T-CELL MEDIATED IMMUNE RESPONSES IN CHILDREN AT RISK OF HIV INFECTION

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EX BIBL. UNIV. EDINBURGEN

DECLARATION

I declare that the work presented in this thesis was carried out by me, and I composed this thesis except where otherwise indicated.

Marian Aldhous

March 1997

DEDICATION

This thesis is dedicated to God, the Lord and father of our saviour, Jesus Christ. "For in Him we live and move and have our being " (Acts 17 v28).

This thesis is also dedicated to my parents, John and Heather Aldhous, who have supported and encouraged me in all that I've done.

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ABSTRACT

This study set out to describe the changes in T-cell immune responses in a cohort of HIV-1-infected children. The responses were investigated in relation to their clinical progression for up to four years.

The main response studied was cytotoxic T lymphocyte (CTL) activity which, in adults, is thought to be one of the major mechanisms by which primary HIV-1-viraemia is cleared and clinical stability maintained. CTL activity was measured against four HIV gene products (*gag, tat, pol, env*). Three patterns of CTL activity were seen in the HIV-1-infected children: (i) CTL activity against HIV-*pol*, -*gag* and/or -*env*; (ii) CTL activity against HIV-*tat* and/or -*pol*; (iii) no CTL activity detected. These different patterns appear to relate to different patterns of clinical disease progression. CTL activity was also detected in children born to HIV-1-infected mothers, who were presumed to have been exposed, but are uninfected. The the specificity of response in these children was predominantly to HIV-*env*. Stimulation of PBMCs with peptides of specific CTL epitopes of HIV-1 resulted in a change in specificity of CTL recognition. These data suggest that the specificity of the CTL response may be related to clinical progression of the child, and that different CTL responses are obtained by different methods of stimulation of PBMCs.

Changes in lymphocyte surface marker expression in peripheral blood were investigated to see if any phenotype was related to CTL activity, or was indicative of the infection status of the child, or disease progression. Activated and memory CD8 cell subpopulations were markedly increased soon after birth in HIV-1-infected children and remained so throughout infection. An increase in the proportion of activated or memory CD4 cells may indicate an imminent and accelerated CD4 cell loss, and subsequent progression to AIDS. The lymphocyte phenotypes in HIV-1-uninfected children remained within normal ranges. CTL activity was not related to any population in peripheral blood, but was related to by a further expansion of memory, activated, CD8 cells in culture.

Proliferative responses were also studied, using tetanus toxoid, PHA and a combination of recombinant HIV-1 proteins as stimuli. Proliferation to tetanus and PHA in the HIV-1-infected children did not differ from responses seen in exposed uninfected children, donor and

cord blood controls.

In one HIV-1-infected child, the possible down regulation of surface CD4 expression on T cells was investigated by developing a method using reverse transcription of RNA followed by a polymerase chain reaction (RT-PCR) to detect messenger RNA (mRNA) for CD4.

The results indicate that the HIV-1-infected children showed anti-HIV responses through different mechanisms. It is possible that these may contribute to the immunopathogenic effects seen in HIV infection and affect the clinical patterns of disease progression in individual children.

ABBREVIATIONS

ADCC	antibody-dependent cell cytotoxicity
AIDS	Acquired Immunodeficiency Syndrome
APC	antigen presenting cell
bp	base pairs
BSA	bovine serum albumin
°C	degrees Celsius
CCR	CC (β)-chemokine receptor
cDNA	complementary DNA
CMV	cytomegalovirus
^{51}Cr	γ-emitting radioactive isotope of Chromium
CSF	cerebrospinal Fluid
CTL	cytotoxic T lymphocyte
CXCR	CXC (α)-chemokine receptor
DEPC	diethyl procarbamine
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide tris phosphate (a mixure of deoxyadenosine 5'trisphosphate (dATP), deoxycytidine 5'trisphosphate (dCTP), deoxyguanosine 5'trisphosphate (dGTP) and deoxythymidine 5'trisphosphate (dTTP))
dsDNA	double stranded deoxyribonucleic acid
DT	Diphtheria toxin
DTT	dithiothreitol
E. coli	*Eschericia coli*
EBV	Epstein Barr Virus
ER	endoplasmic reticulum
EU	exposed uninfected
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FL-	fluorescence channel

FSC	forward scatter
GALT	gut associated lymphoid tissue
^{3}H	α-emitting radioactive isotope of hydrogen, Tritium
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
HTLV	human T cell leukaemia virus
ICAM	intracellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
Ii	invariant chain
IL-	interleukin-
IVDU	intravenous drug users
IVIgG	intravenous immunoglobulin G
LAV	lymphadenopathy virus
LCMV	lymphocytic choriomeningitis virus
LFA	leucocyte function antigen
LIP	lymphocytic interstitial pneumonia
LPS	lipopolysaccharide
LTR	long terminal repeat
M-tropic	macrophage tropic
µCi	microCurie
µg	microgram
µl	microlitre
mg	milligram
MHC	major histocompatibility complex
MIIC	MHC class II compartment
MIP	macrophage inflammatory protein
ml	millilitre
mRNA	messenger RNA
ng	nanogram
NK	natural killer cell
NSI	non-syncytium inducing
PAF	paraformaldehyde

PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCP	*pneumocystis carinii* pneumonia
PCR	polymerase chain reaction
PE	phycoerithrin
PerCP	peridinal chlorophyll protein
pfu	plaque forming units
pg	picogram
PGL	persistent generalised lymphadenopathy
PHA	phytohaemagglutinin
PPD	purified protein derivative
PWM	pokeweed mitogen
RANTES	regulated upon activation, normal T expressed and secreted
RD1	rhodamine
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Rose Park Memorial Institute
RT	reverse transcriptase
SCID	severe combined immunodeficiency
SI	syncytium inducing
SIV	simian immunodeficiency virus
SSC	side scatter
ssDNA	single stranded deoxyribonucleic acid
ssRNA	single stranded ribonucleic acid
T-tropic	T-cell line tropic
TAP	transporter of antigenic peptides
Taq	Taq polymerase
TCA	trichloroacetic acid
TCR	T-cell receptor
Th-	T-helper
TT	tetanus toxoid
v/v	volume per volume
w/v	weight per volume

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1.1 INTRODUCTION TO HIV

1.1.1 ORIGINS, DISCOVERY AND DISTRIBUTION OF HIV

The human immunodeficiency virus (HIV) is one of a family of single-stranded RNA viruses. This family was called retroviruses because they reverse the normal cellular process of DNA to RNA transcription (Alberts *et al.*, 1989). RNA is transcribed to DNA using a reverse transcriptase enzyme before being integrated into the host genome, from where active replication of the virus can take place (Alberts *et al.*, 1989).

Lentiviruses are a genus of retroviruses which are associated with diseases with long periods of clinical latency, from which they derive their name. Other lentiviruses include maedi-visna of sheep, simian immunodeficiency virus (SIV) of primates, equine infectious anaemia virus of horses, caprine arthritis-encephalitis virus of goats, feline immunodeficiency virus of cats and bovine immunodeficiency virus of cattle (Haase, 1986; Letvin, 1990). These viruses have similar genetic structures based around a *gag* gene coding for the cone shaped core, a *pol* gene which encodes for reverse transcriptase (RT) and the other enzymes involved in integration of virus into the host genome, and an *env* gene which encodes for the envelope proteins. These viruses affect the immune system by infecting T cells and/or macrophages, thus causing persistent viraemia and immunosuppression, but often only stimulate a weak antibody response (Haase, 1986; Letvin, 1990).

The acquired immune deficiency syndrome (AIDS) was first described in 1981 in homosexual men in the United States. It was shown that these men were lacking T-lymphocytes which expressed the CD4 molecule. They were also found to be susceptible to opportunistic infections that would normally be kept under control by a healthy immune system (Gottlieb *et al.*, 1981; Centers for Disease Control, 1982). Montagnier's group in France isolated a novel retrovirus from the lymph node, but not peripheral blood, of one such patient with enlarged lymph nodes (lymphadenopathy), and named it as lymphadenopathy virus (LAV) (Barré-Sinoussi *et al.*, 1983). Concurrently, Gallo's group in the USA, isolated and characterised the same retrovirus from both peripheral blood and lymph nodes of other patients with AIDS. These isolates were found to share some properties of the human T cell leukaemia virus types 1 and 2 (HTLV-I and HTLV-II), but were morphologically,

biologically and antigenically distinct (Popovic *et al.*, 1984; Levy, 1993). Gallo's group named the virus HTLV-III, but later the name was changed to the human immunodeficiency virus (HIV, Clavel *et al.*, 1987).

Thus far two major human immunodeficiency viruses have been isolated, HIV-1 and HIV-2. HIV-2 was first isolated from patients in West Africa. These individuals presented with immunological abnormalities identical to those with AIDS, but had no antibodies for HIV-1 (Clavel *et al.*, 1986). Isolation and further characterisation of this new virus from more patients showed that, although related to HIV-1, it was closer to SIV, the simian counterpart of HIV (Clavel *et al.*, 1987; Marx *et al.*, 1991). The similarity of HIV-2 to SIV has lead to theories of SIV transmission to humans from monkeys (and adaption to HIV-2), followed by a rapid evolution to HIV-1 and escape from an isolated human population (Marx *et al.*, 1991; Gardner and Luciw, 1988).

HIV-2 is endemic in West Africa but is found infrequently in individuals from elsewhere, while HIV-1 has been isolated from patients from all over the world. One of the features of HIV-1 is its high sequence variability. A number of groupings have been made, based on serological and genotypic analysis, of *env* and *gag* sequences of HIV-1 isolates from different parts of the world. Highly divergent strains have been found in Cameroon, Gabon and France. Other strains of virus have been divided into subtypes denoted A to H (reviewed in Myers, 1994). The predominant subtype found in Europe and the United States is a subtype B virus. There is some evidence that the different subtypes of virus may be transmitted between individuals with varying efficiency (Kunanusont *et al.*, 1995).

HIV-1 sequence variation is also seen at the patient level, both within a single individual (Holmes *et al.*, 1992; Meyerhans *et al.*, 1989) and between individuals infected with virus from the same source (Simmonds *et al.*, 1991). Additional pressure on the virus to mutate could be exerted by the immune responses to HIV-1; sequence variation could be one of the mechanisms by which HIV-1 evades recognition by the immune system (Nowak *et al.*, 1990; Phillips *et al.*, 1991; Klenerman *et al.*, 1995; Meier *et al.*, 1995).

1.1.2 STRUCTURE OF HIV-1

The structure of HIV-1 is presented as a schematic diagram in figure 1.1. HIV-1 is a typical lentiviral virion (Haase, 1986; Letvin, 1990). The cone-shaped core is composed of the p24*gag* protein and contains two copies of the single (sense)-stranded viral RNA (ssRNA), closely associated with the viral RNA-dependent reverse transcriptase (RT) proteins p66*pol* and p53*pol*, and the nucleocapsid proteins p7*gag* and p9*gag*. Surrounding the core in the inner portion of the viral membrane is the myristoylated protein p17*gag*, which forms the matrix of the virion. Surrounding this is the lipid bilayer membrane, in which are embedded seventy-two external spikes formed by the envelope glycoproteins, gp41 (the transmembrane portion) and gp120 (surface protein). The gp120 has several hypervariable regions (denoted V1 to V5) and constant domains (C1 to C4). The hypervariable regions (particularly the V3 loop) may affect viral interaction with the host cell. The lipid bilayer is also interspersed with other host proteins such as the major histocompatibility complex (MHC) class I and II antigens (Greene, 1992; Levy, 1993). The virion also contains other viral proteins, such as the regulatory proteins vpr, nef and vif. The functions of the different proteins are summarised in table 1.1.

1.1.3 LIFE-CYCLE OF HIV-1

HIV-1 binds to the CD4 receptor via gp120. The exact mechanism of viral entry has not been elucidated, but it is now known that a second receptor is required for viral entry. This has been shown to be a family of receptors for small chemotactic peptides called β-chemokines (Deng *et al.*, 1996; Dragic *et al.*, 1996; Alkhatib *et al.*, 1996). Once in the cytoplasm, the viral core is uncoated and the ssRNA is reverse transcribed to single-stranded DNA (ssDNA). The action of RT and ribonuclease converts the ssDNA into double-stranded DNA (dsDNA). This is then translocated as a nucleoprotein complex to the nucleus while still associated with the viral p17*gag* (matrix) proteins (Gallay *et al.*, 1996). There, the integrase enzyme, within the nucleoprotein complex, inserts the DNA into the host genome, where the virus can remain latent within resting cells (Chun *et al.*, 1995), but would be actively transcribed once the cell became activated.

Figure 1.1 **Structure of HIV-1**

A schematic diagram of the structure of the HIV-1 virion.

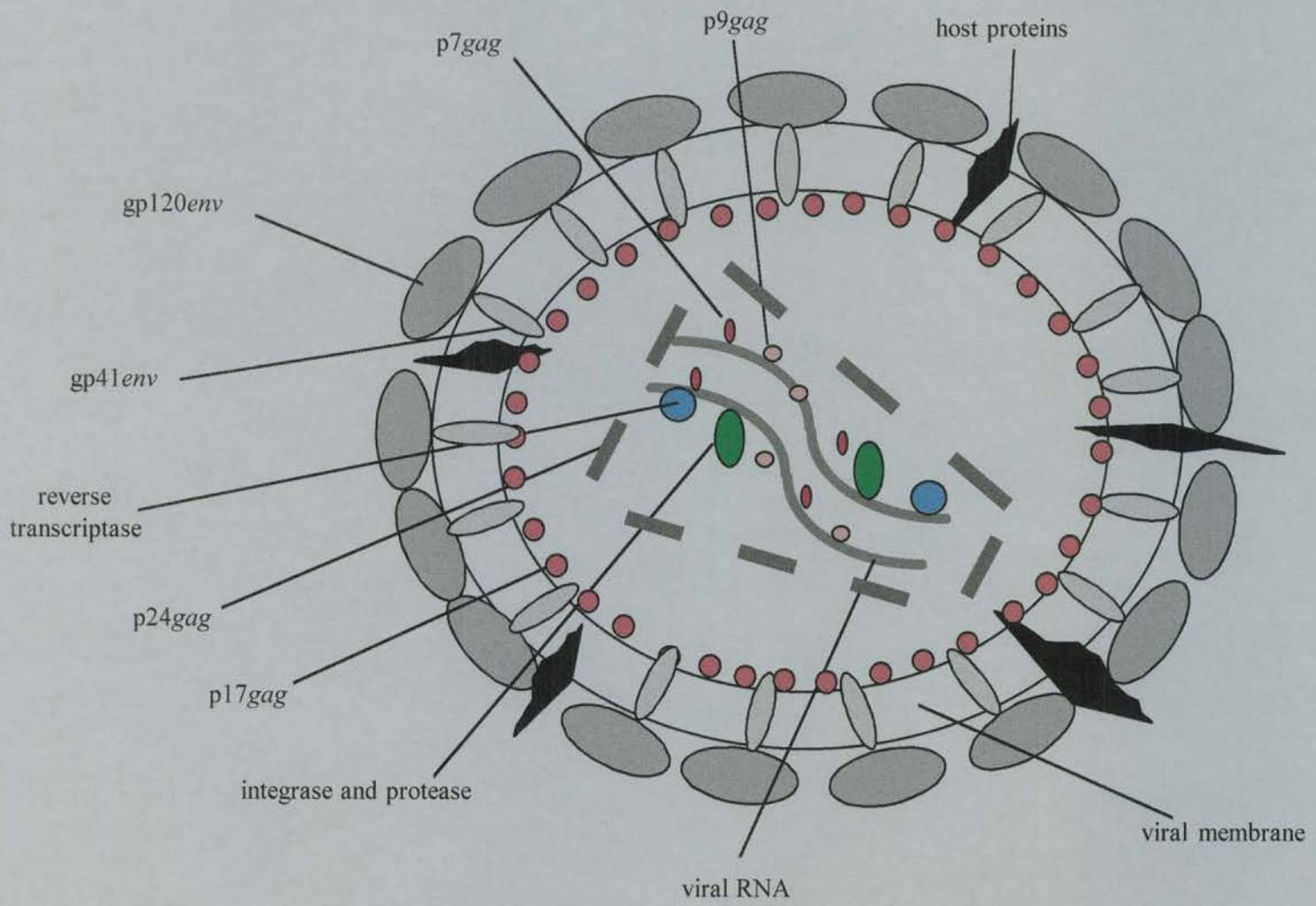

p7*gag*
p9*gag*
host proteins
gp120*env*
gp41*env*
reverse
transcriptase
p24*gag*
p17*gag*
integrase and protease
viral RNA
viral membrane

Table 1.1 **Gene products of HIV-1**

Gene	Protein (size)		Function
gag	53 kD precursor, cleaved by protease to give p7, p9, p17, p24		p24 is the major core protein. p17 is the myristoylated protein lining the inner membrane of virion to stabilise the virion. p9 is an RNA binding protein. p7 binds RNA through 'Zinc-finger' motif, and also binds vpr.
pol	precursor	RT p63, p55	generates the first-strand DNA copy of viral RNA.
		ribonuclease H	degrades the RNA template for second-strand copy of DNA synthesis.
		integrase p11	integrates viral cDNA into the host genome.
		protease p15	used in post translational processing of viral proteins.
env	gp160 kD precursor, cleaved by protease to give gp120 and gp41		gp120, the envelope surface protein, which contains variable and constant regions which affect binding and cellular tropism. gp41, the envelope transmembrane protein.
tat	15 kD ***t***rans-***act***ivator		viral transcription transactivator.
rev	19 kD ***re***gulator of ***v***iral mRNA		mediates export of viral mRNA to cytoplasm and activates gene expression, by functioning as a switch, to allow 'early' (multiply spliced) or 'late' (singly spliced) gene products to be expressed.
nef	25-27 kD ***ne***gative ***f***actor		initially thought to be a negative regulator of gene expression. Found to down regulate surface CD4 expression.

vif	23 kD	***v***iral ***i***nfectivity ***f***actor	promotes virion maturation and infectivity.
vpu	16 kD	***v***iral ***p***rotein ***U***	facilitates proper maturation and release of HIV-1 from cells. Degrades CD4 in the endoplasmic reticulum. Found only in HIV-1.
vpr	14 kD	***v***iral ***p***rotein ***R***	promotes nuclear localisation of the pre-integration complex. Inhibits cell division and arrests infected cells at G2/M phase of the cell cycle.
vpx	15 kD	***v***iral ***p***rotein ***X***	strong homology with HIV-1 vpr but found only in HIV-2/SIV

Table of the genes and functions of the gene products in HIV-1

Sources: (Greene, 1992; Levy, 1993; Myers *et al.*, 1995; Rosen, 1992; Antoni *et al.*, 1994; Schwartz *et al.*, 1995; Subbramanian and Cohen, 1994; Willey *et al.*, 1992)

The genomic organisation of HIV-1 is shown in figure 1.2. Induction of HIV-1 transcription occurs through activation of transcriptional factors, e.g. the NF-κB family of enhancer binding proteins. These proteins normally regulate genes involved in cell growth. The proteins bind to, and activate, the duplicated κB enhancer element located in the U3 region of the long terminal repeat (LTR) of proviral HIV-1. Other host factors required for HIV-1 expression are Sp1, a gene regulatory protein which binds to the promoter of various 'housekeeping' genes, and the TATA factor (TFIID), which binds to the TATA box sequence TTATAA and is critical for promoter activity and for determining the exact point of RNA chain initiation (Antoni *et al.*, 1994; Alberts *et al.*, 1989).

Binding of cellular transcription factors to the LTR allows transcription of the whole genome. This transcript is then multiply spliced to produce short RNA transcripts of about 2 kilobases long. These encode the regulatory proteins tat, rev and nef. Tat up-regulates the transcription of early genes, through an interaction with an RNA 'stem-loop' structure called the 'transactivation response element' (TAR), a sequence occurring in the LTR. This tat-TAR interaction allows longer RNA transcripts to be produced, in larger amounts (Rosen, 1992; Greene, 1992; Antoni *et al.*, 1994).

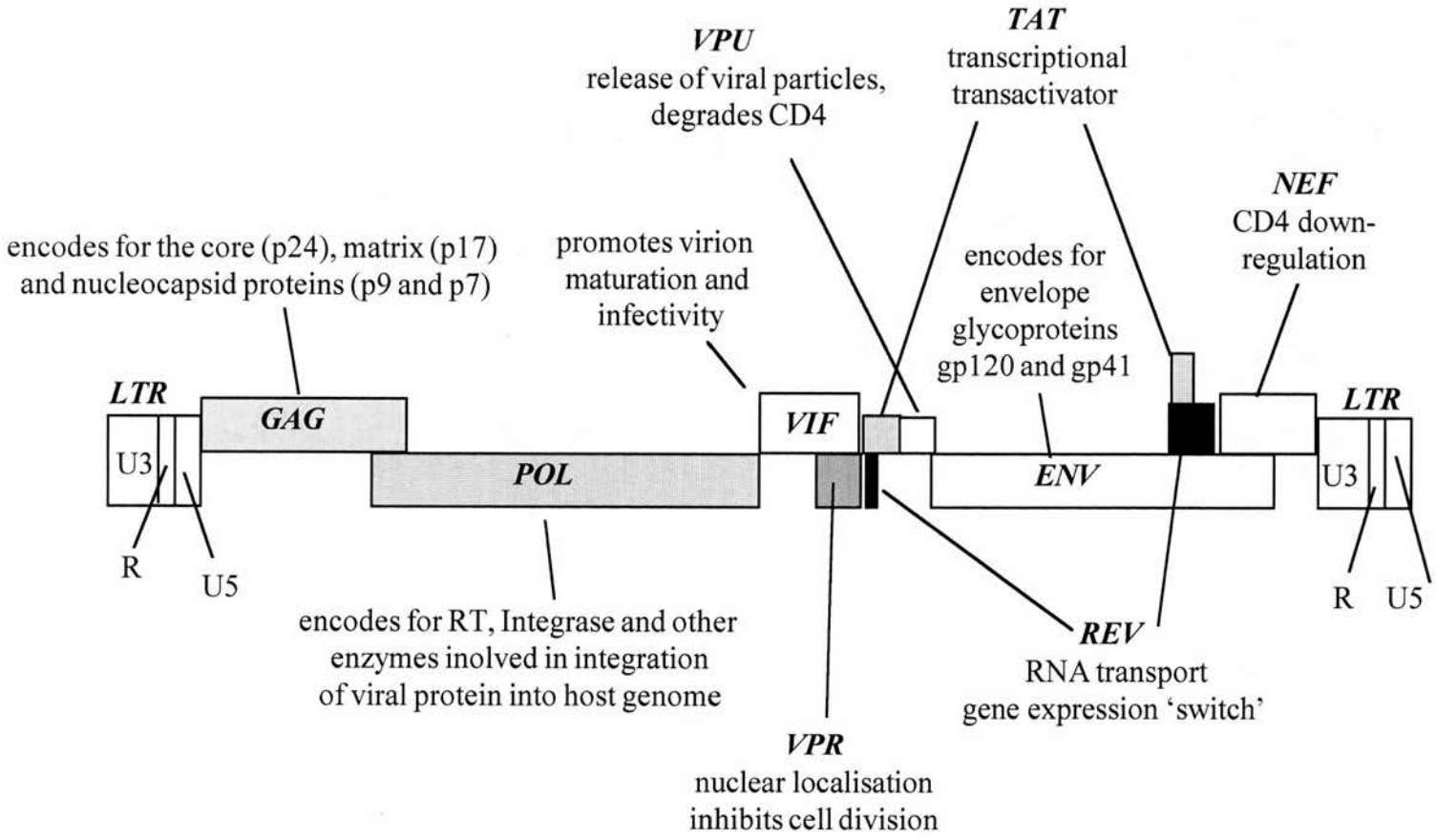
The assembly of infectious HIV-1 virions requires structural and enzymatic proteins. The 'switch' between transcription of early and late gene products depends on rev. Rev exerts its regulatory activity post-transcriptionally, by activating the nuclear export of unspliced and singly spliced HIV-1 mRNA (encoding the gene products of gag, pol and env, and viral ssRNA). Rev functions through interaction with an RNA motif called the rev response element (RRE), which is another stem-loop structure located in the *env* gene. Due to mRNA splicing, RRE is only present in the RNA species whose expression is controlled by rev. An absence of rev produces unspliced and singly spliced mRNAs that remain and are degraded in the nucleus (Greene, 1992; Rosen, 1992; Antoni *et al.*, 1994).

Aggregation of the ribonucleoprotein core in the cytoplasm is followed by acquisition of the lipid bilayer and addition of the env proteins at the cell surface. The final budding of the virion through the plasma membrane of the cell, occurs by the cleavage mediated by HIV-1 protease and myristoylation of p17. Vpu promotes efficient release of budding virus from the cell surface (Greene, 1992; Subbramanian and Cohen, 1994).

Figure 1.2 **Genomic organisation of HIV-1**

The linear arrangement of the HIV-1 genes and proteins encoded by them. The different proteins are produced by frameshifting of the ribosome and differential splicing of the RNA by the viral proteases.

Source: Myers *et al.*, 1995


VPU
release of viral particles, degrades CD4
TAT
transcriptional transactivator
NEF
CD4 down-regulation
encodes for the core (p24), matrix (p17) and nucleocapsid proteins (p9 and p7)
promotes virion maturation and infectivity
encodes for envelope glycoproteins gp120 and gp41
LTR
GAG
VIF
LTR
U3
POL
ENV
U3
R
U5
R
U5
encodes for RT, Integrase and other enzymes inolved in integration of viral protein into host genome
REV
RNA transport
gene expression 'switch'
VPR
nuclear localisation
inhibits cell division

1.1.4 CELLULAR TROPISM OF HIV-1

HIV-gp120 was shown to interact with the CD4 molecule (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984; Maddon *et al.*, 1986), and HIV-1 was found to have the ability to infect cells (particularly T lymphocytes) which expressed CD4. This correlated with the observations that these cells were dramatically decreased in the first cases of AIDS (Gottlieb *et al.*, 1981; Centers for Disease Control, 1982). HIV-1 has also been isolated from other lymphoid cells such as monocyte-macrophages (Pantaleo *et al.*, 1993), B cells (Monroe *et al.*, 1988) and follicular dendritic cells (Embretson *et al.*, 1993). HIV-1 has also been isolated from other tissues such as the brain (Wiley *et al.*, 1986; Cheng-Mayer *et al.*, 1987) and from haemopoietic cells (Folks *et al.*, 1988).

Virus isolates of HIV-1 can differ in their biological properties, including their ability to infect cells (Cheng-Mayer *et al.*, 1990). The third hypervariable region (V3 loop) of gp120 is thought to determine the cellular tropism of a particular viral isolate (Cann *et al.*, 1992). Virus isolates which preferentially infect macrophages (macrophage- or M-tropic viruses) but also infect activated T cells in peripheral blood, are present within an individual throughout HIV-1 infection. It is these variants which are transmitted (Wolinsky *et al.*, 1992) and which play a role in establishing primary HIV-1 infection (Zhu *et al.*, 1993; Popovic and Gartner, 1987). M-tropic variants do not infect T-cell lines and are non-syncytium-inducing (NSI) in culture (Schuitemaker *et al.*, 1991). Viruses which preferentially infect T-cell lines (T-cell- or T-tropic) are syncytium-inducing (SI), i.e. they form giant multi-nucleated cells in culture. These variants have been isolated from some individuals after they have been infected for some time. The emergence of an SI variant has been associated with disease progression (Schuitemaker *et al.*, 1992). Amino acid changes which increase the net positive charge of the V3 loop are thought to be responsible for the change in virus phenotype from NSI to SI (de Jong *et al.*, 1992).

HIV-1 has also shown an ability to infect cells that do not express CD4 (Harouse *et al.*, 1991; Tateno *et al.*, 1989) and an inability to infect cells that express high levels of CD4 (Maddon *et al.*, 1986; Evans *et al.*, 1987). Therefore, other receptors are required for HIV-1 entry into cells. These have now been identified as the receptors for chemotactic peptides, known as the α- and β-chemokines. These chemokines attract other cells: α-chemokines attract

neutrophils, whereas β-chemokines attract lymphocytes. The receptors for these chemokines have been described as GTP-binding proteins (G proteins), which are folded in such a way to give seven transmembrane domains (Feng *et al.*, 1996). The receptors for α-chemokines are denoted C-X-C-chemokine receptors (CXCR), while the receptors for β-chemokines have been denoted as C-C-chemokine receptors (CCR). The cell tropism of the viral strains has an effect on which receptor type is used for virus entry into cells. M-tropic viruses use the CC-chemokine receptors, CCR5 being the receptor originally described for viral entry (Deng *et al.*, 1996; Dragic *et al.*, 1996), although there is some evidence that other receptors in this family may be used (Doranz *et al.*, 1996; Choe *et al.*, 1996). Conversely, T-tropic viruses use the CXCR4 receptor, also known as Fusin, for viral entry (Alkhatib *et al.*, 1996).

1.2 HIV-1 INFECTION IN ADULTS AND CHILDREN

1.2.1 TRANSMISSION OF HIV-1

Transmission of HIV-1 in adults, occurs through sexual contact or through exposure to contaminated blood and blood products. AIDS was first described in homosexual men (Gottlieb *et al.*, 1981; Centers for Disease Control, 1982), and although heterosexual transmission accounted for only a small proportion of cases, these were particularly reported in the sexual partners of those in high risk groups (Harris *et al.*, 1983; Masur *et al.*, 1982). Since then, many cases of heterosexual transmission have been described (Centers for Disease Control, 1991). In the developing world, the most common route of HIV-1 transmission is through heterosexual contact (Chin, 1990).

AIDS was soon identified in haemophiliacs and HIV-1 has been isolated from concentrated factor VIII, made before heat treatment of batches was introduced (Zhang *et al.*, 1991). Many haemophiliacs treated with contaminated factor VIII were infected with HIV-1 (Simmonds *et al.*, 1988). Similarly, blood transfusion associated transmission of HIV-1 also occurred. Since the introduction of blood donor screening for HIV-1 antibodies, the transmission of HIV-1 through blood products and transfusions is rare (Donegan *et al.*, 1990).

Intravenous drug users (IVDUs) are another defined group of people at risk of HIV-1 infection, particularly when needle-sharing is common (Robertson *et al.*, 1986; Brettle *et al.*, 1987). Needle-stick injuries have also been described as a route of HIV-1 transmission (Lange *et al.*, 1990). In all of these routes, transmission appears to depend on different factors, particularly the viral load of the index case (Lange *et al.*, 1990).

1.2.1.1 Mother-child transmission of HIV-1

Vertical transmission from mother to child during pregnancy, child-birth or through breast-feeding is the cause of most paediatric HIV-1 infection. Mother-child transmission of both HIV-1 and HIV-2 has been reported (European Collaborative Study, 1991; European Collaborative Study, 1992; Blanche *et al.*, 1989; Morgan *et al.*, 1990). The transmission rate is estimated as the number of HIV-infected children expressed as a percentage of the total number of children born to HIV-infected mothers in a defined cohort. Transmission rates in different cohorts have been estimated at between 13 and 39% of the total children (European Collaborative Study, 1991; European Collaborative Study, 1992; Blanche *et al.*, 1989; Morgan *et al.*, 1990). Factors affecting the transmission of virus have been reported to include low maternal CD4 count (Ryder *et al.*, 1989; Blanche *et al.*, 1989) and p24 antigenaemia (Ryder *et al.*, 1989). In some cohorts a protective effective of maternal anti-gp120 antibodies has been seen (Peuchmar *et al.*, 1991; Devash *et al.*, 1990), but this has not been confirmed in other studies (Halsey *et al.*, 1992). A more recent study indicated that transmission was more dependent upon the tropism of the maternal HIV strain: monocytes-macrophages from uninfected children, born to HIV-1-infected mothers, were uninfectable *in vitro* with the virus isolated from their mother (Ometto *et al.*, 1995). The timing of transmission (i.e. early or late in pregnancy, or at birth) is not clear. Studies on foetal or placental material have been contradictory: virus has been isolated from 8-week gestation foetuses (Lewis *et al.*, 1990) and from 12 week foetuses (Backé *et al.*, 1993) suggesting that transmission occurs early in pregnancy. However, in these studies, possible contamination by maternal blood was not excluded (Brossard *et al.*, 1995). Other studies suggest that transmission occurs later on in pregnancy (Brossard *et al.*, 1995; Ehrnst *et al.*, 1991; Peuchmar *et al.*, 1991) or at delivery. Strong evidence for infection at delivery has been shown by a study of twins, which indicated that exposure to maternal blood and mucous

during delivery could increase transmission, as the first-born of twins discordant for HIV-1 infection was more likely to be infected (Goedert *et al.*, 1991). This was further supported by studies that showed that Caesarean section, as opposed to vaginal delivery, decreased the transmission rate (European Collaborative Study, 1992; Goedert *et al.*, 1991). More recently prophylactic AZT treatment of the mother during the last trimester of pregnancy, and of the child during the first six weeks after birth, has been shown to reduce the transmission of HIV-1 (Sperling *et al.*, 1996), also indicating that transmission occurs late on in pregnancy. Post-natal transmission through breast-feeding, estimated at approximately 15% has also been reported (European Collaborative Study, 1992; Blanche *et al.*, 1989; van de Perre *et al.*, 1991), which is supported by the isolation of HIV-1 from colostrum (Vonesch *et al.*, 1992).

1.2.2 LABORATORY DIAGNOSIS OF HIV-1

HIV-1 infection is diagnosed, in adults, with an anti-HIV-1 antibody test using enzyme-linked immunosorbent assay techniques and confirmed by western blotting (Margolick *et al.*, 1992; Simmonds *et al.*, 1988). The polymerase chain reaction (PCR) or virus culture from peripheral blood mononuclear cells (PBMCs) are techniques used for confirmation of HIV-1 infection.

Diagnosis of HIV-1 infection in children cannot depend on the detection of antibodies, due to the presence of maternal antibodies. These are passively transferred across the placenta late on in pregnancy and can persist for up to 18 months of age. After this time, any circulating anti-HIV-1 antibody is presumed to be the child's own response to HIV-1 infection, and thus its presence can be used diagnostically (Centers for Disease Control, 1987). Other techniques have been developed to diagnose HIV-1 infection as soon as possible after birth. The most widely used are PCR (de Rossi *et al.*, 1991; de Rossi *et al.*, 1992; Rogers *et al.*, 1989), the culture of HIV-1 from PBMCs (Eschaich *et al.*, 1991; Kline *et al.*, 1993; de Rossi *et al.*, 1991), and p24 antigen levels (de Rossi *et al.*, 1991; Rogers *et al.*, 1989). Comparison of these techniques has shown PCR to be more sensitive than virus culture (Eschaich *et al.*, 1991; de Rossi *et al.*, 1991), which in turn was found to be more sensitive than p24 antigen assays (Kline *et al.*, 1993; Rogers *et al.*, 1989). Other techniques have been developed, such as *in vitro* antibody production (Amadori *et al.*, 1988), IgA-

specific anti-HIV-1 antibodies (Weiblen *et al.*, 1988), and enzyme-linked immunospot assays (Nesheim *et al.*, 1993), but these have been found not to be as sensitive or reliable (de Rossi *et al.*, 1991). In some children, diagnosis was not possible at birth, although, in all these assays, sensitivity increased with age. Thus, a negative result is not necessarily diagnostic of the absence of HIV-1 infection (Cassol *et al.*, 1992; de Rossi *et al.*, 1991; Rogers *et al.*, 1989), but may be a reflection of the timing of vertical transmission. Bryson *et al.*, (1992) defined *in utero* transmission of HIV-1 as having occurred if HIV-1 can be detected in cord or peripheral blood within 48 hours of birth, using co-culture or PCR techniques. Likewise, they defined *intrapartum* transmission of HIV-1 as having occurred if HIV-1 cannot be isolated from cord or peripheral blood during the first week of life, but can be isolated subsequently, and if the child was not breast-fed.

1.2.3 CLINICAL MANIFESTATIONS OF HIV-1 INFECTION

In approximately 5-10% of HIV-1-infected adults, an acute viral illness similar to infectious mononucleosis, has been described, with distinct clinical features including fever, lethargy, headache and lymphadenopathy (reviewed in Tindall and Cooper, 1991). After primary infection there is usually a period where the individual is clinically asymptomatic, as is common in lentiviral infections. The length of this asymptomatic period varies between people (Bird, 1992). Non-specific signs and symptoms, e.g. persistent generalised lymphadenopathy (PGL), tend to appear with increasing frequency before the onset of more severe infections resulting from compromised immune function. Diseases associated with the onset of AIDS include opportunistic infections e.g. *Pneumocystis carinii* pneumonia (PCP), cytomegalovirus infection (CMV), toxoplasmosis, oesophageal candidiasis; neoplasms e.g. Kaposi's sarcoma, non-Hodgkins lymphoma and an increased incidence of bacterial and mycobacterial infections (Moore *et al.*, 1991; Farizo *et al.*, 1992). Other symptoms include an increased incidence of allergies (Carr *et al.*, 1991), abnormal endocrine function (Grinspoon and Bilezikian, 1992), cardiomyopathy (Cohen *et al.*, 1986) and haematological abnormalities (Doweiko, 1993). AIDS indicator diseases occur with similar frequencies within different risk groups, but the spectrum of diseases may be different between risk groups, and may reflect other socio-economic differences of these populations, unrelated to HIV-1 infection (Margolick *et al.*, 1992; Farizo *et al.*, 1992). For example, an increased

incidence of bacterial infections (pneumonia, sepsis, pulmonary mycobacteria) is seen in IVDUs compared to other risk groups (Farizo *et al.*, 1992; Selwyn *et al.*, 1992). Kaposi's Sarcoma is seen almost exclusively in homosexual men. There is evidence this is caused by another infectious agent belonging to the Herpes family of viruses (Kaposi's sarcoma herpes virus or human herpes virus 8), and that this is also transmitted sexually (Volberding, 1992; Schalling *et al.*, 1995).

In children, the clinical pattern of diseases is different from that seen in adults. In particular, a biphasic clinical pattern of HIV-1-disease progression has been noted in perinatally infected children: those who progress to AIDS and die within about two years of birth, and those who progress more slowly (Galli *et al.*, 1995; European Collaborative Study, 1991; Duliège *et al.*, 1992; Blanche *et al.*, 1994). In one study, children who had been defined as infected *in utero*, were also those who progressed more quickly (Dickover *et al.*, 1994). Other studies have suggested that the rate of progression is due to the virus strain transmitted and, therefore, the rate of progression in the child is directly related to that of the mother (Tovo *et al.*, 1994; Blanche *et al.*, 1994). The spectrum of clinical disease in children is different from that found in adults. In children an increased incidence of recurrent bacterial infections is seen (The National Institute of Child Health, 1991). Encephalopathy, cardiomyopathy and failure to thrive are more common in infected children than in adults (Brouwers *et al.*, 1995; Lipshultz *et al.*, 1992). The range of opportunistic infections is also different from that seen in adults: an increased incidence of candidiasis, PCP and lymphocytic interstitial pneumonia (LIP, pneumonia associated with an influx of lymphocytes in the interstitial spaces) is seen, with a decreased incidence of toxoplasmosis and CMV infections (Neumann *et al.*, 1994; Turner *et al.*, 1993; Tovo *et al.*, 1992; Scott *et al.*, 1989).

Onset of an AIDS-defining illness in a child within 6 months of birth, particularly PCP and lymphoma, has a very poor prognosis (Galli *et al.*, 1995; Turner *et al.*, 1993; Tovo *et al.*, 1992; Scott *et al.*, 1989). However, the diagnosis of LIP or bacterial infections, within the same time period, does not have the same severe prognosis and the child could live for several years (Italian Register for HIV Infection in Children, 1994). The onset of clinical signs is significantly later in longer surviving children but the occurrence of severe diseases still occurs (Galli *et al.*, 1995; Italian Register for HIV Infection in Children, 1994).

1.3 THE IMMUNE SYSTEM AND RESPONSES TO HIV-1

1.3.1 INNATE IMMUNITY

The main components of the innate, or natural, immune system are physical barriers, such as skin and mucosal surfaces, non-lymphoid cells such as granulocytes, mast cells, macrophages and Complement. Granulocytes are phagocytic cells which contain bactericidal enzymes. These cells are involved in the clearing of extracellular bacteria. Mast cells and basophils have granules that contain potent mediators, which are released on encounter with an allergen, to induce an inflammatory immune response. Natural killer (NK) cells are involved in killing tumour cells or virally-infected cells. These demonstrate cytotoxic activity without the need for prior activation. Macrophages and monocytes have a central role as antigen presenting cells. These cells phagocytose or endocytose bacteria and viruses, and process the bacterial or viral antigens. Peptides derived from these antigens are presented on the surface of the cells to T lymphocytes in such a way that antigen-specific responses can be elicited (see 1.3.2.2.1). Formation of antibody-antigen complexes can lead to the activation of the Complement system. This is a complex system of enzymes and plasma glycoproteins which act in an enzyme cascade in inflammatory reactions. These reactions ultimately lead to the destruction of bacteria or cells by lysis.

1.3.2 ACQUIRED IMMUNITY AND RESPONSES TO HIV-1

Acquired immunity is antigen-specific and develops in response to the different antigens encountered. The major effector cells of specific immune responses are the lymphocytes. These are found mainly within lymphoid tissues, but a proportion circulate through peripheral blood. Lymphoid tissues include the thymus, spleen, tonsils, regional lymph nodes and gut-associated lymphoid tissue (GALT), such as Peyers patches and the appendix. These tissues are important in generating and maintaining the many antigen-specific responses required from lymphocytes in a mature immune system. Lymphocytes are broadly classed into T and B lymphocytes.

1.3.2.1 B Lymphocytes

B lymphocytes (B cells) develop from haemopoeitic stem cells. During development, a complex series of differentiation events, including gene rearrangement, leads to the expression of immunoglobulin (Ig) on the cell surface. Activation by antigen allows the B cell to differentiate further into an antibody-secreting cell (a primary antibody response, also known as seroconversion) or a memory cell. Re-stimulation of the resting memory cell, by the same antigen, leads to more antibodies being produced (a secondary antibody response). The secondary response occurs more rapidly, and produces larger quantities of high affinity antibodies than the primary response. There are 5 classes of antibody produced by B cells:-

(i) IgG is the most abundant class of antibody, and is important in the secondary immune response to antigens. This is the only class of antibody which crosses the placenta during pregnancy, and is important in the neonate during the first weeks of life. This antibody binds to antigens to form antibody-antigen (immune) complexes. When this occurs on the surface of bacteria, this is known as opsonisation. The opsonised bacteria are then removed by macrophages or lysed by Complement.

(ii) IgA covers all the external mucosal surfaces and secretory IgA occurs in mucosal secretions. This is the primary antibody in mucosal immunity and is particularly important in the gut and lungs.

(iii) IgM is the antibody initially formed on the surface of B cells. Further maturation of the B cell leads to expression of IgD. IgM is a potent activator of the Complement system.

(iv) IgD acts almost exclusively as membrane receptors for antigen.

(v) IgE is primarily involved in immunity against parasitic infections and allergic reactions and binds to mast cells causing a release of histamines.

Antibodies to HIV-1 can be detected within approximately two weeks of exposure (Ariyoshi *et al.*, 1992), with antibodies to different HIV-1 proteins actively being produced from B cells (Vendrell *et al.*, 1991; Delfraissy *et al.*, 1992). Increased anti-p24 antibody is associated with a better clinical outcome than anti-gp120 at seroconversion (Cheingsong-Popov *et al.*, 1991). Neutralising antibodies are defined as those which block the infectivity of the virus *in vitro* (Poignard *et al.*, 1996). Neutralising antibody epitopes have been mapped on gp120, and include the V3 loop, the V1/V2 segment, the C4 domain, the CD4-binding site and epitopes

which are unmasked once gp120 has bound to CD4 (reviewed in Poignard *et al.*, 1996). An epitope has also been defined on gp41 and is denoted the 2F5 epitope (Poignard *et al.*, 1996). However, many of these neutralisation sites were analysed using laboratory-adapted strains of HIV-1 (Weiss *et al.*, 1986; Broliden *et al.*, 1993). Only a specific CD4-binding site epitope and the 2F5 epitope have been found to cross neutralise a broad spectrum of primary isolates (Poignard *et al.*, 1996).

1.3.2.2 **Cell mediated responses**

1.3.2.2.1 Antigen presentation

To elicit a cell mediated response, the antigen has to be presented to T lymphocytes in a certain way so as to be recognised by the effector cell. Antigen presenting cells (APCs) process antigens in two different ways, depending on the source of the antigen (reviewed in Germain and Margulies, 1993). These are outlined in figure 1.3, which shows the different pathways for processing exogenous and endogenous antigens.

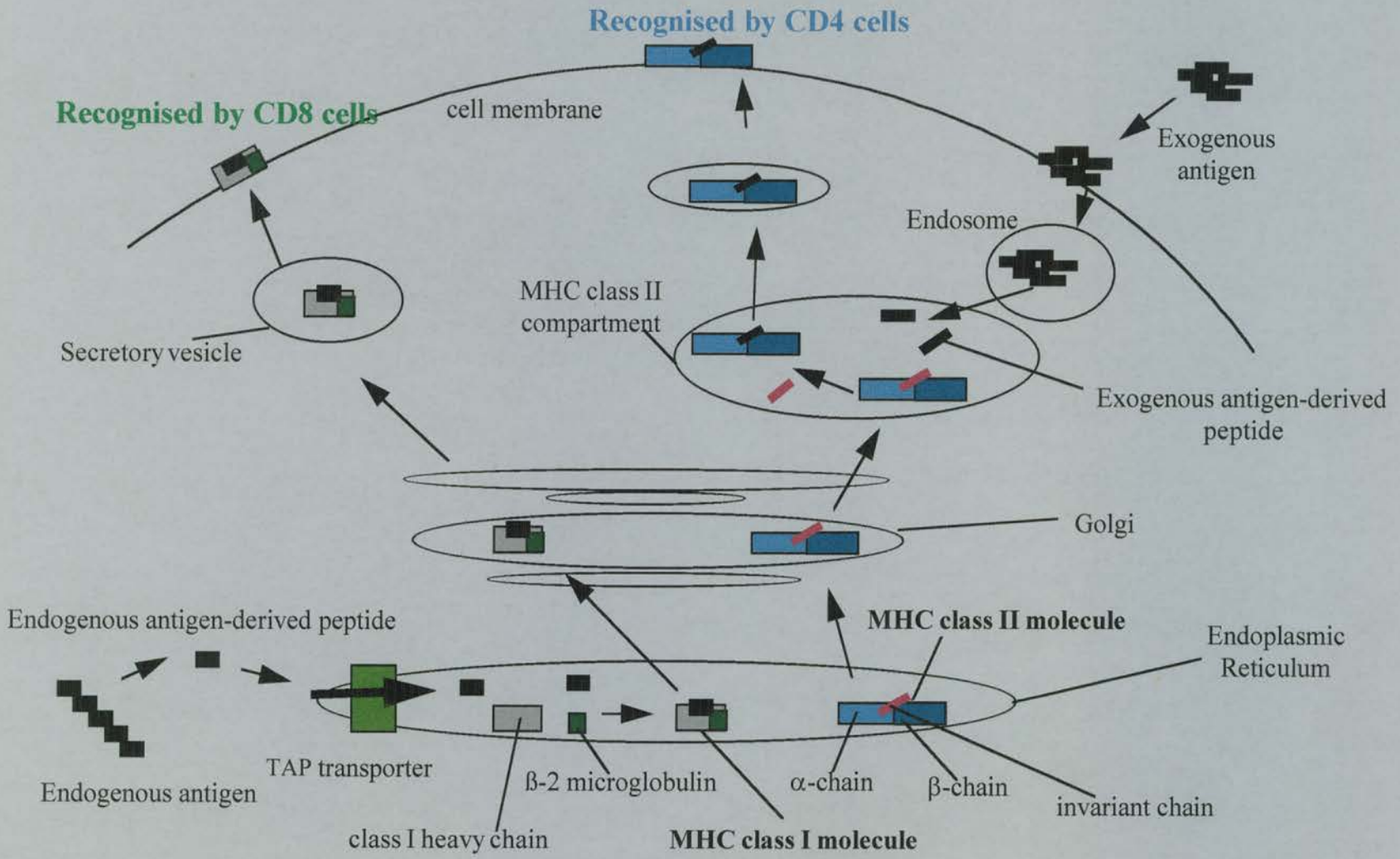
Exogenous antigens (e.g. bacteria, viruses that have not infected cells, extracellular proteins) are endocytosed into the APC. These are degraded in endosomes (an intracellular compartment) by proteases to produce peptides. The peptides are translocated to another compartment, called the MHC class II compartment (MIIC), which contains the major histocompatibility complex (MHC) class II molecule. The MHC class II molecule consists of an α-chain and a ß-chain, which, when associated non-covalently, make a groove into which peptide can bind. When in the endoplasmic reticulum (ER), these α- and ß- subunits form a non-covalently bound trimer with a glycoprotein called invariant chain (Ii) (Germain and Margulies, 1993). MHC class II (i.e. the α/ß/Ii trimer) is transported from the ER, through the golgi to the MIIC. There the peptides derived from the exogenous antigen are loaded onto the groove, displacing the invariant chain, and are then transported to the cell surface (Germain and Margulies, 1993). There the peptide-MHC class II complex can be recognised by T cells expressing CD4, to induce an immune response.

Endogenous antigens (i.e. those which arise from within the cell, such as viral proteins,

Figure 1.3 **Antigen processing pathway**

The different pathways involved in the processing of antigen by antigen presenting cells, prior to recognition by T cells. Processing of exogenous antigen by the MHC class II pathway, for recognition of antigen by CD4 cells, is shown on the right hand side in blue. Processing of endogenous antigen by the MHC class I pathway, for recognition by CD8 cells, is shown on the left hand side in grey.

Source: adapted from Paul, 1993


Recognised by CD4 cells
Recognised by CD8 cells
cell membrane
Exogenous antigen
Endosome
MHC class II compartment
Secretory vesicle
Exogenous antigen-derived peptide
Golgi
Endogenous antigen-derived peptide
MHC class II molecule
Endoplasmic Reticulum
TAP transporter
ß-2 microglobulin
α-chain
β-chain
invariant chain
Endogenous antigen
class I heavy chain
MHC class I molecule

intracellular microbial proteins) are degraded in the cytosol by a proteosome. The resultant peptides, usually about nine amino acids long, are then transported into the rough ER. This is accomplished by a heterodimeric, membrane-associated 'transporter of antigenic peptides' (TAP). Once in the ER, the peptides encounter the newly synthesised MHC class I molecule, which consists of a glycoprotein heavy chain, non-covalently associated with a small glycoprotein called ß-2 microglobulin. ß-2 microglobulin is not anchored to the membrane and can exist either in association with the MHC class I heavy chain, or free in plasma and tissue fluids. The heavy chain is folded in such a way as to produce three domains: the α-1 and α-2 domains form a groove into which peptides of approximately nine amino acids can bind, while the α-3 domain binds CD8. The heavy chain requires the binding of ß-2 microglobulin and peptide for release from the ER into the Golgi. The bound peptide-MHC-class I complex is then transported through the Golgi, into a secretory vesicle and thence to the cell surface (Germain and Margulies, 1993). Once at the cell surface, the bound peptide is recognised by T cells expressing CD8, to induce an effector response.

HIV-1 infection of macrophages (Pantaleo *et al.*, 1993; Zhu *et al.*, 1993) and dendritic cells (reviewed in Cameron *et al.*, 1996) could affect their ability to present antigen to T cells. Cultured dendritic cells infected with HIV-1, have been reported to transmit HIV-1 to co-cultured autologous T cells, in the presence of an immune stimulant (Tsunetsugu-Yokota *et al.*, 1995; Cameron *et al.*, 1996).

1.3.2.2.2 T lymphocytes

T lymphocytes develop from precursor cells in haemopoietic tissue. These migrate to the thymus, where the cells undergo differentiation into one of two lineages: those that express CD4 (CD4 cells) or those that express CD8 (CD8 cells). T cells differ from B cells in that T cells only recognise antigen which has been processed by APCs. Other cell surface molecules are also necessary for the interaction of the T cell with APCs or its target cell, such as the leucocyte function antigen (LFA-1, or CD11a) interaction with the intercellular adhesion molecule (ICAM-1, Paul, 1993).

1.3.2.2.2.1 CD4 cell responses

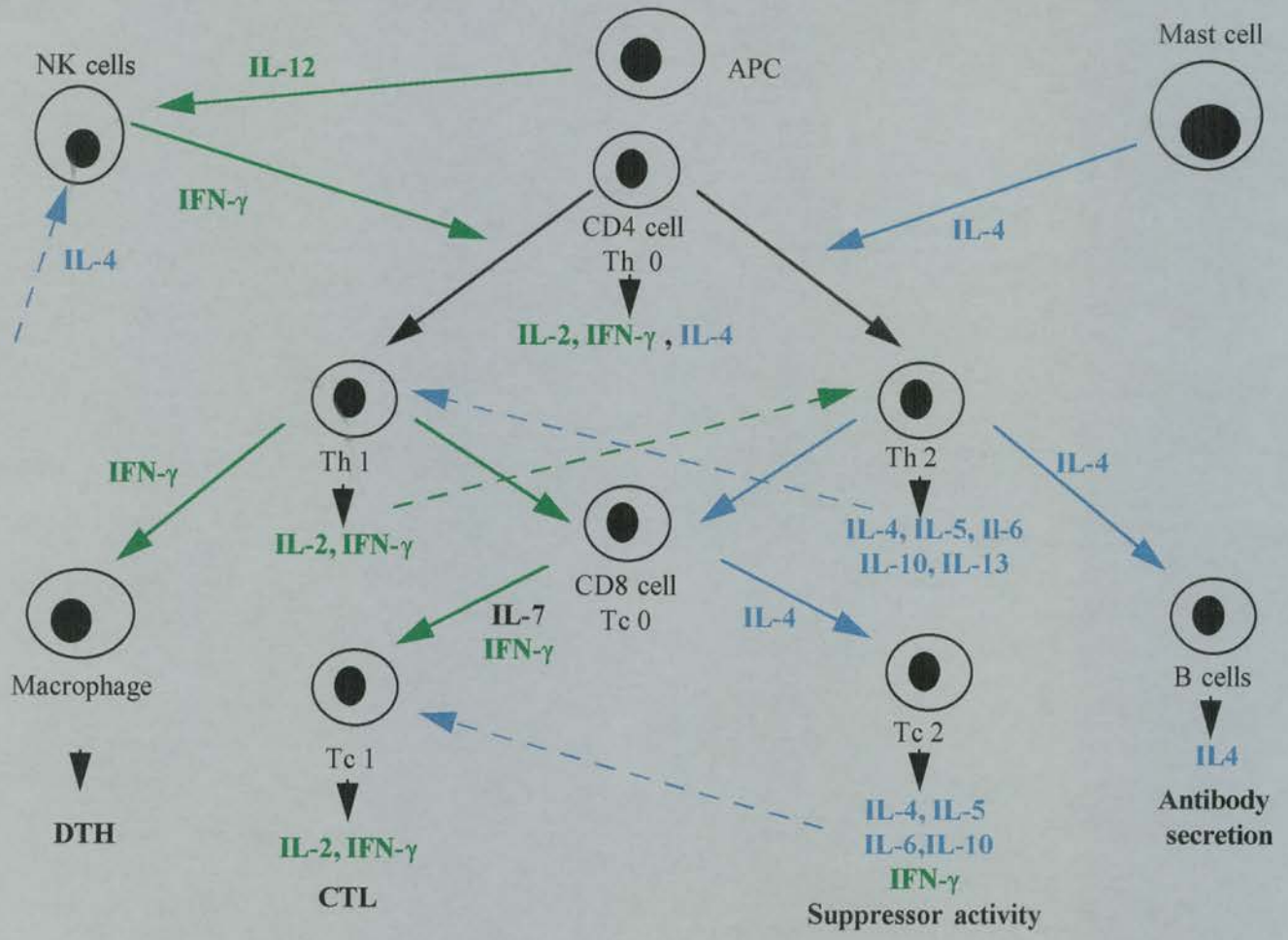
CD4 cells recognise peptide antigens presented by MHC class II molecules. The T-cell receptor (TCR) associates with the CD4 molecule to form a complex which recognises the peptide presented on the MHC class II molecule. The recognition of antigen by the CD4 molecule causes signal transduction acting through intracellular tyrosine kinases. This results in activation of the CD4 cell and the subsequent production of cytokines (low molecular weight 'messenger peptides'). Initial studies in the mouse showed that the influence of different cytokines levels on this activated CD4 cell allowed it to differentiate into either a T-helper 1 (Th-1) cell or a T-helper 2 (Th-2) cell. Th-1 cells produce interleukin 2 (IL-2) and gamma-interferon (IFN-γ), whereas Th-2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13. Figure 1.4 shows the affect of cytokine production on the differentiation of CD4 cells, and the subsequent affect of cytokines on other cells (Kemeny *et al.*, 1994; Kos and Engleman, 1996; Mosmann and Sad, 1996; Romagnani, 1992). The production of Th-1 cytokines is associated with responses mediated by effector cells, whereas Th-2 cytokines are associated with responses mediated by antibodies, secreted factors or Complement (Clerici and Shearer, 1993). The cytokine production is mutually inhibitory, in that Th-1 cytokines inhibit the production of Th-2 cytokines and *vice versa* (see figure 1.4). The dichotomy of the T-helper responses is important in the immune responses to different pathogens. Th-1 responses are effective against protozoa, fungi, bacteria and viruses, and are involved in autoimmunity and transplant rejection. Th-2 responses are effective against parasites, are involved in allergic reactions and in the control of pregnancy (Mosmann and Sad, 1996).

CD4 responses can be, to a certain extent, assessed by the use of proliferation assays to specific exogenous soluble antigens. The addition of exogenous antigen to PBMCs in culture, allows the APCs present to process the antigen for recognition by CD4 cells. The CD4 cells become activated and release cytokines, activating other cells in the culture. The resulting level of proliferation is then measured by the incorporation of ^{3}H-thymidine into replicating DNA. The total proliferative capacity of T cells can be assessed by measuring the responses to T cell mitogens such as phytohaemagglutinin (PHA). This is a plant lectin which acts by stimulating the TCR, but the ability to do so is dependent on the expression and function of the TCR (Paul, 1993).

Figure 1.4 **CD4 cells in immune responses**

The role of CD4 cells in cytokine production, the differentiation of Th-1 or Th-2 responses and the subsequent effects on CD8 cells, macrophages and B cells. Th-1 cytokines are shown in green. Solid green arrows indicate the stimulatory effect of Th-1 cytokines. Dotted green arrows indicate the inhibitory effect of Th-1 cytokines. Th-2 cytokines are shown in blue. Solid blue arrows indicate the stimulatory effect of Th-2 cytokines. Dotted blue arrows indicate the inhibitory effect of Th-2 cytokines.

Source: adapted from Kemeny *et al.*, 1994; Romagnani, 1992; Mosmann and Sad, 1996; Kelso, 1995


NK cells
IL-12
APC
Mast cell
IFN-γ
IL-4
CD4 cell
Th 0
IL-4
IL-2, IFN-γ , IL-4
Th 1
Th 2
IFN-γ
IL-4
IL-2, IFN-γ
IL-4, IL-5, Il-6
IL-10, IL-13
CD8 cell
Tc 0
IL-7
IFN-γ
IL-4
Macrophage
B cells
Tc 1
Tc 2
DTH
IL-2, IFN-γ
CTL
IL4
Antibody
secretion
IL-4, IL-5
IL-6,IL-10
IFN-γ
Suppressor activity

In HIV-1 infection, the depletion or dysfunction of CD4 cells increases the likelihood of opportunistic infections. Many studies have assessed the function of PBMCs *in vitro*, before the loss of CD4 cell numbers *in vivo*. Proliferative responses to recall antigens such as Tuberculin purified protein derivative (PPD), tetanus toxoid (TT), *Candida* antigens, diphtheria toxoid (DT), *Cryptococcus* antigens and influenza antigens have all been studied and found to be reduced in HIV-1-infected adults (Pontesilli *et al.*, 1995; Ranki *et al.*, 1989; van Noesel *et al.*, 1990; Hoy *et al.*, 1988; Clerici *et al.*, 1989) and children (Borkowsky *et al.*, 1990; Chirmule *et al.*, 1995). Proliferation to HIV-1 antigens has been found in a small proportion of HIV-1-infected adults (Pontesilli *et al.*, 1995; Ranki *et al.*, 1989) and children (Borkowsky *et al.*, 1990). Lymphoproliferative responses to mitogens such as phytohaemagglutinin (PHA), pokeweed mitogen (PWM) and anti-CD3 antibodies (α-CD3) are lost later in disease in adults (Hoy *et al.*, 1988; van Noesel *et al.*, 1990; Janossy *et al.*, 1993; Gruters *et al.*, 1991; Hoffman *et al.*, 1989) and children (Chirmule *et al.*, 1995). A pattern of lymphoproliferative defects has been established in asymptomatic adults before CD4 cell loss is seen: recall responses to HIV-1 are lost first, then to other antigens, followed by responses to allogenic antigens, followed by responses to mitogens (Janossy *et al.*, 1993; Clerici *et al.*, 1989), PWM responses being lost before PHA responses (Hoffman *et al.*, 1989). The loss of proliferative responses is associated with an increased likelihood of clinical progression (Giorgi *et al.*, 1987; Chirmule *et al.*, 1995). Several mechanisms to explain the loss of proliferative function have been postulated, such as a switch from the production of Th-1 to Th-2 cytokines by CD4 cells (Clerici and Shearer, 1993), although Th-2 cytokine production has also been found to be dysregulated (Diaz-Mitoma *et al.*, 1995). Another possible mechanism is the selective loss or dysfunction of memory cells (Janossy *et al.*, 1993; van Noesel *et al.*, 1990; Gruters *et al.*, 1991) possibly through infection of these cells with HIV-1 (Schnittman *et al.*, 1990; Cayota *et al.*, 1990; Cayota *et al.*, 1993), though this has not been found in all studies (Giorgi *et al.*, 1987; Hoy *et al.*, 1988).

1.3.2.2.2.2 CD8 responses

CD8 cells recognise peptide antigens (of approximately nine amino acids long) which are presented by MHC class I molecules. These peptides, which fit into the peptide binding groove of the class I molecule, have amino acid motifs which are specific for that particular

HLA-type (Nixon and McMichael, 1991). Particular amino acids bind the peptide into the groove (anchor residues), while other residues interact with the T-cell receptor (Paul, 1993). It has also been shown that MHC processing and presentation of peptide can be mimicked using exogenous synthetic peptides corresponding to the epitope sequence (Townsend *et al.*, 1986). In addition, amino acid changes within an epitope can abolish a specific CTL response (Townsend *et al.*, 1986), either by preventing the binding of peptide to the HLA molecule (Couillin *et al.*, 1995), by non-recognition by the effector cells (Phillips *et al.*, 1991) or by antagonising the CTL response to the corresponding wild type variant (Klenerman *et al.*, 1994; Bertoletti *et al.*, 1994).

The recognition of peptide leads to activation of the CD8 cell which subsequently can mature and differentiate into a cytotoxic T lymphocyte (CTL). A second co-stimulatory signal is required and occurs through the interaction of a surface molecule, CD28, with its ligand B7, usually expressed on APCs. Differentiation of CTLs requires IL-2, normally produced from Th-1 cells (see figure 1.4) (Paul, 1993). The exact mechanism by which mature CTLs kill target cells is not clear (Paul, 1993), but various mechanisms have been postulated: the CTL binds to its target cell and releases cytotoxic granules containing perforin, a potent cytolytic protein. The release of other cytokines, or the induction of apoptosis (programmed cell death), are other mechanisms by which cell lysis may occur (Paul, 1993).

