active individuals, who only differed from the EU cohort by lacking exposure to an HIV⁺ partner and were bled at the same time.

3.4.2.b Recall Responses

Reactivity to the recall antigens, PPD and TT, varied greatly between individuals and the level of responses to PPD were generally higher than to TT. This may be due to the vaccination which primed this immunity, i.e. the BCG vaccine is perhaps a better vaccine at eliciting T_H cell immunity than the tetanus vaccine. Indeed, the BCG vaccine is only given once, but the tetanus vaccine requires regular life long boosts to maintain activity (Mims *et al.* (1993)).

The PPD antigen, used in this assay, may have just been a better antigen than the TT. An alternative source of TT would help ascertain this, although some of the TT reactive individuals did show good responses and children, who have received a vaccine more recently, did respond well to this antigen (Dr. M. Aldhous, personal communication).

The amount of IFN- γ produced was also higher in response to PPD than TT, and this may reflect either the increased proliferative response, or the differing cytokine profiles known to be induced by these antigens (ElGhazali *et al.* (1993)); PPD induces a type 1 response, with IFN- γ , but no IL-4 and in contrast, TT is thought to induce a more T_{H2} response, inducing both IFN- γ and IL-4. Alternative monitoring of the cytokines produced would be required to confirm if the two antigens induced different profiles in this study, as the IL-4 assay used did not appear to be sensitive enough. The standards for the IL-4 ELISA worked well, but the samples either had a factor in the culture supernatant which interfered with the assay in some way, or the level of IL-4 was just not high enough to be detected by this method.

3.4.2.c Allo Responses

Reactivity to alloantigens was first shown highlighted as a potential protective mechanism in vaccine trials in macaques. Several groups (Stott (1991); Langlois *et al.* (1992); Chan *et al.* (1995)) showed that protective immunity could be accounted for by anti-MHC responses and this correlated with measurable anti-MHC antibodies (Chan *et al.* (1995)). However, a recent study of Nairobian prostitutes (Luscher *et al.* (1998)) showed that although anti-HLA class I antibody was seen in a small percentage of these high risk individuals (12%),

the proportion of these individuals did not differ who were HIV⁺ compared to HIV-negative. Alloantigen-specific T cells have also been shown to affect HIV replication *in vitro* (Brühl *et al.* (1996)), predominately mediated by CD8⁺ cells. The inhibition of HIV replication in PHA blasts occurred for both cells from a different donor and from the same donor (autologous). The suppression effect could also be seen when the cells were physically separated by a semi-permeable membrane, hence in a non-lytic mechanism comparable to that reported by others (Walker *et al.* (1986); Moriuchi *et al.* (1996); Walker *et al.* (1991))(see Section 1.6.2.g).

Despite a modest increase in the numbers of EUs in this cohort responding to the alloantigen compared with donors, this was not statistically significant and may reflect the limited numbers assessed. However, there was a significant increase in the amount of IFN- γ produced in response to the alloantigen by the EUs. This is consistent with results of Barcellini and colleagues (1995), who assessed the cytokine production of high risk, yet HIV-negative IDUs, following PHA stimulation. They showed increased IFN- γ production in the IDU-EUs in response to mitogen, compared to controls. Unlike the difference seen for allo stimulation, no differences in IFN- γ production in response to mitogen was observed in the Edinburgh cohort. However, the assay was not optimal for mitogenic responses, unlike that of Barcellini *et al.* (1994), and this may explain the difference, as the IFN- γ may have all be degraded, or utilised in the longer unoptimal assay.

Barcellini *et al.* (1995) also reported impaired PHA-induced IL-4 and IL-10 and increased IL-12 production in the EU IDUs compared to normal healthy controls. This pattern of cytokine production is consistent with a predominant type 1 response, but they also showed a decreased, although not significant, IL-2 production. A reduction in IL-2 suggests impaired CD4⁺ T cell function in the EUs. However, injecting drug misuse has been shown to cause impaired T cell function (Mientjes *et al.* (1991)) which may explain the suppression seen. Proliferative responses were not monitored, which would have confirmed the apparent impaired lymphocyte function.

If the IFN- γ responses to alloantigen in the EUs reflect real differences compared to low risk controls, could this be a potential protective mechanism from heterosexual HIV transmission? Classically, the response to alloantigens is of a T_H phenotype, including the production of IL-2 and IFN- γ (Woloszczuk *et al.* (1986); Kotlan *et al.* (1988)). Interferons are known to induce antiviral activity against a range of viruses (Aboud and Huleihel (1981); Chatterjee, Cheung and Hunter (1982); Whitaker-Dowling *et al.* (1983)). IFN- γ has been

shown to inhibit HIV replication in macrophages (Hammer *et al.* (1986); Koyanagi *et al.* (1988); Hartshorn *et al.* (1987); Kornbluth *et al.* (1990); Meylan *et al.* (1993)) and some T cell lines (Hartshorn *et al.* (1987); Nakashima, Yoshida and Yamamoto (1986)), but in PBMCs IFN- γ only has anti-HIV effects in the presence of other mediators (Wong *et al.* (1988)).

Reports concerning the inhibition of HIV replication in macrophages have been conflicting, with regard to the timing of infection relative to IFN- γ treatment. Koyanagi *et al.* (1988) reported enhanced infection with pretreatment of cells with IFN prior to HIV infection, compared to a reduced infection in post-treated cells. In contrast, Meylan and colleagues (1993) showed a reduction in replication for both pre-and post-treated cells. The discrepancy may reflect differences in both the length of IFN- γ pretreatment before infection (3 days versus 18 hours) and the protocol for macrophage culture. Using a single replication cycle study, Meylan (1993) also showed that the effect of interferon occurred at an early step of the virus life cycle. However, others (Emilie *et al.* (1992)) have reported an antagonistic effect of IFN- γ on TAT mediated transactivation of the viral LTR, which occurs later in the viral replication cycle. It is possible that interferon has multiple effects on both the cell and the viral life cycle.

Despite current controversies, IFN mediated inhibition would suppress further viral synthesis and perhaps allow immune control mechanisms time to contain the infection. As macrophage infection is thought to be an important reservoir for viral dissemination (Gendelman *et al.* (1989)), mechanisms which prevent this spread could be critical in controlling initial infection. IFN- γ is produced by both CD4⁺ and CD8⁺ cells and is potentially a factor involved in *in vivo* non-lytic suppression of HIV replication; overlooked by *in vitro* methods of non-lytic suppression, involving PBMCs in which little suppression is seen (Wong *et al.* (1988)).

IFNs are also known to upregulate MHC Class I and II expression, further augmenting any immune response to control the infection. High levels of IFN- γ would also create a selection for a type 1 response and against a type 2 response (see Section 1.6.2), thought to be protective in HIV infection (Clerici and Shearer (1993); Clerici and Shearer (1994)). Further analysis of the other cytokines, including IL-12, by more sensitive methods such as ELISPOT (Versteegen, Logtenberg and Ballieux (1988)), or RT-PCR for mRNA would confirm if a type 1 response is occurring. Intracellular fluorescent staining for

cytokines is also now possible (Caruso *et al.* (1996)) and co-staining would also allow the responding cells to be identified as either CD4⁺, or CD8⁺ cells.

However, before attributing a major role to alloantigen induced IFN- γ production in protection from heterosexual HIV transmission, it should be noted that three of the donors also produced high levels of IFN- γ in response to alloantigen. Therefore the response in the EUs is not unique and may reflect priming to alloantigens. Previous priming of allo-specific responses may have selectively expanded, or induced cells to produce a strong type 1 response, hence explaining the lack of a difference in proliferative responses to alloantigen in the two groups, but a difference in the IFN- γ production. Sexually active women are exposed to alloantigens in the form of sperm and other cells in the seminal fluid. The EUs may also have encountered alloantigens from exposure to HIV too, which has been seen to carry high quantities of cellular proteins, including those of the MHC, on the viral membrane (Henderson *et al.* (1987); Arthur *et al.* (1992)). A strong allogeneic response may therefore protect from HIV infection by destroying allogeneic virally-infected cells, or virus. As the sex ratio differed in the Donors and EUs, mostly men in the donor group and mostly women in the EUs (see Table 3.1), it is possible that the cohorts may have differences in the level of allogeneic responses, due to differences in exposure. However, of the three donors with high IFN- γ production in response to alloantigen, two were male, although they may have other factors in their life which may have lead to alloantigen exposure, such as a blood transfusion.

The age difference, as discussed previously (see Section 3.4.2.a) may also effect the chance of heterosexual exposure, as younger people are known to be more sexually active and also promiscuous (Johnson *et al.* (1992)). However, the sexual activity of the donors was not assessed and the EUs are not known to be highly promiscuous as they were often in long term relationships.

Allogeneic responses have also been suggested to be involved in protection from vertical transmission, as children with a higher degree of discordant HLA types to their mother have been shown to have a reduced risk of infection from HIV (MacDonald *et al.* (1998)). As children inherit half of their genetic material from their mother and hence will be homologous for half of their HLA alleles, presumably unrelated heterosexual couples are more likely to have different HLA haplotypes and will inevitably mount some form of allogeneic response. Factors which control the level of protection from this are unclear, but may involve the dose and type of exposure, virally infected cells, or free virus. Perhaps previous heterosexual

exposure to alloantigens may boost the level of response, but as the promiscuity of the EU cohort compared to the donors is unknown, this could not be addressed.

Expression of a rare HLA type, less likely to be shared with the partner, may also be involved. The latter point is supported by Plummer and colleagues (Plummer *et al.* (1993)), who in a study of prostitutes in Nairobi, Kenya, showed that the 'resistant' individuals seemed to possess rare HLA types for the local population. More detailed analysis would be needed to confirm the observations made here, including: studying more individuals, extended cytokine analysis, determination of the responding cell population, and the level of discordant HLA types in concordant compared to discordant couples in the Heterosexual Study.

3.4.2.d HIV-specific Responses

Proliferative responses to HIV have been reported in EUs, including health care workers (Clerici *et al.* (1994b)), IDUs (Beretta *et al.* (1996)), sexually exposed individuals (Mazzoli *et al.* (1997); Ranki *et al.* (1989); Clerici *et al.* (1992); Kelker *et al.* (1992)) and children born to infected mothers (Borkowsky *et al.* (1990); Clerici *et al.* (1993b)). To monitor the presence of any HIV-specific reactivity in the Edinburgh cohort, a recombinant HIV protein cocktail was developed. Preliminary experiments in controls showed little reactivity to the concentrations of antigens used, but later, following the use of the cocktail in assays using EUs and donor controls, a high degree of reactivity in the donor population was seen. The donor responses were particularly marked in the lowest concentration of the proteins and perhaps reflects a more optimal level of reactivity to the antigen they were responding to. This was found to be due to the recombinant proteins derived from *E.coli*, suggesting reactivity to a contaminant *E.coli* protein, but when the *E.coli* derived proteins were subsequently removed, few samples were then available for screening. However, reactivity to the modified gp120/p24 cocktail was seen in some HIV-infected children (Dr. M. Aldhous, personal communication), suggesting that this modified cocktail functioned as a HIV-specific antigen.

Coincident with the change of the proteins in the cocktail, it was also decided to make an alternative antigen for inclusion in the assay. HIV was cultured in the CEM cell line, known to not to express MHC Class II and hence reducing any potential allogeneic reactivity to the virus. The strain of virus used was a patient derived virus from an Edinburgh infected haemophiliac (HIV_{CS8}). The virus was heat inactivated, then purified by ultracentrifugation over a sucrose gradient. The amount of total protein and p24 protein was then assessed and

preliminary results showed no cross reactivity in the donors tested. However, during the time taken to produce the antigen, the samples from the EUs were no longer available.

Some of the EUs did show responses to the initial HIV cocktail, in particular C5451, who showed marked responses to all concentrations of the proteins. It is possible that some of these responses were genuine anti-HIV responses, but due to the non-specific activity in the donors this could not be confirmed. Others have shown that to detect HIV-specific responses, IL-2 production needs to be measured (Mazzoli *et al.* (1997); Clerici *et al.* (1992); Clerici *et al.* (1993b); Clerici *et al.* (1994b)), although proliferative responses have been shown to reactive HIV peptides (Kelker *et al.* (1992)), proteins (Borkowsky *et al.* (1990); Ranki *et al.* (1989); Kelker *et al.* (1992)) and inactivated virus (Ranki *et al.* (1989)). Cytokine production in the absence of proliferation was suggested by the numbers of EUs responding to the rHIV cocktail with measurable IFN- γ production, but without comparable proliferation. Some of the responses were perhaps due to spontaneous IFN- γ production, also seen when no antigen was included (see Appendix 3.4), but this was not true for all and even those who did have spontaneous production of cytokines, supernatant from some of the other antigens gave negative IFN- γ results. More detailed analysis would be needed to confirm any association of cytokine production with anti-HIV reactivity.

As well as including a whole virus antigen, one could also add reactive peptides as a further source of HIV-specific antigen, although the initial rationale for using whole proteins was to provide a broadly reactive antigen to all individuals. The peptide approach is limited by the restricted number of T cell and MHC-binding epitopes included, which may not be bound by the HLA molecules of an individual. The addition of anti-IL-2 receptor to measure IL-2 production, may allow detection of HIV-specific responses, which some have suggested can not be by proliferation assays (Clerici *et al.* (1992); Pinto *et al.* (1995)).

3.4.3 CTL Proposal

The cessation of sample collection prevented a strategy established for assaying CTL activity in follow up samples from being performed. This meant that the best targets, HIV-infected PHA blasts, or rVV infected BCLs expressing HIV proteins was not established.

3.4.4 Conclusions

The exposed, uninfected individuals within the Edinburgh Heterosexual Partner Study were shown not to be harbouring a low level infection in the absence of seroconversion, as

confirmed by PCR. This was further supported by analysis of the lymphocyte, which as highlighted by a known seroconverter, would have shown a marked perturbation from normal if a pre-seroconversion sample had been obtained.

The expression of the activation marker HLA-DR was elevated in EUs compared to donors and may explain some of the differences in immune function suggested by some of the proliferation assays. However, perhaps due to restricted sample size, no significant differences in immunological responses to the mitogen, PHA, or recall antigens, PPD and TT were seen in the EUs compared to normal donors. In contrast, the EUs had elevated IFN- γ production in response to alloantigen when compared to normal donors, supportive of the suggested protective role of allo responses in transmission and also of the type 1 response in EUs. However, this requires further investigation, including analysis of other cytokines. HIV-specific reactivity was investigated, but due to problems of antigen contamination, no conclusions could be drawn from this and the cessation of sample collection prevented further investigation of modifications made. However, the persistent reactivity to the HIV cocktail of one EUs in particular (C5451) shows that the cohort warrants further study, making one, or all of the suggested changes.

Other factors may also be involved in protecting individuals from HIV infection. These include the many known risk factors associated with heterosexual transmission (Spira *et al.* (1984)) and genetic factors such as HLA type, which has been seen to affect progression to AIDS (Just (1995)). Also, viral factors may influence the transmission, including the viral strain and dose of virus, both of which are influenced by the infection status of the transmitting individual. Some of these factors will be addressed in later chapters.

Chapter 4

Genetic Factors

CHAPTER 4 - GENETIC FACTORS

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4.4.4.a HLA

4.4.4.b CCR Mutations

4.1. INTRODUCTION

Individuals in a population differ in their *fertility* and *viability* and the combined effect results in the individual's *fitness* in the population and environment. It is known that for most infectious diseases individuals vary in their susceptibility, or viability to that disease and it is theoretically possible that within a population some individuals will be resistant to that agent. However, unless the whole population is exposed to the agent the resistant traits may go undiscovered, as lack of infection does not therefore mean resistance. Since the beginnings of HIV research, factors which protect individuals have been sought, in the hope that they may aid our understanding of the disease and in so doing help to find a cure. This has been impeded by the fact that, due to the nature of the infection, it would be impossible to distinguish individuals that had been exposed and were resistant to the virus. Studies of high risk individuals do not always guarantee actual exposure and may again mask true factors, but cohort studies of individuals with a known risk may help overcome this.

The genes of the MHC are some of the most polymorphic known (Klein (1986b)). The human genes and the proteins they encode, the HLA, are distributed in both an ethnic and geographical manner and the diversity generated by exposures to a vast array of antigens, including infectious diseases (Klein (1986b)). Several infectious diseases are known to have HLA associations, either in a protective, or susceptible role and include: hepatitis B and C (Czaja *et al.* (1993); Carbonara *et al.* (1983)), malaria (Hill *et al.* (1991)) and the mycobacterial infections, tuberculosis and leprosy (Mehra (1990)).

Studies of the murine model to AIDS have shown that susceptibility was affected by the H-2 genes, the mouse equivalent to HLA (Makino *et al.* (1990)). HIV⁺ individuals have also been investigated widely to ascertain if any HLA types correlate with HIV infection, or its associated illness (reviewed in (Just (1995))).

Studies of highly HIV exposed, yet uninfected individuals, have recently highlighted an additional genetic factor, which is involved in protection from infection in these EUs. Paxton and colleagues (1996) identified two homosexual men, who despite extensive exposure to HIV, remained persistently seronegative. Purified CD4⁺ T cells from these men were resistant to *in vitro* infection with a range of primary isolates, but infectable with TCLA strains. It was subsequently shown that non-TCLA strains use the C-C chemokine receptor-5 (CCR-5, see Section 1.5.2) as a co-receptor (Alkhatib *et al.* (1996); Choe *et al.* (1996); Deng *et al.* (1996); Dragic *et al.* (1996)) and these two EU individuals were homozygous for a 32bp deletion (Δ 32) in the CCR-5 gene (Liu *et al.* (1996)). This mutation results in a frame-shift

leading to premature termination of translation (see Section 1.7.3.a). The deletion is present in Caucasian populations at an allele frequency of ~ 10% (Liu *et al.* (1996); Dean *et al.* (1996); Samson *et al.* (1996b)), but absent from all other ethnic backgrounds studied.

Extensive screening of HIV-exposed uninfected and infected cohorts revealed that homozygosity for the $\Delta 32$ deletion conferred a significant level of protection from infection in homosexuals and haemophiliacs, being present almost exclusively in the uninfected individuals (Dean *et al.* (1996); Biti *et al.* (1997); O'Brien *et al.* (1997); Theodorou *et al.* (1997); Balotta *et al.* (1997)). In addition, heterozygosity was shown to correlate with a slower progression to AIDS in HIV⁺ homosexuals, although it conferred no protection against infection following homosexual contact. Another mutation, in the CCR-2 chemokine receptor, (G→A position 190; valine to isoleucine change position 64 (64I)) has also been shown to affect progression (Smith *et al.* (1997); Kostrikis *et al.* (1998); Rizzardì *et al.* (1998)) (see Section 1.7.3.a), although no protective effect from infection was seen for this mutation in homosexual and haemophilic cohorts (Smith *et al.* (1997)). The effect of this mutation was only detected in seroconverter cohorts (Michael *et al.* (1997)), indicating that it acts early in infection.

Kostrikis *et al.* (1998) showed that the CCR-2-64I mutation was in 100% disequilibrium with a mutation in the putative promoter region for *CCR-5* (C→T at nucleotide 927 of *CCR-5* gene; see Section 1.7.3.a). Hence, postulating a possible mode of action on delaying progression, which is otherwise unclear from the apparent conserved mutation in CCR-2. Dr. Mary Carrington and colleagues (Martin *et al.* in preparation) have recently described some additional polymorphisms in the *CCR-5* promoter (see Figure 4.1) and identified a particular combination of these polymorphisms (termed P1 allele; see Figure 4.1), which when homozygous leads to more rapid progression. Both the *CCR-5* $\Delta 32$ and CCR-2 64I mutations were shown always to occur on a P1 allele, hence creating a CCR-2/P1/CCR-5 haplotype. P1 alleles were therefore ascribed to be either WT/P1/WT, 64I/P1/WT, or WT/P1/ $\Delta 32$, as 64I and $\Delta 32$ never occur on the same gene (Smith *et al.* (1997)). The P1,P1 homozygotes which were seen to rapidly progress were all of homozygotes for the WT/P1/WT haplotype.

The risk of acquiring HIV following heterosexual exposure has been shown to be less than that for homosexual contact (Giesecke *et al.* (1992)), presumably reflecting differences between the two modes of transmission. In Edinburgh, a cohort of heterosexual couples discordant for HIV serostatus and selected for repeated exposure to HIV, but who remain uninfected have been identified. In order to assess the effect of genetic variation at the *CCR-5*

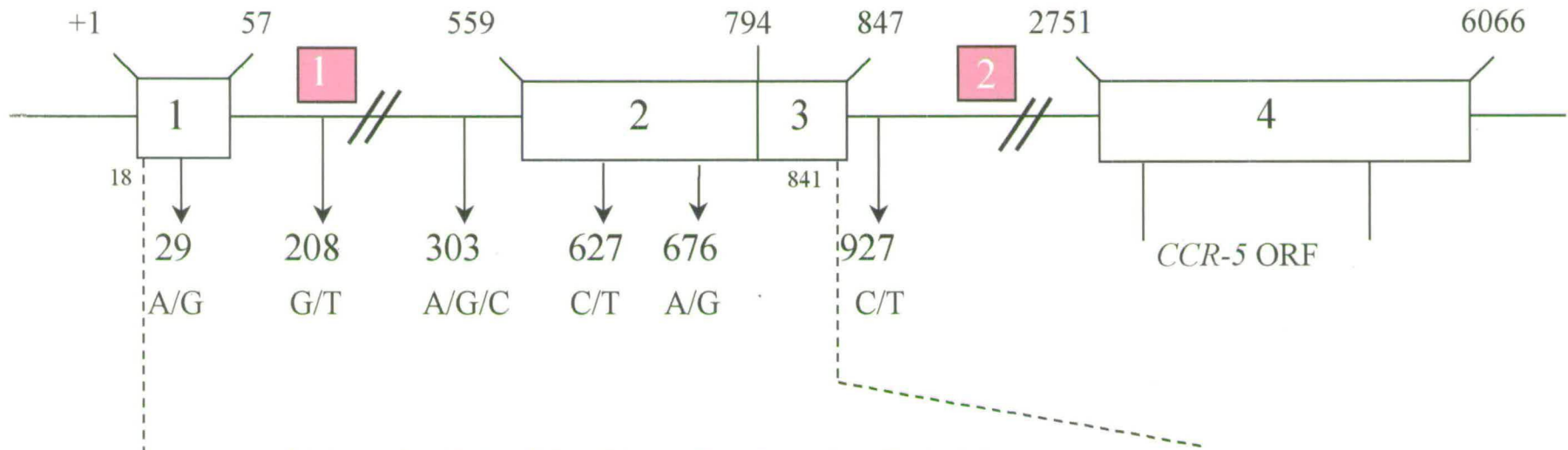
Figure 4.1.

Diagrammatical Representation (not to scale) of the *CCR-5* Gene and Promoter Showing Polymorphisms

Adapted from Mummidi *et al.* (1998) and Martin *et al.* in preparation.

The diagram shows the *CCR-5* locus on chromosome 3. The four exons (open boxes) and two introns (pink boxes) are shown and numbered. Arrows indicate the polymorphisms identified in the promoter region, contained within the region from exon 1 to the end of exon 3. It is unknown if the 927 C→T mutation, out with this region, has any functional role. The *CCR-5* open reading frame (*CCR-5* ORF) is marked in exon 4.

The region amplified +18-+841, indicated with a dashed line, shows the polymorphisms found by Dr. Mary Carrington and colleagues (Martin *et al.*, in preparation). The polymorphisms are divided into alleles, P1-10, depending upon the combination. P1-4 are the most common and P5-10 are derived from single mutations from one of the more common P1-4.



208 612 626 627 630 647 676 684 714 811

P1	G	T	C	C	C	C	A	T	C	G
P2	G	T	C	T	C	C	A	T	C	G
P3	T	T	C	T	T	C	A	T	C	G
P4	T	T	C	T	C	C	G	T	C	G
P5	-	G	C	T	C	C	A	T	C	G
P6	-	T	C	C	T	C	A	C	C	G
P7	-	T	A	C	C	C	A	T	C	G
P8	-	T	C	T	C	A	A	T	C	G
P9	-	T	C	T	C	C	A	T	C	A
P10	-	T	C	T	C	C	G	T	G	G

locus on heterosexually acquired infection, these EUs have been screened for the presence of the *CCR-5* deletion. The frequency obtained was then compared to those observed in heterosexually HIV-infected individuals and low risk controls. The effect of the recently described mutation in the *CCR-2* was also assessed and, in collaboration with Dr. Carrington, the group was also screened for the *CCR-5* promoter polymorphisms (P1-10) described above.

Finally, to determine if any HLA types correlated with HIV infection following heterosexual exposure in the Edinburgh cohort, the heterosexual contacts with HLA types available were also compared, in terms of those who became infected and those who remain uninfected. The HIV⁺ index partners were also assessed to see if any of the above genetic factors correlated with heterosexual transmission.

4.2. METHODS

4.2.3 HLA Typing

The majority of samples were typed by serological methods by a standard two-stage complement dependent microlymphocytotoxicity technique (Klein (1986b)) using sera from the UK National Transplant Service. This serotyping was performed by Dr. P.L. Yap and staff (SNBTS, Edinburgh). Some of the EUs were typed by PCR-sequence-specific oligonucleotide typing based methodology (Bidwell (1994)) at the Northern Ireland Regional Tissue Typing Service, Belfast (service kindly provided by Dr. D. Middleton).

4.2.2 Development of CCR Genotyping Assays

4.2.2.a *CCR-5* Pilot Approach

Following the discovery of the 32bp deletion in the *CCR-5* gene, a method was sought to genotype the Edinburgh EU cohort, as the deletion was too small to detect a difference by standard PCR methods. Primers to amplify the whole *CCR-5* gene were obtained (see Section 2.14.2) and a restriction enzyme sought, using the published gene sequence (Genbank ascension number X91492) and Gene Jockey Sequence Processor (for the Apple Macintosh, Biosoft, Cambridge, UK), which would provide discrete banding patterns in the three different genotypes (WT/WT, WT/ Δ 32, Δ 32/ Δ 32). The restriction fragment length polymorphisms (RFLP) would then allow the groups to be screened and genotyped. The restriction enzyme Asp 700 was found to cut within the Δ 32 deletion site, hence when the deletion was present different fragments would be produced.

Figure 4.2 shows the *CCR-5* gene and the amplification products produced. More detailed description of the methods can be found in Section 2.14. Primers for the whole *CCR-5* gene were 28 and 29 and the Asp 700 restriction sites and fragments produced are shown for the WT and $\Delta 32$ alleles. Individuals heterozygous for the $\Delta 32$ deletion would generate both sets of fragments and hence be distinguishable from wild types. However, when performed only partial restriction digests of some of the fragments occurred making genotyping difficult to perform. Alterations in the amount of enzyme and the incubation time were made, but only partial digestion was still achieved.

A combined restriction digest of BglII and EcoRI was explored as an alternative. Each enzyme had one restriction site in the amplified *CCR-5* gene and the region containing the potential $\Delta 32$ site was small enough so the difference between the WT and $\Delta 32$ alleles could be distinguished (see Figure 4.2).

4.2.2.b Direct PCR approach

Following the successful establishment of a RFLP based genotyping assay, a less laborious direct PCR approach was adopted after collaboration with Dr. Linqi Zhang (Aaron Diamond Research Center, NY). This involved PCR amplification of a smaller region encoding the potential $\Delta 32$ site, using primers C and D (see Figure 4.2.a) and resolution of the products on special high resolution agarose gel (3% Metaphor gel). Standard agarose would have been too brittle for use at the high concentration required. Fragments of 189bp were generated for the WT allele and 157bp for the $\Delta 32$ allele and could easily be distinguished along with heterozygotes. Figure 4.3.a shows a typical result obtained for WT/WT, WT/ $\Delta 32$ and $\Delta 32/\Delta 32$ individuals.

4.2.2.c Confirmation Digest

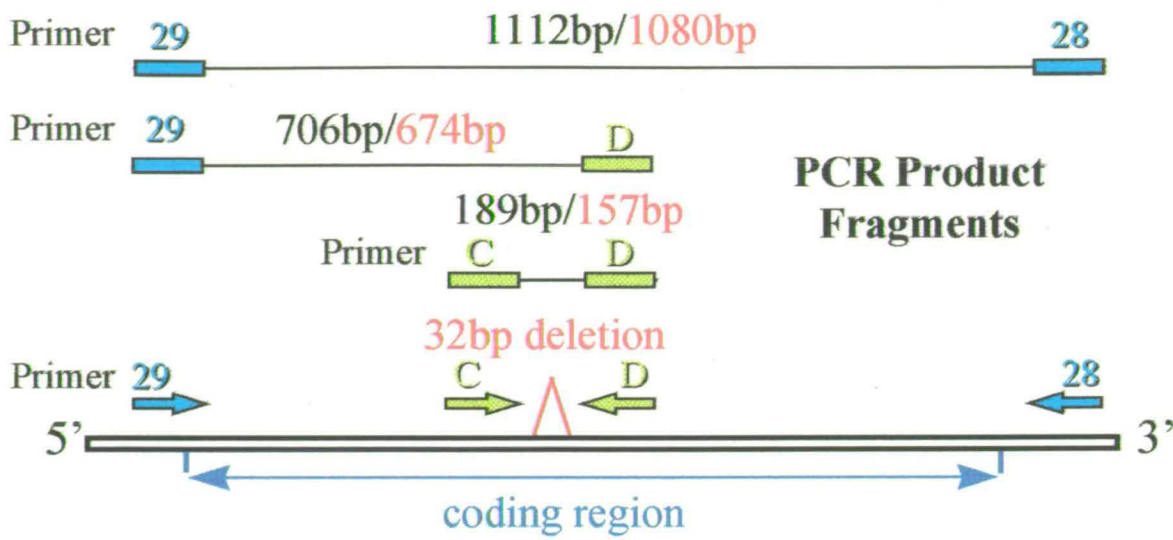
To confirm the results obtained above, random samples from each experiment were amplified using different primers in a different laboratory. The primers used were 29 and D and Figure 4.2.a shows the amplification products formed. These were then digested with BglII and the resulting fragments resolved on a 3% Metaphor gel. Figure 4.2.b shows the fragment sizes formed and Figure 4.3.b shows a typical result for a WT/WT, WT/ $\Delta 32$ and $\Delta 32/\Delta 32$ individual.

Figure 4.2.

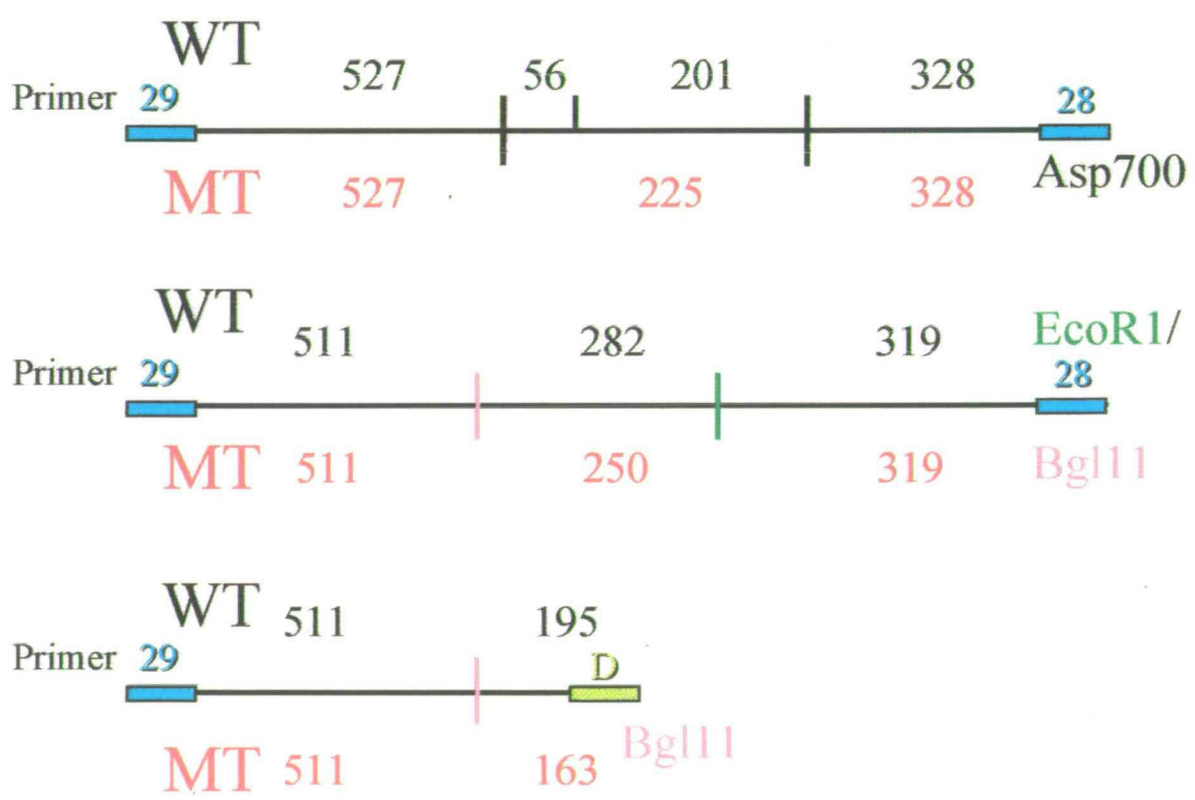
A diagrammatical representation (not to scale) of the CCR-5 gene.

The PCR amplification product and restriction digest fragment sizes from genes wild type (WT) at the *CCR-5* gene are given in black and those from genes containing a 32bp deletion in the *CCR-5* gene are given in red.

- a.) Shows the primer locations ($\Leftarrow \Rightarrow$) and a schematic depiction of the PCR products and their sizes ($\text{---}^{189\text{bp}}\text{---}$).
- b.) Shows the various restriction digests performed on the various PCR products (indicated by their size and primers shown on the ends). The vertical lines correspond to the point of the restriction site for the enzyme. The sizes of the resultant fragments ($\text{---}^{311} \text{---} \text{---}^{125} \text{---}^{\text{BglIII}}$) are shown above the line for the WT gene in black and below for the $\Delta 32$ gene in red. The restriction enzymes used to digest the fragments are shown at the end of the fragment and are colour coded with their corresponding cut; Asp700-black, EcoRI-green, BglIII-pink.



a. CCR-5 gene



b. Restriction Digest Products

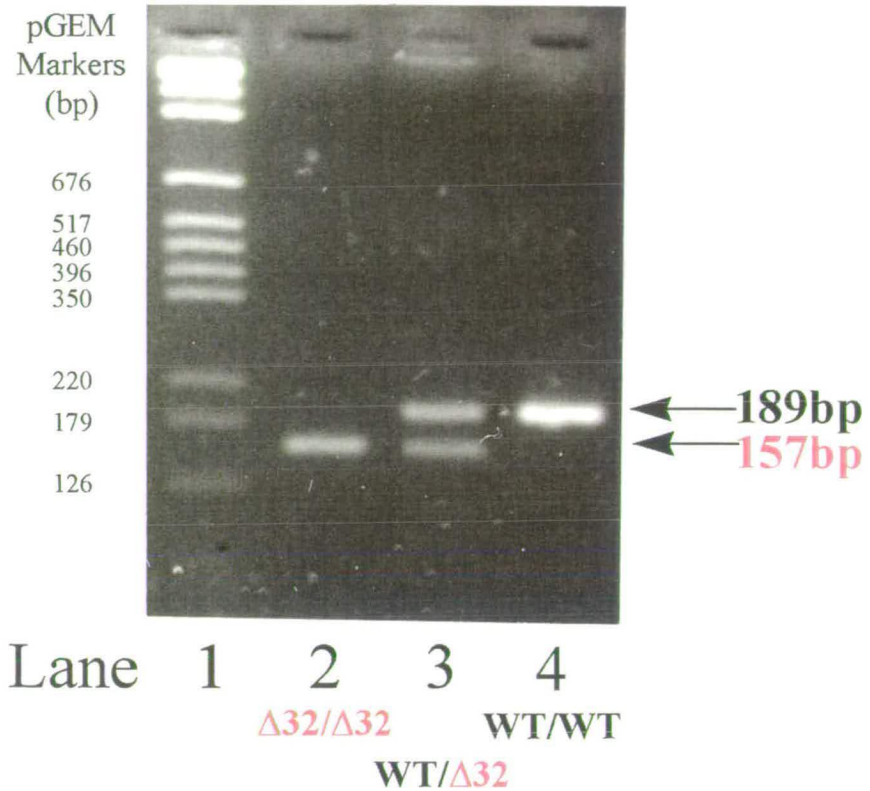
Figure 4.3.

CCR-5 Genotyping

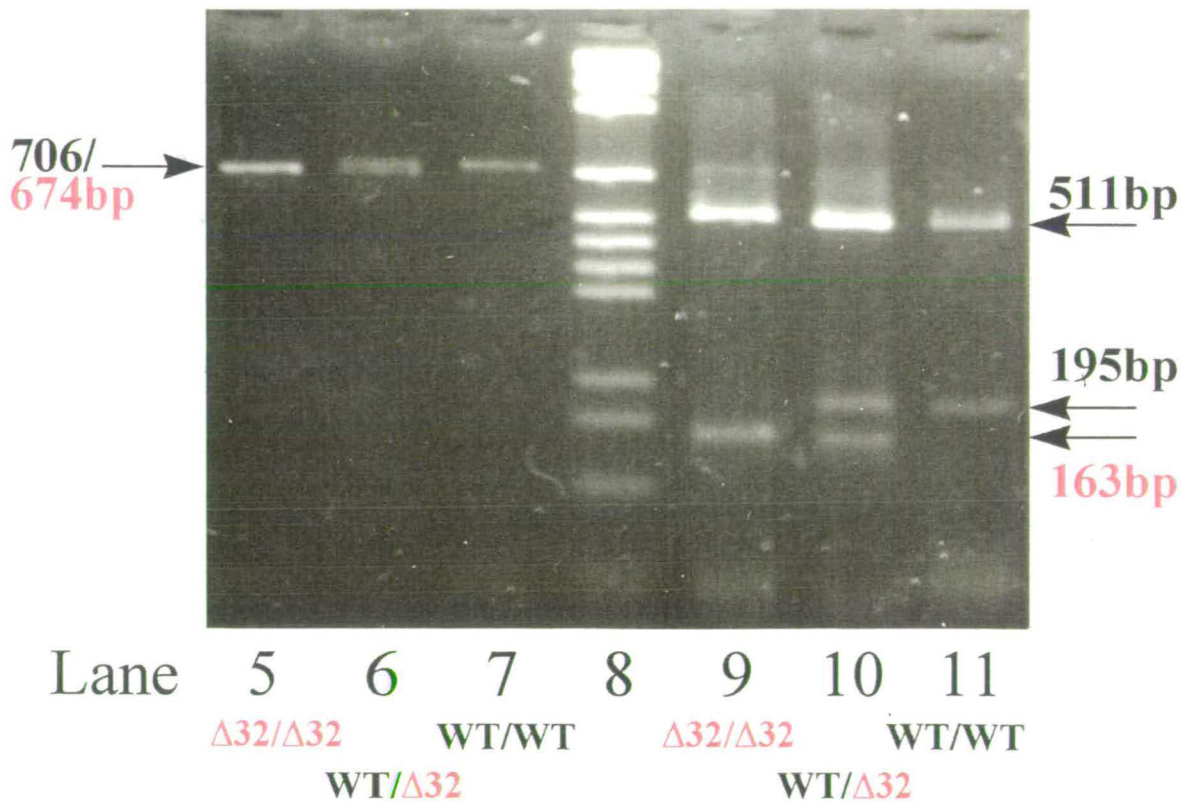
The photographs show a typical result from analysis of the PCR products of the *CCR-5* gene on a 3% Metaphor gel. PCR product and fragment sizes are shown in black for wild-type *CCR-5* genes and red for the 32bp deletion in the *CCR-5* gene.

- a.) Typical results using primers 'C' and 'D'. Lane 1 contains pGEM markers and lanes 2-4 the products from various individuals; lane 2 ($\Delta 32/\Delta 32$) a homozygote for the 32bp deletion in *CCR-5*; lane 3 (WT/ $\Delta 32$) a heterozygous individual and lane 4 (WT/WT) a homozygous wild-type individual.
- b.) Typical results using primers '29' and 'D' (lanes 5-7) and the products obtained from its subsequent digest with BglIII (lanes 9-11). Lane 8 contains pGEM markers. Lanes 5/9 show a $\Delta 32/\Delta 32$ individual; lanes 6/10 show a WT/ $\Delta 32$; lanes 7/11 show a WT/WT individual.

a. CCR-5 Genotyping PCR



b. CCR-5 Genotype Confirmation Digest



4.2.2.d CCR-2

Following the reports of the 64I mutation in CCR-2, it was decided to assess the Heterosexual cohort, screened for the $\Delta 32$ mutation in CCR-5, for this mutation too and analyse if it had any effect on heterosexual HIV transmission. The primers used were already published (Smith *et al.* (1997)) and it was found that identical conditions to the CCR-5 PCR genotyping assay gave successful amplification if the annealing temperature was increased to 60°C for all cycles and the primer concentration was reduced to 0.1 μ M.

The A primer was designed to contain a mis-matched A base, shown in Figure 4.4, which was then present in the amplified 128bp product. If the mutation which caused the V64I mutation (position 190, G→A) was present in the amplified product with the mis-match A from the primer, a restriction site for the BsaBI enzyme was formed. Subsequent digestion with this enzyme results in a RFLP when the mutation (position 190, G→A) was present. The restriction digest products are shown in Figure 4.4.b and a typical result for a WT/WT, WT/64I and 64I/64I individual shown in Figure 4.5. As the restriction fragments were relatively small, the gels had to be run for prolonged periods and under chilled conditions to maintain resolution (see Section 2.17.2). The 18bp fragment was too small to be clearly resolved.

4.2.3 CCR-5 Sequencing

The whole CCR-5 gene was amplified using primers 28 and 29 and the product purified as outlined in the Material and Methods (see Section 2.15.1). The gene was then sequenced using Taq cycle sequencing (see Section 2.15.2) and the four primers, 28, 29, C and D. This resulted in 4 pieces of overlapping sequence that were joined and the resulting sequenced translated and aligned with the CCR-5 consensus sequence for comparison (see Section 2.19).

4.2.4 CCR-5 Promoter Polymorphisms

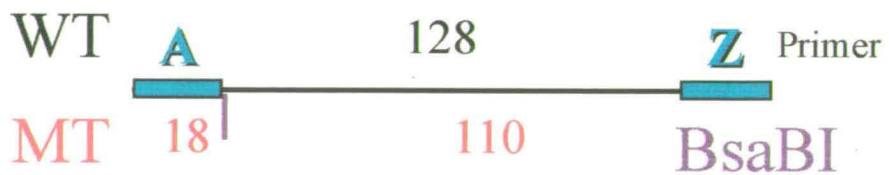
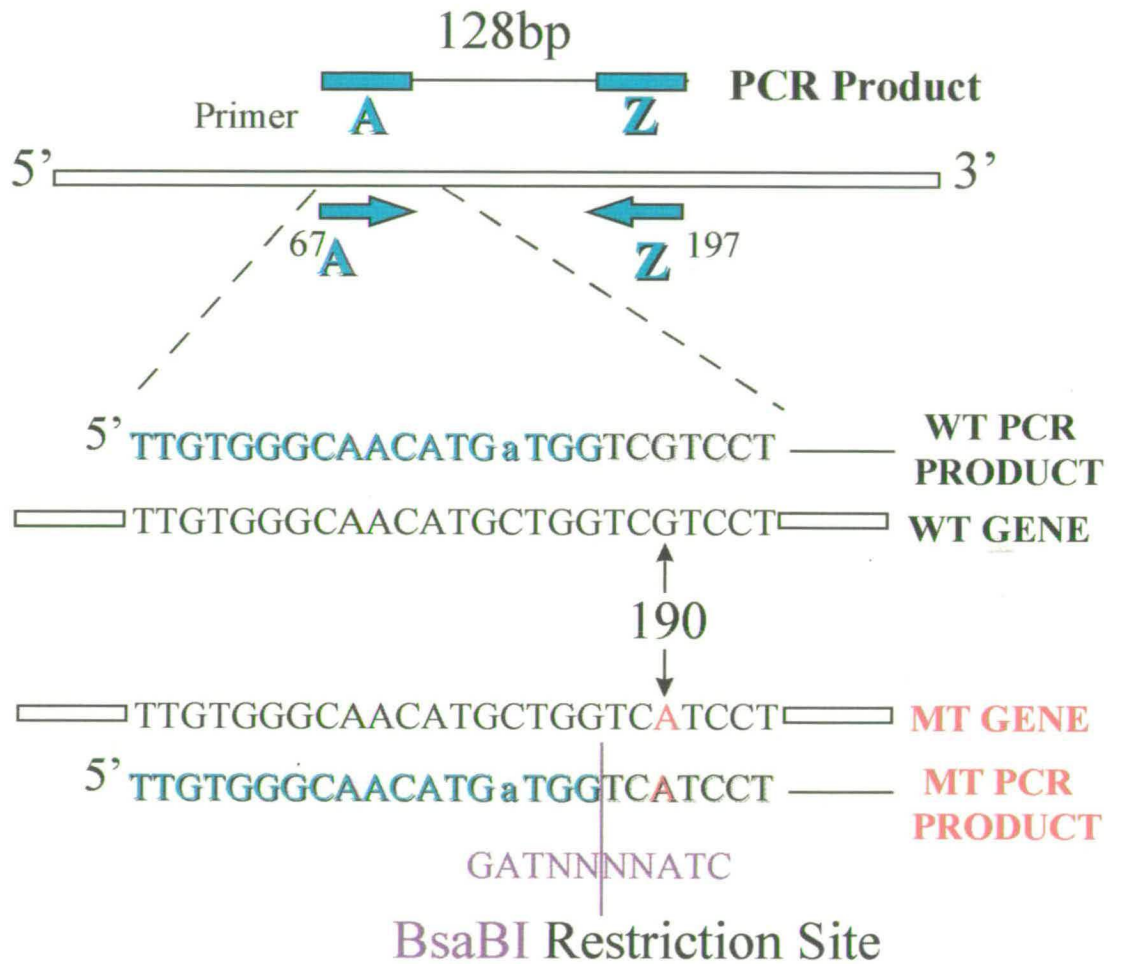
CCR-5 promoter polymorphisms were performed by Dr. Mary Carrington and colleagues (National Cancer Institute, Frederick, MD, USA) using samples described above (see Section 4.1). The polymorphisms were defined as alleles P1-10, as described earlier (see Section 4.1 and Martin *et al.* in preparation). The alleles were determined by PCR-single stranded conformation polymorphism analysis, where PCR amplified products are digested with a panel of restriction enzymes to generate a unique pattern for each allele.

Figure 4.4.

A diagrammatical representation (not to scale) of the CCR-2 gene.