CTL responses are measured by the stimulation of PBMCs in culture, using specific or non-specific stimuli. Target cells express the specific viral antigens and are labelled with ^{51}Cr chromium. The ability of the CTLs to recognise and lyse target cells is measured by the release of ^{51}Cr into culture supernatant.

CTL activity, mediated by MHC-restricted CD8 cytotoxic T lymphocytes has been found in HIV-1 infection, to both structural and regulatory gene products (Watret *et al.*, 1993; Lamhamedi-Cherradi *et al.*, 1992; Walker *et al.*, 1988; Walker *et al.*, 1987; Hoffenbach *et al.*, 1989; Koup *et al.*, 1989; Ho *et al.*, 1993). CTLs detected soon after infection with HIV-1 are involved in the control of primary viraemia (Borrow *et al.*, 1994; Lamhamedi-Cherradi *et al.*, 1995). These are maintained during the asymptomatic phase of infection, but are lost with disease progression (O'Toole *et al.*, 1992; Pantaleo *et al.*, 1990a). Specific CTL epitopes have been mapped in different proteins (Nixon *et al.*, 1988; Brander *et al.*, 1995).

CTL activity is mediated by (phenotypically) activated cells expressing CD38 and HLA-DR (Ho *et al.*, 1993; Watret *et al.*, 1993; Pantaleo *et al.*, 1990a; van Baalen *et al.*, 1993). Loss of CTL activity has been associated with several mechanisms, such as an inability of cells to clonogenically expand *in vitro* (Pantaleo *et al.*, 1990a; Pantaleo *et al.*, 1990b; Watret *et al.*, 1993), although it has been found that general cytolytic function is still present even if HIV-1-specific lysis has decreased (Pantaleo *et al.*, 1990a). Mutations within HIV-1, to evade recognition of the virus by CTLs, have also been described (Phillips *et al.*, 1991). In addition, HIV-1 variants, with mutations at specific CTL epitopes have been found, which prevent recognition of wild-type sequences by CTLs (Meier *et al.*, 1995; Klenerman *et al.*, 1994).

The importance of CTLs in the immune response against HIV-1 infection has prompted studies of infusion of autologous cells into patients (Klimas, 1992; Herbermann, 1992; Koenig *et al.*, 1995). Preliminary results of studies which infused mitogen- or lymphokine-activated cells showed good clinical responses in patients with AIDS (Herbermann, 1992; Klimas, 1992). However, the infusion of a single CTL clone into a patient did not prevent disease progression (Koenig *et al.*, 1995). CTLs have been detected in various organs, such as lung (Plata *et al.*, 1987), cerebrospinal fluid (CSF) (Sethi *et al.*, 1988), lymph nodes (Hoffenbach *et al.*, 1989), and spleen (Cheynier *et al.*, 1994). Increased destruction of cells by CTLs may contribute to the pathological damage seen in these tissues (Embretson *et al.*, 1993; Pantaleo *et al.*, 1993; Zinkernagel, 1995). CTLs release cytokines on encounter with their antigens which could affect the way in which other cells react to HIV-1, and possibly increase HIV-1 transcription (Jassoy *et al.*, 1993; Bollinger *et al.*, 1993).

HIV-1-specific CTL activity has also been studied in children, but to a lesser extent than in adults (Luzuriaga *et al.*, 1991; Cheynier *et al.*, 1992; Buseyne *et al.*, 1993; McFarland *et al.*, 1994; Luzuriaga *et al.*, 1995). In some studies, the CTL activity appeared to be of a similar pattern to that found in adults (Cheynier *et al.*, 1992; McFarland *et al.*, 1994), whereas others have found a less consistent detection pattern (Buseyne *et al.*, 1993; Luzuriaga *et al.*, 1991; Luzuriaga *et al.*, 1995).

CTLs are thought to lyse HIV-1-infected cells. However, CD8 cells have also been shown to release an antiviral factor which suppresses HIV-1 replication in CD4 cells (Walker and

Levy, 1989; Walker *et al.*, 1989). This suppression was found to be non-MHC restricted, mediated by a soluble factor and decreased with clinical progression of the individual (Mackewicz *et al.*, 1991; Mackewicz and Levy, 1992). There has been a suggestion that this CD8 antiviral factor may be released by CTLs (Bollinger *et al.*, 1993; Tsubota *et al.*, 1989). Candidates for this antiviral factor include IL-16 (Baier *et al.*, 1995) and small chemotactic peptides (β-chemokines), RANTES, MIP-1α and MIP-1β (Cocchi *et al.*, 1995). However, although these have some anti-HIV-1 activity, these are not the same as the antiviral factor originally described (Levy *et al.*, 1996).

1.4 IMMUNOLOGICAL MARKERS OF HIV-1 DISEASE PROGRESSION

Although it was thought that viral latency was a feature of the asymptomatic phase of infection, it is now clear the virus is actively replicating and causing pathological changes in lymphoid tissue (Pantaleo *et al.*, 1993; Embretson *et al.*, 1993). This was also indicated by the rapid turnover of CD4 cells and plasma virions (Ho *et al.*, 1995; Wei *et al.*, 1995) although peripheral blood cell numbers are maintained. It has been suggested that these cell numbers were maintained by proliferation of the cells rather than replenishment from the thymus (Ho *et al.*, 1995).

As was found in the earliest cases, those who presented with AIDS-defining illnesses had very few circulating CD4 cells (Gottlieb *et al.*, 1981; Centers for Disease Control, 1982). As the decrease in CD4 cell count, in peripheral blood, occurs later on in the asymptomatic phase of HIV-1 infection, it can therefore be used as a predictor of onset of AIDS (Phillips *et al.*, 1992). However individual CD4 cell count variability can make these measurements less reliable (Aledort *et al.*, 1992; Hoover *et al.*, 1992). In both adults and children different serological, virological and immunological factors have been extensively studied to identify surrogate, prognostic markers which may predict the loss of CD4 cells and clinical progression.

1.4.1 SEROLOGICAL MARKERS

Total immunoglobulin (Ig) levels have been studied in individuals. These, particularly IgA levels in serum, have been found to increase with disease progression, a phenomenon found in both adults (Fernandez-Cruz *et al.*, 1990; Chaisson *et al.*, 1992) and children (d'Arminio Montforte *et al.*, 1990). Specific anti-HIV-1 antibodies have been found to be lost with disease progression in both adults and children, and are associated with an increased risk of severe clinical disorders (Fernandez-Cruz *et al.*, 1990; Duliège *et al.*, 1992; van de Perre *et al.*, 1992).

Measurement of substances which are reflective of cell turnover, such as ß-2 microglobulin (the light chain subunit of the MHC-class I molecule) and neopterin (a compound derived from stimulated macrophages), are also increased in the serum of both adults and children with disease progression and decreased CD4 counts (Fernandez-Cruz *et al.*, 1990; Fahey *et al.*, 1990; Chaisson *et al.*, 1992; Ellaurie *et al.*, 1992).

1.4.2 VIROLOGICAL MARKERS

An increased viral load, as measured by plasma RNA levels, or the ability to culture HIV-1 from plasma or cells (Eschaich *et al.*, 1991; Venet *et al.*, 1991; Bouscarat *et al.*, 1996), and increased HIV-1 replication (Michael *et al.*, 1992) all appear to be associated with clinical disease progression and decreased CD4 levels. In both adults and children, p24 antigenaemia has also been found to be increased with low CD4 levels in some studies (Ellaurie *et al.*, 1992; Fernandez-Cruz *et al.*, 1990) but not in others (Italian Register for HIV Infection in Children, 1994; Duliège *et al.*, 1992; Venet *et al.*, 1991). Direct comparison of plasma p24 and viral RNA in children undergoing anti-retroviral therapy showed that these markers do not vary in the same way (Bush *et al.*, 1996), implying that the p24 levels could include levels of antigen from defective virus, whereas RNA levels are more indicative of viral replicative ability.

1.4.3 LYMPHOCYTE SURFACE MARKERS

The expression of different antigens on the surface of CD4 and CD8 lymphocytes has been shown to be associated with particular cellular functions. These antigens and their associated functions are summarised in table 1.2. There appears to be a large amount of overlap in the expression of different antigens on the same cells. No one surface marker is definitive for a particular function. For example, there is not a 'memory' phenotype as such, but both CD45RO and CD11a (and other antigens) have been described as being expressed on memory cells (Sanders *et al.*, 1988a).

The expression of these markers in HIV-1 infection have been assessed at different stages and are summarised in table 1.3. The measurement of the expression of two antigens may be more informative or predictive of disease progression, than the measurement of single antigens. For example, an increase in CD8 cells expressing HLA-DR, but not CD38, is found in asymptomatic infected adults and long term survivors (Giorgi and Detels, 1989; Ho *et al.*, 1993; Giorgi *et al.*, 1994), while an increase in CD8 cells expressing CD38, but not HLA-DR, is associated with onset of AIDS in adults (Levacher *et al.*, 1992). However, anti-HIV-1 specific CTLs are been shown to be CD8 cells that express both CD38 and HLA-DR (Ho *et al.*, 1993). The increased CD8 cell number seen in HIV-1 infection, has been reported to be due to an increase in the proportion of phenotypically activated and memory effector cells, expressing CD45RO, CD38, HLA-DR and CD57 (Choremi-Papadopoulou *et al.*, 1994; Vingerhoets *et al.*, 1995; Landay *et al.*, 1993; Kestens *et al.*, 1994; Prince and Jensen, 1991) and a decreased proportion of CD8 cells expressing CD28 or CD45RA (Borthwick *et al.*, 1994; Landay *et al.*, 1993; Prince and Jensen, 1991).

1.5 HIV-1-EXPOSED INDIVIDUALS

HIV-1 has been detected by PCR and *in situ* hybridisation in individuals who remained negative to HIV-1 antibodies (Pezzella *et al.*, 1989). There have also been reports of children born to HIV-1-infected mothers, from whom HIV-1 has been detected by PCR or isolated by culture and who have later become seronegative (European Collaborative Study, 1991; de

Table 1.2 **Expression of lymphocyte surface markers**

Antigen	Expression and functional role
CD45	Leucocyte common antigen. Different isoforms are expressed through differential splicing of the mRNA (Wallace and Beverley, 1990; Clark and Ledbetter, 1989). The antigen has tyrosine phosphatase activity in the cytoplasm which is involved in cell signal transduction.
CD45RO	The isoform of CD45 expressed on memory or immunologically primed T cells (Akbar *et al.*, 1988; Sanders *et al.*, 1988b; Merkenschlager *et al.*, 1988; Akbar *et al.*, 1991; Beverley, 1990; Sanders *et al.*, 1988a). Determines cytokine release from CD4 cells (Akbar *et al.*, 1991). Expressed on cytotoxic CD8 effector cells (Vanham *et al.*, 1991; Merkenschlager and Beverley, 1989; de Jong *et al.*, 1991). Memory cells recirculate through the tissues to lymph node (Picker *et al.*, 1993; Mackay, 1991).
CD45RA	Isoform of CD45 expressed on immunologically naïve cells (de Jong *et al.*, 1991), which once primed, express the CD45RO moiety through a CD45RA+ CD45RO+ double positive intermediate (Akbar *et al.*, 1988; Akbar *et al.*, 1991; Beverley, 1990; Picker *et al.*, 1993; Yamashita and Clement, 1989). This unidirectional switch appears to be true for CD4 cells, but not CD8 cells (Sohen *et al.*, 1990; Rabin *et al.*, 1995; Okumura *et al.*, 1993; Scala *et al.*, 1995). Elimination of stimulating antigen may allow the CD45RO cells to revert to CD45RA cells, while retaining memory for antigen (Beverley, 1990). These cells induce CD8 cells to suppress immunoglobulin production, hence called 'suppressor-inducer' cells (Akbar *et al.*, 1988; Merkenschlager and Beverley, 1989; Morimoto *et al.*, 1985; Schnittman *et al.*, 1990). Naïve lymphocytes recirculate through high endothelial venules to the lymph node (Picker *et al.*, 1993; Mackay, 1991).
HLA-DR	Product of the DR locus of MHC class II gene. Detectable on macrophages and B cells, and induced on immunologically activated T cells (Pantaleo *et*

al., 1990b; Ziegler-Heitbrock *et al.*, 1988; Miyawaki *et al.*, 1991; Picker *et al.*, 1993).

CD11a — α chain of leucocyte function antigen 1 (LFA-1). Initially described as being able to differentiate between CD8 cells with cytotoxic and suppressor functions, based on the intensity of expression (Sohen *et al.*, 1990; Morimoto *et al.*, 1987). Expressed on memory cells (Rabin *et al.*, 1995; Picker *et al.*, 1993; Sanders *et al.*, 1988a).

CD57 — Expressed on T cells and NK cells (Remy *et al.*, 1991; Yabuhara *et al.*, 1990; Wang *et al.*, 1994). Cells expressing this marker are important against viral infections (Bonagura *et al.*, 1992). Suppresses generation of EBV-specific CTL through a cell-mediated action (Wang *et al.*, 1994). Possibly a marker for suppressor or anergic T cell subpopulations (Wang *et al.*, 1994).

CD38 — Normally expressed on activated or immature T and B cells and monocytes (Kestens *et al.*, 1992; Giorgi and Detels, 1989; Fletcher *et al.*, 1992; Ziegler-Heitbrock *et al.*, 1988; Picker *et al.*, 1993; Malavasi *et al.*, 1994). Possibly involved in cell activation and/or cell adhesion (Malavasi *et al.*, 1994).

CD28 — Expressed at low level on thymocytes, more so on T cells and is up-regulated by activation, with an increase in Th-1 cytokine production and CD25 (the IL-2 receptor) expression (Linsley and Ledbetter, 1993). Interacts with the B7 ligand on APCs; acts as a co-stimulatory signal for T cell activation, required for antigen-specific T cell responses (Linsley and Ledbetter, 1993). Absence of CD28 co-stimulation leads to T cell anergy and/or clonal inactivation (Linsley and Ledbetter, 1993), which can be reversed on addition of IL-2 (Vingerhoets *et al.*, 1995; Linsley and Ledbetter, 1993). Signal transduction occurs through calcium-dependent and -independent pathways.

Functions of antigens expressed on the surface of lymphocytes

Table 1.3 **Lymphocyte surface markers in HIV-1 infection**

Antigen	Change seen in HIV-1 infection
CD4	Number and proportion of CD4 cells decreased at seroconversion (Zaunders *et al.*, 1995) and further decrease with disease progression (Levacher *et al.*, 1992; Zaunders *et al.*, 1995). Onset of AIDS associated with low CD4 cell number.
CD8	Proportion and number of CD8 cells increased at seroconversion (Yagi *et al.*, 1991; Zaunders *et al.*, 1995) and throughout disease (Giorgi and Detels, 1989; Scala *et al.*, 1995; Zaunders *et al.*, 1995). Loss of CD8 cells seen before onset of AIDS (Margolick *et al.*, 1995).
CD45RO	Decreased expression on CD4 cells of adults in some studies (Shearer and Clerici, 1991; van Noesel *et al.*, 1990; Klimas *et al.*, 1991; Zaunders *et al.*, 1995) but not in others (Giorgi and Detels, 1989; Vanham *et al.*, 1991; Watret *et al.*, 1993; Kestens *et al.*, 1994). Expressed on CD4 cells which were found to be preferentially infected with HIV-1 (Schnittman *et al.*, 1990; Cayota *et al.*, 1990). Expressed on CD4 cells which also show an increased expression of CD38 and HLA-DR (Choremi-Papadopoulou *et al.*, 1994; Kestens *et al.*, 1994), which correlated with a decreased CD4 level and presence of HIV-1 antigen (Kestens *et al.*, 1994). Increased expression on CD8 cells of both adults and children (Froebel *et al.*, 1991; Plaeger-Marshall *et al.*, 1993; Rabin *et al.*, 1995; Watret *et al.*, 1993; Bouscarat *et al.*, 1996; Landay *et al.*, 1993). Cells also show increased co-expression of CD38 or HLA-DR (Bouscarat *et al.*, 1996; Choremi-Papadopoulou *et al.*, 1994; Prince and Jensen, 1991; Zaunders *et al.*, 1995) and CD57 (Prince and Jensen, 1991).
CD45RA	Decreased expression on CD4 cells of adults and children in some studies (Plaeger-Marshall *et al.*, 1993; Plaeger-Marshall *et al.*, 1994; Zaunders *et al.*, 1995), no change in others (Fletcher *et al.*, 1992). Decreased expression on

CD8 cells of both adults and children (Plaeger-Marshall *et al.*, 1993; Rabin *et al.*, 1995; Scala *et al.*, 1995; Landay *et al.*, 1993; Plaeger-Marshall *et al.*, 1994; Prince and Jensen, 1991; Roederer *et al.*, 1995; Zaunders *et al.*, 1995).

HLA-DR — Increased expression on CD4 and CD8 cells of adults and children (Vanham *et al.*, 1991; Kestens *et al.*, 1992; Ziegler-Heitbrock *et al.*, 1988; Giorgi and Detels, 1989; Pantaleo *et al.*, 1990b; Miyawaki *et al.*, 1991; Bouscarat *et al.*, 1996; Levacher *et al.*, 1992; Landay *et al.*, 1993; Kestens *et al.*, 1994; Plaeger-Marshall *et al.*, 1994; Prince and Jensen, 1991; Zaunders *et al.*, 1995; Stites *et al.*, 1989). This increased expression is less marked with the onset of AIDS (Levacher *et al.*, 1992). Expressed on CD8 cells which thought to be responsible for suppression of HIV-1 replication (Landay *et al.*, 1993).

CD11a — Increased expression on CD8 cells of adults (Watret *et al.*, 1993; Cavallin *et al.*, 1993; Scala *et al.*, 1995; Zaunders *et al.*, 1995). Possible relation to increased IgG production due to decreased number of suppressor cells (Morimoto *et al.*, 1987; Cavallin *et al.*, 1993). Increased proportion of CD8 cells expressing CD45RA and CD11a seen which correlates to decreased CD4 levels (Scala *et al.*, 1995).

CD57 — Increased expression on CD8 cells of both adults and children (Ziegler-Heitbrock *et al.*, 1988; Kestens *et al.*, 1992; Bouscarat *et al.*, 1996; Levacher *et al.*, 1992; Gupta, 1986; Plaeger-Marshall *et al.*, 1993; Landay *et al.*, 1993; Plaeger-Marshall *et al.*, 1994; Prince and Jensen, 1991), with decrease in NK activity (Gupta, 1986). Suppresses anti-HIV-1 CTL activity and replication (Joly *et al.*, 1989; Plaeger-Marshall *et al.*, 1992). Also suppresses NK and LAK activity through release of a soluble factor (Sadat-Sowti *et al.*, 1991).

CD38 — Increased expression on CD4 and CD8 cells of both adults and children (Kestens *et al.*, 1992; Bird and Watret, 1992; Plaeger-Marshall *et al.*, 1993; Landay *et al.*, 1993; Kestens *et al.*, 1994; Plaeger-Marshall *et al.*, 1994). This increase correlates with disease progression and can be used in disease

	staging (Giorgi *et al.*, 1994; Bouscarat *et al.*, 1996; Levacher *et al.*, 1992; Prince and Jensen, 1991). The level of co-expression of HLA-DR on CD8 cells expressing CD38 correlates with viral load (Bouscarat *et al.*, 1996). The intensity of CD38 expression on CD8 cells in children increased with disease progression, and occurred on CD8 cells which were co-expressing HLA-DR (Plaeger-Marshall *et al.*, 1994). Expressed on anti-HIV-1-specific CTLs (Ho *et al.*, 1993).
CD28	Decreased expression on CD4 and CD8 cells of adults (Caruso *et al.*, 1994; Bouscarat *et al.*, 1996; Choremi-Papadopoulou *et al.*, 1994; Vingerhoets *et al.*, 1995; Borthwick *et al.*, 1994). Loss of CD28 expression parallels the increase of HLA-DR, CD45RO and CD38 expression on CD4 cells but not CD8 cells (Choremi-Papadopoulou *et al.*, 1994). Expressed on CD8 cells thought to be responsible for suppression of HIV-1 replication (Landay *et al.*, 1993).

Changes in antigen expression on lymphocytes as a consequence of HIV-1 infection

Rossi *et al.*, 1991; Bryson *et al.*, 1995; Roques *et al.*, 1995; Rogers *et al.*, 1989). This could be due to infection with defective virus. However, there is evidence that active immune responses occur in exposed individuals (including those from whom no evidence of HIV-1-infection is seen) which may clear virus before infection becomes established (reviewed in Rowland-Jones and McMichael, 1995). HIV-1-specific CTL activity has been detected in adults (Langlade-Demoyen *et al.*, 1994; Pinto *et al.*, 1995) and children (Cheynier *et al.*, 1992; Luzuriaga *et al.*, 1991; Rowland-Jones *et al.*, 1993) as have lymphoproliferative responses to HIV-1 antigens (Kelker *et al.*, 1992; Borkowsky *et al.*, 1990; Clerici *et al.*, 1994; Clerici *et al.*, 1993b).

More recently, individuals have been identified who appear to be resistant to HIV-1 infection. These individuals were first described as having high plasma levels of the β-chemokines RANTES, MIP-1α and MIP-1β (Paxton *et al.*, 1996). Further investigation showed that they have a 32 bp deletion in both alleles for the chemokine receptor CCR5, which causes aberrant folding of the protein, leading to the non-expression of the receptor on the cell surface (Samson *et al.*, 1996; Liu *et al.*, 1996). CCR5 has been shown to be the co-receptor for entry of M-tropic viruses, and the cells from these individuals were found to be uninfectable with M-tropic strains of HIV-1. Heterozygosity for this mutation (i.e. one normal allele and one allele containing the 32 bp deletion), gives a lower level of expression of CCR5 on the cell surface, but those receptors that are present allow the entry and replication of HIV-1 (Dean *et al.*, 1996; Huang *et al.*, 1996).

1.6 EDINBURGH COHORT OF HIV-1-INFECTED DRUG USERS

The Edinburgh cohort of HIV-1-infected intravenous drug users (IVDUs) has been studied since 1985 when it was found that many were infected with HIV-1. Retrospective analysis of stored blood samples showed that the first IVDUs were infected in 1983 and HIV-1 was transmitted further by the sharing of needles (Robertson *et al.*, 1986; Brettle *et al.*, 1987). Phylogenetic analysis of HIV-1 isolates from this cohort indicates that they were all infected with a similar strain of HIV-1 (Holmes *et al.*, 1995). Approximately one third of these drug

users are women, some of whom have borne children since becoming infected with HIV-1. The children are seen at regular intervals at a follow-up clinic (Mok *et al.*, 1989a; Mok *et al.*, 1989b), when clinical, virological and immunological markers of disease are monitored.

1.6.1 IMMUNOLOGICAL STUDIES IN CHILDREN AND OVERVIEW OF THESIS

As HIV-1 was first identified in homosexual men and was less widely spread in the heterosexual population, most initial cohort studies were carried out in homosexual men. Many functional studies have been performed in adults, analysing different immunological responses (see 1.3). However, fewer studies of immunological function have been carried out in children. Those which have been done, have been cross-sectional studies of one particular aspect (e.g. CTL activity, lymphocyte surface marker expression or proliferative responses). Longitudinal studies in children have, mostly, been confined to clinical studies of the natural history of HIV-1 infection.

Children are born with an intact, but 'virgin', cellular immune system. Therefore, in the first few months after birth, innate immune mechanisms such as NK cells (which do not require prior activation to function) have an important role against infection (Yabuhara *et al.*, 1990). Maternal antibodies, which cross the placenta before birth, and the antibodies in breast milk, particularly colostrum, would have some effect against specific antigens encountered during this time, until the child is able to make antibodies for itself (Wilson, 1986; Pabst and Kreth, 1980). However, even these mechanisms may be reduced, compared to adult levels, as different aspects of the immune system mature at different rates (Pabst and Kreth, 1980). Exposure to different environmental, food and bacterial antigens, causes the immune system to become activated and to develop immunological memory (Cummins *et al.*, 1994; Pabst and Kreth, 1980). Little is known about the development of cell-mediated responses in immunologically naïve individuals. The measurement of surface markers on lymphocytes, in children, has indicated that the expression of some of these markers is age-related (Hayward *et al.*, 1989) and indicate the immaturity of these lymphocytes.

It was decided to prospectively follow the children born to the HIV-1-infected mothers in the

Edinburgh IVDU cohort to investigate changes in T-cell mediated immunity, comparing those in infected and exposed uninfected children. In addition, lymphocyte surface marker analysis was performed to see whether changes in marker expression were related to clinical disease progression.

The results of the lymphocyte surface marker analysis, together with the clinical profile of the children, are presented in chapter 3. These are described first, as the functional responses are related to clinical disease progression in the children. The main T-cell mediated response studied was CTL activity, and these results are presented in chapter 4. As an example of another T-cell mediated response, the proliferative capacity of the cells to different stimuli is presented in chapter 5. During the study, one child was noted who had a disproportionate level of CD3 cells compared to CD4 or CD8 cells. These cells were further investigated and the results are presented in chapter 6. Chapter 7 brings all these results together and discusses their relevance to each other, in the light of other studies in children.

Some of the data presented in this thesis has already been published, and the papers are presented in the appendix. The data presented in the bulk of this thesis were collected by me, whereas the published papers include data collected by other people as part of collaborative studies on these children.

1.6.2 DEFINITIONS

Throughout this thesis several terms are used, in relating to children, which need to be clearly defined.

HIV-1-INFECTED — those children who have been confirmed to be infected with HIV-1 (see 1.2.2).

HIV-1-EXPOSED — a term used for all children born to HIV-1-infected mothers, including the known infected children. These children, by virtue of their mothers HIV-1-status, are presumed to have been exposed to HIV-1 *in utero*.

EXPOSED UNINFECTED those children, born to HIV-1-infected mothers, who have been confirmed to be uninfected with HIV-1 and have lost all maternal antibody.

INDETERMINATE those children, born to HIV-1-infected mothers, but who have not lost maternal antibody. These children do not have any other indicators of HIV-1 infection. During analysis of the results in this thesis, children who were initially indeterminate were reclassified as HIV-1-infected or exposed uninfected.

Chapter 2 # MATERIALS AND METHODS

2.0 GENERAL MATERIALS

2.0.1 **Cell culture reagents**

2.0.2 **Disposable tissue culture plastics**

2.1 SEPARATION OF LYMPHOCYTES

2.2 FREEZING AND THAWING OF CELLS

2.2.1 **Freezing cells**

2.2.2 **Thawing cells**

2.3 LYMPHOCYTE SURFACE MARKER ANALYSIS

2.3.1 **Two colour analysis**

SimulSET™ programme

2.3.2 **Three colour analysis**

Two-step method

One-step method

LYSIS™II and PAINT-a-GATE™ Programmes

2.4 GENERATION OF EBV-TRANSFORMED B CELL LINES

2.5 CALIBRATION OF HIV-VACCINIA CONSTRUCTS

2.6 CTL ASSAYS

2.6.1 **Generation of CTL effector cells**

Co-culture of cells with autologous PHA-stimulated cells

Peptide stimulation

2.6.2 **Generation of target cells**

Vaccinia constructs

Peptide

2.6.3 **CTL assay**

2.6.4 **Immunofluorescent staining of B-cell lines infected with vaccinia-HIV-**

constructs

2.7 CYTOKINE ELISAS

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2.0 GENERAL MATERIALS

2.0.1 CELL CULTURE REAGENTS

RPMI culture medium was obtained from Protein Fractionation Centre, Edinburgh, UK.
Penicillin/Streptomycin was obtained from Gibco, Life Technologies Ltd, Paisley, UK, and stored at -20°C before use.
Glutamine was obtained from Gibco and stored at -20°C before use.
Foetal Calf Serum (FCS) was obtained from Globepharm Ltd, Esher, UK. This was heat inactivated for 1 hour at 56°C before use and batch tested for growth of cell lines. The same batch was used for CTL assays throughout this study, which was stored frozen at -20°C before use.
RPMI denotes RPMI medium, containing Penicillin (100 units/ml), Streptomycin (100 µg/ml), and Glutamine (2 mM), and R10 denotes RPMI (as above) containing 10% (v/v) heat-inactivated FCS.
Saline (0.9% w/v) was obtained from Baxter Healthcare Ltd, Thetford, UK.
Phosphate buffered saline (PBS) tablets were obtained from Oxoid, Unipath, Basingstoke, UK. PBS was made up at 1 tablet/100 ml distilled water.

2.0.2 DISPOSABLE TISSUE CULTURE PLASTICS

Unless otherwise indicated, tissue culture materials were obtained from Costar, High Wycombe, UK at tissue culture grade. These include:

stripettes (25ml, 10ml, 5ml, 2ml, 1ml)
clusters (6-,24-,48-,96-well)
flasks (225cm^2, 75cm^2, 25cm^2)
filters (0.8µm, 0.45µm, 0.22µm)

Other plastics were obtained from Bibby Sterilin Ltd, Stone, UK. These include:

Pastettes
7ml Bijoux
10ml centrifuge tubes

Universal containers

Cryovials were obtained from Nunc (UK).

2.1 SEPARATION OF LYMPHOCYTES

Materials:

Lymphoprep (Nycomed, Birmingham, UK)

Saline

R10

Heparinised whole blood was diluted 1:2 with saline and layered on to Lymphoprep (5ml) in a 10ml tube, taking care not to mix the blood at the interface. The tubes were centrifuged at 2100 rpm for 30 minutes, at room temperature, with the brake off. The buffy layer, containing the peripheral mononuclear cells (PBMCs), was carefully removed with a pastette and transferred to another 10ml tube. Sterile saline or PBS was added to make the volume up to 10ml and the tubes were centrifuged again at 1700 rpm for 15-20 minutes. The cell pellet was resuspended in 10ml saline and centrifuged at 1100 rpm for 5 minutes. The cell pellet was then resuspended in R10 for use as required.

2.2 FREEZING AND THAWING OF CELLS

2.2.1 FREEZING CELLS

Materials:

R10

Freezing mix, which contains 40% (v/v) RPMI, 50% (v/v) FCS and 10% dimethyl sulphoxide (DMSO).

Freezing box containing propan-2-ol, which controls the rate of freezing of the cells to -1°C per minute when placed in a -70°C freezer (Nunclon, UK).

Cells were spun down, washed in R10 and counted. The cell pellet was suspended in 1ml freezing mix, transferred to a cryotube which was put into a freezing box. Cells were subsequently stored in the vapour phase of liquid nitrogen.

2.2.2 THAWING CELLS

Materials:

R10

Cells were thawed quickly at 37°C in a water bath and transferred to a 10ml tube. An equal volume of R10 was added dropwise, with shaking. The cells were left to equilibrate over several minutes. Another equal volume of R10 was added dropwise, with shaking. The cells were left to equilibrate over several minutes. The cells were washed twice in R10, counted and used as required.

2.3 LYMPHOCYTE SURFACE MARKER ANALYSIS

2.3.1 TWO COLOUR ANALYSIS

Unless otherwise indicated, the antibodies were obtained from Becton Dickinson (BD), Oxford, UK.

-PE denotes that the antibody was conjugated to phycoerithrin.

-FITC denotes that the antibody was conjugated to fluorescein isothiocyanate.

-RD1 denotes that the antibody was conjugated to rhodamine.

-PerCP denotes that the antibody was conjugated to peridinal chlorophyll protein

Materials:

A panel of antibodies was used (see below).

FACSLyse™ was obtained from BD

Saline or PBS

1% (w/v) paraformaldehyde in PBS (1% PAF)

Round-bottomed tubes (4ml) were obtained from A&J Beveridge, Edinburgh, UK.

Antibody panel:

LeucoGATE™	anti-CD14-PE/anti-CD45-FITC
Control	anti-IgG1-PE/anti-IgG2-FITC
CD3/DR	anti-CD3(Leu4)-PE/anti-HLA-DR-FITC
CD3/CD8	anti-CD3(Leu4)-PE/anti-CD8(Leu2a)-FITC
CD3/CD4	anti-CD3(Leu4)-PE/anti-CD4(Leu3)-FITC
CD4/RO	anti-CD4(Leu3)-PE/anti-CD45RO(UCHL1)-FITC (Dako,High Wycombe,UK)
CD4/DR	anti-CD4(Leu3)-PE/anti-HLA-DR-FITC
CD4/RA	anti-CD4(Leu3)-PE/anti-CD45RA(Leu18)-FITC
CD8/RO	anti-CD8(Leu2a)-PE/anti-CD45RO(UCHL1)-FITC (Dako)
CD8/DR	anti-CD8(Leu2a)-PE/anti-HLA-DR-FITC
CD8/CD11a	anti-CD11a(S6F1)-RD1/anti-CD8-FITC (Coulter, Luton,UK)
CD8/CD57	anti-CD8(Leu2a)-PE/anti-CD57(Leu7)-FITC
CD8/RA	anti-CD8(Leu2a)-PE/anti-CD45RA(Leu18)-FITC

Each antibody mix (10µl) was added to diluted whole blood (100µl) in a separate round-bottomed tube and incubated at room temperature for 15 minutes. Red cells were lysed by the addition of FACSLyse™ (2ml) and incubating at room temperature for a further 10 minutes. The tubes were centrifuged at 1100rpm for 5 minutes, washed in saline or PBS, and centrifuged again. The cells were fixed in 1% PAF (400µl) and stored at 4°C until they were analysed on a FACScan™ (BD) using the SimulSET™ programme (BD, detailed below).

2.3.1.1 SimulSET™ programme

This programme uses automatic instrument control settings to distinguish lymphocyte populations from the other cell populations in whole blood. This is by virtue of the cell size which is detected by the forward scatter (FSC), the cell granularity which is detected by side scatter (SSC) and an antibody combination of anti-CD45 and anti-CD14 (the leucoGATE™ tube). All leucocytes express CD45 (hence the leucocyte common antigen) but at different

intensities. Lymphocytes and monocytes are of a similar size and granularity, but monocytes are distinguished by the high intensity of expression of both CD14 and CD45. Lymphocytes do not express CD14. Granulocytes are recognised by their increased size, granularity and by a lower intensity of expression of CD45 than that shown by lymphocytes. Once the lymphocyte population had been defined a 'gate' was drawn around this population. For subsequent tubes, all cells within this gate were counted and all other cells were regarded as irrelevant. An example of the printout with these definitions is presented in figure 2.1.

The control tube used irrelevant antibodies to correct for background staining. The simulset programme automatically set markers, such that less than 1% of the cells were above background staining. These markers were used for subsequent tubes to define those cells which expressed the antigen being studied. The percentage of the cells within the lymphocyte gate which fell into each quadrant was automatically given by the programme. Quadrant 1 contained those cells which were only expressing the antigen detected by the PE-conjugated antibody; quadrant 2 contained cells which were expressing both the antigens of interest; quadrant 3 contained the cells which were not expressing either of the antigens of interest; quadrant 4 contained those cells which were only expressing the antigen detected by the FITC-conjugated antibody (see fig 2.1)

Throughout these experiments the percentage of CD4 (or CD8) cells which co-expressed the antigen of interest was calculated:

$$\text{percentage of CDX+ CD4 cells} = \frac{\text{CDX+ CD4 x 100}}{\text{total CD4}}$$

where the value for CDX+ CD4 is from quadrant 2, and the total CD4 value was obtained by addition of quadrant 2 to quadrant 1 or 4, depending on the fluorochrome used for CD4 in that particular antibody combination.

Figure 2.1 **SimulsSET™ Software**

An example of the printout obtained from the SimulSETTM software for the a) LeucoGATE™, b) Control tubes, and c) the CD3/CD4 tube.

a) LeucoGATE™ tube.

The left hand box shows the forward (FSC) and side-scatter (SSC). The leucocyte populations are indicated within this box: the lymphocytes are shown as the green population, the monocytes as the red population, the granulocytes as the blue population and cell debris in pink. The line drawn around the green population is the lymphocyte gate.

The right hand box shows the staining of the cells which fell within the lymphocyte gate. The *y*-axis shows the staining from anti-CD14, and the *x*-axis shows the staining from the anti-CD45. A few contaminating cells show the differences in staining for the respective populations: the red population (monocytes) stained well for both antibodies. A few granulocytes (blue population) are shown to the left of the main lymphocyte (green) population. These granulocytes do not stain for anti-CD14. The intensity of staining for anti-CD45 was less for granulocytes than that for the lymphocytes. The cell debris did not stain for either anti-CD14 or anti-CD45.

b) Control tube

This box shows the non-specific background staining of the cells. Markers have been set such that 99% of the cells are unstained and fall within quadrant 3. The quadrants are shown. The numbers (denoted %L) are the percentages of the gated lymphocytes which fell into each quadrant.

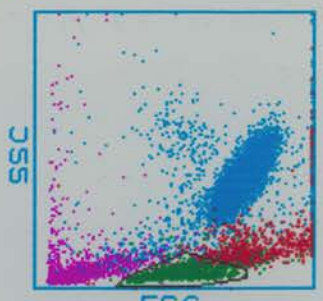
c) CD3/CD4 tube

This box shows the way in which the gated lymphocytes expressed CD3 and CD4, above the background staining set by the markers . The cells within quadrant 1 (upper left) are those which expressed CD4 but not CD3. The cells within quadrant 2 (upper right) are those cells which expressed both CD3 and CD4. The cells within quadrant 3 (lower left) are those which expressed neither CD3 nor CD4. The cells within quadrant 4 (lower right) are those which expressed CD3 but not CD4. The numbers (denoted %L) are the percentages of the gated lymphocytes which fell into each quadrant.

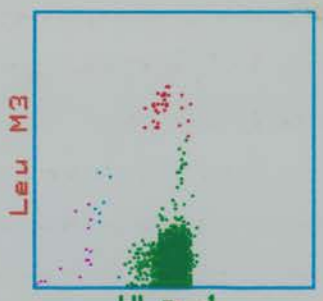
a) LeucoGATE [1]

P201296001

Gate:	FSC	SSC
	74	0
	82	8
	138	32
	194	16
	194	8
	146	0
Mean:	121	12



Gated Events : 3425
Total Events : 15000



Quadrant correction: Yes

Gate Description :	% Lymphs	% Monos	% Grans	% Debris	% Lymphs of total
	99	1	0	0	99

>> Operator defined gates <<

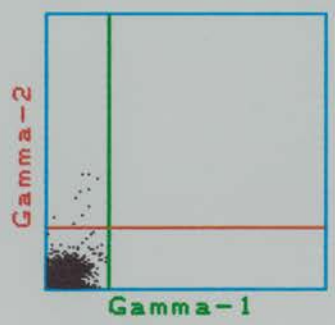
Three Part Differential	% of Leucocytes
Lymphocytes	36
Monocytes	9
Granulocytes	55

b) Control [1]

P201296002

Quad	Cell Type	Corr % L
1	NSS PE	0
2	NSS ++	0
3	Unstained	100
4	NSS FITC	0

Markers:
FL1 56
FL2 56



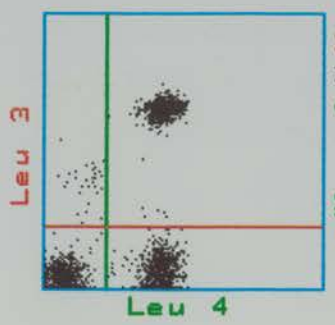
FSC Mean : 120 Gated Events : 2086
SSC Mean : 12 Total Events : 10000

c) CD3/CD4 [1]

P201296005

Quad	Cell Type	Corr % L
1	CD3-CD4+	1
2	CD3+CD4+	36
3	UNSTAINED	34
4	CD3+CD4-	28

Markers:
FL1 56
FL2 56



Subset Label	Corr % L
** CD4+ CELLS	36 ok

FSC Mean : 121 Gated Events : 2352
SSC Mean : 12 Total Events : 10000

2.3.2 THREE COLOUR ANALYSIS

2.3.2.1 Two-step method

This was carried out in two steps, using two layers of antibodies.

Materials:

first and second layer antibodies (see below)

FACSLyse™

Saline

1% PAF

Analysis of CD8 cells co-expressing CD45RO and CD38 used a combination of first layer antibodies:

anti-CD8-PE

anti-CD45RO-Biotin

anti-CD38

These were all obtained from Dr. G. Janossy, Royal Free Hospital, London, UK.

The second layer antibodies were:

goat-anti-mouse-FITC

streptavidin-Tricolour

These were obtained from Bradsure Biologicals, Market Harborough, UK

Analysis of CD8 cells co-expressing CD3 and CD28 used a combination of first layer antibodies:

anti-CD3-FITC (Bradsure)

anti-CD8-Biotin (Bradsure)

anti-CD28-PE

The second layer antibody was:-

streptavidin-Tricolour

The first layer antibody mix (10µl) was added to diluted whole blood (100µl) and incubated at room temperature for 10 minutes, then the cells were washed in saline and centrifuged at 1500 rpm for 5 minutes. The second layer antibody mix (10µl) was added and incubated for 10 minutes at room temperature. FACSLyse™ (2ml) was added and incubated at room temperature for 10 minutes, following which the cells were centrifuged at 1100 rpm for 5

minutes, washed in saline, and centrifuged again at 1100 rpm for 5 minutes. The cells were then fixed in 1% PAF (400µl) and stored at 4°C until analysed on FACScan™ using the LYSIS™II and PAINT-a-GATE™ programmes (BD).

2.3.2.2 One-step method

This was carried out in the same way as above only using directly conjugated antibodies.

Analysis of CD8 cells co-expressing CD45RO and CD38 used a combination of antibodies:

anti-CD8-PerCP
anti-CD38-FITC (Coulter)
anti-CD45RO-PE (Dako)

Analysis of CD8 cells co-expressing CD3 and CD28 used a combination of antibodies:

anti-CD8-PerCP
anti-CD3-FITC (Bradsure)
anti-CD28-PE

For both these antibody combinations, a control tube was set up, using the antibodies from the two-colour analysis.

The antibody mix (10µl) was added to diluted whole blood (100µl) and incubated at room temperature for 15 minutes. FACSLyse™ (2ml) was added and incubated at room temperature for 10 minutes. The cells were centrifuged at 1100 rpm for 5 minutes, washed in saline, and centrifuged again at 1100 rpm for 5 minutes. The cells were fixed in 1% PAF (400µl) and stored at 4°C until analysed on FACScan™ using the LYSIS™II and PAINT-a-GATE™ programmes (BD).

2.3.2.3 LYSIS™II and PAINT-a-GATE™ Programmes

Cells were acquired on a FACScan™, using the LYSIS™II programme (BD, instrument

control settings were those defined by Dr. M. Armitage). As in the two-colour analysis a lymphocyte gate could be defined by size and granularity of the cells (from FSC and SSC). The control tube was used to adjust the detector settings, so that the background fluorescence on each channel (FL-1, FL-2 or FL-3) was less than 10.

The cells were analysed using the PAINT-a-GATE™ programme (BD), which uses overlapping colours to identify different populations of cells expressing one or more of the antigens of interest. Histograms were used to define background values from the control tube, for each fluorescent channel (FL-1, FL-2, FL-3) and markers were set. As in the two-colour analysis, the markers were used to define cells expressing the antigens. The 'autopaint' facility within PAINT-a-GATE™ allowed cells expressing more than one antigen to be defined by different colours and the percentage of cells expressing each colour was automatically calculated. As in the two-colour method, the percentage of CD8 cells expressing one or more antigen was calculated using the same equation (2.3.1).

2.4 GENERATION OF EBV-TRANSFORMED B CELL LINES

Materials:

Epstein-Barr Virus (EBV) supernatant from B958 Marmoset cell line (obtained from S. Lockett). B958 cells were cultured to confluence and then starved (i.e. fresh medium was not added) for 7 days. The culture supernatant was collected and stored in liquid nitrogen. A 1:10 dilution of the supernatant was found to transform 1 x 10^6 PBMCs, freshly isolated from cord blood (S. Lockett, personal communication).

R10

Cyclosporin A (10µg/ml)

Freshly separated PBMCs (as described in 2.1) were suspended in R10 (2ml) in a bijou. EBV supernatant (200µl) and Cyclosporin A (20µl) was added. The cells were left for one week, and then fed by removing medium (1ml) and replacing with fresh R10 (1ml). This was repeated every week until the cells transformed. New cell lines were cultured for at least two months from addition of EBV, to ensure that the cell-lines were established. Lines were frozen down (at more than 1 x 10^7 cells/vial) and stored in the vapour phase of liquid

nitrogen. B-cell lines, throughout this thesis, are referred to as the patient number with a prefix of 'E', e.g. P125 goes to EP125. B cell-lines were thawed and grown up on receipt of a sample for a CTL assay (see 2.6).

2.5 CALIBRATION OF VACCINIA-HIV CONSTRUCTS

Materials:

TK143- cell line

R10

10% (v/v) Formalin

Crystal Violet (0.5% (w/v) in 20% methanol, and filtered through a 0.45µm filter)

Vaccinia constructs used in CTL assays (see 2.6.2.1).

0.25% (v/v) Trypsin (obtained from Gibco) in RPMI

TK143- cells were grown on a monolayer in 75cm^2 flasks, one flask used for each construct to be tested. Cells were trypsinised and washed in R10. Single cell suspensions were made by pipetting cells vigorously. The cells recovered from each flask, were each resuspended in R10 (30ml), divided into the wells of a 6-well plate and incubated overnight at 37°C. Virus stock was diluted in RPMI in 10-fold dilutions from 1:10^3 to 1:10^{10}. R10 was removed from the cell monolayers in the 6 well plates. Virus (1ml) was added to each monolayer using dilutions from 1:10^6 to 1:10^{10}, with one well containing RPMI alone as a control. The plates were incubated for 1 hour at 37°C, rocking every 10 minutes. Virus or medium was removed and R10 (5ml) added, after which the plates were incubated at 37°C for 2-3 days. The medium was removed and the cells fixed in 10% Formalin for 20 minutes at room temperature. Crystal Violet was used to visualise plaques by adding for 2 minutes, after which it was rinsed off with tap water. A well with a countable number of plaques was identified and these were counted (y). The number of plaque forming units (pfu) is calculated as y multiplied by the dilution factor.

2.6 CTL ASSAYS

2.6.1 GENERATION OF CTL EFFECTOR CELLS

2.6.1.1 Co-culture of cells with autologous PHA-stimulated cells

Materials:

R10

PHA-medium - R10 containing 5µg/ml Phytohaemagglutinin (PHA-P)

Lymphocult (Biotest, UK)

Freshly separated PBMCs were suspended in R10 (1ml) in a 10ml tube. 10% (100µl) of the cells were added to PHA-medium (1ml) in another 10ml tube. Both tubes were incubated at 37°C for 2 days. The tubes were spun at 1100 rpm for 5 minutes, the cell pellets were combined and resuspended in R10 (2ml) in a 24-well plate. This is counted as day 0 of culture. The surrounding wells were filled with sterile distilled water (2ml) to limit evaporation. The culture was incubated at 37°C for 7 days. Cells were put into a 10ml tube and spun at 1100 rpm for 5 minutes. Supernatant was kept for cytokine analysis. The cells were resuspended in R10 (2ml) containing 5% Lymphocult. Lymphocult was added (100µl) every 3-4 days until the cells were used in a CTL assay. It was aimed for CTL assays to be performed between 10 and 15 days after combination of cells in culture.

2.6.1.2 Peptide stimulation

Materials:

R10

Peptides (of between eight and ten amino acids) of CTL epitopes (obtained as a gift from Dr. Sarah Rowland-Jones, Institute of Molecular Medicine, Oxford).

Lymphocult

Interleukin-7 (IL-7) at a concentration of 1µg/ml

Freshly separated PBMCs were suspended in R10 (2ml) in a 24-well plate. All the HLA-specific peptides available (20µl of 1mg/ml peptide solution) were added, according to tissue type of cells. The surrounding wells were filled with distilled sterile water (2ml) to limit

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evaporation. The plate was incubated at 37°C. Lymphocult (200µl) was added every 3-4 days until the cells were used in a CTL assay. It was aimed for CTL assays to be performed after 14 days in culture.

On some occasions, IL-7 (50µl) was added to the peptide stimulated cultures on day 0 (i.e. as the cells were stimulated) and then, concurrently with the addition of Lymphocult.

2.6.2 GENERATION OF TARGET CELLS

2.6.2.1 Vaccinia constructs

Materials:

R10

Vaccinia vectors: p55-gag from HIV-1_{IIIB} (ARP253). Donated to the MRC AIDS Reagent Programme, NIBSC, UK by Professor A. McMichael and Dr. D. Nixon.

Wild type vaccinia, (vSC8), expressing ß-galactosidase. Donated to the MRC AIDS Reagent Programme, NIBSC, UK by Dr. B. Moss.

pol (VVTG3167), tat (VVTG3196) and env (VVTG1132) from HIV-1_{BRU}. These were obtained from Transgène, Strasbourg, France.

These constructs were kindly grown by Prof. F. Gotch, Chelsea and Westminster Hospital, London, UK.

^{51}Cr Sodium Chromate (CJS4), Amersham Life Sciences, Amersham, UK.

The autologous B cell-line, corresponding to the sample in culture for CTL, was thawed and grown as required. Cells were washed and resuspended at 1 x 10^6 cells/ml in R10. 6 x 1ml aliquots were put into 10ml tubes, and 1 x 10^6 pfu of each of the vaccinia-HIV constructs added (1 construct per tube): gag, tat, pol, env, and vaccinia control, leaving one tube as a medium control. These were incubated overnight at 37°C. Cells were washed twice in RPMI and incubated with 100µCi ^{51}Cr for 1 hour at 37°C. Cells were washed three times in RPMI, and resuspended at 5 x 10^4 cells/ml.

2.6.2.2 Peptide

Materials:

R10

The same peptides used to stimulate the cells

The autologous B cell-line, corresponding to the sample in culture for CTL, was thawed and grown as required. Cells were washed and resuspended at 1 x 10^6 cells/ml in R10. 1ml aliquots were put into 10ml tubes, one for each HLA-locus for which peptide was available and incubated overnight at 37°C. These were spun down and incubated with 100µCi ^{51}Cr for 1 hour at 37°C. The cells were washed once in RPMI and 2µl of each peptide was added to each of the tubes for each HLA-locus. Cells were incubated for 1 hour at 37°C, washed twice in RPMI and resuspended at 5 x 10^4 cells/ml.

2.6.3 CTL ASSAY

Materials:

R10

RPMI

5% (v/v) Triton-X

96-well U-bottomed plate

'Spot-on' filtermats (1205/402), obtained from Wallac and Berthold, UK

Bags for filtermats, obtained from Wallac and Berthold

'ß-Scint' for filtermats, obtained from Wallac and Berthold

Aliquots (100µl) of target cells were added to a 96-well U-bottomed plate in duplicate for sample lysis. For spontaneous lysis, aliquots were added in quadruplicate to RPMI (100µl). For total lysis, aliquots were added in quadruplicate to 5% Triton-X (100µl). The effector cells were harvested, spun down and resuspended in R10 (2ml) and counted. The effector:target (E:T) ratio was calculated by dividing the number of cells/ml by 5 x 10^4. If the E:T ratio was less than 5:1, the assay was abandoned (except for peptide stimulated effector cells). Cell suspension (100µl) was added to the duplicate wells of target cells. The remaining effector cells were analysed phenotypically for effector cell populations as described in 2.3, omitting the lysis step. The plate was incubated for 4 hours at 37°C.

Supernatant (35µl) was carefully removed (so as not to disturb cells), dotted onto filter mats and dried. When dry these were bagged and counted on a Betaplate scintillation counter (Wallac-Berthold). Results were calculated as:

$$\% \text{ specific lysis} = \frac{\text{(sample release - spontaneous release)}}{\text{(total release - spontaneous release)}} \times 100$$

Specific lysis of greater than 10% above medium or vaccinia controls was considered a positive specific result.

2.6.4 IMMUNOFLUORESCENT STAINING OF B-CELL LINES INFECTED WITH VACCINIA-HIV CONSTRUCTS

Materials:

19mm circular coverslip

Poly-L-Lysine diluted 1:4 in ultra-pure water

PBS

RPMI

Cold acetone:methanol (1:1) stored at -20°C

1% paraformaldehyde (w/v) in PBS (1% PAF)

1% bovine serum albumin (w/v) in PBS (1% BSA)

Parafilm

Mowiol mounting medium containing DABCO

mouse anti-tat antibody ADP3021 donated to the MRC AIDS Reagent project by Dr. Helland and Dr. Szilvay, Bergen, Norway.

mouse anti-RT antibody ADP381 donated to the MRC AIDS Reagent project by Dr. Ferns and Dr. Tedder, London, UK.

mouse anti-gp160/gp41 antibody ADP306 donated to the MRC AIDS Reagent project by Dr. Daniels, London, UK.

mouse anti-p55/p18 antibody ADP315 donated to the MRC AIDS Reagent project by Dr. Ferns, London, UK.

goat anti-mouse-FITC antibody

Microscope slides

Nail varnish

Photographic film (35mm) 1600ASA

B-cells were infected with vaccinia-HIV constructs overnight as for the CTL assay targets (see 2.6.2.1). A coverslip was used for each vaccinia-construct-infected line tested. One coverslip was placed in each well of a 6-well plate. Poly-L-Lysine was added to a level which covered the coverslip, and incubated at 37°C for 20-30 minutes. The coverslip was washed 2-3 times in PBS, using a vacuum pump to remove the liquid. The plates were allowed to dry in air for approximately 30 minutes. The vaccinia-construct-infected B cells were spun down and resuspended in RPMI (500µl). The cells were placed on the coverslip and incubated for 1 hour at 37°C. The cells were washed once in PBS, and excess cold methanol/acetone was added for 2 minutes. The cells were washed twice in PBS, and excess 1% PAF was added and incubated at 4°C for 1 hour. The PAF was removed and excess 1% BSA was added for 10 minutes at room temperature. A damp tissue was laid on a box lid, and strips of parafilm were laid on top, labelled according to the wells in each plate. Drops of diluted antibody (1:100 in PBS, 95µl) were placed on the parafilm, the appropriate antibody for each construct. The coverslip was removed from the plate using forceps, drained on a piece of tissue and placed cell-side down on top of the drop of antibody. The box lid was then covered with foil and incubated at room temperature for 20-30 minutes. The coverslip was "floated" off using PBS and a pastette (i.e. PBS was gently squirted under the coverslip until it floated, as, if it was pulled off, the cells would be removed). The coverslip was put back into the 6-well plate, which still contained the 1% BSA, cell-side up. The cells were washed 2-3 times in PBS. Drops of diluted (1:100 in PBS) goat-anti-mouse-FITC antibody (95µl) were placed on the parafilm, as before. The coverslip was removed from the plate using forceps, drained on a piece of tissue and placed cell-side down on top of the drop of antibody. This was all covered with foil and incubated at room temperature for 20-30 minutes. The coverslip was "floated" off using PBS and put back into the 6-well plate, still containing the 1% BSA, cell-side up. The cells were washed 2-3 times in PBS. Microscope slides were labelled and a drop of Mowiol was put onto the slide. The coverslip was drained and placed cell-side down onto the Mowiol. The edges were sealed with nail varnish to prevent drying out. Cells were visualised under a fluorescent microscope and photographs were taken.

2.7 CYTOKINE ELISAS

Cytokine kits for IL-2, IL-4, IL-6 and IFN-γ were obtained from Genzyme and used according to the manufacturers instructions.

2.8 PROLIFERATION ASSAYS

Materials:

RPMI containing 10% human AB serum (R10/AB)

^{3}H-thymidine (TRA-310) obtained from Amersham, UK

Recombinant Tetanus Toxoid from *Clostridium tetanii*, obtained from Calbiochem Novobiochem, UK.

Phytohaemagglutinin (PHA-P)

A 'cocktail' of recombinant HIV-1 proteins (rHIV cocktail) was made, all recombinants obtained from the MRC AIDS Reagent Project. This cocktail contained:-

recombinant HIV-1_{SF} gp120 (expressed in CHO cells), >90% pure. ARP629 (donated by Dr. K. Steimer, Chiron Corporation.)

recombinant HIV-1_{MN} gp120 (expressed in Baculovirus), >95% pure ARP646.

recombinant HIV-$1_{LAI/BRU}$ gag p24 (expressed in Baculovirus), >90% pure. ARP620.

recombinant HIV-1_{LAV} Reverse Transcriptase (p66) (expressed in *E.coli*) ARP631.1 (donor Dr. D. Stammers, Glaxo Wellcome, UK.)

recombinant HIV-1_{IIIB} nef (expressed in *E.coli*), >90% pure. EVA650 (donor Dr.V Erfle, Munich, Germany.)

recombinant HIV-1_{IIIB} tat (expressed in *E.coli*). >90% pure. EVA658 (donor Dr. J. Raina, Agmed Corps, USA.)

Glass filtermats (1205/401) obtained from Wallac and Berthold

Bags for filtermats, obtained from Wallac and Berthold

'ß-Scint' for filtermats, obtained from Wallac and Berthold

Tetanus toxoid (TT) and PHA were made up at concentrations of 5µg/ml and 1µg/ml, which would give final concentrations in the assay, of 2.5µg/ml and 0.5µg/ml. The rHIV-1 proteins were combined at concentrations of 2.5 and 0.5 µg/ml of each protein, to give final concentrations, in the assay, of 1.25µg/ml and 0.25µg/ml. Aliquots (100µl) of each concentration of TT, PHA and rHIV cocktail were plated out into a 96-well U-bottomed plate in quadruplicate, with medium in sexlicate as control. Freshly separated PBMCs were counted and suspended in R10/AB at a concentration of $1x10^6$ cells/ml. Cells (100µl) were added to each antigen. The plate was incubated at 37°C for 6 days, and then labelled with ^{3}H-thymidine at 1µCi/well, incubated for a further 18 hours, and harvested with distilled water onto filtermats. The filtermats were dried, bagged and counted on a Betaplate counter.

For the medium control, the highest and the lowest counts from the six wells were discarded and only four wells were used in the analysis. The geometric means for all the wells were calculated for each stimulus and the medium control. The stimulation index was calculated for all the stimuli as:-

$$\text{stimulation index} = \frac{\text{geometric mean cpm of stimulus}}{\text{geometric mean cpm of medium}} \times 100$$

2.9 DETECTION OF A SYNCYTIUM-INDUCING (SI) VIRAL VARIANT

Materials:

R10

R10 containing 2.5µg/ml Polybrene (Aldrich, UK)

MT2 cell line

The MT2 cell line was maintained in R10, in a log phase. Culture supernatants (0.5ml) were obtained from Dr. M. Arnott, Medical Microbiology, University of Edinburgh. These were separated PBMCs from children, which had been cultured with $2x10^6$ PHA-blasted donor PBMCs. The cultures had been fed with fresh PHA-blasts every week and tested for p24 antigen. These supernatants were cultured with 0.25 x 10^6 MT2 cells in R10/2.5µg/ml polybrene (2.5ml) in a 24-well plate. The other wells in the plate were filled with sterile

water (2ml) to reduce evaporation. The culture was incubated for 7 days at 37°C. The cells were checked for syncytia formation using an inverted microscope. Culture supernatant (1ml, including the cells) was transferred to fresh R10/2.5µg/ml polybrene medium (1.5ml) in a clean 24-well plate. Cultures were discarded after 4 weeks.

2.10 REVERSE TRANSCRIPTASE (RT) ASSAY

Materials:

5X RT mix:-

750 mM potassium chloride, KCl

50mM magnesium chloride $MgCl_2$

50mM Tris/HCl pH 8.0

2.5mM ethylene glycol tetraacetic acid EGTA

0.5% (v/v) Triton X-100

125µg/ml Bovine Serum Albumin (BSA)

10% ethane-diol in DEPC-water (0.1% diethyl procarbamine in distilled water and sterilised)

This was made up in batches and stored at -20°C

100mM dithithreitol (DTT)

75µg/ml poly-rA-oligo-$dT_{(12-18)}$ (Pharmacia 27-7878-02)

1 µCi/well ^{3}H-Thymidine-5'-Trisphosphate (^{3}H-TTP) (Amersham TRK324)

Trichloroacetic acid (TCA, 100% (w/v)) diluted to 10% (v/v) or 5% (v/v) in DEPC-water.

Ribonucleic acid (RNA) type lll from Bakers yeast diluted to 150µg/ml in 10% TCA (above)

5% TCA (above)

3% sodium pyrophosphate

70% methanol or ethanol (Hayman Ltd, Witham Essex,UK) AR grade

4ml round bottomed tubes

DEAE-impregnated filtermats (Wallac 1205/405)

Nitrogen gas

The final volume (V_f) for 2.5X reaction mix was calculated for the number of samples to be assayed. The figure in brackets is an example to give a final volume of 500µl of 2.5X reaction mix.

1/10 V_f (50µl) of ^{3}H-TPP was placed in a 4ml round-bottomed tube. Approximately half the volume was evaporated off under a nitrogen gas stream to remove the ethanol. 1/20 V_f (25µl) of DTT, 1/13.3 V_f (37.5µl) of poly rA-oligo-dT and 1/2 V_f (250µl) 5X RT mix was added. The volume was made up to V_f with DEPC-treated water (162.5µl). 2.5X reaction mix was added (10µl/well) to the inner 60 wells of a 96-well V-bottomed plate. Samples were assayed in triplicate, and at two concentrations (neat and diluted 1:3 in PBS). Sample (15µl) was added to each well. The plate was sealed with a plate sealer and incubated at 37°C for 48 hours. The reaction was stopped with 10% TCA containing 50µg/ml RNA (150µl/well). The plate was placed on ice for 15 minutes and then harvested with 5% TCA, 3% sodium pyrophosphate and 70% methanol in successive washes, onto DEAE-impregnated filtermats. The filtermats were dried, bagged with scintillant and counted on a beta-plate counter.

2.11 CELL SEPARATION USING MINIMACS COLUMNS

Materials:

PBS containing 0.2% BSA - staining buffer

PBS containing 0.5% BSA - running buffer

PBS

mouse anti-CD4 (QS410 supernatant obtained from Donald Innes)

anti-mouse-IgG immunomagnetic beads obtained from Binding Site, Birmingham, UK.

Mini-MACS separation columns obtained from Eurogenics, UK.

Screw top tubes (2ml).

Cells were counted, a small aliquot was taken for FACScan analysis (see 2.3.1) and the rest were suspended in staining buffer (100µl). CD4+ve cells were removed by positive selection, i.e. an anti-CD4 antibody was added (1ml), mixed and incubated at 4°C for 40

minutes. Cells were washed in PBS and resuspended in staining buffer (100µl). Magnetic beads (20µl) were added, mixed and incubated for 10 minutes at 4°C. Cells were mixed again and incubated at 4°C for a further 10 minutes. The cell/bead suspension was added to a primed mini-MACS column fitted in the magnet, followed by running buffer (500µl) and the fraction collected. The column was washed through with running buffer (2 x 500µl) and collected into a 2ml tube. This was the 'negative' fraction (i.e. containing cells not expressing CD4). The mini-MACS column was removed from the magnet and washed through with running buffer (3 x 500µl), eluting the cells which were attached to beads into another 2ml tube. This was the 'positive' fraction (i.e. cells expressing CD4). The cells were centrifuged at 3000 rpm for 5-10 minutes. Supernatant was removed and the cell pellet was snap frozen at -70°C ready for extraction.