- a.) Shows the primer locations (\rightleftharpoons) and a schematic depiction of the PCR product ($\text{---}^{189\text{bp}}\text{---}$). The enlarged segment of the gene and PCR product depicts the mismatch base in the primer 'A', depicted by a lower case letter (a) and this and the remaining primer sequence are shown in turquoise. The G→A (position 190) mutation in the CCR-2 gene is shown in red and the restriction site for the BsaBI enzyme given in purple ($\text{---}^{\text{128}}\text{---}^{\text{116}}\text{---}^{\text{BsaBI}}$).
- b.) The PCR amplification product and restriction digest fragment sizes from individuals wild type (WT) at the CCR-2 gene are given in black and those from individuals containing the G→A (position 190) in the CCR-2 gene are given in red. The BsaBI restriction enzyme cut is coloured in purple ($\text{---}^{\text{128}}\text{---}^{\text{116}}\text{---}^{\text{BsaBI}}$).

a. CCR-2 gene



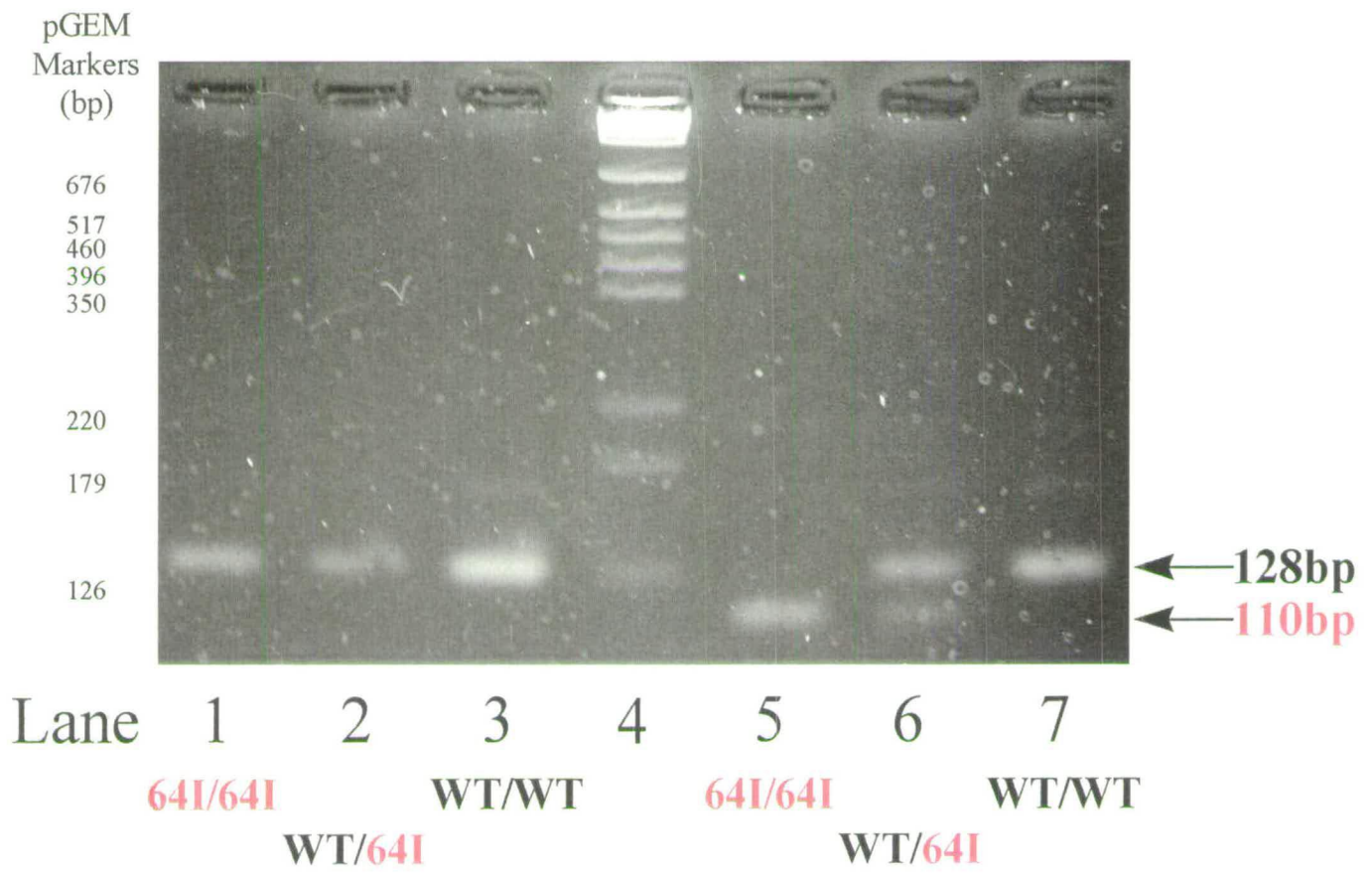
b. Restriction Digest Products

Figure 4.5.

CCR-2 Genotyping

The photographs show a typical result from resolving the PCR products of the CCR-2 genotyping assays on a 4% Metaphor gel. PCR product and fragment sizes are shown in black for wild-type *CCR-2* genes and red for the G→A (position 190) mutation causing a valine to isoleucine a-a change (a-a 64, 64I) in CCR-2.

Typical results using primers 'A' and 'Z' (lanes 1-3) and the products obtained from its subsequent digest with BsaBI (lanes 5-7). Lane 8 contains pGEM markers. Lanes 1/5 show an individual who is homozygous for the 64I mutation in CCR-2 (64I/64I); lanes 2/6 show a heterozygous individual (WT/64I); lanes 3/7 show a homozygous wild-type individual for CCR-2 (WT/WT).



4.3. RESULTS

4.3.1 HLA Phenotypes

HLA results were available for many of the individuals in the Heterosexual Study. Table 4.1 lists the number and the extent of typing for the individuals of interest. The full HLA phenotypes for these individuals are listed in Appendix 4.1-4.4.

Of the EUs, 23 had been solely DNA typed, 22 solely serotyped and 11 had been typed by both methods. Six of the 11 gave the same HLA type and 5 gave either different, or an unclear HLA type, usually for only one allele. Four of the solely DNA typed individuals (2 full; 2 B/DR) also had ambiguous results at one or more locus (see Appendix 4.1). These unclear results were excluded from the analysis when a clearly defined HLA type was required, but included when analysing for mis-match if the possible choices clearly differed from the other individual's HLA type (see later).

Three of the indexes were represented in both groups as they had concordant and discordant relationships. They were excluded for comparisons of the two groups, but included in the mis-match analysis between couples (see later).

For comparisons of the heterosexually exposed groups, HIV⁺ concordant contacts and EUs, any EUs PCR-typed as A68 were included in the broader A28 group from which it was split (Hurley *et al.* (1997)). Likewise DR12 was grouped with DR5 and DR13/14 with DR6 (see Table 4.2). For comparisons of EUs to published population control frequencies from the area, further limiting groups were needed as the control population had been less clearly typed. Individuals expressing A30 and A31 were pooled as in the control group and the following narrower specificities used: B62=>B15; B39=>B16; B57=>B17; B40/50=>B21; B55=>Bw22; B60=>B40 and DR11/12=>DR5 (see Table 4.2). The individual expressing A33 (C3781: A2,33) was excluded from this analysis as this allele was not typed and could not be grouped with a broader specificity, as was the B53 expressing individual (C4631: B51,53) and the individual expressing DR9 (C5212: DR9,13). The numbers in each group are shown in Table 4.1, with the actual numbers in the two groups compared shown in Table 4.1.b.

4.3.1.a Heterosexually Exposed HIV⁺ and Uninfected Contacts

The numbers of individuals expressing each A, B and DR allele in the two groups were compared by Fisher's Exact test on a 2x2 contingency table. An example of the tables formed is shown below (see Table 4.3) along with the calculation.

Table 4.1.**Details of Groups in HLA Analysis****A. Numbers Typed**

	Total	Full A/B/DR Typing	Others*	
Contacts				
EUs	56	35	A + DR: 4 B + DR: 14	B: 2 DR: 1
HIV ⁺ s	17	16	A + B: 1	
Indexes				
TRIs	22	19	DR: 1	A + B: 1
NTRIs	34	32	A + B: 2	

* Individuals with only partial typing.

B. Numbers Used in Analysis

Total Numbers of Individuals in Comparisons*

	EUs	HIV ⁺ s	EUs [†]	Controls	TRI	NTRI
A	39	17	38	264	18	30
B	51	17	50	264	18	30
DR	54	16	53	264	17	28

* Numbers of individuals remaining due to partial typing and after exclusions due to unclear typing.

† Two individuals removed, one from each group, due to lack of equivalent typing in control group.

Table 4.2.

Details of Relationships of Broad and Narrow HLA Types Used*

Broad HLA	Narrow Split
A28	A68 [#]
A30/31	A30 [†]
	A31 [†]
B15	B62 [†]
B16	B39 [†]
B17	B57 [†]
B21	B49 [†]
	B50 [†]
Bw22	B55 [†]
B40	B60 [†]
DR5	DR11 [†]
	DR12 [#]
DR6	DR13 [#]
	DR14 [#]

* Described in Hurley *et al.* (1997).

[#] Grouped with broader HLA type in comparisons of EUs versus HIV⁺s and EUs versus Controls

[†] Grouped with broader HLA type in comparison of EUs and Controls only.

Table 4.3.

2x2 Contingency Table for EUs and HIV⁺s for HLA B27

A	Number of Individuals Expressing B27	Numbers not expressing B27	Total
EUs	2	49	51
HIV ⁺ s	3	14	17

P=0.0832, Exact test.

To calculate the probability of observing the actual number, or a more extreme distribution, when the initial 2x2 table had a P<0.10, the values were shifted as in Table 4.3.b.

B	Number of Individuals Expressing B27	Numbers not expressing B27	Total
EUs	1	50	51
HIV ⁺ s	4	13	17

P=0.016, Exact test.

This was continued until one group was zero, in fact the next one in this case. The exact probability is then the sum of all the values obtained, i.e. $0.0832 + 0.0116 + 0.0006 = 0.0954$, which becomes P=0.10, Exact test (one tailed). A similar analysis was performed for all the antigens compared, but only to more extreme values when the initial 2x2 revealed a P<0.10.

Only DR11 was significantly different in the two groups (EUs 14/56, HIV⁺s 0/16; P<0.02, Exact test (one tailed)), but once corrected for the number of tests ($P_{\text{corr}} = \text{original P} \times \text{number of antigens tested}$ (Hawkins (1981))) was no longer so ($P_{\text{corr}}=0.18$, Exact test (one tailed)). Those which although not formally significant, but showed a difference, included B27 which was increased in the HIV-infected contacts compared to EUs (EUs 2/51, HIVs 3/17; P=0.10, Exact test (one tailed)). Also increased in the infected group was A26 (EUs 1/39, HIV⁺s 2/17; P<0.09, Exact test (one tailed)) and DR3 (EUs 16/54, HIV⁺s 8/16; P=0.11,

Exact test (one tailed)). Both expression of A3 and DR4 was increased in EUs compared to the infected contacts (A3: EUs 13/39, HIV⁺s 2/17; $P < 0.09$, Exact test (one tailed); DR4: EUs 19/54, HIV⁺s 2/16; $P = 0.07$, Exact test (one tailed)). However, as for DR11, all of these were even less significant once the correction for the number of tests was performed (data not shown).

4.3.1.b EUs and Population Controls

The main problem with analysis of MHC data arises from the large number of alleles at each locus, such that the numbers of each allele are typically small and large numbers of individuals are required to show differences between two groups. As the number of heterosexually infected contacts typed was so low, it was unlikely to show any small differences that existed between the groups. It was therefore decided to compare the antigen frequencies of the EUs to those of a published population control study in the Edinburgh area (Jazwinska and Kilpatrick (1987)). This study involved 264 parents of babies born in the maternity ward of an Edinburgh hospital. The antigen frequencies reported are shown in Appendix 4.4 and as explained earlier involved a more limited typing than performed on the heterosexual cohort. This meant several antigens had to be grouped in the EUs to broader specificities (see Section 4.3.1).

The more significant results are summarised in Table 4.4. The most striking differences are in the frequencies of DR5 and DR6 (see Table 4.4; DR5: $P = 0.005$; DR6: $P = 0.002$, Exact test (one tailed)), with DR5 being higher in EUs and DR6 higher in Controls. The majority of the EUs expressing DR5 were in fact DR11 (14/15). However, as the control population was typed with a lower specificity and hence, the numbers of Controls expressing DR11/12 was unknown, it could not be confirmed that the difference was due to DR11 alone.

B7 and DR1 were also significantly different in the two groups (see Table 4.4; B7 $P = 0.01$; DR1 $P = 0.03$), with B7 increased in Controls and DR1 increased in EUs. However, the corrected values were not significant for B7 and DR1, but remained so for DR5 and DR6 (see Table 4.4; B7: $P_{\text{corr}} = 0.18$; DR1: $P_{\text{corr}} = 0.24$; DR5: $P_{\text{corr}} = 0.04$; DR6: $P_{\text{corr}} < 0.02$).

4.3.1.c Indexes

The indexes were divided into those who transmitted HIV heterosexually (Transmitting Indexes (TRIs)) and those who did not transmit (Non-TRIs (NTRIs)). The numbers HLA typed in each group are shown in Table 4.1 and the actual HLA types for the

Table 4.4.**Comparisons of HLA Antigen Frequencies in EUs and Population Controls**

	Number of EUs Expressing Antigen/Total (%)	Number of Controls Expressing Antigen/Total (%)	P Exact Test (one tailed)	P _{corr} * Exact Test (one tailed)
A11	9/38 (32)	33/264 (13)	0.06	0.72
B7	8/50 (16)	86/264 (33)	0.01 [#]	0.18
B14	8/50 (16)	22/264 (8)	0.08	NS
B18	4/50 (8)	6/264 (2)	0.06	NS
B35	10/50 (20)	32/264 (12)	0.10	NS
DR1	11/53 (21)	27/264 (10)	0.03 [#]	0.24
DR4	19/53 (36)	66/264 (25)	0.07	0.56
DR5 (11/12)	15/53 (28)	33/264 (13)	0.005 [#]	0.04 [#]
DR6 (13/14)	8/53 (15)	93/264 (35)	0.002 [#]	0.02 [#]

* Corrected for the number of tests by multiplying the original P value by the number of antigens tested (Hawkins (1981)).

[#] P<0.05

NS - non-significant, i.e. P_{corr}>1.00

individuals are shown in Appendix 4.2-4.3. Only A24 showed any remote difference between the two groups, but this was not significant (TRIs 1/18, NTRIs 7/30; $P=0.11$, Exact test (one tailed)).

4.3.2 Mismatch HLA Types

Full HLA-A/B/DR types were available for 11 concordant couples and 30 discordant couples. The HLA types were scored according to the degree of mismatch of the contact to the index (see Table 4.5). For example, Index 4681 (I4681) - A1,33; B7,37; DR1,1 and Contact 4681 (C4681) - A1,1; B62,62; DR1,1 would be scored 3, as C4681 would have been mismatched at the A33, B7 and B37 of her partner. The minimum mismatch would have theoretically been 0, but was in fact 1 and the maximum was 6. Individuals with unclear HLA typing were included if the possible phenotypes differed, e.g. C3022 was typed B44, 40/41, but her partner was B13,14, so either would have been a mismatch.

The mean scores were 3.5 for the concordant couples and 4.5 for the discordant couples (see Table 4.5) and this difference was significant following a t test of the square root of the mismatch scores ($t=2.51$, $P<0.02$), or using the non-parametric Mann-Whitney U/Wilcoxon Rank Sum W Test ($z=2.34$, $P<0.02$). This shows that discordant couples had a significantly higher degree of mismatch at the HLA loci than concordant couples.

4.3.3 CCR-5

4.3.3.a Genotypes

A total of 58 (44 female, 14 male; see Appendix 4.5 for individual results) EUs and 86 (65 female, 21 male; see Appendix 4.6 for individual results) heterosexually infected HIV⁺ individuals were genotyped by PCR for a 32 bp deletion ($\Delta 32$) *CCR-5* (see Section 2.14.3). Control samples from 50 individuals (see Appendix 4.7 for individual results), selected for a study of polycystic kidney disease without regard for risk of HIV infection, were also analysed (kindly provided by Prof. Alan Wright, MRC Human Genetics Unit, Edinburgh). All subjects were of Caucasian origin and 95% reside in Central Scotland.

Individuals were either homozygous wild type (WT/WT) at this locus, homozygous for $\Delta 32$ ($\Delta 32/\Delta 32$), or heterozygous for the mutation (WT/ $\Delta 32$). A total of three homozygotes for the $\Delta 32$ mutation were observed; all were HIV-negative, two were from the control group and one in the EUs (see Table 4.6).

Table 4.5.

HLA Mismatch Scoring for Heterosexual Couples

Concordant Pairs (n=11)		Discordant Pairs (n=30)	
ID	Mismatch Score	ID	Mismatch Score
3152	4	3021	6
3891	5	3022	4
4101	5	3061	6
4151	6	3073	1
4241	3	3141	5
4461	3	3241	6
4681	3	3311	5
4691	3	3351	5
4831	1	3372	5
4901	2	3672	4
5291	3	3723	5
		3781	4
		3791	4
		3961	6
		4051	5
		4141	6
		4191	4
		4311	4
		4331	5
		4401	5
		4431	4
		4441	4
		4471	4
		4631	3
		5051	3
		5052	4
		5191	5
		5231	4
		5351	4
		5411	5
Mean	3.5	Mean	4.5
Median	3	Median	4.5

Table 4.6.

CCR-5 Genotypes and Allele Frequencies in Controls, EUs and HIV⁺ Cohorts

	Number			%			$\Delta 32$ Allele Freq (%) (95% CI) [†]
	WT/WT*	WT/ $\Delta 32$	$\Delta 32/\Delta 32$	WT/WT*	WT/ $\Delta 32$	$\Delta 32/\Delta 32$	
EUs n=58	40	17	1	69	29	2	16 (10-24)
HIV⁺ n=86	63	23	0	73	27	0	13 (8-19)
Controls n=50	38	10	2	76	20	4	14 (8-22)

* WT/WT: homozygous wild-type CCR-5 genotype: $\Delta 32/\Delta 32$ for the homozygous mutant for the 32bp deletion in the CCR-5 gene and WT/ $\Delta 32$ for the heterozygote.

[†] 95% Binomial Confidence Intervals (CI).

Table 4.7 summarises all the combinations and comparisons of genotype frequencies performed. There was no difference in the genotype frequencies amongst the EUs, heterosexually infected and control individuals (see Table 4.7.a; $\chi^2=0.69$, $P=0.71$, combining homozygous mutant individuals with heterozygotes, due to low numbers of homozygotes; $\chi^2=1.12$, $P=0.57$, comparing WT/WT and heterozygotes only, i.e. excluding $\Delta 32/\Delta 32$ homozygotes).

Direct comparison of the groups heterosexually exposed to HIV (EUs and HIV⁺s), also revealed no difference in the *CCR-5* genotype frequencies (see Table 4.7.c), neither were there any difference between the control group and either the EUs, or HIV⁺s (see Table 4.7.b). The apparent discrepancy in the two tests used (see Table 4.7.b/c; Chi-squared/ Fisher's Exact Test) comes from computational differences. The Exact test was computed manually and following the test on the initial 2x2 square, requires that more extreme tables are constructed and the sum of all the results giving the 'exact' test (see Section 4.3.1 for an example). This was therefore only performed on results which gave an initial $P < 0.10$. The χ^2 result therefore provides a more accurate reflection of the probability in the non-significant results, but generally has reduced power for smaller sample sizes, hence the use of the Exact test.

4.3.3.b Allele Frequencies

The mean allele frequencies were obtained from the following formula:

$$\text{Mutant Allele frequency} = \frac{\text{Number of mutant alleles}}{\text{Total number of alleles (N)}} \\ (p)$$

where the mutant allele frequency is p and the wild type allele frequency is q and $p+q=1$. The Hardy Weinberg theory states that, in the absence of selection, migration, or mutation affecting the locus in question the relationship between the allele frequencies and genotype frequencies is given by: $p^2 + 2pq + q^2 = 1$, where p^2 is the proportion of homozygotes for the mutant allele, $2pq$ is the proportion of heterozygotes and q^2 is the proportion of homozygous wild type individuals.

The difference between the mean allele frequencies are summarised in Table 4.6. Allele frequencies obtained of 16% for EUs, 13% for HIV⁺s and 14% for Controls were obtained (see Table 4.6) and no significant differences were detected between any group (see

Table 4.7.**Probabilities obtained from Comparisons of Genotype Frequencies**

A. EUs/ HIV⁺s and Controls*	χ^2 P
CCR-5[†]	
WT/WT vs WT/ Δ 32 + Δ 32/ Δ 32	0.71
WT/WT vs WT Δ 32	0.57
CCR-2[§]	
WT/WT vs WT/64I + 64I/64I	0.25
WT/WT vs WT/64I	0.30
CCR-2/CCR-5	
WT/WT vs WT/ Δ 32, 64I/WT, 64I/ Δ 32, Δ 32/ Δ 32, 64I/64I	0.83

B. Control Versus EUs and HIV⁺s*	Control / EU		Control / HIV⁺	
	χ^2 P [§]	Fisher Exact Test P [¶]	χ^2 P [§]	Fisher Exact Test P [¶]
CCR-5[†]				
WT/WT vs WT/ Δ 32 + Δ 32/ Δ 32	0.55	0.12	0.88	0.15
WT/WT vs WT Δ 32	0.41	0.10	0.58	0.13
CCR-2[§]				
WT/WT vs WT/64I + 64I/64I	0.37	0.12	0.91	0.19
WT/WT vs WT/64I	---	---	0.96	0.20
CCR-2/CCR-5				
WT/WT vs WT/ Δ 32, 64I/WT, 64I/ Δ 32, Δ 32/ Δ 32, 64I/64I	0.86	0.15	1.00	0.14

* Chi-squared/ Fisher's Exact test analysis of genotyping data

[†] WT/WT: homozygous wild-type CCR-5 genotype; Δ 32/ Δ 32 for the homozygous mutant for the 32bp deletion in the CCR-5 gene and WT/ Δ 32 for the heterozygote.

[§] WT/WT: homozygous wild-type CCR-2 genotype; 64I/64I for the homozygous mutant for the valine to isoleucine a-a change and WT/64I for the heterozygote.

[¶] Calculated from 2x2 table by either pooling, or excluding homozygous mutants with heterozygotes.

[§] Calculated with Yate's correction for contingency

[¶] Probability for Exact test is underestimated when P>0.10, see Section 4.3.1.a for explanation.

C. EUs versus HIV ⁺ s*	Both Sexes		Females Only		Males Only	
	χ^2 P [§]	Fisher Exact Test P [£]	χ^2 P [§]	Fisher Exact Test P [£]	χ^2 P [§]	Fisher Exact Test P [£]
CCR-5[†]						
WT/WT vs WT/ Δ 32 + Δ 32/ Δ 32	0.71	0.13	0.86	0.16	0.94	0.26
WT/WT vs WT Δ 32	0.83	0.14	0.98	0.17	---	---
CCR-2[§]						
WT/WT vs WT/64I + 64I/64I	0.16	0.08 [£]	0.06	0.02 ^{#£}	0.80	0.32
WT/WT vs WT/64I	0.21	0.13 [£]	0.08	0.04 ^{#£}	---	---
CCR-2/CCR-5						
WT/WT vs WT/ Δ 32, 64I/WT, 64I/ Δ 32, Δ 32/ Δ 32, 64I/64I	0.66	0.12	0.61	0.13	1.00	0.27

* Chi-squared and Fisher's Exact test of genotyping data

P<0.05

[†] WT/WT: homozygous wild-type CCR-5 genotype; Δ 32/ Δ 32 for the homozygous mutant for the 32bp deletion in the CCR-5 gene and WT/ Δ 32 for the heterozygote.

[§] WT/WT: homozygous wild-type CCR-2 genotype; 64I/64I for the homozygous mutant for the valine to isoleucine a-a change and WT/64I for the heterozygote.

[‡] Calculated from 2x2 table by either pooling, or excluding homozygous mutants with heterozygotes.

[§] Calculated with Yate's correction for contingency

[£] Probability for Exact test is underestimated when P>0.10, see Section 4.3.1.a for explanation.

Table 4.8.a). Combining all groups, the overall frequency of the $\Delta 32$ allele in this population was 14% (9-23% (95% Binomial Confidence Intervals (CI))).

From the allele frequencies obtained, Hardy Weinberg predicted genotype frequencies were calculated for the three groups (data not shown). The actual values obtained fitted the expected ones from the various allele frequencies.

4.3.3.c Single Sex Analysis

Due to the known heterogeneity in the relative risk of male-to-female and female-to-male heterosexual transmission (see section 1.3.3), the contacts were assessed separately according to sex (see Table 4.9). The sex of the Controls was not known and comparisons with them was therefore not performed.

As the majority of the contacts were female, the analysis of males only carried little statistical weight due to the low numbers (see Table 4.7.c). However, there was still no significant difference when female only contacts were analysed separately (see Table 4.7.c). The allele frequencies (see Table 4.9; females only - EUs 16%, HIV⁺s 13%; males only - EUs 18%, HIV⁺s 14%), also did not differ between the groups for either sex (see Table 4.8.a; females only - $z=0.59$, $P=0.56$; males only - $z=0.40$, $P=0.69$).

4.3.4 CCR-2

4.3.4.a Genotypes

The same groups screened for the deletion in the *CCR-5* gene (see Section 4.3.3; see Appendix 4.5, 4.6 and 4.7 for individual results) were also assessed for their genotype for a mutation in *CCR-2*, causing a valine to isoleucine amino acid change at position 64 (64I).

Only one homozygote for the 64I mutation was seen and this was in the HIV⁺ group (see Table 4.10). As for *CCR-5*, no significant heterogeneity was observed among the genotype frequencies of the three groups (see Table 4.7.a; $\chi^2=2.79$, $P=0.25$, combining 64I/64I individuals; $\chi^2=2.38$, $P=0.30$, excluding 64I/64I individuals). However, a higher frequency of heterozygotes were observed in HIV⁺s than EUs (see Table 4.10); a difference which was close to significance (see Table 4.7.c; $P<0.08$, Exact test (one-tailed) pooling 64I/64I homozygotes, $P=0.13$, Exact test (one-tailed) excluding them).

Table 4.8.**Probabilities Obtained When Comparing Allele Frequencies****A. CCR-5**

CCR-5	Both Sexes		Females Only		Males Only	
	z*	P†	z	P	z	P
EU vs HIV ⁺	0.71	0.48	0.59	0.56	0.40	0.69
EU vs Control	0.48	0.63	---	---	---	---
HIV ⁺ vs Control	0.15	0.88	---	---	---	---
TRI [§] vs NTRI [‡]	0.93	0.35	0.10	0.92	1.08	0.28

B. CCR-2

CCR-2	Both Sexes		Females Only		Males Only	
	z	P	z	P	z	P
EU vs HIV ⁺	1.77	0.08	2.33	0.02 [§]	0.16	0.87
EU vs Control	1.18	0.24	---	---	---	---
HIV ⁺ vs Control	0.50	0.62	---	---	---	---
TRI vs NTRI	1.47	0.14	0.81	0.42	1.27	0.20

* Standardised normal deviate (z) obtained after comparing allele frequencies

† Probability (P) of obtaining z in two-tailed test

§ TRansmitting Index

‡ Non-TRansmitting Index

§ P<0.05

Table 4.9.**CCR-5 Genotypes and Allele Frequencies in EUs and HIV⁺ Cohorts - Single Sex****A. Females only**

Females only	Number			%			Δ 32 Allele Freq (%) (95% CI) [†]
	WT/WT*	WT/ Δ 32	Δ 32/ Δ 32	WT/WT*	WT/ Δ 32	Δ 32/ Δ 32	
EUs n=44	31	12	1	71	27	2	16 (9-25)
HIV⁺ n=65	48	17	0	74	26	0	13 (8-20)

B. Males only

Males only	Number			%			Δ 32 Allele Freq (%) (95% CI) [†]
	WT/WT*	WT/ Δ 32	Δ 32/ Δ 32	WT/WT*	WT/ Δ 32	Δ 32/ Δ 32	
EUs n=14	9	5	0	64	36	0	18 (6-36)
HIV⁺ n=21	15	6	0	71	29	0	14 (6-28)

* WT/WT: homozygous wild-type CCR-5 genotype; Δ 32/ Δ 32 for the homozygous mutant for the 32bp deletion in the CCR-5 gene and WT/ Δ 32 for the heterozygote.

[†] 95% Binomial Confidence Intervals (CI).

Table 4.10.

CCR-2 Genotypes and Allele Frequencies in Controls, EUs and HIV⁺ Cohorts

	Number			%			64I Allele Freq (%) (95% CI) [†]
	WT/ WT*	WT/ 64I	64I/ 64I	WT/ WT*	WT/ 64I	64I/ 64I	
EUs n=58	54	4	0	93	7	0	3 (1-8)
HIV⁺ n=86	72	13	1	84	15	1	9 (5-14)
Controls n=50	43	7	0	86	14	0	7 (3-14)

* WT/WT: homozygous wild-type CCR-2 genotype; 64I/64I for the homozygous mutant for the valine to isoleucine a-a change and WT/64I for the heterozygote.

[†] 95% Binomial Confidence Intervals (CI).

4.3.4.b Allele Frequencies

Allele frequencies of 3% for EUs, 9% for HIV⁺s and 7% for Controls (see Table 4.10) were obtained. The statistical differences between the groups are summarised in Table 4.8.b. The frequency obtained for the control group did not differ from either group, but the difference between the heterosexually exposed cohorts (EUs and HIV⁺s) approached significance (see Table 4.8.b; $z=1.77$, $P=0.08$).

Hardy Weinberg predicted frequencies did not differ from those obtained, except for the CCR-2 frequencies of the NTRIs (both sexes). Expected frequencies of 30.4, 7.1 and 0.4 for WT/WT, WT/64I and 64I/64I respectively were calculated from the allele frequencies (WT: 89%; 64I: 11%) which differed significantly from the observed frequencies of 32, 4 and 2 ($P<0.04$, Exact test calculated as outlined in Weir (1990)). The departure was a higher than expected frequency of homozygotes for the 64I mutation in the NTRIs. When males only were analysed, a significant departure was no longer seen.

4.3.4.c Single Sex Analysis

As for CCR-5, the genotypes and allele frequencies were determined for males and females separately (see Table 4.11). The increased number of heterozygotes seen when both sexes were used, was more marked in the female only analysis (see Table 4.11.a; 2% heterozygotes in EUs compared to 14% in HIV⁺s) and the difference was significant (see Table 4.7.c; $P=0.02$, Exact test (one-tailed), pooling 64I/64I homozygotes; $P=0.04$, Exact test (one-tailed) excluding them). The CCR-2 64I mutant allele thus appears to be a risk factor for heterosexual infection of females ($RR=1.6$ (95% CI 1.3-2.1)). No difference between the two groups was seen when males only were included (see Table 4.11.b; 21% heterozygotes in EUs compared to 19% in HIV⁺s (see Table 4.7.c)).

The allele frequencies also differed significantly between the female EUs and HIV⁺s (see Table 4.11.a; 1% in EUs, 8% in HIV⁺s; see Table 4.8.b; $z=2.33$, $P<0.02$), but not when males only were considered (see Table 4.11.b; 11% in EUs, 10% in HIV⁺s; see Table 4.8.b; $z=0.16$, $P=0.87$).

4.3.5 Combined CCR-2/5 Analysis

Individuals WT at both the CCR-2 and CCR-5 loci were compared to those of all other possible genotypic combinations (WT/ Δ 32, WT/64I, 64I/ Δ 32, Δ 32/ Δ 32 and 64I/64I).

Table 4.11.**CCR-2 Genotypes and Allele Frequencies in EUs and HIV⁺ Cohorts - Single Sex****A. Females only**

Females only	Number			%			64I Allele Freq (%) (95% CI) [†]
	WT/ WT*	WT/ 64I	64I/ 64I	WT/ WT*	WT/ 64I	64I/ 64I	
EUs n=44	43	1	0 [§]	98	2	0	1 [‡] (0-6)
HIV⁺ n=65	55	9	1 [§]	85	14	2	8 [‡] (4-14)

B. Males only

Males only	Number			%			64I Allele Freq (%) (95% CI) [†]
	WT/ WT*	WT/ 64I	64I/ 64I	WT/ WT*	WT/ 64I	64I/ 64I	
EUs n=14	11	3	0	79	21	0	11 (2-28)
HIV⁺ n=21	17	4	0	81	19	0	10 (3-22)

* WT/WT: homozygous wild-type CCR-2 genotype; 64I/64I for the homozygous mutant for the valine to isoleucine a-a change and WT/64I for the heterozygote.

[†] 95% Binomial Confidence Intervals (CI).

[§] P<0.02, Exact test comparing the CCR-2 genotype frequencies in the two cohorts, pooling samples to avoid low expected values.

[‡] z=2.33, P<0.02, difference between the allele frequencies established from the standardised normal deviate.

No significant difference was observed between the three groups (see Table 4.7.a; $\chi^2=0.38$, $P=0.83$), or between the Controls and either the EUs, or HIV⁺s (see Table 4.7.b). The difference between the heterosexually exposed cohorts only (EUs and HIV⁺s) was also not significant, even during single sex analysis (see Table 4.7.c).

4.3.6 HIV⁺ Index Partners

Samples were obtained from HIV⁺ index partners from the Heterosexual study and divided into those who transmitted virus to their heterosexual partner (TRIs; $n=19$ (3 female, 16 male; see Appendix 4.8 for individual results) and those who did not transmit (NTRIs; $n=38$ (10 female, 28 male; see Appendix 4.9 for individual results).

4.3.6.a *CCR-5 Genotypes*

No homozygotes for the $\Delta 32$ deletion in *CCR-5* were seen (see Table 4.12) and despite an increased number of WT/ $\Delta 32$ heterozygotes in the NTRIs (NTRIs 34%, TRIs 21%), no significant difference between the *CCR-5* genotypes in two groups was seen (see Table 4.13).

When the groups were divided into single sexes (see Table 4.14), the low numbers of females meant analysis of them carried little statistical weight (see Table 4.13). In the males only group (see Table 4.14.b) the increase in heterozygotes in the NTRIs was more marked (NTRIs 36%, TRIs 19%), but this was again not significant (see Table 4.13).

4.3.6.b *CCR-5 Allele Frequencies*

Allele frequencies for the $\Delta 32$ mutation of 11% in TRIs and 17% in NTRIs were obtained, but the difference was not significant (see Table 4.8; $z=0.93$, $P=0.35$). When males only were considered, the difference was again increased (see Table 4.14; TRIs 9%, NTRIs 18%), but this was also not significant (see Table 4.8; $z=1.08$, $P=0.28$).

4.3.6.c *CCR-2 Genotypes*

Two homozygous 64I/64I individuals were seen in the NTRIs (see Table 4.15) and an increased number of WT/64I heterozygotes were seen (see Table 4.15; NTRIs 11%, TRIs 5%), but this was not significant (see Table 4.13). As for *CCR-5*, separate analysis of the

Table 4.12.

CCR-5 Genotypes and Allele Frequencies in HIV⁺ Indexes

	Number			%			$\Delta 32$ Allele Freq (%) (95% CI) [†]
	WT/WT*	WT/ $\Delta 32$	$\Delta 32/\Delta 32$	WT/WT*	WT/ $\Delta 32$	$\Delta 32/\Delta 32$	
TRI[§] n=19	15	4	0	79	21	0	11 (3-24)
NTRI n=38	25	13	0	66	34	0	17 (10-27)

* WT/WT: homozygous wild-type CCR-5 genotype: $\Delta 32/\Delta 32$ for the homozygous mutant for the 32bp deletion in the CCR-5 gene and WT/ $\Delta 32$ for the heterozygote.

[†] 95% Binomial Confidence Intervals (CI).

[§] TRansmitting Index

^{||} Non-TRansmitting Index

Table 4.13.

Probabilities obtained from Comparisons of Genotype Frequencies

TRI versus NTRI*	Both Sexes		Females Only		Males Only	
	χ^2 p [§]	Fisher Exact Test p [£]	χ^2 p [§]	Fisher Exact Test p [£]	χ^2 p [§]	Fisher Exact Test p [£]
CCR-5[†]						
WT/WT vs WT/ Δ 32 + Δ 32/ Δ 32	0.47	0.15	XXX	0.50	0.40	0.14
WT/WT vs WT Δ 32	---	---	---	---	---	---
CCR-2[§]						
WT/WT vs WT/64I + 64I/64I	0.48	0.20	XXX	0.77	0.53	0.22
WT/WT vs WT/64I	0.82	0.32	XXX	1.00	0.72	0.29
CCR-2/CCR-5						
WT/WT vs WT/ Δ 32, 64I/WT, 64I/ Δ 32, Δ 32/ Δ 32, 64I/64I	0.21	0.10 [£]	XXX	0.49	0.19	0.10 [£]

* Chi-squared and Fisher's Exact test of Tables 4.X., 4.X, etc

P<0.05

[†] WT/WT: homozygous wild-type CCR-5 genotype; Δ 32/ Δ 32 for the homozygous mutant for the 32bp deletion in the CCR-5 gene and WT/ Δ 32 for the heterozygote.

[§] WT/WT: homozygous wild-type CCR-2 genotype; 64I/64I for the homozygous mutant for the valine to isoleucine a-a change and WT/64I for the heterozygote.

[‡] Calculated from 2x2 table by either pooling, or excluding homozygous mutants with heterozygotes.

[§] Calculated with Yate's correction for contingency

XXX - n \leq 1 for one of groups so test not valid

[£] Probability for Exact test is underestimated when P>0.10, see Section 4.3.1.a for explanation.

Table 4.14.**CCR-5 Genotypes and Allele Frequencies in HIV⁺ Indexes - Single Sex****A. Females only**

Females only	Number			%			$\Delta 32$ Allele Freq (%) (95% CI) [†]
	WT/WT*	WT/ $\Delta 32$	$\Delta 32/\Delta 32$	WT/WT*	WT/ $\Delta 32$	$\Delta 32/\Delta 32$	
TRI[§] n=3	2	1	0	67	33	0	17 (0-60)
NTRI[‡] n=10	7	3	0	70	30	0	15 (3-38)

B. Males only

Males only	Number			%			$\Delta 32$ Allele Freq (%) (95% CI) [†]
	WT/WT*	WT/ $\Delta 32$	$\Delta 32/\Delta 32$	WT/WT*	WT/ $\Delta 32$	$\Delta 32/\Delta 32$	
TRI[§] n=16	13	3	0	81	19	0	9 (2-25)
NTRI[‡] n=28	18	10	0	64	36	0	18 (9-30)

* WT/WT: homozygous wild-type CCR-5 genotype; $\Delta 32/\Delta 32$ for the homozygous mutant for the 32bp deletion in the CCR-5 gene and WT/ $\Delta 32$ for the heterozygote.

[†] 95% Binomial Confidence Intervals (CI).

[§] TRansmitting Index

[‡] Non-TRansmitting Index

Table 4.15.

CCR-2 Genotypes and Allele Frequencies in HIV⁺ Indexes

	Number			%			64I Allele Freq (%) (95% CI) [†]
	WT/ WT*	WT/ 64I	64I/ 64I	WT/ WT*	WT/ 64I	64I/ 64I	
TRI[§] n=19	18	1	0	95	5	0	3 (0-14)
NTRI[‡] n=38	32	4	2	84	11	5	11 (5-20)

*WT/WT: homozygous wild-type CCR-2 genotype; 64I/64I for the homozygous mutant for the valine to isoleucine a-a change and WT/64I for the heterozygote.

[†] 95% Binomial Confidence Intervals (CI).

[§] TRansmitting Index

[‡] Non-TRansmitting Index

females only (see Table 4.16.a) was inhibited by the low numbers (see Table 4.13) and the differences between the males only (see Table 4.16.b) was not significant (see Table 4.13).

4.3.6.d CCR-2 Allele Frequencies

Allele frequencies of 3% in TRIs and 11% in NTRIs were obtained (see Table 4.15), but the difference was not formally significant (see Table 4.8.b; $z=1.47$, $P=0.14$). Analysis of males only yielded the same allele frequencies (see Table 4.16.b), but the lower numbers reduced the significance (see Table 4.8.b; $z=1.27$, $P=0.20$).

4.3.6.e Combined CCR-2/5 Analysis

When comparing the frequency of individuals WT at both the *CCR-2* and *CCR-5* loci to all other genotypic combinations (as outlined above) (20 WT:WT, 18 non-WT/WT for NTRIs; 14 WT/WT, 5 non-WT/WT for TRIs) the difference between the two groups was increased (see Table 4.13; $P=0.10$, Exact test (one tailed)), thus the two mutations appeared to have an additive effect on the probability of heterosexual transmission from HIV⁺ indexes.

4.3.7 CCR Genotypes and Exposure

The availability of quantitative data on exposure levels with the EU group allowed us to investigate whether the *CCR-2* and *CCR-5* genotypes differed with respect to the average 'at risk' exposures accumulated without seroconversion.

The level of 'at risk' exposure, determined as outlined in Section 2.2.1.a, ranged from less than 50 contacts to over 1000. For *CCR-5*, the range of exposures for both WT/WT and WT/ Δ 32 was evenly spread with no difference with respect to genotype (see Figure 4.6.a). Median values of 131 and 118 for the WT/WT and WT/ Δ 32 respectively were obtained. The accumulated mean exposure for WT/WT and WT/ Δ 32 heterozygotes did not differ significantly (WT/WT: 280, WT/ Δ 32: 216; $t=0.14$, $P=0.89$ following log transformation). When the female contacts were analysed separately, the median accumulated exposures were: WT/WT - 100, WT/ Δ 32 - 129, but the difference was again not significant (Mean values WT/WT: 222, WT/ Δ 32: 217; $t=0.19$, $P=0.85$ following log transformation).

The low numbers of EUs expressing the *CCR-2* 64I mutation (4 WT/64I) meant that little could be concluded from the exposure data relative to the *CCR-2* genotype. Figure 4.6.b shows the distribution with median values of 127 WT/WT and 150 WT/64I. The mean

Table 4.16.**CCR-2 Genotypes and Allele Frequencies in HIV⁺ Indexes - Single Sex****A. Females only**

Females only	Number			%			64I Allele Freq (%) (95% CI) [†]
	WT/ WT*	WT/ 64I	64I/ 64I	WT/ WT*	WT/ 64I	64I/ 64I	
TRI[§] n=3	3	0	0	100	0	0	0 (0-43)
NTRI[‡] n=10	9	0	1	90	0	10	10 (1-32)

B. Males only

Males only	Number			%			64I Allele Freq (%) (95% CI) [†]
	WT/ WT*	WT/ 64I	64I/ 64I	WT/ WT*	WT/ 64I	64I/ 64I	
TRI[§] n=16	15	1	0	94	6	0	3 (0-16)
NTRI[‡] n=28	23	4	1	82	14	4	11 (4-22)

* WT/WT: homozygous wild-type CCR-2 genotype; 64I/64I for the homozygous mutant for the valine to isoleucine a-a change and WT/64I for the heterozygote.

[†]95% Binomial Confidence Intervals (CI).

[§] TRansmitting Index

[‡] Non-TRansmitting Index

Figure 4.6.

HIV Exposure Among EUs Related to CCR-2 and CCR-5 Genotype

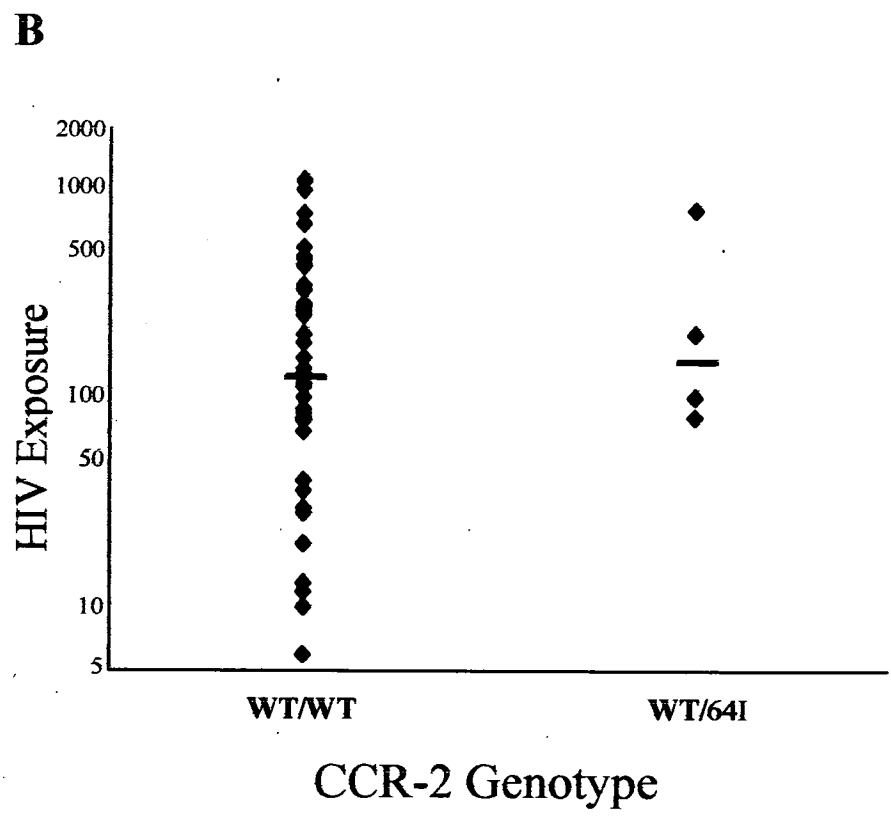
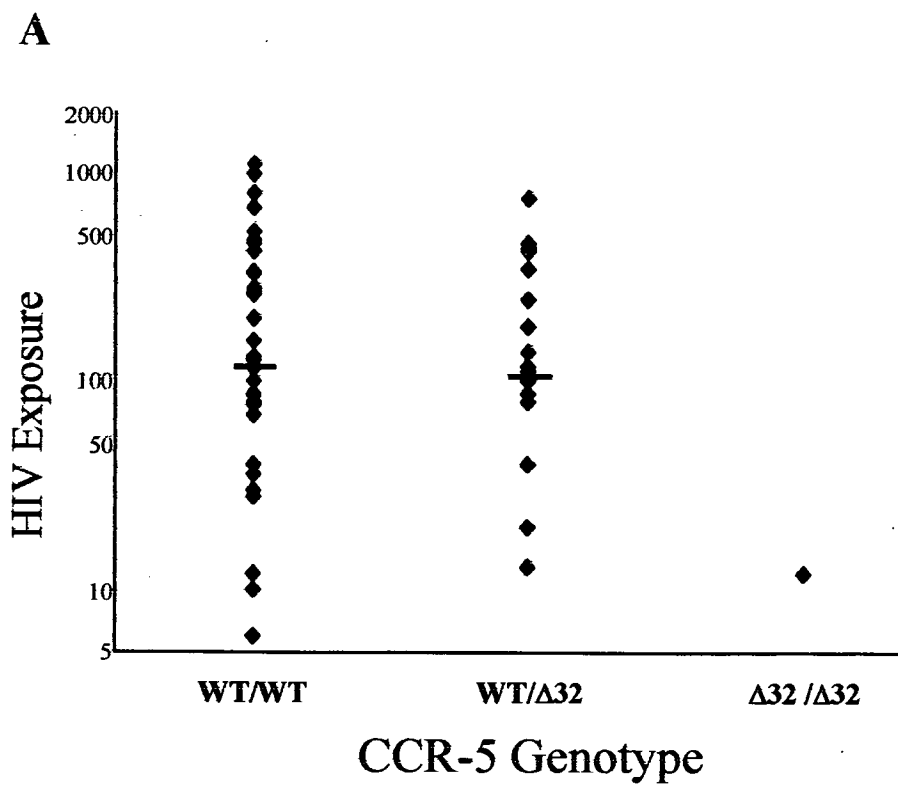
HIV exposure levels (plotted on a log scale) are the number of 'at risk' exposures for each contact individual, estimated as outlined in Section 2.2.1.a. Median values are represented as horizontal bars.

a) CCR-5 genotypes

WT/WT for homozygous wild type, $\Delta 32/\Delta 32$ for the homozygous mutant for the 32bp deletion in the CCR-5 gene and WT/ $\Delta 32$ for the heterozygote. Median exposure values shown : WT/WT: 131, WT $\Delta 32$: 118. Mean exposure values: WT/WT: 280, WT/ $\Delta 32$: 216 (t=0.14, P=0.89).

b) CCR-2 Genotypes

WT/WT for homozygous wild type and WT/64I for the heterozygote for the isoleucine mutation at amino acid position 64. Median exposure values shown : WT/WT: 127, WT/64I: 150. Mean exposure values: WT/WT: 252, WT/64I: 295 (t=0.57, P=0.57).



accumulated values (WT/WT: 252, WT/64I: 295) did not differ significantly as expected from the low numbers of heterozygotes ($t=0.57$, $P=0.57$ following log transformation).

4.3.8 CCR-5 Sequencing

Following the discovery that the $\Delta 32$ deletion in *CCR-5* had no effect on heterosexual transmission in this cohort, it was decided to sequence several of the EUs with the highest exposures to see if they had any other alterations in their *CCR-5* locus that may explain their seronegative status. Five individuals were chosen: C3361 (female; WT/WT *CCR-2* and 5; Exposures: 990); C4191 (female; WT/ $\Delta 32$ *CCR-5*, WT/WT *CCR-2*; Exposures: 756); C4331 (male; WT/WT *CCR-5*, WT/64I *CCR-2*; Exposures: 798); C4421 (male; WT/WT *CCR-2* and 5; Exposures: 1096); C5191 (female; WT/WT *CCR-2* and 5; Exposures: 1132).

The whole *CCR-5* gene was amplified, as described earlier (see Section 4.2.3). All four individuals homozygous wild-type for the $\Delta 32$ mutation in *CCR-5* had identical amino acid sequence to that of the consensus, with no other deletions, insertions, or mutations apparent. The resulting amino acid sequences are given in Appendix 4.10. The WT/ $\Delta 32$ individual (C4191) gave an unclear signal after the mutation site as expected, as half of the products would have been from the WT gene and half from the $\Delta 32$ gene. Limiting dilutions, to allow amplification from a single allele, were not performed.