2.12 EXTRACTION OF DNA AND RNA

2.12.1 EXTRACTION OF DNA/RNA

Materials:

TNE buffer 0.5% SDS + 10%

0.11M sodium chloride NaCl

55mM Tris pH 8.0

1.1mM etheylene diamine tetraacetic acid EDTA pH 8.0

0.55% sodium dodecyl sulphate SDS

TNE buffer 0.1% SDS

0.1M NaCl

50mM Tris pH 8.0

1mM EDTA pH 8.0

0.1% SDS

Phenol containing hydroxychinolin (1g/kg phenol) and saturated with T10E1 (Phenol/TE)

Chloroform:isoamyl alcohol 50:1 (v/v)

Phenol/TE:chloroform/isoamyl alcohol 1:1 (v/v)

3M sodium acetate pH 5.2

80% ethanol

Absolute ethanol

Proteinase K (10mg/ml)

Poly A (2mg/ml)

Conical screw cap tubes (1.5ml)

Fine-tipped pastette

A solution of TNE buffer 0.5% SDS containing 1mg/ml proteinase K and 40µg/ml poly A was made up from: 9 volumes of TNE buffer 0.5% SDS + 10% added to 1 volume of proteinase K and 20µl/ml poly A. The solution was pre-incubated at 37°C for 10 minutes to inactivate RNAases. The samples to be extracted (100µl plasma/serum/cell pellet) were put into small conical tubes. TNE 0.5% SDS/proteinase K/poly A solution (0.4ml) was added to each extraction tube. The tubes were vortexed thoroughly and incubated at 37°C for 2 hours. Phenol/TE (450µl) was added to each tube, vortexed thoroughly, and centrifuged at 13000 rpm for 15 minutes. The upper layer (water phase) was transferred, using a fine-tipped pastette, to a fresh tube containing phenol/chloroform (1:1, 450µl) and TNE 0.1 SDS (75µl). The tubes were vortexed thoroughly and centrifuged at 13000 rpm for 10 minutes. The water phase was transferred to fresh tube containing 450µl chloroform:isoamyl alcohol, mixed extensively and centrifuged at 13000 rpm for 10 minutes. The water phase was transferred to a fresh tube containing 3M sodium acetate pH 5.2 (40µl). These were mixed, absolute ethanol (800µl) was added and allowed to precipitate overnight at -20°C. The tubes were centrifuged at 13000 rpm for 20 minutes. The DNA/RNA pellet was washed with 80% ethanol (1ml) and centrifuged at 13000 rpm for 5 minutes. The precipitate was dried on a hot block at 40°C, before resuspension in 20µl DEPC-water.

2.12.2 EXTRACTION OF RNA USING STRATAGENE MICRO RNA ISOLATION KIT

Materials: (all the reagents were obtained as a kit from Stratagene, UK)

'Solution D', a denaturing solution containing guanidine isothiocyanate and ß-mercaptoethanol (7.2µl/ml solution D).

2M sodium acetate

water-saturated phenol

chloroform:isoamylalcohol (1:1)

Glycogen

Isopropanol

75% ethanol

DEPC-treated water

Conical screw cap tubes (1.5ml)

Fine-tipped pastettes

Volume D is the volume of solution D used in the extraction. This volume is adjusted according to the number of cells in the sample: 100µl of solution D is required for every 10^6 cells processed, and all other reagent volumes are relative to volume D. The volume quoted is that used for 1 x 10^6 cells, as an example.

Solution D (100µl) was added to extraction tubes containing the sample. To this 2M sodium acetate (10µl) was added. Water-saturated phenol (100µl) was then added, ensuring the lower phenol layer was used. Chloroform:isoamyl alcohol was added (20µl). The tubes were capped and vortexed vigorously. The tubes were centrifuged for 5 minutes at 14000 rpm so that two phases were visible. The upper layer was transferred to clean tubes, using a fine-tipped pastette. Glycogen was added as an RNA carrier (1µl). Isopropanol (100µl) was added to the RNA solution and mixed well. The tubes were centrifuged at 14000 rpm for 30 minutes. The supernatant was removed and pellet washed in 200µl of 75% ethanol (100µl), and the pellet dried on a hot block at 40°C rapidly. The RNA was resuspended in 20µl DEPC-water.

2.12.3 QUANTIFICATION OF RNA OR DNA

Materials:

DEPC-water

Quartz cuvette

Absorbance spectrophotometer

A small aliquot (5µl) of the resuspended RNA or DNA was diluted in DEPC-water (700µl). The spectrophotometer was zeroed against DEPC-water at 260 and 280nm. The same cuvette

was used for the sample. The absorbance at 260 and 280nm was measured for each sample (Ausubel *et al.*, 1997).

The amount of DNA (µg/µl) was calculated as:- A_{260} x 50 x dilution factor x 1000

The amount of RNA (µg/µl) was calculated as:- A_{260} x 40 x dilution factor x 1000

2.13 RT-PCR FOR CD4, ACTIN AND HIV-1

2.13.1 REVERSE TRANSCRIPTION OF mRNA TO cDNA

2.13.1.1 Reverse Transcription using AMV-RT

Unless otherwise indicated all materials are from Promega, Southampton, UK.

Materials:

RNA (0.5µg)

0.5µg oligo $(dT)_{15}$ primer

RT buffer (50mM Tris-HCl pH 8.3, 5mM KCl, 10mM $MgCl_2$, 10mM DTT, 0.5mM Spermidine)

0.66mM dNTPs

13 U RNAsin (an RNAase inhibitor)

10 U AMV Reverse transcriptase

DEPC-water

0.5 ml Eppendorf tubes

GeneE Thermocycler (ABI, UK)

The above reagents were combined in 0.5ml eppendorf tubes, at the above concentrations in a final volume of 20µl. A control containing DEPC-water instead of RNA (RNA-ve) was also used. The tubes were incubated on a thermocycler at 42°C for 1 hour.

2.13.1.2 Reverse Transcription using Expand™-RT

Materials:

0.5µg oligo $(dT)_{15}$ primer

0.5µg RNA

DEPC-water to make up to total volume of 20µl

Reverse transcriptase buffer (50mM Tris-HCl; 40mM KCl; 5mM $MgCl_2$; 0.5% Tween 20 (v/v), pH 8.3), obtained from Boehringer Mannheim, UK.

10mM DTT, obtained from Boehringer Mannheim, UK.

0.66mM dNTP

13 U RNAsin (RNAase inhibitor)

50 U Expand™ Reverse transcriptase, obtained from Boehringer Mannheim, UK.

0.5ml Eppendorf tubes

GeneE Thermocycler

The RNA, oligo-dT primer and DEPC-water were combined in an eppendorf tube, and were denatured at 65°C for 10 minutes on a thermocycler. The tubes were immediately cooled on ice. The reagents were added to the tube, to give the final concentrations (as above). A control containing DEPC-water instead of RNA (RNA-ve) was also used. The tubes were incubated on a thermocycler at 42°C for 1 hour.

2.13.2 PCR FOR CD4

2.13.2.1 Primary PCR

Materials:

2µl cDNA (from 2.13.1)

Taq polymerase reaction buffer (50mM KCl, 10mM Tris-HCl pH 9.0, 0.1% Triton X-100)

0.5µM CD4 primer (sense, see below)

0.5µM CD4 primer (antisense, see below)

33µM dNTPs

2.5 U Taq DNA polymerase (Taq)

GeneE thermocycler

The above reagents were combined in 0.5ml eppendorf tubes, at the above concentrations in a final volume of 20µl. A control containing DEPC-water instead of cDNA (cDNA-ve) was also used. The tubes were incubated on a thermocycler with the following programme:-

4 minutes at 94°C (denaturing)

1 minute at 94°C, 1.5 minutes at 50°C, 2 minutes at 72°C, for 30 cycles

10 minutes at 72°C (extension)

2.13.2.2 Secondary PCR

Materials:

2µl primary product (from 2.13.2.1)

Taq polymerase reaction buffer (50mM KCl, 10mM Tris-HCl pH 9.0, 0.1% Triton X-100)

0.5µM CD4 primer (sense, see below)

0.5µM CD4 primer (antisense, see below)

33µM dNTPs

2.5 U Taq DNA polymerase (Taq)

The above reagents were combined in 0.5ml eppendorf tubes, at the above concentrations in a final volume of 20µl. These were incubated on a thermocycler using the same programme as used in the primary PCR.

CD4 primer sequences:-

Sense	CD4-1	5' CCA CAA TGA ACC GGG GAG TCC CTT TTA GGC (+,70bp)
	CD4-3	5' GTG GTG CTG GGC AAA AAA GGG G (+,155bp)
	CD4-5	5' GAC CTG AAG AAC AAG GAA GTG TC (+,837bp)
	CD4-6	5' AGA TGG GCA AGA AGC TCC CGC (+,891bp)
Antisense	CD4-2	5' GCA CTG GCA GGT CTT CTT CTC ACT GAG (-,1416bp)
	CD4-4	5' GGT GCC GGC ACC TGA CAC AGAA (-,1350bp)

All primers were synthesised by Oswel DNA Services, Southampton UK.

Primer combinations:

Primary PCR:	CD4-1 and CD4-2, giving a primary product of 1346bp
	CD4-5 and CD4-2, giving a primary product of 579bp
Secondary PCR:	CD4-3 and CD4-4, giving a secondary product of 1195bp
	CD4-6 and CD4-4, giving a secondary product of 459bp

Primers CD4-1 - CD4-4 and PCR programme were obtained from Fomsgaard *et al* (1992). Primers 5 and 6 were designed from the cDNA sequence as in Maddon *et al* (1985) (see chapter 6).

When PCR reactions for CD4 were performed in parallel with those for HIV-1 and ß-Actin, the thermocycler programme for ß-Actin was used (see 2.13.3).

2.13.3 PCR FOR ACTIN

2.13.3.1 Primary PCR

Materials:

2µl cDNA (from 2.13.1)

Taq polymerase reaction buffer (50mM KCl, 10mM Tris-HCl pH 9.0, 0.1% Triton X-100)

1µM ß-Actin primer pair (from Stratagene, see below)

33µM dNTPs

2.5 U Taq DNA polymerase (Taq)

The above reagents were combined in 0.5ml eppendorf tubes, at the above concentrations in a final volume of 20µl. A control containing DEPC-water instead of cDNA (cDNA-ve) was also used. The tubes were incubated on a thermocycler with the following programme (manufacturers recommendation):-

5 minutes at 94°C (denaturing)

5 minutes at 60°C (annealing)

1.5 minutes at 72°C, 45 seconds at 94°C, 45 seconds at 60°C, for 35 cycles

10 min at 72°C (extension)

Actin primer sequences (obtained from Stratagene):

Sense 5' TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA (+, 1038bp)

Antisense 5' CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG (-, 1905bp)

These gave a product of 661bp

2.13.3.2 **Secondary PCR**

Materials:

2µl primary product (from 2.13.3.1)

Taq polymerase reaction buffer (50mM KCl, 10mM Tris-HCl pH 9.0, 0.1% Triton X-100)

0.25µM ß-Actin primer (sense, see below)

0.25µM ß-Actin primer (antisense, see below)

33µM dNTPs

2.5 U Taq DNA polymerase (Taq)

The above reagents were combined in 0.5ml eppendorf tubes, at the above concentrations in a final volume of 20µl. These were incubated on a thermocycler using the same programme.

Actin primer sequences (synthesised by Oswel DNA Services, Southampton, UK):

Sense 5' GCC CTG GAC TTC GAG CAA GAG ATG GCC A (+, 1231bp)

Antisense 5' CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG (-, 1905bp)

These gave a product of 501bp

2.13.4 **PCR FOR HIV-1**

2.13.4.1 **Primary PCR**

Materials:

2µl cDNA (from 2.13.1)

Taq polymerase reaction buffer (50mM KCl, 10mM Tris-HCl pH 9.0, 0.1% Triton X-100)

0.5µM HIV-1 primer (sense, see below)

0.5µM HIV-1 primer (antisense, see below)

33µM dNTPs

2.5 U Taq DNA polymerase (Taq)

GeneE thermocycler

The above reagents were combined in 0.5ml eppendorf tubes, at the above concentrations in a final volume of 20µl. A control containing DEPC-water instead of cDNA (cDNA-ve) was also used. The tubes were incubated on a thermocycler with the following programme:-

25 seconds at 94°C, 35 seconds at 55°C, 2.5 minutes at 68°C, for 30 cycles

7 min at 68°C (extension)

Primer sequences

Sense NARS 5' CTC TAG CAG TGG CGC CCG AAC AGG GG (+, 173bp)

Antisense 534A 5' CTA TGA TTA CTA TGG ACC AC (-, 5725 bp)

These gave a product of 215bp

2.13.4.2 **Secondary PCR**

Materials:

2µl primary product (from 2.13.41)

Taq polymerase reaction buffer (50mM KCl, 10mM Tris-HCl pH 9.0, 0.1% Triton X-100)

0.5µM HIV-1 primer (sense, see below)

0.5µM HIV-1 primer (antisense, see below)

33µM dNTPs

2.5 U Taq DNA polymerase (Taq)

The above reagents were combined in 0.5ml eppendorf tubes, at the above concentrations in a final volume of 20µl. These were incubated on a thermocycler using the same programme.

Primer sequences

Sense M661 5' CCA GAG GAG CTC TCT CGA CGC AGG (+, 217bp)

Antisense 534A 5' CTA TGA TTA CTA TGG ACC AC (-, 5725 bp)

These gave a product of 179bp

When PCR for HIV-1 was carried out in parallel with those for CD4 and ß-Actin, the ß-Actin programme was used.

2.13.5 GEL ELECTROPHORESIS

Materials:

Agarose (electrophoresis grade) obtained from Flowgen, UK

10X TBE buffer: 540g Tris Base

275g Boric Acid

200ml 0.05M EDTA pH 8.0

made up to 5 litres with distilled water. This was diluted as required.

Ethidium Bromide (10mg/ml)

A 1.3% agarose (w/v) solution in TBE buffer was made by heating the agarose in the buffer. The solution was allowed to cool with mixing. While still liquid, ethidium bromide was added (3µl/100ml agarose solution). The gel was poured on to a gel tray with combs as required and allowed to set. The samples (10µl) were loaded into each well, and a well for for pGEM markers was also used. These markers give DNA bands of specific sizes: 2645bp, 1605bp, 1198bp, 676bp, 517bp, 460bp, 396bp, 350bp, 222bp, 179bp, 126bp, 75bp, 65bp, 51bp and 36bp. The gel was put into a gel tank containing TBE buffer and run at 150V for 1.5 hours. The DNA was visualised on a UV light box and photographed.

2.14 DATA ANALYSIS

Throughout this thesis, data analysis and statistics were performed using either the Microsoft Excel programme on Windows 3.1 Microsoft Office (copyright Microsoft Corporation), or by using Minitab (Minitab Data Analysis Software, Minitab Inc., version 7.2, 1989).

Chapter 3 LYMPHOCYTE SURFACE MARKERS

3.1 **INTRODUCTION**

3.2 **METHODS**

3.3 **RESULTS**

3.3.1 **CD4 and CD8 cells**

CD4 cells

CD8 cells

3.3.2 **Co-expression of activation and memory markers on CD4 and CD8 cells**

CD4 subpopulations

CD8 subpopulations

3.3.3 **Co-expression of CD38 and CD45RO on CD8 cells by triple-coloured immunofluorescence (CD38/CD45RO/CD8 triple)**

Comparison of CD38/CD45RO/CD8 triple with CD4%

3.3.4 **Relation of lymphocyte surface markers to viral markers**

Relation of CD8 subpopulations to viral culture

Relationship between lymphocyte marker and viral phenotype

3.4 **DISCUSSION**

3.4.1 **CD4 cells and subpopulations**

3.4.2 **CD8 cells and subpopulations**

3.4.3 **Fast progressors**

3.4.4 **Relation of lymphocyte surface markers to progression and diagnosis of HIV-1 infection**

3.1 INTRODUCTION

During the asymptomatic phase of infection in adults, despite a lack of detectable virus in the periphery, HIV-1 has been shown to cause pathological changes within lymphoid tissue (Pantaleo *et al.*, 1993; Embretson *et al.*, 1993). This is also indicated by the rapid turnover of CD4 cells and plasma virions (Ho *et al.*, 1995; Wei *et al.*, 1995) although cell numbers within peripheral blood are maintained. Those individuals who develop AIDS-defining illnesses usually have very few circulating CD4 cells (Centers for Disease Control, 1982). The CD4 cell count in peripheral blood decreases later on in the asymptomatic phase of HIV-1 infection and can, therefore, be used as a predictor of the onset of AIDS, or for determining the timing of prophylactic therapy for opportunistic infections (Phillips *et al.*, 1992). However, in some, AIDS can develop despite quite high levels of circulating CD4 cells, while others can remain healthy with a negligible CD4 cell count (J.A. Whitelaw, personal communication). In both adults and children different serological, virological and immunological factors have been studied to identify prognostic markers, which would more specifically predict the loss of CD4 cells and, hence, clinical progression.

The expression of different antigens on the surface of CD4 and CD8 lymphocytes is associated with particular cellular functions (summarised in table 1.2). Many of these have been assessed at different stages of HIV-1 infection in adults, both as markers of disease progression and to identify antigen expression which may give some clues to the mechanisms involved in the immunopathogenesis of HIV-1 (summarised in table 1.3).

In HIV-1-infected children there have been fewer studies of lymphocyte surface marker expression. One complication of this type of study, is the fact that some of the markers are indicators of cell maturity and the levels of expression change naturally with the increasing age of the child (Hayward *et al.*, 1989). For this reason, conclusions drawn from studies in HIV-1-infected adults cannot be extrapolated to children. This study was undertaken to monitor, longitudinally, the percentages of CD4 and CD8 T lymphocytes in peripheral blood, together with the clinical pattern of HIV-1 infection in this cohort of children. The expression of activation and memory markers on both CD4 and CD8 cells was also measured, by dual-colour and triple-colour immunofluorescence, to ascertain whether the expression of these markers differed from that in adults. The marker expression was related to the clinical

pattern and the CD4 level, to determine whether any markers were indicative of HIV-1 infection in children, or predictive of disease progression.

One of the main problems encountered in studying children is the availability of normal controls, as it is considered unethical to take blood from a child for a study from which it will not directly benefit. To overcome this, residual peripheral blood samples were obtained from 168 children (aged 0 - 122 months) who were HIV-1-uninfected, not suffering from any immunological defect or infectious disease, and who had had blood samples taken for other reasons. From these data, charts of centiles were calculated for the lymphocyte surface markers studied here (by Professor G. Raab, Napier University, Edinburgh), which showed the normal age-related changes seen in these markers (Aldhous *et al.*, 1994a, appendix). The data from the infected children were plotted on these charts, as shown in the results.

Part of the data (up to July 1994) has been published (Aldhous *et al.*, 1996, appendix). The data presented here includes and continues that study. Several children have now progressed clinically.

3.2 METHODS

Lymphocyte surface markers were measured using dual- or triple-colour immunofluorescence and flow cytometry (see 2.3.1 and 2.3.2 respectively). Briefly, diluted whole blood (100µl) was incubated for 15 minutes with each antibody combination (10µl) at room temperature. After red cell lysis and washing in PBS, the cells were fixed in 1% paraformaldehyde in PBS. Cells were acquired on a FACScan™ and analysed using SimulSET™, LYSIS™II and PAINT-a-GATE™ programmes (Becton Dickinson), as described in 2.3.3.1 and 2.3.2.3.

3.3 RESULTS

The normal range charts were constructed as age-related centiles, the normal range being

defined as those values which fall between the 3rd and the 97th centiles (Aldhous *et al.*, 1994a, appendix). Values for fifteen HIV-1-infected children were plotted on the normal range charts for each marker, and the data presented for those which showed changes outwith the normal ranges. Details of the children are outlined in table 3.1. This table includes the route of infection, the age range over which the child was monitored and the overall clinical status during the study period.

3.3.1 CD4 AND CD8 CELLS

3.3.1.1 CD4 cells

For each child, the percentage of lymphocytes expressing CD4 (CD4%) was plotted on the normal range charts and are presented in figure 3.1 (a-c). The first and last CD4% recorded are shown for each child in table 3.2, together with the relevant age-centile and the length of time over which the children were studied. The highest and lowest CD4% observed are also given, to show the range over which the CD4% fluctuated during the study period. The children fell into three main groups and each group is presented separately:-

I Those children of greater than 60 months (5 years) of age, whose CD4% remained steady (see figure 3.1(a), P6, P27, P69 and P121). Two of these can be classified as long-term non-progressors as they had CD4% within the normal range (~10-25th and ~3rd-5th centiles for P69 and P27 respectively) at 108 months (9 years) of age. From table 3.2, the first and last CD4% for P6, P27 and P121 were within 2% of each other, corresponding to the crossing of one centile. For P69, a drop of 10% was seen which corresponds to the crossing of two centiles.

II Those children of over 60 months (5 years) of age whose CD4% decreased (see figure 3.1(b), P92, P110, P116, CB and AF). Of these, three have died and one has AIDS with recurrent *Candida* infections. From table 3.2, the first and last CD4% showed a large decrease of over 20% for three of these children (P92, P110 and P116), with a corresponding drop to below the third centile. For CB and AF, the CD4% was consistently below the third centile. For all these children the fluctuations appeared less than those seen in the children of group I.

Table 3.1 **Clinical details of the HIV-1-infected children**

Child	Infection route	Age range of study (months)	Clinical/other information
P6	vertical	72 - 129	non-specific signs and symptoms
P27	vertical	62 - 120	thrombocytopenia; LIP; non-specific signs and symptoms
P69	vertical	78 - 137	non-specific signs and symptoms
P121[+]	unknown	24 - 78	non-specific signs and symptoms.
P92	vertical	46 - 104	cerebral atrophy; recurrent candidiasis; non-specific signs and symptoms
P110	vertical	15 - 72	non-specific signs and symptoms
P116[§]	vertical	31 - 77	recurrent respiratory infections (LIP); HIV-1 encephalopathy; disseminated Herpes zoster; died at 78 months
CB	horizontal[¶]	93 - 108	thrombocytopenia; HIV-1 encephalopathy; disseminated Herpes Zoster; died at 110 months
AF	vertical	80 - 114	Salmonella meningitis and septicaemia; failure to thrive; diarrhoea; died at 120 months
P125	vertical	birth - 48	recurrent upper respiratory tract infections
P131	vertical	5 days - 35	clinically well
P132	vertical	7 days - 26	clinically well; sibling of P125

P142[++]	unknown	41 - 60	non-specific signs and symptoms.
P126	vertical	3 weeks - 13	HIV-1 encephalopathy; cerebral toxoplasmosis; died at 17 months
P130	vertical	2 - 36	thrush; pneumonia; non-specific signs and symptoms; developmentally delayed

---

LIP lymphocytic interstitial pneumonitis

§ P116 African child, probably infected with a non-subtype B virus

\+ P121 Romanian child, possibly infected with a non-subtype B virus

++ P142 Romanian child, possibly infected with a non-subtype B virus. Also infected with Hepatitis B

¶ horizontal infection denotes this child was infected by blood transfusion

Figure 3.1 **Percentage of CD4 lymphocytes**

The percentage of CD4 lymphocytes (y-axis), for each child, was plotted on age-related normal range charts, against the age in months (x-axis).

a) Children over 60 months of age with a steady CD4%. P6 is shown as a black line; P27 in green; P69 in blue and P121 in red. These children were classed as group I.

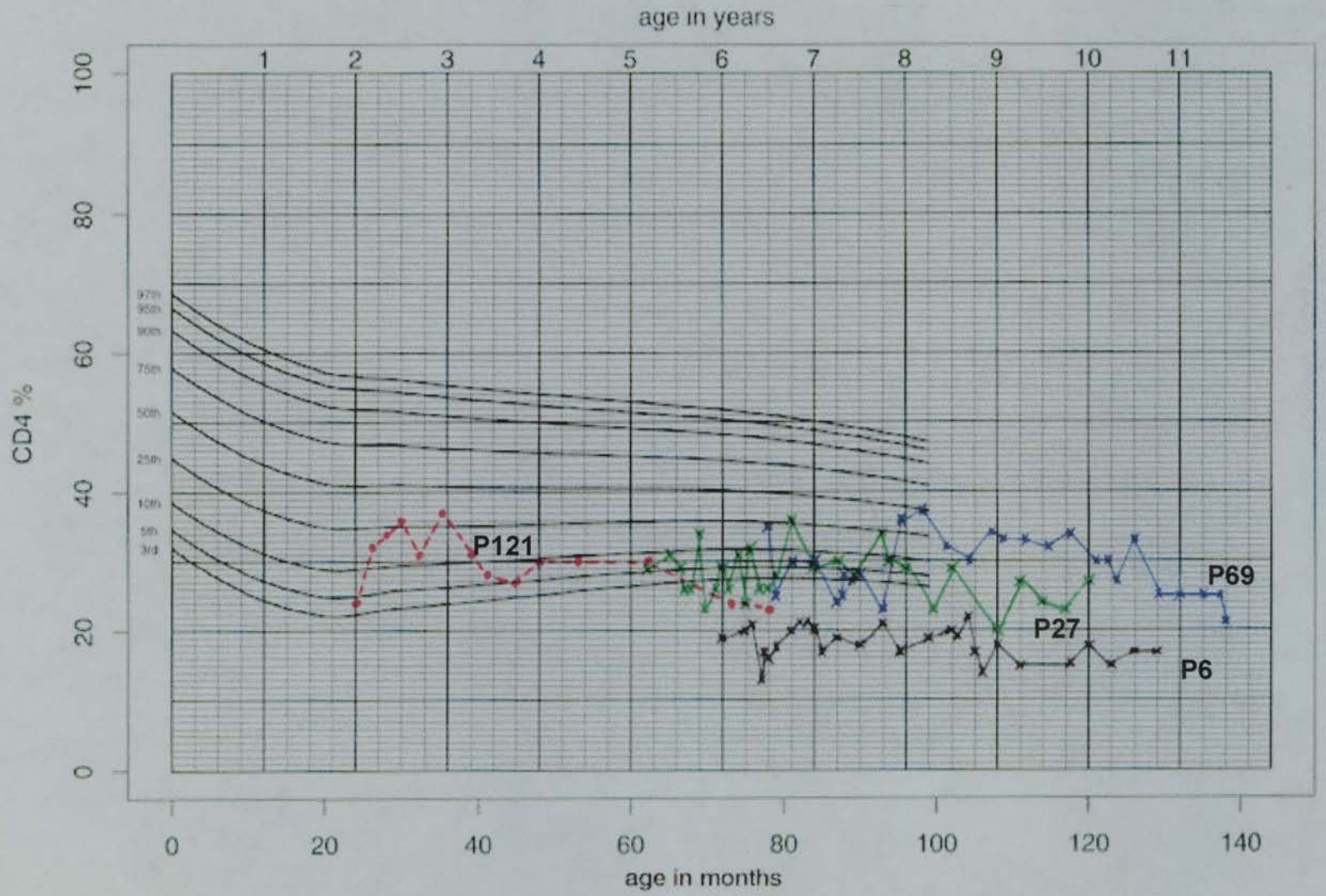

age in years
1
2
3
4
5
6
7
8
9
10
11
CD4 %
0
20
40
60
80
100
97th
95th
90th
75th
50th
25th
10th
5th
3rd
P121
P69
P27
P6
0
20
40
60
80
100
120
140
age in months

Figure 3.1 **Percentage of CD4 lymphocytes**

b) Children over 60 months of age whose CD4% has decreased. AF is shown as a black line, P92 and CB are both shown in red (CB is the shorter line), P116 is the green line and P110 in blue. The arrows denote the time at which an SI variant of HIV-1 was first isolated from these children. These children were classed as group II.

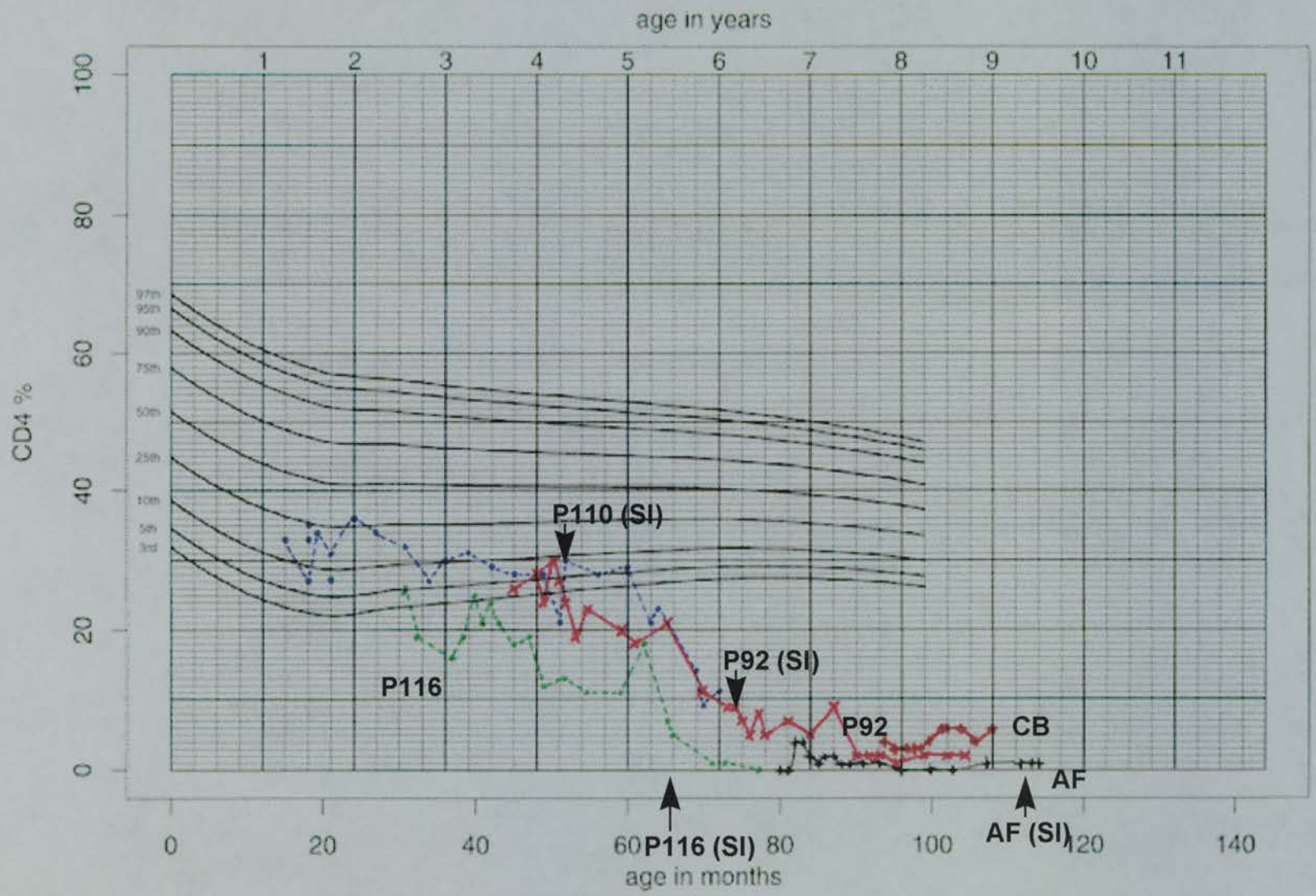
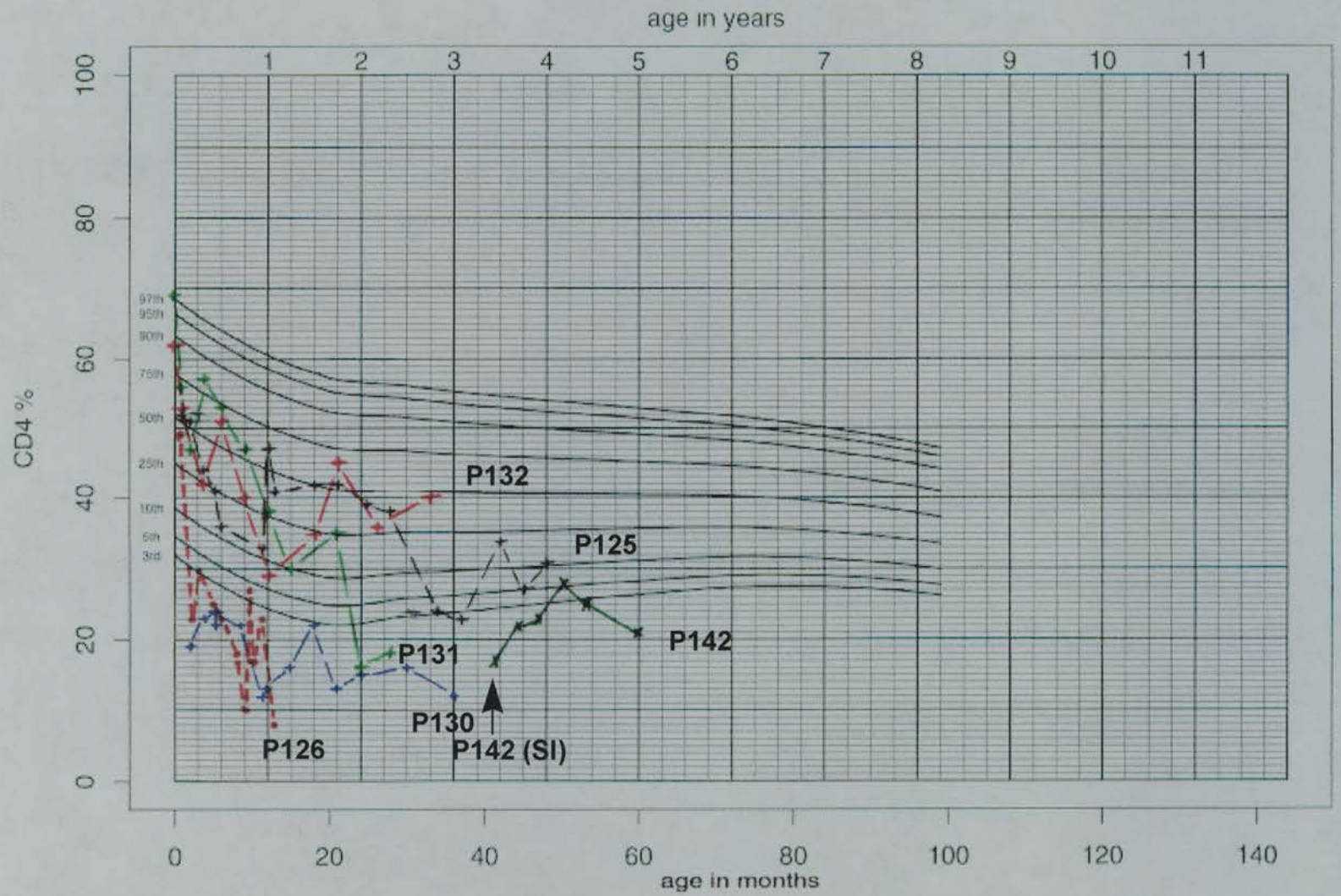

age in years
1
2
3
4
5
6
7
8
9
10
11
CD4 %
100
80
60
40
20
0
97th
95th
90th
75th
50th
25th
10th
5th
3rd
P110 (SI)
P116
P92 (SI)
P92
CB
AF
AF (SI)
P116 (SI)
0
20
40
60
80
100
120
140
age in months

Figure 3.1 **Percentage of CD4 lymphocytes**

c) Children under 60 months of age. P125 is shown as a black line, P131 as a green line, P132 as a red line and P142 as a dark green line. These children were classed as group III.

P126 is shown as a red line and P130 as a blue line. These two children were classed as fast progressors.


age in years
1
2
3
4
5
6
7
8
9
10
11
CD4 %
0
20
40
60
80
100
97th
95th
90th
75th
50th
25th
10th
5th
3rd
P132
P125
P142
P131
P130
P126
P142 (SI)
0
20
40
60
80
100
120
140
age in months

+ Centiles as calculated in Aldhous *et al.*, 1994a, appendix.

§ CD4 group according to CD4% profile at a particular age, as defined in 3.3.1.1, and shown in figures 3.1(a-c).

The centile figure underneath each value for the percentage refers to the centile corresponding to the age of the child when the measurement was made. The same percentage value may give a different centile, according to the age-related changes in percentage of cells.

<3rd denotes a value of less than 10% below the value of the 3rd centile at that age.

<<3rd denotes a value of more than 10% below the value of the 3rd centile at that age.

>97th denotes a value of less than 10% above the value of the 97th centile at that age.

>>97th denotes a value of more than 10% above the value of the 97th centile at that age.

Table 3.2 **CD4 and CD8 percentages in peripheral blood of children**

Child	Time of study of child (months)	CD4% first - last (range) centiles[+]		CD8% first - last (range) centiles[+]		CD4% grouping[§]
P6	57	19 - 17	(29 - 13)	54 - 63	(75 - 53)	I
		< 3rd	(5th - <3rd)	>97th->>97th	(>>97th - >97th)	
P27	58	29 - 27	(36 - 20)	52 - 53	(59 - 42)	I
		5th-3rd	(25th - <3rd)	> 97th	(>>97th - 95th)	
P69	59	35 - 25	(37 - 23)	46 - 53	(59 - 41)	I
		25th-3rd	(50th - <3rd)	>97th	(>>97th - 90th)	
P121	54	24 - 23	(37 - 23)	38 - 61	(61 - 38)	I
		5th-<3rd	(25th - <3rd)	97th->>97th	(>>97th - 97th)	
P92	58	26 - 2	(30 - 1)	43 - 53	(72 - 43)	II
		5th-<<3rd	(10th - <<3rd)	>97th	(>>97th - >97th)	
P110	57	33 - 11	(36 - 9)	35 - 57	(68 - 28)	II
		>10th-<<3rd	(25th - <<3rd)	95th->>97th	(>>97th - 75th)	
P116	46	26 - 0	(26 - 0)	37 - 39	(65 - 33)	II
		5th-<<3rd	(5th - <<3rd)	97th-90th	(>>97th - 90th)	

CB	15	4 - 6	(6 - 43)	81 - 64	(83 - 64)	II
		<<3rd	(<<3rd)	>>97th->97th	>>97th->97th	
AF	34	0 - 1	(4 - 0)	59 - 39	(78 - 11)	II
		<<3rd	(<<3rd)	>>97th-75th	(>>97th - <<3rd)	
P125	48	52 - 31	(52 - 23)	27 - 41	(57 - 26)	III
		50th-10th	(50th - 3rd)	75th-97th	(>>97th - 75th)	
P131	35	69 - 14	(69 - 14)	18 - 30	(36 - 17)	III
		97th-<3rd	(97th - <3rd)	10th-90th	(97th - 10th)	
P132	26	62 - 36	(62 - 29)	22 - 30	(32 - 22)	III
		90th-10th	(90th - 10th)	>25th-<90th	(>90th - 10th)	
P142	19	17 - 21	(28 - 17)	26 - 53	(53 - 26)	III
		<3rd	(5th - <3rd)	50th->97th	(>>97th - 50th)	
P126	13	49 - 8	(49 - 8)	49 - 49	(29 - 59)	fast
		50th-<<3rd	(50th - <<3rd)	>97th->>97th	(75th - >>97th)	
P130	34	19 - 12	(24 - 12)	37 - 64	(64 - 34)	fast
		<<3rd	(<3rd - <<3rd)	95th->>97th	(>>97th - 90th)	

III All the children of less than 60 months of age were, initially, grouped together (P125, P126, P130, P131, P132, P142, see figure 3.1(c)). All, except one (P142), were followed from birth. Four children had a CD4% below normal levels (P126, P130, P131 and P142). P131 showed a steadily decreasing CD4% from birth, but was within normal ranges until 21 months of age. From table 3.2, it can be seen that this child had the highest decrease in CD4%. P142, whose first sample was received at 41 months of age, had a stable CD4% at just below the third centile. P125 and P132 have also decreased in CD4%, from the 50th and 90th centile, respectively, to the 10th centile (table 3.2).

Two of these children (P126 and P130) were, subsequently, grouped separately, as both had CD4% below the 3rd centile within 3 months of birth. P126 died of AIDS at 17 months, and P130 was developmentally delayed, though it is not clear whether or not this is due to HIV-1. These children were classed as fast progressors (Dr. J. Mok, personal communication) and are referred to as such for the rest of the study.

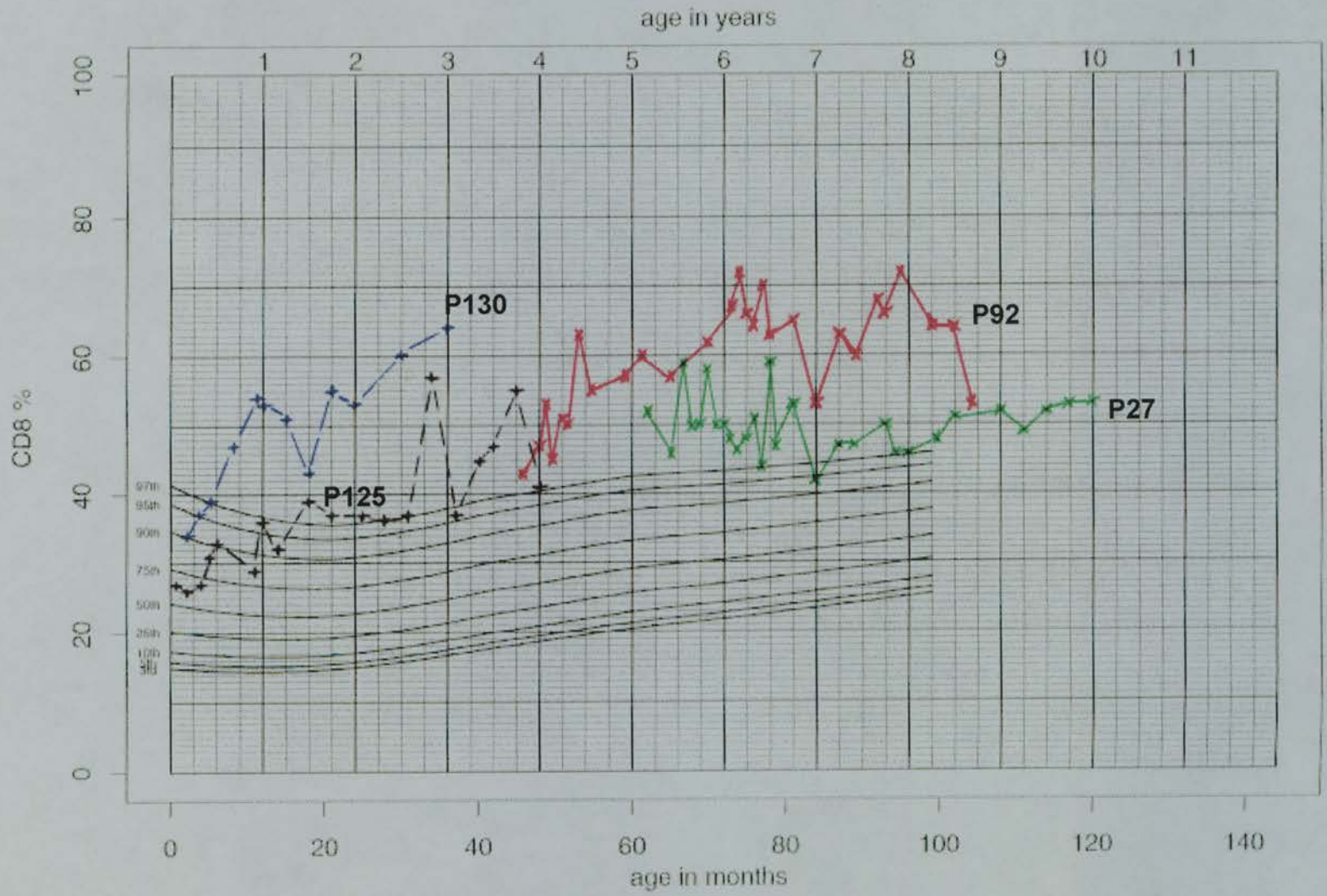
3.3.1.2 CD8 cells

For each child, the percentage of lymphocytes expressing CD8 (CD8%) was plotted on the normal range charts. As for the CD4%, the first and last CD8% recorded are shown for each child in table 3.2, together with the relevant age-centile. The highest and lowest CD8% observed are also given, to show the range over which the CD8% fluctuated during the study period. Figure 3.2 shows representative plots from the slow progressors: group I (P27), group II (P92) and group III (P125). The CD8% from a fast progressor is also shown (P130). The CD8% was found to be increased in all the older children (groups I and II) above the normal range. The CD8% in the group I children remained steady, at or above the 97th centile. The CD8% from the group II children was found to decrease, after the CD4% decreased, and up to two years before death. In the group III (younger) children the CD8% crossed the 97th centile at about 24 months of age. An exception to this was seen in the fast progressors, as these children showed a CD8% above the normal range at a much earlier age (between 6 and 9 months) than the other group III children.

Figure 3.2 **Percentage of CD8 lymphocytes**

The percentage of CD8 lymphocytes (y-axis), for each child, was plotted on age-related normal range charts, against the age in months (x-axis).

Representative lines from each group are shown: P27 is shown in green (group I), P92 is shown in red (group II), P125 is shown in black (group III) and P130 is shown in blue (fast progressor).


age in years
1
2
3
4
5
6
7
8
9
10
11
CD8 %
0
20
40
60
80
100
P130
P125
P92
P27
97th
95th
90th
75th
50th
25th
10th
3rd
0
20
40
60
80
100
120
140
age in months

3.3.2 CO-EXPRESSION OF ACTIVATION AND MEMORY MARKERS ON CD4 AND CD8 CELLS

3.3.2.1 CD4 subpopulations

The percentages of CD4 cells co-expressing CD45RO, CD45RA or HLA-DR (CD4 subpopulations) were calculated and plotted on the normal range charts (data not shown). The CD4 subpopulations remained within normal ranges throughout infection. There were a few children who showed a sustained increase in the percentage of CD4 cells expressing HLA-DR or CD45RO. These children were all from group II (P92, P116 and CB). The increase in expression of these markers occurred after, or at the same time as the overall CD4% started to decrease.

3.3.2.2 CD8 subpopulations

The percentage of CD8 cells co-expressing CD45RO, CD45RA, HLA-DR, CD11a and CD57 (CD8 subpopulations) were calculated and plotted on the normal range charts. Figures 3.3(a-e) show representative plots from group I (P27), group II (P92) and group III (P125). The results from a fast progressor are also shown (P130).

With a few exceptions, the expression of CD45RO, CD45RA and HLA-DR on CD8 cells was outwith normal ranges throughout the study in these children (see figures 3.3(a-c)). For CD45RO, CD45RA expression, three children showed levels within normal ranges (P69, P116 and P121, not shown) and for HLA-DR expression, two children (P69 and P121, not shown) showed levels within normal ranges. In all the children the expression of CD11a was outwith the normal range throughout infection (see figure 3.3(d)). The levels of CD57 expression on CD8 cells were within the normal ranges for all, but one, of the children. The exception was one of the fast progressors (P130), who showed markedly raised and sustained levels above the 97th centile (see figure 3.3(e)). Another child (P116) fluctuated in and out of the top of the normal range (not shown).

Figure 3.3 **CD8 subpopulations**

The percentage of CD8 cells expressing the marker of interest (y-axis), for each child, was plotted on age-related normal range charts, against the age in months (x-axis).

Legend as for figure 3.2.

a) percentage of CD8 cells expressing CD45RO

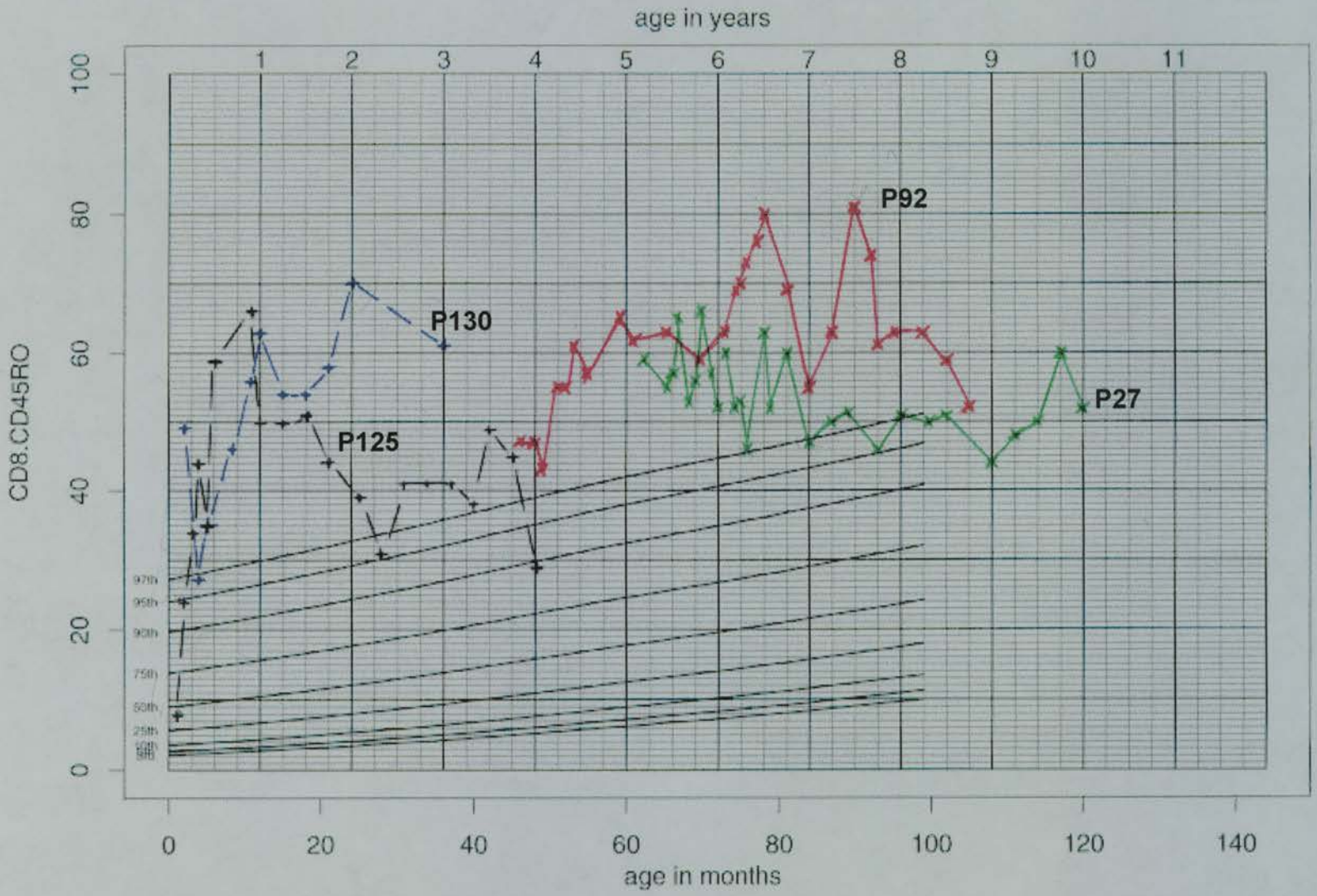

age in years
1
2
3
4
5
6
7
8
9
10
11
CD8.CD45RO
0
20
40
60
80
100
P130
P125
P92
P27
97th
95th
90th
75th
50th
25th
10th
0
20
40
60
80
100
120
140
age in months

Figure 3.3 **CD8 subpopulations**

b) percentage of CD8 cells expressing CD45RA

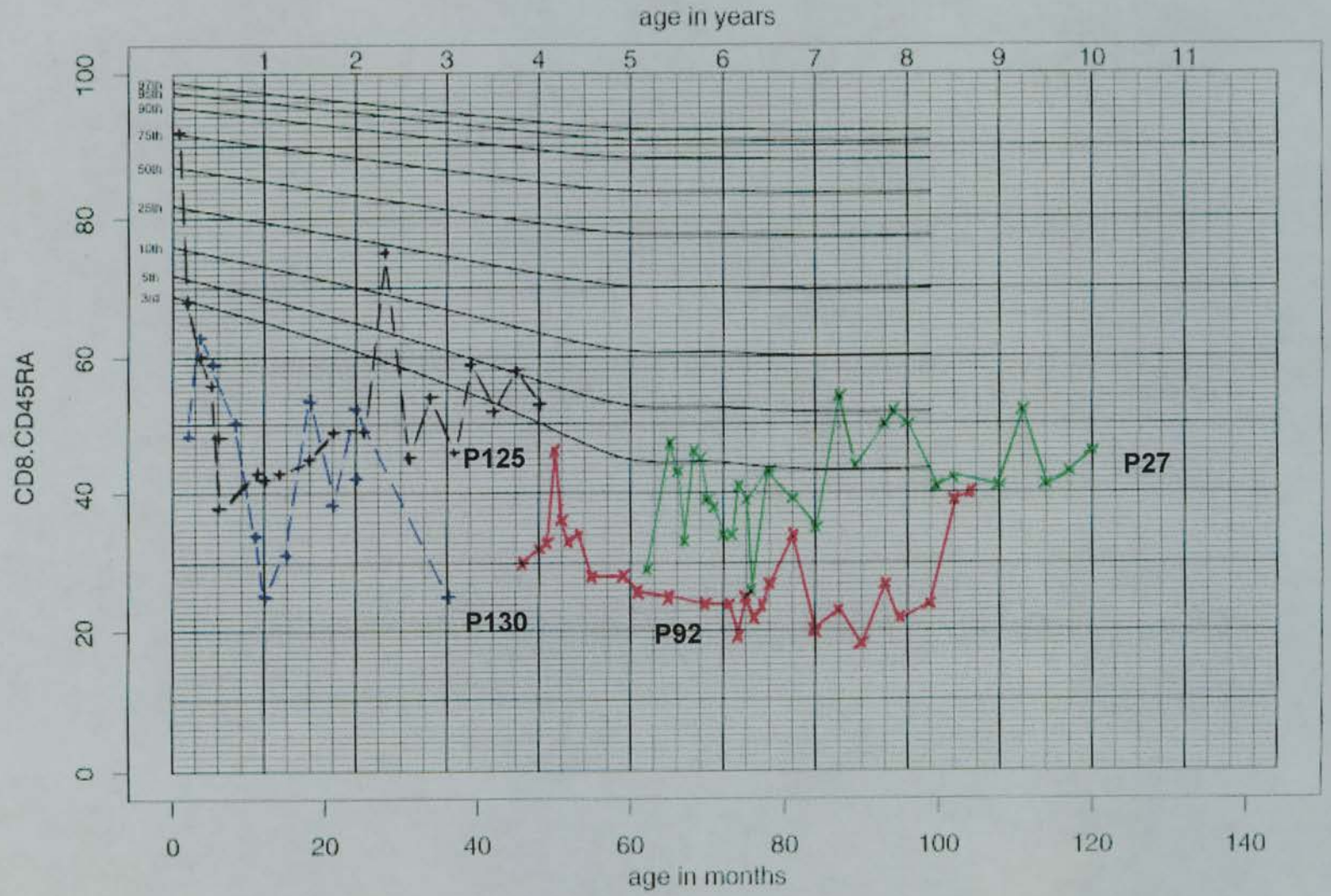

age in years
1
2
3
4
5
6
7
8
9
10
11
CD8.CD45RA
0
20
40
60
80
100
P125
P130
P92
P27
0
20
40
60
80
100
120
140
age in months

Figure 3.3 **CD8 subpopulations**

c) percentage of CD8 cells expressing HLA-DR

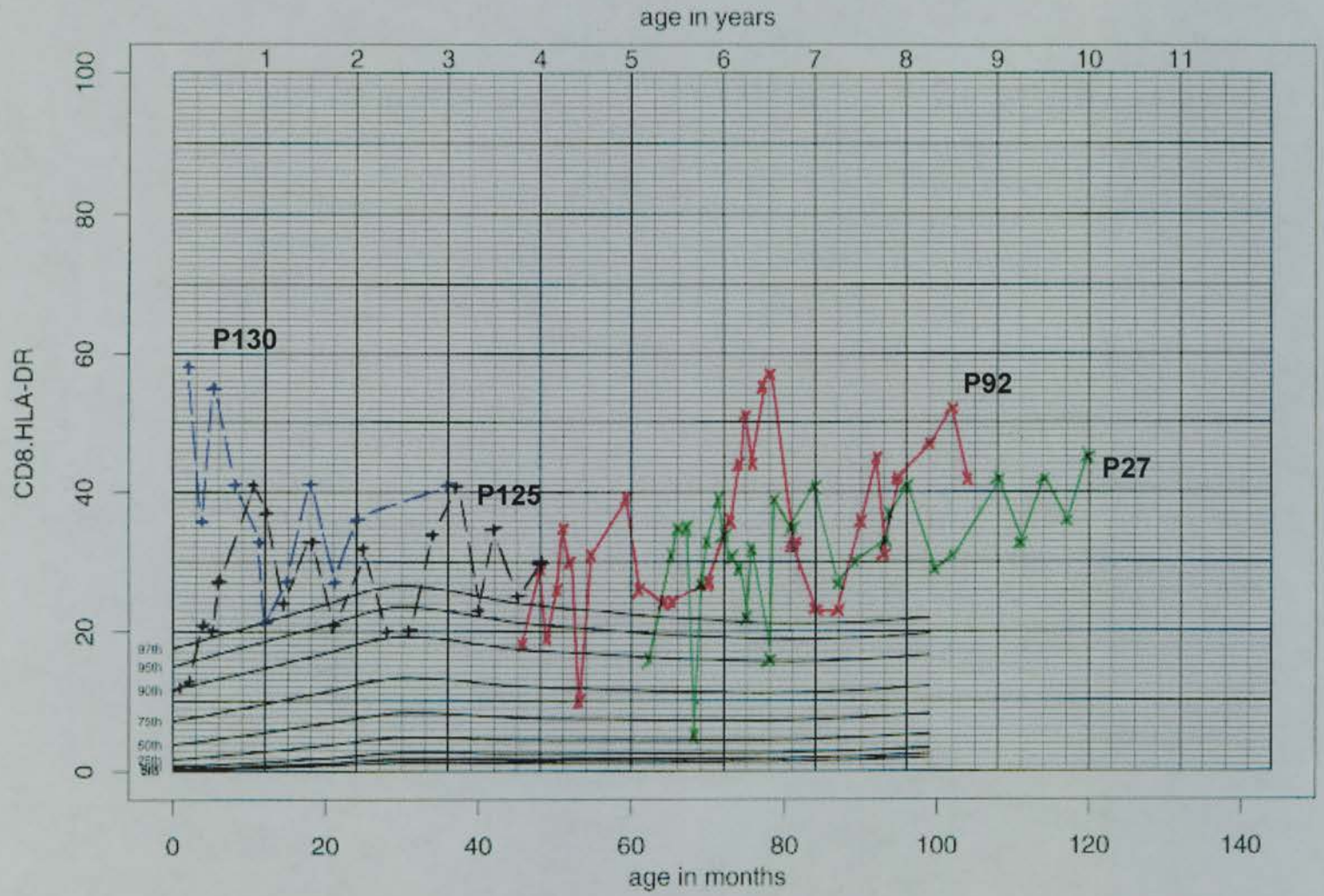

age in years
1
2
3
4
5
6
7
8
9
10
11
CD8.HLA-DR
0
20
40
60
80
100
P130
P125
P92
P27
97th
95th
90th
75th
50th
25th
5th
0
20
40
60
80
100
120
140
age in months

Figure 3.3 **CD8 subpopulations**

d) percentage of CD8 cells expressing CD11a

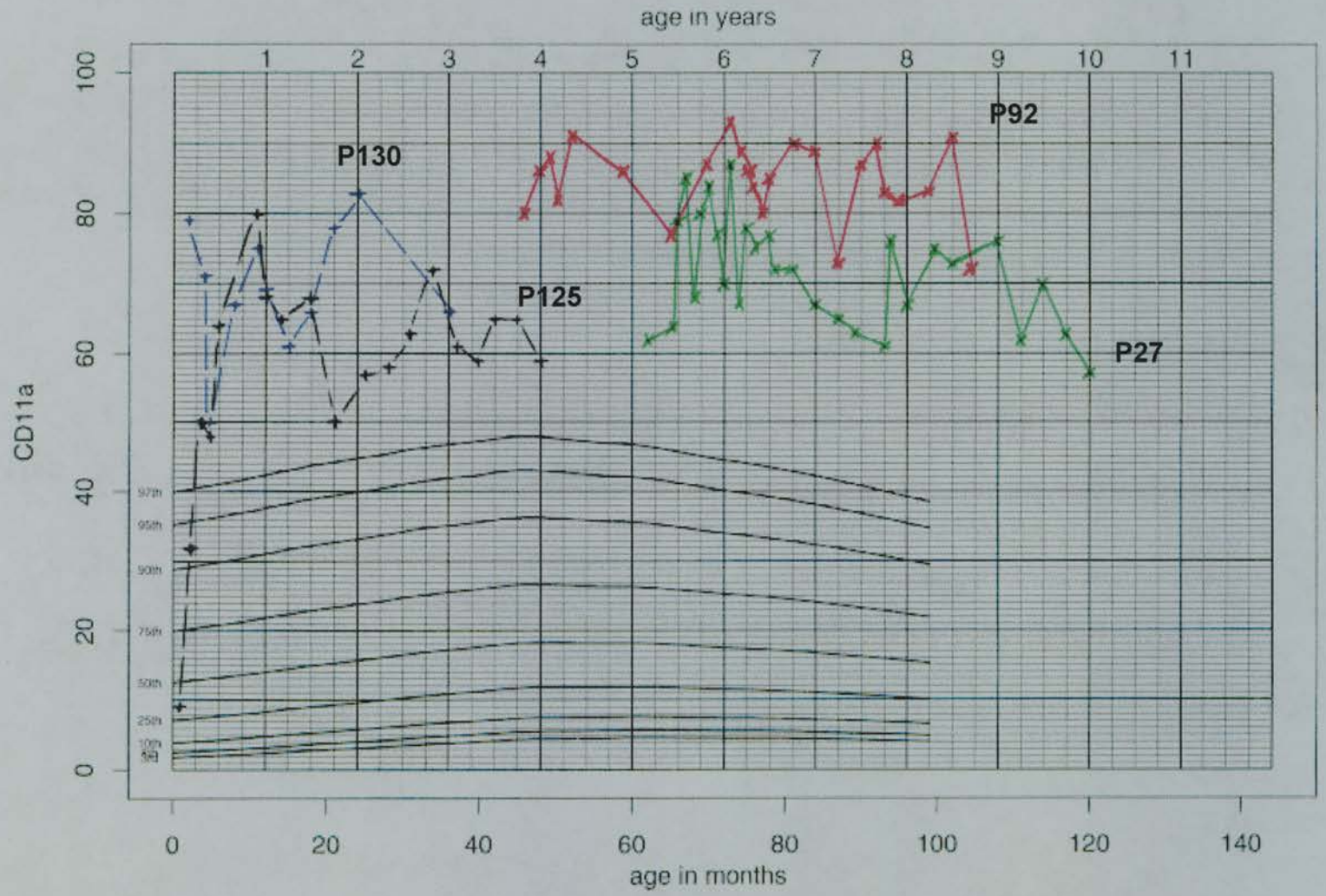
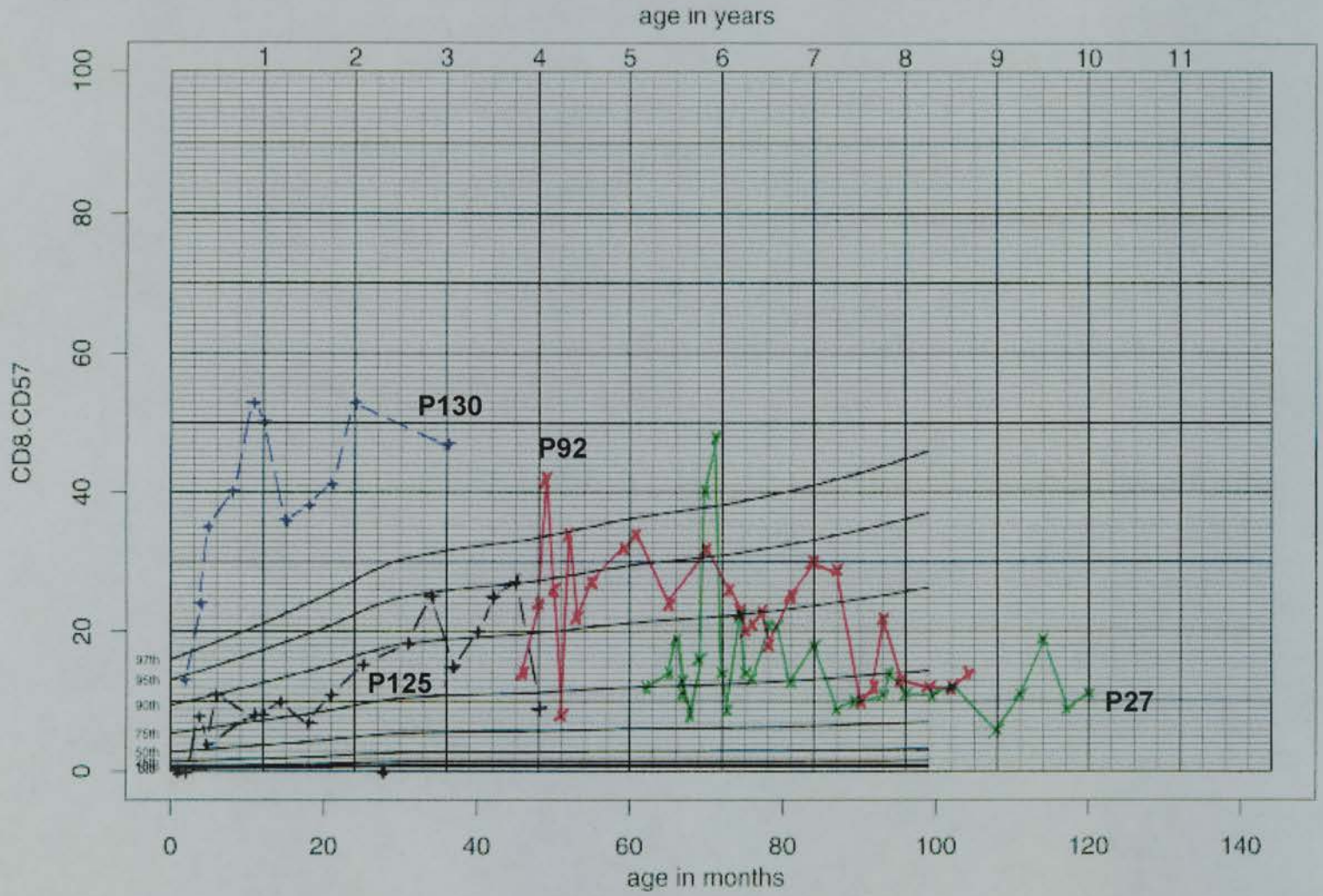

age in years
1
2
3
4
5
6
7
8
9
10
11
P130
P125
P92
P27
CD11a
0
20
40
60
80
100
97th
95th
90th
75th
50th
25th
10th
3rd
0
20
40
60
80
100
120
140
age in months

Figure 3.3 **CD8 subpopulations**

e) percentage of CD8 cells expressing CD57


age in years
1
2
3
4
5
6
7
8
9
10
11
CD8.CD57
0
20
40
60
80
100
P130
P92
P125
P27
97th
95th
90th
75th
50th
0
20
40
60
80
100
120
140
age in months

3.3.3 CO-EXPRESSION OF CD38 AND CD45RO ON CD8 CELLS BY TRIPLE-COLOURED IMMUNOFLUORESCENCE (CD38/CD45RO/CD8 TRIPLE)

An increase in the expression of CD38 and CD45RO on CD8 cells (Prince and Jensen, 1991) has been shown to be associated with an increased likelihood of CD4 cell loss in adults (Bofill *et al.*, 1996). These markers were studied by triple-colour immunofluorescence to see whether the same holds true in children. No normal ranges for these markers have been established in children, so the results are shown as the comparison of marker expression in HIV-1-infected and -exposed uninfected (EU) children at different ages.