4.3.9 CCR-5 Promoter Mutations

The heterosexually exposed individuals (EUs and HIV⁺s) and the populations controls were screened, in collaboration with Dr. M. Carrington, for the presence of different *CCR-5* promoter alleles (see Appendix 4.5, 4.6 and 4.7 for individual results). The alleles which Dr. Carrington and co-workers have described relate to different combinations of polymorphisms in the *CCR-5* promoter region (see Section 4.1 and Figure 4.1). The frequencies obtained for the alleles are shown in Table 4.17 and the numbers screened are slightly lower than those previously screened for the *CCR-2* and *CCR-5* mutations, due to limited samples remaining (see Table 4.17).

Of the 10 alleles described by Dr. Carrington (P1-10), P5-10 are rare and were not seen for any of the Edinburgh cohort screened. The frequency of the P3 allele is also rare in Caucasians (0.14%, Martin *et al.* in preparation) and was also not seen for any individual in the Edinburgh cohort (see Table 4.17).

Table 4.17.

**Frequency of CCR-5 Promoter Mutation Alleles and Composite
CCR-2/P1/CCR-5 Bearing Haplotypes.**

	Number*			%#		
	EU (n=51)	Control (n=48)	HIV ⁺ s (n=70)	EU	Control	HIV ⁺ s
WT/P1/WT	41	35	52	40	36	37
P1 [§] 64I/P1/WT	3	7	15	3	7	11
WT/P1/Δ32	19	13	22	19	14	16
P1	63	55	89	62	57	64
P2	6	13	9	6	14	6
P4	33	28	42	32	29	30
Total [†]	102	96	140			

* Number of Alleles in different risk groups

Allele Frequency as % of total

§ Frequency of CCR-5-P1 bearing haplotypes: CCR-2/P1/CCR-5

† Total Number of Alleles (2 x number of individuals)

For the P1, P2 and P4 alleles, similar frequencies were obtained between the groups, except for the P2 allele, which was elevated in the population controls (14% Controls compared to 6% in EUs and HIV⁺s; see Table 4.17). The same difference was reflected when the allele frequencies were compared and the P2 allele approached a significant difference when the Controls were compared to HIV⁺s and EUs ($z=1.83$, $P=0.067$ EUs versus Controls; $z=1.85$, $P=0.064$ HIV⁺s versus Controls; see Table 4.18). No other comparison revealed a significant difference (see Table 4.18).

When the distribution of the three alleles (P1,2,4) were compared between all three groups together and two groups separately, no significant differences were seen, although lower probabilities were obtained when comparing the Controls (see Table 4.19). In contrast, the EUs and HIV⁺s were very similar, reflected in the very high probability ($\chi^2=0.16$, $P=0.92$; see Table 4.19) and hence the lower probabilities seen in the analysis including the Controls, probably reflects the observed difference in the P2 allele (see Table 4.19).

As described earlier (see Section 4.1), the CCR-2-64I and CCR-5- $\Delta 32$ mutations, only occur on a P1 allele. This was supported by the lack of any individuals homozygous for the P2, or P4 alleles which expressed the 64I, or the $\Delta 32$ mutations and all the 64I/64I and $\Delta 32/\Delta 32$ homozygotes were also homozygous for the P1 allele. The P1 allele could therefore be divided into three different CCR-2/P1/CCR-5 haplotypes: WT/P1/WT, 64I/P1/WT and WT/P1/ $\Delta 32$, as the CCR-2 and CCR-5 mutations never appear on the same allele (see Section 1.7.3.a). The frequency of the three P1 haplotypes for the three groups are summarised in Table 4.17. As expected from the previous analysis in this Chapter, the haplotype expressing the CCR-2-64I mutation was elevated in the HIV⁺s and decreased in the EUs ($\chi^2=5.22$, $P=0.07$ and $z=2.28$, $P=0.02$ for EUs and HIV⁺s alone; see Table 4.18 and 4.20). No other significant differences were seen for the CCR-2/P1/CCR-5 haplotypes (see Table 4.18 and 4.20).

The distribution of the genotypes for the promoter alleles are summarised in Table 4.20 for the three groups. As for the allele frequencies, the most striking difference is again in the population controls, which show a significantly higher frequency of the P1,P2 genotype than the other two groups (21% Controls; 8% EUs and 7% HIV⁺s (see Table 4.20); $\chi^2=6.19$, $P=0.045$ (see Table 4.21)). The comparisons between just Controls and one of the other groups also approach significance and extent of significance may just reflect the smaller

Table 4.18.**Probabilities Obtained When Comparing CCR-5 Promoter Mutation Allele Frequencies**

A	P1		P2		P4	
	z*	P†	z	P	z	P
EU vs HIV ⁺	0.29	0.77	0.17	0.87	0.39	0.70
EU vs Control	0.64	0.52	1.83	0.067	0.49	0.62
HIV ⁺ vs Control	0.97	0.33	1.85	0.064	0.14	0.89

B	WT/P1/WT [§]		64I/P1/WT		WT/P1/Δ32	
	z*	P†	z	P	z	P
EU vs HIV ⁺	0.48	0.63	2.28	0.02 [#]	0.60	0.56
EU vs Control	0.54	0.60	1.40	0.16	0.97	0.33
HIV ⁺ vs Control	0.11	0.87	0.89	0.37	0.46	0.65

* Standardised normal deviate (z) obtained after comparing allele frequencies

† Probability (P) of obtaining z in two-tailed test

[#] P<0.05

[§] Composite P1 haplotypes for CCR-2/P1/CCR-5 mutations (see Section 4.X)

Table 4.19.

Probabilities for Comparisons of CCR-5 Promoter Mutation Alleles

A. P1/P2/P4*	χ^2 P
EU/HIV ⁺ s/Controls	0.28
EU/HIV ⁺ s	0.92
EU/Controls	0.19
Control/HIV ⁺ s	0.18

B. CCR-2/CCR-5-P1/CCR-5	χ^2 P
EU/HIV ⁺ s/Controls	0.25
EU/HIV ⁺ s	0.07
EU/Controls	0.26
Control/HIV ⁺ s	0.76

* Comparisons of distribution of promoter mutation alleles in different groups

† Comparisons of distribution of CCR-5-P1 bearing haplotypes: WT/P1/WT, 64I/P1/WT, WT/P1/Δ32

Table 4.20.

Frequency of CCR-5 Promoter Mutation Genotypes.

	Number *			%		
	EU	Control	HIV ⁺ s	EU	Control	HIV ⁺ s
P1,P1	19	16	29	37	33	41
P1,P2	4	10	5	8	21	7
P1,P4	21	13	26	41	27	37
P2,P2	1	0	1	2	0	1
P2,P4	0	3	2	0	6	3
P4,P4	6	6	7	12	13	10
Total [†]	51	48	70			

* Number of Individuals bearing given genotypes for CCR-5 Promoter Mutations Alleles

† Total number of Individuals in each group.

Table 4.21.**Probabilities obtained from Comparisons of CCR-5 Promoter Mutation Genotype Frequencies**

	EUs/HIV⁺s/Controls	EUs/HIV⁺s		EUs/Controls		Controls/HIV⁺s	
	χ^2	χ^2	Fisher Exact Test	χ^2	Fisher Exact Test	χ^2	Fisher Exact Test
	P	P [§]	P [£]	P [§]	P [£]	P [§]	P [£]
P1,P1 vs not P1,P1	0.67	0.78	0.13	0.84	0.15	0.49	0.10
P1, P2 vs not P1,P2	0.045	0.84	0.27	0.12	0.06 [£]	0.056	0.029 ^{£#}
P1,P4 vs not P1,P4	0.32	0.79	0.14	0.21	0.10 [£]	0.35	0.17 [£]
P2,P2 vs not P2,P2	0.65	0.62	0.49	0.98	0.52	0.85	0.59
P2,P4 vs not P2,P4	0.19	0.62	0.33	0.22	0.11	0.66	0.24
P4,P4 vs not P4,P4	0.91	0.99	0.22	0.84	0.24	0.90	0.21

* Chi-squared and Fisher's Exact test of genotyping data

§ Calculated with Yate's correction for contingency

£ Probability for Exact test is underestimated when P>0.10, see Section 4.3.1.a for explanation.

P<0.05

numbers compared (see Table 4.21). The elevated P2 allele frequency in the Controls seems therefore to reflect an increase in the P1,P2 genotype. No other significant differences were seen for any other genotype (see Table 4.21).

Analysis of the HIV⁺s and EUs for male and females only did not show any differences from the close associations seen for the analysis of both sexes together. The only exception was the difference in the 64I/P1/WT haplotype and this merely reflected the previous results seen for the CCR-2 mutation (see Section 4.3.4.c).

4.4. DISCUSSION

4.4.1 Establishment of Chemokine Receptor Genotyping Assay

The use of the restriction enzyme Asp 700 in generating RFLPs to genotype individuals for the $\Delta 32$ mutation in *CCR-5* was confounded by the inability to completely digest the product. This can often be due to too little enzyme, or too much product, or too short an incubation time. These factors were investigated and still only a partial digest was achieved. It was decided that a 'difficult' site may exist for this enzyme and an alternative strategy involving different enzymes used instead.

The decision to use the direct PCR method to screen the cohorts was due to the saving in cost and time in the less laborious approach. It also enabled the screening of individuals who only had plasma samples available, as it required lower concentrations of DNA to amplify from.

4.4.2 HLA

The HLA phenotypes in individuals heterosexually exposed to HIV were compared according to infection status, either HIV⁺, or EU. A striking difference was seen for DR11, which was increased in the EUs compared to HIV⁺ contacts ($P < 0.02$). DR11 is a split from DR5 (Hurley *et al.* (1997)) which has been linked in many studies to Kaposi's sarcoma (KS), both classical (Pollack, Safai and Dupont (1983)) and AIDS related (Pollack, Safai and Dupont (1983); Friedman-Kien *et al.* (1982)). DR5 has also been linked to the presence of persistent generalised lymphadenopathy PGL (dePaoli *et al.* (1986); Raffoux *et al.* (1987)) and thrombocytopenia (Raffoux *et al.* (1987)) in HIV⁺ individuals. The genetic background seems to affect the associations, with individuals of a Black, Ashkenazi Jewish, or Hispanic background apparently showing the association of DR5 and KS and other ethnic backgrounds

not (Friedman-Kien *et al.* (1982); Prince *et al.* (1984)). The ethnic effect on DR5 as a risk factor for HIV infection was further supported by a study involving non-KS HIV⁺ individuals of both Caucasian and African American decent (Cruse *et al.* (1991)). They showed an association of DR5 with HIV serostatus in both groups, but this was not significant in either group in corrected values. However, while the two splits of DR5, DR11 and DR12, were significant as risk factors for HIV infection in African Americans in corrected values, only DR12 was in Caucasians. As the individuals investigated in the Edinburgh cohort were all Caucasians, this may explain the discrepancy of DR5 being a risk factor for HIV infection in other studies, but one of its splits, DR11, apparently acting as a protective factor in this study. As DR12 was not typed in the infected individuals, one can only infer from the DR5 frequency that this was elevated in the heterosexually infected individuals (2/16) compared to EUs (1/54), but this not significant. Once corrected for the number of tests, the DR11 frequency difference was also not significant. This highlights the major limitation of this study, in that the number of individuals HLA typed was limited, particularly in the HIV⁺ individuals, relative to the number of antigens tested making it difficult to establish statistical significance.

When compared to population control frequencies the DR5 levels in the EUs (15/53, 14/15 DR11) were also significantly increased, even after correction for the number of tests ($P=0.005$, $P_{\text{corr}}=0.04$). What is not clear is if the association is related to DR11, as the control group was typed for only DR5, rather than DR11 and DR12, but it certainly warrants more investigation to establish whether the DR11 antigen is acting as a protective factor in the EUs. It is possible an opposing effect of the components of the DR5 group, DR11 and DR12, may be occurring, which would explain the discrepancy of this study which appears to suggest a protective effect of DR5 rather than as a risk factor. One study on mother to child transmission revealed an association of DR11 in the mother with an increase risk of transmission (Fabio *et al.* (1992)). However, the factors affecting transmission may differ from those affecting infection.

The antigens A3 and DR4 were also increased in EUs compared to heterosexually HIV-infected individuals and although not formally significant, DR4 as a protective factor was in agreement with a study of Italian haemophiliacs (Fabio *et al.* (1990)). Antigens increased in the HIV⁺s, i.e. potential risk factors, were A26, B27 and DR3, but none were formally significant. DR3 has been widely reported as a factor associated with disease progression in HIV⁺s and also as a risk factor for infection, usually in association with A1

and B8 with which it is in linkage disequilibrium (Steel *et al.* (1988); Fabio *et al.* (1990); Kaplan *et al.* (1990); Kaslow *et al.* (1990); Mallal *et al.* (1990); McNeil *et al.* (1996)). The numbers expressing A1B8DR3 did not differ in the two heterosexually exposed groups examined here (EUs 5/35; HIV⁺s 3/17).

Other risk factors could be inferred from their under representation in EUs compared to population control frequencies, although I feel care should be taken in such conclusions, especially if they are not supported in HIV⁺ individuals. DR6 was significantly higher in Controls than EUs, even after correction for the number of tests ($P=0.002$, $P_{\text{corr}}<0.02$), but this was not seen in the HIV⁺ contacts. The difference may reflect differences in the methods used to obtain the results and highlights a problem in the use of these controls. The potential differences in the both the different typing methods and also the fact that the results were generated in different laboratories, are a major limitation. Different conditions, although unlikely, may yield different results and therefore all of the apparent differences seen in this study would need to be confirmed, by re-analysis of the cohorts studied, using the same methods and under the same conditions.

In contrast, Cruse and colleagues (1991) showed DR6 as a protective factor, but only in African Americans. DR6, like DR5, is a broader antigen type and is composed of the splits DR13 and DR14 and it is possible that the protective effect seen in the African Americans is due to one of these splits and DR6 as a risk factor in the Edinburgh cohort is due to the other. This could not be confirmed as only some of the Edinburgh EUs were typed specifically for these splits. In support of this is an Italian study of children born to HIV⁺ mothers, who showed DR13 as a risk factor for infection and DR14 as a protective factor (Greggio *et al.* (1993)). More extensive typing in the Edinburgh groups would be required to explain the apparent decrease of DR6 in the EUs. B7 was also increased in the Controls and DR1 in the EUs, but neither were significant in corrected values.

No antigen was shown to correlate with transmission when comparing HIV⁺ indexes who had either transmitted heterosexually (TRIs), or not transmitted (NTRIs). This may reflect the limited numbers available for comparison. Only one other study has reported an effect of HLA on transmission and this was the mother child report mentioned earlier (Fabio *et al.* (1992)), which found DR11 to be associated with increased infectiousness. No association was found in the same study when looking at heterosexual couples, but this study may also have been limited by its size.

An effect of HLA on transmission may be merely a reflection of disease status in the transmitting individual. Associations of disease status and HLA have been widely reported (Mann *et al.* (1991); Steel *et al.* (1988); Jeannet *et al.* (1989); Cruse *et al.* (1991); Kaplan *et al.* (1990); Kaslow *et al.* (1990); Mallal *et al.* (1990); Louie, Newman and King (1991); Donald *et al.* (1992); McNeil *et al.* (1996)), as has the effect of disease status on transmission risk (Laga *et al.* (1989); Fiore *et al.* (1997)). This is suggested in the indexes, for example A1B8DR3 is increased in the TRIs (TRIs 4/16; NTRIs 3/28) and B27 increased in the NTRIs (TRIs 0/17; NTRIs 3/30), both having been shown to be important disease progression markers in the Edinburgh area (Steel *et al.* (1988); McNeil *et al.* (1996)). However, no significant differences were seen.

The associations of HLA with HIV infection and disease may act in a variety of ways and be dependent on the HLA type and the strain of the infecting virus. Both of which may account for the regional and ethnic differences in HIV/HLA associations. The most apparent mechanism for HLA to affect HIV infection and disease is by a direct affect on antigen presentation. The HLA antigens have discrete differences in the peptides they bind and present to the immune system to induce responses. The peptides corresponding to HLA antigens may be absent from some strains, or more prevalent in others. Some may induce different responses, such as cytotoxicity versus suppressor phenotypes of CTLs. This could then have a positive, or negative effect on the course of HIV infection. The affinity of peptide binding may also be important, whereby precise peptide sequences could also effect antigen presentation (Callahan *et al.* (1990)).

Molecular mimicry between HIV proteins, particularly gp120 (Golding *et al.* (1988); Young (1988)) is another possible mechanism by which HLA phenotype can influence the response to the virus. This may even induce a potential autoimmune response, suggested by some as the cause of AIDS (Kion and Hoffman (1991)). Studies of individuals genetically susceptible to autoimmune diseases, such as rheumatoid arthritis (Klein (1986b)), suggest that certain HLA types might be associated with aberrant immune responses and could thus affect susceptibility to HIV infection and disease. Notably, the A1B8DR3 phenotype has been linked to increased antibody production (Kallenberg *et al.* (1988)), perhaps reflecting a predisposition to a strong Type 2 response and hence a poor prognosis (Clerici and Shearer (1993); Clerici and Shearer (1994)). It is most likely that a variety of mechanisms are responsible for the diversity of effects of HLA on HIV, with different alleles associated with each mechanisms.

4.4.2.a Mismatch HLA Types

To investigate if discordance at the HLA loci between heterosexual couples had any effect on HIV transmission, the HLA types of the concordant and discordant couples of the heterosexual study were investigated. Mismatched HLA types could induce a stronger allogeneic immune response to the index partner's cells, which if infected may destroy a potential source of infection. The virus itself is known to 'acquire' host proteins as it buds from cells, including those of the MHC (Henderson *et al.* (1987); Arthur *et al.* (1992)). Virus from the index partner will hence contain HLA molecules of that individual. Therefore even if the contact is exposed to free virus, as opposed to cell-associated, they may still mount an allogeneic immune response to the host proteins contained within the viral envelope. It is perhaps of particular importance that the majority of the couples in the heterosexual study are in long term relationships rather than multiple single contacts. So if allogeneic responses are protective, then they could have a long term protective mechanism. Despite restricted numbers, the EUs had a clearly higher degree of discordance at the HLA-A/B and DR loci than the heterosexually HIV-infected contacts. It is perhaps also pertinent that several of the contacts, with multiple relationships in the study, became infected shortly after a partner change (Rona Wyld, personal communication). This may then reflect a lack of protective allogeneic responses, although other factors are also possible, such as a change in the frequency of sexual intercourse and a potential change in sexual practises with the new partner.

A similar report of mother-child transmission noted that Class I concordance was associated with an increased risk of perinatal HIV transmission, with increased concordance carrying an increased risk (MacDonald *et al.* (1998)). Earlier studies in macaques had also revealed an association of anti-human MHC responses, induced by immunisation with human cells, correlating with protection from SIV challenge, if the virus was grown in the same cell line as they were immunised with (Stott (1991); Chan *et al.* (1992)). Direct immunisation with purified Class I molecules was later shown to protect macaques from a cell-free virus challenge when the virus expressed the same Class I molecule (Chan *et al.* (1995)), confirming the role of MHC in the protection. Although the monkeys were protected by a xenogenic response, studies in humans have shown that allogeneic responses are in fact more vigorous than xenogenic anti-pig ones (Dorling, Binns and Lechler (1996)).

No study of heterosexual transmission has previously shown an association of HLA discordance and protection from infection, although Plummer and colleagues (Plummer *et al.* (1993)) reported in a study of Nairobi prostitutes, that those with rare HLA types for the area, which would probably have had a high mismatch as defined in this study, had a reduced risk of HIV infection. Anti-lymphocyte antibodies have been detected in women immunised with their husband's cells as treatment for recurrent spontaneous abortions. When investigated their serum had *in vitro* anti-HIV activity in a complement independent manner, suggesting direct virolysis (Beretta *et al.* (1996)).

Further study of the Edinburgh cohort would further confirm the effect of HLA concordance between couples on HIV transmission, including trying to gain more complete typing to increase numbers and also extend to further HLA loci such as Cw, DQ and even the TAP alleles. However, the strong association, even with the limited numbers, provides clear evidence of a potential protective mechanism from heterosexual HIV transmission and support for the investigation of anti-HLA vaccine strategy (Shearer, Clerici and Dalgleish (1993)).

4.4.3 Chemokine Receptor Polymorphisms.

4.4.3.a CCR-5

An overall frequency for the mutant CCR-5 allele of 14% (9-23%) was obtained, which is higher than that previously published for Continental European Caucasian populations (9.2%, n=704 and 9.8%, n=122) (Samson *et al.* (1996b); Liu *et al.* (1996)) and significantly higher than the 8% found in an American population (n=637) (Huang *et al.* (1996)). The global distribution of the $\Delta 32$ allele is now known (Martinson *et al.* (1997)) and appears to decrease from Iceland south-easterly across Europe, to lower frequencies in the Middle East. It is possible that, although the American sample was of Caucasians, the allele frequency was reduced in this population due to racial admixture. Africans, along with several other non-Caucasian races studied (Huang *et al.* (1996); Liu *et al.* (1996); Samson *et al.* (1996b)) lack the $\Delta 32$ mutation in CCR-5 and when racial mixing occurs, as may have occurred in the United States, the frequency of such a mutation is reduced in the total population. The subsequent departure from Hardy-Weinberg Equilibrium, seen in the US population (Huang *et al.* (1996)) is probably due to admixture, a process known as the Wahlund effect (Hartl and Clark (1997)).

Previous results established a substantial protective effect of homozygosity for the CCR-5 mutant allele from HIV-1 infection following homosexual contact (Dean *et al.* (1996); Huang *et al.* (1996)). However, reports of several $\Delta 32/\Delta 32$ HIV⁺ individuals now exist (Balotta *et al.* (1997));

Biti *et al.* (1997); O'Brien *et al.* (1997); Theodorou *et al.* (1997)), but they are extremely rare and may involve infection with a virus dependent on CXCR-4 usage. We also only found $\Delta 32/\Delta 32$ individuals in our HIV-negative groups, but we did not observe the departure from Hardy-Weinberg predicted frequencies seen in EU homosexuals (Huang *et al.* (1996)), possibly because of a lower sample size. However, due to the increased occurrence of the deletion in the Scottish population, it would have been more likely that $\Delta 32/\Delta 32$ individuals were exposed and hence a greater chance of seeing an effect.

While individuals heterozygous for the $\Delta 32$ in *CCR-5* have been shown to have a slower rate of progression in several US cohorts (Dean *et al.* (1996); Eugen-Olsen *et al.* (1997); Meyer *et al.* (1997); Stewart *et al.* (1997)), no clear effect of heterozygosity on transmission risk has been seen in homosexuals, or haemophiliacs, despite an early report of a reduced frequency of heterozygotes in HIV⁺ individuals (Samson *et al.* (1996b)). Recently, Hoffman *et al.* (Hoffman *et al.* (1997)) reported from a small scale study that heterosexual (n=29), but not homosexual couples (n=25) showed an increase in WT/ $\Delta 32$ heterozygotes in the exposed uninfected partners versus the HIV⁺ partners. Our study group is substantially larger and we have found no significant difference in the *CCR-5* $\Delta 32$ genotype distribution of our heterosexually exposed uninfecteds compared to heterosexually infected individuals. Hoffman and colleagues did not compare equally exposed cohorts, as some of their HIV⁺ subjects became infected by other modes of transmission, thus they were not able to directly assess the effect of heterozygosity on heterosexually acquired infection.

In order to address the effects of heterozygosity at the *CCR-5* locus on infection more sensitively, the distribution of WT/ $\Delta 32$ heterozygotes was analysed with regard to the level of 'at risk' exposure, as it has been shown that increased exposure confers to an enhanced risk of infection in this cohort (Fielding *et al.* (1995); Robertson *et al.* (1998)). There was no evidence for any increase in the mean exposure accumulated for WT/ $\Delta 32$ heterozygous EUs. Indeed, the highest levels of exposures (>1000 unprotected contacts) were seen in individuals who were wild-type at the *CCR-5* locus. Thus heterozygosity for the $\Delta 32$ mutant *CCR-5* does not appear to provide significant protection against heterosexual HIV transmission in this study. As the frequency of homozygotes for the mutant *CCR-5* does not appear to be greater than 1% in any Caucasian population and it is absent from non-Caucasian populations, the $\Delta 32$ mutation may not therefore contribute substantially to variation in susceptibility to heterosexual infection.

The clear effect of the $\Delta 32$ mutation on homosexual transmission, but not on heterosexually acquired infection may result from differences in infection in these two risk

groups. Both involve transmission to a mucosal surface, but the mucosa at the two sites, vaginal and rectal, have been reported to be different. The vaginal epithelium has been shown to contain Langerhans' cells (LCs), but these were absent from the rectal epithelium (Hussain and Lehner (1995)). In monkey studies, using intravaginal inoculation of SIV, the first cells to be infected are dendritic in nature and most likely are LCs (Spira *et al.* (1996)). The primary cells infected via rectal exposure are most likely macrophages and T cells. Anal sex is also more likely to involve trauma and hence exposure to blood, which may again alter the cell type infected following exposure .

It is possible that there are differences in co-receptor usage by the virus infecting the different cells and the level of expression may also differ in different cell types and tissues. *In vitro* studies have shown that dendritic cells (DCs) do express CCR-5 and CCR-3, but not CXCR-4 (Rubbart *et al.* (1998)). However, the culturing process may change the expression patterns and this may therefore not reflect the *in vivo* expression. This was supported by a study of infection of DCs with viruses of different tropisms (Granelli-Piperno *et al.* (1998)). Immature DCs, for example LCs, were only able to support M-tropic viral infection, but mature DCs were infectible with M-tropic and T-tropic virus. The inferred difference in CCR expression in the latter study was confirmed by a recent study of the CCR expression on epidermal LCs (Zaitseva *et al.* (1997)). The freshly isolated cells, which resembled resident mucosal LCs, only expressed CCR-5, where as cultured cells expressed both CCR-5 and CXCR-4.

It is possible that other, as yet unknown, receptors maybe more important in LC infection, as suggested by Ruppert *et al.* (1998). They showed that although DCs did not express CXCR-4, they were still sensitive to SDF-1 and that DCs from $\Delta 32/\Delta 32$ individuals were still infectible with M-tropic virus, which was sensitive to SDF-1 inhibition. This infers than a non-CXCR-4 SDF receptor may be present in DCs and important for HIV infection. Despite potentially different mechanisms, most primary infections involve a macrophage tropic, or NSI, non-TCLA strain of virus and which continue to be abundant in most individuals throughout infection (Zhu *et al.* (1993); Zhang *et al.* (1993)), but the co-receptor requirements still may differ. For example, while CCR-2 and CCR-3 have been shown to act as co-receptors in only a few *in vitro* systems (Choe *et al.* (1996); Doranz *et al.* (1996); Frade *et al.* (1997)), it is possible that they may be involved *in vivo*.

To determine if other mutations in *CCR-5* existed in this cohort, which would account for the lack of infection, five individuals with the highest exposure were chosen and the entire

1.1kb coding region of the *CCR-5* gene was sequenced. All four WT/WT individuals had amino acid and nucleotide sequences identical to the published consensus and the WT/ Δ 32 heterozygote had a mixed sequence as expected. This confirmed that no further mutations, such as the recently reported point mutation (T→A, position 303) (Quillent *et al.* (1998)), or other rarer ones (Ansari-Lari *et al.* (1998)), were involved in the lack of infection in these four individuals.

It is possible that polymorphisms in the recently described promoter region of the receptor (Mummidi *et al.* (1997); Guignard *et al.* (1998)), upstream of the region sequenced, may be present in the EUs and several have been reported (Mummidi *et al.* (1997); Kostrikis *et al.* (1998); Mummidi *et al.* (1998)) and have been shown to effect progression in HIV⁺ individuals. Some of these mutations were screened in this cohort and will be discussed later (see Section 4.4.3.b).

Such mutations may effect the level of receptor expression, which has been shown to be important in infection (Moore (1997)). Wu and colleagues (1997b) showed that the level of *CCR-5* receptor expression varies widely between individuals and heterozygotes for the Δ 32 deletion had reduced expression. Low *CCR-5* expression then correlated with reduced infectability of T cells with M-tropic HIV in *in vitro* studies. It is therefore possible that the EUs have altered expression levels of co-receptor, either due to promoter polymorphisms, or differences in the factors which regulate and induce expression and this may then account for their apparent lack of infection. This was recently confirmed by Paxton *et al.* (1998), who showed that WT/WT EUs had reduced *CCR-5* expression and increased susceptibility to the β -chemokines, known to have HIV suppressive properties. Similar studies of the level of *CCR-5* expression in the Edinburgh cohort, to determine if this is also involved in heterosexual transmission, would be of value.

The choice of cell type for such studies remains a subject of debate. For practical purposes, PBMCs would be an obvious choice, but resting T cells do not express measurable level of receptor, this requires mitogenic and IL-2 activation (Bleul *et al.* (1997)) and resting T cells can not be infected. Is this then a true reflection of *in vivo* expression? The cells under such stimuli would most likely be producing maximal levels and may mask any true natural difference that may occur physiologically. One could also question the relevance of PBMCs when trying to look at heterosexually acquired infection, as these are most likely not the primary cell type infected (Spira *et al.* (1996)). STDs are a major risk factor for infection (Greenblatt *et al.* (1988); Laga *et al.* (1993)) and most likely involve recruitment of T cells

and could therefore explain the enhanced risk. However, the level of STDs was low in the Edinburgh Study (Fielding *et al.* (1995)), probably due to the low rates of partner change and therefore T cells are not likely to be the primary infected cell type in this cohort.

Dendritic cells are difficult to culture, with methods varying widely and are likely to influence the expression of receptors. As in most cases such compromises are probably necessary to unravel the answers and animal models can help clarify and confirm the findings.

An extensive study recently described by Zhang and colleagues (1998) provided further understanding into the *in vivo* expression of the chemokine receptors, CXCR-4, CCR-3 and CCR-5. They showed evidence to support the theory that the receptors may be differentially regulated both in different tissues and different cell types within the same tissues. In support of the lack of association of $\Delta 32$ in CCR-5 in heterosexually compared to homosexually acquired infection, they saw no CCR-5⁺ cells in the vagina and very few in the cervix (T lymphocytes), but CCR-5⁺ cells were frequently identified in the rectum (T lymphocytes) and colon (T lymphocytes and macrophages). Perhaps other receptors, such as CCR-3, or others not screened in this study, are therefore utilised as co-receptors following heterosexual HIV transmission.

4.4.3.a.i Indexes

Despite an increased number of WT/ $\Delta 32$ heterozygotes in the NTRIs, no statistical difference was seen from TRIs. This probably reflects the limited sample size studied here, but was an attempt to see if the affect seen on progression (Dean *et al.* (1996); Eugen-Olsen *et al.* (1997); Meyer *et al.* (1997); Stewart *et al.* (1997)) was mirrored in transmission. If the difference was supported in a larger study, it may highlight a potential correlation of the affect of $\Delta 32$ on viral load and progression (Meyer *et al.* (1997)) and the effect of viral load on transmission (Fiore *et al.* (1997)). The viral load in these individuals could not be assessed due to the lack of suitable samples, but a low CD4 count in the index was found to correlate with increased risk of HIV transmission in this study (Fielding *et al.* (1995)).

The mechanism by which heterozygosity for $\Delta 32$ may effect viral load and progression is unresolved, but probably involves the kinetics of replication, particularly following primary infection. If heterozygosity for $\Delta 32$ reduces the receptor level expression on average per cell (Wu *et al.* (1997b); Paxton *et al.* (1998)) and thus reducing the probability of infection per cell, it would be expected to have profound effects on the resulting viral load and

hence potential 'seeding' of the individual prior to immune control (Phillips (1996)). A lower viral load in primary infection has been seen as a significant indicator of an extended survival time and lack of progression (Ruiz *et al.* (1996)). A reduced viral load has also been seen to reduce the risk of transmission (Fiore *et al.* (1997)), which might lead to the expectation that the $\Delta 32$ heterozygotes would be less infectious, on average.

This was supported by a study of the humanised mouse, whereby human lymphocytes are adoptively transferred to a mice lacking it's own, forming a mouse with a human T cell system. Mice derived from T cells of WT/ $\Delta 32$ heterozygotes showed delayed replication of M-tropic HIV compared to mice derived from WT/WT individuals (Picchio, Gulizia and Mosier (1997)). This was in contrast to only a small and varied effect seen in *in vitro* cultures of M-tropic infection of T cells from $\Delta 32$ heterozygotes compared to WT/WT cells, suggesting that the *in vivo* replication dynamics can not be inferred from *in vitro* cultures (Picchio, Gulizia and Mosier (1997)).

4.4.3.b CCR-2

A more recently discovered point mutation in CCR-2, causing a single amino acid change (V64I) has been described and shown to occur in individuals of most ethnic backgrounds: allele frequency - 9.8% Caucasians, 15.1% African Americans, 17.2% Hispanics and 25% in Asians (Smith *et al.* (1997)). The mutation has also been shown to affect the rate of progression in seroconversion cohorts (Smith *et al.* (1997); Kostrikis *et al.* (1998); Rizzardi *et al.* (1998)), but not seroprevalent cohorts (Kostrikis *et al.* (1998); Michael *et al.* (1997)). This is possibly due to the lack of recruitment of a few rapidly progressing individuals in seroprevalent cohorts, who may have died before referral to such as study. We have analysed the distribution of the CCR-2 mutation in our groups and have found a significant difference between genotypes at CCR-2 in heterosexually exposed females. In this case, a lower frequency of individuals heterozygous for the 64I mutation in CCR-2 was observed in EUs compared to heterosexually infected HIV⁺ subjects, when females only were analysed. Thus, CCR-2 64I, instead of giving protection, is acting as a risk factor for heterosexual infection of females in this cohort (RR=1.6, P<0.05).

Female-to-male transmission is less frequent than male to female (European Study Group (1992); Fielding *et al.* (1995); Giasecke *et al.* (1992)), so substantially larger numbers of male contacts would be required to detect any effect of genotype. This difference justifies the analysis of the sexes separately. The majority of contacts in this study were female and the significance of the difference between genotypes is reduced (P<0.08) when male contacts were included. The

difference in the infection risk for 64I heterozygotes was not seen in homosexual contacts either (Kostrikis *et al.* (1998); Michael *et al.* (1997); Smith *et al.* (1997)) and perhaps reflects contrasting mechanisms of transmission in these risk groups as discussed previously for CCR-5 (see 4.4.3.a).

The effect of the 64I mutation on progression and heterosexual infection is clear, but the mechanism by which it exerts its effect is not. The valine to isoleucine mutation makes the first transmembrane domain of the receptor identical to the corresponding region in CCR-5 (Smith *et al.* (1997)). This raised the possibility that this mutation makes CCR-2 more like CCR-5 and perhaps then more utilisable as a co-receptor for entry of HIV. However, *in vitro* studies have shown that the 64I mutation does not alter the receptor's properties, either as a chemokine receptor, or as an HIV co-receptor (Kostrikis *et al.* (1998); Lee *et al.* (1998)). A study of CCR-2 and CCR-5 chimeras has shown that the amino terminal domain and the first extracellular loop seem to be the critical regions involved in M-tropic HIV binding to CCR-5 (Rucker *et al.* (1996)). Chimeras containing the first transmembrane domain of CCR-5 in a CCR-2 background were utilisable by the M-tropic JR-FL, but only if the CCR-5 amino terminal was also present. Other studies (Atchison *et al.* (1996); Doranz *et al.* (1997); Kuhmann *et al.* (1997); Picard *et al.* (1997); Wu *et al.* (1997a); Dragic *et al.* (1998); Rabut *et al.* (1998)) have also showed the amino terminus and first and second extracellular loops to be the critical ones in the virus: co-receptor interaction.

It is also apparent that different strains of virus have different requirements on the region of the co-receptor they bind to, with dual tropic viruses being less tolerant to changes in CCR-5, particularly in the amino terminus (Rucker *et al.* (1996); Doranz *et al.* (1997); Picard *et al.* (1997); Wu *et al.* (1997a); Rabut *et al.* (1998)). However, although the conserved valine to isoleucine substitution occurs in a region not apparently involved in HIV binding, it is possible that such a mutation can affect the overall function, as seen in other proteins (Dawson, Morris and Latchman (1996)), possibly by altering the secondary structure. Further studies into the properties of the mutant CCR-2 receptor will help confirm, or contrast the apparent lack of effect of this mutation on its co-receptor usage by HIV.

While a direct mechanism by which a single base substitution in the transmembrane region of CCR-2 may affect transmission is not obvious, a recent discovery by Kostrikis *et al.* (1998) raised an alternative possibility. They showed that the 64I mutation in CCR-2 is in 100% linkage disequilibrium with a mutation, 59653T, in the promoter region of the CCR-5 gene, although rare exceptions have recently been shown (Mummidi *et al.* (1998)). The close association of the two receptors' genes (~17.5kb apart (Samson *et al.* (1996a))) means that mutations arising in one chromosome are unlikely to be transferred to the other by recombination. Thus if a further mutation

arises in the background of the other, then they will always be present together. A similar phenomenon is seen for HLA genes, e.g. A1B8DR3. The 64I mutation and the $\Delta 32$ mutation are never seen on the same chromosome, confirmed by the absence of double homozygous mutants (64I/64I with $\Delta 32/\Delta 32$) (Smith *et al.* (1997)).

A mutation in the *CCR-5* gene promoter region may affect the level of *CCR-5* expression, which has been seen as being important for infection (Wu *et al.* (1997b)) and clearly *CCR-5* promoter mutations could have various effects on *CCR-5* tissue expression and distribution. Thus, the *CCR-2* 64I mutation may act as a marker for this, or possibly other mutations affecting the expression of *CCR-5* in this population and explain the apparent increased risk of heterosexual infection in individuals bearing the 64I mutation.

Mummidi and colleagues (1998) recently described several mutations in the promoter region and the history of their occurrence in the human population. For example, the $\Delta 32$ mutation in *CCR-5*, being limited to Caucasians, is probably a much more recent mutation than the 64I mutation in *CCR-2*, which is present in a wide variety of different ethnic backgrounds.

Recently, some of these mutations were screened for in this cohort in collaboration with Dr. Mary Carrington. None of the three alleles seen in this cohort (P1,2,4) were found to be different in heterosexually HIV⁺ individuals compared to the EUs. However, the P2 allele, in particular the P1,P2 genotype was found to be elevated in the population controls compared to the heterosexually exposed groups. The population controls were from a study of polycystic kidney disease, which was not previously thought to be effected by *CCR-5* (Dr. Alan Wright, personal communication). Whether this genotype has any clinical significance in their disease, is completely unknown and requires further investigation of this unexpected finding. The effect of all the promoter mutations is as yet still unclear and it is possible that certain combinations may relate to different levels of *CCR-5* expression. If some related to increased, or decreased expression, they may reveal differences if grouped, but this requires further study. The 59653, or 927 C→T mutation in the *CCR-5* promoter region (Kostrikis *et al.* (1998); Mummidi *et al.* (1998)) in disequilibrium with the *CCR-2*-64I mutation has not yet been screened in for in this cohort.

The actual relevance of these polymorphisms in the *CCR-5* promoter region have yet to be clarified, in terms of the *CCR-5* expression level. Mummidi *et al.* (1998) assessed the *in vitro* *CCR-5* expression in 64I/64I individuals compared to matched controls and no difference was seen, in support of preliminary reports earlier by Kostrikis *et al.* (1998). This was recently confirmed in 64I heterozygotes (Lee *et al.* (1998)), where *CCR-5* expression was not affected, but

CXCR-4 expression was decreased. A decrease in CXCR-4 may increase the chance of interaction with CCR-5 and hence, increase the chance of infection. However, as noted earlier (Zhang *et al.* (1998)), the *in vivo* expression of the chemokine receptors can vary in different tissues and cell types and this may be masked in these *in vitro* studies of PBMCs.

Differential regulation in various cells and tissues may also explain the apparent contradiction between 64I acting as a risk factor for infection and a marker for a slower disease progression in HIV⁺s. Perhaps the LCs involved in heterosexual infection have an increased expression of CCR-5 in the presence of other stimuli present, but that T cells and macrophages are stimulated to reduce the expression. It could be that the mutation causes exhaustive expression of CCR-5 following infection and the level of CCR-5 may then be reduced via a negative feed-back mechanism.

In conclusion, the presence of the 64I alteration in CCR-2 appears to have an increased risk for infection in heterosexual transmission. This mutation appears to act as a marker for a mutation in the regulatory region of the *CCR-5* gene (Kostrikis *et al.* (1998); Mummidi *et al.* (1998)), but further understanding of the functional effects of both mutations are required before this result can be more clearly defined. However, it is possible that a mutation of this kind in the regulatory region of the *CCR-5* gene, or other mutations in the *CCR-5* gene, may alter the level and expression of CCR-5 in different tissues and cell types and hence may effect the risk of heterosexual transmission. Alternatively, the mutation may effect the expression of other co-receptors, such as the decrease in CXCR-4 seen in PBMCs (Lee *et al.* (1998)) and may increase the potential interaction with the CCR-5 receptor, hence increase the chance of infection in CCR-2-64I heterozygotes.

4.4.3.b.i Indexes

As mentioned previously, host factors acting in the index, such as the HLA type and the $\Delta 32$ mutation in *CCR-5*, have been shown to influence progression (Steel *et al.* (1988); Dean *et al.* (1996); Kaslow *et al.* (1996); McNeil *et al.* (1996); Eugen-Olsen *et al.* (1997); Meyer *et al.* (1997); Smith *et al.* (1997); Stewart *et al.* (1997); Kostrikis *et al.* (1998); Rizzarda *et al.* (1998)). More recently, a mutation in the CXCR-4 ligand, SDF, was also reported and shown to affect progression (Winkler *et al.* (1998)). The effects of the CCR-2 genotype of the HIV⁺ index patients on the probability of transmission were also then assessed. As for the $\Delta 32$ deletion in *CCR-5*, no significant effect was seen for the 64I mutation either. Although when 64I and $\Delta 32$ mutations were analysed together an additive effect was seen. This suggests that both mutations are having a

positive effect on a lack of heterosexual transmission and that perhaps the study is too small to detect this effect for each mutation, but the combined effect is more significant due to greater numbers. Larger studies would be needed to confirm this. As described earlier for *CCR-5* (see 4.4.3.a.i), this most likely reflects a more stable clinical status in the NTRIs, possibly by a stable low viral load, although this was not monitored for this cohort.

Viral strains from the Edinburgh IDUs have been shown to be closely related and fall into a single cluster within the B subtype (Leigh Brown *et al.* (1997)), indicating viral variation is unlikely to be an important factor affecting the probability of transmission in this cohort. The disease status of the transmitting index has been shown to affect the transmission probability, as discussed previously for *CCR-5* (see 4.4.3.a.i).

4.4.4 Conclusions

4.4.4.a HLA

- 1.) DR5 appears to be acting as a protective genetic factor in heterosexual HIV transmission, possibly through the DR11 split and DR6 is suggestive of a risk factor, although this was not reported in heterosexually infected HIV⁺s. These associations need confirmation through more complete HLA typing using the same methodology for all groups.
- 2.) Mismatch HLA types between heterosexual couples also appears to protect heterosexual transmission and is perhaps mediated by allogeneic responses in the contact and requires further elucidation.

4.4.4.b CCR Mutations

- 1.) The $\Delta 32$ deletion in the *CCR-5* does not seem to be involved in protection from infection in this cohort. However, the 64I mutation in *CCR-2* appears to act as a risk factor for male to female heterosexual HIV transmission, by an as yet undefined mechanism.
- 2.) Polymorphisms in the *CCR-5* promoter region also do not appear to be involved in heterosexual HIV transmission.

The work described in this chapter has been submitted in part for publication in the *Journal of Infectious Diseases* (Lockett *et al.* submitted for publication).

Chapter 5

Viral Factors

CHAPTER 5 - VIRAL FACTORS

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5.1. INTRODUCTION

Many studies have revealed differences in susceptibility to heterosexual infection for the contact, be that genetic (see Section 1.7.3), immunological (see Section 1.7.2), or due to other risk factors, such as anal sex, sexually transmitted diseases (see Section 1.3.5 and reviewed in Royce *et al.* (1997)). Similar studies have identified factors which relate to increased infectiousness of the index, such as the disease status, which appears to relate to the viral load, sexually transmitted diseases and sex during menses (female-male transmission) (see Section 1.3.5 and reviewed in Royce *et al.* (1997)). Indexes are often found to be transmitters (or Transmitting Index (TRI)), or non-transmitters (or NTRI), but the factors which govern this, other than the already mentioned properties are unclear and difficult to examine. Some studies have investigated the presence of HIV in semen and vaginal/cervical secretions (see Section 1.3.3), but these studies are often met with poor compliance and the precise relationship with transmission is poorly understood.

Molecular epidemiological studies of transmission sets (Kleim *et al.* (1991); Holmes *et al.* (1992); Wike *et al.* (1992); Wolinsky *et al.* (1992); Scarlatti *et al.* (1993); Zhang *et al.* (1993); Zhu *et al.* (1993); van't Wout *et al.* (1994); Briant *et al.* (1995); Zhu *et al.* (1995); Janini *et al.* (1998); Wade, Lobidel and Leigh Brown (1998)), have shown that the virus found in the contact is highly related to the index and while in some cases can reflect only a minor variant in the index (Wolinsky *et al.* (1992)), this is not always the case (Scarlatti *et al.* (1993)). The transmitted variant is almost always a predicted NSI variant (Roos *et al.* (1992); Zhu *et al.* (1993); van't Wout *et al.* (1994)) and only rarely have SI transmissions been seen (Roos *et al.* (1992)). It is therefore possible that as well as the amount of virus present in the index, reflected by the viral load, the type of virus present in the index may be involved in transmission. This may be of particular importance where infection is restricted to a particular cell type, such as may occur in sexual transmission (Spira *et al.* (1996); Granelli-Piperno *et al.* (1998)).

As part of a molecular epidemiological study of father-mother-child transmission, Dr. Christopher Wade (Wade (1997) PhD thesis) identified potential transmitted variants from the father, the Index. The Index had heterosexually transmitted virus to two female Contacts and they had subsequently infected their offspring. It seemed therefore that the Index was a clear 'TRI' and the viral variants seen by Dr. Wade were transmissible by both heterosexual and subsequently by vertical modes. However, during the study of the discordant contacts in the Edinburgh Heterosexual Study, two additional Contacts were found, who despite 'at risk'

exposure, remained uninfected. One of the Contacts, C3153, was a homozygote for the $\Delta 32$ mutation in the *CCR-5* gene and was therefore expected to be protected from infection with CCR-5 restricted virus (see Section 1.7.3). The final Contact was a heterozygote for the $\Delta 32$ mutation, but there is little evidence to suggest this may protect from infection (see Section 1.7.3).

The purpose of the first part of this study was to isolate virus from the Index to determine if the later uninfected Contacts were truly protected from infection with this virus. To try and elucidate whether this protection was CCR-5 restricted, other viruses with known co-receptor usage were also to be investigated. In order to determine whether other individuals in the EU cohort were also resistant to infection, it was intended that they would also be screened, where samples were available.

The second side of this study was to expand on the sequence information gained by Dr. Wade during his study, to see if the potential transmission variants were still present, or if the lack for them may account for the change in status of the Index from a TRI, to a NTRI. This would then also allow the predicted NSI/SI phenotype of the virus present at the time of exposure to the two later Contacts to be determined, to see if this also related to the lack of transmission.

5.2. METHODS

5.2.1 Background

The Index, I3151, had 4 relationships during the Heterosexual Study; two were concordant (C3151 and C3152), i.e. they became HIV⁺ following heterosexual contact with the Index and the remaining two were discordant (C3153 and C3154). Dates of relationships are detailed in Table 5.1 and the course of the Index's infection relative to the Contacts' exposure times are shown in Figure 5.1, along with the time of sampling. Detailed sequence analysis of the virus infecting the three HIV⁺ individuals (I3151/C3151/C3152) as they were investigated as part of a transmission group of father-mother-child transmission; both infected Contacts gave birth to HIV⁺ children (Wade (1997) PhD thesis and in preparation).

Genotypes for the CCR mutations are given in Table 5.1. C3153 was homozygous for the $\Delta 32$ mutation in the CCR-5 gene and C3154 was heterozygous. C3152 was wild type for both mutations, but no sample was available to test the genotype of C3151, who died in 1989. I3151 was wild type for both mutations.

Table 5.1.

Details of Index (I3151) and Heterosexual Contacts

	HIV Serostatus	DOB*	Dates of Relationship	CCR-5† Genotype	CCR-2§ Genotype
I3151	+	09.05.64	-----	WT/WT	WT/WT
C3151	+	06.05.63	12/82-08/87	unknown	unknown
C3152	+	20.12.72	12/89-06/92	WT/WT	WT/WT
C3153	-	08.04.62	02/93-06/93	Δ 32/ Δ 32	WT/WT
C3154	-	09.06.78	10/94-05/95	WT/ Δ 32	WT/WT

* Date of Birth (DOB).

† WT/WT: homozygous wild-type CCR-5 genotype; Δ 32/ Δ 32 for the homozygous mutant for the 32bp deletion in the CCR-5 gene and WT/ Δ 32 for the heterozygote.

§ WT/WT: homozygous wild-type CCR-2 genotype; 64I/64I for the homozygous mutant for the valine to isoleucine a-a change and WT/64I for the heterozygote.

^{||} Date from start of relationship to last interview. Both relationships were ongoing at the time of interview, but no follow-up was achieved and the relationships were only considered during these dates, although the actual relationship extended beyond these dates.

Figure 5.1.

Time Scale of Index and Contacts Relationships, Infection Times and Sampling Points

The diagram shows the 12 year period I3151 HIV⁺ status relating to his contacts with the four heterosexual partners, shown with a line. The relationship with C3151 commenced prior to seroconversion, shown with a dot. The year of seroconversion is shown for I3151 and C3151 and the date of discovery of seropositivity of C3152 are shown with an open arrow. The approximate dates of available samples are shown with a closed arrow and samples analysed by Dr. Wade are indicated by F (father) followed by the year after seroconversion. Later samples assessed in this study are indicated with a * and numbered 1-3. Sample 1 was obtained on the 17/09/94 (10 years post seroconversion); Sample 2 was obtained on 26/02/95 and Sample 3 was obtained on 24/07/95 (both 11 years post seroconversion).

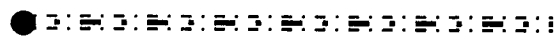
12/82

C3151

C3152

C3153

C3154



**Seroconversion of
I3151 and C3151**

C3152 HIV⁺



1984

1986

1988

1990

1992

1994

1996

↑
F0

↑
F5

↑
F6

↑
F7

↑
F8

↑
F9

↑
*1

↑
*2

↑
*3

5.2.2 Infectivity Assay

5.2.2.a Virus Isolation From Index

Several attempts were made to isolate virus from two cryopreserved samples stored in the Molecular Epidemiology Respository, Edinburgh for use in an infectivity assay. Both plasma and PBMCs were available for both samples. Early attempts involved a standard co-culture with donor PHA blasted PBMCs with the Index's cells (see Section 2.9.2.a), including attempts made in collaboration with Dr. Myra Arnott (Department of Medical Microbiology, University of Edinburgh). Further attempts, involving co-culture of PBMCs with the cell line, PM1, known to be permissive to growth of NSI and SI HIV viral isolates, were also tried (see Section 2.9.2.b).

Additional attempts at isolation were carried out using plasma and PM1 cells (see Section 2.9.2.c), CD8⁺ cell depleted PBMCs (see Section 2.9.2.c) and the U87-CD4-CCR-5 cell line with both plasma and PBMCs (see Section 2.9.2.d).

5.2.2.b Viral Stocks

High titre stocks were produced as outlined in the Material and Methods (see Section 2.9.3 and 2.9.4). The TCID₅₀ was determined in the PM1 cell line as it was reproducible and allowed TCLA and non-TCLA adapted strains to be grown in the same cell line (see Section 2.9.5). Infection was determined by p24 staining and immunofluorescent microscopy (see Section 2.10.2 and 2.8.2). Representative results for the different viruses are shown in Figure 5.2. The p24-FITC staining usually occurs just under the plasma membrane and often occurs in bright 'blobs' of concentrated staining. The SI virus shown in Figure 5.2.b shows a syncytium of two fused cells, resulting in a larger than average cell; this was only seen for the SI virus stocks. The mock infected and negative controls were repeatedly negative. Wells were scored as positive, or negative; variation in the extent of staining was not considered significant.

5.2.3.c Co-receptor Usage by Viral Strains

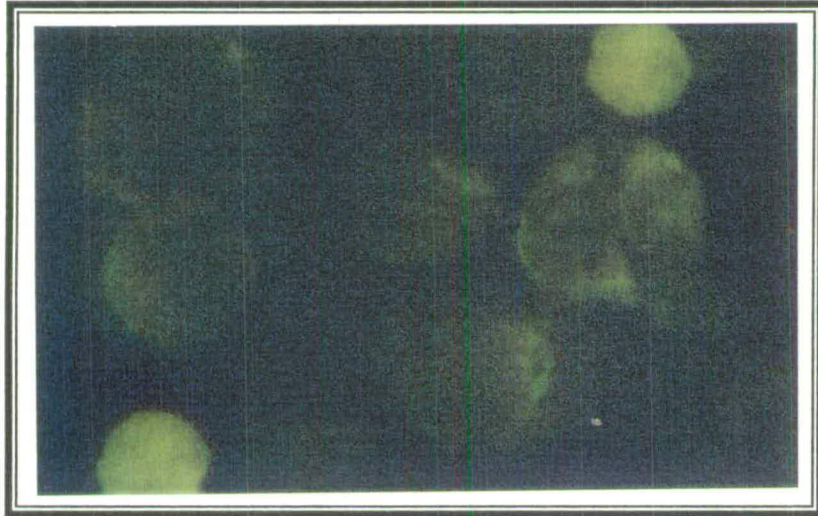
The co-receptor usage of the strains used in the infectivity assay were determined by growth the U87 cell line stably transduced with the CD4 receptor and transfected with the chemokine receptors, CCR-1/2/3/5 and CXCR-4. These were kindly obtained from Dr. Paul Clapham (Chester Beatty Laboratories, ICRF, London) via the MRC AIDS Repository, South

Figure 5.2.

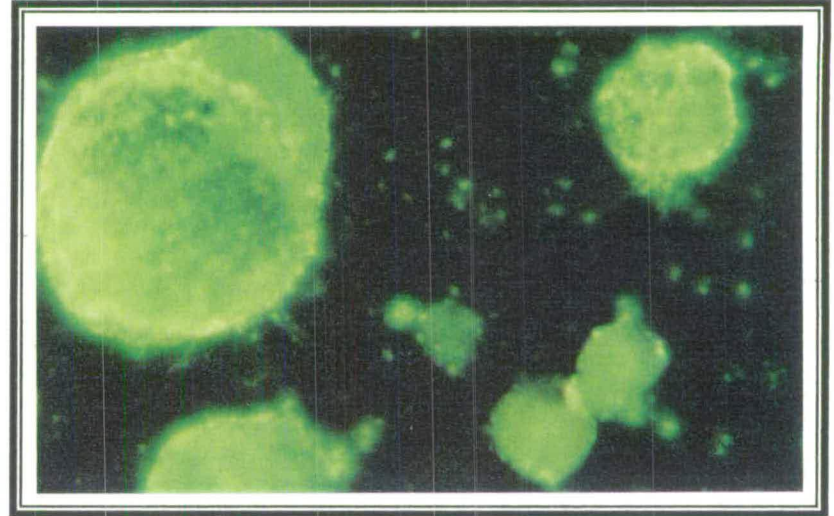
Immunofluorescent p24 Staining of PM1 Infected Cells

The following pictures show representative immunofluorescent staining of PM1 cells infected with various HIV isolates, stained with anti-p24 monoclonal antibody conjugated to the fluorochrome FITC (KC57:FITC, see Section 2.8.2). The staining was used to calculate the 50% Tissue Culture Dose (TCID₅₀) of the viral stocks for use in an infectivity assay (see Section 2.9.5 and 2.9.7).

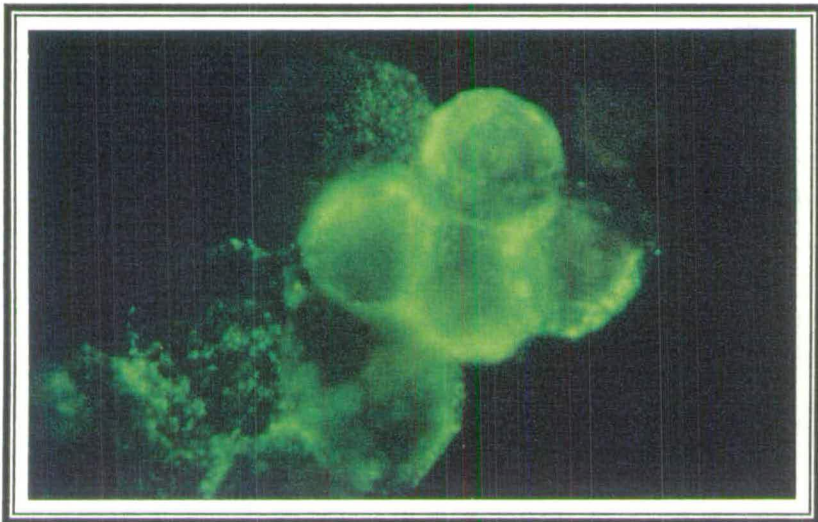
- a.) Mock infected cells to ensure no no-specific staining.
- b.) HIV_{C98} infected cells (SI isolate).
- c.) HIV_{Bal.} infected cells (NSI isolate).
- d.) HIV_{139.6b} infected cells (NSI isolate).



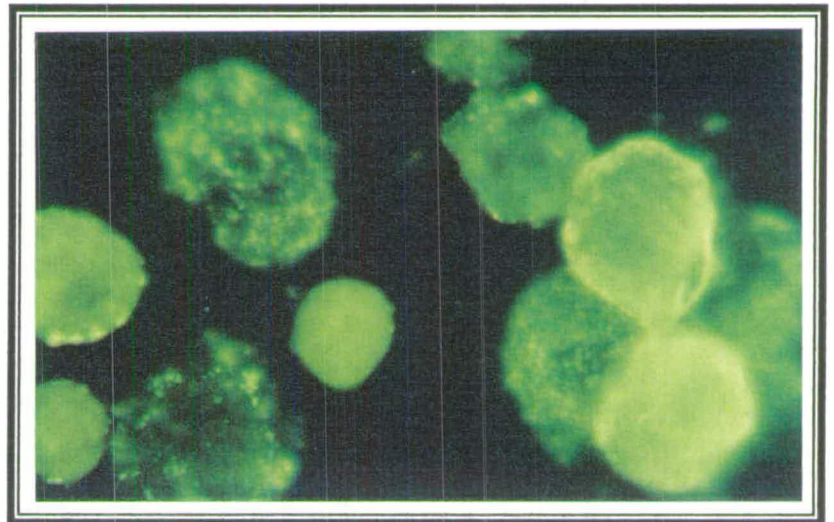
a.) Mock



b.) SI virus



c.) NSI virus



d.) NSI virus

Mimms. Further details of the cell lines and their culture can be seen in the Material and Methods (see Section 2.6.6).

Cells were infected and performed in duplicate (see Section 2.9.6) and the values obtained were consistent for each replicate. Culture supernatant was stored and p24 antigen detected by ELISA (see Section 2.10.2).

5.2.2.d PBMC Samples for infection

22 PBMC samples from EUs were obtained from cryopreserved storage (16 at Medical Microbiology and 6 from storage at the Centre for HIV Research, King's Buildings). All samples were thawed and PHA stimulated and subsequently cultured with Lymphocult which contains IL-2 (see Section 2.6.3). Of the 16 samples from Medical Microbiology, none grew despite every effort. Of the remaining 6 samples, all grew successfully and were expanded for a total of 8 days of culture, following which they were CD8 depleted (see Section 2.6.4). Samples from three Donors (blood packs, see Section 2.2.3) were expanded and depleted in parallel and in duplicate as the EUs samples were performed in batches. This allowed the Donors to reflect the reproducibility of the assay. The EUs and the Donors are listed in Table 5.2 along with the genotypes for the chemokine receptor polymorphisms (see Chapter 4). The EUs were assayed across the three batches; Batch 1 - C3153; Batch 2 - C4311, C5052, C5191; Batch 3 - C5411, C5491.

5.2.2.e CD8⁺ Cell Depletion

To assess the efficiency of the CD8⁺ cell depletion, cells were stained with an anti-CD8 monoclonal antibody conjugated to the fluorochrome FITC and assessed by flow cytometry for the presence of staining (see Section 2.8.2). An irrelevantly conjugate antibody was used to assess the background fluorescence.

5.2.2.f Infectivity Assay

The infectivity assay involved infection of CD8⁺ cell depleted PHA blasted PBMCs using the following viral strains: HIV_{BaL}, HIV_{C98}, HIV_{IIB}, HIV_{139.6b} (see Section 2.9.7). All infections were performed at a m.o.i. of 0.001, equivalent to 1 TCID₅₀ unit/ 1000 cells, except HIV_{BaL} which was also performed at a m.o.i =0.01. Further details of the infection are given in the Materials and Methods (see Section 2.9.7). At various time points throughout the

Table 5.2.

CCR Genotypes of Individuals Used in Infectivity Assay.

	CCR-2 Genotype*	CCR-5 Genotype†
C3153‡	WT/WT	Δ32/Δ32
C4311	WT/WT	WT/WT
C5052	WT/WT	WT/WT
C5191	WT/WT	WT/WT
C5491	WT/WT	WT/Δ32
C5411	WT/WT	WT/WT
BCA#	WT/WT	WT/WT
BCB	WT/WT	WT/WT
BCC	WT/64I	WT/WT

* Genotype for valine to isoleucine amino acid mutation at position 64 of CCR-2 (64I); wild type homozygous (WT/WT), mutant homozygous (64I/64I) and heterozygous (WT/64I).

† Genotype for 32 base pair deletion in *CCR-5* gene (Δ32); wild type homozygous (WT/WT), mutant homozygous (Δ32/Δ32) and heterozygous (WT/Δ32).

‡ Exposed uninfected heterosexual contact of HIV⁺ index patient.

Donor (buffy coat derived).

infection, supernatant was removed and stored at -70°C and fresh culture media added. The culture supernatants were later assessed for p24 production via ELISA (see Section 2.10.2).

5.2.3 Sequence Analysis

5.2.3.a *Polymerase Chain Reaction (PCR)*

Viral RNA was extracted from plasma (see Section 2.11.2) and primer specific cDNA synthesis performed using primers HIV *gag* (p17) and *env* (V3) specific primers (see Section 2.12.3). Limit dilution nested PCR was performed to gain products from a single molecule (see Section 2.12).

5.2.3.b *Sequence Analysis*

The PCR amplified products were then sequenced using a direct solid phase automated sequencing approach for both the sense and antisense strands of *gag* and *env* (see Section 2.13). The completed sequences were processed using the STADEN package and aligned and translated using the GDE package (see Section 2.19). The sequences were then compared to those previously obtained from the Index (Wade (1997) PhD thesis) and to those of other subtype B HIV-1 isolates, including potential contaminants from cloned material from within the Centre.

5.2.3.c *V3 Loop Analysis*

The predicted NSI/SI phenotype was deduced from the predicted amino acid sequence obtained for the V3 loop by the method of Donaldson *et al.* (Donaldson *et al.* (1994)). A model to predict the CCR-5 co-receptor usage was deduced by alignment of V3 loops from isolates with known co-receptor usage and then applied to that of the V3 loop from the Index.

5.2.3.d *Phylogenetic Analysis*

Phylogenetic reconstructions were performed by the neighbour joining method, using the subtype D isolate HIV_{ELI} as an outgroup (see Section 2.19). Bootstrap resampling was calculated to assign support with 100 replicates (see Section 2.19). Mean pairwise distances for the Index were calculated within samples (intra) and between samples (inter) (see Section 2.19).

5.3. RESULTS

5.3.1 Infectivity Assay

5.3.1.a Index Virus Isolation

Several different methods were used to try and isolate virus from the Index for use in the infectivity assay (see Section 5.2.2). Initial attempts using a standard PHA blast co-culture failed on repeated attempts and even when performed in collaboration with Dr Myra Arnott (Medical Microbiology, University of Edinburgh). The SI and NSI permissive cell line, PM1, was then used in case the problems were associated with the use of PBMCs, but this also failed. Attempts to isolate virus from plasma both using PM1 and CD8 depleted PHA blasts also failed and a final attempt using the U87-CD4-CCR-5 cell line with both plasma and PBMCs failed yet again.

5.3.1.b Co-receptor usage of viral stocks

The NSI/SI phenotype of the viral stocks was known, but with the discovery of the co-receptors, it was considered important to determine which co-receptors the viruses were capable of utilising. This was particularly important with reference to the $\Delta 32/\Delta 32$ individual, whose cells may be infectible with a CCR-5 dependent virus, if it could utilise another receptor too. The results are given in Table 5.3 for each virus with each cell line. Mock infected cells were repeatedly negative.

HIV_{MB} showed weak p24 production following infection of the U87-CD4 cells alone and those expressing CCR-1 at day 5 of infection, but this was very low for this normally very virulent strain and probably represents values below physiological levels. In contrast, the replication in the cell line expressing CXCR-4 was extremely high and the p24 production was greater than that measurable by this assay, as precise values were not required a dilution series was not performed. Therefore, HIV_{MB} is primarily restricted to usage of the co-receptor CXCR-4. The other TCLA-adapted strain, HIV_{CS8} also replicated well in the cell line expressing CXCR-4, but in contrast to HIV_{MB}, replicated well in the cell line expressing CCR-5 and weakly in the cell line expressing CCR-3 too.

The M-tropic strain HIV_{BaL} replicated less virulently than the TCLA strains, as shown by reduced levels of p24 produced and was restricted to CCR-5 usage. The patient derived clone, HIV_{139.6b}, showed persistent low levels of p24 in all assays, possibly representing excessive protein compared to competent virus, which was not efficiently removed by the washing stage (see Section 2.9.6). However, as predicted by the non-TCLA phenotype, only

Table 5.3.

P24 ELISA Results following Infection of U87-CD4 Cell Lines*

HIV [†]	U87-CD4		CCR-1		CCR-2b		CCR-3		CXCR-4		CCR-5	
	D5 [†]	D8	D5	D8	D5	D8	D5	D8	D5	D8	D5	D8
III B	0.2	0.1	0.1	0.0	0.0	0.0	0.0	0.0	Max [§]	Max	0.0	0.0
C98	0.0	0.0	0.0	0.0	0.0	0.0	0.8	2.8	129.2	Max	28.9	Max
BaL	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.4	3.9
139.6b	0.3	0.3	0.4	0.3	0.2	0.1	0.2	0.2	0.2	0.1	2.2	4.1

* Results of p24 ELISA (see Section 2.10.2), following infection of U87-CD4 cell line with and without chemokine receptors (see Section 2.6.6)

[†] Strains of HIV used to infect cells at an multiplicity of infection of 0.01 (see Section 2.9.6).

[†] Values recorded as average of duplicate in ng/ml at day 5 and day 8 of infection.

[§] Greater than saturating amount of p24 for ELISA (316ng/ml).

cells expressing CCR-5 showed efficient replication and an increase of p24 over time, reflective of active replication.

5.3.1.c CD8⁺ Cell Depletion

Representative staining for the Donors before and after depletion are shown in Figure 5.3. A monoclonal antibody specific for CD4 was also used in later samples and is also shown. The depletion clearly has removed the vast majority of the CD8⁺ cells and the batch to batch and sample to sample variation was low for the Donors shown here and Donor B gave similar results. It appears that a very small proportion of CD8⁺ cells may have remained, although most of these are staining with a much lower intensity than the main population seen in the undepleted sample (intensity $>10^2$).

The EUs show a more varied efficiency of CD8 depletion, with some individuals showing good depletion (C5191, C5411 and C5491) and others showing less efficient depletion (C3153 and C4311 and C5052) (see Figure 5.4). Due to limited numbers of cells, a pre-depletion sample was only assessed for C5411 and shows that a similar number, to that seen for the Donors, of the expanded population were CD8⁺ and therefore does not mean that more cells needed depletion in this EU, but this can not be ruled out for the remaining individuals.

5.3.1.d Infectivity Assay

As a final check on the validity of the protocol, two Donors were used as controls prior to commencing the main assay (see Figure 5.5). The infection with HIV_{IMB} and HIV_{139.6b} showed a transient rise in p24 levels, followed by a dip, which rose again by day 13 (see Figure 5.5). Infection with HIV_{C98} resulted in a constant rise in infection, peaking as for HIV_{IMB} and HIV_{139.6b} at day 13 (see Figure 5.5.a). The p24 production from HIV_{BaL} infected cells was much lower than the other strains, but showed a steady rise in p24 production over the course of infection in both Donors (see Figure 5.5.b).

The two Donors showed similar replication dynamics, but different levels of infection, with Donor D showing an increased in p24 production following infection with HIV_{IMB}, HIV_{C98} and HIV_{BaL} and Donor E showing increased production for HIV_{139.6b} (see Figure 5.5.a and 5.5.b). This could reflect either variation in experiments, or individual variation from

Figure 5.3.

CD8⁺ Cell Depletion of Donor Cells

The cells were stained before and after CD8⁺ cell depletion of PHA-blasts (see Section 2.6.4) with CD8 specific monoclonal antibody conjugated to the fluorochrome FITC and analysed on a FACScan (see Section 2.8.1). The intensity of the fluorochrome and hence the CD8 staining is shown on the x-axis and the number of cells staining at the given intensity is shown on the y-axis. The intensity is compared to an irrelevantly conjugated control antibody to assess the background fluorescence (Control - shown in grey). CD8 staining is shown in green and some of the later samples were also stained with an anti-CD4 monoclonal:FITC (blue). Undepleted and post CD8 depletion staining is shown for Donor A and Donor C for the Batch 1 (A - top row) and Batch 3 (B - bottom row).

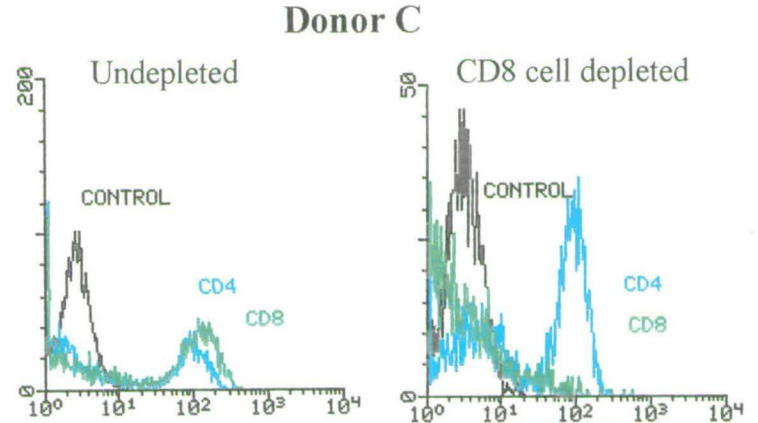
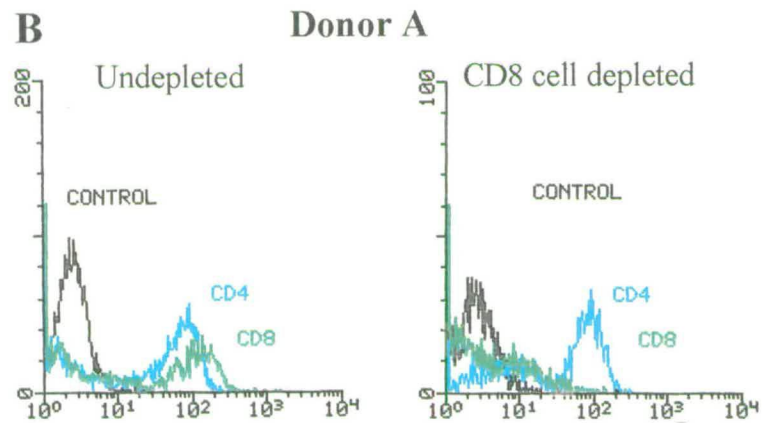
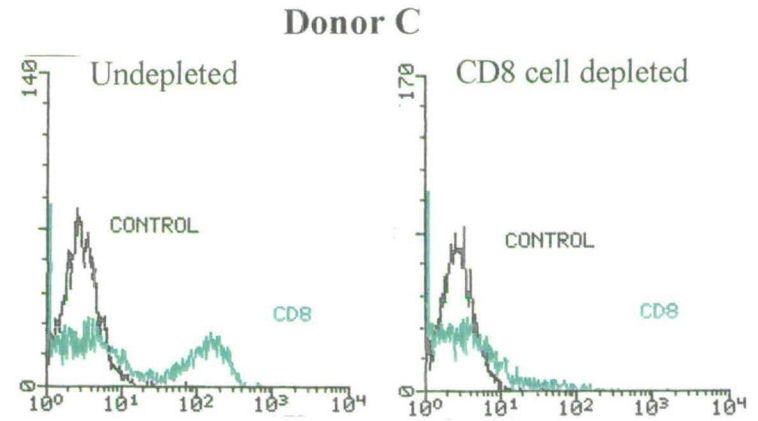
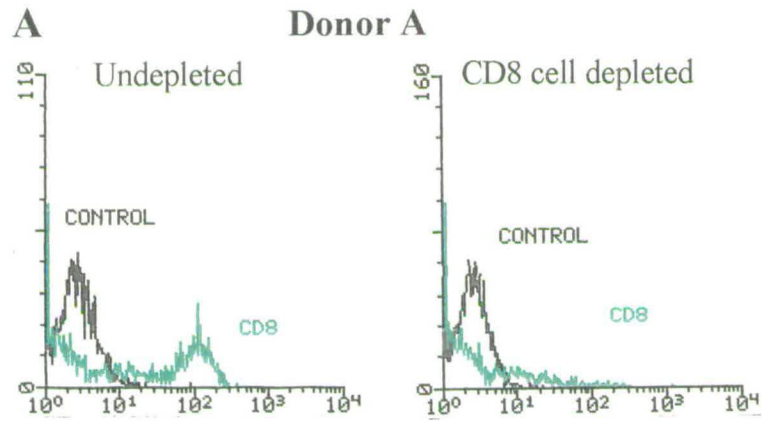


Figure 5.4.

CD8⁺ Cell Depletion of EU Cells

The cells were stained following CD8⁺ cell depletion of PHA-blasts (see Section 2.6.4) with CD8 specific monoclonal antibody conjugated to the fluorochrome FITC and analysed on a FACScan (see Section 2.8.1). The intensity of the fluorochrome and hence the CD8 staining is shown on the x-axis and the number of cells staining at the given intensity is shown on the y-axis. The intensity is compared to an irrelevantly conjugated control antibody to assess the background fluorescence (Control - shown in grey). CD8 staining is shown in green and some of the later samples were also stained with an anti-CD4 monoclonal:FITC (blue). Undepleted and post CD8 depletion staining is shown for C5411 and the remaining individuals the post-depletion staining only is given.

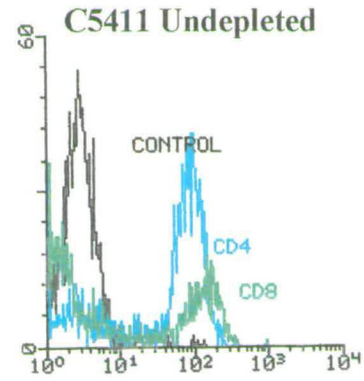
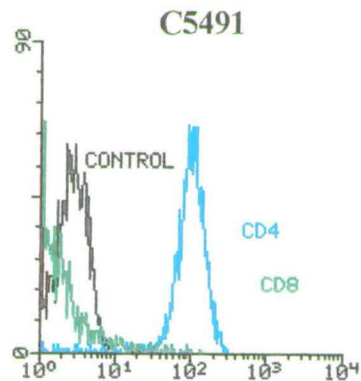
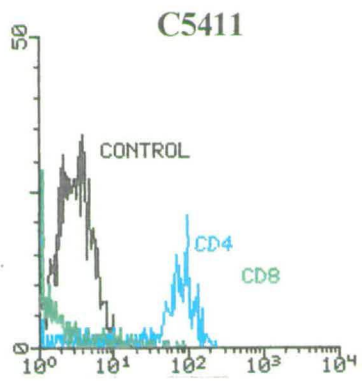
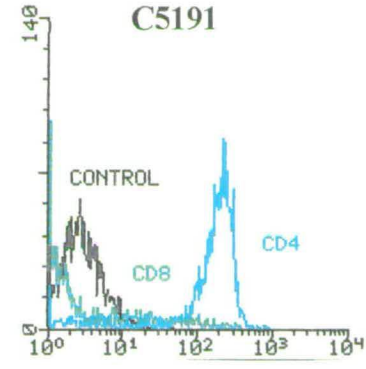
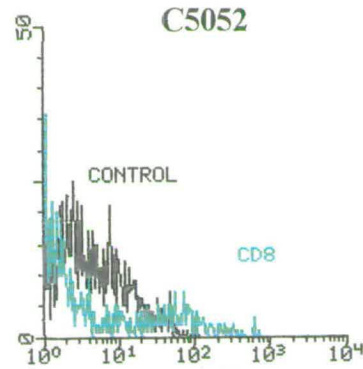
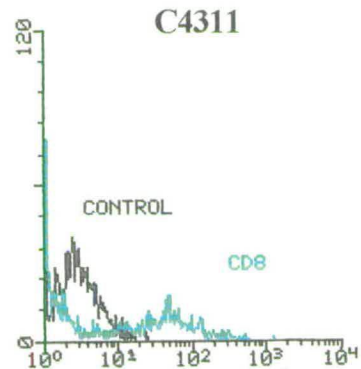
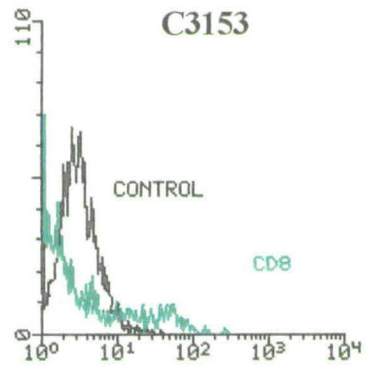


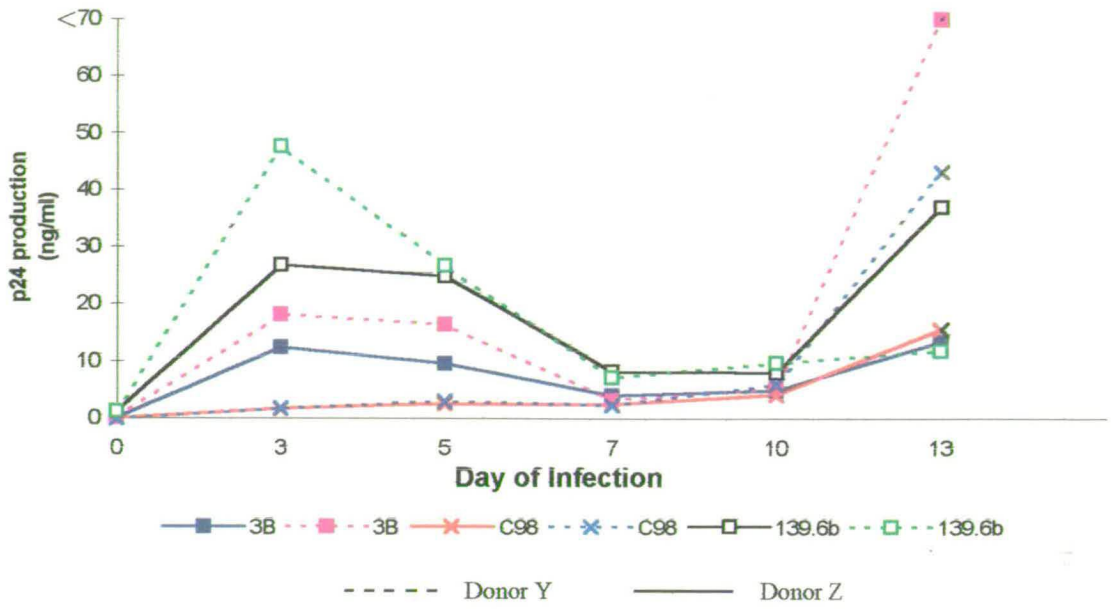
Figure 5.5.

Infectivity Assay - Control Assay

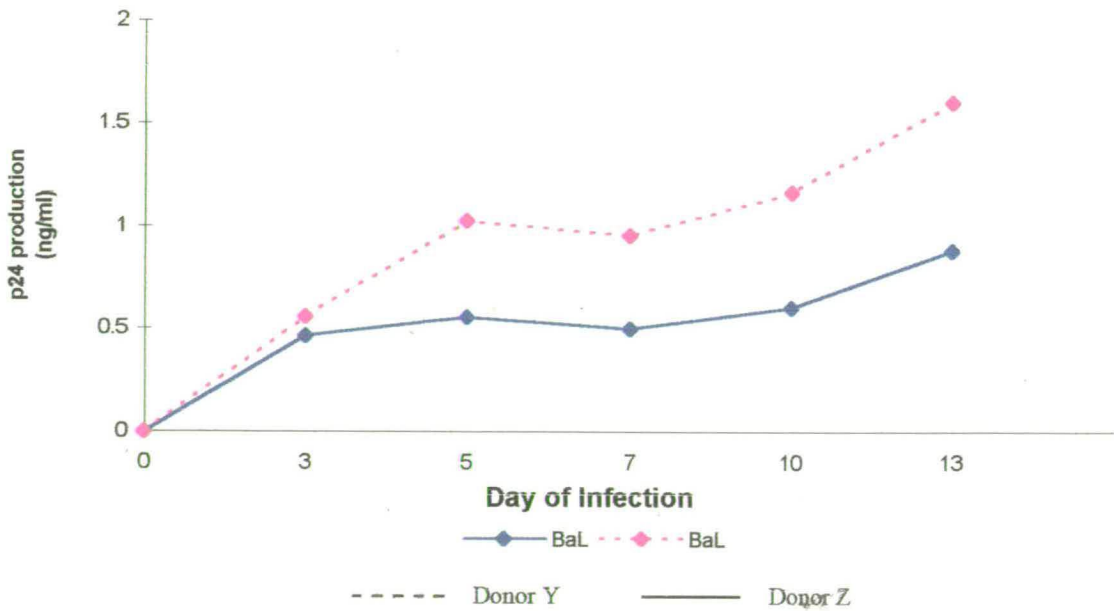
The graphs show the p24 production (ng/ml) following infection of CD8 depleted PBMCs from two donors (Donor D (dashed line) and Donor E (solid line) with different strains of HIV at a m.o.i. of 0.001 (see Section 2.9.7). This assay was performed prior to the main assay to confirm the validity of the protocol.

- a.) Shows the results for HIV_{III}B (3B), HIV_{C98} (C98) and HIV_{139.6b} (139.6b)
- b.) Shows the results for HIV_{BaL} (BaL)

A



B



person to person. However, the assay clearly resulted in measurable infection and if the EUs were resistant to infection, then this would be expected to be apparent.

The cultures by the end of the infection period were overgrown and the media appeared yellow, reflective of exhaustion, so it was decided to reduce the cell concentration from 5×10^5 to 3×10^5 , which was well within the range which would result in cell growth and viral culture. This would also increase the number of parameters able to be investigated as cell number was very limiting for the EUs.

To try and establish peak infection and to make sampling easier with multiple experiments, it was decided to sample the main assay every 3 days from day 3 until day 15. To account for variation from experiment to experiment, where sufficient cells were available following depletion, duplicates were also performed. Also, as the initial numbers of individuals to be assayed were to be fairly large, the assays were performed in three batches on three consecutive days. To rule out day to day differences, three Donors (Donor A, B and C) were used repeatedly with the first and last batch.

Finally, to assess if the $\Delta 32/\Delta 32$ homozygous individual (C3153) was truly resistant to CCR-5 restricted infection a higher m.o.i. was also performed for HIV_{BaL} (m.o.i.=0.01, as well as the standard m.o.i =0.001 used for all the other strains). The day 0 value was the average of the p24 value obtained for the inoculum used for batch one and two, as the sample from the third batch was inadvertently stored incorrectly and was therefore not assayed.

5.3.1.d.i Mock Infection

The mock infections were negative for all individuals at all times.

5.3.1.d.ii HIV_{BaL} Infection

The results are summarised in Figure 5.6 and show that the variation in infection is similar for both EUs and Donors and with, or without CD8 depletion (for the Donors). The range of responses is similar to that seen in the test assay (test assay: max - 0.9-1.6 ng/ml; main assay - 1.4ng/ml). Of those assays which showed apparent infection, most peaked at day 12 and often dipped by day 15. Some of the individuals in all groups clearly did not show productive infection, but this is unclear when all the data is presented. Figure 5.7 shows the data in the three groups: Donors undepleted/ Donors CD8 depleted and EUs depleted.

Overall the undepleted Donors did not show a vast production of p24 (see Figure 5.7.a), but a clear rise can be seen for Donor A and almost identical results are obtained on

Figure 5.6.

Infectivity Assay with HIV_{BaL} (m.o.i.=0.001)

The following graph summarises all the p24 results (ng/ml) obtained following infection with HIV_{BaL} at a multiplicity of infection (m.o.i) of 0.001 (see Section 2.9.7). For the Donors (A-C) results are presented for both undepleted PBMCs (un) and CD8 depleted PBMCs (see Section 2.6.4) and for assays performed on different days (batch 1 (1) and batch 3 (3) to assess day to day variation. All the EU samples are for CD8 depleted assays and they are identified by their Heterosexual Study number (C -contact) followed by the partner number (see Section 2.2.1.a). Where sufficient cells were available, duplicate experiments were performed and are indicated by /1 and /2 respectively. Hence DonorB3un/1 indicates Donor B, for batch 3, with undepleted cells and the first of two duplicate assays performed on the same day.

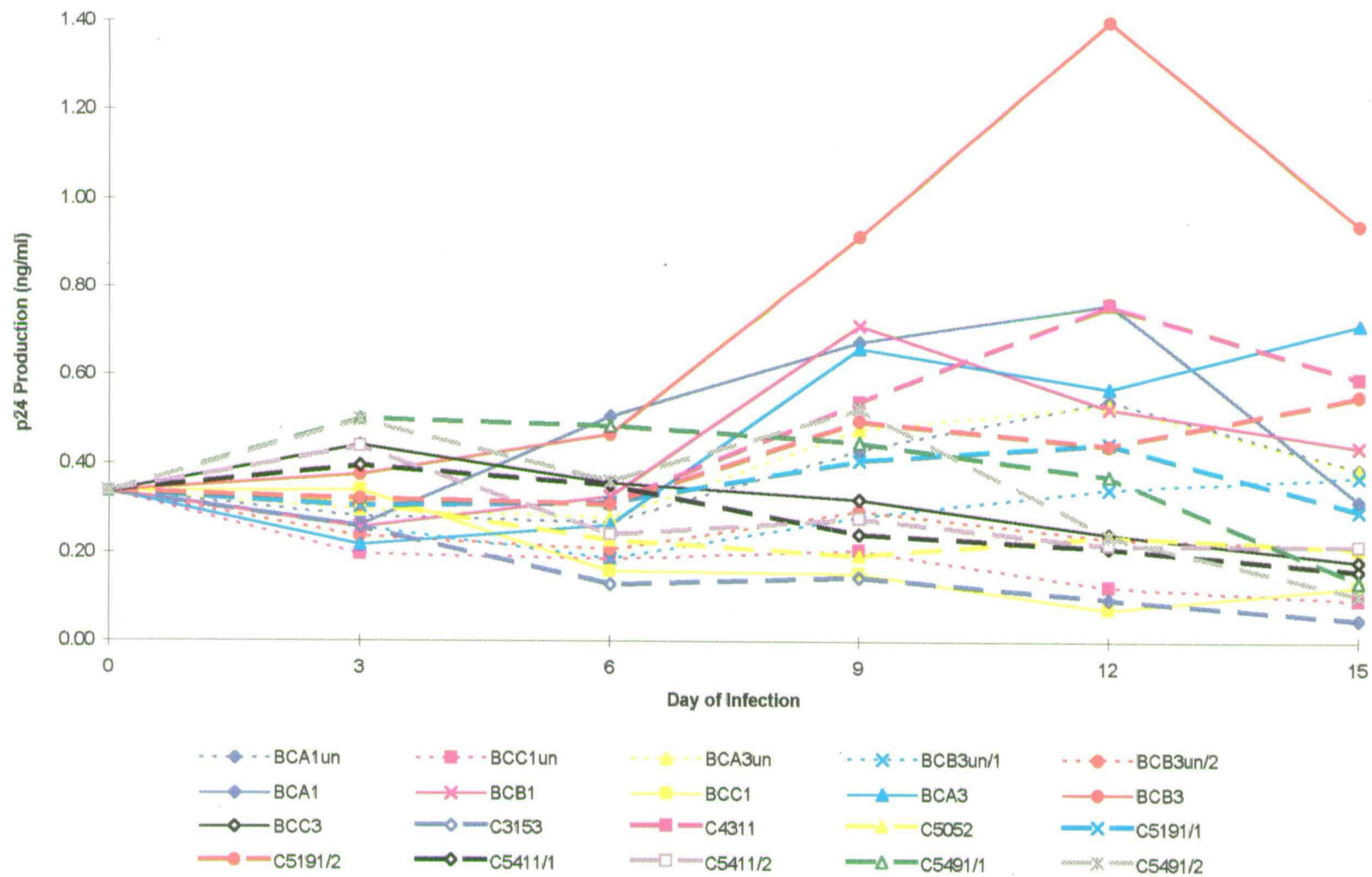
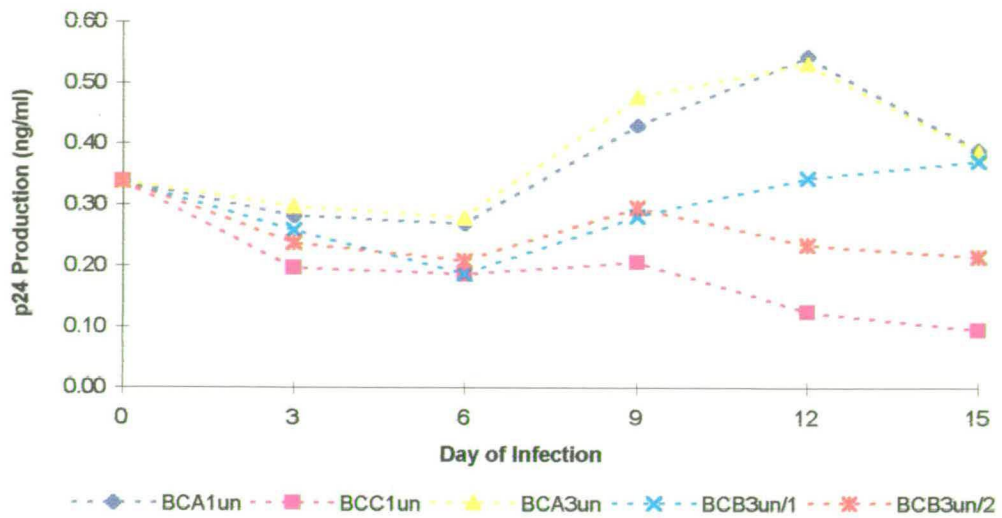
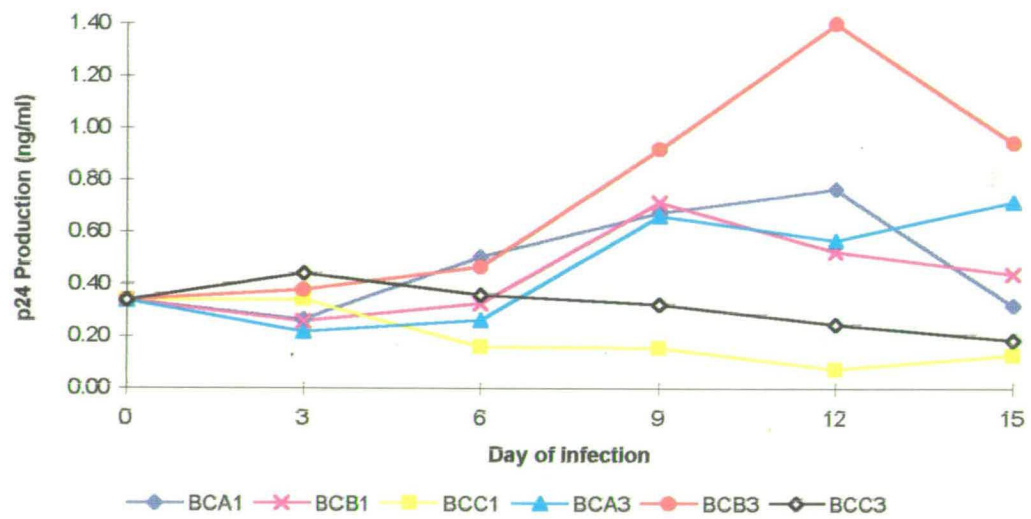
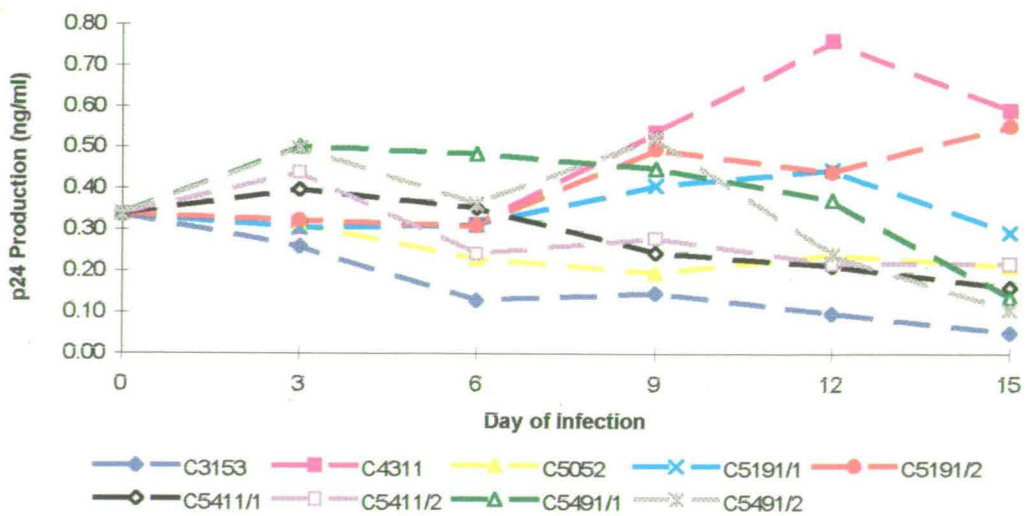


Figure 5.7.

Infectivity Assay with HIV_{BaL} (m.o.i.=0.001) - Separate Groups

The graphs show the p24 production (ng/ml) following infection with HIV_{BaL} at a multiplicity of infection (m.o.i) of 0.001 (see Section 2.9.7). For the Donors (A-C) results are presented for undepleted PBMCs (un) and CD8 depleted PBMCs (see Section 2.6.4) and for assays performed on different days (batch 1 (1) and batch 3 (3)) to assess day to day variation. All the EU samples are for CD8 depleted assays and they are identified by their Heterosexual Study number (C - contact) followed by the partner number (see Section 2.2.1.a). Where sufficient cells were available, duplicate experiments were performed and are indicated by /1 and /2 respectively. Hence DonorB3un/1 indicates Donor B, for batch 3, with undepleted cells and the first of two duplicate assays performed on the same day.

- a.) Shows the results for undepleted PBMCs (un) for Donors A-C.
- b.) Shows the results for CD8 depleted PBMCs for Donors A-C.
- c.) Shows the results for the EUs for CD8 depleted PBMCs.

A**B****C**

the two separate occasions (batch 1 and 3). Donor B gave similar results up to day 6 and then one of the duplicate experiments shows a rise in infection and the other a small drop. This perhaps shows the well to well difference which could occur, one may have more CD8 suppressor cells for instance. The final Donor, Donor C, does not appear to be infected with HIV_{BaL}, as no apparent rise in p24 production was seen over the period of infection.

For the assays where the CD8⁺ cells were depleted (see Figure 5.7.b), the Donors show a range of responses. Donor A shows an intermediate level of p24 production and the batch to batch results are similar, although not identical. Donor B gave a similar result to Donor A for batch 1 and a higher level of replication in batch 3, further indicating the presence of batch to batch variation. Donor C, as for the undepleted cells, does not show an apparent infection with HIV_{BaL}, with similar results obtained for each of the two assays.

The most obvious result for the EUs following infection with HIV_{BaL} (see Figure 5.7.c) is that the $\Delta 32/\Delta 32$ homozygote, C3153, showed no evidence of infection. C5052 and C5411 also do not appear to show evidence of productive infection. C4311 and C5191 in contrast do appear to have become infected with HIV_{BaL} and C5491 shows evidence of a low level of p24 production, as otherwise the level of p24 would continue to fall, due to the removal and addition of fresh media at each of the time points.

5.3.1.d.iii HIV_{C98} Infection

The overall level of infection with HIV_{C98}, was markedly lower than that seen in the test assay (test assay: max - 15.6-43.0 ng/ml; main assay: max - 4.3ng/ml) (see Figure 5.8 and 5.5). As for HIV_{BaL} most infections peaked at day 12 and were often starting to dip by day 15. The further obvious difference for this data, is that the majority of EUs showed enhanced infection over the Donors. This was not a batch difference as representatives from all three batches were present in this elevated set.