The HIV-1-infected children (113 results from 18 individuals) and EU children (123 results from 60 individuals) were plotted against age (in months). The total CD8% was found to be increased in the infected children, compared to the EU (data not shown), in a similar way to that seen in the dual-colour data. The proportion of CD8 cells expressing CD45RO was also found to be increased in HIV-1-infected children, in a similar pattern to that seen by dual colour fluorescence. This confirms that the results seen using this triple-colour system are comparable to those results obtained by dual-colour immunofluorescence.

The percentage of CD8 cells expressing CD38 was plotted against age (months) and are presented in figure 3.4. The expression of this marker decreased with increasing age, with a great deal of overlap between the groups of children. A linear regression analysis showed that the expression of this marker was significantly age dependent ($p<0.001$), but no difference was seen between HIV-1-infected and EU children ($p = 0.16$, S. Hutchinson and S. Lewis, MRC BIAS, personal communication).

The percentage of the CD8 cells expressing both CD45RO and CD38 together was plotted against age (months) and are shown in figure 3.5. A regression analysis showed that, for the EU children there was no age-relation in the expression of this marker ($p = 0.15$). By comparison, the HIV-1-infected children showed a significantly raised expression of this marker ($p<0.001$, S. Hutchinson and S. Lewis, MRC BIAS, personal communication). The outlying point in the 73-84 month age band was due to higher levels of expression of this marker in one child. When this child was removed, the slope of the regression line was still

Figure 3.4 **CD38 expression on CD8 cells in HIV-1-exposed children**

The percentage of CD8 cells expressing CD38 (*y*-axis) for the infected (HIV+ve) and exposed uninfected (EU) children were analysed against age (*x*-axis), in age bands by month (0-1, 2, 3, 4, 5-8, 9-12, 13-18, 19-24, 25-36, 37-48, 49-60, 61-72, 73-84, 85-96, 97-108, and over 108 months). The geometric mean and 95% confidence limits were calculated for each age band. One confidence limit is shown for each data set, the upper for the HIV+ve and the lower for the EU children.

Linear regression lines were drawn for each data set and are shown, with the equations, on the chart.

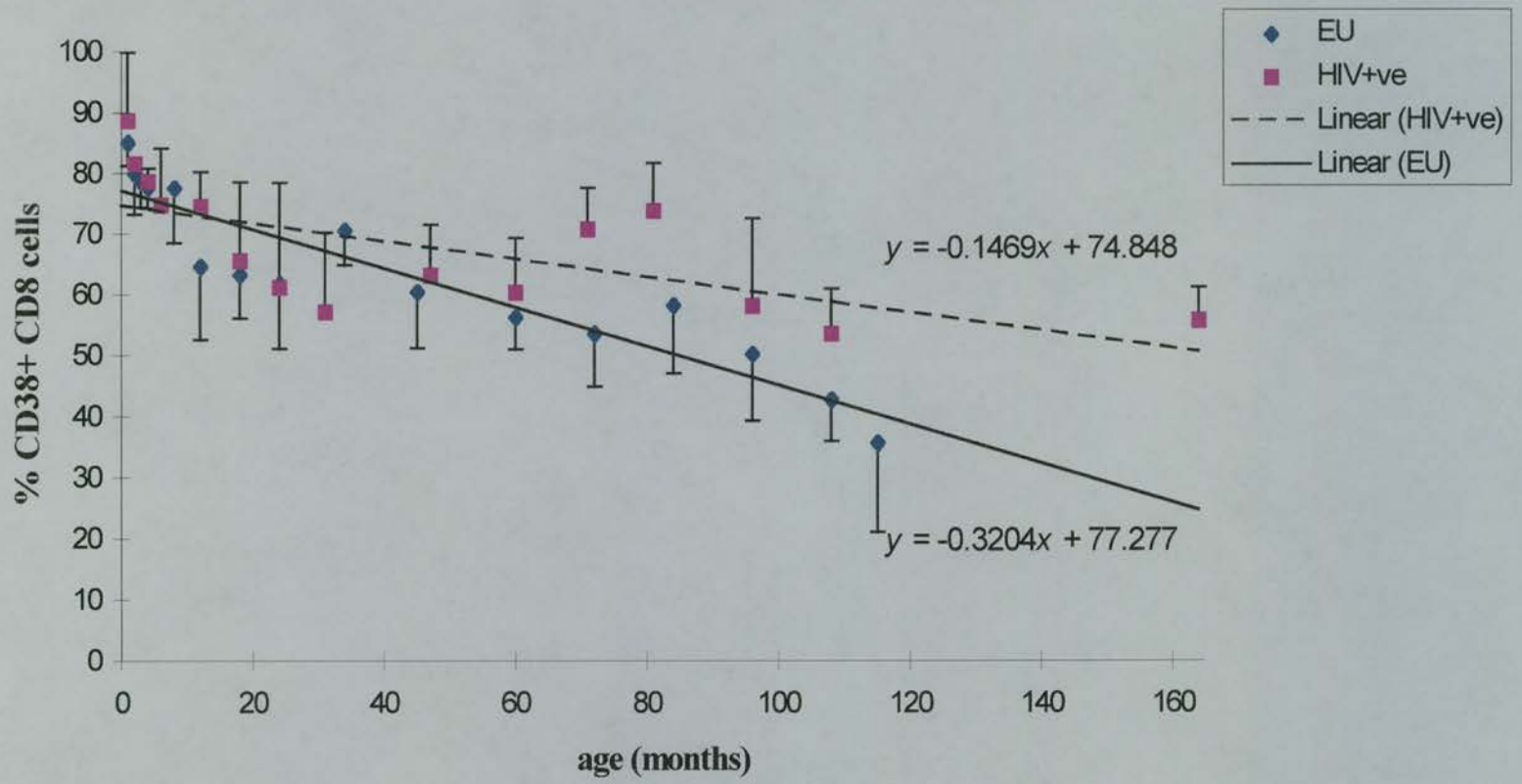
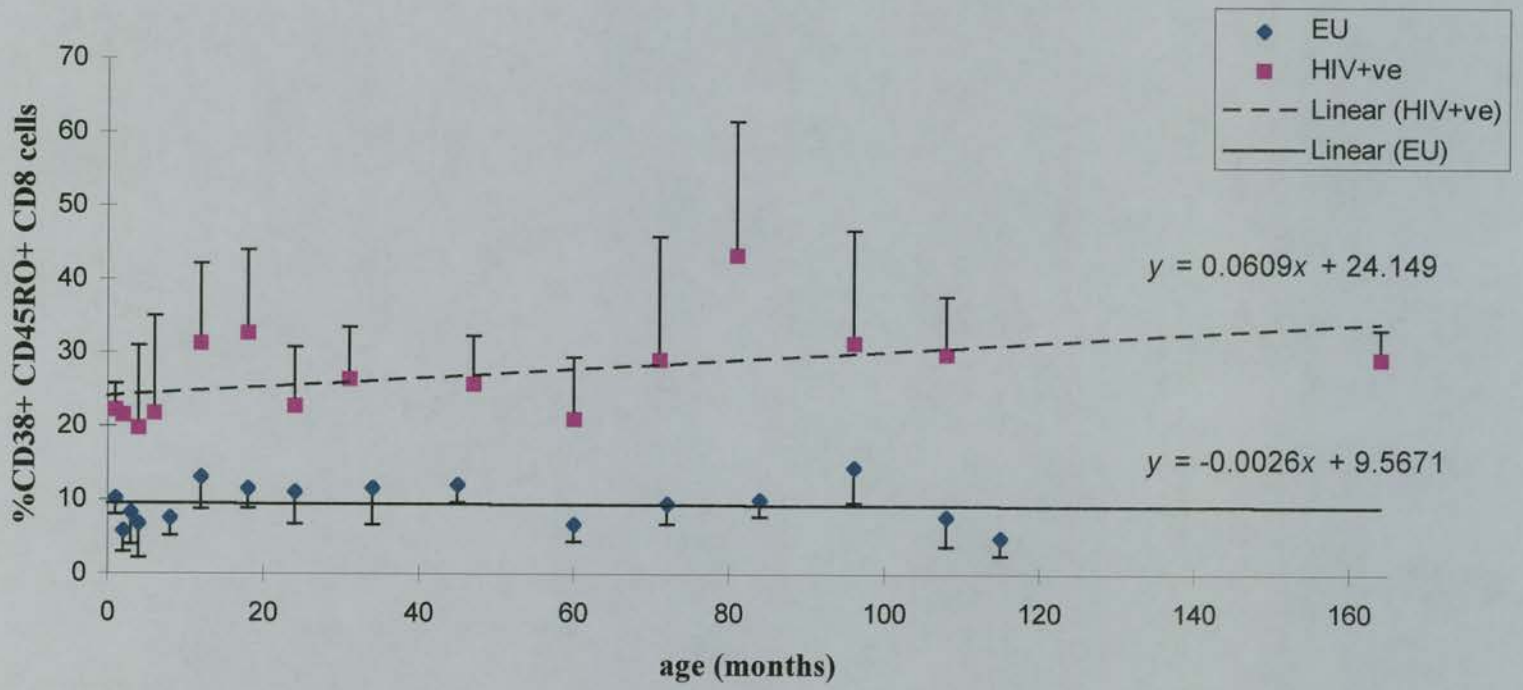

EU
HIV+ve
Linear (HIV+ve)
Linear (EU)
y = -0.1469x + 74.848
y = -0.3204x + 77.277
% CD38+ CD8 cells
age (months)
0
10
20
30
40
50
60
70
80
90
100
0
20
40
60
80
100
120
140
160

Figure 3.5 **Co-expression of CD38 and CD45RO on CD8 cells in HIV-1-exposed children**

The percentage of CD8 cells co-expressing CD38 and CD45RO (*y*-axis) for the infected (HIV+ve) and exposed uninfected (EU) children were analysed against age (*x*-axis) in the same age bands as described in figure 3.4.

Legend is as for figure 3.4


EU
HIV+ve
Linear (HIV+ve)
Linear (EU)
y = 0.0609x + 24.149
y = -0.0026x + 9.5671
%CD38+ CD45RO+ CD8 cells
age (months)
0
10
20
30
40
50
60
70
0
20
40
60
80
100
120
140
160

found to increase (not shown). This marker differentiated between infected and EU children with a cut off level at about 20% expression.

3.3.3.1 Comparison of CD38/CD45RO/CD8 triple with CD4%

As the expression of this triple, in HIV-1-infected adults, is associated with a decreased CD4% and disease progression (Bofill *et al.*, 1996), the percentage of CD8 cells co-expressing CD45RO and CD38, in the infected children, was compared with the CD4%. The CD4% data approximated to a normal distribution, but the data for the triple did not. A normal distribution was obtained by taking the square-root of each of the values. The square-root of the triple was plotted against CD4% and is presented in figure 3.6. A linear regression line was plotted through the points, which showed that the highest expression of the triple was seen in those children with the lowest CD4%. Correlation values (r, calculated using Microsoft Excel) for the comparison of the CD4% with the transformed data, and a correlation value of $r > 0.45$ (for $n = 17$ children) was taken to be significant (Wardlaw, 1985). A negative correlation was found between the CD4% and the square-root of the CD38/CD45RO/CD8 triple ($r = -0.523$) in the HIV-1-infected children.

3.3.4 RELATION OF LYMPHOCYTE SURFACE MARKERS TO VIRAL MARKERS

3.3.4.1 Relation of CD8 subpopulations to viral culture

The children followed from birth (P125, P126, P130, P131, P132) were routinely monitored, by virus culture, for the diagnosis of HIV-1 infection. The virus-culture results (from Dr. M. Arnott, Dept Medical Microbiology, University of Edinburgh) were compared with the corresponding expression of the CD8 subpopulations at the same time points. The results for the five children are presented in table 3.3. In two children (P125 and P126), an increase in the CD8 subpopulations occurred at the same time that virus was first isolated. In one child (P131), the increase in CD8 subpopulations was seen consistently over four months, prior to HIV-1 being isolated in culture. In one child (P132), at one month of age, the CD8

Figure 3.6 **CD38/CD45RO/CD8 triple against CD4%**

The square-root of the triple marker was plotted against the CD4% for the infected children.

A linear regression line was drawn and is shown with the equation.

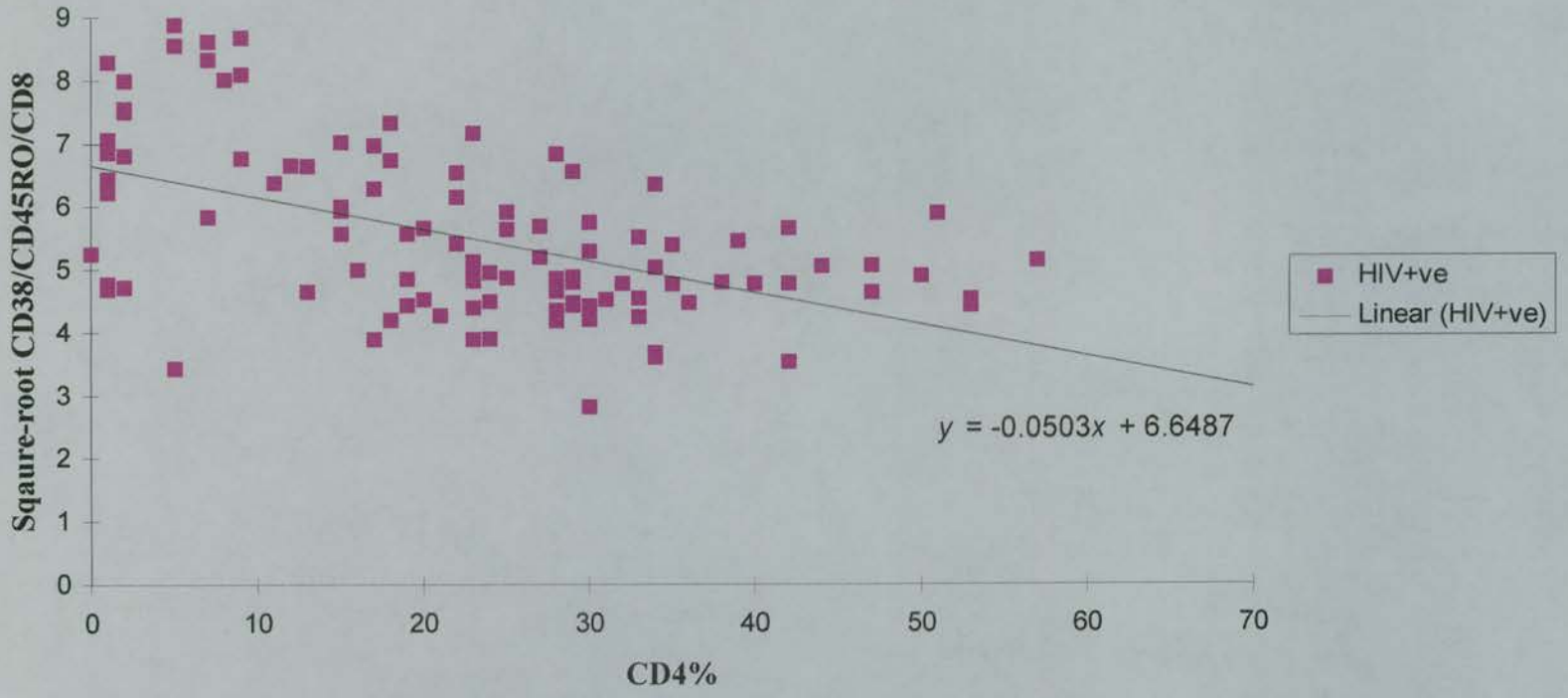

Sqaure-root CD38/CD45RO/CD8
CD4%
HIV+ve
Linear (HIV+ve)
y = -0.0503x + 6.6487
0
1
2
3
4
5
6
7
8
9
0
10
20
30
40
50
60
70

Table 3.3 **CD8 subpopulations and HIV-1 culture in the diagnosis of HIV-1 infection in children**

		Age at testing								
		Neonatal (weeks)				(months)				
Child		**cord**	**1**	**2**	**3**	**1**	**2**	**3**	**4**	**6**
P125	CD8 subsets	norm			norm		↑		↑	↑
	virus culture[§]				neg		**pos**		**pos**	**pos**
P126$_+$	CD8 subsets				↑		↑	↑		↑
	virus culture				**pos**		**pos**	**pos**		**pos**
P130[+]	CD8 subsets						↑		↑	
	virus culture				**pos**		**pos**		**pos**	
P131	CD8 subsets		norm			↑	↑		↑	↑
	virus culture					neg	neg		neg	**pos**
P132	CD8 subsets		norm			norm			↑	
	virus culture		neg			**pos**			**pos**	

§ virus culture results form Dr. M Arnott, Dept Medical Microbiology, University of Edinburgh

\+ identified as a fast progressor

norm all CD8 subpopulations in normal ranges

↑ one or more CD8 subpopulation outwith normal range

neg culture HIV-negative

pos culture HIV-positive

subpopulations were within normal ranges, but HIV-1 was isolated from the same blood sample. In another child (P130), where virus was isolated at 3 weeks of age, no blood was available for subpopulation analysis. Thus in three of five children, an increase in one or more CD8 subpopulations was seen at the same time or before virus was isolated in culture.

3.3.4.2 Relationship between lymphocyte marker and viral phenotype

The emergence of a syncytium-inducing (SI) strain of HIV-1 has been associated with an increased likelihood of onset of AIDS (Schuitemaker *et al.*, 1992). To ascertain whether there was any association between the presence of SI viral phenotypes and any lymphocyte phenotype, virus-culture supernatant (from Dr. M. Arnott) was co-cultured with MT2 cells (as described in 2.9). Syncytia were seen in the MT2-cultures from five of fourteen children tested. Of these, four children were from group II (P92, P110, P116, and AF) and one was from group III (P142). These results are marked by arrows on figures 3.1(b) and 3.1(c). The time at which the SI variant was detected varied relative to the fall in CD4%. In two children (AF and P116), the CD4% had started to decrease before the emergence of the SI variant. In another two children (P110 and P142) an SI phenotype emerged before the loss of CD4 cells: for one of these (P142), the CD4% was still steady at 60 months of age. For a fifth child (P92) an SI phenotype was seen at the first time of testing at 74 months of age, but the CD4% had already decreased to 9%. Attempts to phenotype the virus in small samples of archive material from this child (P92) were unsuccessful. The emergence of an SI variant of HIV-1 was not associated with any change in the level of expression of any CD4 or CD8 subpopulation. No SI variants were seen in the group I children, or the other group III children including the fast progressors.

3.4 DISCUSSION

The use of flow cytometry in monitoring the lymphocyte surface markers in peripheral blood is well established, and is routinely used to measure CD4 lymphocyte counts in HIV-1 infection (Landay *et al.*, 1990). In adults, the CD4 percentage and cell count in peripheral

blood decrease during the asymptomatic stage of HIV-1 infection, and this loss is associated with the onset of AIDS (Taylor *et al.*, 1989; Hoover *et al.*, 1992). In adults, the expression of different antigens on CD4 and CD8 cells has been used as surrogate markers to predict the loss of CD4 cells and disease progression (Levacher *et al.*, 1992; Zaunders *et al.*, 1995; Kestens *et al.*, 1994). This study was undertaken to investigate these surface markers on the CD4 and CD8 cells of HIV-1-infected children, to determine whether any particular marker was indicative of HIV-1 infection in children or was associated with disease progression.

The expression of some of the markers studied is related to age and, therefore, the age-related changes in normal children were defined (Aldhous *et al.*, 1994a, appendix). Normal range charts of the percentage of cell expressing a particular marker at different ages were constructed as age-related centiles. The results obtained from the HIV-1-infected children were plotted on these charts. Throughout this study, the results were presented as percentages of lymphocytes, rather than absolute counts, as percentages are less likely to fluctuate and, therefore, give more consistent results longitudinally (Taylor *et al.*, 1989). In addition, the error in measuring the white blood cell counts, from which absolute counts are calculated, has been shown to increase significantly the error of reported absolute values of CD4 and CD8 cell numbers (Hoover *et al.*, 1992).

3.4.1 CD4 CELLS AND SUBPOPULATIONS

The children could be easily divided into three groups according to age and CD4%. The children in group I had CD4% which were steady at ages over 60 months. These children are clinically stable, and none have any AIDS-defining illnesses. The children under 60 months of age were all, initially, grouped together (group III). No clear pattern of CD4% change has emerged in these children, apart from the two who were classed as fast progressors; these two children will be discussed separately. The five children in group II have all shown a CD4% decrease. Of these, four have developed AIDS, but the defining conditions occurred once the CD4% had decreased to under 5% of the total lymphocytes. This indicates that there may be some mechanism by which the immune system remains functional, despite a low level of CD4 cells in peripheral blood. CD4 and CD8 cells originate in the thymus from stem cells which express both molecules on the cell surface

(Levy *et al.*, 1988). In adults the thymus is obsolete, but in children it is still functional (George and Ritter, 1996). It is possible that cells emerging from the thymus may traffic directly into tissues rather than remain in the periphery, from where the blood was sampled, thus giving a consistently low CD4%.

Due to fluctuations in the CD4%, it is not always clear whether a drop in CD4% is transient, or a result of HIV-1 disease progression. Hence repeated blood samples are recommended, to verify sudden drops in the CD4 level. However, an accompanying increase in activation or memory markers (HLA-DR and CD45RO) on CD4 cells indicated that the CD4% decrease was due to disease progression. Memory cells (expressing CD45RO) have been reported to be infected preferentially with HIV-1 (Schnittman *et al.*, 1990; Cayota *et al.*, 1990) and these cells are increasingly activated during infection (Kestens *et al.*, 1994). Therefore, an increase in activated or memory cells could contribute to the mechanisms of CD4 cell loss. It has since been suggested that both memory and naïve CD4 cells can be infected with HIV-1. The same study also claimed that HIV-1 replication depends on the differentiation stage of the cells, the disease stage of the individual and the activation signal used (Cayota *et al.*, 1993). This could also explain the differences between those studies which have seen a selective loss of CD4 cells expressing memory markers (Shearer and Clerici, 1991; van Noesel *et al.*, 1990; Klimas *et al.*, 1991) and those, including this one, which did not (Giorgi and Detels, 1989; Vanham *et al.*, 1991; Watret *et al.*, 1993; Kestens *et al.*, 1994).

3.4.2 CD8 CELLS AND SUBPOPULATIONS

In adults, an increased percentage of CD8 cells has been reported at seroconversion (Yagi *et al.*, 1991; Zaunders *et al.*, 1995), which was maintained throughout disease (Giorgi and Detels, 1989; Scala *et al.*, 1995; Zaunders *et al.*, 1995). This increase was accompanied by an increased proportion of activated or memory CD8 cells, which may be involved in the immune response against HIV-1 (Rowland-Jones *et al.*, 1993). In the slow progressing children studied here, the CD8% was increased in the children of over 60 months of age, and remained so throughout the study, only decreasing in a few children before death. For the children followed from birth, the CD8% was within normal levels until about 24 months. This may be a reflection of an immature immune system showing a delayed response

following perinatal infection. However, the levels of activation and memory markers on the CD8 cells were increased at a much earlier age in these children, and remained so throughout infection, although the levels both within and between children varied.

The percentage of CD8 cells expressing CD45RO were found to be within normal levels in four children, three of whom became infected overseas. This raises the possibility that these children are infected with non-subtype B HIV-1 strains, which may produce different immune responses in individuals. However, the fourth child is a long-term survivor from the Edinburgh cohort. In adults, cases of long-term survivors with normal CD4 counts and a low HIV-1 burden have been described (Ferbas *et al.*, 1995; Dalod *et al.*, 1996; Lamhamedi-Cherradi *et al.*, 1995). These patients also had a low level of activated CD8 cells and a lack of anti-HIV-1 functional responses, similar to those seen in this child.

In adults, CD8 cells have been described as being chronically activated by virtue of the increased co-expression of CD45RO and CD38, and the increased co-expression of CD45RO and HLA-DR (Prince and Jensen, 1991). The co-expression of CD45RO and CD38 on CD8 cells was markedly raised in children with HIV-1 infection, compared to EU children. The expression of CD38 on CD8 cells, by itself, cannot be used as an independent marker of disease progression in children as it can in adults (Levacher *et al.*, 1992; Bofill *et al.*, 1996). This is because the CD38 expression, induced by activation of the cell is not easily distinguished from the residual CD38 expression on immature cells. In EU children from birth and up to about 24 months of age, CD38 was expressed on 90-100% of CD8 cells and the levels of expression decreased with age (figure 3.4). This confirms the findings of Hulstaert *et al.*, (1994), who also showed that in adults, only about 40% of the CD8 cells express CD38 constitutively. Thus, the induction of CD38 due to activation of cells is more easily measured in adults than in children. Plaeger-Marshall *et al.*, (1994) described a population of cells which were brightly stained for CD38, in HIV-1-infected children, and interpreted this as defining a population of activated cells. In the HIV-1-infected children studied here, no brightly staining cells were seen. Instead, the CD38 expression on activated cells, rather than immature cells, was distinguished by the co-expression of CD45RO. The increase in expression of the triple marker in the infected, compared to the EU children, was more marked than that of either CD45RO or CD38 expression on CD8 cells alone. This is because the expression of both markers individually (i.e. CD45RO or CD38 on CD8 cells)

is age-related and, to account for this, normal age-related ranges were required. The co-expression of both markers together is not age-related, as, in EU children, the increase in expression of one marker occurred as the expression of the other decreased. There was an inverse relationship between the CD4% and the proportion of these activated CD8 cells. The exact nature of this relationship requires further study, but the co-expression of CD38 and CD45RO on CD8 cells may be a useful marker for disease progression in children.

Rabin *et al.*, (1995) defined naïve CD8 cells as expressing high levels of CD45RA and CD62L (L-selectin) but expressing CD11a only dimly. They showed that this population was decreased in HIV-1-infected adults and children, and that the numbers of cells with this phenotype correlated with the CD4 count. In the children studied here, the co-expression of CD11a with CD45RA was not monitored and the expression of CD45RA on CD8 cells alone was not related to the changes in the percentage of CD4 cells, as found in other studies (Rabin *et al.*, 1995; Scala *et al.*, 1995; Roederer *et al.*, 1995). The CD45RA expression on CD8 cells was found to change in a reciprocal manner to the CD45RO expression (i.e. an increase in CD45RO was accompanied by a decrease in CD45RA expression) confirming that the CD8 cells expressed one or other of the isoforms (Wallace and Beverley, 1990).

CD11a is the α-chain of LFA-1, an adhesion molecule which binds to its ligand, ICAM-1. This LFA-1-ICAM-1 binding is essential for interaction of T cells with their targets (Springer, 1990). The expression of CD11a on CD8 cells was markedly increased outwith normal ranges in the infected children, indicating that these CD8 cells may be readily interacting with other cells.

The increased expression of activation, memory and markers of cell-cell interactions on CD8 cells indicates that these cells are involved in immune mechanisms in response to HIV-1 infection in these children.

3.4.3 FAST PROGRESSORS

Two children (P126 and P130) showed different levels of expressions of markers, compared with the other children of less than 60 months of age. Clinically, these were classed as fast

progressors as one had developed AIDS and died at 17 months, and the other was developmentally delayed. A low CD4% was seen in both children by 3 months of age, but neither showed any marked or sustained increase in the expression of any activation or memory markers on CD4 cells. This was unexpected, given the increase in activation markers seen on the CD4 cells of the older children who progressed clinically. However, a recent report indicated high levels of HIV-1 provirus in CD45RA+ (naïve) CD4 cells in children who also showed rapid CD4 cell loss (Sleasman *et al.*, 1996). The authors suggested that, although thymocytes also express CD45RO and are infectable with HIV-1 *in vitro*, HIV-1-infected thymocytes are probably deleted intrathymically, and that in *in utero* infected children, the CD45RA+, as well as CD45RO+ CD4 cells appear to be a target for HIV-1 infection (Sleasman *et al.*, 1996). Thus CD4 cell depletion is through the loss of both CD45RA+ and CD45RO+ cells, and hence, these children would not show any disproportionate increase in either of these subpopulations, as was seen in the two children studied here.

The percentage of CD8 lymphocytes was increased in the fast progressors at a much earlier age than that seen in the other children followed from birth. Studies in *SCID/hu* mice (mice with severe combined immunodeficiency (SCID), with human foetal liver and thymus tissue implanted under the kidney capsule (Aldrovandi *et al.*, 1993)) have indicated that HIV-1 infection in these organs leads to a severe depletion of CD4 cells with a corresponding increased proportion of CD8 cells emerging from the thymus (Bonyhadi *et al.*, 1993; Aldrovandi *et al.*, 1993; Su *et al.*, 1995). One possibility is that these children were infected with strains of virus which have targeted the thymus as opposed to other organs. This is unlikely, as comparison of the viral sequences from one of these fast progressing children (P130) with those from two other group III children (P125 and P132), has shown that there is no more difference between these variants, than between other HIV-1 variants from the Edinburgh drug users (C.M. Wade, Dr A. Leigh Brown, personal communication). Alternatively, these results may reflect a difference in timing of transmission of HIV-1, with the two fast progressors being infected at an earlier time point perinatally (i.e. *in utero* as opposed to *intrapartum*), causing an earlier change in T cell phenotypes. The first definition of *in utero* infection, by Bryson *et al* (1992), was that if virus was isolated (by PCR or culture) from samples taken within the first 48 hours of birth, then the child was infected *in utero*. If no virus was isolated during this time period, but was isolated from a sample taken

at a later date, then the child was infected *intrapartum*. Thus far, no direct method has been found to distinguish between *in utero* and *intrapartum* infection. In this study, neither child was tested for virus culture or PCR during the first 48 hours after birth, so it cannot be ascertained whether these children were infected *in utero*, according to this definition. However, the CD4% and CD8% were also outwith normal ranges at an earlier age than those seen in the other children followed from birth. Therefore, fast progressors were considered to have been infected *in utero*, on the basis of virus isolation, a markedly decreased percentage of CD4 cells, an increased percentage of CD8 cells and increased CD8 subpopulations at an early age.

3.4.4 RELATION OF LYMPHOCYTE SURFACE MARKERS TO PROGRESSION AND DIAGNOSIS OF HIV-1 INFECTION

There appears to be some factor which influences clinical progression at between approximately 48 and 60 months (4-5 years) of age, as the children either continue with a steady CD4%, or they start to lose CD4 cells at a faster rate. One possibility is the emergence of an SI virus phenotype, which, in some studies, has been associated with an increased progression to AIDS (Schuitemaker *et al.*, 1992). These virus variants kill cells by causing the formation of syncytia (giant multi nucleated cells in susceptible cell lines *in vitro*). Five children studied here had an emergent SI variant of HIV-1. In these children, there were those whose CD4 cell loss started before the emergence of the SI variant, and those from whom an SI variant was seen before the loss of CD4 cells. These results indicate the tension and balance between the immune system and HIV-1. The timing of the emergence of the SI variant may have some effect on the immune response. It is not clear in these cases whether the SI variant directly caused the loss of CD4 cells (whether syncytia are formed *in vivo* is not clear), or whether the loss of CD4 cells allowed an SI variant to emerge due to decreased immune function or surveillance; in these children there is evidence for both scenarios. For the latter scenario, the children whose CD4% had already decreased, both died within 12 months of the SI phenotype emerging. This suggests that in an already compromised immune system, a more virulent virus cannot be overcome immunologically. For the former scenario, the other three children are still alive, at up to 30 months after the SI virus was first seen. This supports the suggestion that the SI variant may have an effect

by increasing the loss of CD4 cells (Schuitemaker *et al.*, 1992). None of the children with a steady CD4% (or a very slow decline) past 60 months of age, have shown an SI phenotype.

The expression of different markers on CD4 and CD8 cells was examined to identify any marker which was indicative of HIV-1 infection. Plaeger-Marshall *et al.*, (1994) did not find any surface marker that was clearly diagnostic for HIV-1 infection in children. However, in HIV-1-infected adults, Zaunders *et al.*, (1995) did find an increased expression of activation and memory markers on CD8 cells contributed to HIV-1 diagnosis in adults. In the children studied here, an increase in activation and memory markers (CD45RO, HLA-DR and CD11a) was seen in infected children soon after birth which could be indicative of HIV-1 infection, particularly as the increased expression of these markers was maintained. However, these markers were increased at about 3 months of age, whereas in adults, the increase in markers were seen at about the same time as seroconversion (Zaunders *et al.*, 1995). This could be reflective of the immaturity of the immune system in these children. In other viral infections the increased expression of these markers has been found to be transient, i.e. the levels of the CD8 subpopulations returned to within normal ranges once the infection was cleared (Watret *et al.*, 1993; Miyawaki *et al.*, 1991; and unpublished results). This indicates that these cells were stimulated by viral antigens, through some undefined mechanisms, and that once the infecting agent was removed, the cells were removed (Akbar *et al.*, 1994) and the marker expression returned to normal levels. Likewise, the non-activation of CD8 cells in EU children, indicates that there is no infectious agent (such as HIV-1) to stimulate these cells. Therefore, the levels of subpopulations within normal ranges indicate that these children are not infected with HIV-1.

In summary, this study showed that increased expression of activation markers on CD8 cells can be used as an indication of HIV-1 infection in children born to HIV-1-infected mothers. A decrease in CD4% concurrent with an increased expression of activation markers on the CD4 cells can indicate disease progression. The relation of lymphocyte surface markers to the functional capacity of CD4 and CD8 cells in immune responses will be further examined in chapters 4 and 5.

Chapter 4 CTL RESPONSES IN HIV-1 EXPOSED CHILDREN

4.1 **INTRODUCTION**

4.2 **METHODS**

4.3 **RESULTS**

4.3.1 **Longitudinal CTL activity in children born to HIV-1-infected mothers**

HIV-1-infected children

HIV-1-exposed, uninfected (EU) children

Effect of method variation on CTL specificity

4.3.2 **Relation of CTL activity to disease progression in HIV-1-infected children**

Fast progressors

4.3.3 **Non-detection of CTL activity in HIV-1-infected children**

Expression of HIV-1-gene product from vaccinia-constructs by B cells

Comparison of B cell line function

4.3.4 **CTL assays using peptide stimulated effector cells**

CTL-ve children

CTL+ve children

HIV-1-uninfected child

4.3.5 **CD8 subpopulations in effector cells**

4.3.6 **Cytokine production from CTL cultures**

4.4 **DISCUSSION**

4.4.1 **Specificity of CTL response from bulk-cultured effector cells**

4.4.2 **CTL activity and clinical progression**

4.4.3 **Non-detection of CTL activity**

4.4.4 **Specificity of CTL response from peptide-stimulated effector cells**

4.4.5 **CTL activity, lymphocyte phenotypes and cytokines**

4.4.6 **CTL activity in EU children**

4.1 INTRODUCTION

Cytotoxic T lymphocytes (CTLs) play a significant role in the immune response to viral infections. In some, such as influenza or Epstein-Barr virus infections, CTL activity is responsible for clearing virus from the body (McMichael *et al.*, 1983; Tomkinson *et al.*, 1989); in others, such as Hepatitis B, there is evidence that CTLs are involved in the destruction of the liver tissue (Chisari and Ferrari, 1995). In HIV-1 infection, the CTL response is thought to play a major role in the clearing of primary viraemia in adults (Lamhamedi-Cherradi *et al.*, 1995; Brander *et al.*, 1995). This CTL activity persists in seropositive asymptomatic individuals, decreasing with clinical disease progression (Hoffenbach *et al.*, 1989; Koup *et al.*, 1989). More recently it has been suggested that CTLs may cause some of the pathological damage seen in HIV-1 infection (Zinkernagel, 1995).

Initial studies in HIV-1-infected adults identified different regions of the HIV-1 virion which elicited a CTL response, such as HIV-*gag* (Nixon *et al.*, 1988; Koup *et al.*, 1989), HIV-*pol* (Walker *et al.*, 1987; Walker *et al.*, 1988) and HIV-*env* (Hoffenbach *et al.*, 1989). Since then studies have focused on HIV-*gag* and HIV-*pol*, as these are more highly conserved across HIV-1 strains than HIV-*env*. CTL epitopes within HIV-1 have been defined (van Baalen *et al.*, 1993; Meyerhans *et al.*, 1991; Brander *et al.*, 1995). These are specific sequences of viral peptides which correspond to the peptide binding motifs defined for different MHC-class I molecules (reviewed in Brander and Walker, 1995).

Little work on HIV-1-specific CTLs has been done in children. Vertical HIV-1 infection takes place in the context of a developing and immature immune system. Little is known about the differences between CTL responses to viruses transmitted *in utero* compared to those transmitted *intrapartum*. Although the cellular immune system is thought to be functionally mature at birth, little is known about the development of CTL responses in immunologically naïve individuals. Therefore, children are an important group to study, as the conclusions drawn from studies of CTL responses in adults need to be verified in children.

A longitudinal, prospective study of HIV-1-specific CTL activity in children, born to HIV-1-infected mothers, was undertaken to address some of these questions. Some of these children are HIV-1-infected, and the others are presumed to have been exposed to HIV-1, but

remained uninfected (EU children). CTL responses to different HIV-1-proteins, specifically HIV-*gag*, -*tat*, -*pol* and -*env* were measured. Possible relationships between the specificity of the CTL response, the age of the children, and clinical disease progression were investigated. The phenotypes of lymphocytes in whole blood and the effector cell populations were also examined, to determine whether it was possible to identify specific surface markers which were associated with the presence of CTL activity. The production of cytokines in culture was measured to see whether the levels of Th-1 or Th-2 cytokines were related to the development of CTL responses.

Some of the results have been published (Aldhous *et al.*, 1994b; Froebel *et al.*, 1994, appendix). The published results for the HIV-1-infected children have been included and extended in the longitudinal analysis presented here. The results for the exposed uninfected children in these papers have not been included as these were performed before the start of this thesis.

4.2 METHODS

Peripheral blood samples (approximately 2 ml) were obtained from 33 children. CTL assays were performed using separated PBMCs which had been co-cultured with autologous PHA-activated cells as effector cells (bulk culture method). Autologous EBV-transformed B-cell lines, infected with vaccinia-HIV constructs expressing the gene products from HIV-*gag*, -*tat*, -*pol* and -*env*, were used as target cells, in a 4-hour ^{51}Cr chromium release assay (as described in 2.6.1.1, 2.6.2.1 and 2.6.3).

For some children, effector cell populations were also stimulated with HIV-1 peptides, and assayed against target cells infected with vaccinia-HIV constructs or pulsed with peptide (as described in 2.6.1.2, 2.6.2. and 2.6.3).

4.3 RESULTS

Details of the HIV-1-infected children are summarised in table 4.1. In this table the children are grouped according to their CD4% profile, as outlined in chapter 3 (3.3.1). The age range over which each child was studied, the number of CTL assays carried out for each child and the specificity of the responses are also given.

4.3.1 LONGITUDINAL CTL ACTIVITY IN CHILDREN BORN TO HIV-1-INFECTED MOTHERS

4.3.1.1 HIV-1-infected children

Sequential CTL assays were carried out in 13 HIV-1-infected children over different periods of time (range 10 - 46 months, table 4.1). For the bulk culture method, the frequency of the responses is presented in table 4.2. CTL activity was detected in 9/13 (69%) children (CTL+ves) on at least one occasion. CTL activity was not detected in 4/13 (31%) infected children; these are called CTL-ve throughout this chapter. The specificity of the CTL response in individual children changed on different occasions of testing, and all the CTL+ve children except one (P142, who was tested three times) had occasions when CTL activity was not detected.

The most frequent response was seen to HIV-*pol*, followed by HIV-*gag* and -*tat*, with responses to HIV-*env* seen infrequently (see table 4.2). The CTL+ve children could be divided into two groups by CTL specificity: those with CTL activity to HIV-*pol*, HIV-*gag* and HIV-*env*, (P6, P27, P130, P131 and P142) and those with predominant CTL activity to HIV-*pol* and HIV-*tat* (P92, P110, P121 and P126). All but one child (P131) showed CTL activity to HIV-*pol*.

4.2.1.2 HIV-1-exposed, uninfected (EU) children

CTL assays were carried out in 20 children, born to HIV-1-infected mothers, and so were

Table 4.1 **HIV-1-infected children studied for CTL activity**

Child	Age range[+] (months)	CTL+ve / total assays[++]	(specificity)	Disease Progression
Group I[+++]				
P6	79 - 126	3/6	*pol/gag*	
P27	71 - 120	5/8	*pol/gag/env*[¶]	
P121	28 - 53	2/4	*tat/pol*	
P69	88 - 132	0/7		
Group II				
P92	53 - 102	4/6	*gag*[§]*/tat/pol*	AIDS
P110	21 - 70	4/6	*tat/pol*	low CD4%
P116	40 - 52	0/4		died at 78 months
Group III				
P125	4 - 42	0/4		
P131	2 - 28	1/2	*gag*	
P132	9 - 26	0/1		
P142	47 - 60	3/3	*gag/pol/env*	
Fast progressors[++++]				
P126	3 - 13	1/4	*tat/pol*	died at 17 months
P130	4 - 30	2/3	*gag/pol*	developmentally delayed

[+] Ages at first and last CTL assays

[++] Bulk culture assay

[+++] Groups based on CD4% and age, as outlined in chapter 3

[++++] Children defined as clinical fast progressors (see chapter 3)

[¶] a borderline response to HIV-*env* (10.7%) was seen on one occasion

[§] a borderline response to HIV-*gag* (10.1%) was seen on one occasion

Table 4.2 **Frequency of CTL response in HIV-1-exposed children**

HIV-1-protein	Infected children (total number = 13) Number	Cases	Uninfected children (total number = 20) Number	Cases
HIV-gag	6	P6, P27, P130, P131, P142, P92[§]	2	P140, P145
HIV-tat	4	P92, P110, P121, P126	1	P145
HIV-pol	8	P6, P27, P121, P110, P92, P126, P130, P142	3	JR, P133, P135
HIV-env	2	P142, P27[¶]	6	P133, P135, P141, P145, P146, P148
CTL+ve children	**9**		**7**	
CTL-ve children	**4**	P69, P116, P125, P132	**13**	TA, P107, JF, P122, JC, P127, JR, P128, TS, JJH, P129, P139, P143

Number of infected or EU children with responses to the different HIV-1-genes, on any occasion of assay, is shown.

presumed to have been exposed to HIV-1. Those confirmed as being uninfected had no indicators of HIV-1 infection, i.e. they were negative for PCR, HIV-1 antigen (p24) and virus culture (results from Dr. M. Arnott, Dept. Medical Microbiology, University of Edinburgh) and had levels of CD8 subpopulations within normal ranges in whole blood (see chapter 3). Those whose infection status was not confirmed (i.e. indeterminate, see 1.6.2), only had maternal antibody as an indication of exposure to HIV-1, and these were grouped with the EU children. CTL activity was detected in 7/20 (35%) of the EU children (see table 4.2). To determine whether CTL responses to HIV-1 were detected more frequently in infected or EU children, a chi-squared analysis was performed. This showed that there was no significant difference in the number of CTL+ve and CTL-ve children between the groups (χ^2 = 2.453, p = 0.245).

4.3.1.3 Effect of method variation on CTL specificity

The CTL assays were carried out after different numbers of days in culture and at different effector:target (E:T) ratios. To assess whether these methodological differences affected which gene product was detected, the CTL+ve results, from both infected and EU children, were combined and analysed for day of assay and the E:T ratio. These results are presented in table 4.3. In addition, whether the age of the child was important in the detection of a CTL response, was also investigated and is included in table 4.3.

CTL assays were performed after culturing the cells for between 10 and 23 days (see 2.5.1.1). A previous result had shown there was a difference in the specificity of the response, when effector cells from the same culture were assayed on different days (Aldhous *et al.*, 1994b, appendix). To see whether the CTL specificity was associated with the time of culture of the effector cells, the day of assay were compared for each HIV-gene product (table 4.3). There was no correlation between the construct recognised and the day of assay.

The E:T ratios (i.e. the number of effector cells per target cell) of the assays were between 10:1 and 54:1, depending on the number of cells recovered from the culture. To see whether the number of effector cells was associated with the specificity of recognition, the mean E:T ratios for the different HIV-gene products recognised in the CTL assay were compared (table

Table 4.3 **CTL specificity and methodology**

Variable		Specificity of CTL Response			
		gag	tat	pol	env
Day of assay	mean	13.6	14.4	15.4	14.7
	range	(10 - 18)	(10 - 21)	(11 - 23)	(10 - 18)
E:T ratio	mean	28	21.8	24.1	29.1
	range	(13 - 49)	(10 - 41)	(13 - 54)	(14 - 49)
Age (months)	mean	47.1	36.9	47.8	26.8
	range	(2 - 108)	(3 - 78)	(6 - 117)	(6 - 75)

The CTL specificity from both infected and EU children was assessed for methodological differences.

4.3). There was no correlation between the E:T ratio and the specificity of construct recognition.

Initial results using the bulk culture method, had indicated that there may be a relationship between the age of the child and the specificity of construct recognition. It appeared that HIV-*tat* and HIV-*pol* were recognised in the younger children, and that a response to HIV-*gag* developed later in infection (Aldhous *et al.*, 1994b, appendix). Since then, CTL activity to HIV-*gag* has also been detected in younger children. To see whether the age of the child was associated with which HIV-gene product was recognised, the mean age for the different CTL specificities were compared (table 4.3). There was no correlation between the age of the child and the specificity of construct recognition. CTL responses were detected in children as young as two months, showing there is no age 'threshold' before which responses develop.

4.3.2 RELATION OF CTL ACTIVITY TO DISEASE PROGRESSION IN HIV-1-INFECTED CHILDREN

The HIV-1-infected children were divided into groups according to age and CD4% (groups I, II, III and fast progressors) as defined in chapter 3. Disease progression was defined as those children in whom AIDS-defining diseases had been diagnosed, or had a low CD4% (see table 4.1).

Group I contained those children of over 60 months of age, with a steady CD4%. Representative results from CTL+ve and CTL-ve children (P27 and P69, respectively) are shown in figure 4.1 (a) and (b), and the results for each child are summarised in table 4.1. CTL responses were seen in three of four children, predominantly to HIV-*pol*, -*gag* and -*tat*, with a borderline response to -*env* in one child. CTL activity was lost in all these children while the CD4% was still steady. No CTL activity was detectable after 108 months from one child (P27, figure 4.1(a)), although the child was followed for a further 6 months and two more occasions of assay. Similarly, CTL responses were detected to HIV-1 detected from another child (P6) at 93 months, and was followed for a further 30 months, but no CTL activity was detected on two more occasions. Likewise, in another child (P121), CTL activity

Figure 4.1 **CTL activity in relation to CD4% profile in slow progressors**

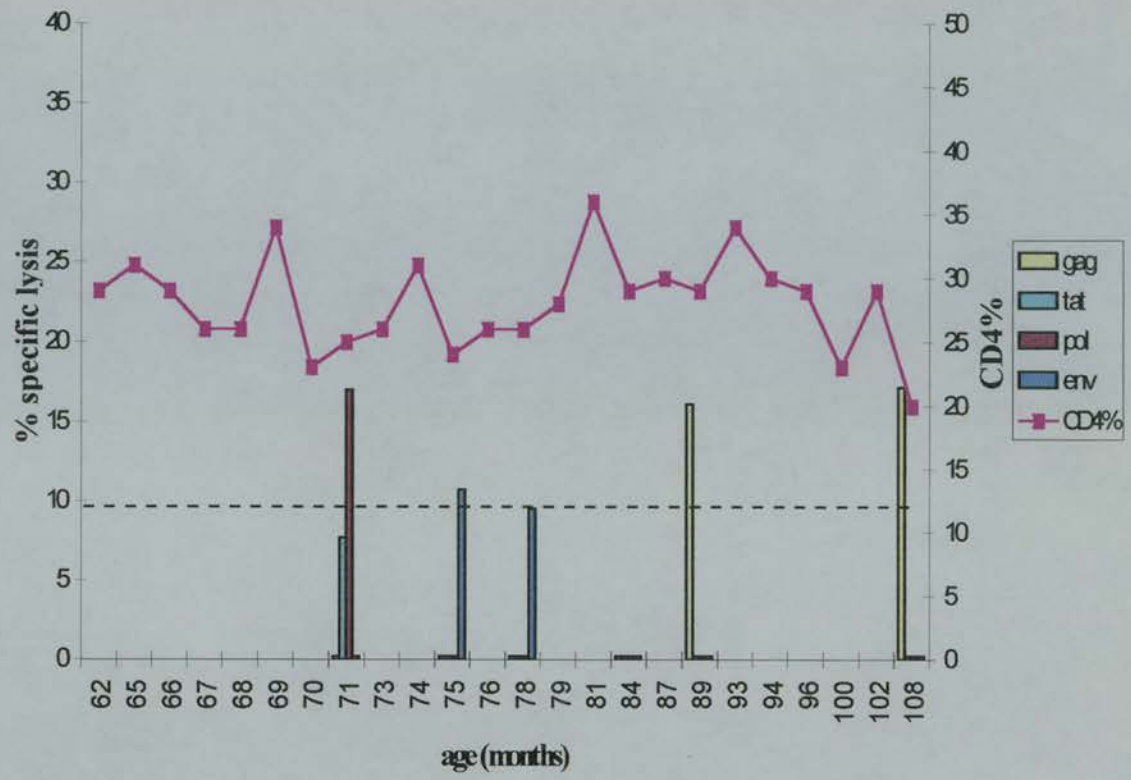
The specific lysis of target cells (i.e. after subtraction of background lysis from medium and vaccinia controls) infected with different constructs (gag, tat, pol, env) is shown. The horizontal line is drawn at 10% specific lysis, above which the result is considered to be positive. The age at which each assay was done is shown on the *x*-axis (this axis is not linear). The corresponding CD4% for the blood sample at that age is also plotted (right *y*-axis).

Representative results from the group I children (those with a steady CD4%, at ages older than 60 months) are shown.

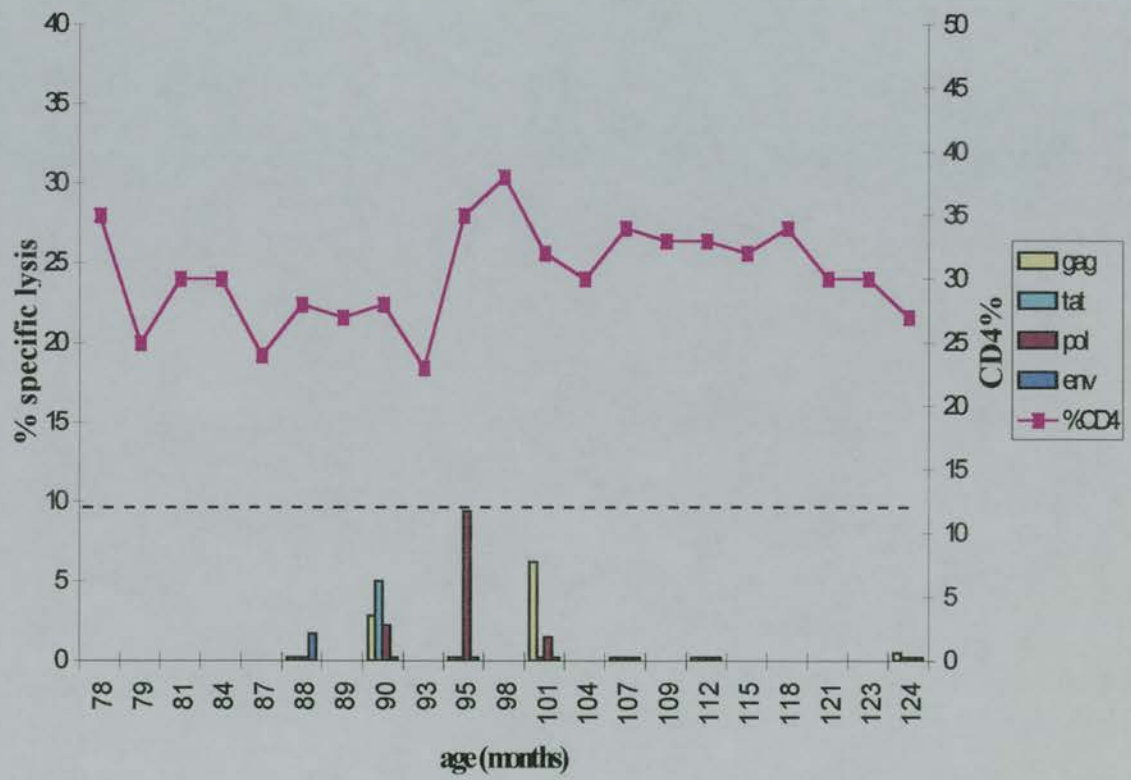
(a) CTL+ve child (P27 is presented. Similar results were also seen in P6 and P121).

(b) CTL-ve child (P69 is presented).

(a)


% specific lysis
CD4%
age (months)
gag
tat
pol
env
CD4%
0
5
10
15
20
25
30
35
40
45
50
62
65
66
67
68
69
70
71
73
74
75
76
78
79
81
84
87
89
93
94
96
100
102
108

(b)


% specific lysis
CD4%
age (months)
gag
tat
pol
env
%CD4
0
5
10
15
20
25
30
35
40
45
50
78
79
81
84
87
88
89
90
93
95
98
101
104
107
109
112
115
118
121
123
124

was detected at 28 and 35 months, but not on two further occasions at 45 and 53 months. The fourth child in this group (P69) showed no CTL activity (see figure 4.1(b)) but still had a steady CD4%.

Group II contained those children of over 60 months whose CD4% had decreased. Representative results from CTL+ve and CTL-ve children (P92 and P116, respectively) are shown in figure 4.1 (c) and (d), and the results for each child are summarised in table 4.1. CTL responses were detected in two of three children to HIV-*pol* and HIV-*tat*, but, apart from one borderline response, not to HIV-*gag* or -*env*. The CTL+ve children maintained responses while the CD4% was decreasing. For one child (P92, shown in figure 4.1(c)), CTL activity was lost at 78 months, once the CD4% had decreased to 9%. For the other child (P110, not shown) the CTL response was maintained despite a low CD4%. The third child in this group (P116) showed no CTL activity, but whose CD4% still decreased (figure 4.1(d)).

Group III contained those children younger than 60 months, who were not fast progressors. Representative results from CTL+ve and CTL-ve children (P142 and P125, respectively) are shown in figure 4.1 (e) and (f), and the results for each child are summarised in table 4.1. CTL responses were detected in two of four children to HIV-*pol*, HIV-*gag* and HIV-*env*. One of these (P142) showed CTL activity to target cells infected with each of these three vaccinia-HIV constructs (figure 4.1(e)). Another child (P131) showed CTL activity to HIV-*gag* at one time point. The other two children (P125, shown in figure 4.1(f), and P132) were consistently CTL-ve.

4.3.2.1 Fast progressors

To assess whether the specificity of the CTL responses was different in fast progressors, the results of two such children (P126 and P130) are shown in figure 4.2 (a) and (b), respectively, and summarised in table 4.1. Both children were HIV-1-culture positive at 3 weeks of age and both had a CD4% below the 3rd centile at 2 months of age (see chapter 3). One child (P126) showed CTL activity to HIV-*tat* and HIV-*pol*, on one occasion at 6 months of age, but not at 3, 9 and 13 months of age. This child died of AIDS at 17 months.

Figure 4.1 **CTL activity in relation to CD4% profile in slow progressors**

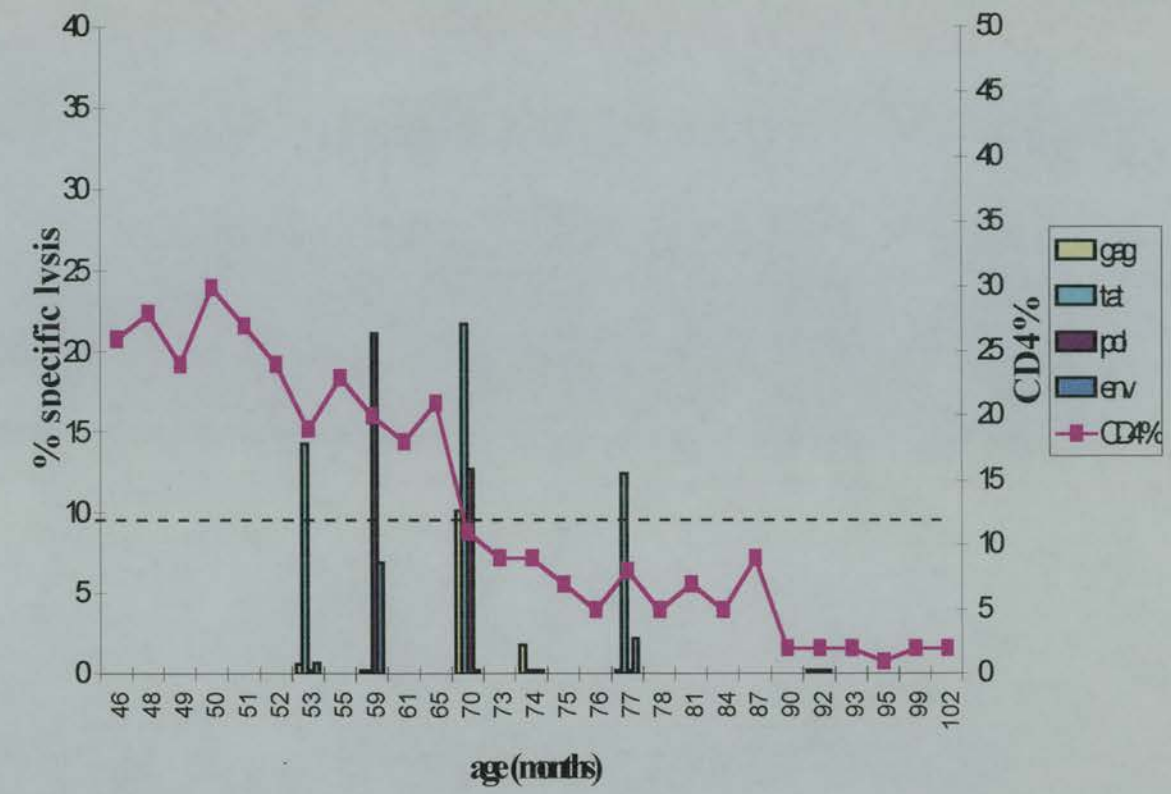
Representative results from group II children (those with a decreased CD4% at ages older than 60 months) are shown.

(c) CTL+ve child (P92 is presented. Similar results were also seen in P110).

(d) CTL-ve child (P116 is presented).

The legend is the same as for figure 4.1 (a) and (b).