The undepleted Donors generally showed a poor level of infection, with this normally virulent strain (see Figure 5.9.a). Donor A showed little evidence of infection and again, as for HIV_{BaL} gave very similar results from batch to batch. Donor B showed a small rise in p24 production, although only very slightly. In contrast, Donor C which was not infected with HIV_{BaL}, showed the clearest evidence of infection with HIV_{C98}, but this was not much above the other two Donors.

Consistent with the undepleted assay, Donor A showed no apparent infection with HIV_{C98} (see Figure 5.9.b). Donors B and C did show a small rise in p24 production over the

Figure 5.8.

Infectivity Assay with HIV_{C98} (m.o.i.=0.001)

The following graph summarises all the p24 results (ng/ml) obtained following infection with HIV_{C98} at a multiplicity of infection (m.o.i) of 0.001 (see Section 2.9.7). For the Donors (A-C) results are presented for both undepleted PBMCs (un) and CD8 depleted PBMCs (see Section 2.6.4) and for assays performed on different days (batch 1 (1) and batch 3 (3) to assess day to day variation. All the EU samples are for CD8 depleted assays and they are identified by their Heterosexual Study number (C -contact) followed by the partner number (see Section 2.2.1.a). Where sufficient cells were available, duplicate experiments were performed and are indicated by /1 and /2 respectively. Hence DonorB3un/1 indicates Donor B, for batch 3, with undepleted cells and the first of two duplicate assays performed on the same day.

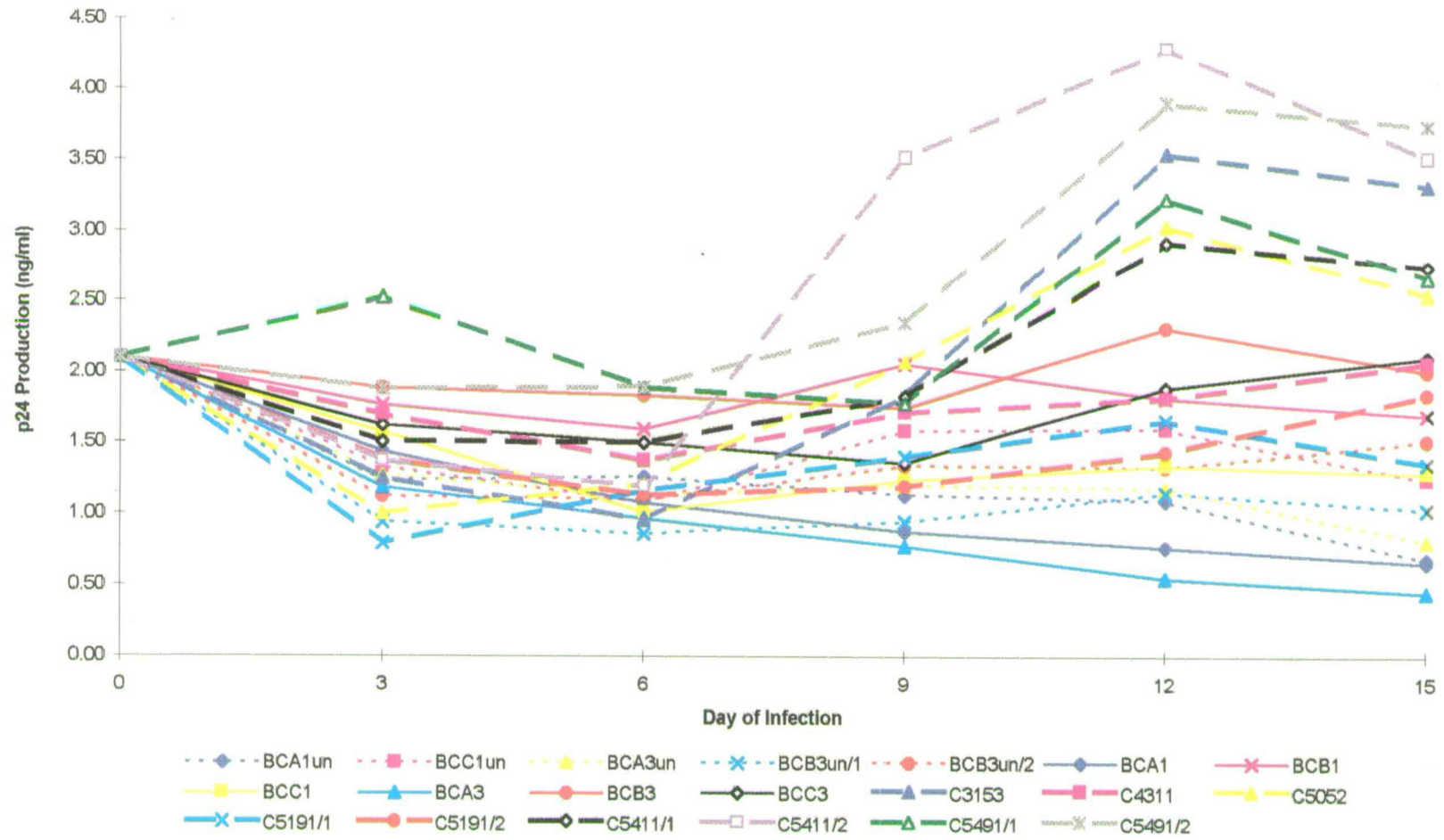
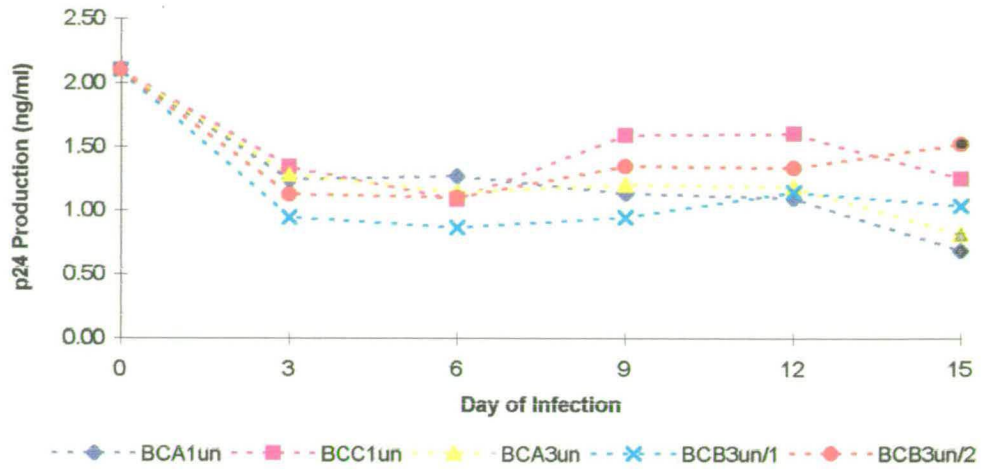
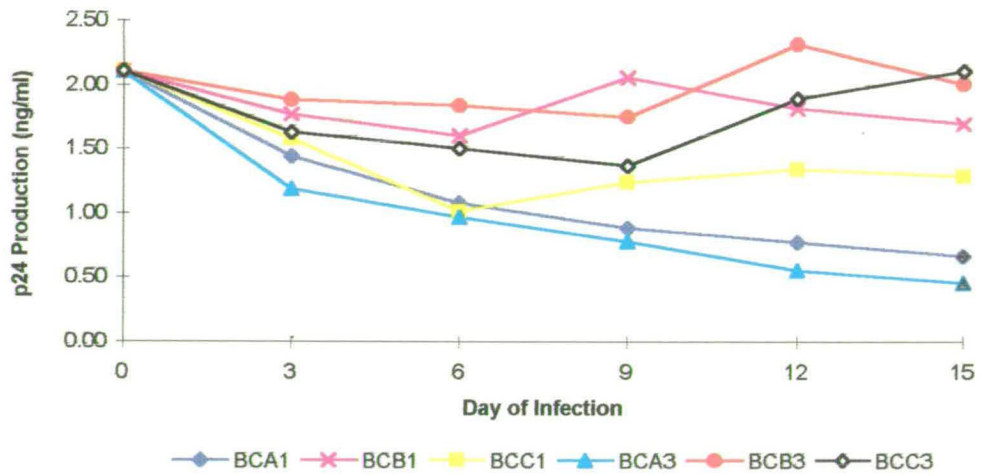
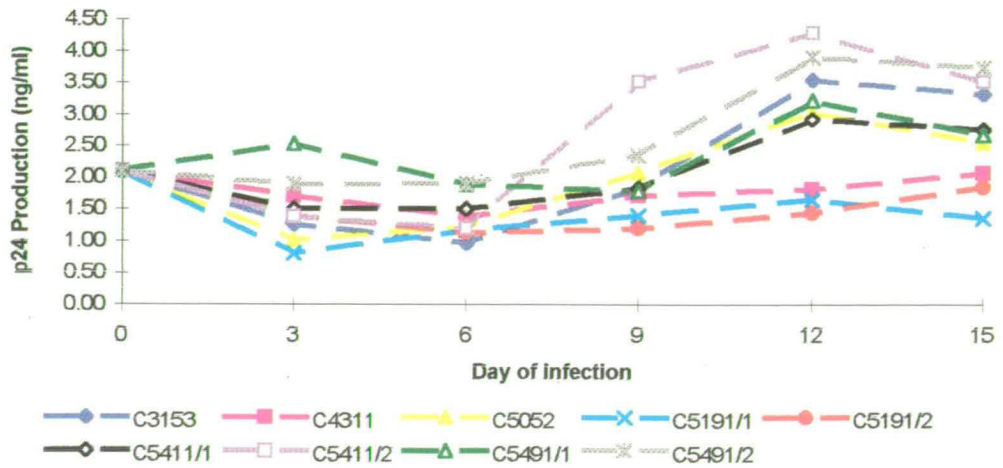


Figure 5.9.

Infectivity Assay with HIV_{CR8} (m.o.i.=0.001) - Separate Groups

The graphs show the p24 production (ng/ml) following infection with HIV_{CR8} at a multiplicity of infection (m.o.i) of 0.001 (see Section 2.9.7). For the Donors (A-C) results are presented for undepleted PBMCs (un) and CD8 depleted PBMCs (see Section 2.6.4) and for assays performed on different days (batch 1 (1) and batch 3 (3)) to assess day to day variation. All the EU samples are for CD8 depleted assays and they are identified by their Heterosexual Study number (C - contact) followed by the partner number (see Section 2.2.1.a). Where sufficient cells were available, duplicate experiments were performed and are indicated by /1 and /2 respectively. Hence DonorB3un/1 indicates Donor B, for batch 3, with undepleted cells and the first of two duplicate assays performed on the same day.

- a.) Shows the results for undepleted PBMCs (un) for Donors A-C.
- b.) Shows the results for CD8 depleted PBMCs for Donors A-C.
- c.) Shows the results for the EUs for CD8 depleted PBMCs.

A**B****C**

period of infection, but this was again, not as marked as that expected from the test assay (see Figure 5.9.b and 5.5.a).

The EUs appeared to fall into two different groups with regard to HIV_{CR8} infection (see Figure 5.9.c). One group, including: C3153, C5052, C5411 and C5491, showed a clear rise in p24 production over the course of infection and this was still well below the values recorded for the test assay (see Figure 5.5.a). The other group including: C4311 and C5191, only showed a very slight rise in p24 production, but did not show a progressive decrease expected from the lack of any p24 production, due to the sequential sampling and addition of fresh media. Hence, the second group appear to be undergoing a very low level of replication.

5.3.1.d.iv HIV_{IIIb} Infection

Only one individual assay appeared to show a persistent, although only small, rise in p24 production, after day 9 (C5411) and this was not mirrored in the repeat sample for this assay (see Figure 5.10). The high values seen early in infection for some of the assays are in fact all from the same batch (batch 3) and apart from one slight blip at day 12 for C5491/1, then all still show no evidence of productive infection. These results are in stark contrast to those of the test infection with HIV_{IIIb} (see Figure 5.4.a), which showed a large production of p24 in both of the Donors tested. This suggests a significant problem in these later assays. Analysis of the groups separately (see Figure 5.11) reflects the results shown when all the data were presented together.

5.3.1.d.v HIV_{BaL} Infection - $m.o.i.=0.01$

A higher multiplicity of infection for the CCR-5 restricted HIV_{BaL} was included to confirm the expected resistance of the $\Delta 32/\Delta 32$ individual (C3153). As for HIV_{IIIb}, some of the earlier results showed markedly higher values than other assays and again were from the third batch of assays (see Figure 5.12); although not all of the results from batch 3 behaved in this way. Apart from these outliers, the rest of the assays show similar variation between the EUs and Donors and for undepleted and depleted assays (see Figure 5.12).

The analysis of the groups separately, shows the results more clearly (see Figure 5.13). For the undepleted samples from the Donors, no apparent infection was seen (see Figure 5.13.a) and the depleted samples gave similar results, if the apparent increase at day 3 for Donor B and C for batch 3 is ignored (see Figure 5.13.b). Not surprisingly all but one of

Figure 5.10.

Infectivity Assay with HIV_{III} (m.o.i.=0.001)

The following graph summarises all the p24 results (ng/ml) obtained following infection with HIV_{III} at a multiplicity of infection (m.o.i) of 0.001 (see Section 2.9.7). For the Donors (A-C) results are presented for both undepleted PBMCs (un) and CD8 depleted PBMCs (see Section 2.6.4) and for assays performed on different days (batch 1 (1) and batch 3 (3) to assess day to day variation. All the EU samples are for CD8 depleted assays and they are identified by their Heterosexual Study number (C -contact) followed by the partner number (see Section 2.2.1.a). Where sufficient cells were available, duplicate experiments were performed and are indicated by /1 and /2 respectively. Hence DonorB3un/1 indicates Donor B, for batch 3, with undepleted cells and the first of two duplicate assays performed on the same day.

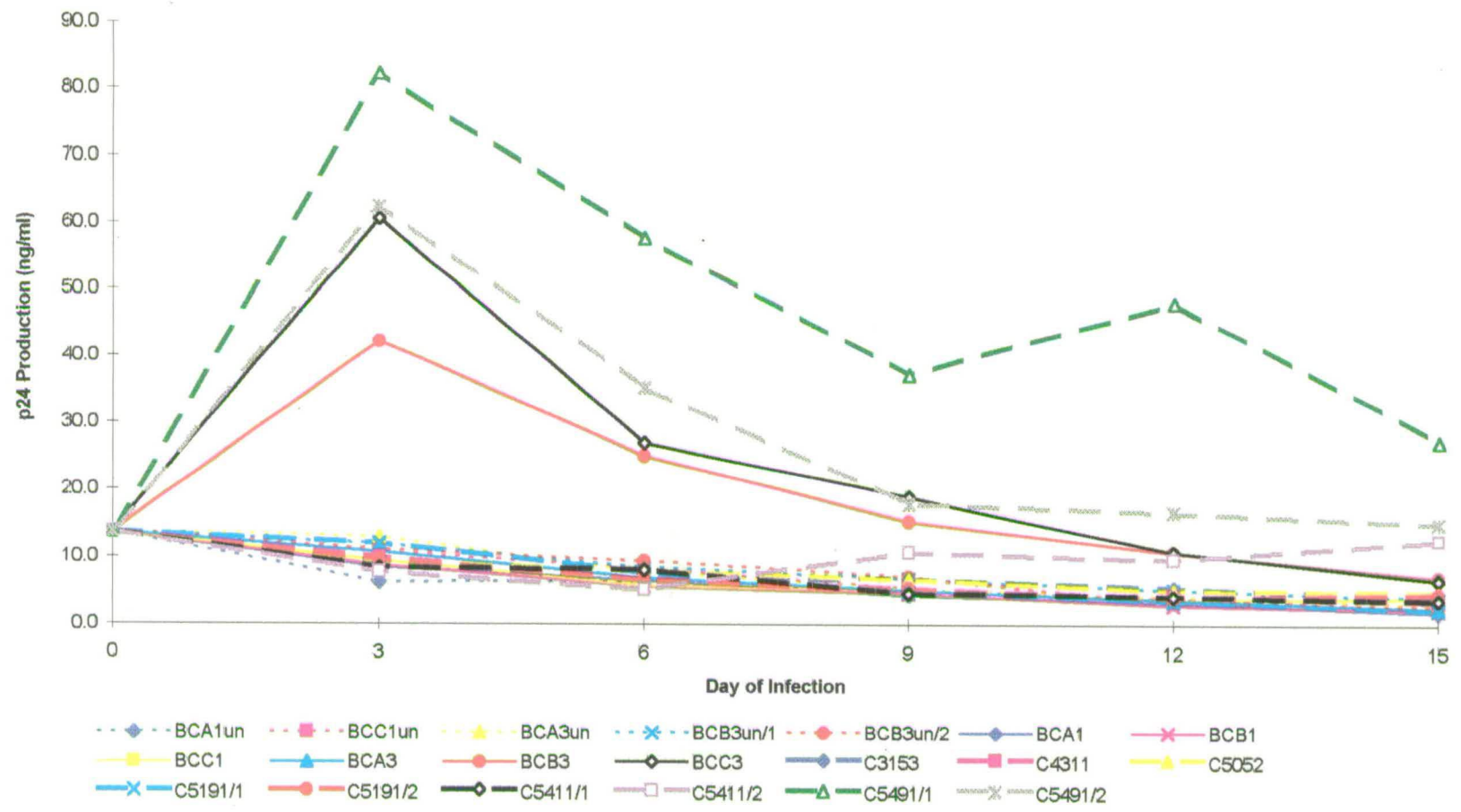


Figure 5.11.

Infectivity Assay with HIV_{III} (m.o.i.=0.001) - Separate Groups

The graphs show the p24 production (ng/ml) following infection with HIV_{III} at a multiplicity of infection (m.o.i) of 0.001 (see Section 2.9.7). For the Donors (A-C) results are presented for undepleted PBMCs (un) and CD8 depleted PBMCs (see Section 2.6.4) and for assays performed on different days (batch 1 (1) and batch 3 (3)) to assess day to day variation. All the EU samples are for CD8 depleted assays and they are identified by their Heterosexual Study number (C - contact) followed by the partner number (see Section 2.2.1.a). Where sufficient cells were available, duplicate experiments were performed and are indicated by /1 and /2 respectively. Hence DonorB3un/1 indicates Donor B, for batch 3, with undepleted cells and the first of two duplicate assays performed on the same day.

- a.) Shows the results for undepleted PBMCs (un) for Donors A-C.
- b.) Shows the results for CD8 depleted PBMCs for Donors A-C.
- c.) Shows the results for the EUs for CD8 depleted PBMCs.

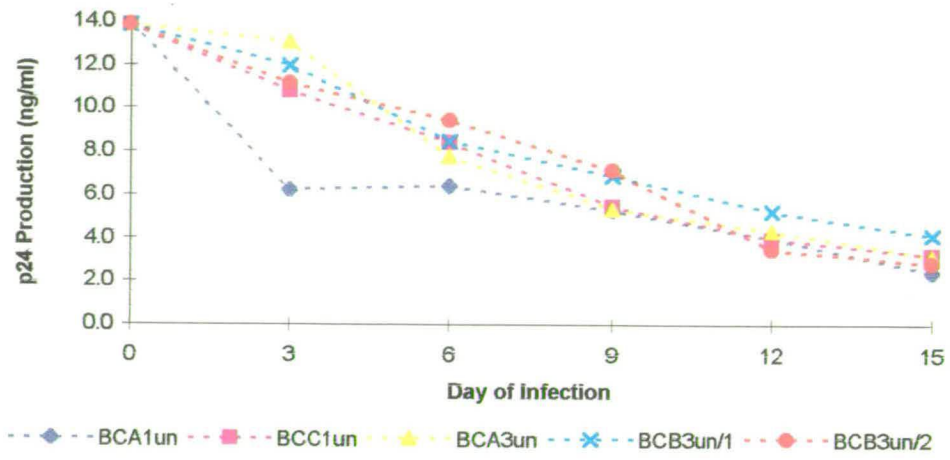
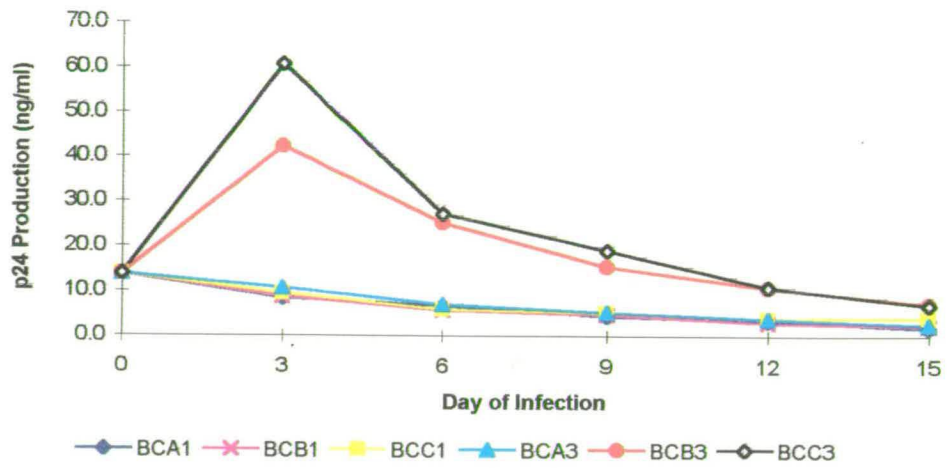
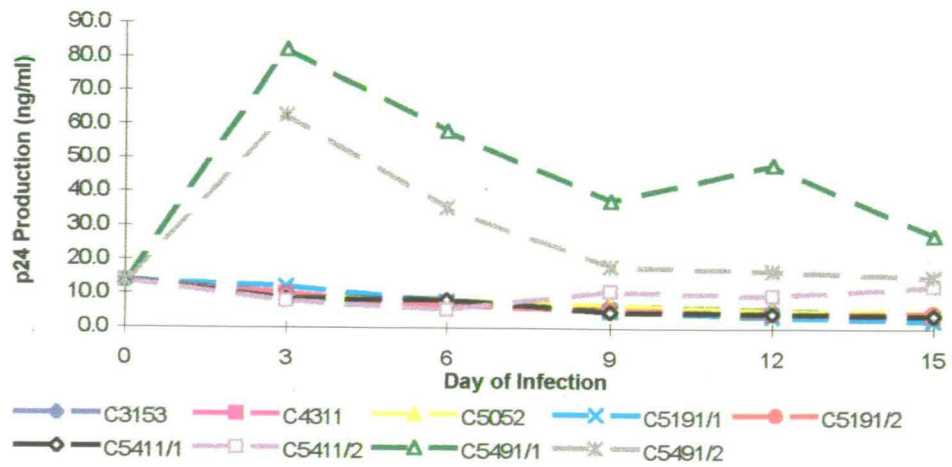
A**B****C**

Figure 5.12.

Infectivity Assay with HIV_{BaL} (m.o.i.=0.01)

The following graph summarises all the p24 results (ng/ml) obtained following infection with HIV_{BaL} at a multiplicity of infection (m.o.i) of 0.01 (see Section 2.9.7). For the Donors (A-C) results are presented for both undepleted PBMCs (un) and CD8 depleted PBMCs (see Section 2.6.4) and for assays performed on different days (batch 1 (1) and batch 3 (3) to assess day to day variation. All the EU samples are for CD8 depleted assays and they are identified by their Heterosexual Study number (C -contact) followed by the partner number (see Section 2.2.1.a). Where sufficient cells were available, duplicate experiments were performed and are indicated by /1 and /2 respectively. Hence DonorB3un/1 indicates Donor B, for batch 3, with undepleted cells and the first of two duplicate assays performed on the same day.

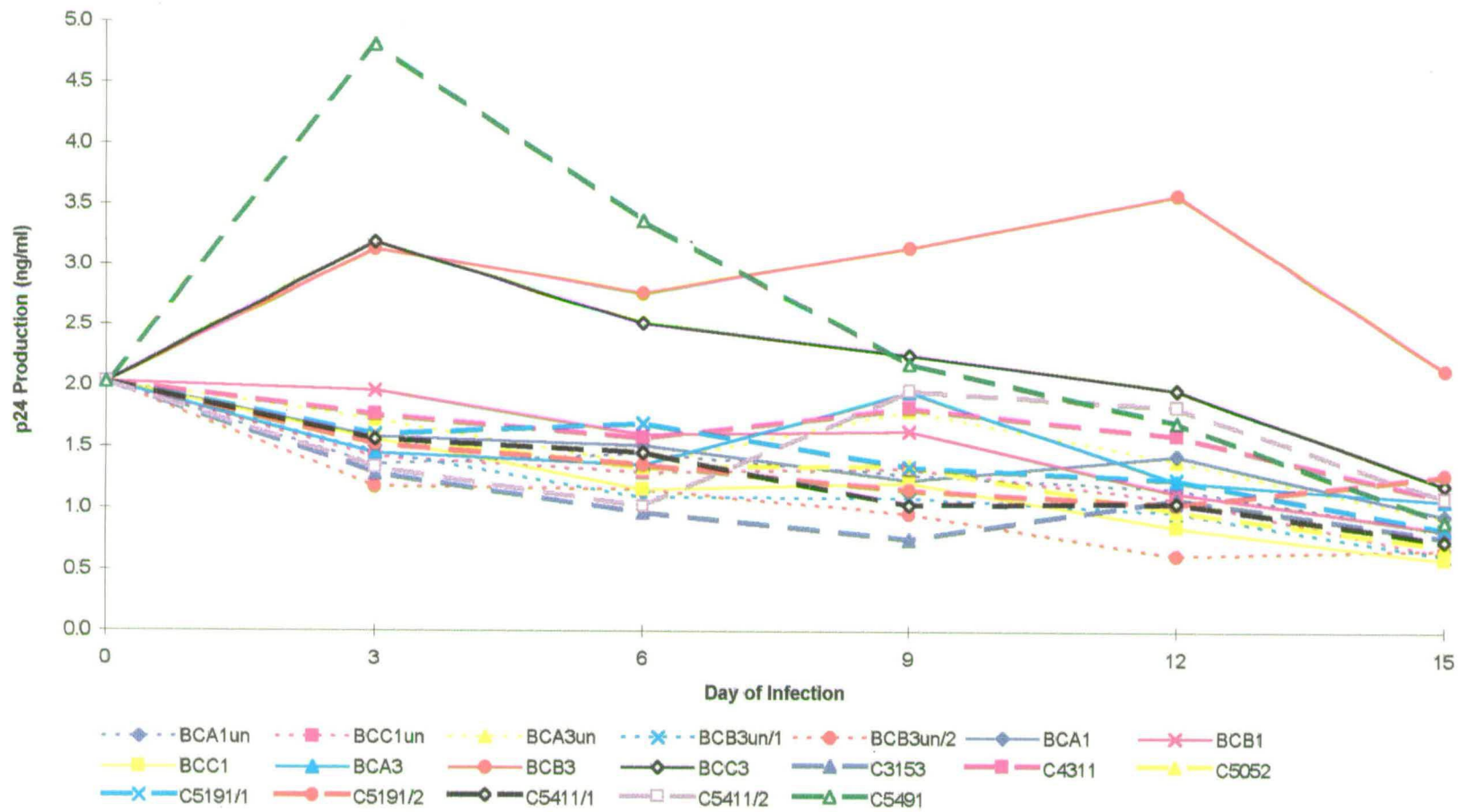
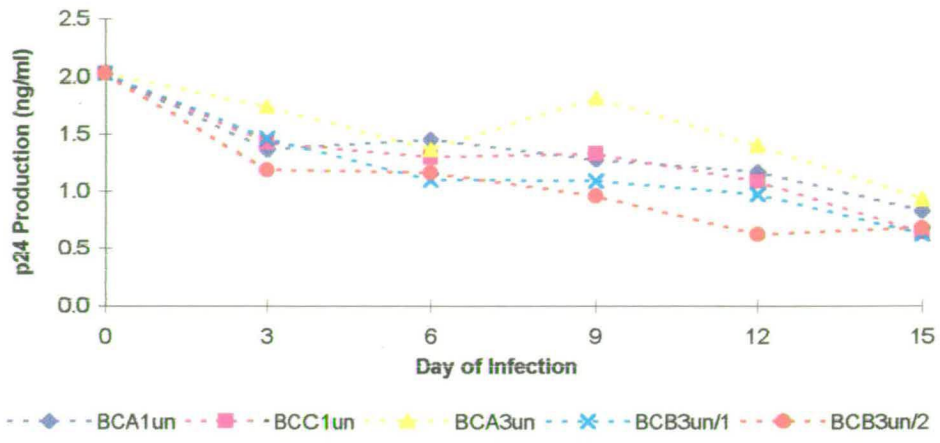
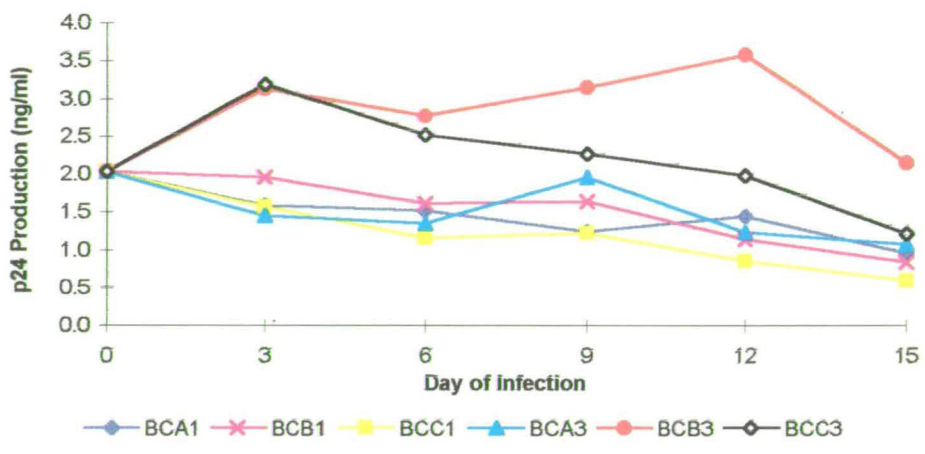
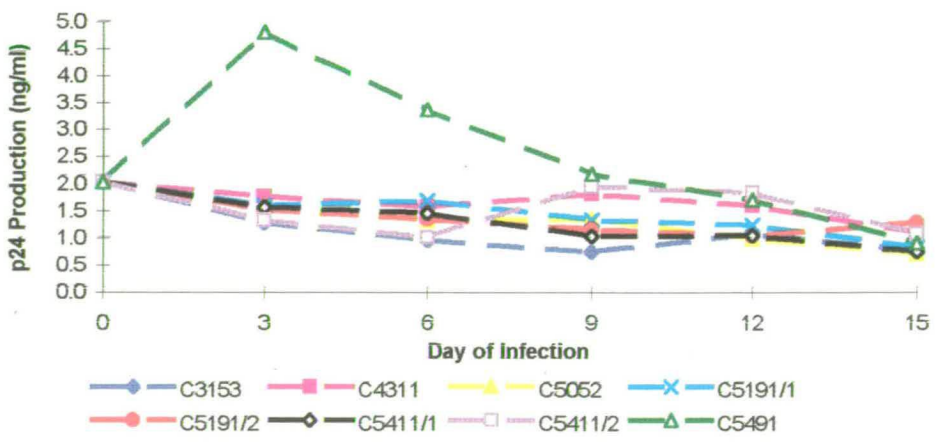


Figure 5.13.

Infectivity Assay with HIV_{BaL} (m.o.i.=0.01) - Separate Groups

The graphs show the p24 production (ng/ml) following infection with HIV_{BaL} at a multiplicity of infection (m.o.i) of 0.01 (see Section 2.9.7). For the Donors (A-C) results are presented for undepleted PBMCs (un) and CD8 depleted PBMCs (see Section 2.6.4) and for assays performed on different days (batch 1 (1) and batch 3 (3)) to assess day to day variation. All the EU samples are for CD8 depleted assays and they are identified by their Heterosexual Study number (C - contact) followed by the partner number (see Section 2.2.1.a). Where sufficient cells were available, duplicate experiments were performed and are indicated by /1 and /2 respectively. Hence DonorB3un/1 indicates Donor B, for batch 3, with undepleted cells and the first of two duplicate assays performed on the same day.

- a.) Shows the results for undepleted PBMCs (un) for Donors A-C.
- b.) Shows the results for CD8 depleted PBMCs for Donors A-C.
- c.) Shows the results for the EUs for CD8 depleted PBMCs.

A**B****C**

the assays for the EUs (C5411/2) also showed no apparent rise in p24 production and the rise in this individual was not repeated in the duplicate (see Figure 5.12.c).

5.3.1.d.vi HIV_{139.6b} Infection

Similarly unexpected results were obtained for some of the batch 3 assays following infection with HIV_{139.6b} (see Figure 5.14). However, for the assay as a whole, no real evidence of productive infection was seen for any individual, unlike that seen for the Donors assessed in the test assay (see Figure 5.5.a). This is shown more clearly when the groups are presented separately (see Figure 5.15). There were insufficient cells from C3153 to infect with this isolate.

5.3.2 Sequence Analysis of Virus Present in Index

Due to the inability to obtain virus by culture from the Index for use in the infectivity assay, it was decided to sequence the *env* gene, containing the region encoding the V3 loop. The likely NSI/SI phenotype of the virus was then ascertained, to determine if, at the times of the Contacts' exposure, the Index was infected with viral variants which would be able to infect them. This was of particular interest for C3153, who was homozygous for the $\Delta 32$ mutation in *CCR-5*. A predicted pattern of V3 amino acid sequences, which related to *CCR-5* co-receptor usage, was also constructed from published data and applied to the viral variants found in the Index, to determine if lack of *CCR-5* usage may have accounted for the lack of transmission.

In addition, due to extensive sequence data on the Index from Dr. Wade's study (Wade (1997) PhD thesis), it was possible to relate viral variants present at the times of transmission to the first two Contacts (C3151 and C3152) to later variants. This then allowed investigation of whether major change had occurred between the time when the Index did and did not transmit, to determine if this could account for the lack of transmission to the later Contacts. To support the *env* sequencing, the p17 region of the *gag* gene was also sequenced and compared in an analogous way to that of *env*. Phylogenetic analysis was performed on the sequence data to determine the degree of relatedness of the virus at the different time points and to screen for potential contamination.

The phylogenetic analysis was initially used to check for potential contamination, or mis-labelling. This revealed that the last sample (Sample 3; see Figure 5.1) was not from the Index as thought and was excluded from all other analysis of this data. A single contaminant

Figure 5.14.

Infectivity Assay with HIV_{139.6b} (m.o.i.=0.001)

The following graph summarises all the p24 results (ng/ml) obtained following infection with HIV_{139.6b} at a multiplicity of infection (m.o.i) of 0.001 (see Section 2.9.7). For the Donors (A-C) results are presented for both undepleted PBMCs (un) and CD8 depleted PBMCs (see Section 2.6.4) and for assays performed on different days (batch 1 (1) and batch 3 (3) to assess day to day variation. All the EU samples are for CD8 depleted assays and they are identified by their Heterosexual Study number (C -contact) followed by the partner number (see Section 2.2.1.a). Where sufficient cells were available, duplicate experiments were performed and are indicated by /1 and /2 respectively. Hence DonorB3un/1 indicates Donor B, for batch 3, with undepleted cells and the first of two duplicate assays performed on the same day.

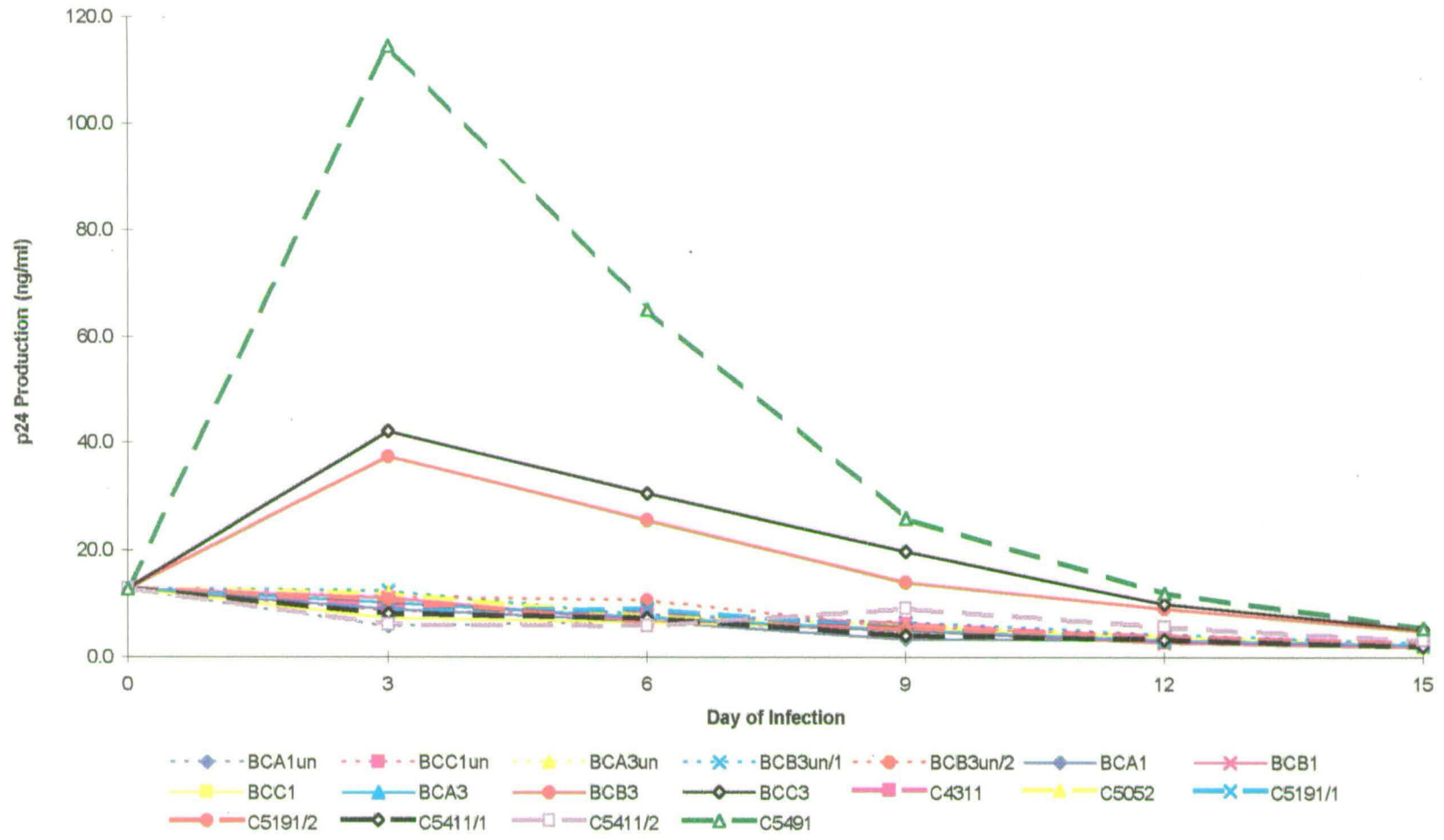
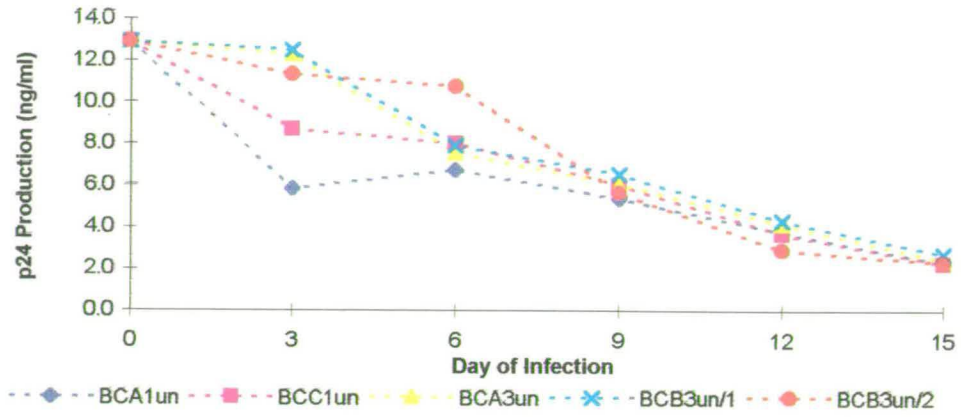
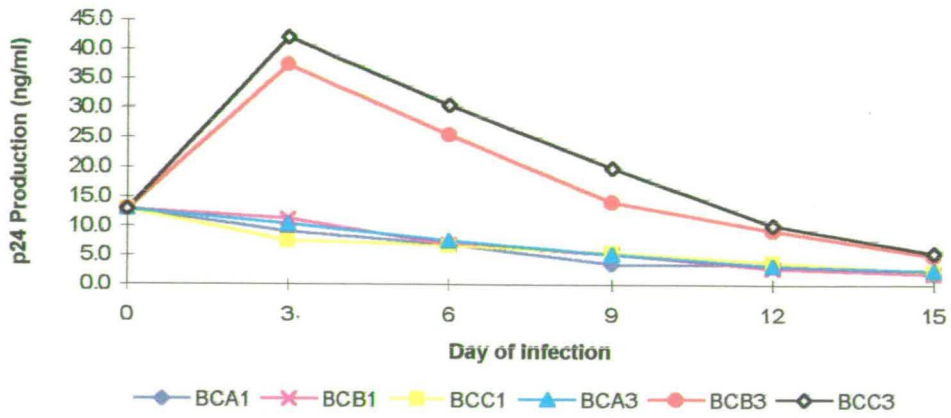
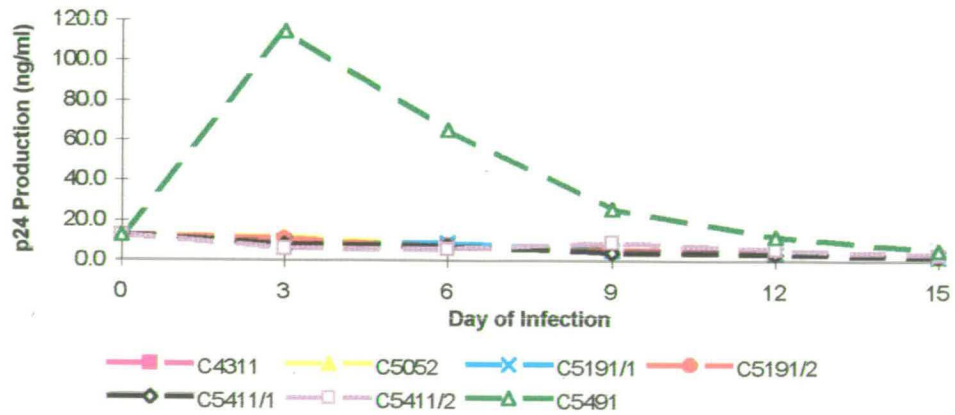


Figure 5.15.

Infectivity Assay with HIV_{139.6b} (m.o.i.=0.001) - Separate Groups

The graphs show the p24 production (ng/ml) following infection with HIV_{139.6b} at a multiplicity of infection (m.o.i) of 0.001 (see Section 2.9.7). For the Donors (A-C) results are presented for undepleted PBMCs (un) and CD8 depleted PBMCs (see Section 2.6.4) and for assays performed on different days (batch 1 (1) and batch 3 (3)) to assess day to day variation. All the EU samples are for CD8 depleted assays and they are identified by their Heterosexual Study number (C - contact) followed by the partner number (see Section 2.2.1.a). Where sufficient cells were available, duplicate experiments were performed and are indicated by /1 and /2 respectively. Hence DonorB3un/1 indicates Donor B, for batch 3, with undepleted cells and the first of two duplicate assays performed on the same day.

- a.) Shows the results for undepleted PBMCs (un) for Donors A-C.
- b.) Shows the results for CD8 depleted PBMCs for Donors A-C.
- c.) Shows the results for the EUs for CD8 depleted PBMCs.

A**B****C**

was also found for one of the *env* sequences from sample 1 and was also excluded (see Section 5.3.2.e).

5.3.2.a NSI/SI Phenotype

The predicted NSI/SI phenotype was determined, relating the net charge of the V3 loop and degree of variation from the subtype B consensus sequence. Figure 5.16 shows the predicted association of the charge and heterogeneity to NSI/SI phenotype as deduced by Donaldson *et al.* (1994), along with the locations of several well defined isolates. The properties of predicted V3 isolates sequenced from samples F0-9, along with isolates from sample 1 and 2 of this study (see Figure 5.16) are shown. The number with each property are summarised in Table 5.4.

The majority of isolates from sample 1 and 2 and F0-9 are predicted to be of an NSI phenotype (see Figure 5.16 and Table 5.4; 42/51 F0-9 and 21/23 Sample 1-2). One isolate from both F0-9 and sample 1-2 was a borderline value of +4 charge and 5 amino acid differences from subtype B and 8 isolates from F0-9 and 1 from sample 1-2 gave a predicted SI phenotype, although still relatively borderline compared to the clear SI isolates such as HIV_{MN} and HIV_{LAI}. From the F9 sample, when C3153 was exposed, none of the isolates sequenced had a predicted SI phenotype (0/12). In fact, all the potential and borderline SI isolates were only found in the F5 and F6 time points and none in the later samples (F7-9).

5.3.2.b CCR-5 Co-receptor Usage

Due to the inability to isolate virus from the Index patient, it could not be ascertained by culture, what the co-receptor usage of virus present around the time of exposure of the uninfected Contacts was. From published sequence and co-receptor usage, a model of important amino acid residues was constructed which related to CCR-5 co-receptor usage *in vitro*. By alignment of V3 loop sequences, potential residues involved in CCR-5 usage were determined (see Table 5.5).

From the work of Speck *et al.* (1997) studying the effects of amino acid substitutions in the V3 loop on the effect of co-receptor usage, it is apparent that a change from the subtype B consensus at position 13 of the loop from histidine (H) when present with a change from isoleucine at position 32 (I), appears to result in a loss of CCR-5 co-receptor usage. Other mutations also seem to affect the properties of the viruses and CXCR-4 usage, but with less consistency. To test if the association with CCR-5 usage was true for other isolates, the V3

Figure 5.16.

Predicted SI/NSI Phenotype of Viral Variants of Index Based on V3 Loop Sequences

Graph based on that compiled by Donaldson *et al.* (1994).

Diagram shows the predicted SI/NSI phenotype of virus present in the Index based on the predicted overall charge and the degree of difference from the subtype B consensus sequence (CTRPNNNTRKSIHGPGRAFYTTEIIGDDIRQAHC) (Dighe, Korber and Foley (1997). The charge was deduced by assigning a +1 for arginine and lysine (R and K) and -1 charge to glutamate and aspartate residues (E and D). The potential charge contribution by histidine residues was discounted (H). The line divides the estimated split between an SI and NSI phenotype. Several well defined isolates are shown with * followed by their name. Isolates from the Index sequenced by Dr. Chris Wade (Wade (1997) PhD Thesis) are shown with a dot and those sequenced in this study are shown with a X. Where two samples gave the same values, the symbols were placed side by side. The actual number of sequences with the predicted values shown are given in Table 5.4.

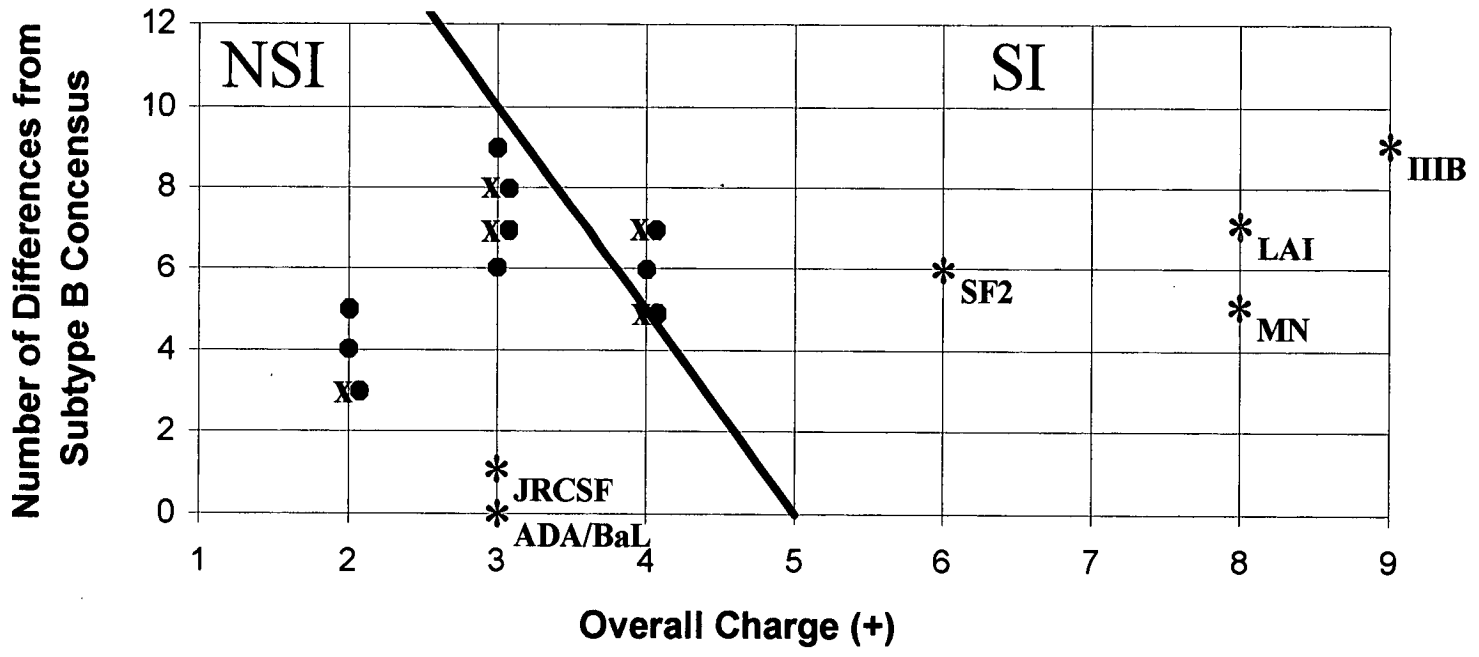


Table 5.4.

Number of Sequences Obtained with Given Charge and Heterogeneity From Subtype B Consensus of V3 Loop as Shown in Figure 5.16.

Overall Charge (+)	Number of Differences from Subtype B Consensus	Number of Sequences from Index with These Properties	
		F0-F9 Samples* (F9 only)	Sample 1 and 2†
2	3	4 (4)	14
2	4	2	0
2	5	5	0
3	6	2	0
3	7	17 (2)	4
3	8	11 (5)	3
3	9	1	0
4	5	1	1
4	6	7	0
4	7	1	1

* Samples sequenced by Dr. Wade. Number in brackets indicates number of sequences obtained at 9 years post seroconversion, the time of exposure of C3153 (see Figure 5.1).

† Samples sequenced in this study at time of exposure of C3154 (see Figure 5.16).

Table 5.5. - Published V3 Loop Sequences and Growth and Co-receptor Properties

	1	11	21	31	P*	M*	T*	CCR-5 [#]	CXCR-4 [#]	SI/NSI [†]	Score [§]	Reference
Consensus [§]	CTRPNNNTRKSIHI--GPGRAFYTTEIIGDIRQAHC											
NL43	R .QR.....	V.I.K.-.NM.....		+	-	+	-	+	SI	0	1, 2
123	R.. S	R..-Q.V.NL.....		+	-	+	-	+	SI	0	1
126	R..-Q.V.NL.....		+	+	-	+	-	NSI	1	1
134	S	R..-Q.V.NL.....		+	-	+	-	+	SI	0	1
241	R.. S	R..-Q.....		+	+	+	+	+	SI	1	1
242	R.. S	R..-.....		+	+	-	+	-	NSI	1	1
254	R.. S	R..-Q.V.....		+	+	+	+	+	SI	1	1
255	R.. S	R..-Q.V.L.....		+	-	+	-	+	SI	0	1
256	R.. S	R..-Q.V.N.....		+	+	+	-	+	SI	1	1
HXB2R/IIIB	R.R.QR.....	V.I.K.-.NM.....		+	-	+	-	+	SI	0	3, 4, 5, 6, 7, 8, 9
SF-2	Y	H..R.....K.....		+	-	+	+	+	SI	1	3
SF2Mu3	Y	A..D.....K.....		+	+	+	+	+	SI	1	3
SF-162	T	A..D.....		+	+	-	+	-	NSI	1	3, 4, 6
SF162Db1	TR	V..A..D.....		+	-	+	+	+	SI	1	3
BaL		+	+	-	+	-	NSI	2	4, 6, 9
JR-CSF	S		+	+	-	+	-	NSI	2	1, 2
JR-FL		+	+	-	+	-	NSI	2	2, 4, 5, 6, 9
89.6	RRL S	ARRN.....		+	+	+	+	+	SI	1	5
YU2	N	L.....		+	+	-	+	NT	NSI	1	10
ADA		+	+	-	+	-	NSI	2	4, 5, 6, 8, 9, 10.
RF	TK	VI.A.Q.....K.....		+	-	+	+	+	SI	1	4, 6
GUN WT	T	HAIEK..N.....		+	+	+	+	+	SI	1	7
GUN-1 V	T	S...HAIEK..N.....		+	-	+	-/+	+	SI	1	7
GUN V.Es.1	T	HAIEK..N.....		+	+	+	+	+	SI	1	7
GUN V.Es.2	T	S...INAIEK..N.....		+	+	+	+	+	SI	1	7
GUN V.Es.3	T	S..T.QAIEK..N.....		+	-	+	+	+	SI	1	7
GUN V.Es.4	T	S...LHAIEK..N.....		+	-	+	+	+	SI	1	7

* Replication in PBMCs (P), Macrophages (M), or T cell lines (T). [#] Replication in cell line expressing CD4 and CCR-5, or CXCR-4. [†] Syncytium-inducing (SI), or Non-SI (NSI)

[§] Presence of histidine (H) at position 13 and isoleucine at position 32, shown in bold. If both present score =2, if one score =1, etc. NT - not tested

^{||} 1: Speck *et al.* (1997); 2: Zhang *et al.* (1996); 3: Cheng-Mayer *et al.* (1997); 4: Feng *et al.* (1996); 5: Doranz *et al.* (1996); 6: Alkhatib *et al.* (1996); 7: Dittmar *et al.* (1997); 8: Bjorndal *et al.* (1997); 9: Deng *et al.* (1996); 10: Choe *et al.* (1996).

[§] Subtype B consensus from Digne, Korber and Foley (1997). Identity with the consensus is shown with a dot and alignment gaps are shown with a dash.

loops of published viruses with known co-receptor usage were aligned. As can be seen from Table 5.5, of the isolates able to utilise CCR-5 as a co-receptor, all had either the H at position 13 and/or I at position 32. Those isolates unable to use CCR-5 had changes from both H and I at position 13 and 32 respectively.

No other association could be seen from this analysis and it was clear that CCR-5 usage by both T- and M-tropic viruses was possible. It also seemed to be the case that CCR-5 usage did not relate to an ability to infect macrophages in the studies assessed here and no V3 loop sequences were apparent from this analysis that related to the ability to infect macrophages.

Having confirmed the H/I association in other isolates, the predicted V3 loop regions of viral isolates sequenced from the Index were aligned and assessed for the predicted CCR-5 receptor usage (see Table 5.6). Of the samples sequenced by Dr. Wade (F5-9 for *env*), all had both H at position 13 and I at position 32, hence predicted to be able to utilise CCR-5 as a co-receptor. The sequences for F9, around the time of C3153's exposure and isolates from sample 1 and 2 of this study (see Figure 5.1) are shown in Table 5.6, where it shows that all isolates found in later samples also had a predicted ability to utilise CCR-5 on this basis.

5.3.2.c Changes in Virus from Index

During a father-mother-child transmission study involving the Index, four subgroups of viral populations were found for *gag* and *env* during the nine years from seroconversion (A-D) (Wade (1997) PhD thesis). Representative predicted amino acid sequences across this study time for each of these groups are given in Figure 5.17 for *env* and 5.18 for *gag* (nucleotide sequence given in Appendix 5.1 and 5.2). To determine if any major changes in the viral population infecting the Index had occurred, during the later stages of infection, which may explain the lack of transmission to his later partners (C3153 and C3154), sequences from the later time points (10 and 11 years post seroconversion; sample 1 and 2 respectively) were aligned to the earlier ones. From the predicted amino acid sequences, the group with which the sequence bore most homology is indicated, unless similarity to more than one is seen, when both are given, or if too much divergence was seen, no group was assigned (see Figure 5.17 and 5.18).

More variation was seen in *env* than *gag*, both during the previous study and also in the later samples studied here. For *env*, the A subgroup was only seen in the earliest samples (F5 and F6), but in the F9 sample, during C3153's exposure, all the remaining groups were

Figure 5.17.

Alignments of the Predicted Amino Acid Sequence of the V3 Loop and Flanking Regions

The diagram shows the predicted amino acid sequences for the sequenced region of the *env* gene for multiple sequences at the given sample points (see Figure 5.1). The sequences are aligned to the first variant sequenced at 5 years post seroconversion (F5), with identical amino acids to this sequence shown with a dot. Alignment gaps are noted with a dash and stop codons represented with an asterisk. The sequences provided by Dr. Wade are examples of the 4 major subgroups found during his study (A-D). The remaining sequences are those analysed here with the sample point and the sequence number both given. The sub-group with which they bear most homology to are given after the sequence, although most show additional variation from these initial sub-groups. Where similarity to more than one sub-group is seen, both are indicated.

	1	11	21	31	41	51	61	71	81	91	101	111	121
F5.A	V	N	E	H	G	R	C	T	E	F	I	V	F
F6.A	I	I	I	S	G	I	A	R	A	R	M	S	S
F6.B				S		E	L	A	G	K			
F7.B	I			N		E	L	A	G	K	V		T
F9.C			K	G	H	E	E	H					T
F9.C	I		K	G	H	E	E	R	K	T	H		T
F6.D				S				K	A				T
F9.D			K	S	R	E		K	A				T
1.1	S			G		E		S	K				T
1.2	S		K	S		A		S	K	S			T
1.3			S	N		E	L	I	A	K			T
1.4			S	N		E	L	I	A	K			T
1.5			S	N		E	L	I	A	K	D		T
1.6			S	N		E	L	I	A	K			T
1.7			S	N		E	L	I	A	K			T
1.8			S	N		E	L	I	A	K			T
1.9			P	N		E	L	I	A	K			T
1.10			S	N		E	L	I	A	N			T
1.11			S	N		E	L	I	A	K			T
1.12			S	N		E	L	I	A	K			T
1.13	T	S	V	N	R	S		D	I	I	A	E	K
1.14			S	N		E	L	I	A	N			T
1.15			S	N		E	L	I	A	K			T
2.1			S	N		E	L	I	A	K			T
2.2	I		K	G	H	E		E	H	K			T
2.3				N		E	L	I	A	K			T
2.4			K	G	H	E		E	A	N	G	M	T
2.5				S	R	E		S	K	A			T
2.6				S		D		S	R		E		T
2.7				G		E		S	K				T
2.8				S	R	E		S	K	A			T
2.9			K	G	H	E		E	H				T

Figure 5.18.

Alignments of the Predicted Amino Acid Sequence of the p17 Region of the *gag* Gene and Flanking Regions

The diagram shows the predicted amino acid sequences for the sequenced region of the *gag* gene for multiple sequences at the given sample points (see Figure 5.1). The sequences are aligned to the first variant sequenced at the year of seroconversion (F0), with identical amino acids to this sequence shown with a dot. Alignment gaps are noted with a dash. The sequences provided by Dr. Wade are examples of the 4 major subgroups found during his study (A-D). The remaining sequences are those analysed here with the sample point and the sequence number both given. The sub-group with which they bear most homology to are given after the sequence, although most show addition variation from these initial sub-groups. Where similarity to more than one sub-group is seen, both are indicated.

	1	11	21	31	41	51	61	71	81	91	101	111	121	131	
F0.A	G	G	F	S	L	E	T	V	K	I	N	N	Q	M	T
F7.B	K	W	R	E	L	E	R	R	E	E	Q	N	K	S	K
F9.B	K	I	V	A	S	R	E	L	E	R	K	A	Q	A	A
F6.C	K				S	P									
F8.C	K				S	P									
F5.D	K				K										
F9.D	K			M	K					T			M		
1.1	K							R			N	VSQ	L	B	
1.2				S				R			N	VSQ		B/C	
1.3				S				R		T	N	VSQ		B/C	
1.4	K				S	P					N	VSQ	L	B	
1.5	K				S			R			N	VSQ	L	B/C	
1.6	K			M	K			T		T			M	D	
1.7	K				S			R			N	VSQ	L	B/C	
1.8	K				S			R	I		N	VSQ	L	B/C	
1.9	K							R			N	VSQ	L	B	
1.10	K				S			R			N	VSQ	L	B/C	
1.11	K				S			R		V	N	VSQ	M	B/C	
2.1				M	K			T		T				D	
2.2	K				S			R			N	VSQ		B/C	
2.3	K			H	M	H		K		T				D	
2.4				M	K			T		T			M	D	
2.5				M	K			T		T				D	
2.6					S			R		T	N	VSQ		B/C	
2.7					S	P						Q		C	

seen (Wade (1997) PhD thesis). This was also reflected in the later time points where variants with homology to all groups were seen, along with some which showed similarity to two groups (see Figure 5.17). One variant was seen which had a stop codon in the V3 region (2.4; see Figure 5.17) and may not have produced a functional gp120 protein.

As for *env*, the first group seen for *gag*, A (F0), was only seen early in infection and was not seen in later samples (F5-F9) (Wade (1997) PhD thesis). The remaining groups were seen across the later time points, but at F9 only group B and D were seen, although only 3 sequences were obtained for this time. However, group C was seen in the preceding sample (F8), where more sequences were analysed (Wade (1997) PhD thesis). In the samples analysed in this study (1 and 2), variants with homology to groups B-D were seen, along with B/C variants (see Figure 5.18). One additional change not previously seen was a VSQ insertion (position 105-8 as numbered in Figure 5.18). A Q insertion was seen previously, but not the VSQ which seems to predominate in the later time points.

5.3.3.d Mean Pairwise Distances

The intra sample diversity and the inter sample diversity was calculated over time for the V3 region of *env* and the p17 region of *gag* sequenced (see Table 5.7). Overall, the diversity in V3 was greater than *gag* (mean intra sample diversity: 3.7% *env*; 2.2% *gag*; see Table 5.7) and the variation early in infection is less than later time points (less than 5 years post seroconversion for *env* and less than 6 years for *gag*). However, most of these earlier time points had few sequences on which to perform the analysis.

The greatest intra sample diversity was seen in the F8 sample 8 years post seroconversion for *env* (6.6%) and at sample 2 for *gag* (3.4%), although the overall variation seen was not that great at any of the time points. From the time when the three later partnerships occurred (F6 onwards, see Figure 5.1), the degree of intra sample variation in *env* was similar and despite a slight increase in the later samples, this was comparable for *gag* (see Table 5.7).

The inter sample variation did not differ greatly from than of the intra sample values, reflected in the mean values (5.1% for *env* and 2.9% for *gag*) as there appeared to be as much variation within the sample points as there was over time (see Table 5.7). The greatest diversity was seen between the F8 and sample 2 time points (7.6% for *env* and 4.6% for *gag*; see Table 5.7), but as for the intra sample values these did not differ widely from the mean.

Table 5.7.**Mean Pairwise Distances (%) for Intra and Inter Sample Diversity from Index**

Sample*	<i>env</i>		<i>gag</i>	
	n [†]	MPD [#]	n [†]	MPD [#]
F0	0	---	3	0.20
F5	2	1.42	3	1.62
F6	12	3.17	11	1.80
F7	17	3.74	15	2.61
F8	10	6.63	9	2.10
F9	12	4.63	3	2.65
1	14	2.31	11	3.25
2	9	4.26	7	3.36
Mean Intra sample Variation[§]	3.74		2.20	
(Range)[‡]	1.42-6.63		0.20-3.36	
Mean Inter sample Variation[§]	5.07		2.87	
(Range)[‡]	3.32-7.56		1.37-4.58	

* Sample from Index (see Figure 5.1).

† Number of sequences for each sample.

Mean Pairwise Distance (%) between sequences within sample (intra sample) calculated using a two parameter correction for multiple hits (Kimura (1980)).

§ Mean of all % distances calculated for inter /intra sample variation, calculated as above.

‡ Range of differences in analysis (samples with the given value).

5.3.2.e Phylogenetic Analysis

Neighbour joining phylogenetic trees were reconstructed from the *env* and *gag* nucleotide sequence data sets of the Index (see Figure 5.19 and 5.20). The trees were rooted with the subtype D virus HIV_{ELI} as the outgroup. Several reference subtype B strains were included in the analysis, along with some strains obtained from Scottish IDUs and related heterosexual transmissions (Holmes *et al.* (1995)). Due to work carried out at the Centre on cloned material from Edinburgh haemophiliacs, which involves high concentrations of viral DNA, sequences from these clones were included to check for potential contamination, although every effort was carried out to avoid this. As this work only involves the *env* gene, these were not included in the *gag* analysis, although a few sequences from Edinburgh Haemophiliacs were included for comparison.

The four groups described by the father-mother-child transmission study (Wade (1997) PhD thesis) can be seen for the *env* tree (see Figure 5.19) and it is clear that the later samples 1 and 2 yielded variants which fall into all but the earlier A group. The grouping of the all the Index virus is very strongly supported, with an 80% bootstrap (>70% normally deemed significant) for the main branch node for the cluster.

The final sample obtained and sequenced from the Index (sample 3; see Figure 5.1) is widely divergent from the other sequences and such strong departure would have been highly unlikely in the few months between the samples and suggests that that either contamination of the sample occurred, or a sample mix-up occurred and this sample was not from the Index. The sample did not cluster with the most likely source of contamination, the haemophiliac clones, unlike a single sequence from the first sample, which although not closely clustering with the clones, definitely appeared to be a contaminant and was excluded from all other analysis within this chapter.

The diverse nature of the sequences from sample 3 were more marked in the *gag* tree (see Figure 5.20) and strongly suggest that the sample was not from the Index as the chance of contaminating both *gag* and *env* without contaminating sample 2 which was processed in parallel is extremely unlikely. The sequences from this time point were excluded from all other analysis contained within this chapter.

The four groups can also be seen in the *gag* tree (see Figure 5.20) and sequences from samples 1 and 2 do fall into these clusters. There is also an additional cluster, which appears to represent the divergent B/C group seen in the amino acid alignment (See Figure 5.18). The lower degree of diversity compared to *env* is reflected in the difference in the scale (shown on

Figure 5.19.

Neighbour Joining Phylogenetic Tree for *Env*

A rooted phylogenetic tree obtained by the neighbour joining reconstruction method (see Section 2.19) for the V3 sequences obtained from the Index. The tree is rooted with the subtype D virus HIV_{ELI} (ELI) as the outgroup.

The scale bar corresponds to 10% nucleotide sequence divergence and % at the node is the % of bootstrap replicates in which the node occurs in this position in 100 reconstructions (see Section 2.19).

The subtype B reference strains are indicated by their name and the viruses derived from Scottish IDU and related heterosexual transmissions are indicated by IDU and HS respectively (Holmes *et al.* (1995)) The clones derived from Scottish Haemophilacs are grouped, as they fall into one discrete cluster (Ashelford (1996) PhD Thesis).

The sequences obtained and analysed by Dr. Wade (Wade (1997) PhD Thesis) are labelled F (father) followed by the year of sampling post seroconversion (see Figure 5.1). The four sub-groups identified in that analysis are shown labelled A-D. Later samples from the index are marked with an asterisk and the sample number 1-3 (see Figure 5.1). The sequence obtained from sample 1 which is a likely contaminant is marked with a #.

Figure 5.20.

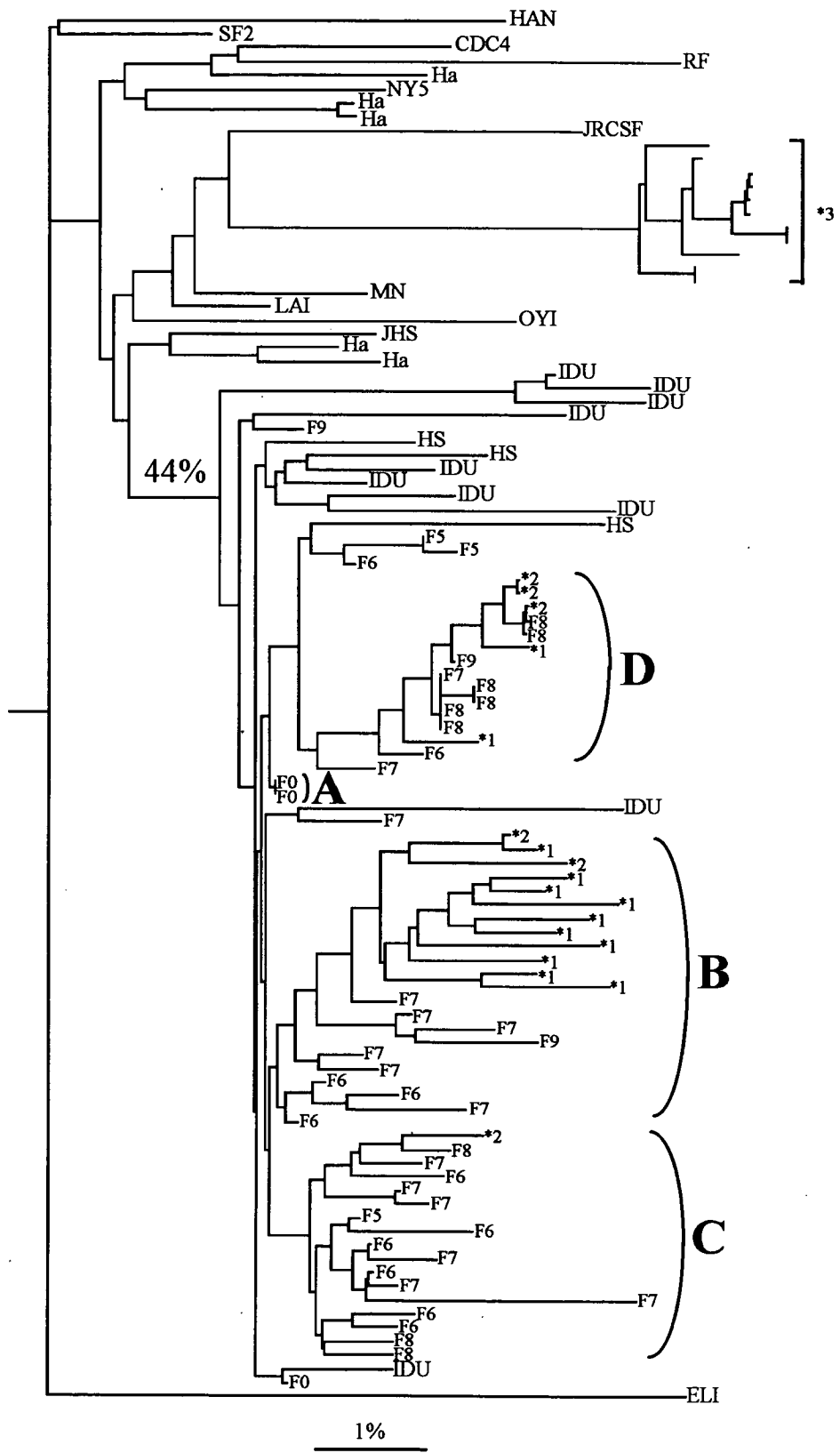
Neighbour Joining Phylogenetic Tree for Gag

A rooted phylogenetic tree obtained by the neighbour joining reconstruction method (see Section 2.19) for the p17 gag sequences obtained from the Index. The tree is rooted with the subtype D virus HIV_{ELI} (ELI) as the outgroup.

The scale bar corresponds to 1% nucleotide sequence divergence and % at the node is the % of bootstrap replicates in which the node occurs in this position in 100 reconstructions (see Section 2.19).

The subtype B reference strains are indicated by their name and the viruses derived from Scottish IDU and related heterosexual transmissions are indicated by IDU and HS respectively (Holmes *et al.* (1995)). Sequences derived from Scottish Haemophilacs are labelled as Ha (Ashelford (1996) PhD Thesis).

The sequences obtained and analysed by Dr. Wade (Wade (1997) PhD Thesis) are labelled F (father) followed by the year of sampling post seroconversion (see Figure 5.1). The four sub-groups identified in that analysis are shown labelled A-D. Later samples from the index are marked with an asterisk and the sample number 1-3 (see Figure 5.1).



Figures 5.19 and 5.20). This also may explain the reason for the lack of the discrete cluster of Index variants seen for *env*, as other Edinburgh samples fall within the Index sequences, although the bootstrap support for this is not significant. This intermingling of IDU and Index sequences has already been observed by Dr. Wade (personal communication). The bootstrap support for the node dividing the Scottish IDUs and their related heterosexual transmissions is not significant at 44%, but is high considering the low degree of variation in this region.

5.4. DISCUSSION

5.4.1 Infectivity Assay

5.4.1.a Isolation of Virus from Index

It is unclear why the repeated attempts at viral isolation failed. It is possible that some inhibitory factor was present in the culture media, most likely the serum. The C-C chemokine, RANTES, is a high concentration in human serum (Donald Innes, personal communication) and it is possible that foetal calf serum may contain an analogous factor. However, the failure to isolate virus in collaboration with Dr. Myra Arnott at the Department of Medical Microbiology, using different reagents, which are frequently used for viral isolations in the same manner, suggests that this is unlikely to be true.

It is possible that some process in the storage of the cells had reduced their viability, but all cells were stored continually in liquid nitrogen for PBMCs and -70°C for plasma, in an identical manner to those I stored and achieved reliable recovery from (see Section 5.2.2.d). The samples had been stored for at least a year before the first culture attempts and the length of storage time can also affect the ability to culture (Dr. Myra Arnott, personal communication).

It is also possible that the copy number of virus present within the samples was very low and hence may explain the failure. In support on this the Index was known to be asymptomatic at the time of sampling and this is known to relate to a low viral load (Venet *et al.* (1991); Ruiz *et al.* (1996)) and low number of infected PBMCs (Simmonds *et al.* (1990b)). An additional sample was found later (Sample 1; see Figure 5.1) and used in the sequence analysis, but as this was after the infectivity assay had been performed, viral isolation was not attempted. After repeated attempts, it was decided to perform the assay with only well defined strains used within the Centre and an alternative, molecular, strategy was used to investigate the Index's virus.

5.4.1.b Co-receptor Usage

In keeping with the expected co-receptor usage from published data of other primary isolates (Alkhatib *et al.* (1996); Doranz *et al.* (1996); Feng *et al.* (1996); Cheng-Mayer *et al.* (1997); Dittmar *et al.* (1997); Speck *et al.* (1997)), the SI primary isolate derived from an Edinburgh Haemophilic (HIV_{CS8}), could utilise both CCR-5 and CXCR-4, a low level of infection was also seen for CCR-3 expressing cells. The TCLA strain HIV_{IMB} was restricted to CXCR-4, as shown by several other groups (Alkhatib *et al.* (1996); Deng *et al.* (1996); Doranz *et al.* (1996); Feng *et al.* (1996); Björndal *et al.* (1997); Cheng-Mayer *et al.* (1997); Dittmar *et al.* (1997)) and the two NSI strains, HIV_{BaL} and HIV_{139.6b}, were restricted to CCR-5 usage as a co-receptor for entry. This is again in agreement with other reports regarding HIV_{BaL} (Alkhatib *et al.* (1996); Deng *et al.* (1996); Feng *et al.* (1996)) and is agreement with other unpublished data for HIV_{139.6b} (Dr. Robert Walker, personal communication). This provided a range of viruses, with differing co-receptor restrictions, to assess the infectibility of the EUs studied.

5.4.2.c PHA Blasts for Infection

The high degree of failure to grow the cells, obtained from the Department of Medical Microbiology, was surprising and severely limited the extent of the infectivity assay. It is most likely a reflection of both the age of the samples, some were stored for many years, and also the storage conditions. It was discovered that a proportion of samples were being incorrectly stored for a time and it is possible that other problems may have also effected the viability. The poor viability of these samples seems a more plausible explanation for the failure to culture than the culture conditions, as all the samples stored at the Centre for HIV Research, King's Buildings grew well.

The numbers of individuals able to be assayed may have been increased if I had not mixed samples to maximise the number of cells achieved. This meant that cells stored at the Centre may have grown if not cultured with the Medical Microbiology cells. The final Contact of the Index studied here, C3154, was one such individual and meant that she was unable to be included in the infectivity assay.

5.4.1.d CD8⁺ Cell Depletion

The depletion method used was not 100% successful and varied from individual to individual. However, it is a similar method to that used by others for similar assays (Connor *et*

al. (1996); Paxton *et al.* (1996); Pal *et al.* (1997); Schwartz *et al.* (1997); Wu *et al.* (1997b); Paxton *et al.* (1998)). Alternative methods could have been more successful, such as cell sorting, whereby a FACScan is used to actively positive, or negatively select cell populations that have been stained with particular antibodies. An alternative magnetic depletion based method is using a MiniMACS System (Miltenyi Biotec, Surrey). This has been shown to be a highly efficient method of depletion (Dr. Marian Aldhous, personal communication), but is very laborious and not well suited to large numbers of depletions.

A further depletion of the depleted population, using the same technique may have improved the efficiency, but it may have also reduced the viability of the cells, which need to be manipulated as little as possible.

5.4.1.e Infectivity Assay

It is clear from the results for the majority of isolates, all but HIV_{BaL} (m.o.i. =0.001) that something was clearly wrong with the assay. HIV_{C98} did appear to work, but only showed a much lower level of infection (p24 production) than was seen for the test run. It was not a problem of a particular batch as all behaved in an analogous manner, which was expected as all were treated identically.

There are several possible reasons for the failure of the main assay. Most seem unlikely as they were controlled for, but can not be ruled out. The first possibility is variation in the culture conditions, i.e. the medium and plastic-ware. The same source was used throughout and the same supplier was used for the media and PHA, etc. and therefore seems unlikely. In support of this is the fact that the cultures themselves did appear to be healthy and viable even at the end of the culture period.

The depletion efficiency may have varied between the two main assay and the test assay and as this was not measured for the test assay can not be ruled out. However, routinely similar results have been achieved with this method, and the fact that the undepleted cells did show replication in the HIV_{BaL} experiments suggests that this is unlikely to be the reason for the widespread failure of the other assays. Previous experiments have also shown infection in undepleted cells using these isolates.

It is possible that the virus used in the assays may have lost activity during storage as the main assay was performed a few months after the test assay. To minimise variation in the viral stock, the high titre stock was pooled and mixed thoroughly and aliquoted into small volumes. This enabled a vial to be removed and used, to prevented freeze-thawing, which

would have reduced the viral titre. It seems unlikely that the viral stocks could have deteriorated over this time, when they had been correctly stored, throughout the time between assays.

A more plausible explanation is a problem in the assay used to assess the viral replication, the p24 ELISA. New reagents were required during the time between the test and the main assay. However, all new reagents were tested in parallel with the old stocks, for variation in the standard curve using recombinant p24 and no vast difference was seen for the new reagents. It is possible that the viral derived p24 may behave in a different way to the recombinant, which will not have other proteins present which may interfere with the binding of the p24 to the detection antibody. It is equally possible that the ELISA reagents used for the test assay, were perhaps incorrectly measuring the p24 content instead, but as only a limited amount of culture supernatant was available for all these cultures this could not be ascertained.

The detergent (empigen) lysis of the virus, which is performed prior to the ELISA to release the viral p24, which is contained within the viral envelope (see Section 2.10.2) may have been a problem and was suggested by the early peak and subsequent drop in p24 content for the HIV_{1MB} and HIV_{139.6b} in the test assay. The p24 values of the inoculum were far lower than those of the first time point and suggest, either an initial rise in infection, or an under estimate of the amount of p24 added. As the infection kinetics of a typical viral culture are thought to peak at 10-14 days (Coligan *et al.*(1996)), the latter seems a plausible explanation. Whether, the virus dilution was not mixed sufficiently, when the sample was taken, or whether the empigen lysis was not effective, is unclear, but either are possible, although both seem unlikely. I routinely took such samples after I performed the experiment, in case I ran out and routinely mixed dilutions of any sort. Also, the addition of empigen results in a clear colour change and hence would have been obvious if omitted and all the time points were analysed at the same point, so if the reagent had expired, all the results for that assay would have been effected.

The unexpected results seen for some of the batch 3 assays, may reflect a mistake in the dilution, although it was checked and similar to those performed for the first two batches. Again, it is possible that viral stock aliquots were different in some way, but as explained above this seems unlikely. Although, it does appear a higher amount of p24 was added to those cultures than others. However, in light of the poor response of this assay, the apparent increase in virus did not then result in a better infection.

A final possible explanation of the failure of the main assay was the decrease in the cell number from 5×10^5 to 3×10^5 , hence the decrease in the amount of virus as the m.o.i. is dependent upon the cell number. However, the cells appeared to be growing throughout the culture and did not reach saturation, as in the test assay. There was also visible evidence of apparent syncytia in many of the HIV_{CR8} infected wells and some of the HIV_{IMB} wells. Also the cell number and m.o.i was within range used by others (Paxton *et al.* (1996); Aarons *et al.* (1997); Pal *et al.* (1997); Wu *et al.* (1997b)) and some even perform the experiment on a 96-well basis (Paxton *et al.* (1998)).

In attempt to verify the results, I extracted the cell pellet stored at the termination of the culture, but I was unable to get consistent positive results for the control HLA-DQ α (see Section 2.12.1), suggesting that the cell number was too low to enable a reliable extraction and PCR amplification.

5.4.1.e.i HIV_{BaL} Infection

In contrast to all the other viruses, the HIV_{BaL} infection at the m.o.i. of 0.001 gave similar results in both the test and main assay, whether this was due to the low values, which meant that differences on the p24 ELISA were not seen, is unclear.

What is clear is that the individuals studied here varied in their susceptibility, which was not limited to the EUs. This variation was unlikely to reflect the differences in the CD8 depletion, as some of the individuals, who showed good depletion (Donor C; C5411), did not show an apparent infection and others with a less efficient depletion (C4311), showed a good infection. Also two of the undepleted Donors (A and C) also showed infection, comparable to that of others and even the worst depletion had many fewer CD8⁺ cells than these undepleted samples.

The lack of apparent HIV_{BaL} infection in C3153 supports the role of homozygosity for the $\Delta 32$ in the *CCR-5* gene as being protective from infection with CCR-5 restricted virus (Dean *et al.* (1996); Liu *et al.* (1996); Samson *et al.* (1996b)). However, the other EUs (C5411, C5052) which showed an apparent lack of infection were wild-type for both the $\Delta 32$ in *CCR-5* and the 64I mutation in the CCR-2 receptor. C5491, who gave an indeterminate result was heterozygous for the $\Delta 32$ in *CCR-5*, but the result was not sufficiently clear in this assay to form any conclusion on the possible effect this may have had. The lack of infection was not restricted to EUs, as Donor C also showed no evidence of infection and they were found to be heterozygous for the 64I mutation in the CCR-2 receptor. It is still unclear if this

mutation has any physiological role (see Section 4.4.3.b) and contradicts the apparent additional risk of heterosexual infection in individuals bearing this mutation (see Section 4.3.4). However, the effect on heterosexual infection may involve different cells, to the PBMCs studied here and any effect the CCR-2 mutation may have, be it as a marker for another mutation, or a direct effect itself, may differ in different cell types.

A possible reason for the differences in infectibility of these individuals is the level of surface CCR-5 expression. The level of CCR-5 expression is known to vary between individuals (Moore (1997)) and reduced expression has been shown to be related to reduced infectibility with M-tropic strains (Wu *et al.* (1997b); Paxton *et al.* (1998)). Heterozygotes for the $\Delta 32$ deletion in the *CCR-5* gene express lower levels of surface CCR-5 (Wu *et al.* (1997b); Paxton *et al.* (1998)) and a recent study by Paxton *et al.* (1998) showed that homozygous wild-type EUs also had reduced expression, which related to reduced infection with M-tropic strains of HIV. This may be related to, or have an additional effect to the reported association of resistance to infection being related to high levels of β -chemokine production (Paxton *et al.* (1996); Pal *et al.* (1997); Zagury *et al.* (1998); Paxton *et al.* (1998)). Presumably, reduced receptor expression may relate to a reduction in targets for the chemokine and therefore show a higher level of free chemokine. Conversely, higher levels of chemokine production may mean that lower expression of receptor is needed to ensure correct signalling and receptor:chemokine interaction. The effectiveness of the chemokine inhibition on infection may also be increased in individuals expressing lower levels of surface receptor, due to increased competition for a reduced number of sites. All of these factors may be involved in some individuals, with differences from individual to individual too. Whether the differences in receptor level expression are due to polymorphisms in other areas, such as the *CCR-5* promoter region, has not yet been fully elucidated, but screening of the CCR-5 expression level in Edinburgh Heterosexual EU cohort may help explain the apparent protection from infection.

The higher concentration of HIV_{BaL} (m.o.i.=0.01) did not show a clear infection and may require the removal of residual virus following infection, as this is the most likely reason that any infection was masked, as HIV_{BaL} only establishes a low level infection by comparison with HIV_{IBB}.

5.4.1.e.ii HIV_{C98} Infection

The infection with HIV_{C98} showed an almost inverse relationship of successful infections from that of HIV_{BaL}. Of the EUs which showed a lack of, or poor replication with HIV_{BaL}, they then showed clear infection with HIV_{C98} (C3153, C5052, C5411 and C5491) and for those individuals which showed replication with HIV_{BaL} (C4311 and C5191), poor replication with HIV_{C98} was seen. A similar picture was seen for the Donors, with Donor A showing infection with HIV_{BaL} and not HIV_{C98} and Donor C the opposite, but the difference was not as marked as seen in the EUs. This intriguing difference may again be related to the receptor expression levels, or even the presence of inhibitory chemokines. HIV_{C98} is a virus able to utilise either CCR-5, or CXCR-4, but as seen from the infection studies with the U87-CD4 cells (see Section 5.3.1.b), the replication with CXCR-4 is apparently more virulent. If the level of CCR-5 was reduced, either due to competition from inhibitory chemokines, or due to reduced expression, the virus would have an increased chance of utilising the CXCR-4 receptor, which it may do more readily and/or quickly. However, where the CCR-5 level is higher, then as the virus can bind to either receptor, the increased expression may mean that more virus infects the cells using CCR-5, hence slowing the infection.

The culture conditions of the PBMCs are known to effect the level of receptor expression for both CCR-5 and CXCR-4 (Bleul *et al.* (1997)), but the cells in this study were treated in an analogous manner and batch to batch samples showed similar patterns of infection, therefore, this is unlikely to be the explanation for the differences in infections. A further interesting difference in expression was also reported by Bleul and co-workers (1997), they showed that naïve T cells (CD26^{low} CD45RA⁺ CD45RO⁻) expressed CXCR-4 and memory/ activated T cells (CD26^{high} CD45RA⁻ CD45RO⁺) expressed CCR-5, hence a difference in the proportion of naïve versus memory cells may effect the response to *in vitro* activation and the final level of receptor expression.

The despite the many problems with the infectivity assay, the differences in susceptibility to the CCR-5 restricted HIV_{BaL} and the CXCR-4 and CCR-5 using HIV_{C98}, highlight that important differences in receptor expression and chemokine inhibition may possibly be occurring. The receptor expression could be investigated by flow cytometry, utilising the co-receptor specific monoclonal antibodies which are now more widely available. The production of chemokines could be monitored by ELISA in response to stimulation, with and without infection. The infectivity assay would be better if it could be performed on a smaller scale too, such as performed by Paxton *et al.* (1998), but this would require the use of

a more sensitive p24 ELISA which could monitor smaller volumes at a more sensitive level. This may also improve the batch to batch variation of reagents which may have occurred for the in-house ELISA used in this study.

A smaller scale study may also permit the study of the inhibitory effects of CD8⁺ cells on HIV infection, by performing assays with and without depletion. Depletion systems are also available which allow the detachment of the magnetic bead and hence the CD8⁺ cells could even be titrated in to see at what level they exert an effect. A smaller study may also allow the inclusion of more viruses, which were limited by the cell numbers in this study.

5.4.2 Sequence Analysis

5.4.2.a NSI/SI Virus

Several studies have shown an association of NSI phenotype with transmission (Roos *et al.* (1992); Zhu *et al.* (1993); van't Wout *et al.* (1994)) and it has been proposed to be linked to the initial infected cell type, thought to be a macrophage, or dendritic cell (Granelli-Piperno *et al.* (1998)). During the period of time when infection of the first Contact occurred, the NSI/SI phenotype of the Index's virus could not be determined, due to the lack of *env* sequences. The majority of sequences obtained at all other time points were predicted to be NSI based on the V3 sequence using the method of Donaldson *et al.* (Donaldson *et al.* (1994)). A few borderline SI variants were seen, although none were found at the time of exposure to the $\Delta 32/\Delta 32$ homozygote, C3153. While this may reflect a sampling effect, 12 sequences were obtained and no SI variants were seen in the preceding two samples either (both F8; 9 sequences).

The lack of SI variants at the time of C3153's exposure is important as homozygosity for the $\Delta 32$ mutation in the CCR-5 co-receptor has been strongly linked to protection from HIV infection (see Section 1.7.a.i; (Dean *et al.* (1996); Huang *et al.* (1996); Liu *et al.* (1996); Samson *et al.* (1996b))). The apparent NSI dependency and hence, presumed CCR-5 usage of transmitting viruses, is further supported by the protective effect of the CCR-5 mutation, as if CXCR-4 was also utilised during transmission, more $\Delta 32/\Delta 32$ homozygotes would be HIV⁺ instead of the very few rare reported events (Biti *et al.* (1997); O'Brien *et al.* (1997); Theodorou *et al.* (1997); Balotta *et al.* (1997)). It is not yet clear if infection of these $\Delta 32/\Delta 32$ individuals is due to SI, T-tropic variants, or if it is due to additional co-receptor usage, such as CCR-3, by NSI, M-tropic strains. However, the reports of protection in very highly

exposed uninfected homosexuals who were homozygous for $\Delta 32$ (Huang *et al.* (1996); Liu *et al.* (1996)), shows a strong dependency on CCR-5 usage during infection. As some of these EUs have had widespread exposure to HIV, if alternatives were possible one would have thought they would have been utilised in these individuals.

5.4.2.b Co-receptor Usage Model

If virus isolation from the Index had been successful, it would have been possible to determine if the virus was able to infect the EU contacts, or if they were intrinsically protected by $\Delta 32/\Delta 32$ homozygosity in the case of C3153, or some other mechanism in C3154. However, due to repeated failure to do this, an attempt to determine a model of co-receptor usage was investigated. This was based on the predicted amino acid sequence of the V3 loop, as it had been shown in culture that switching the V3 loop of a CCR-5 independent virus with a dependent virus, was sufficient for a change in co-receptor usage to that defined by the virus from which the V3 loop only was derived (Cocchi *et al.* (1996)).

Variation at position 13 and 32 of the V3 loop from H and I respectively was found to correlate with a loss in ability to use CCR-5 as a co-receptor for entry *in vitro*. Many SI viruses, particularly primary isolates, have been shown to be able to use CCR-5 and CXCR-4 as co-receptors for entry (Alkhatib *et al.* (1996); Doranz *et al.* (1996); Feng *et al.* (1996); Cheng-Mayer *et al.* (1997); Dittmar *et al.* (1997); Speck *et al.* (1997)) and only viruses very strongly adapted by long term passage in T cell lines (T Cell Line Adapted; TCLA) are unable to utilise CCR-5 in these *in vitro* systems (Alkhatib *et al.* (1996); Deng *et al.* (1996); Doranz *et al.* (1996); Feng *et al.* (1996); Zhang *et al.* (1996); Björndal *et al.* (1997); Cheng-Mayer *et al.* (1997); Dittmar *et al.* (1997); Speck *et al.* (1997)) It appears that these CXCR-4 dependent strains have variation from both H at position 13 and I at position 32, but many other SI strains can still utilise CCR-5 and do not have these changes. It is also therefore apparent that CCR-5 usage is not restricted to those cells capable of infecting macrophages and other as yet undefined factors govern this. Therefore, SI viruses capable of using CCR-5 are not all dual tropic viruses and some are still classical T-tropic strains. Hence the model which became apparent from the analysis of known V3 loops is more relevant to *in vitro* culture properties than to transmission. This is because the ability to utilise CCR-5 does not simply imply the ability to be capable of infecting cells, such as macrophages, which may depend upon CCR-5 usage and other factors.

A recent report by Follis and colleagues (1998) also questioned the distinction of TCLA adapted strains being unable to utilise CCR-5. They adapted a primary SI isolate, which was able to utilise CCR-5 and CXCR-4 as expected, to become a TCLA strain, but this strain was still able to utilise CCR-5 and CXCR-4. The most obvious answer is that the adapted strain was not a classic TCLA, which are known to be sensitive to neutralisation by neutralising antibodies, unlike primary isolates, which are insensitive (Wrin and Nunberg (1994); McKnight *et al.* (1995); Wrin *et al.* (1995); Mascola *et al.* (1996)). However, in keeping with the TCLA association, the adapted strain was sensitive to neutralisation, unlike the primary isolate from which it was derived (Follis *et al.* (1998)). It has also been shown by others that neutralisation sensitivity is independent of co-receptor usage (LaCasse *et al.* (1998); Montefiori *et al.* (1998); Trkola *et al.* (1998)). Hence, co-receptor usage is not as simple as M-tropism relating to CCR-5 usage, or TCLA strains, which become neutralisation sensitive, losing the ability to utilise CCR-5. It is apparent that these factors are anything but simple and are most likely multi-factorial and possibly independent of one another. Therefore, the likelihood of a model being broadly applicable seems, in light of these findings, highly unlikely, although the V3 loop of the TCLA strain, which was still able to utilise CCR-5, did only have a H→R change a position 13 and was still I at position 32, so would have been expected to use CCR-5 by the model presented here.

In order, to address the receptor usage of the viral variants found infecting the Index, further attempts at culturing virus could be tried from different samples, but if this still failed an alternative would be to clone the *env* gene into a vector and study the receptor usage of the *env* gene alone. This could then allow dissection of the regions of *env* associated with receptor usage, in that the minimal regions which conferred use could be determined. This would allow, for example, the different V3 loops to be put in a similar background and determine the co-receptor usage relative to the original V3 loop. This approach would be more informative than a direct culture approach, which may select for specific variants and could also result in a mixed population. A mixed population could then yield confusing results, as it may contain variants which are capable of using CCR-5 and CXCR-4, or individual variants which can use only one. Both these situations would give the same result, as growth would be seen in CCR-5⁺ and CXCR-4⁺ cell lines with both such possibilities. This was shown recently by Scarlatti *et al.* (1997), who studied the evolution of co-receptor usage over time in a cohort of HIV⁺ patients. They found some isolates which could use CXCR-4 and CCR-5, which when

passaged in cells only expressing one receptor, could not always then grow in cells expressing the other, from which they concluded a mixed population had been present in the isolate.

Scarlati *et al.* (1997) also found that virus present during the asymptomatic phase was usually CCR-5 dependent and that in individuals who progressed, the majority of later isolates could then use CXCR-4 and occasionally CCR-3 and gradually lost the ability to use CCR-5. A similar pattern was reported by Connor *et al.* (1997), who also showed a predominance of CCR-5 restricted usage during the asymptomatic phase and disease progression was associated with an expanded co-receptor repertoire to include: CXCR-4, CCR-5, CCR-3 and CCR-2b. These studies support the CCR-5 usage by the asymptomatic Index studied here.

Scarlati *et al.* (1997) also presented the V3 loop regions of some CXCR-4 restricted viruses which did not show the changes at positions 13 and 32, predicted by the model to relate to loss of CCR-5 usage. It is possible that a sequencing bias occurred, as they only sequenced the viral isolate cultured and not a single clone, so it could not be directly confirmed that the culture properties of the viral isolate related exactly to that of the sequenced variant. However, as no CCR-5 usage was seen for the isolate and the consensus sequence would have most likely reflected the major variant, then it is unlikely that a variant too low to establish infection in culture would then be the variant sequenced. This therefore casts doubt on the relevance of the model on patient derived isolates and seems to reflect the properties of highly adapted laboratory strains, which have little relevance to *in vivo* variants.

The limitations of the V3-CCR-5 usage model was further highlighted by a study by Bjorndal and colleagues (1997), who assessed the co-receptor usage of a diverse panel previously described (Gao *et al.* (1996)) primary isolates representing a variety of different HIV-1 subtypes. The model was again questioned as several of the different subtypes, which are diverse in the V3 region did not show changes at both position 13 and 32, but apparently were unable to utilise CCR-5. It is possible that the differences in the different systems, i.e. differences in the cells transfected with the chemokine receptors may be responsible for the apparent discrepancy and a well defined standardised cell type and system would be required to investigate any true association of V3 with co-receptor usage, much like the use of the MT-2 cell line for defining SI phenotype (Koot (1992)).