(c)


% specific lysis
CD4%
age (months)
gag
tat
pol
env
CD4%

(d)

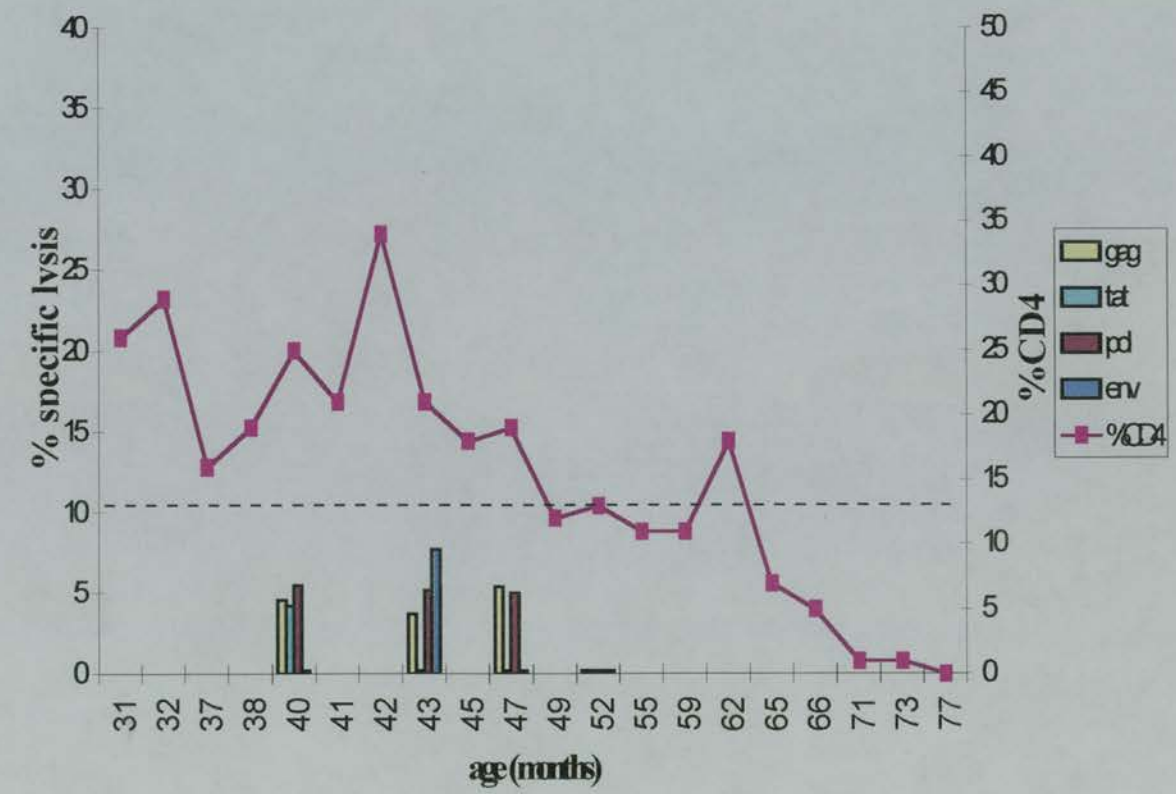

% specific lysis
%CD4
age (months)
gag
tat
pol
env
%CD4

Figure 4.1 **CTL activity in relation to CD4% profile in slow progressors**

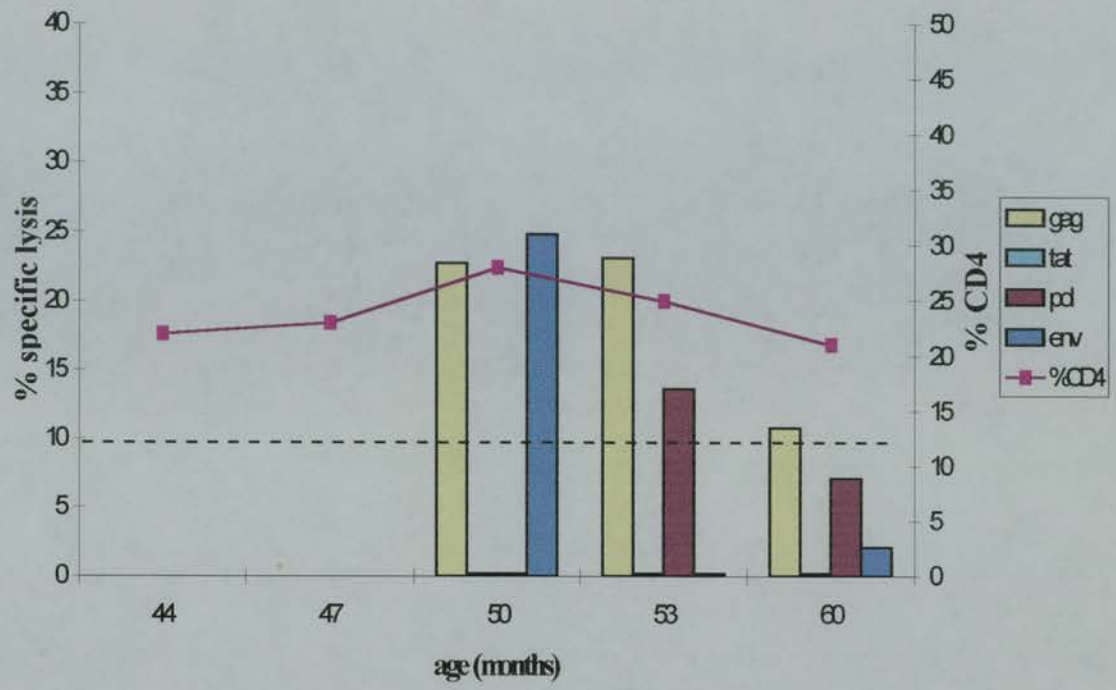
Representative results from group III children (those children younger than 60 months, not including the fast progressors) are shown.

(e) CTL+ve child (P142 is presented. Similar results were also seen in P131).

(f) CTL-ve child (P125 is presented. Similar results were also seen in P132).

The legend is the same as for figure 4.1 (a) and (b).

(e)


% specific lysis
% CD4
gag
tat
pol
env
%CD4
44
47
50
53
60
age (months)

(f)

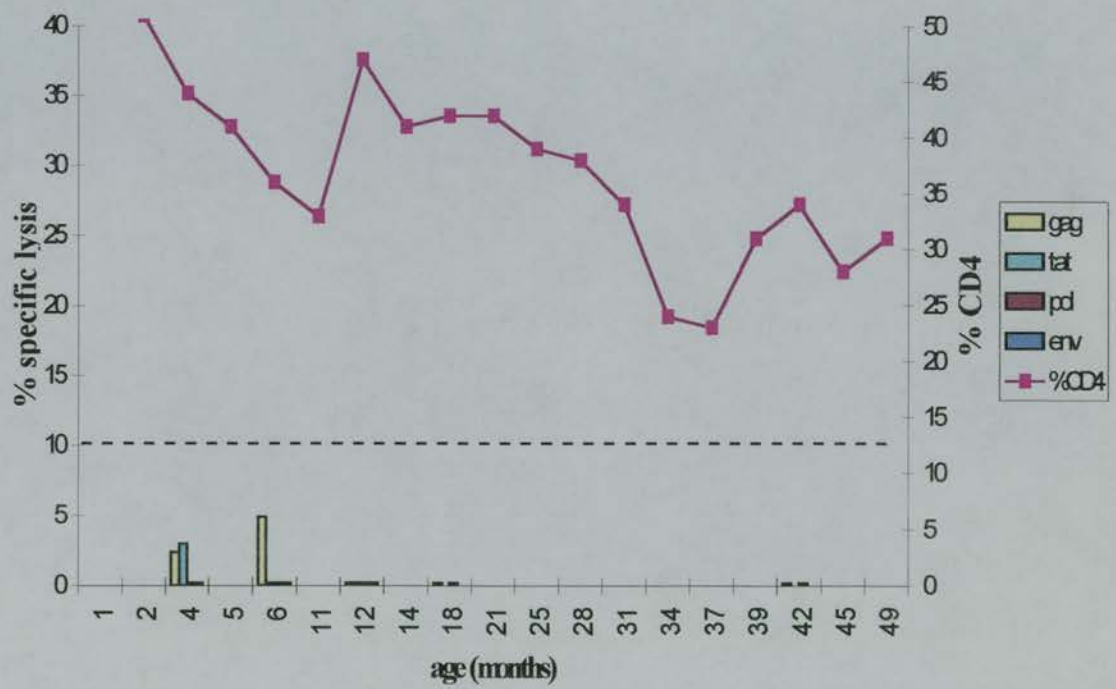

% specific lysis
% CD4
gag
tat
pol
env
%CD4
1
2
4
5
6
11
12
14
18
21
25
28
31
34
37
39
42
45
49
age (months)

Figure 4.2 **CTL activity in fast progressors**

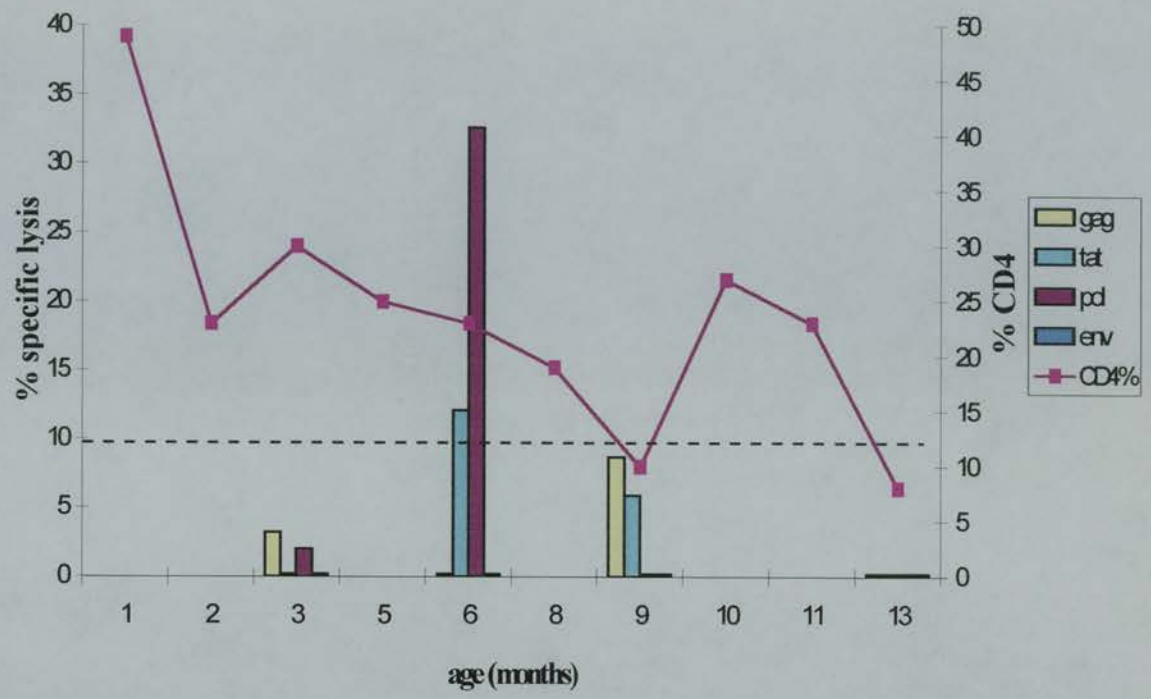
Results from the fast pogressors are shown.

(a) CTL activity in P126

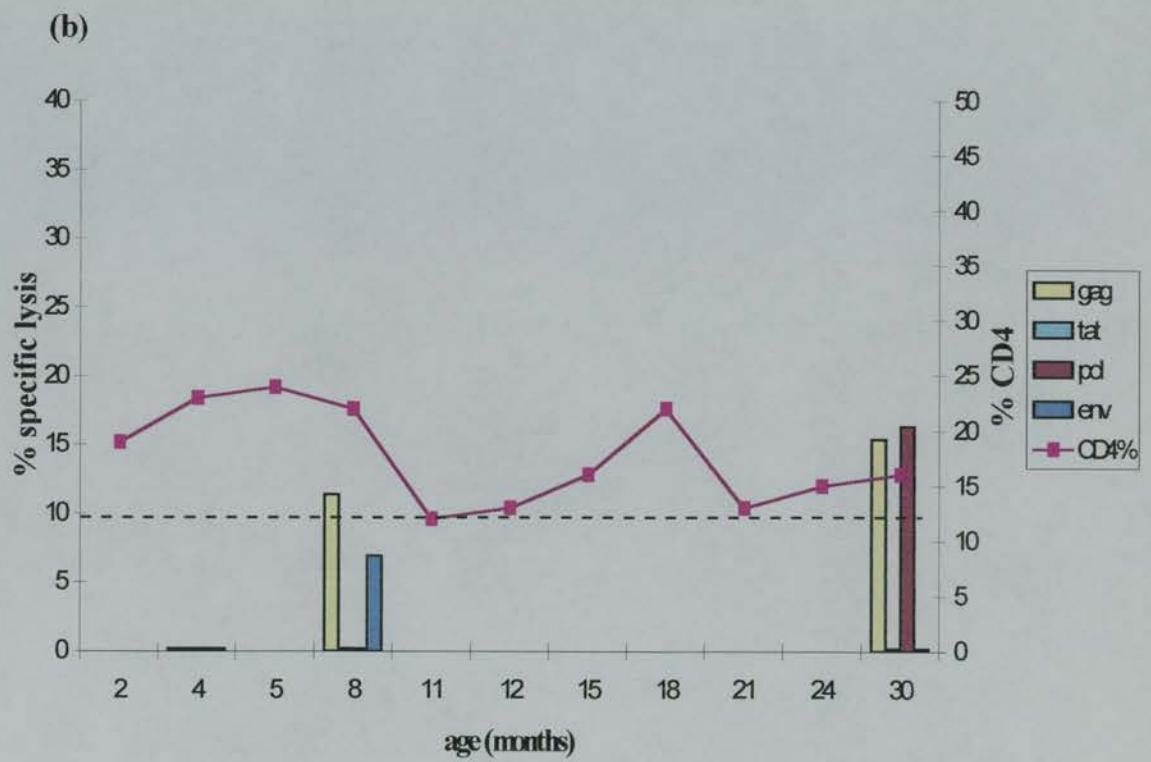
(b) CTL activity in P130

The legend is the same as for figure 4.1.

(a)


% specific lysis
% CD4
gag
tat
pol
env
CD4%
age (months)

(b)


% specific lysis
% CD4
gag
tat
pol
env
CD4%
age (months)

The other child (P130) showed CTL activity to HIV-*gag* at 8 months, and to HIV-*gag* and HIV-*pol* at 30 months. These CTL responses, in terms of specificity, were not different from those seen in children from groups I, II or III.

4.3.3 NON-DETECTION OF CTL ACTIVITY IN HIV-1-INFECTED CHILDREN

Four HIV-1-infected children (P69, P116, P125, and P132) were consistently negative for CTL activity using the bulk culture method. These children were further investigated to determine whether this was due to some methodological differences between these and the CTL+ve children. The expression of the HIV-gene product on the surface of the target cells, and whether the B cell line functioned efficiently as a target cell were both investigated. In addition, the effect of an alternative method of stimulation of the CTL effector cells was assessed (see 4.3.4).

4.3.3.1 Expression of HIV-gene product from vaccinia-constructs by B cells

Non-expression of the HIV-gene product, after infection with the vaccinia-constructs, on the surface of the cells, could be one explanation for the lack of CTL activity in these children. To check whether this was the case, aliquots of B-cells were infected with each vaccinia-HIV construct overnight. These cells were stained with anti-HIV-1 antibodies (α-p55/p17, α-gp120, α-tat and α-RT), followed by a FITC-conjugated goat anti-mouse antibody and visualised by fluorescent microscopy, as described in 2.7.2. B-cells from both a CTL-ve (P69) and a CTL+ve (P110) child, were used. Representative results are presented in figure 4.3 and show that both cell lines expressed the HIV-1-proteins efficiently on the cell surface.

4.3.3.2 Comparison of B cell line function

One CTL-ve child (P125) was found to be the same HLA-type as a CTL+ve child (P92). To assess whether the B cells from the CTL-ve child functioned effectively as target cells, CTL

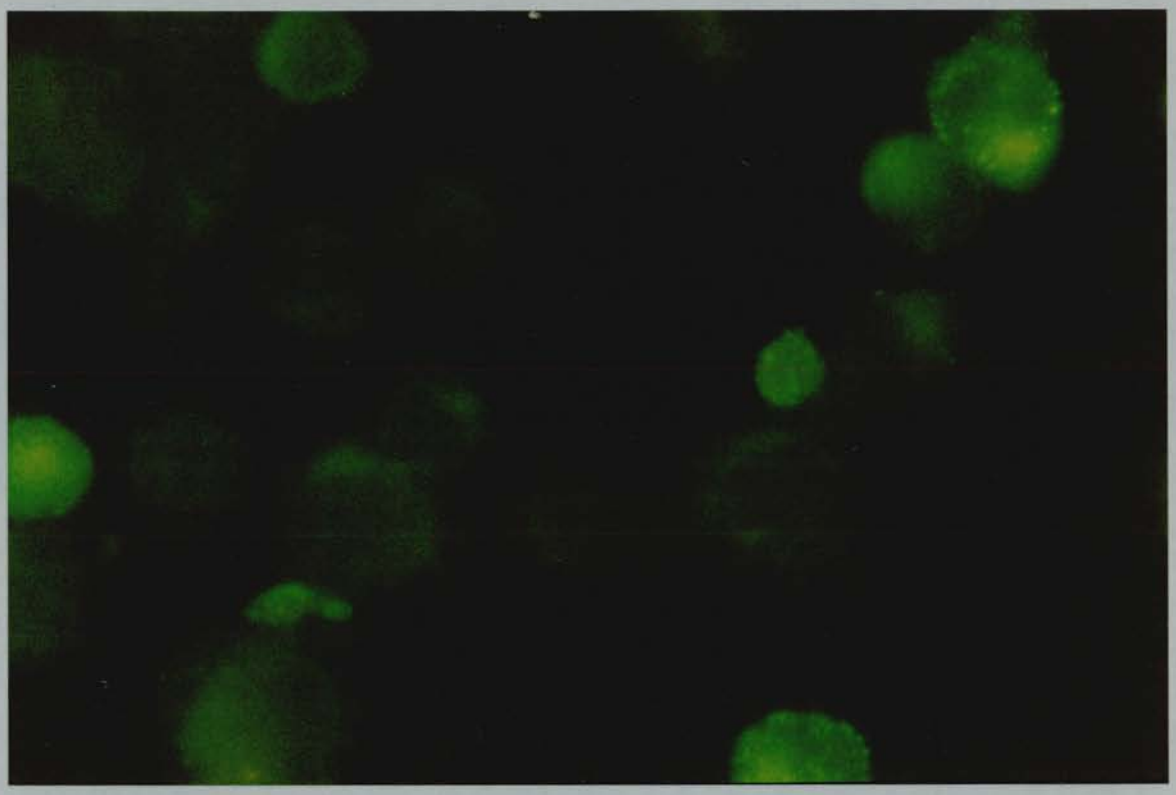
Figure 4.3 **Expression of HIV-constructs by B cells**

Representative photographs of the cells infected with the vaccinia-constructs. The proteins expressed on the surface of the cell were detected by an anti-HIV-1 antibodies and FITC-conjugated goat-anti-mouse, and visualised by fluorescent microscopy. The brightly staining cells are those which express the protein of interest on the cell surface.

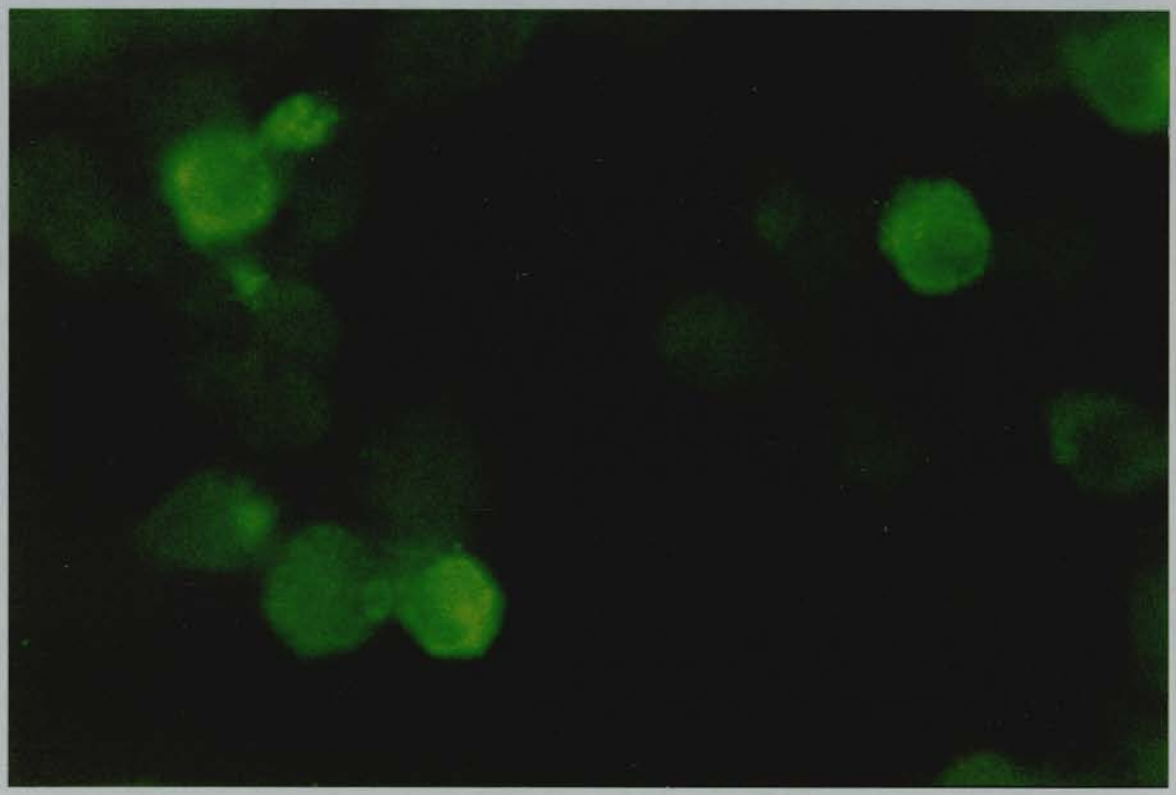
(a) a B-cell line from a CTL+ve child (P110) infected with HIV-*env*, stained with an anti-gp120 antibody

(b) a B-cell line from a CTL-ve child (P69) infected with HIV-*env*, stained with an anti-gp120 antibody

a)



b)



assays were carried out using B cells from both children as targets. These were infected with vaccinia-HIV constructs (HIV-*gag*, HIV-*pol* or the vaccinia-construct control *(vac)*) and uninfected aliquots were used as medium controls *(med)*. Effector cells were from the CTL-ve child (P125). Two different methods were used to stimulate the effector cells: the bulk culture method, and specific stimulation by a pool of HIV-1-peptides (see 4.3.4). The results are presented in figure 4.4. Lysis was seen to the medium control and vaccinia-construct-infected targets, but no HIV-1-specific lysis was seen above these levels. Therefore, the non-detection of CTL in this child (P125), is probably due to an absence of CTLs or non-recognition by the effector cells, rather than deficient target cell function.

4.3.4 CTL ASSAYS USING PEPTIDE STIMULATED EFFECTOR CELLS

In the bulk culture assay, PBMCs were cultured with autologous PHA-activated cells to stimulate the reactivation of memory cells, and the differentiation of precursor CTLs, without inducing apoptosis. However, such a polyclonal stimulation would cause any non-HIV-1-specific memory CTLs to expand as well, which could mask a low number of HIV-1-specific CTLs. Therefore, a method which would be more HIV-1-specific was tried using peptides. Short amino acid sequences in different HIV-1 proteins have been identified as CTL epitopes, specific for particular MHC-class I molecules. Depending on affinity, these peptides would bind to the class-I MHC-molecule, displacing the peptide already bound. This binding mimics the expression of endogenously processed antigen, stimulating the expansion of HIV-1-specific memory CTL populations. Using this method, the numbers of non-HIV-1-specific memory CTL cells would be reduced in culture, thus enabling a lower level of HIV-1-specific CTL responses to be detected.

All the HIV-1-infected children were tissue-typed by a PCR method (Krausa *et al.*, 1995), with the help of P. Krausa, (Institute of Molecular Medicine, Oxford). Peptides of 8-10 amino acids long, corresponding to defined CTL epitopes, were kindly donated by Dr. S. Rowland-Jones (Institute of Molecular Medicine, Oxford). All the available peptides, specific for the HLA-type of each child, were selected and pooled to stimulate PBMC effector cells (as described in 2.6.1.2). These same peptides were used to pulse B-cells (peptide-pulsed targets), only this time the peptides for each MHC class I molecule were pooled separately

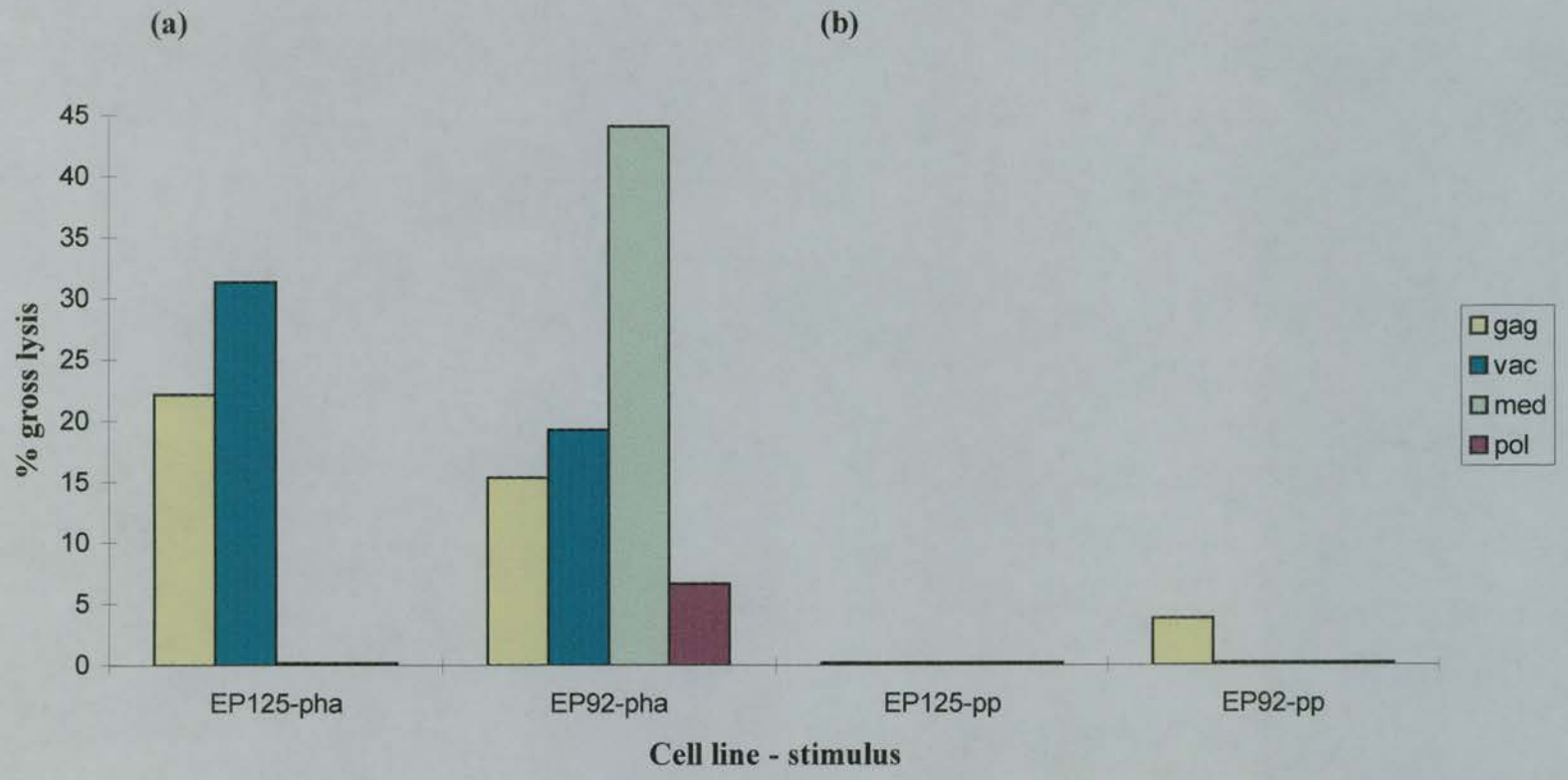
Figure 4.4 **Comparison of target cells**

CTL using effector cells from a CTL-ve child (P125) assayed against autologous target cells (EP125) and those from a CTL+ve child (EP92). Target cells were B cells infected with vaccinia constructs (HIV-*gag*, vac, or HIV-*pol*) and a medium control (med).

(a) effector cells stimulated using the bulk culture method and assayed against target cells, shown as EP125-pha and EP92-pha.

(b) effector cells stimulated with a pool of HIV-1-peptides specific to the tissue type of the child and assayed against targets cells, shown as EP125-pp and EP92-pp.

In both these experiments the % gross lysis is presented, i.e. before subtraction of the lysis from medium and vaccinia controls.


(a)
(b)
45
40
35
30
25
20
15
10
5
0
% gross lysis
gag
vac
med
pol
EP125-pha
EP92-pha
EP125-pp
EP92-pp
Cell line - stimulus

(as described in 2.6.2.2). Vaccinia-HIV constructs were also used as target cells in the CTL assay (as described in 2.6.2.1 and 2.6.3). The HLA-types of the children and the peptides used to stimulate the cells are shown in table 4.4.

4.3.4.1 CTL-ve children

Pools of HIV-1-peptides were used to stimulate effector cells in three CTL-ve children (P69, P125 and P132) to see if an HIV-1-specific CTL response could be elicited. Peptide stimulated effector cells were used against the vaccinia-HIV construct infected targets as well as peptide-pulsed target cells. The results are shown in figure 4.5.

PBMCs from P69 were tested on three occasions. From the first assay, at 126 months of age, CTL activity was detected against pp 2 (corresponding to a *nef* peptide presented on HLA-B18) but not to pp 1 (*pol* and *gag* peptides, presented on HLA-A2). On the second time of testing, at 129 months of age, no CTL activity was seen at all. The third time of testing, at 132 months of age, showed a weak CTL response to pp 1 (*pol* and *gag* peptides, presented on HLA-A2), but not to pp 2 (*nef* peptide, presented on HLA-B18). No CTL activity was detected on any occasion against the vaccinia-HIV construct-infected target cells. The CTL assays were carried out after the same time in culture of the effector cells (13 or 14 days), but the E:T ratios varied (2:1, 44:1 and 11:1, respectively).

PBMCs from P125 were tested once, but no CTL activity was detected to the peptides. However, a weak CTL response was seen against target cells infected with the vaccinia-HIV-*pol* construct.

PBMCs from P132 were tested on two occasions, but no CTL activity was detected to either peptide-pulsed- or vaccinia-HIV construct-infected target cells.

4.3.4.2 CTL+ve children

Pools of HIV-1-peptides were also used to stimulate the PBMCs from four CTL+ve children

Table 4.4 **HLA-type and peptide pools used for stimulation of effector cells**

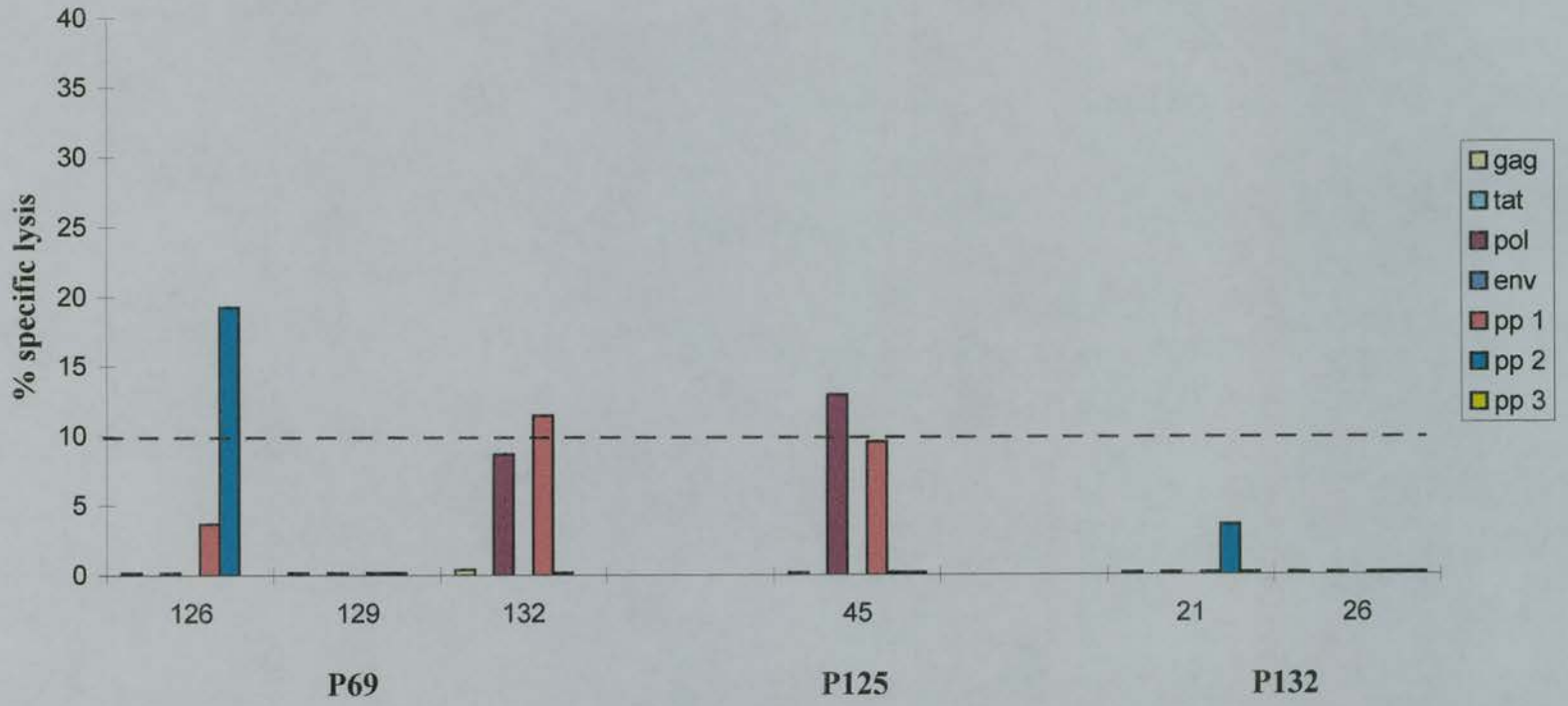
Child	Previous CTL result (bulk culture)	HLA-type	HLA-locus and pooled peptides (pp) used					
			pp 1		pp 2		pp 3	
P69	Negative	A1/A2 B18/B49	A2	*pol* *gag* (p17) *nef*	B18	*nef*		
P125	Negative	A2/A25 B7/B18	A25	*gag* (p24) *gag* (p24)	B7	*env* (gp120) *nef*	B18	*nef*
P132	Negative	A1/A25 B18/B57	A25	*gag* (p24) *gag* (p24)	B18	*nef*	B57	*nef*
P6	HIV-*pol*,-*gag*	A1/A24 B8/B58	A24	*env* (gp41) *nef*	B8	*gag* (p17/p24) *pol* *env* (gp41) *nef*		

P27	HIV-*pol*, -*gag*	A2/26 B44/B57	A2	*pol* *gag* (p17)	B57	*nef*		
P110	HIV-*pol*, -*tat*	A2/A3 B44/B45	A2	*pol* *gag* (p17)	A3	*gag* (p17) *env* (gp120/gp41) *nef*		
P131	HIV-*gag*	A11/A24 B7/B15	A24	*env* (gp41) *nef*	B7	*env* (gp120) *nef* *gag* (p24)		
P140 (HIV-ve)	HIV-*gag*	A1/A3 B7/B35	A3	*gag* (p17) *env* (gp120/gp41) *nef*	B7	*env* (gp120) *nef* *gag* (p24)	B35	*pol* *gag* (p17/p24) *nef*

HIV-1-peptides used for the stimulation of CTL effector cells are shown according to the HLA-type for each child.

Figure 4.5 **CTL activity from peptide stimulated effector cells in CTL-ve children**

The specific lysis of targets by peptide stimulated effector cells from CTL-ve children. Autologous target cells were B cells, either infected with vaccinia-HIV constructs (*gag*, *tat*, *pol* and *env*), or pulsed with peptide (pp 1, pp 2, pp 3, as defined in table 4.4). The children and the ages at which the assays were carried out are shown.


% specific lysis
40
35
30
25
20
15
10
5
0
gag
tat
pol
env
pp 1
pp 2
pp 3
126
129
132
45
21
26
P69
P125
P132

(P6 and P27 from group I, P110 from group II and P131 from group III). These experiments were done separately (i.e. not at the same time as the bulk-culture method), to investigate whether the different method of stimulation caused the specificity of the CTL response to differ from that previously seen using the bulk-culture method. The results are shown in figure 4.6 (a).

No CTL activity was seen for P6 (at 126 months of age), to either peptide-pulsed or vaccinia-HIV-1 construct-infected target cells. This confirmed the observations from the two previous assays using the bulk-culture method (not shown), that this child had lost CTL activity.

For P27, no CTL activity was seen when the effector cells were stimulated with peptides, at 111 months of age, although CTL activity to target cells infected with vaccinia-HIV-*gag* had been seen three months previously, from the bulk culture method. For the ensuing sample from this child, at 114 months, the PBMCs were stimulated using the bulk culture method and assayed against peptide-pulsed and vaccinia-construct-infected target cells. No CTL activity was detected to the peptide-pulsed target cells, but borderline CTL activity to target cells infected with the vaccinia-HIV-*pol* construct was seen (figure 4.6, P27, 114+). However, CTL activity was absent when this was repeated at 117 months (see figure 4.6, P27 117++).

Peptide stimulation of PBMCs from P110, at 64 months of age, elicited a CTL response to target cells pulsed with the peptides from pp 2 *(gag*, *env*, and *nef*, presented on HLA-A3). CTL activity was also seen to target cells infected with the vaccinia-HIV-*gag* construct, having previously shown no CTL activity to this target using the bulk-culture method. Interleukin 7 (IL-7) was added to the peptide-stimulated cultures (described 2.6.1.2) on two subsequent occasions, at 69 and 70 months, as this has been reported to enhance antigen-specific CTL effector cell production (Carini and Essex, 1994). On the first time of testing with IL-7 added (figure 4.6, P110, 69§), no CTL activity to peptide-pulsed target cells was detected, but CTL activity to target cells infected with the vaccinia-HIV-*pol* and -*env* constructs was seen. On the second time of testing with IL-7 added, CTL activity to pp 1 (*pol* and *gag* peptides, presented on HLA-A2) was detected, as well as responses to target cells infected with the vaccinia-HIV-*gag*, -*pol* and -*env* constructs (figure 4.6(a), P110, 70§). There was a slight difference in culture time of the effector cells between these two occasions

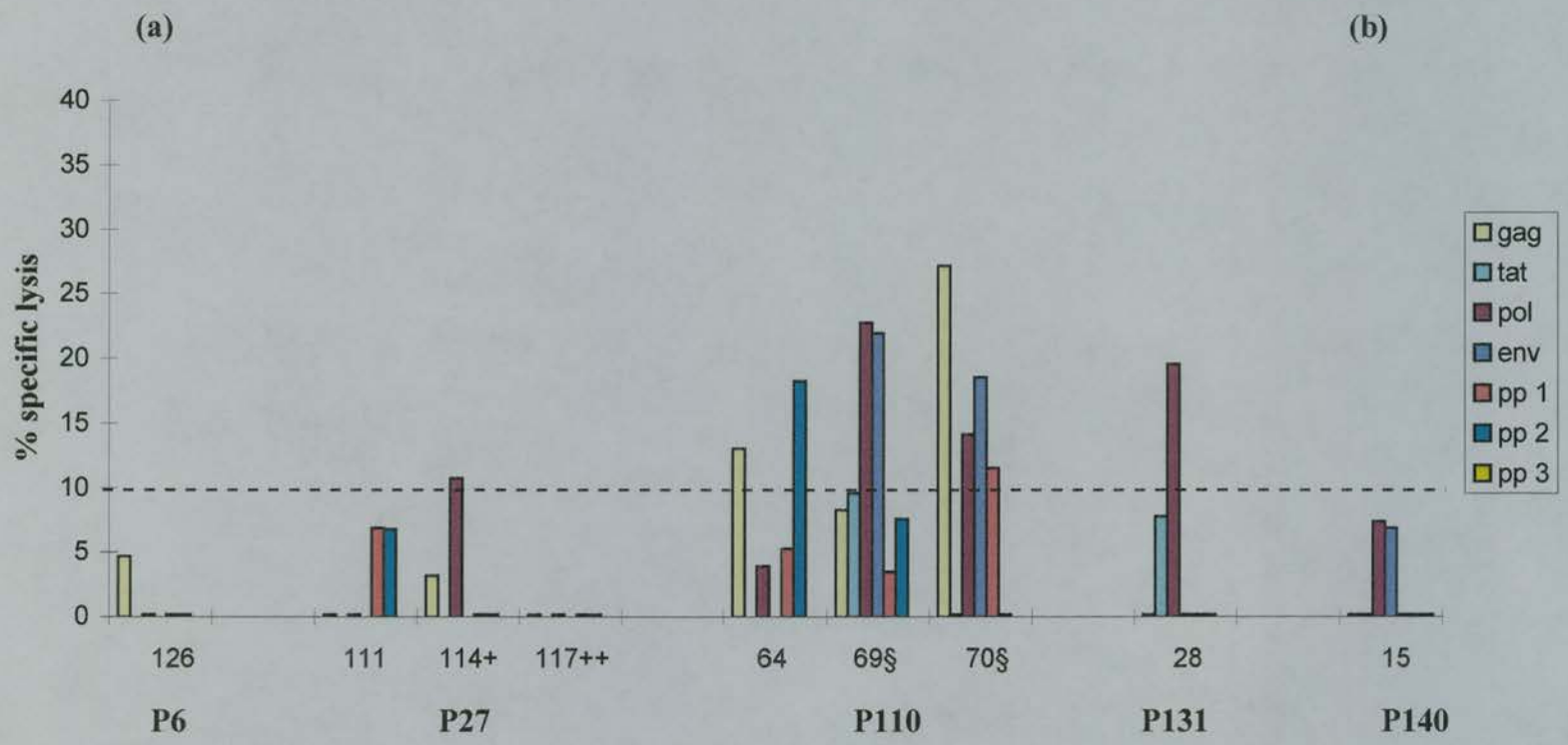
Figure 4.6 **Peptide stimulation in CTL+ve children**

The specific lysis, by peptide-stimulated effector cells, of autologous target cells: B cells, either infected with vaccinia-HIV constructs (*gag, tat, pol* and *env*), or pulsed with peptide (pp 1, pp 2, pp 3, as defined in table 4.4). The ages (months) at which the CTL assays were carried out for each child are shown.

(a) from CTL+ve children
(b) an HIV-1-uninfected child

\+ denotes stimulation of the childs' (P27) effector cells by bulk culture method
++ denotes a repeat of the above experiment at a later time point

§ denotes addition of IL-7 to the culture of effector cells


(a)
(b)
% specific lysis
40
35
30
25
20
15
10
5
0
gag
tat
pol
env
pp 1
pp 2
pp 3
126
111
114+
117++
64
69§
70§
28
15
P6
P27
P110
P131
P140

of testing (19 days compared to 14 days, respectively). However, for the peptide-stimulated CTLs no activity to the target cells infected with the vaccinia-HIV-*tat* construct was seen, this specificity having been seen previously using the bulk-culture method.

No CTL activity to peptide-pulsed target cells was seen from P131. However, a response to target cells infected with the vaccinia-HIV-*pol* construct was seen, this child not having previously shown any responses to this target.

4.3.4.3 HIV-1-uninfected child

In a 15 month uninfected child (P140), the peptide assay was used to try to stimulate an HIV-1-specific CTL response. This child had been consistently CTL-ve, apart from one response to HIV-*gag* at 9 months old. The results are shown in fig 4.6 (b). No CTL activity was seen.

4.3.5 CD8 SUBPOPULATIONS IN EFFECTOR CELLS

Various studies in HIV-1-infected adults have described the phenotype of the CTL effector cells as CD8 cells expressing combinations of HLA-DR, CD38, CD45RO or CD11a (Ho *et al.*, 1993; van Baalen *et al.*, 1993; Watret *et al.*, 1993). In whole blood, the proportion of CD8 cells expressing these antigens is markedly increased in HIV-1-infected children (chapter 3). The marker expression on whole blood lymphocytes and on effector cells, from HIV-1-infected children, was investigated to ascertain whether a specific phenotype on peripheral blood lymphocytes or on effector cells was associated with CTL activity.

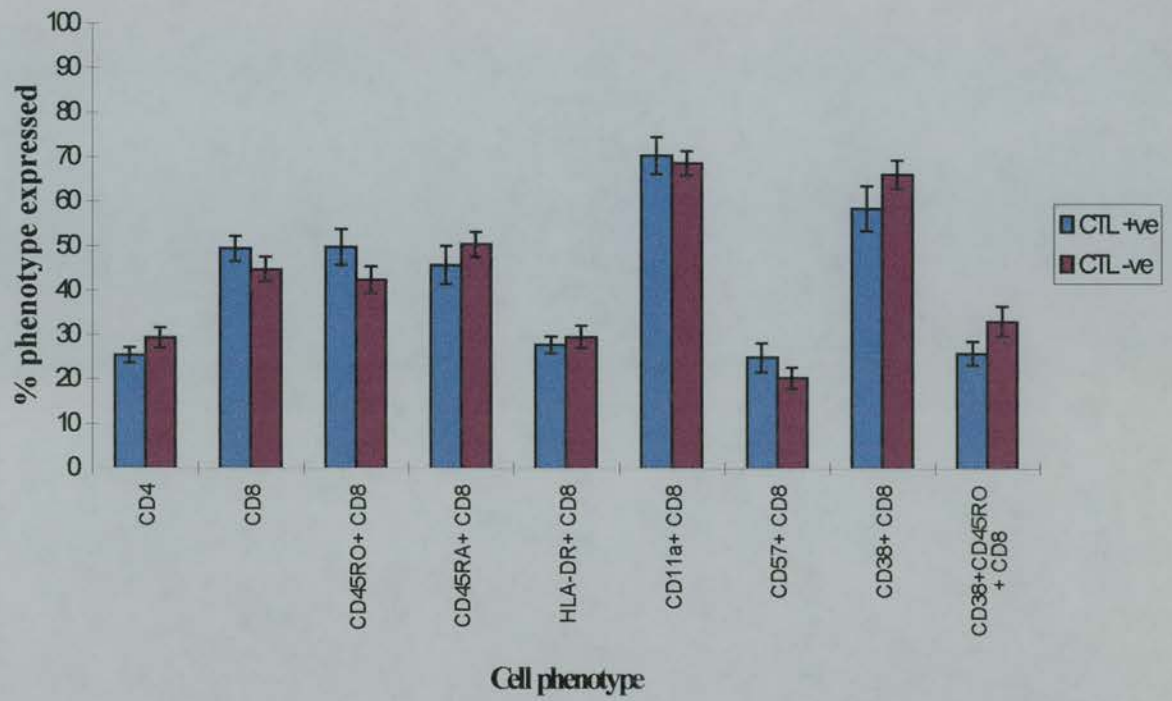
The surface markers of CD8 cells in whole blood, and in the effector cell population used in the CTL assay, were measured (as described in 2.3.1). The levels of marker expression were compared between the cultures which were CTL+ve (n = 16) and those which were CTL-ve (n = 25), from HIV-1-infected children. The results for whole blood are shown in figure 4.7 (a) and for effector cells in figure 4.7 (b). Pooled two-sample t-tests were used to compare the levels of marker expression. In whole blood, no significant differences were

Figure 4.7 **Phenotypes of cells from CTL+ve and CTL-ve cultures**

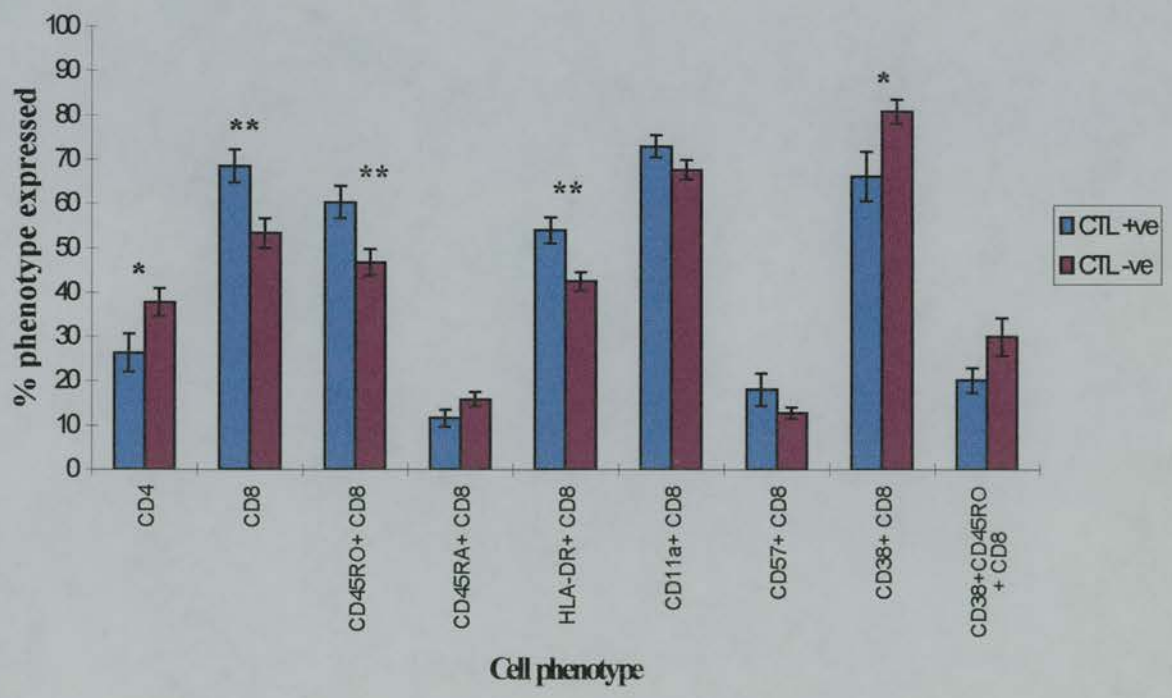
The percentage marker expression (mean ± sem) for the CTL+ve and CTL-ve cultures are shown (*y*-axis). The markers are defined on the *x*-axis.

(a) in whole blood (i.e. cells put into culture).
(b) on effector cells used in the CTL assay.

(a)


% phenotype expressed
100
90
80
70
60
50
40
30
20
10
0
CD4
CD8
CD45RO+ CD8
CD45RA+ CD8
HLA-DR+ CD8
CD11a+ CD8
CD57+ CD8
CD38+ CD8
CD38+CD45RO + CD8
CTL+ve
CTL-ve
Cell phenotype

(b)


% phenotype expressed
100
90
80
70
60
50
40
30
20
10
0
*
**
**
**
*
CD4
CD8
CD45RO+ CD8
CD45RA+ CD8
HLA-DR+ CD8
CD11a+ CD8
CD57+ CD8
CD38+ CD8
CD38+CD45RO + CD8
CTL+ve
CTL-ve
Cell phenotype

found in marker expression between the two groups. However, in the effector cell populations a significant increase in CD8% (** $p<0.01$) and significant decrease in CD4% (* $p<0.05$) was seen in CTL+ve compared to CTL-ve cultures. The proportion of CD8 cells expressing CD45RO and HLA-DR was significantly increased (** $p<0.01$ respectively), while the proportion of CD8 cells expressing CD38 was significantly decreased (* $p<0.05$) on the CTL+ve effector cells, compared to the CTL-ve effector cells.

4.3.6 CYTOKINE PRODUCTION FROM CTL CULTURES

CTL activity has been reported to be associated with the production of Th-1 cytokines from CD4 cells (Kemeny *et al.*, 1994). It has been suggested that the loss of CTL activity is related to a decrease in the production of Th-1 cytokines from CD4 cells *in vivo* (Clerici and Shearer, 1993). Therefore the relationship between the production of Th-1 or Th-2 cytokines and the presence of CTL effector cells was investigated.

CTL culture supernatants from HIV-1-infected and EU children taken at day 7 were analysed for Th-1 cytokines (IL-2 and IFN-γ) and Th-2 cytokines (IL-4 and IL-6), using commercial kits (Genzyme) according to the manufacturers instructions. Results were obtained for IL-6, but not for the other cytokines. The IL-6 levels from CTL+ve and CTL-ve cultures are shown in figure 4.8 (a) and were compared by a Mann-Whitney statistical test. There was no significant difference in the IL-6 level between the two groups (medians = 66.5 pg/ml and 156 pg/ml, respectively, $p = 0.198$). The levels of IL-6 in cultures from EU and HIV+ve children are shown in figure 4.8 (b). Comparison of the groups by a Mann-Whitney statistical test, showed that the supernatants of CTL cultures from HIV+ve children had significantly less IL-6 than those from EU children (medians = 61 pg/ml and 358 pg/ml respectively, $p < 0.01$).

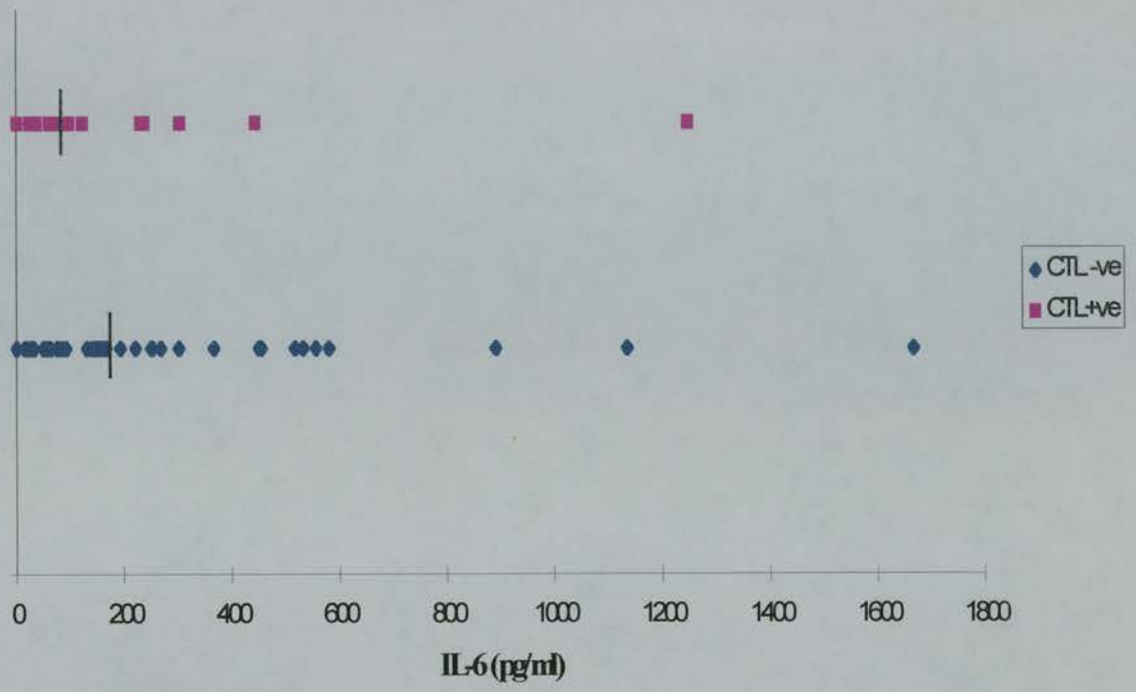
4.4 DISCUSSION

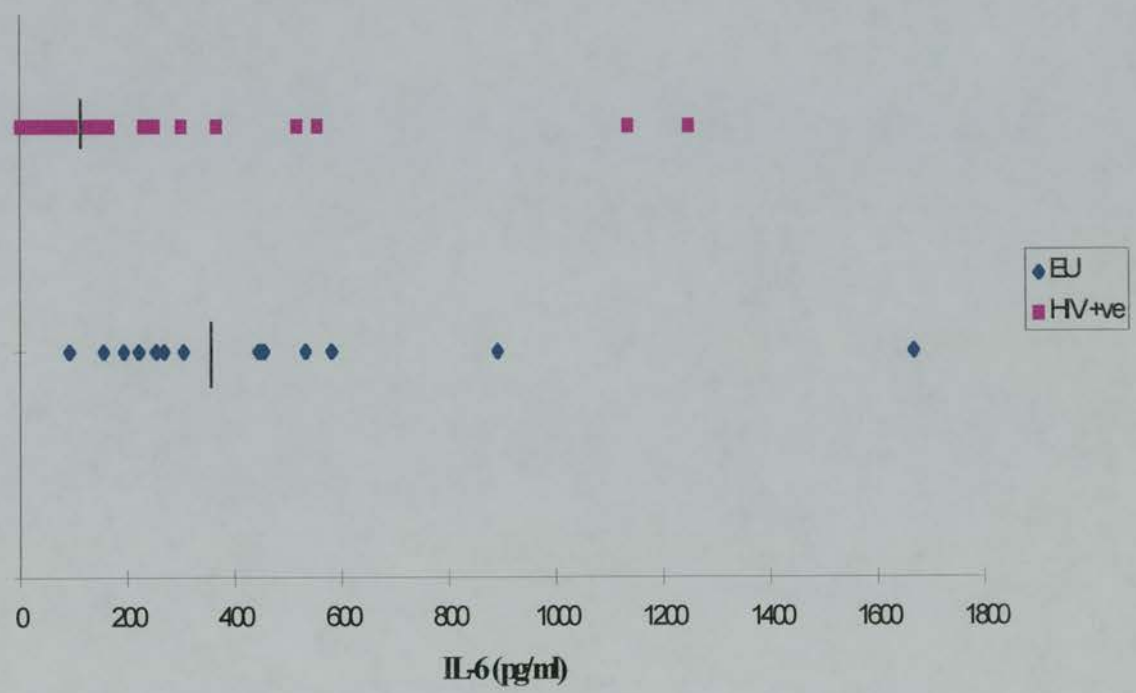
HIV-1-specific CTL activity was studied in infected children to investigate how these

Figure 4.8 **IL-6 levels in supernatants**

The levels of IL-6 (pg/ml) detected in culture supernatants are shown. The levels are presented horizontally, and the vertical bars denote the median level in each group.

(a) CTL+ve and CTL-ve cultures
(b) HIV+ve and EU children


CTL-ve
CTL+ve
0
200
400
600
800
1000
1200
1400
1600
1800
IL-6 (pg/ml)


EU
HV+ve
0
200
400
600
800
1000
1200
1400
1600
1800
IL-6 (pg/ml)

responses changed longitudinally. The presence and specificity of the CTL response was studied in relation to methodological differences and the clinical progression of the children. The effect of stimulation with specific HIV-1-peptides was also investigated. In addition, the phenotypes of the effector cells and the production of cytokines in the CTL cultures were analysed. CTL responses were also studied in 20 EU children, to see whether HIV-1-specific CTL responses developed after *in utero* or *intrapartum* exposure to HIV-1.

4.4.1 SPECIFICITY OF CTL RESPONSE FROM BULK-CULTURED EFFECTOR CELLS

The main method used in this study was the bulk culture method to allow the expansion and differentiation of memory CTL cells. The specificity of these secondary (restimulated) CTL responses varied, both within and between children. This was not due to methodological variation, such as the day of assay or the E:T ratio; these parameters were all comparable to those used in other studies of children (Luzuriaga *et al.*, 1995; Buseyne *et al.*, 1993; Cheynier *et al.*, 1992; Luzuriaga *et al.*, 1991; McFarland *et al.*, 1994). Others have found in children, that the primary CTL response (the CTL activity detected from freshly isolated, unstimulated PBMCs) and the secondary CTL response from the same blood sample, do not show the same specificity of target recognition (Buseyne *et al.*, 1993; Cheynier *et al.*, 1992; McFarland *et al.*, 1994). Therefore, the differences in specificity detected here are probably a reflection of the expansion of different cell populations during culture; some populations may give detectable killing before others, as different cell populations grow at different rates.

The most abundant proteins in the HIV-1 virion are those encoded by *env* and *gag*. Therefore, it was expected that CTL responses to HIV-*gag* and HIV-*env* would predominate. However, the most frequently recognised construct was HIV-*pol*, which was detected in all but one, of the CTL+ve infected children. This indicates that *pol* contains major CTL epitopes which are conserved across different HIV-1 variants in children, as has been shown in adults (Walker *et al.*, 1988; Lamhamedi-Cherradi *et al.*, 1992; Lamhamedi-Cherradi *et al.*, 1995). CTL activity to *pol* has rarely been reported in children (McFarland *et al.*, 1994; Buseyne *et al.*, 1993). In one study, this could be because the CTL activity was measured early, on day 7 of culture (McFarland *et al.*, 1994). In the children studied here, and from

studies in adults (Lamhamedi-Cherradi *et al.*, 1992; Lamhamedi-Cherradi *et al.*, 1995), the CTL activity was measured later, giving more time for the expansion of *pol*-specific effector cells.

CTL activity to HIV-*gag* was seen in five infected children, with a borderline response in another child. CTL activity to HIV-*env* was seen in two infected children, and one of these was a borderline response. This contrasts with published studies in both adults and children, which have reported high frequencies of CTL activity to both *gag* (Ho *et al.*, 1993; Walker *et al.*, 1987; Koup *et al.*, 1989; Buseyne *et al.*, 1993; McFarland *et al.*, 1994) and *env* (Hoffenbach *et al.*, 1989; Koup *et al.*, 1989; Luzuriaga *et al.*, 1995; Cheynier *et al.*, 1992). Sequence variation between the virus used in the construct and that found in the children, could be such that few cross-reactive epitopes are recognised by the effector cells, and may explain the lower proportion of HIV-*gag*-specific responses seen in these children, compared with that found in other cohorts (Buseyne *et al.*, 1993; Cheynier *et al.*, 1992; McFarland *et al.*, 1994). To verify this, the viral sequences in the children would have to be compared with the construct sequence.

The vaccinia-HIV-*gag* construct used in this study encoded the p55 *gag*-precursor protein and cells infected with this construct expressed p55 on the cell surface (Nixon *et al.*, 1988; Walker *et al.*, 1987). Initial studies reported that this p55 precursor was not further processed to produce mature HIV-1-*gag* proteins, p24, p17 and p15 (Nixon *et al.*, 1988; Walker *et al.*, 1987), but has been successfully used in CTL assays in adults (Nixon *et al.*, 1988). Whether this lack of further processing of the p55 protein contributed to the non-recognition of the target cells by the effector cells from children, is not clear, but could be investigated by comparison with another vaccinia-HIV-*gag* construct (Gowda *et al.*, 1989).

Differences in viral populations between individuals could affect CTL recognition, particularly as there is more variation in *env* than in other parts of the HIV-1 virion (Simmonds *et al.*, 1991). A study by Robertson *et al.*, (1994) compared *env*-specific cytotoxic responses (CTL and ADCC) to HIV-1_{MN} and HIV-1_{LAI} in adults, and found some degree of cross-reactivity between strains. In the infected children studied here, the frequency of responses to HIV-*env* was lower than that found in other cohorts. The reasons for this require further investigation.

CTL activity to HIV-*tat* has not been previously described in children. This response was unexpected as, in adults, *tat*-specific CTLs have been detected, but at such a low frequency that it was concluded that HIV-*tat* was not a major CTL epitope (Lamhamedi-Cherradi *et al.*, 1992). These results highlight the differences seen from studying CTL responses in children as opposed to adults. The MHC restriction and possible epitope sequences specific to this CTL response require further investigation.