A different consensus model was recently proposed by Xiao and colleagues (1998). They reported that CCR-5 usage correlated with an uncharged residue at position 11 of the V3 loop (usually serine or glycine), the presence of the GPG motif and a negatively charged

residue at position 25 (usually glutamic, or aspartic acid) which was confirmed using site directed mutagenesis. When this motif was applied to the sequences used to form the model in this study (see Table 5.8), then many strains were unassigned and Xiao's model seems less sound than the one proposed in this study. For the isolates in the study by Speck *et al.* (1997) (e.g. 123/126/134/241), some are identical in their predicted properties at position 11/GPG and 25, but show actual differences in co-receptor usage (see Table 5.8 and 5.5). It therefore seems apparent that the predicted model is very dependent upon which sequences were used to form it and clearly the actual correlates of CCR-5 usage are not simply obtained from known associations.

5.4.2.c Phylogenetic Analysis

The heterosexual transmission to the first Contact, C3151, occurred very early in the infection of the Index, around the time of seroconversion. This would therefore have coincided with the primary viraemia in the Index and the high viral load found at this time (Jacquez *et al.* (1994)). It is clear from several studies that a high viral load in the index is associated with an increased risk of transmission (Lee *et al.* (1996); Fiore *et al.* (1997); Ragni, Faruki and Kingsley (1998)), presumably by an increase in the seminal viral load and hence potential inoculum to the Contact. Although only limited sampling occurred at this time, the virus is most likely to have been NSI in nature (Roos *et al.* (1992); Zhu *et al.* (1993); van't Wout *et al.* (1994)). It is also known that a high degree of identity is present in the viral population in the early stages of infection (Kleim *et al.* (1991); Wike *et al.* (1992); Wolfs *et al.* (1992); Wolinsky *et al.* (1992); Scarlatti *et al.* (1993); Zhang *et al.* (1993); Zhu *et al.* (1993); van't Wout *et al.* (1994)), although a few cases of transmission of multiple variants have been reported (van't Wout *et al.* (1994); Briant *et al.* (1995); Zhu *et al.* (1995); Janini *et al.* (1998); Wade, Lobidel and Leigh Brown (1998)). Hence the virus sampled early in the Index is highly likely to be related to the transmitted variant, confirmed in the F0 gag sample which was found to cluster with the first mother-child pair in phylogenetic reconstructions (Wade (1997) PhD thesis). Even in the first sample for *env* (F5), the virus was still shown to cluster with the virus in the group A of Index variants, presumably linked to the earlier viral population which was transmitted (Wade (1997) PhD thesis).

By the time the Index transmitted to the next Contact, C3152, he had been infected for approximately 6-8 years, was clinically stable and in the asymptomatic stage of disease. It is therefore unlikely that transmission was related to a high viral load, as this has been shown to

Table 5.8. : Predicted CCR-5 Sequence from Model Predicted by Xiao *et al.* (1998)

	1	11	21	31	11*	GPG*	25*	Predicted CCR-5#	Actual CCR-5#
Consensus [§]	CTRPNNNTRKSIHI--GPGRAFYTTEIIGDIRQAH								
NL43		R.QR	V.I.K.-.NM		u	v	+	-/+	-
123		R.S	R.-Q.V.NL		u	v	u	-/+	-
126			R.-Q.V.NL		u	v	u	-/+	+
134		S	R.-Q.V.NL		u	v	u	-/+	-
241		R.S	R.-Q		u	v	u	-/+	+
242		R.S	R.-		u	v	+	+	+
254		R.S	R.-Q.V		u	v	u	-/+	+
255		R.S	R.-Q.V.L		u	v	u	-/+	-
256		R.S	R.-Q.V.N		u	v	u	-/+	+
HXB2R/IIIB		R.R.QR	V.I.K.-.NM		+	v	+	-	-
SF-2		Y	H.R	K	u	v	+	-/+	+
SF2Mu3		Y	A.D	K	u	v	-	+	+
SF-162		T	A.D		u	v	-	+	+
SF162Db1		TR	V.A.D		u	v	-	+	+
BaL					u	v	+	+	+
JR-CSF	S				u	v	+	+	+
JR-FL					u	v	+	+	+
89.6		RRLS	ARRN		+	v	u	-/+	+
YU2		N	L		u	v	+	+	+
ADA					u	v	+	+	+
RF		TK	VI.A.Q	K	u	v	u	-/+	+
GUN WT		T	HAIEK	N	u	v	+	-/+	+
GUN-1 V		T.S	HAIEK	N	u	n	+	-/+	-/+
GUN V.Es.1		T	HAIEK	N	u	v	+	-/+	+
GUN V.Es.2		T.S	INAIEK	N	u	n	+	-/+	+
GUN V.Es.3		T.S	T.QAIEK	N	u	n	+	-/+	+
GUN V.Es.4		T.S	LHAIEK	N	u	n	+	-/+	+

* Model predicts that CCR-5 usage correlates with an uncharged (u) residue at position 11, the GPG motif -y- yes/ n-no and a negatively (-) charged residue at position 25, rather than u, or positive (+).

Predicted CCR-5 usage from Xiao *et al.* (1998) model (+: CCR-5 used/ -: CCR-5 not used/ -/+: unclear from properties. Actual CCR-5 usage as for Predicted except -/+; low level usage.

be a strong prognostic marker of disease progression (Venet *et al.* (1991); Ruiz *et al.* (1996)). As the Index then remained asymptomatic during the remaining period of the study (until 1996), the disease status does not reflect the difference in transmission compared to non-transmission in the later HIV⁺ Contact as it did for the first.

During the phylogenetic reconstructions performed by Dr. Wade (Wade (1997) PhD thesis), the virus group which clustered with the second mother (C3152)-child pair was group D, so further sequence analysis of later time points was performed to see if this virus group was no longer represented in the Index at the time of the later relationships, which may explain the lack of transmission. However, at all time points relating to their exposure period, virus closely related to the group D variants, which were linked to the second heterosexual transmission by Dr. Wade, were still present in the Index. This was true for analysis of both *gag* and *env* regions of the virus. Hence, the loss of potentially transmissible virus was unlikely to be the reason for the lack of transmission to the later Contacts (C3153 and C3154), as genetically related virus to the transmitted variant was still present at the time of their exposure.

5.4.2.c.i Contamination Screening

The discovery of a single contaminant from other material present in the Centre, highlights the importance of such screening when forming conclusions from sequence data, particularly when working from single copy amplifications. The phylogenetic reconstruction also clearly highlighted that the Sample 3 sequences were too divergent, for both *gag* and *env*, to represent those from the Index. This sample was most likely a sample mix-up, which although every care is taken to avoid, can still happen. Sample mixing and contamination was recently described by Frenkel *et al.* (1998), who showed that most PCR-positive, yet antibody negative reports in an earlier study of children born to HIV⁺ mothers, were proved to either a sample mix-up, or contamination. This further highlights the importance of such checks when performing analysis of this kind (Learn *et al.* (1996); Frenkel *et al.* (1998)).

5.4.3 Conclusions

Two of the four heterosexual contacts of an HIV⁺ Index, have possible explanations for their serostatus. The first Contact, C3151, was HIV⁺ and most likely became infected during the primary infection of the Index coincident with a high viral load. The third Contact,

C3153, was uninfected and was probably protected by the fact that she was homozygous for the $\Delta 32$ deletion in the CCR-5 gene. As transmission seems to be limited to NSI variants and she was shown to be protected from *in vitro* infection with an NSI, but not SI variant, she was highly unlikely to have become infected from sexual contact with this Index.

It is still unclear why the remaining two Contacts were HIV⁺, for C3152, and HIV-negative for C3154. It was shown that it was unlikely to be due to a change in the viral population in the Index, as virus, related to the variant transmitted to C3152, was still present when the final two uninfected Contacts were exposed. It may be that other factors than those studied here related to their increased, or decreased susceptibility, such as the level of co-receptor expression. C3154 was a heterozygote for the $\Delta 32$ mutation in CCR-5 and C3152 was WT homozygous, which may mean that the level of CCR-5 expression differed in these two individuals (Moore (1997); Wu *et al.* (1997b); Paxton *et al.* (1998)), but this could not be confirmed. Immune defence mechanisms controlled by the individual's HLA phenotype could also have explain the difference, but it is also possible that it could just been their good, or bad fortune.

A study of the infectibility of other EUs revealed an inverse relationship between the infection with a CCR-5 restricted isolate and a CCR-5, or CXCR-4 restricted one. This may reflect the level of co-receptor expression, but as the overall nature of this assay was poor, further study would be needed to clarify and confirm these findings.

Chapter 6

General Discussion

CHAPTER 6 - GENERAL DISCUSSION

6.1. HLA

6.2. OTHER IMMUNE RESPONSES

6.3. CHEMOKINE RECEPTORS

6.4. FACTORS AFFECTING TRANSMISSION FROM THE HIV⁺ INDEX

6.5. DIFFERENCES FROM AFRICAN COHORTS

6.6. CONCLUSIONS

Heterosexual transmission is by far the major mode of HIV transmission on a global scale (approximately 70%)(Expert Group of the Joint United Nations Programme of HIV/AIDS (1997)) and is the most rapidly increasing risk factor for new infections even in Western countries (Balfe (1998)), where transmission initially occurred predominately in homosexuals and IDUs. Although the transmission rate in the Edinburgh Heterosexual cohort was not high (Fielding *et al.* (1995); Robertson *et al.* (1998)), studying such a cohort can help to further our understanding of the factors which affect heterosexual HIV transmission. The mechanisms governing protection may differ from the more widely studied homosexual transmission, which globally is much less common. The Edinburgh Heterosexual Partner study was a well defined cohort and was not confounded by viral variation, as much of the index patients were infected with fairly similar subtype B virus (Holmes *et al.* (1995); Leigh Brown *et al.* (1997)).

6.1. HLA

Several associations related to HLA have been found in this study which deserve attention in other cohorts. The increased frequency of DR5 in the EUs suggests that this HLA type may be a protective one for HIV transmission, possibly through DR11, and warrants further study. The same is true for the decreased frequency of DR6 in the EUs, implicating DR6 as a potential risk factor for acquiring HIV infection through heterosexual contact and which requires confirmation in a larger cohort study. These HLA types may be more or less likely to mount a particular immune response to a particular antigen, with either a protective, or deleterious effect. The mimicry of HIV proteins to those of the MHC, may also be linked to a particular HLA type and again, may result in a protective response against a particular protein, perhaps due to prior exposure to a mis-matched HLA antigen. In contrast, the mimicry may induce inappropriate anti-self responses and increase the risk of HIV infection and disease.

The other HLA based associations are potentially linked to each other. The finding of a higher degree of mis-matched HLA antigens in discordant compared to concordant couples, if substantiated in a larger study, may reflect the increase of potentially protective allogeneic immune responses. This was supported by the finding of an increase in IFN- γ production in response to alloantigen in EUs compared to controls and may reflect *in vivo* priming of allogeneic responses. Alternatively, it may be that the EUs mounted a strong type 1 CMI response to the antigen and this type of response has been suggested in other EU cohorts to be

a potential protective mechanism (Clerici and Shearer (1993); Clerici and Shearer (1994); Barcellini *et al.* (1995)). The IFN- γ production may then be more likely in EUs with a high degree of HLA mis-match with their partner and could work in a variety of ways. IFN- γ itself has been shown to be able to inhibit replication of HIV (Hammer *et al.* (1986); Nakashima, Yoshida and Yamamoto (1986); Koyanagi *et al.* (1988); Hartshorn *et al.* (1987); Wong *et al.* (1988); Kornbluth *et al.* (1990); Meylan *et al.* (1993)), particularly in macrophages and may also occur in DCs. IFN- γ is also known to be a strong inducer of CMI and may augment lytic reactions to both virus and HIV-infected cells, both of which would contain 'foreign' MHC proteins. Both macrophage and NK cell mediated lysis are activated by IFN- γ and are therefore both potential protective mechanisms, because if an HIV-infected cell, either self, or foreign (e.g. seminal lymphocyte) is destroyed, this would prevent further viral replication and hence, help control an infection.

The importance of allogeneic responses has also been seen in monkey studies (Stott (1991); Langlois *et al.* (1992); Chan *et al.* (1995)) and the suggestion of an allogeneic vaccine, which may protect from HIV infection, is further supported by the preliminary findings of this thesis. Despite a recent report which questioned the role of anti-MHC antibody responses (Luscher *et al.* (1998)), the increased production of IFN- γ seen in this thesis suggests that CMI responses are also potentially induced and may be protective. The presence of proliferative response to alloantigen were not studied by Luscher and colleagues and therefore the potential of an allogeneic vaccine in light of these findings is still substantial.

6.2. OTHER IMMUNE RESPONSES

The role of HIV-specific immune responses in protection from HIV transmission could not be confirmed in this study, but the presence of both HIV-specific proliferative and CTLs in other EUs (Ranki *et al.* (1989); Borkowsky *et al.* (1990); Clerici *et al.* (1992); Kelker *et al.* (1992); Clerici *et al.* (1993); Clerici *et al.* (1994); de Maria, Cirillo and Moretta (1994); Langlade-Demoyen *et al.* (1994); Pinto *et al.* (1995); Rowland-Jones *et al.* (1995); Mazzoli *et al.* (1997)), suggest that such responses are protective in some individuals. It is possible that a strong allogeneic response may also delay viral replication long enough for HIV-specific ones to be generated and the two may therefore complement each other in terms of protection. It was for this reason that I hoped to monitor a wide variety of responses and potentially different mechanisms to see if they had a combined, or additive effect.

The importance of non-lytic suppression in EUs was suggested by the high IFN- γ production in response to alloantigen. As described above, the IFN- γ has anti-HIV activity in many cells, although has only been seen to be effective in inhibiting replication in PBMCs in the presence of other factors (Wong *et al.* (1988)). The precise factors involved in non-lytic suppression are still controversial, although the role of the β -chemokines clearly deserves investigation, in light of the findings of the potential protective role of a lack of, or reduced CCR-5 expression in homosexual EUs (Dean *et al.* (1996); Biti *et al.* (1997); O'Brien *et al.* (1997); Theodorou *et al.* (1997); Balotta *et al.* (1997); Paxton *et al.* (1998)). These and other chemokines to as yet undiscovered receptors could be used as receptor antagonists, blocking viral entry by preventing binding. Partial inhibitors, which lack the full functional properties of the chemokines may prove to reduce the potential side effects of the actual ligands.

The multitude of effects, e.g. non-contact mediated versus contact mediated (see Section 1.6.2.g), suggests that several factors are involved in non-lytic suppression and may differ from study to study depending upon the culture conditions and the cell type used. Also, as cytokines are generally both pleiotropic and redundant in their other actions in the body it is likely that several factors are involved in the non-lytic suppression of HIV to explain the contrasting results reported. However, the broad cross reactivity with different stains of HIV and even other retroviruses (Mackewicz, Ortega and Levy (1991); Mackewicz and Levy (1992); Walker *et al.* (1991)), along with the discovery of a similar mechanism in the natural, non-pathogenic infection of African green monkeys with SIV_{AGM} (Ennen *et al.* (1994)), stress the importance of this mechanism, being both a potential protective one and also one which could be utilised for both prophylactic and therapeutic purposes.

Mucosal immunity may also be important in heterosexual transmission and HIV-specific secretory IgA have been reported in EUs (Mazzoli *et al.* (1997)). Cervical CTLs have been seen in HIV⁺ individuals (Musey *et al.* (1997)) and it could be investigated in EUs too. Other innate defence mechanisms may also prevent transmission, such as neutrophils which have been shown to have anti-HIV activity (Klebanoff and Coombs (1992)). NK cells are also a potentially very important defence against viral infections and may be involved in allogeneic responses too, as they are known to be both activated by and also to produce IFN- γ . These innate mechanisms are often overlooked, often due to difficulties in studying them, but their importance is highlighted by the lower rate of heterosexual transmission compared to other modes.

6.3. CHEMOKINE RECEPTORS

No protective effect of the $\Delta 32$ mutation in the *CCR-5* gene was seen in this heterosexual cohort, compared to the protective effect of homozygosity seen in homosexual cohorts (Dean *et al.* (1996); Biti *et al.* (1997); O'Brien *et al.* (1997); Theodorou *et al.* (1997); Balotta *et al.* (1997)) and may come from differences in the mode of transmission in these two different risk groups. LCs are lacking in the rectal mucosa (Hussain and Lehner (1995)) and suggests that different cell types may be infected following homosexual contact and hence may explain the increased frequency of the $\Delta 32$ mutation in the homosexual EU cohorts. Perhaps other factors are involved in heterosexual transmission, such as the receptor expression level, hence the lack of selection for $\Delta 32$ homozygotes and that receptor level expression is less critical on the potential cells infected in homosexual HIV transmission. However, Paxton (1998) did note a decreased *CCR-5* expression in EU homosexuals compared to normal controls, therefore this is not a simple answer. Perhaps the act of anal sex, which may be more likely to cause damage and contact with blood, could then induce an upregulated expression of the co-receptor, but this does not occur commonly during heterosexual contact. This is support with the finding that the practise of anal sex in heterosexual couples is seen as a high risk factor for HIV transmission (European Study Group (1992); Fielding *et al.* (1995)).

It remains to be elucidated if heterosexual transmission is dependant upon *CCR-5* usage, or if other receptors may also be utilised, such as *CCR-2* and *CCR-3*, or one of the newly described (Liao *et al.* (1997)), or yet to be described receptors. This is supported by the finding by Zhang *et al.* (1998) of few *CCR-5*⁺ cells in the vaginal and cervical mucosa, suggesting that some other factor may be involved, but that in the rectal mucosa, *CCR-5*⁺ cells were more common.

Clearly, *CCR-5* usage on its own does not confer M-tropism (Alkhatib *et al.* (1996); Doranz *et al.* (1996); Feng *et al.* (1996); Cheng-Mayer *et al.* (1997); Dittmar *et al.* (1997); Speck *et al.* (1997)), so perhaps this is limited by further factors, which are as yet unknown and may then differ in infection of macrophages, T cells and DCs. While tropism is strongly linked to the V3 loop, the V1 and V2 regions have also been shown to be important (Boyd *et al.* (1993); Groenink *et al.* (1993); Sullivan *et al.* (1993)) and the precise mechanisms of both co-receptor interaction and tropism determinants require further understanding.

A much larger cohort would be required to detect an effect of homozygosity for $\Delta 32$ on heterosexual HIV transmission in the Edinburgh EUs. However, although the sample size was small, the allele frequency is elevated in this population, therefore any affect would have

been expected to be more detectable in this population. It is still not known why the frequency of $\Delta 32$ allele is so high in Caucasian populations. It is possibly just a founder effect and due to the apparent lack of a deleterious effect on individuals bearing it, no selection against it has allowed it to persist. It is also possible that it conferred a selective advantage to a previous, unknown pathogen, such as is seen by the similar mutation in the Duffy antigen, which is utilised by the malaria parasite (Mallinson *et al.* (1995)).

With regard to CCR-2, further understanding as to the effect of the 64I mutation on heterosexual transmission is required, before the apparent increased risk to individuals bearing this mutation can be fully explained. Its linkage disequilibrium with the 927T mutation in the CCR-5 promoter (Kostrikis *et al.* (1998)) seems an incomplete explanation, in light of the fact it is in an apparent intron and no difference in CCR-5 expression was seen in cells from 64I individuals. However, surprisingly a decrease in CXCR-4 was seen and may mean that other receptors may also be effected. As explained for the CCR-5 mutation, the cell type infected may differ from those studied and also differ in different risk groups and therefore the 64I mutation as a risk factor needs confirming in other heterosexual cohorts and the effect of the mutation on HIV replication and receptor level expression needs to be studied in a variety of different cell types.

6.4. FACTORS AFFECTING TRANSMISSION FROM THE HIV⁺ INDEX

In an attempt to investigate if the known associations with disease progression, also affected transmission, the HLA types and CCR genotypes were determined in indexes who either transmitted to their heterosexual partner (TRI), or did not transmit (NTRI). No significant associations were found, although the sample size was limited. An additive effect of the two CCR mutations was seen, which suggested they were both associated with a reduced risk of transmission. The known relationship of these mutations with viral load (Meyer *et al.* (1997)) and the association of viral load on risk of transmission (Fiore *et al.* (1997)) is a possible explanation of this result which would require investigation in a larger cohort.

The effect of viral variation was studied in more detail in an index with four heterosexual partners, to determine if changes in the viral population could account for the differences in transmission to the four contacts. The first contact was most likely infected during, or around the time of seroconversion of the index and hence explains the increased chance for transmission. The third contact was homozygous for the $\Delta 32$ mutation in CCR-5

and her PBMCs were shown to resist M-tropic, CCR-5 restricted virus infection in an *in vitro* assay. This may explain her apparent protection as CXCR-4 restricted strains were not seen in the index. The difference in transmission to the remaining two contacts was not obvious, but was shown not to be due to differences in potential transmissible viral variants.

The final EU contact was heterozygous for the $\Delta 32$ mutation, but the effect this may have had on transmission was not clear from the results presented in this thesis. It is possible that other factors may have protected the final contact, or increased the risk of transmission to the second contact, such as the receptor level expression, the presence of immune responses, or other genetic factors.

6.5. DIFFERENCES FROM AFRICAN COHORTS

Despite the fact that many epidemiological based studies have been carried out in Africa, such studies are often difficult to perform and maintain in often difficult regions and important discoveries may come from studying a European cohort which could then aid our understanding of African cohorts. However, it should be noted that important differences between the cohorts may exist. For example, socio-economic differences which result in a lower standard of nutrition, healthcare and sanitation for an African population, could all affect the general health and well-being of individuals and make them more susceptible to infectious diseases in general. The higher incidence of disease may include sexually transmitted diseases, which are known to increase the risk of HIV transmission (Greenblatt *et al.* (1988); Laga *et al.* (1993)) and may even result in different cell types being exposed to HIV after recruitment to a site of inflammation. The importance of this is highlighted by the possible differences between homosexual and heterosexual infection (see Section 6.3).

The different subtypes of HIV-1 have been suggested to differ in their efficiency of heterosexual spread (Kunanusont *et al.* (1995); Williamson *et al.* (1995); Mastro *et al.* (1997)) and also DC-tropism (Soto-Ramirez *et al.* (1996); Essex *et al.* (1997)) although CCR-5 usage has been shown not to differ in different subtypes (Gao *et al.* (1996); Zhang *et al.* (1996); Cheng-Mayer *et al.* (1997); Dittmar *et al.* (1997)). It is therefore possible that this may also depend upon usage of other receptors, although potential differences in the ability to replicate in DCs still remain controversial (Dittmar *et al.* (1997); Pope *et al.* (1997b); Pope *et al.* (1997a)). The effect of subtype on transmission was recently reported in a Swedish cohort (Sönnnerborg *et al.* (1998)), where they saw an increase in non-subtype B infections and a concurrent increase in heterosexually acquired infections too. Although some of this data were

acquired from immigrants into Sweden and may be confounded by other factors associated with differences in susceptibility in individuals of different race. It remains to be seen if the introduction of non-subtype B virus into the Swedish population results in substantial spread.

The different genetic makeup of African versus Caucasian populations may also affect the risk of transmission. This may result from differences in HLA types directing different immune responses, or even the CCR mutations. The $\Delta 32$ mutation in CCR-5 is not found in persons of African descent suggesting an explanation for the apparent increased heterosexual transmission of HIV in African populations. However, even in the Scottish population with a high $\Delta 32$ allele frequency (14%), only 2% of the population would be predicted to be homozygotes by the Hardy-Weinberg law. It seems unlikely that the difference in transmission can be accounted for by 2% of individuals. The lack of a protective effect of heterozygosity for the $\Delta 32$ mutation described in this thesis and recently in another heterosexual cohort (O'Brien *et al.* (1998)), suggests that the $\Delta 32$ mutation is unlikely to account for the intercontinental differences in HIV-1 incidence.

Individuals of African descent have been shown to have an increased frequency of the 64I mutation in CCR-2 (15.1% African Americans compared to 9.8% Caucasians (Smith *et al.* (1997))) and if this is a risk factor for acquiring HIV infection heterosexually may account for some of the increased incidence in African populations. This would require confirmation in an African heterosexual cohort, of HIV and HIV-negative individuals.

6.6. CONCLUSIONS

Several different mechanisms of protection from heterosexual HIV transmission were investigated in this thesis and both genetic and immunological differences were seen in EUs compared to HIV⁺ individuals and controls. It is unlikely that any one factor will be protective in all individuals, in all risk groups which stresses the importance of investigating several factors in several different cohorts. As with any infectious disease the paradigms of route, dose and susceptibility are also crucial to HIV transmission and must be considered in a varied range of individuals if the most important protective factors are ever to be determined, with the long term aim of preventing, or reducing HIV transmission world-wide.

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Appendix

APPENDIX

- Appendix 1.1. Example of Interview Performed at Recruitment**

- Appendix 3.1. Percentage Subset Population of Lymphocytes in PBMCs**
- Appendix 3.2. Stimulation Indices Obtained Following Lymphoproliferation Assays**
- Appendix 3.3. Range of Values Obtained for Lymphoproliferation Assay in Counts per Minute (cpm)**
- Appendix 3.4. IFN- γ Production Obtained Following Lymphoproliferation Assays**
- Appendix 3.5. IFN- γ Production Obtained Following Lymphoproliferation Assays - Adjusted Values**

- Appendix 4.1. HLA Types of Exposed Uninfected (EUs) Individuals**
- Appendix 4.2. HLA Types of Individuals Studied**
- Appendix 4.3. HLA Types of Non-Transmitting Indexes (NTRIs)**
- Appendix 4.4. Frequency of HLA Types in Population Controls**
- Appendix 4.5. Genotypes for CCR Polymorphisms and Mutations in EUs**
- Appendix 4.6. Genotypes for CCR Polymorphisms and Mutations in Heterosexually HIV-Infected Individuals**
- Appendix 4.7. Genotypes for CCR Polymorphisms and Mutations in Controls**
- Appendix 4.8. Genotypes for CCR Mutations in Transmitting Indexes**
- Appendix 4.9. Genotypes for CCR Mutations in Non-Transmitting Indexes**
- Appendix 4.10. Nucleotide Sequence for *CCR-5* Gene**

- Appendix 5.1. Nucleotide Sequence of *env* Gene (V3) From I3151**
- Appendix 5.2. Nucleotide Sequence of *gag* Gene (p17) From I3151**

Appendix 1.1.

Example of Interview Performed at Recruitment.

The following interviews are examples of the questions asked at recruitment into the Heterosexual Partner Study (see Section 1.8.2).

a.) Female Contact

b.) Male Contact

A.

INITIAL INTERVIEW - FEMALE CONTACT

1. Contact Code Number

2. Participating Centre

3. Date of Interview

4. Interviewer Code

PERSONAL DETAILS

First, I'd like to ask you some questions about yourself:

5. What is your date of birth?

6. Where were you born?

- (1 = UK 2 = Eire 3 = European, specify_____
- 4 = Uganda 5 Other African, specify_____
- 6 = Other, specify_____

7. Ethnic Origin

- 1 = white 2 = African, specify_____
- 3 = Indian Sub-continent 4 = West Indian/Guyana
- 5 = Other or mixed, specify_____

PROBABLE MODE OF TRANSMISSION

8. Have you ever injected any substance or drug under your skin or into your vein?

0 = No 1 = Yes 9 = NK

9. When was the last time? (mmyy) 9999 = NK/NA

10. Have you ever shared works with others?

0 = No 1 = Yes 9 = NK

11. When did you last share? (mmyy)

12. Have you ever had a blood transfusion or blood products? (mmyy) never = 0000 DK = 9999

13. Have you ever had an injury which brought you in contact with human blood? eg needlestick or sharps injury.

0 = No 1 = Yes 9 = DK

If yes, when (mmyy)

14. Do you have a current partner? (last 6 months)

0 = no 1 = yes

15. Do you know his HIV status?

0 = negative 1 = positive
2 = never tested 9 = DK/NA

16. How many other sexual partners have you had in

a) the last 6 months

b) the last 5 years

17. How many of these were

- a) haemophiliac
- b) bisexual
- c) drug users
- d) HIV/Aids

18. What is your relationship to (Name of Index)?

- 1 = Spouse
- 2 = Cohabitee
- 3 = Regular girlfriend
- 4 = Casual girlfriend
- 5 = Other, specify
- 9 = Missing

Regular girlfriend = sex more than three times
 Casual girlfriend = sex less than three times

GENERAL HEALTH

Now I'd like to ask you some questions about your general health:-

19. Have you ever suffered from a glandular fever like illness?

- 0 = No
- 1 = Yes
- 9 = DK

If Yes, when (mmyy)

--	--	--	--

20. Was that confirmed by a blood test?

21. Have you had any of the following in the last 6 months?

- | | | |
|-------|---------------------|--------------------------|
| Code: | 01 = rash | 05 = flu like illness |
| | 02 = fever | 06 = day or night sweats |
| | 03 = diarrhoea | 07 = loss of appetite |
| | 04 = swollen glands | 08 = fatigue |

Condition	Code	Date started	No. of weeks
.....	[][]	[][][][][][][][]	[][]
.....	[][]	[][][][][][][][]	[][]
.....	[][]	[][][][][][][][]	[][]
.....	[][]	[][][][][][][][]	[][]
.....	[][]	[][][][][][][][]	[][]
.....	[][]	[][][][][][][][]	[][]
.....	[][]	[][][][][][][][]	[][]
.....	[][]	[][][][][][][][]	[][]

22. Have you ever suffered from:-

	Most recent year	0=No 1=Yes 9=NK/NA	Times in last 5yrs 00=No 99=NK/NA
a) Abnormal cervical smear	<input type="checkbox"/>	<input type="text"/>
b) Cervical erosion/cervicitis	<input type="checkbox"/>	<input type="text"/>
c) Salpingitis/P.I.D. (infection of the tubes)	<input type="checkbox"/>	<input type="text"/>
d) Chlamydia/NSU contact	<input type="checkbox"/>	<input type="text"/>
e) Herpes simplex (around the vulva)	<input type="checkbox"/>	<input type="text"/>
f) Warts (around the vulva)	<input type="checkbox"/>	<input type="text"/>
g) Gonorrhoea	<input type="checkbox"/>	<input type="text"/>
h) Syphilis	<input type="checkbox"/>	<input type="text"/>
i) Hepatitis A	<input type="checkbox"/>	<input type="text"/>
B	<input type="checkbox"/>	<input type="text"/>
C	<input type="checkbox"/>	<input type="text"/>

PREGNANCY AND CONTRACEPTION

23. Now I would like to talk about the length of your relationship with (name).

- a) When did your sexual relationship with (name) begin? (mm/yy)
- b) Is your relationship continuing now?
0 = no 1 = yes 9 = DK
- c) If No, when was the last time you had sexual contact? (mmyy) NA = 9999
- d) Have you ever been pregnant by (name)?
0 = no 1 = yes 9 = DK
- If yes, total number of pregnancies (including miscarriage and abortions)
9 = NA

24. When did you first learn that your partner was HIV infected. (mm/yy) DK = 9999

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25. During the last 5 years of your relationship (or total period if less than 5 years) with (name) what contraception have you used? Here is a list of methods.

	198	198	199	199	199	199	Months of Use
a) Abstinence	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
b) Sheath or sheath + spermicide	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
c) Pill	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Code number of months for which each method was used in each year. Where more than one method used, eg, pill and condom, code both. Code abstinence for periods of no sexual contact.

SEXUAL PRACTICES

The next few questions are about your sexual activities in your relationship with (Name). These are very personal questions but they are a very important part of our research as to how people can be protected from the A.I.D.S. virus. You can leave out any questions you want to, but all your answers are completely confidential.

27. Over the period of your relationship with (name) on average how often would you do these things?

	Never	Once	<1/	<1/wk	1-2	>3/	DK/
			1/	to	/wk	/wk	NA
			1/mth				
Vaginal intercourse	0	1	2	3	4	5	9
in last 6/12	0	1	2	3	4	5	9

28. On average how many times a year do you have unprotected intercourse with (Name) during menstruation?

--	--

29. Have you ever noticed any bleeding from the vulva or vagina after intercourse? (other than during period) 1 = No 2 = Yes 9 = DK

30. On average during your relationship, how often do you have unprotected rectal or anal intercourse with (name)?

	Never	Once	<1/ mth	1/wk to 1/mth	1-2 /wk	>3/ /wk	DK/ NA	
On average	0	1	2	3	4	5	9	<input type="checkbox"/>
last 6/12	0	1	2	3	4	5	9	<input type="checkbox"/>

31. Have you ever noticed any bleeding after anal intercourse?

32. Since 1979 have you had sexual intercourse with anyone from a foreign country?

Country	Code	No. of contacts
.....	<input type="text"/>	<input type="text"/>
.....	<input type="text"/>	<input type="text"/>

HIV Antibody Test (from clinical notes)

Last negative

First Positive

CLINICAL STATUS AT INTERVIEW (CDC codes)

(from clinical notes)

00=not infected

01=acute infection (I)

02=asymptomatic infection (II)

03=PGL (III)

04=constitutional disease (IVA)

05=neurological disease (IVB)

06=AIDS - secondary infectious disease (IV C1)

07=other infectious disease (IV C2)

08=AIDS cancers (IVD)

09=other conditions (IVE)

99=NK

B, INITIAL INTERVIEW - MALE CONTACT

- 1. Contact Code Number
- 2. Participating Centre
- 3. Date of Interview
- 4. Interviewer Code

PERSONAL DETAILS

First, I'd like to ask you some questions about yourself:

- 5. What is your date of birth?
- 6. Where were you born?
1 = UK 2 = Eire 3 = European, specify _____
4 = Uganda 5 Other African, specify _____
8 = Other, specify _____
- 7. Ethnic Origin
1 = white 2 = African, specify _____
3 = Indian Sub-continent 4 = West Indian/Guyana
5 = Other or mixed, specify _____

PROBABLE MODE OF TRANSMISSION

- 8. Have you ever injected any substance or drug under your skin or into your vein?
0 = No 1 = Yes 9 = NK
- 9. When was the last time? (mmyy) 9999 = NK/NA
- 10. Have you ever shared works with others?
0 = No 1 = Yes 9 = NK/NA

11. When did you last share? (mmyy)

12. Have you ever had a blood transfusion or blood products? (mmyy)

13. Have you ever had an injury which brought you in contact with human blood? e.g. needlestick or sharps injury.

0 = No, 1 = Yes
9 = N/K

If yes, when (mmyy)

14. Do you have a current partner? (last 6 months)
0 = no 1 = yes

15. Do you know her/his HIV status?
0 = negative 1 = positive
2 = never tested 9 = DK

16. How many other sexual partners have you had in
a) in the last 6 months?

Male
Female

b) in the last 5 years?

Male
Female

17. How many of these were
a) haemophiliac
b) bisexual
c) drug users
d) HIV/Aids

18. Have you ever had sex with a man
0 = No 1 = Yes 9 = DK

If yes;
When was the last time (mmyy)
9999 = DK/NA

19. What is your relationship to (Name of Index Case)?

- 1 = Spouse
- 2 = Cohabitee
- 3 = Regular boyfriend
- 4 = Casual boyfriend
- 5 = Other, specify
- 9 = Missing

Regular boyfriend = sex more than three times
 Casual boyfriend = sex less than three times

GENERAL HEALTH

Now I'd like to ask you some questions about your general health:-

20. Have you ever suffered from a glandular fever like illness?

- 0 = No
- 1 = yes
- 9 = DK

If yes, when (mmyy)

--	--	--	--

21. Was that confirmed by a blood test?

22. Have you had any of the following in the last 6 months?

- | | | |
|-------|-----------------------|--------------------------|
| Code: | 01 = rash | 06 = cold sores |
| | 02 = fever | 07 = shingles |
| | 03 = diarrhoea | 08 = day or night sweats |
| | 04 = swollen glands | 09 = loss of appetite |
| | 05 = flu like illness | 10 = fatigue |

Condition	Code	Date started	No. of weeks
.....	□□	□□□□□□□□	□□
.....	□□	□□□□□□□□	□□
.....	□□	□□□□□□□□	□□
.....	□□	□□□□□□□□	□□
.....	□□	□□□□□□□□	□□
.....	□□	□□□□□□□□	□□
.....	□□	□□□□□□□□	□□
.....	□□	□□□□□□□□	□□

23. Have you ever suffered from:-

	Most recent year	0=No 1=Yes 9=NK/NA	Times in last 5yrs 00=No 99=NK/NA
a) Balanitis	<input type="checkbox"/>	<input type="checkbox"/>
b) Proctitis	<input type="checkbox"/>	<input type="checkbox"/>
c) NSU	<input type="checkbox"/>	<input type="checkbox"/>
d) Herpes Simplex - penile	<input type="checkbox"/>	<input type="checkbox"/>
perianal		<input type="checkbox"/>	<input type="checkbox"/>
e) Warts - penile	<input type="checkbox"/>	<input type="checkbox"/>
perianal	<input type="checkbox"/>	<input type="checkbox"/>
f) Gonorrhoea - urethral	<input type="checkbox"/>	<input type="checkbox"/>
rectal	<input type="checkbox"/>	<input type="checkbox"/>
g) Syphilis	<input type="checkbox"/>	<input type="checkbox"/>
i) Hepatitis A	<input type="checkbox"/>	<input type="checkbox"/>
B	<input type="checkbox"/>	<input type="checkbox"/>
C	<input type="checkbox"/>	<input type="checkbox"/>

24. Now I would like to talk about the length of your relationship with (name).

a) When did your sexual relationship with (name) begin? (mm/yy)

b) Is your relationship continuing now?
0 = no 1 = yes 9 = DK

c) If No, when was the last time you had sexual contact? (mmyy)

25. When did you first learn that your partner was HIV infected. (mm/yy)

26. During the last 5 years of your relationship (or total period if less than 5 years) with (name) what contraception have you used? Here is a list of methods.

	198 ⁸	198 ⁹	199 ⁰	199 ¹	199 ²	199 ³	Months of use
a) Abstinence	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="text"/>
b) Sheath or sheath + spermicide	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="text"/>
c) Pill	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="text"/>

Code number of months for which each method was used in each year. Where more than one method used, eg, pill and condom, code both. Code abstinence for periods of no sexual contact.

SEXUAL PRACTICES

The next few questions are about your sexual activities in your relationship with (Name). These are very personal questions but they are a very important part of our research as to how people can be protected from the A.I.D.S. virus. You can leave out any questions you want to, but all your answers are completely confidential.

27. Over the period of your relationship with (name) on average how often would you do these things?

	Never	Once	<1/ mth	<1/wk to 1/mth	1-2 /wk	>3/ /wk	DK/ NA	
Vaginal intercourse in last 6/12	0	1	2	3	4	5	9	<input type="text"/>
	0	1	2	3	4	5	9	<input type="text"/>

28. On average how many times a year do you have unprotected intercourse with (Name) during menstruation?

29. Have you ever noticed any bleeding from the vulva or vagina after intercourse? (other than during period) 0 = No 1 = Yes 9 = DK

30. On average during your relationship, how often do you have unprotected rectal or anal intercourse with (name)?

	Never	Once	<1/ mth	1/wk to 1/mth	1-2 /wk	>3/ /wk	DK/ NA	
On average	0	1	2	3	4	5	9	<input type="checkbox"/>
last 6/12	0	1	2	3	4	5	9	<input type="checkbox"/>

31. Have you ever noticed any bleeding after anal intercourse?

32. Since 1979 have you had sexual intercourse with anyone from a foreign country?

Country	Code	No. of contacts
.....	<input type="text"/>	<input type="text"/>
.....	<input type="text"/>	<input type="text"/>

HIV Antibody Test (from clinical notes):

	Mo	Yr
Last negative	<input type="text"/>	<input type="text"/>
First Positive	<input type="text"/>	<input type="text"/>

CLINICAL STATUS AT INTERVIEW (CDC codes)
(from clinical notes)

- 00=not infected
- 01=acute infection (I)
- 02=asymptomatic infection (II)
- 03=PGL (III)
- 04=constitutional disease (IVA)
- 05=neurological disease (IVE)
- 06=AIDS - secondary infectious disease (IV C1)
- 07=other infectious disease (IV C2)
- 08=AIDS cancers (IVD)
- 09=other conditions (IVE)
- 99=NK

Appendix 3.1.

Percentage Subset Population of Lymphocytes in PBMCs

The following Tables show the % of lymphocytes of the subsets shown (see 2.8.1). The CD4:CD8 cell ratio is also given along with the number of individuals assessed (n), the group mean and standard deviation (SD). Assays not performed are listed as Not Done (ND).

a.) Results for the Exposed Uninfecteds (EUs)

b.) Results for the Donors (D)

A

	% CD3:CD4	% CD3:CD8	% DR	%CD45 RO	% DR:RO	CD4:CD8
C3154	37	30	ND	ND	ND	1.23
C3942	33	20	ND	ND	ND	1.65
C4051	17	53	20	44	5	0.32
C4371	26	23	20	23	3	1.13
C4401	43	47	ND	ND	ND	0.91
C5191	39	33	21	30	6	1.18
C5411	51	27	15	47	2	1.89
C5451	29	27	40	20	1	1.07
C5621	60	15	17	51	8	4.00
C5711	38	15	ND	ND	ND	2.53
C5721	40	28	11	24	2	1.43
C5731	28	27	22	33	4	1.04
C5761	49	22	17	39	3	2.23
n	13	13	9	9	9	13
Mean	37.69	28.23	22.17	35.83	4.17	1.49
SD	11.53	11.11	9.02	13.20	2.64	1.04

B

	% CD3:CD4	% CD3:CD8	% DR	%CD45 RO	% DR:RO	CD4:CD8
D1	44	19	12	22	3	2.32
D2	45	20	14	41	3	2.25
D3	50	23	14	42	4	2.17
D4	42	18	23	37	10	2.33
D5	39	13	14	32	3	3.00
D6	26	43	11	24	2	0.60
D7	52	25	11	30	2	2.08
D8	30	21	17	16	4	1.43
D9	41	25	12	45	3	1.64
D10	23	24	ND	ND	ND	ND
D11	35	33	ND	ND	ND	ND
D12	47	25	ND	ND	ND	ND
D13	44	26	ND	ND	ND	ND
n	13	13	9	9	9	9
Mean	39.85	24.23	14.22	32.11	3.78	1.98
SD	8.97	7.40	3.80	9.99	2.44	0.68

Appendix 3.2.

Stimulation Indices Obtained Following Lymphoproliferation Assays

The following Tables show the stimulation indices (SI) obtained following proliferation to various given antigens at the concentrations shown (see 2.7.1). SI was determined by the following equation:

$$SI = \frac{\text{CPM obtained from cells alone (medium only)}}{\text{CPM obtained cells with antigen}}$$

The geometric mean counts per minute (cpm) obtained for the medium only (No Antigen) are also given along with the 95% Confidence Intervals (CI). Assays not performed are listed as Not Done (ND).

The number of individuals assessed for each antigen (n) are given along with the mean, standard deviation (SD) and the median of the group for each antigen.

- a.) Results for the Exposed Uninfecteds (EUs)
- b.) Results for the Donors (D)

A

	PHA 5 µg/ml	PHA 1 µg/ml	ALLO 1:1	PPD 100 U/ml	TT 2.5 µg/ml	TT 1.25 µg/ml	rHIV 1.25 µg/ml	rHIV 0.625 µg/ml	rHIV 0.125 µg/ml	No Antigen cpm (+/- 95% CI)
C3154	50.27	3.41	15.41	1.04	49.99	33.32	1.30	1.17	ND	88.92 (84.86-93.16)
C3942	163.44	11.06	21.65	0.62	4.10	1.03	1.15	1.46	ND	77.42 (63.11-94.96)
C4051	1085.40	219.08	448.27	439.60	4.58	12.79	3.85	4.51	ND	34.44 (26.90-44.11)
C4371	483.02	179.98	59.40	6.94	13.42	11.87	4.89	3.97	3.23	55.55 (46.32-66.62)
C4401	185.68	122.09	164.60	164.84	126.67	107.82	ND	ND	ND	65.39 (50.77-84.22)
C4712	2064.87	225.36	11.92	29.59	1.16	1.36	2.31	1.93	0.89	96.74 (74.28-125.99)
C5191	469.46	61.28	378.74	146.72	7.88	33.80	4.61	5.91	5.77	44.94 (34.23-59.01)
C5411	226.72	41.55	51.53	178.12	1.02	1.02	1.10	1.10	2.90	90.43 (66.95-122.16)
C5451	238.24	91.42	39.38	0.81	1.80	1.85	8.38	13.16	11.44	75.97 (60.03-96.16)
C5621	124.07	74.24	305.79	115.49	0.66	1.28	0.92	1.71	13.53	112.73 (97.66-130.12)
C5711	574.60	261.85	66.64	40.42	36.53	43.23	1.79	2.52	ND	95.81 (85.00-108.00)
C5721	0.53	16.87	ND	98.45	41.83	272.62	1.52	8.94	ND	32.31 (26.29-39.71)
C5731	338.27	95.40	283.13	134.00	181.61	152.93	0.28	0.57	0.92	113.68 (90.88-142.19)
C5761	333.67	39.23	19.72	29.09	46.71	41.39	ND	ND	ND	129.82 (79.20-203.07)
n	14	14	13	14	14	14	12	12	7	
Mean	452.73	103.06	143.55	98.98	37.00	51.16	2.68	3.91	5.53	
SD	538.46	86.10	155.64	117.97	54.01	77.87	2.34	3.80	5.07	
Median	285.97	85.83	59.40	69.44	10.65	23.06	1.66	2.23	3.23	

B

	PHA 5 µg/ml	PHA 1 µg/ml	ALLO 1:1	PPD 100 U/ml	TT 2.5 µg/ml	TT 1.25 µg/ml	rHIV 1.25 µg/ml	rHIV 0.625 µg/ml	rHIV 0.125 µg/ml	No Antigen cpm (+/- 95% CI)
D1	297.60	96.80	13.32	1000.96	3.55	6.89	2.44	2.45	21.46	52.75 (42.53-65.90)
D2	142.20	48.80	9.75	458.10	1.14	1.06	4.63	6.97	10.22	80.4 (61.57-105.00)
D3	199.58	31.41	15.11	148.24	0.87	1.87	2.21	1.00	3.08	75.75 (31.04-184.88)
D6	252.56	48.75	89.49	74.20	0.57	0.87	3.98	3.87	24.95	40.30 (34.03-45.08)
D7	611.13	126.75	411.14	1426.79	226.45	110.33	2.82	3.56	26.05	37.79 (29.84-47.79)
D8	147.24	32.31	36.86	3.98	107.48	56.94	1.86	1.60	4.17	66.28 (56.82-77.79)
D9	83.05	72.96	165.79	439.84	1.53	2.13	7.31	6.59	13.55	62.80 (46.79-84.33)
D14	107.52	16.70	10.76	20.79	1.02	1.73	2.15	2.82	5.10	69.65 (58.84-82.45)
D15	837.02	30.19	81.88	7.08	2.13	13.03	3.05	2.10	1.84	32.03 (22.08-46.46)
D16	110.84	28.52	122.50	146.59	1.94	9.22	1.88	3.06	4.33	34.35 (22.58-52.26)
D17	38.12	8.20	227.26	1.61	1.66	1.15	1.32	1.04	1.78	57.76 (32.64-103.05)
D18	573.81	23.28	20.24	8.22	61.95	52.72	1.88	1.60	5.42	50.72 (27.77-92.65)
n	12	12	12	12	12	12	12	12	12	
Mean	283.43	47.06	100.34	311.37	34.19	21.50	2.96	3.06	10.15	
SD	253.29	35.15	120.40	459.95	69.20	34.33	1.67	1.97	9.13	
Median	173.41	31.86	59.37	110.40	1.80	4.51	2.36	2.64	5.26	

Appendix 3.3.

Range of Values Obtained for Lymphoproliferation Assays in Counts Per Minute (cpm)

The following table shows the range of geometric mean values (cpm) obtained for the different antigens. The lowest and highest values recorded are given for the EUs and Donors, with the individual indicated. 95% Confidence Intervals (CI) for the variation from the geometric mean. The geometric mean values were then used to calculate the Stimulation Index (SI) for each antigen (see Appendix 3.2).

	EU	low [£] (cpm)	95% CI [#]	EU	high [£] (cpm)	95% CI	Donor	low [£] (cpm)	95% CI	Donor	high [£] (cpm)	95% CI
No Antigen	C4051	34.4	26-44	C5761	126.8	79-203	D15	32.0	22-46	D2	80.4	62-105
PHA (5mg/ml)	C3154	4470.2	4028-4961	C4712	199755.6	181514-219830	D17	2201.7	1750-2770	D18	29103.7	25134-33701
PHA (1mg/ml)	C3154	303.0	171-538	C4712	21801.1	17872-26574	D17	473.5	426-525	D1	5106.4	3600-7243
Allo (1:1)	C4712	1152.8	738-1801	C5621	34470.4	27367-43418	D1	1702.0	408-1210	D7	15524.7	14272-16887
PPD (100/ml)	C5451	61.8	52-73	C5411	16107.1	11461-22637	D17	93.2	69-127	D7	53875.6	38512-75369
TT (2.5mg/ml)	C5621	74.2	35-157	C5731	20645.6	13373-31873	D6	22.8	15-36	D7	8550.8	5891-12411
TT (1.25mg/ml)	C5411	92.6	52-164	C5731	17385.0	13265-22785	D6	34.9	26-46	D7	4166.1	3131-5543
rHIV (1.25mg/ml)	C5731	31.9	29-35	C5451	636.7	537-755	D16	64.7	49-86	D9	459.4	343-615
rHIV (0.625mg/ml)	C5731	64.2	28-148	C5451	869.2	390-1939	D17	59.9	38-95	D2	560.7	353-890
rHIV (0.125mg/ml)	C4712	85.9	60-122	C5621	1525.2	160-3060	D15	58.8	42-82	D1	1132.0	613-2090

[£] Range of geometric mean counts per minute (cpm), lowest value for group (low) to highest value for group (high)

[#] 95% Confidence Interval (CI) of variation from geometric mean

Appendix 3.4.

IFN- γ Production Obtained Following Lymphoproliferation Assays

The following Tables show the IFN- γ produced (pg/ml) following proliferation to various given antigens at the concentrations shown (see 2.7).

Assays not performed are listed as Not Done (ND) and data excluded due to inconsistent replicates are indicated with a blank.

The number of individuals assessed for each antigen (n) are given along with the mean, standard deviation (SD) and the median of the group for each antigen.

- a.) Results for the Exposed Uninfecteds (EUs)
- b.) Results for the Donors (D)

A

	No Antigen	PHA 5 µg/ml	PHA 1 µg/ml	ALLO 1:1	PPD 100 U/ml	TT 2.5 µg/ml	TT 1.25 µg/ml	rHIV 1.25 µg/ml	rHIV 0.625 µg/ml	rHIV 0.125 µg/ml
C3154		1978.3	144.0	6584.7	189.7	994.0	1154.2	511.8	970.8	ND
C3942		3688.2		22300.0	0.0		0.0		0.0	ND
C4051	0.0		12450.0	>75000.0	15400.0	0.0	0.0		441.0	ND
C4371	0.0	29900.0	2625.2	56500.0	940.9	880.1	545.0	>1500.0		
C4401	421.5	28000.0	23400.0	42500.0	4588.4	>1500.0	>1500.0	ND	ND	ND
C4712	0.0	17400.0	10500.0	>1500.0	1402.3	0.0	0.0	0.0	0.0	
C5191	594.6	9780.0	5373.7	>75000.0	1602.8	380.5	854.7	396.4		264.9
C5411	283.2	31200.0	36400.0	>75000.0	19800.0	0.0	0.0	270.3		549.5
C5451	1108.6	4213.9	1665.5	12900.0	344.7			0.0	0.0	1473.1
C5621	0.0	35300.0	19500.0	2096.5	>1500.0	0.0	0.0	0.0	0.0	800.3
C5711		29600.0	5183.0	59000.0	909.8	213.7	454.8	0.0	0.0	ND
C5721	0.0	3606.8	1179.8	6000.9	1162.4	0.0	284.8	0.0	0.0	ND
C5731	0.0	>75000.0	11700.0	12000.0	10200.0	>1500.0	>1500.0		>1500.0	>1500.0
C5761	0.0	6163.0	2610.0	36600.0	947.8	>1500.0	>1500.0	ND	ND	ND
n	11	13	13	14	14	12	13	9	9	5
Mean	218.9	21217.7	10210.1	34498.7	4213.5	580.7	599.5	297.6	323.5	917.6
SD	362.4	20475.4	10697.9	28990.1	6304.1	650.6	627.5	493.1	552.8	552.9
Median	0.0	17400.0	5373.7	29450.0	1282.4	297.1	454.8	0.0	0.0	800.3

B

	No Antigen	PHA 5 µg/ml	PHA 1 µg/ml	ALLO 1:1	PPD 100 U/ml	TT 2.5 µg/ml	TT 1.25 µg/ml	rHIV 1.25 µg/ml	rHIV 0.625 µg/ml	rHIV 0.125 µg/ml
D1	0.0	19600.0	1102.6	0.0	8382.4	0.0	0.0	0.0	0.0	>1500.0
D2	0.0	38600.0	15600.0	341.0	24900.0		393.3	297.9	187.3	314.3
D3	672.2	25950.0	16450.0	0.0			0.0	0.0	182.5	345.4
D6	0.0	33600.0	3019.2	56700.0	1807.9	0.0	0.0	0.0	0.0	>1500.0
D7	203.3	70100.0	24100.0	>75000.0	26300.0	>1500.0	939.2	214.6	0.0	>1500.0
D8		15200.0	8960.0	68200.0	0.0	683.1	570.0		0.0	
D14	0.0	36700.0	701.6	279.3	134.8	0.0	0.0	0.0	0.0	264.7
D15	217.1	13100.0	782.5	233.6	280.5	136.6	84.2		0.0	
D16	0.0	5137.9	361.6	367.9	763.8	0.0		0.0	0.0	
D17		3727.4	1515.6	1629.5	0.0	0.0	0.0		0.0	
n	8	10	10	10	9	8	9	7	10	6
Mean	136.6	26171.5	7259.3	20275.1	6952.2	290.0	220.7	73.2	37.0	904.1
SD	236.4	19806.9	8576.1	32289.3	10902.5	542.8	612.4	127.3	78.0	653.3
Median	0.0	22775.0	2267.4	354.4	763.8	0.0	0.0	0.0	0.0	922.7

Appendix 3.5.

IFN- γ Production Obtained Following Lymphoproliferation Assays - Adjusted Values

The following tables show the IFN- γ produced (pg/ml) following proliferation to various given antigens at the concentrations shown (see 2.7), with any spontaneous production (No Antigen; see Appendix 3.4) subtracted.

Assays not performed are listed as Not Done (ND) and data excluded due to inconsistent replicates are indicated with a blank.

The number of individuals assessed for each antigen (n) are given along with the mean, standard deviation (SD) and the median of the group for each antigen.

- a.) Results for the Exposed Uninfecteds (EUs)
- b.) Results for the Donors (D)

A

	PHA 5 µg/ml	PHA 1 µg/ml	ALLO 1:1	PPD 100 U/ml	TT 2.5 µg/ml	TT 1.25 µg/ml	rHIV 1.25 µg/ml	rHIV 0.625 µg/ml	rHIV 0.125 µg/ml
C4051		12450.0	>75000.0	15400.0	0.0	0.0		441.0	ND
C4371	29900.0	2625.2	56500.0	940.9	880.1	545.0	>1500.0		
C4401	27578.5	22978.5	42078.5	4166.9	1078.5	1078.5	ND	ND	ND
C4712	17400.0	10500.0	>1500.0	1402.3	0.0	0.0	0.0	0.0	
C5191	9185.4	4779.1	74405.4	1008.2	0.0	260.1	0.0		0.0
C5411	30916.8	36116.8	74716.8	19516.8	0.0	0.0	0.0		266.3
C5451	3105.3	556.9	11791.4	0.0			0.0	0.0	364.7
C5621	35300.0	19500.0	2096.5	>1500.0	0.0	0.0	0.0	0.0	800.3
C5731	>75000.0	11700.0	12000.0	10200.0	>1500.0	>1500.0		>1500.0	>1500.0
C5761	6163.0	2610.0	36600.0	947.8	>1500.0	>1500.0	ND	ND	ND
n	9	10	10	10	9	9	6	5	5
Mean	26061.0	12381.7	38668.9	5508.3	551.0	542.6	250.0	388.2	586.3
SD	21805.1	1148.6	30519.7	7016.4	680.6	649.8	612.4	650.2	586.5
Median	27578.5	11100.0	39339.3	1451.2	0.0	260.1	0.0	0.0	364.7

B

	PHA 5 µg/ml	PHA 1 µg/ml	ALLO 1:1	PPD 100 U/ml	TT 2.5 µg/ml	TT 1.25 µg/ml	rHIV 1.25 µg/ml	rHIV 0.625 µg/ml	rHIV 0.125 µg/ml
D1	19600.0	1102.6	0.0	8382.4	0.0	0.0	0.0	0.0	>1500.0
D2	38600.0	15600.0	341.0	24900.0		393.3	297.9	187.3	314.3
D3	25277.8	15777.8	0.0			0.0	0.0	0.0	0.0
D6	33600.0	3019.2	56700.0	1807.9	0.0	0.0	0.0	0.0	>1500.0
D7	69896.7	23896.7	74796.7	26096.7	1296.7	735.9	0.0	0.0	1296.7
D14	36700.0	701.6	279.3	134.8	0.0	0.0	0.0	0.0	264.7
D15	12882.9	564.4	0.0	63.4	0.0	0.0		0.0	
D16	5137.9	361.6	367.9	763.8	0.0		0.0	0.0	
n	8	8	8	7	6	7	7	8	6
Mean	30211.9	7628.0	16560.6	8878.4	216.1	161.3	42.6	23.4	812.6
SD	19873.8	9328.4	30742.6	11714.7	529.4	292.7	112.6	66.2	691.1
Median	29438.9	2060.9	310.1	1807.9	0.0	0.0	0.0	0.0	805.5

ID	SEX	A		B		DR	
C3021	F	2	24	7	62	1	4
C3022	F	3	31	44	<i>40/41</i>	4	6
C3061	F	11	24	35	51	11	11
C3073	F	2	11	8	35	3	3
C3131	M	2	11	7	37	4	11
C3141	F	2	28	21	44	11	15/2
C3153	F			7	35	1	7
C3154	F			49	<i>35/15/50/53</i>	1	11
C3241	F	1	3	8	35	3	13
C3292	F			8	15	2	3
C3311	M	3	24	5	57	4	7
C3321	F	3	<i>3/11</i>	35	44	1	2
C3351	F	1	<i>1/23</i>	8	44	3	<i>3/6</i>
C3361	F			44	44	7	7
C3372	F	2	32	14	44	3	7
C3622	F			39	44	4	11
C3631	M	1	3	7	7	2	11
C3672	F	1	2	39	39	3	4
C3723	F	3	11	15	<i>42/7/67</i>	4	14
C3781	F	2	33	14	35	3	6
C3791	F	3	11	35	37	1	4
C3851	F	1	29	8	44	3	7
C3942/5041	F	2	24	44	44	4	11
C3961/5191	F	3	30	14	18	3	13
C4051	M	1	2	62	37	2	6
C4141	M	2	2	62	62	1	11
C4191	F	31	<i>31/32</i>	14	60	4	7
C4261	F			15	55	2	4
C4272	F			39	40	7	13
C4311	F	2	29	44	60	3	4
C4331	M	1	3	14	51	2	6
C4371	F	1	2	8	44	3	4
C4401	F	24	68	40	15	4	11
C4421	M			27	44	4	4
C4431	M	1	2	14	14	2	11
C4441	M	2	2	44	55	11	11
C4471	M	2	29	44	44	4	7
C4631	M	1	2	51	53	3	3
C4882	M			8	18	3	<i>3/6</i>
C5051	F	2	11	13	55	4	7
C5052	F	2	11	35	8	1	3
C5191	F	3	30	14	18	3	13
C5211	F	2	3	<i>50/35</i>	<i>51/52</i>	2	7
C5212	F	11	68	27	15	9	13
C5221	F			18	44	1	3
C5231	F	1	2	8	49	3	4
C5341	F	3	30	14	18	3	8
C5351	F	2	3	44	44	2	11
C5411	F	1	<i>28/68</i>	8	<i>35/52</i>	1	2
C5451	M	1	3	35	17	1	7
C5471	M	1	3	7	8	3	11
C5491	F	1	23	8	44	2	7
C5531	F	11	11	7	51	7	11
C5691	F			7	44	4	12
C5711	F			7	7	2	2
C5721	F			51	44	1	7
C5731	M			14	35	7	13

Numbers in *italics* indicate those with undefined/ inconsistent typing

Appendix 4.2.

HLA Types of Individuals Studied

The following tables show the HLA types of individuals studied (see Section 4.2.3)

a.) Heterosexually HIV-Infected Contacts

b.) Transmitting Indexes

A. HLA Types of Heterosexually HIV-Infected Contacts

ID	SEX	A		B		DR	
C3152	F	29	29	35	35	2	6
C3562/14631	F	1	2	8	39	1	3
C3611	F	2	11	7	8	2	3
C3891	F	25	29	44	51	4	5
C4001	M	2	28	7	27	3	8
C4061	F	2	11	7	27	2	2
C4101	F	24	26	14	60	3	7
C4151	F	2	3	8	18	3	3
C4211	F	2	2	7	8	2	3
C4241	F	2	29	44	35	7	7
C4461	M	1	9	8	13	3	5
C4681	F	1	2	62	62	1	1
C4691	F	26	28	35	44	2	6
C4831	M	2	28	27	44	1	7
C4901	F	2	30	13	13	4	7
C5011	F	1	31	57	60		
C5291	F	1	3	8	14	3	6

B. HLA Types of Transmitting Indexes

HET No.	SEX	A		B		DR	
I3071*	M	2	11	8	35	3	5
I3151*	M	2	2	49	55	2	4
I3372*	M	1	31	44	27	4	11
I3562	M	1	2	8	39	1	3
I3571	M	2	2	47	47	1	4
I3711	F	2	11	7	60	2	2
I3891	M	3	26	39	53	1	4
I3991	M	1	24	8	60	3	4
I4001	F	3	3	7	44		
I4101	M	3	28	35	37	3	6
I4151	M	1	11	7	44	2	4
I4211	M					4	7
I4241	M	11	29	7	7	3	3
I4341	M	3	29	7	44		
I4461	F	1	3	3	60	3	4
I4681	M	1	33	7	37	1	1
I4691	M	1	28	44	60	6	11
I4831	F	1	2	44	44	7	7
I4861	M	2	28	14	62	4	7
I4901	M	1	2	13	55	4	7
I5011	M	2	3	7	44	2	7
I5291	M	1	2	7	8	2	3

* Indicates indexes who had both concordant and discordant relationships and were excluded from some of the analysis

Appendix 4.3.

HLA Types of Non-Transmitting Indexes (NTRIs)

The following table shows the HLA types of individuals studied (see Section 4.2.3)

HET No.	SEX	A		B		DR	
I3021	M	3	30	13	14	6	7
I3061	M	1	2	7	44	1	7
I3071*	M	2	11	8	35	3	5
I3141	M	1	25	35	51	2	4
I3151*	M	2	2	49	55	2	4
I3241	M	11	24	7	14	4	7
I3311	F	2	11	62	38	4	11
I3321	M	1	2	7	8		
I3351	M	11	26	35	44	1	4
I3361	M	2	24	14	44	4	7
I3372*	M	1	31	44	27	4	11
I3631	F	3	29	7	44		
I3672	M	1	11	18	37	1	3
I3721	M	1	33	27	38	5	5
I3781	M	1	33	7	55	4	6
I3791	M	3	10	7	18	1	3
I3961	M	1	31	22	37	4	7
I4051	F	1	24	8	14	4	7
I4141	F	3	24	7	27	3	5
I4191	M	1	24	8	8	3	7
I4271	M	2	3	44	45	8	11
I4311	M	2	3	44	14	6	11
I4331	F	1	2	44	47	10	11
I4401	M	11	24	7	57	5	6
I4431	F	11	33	14	51	2	4
I4441	F	1	2	8	44	3	4
I4471	F	25	31	27	51	4	4
I4631/C3562	F	1	2	8	39	1	3
I4881	F	2	3	44	44	6	11
I5051	M	2	30	57	47	7	7
I5191	M	1	3	7	57	2	7
I5231	M	3	11	7	7	2	3
I5351	M	2	28	44	62	3	4
I5411	M	2	24	8	62	7	11

* Indicates indexes who had both concordant and discordant relationships and were excluded from some of the analysis.

Appendix 4.4.

Frequency of HLA Types in Population Controls

The following table shows the frequency of HLA types in a group of population controls (see Section 4.3.1.b).

HLA Antigen Frequencies In Population Controls*

	Individuals Expressing Antigen	Individuals Not Expressing Antigen
A1	97	167
A2	131	133
A3	81	183
A11	33	231
A23	13	251
A24	37	227
A25	11	253
A26	11	253
A28	17	247
A29	18	246
A30/31	16	248
A32	16	248
B5	21	243
B7	86	178
B8	80	184
B13	14	250
B14	22	242
B15	27	237
B16	6	258
B17	27	237
B18	6	258
B21	19	245
Bw22	11	253
B27	22	242
B35	32	232
B37	5	259
B40	25	239
Bw41	1	263
B44	73	191
B45	2	262
B47	5	259
DR1	27	237
DR2	77	187
DR3	83	181
DR4	66	198
DR5	33	231
DR6	93	171
DR7	86	178
DR8	10	254

* Numbers derived from those published by Jazwinska and Kilpatrick (1987).

Appendix 4.5.

Genotypes for CCR Polymorphisms and Mutations in EUs

The following table shows genotypes of the individuals studied (see Chapter 4).

ID	SEX	CCR-5 Genotype [*]	CCR-2 Genotype [#]	CCR-5 Promoter Genotype [‡]
C3021	F	WT/WT	WT/WT	1,1
C3022	F	WT/WT	WT/64I	1,1
C3061	F	WT/WT	WT/WT	1,4
C3073	F	WT/WT	WT/WT	1,4
C3131	M	WT/WT	WT/WT	4,4
C3141	F	WT/WT	WT/WT	1,1
C3153	F	Δ32/Δ32	WT/WT	1,1
C3154	F	WT/Δ32	WT/WT	1,4
C3241	F	WT/WT	WT/WT	1,4
C3292	F	WT/WT	WT/WT	1,2
C3311	M	WT/Δ32	WT/WT	1,4
C3321	F	WT/Δ32	WT/WT	1,4
C3351	F	WT/Δ32	WT/WT	1,4
C3361	F	WT/WT	WT/WT	2,2
C3372	F	WT/WT	WT/WT	1,4
C3561	F	WT/WT	WT/WT	1,1
C3622	F	WT/WT	WT/WT	1,1
C3631	M	WT/Δ32	WT/WT	1,4
C3672	F	WT/WT	WT/WT	1,1
C3721	F	WT/WT	WT/WT	NT
C3723	F	WT/Δ32	WT/WT	1,1
C3781	F	WT/WT	WT/WT	1,4
C3791	F	WT/WT	WT/WT	1,4
C3851	F	WT/Δ32	WT/WT	1,4
C3942/5041	F	WT/Δ32	WT/WT	1,4
C4051	M	WT/WT	WT/WT	NT
C4141	M	WT/WT	WT/WT	1,1
C4191	F	WT/Δ32	WT/WT	1,4
C4261	F	WT/WT	WT/WT	1,1
C4272	F	WT/WT	WT/WT	1,2
C4311	F	WT/WT	WT/WT	NT
C4331	M	WT/WT	WT/64I	NT
C4371	F	WT/WT	WT/WT	NT
C4401	F	WT/WT	WT/WT	1,1
C4421	M	WT/WT	WT/WT	1,4
C4431	M	WT/Δ32	WT/64I	1,1
C4441	M	WT/WT	WT/WT	1,1
C4471	M	WT/WT	WT/WT	NT
C4631	M	WT/WT	WT/WT	1,4
C4882	M	WT/Δ32	WT/64I	1,1
C5051	F	WT/Δ32	WT/WT	1,1
C5052	F	WT/WT	WT/WT	1,4
C5111	F	WT/WT	WT/WT	NT
C5191	F	WT/WT	WT/WT	4,4
C5211	F	WT/WT	WT/WT	4,4
C5212	F	WT/WT	WT/WT	1,1
C5221	F	WT/Δ32	WT/WT	1,4
C5231	F	WT/WT	WT/WT	1,1
C5341	F	WT/WT	WT/WT	4,4
C5351	F	WT/Δ32	WT/WT	1,1
C5411	F	WT/WT	WT/WT	1,4
C5451	M	WT/Δ32	WT/WT	1,1
C5471	M	WT/WT	WT/WT	4,4
C5491	F	WT/Δ32	WT/WT	1,2
C5531	F	WT/WT	WT/WT	1,4
C5691	F	WT/WT	WT/WT	1,4
C5711	F	WT/Δ32	WT/WT	1,2
C5721	F	WT/WT	WT/WT	4,4

* CCR-5 Genotype for Δ32 Mutation (see Section 4.2.3.b)

CCR-2 Genotype for 64I mutation (see Section 4.2.3.d)

‡ Genotype for CCR-5 Promoter mutations (see Section 4.1)

NT - Not Tested

Appendix 4.6.

Genotypes for CCR Polymorphisms and Mutations in Heterosexually HIV-infected Individuals

The following table shows genotypes of the individuals studied (see Chapter 4).

ID	SEX	CCR-5 Genotype [*]	CCR-2 Genotype [#]	CCR-5 Promoter Genotype [£]
H1	F	WT/WT	WT/WT	1,2
H2	F	WT/Δ32	WT/WT	1,1
H3	M	WT/Δ32	WT/WT	1,1
H4	F	WT/Δ32	WT/WT	1,1
H5	F	WT/Δ32	WT/64I	1,1
H6	M	WT/Δ32	WT/WT	1,4
H7	F	WT/WT	WT/WT	1,4
H8	F	WT/WT	WT/WT	1,4
H9	M	WT/WT	WT/64I	1,1
H10	F	WT/WT	WT/64I	1,4
H11	F	WT/Δ32	WT/WT	1,4
H12	F	WT/WT	WT/WT	1,1
H13	F	WT/WT	64I/64I	1,1
H14	M	WT/Δ32	WT/WT	1,1
H15	F	WT/WT	WT/64I	1,4
H16	F	WT/WT	WT/WT	1,4
H17	F	WT/Δ32	WT/WT	1,1
H18	F	WT/WT	WT/WT	NT
H19	F	WT/WT	WT/WT	NT
H20	F	WT/Δ32	WT/WT	NT
H21	F	WT/WT	WT/WT	NT
H22	F	WT/WT	WT/WT	NT
H23	F	WT/WT	WT/WT	NT
H24	F	WT/WT	WT/WT	NT
H25	F	WT/WT	WT/WT	4,4
H26	F	WT/WT	WT/WT	1,1
H27	F	WT/Δ32	WT/WT	1,4
H28	F	WT/WT	WT/WT	4,4
H29	F	WT/WT	WT/64I	1,1
H30	F	WT/WT	WT/WT	1,4
H31	F	WT/WT	WT/WT	2,4
H32	F	WT/Δ32	WT/64I	1,1
H33	F	WT/Δ32	WT/WT	1,2
H34	F	WT/WT	WT/WT	NT
H35	F	WT/Δ32	WT/WT	1,4
H36	F	WT/WT	WT/WT	1,2
H37	F	WT/Δ32	WT/WT	1,1
H38	F	WT/WT	WT/WT	NT
H39	F	WT/WT	WT/WT	4,4
H40	F	WT/Δ32	WT/WT	1,4
H41	F	WT/WT	WT/WT	NT
H42	F	WT/WT	WT/WT	1,1
H43	F	WT/WT	WT/WT	NT
H44	F	WT/WT	WT/WT	NT
H45	F	WT/WT	WT/64I	1,4
H46	F	WT/WT	WT/WT	1,1
H47	F	WT/WT	WT/WT	2,2
H48	F	WT/WT	WT/WT	1,4
H49	F	WT/WT	WT/WT	1,1
H50	F	WT/WT	WT/WT	4,4
H51	F	WT/WT	WT/64I	1,4

ID	SEX	CCR-5 Genotype*	CCR-2 Genotype [#]	CCR-5 Promoter Genotype [£]
H52	F	WT/Δ32	WT/WT	1,4
H53	F	WT/WT	WT/WT	NT
H54	F	WT/WT	WT/WT	2,4
H55	F	WT/WT	WT/WT	NT
H56	F	WT/WT	WT/64I	1,4
H57	F	WT/WT	WT/WT	1,4
H58	F	WT/WT	WT/WT	4,4
H59	F	WT/WT	WT/WT	1,2
H60	F	WT/Δ32	WT/WT	1,1
H61	F	WT/WT	WT/WT	1,4
H62	F	WT/Δ32	WT/WT	1,1
H63	F	WT/WT	WT/WT	1,4
H64	F	WT/WT	WT/WT	1,1
H65	F	WT/WT	WT/64I	1,1
H66	F	WT/WT	WT/WT	1,1
H67	F	WT/Δ32	WT/WT	1,1
H68	F	WT/Δ32	WT/WT	1,1
H69	M	WT/WT	WT/WT	1,4
H70	M	WT/WT	WT/64I	1,1
H71	M	WT/WT	WT/WT	NT
H72	M	WT/WT	WT/WT	1,1
H73	M	WT/WT	WT/WT	1,4
H74	M	WT/WT	WT/WT	NT
H75	M	WT/WT	WT/WT	4,4
H76	M	WT/Δ32	WT/64I	1,1
H77	M	WT/Δ32	WT/WT	1,1
H78	F	WT/WT	WT/WT	1,4
H79	M	WT/WT	WT/WT	4,4
H80	M	WT/WT	WT/WT	1,4
H81	M	WT/WT	WT/WT	1,4
H82	M	WT/Δ32	WT/WT	1,2
H83	M	WT/WT	WT/WT	1,4
H84	M	WT/WT	WT/WT	1,4
H85	M	WT/WT	WT/WT	1,1
H86	M	WT/WT	WT/64I	1,1

* CCR-5 Genotype for Δ32 Mutation (see Section 4.2.3.b)

[#] CCR-2 Genotype for 64I mutation (see Section 4.2.3.d)

[£] Genotype for CCR-5 Promoter mutations (see Section 4.1)

NT - Not Tested

Appendix 4.7.

Genotypes for CCR Polymorphisms and Mutations in Controls

The following table shows genotypes of the individuals studied (see Chapter 4).

ID	CCR-5 Genotype [*]	CCR-2 Genotype [#]	CCR-5 Promoter Genotype [£]
C1	WT/WT	WT/WT	1,1
C2	WT/ Δ 32	WT/WT	1,1
C3	Δ 32/ Δ 32	WT/WT	1,1
C4	WT/WT	WT/WT	4,4
C5	WT/WT	WT/64I	1,1
C6	WT/WT	WT/WT	1,4
C7	WT/WT	WT/64I	1,2
C8	WT/WT	WT/WT	1,1
C10	WT/WT	WT/WT	2,4
C11	WT/WT	WT/WT	4,4
C12	WT/WT	WT/WT	4,4
C13	WT/ Δ 32	WT/WT	1,1
C14	WT/WT	WT/WT	1,2
C15	WT/WT	WT/64I	1,1
C16	WT/WT	WT/WT	1,4
C17	WT/WT	WT/WT	2,4
C18	WT/ Δ 32	WT/WT	1,1
C20	WT/WT	WT/WT	1,4
C21	WT/WT	WT/64I	1,4
C22	WT/WT	WT/WT	1,4
C23	WT/WT	WT/WT	1,2
C24	WT/WT	WT/WT	4,4
C25	WT/WT	WT/64I	1,1
C26	WT/WT	WT/WT	1,1
C27	WT/WT	WT/WT	1,1
C28	WT/ Δ 32	WT/WT	1,2
C29	Δ 32/ Δ 32	WT/WT	1,1
C30	WT/WT	WT/WT	2,4
C31	WT/ Δ 32	WT/WT	1,4
C32	WT/WT	WT/WT	1,4
C33	WT/ Δ 32	WT/WT	1,4
C34	WT/WT	WT/WT	1,1
C36	WT/WT	WT/WT	1,2
C37	WT/WT	WT/64I	1,2
C38	WT/WT	WT/WT	NT
C39	WT/WT	WT/64I	1,1
C40	WT/WT	WT/WT	1,2
C42	WT/ Δ 32	WT/WT	1,2
C43	WT/WT	WT/WT	4,4
C44	WT/WT	WT/WT	1,1
C45	WT/WT	WT/WT	1,4
C46	WT/ Δ 32	WT/WT	NT
C47	WT/WT	WT/WT	1,4
C48	WT/ Δ 32	WT/WT	1,4
C49	WT/WT	WT/WT	1,2
C50	WT/ Δ 32	WT/WT	1,4
C51	WT/WT	WT/WT	1,4
C52	WT/WT	WT/WT	1,1
C53	WT/WT	WT/WT	1,2
C54	WT/WT	WT/WT	4,4

^{*} CCR-5 Genotype for Δ 32 Mutation (see Section 4.2.3.b)

[#] CCR-2 Genotype for 64I mutation (see Section 4.2.3.d)

[£] Genotype for CCR-5 Promoter mutations (see Section 4.1)

NT - Not Tested

Appendix 4.8.

Genotypes for CCR Mutations in Transmitting Indexes

The following table shows genotypes of the individuals studied (see Chapter 4).

ID	SEX	CCR-5 Genotype*	CCR-2 Genotype#
I3071 [£]	M	(WT/WT)	(WT/WT)
I3151 [£]	M	(WT/WT)	(WT/WT)
I3372 [£]	M	(WT/WT)	(WT/WT)
I3451	M	WT/ Δ 32	WT/WT
I3562	M	WT/WT	WT/WT
I3571	M	WT/ Δ 32	WT/WT
I3611	M	WT/WT	WT/WT
I3711	F	WT/WT	WT/WT
I3891	M	WT/WT	WT/WT
I4061	M	WT/WT	WT/WT
I4101	M	WT/WT	WT/WT
I4151	M	WT/WT	WT/WT
I4211	M	WT/WT	WT/WT
I4241	M	WT/WT	WT/WT
I4341	M	WT/WT	WT/WT
I4461	F	WT/ Δ 32	WT/WT
I4691	M	WT/WT	WT/WT
I4831	F	WT/WT	WT/WT
I4861	M	WT/WT	WT/WT
I4901	M	WT/WT	WT/WT
I5011	M	WT/ Δ 32	WT/WT
I5291	M	WT/WT	WT/64I

* CCR-5 Genotype for Δ 32 Mutation (see Section 4.2.3.b)

CCR-2 Genotype for 64I mutation (see Section 4.2.3.d)

[£] Had both Concordant and Discordant relationships and were excluded from analysis

Appendix 4.9.

Genotypes for CCR Mutations in Non-Transmitting Indexes

The following table shows genotypes of the individuals studied (see Chapter 4).

ID	SEX	CCR-5 Genotype*	CCR-2 Genotype#
I3021	M	WT/WT	WT/WT
I3061	M	WT/WT	WT/WT
I3071 [‡]	M	(WT/WT)	(WT/WT)
I3131	F	WT/WT	WT/WT
I3141	M	WT/Δ32	WT/WT
I3151 [‡]	M	(WT/WT)	(WT/WT)
I3241	M	WT/WT	WT/WT
I3291	M	WT/WT	WT/64I
I3321	M	WT/WT	WT/WT
I3351	M	WT/Δ32	WT/WT
I3372 [‡]	M	(WT/WT)	(WT/WT)
I3621	M	WT/Δ32	WT/64I
I3631	F	WT/WT	WT/WT
I3672	M	WT/Δ32	WT/WT
I3721	M	WT/Δ32	WT/WT
I3781	M	WT/WT	WT/WT
I3791	M	WT/Δ32	WT/WT
I3851	M	WT/Δ32	WT/WT
I3942	M	WT/WT	WT/WT
I4051	F	WT/WT	WT/WT
I4141	F	WT/WT	WT/WT
I4191	M	WT/WT	64I/64I
I4261	M	WT/WT	WT/WT
I4271	M	WT/WT	WT/WT
I4311	M	WT/WT	WT/WT
I4331	F	WT/Δ32	WT/WT
I4371	M	WT/WT	WT/64I
I4401	M	WT/WT	WT/WT
I4421	F	WT/WT	WT/WT
I4431	F	WT/WT	WT/WT
I4441	F	WT/Δ32	WT/WT
I4471	F	WT/Δ32	WT/WT
I4631/C3562	F	WT/WT	64I/64I
I4881	F	WT/WT	WT/WT
I5041	M	WT/WT	WT/WT
I5051	M	WT/Δ32	WT/WT
I5111	M	WT/Δ32	WT/WT
I5191	M	WT/Δ32	WT/WT
I5211	M	WT/WT	WT/64I
I5231	M	WT/WT	WT/WT
I5351	M	WT/WT	WT/WT
I5411	M	WT/WT	WT/WT

* CCR-5 Genotype for Δ32 Mutation (see Section 4.2.3.b)

CCR-2 Genotype for 64I mutation (see Section 4.2.3.d)

[‡] Had both Concordant and Discordant relationships and were excluded from analysis

Appendix 4.10.

Nucleotide Sequence of *CCR-5* Gene

Nucleotide sequences obtained following sequencing of the *CCR-5* gene (see Section 4.2.3).

CCR-5 denotes the published consensus sequence (Genbank accession number: X91492). The sequences obtained for the four exposed uninfected individuals are given, identified by their contact identification (e.g. C5191) and does not start until position 248 on the consensus sequence (denoted with a dash). Dots indicate the sequence was identical to that of the consensus.

	1	11	21	31	41	51	61	71	81	91	101	111	120
CCR-5	GAAATTCOCCCAACAGAGCCAAAGCTCCACTAGTGGACAGGGAAGCTAGCAGCAAACTCCCTTCACACAAAATCTCAATGCTTGGCCAAAAAGAGAGTTAAATCAATGTAGACATC												
C3361	-----												
C4331	-----												
C4421	-----												
C5191	-----												
	121	131	141	151	161	171	181	191	201	211	221	231	240
CCR-5	TATGTAGCCAAATAAAAACCTATTGATGTATAAAAACAGTTGCATTTCAGAGGGCAACTAAAACATCTTAGGACTTTATAAAGATCACTTTTATTTATGACAGGGTGGAAACAAGA												
C3361	-----												
C4331	-----												
C4421	-----												
C5191	-----												
	241	251	261	271	281	291	301	311	321	331	341	351	360
CCR-5	TGGATTATCAAGTGTCAAGTCCAATCTATGACATCAATATATATACATCGGAGCCCTGGCCAAAAATCAAATGGAAGCAATCGCAGCCGGCTCTGGCTCCGCTACTCAGCTGGTGT												
C3361	-----												
C4331	-----												
C4421	-----												
C5191	-----												
	361	371	381	391	401	411	421	431	441	451	461	471	480
CCR-5	TCACTTTGGTTTTGTGGCCACATGCTGGTCACTCCATCTGATAAAGCTGCAAAAGGCTGAAGAGCATGACTGACATCTACTCTCAACCTGGCCATCTCTGACCTGTTTTCTCTC												
C3361	-----												
C4331	-----												
C4421	-----												
C5191	-----												
	481	491	501	511	521	531	541	551	561	571	581	591	600
CCR-5	TTACTGTCCCTTC TGGGCTACTATGCTGGCCGCCAGTGGGACTTTGGAAATACAATGTCTCACTCTTGACAGGCTCTATTTTATAGGCTTCTCTCTGGAAATCTTTCTTCAATCC												
C3361	-----												
C4331	-----												
C4421	-----												
C5191	-----												
	601	611	621	631	641	651	661	671	681	691	701	711	720
CCR-5	TCTGACAAATCGATAGGTACCTGGCTGCTGTCATGCTGTGTTGCTTTAAAAGCCAGGACGGTCACTTTGGGGTGGTGACAAGTGTGATCACTTGGGTGGTGGCTGTGTTGGCGTCTC												
C3361	-----												
C4331	-----												
C4421	-----												
C5191	-----												
	721	731	741	751	761	771	781	791	801	811	821	831	840
CCR-5	TCCCAGGAATCACTTTTACCAGATCTCAAAAAGAGGCTTCTTACACCTGCGAGCTCTCAATTTCCATACAGTCAGTATCAATCTCGAAGAAATTCAGACATTAAGATAGTCATCT												
C3361	-----												
C4331	-----												
C4421	-----												
C5191	-----												
	841	851	861	871	881	891	901	911	921	931	941	951	960
CCR-5	TGGGGCTGGTCTGGCCGCTGTTGTCATGGTCACTGCTACTCGGGAATCCTAAAAACTCTGCTTCGGTGTGCAAAATGAGAAGAAGAGGCACAGGGCTGTGAGGCTTATCTTCAACATCA												
C3361	-----												
C4331	-----												
C4421	-----												
C5191	-----												
	961	971	981	991	1001	1011	1021	1031	1041	1051	1061	1071	1080
CCR-5	TGATTGTTAATTTCTCTCTGGCTCCCTACAACATTTGCTCTCTCTGAACACCTTCCAGGAATCTTTGGCCTGAATAATGCGATAGCTCTAACAGTTGGACCAAGCTATGCAAGG												
C3361	-----												
C4331	-----												
C4421	-----												
C5191	-----												
	1081	1091	1101	1111	1121	1131	1141	1151	1161	1171	1181	1191	1200
CCR-5	TGACAGAGACTTTGGGATGACGCACTGCTGCATCAACCCCATCATCTATGCTTTGTGGGGAGAAGTTCAGAACTACCTCTTAGTCTCTTCGAAAGACATGGCCAAAGCTTCT												
C3361	-----												
C4331	-----												
C4421	-----												
C5191	-----												
	1201	1211	1221	1231	1241	1251	1261	1271	1281	1291	1301	1311	1320
CCR-5	GCAATGCTGTTCTAATTTCCAGCAAGAGGCTCCCGAGCGCAAGCTCAGTTTACACCGATCCACTGGGAGCAGGAAATATCTCTGGGCTGTGACAGGACTCAAGTGGGCTGGTG												
C3361	-----												
C4331	-----												
C4421	-----												
C5191	-----												
	1321	1331	1341	1351	1361	1371							
CCR-5	ACCCAGTCAGAGTTGTGCACATGGCTTAGTTTTTATACACAGCTGGGCTGGGGGT												
C3361	-----												
C4331	-----												
C4421	-----												
C5191	-----												

	1	11	21	31	41	51	61	71	81	91	101	111	120
CCR-5	GAATTCGCCCAACAGAGCAAGCTCTCCATCTAGTGGACAGGGAAGCTAGCAGCAAACTTCCTTCACACAAAACCTCAATTGCTTGCCAAAAAGAGAGTTAAATTCATGTAGACATC												
C3361												
C4331												
C4421												
C5191												
	121	131	141	151	161	171	181	191	201	211	221	231	240
CCR-5	TATGTAGGCAATAAAAACCTATGATGTATAAAACAGTTTCATTCATGGAGGCAACTAAATACATTCAGGACTTTATAAAAGATCACATTTTATTTATGACACGGGTGGAACAAGA												
C3361												
C4331												
C4421												
C5191												
	241	251	261	271	281	291	301	311	321	331	341	351	360
CCR-5	TGGATTATCAAGTGTCAAGTCCAATCTATGACATCAATATATATACATCGGAGCCCTGCCAAAAATCAATGTGAAGCAATGCCAGCCCGCTCCCTCCCGCTACTACATGGTGT												
C3361												
C4331												
C4421												
C5191												
	361	371	381	391	401	411	421	431	441	451	461	471	480
CCR-5	TCATCTTTGGTTTGTGGGCAACATGCTGGTCATCTCATCTGATAAACTGCAAAAGGCTGAAGAGCATGACTGACATCTACCTGCTCAACTGGCCATCTCGACCTGTTTTCTCTC												
C3361												
C4331												
C4421												
C5191												
	481	491	501	511	521	531	541	551	561	571	581	591	600
CCR-5	TTACTGTCCCTCTCGGGTCACTATGCTGCCGCCAGTGGACTTTGSAATACAAATGTGTCAACTCTTGACAGGGCTCTATTTATAGGCTTCTCTCTGGAACTTCTTCATCATCC												
C3361												
C4331												
C4421												
C5191												
	601	611	621	631	641	651	661	671	681	691	701	711	720
CCR-5	TCCGTGACATGATAGTACTGGCTGCTGCTCCATGCTGTTTGGCTTTAAAAGCCAGGACGGTCACCTTTGGGGTGGTGAACAAGTGTGATCAGTTGGGTGGTGGCTGTTTGGCTCTC												
C3361												
C4331												
C4421												
C5191												
	721	731	741	751	761	771	781	791	801	811	821	831	840
CCR-5	TCCGAGAAATCACTTTACCAAGATCTCAAAAAGAGGCTTTCATTACACTGCAGCTCTCAATTTCCATACAGTCAGTATCAAATCTGGAGAAATCCAGACATTAAGATAGTCACTC												
C3361												
C4331												
C4421												
C5191												
	841	851	861	871	881	891	901	911	921	931	941	951	960
CCR-5	TGGGGTGGTCC TGCCGCTGCTTTGTCAATGCTCTGCTACTCGGGAATCCTAAAAACTGCTTCGGTGTGAAATGAGAGAAAGGCACAGGGCTGTGAGGCTTATCTTCAOCATCA												
C3361												
C4331												
C4421												
C5191												
	961	971	981	991	1001	1011	1021	1031	1041	1051	1061	1071	1080
CCR-5	TGATTGTTATTTCTCTCTGGCTCCCTACAACATTCCTCTCTGAAACACTTCAGGAAATCTTTGGCCTGAATAATGCAATAGCTCTAACAGGTGGACCAAGCTATGCAAGG												
C3361												
C4331												
C4421												
C5191												
	1081	1091	1101	1111	1121	1131	1141	1151	1161	1171	1181	1191	1200
CCR-5	TGACAGAGACTTTGGGATGACGCACTGTCATCAACCCATCACTATGCTTTGTGGGGAGAAATCAGAACTACCTTTAGTCTCTTCCAAAAGCAGATTGCCAAACGCTTCT												
C3361												
C4331												
C4421												
C5191												
	1201	1211	1221	1231	1241	1251	1261	1271	1281	1291	1301	1311	1320
CCR-5	GCCAAATGCTGTTCTATTTCCAGCAAGAGCTCCCGAGCGAGCAAGCTCAGTTTACACCCGATCCACTGGGAGCAGGAAATATCTGTGGCTTGTGACAGGACTCAAGTGGGCTGGTG												
C3361												
C4331												
C4421												
C5191												
	1321	1331	1341	1351	1361	1371							
CCR-5	ACCCAGTCAGAGTTGTGCACATGGCTTAGTTTTTCATACACAGCCTGGGCTGGGGT												
C3361												
C4331												
C4421												
C5191												

Appendix 5.1.

Nucleotide Sequence of *env* Gene (V3) From I3151

Nucleotide sequences obtained following sequencing of the *env* gene (see Section 2.13 and 2.19).

The sequences are aligned to the first variant sequenced at 5 years post seroconversion (F5), with identical nucleotides to this sequence shown with a dot and alignment gaps noted with a dash. The sequences are labelled according to the samples number (1/2) and the individual sequence (.X).

	1	11	21	31	41	51	61	71	81	91	101	111	120
F5.1	TTTCAGGGAC	AATGCTAAAG	TCATAATAGT	ACAGCTGAAT	GAAACTGTAG	AAATTAATTG	TACAAGACCC	CACAGCAATA	CAAGTAAAGG	TATACATATA	GGACCAGGCA	GAGCATCTTA	
1.1T.AATAGG..AG	
1.2T.AATAG..A	
1.3ATA..A	
1.4ATA..A	
1.5ATA..AC	
1.6ATA..A	
1.7ATA..A	
1.8ATA..A	
1.9ATA..A	
1.10ATA..A	
1.11ATA..A	
1.12ATA..A	
1.13ATA..A	
1.14	C.....AA	C.....TTA..AAGT
1.15CTA..A	
2.1ATA..A	
2.2ATA..A	
2.3ATA..A	
2.4ATA..A	
2.5ATA..A	
2.6ATA..AG	
2.7ATA..A	
2.8ATA..A	
2.9ATA..AG	

	121	131	141	151	161	171	181	191	201	211	221	231	240
F5.1	TGCAACAGGA	AGAATAACAG	GAGATATAAG	ACAGCCACAT	TGTAACCTTA	GTAGAACAAA	ATGGAATGAC	ACTTTACAC	GGATAGTTAT	AAAGTTAAGA	GAACAATTTG	GAATTAATAA	
1.1AC	
1.2GCT	
1.3GATT	
1.4GATT	
1.5GATT	
1.6GATT	
1.7GATT	
1.8GATT	
1.9GATT	
1.10GATT	
1.11GATT	
1.12GATT	
1.13GACTG	
1.14CGATGT	
1.15GGATC	
2.1CGATT	
2.2CAT	
2.3CAT	
2.4CAT	
2.5AT	
2.6ACT	
2.7AT	
2.8CAT	
2.9CAT	

	241	251	261	271	281	291	301	311	321	331	341	351
F5.1	AACAATAGCC	TTTAATCGAT	CCTCAGGAGG	GGACCCAGAA	ATTGTAATGC	ACAGTTTTAA	TTGGGAGGG	GAATTTTTCT	ACTGTAATTC	ATCACAACCTG	TTTAATAGTA	CTTGG
1.1A
1.2A
1.3A
1.4A
1.5A
1.6A
1.7A
1.8A
1.9A
1.10A
1.11AA
1.12A
1.13T
1.14A
1.15A
2.1A
2.2AC
2.3A
2.4AC
2.5A
2.6A
2.7A
2.8A
2.9AC

Appendix 5.2.

Nucleotide Sequence of *gag* Gene (p17) From I3151

Nucleotide sequences obtained following sequencing of the *gag* gene (see Section 2.13 and 2.19).

The sequences are aligned to the first variant sequenced at 5 years post seroconversion (F0), with identical nucleotides to this sequence shown with a dot and alignment gaps noted with a dash. The sequences are labelled according to the samples number (1/2) and the individual sequence (.X).

	1	11	21	31	41	51	61	71	81	91	100
FO.1	GGGAGCTAGA	ACGATTGCGA	GTTAATCCTG	GCCTATTAGA	AACATCAGAA	GGCTGTAGAC	AAATACTGGA	ACAGCTACAA	CCAGCCCTTC	AGACAGGATC	
1.1C.....T.....T.....
1.2	G.....T.....
1.3C.....C.....T.....T.....
1.4C.....T.....T.....	C.....
1.5G.....T.....T.....
1.6	A.....A.....
1.7C.....T.....T.....
1.8C.....C.....T.....
1.9C.....T.....
1.10C.....C.....T.....
1.11C.....T.....T.....
2.1	A.....
2.2C.....
2.3	C.....	A.....	C.....	A.....
2.4	A.....
2.5	A.....
2.6	A.....	T.....
2.7	G.....	T.....
	101	111	121	131	141	151	161	171	181	191	200
FO.1	AGAAGAACTT	AGATCATTAT	TTAATACAGT	AGCAACCCCTC	TATTGTGTGC	ATCAAAAGAT	AGATGTAATA	GACACCAAGG	AAGCTTTAGA	GAAGATAGAG	
1.1
1.2	G.....
1.3	G.....	A.....
1.4	G.....
1.5
1.6C.....	A.....
1.7	G.....	A.....
1.8	G.....	A.....	A.....
1.9	G.....	A.....
1.10	G.....	C.....
1.11	G.....	A.....
2.1C.....	A.....
2.2
2.3
2.4C.....	A.....
2.5C.....	A.....
2.6
2.7
	201	211	221	231	241	251	261	271	281	291	300
FO.1	GAAGAGCAAA	ACAAAAGTAA	GAAAAAGCA	CAGCAAGCAG	CAGCTGGCAC	AGGAAACAGC	AGCCAGGTCA	G-----	CCAAAATTAC	CCTATAGTGC	
1.1
1.2	A.....	A.....	C.....
1.3	A.....	A.....	C.....
1.4GA.....	A.....	A.....	C.....
1.5G.....	A.....	A.....	C.....
1.6C...G	A.....	A.....	C.....
1.7	G.....C.....	A.....	A.....
1.8G.....	A.....	A.....
1.9	A.....	A.....
1.10	A.....	A.....
1.11	A.....	A.....
2.1
2.2C.....
2.3C.....
2.4C.....
2.5C.....
2.6G.C.....
2.7G.....
	301	311	321	331	341						
FO.1	AGAAGATCCA	GGGGCAAATG	GTACATCAGG	CCATATCACC	TAGA						
1.1C.T.....						
1.2						
1.3						
1.4C.T.....						
1.5C.T.....						
1.6G.....						
1.7C.T.....						
1.8C.T.....						
1.9C.T.....						
1.10C.T.....						
1.11G.....						
2.1						
2.2						
2.3						
2.4G.....						
2.5						
2.6						
2.7						