4.4.2 CTL ACTIVITY AND CLINICAL PROGRESSION

The loss of CTL activity in progressors (Rinaldo *et al.*, 1995; Klein *et al.*, 1995; Ho *et al.*, 1993) and the relation of CTL to the control of primary viraemia (Borrow *et al.*, 1994; Lamhamedi-Cherradi *et al.*, 1995; Koup *et al.*, 1994) have been well documented and indicate that CTL activity is beneficial in the HIV-1-infected individual. However the detection of CTLs from lung (Plata *et al.*, 1987), CSF (Sethi *et al.*, 1988) and splenic tissue (Cheynier *et al.*, 1994) of patients with AIDS suggest that these may contribute to the tissue degradation seen in HIV-1 infection (Pantaleo *et al.*, 1993; Embretson *et al.*, 1993). The ability of CTLs to kill uninfected, activated CD4 cells *in vitro* has been described (Bienzle *et al.*, 1996) and is associated with CD4 depletion *in vivo* (Grant *et al.*, 1994). It has also been reported that, *in vitro*, CD8 cells can become infected with HIV-1 in the process of killing HIV-1-infected target cells. These CD8 cells in turn, were able to infect CD4 cells, but were not recognised as being infected themselves and therefore were not removed by any mechanism (de Maria *et al.*, 1994b). Furthermore, HIV-1-infected CD8 cells have been isolated from the lung (Semenzato *et al.*, 1995) and peripheral blood (Livingstone *et al.*, 1996) of infected patients, suggesting that CTLs could spread virus to different organs.

In the children studied here, all the CD4% groups contained CTL+ve and CTL-ve children, but only one of the CTL-ve children the child (from group II) has progressed clinically. The CTL+ve children who progressed, all showed CTL responses to HIV-*tat* from the bulk-culture method. This was unexpected, as Torpey *et al.*, (1993) postulated that CTL activity to HIV-*tat* may be beneficial, as an attempt on the part of the immune system, to destroy HIV-1-infected cells before the production of mature virions. However, these results indicate that CTL activity to HIV-*tat* does not prevent disease progression. It is possible that *in vitro*

detection of *tat*-specific CTL may indicate an increased level of HIV-1-replication *in vivo*, therefore reflecting an increased likelihood of disease progression.

It could be postulated that CTL activity is beneficial up to a certain point in the natural history of disease. In the children studied here, three lost CTL activity before the CD4% showed any substantial decline, yet have remained clinically stable. Conversely, two other children, continued to show CTL activity once the CD4% decline had started. These two, and one other child who also showed vigorous CTL activity, have developed SI variants of HIV-1, which have been claimed to be predictive for the onset of AIDS (Schuitemaker *et al.*, 1992). If these SI variants emerged as an attempt by the virus to evade recognition by CTLs (Phillips *et al.*, 1991), this suggests that CTL activity may contribute to the selective pressure leading to the emergence of a more virulent viral strain.

4.4.3 NON-DETECTION OF CTL ACTIVITY

Four children were consistently CTL-ve using the bulk-culture method and the loss of CTL activity was seen in five children. The expression of the HIV-gene products on the surface of B cells infected with the vaccinia-HIV constructs, were comparable in two children, as found elsewhere (Gowda *et al.*, 1989). Therefore, the difference in CTL recognition must be at the effector cell level. Joly *et al.*, (1989) reported that a soluble factor released by CD57+ CD8 cells could suppress CTL recognition of HIV-1-infected cells by an unknown mechanism, and that this cell population expands with disease progression. However, in these children, there was no increase in the proportion of CD57+ CD8 cells outwith normal levels in whole blood (see chapter 3), nor in the effector cell population. Effros *et al.*, (1996) reported that, in HIV-1-infected individuals, a population of CD8 cells which did not express CD28, were found to have become senescent, possibly due to persistent viral stimulation. It was suggested that these senescent cells may be immunologically exhausted. It is possible that in the children who lost CTL activity, that exhaustion of CTL clones had occurred.

A study by Wolinsky *et al.* (1996), suggested that a low level of diversity of viral sequence, within an individual, is related to the loss or non-appearance of CTL responses and subsequent progression to AIDS. This was seen in two children (P125 and P132), who were

born to the same mother. Viral sequences from the mother and the two children indicated that these virus strains were more closely related than those found in the wider Edinburgh IVDU cohort (~3% divergence as opposed to ~5% divergence, respectively). The intra-child viral diversity was higher in P125 than that found in P132, (~2% as opposed to ~0.5%, C.M. Wade, J Virol 1997, submitted). One child (P125) showed CTL activity, but only when the cells were stimulated with HIV-1-peptides. The other child showed no CTL activity, thus supporting the view that higher viral diversity and CTL activity are related. These children differ from the individuals in Wolinsky's study, in that neither has progressed clinically.

As one of the CTL-ve children (P116) was born in central Africa, it is likely this child was infected with a non-subtype B virus, but could be infected with a subtype A or D virus, these being more prevalent (Myers, 1994). The subtype identification of this virus has not been performed due to a lack of material. Differences in viral sequence at the CTL epitopes may explain the non-recognition of B-subtype CTL targets in this child. A few cross-reactive CTL epitopes between HIV-1 subtypes have been defined, corresponding to one of the childs' HLA loci, B14 (Korber *et al.*, 1995), but not the other loci, which could limit the generation of a CTL response. This child was the only CTL-ve child to progress to AIDS, and has since died.

A few studies in HIV-1-infected adults have associated the non-detection of CTL activity with a low virus burden (Ferbas *et al.*, 1995; Dalod *et al.*, 1996). It was suggested that the low virus load would be reflected by a lower proportion of cells expressing HIV-1-antigens, which would be insufficient to stimulate a CD8 cell-mediated response. One child was consistently CTL-ve using the bulk-culture method. Repeated attempts to culture virus from PBMCs from this child have been unsuccessful. Plasma HIV-RNA, but not HIV-antigen (p24), has been detected (Dr. M Arnott, personal communication), but there is also some evidence that the plasma virus may have been neutralised by antibody (data not shown). In the case described by Dalod *et al.*, (1996) CTL activity was detected once the patient had detectable virus levels. In the child in this study, CTL responses were detectable when the cells were specifically stimulated using HLA-specific peptides, indicating that memory HIV-1-specific CTLs do exist, but at a low level. If the viral levels increase in this child, a further expansion of these CTLs could occur and CTL activity may then become detectable using the bulk-culture method. As was found in the adult studies (Ferbas *et al.*, 1995; Dalod *et al.*,

1996), this child has remained clinically stable, despite the lack of CTL activity.

4.4.4 SPECIFICITY OF CTL RESPONSE FROM PEPTIDE STIMULATED EFFECTOR CELLS

Stimulation of effector cells by HIV-1-peptides in the CTL+ve children showed a change in the specificity of response, from that observed previously by the bulk-culture method. Ahearne *et al.*, (1995) showed an increased CTL response to the target cells which expressed the same proteins as those used for effector cell stimulation (Ahearne *et al.*, 1995). Therefore, an increased response to peptide-pulsed target cells was expected. However, Buseyne *et al.*, (1994) found that peptide-pulsed B cells were not lysed by the effector cells as frequently as target cells infected with a vaccinia-HIV construct expressing the same sequence. This was borne out in one child, where the vaccinia-HIV construct-infected target cells were preferentially recognised over peptide-pulsed target cells. More interestingly, CTL responses were seen, in two children, to the vaccinia-HIV-*pol* construct, when peptides used to stimulate the cells in those cultures had included those from the *gag*, *env* and *nef* regions, but not the *pol* region of HIV-1. It is possible that *pol*-specific CTL clones were stimulated by endogenous HIV-1 expression during activation of the cells in culture. However, these results indicate that the method of stimulation of the effector cells affect the specificity of the response, indicating that studies using different methods may not be comparable.

The addition of IL-7 to the peptide-stimulated cultures of one child, enhanced the CTL response to the vaccinia-HIV construct-infected target cells. It has been suggested that addition of IL-7 allows the CTL precursors to mature faster than would occur without IL-7 (Carini and Essex, 1994; Ferrari *et al.*, 1995). This could explain the difference in specificity between these experiments, as the cells were cultured for different lengths of time.

4.4.5 CTL ACTIVITY, LYMPHOCYTE PHENOTYPES AND CYTOKINES

The phenotypes of blood and effector cells for all the CTL+ve and CTL-ve occasions in the infected children were compared. The effector cells from the CTL+ve occasions contained

an increased proportion of phenotypically activated and memory CD8 cells, supporting the findings from studies in adults (Ho *et al.*, 1993; Watret *et al.*, 1993; van Baalen *et al.*, 1993). Non-detection of CTL activity was accompanied by a lack of expansion of HLA-DR+ CD8 cells, confirming other studies (Watret *et al.*, 1993; Pantaleo *et al.*, 1990b). This also fits with the observations of Mahalingham *et al.*, (1995), who showed, in HIV-1-infected adults, that although cells may express different activation markers, only those which express HLA-DR actively proliferate. The decreased CD38 expression on the CTL+ve effector cells was surprising. In adults, circulating HIV-1-specific CTLs have been shown to be within the CD38+ HLA-DR+ CD8 cell population (Ho *et al.*, 1993). However, the expression of CD38 on CD8 cells in whole blood, is different in children from that in adults (see chapter 3) and reflects the immaturity, rather than activation, of these cells. Therefore, the decrease in CD38 expression on the CTL+ve effector cells may be an indication of the maturation and differentiation of these cells in culture. Alternatively, the increased expression of CD38 on the CTL-ve effector cells may reflect an increased occurrence of circulating CD38+ CD8 cells, not expressing HLA-DR, which do not have CTL effector function *in vivo* (Ho *et al.*, 1993; Giorgi *et al.*, 1994). Studies in adults also showed an increased expression of CD11a+ (S6F1) CD8 cells within the effector population (Sohen *et al.*, 1990; Watret *et al.*, 1993), which was not seen here. It is possible that, as the expression of this marker is very high in the whole blood of children (see chapter 3), there is no further expression possible in culture.

CTL differentiation has been associated with the production of cytokines produced by the CD4 cells during culture, with Th-1 cytokines enhancing and Th-2 cytokines inhibiting cell mediated immunity (Clerici and Shearer, 1993). A significantly higher amount of IL-6 was produced in the cultures from EU children, regardless of whether they were CTL+ve or not. The serum levels of IL-6 have been shown to be raised in HIV-1-infected children (Rautonen *et al.*, 1991), and therefore it was expected that the cultures from the infected children would produce more IL-6. Although IL-6 is a Th-2 cytokine, it is an inducer of T cell growth (Kuhweide *et al.*, 1990), and so would be produced and used, during CTL effector growth in culture. As the cytokine levels were measured after day 7 in culture, the decreased level of IL-6 in the cultures from HIV-1-infected children may indicate increased usage of this cytokine, possibly by increased cell turnover, rather than lower production, as a higher proportion of cells from infected children expressed activation markers (see chapter 3).

4.4.6 CTL ACTIVITY IN EU CHILDREN

Anti-HIV-1 CTL activity was also detected in EU children. Unlike those found in the infected children, most of the responses were to HIV-*env*, possibly reflecting the abundance of this protein in the virion. Responses to HIV-1 proteins (*gag*, *env*, *pol* and *nef*) have been detected in other studies of children born to HIV-1-infected mothers (Rowland-Jones *et al.*, 1993; Cheynier *et al.*, 1992; de Maria *et al.*, 1994a) and also other exposed uninfected individuals, such as health care workers (Pinto *et al.*, 1995), prostitutes (Rowland-Jones *et al.*, 1995) and the uninfected sexual partners in HIV-1-discordant couples (Langlade-Demoyen *et al.*, 1994). It was suggested that the CTL responses within all these individuals, were induced from exposure to the virus and had a protective effect in the prevention of HIV-1 infection.

Previous results had identified two exposed uninfected children with CTL activity coinciding with raised CD8 subpopulations in whole blood. Repetition of these assays, at a later date, showed no CTL activity and CD8 subpopulations within normal ranges. Therefore, it was suggested that these children had cleared virus and become uninfected (Rowland-Jones *et al.*, 1993; Aldhous *et al.*, 1994b, appendix). The increased CD8 subpopulations indicated that the cells involved in the immune response were activated. Similar observations have been made in other viral infections, which were cleared by CTL activity and accompanied by a transient increase in CD8 subpopulations (Miyawaki *et al.*, 1991; Watret *et al.*, 1993; Byrne and Oldstone, 1984). Direct evidence that children can clear virus has come from Bryson *et al.*, (1995) who described a child infected with HIV-1, by being positive for virus culture and PCR, who then became negative for PCR at a later date. No assays of immune function were performed in this child, so the mechanisms by which clearance of HIV-1 took place, are not known. The clearance of HIV-1 in children may be facilitated by a low viral load, or a defective virus, together with an effective immune response. In the EU children studied here, the lymphocyte surface markers in whole blood were all within the normal ranges shown in chapter 3. Therefore, it is unlikely that these children were in the process of actively clearing virus; rather that the CTL responses were from the restimulation of circulating, memory CTLs. To induce a CTL response, CD8+ CTL precursor cells have to be stimulated by another cell, which has endogenously processed virus and presented it on the cell surface (Paul, 1993). This suggests that CTL responses are a result of exposure to, and possible

clearance of, replicating virus, although CTL responses in mice have been elicited from immunisation with purified HIV-1 proteins (Takahashi *et al.*, 1990).

In summary, this longitudinal study followed the CTL activity to different HIV-1-proteins in a cohort of HIV-1-infected children. Different specificities of target recognition by CTLs were seen, although all but one, of the CTL+ve children recognised HIV-*pol*. There was no association between the presence of CTL activity and disease progression. However, a possible association between the specificity of responses and disease progression was seen, with HIV-*tat* rather than HIV-*gag*, being recognised in the children who progressed clinically. This would need to be confirmed in other cohorts of children. The method of stimulation of CTLs has an effect on the specificity of response of the effector cells: stimulation of the PBMCs in culture with HIV-1-peptides allowed a more HIV-1-specific cell population to grow. The CTL response was found to be associated with the expansion of a population of phenotypically activated, memory CD8 cells. However there was no phenotype in whole blood which could predict the presence of CTLs in the child. CTL responses were not associated with the levels of IL-6 in culture.

Chapter 5 LYMPHOCYTE PROLIFERATION

5.1 INTRODUCTION

5.2 METHOD

5.3 RESULTS

5.2.1 **Lymphocyte proliferation to PHA**

5.3.2 **Lymphocyte proliferation to tetanus toxoid**

Relationship of TT responses to age in EU children

5.3.3 **Lymphocyte proliferation to recombinant HIV-1 cocktail**

5.3.4 **Relationship between proliferative responses and PBMC subpopulation**

5.3.5 **Responses in HIV-1-infected children**

5.3 DISCUSSION

5.1 INTRODUCTION

The phenomenon of CD4 cell loss in HIV-1 infection has been well documented: indeed it was part of the early descriptions of HIV-1 infection (Gallo *et al.*, 1984; Barré-Sinoussi *et al.*, 1983). The CD4 cell plays a central role in the induction of an immune response, through recognition of foreign antigen and secretion of cytokines (see 1.2.2.3.1). In HIV-1 infection, it is possible that functional defects of CD4 cells may occur prior to cell death, which would affect its ability to regulate the other aspects of the immune repertoire (Romagnani, 1992; Kos and Engleman, 1996; Mosmann and Sad, 1996; Kemeny *et al.*, 1994).

Previous studies in HIV-1-infected adults have used lymphocyte proliferation to assess the biological responses of T cells to various stimuli, including HIV-1. In these adult studies, the CD4 cell depletion was preceded by a loss of T cell function, as shown by an inability of lymphocytes to proliferate to antigens and mitogens (Clerici *et al.*, 1989; Klimas *et al.*, 1991; Ranki *et al.*, 1989). This reduction in proliferative responses was found to occur in a specific pattern: the loss of responses to HIV-1 was seen first, then recall antigens followed by allogenic antigens and finally by a loss of responses to mitogens (Clerici *et al.*, 1989; Ranki *et al.*, 1989; Bentin *et al.*, 1989).

Only a few studies have examined the T cell proliferative responses in HIV-1-infected children. A decreased T cell responsiveness and decreased CD4 cell count have been associated with an increased likelihood of onset of an AIDS-defining illness (Chirmule *et al.*, 1995). A similar pattern of loss of proliferative response to that seen in adults, has also been described (Johnson *et al.*, 1991).

Lymphocyte proliferation was studied in the Edinburgh cohort of children, to ascertain whether the responses were associated with clinical progression in the infected children, and also, if any loss of response was related to changes in specific lymphocyte populations. Due to the small amount of material available from these children, the range of stimuli used was limited to one mitogen, one recall antigen and a combination of recombinant HIV-1 proteins (rHIV cocktail). Phytohaemagglutinin (PHA) was chosen as the mitogen, as this is a polyclonal T cell mitogen acting through the T cell receptor and CD2 (Coligan *et al.*, 1996).

Tetanus toxoid (TT) was chosen as the recall antigen, as the children in the cohort were immunised against this at 2, 3 and 4 months, with a booster between 4 and 5 years of age (Department of Health, 1992). The recombinant HIV-1 proteins used in the cocktail were: gp120 from two different strains of HIV-1 (HIV-1_{SF2} and HIV-1_{MN}), p24 (from HIV-1_{LAI}), the p66 protein of reverse transcriptase (RT) (from HIV-1_{LAV}), nef and tat (both from HIV-1_{IIIB}).

There is some evidence that lymphoproliferative responses to HIV-1 also occur in HIV-1-exposed but uninfected (EU) individuals. These have been interpreted as evidence of specific immune responses to HIV-1, which may be involved in the prevention of HIV-1 infection in these individuals (Kelker *et al.*, 1992; Borkowsky *et al.*, 1990; Clerici *et al.*, 1994). The proliferative responses in EU children were also studied, to see whether they showed any responses to HIV-1, and if so, for how long these responses remained. As controls, blood samples were obtained from healthy HIV-1-seronegative plasma donors (normal donors) and from the umbilical cords of healthy newborn babies (cord bloods), with no risk factors for HIV-1 infection.

5.2 METHODS

Proliferative responses were assayed using freshly separated PBMCs incubated with either antigen or mitogen in quadruplicate, at a final concentration of $1x10^5$ cells/well, in a 96 well U-bottomed plate. To ascertain the sensitivity of the responses, the mitogen or antigens were added at two concentrations, one within and one below the recommended optimum range (between 1 and 10 µg/ml, Coligan *et al.*, 1996). Control wells contained cells with medium alone. The cells were incubated for 6 days, labelled with 1µCi ^{3}H-thymidine for a further 18 hours, harvested onto filtermats and counted (as described in 2.10).

5.3 RESULTS

5.2.1 LYMPHOCYTE PROLIFERATION TO PHA

The proliferative responses to PHA at concentrations of 2.5µg/ml and 0.5µg/ml are presented in figure 5.1. These are presented as the stimulation index (geometric mean cpm of stimulant / geometric mean cpm of medium), as this corrects for the range of background proliferation observed between individuals. The results for both mitogen concentrations are shown for the cord bloods, normal donors, EU and HIV-1-infected children. The infected children were further divided into groups according to age and CD4% profile as previously described (see chapter 3).

Comparison of the responses at the higher (optimal) concentration, showed that the normal donors had significantly higher stimulation index values than the other three groups (analysis of variance on the log stimulation index values, $p<0.01$). None of these responses were at stimulation index values of less than 100. Of thirteen assays in cord bloods, only one had a stimulation index of less than 3 (i.e. a negative response). There was no significant difference in the stimulation index values when comparing the HIV-1-infected and EU children (by two-sample t-tests on log values). Three children (two EU and one HIV-1-infected) had stimulation index values of less than 3.

At the lower (sub-optimal) concentration, a one way analysis of variance on the log values showed that the normal donors still had significantly higher stimulation index values than the other three groups ($p<0.05$). Of thirteen cord bloods, two had a stimulation index of less than 3. There was no significant difference when the HIV-1-infected and EU children were compared, by a two-sample t-test on the log stimulation index values. Six children (four EU and two infected) had stimulation index values of less than 3.

5.3.2 LYMPHOCYTE PROLIFERATION TO TETANUS TOXOID

The proliferative responses, for the different subject groups, to tetanus toxoid (TT) at concentrations of 2.5µg/ml and 0.5µg/ml are presented in figure 5.2. Comparison of the

Figure 5.1 **Proliferative responses to PHA**

Proliferative responses, shown as the log stimulation index, (*y*-axis) to two mitogen concentrations 2.5 µg/ml and 0.5µg/ml, (*x*-axis) are shown for the different subject groups. The cord bloods (CB, n = 13) are shown as dark blue squares, normal donors (ND, n = 9) as brown triangles, exposed uninfected children (EU, 44 assays from 25 children) as light blue diamonds and the HIV-1-infected children (HIV+ve, 21 assays from 11 children) as circles. The infected children are shown according to the groups relating to age and CD4% profile, defined in chapter 3. The group I children (those with a steady CD4% at over 60 months of age) are shown as green circles; the group II children (those with a decreased CD4% at over 60 months of age) are shown as blue circles; the group III children those of less than 60 months of age, (and includes one fast progressor) are shown as red circles. The horizontal line denotes the cut-off value of 3 for the stimulation index. Above this line responses were defined as positive. The subject groups are presented vertically and the horizontal bars denote the geometric mean value for each subject group.

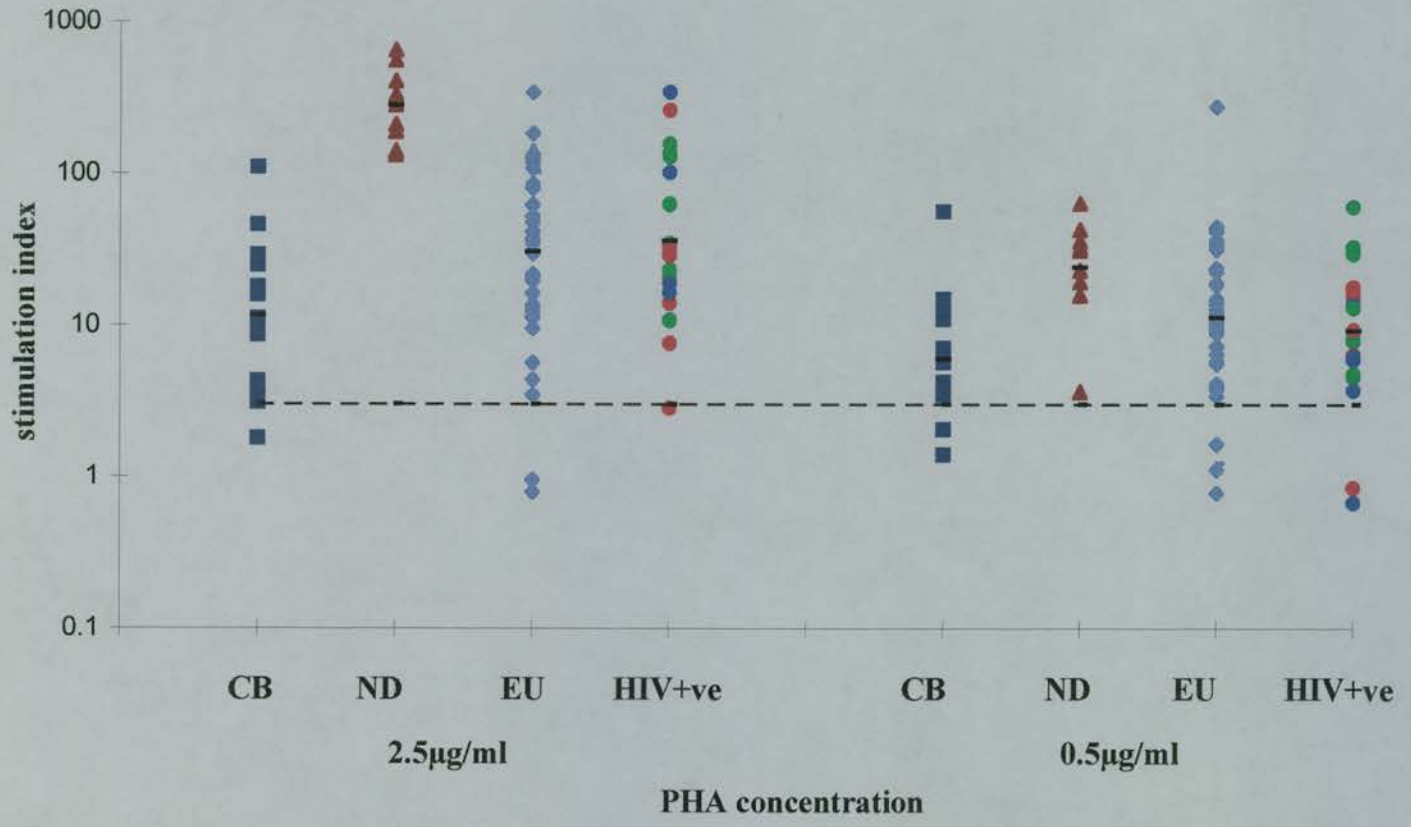
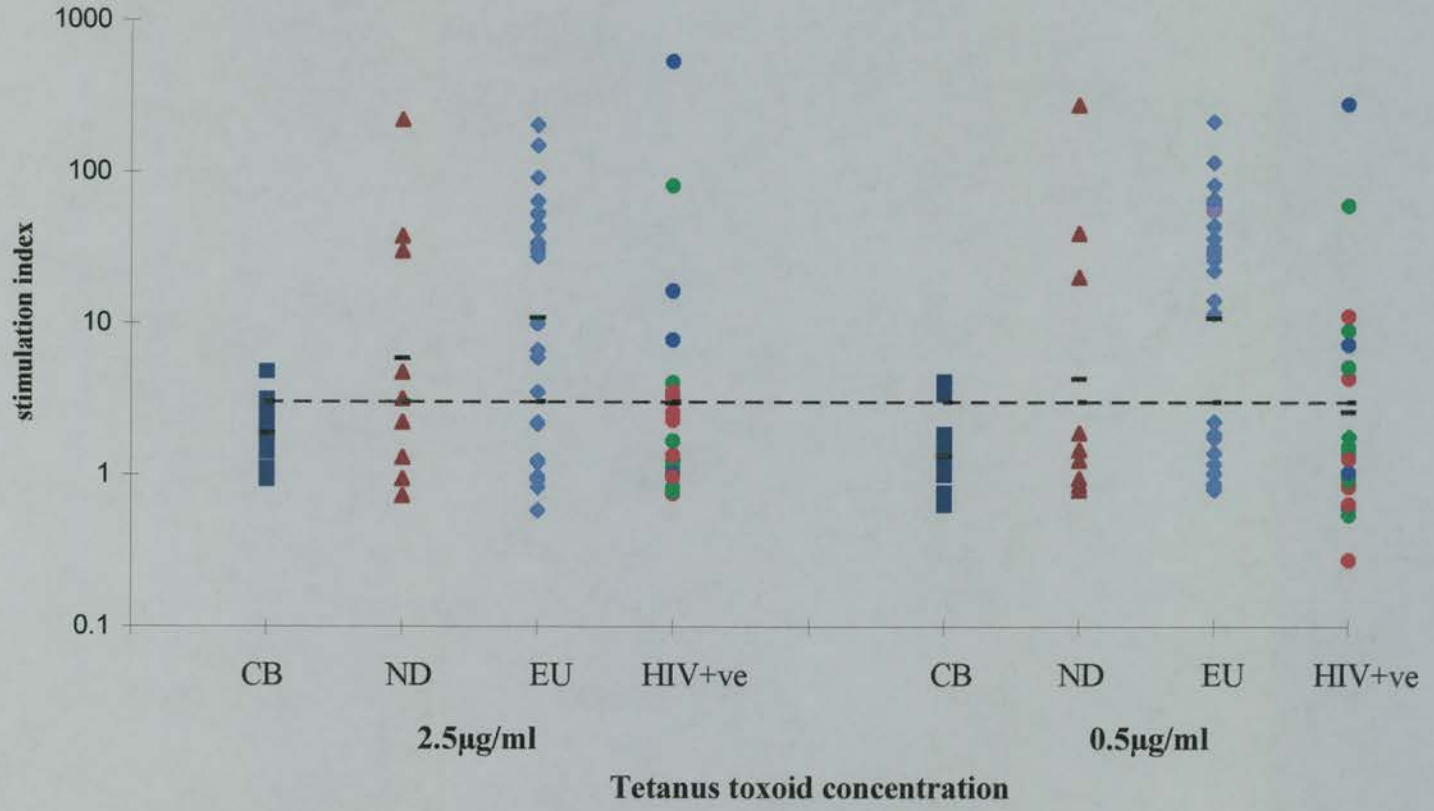

1000
100
10
1
0.1
stimulation index
CB
ND
EU
HIV+ve
CB
ND
EU
HIV+ve
2.5µg/ml
0.5µg/ml
PHA concentration

Figure 5.2 **Proliferative responses to Tetanus toxoid**

The proliferative responses to two antigen concentrations (2.5µg/ml and 0.5 µg/ml) are shown for the different subject groups. The symbols and numbers within each group are as for figure 5.1.


1000
100
10
1
0.1
stimulation index
CB
ND
EU
HIV+ve
CB
ND
EU
HIV+ve
2.5µg/ml
0.5µg/ml
Tetanus toxoid concentration

responses (by analysis of variance on the log stimulation index values) showed there was no significant difference between the groups at the higher antigen concentration. At the lower concentration, a difference was seen ($p<0.05$). The responses in the EU children were significantly higher than those in the HIV-1-infected children ($p<0.05$, by two-sample t-test on log stimulation index values).

5.3.2.1 Relationship of TT responses to age in EU children

The EU children, as a group, were younger in age than the HIV-1-infected children (mean age = 31.4, range 1 - 156 months; mean age = 83.2, range 21 - 137 months, respectively). To see whether there was an age-relation in these TT responses, and whether the lower responses in the infected children could be due to their being older, the EU children were divided into 3 groups: younger than 6 months, 6 - 18 months and older than 18 months. The stimulation index values (geometric mean and range) are shown in table 5.1, together with the numbers within each group with a positive proliferative response. Comparison of the age-groups by analysis of variance (on the log stimulation index values) showed that the children younger than 6 months had significantly reduced responses to tetanus toxoid at both concentrations ($p<0.05$, table 5.1). This difference could reflect the age at which the children were immunised against TT. The stimulation index values for all the children older than 6 months of age were combined and compared to the HIV-1-infected children (all of whom were older than 6 months of age). This is shown in table 5.1, as geometric means and range. The older EU children had significantly higher responses to TT than the HIV-1-infected children ($p<0.01$ at 2.5 µg/ml and $p<0.01$ at 0.5 µg/ml, by two-sample t-tests on the log stimulation index values).

5.3.3 LYMPHOCYTE PROLIFERATION TO RECOMBINANT HIV-1 COCKTAIL

The proliferative responses to the rHIV cocktail at concentrations of 1.25µg/ml and 0.25µg/ml are shown in figure 5.3. Comparison of the subject groups by analysis of variance (on the log stimulation index values) showed that the responses from the cord bloods were

Table 5.1 **Responses to Tetanus Toxoid**

Child group	Stimulation index geometric mean (range)		Responses SI > 3 (n)	p value
TT at 2.5µg/ml				
EU Children				
< 6 months (n = 12)	2.07	(0.96 - 42.08)	4	
6 - 18 months (n = 18)	13.43	(0.93 - 206)	12	0.029¶
> 18 months (n = 14)	8.55	(0.58 - 358)	11	
EU > 6 months (n = 32)	12.33	(0.58 - 358	23	0.0021§
HIV+ve (n = 21)	3.26	(0.75 - 538)	8	
TT at 0.5µg/ml				
EU children				
< 6 months	1.61	(0.46 - 30.5)	1	
6 - 18 months	9.84	(0.88 - 217)	10	0.024¶
> 18 months	10.68	(0.80 - 387)	10	
EU > 6 months	12.24	(0.80 - 387)	20	0.0018§
HIV+ve	2.59	(0.28 - 285)	6	

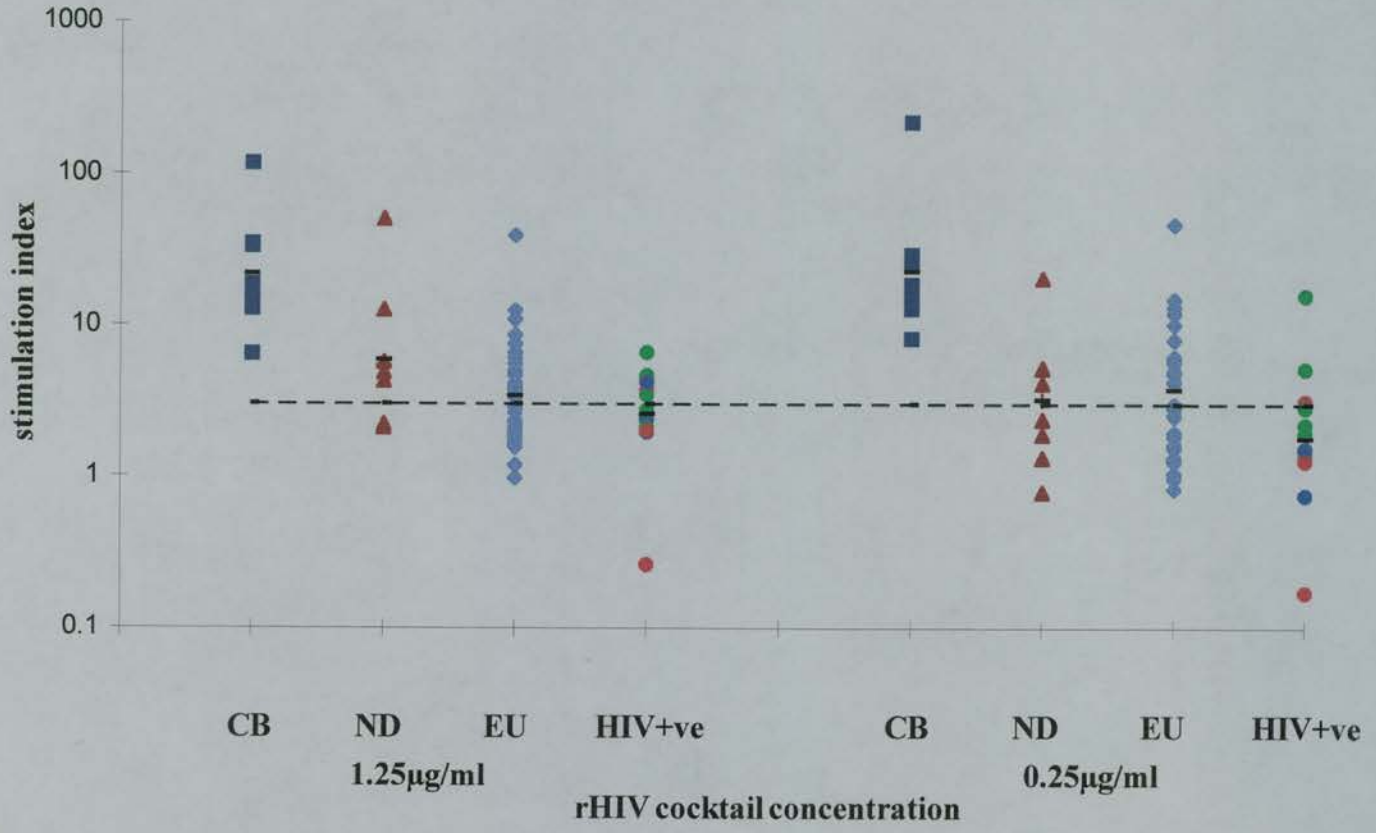
The proliferative responses to tetanus toxoid (TT) are shown at two concentrations.
The geometric mean and range of the stimulation index values (SI) are shown for the different age groups of the EU children, and for the EU and HIV-1 infected (HIV+ve) children of over 6 months.

¶ denotes the p value was obtained from the analysis of variance for these groups using the log stimulation index values.

§ denotes the p value obtained from this test. Statistics were performed on the data using Minitab.

Figure 5.3 **Proliferative responses to rHIV cocktail**

The proliferative responses to two rHIV cocktail (concentrations 1.25µg/ml and 0.25µg/ml) are shown for the different subject groups. The symbols are the same as those in figure 5.1, but the numbers from each group differ: cord bloods (n = 8), normal donors (n = 9), EU (32 assays from 22 children), HIV-1-infected (18 assays from 10 children).


1000
100
10
1
0.1
stimulation index
CB
ND
EU
HIV+ve
CB
ND
EU
HIV+ve
1.25µg/ml
0.25µg/ml
rHIV cocktail concentration

significantly higher than those in the other subject groups, at both concentrations ($p<0.01$). Comparison of the EU and infected children showed a slight decrease in responses in the infected children, which was not significant at the higher concentration ($p = 0.06$), but was significant at the lower concentration ($p<0.01$).

These unexpected results were further investigated by obtaining five more cord bloods, and assaying for proliferation against the rHIV cocktail as before. The cells were also incubated with each recombinant protein individually and with an alternative combination of the two gp120s and p24 (gp120/p24), all at the same concentrations previously used. The results are presented in figure 5.4, and show that proliferative responses were seen against the rHIV cocktail as before, and also against the recombinant proteins tat, nef and RT (p66). These three proteins had been expressed in *Esherichia coli (E.coli)*. The gp120/p24 combination induced no proliferative response in cord bloods, and was, therefore, used in subsequent proliferation assays.

Preliminary results using the gp120/p24 at concentrations 1.25µg/ml and 0.5µg/ml in the cord bloods, EU and HIV-1-infected children are presented in figure 5.5. None of the cord bloods or the EU children showed any responses to these proteins, but two (of six) HIV-1-infected children did show positive responses.

5.3.4 RELATIONSHIP BETWEEN PROLIFERATIVE RESPONSES AND PBMC SUBPOPULATION

To see whether there was a relationship between proliferative responses and the proportions of different lymphocyte populations in whole blood, these were compared for both PHA and TT, for all the subject groups together. The lymphocyte populations measured were: the percentage of total lymphocytes expressing CD3, CD4 or CD8; the percentage of CD4 cells co-expressing either CD45RO or CD45RA, to see whether the proportion of (phenotypically) memory and naïve cells affected the responses; the percentage of CD3 cells co-expressing CD28, to see whether the expression of this co-stimulatory molecule, required for antigen-specific T cell responses (Linsley and Ledbetter, 1993), was related to proliferation to a particular antigen. The correlation coefficients are shown in table 5.2. An absolute r value

Figure 5.4 **Proliferative responses to HIV-1 proteins**

Proliferative responses to two concentrations (1.25µg/ml and 0.25µg/ml) of the rHIV cocktail, the gp120/p24 combination and all the individual HIV-1 proteins are shown for cord bloods (n = 5). The stimulant is indicated on the *x*-axis. The horizontal line denotes the cut-off value of the stimulation index of 3, above which responses were defined as positive. The subject groups are presented vertically and the horizontal bars denote the geometric mean value for each stimulant.

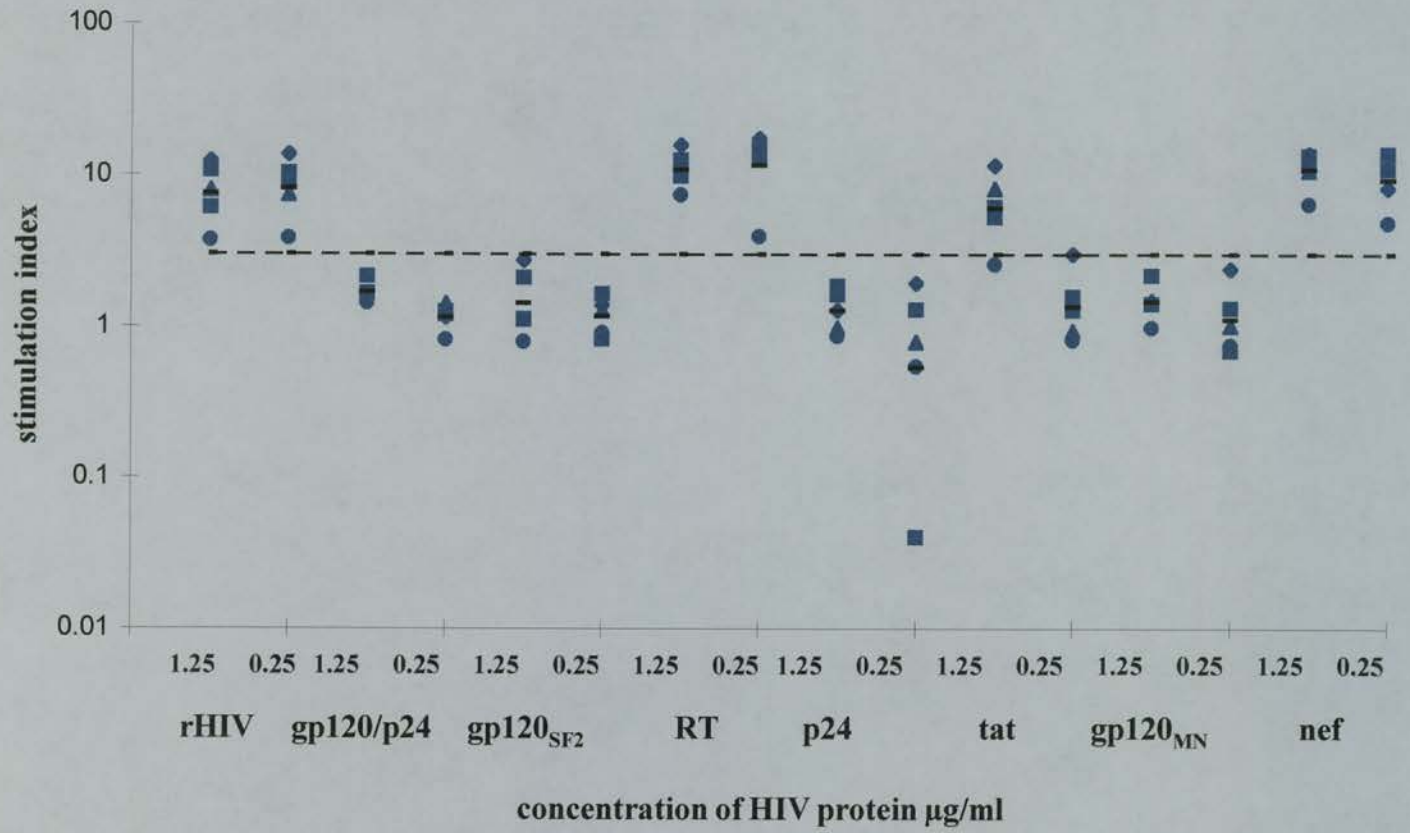
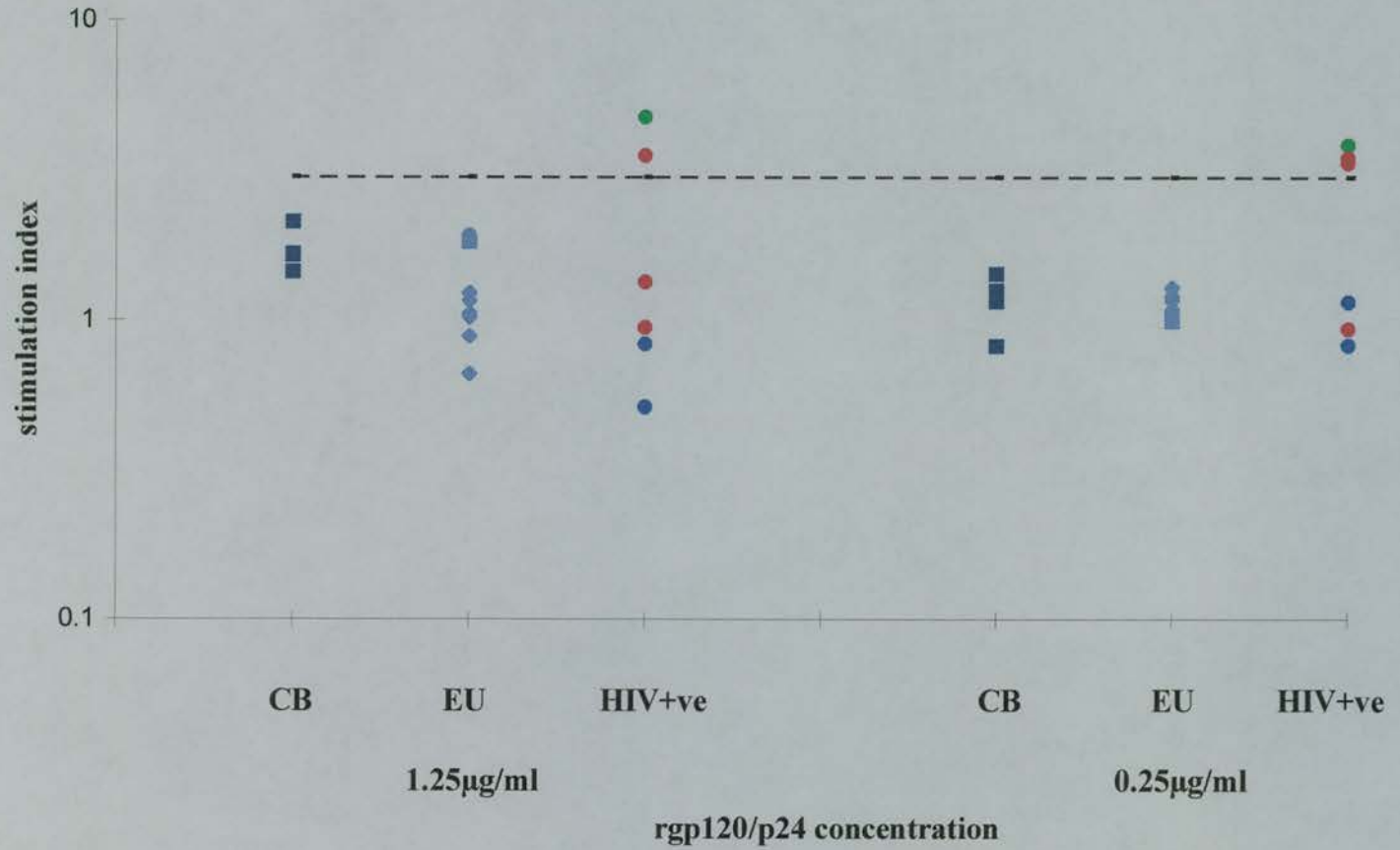

100
10
1
0.1
0.01
stimulation index
1.25 0.25 1.25 0.25 1.25 0.25 1.25 0.25 1.25 0.25 1.25 0.25 1.25 0.25 1.25 0.25
rHIV
gp120/p24
gp120SF2
RT
p24
tat
gp120MN
nef
concentration of HIV protein µg/ml

Figure 5.5 **Proliferative responses to rgp120/p24**

Proliferative responses to two concentrations (1.25µg/ml and 0.25µg/ml) of the gp120/p24 combination are shown for the cord bloods (n = 5), EU children (n = 8) and HIV-1-infected (n = 6) children. The symbols are the same as shown in figure 5.1.


10
1
0.1
stimulation index
CB
EU
HIV+ve
CB
EU
HIV+ve
1.25µg/ml
0.25µg/ml
rgp120/p24 concentration

The proliferative responses for PHA and TT (log stimulation index) were compared with the percentage of cells expressing the marker of interest.

The lymphocyte populations are defined as: the percentage of total lymphocytes expressing CD3, CD4 or CD8; the percentage of CD4+ cells co-expressing either CD45RO or CD45RA (CD45RA+ CD4); the percentage of CD3+ cells co-expressing CD28 (CD28+ CD3)

§ the correlation value (r) was obtained using Microsoft Excel

¶ denotes a significant correlation ($p<0.05$)

Table 5.2 **Proliferative response and lymphocyte population**

Stimulant	Lymphocyte population (%)	correlation value (r)[§]
PHA 2.5µg/ml	CD3	0.100
	CD4	-0.075
	CD8	0.141
	CD45RO+ CD4	0.473[¶]
	CD45RA+ CD4	-0.433[¶]
	CD28+ CD3	-0.022
PHA 0.5µg/ml	CD3	0.158
	CD4	-0.004
	CD8	0.110
	CD45RO+ CD4	0.207
	CD45RA+ CD4	-0.052
	CD28+ CD3	-0.054
TT 2.5µg/ml	CD3	0.024
	CD4	0.145
	CD8	-0.096
	CD45RO+ CD4	0.122
	CD45RA+ CD4	-0.100
	CD28+ CD3	0.113
TT 0.5µg/ml	CD3	0.035
	CD4	0.163
	CD8	-0.100
	CD45RO+ CD4	0.102
	CD45RA+ CD4	-0.118
	CD28+ CD3	0.095

of greater than 0.21 denotes a significant correlation (for n = 87 children, Wardlaw, 1985).

For PHA, there was no correlation between the log stimulation index and percentage of CD3, CD4 or CD8 lymphocytes in PBMCs at either concentration (table 5.2). Correlations between the log stimulation index and the percentages of CD45RO+ and CD45RA+ CD4 cells were seen at the higher concentration (r = 0.473 and r = -0.433, respectively), indicating that the proliferative responses increased as the proportion of CD45RO+ CD4 cells increased. There was no correlation between the log stimulation index and any lymphocyte phenotype at the lower concentration of PHA.

For TT, no lymphocyte population showed any correlation to the log stimulation index.

5.3.5 RESPONSES IN HIV-1-INFECTED CHILDREN

Data from the HIV-1-infected children were presented in figures 5.1 and 5.2 according to the CD4% groups outlined in chapter 3: group I contains the older children who have not yet progressed; group II contains the older children who have a decreased CD4%; group III contains the younger children and includes one of the children classed as a fast progressor. From figure 5.1 it can be seen that all except one group III child, responded to PHA at the higher concentration. At the lower PHA concentration, the same child and a group II child did not respond. The highest responses to PHA were seen in three children, one from each group. There was no association between the level of response to PHA and the groups to which the children belonged.

From figure 5.2 it can be seen that the highest responses to TT were seen in two children from groups I and II. Responses to TT were seen in children from all the groups, and all the groups contained non-responders.

5.3 DISCUSSION

The loss of CD4 cell function before the depletion of CD4 cell number has been documented in HIV-1 infected adults. A consistent progressive pattern of loss of responses to HIV-1 and recall antigens, followed by alloantigens and finally mitogens has been shown in a number of studies in adults (Clerici *et al.*, 1989; Bentin *et al.*, 1989; Hoy *et al.*, 1988; Ranki *et al.*, 1989). This study was undertaken to determine whether proliferative responses to PHA, TT and HIV-1 were lost in a particular pattern in HIV-1-infected children.

The HIV-1-infected children showed similar PHA responses to those seen in EU children. However, the responses to TT were significantly lower in the HIV-1-infected children, compared with the EU children older than 6 months of age. This is consistent with results of a previous study of infected adults, which showed that the loss of proliferative responses to TT could identify early immune dysfunction (Hoy *et al.*, 1988). HIV-1-infected children from all the CD4% groups had reduced responses to TT, which supports the results of a previous study (Chirmule *et al.*, 1995). A study by Clerici *et al.*, (1993a) suggested that age affects the cytokine and proliferative responses to recall antigens in normal children. They reported that PBMCs from cord bloods and children between 6 and 13 months of age did not produce responses to flu, and only a few children showed weak responses to tetanus. In contrast, the results from the EU children in this study, showed that responses to TT reached adult levels by 6 months of age. However, markedly reduced responses to TT were seen in the EU children of less than six months of age. The most likely explanation of this would be that the recall response to this antigen after primary immunisation had not yet developed.

There is evidence indicating that the timing and extent of the PHA response are influenced by the proportions of CD45RO+ and CD45RA+ cells in the culture: CD45RA+ cells respond more readily to stimulation by PHA (Morimoto *et al.*, 1985; Merkenschlager and Beverley, 1989; Akbar *et al.*, 1991), whereas CD45RO+ cells respond readily to recall antigens and more slowly to PHA (Merkenschlager *et al.*, 1988; Merkenschlager and Beverley, 1989). Thus, the kinetics of the proliferative response to PHA, from the CD45RA+ cells, would be faster and would peak earlier, than that of the CD45RO+ cells; a slower response, which would peak later, would be expected from the CD45RO+ cells. A positive correlation was seen between the PHA response and the percentage of CD45RO+ CD4 cells, at the higher

concentration of PHA. Conversely, a negative correlation was seen between the PHA response and the percentage of CD45RA+ CD4 cells, i.e. a higher response to PHA was seen with an increased proportion of CD45RO+ CD4 cells. The cultures from the normal donors contained a higher proportion of CD45RO+ CD4 cells, in the initial cell populations, than those of the other subject groups, and these showed the least variation and the highest stimulation index values. As the cells were incubated for a period of time which was longer than is optimal for this mitogen (3 - 4 days would have been better than 7 days), these higher responses in the normal donors, therefore, could reflect a later peak of the PHA response, due to the higher proportion of CD45RO+ CD4 cells.

Memory CD4 cells respond to recall antigens (Merkenschlager *et al.*, 1988; Merkenschlager and Beverley, 1989), and some studies have correlated the loss of CD4 function, in HIV-1-infection, with the loss of the memory CD4 cell population (van Noesel *et al.*, 1990; Schnittman *et al.*, 1990; Shearer and Clerici, 1991). However, this finding has not been confirmed in all studies (Giorgi *et al.*, 1987; Hoy *et al.*, 1988). Other studies have shown that the induction of a proliferative response to a recall antigen requires activation of the CD28 moiety on the cell by interaction with the B7 antigen on the APC (Linsley and Ledbetter, 1993). They have further suggested that the loss of recall response is due to the loss of surface CD28 expression (Linsley and Ledbetter, 1993; Borthwick *et al.*, 1994; Boise *et al.*, 1995). However, in this study, there was no correlation between recall response to TT and the expression of the memory (CD45RO) marker or CD28 on lymphocytes.

In other studies of HIV-1-infected children, a correlation between proliferative responses and absolute CD4 count has been seen (Chirmule *et al.*, 1995; Johnson *et al.*, 1991). However, both these studies also reported a subset of children with a decrease in recall responses, regardless of the CD4 count. There was no association between CD4% and responsiveness to recall antigen in the HIV-1-infected children studied here, as all the CD4% groups contained responders and non-responders. The highest responses to TT was seen in a group II child, who was losing CD4 cells. This suggests that those circulating CD4 cells that were present, may have had an 'over-reactive' response to antigen, which could lead to cell death through apoptosis or other biochemical mechanisms (Katsikis *et al.*, 1995; Bofill *et al.*, 1995). Another recent study has shown that the addition of intravenous immunoglobulin G (IVIgG) preparations to cultures *in vitro*, can suppress the proliferative response (Andersson *et al.*,

1996). IVIgG is used to prevent bacterial infections (de Martino *et al.*, 1991; Mofenson and Moye Jr, 1993). In the study here, two children in group I and one child in group II, received regular IVIgG infusions every three weeks (Dr. J. Mok, personal communication). The mechanism of IVIgG suppression of proliferation is unknown, but it may have some residual effect on the PBMCs, despite their having been washed before culture.

Proliferative responses were seen to the HIV-1 proteins which had been expressed in *E.coli* (nef, tat and the p66 moiety of reverse transcriptase). It is possible that during the extraction or purification procedure, some of the *E.coli* proteins were also co-purified with the recombinant HIV-1 proteins (Dr. H. Holmes, MRC AIDS Reagent Programme NIBSC, personal communication). However, it was not possible to confirm this, as control extracts were not available. The preparations were greater than 90% pure (MRC ADP Reagents catalogue), and the concentrations of recombinant protein used were 1.25µg/ml and 0.5 µg/ml. If 10% of the protein added to the culture was derived from bacteria, this would mean that the concentration of the bacterial contaminant could be as much as 50ng/ml. One study of proliferative responses to bacterial lipopolysaccharide (LPS), optimised the responses using between 100 and 10,000 ng/ml of antigen, although responses were seen at lower concentrations (Mattern *et al.*, 1994). Therefore, it could be postulated that the concentration of bacterial contaminant, which might be present in these cultures, would be enough to give a measurable proliferative response. Small amounts of bacterial products can induce the release of cytokines from cells which, in turn, could lead to the activation of these cells by autocrine mechanisms (Mattern *et al.*, 1994). Therefore, the responses seen to the rHIV cocktail could be better interpreted as anti-bacterial responses, possibly occurring through some mechanism which does not require prior priming by antigen (Zumia, 1992).

A study of responses to *Cryptococcus neoformans*, in adults without prior exposure, showed that proliferative responses to this organism were seen in normal adults. In HIV-1-infected individuals a loss of proliferative response to this antigen was associated with an increased likelihood of progression to AIDS (Hoy *et al.*, 1988). If the responses to rHIV cocktail, in the children studied here, were interpreted as anti-bacterial responses, then the proliferative responses of the cord bloods may indicate the available vigorous response to bacterial antigens encountered perinatally, possibly by some innate mechanism (Pabst and Kreth, 1980). At the lower concentration of the rHIV cocktail, these responses were comparable in

EU children and normal adults, but decreased in the HIV-1-infected children. This may reflect a decreased proliferative capacity against bacterial antigens in the infected children, which may explain the increased susceptibility to opportunistic bacterial infections observed in other cohorts (Galli *et al.*, 1995).

It is possible that the proteins themselves had an effect on the cells to increase their proliferative activity. Studies have indicated that HIV-tat can act as a T-cell growth factor (Rosen, 1992) and has some homology with snake neurotoxins (Garry and Koch, 1992). However, there is also evidence that HIV-1-proteins may inhibit proliferation. HIV-tat has been shown to inhibit antigen-specific T cell responses (Subramanyam *et al.*, 1993), whereas intracellular HIV-nef has been shown to inhibit activation pathways of PBMCs (Greenway *et al.*, 1995).

Proliferative responses to HIV-1 antigens have been reported in some HIV-1-infected children and adults (Borkowsky *et al.*, 1990; Pontesilli *et al.*, 1995). In adults, responses have been seen to a selection of HIV-1 proteins (gp120, p24 and rev) in individuals with higher CD4 counts, but the specificity of response differed between individuals (Pontesilli *et al.*, 1995). The results presented here are consistent with this study, as proliferative responses to gp120/p24 were seen in two children, both of whom had stable CD4 percentages.

Preliminary results using the p24/gp120 cocktail indicated that there was no substantial proliferation to these antigens in the EU children. Any responses that may have been present were masked by the responses to the probable contaminant in the antigen preparation. Some studies of exposed uninfected individuals (EUs) have detected IL-2 production in response to HIV-1 antigens but not proliferation (Clerici *et al.*, 1992; Clerici *et al.*, 1993b). In one study of 23 cord bloods, from HIV-1-exposed children, IL-2 production in response to HIV-1 antigens was only found in a proportion (8/23). In the same study, 3 of the 15 non-IL-2-producers (20%), but none of those who produced IL-2, were later found to be infected with HIV-1. The authors suggested that exposure to HIV-1 *in utero* can stimulate anti-HIV-1 responses which is protective against HIV-1-infection (Clerici *et al.*, 1993b). However, in that study, the twelve non-IL-2 producers who did not become infected indicate that there must be other mechanisms by which these children remained uninfected.

In conclusion, this study shows that the responses to recall antigen in the HIV-1-infected children were reduced compared to EU children, but that PHA responses were comparable. There was no association between proliferative responses and clinical progression. A correlation between the proliferative response to PHA and the proportion of CD45RO+ CD4 cells in whole blood was seen. Two (of six) infected children, both of whom with a stable CD4% showed responses to recombinant gp120/p24 proteins, indicating that these responses may be associated with the maintenance of disease stability.

Chapter 6 DOWN REGULATION OF CD4 FROM THE SURFACE OF T CELLS IN AN HIV-1-INFECTED CHILD

6.1 INTRODUCTION AND OBJECTIVE

6.2 STRATEGY FOR INVESTIGATION AND CD4 RT-PCR METHOD DEVELOPMENT

6.2.1 CD4 depletion of PBMCS

6.2.2 RNA extraction

6.2.3 Reverse transcription of mRNA to cDNA

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Separate RT and PCR reactions

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6.1 INTRODUCTION AND OBJECTIVE

T lymphocytes are characterised by the expression of CD3 and the T cell receptor (TCR) on the surface of the cells. They are divided, functionally, into those which have a helper function and those which have a cytotoxic or suppressor function. T-helper cells usually express CD4 on the surface of the cells, while cytotoxic/suppressor cells usually express CD8. The CD4 molecule is a 60kD trans-membrane glycoprotein, which, in conjunction with the TCR, recognises antigenic peptide presented on the MHC class II molecule on APCs (see 1.4.2.1 and figure 1.3). The recognition of antigen causes a signal to transduce into the cell, through the cytoplasmic domain of the CD4 molecule. The CD4 molecule then associates with $p56^{lck}$ (a member of the *src* family of tyrosine kinases) within the cytosol (Ravichandran and Burakoff, 1994). Tyrosine kinases are enzymes which phosphorylate proteins (on the tyrosine moiety), and are part of the signal transduction mechanisms within the cell. Intracellular signal transduction leads to the activation of the cell and production of cytokines. These cytokines (small 'messenger' peptides), in turn, affect other cells to produce an immune response (see figure 1.4) (Kemeny *et al.*, 1994; Kos and Engleman, 1996; Mosmann and Sad, 1996; Romagnani, 1992). In HIV-1 infection, progression of disease is characterised by the loss of circulating lymphocytes expressing CD4 on the surface, leading to an increased susceptibility of the individual to opportunistic infections.

The CD4 molecule has also been shown (together with the ß-chemokine receptors) to be the main route for HIV-1 entry into cells. HIV-gp120 binds to the CD4 molecule on the surface of cells, causing a conformational change, followed by fusion of viral and cellular membranes, allowing entry of the viral core into the cell (Antoni *et al.*, 1994). Infection of cells expressing CD4, by HIV-1, can affect the expression of CD4 on the cell surface. Studies in T-cell lines have shown that CD4 expression at the cell surface is down-regulated by HIV-1. The exact mechanisms by which this down-regulation occurs is not clear, but HIV-*nef*, -*vpu* and -*env* have all been implicated. Some (thus far unclear) interaction of *nef* with the cytoplasmic domain of the CD4 molecule (Garcia *et al.*, 1993; Anderson *et al.*, 1994) has been shown to cause endocytosis of surface CD4 which accumulated within endosomes in the cell (Schwartz *et al.*, 1995). There is evidence that HIV-*vpu* then promotes the degradation of the CD4 molecule within these endosomes (Willey *et al.*, 1994; Bour *et al.*, 1995). Crise *et al.*, (1990) showed that, after synthesis of CD4 in HIV-1-infected cells,

complexes of CD4 and gp160 (the envelope precursor protein of HIV-1) were formed. These CD4-gp160 complexes were retained within the endoplasmic reticulum (ER), and thus CD4 was not expressed on the cell surface. However, CD4 molecules not complexed with gp160, were expressed normally at the cell surface (Crise *et al.*, 1990). Chen *et al.*, (1996) showed that the *nef*, *vpu*, and *env*-mediated mechanisms of CD4 down-regulation occurred independently and at different stages of HIV-1 infection within cells. They found that *nef* down-regulated CD4 in the early stages of viral infection of the cell, while the actions of *vpu* and *env* were seen later on in the viral life-cycle. However, maximal CD4 down-regulation was seen when the actions of all three gene products occurred in the same cells. This down-regulation could also occur in PBMCs, but was less efficient than that seen in T cell lines, and was more dependent on the actions of *nef* than *vpu* and *env* (Chen *et al.*, 1996). However, these mechanisms of down-regulation do not affect the *de novo* biosynthesis of CD4 (Schwartz *et al.*, 1995; Sanfridson *et al.*, 1994).

During the study of lymphocyte surface markers (chapter 3), one child was noted whose total percentage of lymphocytes expressing CD3, was markedly greater than the sum of the percentages of lymphocytes expressing CD4 or CD8. This was also seen in the routine monitoring of the CD3, CD4 and CD8 expressing cells in this child using a different set of antibodies (J.A. Whitelaw, personal communication, see table 6.1). Thus, it appeared that a circulating population of T lymphocytes was present in this child which did not express either CD4 or CD8. The child was born in Africa, had consistently shown no CTL activity to HIV-1 (see chapter 4) and had progressed to AIDS by the age of 70 months.

Although, the down-regulation of CD4 has been shown in *in vitro* HIV-1-infected PBMCs, this phenomenon has not been described in cells obtained from HIV-1-infected individuals. Therefore, it was decided to investigate the T cells from this child, to determine whether they were synthesising CD4, but not expressing it on the cell surface, and whether this loss of surface CD4 was associated with HIV-1 infection of the cell.

6.2 STRATEGY FOR INVESTIGATION AND CD4 RT-PCR METHOD DEVELOPMENT

The strategy for investigation is shown in figure 6.1, with the modifications of the methods outlined in italics, and described in detail below.

6.2.1 CD4 DEPLETION OF PBMCS

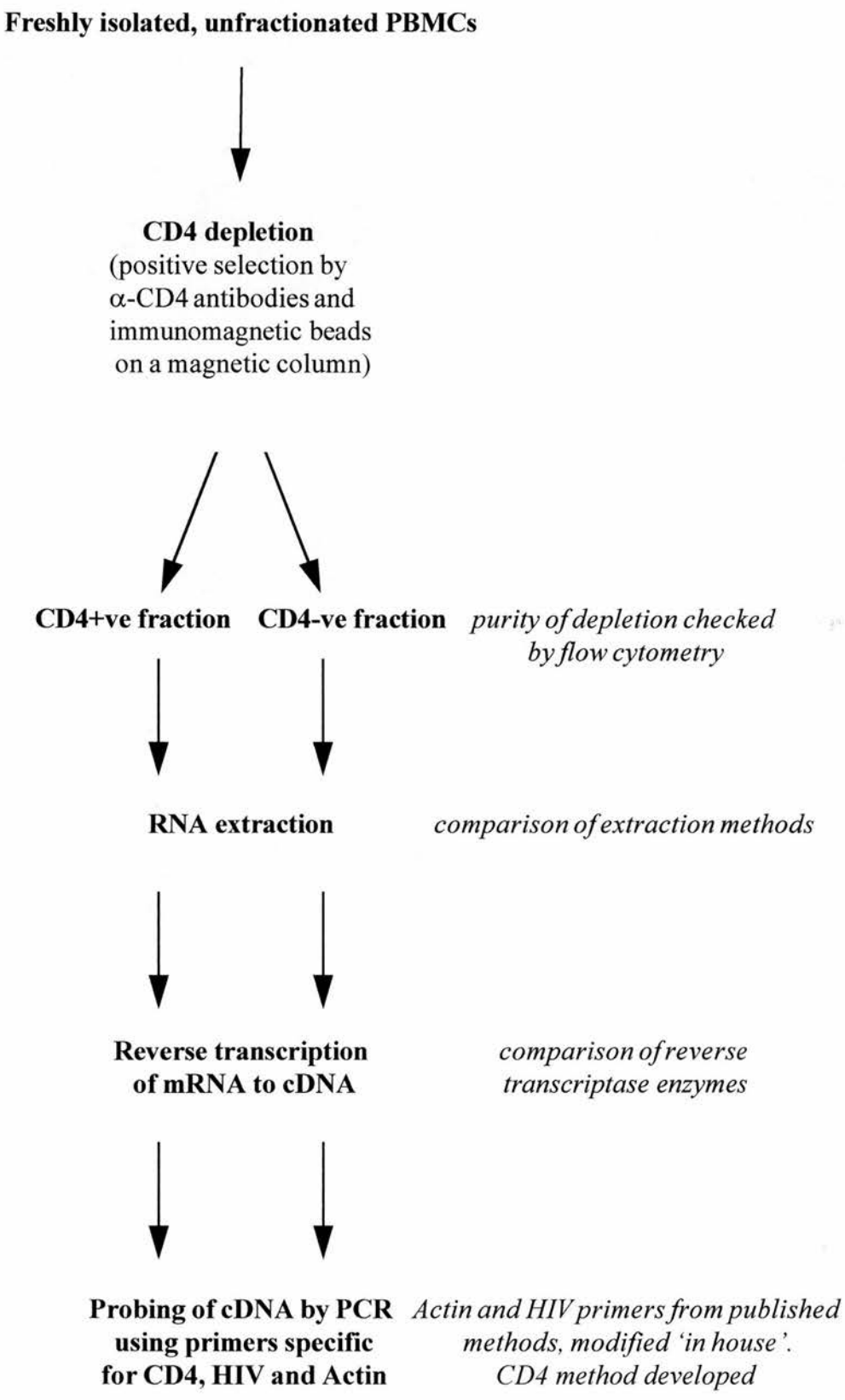
Freshly isolated PBMCs were separated into cells expressing surface CD4 (CD4+ve) and those not expressing CD4 (CD4-ve) fractions. This was done by positive selection using anti-CD4 antibodies and immunomagnetic beads. The CD4 expressing cells were retained on a magnetic column, while the CD4-ve fraction was collected. The CD4+ve fraction was then eluted and collected (described in detail in 2.11). The purity of the eluted fractions was checked by flow cytometry (described in 2.3.1), to show that the CD4-ve fraction contained only cells which were not expressing CD4 on the surface. Cells from the CD4+ve fraction were not checked by flow cytometry, as the surface CD4 molecules were already complexed to antibodies and beads.

6.2.2 RNA EXTRACTION

Initially, DNA and RNA were co-extracted from cells (denoted DNA/RNA) using the method described in 2.12.1. It was envisaged that, in the final experiment, total RNA (without DNA) would be used, from which mRNA could be detected. Detection of mRNA would reflect the cells which were actively synthesising the molecules of interest.

A DNAase step to remove DNA was tried, by incubating the DNA/RNA with DNAase at 37°C for 20 minutes. This was then heated to 80°C for 10 minutes to degrade the DNAase. PCR using the DNAase-treated RNA gave no bands (see below). However, bands were seen when DNA/RNA was used without a DNAase step (see below).

A Micro-RNA Isolation kit was tried from Stratagene, (described in 2.12.2) as this was


Freshly isolated, unfractionated PBMCs
CD4 depletion
(positive selection by
α-CD4 antibodies and
immunomagnetic beads
on a magnetic column)
CD4+ve fraction
CD4-ve fraction
purity of depletion checked
by flow cytometry
RNA extraction
comparison of extraction methods
Reverse transcription
of mRNA to cDNA
comparison of reverse
transcriptase enzymes
Probing of cDNA by PCR
using primers specific
for CD4, HIV and Actin
Actin and HIV primers from published
methods, modified 'in house'.
CD4 method developed

promoted as giving a more efficient extraction of small amounts of RNA. To compare the extraction methods, PBMCs from normal donor cells were used, from which adherent cells had been removed. These cells were 'snap' frozen (i.e. frozen quickly at -70°C, which helps to break up the cells) in aliquots of 10-fold dilutions from 10^7 to 10^2 cells and extracted using the Strategene kit or the phenol/chloroform method (described in 2.12). RT-PCR for ß-Actin was performed on the RNA or DNA/RNA (described in 2.13.2 and 2.13.3.1). The primary PCR products for ß-Actin, (10µl) were visualised by electrophoresis on a 1.3% agarose gel against molecular weight (pGEM) markers. (These are lengths of DNA of known sizes against which the DNA bands of interest are sized, see 2.13.5). The gel was run at 150V for 1.5 hours.

The results are presented in figure 6.2. ß-Actin bands were seen from cells extracted using the Stratagene kit, but not the DNA/RNA from the phenol/chloroform method. This shows that the Stratagene method is more efficient for RNA extraction. The initial PCR experiments used extracted DNA and RNA (denoted DNA/RNA), whereas the later ones used RNA extracted with the Stratagene method (denoted RNA).

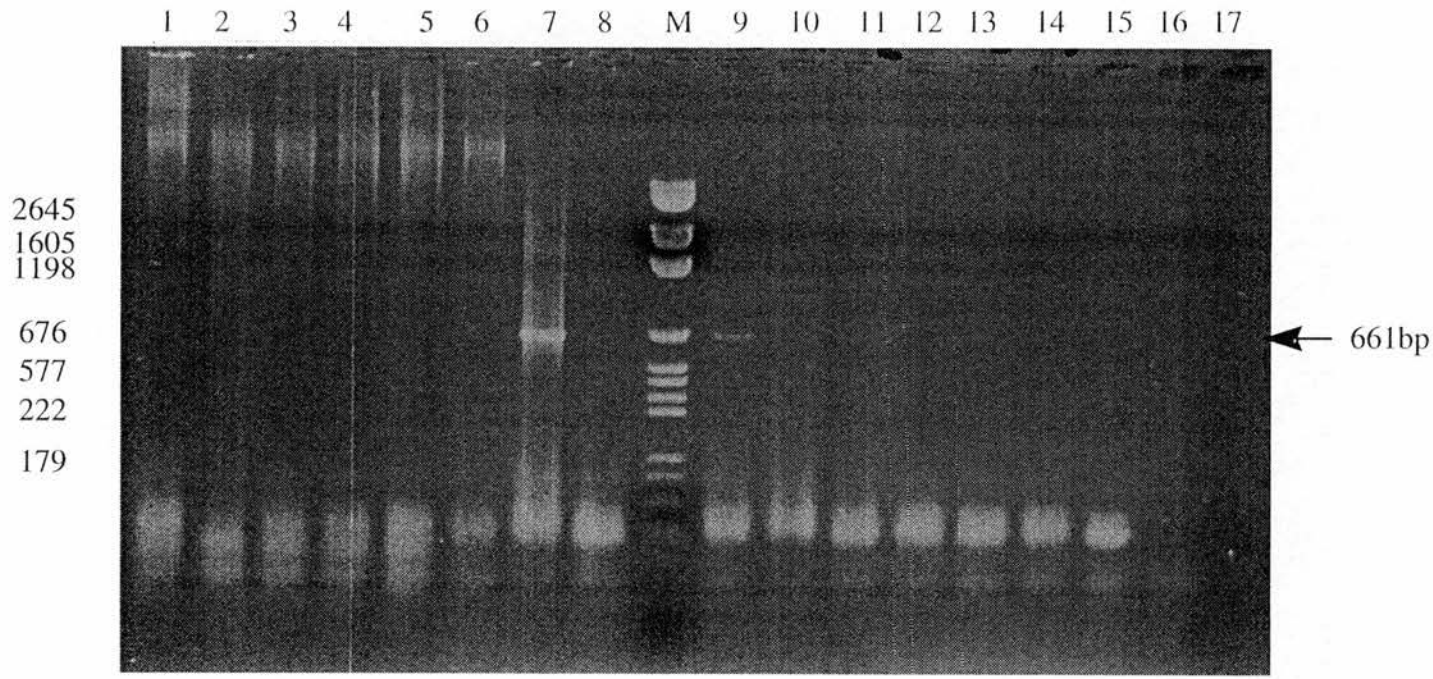
6.2.3 REVERSE TRANSCRIPTION OF mRNA TO cDNA

For CD4, the initial method combined the reverse transcriptase (RT) and primary PCR steps (see 6.2.4.1). When this was unsuccessful, it was decided to separate the RT and PCR steps, as these could allow conditions which would be more optimal for the enzymes used. All mRNA contains a 3'-poly-A tail (Alberts *et al.*, 1989). Therefore, an oligo-dT primer was used which annealed to the 3'-poly-A tail, and RT transcribed the RNA to cDNA (described in 2.13.1).

For most of the experiments, the RT step was done using a reverse transcriptase enzyme obtained from Promega (AMV-RT). However, a different reverse transcriptase enzyme, Expand™-RT (from Boehringer), was also tried (described in 2.12.1.2). This enzyme was engineered with a point mutation so that it has less RNase H activity, and so produces larger amounts of cDNA. This enzyme was compared to AMV-RT, using RNA diluted out in 10-fold steps from neat to 10^{-4} (in DEPC-water). The cDNA was used in a ß-Actin PCR,

Figure 6.2 **Comparison of the methods for extraction of RNA**

PCR gel showing bands of the primary PCR product of ß-Actin are shown (expected product size of 661bp). Lanes 1 to 6 show the PCR product from RNA extracted from PBMCs using the DNA/RNA extraction method. The PBMCs were in 10-fold dilutions in consecutive lanes, from 10^7 cells (in lane 1) to 10^2 cells (in lane 6). Lanes 7, and lanes 9 to 15 show the PCR product from RNA extracted using the Stratagene Kit. Similarly, the PBMCs were in 10-fold dilutions in consecutive lanes, from 10^7 cells in (lanes 7 and 9) to 10^2 cells in (lane 15). Lanes 8, 16 and 17 contained the RNA, cDNA and Taq negative controls, respectively. The numbers on the left hand side correspond to the pGEM markers in the lane marked M.


1 2 3 4 5 6 7 8 M 9 10 11 12 13 14 15 16 17
2645
1605
1198
676
577
222
179
661bp

(described in 2.13.3.1). The primary PCR product (10µl) was electrophoresed on a 1.2% agarose gel, against pGEM markers at 150V for 1.5 hours.

Bands corresponding to the expected product size of ß-Actin (661bp) were seen from cDNA from using both enzymes and are shown in figure 6.3. Those using the Expand™-RT were brighter than those seen from using the AMV-RT. These results indicate that the Expand™-RT is a more efficient enzyme than AMV-RT, for reverse transcription of RNA to cDNA.

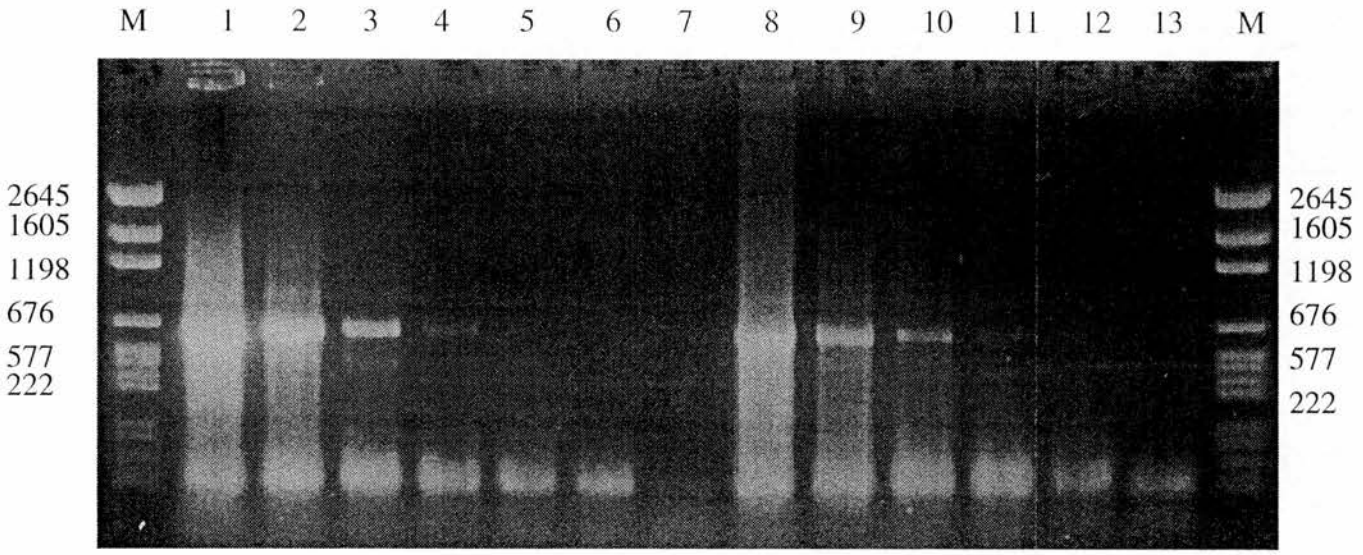
6.2.4 DEVELOPMENT OF A PCR METHOD FOR CD4

6.2.4.1 CD4 RT-PCR using combined RT and PCR steps

A PCR method for CD4 was found in a study which had compared CD4 sequences between humans and different monkey species (Fomsgaard *et al.*, 1992). The primer sequences used in that study were made from the conserved regions of human CD4, which were found to be the same as another published sequence of human CD4 (Maddon *et al.*, 1985). The primers were synthesised (by Oswel DNA Services) and the sequences are given in 2.13.2.2. The outer primers for the primary PCR are denoted CD4-1 (sense) and CD4-2 (antisense). These give an expected product size of 1346bp. The inner (or nested) primers for the secondary PCR are denoted as CD4-3 (sense) and CD4-4 (antisense). These gave an expected product size of 1195bp. DNA/RNA was extracted from normal donor PBMCs (described in 2.12.1). Although the sources of reagents were different, combined reverse transcription and PCR steps were performed as described in the original paper (Fomsgaard *et al.*, 1992). Briefly, DNAase-treated RNA was incubated with the primary PCR primers (CD4-1 and CD4-2) in a PCR buffer containing dNTPs and RNAsin, and both AMV Reverse Transcriptase (AMV-RT) and Taq polymerase (Taq) in a 100µl amplification reaction. The tubes were incubated on a thermocycler at 42°C for 60 minutes (reverse transcription step), followed by 94°C for 4 minutes (to denature the cDNA) and then 30 cycles of 94°C for 1 minute, 50°C for 1.5 minutes (to anneal the primers to the cDNA) and 72°C for 2 minutes (extension step). A secondary PCR was performed from the primary product (10µl), using inner primers (CD4-3 and CD4-4) and the same PCR programme, omitting the initial 60 minute incubation (Fomsgaard *et al.*, 1992). The secondary product (25µl) was electrophoresed on a 0.5%

Figure 6.3 **Comparison of AMV-RT and Expand™-RT enzymes**

PCR gel showing bands of the primary PCR product of ß-Actin are shown (expected product size of 661bp). The PCR products shown in lanes 1 to 6 were from RNA reverse transcribed using the Expand™-RT enzyme. The RNA had been titrated out in 10-fold dilutions, from neat (lane 1) to $1:10^4$ (lane 5). Lane 6 contained no RNA, and lane 7 contained the cDNA negative control. The products in lanes 8 to 13 were from RNA reverse transcribed using the AMV-RT enzyme. The RNA in these lanes had been titrated out in 10-fold dilutions, from neat (lane 8) to $1:10^4$ (lane 12). Lane 13 contained no RNA. The numbers down each side correspond to the bands shown by the pGEM markers are shown in the lanes marked M.



Expand^{TM}-RT **AMV-RT**

agarose gel against pGEM markers. The gel was run at 75V for 2 hours. No bands were seen. This experiment was repeated using DNA/RNA without DNAase treatment, and still no bands were seen.

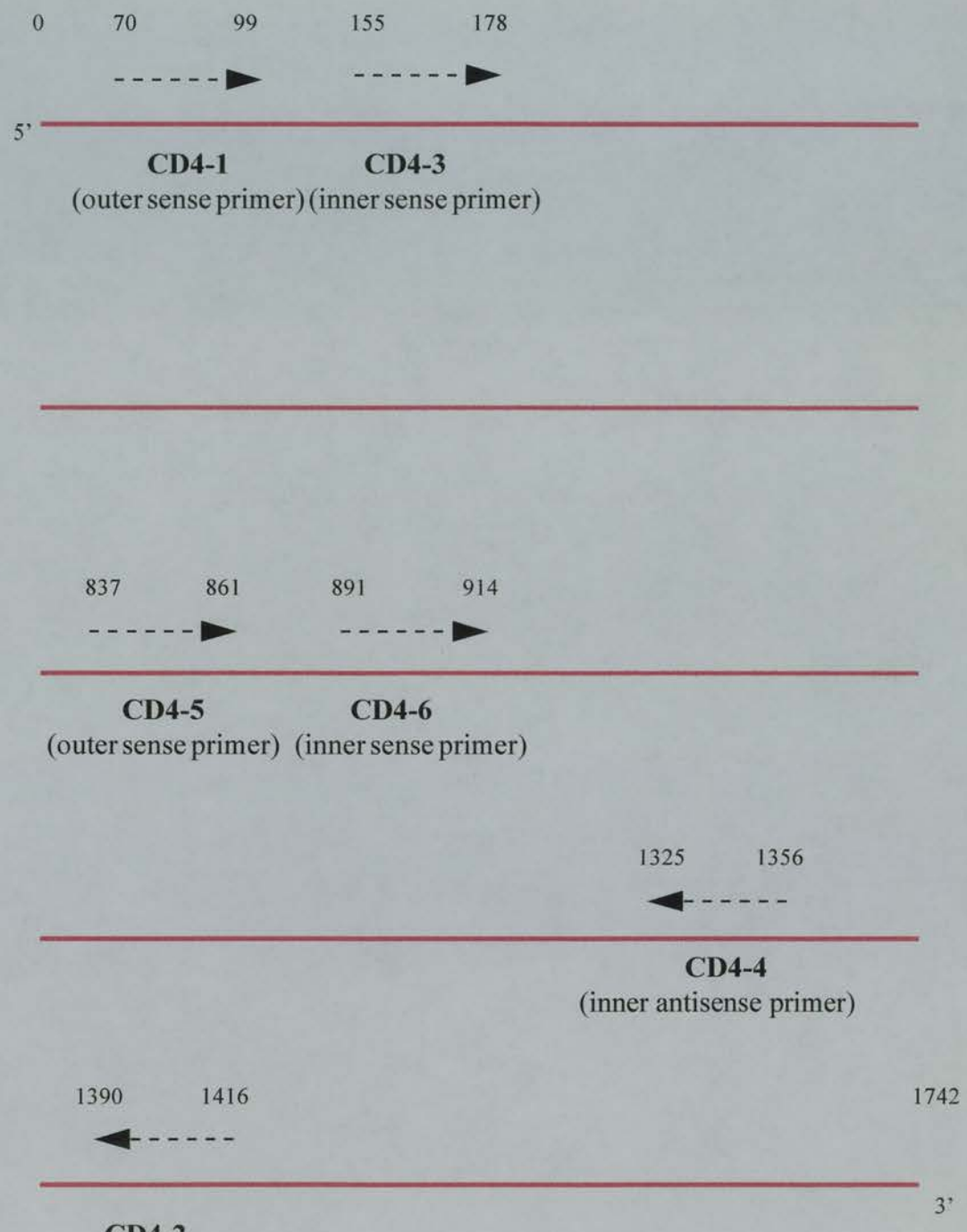
The primary and secondary PCR products from these primers were very large (1346bp and 1195bp respectively) as they were designed to be used to create cDNA libraries from low quality RNA. In a nested PCR, which is driven by Taq polymerase, full length copies may not be produced. Therefore, using a published sequence of CD4 (Maddon *et al.*, 1985), and keeping the two antisense primers from the original method (CD4-2 and CD4-4), two further sense primers were designed (with the help of C.M Wade). This was done by choosing a region in the CD4 gene which was approximately 500bp from CD4-2 (the outer anti-sense primer). Sequences which were rich for C and G bases were chosen, as these are more stable than A-T rich regions. The new primers were denoted CD4-5 (outer sense primer) and CD4-6 (inner sense primer). These gave expected primary and secondary PCR product sizes of 579bp and 459bp respectively. The alignment positions of all the primers, both from the original paper and those designed, are shown in figure 6.4, and the primer sequences are described in 2.13.2.2. These primers were used in the same PCR reaction as used originally (see above) for two attempts but no bands were seen.

6.2.4.2 Separate RT and PCR reactions

Resting PBMCs should contain mRNA for CD4, through a low level of cell turnover. However, it was decided for these experiments, to use RNA extracted from human cell lines which express CD4 and are actively replicating, as there would be more total, and CD4-specific mRNA, available from these lines. The DNA/RNA used in this experiment was prepared (by D. Innes, E. Harvey and A. Alonso) from the following cell lines: C8166 cells (a human T-lymphoblastoid cell-line, which expresses CD4); HeLa cells (a human epithelial-like cell line, which does not express CD4) and HeLa cells transfected with CD4 (HeLa-CD4). Total RNA (1µg), from each cell line, was used in each AMV-RT reaction (described in 2.13.1). The synthesised cDNA was used in PCRs for CD4, using both sets of primers (i.e. to give both the larger (1195bp) and smaller (459bp) secondary PCR products), and the original thermocycler programme (described in 2.13.2). The secondary PCR products (10µl)

Figure 6.4 **Alignment of the CD4 primers**

The 5' to 3' sequence of the DNA of human CD4 is represented by the red line. The positioning of the primers for CD4 are shown by arrows, and the numbers above the arrows indicate the position of the primer by the base number. Arrows pointing 5' to 3' indicate the sense primers. Arrows pointing 3' to 5' indicate the anti-sense primers. The primers from the original CD4 method (Fomsgaard *et al.*, 1992) are indicated as CD4-1 to CD4-4. The new primers synthesised are shown as CD4-5 and CD4-6. The primer sequences are given in 2.13.2.


0
70
99
155
178
5'
CD4-1
(outer sense primer)
CD4-3
(inner sense primer)
837
861
891
914
CD4-5
(outer sense primer)
CD4-6
(inner sense primer)
1325
1356
CD4-4
(inner antisense primer)
1390
1416
1742
3'
CD4-2
(outer antisense primer)

were separated by electorphoresis on a 1.3% agarose gel against pGEM markers, at 150V for 1.5 hours.

Results are shown in figure 6.5. Both sizes of CD4 products were seen from C8166 and HeLa-CD4 cells but not HeLa cells, as expected. No bands were seen for RNA-ve, cDNA-ve and primer-ve controls and only non-specific bands were seen for Taq-ve controls.

6.2.4.3 Titration of HeLa-CD4 RNA

As it was envisaged that only a small amount of RNA would be available in the final experiment, and hence even less CD4-specific mRNA, the limits of detection were investigated. The RT step was carried out using AMV-RT for the HeLa-CD4 DNA/RNA, titrating the total DNA/RNA added from 1µg to 1pg, in 10-fold dilutions in DEPC-water. PCR for CD4 was carried out using both primer sets to see which size of CD4 PCR product would be easier to obtain from small amounts of RNA. The secondary PCR products (10µl) were run on a 1.3% agarose gel against pGEM markers, at 150V for 1.5 hours.

The results are presented in figure 6.6 and show that CD4 bands (1195bp and 459bp) were seen at the dilutions down to 100pg of total DNA/RNA in the RT step, but not below. For the larger CD4 product, some bands were very faint. The primers for the smaller CD4 product (459bp) gave more consistent results, and therefore, were used for the rest of the study. No specific bands were seen in the RNA-ve, cDNA-ve, Taq-ve and primer-ve controls.

6.2.5 PCR FOR ß-ACTIN

To show that the RNA had reverse transcribed, the cDNA from the above experiments was also used in a PCR for β-Actin. β-Actin was used as a positive control as this protein is constitutively expressed as part of the cell cytoskeleton. Therefore, every time a cell divides, mRNA for β-Actin is produced, and is available in larger amounts than mRNA for other molecules.

Figure 6.5 **Detection of CD4 from cell lines**

PCR gel showing bands for CD4 products after an RT-PCR using RNA from different cell lines. Both sets of CD4 primers were used (expected product sizes of 1195bp and 459bp, respectively) and are shown by the arrows. Lanes 1 (1195bp product) and 2 (459bp product) used RNA from C8166 cells; lanes 3 and 4 used RNA from HeLa cells, and no CD4 was seen. Lanes 5 and 6 used RNA from HeLa-CD4 cells. Lanes 7 to 13 contain the negative controls: lanes 7 and 8 (no RNA), 9 and 10 (no cDNA), 11 and 12 (no Taq) and lane 13 (no primer). The numbers on the right hand side correspond to the pGEM markers in the lane marked M.

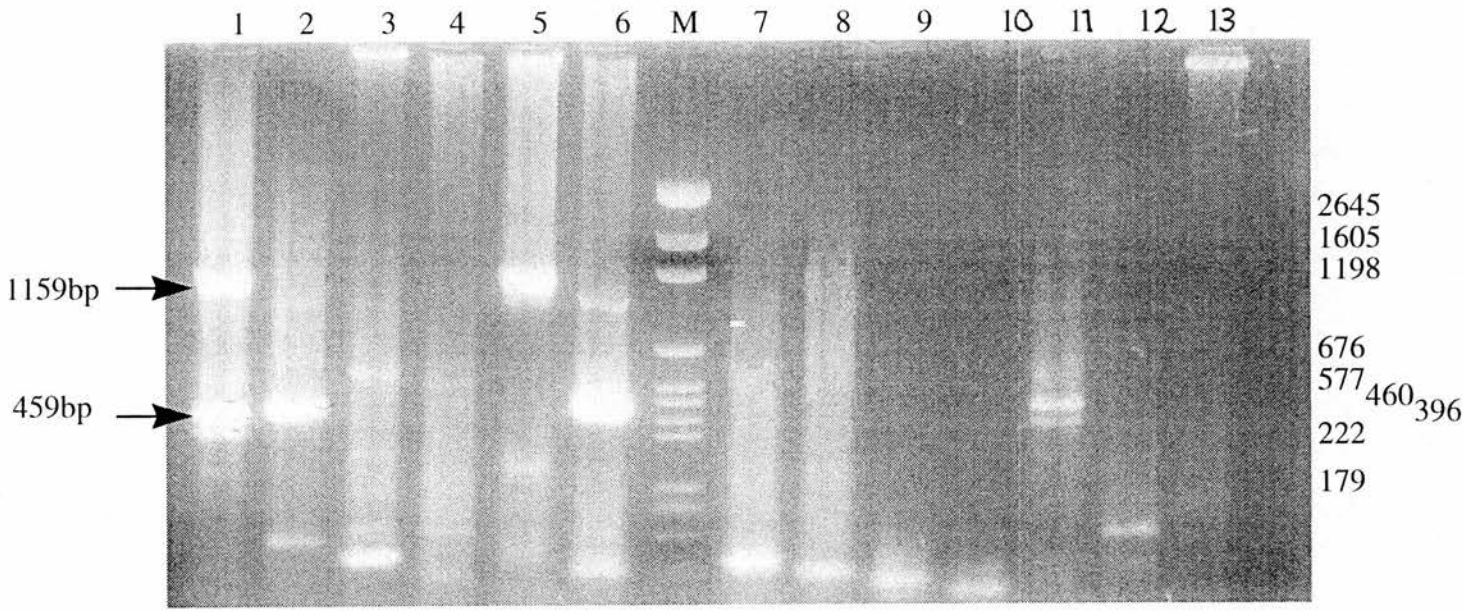
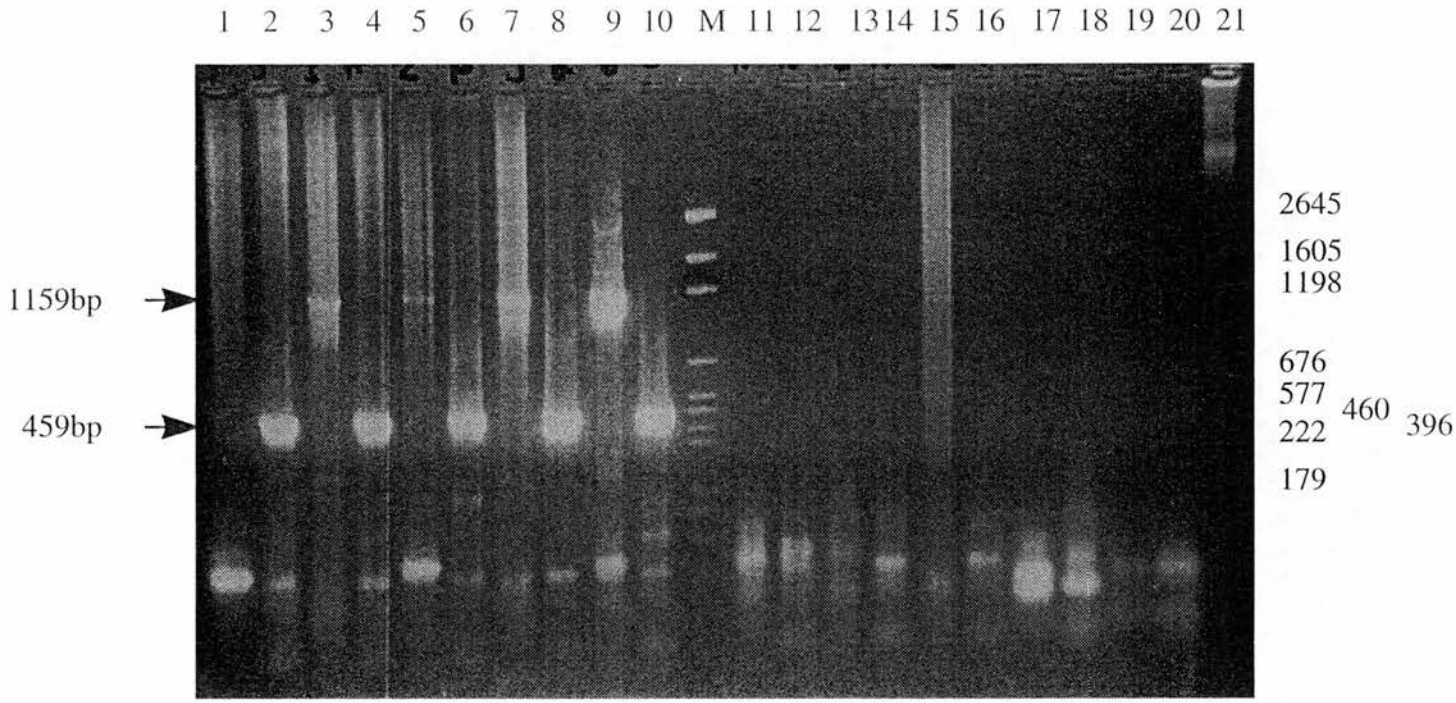

1 2 3 4 5 6 M 7 8 9 10 11 12 13
1159bp
459bp
2645
1605
1198
676
577
460
396
222
179

Figure 6.6 **Limits of detection of CD4**

PCR gel after RT-PCR for CD4 using both primer sets. HeLa-CD4 RNA was titrated out from 1µg (in lanes 1 and 2) to 1pg (lanes 13 and 14) in the RT-reaction. The cDNA was used in a nested PCR. The secondary product is shown. Bands for the larger CD4 product (expected size 1195bp) and the smaller CD4 product (expected size 459bp) are indicated by arrows. The pGEM markers are shown in the lane marked M, and the numbers on the right hand side refer to the sizes of these markers. Lanes 15 to 21 contain the negative controls: lanes 15 and 16 (no RNA), lanes 17 and 18 (no cDNA), lanes 19 and 20 (no Taq) and lane 21 (no primers).


1 2 3 4 5 6 7 8 9 10 M 11 12 13 14 15 16 17 18 19 20 21
1159bp
459bp
2645
1605
1198
676
577
460
396
222
179

The primary PCR for β-Actin was carried out (described in 2.13.3), using primers and a method obtained from Stratagene. The primer sequences are detailed in 2.13.3.1. Bands corresponding to the expected product size (661bp) of β-Actin are shown in figures 6.2 and 6.3.

A secondary primer was also obtained for the β-Actin PCR, in a nested reaction which gave a secondary PCR product of 501bp. The primer was designed by A. Alonso, and synthesised by Oswel DNA Services. Details of the primers are given in 2.13.1.2. The secondary PCR for β-Actin was tried using the primary products obtained in 6.2.3 (comparison of the two RT enzymes). The secondary products (20µl) were electrophoresed on a 1.2% agarose gel against pGEM markers, at 150V for 1.5 hours.

The results are shown in figure 6.7. Bands for all the dilutions of RNA (described in 6.2.3, and legend to figure 6.3) were seen from the secondary PCR of β-Actin. In the lanes containing higher initial amounts of RNA, a second band was also seen with the secondary PCR product. This band was the same size as the primary product for ß-Actin, and was probably residual primary product. As this was the last preliminary experiment, the secondary PCR was only used for the final experiment.

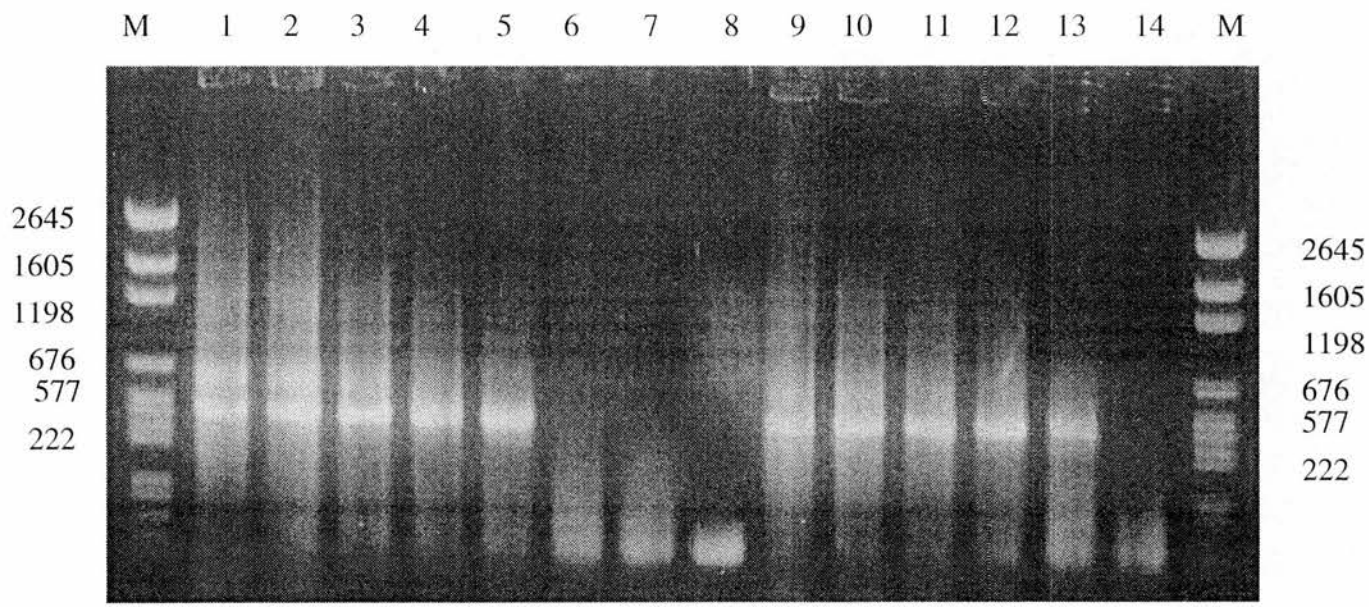
6.2.6 PCR FOR HIV-1

A CD4 cell productively infected with HIV-1 would be more likely to show surface CD4 down-regulation, than a latently infected one. Therefore, as the detection of HIV-1 DNA would not distinguish between latently and productively infected cells, the presence of HIV-1 mRNA was investigated to see whether the virus was actively replicating in the same cell fractions.

This was done using primers for a region of the *env* gene, which produces spliced mRNA, i.e. the regions in the HIV-1 genome are quite distant (the primary sense primer anneals at 173bp, and the antisense primer anneals at 5725bp). When mRNA is made, the exons within the genomic DNA are removed and a small product is given (in this case a product of 215bp) (Zack *et al.*, 1990; Schwartz *et al.*, 1990). A secondary primer was designed by A. Alonso.

Figure 6.7 **Secondary PCR for β-Actin**

PCR gel showing the products of a secondary PCR for ß-Actin. The legend is as for figure 6.3


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 M
2645
1605
1198
676
577
222
2645
1605
1198
676
577
222
Expand™-RT
AMV-RT

The primers and method are described in 2.13.4.

6.2.6.1 Analysis of subtype A/D strains of HIV-1

As the child to be studied was from central Africa, it was necessary to check whether the HIV-1 primers were suitable for subtype A and subtype D viruses, as they had originally been designed using a subtype B strain of HIV-1 (Zack *et al.*, 1990; Schwartz *et al.*, 1990). Three different viruses were used, derived from Zairian patient strains. Two were subtype D viruses (HIV-1_{ELI} and HIV-1_{Z129}) and the other a subtype A virus (HIV-1_{MAL}). Human T-cell lines infected with these subtypes of HIV-1 were available (from the MRC AIDS Reagent Programme): CEM cells infected with HIV-1_{ELI} (called CEM_{ELI} here), H9 cells infected with HIV-1_{Z129} (called $H9_{Z129}$ here), or with HIV-1_{MAL} (called $H9_{MAL}$ here). These cells were snap-frozen and the RNA extracted (described in 2.12.2). The RT step was done using AMV-RT and the resulting cDNA was used in nested PCRs for CD4, HIV-1 and a primary PCR for β-Actin. The PCR products (10µl) were run on a 1.3% agarose gel against pGEM markers, at 150V for 1.5 hours.

The results are shown in figure 6.8. Bands corresponding to the sizes of the expected PCR products were seen for Actin (661bp), CD4 (459bp) and HIV-1 (179bp). PCR products were seen for each of these HIV-1-infected cells lines indicating that the primers were appropriate for recognition sequences from these HIV-1 subtypes.

6.3 EXPERIMENT

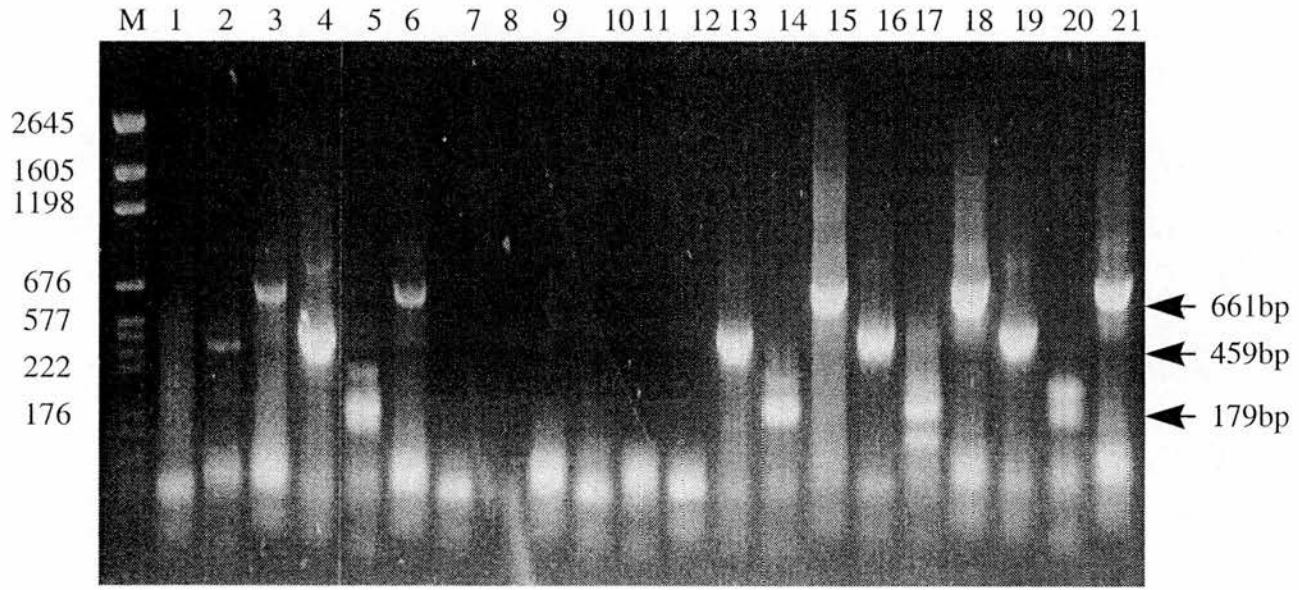
Although the phenomenon of the lymphocyte levels, had been seen over a period of 18 months, only one fresh blood sample was available from the index child.

The blood samples used were as follows:-

Sample P116[§] the HIV-1-infected child under investigation (index child)

Figure 6.8 **Detection of HIV-1 in cell lines infected with HIV-1 subtypes A and D**

PCR gel showing the secondary products of CD4 and HIV-1 and the primary product of ß-Actin (expected product sizes 459bp, 179bp and 661bp, respectively), marked by arrows. The products were loaded in the order of CD4, HIV-1 and ß-Actin. Lanes 1 to 3 contain supernatant from an MT2-culture with cells from the index child; lanes 4 to 6 contain supernatant from an MT2-culture with cells from another HIV-1-infected child; lanes 7 to 12 contain the negative controls: lanes 7 to 9 (no RNA), lanes 10 to 12 (no cDNA); lanes 13 to 21 contain the cell lines infected with different HIV-1 strains: lanes 13 to 15 (CEM_{ELI}); lanes 16 to 18 ($H9_{Z129}$); lanes 19 to 21 ($H9_{MAL}$). The numbers down the left hand side correspond to the pGEM markers in the lane marked M.


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
2645
1605
1198
676
577
222
176
661bp
459bp
179bp

Controls

P69[§] an HIV-1-infected child with a steady CD4%, whose CD4% and CD8% add up to the CD3%

P92[§] an HIV-1-infected child with a very low CD4%, but whose CD4% and CD8% add up to the CD3%

P144 an HIV-1-uninfected child born to an HIV-1-infected mother

CB1[¶] a cord blood as a 'thymocyte control'

$H9_{MAL}$)

$H9_{Z129}$) cells infected with African HIV-1 strains

CEM_{ELI})

[§] For P116, P69 and P92, samples of diluted whole blood (diluted 1:3 in PBS) were available as well as the separated PBMCs.

[¶] The cord blood was included as the earliest available source of CD3 lymphocytes which would be newly emerging from the thymus. These may have residual mRNA expression from the stage of development at which these cells co-express CD4 and CD8 (Marodon and Rocha, 1994).

For each blood sample, the percentages of cells expressing CD3, CD4 and CD8, and the percentage of cells expressing both CD4 and HLA-DR were measured (described in 2.3.1). The expression of HLA-DR on CD4-expressing cells was measured as an indication of the activation state of the cells. It has been suggested that, in HIV-1 infection, only those cells which express HLA-DR are actively replicating (Malhalingham *et al.*, 1995).

PBMCs were isolated from peripheral blood of the index and control children, and separated into CD4+ve and CD4-ve cell fractions by magnetic beads (outlined in 6.2.1 and described in detail in 2.11). The RNA from each cell fraction was extracted using the Stratagene RNA Micro-Isolation kit (described in 2.12.2).

RNA preparations were screened on a Spectrophotometer at wavelengths of 260 and 280nm to measure the amounts of RNA available (described in 2.12.3). The RT-step was done using Expand^{TM}-RT (described in 2.12.2). The amount of total RNA put in the initial RT reaction was adjusted to approximately 0.5µg. The nested PCR reactions for CD4, HIV-1 and ß-Actin were carried out (described 2.13.2, 2.14.3 and 2.14.4). The secondary PCR products (20µl) were separated by electrophoresis on a 1.3% agarose gel at 150V for 1.5 hours.

6.4 RESULTS

The percentages of cells expressing CD3 (CD3%), CD4 (CD4%) and CD8 (CD8%) from the index child, over a period of time, are presented in table 6.1. The numbers in bold represent the difference between the CD3% and the sum of the CD4% and CD8%. Results from two flow cytometry systems, using antibodies from either Becton Dickinson (BD) or Ortho, showed that approximately 20% of CD3 expressing cells did not express CD4 or CD8. For comparison, the CD3%, CD4% and CD8% from an age-matched HIV-1-uninfected child (P86) are also shown. The sample used in the experiment had a lower proportion of the cells under investigation than that seen in previous blood samples. Using the BD antibodies, this proportion was not much different from the population seen in the uninfected child. However, a larger difference was seen from the using the Ortho antibodies.

The CD3%, CD4% and CD8% from the index and the control children are presented in table 6.2. Results using the Ortho antibodies are not presented as these were not available for all the control samples used. The percentage of activated CD4 cells (expressing HLA-DR) are also shown.

Two examples of the FACScan plots of the PBMC populations are shown in figure 6.9(a) and (b), before (A and B), and after (C and D) CD4 cell depletion by immunomagnetic beads. The results presented here are from the index case, P116, (figure 6.9(a)), and from one of the HIV-1-infected control patients, P69 (figure 6.9(b)) and are representative of the separation results for all the samples.

For the index child (P116) both before and after CD4 cell depletion, there were no cells which expressed CD4 on the surface, detectable by flow cytometry (figure 6.9(a)). However, approximately 10% of the gated lymphocytes expressed CD3 but not CD8. These were seen both before and after the CD4 depletion, and are shown in the lower right quadrants of boxes A and C.

For the HIV-1-infected control (P69) before CD4 cell depletion, approximately 50% of the gated cells expressed CD8 figure 6.9(b), (A), and approximately 21% of the gated cells expressed CD4 (B). After CD4 cell depletion, approximately 65% of the gated cells

Table 6.1 **Percentages of CD3, CD4 and CD8 lymphocytes in P116 over time**

Sample date	BD antibodies				Ortho antibodies*			
	CD3	CD4	CD8	CD3+ DN	CD3	CD4	CD8	CD3+ DN
26/11/93	79	11	49	**19**	-	-	-	-
04/03/94	67	11	49	**7**	-	-	-	-
17/06/94	72	18	45	**9**	-	-	-	-
07/10/94	77	7	52	**18**	-	-	-	-
28/10/94	76	5	53	**18**	72	3	34	**35**
18/11/94	-	-	-	-	73	3	45	**25**
20/12/94	-	-	-	-	74	1	49	**24**
03/03/95	70	1	52	**17**	72	1	43	**28**
03/05/95	64	1	47	**16**	61	1	37	**23**
#09/09/95	44	0	39	**5**	57	1	42	**14**
P86	80	47	29	**4**	80	48	27	**5**

Percentages of CD3, CD4 and CD8 lymphocytes are shown.

The results of an age-matched HIV-1-uninfected child (P86) are also shown.

CD3+ DN denotes the difference between the total CD3% and the sum of the CD4% and the CD8% (i.e. CD3 positive, CD4 and CD8 (double) negative).

* The results using the Ortho antibodies were produced with permission of J.A. Whitelaw, HIV Immunology Unit, Royal Infirmary, Edinburgh.

\# Sample used for the index child in the final experiment.

Table 6.2 **Lymphocyte percentages in the index and control children**

Sample	CD3	CD4	CD8	CD3+CD4-CD8-	% DR+ CD4
Index child (P116)	44	0	39	**5**	NA
HIV+ve control (P69)	82	25	54	**3**	13
HIV+ve control (P92)	64	2	64	**0**	NA
HIV-ve control (P144)	68	43	26	**0**	2
Thymocyte control (CB1)	61	43	16	**2**	4

Percentages of lymphocytes in the index and control children.

%DR+ denotes the percentage of activated CD4 cells (expressing HLA-DR).

NA denotes not available

Figure 6.9 **Efficiency of the CD4 cell depletion**

FACScan plots of the separations before (A and B) and after (C and D) CD4 cell depletion.

The results shown are representative of the separation results for all the samples.

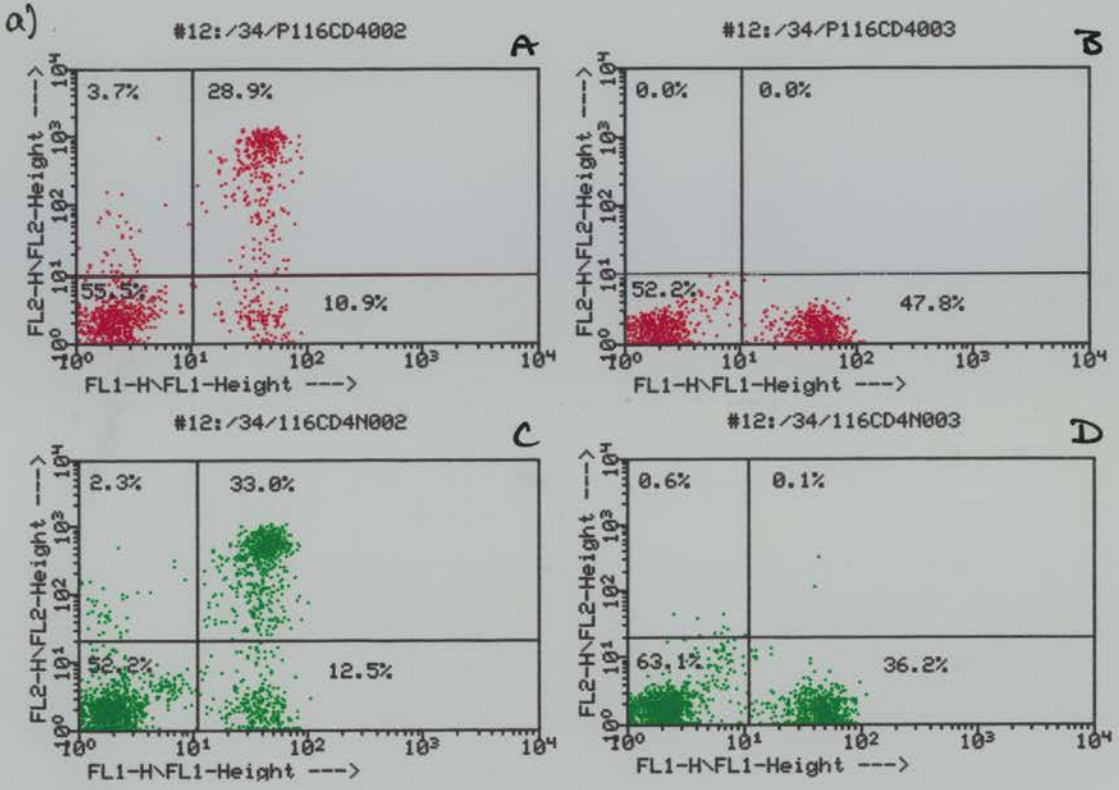
The combination of α-CD3 and α-CD8 antibodies are shown in boxes A and C. Cells expressing CD3 are shown on FL-1 (*x*-axis) and those expressing CD8 are shown on FL-2 (*y*-axis). Quadrant lines are those which were set from a control tube, such that less than 1% of cells showed non-specific staining. The upper right quadrant contains those cells which express both CD3 and CD8.

The combination of α-CD3 and α-CD4 antibodies are shown in boxes B and D. Cells which express CD3 are shown on FL-1 (*x*-axis) and those expressing CD4 are shown on FL-2 (*y*-axis). The upper right quadrant contains those cells which express both CD3 and CD4. The number in each quadrant is the percentage of cells in the lymphocyte gate which fall into that quadrant.

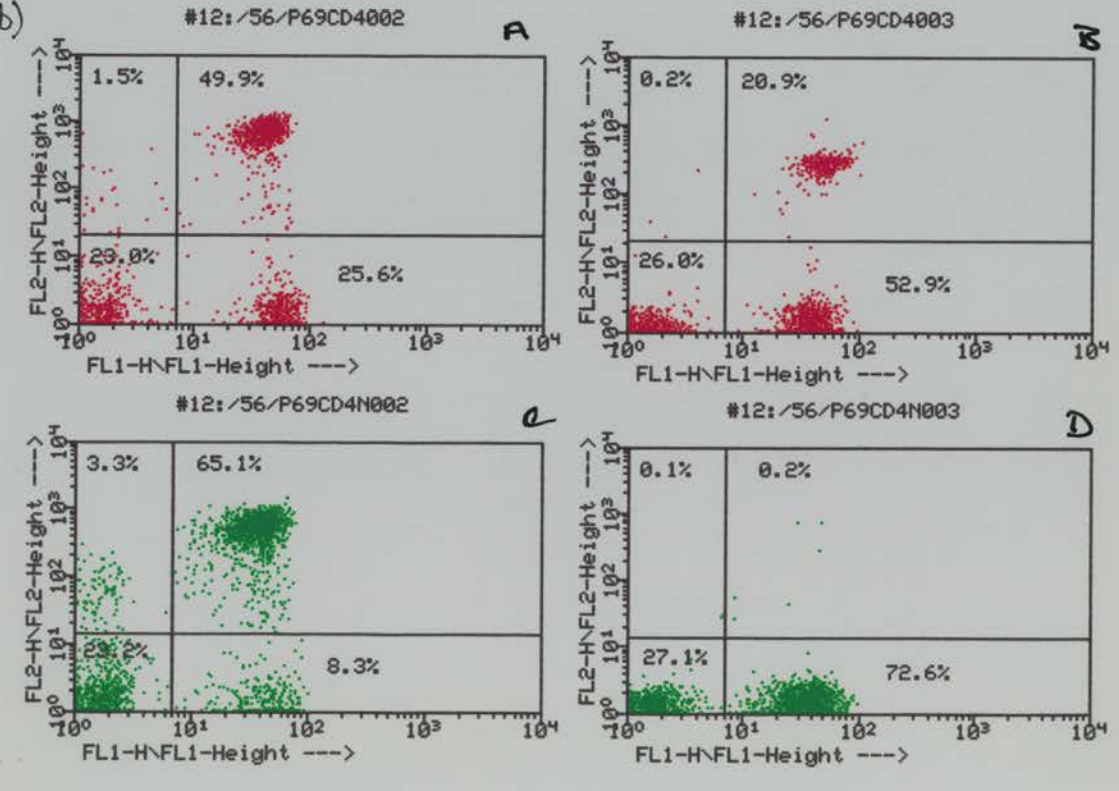
(a) Results for P116, the index child

(b) Results for P69, a control child

a)


#12:/34/P116CD4002
A
3.7%
28.9%
55.3%
10.9%
#12:/34/P116CD4003
B
0.0%
0.0%
52.2%
47.8%
#12:/34/116CD4N002
C
2.3%
33.0%
52.2%
12.5%
#12:/34/116CD4N003
D
0.6%
0.1%
63.1%
36.2%
FL2-H\FL2-Height --->
FL1-H\FL1-Height --->

b)


#12:/56/P69CD4002
A
1.5%
49.9%
23.0%
25.6%
#12:/56/P69CD4003
B
0.2%
20.9%
26.0%
52.9%
#12:/56/P69CD4N002
C
3.3%
65.1%
23.2%
8.3%
#12:/56/P69CD4N003
D
0.1%
0.2%
27.1%
72.6%
FL2-H\FL2-Height --->
FL1-H\FL1-Height --->

expressed CD8 (C), and less than 1% of the gated cells expressed CD4 (D), showing that the CD4 depletion efficiently removed the CD4-expressing cells. This child also had a small proportion of CD3-expressing cells which did not express either CD4 or CD8 figure 6.9(b), C (lower right quadrant).

Results of the RT-PCR for CD4, Actin and HIV-1 are shown in figure 6.10. These are results of the second of two experiments. Similar results were obtained in both experiments but the gel from the second was clearer.

In both experiments, bands corresponding to the product size of β-Actin were detected in all the samples from the children, and the HIV-1-infected cell lines. No bands were observed in the negative controls (as expected). This shows that there was cDNA added to the PCRs and that the RT step was successful.

In both experiments, bands corresponding to the product size of CD4 (459bp) were expected and seen in the CD4+ve fractions for the cord blood (lane 50) and in the diluted whole blood from the one of the HIV-1-infected control children (P69, lane 11) and two of the HIV-1-infected cell lines (lanes 30 and 33). In the second experiment bands were also seen from the CD4+ve fraction of the index child (lane 7) and the other HIV-1-infected control child (P92, lane 21). The first experiment gave an additional band from the HIV-1-infected cell line, CEM_{ELI}, which was absent from the second experiment, but had been seen previously (figure 6.8).

In both experiments, bands for CD4 were expected but not seen, from the diluted whole blood sample from the index child (lane 1), and the CD4+ve fractions of the control HIV-1-infected and uninfected children (lanes 16, 27 and 44).

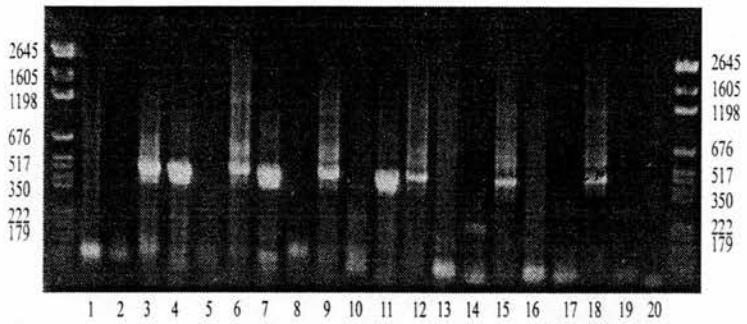
In both experiments, bands corresponding to the product size for CD4 were seen in the CD4-ve fractions from the index child (lane 4) and the cord blood (lane 47). These results indicate that there is mRNA for CD4 present within these cells.

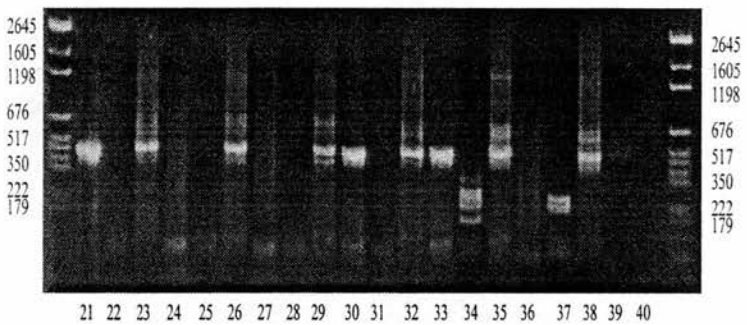
In both experiments, bands corresponding to the product size of HIV-1 (179bp) were expected and seen from the whole blood sample of one of the HIV-1-infected control children

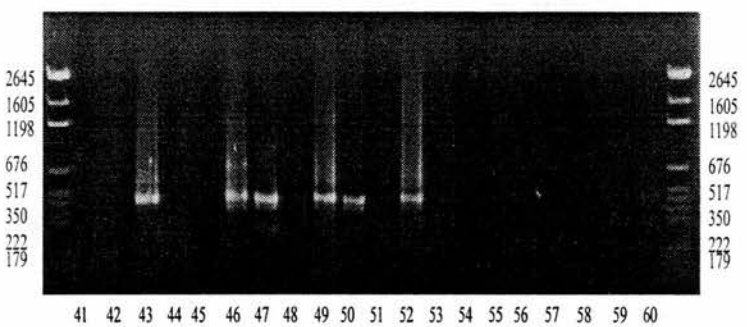
Figure 6.10 **PCR results for CD4, HIV-1 and Actin**

The PCR gel is shown in three sections. At each end of each section, the pGEM markers are shown to size the bands. The sizes of the pGEM bands are shown beside each section. The expected product sizes are 459bp for CD4, 179bp for HIV-1 and 501bp for Actin. The lane numbers are above each lane on the gel, and are described below:-

Lanes	Sample	
1 - 3	P116 diluted whole blood	CD4 / HIV-1 / Actin
4 - 6	P116 CD4-ve fraction	CD4 / HIV-1 / Actin
7 - 9	P116 CD4+ve fraction	CD4 / HIV-1 / Actin
10 - 12	P69 diluted whole blood	HIV-1 / CD4 / Actin
13 - 15	P69 CD4-ve fraction	CD4 / HIV-1 / Actin
16 - 18	P69 CD4+ve fraction	CD4 / HIV-1 / Actin
19	$H9_{MAL}$ CD4 Taq -ve control	
20	$H9_{Z129}$ HIV-1 Taq -ve control	
21 - 23	P92 diluted whole blood	CD4 / HIV-1 / Actin
24 - 25	P92 CD4-ve fraction	CD4 / HIV-1 / Actin
27 - 29	P92 CD4+ve fraction	CD4 / HIV-1 / Actin
30 - 32	$H9_{MAL}$	CD4 / HIV-1 / Actin
33 - 35	$H9_{Z129}$	CD4 / HIV-1 / Actin
36 - 38	CEM_{ELI}	CD4 / HIV-1 / Actin
39	CEM_{ELI} Primer -ve control	
40	Empty	
41 - 43	P144 CD4-ve fraction	CD4 / HIV-1 / Actin
44 - 46	P144 CD4+ve fraction	CD4 / HIV-1 / Actin
47 - 49	CB1 CD4-ve fraction	CD4 / HIV-1 / Actin
50 - 52	CB1 CD4+ve fraction	CD4 / HIV-1 / Actin
53 - 55	RNA -ve control	CD4 / HIV-1 / Actin
56 - 58	cDNA -ve control	CD4 / HIV-1 / Actin
59	CB1 CD4-ve Actin Taq -ve control	
60	CB1 CD4+ve Primer -ve control	


2645
1605
1198
676
517
350
222
179
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
P116 WB P116 4- P116 4+ P69 WB P69 4- P69 4+


2645
1605
1198
676
517
350
222
179
21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
P92 WB P92 4- P92 4+ H9MAL H9Z129 CEMELI


2645
1605
1198
676
517
350
222
179
41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60
P144 4- P144 4+ CB1 4- CB1 4+ RNA -ve cDNA-ve

(P69, lane 10) and from two of the three HIV-1-infected cell lines (lanes 34 and 37).

In both experiments, bands for HIV-1 were expected, but not seen, from the whole blood samples of the index child (P116) and the other HIV-1-infected control child (P92, lanes 2 and 22, respectively); from the CD4+ve fractions of all three HIV-1-infected children (lanes 8, 17 and 28) and from the third HIV-1-infected cell line (lane 31), although this band had been seen previously (figure 6.8).

In both experiments, a band corresponding to the expected product size of HIV-1 was seen in the CD4-ve fraction of one of the HIV-1-infected control children (lane 14). These results indicate that HIV-1 is present and actively replicating within these cells.

6.5 DISCUSSION

A significant proportion of T cells expressing surface CD3, but not CD4 nor CD8, was noted in an HIV-1-infected child. The aim of this study was to see whether these cells were synthesising, but not expressing, the CD4 molecule, and whether this was associated with HIV-1 replication within the same cell population. Down-regulation of surface CD4 by HIV-1 has been reported in T cell lines and in *in vitro* HIV-1-infected PBMCs (Chen *et al.*, 1996), but thus far, not in HIV-1-infected individuals. Therefore, a strategy was developed which would identify any cells which were actively synthesising CD4, but not expressing the protein at the cell surface.

Cells expressing CD4 were positively depleted using anti-CD4 antibodies and immunomagnetic beads. Positive depletion was used (i.e. the CD4 expressing cells were removed) as this would remove monocytes as well as T cells. The remaining CD4-ve fraction should not include any cells expressing CD4 and the flow cytometry results confirmed that the CD4 expressing cells were effectively removed. It was decided not to deplete the PBMCs for CD8 cells as well as CD4 cells, as the cell yield after a second manipulation with magnetic beads could be so low as to be undetectable. Therefore throughout these experiments the CD4-ve fraction includes cells expressing CD3 and CD8,

as well as the cells of interest. Other experiments using cells which were positively depleted for CD8 (i.e. CD8+ve fractions), from normal donor PBMCs did not shown any CD4 mRNA (not shown), nor did the CD4-ve fractions from the HIV-1-infected controls, which shows that there is no cross-reaction with genomic DNA for CD4.

Total RNA from the cells was initially extracted using a phenol/chloroform method which isolated all the nucleic acids from cells. As it was thought, initially, that the amounts of DNA in the preparation may interfere with the RT-PCR, either by increasing the amounts of non-specific sequences or by cross-reaction with genomic DNA, a DNAase step was included. However, the enzyme may not have been efficiently degraded by heating at 80°C for 10 minutes, such that, during reverse transcription, any cDNA could have been removed by residual DNAase activity. An alternative approach, such as re-extracting the RNA by the chloroform/phenol method, was likely to decrease the yield of RNA. Therefore, the Micro-Isolation kit was deemed to be the most efficient method and was shown to give a better yield of PCR product when compared with the phenol/chloroform extracted DNA/RNA.

The reverse transcription of mRNA by an oligo-dT primer annealing to the 3'-poly-A tail, allowed all the available mRNA to be transcribed to cDNA. As this step is not very efficient, the Expand™-RT enzyme was used, which was promoted as giving a better yield of cDNA. The results showed that it gave PCR products from smaller starting amounts of total RNA, than did the AMV-RT. The limiting factor in the final experiment was the small number of cells from an end-stage HIV-1-infected child, when only a very small amount of mRNA would be available. The limit of detection of the CD4 PCR was found to be a starting amount of 100 pg (0.1ng) of total RNA from a CD4-expressing T cell line (HeLa-CD4 cells). However, these HeLa-CD4 cells were actively dividing, and about a third of them expressed surface CD4 (data not shown). As a higher proportion of the total RNA from these cells, would be CD4-specific mRNA than that found in PBMCs from an individual, the starting amount of total RNA was adjusted to approximately 500ng for each sample in the final experiment, to try to ensure that the mRNA levels were above the limit of detection.

The CD4 RT-PCR was adapted from a published method (Fomsgaard *et al.*, 1992), which combined reverse transcription with a primary PCR step, to amplify the specific CD4 sequences directly from mRNA. The initial difficulties encountered could have been due to

the very low level of CD4 mRNA in normal, resting PBMCs. In retrospect it would have been better to start with DNA/RNA from a CD4-expressing cell line. The separation of the RT and PCR steps, the acquisition of new primers and the use of RNA from CD4-expressing cell lines gave bands on a gel of the expected size for CD4. The separation of the RT and PCR steps also allowed the cDNA to be probed for β-Actin as a positive control for the presence of cDNA, and HIV-1, to see whether HIV-1 was replicating in the cells which did not express CD4.

Bands of the same size as the expected product for CD4 were detected in both the CD4+ve and CD4-ve fractions from the index child (P116), indicating that CD4 mRNA was present. However, CD4 mRNA was not detected in the diluted whole blood sample from this child. For the other two HIV-1-infected children, CD4-PCR products were detected in the diluted whole blood, but not the separated cell fractions. The same amount of RNA from the whole blood samples from each HIV-1-infected child was used. The differences between these children, could be because the whole blood for the index child (P116) contained a lower proportion of total lymphocytes (approximately 5%), and the amount of mRNA for CD4 may have been below the limits of detection. The proportions of lymphocytes in the whole blood of the other two HIV-1-infected children were much higher (41% in P92 and 37% in P69). In addition, whole blood contains inhibitors of PCRs, which may have had a proportionally larger effect on the lymphocytes of the index child as there were fewer of them, than in the HIV-1-infected control children.

Despite the low numbers of cells available from the index child, CD4 mRNA was detected in the CD4-ve fraction, showing that these cells were synthesising CD4. The presence of CD4 mRNA may be due to some type of "feed-back mechanisms" of the cell which may detect the non-expression of CD4 at the cell surface, and therefore, direct the cell to increase biosynthesis of CD4. In the other two HIV-1-infected children these feedback mechanisms may not be used to the same extent, as, from the phenotype results, the CD3-expressing cells also expressed either CD4 or CD8. The absence of CD4 mRNA in the CD4+ve fraction of one of the other two HIV-1-infected children (P92) could be due to very low levels of CD4 cells (1% of lymphocytes). For the other HIV-1-infected child (P69), the absence of mRNA for CD4 could be explained by the low proportion of activated CD4 cells, as only the activated cells would be undergoing mRNA synthesis (Malhalingham *et al.*, 1995). Likewise,

the uninfected child (P144) did not show any CD4 mRNA in the separated fractions of the PBMCs. In this child, an even smaller proportion of those cells were activated, compared to the infected children, so most of the cells would not be undergoing mRNA synthesis. The cord blood showed CD4 mRNA in both the CD4+ve and CD4-ve fractions, which was confirmed when the experiment was repeated. This was unexpected as the flow cytometry plots were similar to those shown in figure 6.9 (data not shown). Repetition of the experiment with another cord blood and the RNA from P144 gave similar results (data not shown).

T cells, expressing CD4 or CD8, arise from the same stem-cell population in the thymus, which expresses both antigens on the cell surface (double positive). These cells then mature and become either CD4- or CD8-single positive. Different mechanisms are involved in the control of CD4 gene expression in immature (double positive) and mature (single positive) thymocytes (Hanna *et al.*, 1994) and the down-regulation of CD4 or CD8 expression occurs at different stages of thymocyte development (Marodon and Rocha, 1994). The results from the cord bloods suggest that there may be some residual CD4 mRNA expression in CD8-single positive lymphocytes, which have newly emerged from the thymus. The contribution of the thymus in the production of new CD4-expressing T cells, in older HIV-1-infected children is not clear. However, studies in *SCID-Hu* mice (Aldrovandi *et al.*, 1993) have shown that HIV-1 infection of foetal thymic tissue, leads to a depletion in the number of CD4-expressing T cells emerging from the thymus (Bonyhadi *et al.*, 1993; Aldrovandi *et al.*, 1993; Su *et al.*, 1995). The infection of a human foetal thymocyte cell line not expressing CD4, with HIV-1 *in vitro*, has also been reported (Hatch *et al.*, 1992). These studies have implications for the HIV-1 infection of the foetus *in utero* and has been discussed previously (see 3.4.3). In HIV-1-infected adults, thymic regeneration has been suggested as being unlikely (Ho *et al.*, 1995; Wei *et al.*, 1995). The CD4 mRNA in the CD4-ve fraction from the index child, may be from newly emerged cells from the thymus expressing surface CD8. However, evidence from studies in lymph nodes from HIV-1-infected adults showed the pathogenic effects of HIV-1 infection in the destruction of lymphoid tissue long before the levels of circulating, peripheral CD4 expressing lymphocytes were decreased (Pantaleo *et al.*, 1993; Embretson *et al.*, 1993). The assumption that the same pathogenic effects of HIV-1 in lymphoid tissue of children also occurs, suggests that it is unlikely for the thymus be producing functional cells. Therefore, the detection of CD4 mRNA in the CD4-ve fraction

from the index child, is more likely to be due to the down-regulation of surface CD4.

It is possible that the sequence of the CD4 molecule on the surface of the cells could have changed, such that the CD4 molecule was not recognised by the antibodies used. Polymorphisms within the CD4 molecule have been reported (Dean *et al.*, 1994). It has been previously reported, that the anti-CD4 antibody obtained from Ortho (clone OKT4) does not recognise the CD4 molecule in a small number of individuals. However, in these same individuals, the CD4 molecule was recognised by the anti-CD4 antibody obtained from BD (clone Leu 3a, Levy *et al.*, 1988). Previous results from the Edinburgh cohort have also found that CD4-expressing cells not detectable using the Ortho antibodies, were detectable using the BD antibodies (J.A. Whitelaw and M.C. Aldhous, unpublished observations). Comparison of the results from the index child, using both antibody sets, showed that there was a slight difference in the percentages of lymphocytes expressing the different surface molecules. However, cells expressing CD3, but lacking CD4 and CD8 in the index child, were detected using both sets of antibodies, indicating that the non-expression of surface CD4 is a more probable explanation.

Studies in HIV-1-infected adults have indicated that the turnover of CD4 cells is much higher than was previously thought, with approximately 2×10^9 cells being produced and destroyed per day (Ho *et al.*, 1995; Wei *et al.*, 1995). They suggest that this high cell turnover is driven by the high level of HIV-1 infection and production from these same cells. The detection of CD4 mRNA in whole blood or separated cells from the infected children studied here, indicates that some proportion of the CD4 cells are indeed actively replicating. Conversely, the non-detection of CD4 mRNA from the uninfected child, indicates that CD4 cell turnover could be much less. This was assumed in the studies previously mentioned (Ho *et al.*, 1995; Wei *et al.*, 1995), but the actual rate of cell turnover within an uninfected individual is not known.

Whether the non-expression of CD4 at the cell surface was associated with HIV-1 infection of the cell, is not clear, as HIV-1 was not detected. The non-detection of HIV-1 in the index child (P116) may be due to a low rate of HIV-1 replication. Using *in situ* hybridisation techniques it has been found that as few as 1 in 10^4 or 10^5 cells actively express HIV-1 in PBMCs (Harper *et al.*, 1986) and, as only a small number of cells were available from this

child, this could explain the non-detection of HIV-1. A few unidentified bands appeared on the gels, particularly from the HIV-1 PCR. One possibility is that reduced selectivity of the priming occurred, due to the other mRNAs which would have been made into cDNA. There is also the possibility of different HIV-1 transcripts being given, as the annealing site for the antisense primer encodes for some of the regulatory proteins, such as *vpr* and *vpu*, and these may, or may not, be transcribed depending on which part of the life-cycle the virus was going through (Zack *et al.*, 1990; Schwartz *et al.*, 1990). Bands corresponding to the expected product size of HIV-1 was identified from one of the HIV-1-infected controls (P69), in the diluted whole blood sample and in the CD4-ve population, but not the CD4+ve population and was seen in both experiments. This was unexpected and an explanation for this is not clear. The HIV-1 detected could be from some cell population that does not require CD4 for entry of virus, e.g. B-cells (Monroe *et al.*, 1988). In late-stage HIV-1-infected patients (with CD4 counts under 200 cells/µl), CD8 cells infected with HIV-1 have been described (Livingstone *et al.*, 1996). However, as this child still has a CD4 count of approximately 700 cells/µl, this was deemed unlikely.

The down-regulation of surface CD4 expression has been previously observed in PBMCs and T-cell lines infected with HIV-1 *in vitro* (Chen *et al.*, 1996), but has not been reported from HIV-1-infected individuals. It is possible that a similar population of cells has occurred in other HIV-1-infected individuals. However, this observation may have been missed, due to the emphasis on the routine monitoring of the CD4 count alone (Margolick *et al.*, 1992; Aledort *et al.*, 1992), rather than within the context of the levels of total T lymphocytes and those expressing surface CD8.

Cells expressing CD3 but not CD4 nor CD8 were not seen in any other child. The most obvious difference between the index child and the rest of the cohort, is that this child was born in Africa and is unlikely to be infected with a subtype B virus. It is possible that the down-regulation of CD4 *in vivo* may only occur with non-subtype B viruses. However, the effects of different viral subtypes on immune responses have not been widely studied. There is evidence that different subtypes have different tropism for Langerhans cells (Soto-Ramirez *et al.*, 1996) and that different subtypes may be transmitted more easily by different routes (Kunanusont *et al.*, 1995).

The loss of surface CD4 would have similar effects on immune function, as does the actual loss of T helper cells. Without the CD4-MHC class II interaction, the T helper cell would not be able to recognise the presence of exogenous antigen and, as with the loss of CD4 cells, the individual becomes more susceptible to opportunistic infections. Similarly, the T helper cells could not be activated in response to antigen recognition, so the production of cytokines is dysfunctional, which, in turn, leads to dysfunctional immune responses.

The preliminary results shown here indicate that the down-regulation of CD4 from the surface of the cell can take place *in vivo*, and therefore could contribute to CD4 cell dysfunction in HIV-1-infected patients.

Chapter 7

GENERAL DISCUSSION

7.1 T-CELL MEDIATED IMMUNE RESPONSES IN HIV-1-INFECTED CHILDREN

7.2 T-CELL MEDIATED IMMUNE RESPONSES AND LOSS OF CD4 CELLS

7.3 OTHER FACTORS AFFECTING DISEASE PROGRESSION

7.4 HIV-SPECIFIC IMMUNE RESPONSES IN EXPOSED UNINFECTED CHILDREN

7.1 T-CELL MEDIATED IMMUNE RESPONSES IN HIV-1-INFECTED CHILDREN

This thesis presents the results of the studies of different T-cell mediated immune responses which may be at work in HIV-1-infected children. The lymphocyte surface marker analysis showed that a sustained increase in the proportion of activated and memory CD8 cells occurred soon after birth, and that these can be used as indicators of HIV-1 infection in exposed children (chapter 3). The study of these surface markers in relation to functional assays indicated that the expression of the different antigens on the cell surface in whole blood bore no relation to their functional capacity. The study of CTL responses showed that the presence and specificity of target recognition varied, both within and between children. There was also evidence to suggest that the presence and specificity of the CTL response may contribute to disease progression (chapter 4). The study of proliferative responses showed that, as a group, HIV-1-infected children had reduced responses to TT, but maintained responses to PHA. There was no association between the responses to any antigen and clinical progression (chapter 5). Finally, the down-regulation of CD4 expression on the surface of cells was seen in one child (chapter 6).

There was no correlation between expression of surface markers on T cells and functional activity. This is not entirely surprising as there is still some debate as to the relationship between phenotype and function (Westermann and Pabst, 1996). An increase in the percentage of CD8 lymphocytes expressing activation and memory markers has been seen in newly infected adults (Zaunders *et al.*, 1995), which was associated with CTL responses to the virus (Pontesilli *et al.*, 1994; Hoffman *et al.*, 1989). Therefore, the increased expression of activation and memory markers on CD8 cells, indicate that some immune activation was occurring in these children.

7.2 T-CELL MEDIATED IMMUNE RESPONSES AND LOSS OF CD4 CELLS

Although recent studies of the turnover of CD4 cells *in vivo* indicate that cell killing occurs

rapidly (Ho *et al.*, 1995; Wei *et al.*, 1995), HIV-1 is not classed as a directly cytopathic virus (Zinkernagel, 1995). Therefore other mechanisms must be at work to produce CD4 cell loss. In these children, the different types and extent of the immune responses suggest that different mechanisms are implicated, such as CTL activity (chapter 4), dysfunctional proliferative responses (chapter 5) or surface CD4 down-regulation (chapter 6).

Reports have been published to suggest that HIV-1 preferentially infects CD4 cells with a memory phenotype (Schnittman *et al.*, 1990; Cayota *et al.*, 1990). In some adults, the loss of proliferative responses has been related to a loss of memory CD4 cells (Shearer and Clerici, 1991; van Noesel *et al.*, 1990). In the children studied here, antigen-specific proliferative responses were not related to any phenotype (chapter 5), and an increase in expression of memory or activation markers on CD4 cells was only seen much later in HIV-1 disease, as the CD4% started to decrease (chapter 3). This increase in activation or memory markers may reflect an increased incidence of HIV-infected CD4 cells in the periphery, later on in disease. It has been shown that the CD4 cells in lymph nodes are infected while the numbers of circulating CD4 cells are still steady (Pantaleo *et al.*, 1993; Embretson *et al.*, 1993). Therefore, in measuring the surface markers of lymphocytes in peripheral blood, it has to be remembered that these cells do not necessarily reflect the changes occurring in the tissues. Although there is cell trafficking, only approximately 2% of the total lymphocyte pool are circulating in the periphery at any one time, and the compartments are not in equilibrium (Ho *et al.*, 1995; Wei *et al.*, 1995). Therefore, a decrease in CD4% in peripheral blood may be an indication that the pathological effects of HIV-1 infection (direct or indirect) have already destroyed other lymphoid tissue. From the peripheral circulation, the virus can lodge in other areas of the body. In post-mortem AIDS patients, HIV-1 has been isolated from many different tissues which are not normally associated with lymphoid function, and some are sequestered from immune surveillance (Donaldson *et al.*, 1994).

There was no association between CTL activity and proliferative responses to HIV-1 in individual children. Two children did not respond to HIV-1 at all (i.e. consistently showed no CTL activity and did not proliferate to HIV-1 proteins), while two other children showed both CTL activity and proliferation to HIV-1 proteins. All the other children, for whom data were available, showed CTL activity, but not proliferation, to HIV-1. This suggests that the CTL responses are longer lived than proliferative responses in children. Similarly, the

proliferative responses to other stimuli (TT and PHA) did not correlate with CTL responses, as the responses to TT were reduced while the responses to PHA were retained in most children, regardless of their CTL responses.

One such child showed vigorous CTL activity to HIV-*tat* and -*pol* and a strong proliferative response to TT and PHA, but not HIV-1, despite the strong CTL response. These responses occurred in the context of a rapidly declining CD4% (a drop from 23% to 9% in 6 months). In this child, it is possible that the CTLs are actively killing both infected and uninfected 'bystander' CD4 cells, and hence, are contributing to the loss of CD4 cells. There is evidence that activated CD4 cells are more likely to be killed in this 'bystander' fashion (Grant *et al.*, 1994; Bienzle *et al.*, 1996). The high proliferative responses may be an indication of the ease with which these cells can be activated by other pathogens or stimuli *in vivo*, as if the cells were in some 'hyperactive' state. These antigen-specific responses may activate HIV-1 replication *in vivo*, and hence contribute to disease progression.

Conversely, another child showed an anti-HIV-1 proliferative response, which, although was not high, was above that seen in the controls. This child has consistently appeared to 'ignore' the virus, with a CTL response only induced after peptide stimulation, a lower proportion of (phenotypically) activated CD8 cells than that seen in other children, and a very slow decline in CD4 cells. Other cases of patients with similar non-responsiveness have been described (Dalod *et al.*, 1996; Ferbas *et al.*, 1995). However at some point, the virus levels in these other patients has increased, the immunological responses have become detectable, and an increased rate of CD4 cell loss was seen. The mechanism which causes increased virus levels, and hence the immune responses, in these other patients, was not clear. Whether this child will follow this pattern remains to be seen.

The finding that the down-regulation of surface CD4 expression can occur *in vivo* indicates another mechanism by which CD4 cell depletion occurs (as measured by the decreased proportion of T cells expressing CD4 on the cell surface). This child consistently showed no CTL responses during the study, which was initially assumed to be due to infection with a non-subtype B strain of HIV-1. It was speculated that this child may have CTL activity *in vivo*, even if it was undetectable *in vitro*, due to differences between the viral strains found in the child and those used in the CTL assay. However, the down-regulation of surface CD4

expression indicates that this may have had an impact on the generation of CTLs, due to a decreased T-helper function. If no CD4 is expressed on the cell surface, then this cell cannot interact with APCs via MHC class II and the CD4/T-cell receptor ceomplex (Paul, 1993). Therefore, the cell would be unable to activate cell signalling pathways to produce cytokines (Ravichandran and Burakoff, 1994; Kemeny *et al.*, 1994; Romagnani, 1992; Kos and Engleman, 1996; Mosmann and Sad, 1996), and so no other cells could be induced to provide an immune response to HIV or any other antigen.

7.3 OTHER FACTORS AFFECTING DISEASE PROGRESSION

These studies did not take into account other factors which may influence disease progression, such as maternal virus strain, HLA-type (Just *et al.*, 1995) and presence of the 32bp ß-chemokine receptor mutation (Dean *et al.*, 1996; Huang *et al.*, 1996). Whether there is a difference in the virus strains transmitted to the mothers by different routes, is not clear. Mucosal barriers and mucosal immune responses, may imply that a more virulent strain is required for successful transmission of infection by a sexual route rather than parentally. Indeed, in Thailand, where two epidemics appeared at the same time in almost exclusive risk populations, there are indications that the more virulent virus strain is transmitted sexually (Kunanusont *et al.*, 1995). For eight children, for whom information was available, born to seven HIV-1-infected mothers from the Edinburgh cohort, six mothers had intravenous drug use as their risk group, while one had been a partner of an intravenous drug user. The child whose mother had been infected sexually, did not have remarkably different responses from those observed in the other children. The influence of the maternal immune responses on the selection of the variants transmitted vertically is not clear, though there is evidence that selection occurs as, a minor, monocytotropic variant is often transmitted (Wolinsky *et al.*, 1992).

There have been reports that the rate of disease progression in children is related to the rate of disease progression in their mothers (Tovo *et al.*, 1994). Although the information is limited, this does not seem to be borne out in these children. The older children who did not progress were born in the mid-1980s, soon after their mothers became infected. These

women were part of the original Edinburgh cohort (Mok *et al.*, 1989a; Mok *et al.*, 1989b) and two of three of these mothers have died, after having been infected for several years. For the older children who did progress, born to mothers who were presumed to have been infected at the same time as those in the original cohort, two of three mothers have died, again after having been infected for several years. The younger children were born in the 1990s and all their mothers are still alive, including the mothers of the two children classed as fast progressors, despite one of the children having died. Some of these younger children were born after their mothers had been infected for several years. This could reflect the underlying disease progression in the mothers, which, in turn, increased the likelihood of transmission of HIV-1 (European Collaborative Study, 1991). There were also those who transmitted virus relatively soon after infection, which is similar to the scenario of the original cohort (Mok *et al.*, 1989a; Mok *et al.*, 1989b). However, there may be slight but important differences in the virus strains transmitted (C.M Wade *et al.*, J Virol 1997, submitted), which could account for any differences in clinical disease progression and immune responses in these children. To ascertain this, the virus populations in all the children would have to be isolated and the DNA sequences analysed.

Although the HLA-types for the children are known, the data set is too small for any influence to be seen (Just *et al.*, 1995). Preliminary data for the β-chemokine receptor CCR5 32bp deletion (Samson *et al.*, 1996; Liu *et al.*, 1996), showed that three of the infected children are heterozygous for this mutation, which has been claimed to influence the rate of disease progression in adults (Dean *et al.*, 1996; Huang *et al.*, 1996). However, these three children fall into each of the groups, defined in chapter 3, according to age and CD4%, and two children have also developed an SI variant of the virus, which is associated with disease progression (Schuitemaker *et al.*, 1992). The anti-HIV-specific responses differed in these children, as one showed CTL activity alone, but the other two had both proliferative and CTL responses to HIV-1.

In other viral systems such as lymphocytic choriomeningitis virus (LCMV), the level of the immune response was directly related to the dose of pathogen: i.e. a higher dose of LCMV produced a vigorous CTL response, which cleared virus but led to severe immunodeficiency, whereas a lower dose led to a chronic viral infection, without pathogenic effects (Moskophidis *et al.*, 1993). Other reports of studies of neonatal mice also indicate that a

different dose of pathogen can lead to either induction of tolerance, a Th-1 or a Th-2 response (Ridge *et al.*, 1996; Sarzotti *et al.*, 1996; Forsthuber *et al.*, 1996), all of which would affect the cell mediated-immune responses to that pathogen. In the children studied here, the dose of infecting virus from the mother may vary, according to the stage of her disease. However, the different responses to HIV-1 seen in these children, may indeed be due to the different levels of virus in each child.

7.4 HIV-SPECIFIC IMMUNE RESPONSES IN HIV-EXPOSED UNINFECTED CHILDREN

The detection of HIV-1-specific responses in the EU children, highlights the non-availability of control subjects, as it is considered unethical to take blood samples from children for studies from which they will not directly benefit. Therefore, whether the responses seen in the EU children are truly due to exposure to the virus, or whether some cross-reactive mechanisms have been at work, has not been verified. In a study of HIV-1-exposed adults, CTL responses to HIV-gag were seen in seronegative blood donors with no risk factors for infection (Langlade-Demoyen *et al.*, 1994).

Cell-mediated responses to HIV-1 were seen in the EU children, but these were confined to CTL responses, and not proliferation. This may be purely methodological: some studies have indicated that anti-HIV-1 proliferation can only be detected by measuring cytokine production, and that using ^{3}H-thymidine incorporation is not sensitive enough (Clerici *et al.*, 1992; Clerici *et al.*, 1993b), but this is not the case in other studies (Borkowsky *et al.*, 1990; Pontesilli *et al.*, 1995). Efficient CD4 recognition of HIV-1 produces cytokines, enabling the generation of CTLs, which, in turn, recognise endogenously processed antigen presented on the MHC-class I molecule. Some of the HIV-1-exposed children did recognise processed HIV-proteins in the CTL assay, as has been seen in other studies of exposed but uninfected individuals (Rowland-Jones *et al.*, 1993; Cheynier *et al.*, 1992; de Maria *et al.*, 1994a; Pinto *et al.*, 1995; Rowland-Jones *et al.*, 1995; Langlade-Demoyen *et al.*, 1994). This indicates that these children do have efficient CD4 responses to HIV-1 antigens, despite the lack of proliferation. These results also suggest that these children may have previously actively

processed antigen, either through a low dose exposure, or through an inefficient or abortive infection with HIV-1. Cases of children clearing HIV-1 have been described (Rowland-Jones *et al.*, 1993; Bryson *et al.*, 1995; Roques *et al.*, 1995; Aldhous *et al.*, 1994b). There are also cases described of children from whom HIV-1 has been transiently detected (by PCR or culture), without concomitant clinical infection (European Collaborative Study, 1991; de Rossi *et al.*, 1991; Rogers *et al.*, 1989).

The study of several different immune mechanisms in the same children has not been done before. There have been separate studies of surface markers, CTL activity and proliferation in different cohorts of children, but none trying to relate all these together in the same cohort. The information gained from these children suggests that no one immune mechanism is at work in mediating anti-HIV-1 responses, and that in some cases the anti-HIV-1 responses may contribute to pathogenesis. Therefore, to focus in on one specific set of responses, could be limiting. These results also suggest that no single immune response is responsible for the maintenance of clinical stability, or the induction of clinical progression in these children.

These results need to be confirmed in a larger group of children. In addition, although these children have been monitored for a period of up to four years, the differences in responses show that one cannot extrapolate from one child to the next. Therefore all the children should be studied from birth to death to obtain a clearer picture of the changes and significance of different immune responses during HIV-1 infection.

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APPENDIX

Aldhous M.C., G.M. Raab, K.V. Doherty, J.Y.Q. Mok, A.G. Bird and K.S. Froebel. Age related ranges of memory, activation and cytotoxic markers on CD4 and CD8 cells in children. *J Clin Immunol* **14**: 289-298 (1994a).

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Age-Related Ranges of Memory, Activation, and Cytotoxic Markers on CD4 and CD8 Cells in Children

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The expression of markers defining functional subpopulations on the surface of CD4 and CD8 cells changes with disease. To monitor these changes in children, it is important to establish the age-related normal changes in marker expression due to maturation of the immune system. We have studied the expression of several functionally important molecules on both CD4 and CD8 cells in 168 children (aged 0–122 months) using monoclonal antibodies and flow cytometry. Our results show that the percentage of CD4 cells decreases with age, while the CD8 percentage increases, resulting in a decrease in the CD4/CD8 ratio. The expression of CD45RO and CD29 increases with age, while CD45RA expression decreases, both on CD4 and CD8 cells. The expression of HLA-DR on both CD4 and CD8 cells, and of CD11a and CD57 on CD8 cells, is less clearly age dependent. The relationships between the marker percentages and age were not straightforward; the standard deviations and the skewness, as well as their mean values, varied as a function of age. The changes were modeled for each marker and age-specific centiles are presented.

KEY WORDS: Lymphocyte surface markers; normal children; age-related expression.

INTRODUCTION

The use of monoclonal antibodies (MAbs) and flow cytometry to monitor the CD4 and CD8 T-lymphocyte populations in peripheral blood is well established (1). Molecules defining subpopulations of CD4 and CD8 cells are associated with particular functions, and monitoring these subpopulations can help in understanding the immunopathogenesis of cellular immune responses.

The expression of subpopulation markers on CD4 and CD8 cells has been shown to be abnormal in a number of diseases. Increased expression of CD45RO, a marker of memory (2), on CD8 cells has been shown in infectious mononucleosis (3), in some DNA viral infections (T. Miyawaki, personal communication), and in HIV infection (4, 5), with a decrease in the reciprocal CD45RA "naive" marker (6). In contrast, CD29, also a marker of memory (7), has been shown by some groups to be lost in HIV infection (8, 9), although not confirmed by others (5, 10). CD8 lymphocytes show increased expression of the activation marker, HLA-DR, in infectious mononucleosis (3), and in HIV (5, 10, 11), which, together with an increased expression of CD57, a marker of natural killer activity (12), can be related to cell dysfunction (13, 14). An increase in CD11a, a marker which differentiates between suppressor and cytotoxic CD8 cells (15), has also been seen in HIV infection (16). The further evaluation of CD4 and CD8 subpopulations, both cross-sectionally and longitudinally, are potentially important in the study of cellular immune response mechanisms in both adults and children.

Children are born with immature immune systems (17) and studies of cellular phenotypes in disease states need to take into account the changes in marker expression with age. A number of studies of lymphocytes in normal children have concentrated on T and B cells (18–20), while others have included some CD4 and CD8 subpopulations (21–25). These studies, however, covered only a small number of markers over a limited age range.

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Table I. Details of the Children Studied

Group	Number	Mean age (mo)	Age Range (mo)
HIV positive mother	60	39.0	0–81
Premature	15	1.4	0–6
Preoperative[a]	18	29.7	4–76
Outpatients[b]	75	44.1	0–122

[a]Patients undergoing cardiac catheterization and/or surgery.
[b]Children with noninfectious or nonimmunological diagnoses.

The European Collaborative Study (ECS) (19) has provided age-specific centiles for the CD4 and CD8 cell numbers and percentages, up to 4 years of age in HIV-uninfected children born to HIV-infected mothers. The study presented here extends the age range and, also, defines the age-related expression of immunologically important CD4 and CD8 subpopulations in children.

One problem in determining normal ranges is access to blood from healthy children. We have used residual blood samples obtained from 108 children attending outpatient clinics, with known nonimmunological or infectious diagnoses, and 60 HIV-uninfected children born to infected mothers.

METHODS

Subjects. Residual heparinized peripheral blood samples were obtained from 168 children. Children were excluded whose diagnoses had evidence of immunological involvement such as pyrexia, infections, jaundice, or burns. The diagnoses of those studied, together with numbers and ages, are shown in Table I. The samples were stored at room temperature and processed on the same day as venesection.

Immunofluorescence Analysis. Blood phenotypes were analyzed using standard dual-color flow cytometry with the following monoclonal antibodies: anti-CD4(Leu3a)-fluorescein isothiocyanate (FITC), anti-CD8(Leu2a)-PE, anti-HLA-DR-FITC, anti-CD45RA(Leu18)-FITC (Becton–Dickinson), anti-CD11a(S6F1)-RD1/anti-CD8-FITC, anti-CD29(4B4)-FITC (Coulter), and anti-CD45RO(UCHL1)-FITC (DAKO Ltd). All antibody mixes were diluted 1:2, except for CD11a, which was used at 1:16. Briefly, diluted blood was added to the antibody pair and incubated for 15 min at room temperature. The red cells were lysed using Facslyse (Becton–Dickinson). The samples were centrifuged, washed in saline, fixed in 1% formaldehyde/phosphate-buffered saline, and stored at 4°C until analyzed on a FACScan (Becton–Dickinson). Results are expressed as the percentage of CD4 or CD8 cells coexpressing the antigen studied: $(CD4^+ CDX^+/CD4) \times 100$.

Statistical Analysis of Results. Each child contributed only one sample to the data set. Results below the level of detection of the assay were assigned a value of 1%, which was the detection limit for all markers. Age-related centiles were calculated for each marker by the method described in the appendix. All values beyond three standard deviations from their age-specific mean were checked and/or recalculated from the stored FACScan data.

For all the markers the age centiles allow the data to be transformed to Z scores, which measure the deviation of a value from its age-specific median in standard deviation units. These Z scores were then compared across subgroups to check for any differences.

RESULTS

Our results are presented throughout as centiles, over an age range of 0–100 months. These show the median and ranges (the values between the upper and the lower centiles) within which a defined proportion of the results lie.

The fitted centiles for the CD4 percentage (CD4%), CD8 percentage (CD8%), and CD4/CD8 ratio (dotted lines) are compared to the results for the ECS (solid lines) in Fig. 1. The lines illustrated are the 5th, 25th, 50th (median), 75th, and 95th centiles for each case. Figure 2 shows the data and the fitted centiles for the other 10 subpopulations measured. The predicted values of the age-specific medians are given in Table AI (Appendix).

CD4 and CD8 Populations

In the first 20 months of life, the CD4% decreased with age (the range between the 3rd and the 97th centiles decreased from 34–67 to 25–57%) and then remained fairly steady, while the range became slightly narrower (26–52% at 100 months). The CD8% decreased slightly during the first 20 months (from 15–38 to 14–34%) and then increased steadily with age (25–43% at 100 months). Consequently, the CD4/CD8 ratio also decreased steadily, with the range also becoming narrower with age (from 1.0–3.8 at birth to 0.8–1.8 at 100 months).

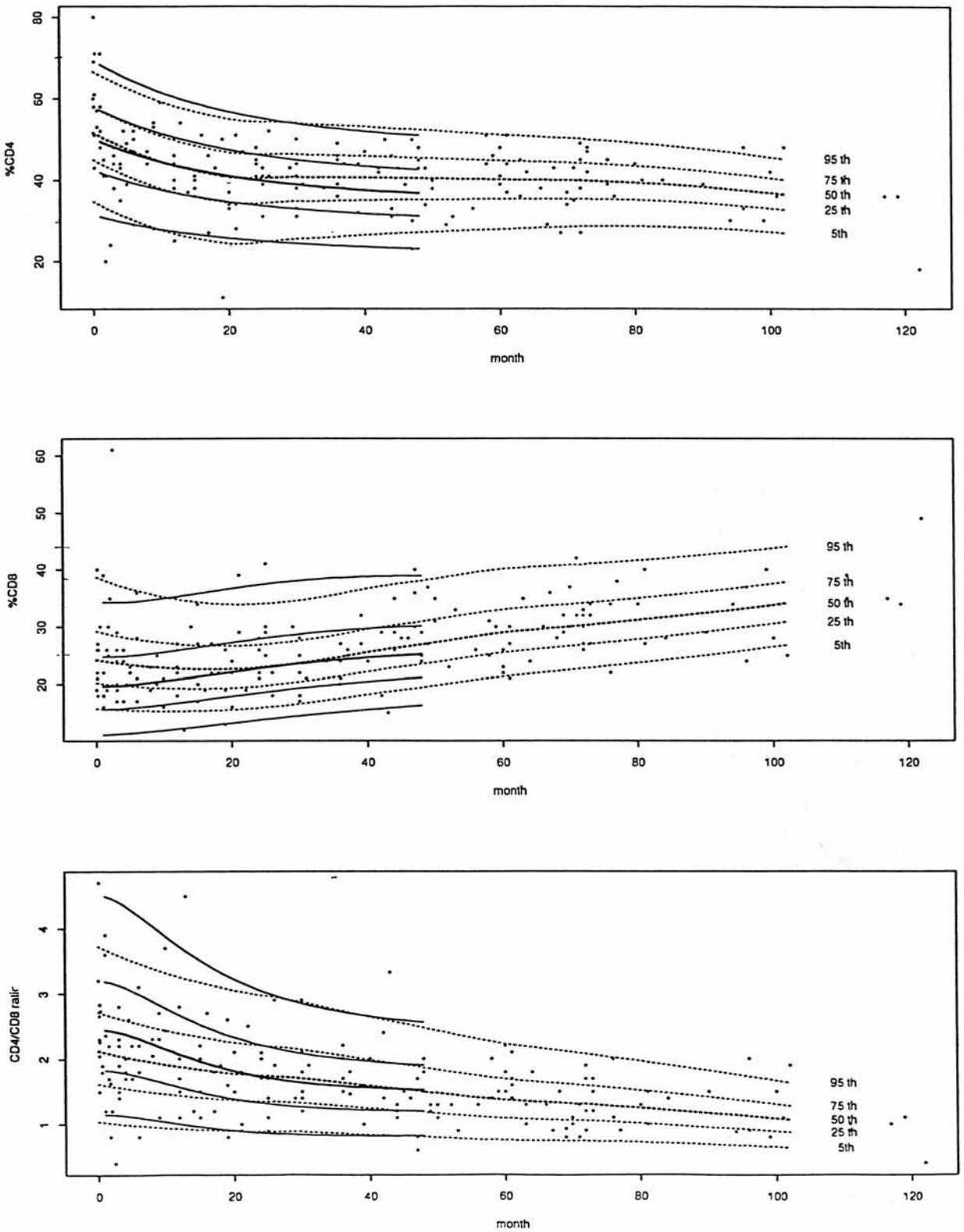

Fig. 1. Centiles for percentage of CD4 and CD8 cells and CD4/CD8 ratio compared to the ECS (19). Solid lines denote results for the ECS. Dotted lines denote our centiles. Lines illustrated are the 5th, 25th, 50th (median), 75th, and 95th centiles.

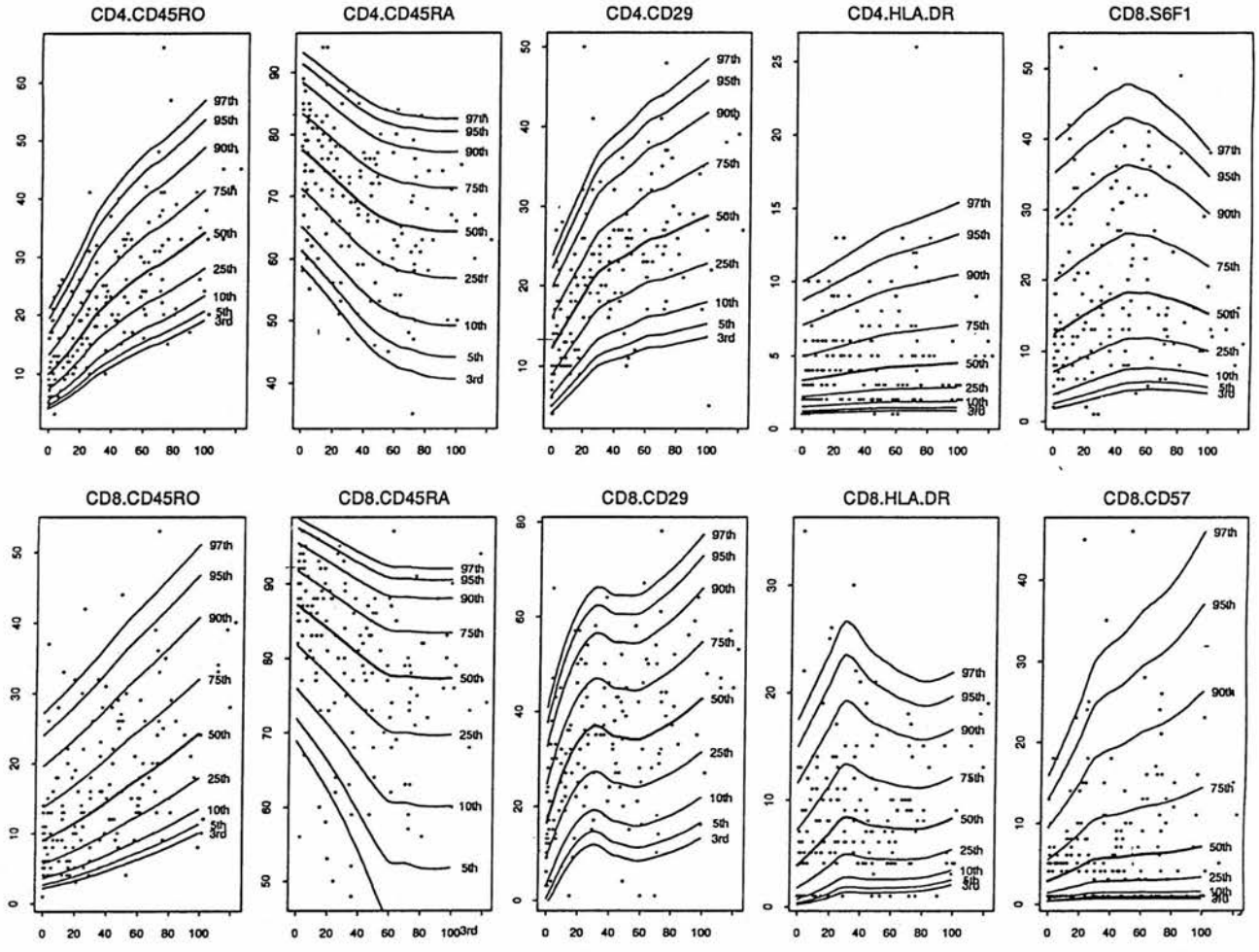

Fig. 2. Data and fitted centiles for marker coexpression. Lines illustrated are the 3rd, 5th, 10th, 25th, 50th (median), 75th, 90th, 95th, and 97th centiles. Axes are percentage coexpression of marker on CD4 or CD8 cells against age in months.

Memory Cell Markers

CD45RO expression on CD4 and CD8 cells increased with age (3–21% at birth to 18–58% at 100 months on CD4 cells and from 3–27% at birth to 10–52% at 100 months on CD8 cells), with the range also increasing, especially on CD4, where there was a lower and narrower range at birth. CD29 expression also increased markedly with age (from 3–24% at birth to 13–48% at 100 months on CD4 cells and from 1–41% at birth to 14–78% at 100 months on CD8 cells), with the rise being steepest in the first 2 years. In contrast, CD45RA expression on CD4 and CD8 cells was high at birth (59–93% on CD4 cells and 69–99% on CD8 cells) and decreased steadily, leveling off at approximately 60 months, and any further decrease was more gradual. The range increased over this time (42–83% on CD4 cells and 44–92% on CD8 cells at 100 months).

Activation Markers

There was no clear relationship of HLA-DR expression on T cells with age. The range from the 3rd to the 97th centiles at birth was 0–10 and 0–17% for CD4 and CD8 cells, respectively. Thereafter the lower centiles were still around zero, but the upper centiles rose to 16% on CD4 cells and 23% on CD8 cells at 100 months. The expression of HLA-DR on CD8 cells seemed to peak during the third year and subsequently leveled off.

Cytotoxic Markers

CD11a expression on CD8 cells showed little relationship with age. A wide scatter occurred at all ages (2–40% at birth, 5–48% at 48 months, and 5–39% at 100 months), and a peak of higher values

Table II. Correlations Between *Z* Scores for Different Markers

	(a) Correlations among CD4%, CD8%, ratio, and CD4 subpopulations						
	CD4	CD8	Ratio	CD4 CD45RO+	CD4 CD45RA+	CD4 CD29+	CD4 HLA-DR+
CD4	*						
CD8	−0.39	*					
Ratio	0.79	−0.86	*				
CD4 CD45RO+	−0.39	0.20	−0.36	*			
CD4 CD45RA+	—[a]	—	—	−0.51	*		
CD4 CD29+	−0.34	0.22	−0.33	0.65	−0.50	*	
CD4 HLA-DR+	−0.21	—	−0.20	0.20	—	0.23	*

	(b) Correlations between CD8 subpopulations					
	CD8 CD45RO+	CD8 CD45RA+	CD8 CD29+	CD8 HLA-DR+	CD8 CD11a+	CD8 CD57+
CD8 CD45RO+	*					
CD8 CD45RA+	−0.46	*				
CD8 CD29+	0.30	−0.20	*			
CD8 HLA-DR+	0.46	−0.21	0.27	*		
CD8 CD11a+	—	—	—	0.22	*	
CD8 CD57+	—	—	0.26	—	0.38	*

[a]Denotes a correlation <0.2 in absolute value.

at about 48 months, with a subsequent decline in the upper centiles was seen. CD57 expression on CD8 cells also had a wide range, with unmeasurable values (i.e., <1%) being common at all ages. The range here was narrower at birth (1–17%) and widened steadily with age (1–46% at 100 months).

Between-Group Analysis of Variance

We carried out an analysis of variance to assess whether the age-corrected values differed by the group of children. All the measurements were transformed into *Z* scores, which give the number of standard deviations above or below the median. The assumption of a normal distribution was reasonable for all markers except for the three which had a substantial number of undetectable results (HLA-DR on CD4 and CD8 cells and CD57 on CD8 cells). For these three markers the analysis of variance was checked against a nonparametric test (Kruskall–Wallis test), but the results were very similar. In two cases (CD8 CD11a+ and CD8 CD45RA+) the *P* value for the test of significance was less than 0.01 (data not shown). However, there was no clear pattern of differences between groups and the actual differences were small. The mean *Z* score for the uninfected children of HIV-infected mothers was never more than 0.5 of a standard deviation away from the overall mean of zero. Data from the groups were therefore pooled for subsequent analysis.

Correlations Between Lymphocyte Subpopulations

Correlations between the *Z* scores, which are above 0.2 are given in Table IIa, for CD4%, CD8%, CD4/CD8 ratio, and CD4 cell subpopulations, and in Table IIb, for CD8 cell subpopulations. These correlations, since they are between *Z* scores, are corrected for the age of the child. The only correlations between subpopulations and the CD4% and CD8% are for CD4 expression of CD45RO and CD29. Within the CD4 population, the "memory markers" CD45RO and CD29 are positively correlated ($r = 0.65$); both correlate negatively with the "naive marker" CD45RA ($r = -0.51$ and $r = -0.50$, respectively). On CD8 cells, the negative correlation between CD45RO and CD45RA expression persists ($r = -0.46$), but the correlation with CD29 and CD45RO is much weaker ($r = 0.30$), as is the negative correlation between CD29 and CD45RA ($r = -0.20$). CD45RO expression also correlates with HLA-DR expression ($r = 0.46$).

DISCUSSION

The phenotypic characterization of CD4 and CD8 subpopulations in peripheral blood is potentially useful in the understanding of the complex relationships between cell types and pathogenic processes (1). It is particularly relevant in diseases of children, when little blood is available for functional studies. Because of the maturation of the immune system

with age, it is important to establish the normal patterns of marker expression in children and to distinguish changes found in disease processes from those which occur naturally. Here we present age-related normal centiles for surface markers which are particularly relevant to immune function.

Previous studies have tended to concentrate on CD4 subpopulations and have a limited range of markers; some have also been limited by the age range of the subjects studied. Because of the increased awareness of the role of CD8 cells in the immune response to diseases, we have included the phenotyping of both CD4 and CD8 subsets. Our results are presented throughout as percentages, rather than absolute counts, as these are subject to less fluctuation and, therefore, give more consistent results longitudinally (26). Error in measuring white counts, from which absolute values are calculated, has been shown to increase significantly the error of reported values (27). It must be noted, however, that for all the subsets studied, double staining of CD8 cells tends to exaggerate the percentage change due to the relatively low number of CD8 cells compared to CD4 cells. This could explain the greater scatter found in the CD8 compared to CD4 populations.

Our results for CD4% and CD8% across the comparable age ranges are consistent with other groups (18–23). The values for CD8%, in this study, are slightly higher but have a narrower standard deviation than in the ECS, especially at the youngest ages, which is reflected in the lower values of the ratio in the first few months of life. However, the ECS showed evidence of considerable between-lab variance for this measurement, which was possibly due to different methods used. The CD4% results show reasonable agreement with the ECS over most of the range. Overall, the agreement between the two sets of centiles is very close, considering the differences in numbers, and this agreement validates the subsequent results of this study.

Our studies have found that the CD4% and absolute counts are high at birth and decrease with age, while the CD8 levels, which are lower at birth than in adults, increase over the same period of time, thus resulting in a net decrease in the CD4/CD8 ratio with age (18–23). We found that the CD4% fell more sharply in the first 20 months than later, after which it declined more slowly. The CD8% fell slightly in the first 18 months and then a steady rise was seen. The combination of these changes resulted in a steady decline in the CD4/CD8 ratio, initially because of the steeper decline in CD4%, relative to CD8%, and subsequently due to the steady rise in CD8%, with a corresponding steady CD4%. In our premature children there were no significant differences in CD4 and CD8 percentages compared to full-term children. This agrees with the findings of Thomas and Linch (28), who studied premature babies and found that those which were normal size for gestational age had no significant differences in their T-lymphocyte populations compared to full-term babies, while babies that were small for gestational age had a significant deficiency in T and B lymphocytes. Wilson *et al.* (29) found that children who were stressed at birth had lymphocyte abnormalities, but these were confined to the B-cell population.

Most other studies of memory markers have focused on CD4 cells and have found that CD29 or CD45RO expression increases with age, with a reciprocal decrease in CD45RA expression (23–25, 30). Our results confirm these findings for CD4 cells, as the expression of both CD45RO and CD29 follows a similar pattern, and the two subpopulations are strongly correlated. However, on CD8 cells, the expression of these markers is different. The percentage of CD8 cells coexpressing CD29 is consistently higher than that coexpressing CD45RO, and there is a weak correlation between the two subpopulations. CD29 and CD45RO have been cited interchangeably as markers of memory or primed cells (31); however our results show that, on CD8 cells, CD29 and CD45RO identify different populations, which only partially overlap and are therefore not interchangeable (32, 33). The expression of CD45RA decreases with age in an approximately reciprocal manner to the increase in CD45RO expression, particularly in the CD8 population, which is consistent with the observations that cells that are CD45RA+ CD45RO− switch to CD45RA− CD45RO+ on maturation or priming (34–36). The decrease in CD45RA expression with age is also consistent with other studies, although the percentage of CD45RA+ CD4 cells neonatally was slightly lower here than in other studies which used cord blood samples (~80% compared with ~91%) (23, 24). We used samples taken during the first few days of life, which may explain the differences and suggests that, even within a very short period of life, lymphocyte maturation events are taking place.

Our results show a slight age relationship in the coexpression of HLA-DR on CD4 and CD8 cells. This is in agreement with Erkeller-Yuksel *et al.* (23), who found an age-related increase in HLA-DR expression on CD3 cells, but not with Parker *et al.* (25), who found no significant age relationship of HLA-DR expression on CD8 cells. We have also shown that the high-density expression of CD11a on CD8 cells is not age related in children. This supports the findings of Morimoto *et al.* (15), who suggested that CD11a is induced or up-regulated on cytotoxic effector cells. Natural killer cytotoxicity, mediated by CD57+ CD8 cells, is thought to be an important means of immune defense against viral infections in children (12). Our results confirm those of other groups, showing an age-related increase in CD57 expression on CD8 cells, with an increase in spread of values with age (12, 21–23). The transient rise in expression of CD29, HLA-DR, and CD57 on CD8 cells at about 30 months is difficult to explain and requires further investigation. It appears approximately 10 months after a slight trough in the CD8 percentage and may be an expression of normal maturational changes, or may reflect subclinical infectious events, such as infection with viruses, e.g., cytomegalovirus (CMV). A recent report suggests that individuals who express high levels of CD57+ CD8 cells are also infected with CMV (37).

The use of hospitalized children raises the possibility that pathological factors would affect lymphocyte subpopulation expression. However, our samples were selected for nonimmunological and noninfectious diagnoses, and any differences found between the groups of children were small, with no group consistently different. Any effect of trauma of hospital attendance and venesection would affect all of the control groups equally and, also, children being monitored for disease.

In conclusion, this study has established normal, age-related values for a number of lymphocyte subpopulations against which children with pathological conditions can be compared. Lymphocyte subset phenotyping is particularly relevant in the monitoring of pediatric HIV infection, where the centiles shown in Fig. 2 can be used to compare the tracks of markers in children at risk and provide diagnostic and prognostic indicators (M.C.A., in preparation). Charts suitable for clinical use, based on Fig. 1, can be obtained from the authors. Alternatively, the Appendix gives information from which charts or Z scores could be calculated.

APPENDIX: DETAILS OF THE METHOD OF CALCULATION OF CENTILE CURVES

Introduction

The method used to fit the centiles to the data in the European Collaborative study (19) was based on the method of Cole (38). This method proceeds by dividing the data into bands by age group, estimating the mean (M), standard deviation (S), and skewness (L) for each band and then fitting smoothed curves to the L, M, and S values. The method used in this paper is very similar but proceeds to estimate the LMS curves directly from the raw data, rather than by dividing them into bands. A similar, but distinct, approach has been adopted by Cole and Green (39).

The Model

For each marker we have a set of n measurements (denoted by y) at a series of ages (x). We model the distribution of y, given x, as follows.

L: At a given age (x) the values of the box–cox transformation of y, with parameter λ, are assumed to be normally distributed. The box–cox transformation is given by

$$\mathrm{bc}(y) = (y^{\lambda} - 1)/\lambda \quad \text{or} \quad \mathrm{bc}(y) = \ln(y) \quad \text{when} \quad \lambda = 0$$

A value of 1 for λ corresponds to a symmetric distribution of y; values less than 1 are positively skewed, with the percentiles farther apart at high values of y, and values greater than 1 correspond to a negatively skewed distribution where the percentiles are farther apart at low values of y. In all cases a single value of λ was found to model the data adequately.

M: The mean of the distribution of $bc(y)$ as a function of x was modeled using a locally weighted regression which produces a smoothed fit to the data [the loess procedure in Splus described by Chambers and Hastie (40, Chap. 8)]. The smoothness of the fit can be adjusted by choosing a locally linear or a locally quadratic fit and by adjusting the smoothing parameter.

S: The standard deviation was allowed to vary as a function of the age x according to the function $\mathrm{sd}(x) = \sigma \exp(1 + bx)$. Positive values of b correspond to a standard deviation which increases with age, and negative values to one that decreases with age.

Table AI. Predicted Values of the Age-Specific Median (Expressed as Percentages)

Age (mo)	CD4	CD8	Ratio	CD45RO		CD45RA		CD29		HLA-DR		CD11a, CD8	CD57, CD8
				CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8		
0	51.58	24.24	2.12	9.97	9.01	77.46	87.12	12.30	16.46	3.31	3.83	NA	2.81
6	47.26	23.12	2.01	11.69	9.72	76.08	86.14	14.26	23.01	3.41	4.67	13.20	3.29
12	43.99	22.50	1.91	13.57	10.46	74.68	85.15	16.22	28.45	3.52	5.56	14.02	3.79
18	41.80	22.33	1.84	15.62	11.24	73.31	84.16	18.16	32.53	3.62	6.51	14.95	4.31
24	40.95	22.70	1.78	17.88	12.08	71.95	83.16	20.18	35.36	3.73	7.57	15.69	4.94
30	41.06	23.56	1.73	20.11	12.94	70.60	82.15	21.81	36.87	3.84	8.37	16.45	5.46
36	40.82	24.71	1.65	21.73	13.85	69.22	81.14	22.67	36.25	3.94	8.23	17.15	5.62
42	40.70	25.97	1.57	23.12	14.81	68.04	80.13	23.37	34.81	4.05	7.83	17.75	5.72
48	40.62	26.99	1.51	24.57	15.81	67.01	79.16	24.05	34.47	4.14	7.58	18.21	5.80
54	40.59	28.09	1.44	25.87	16.80	66.30	78.28	24.84	34.05	4.21	7.49	18.14	5.94
60	40.51	29.08	1.38	27.14	17.74	65.69	77.68	25.63	34.12	4.26	7.37	18.15	6.07
66	40.42	29.75	1.35	28.11	18.66	65.26	77.53	26.06	35.00	4.30	7.28	17.86	6.15
72	40.27	30.32	1.32	28.91	19.59	65.09	77.59	26.34	36.07	4.33	7.27	17.46	6.21
78	39.90	31.03	1.28	29.99	20.56	64.72	77.49	26.89	37.21	4.37	7.26	17.15	6.33
84	39.36	31.77	1.23	31.13	21.55	64.50	77.38	27.45	38.54	4.41	7.40	16.71	6.49
90	38.65	32.54	1.18	32.32	22.57	64.39	77.34	28.00	40.07	4.45	7.67	16.16	6.70
96	37.74	33.35	1.13	33.55	23.63	64.36	77.35	28.56	41.79	4.49	8.02	15.55	6.93
102	36.63	34.21	1.07	34.81	24.72	64.39	77.39	29.11	43.72	4.54	8.44	14.92	7.18
108	35.29	35.15	1.00	36.11	25.86	64.43	77.44	29.67	45.85	4.58	8.89	14.30	7.44
114	33.70	36.17	0.94	37.43	27.05	64.45	77.48	30.2	48.18	4.63	9.33	13.71	7.70
120	31.83	37.29	0.86	38.77	28.29	64.41	77.48	30.79	50.72	4.67	9.72	13.18	7.94

The parameters λ, b, and σ and the parameters of the loess fit were fitted by maximum likelihood with the package Splus. The smoothness of the fit was chosen by comparing the extra variance explained between models and by examining the smoothed curves.

Results

For all the markers locally linear fits were compared to locally quadratic fits. Locally linear fits were found to be adequate for all markers except CD4%, CD4 CD29+, CD8 CD29+, CD4 HLA-DR+, and CD8 CD57+. However, the fits to CD4 CD29+, CD4 HLA-DR+, and CD8 CD57+, all of which gave a rather sharp local peak at about 30 months appeared implausible, and the linear fits have been presented. Thus linear fits were used except for CD4%, CD8 CD29+, and (for consistency) CD8% and CD4/CD8 ratio. The fit to the data was not changed greatly by the choice of the band width for the locally linear regression except for very small values which produced irregular fits. It was increased in some instances to make the plots look smoother.

The fitted values for the median (shown in Figs. 1 and 2) are tabulated in Table AI for six monthly ages.

The values of σ, b, and λ for each marker are given in Table AII. Most of the markers have positively skew distributions. The exceptions are CD4% and (especially) CD45RA on CD4 and CD8 cells, where the upper limit of 100% produces negatively skew distributions. The values of λ for CD4%, CD8%, and their ratios are similar to those found in the ECS.

The values of b are more difficult to interpret, because they refer to the standard deviation of $bc(y)$, which will vary with the mean level of y. Thus, for example, the standard deviation of CD4 CD45RO+ clearly increases with x (Fig. 2), but the b estimate for this marker is negative. This comes about because the levels of y are increasing with x, and a constant increment in $bc(y)$, which in this case is proportional to $y^{0.28}$, will correspond to a larger

Table AII. Fitted Values of the Parameters for Each Marker

	σ	b	λ
CD4%	1.700*e*+01	−6.917*e*−03	1.40
CD8%	4.990*e*−02	−6.109*e*−03	−0.22
CD4/CD8	1.904*e*−01	−5.515*e*−03	0.41
CD4 CD45RO+	2.898*e*−01	−1.106*e*−03	0.25
CD8 CD45RO+	3.794*e*−01	−2.291*e*−03	0.20
CD4 CD45RA+	9.924*e*+01	3.047*e*−04	1.78
CD8 CD45RA+	3.262*e*+05	−5.963*e*−05	3.62
CD4 CD29+	6.854*e*−01	2.067*e*−03	0.58
CD8 CD29+	2.493*e*+00	2.039*e*−03	0.80
CD4 HLA-DR+	2.353*e*−01	1.220*e*−03	0.05
CD8 HLA-DR+	5.811*e*−01	−2.931*e*−03	0.31
CD8 CD11a+	6.343*e*−01	−2.087*e*−03	0.33
CD8 CD57+	4.127*e*−01	1.797*e*−03	0.10

increment in the corresponding raw values, at higher values of *y*.

To obtain a *Z* score for a value *Y* of a marker measured at age *X,* one must first read the median value *(M)* for *X* from Table AI. The *Z* score is then calculated as

$$[bc(Y) - bc(M)]/\sigma\exp(1 + bX)$$

where the appropriate λ in *bc* and the values of *b* and σ are taken from Table AII. The percentile can then be obtained by referring *Z* to tables of the normal distribution.

Similarly, to obtain a percentile point for a marker at a given age, one first obtains the appropriate *Z* statistic from the normal distribution tables. The value of *bc(y)* is then calculated as

$$k = bc(M) + Z\sigma\exp(1 + bX)$$

and the corresponding raw value for the percentile point calculated as $(1 + \lambda k)^{1/\lambda}$.

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Cytotoxic T lymphocyte activity and CD8 subpopulations in children at risk of HIV infection

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SUMMARY

HIV-specific cytotoxic T lymphocytes (CTL) are thought to play a major role in viral control in HIV-infected adults. Changes in the relative proportions of CD8 lymphocyte subpopulations are also thought to be associated with disease progression. Less is known about the relative effectiveness of CTL against different HIV targets, or about the relationship, if any, between CTL activity and CD8 subpopulations. We have measured CTL activity against four HIV gene products (*gag*, *tat*, *pol* and *env*) and expression of CD45RO, CD45RA, HLA-DR, CD29, S6F1, and CD57 surface markers on CD8 cells from nine HIV-infected and 11 HIV-uninfected children. Of nine HIV-infected children, six showed antigen-specific CTL activity on at least one occasion: 4/6 directed against *tat*, 6/6 against *pol*, 1/6 against *env*, and 1/6 against *gag*. However, the specificity of the CTL activity varied between children and within individual children with time. Furthermore, two uninfected children showed CTL activity, one to HIV-*gag*, -*pol* and -*tat*, and the other to HIV-*pol*. All the HIV-infected and two uninfected children had abnormal proportions of CD8 subpopulations in whole blood compared with age-matched controls. There was no correlation between CTL activity and CD8 subsets in whole blood. Five children changed from CTL-positive to CTL-negative (or *vice versa*) during the study. In these, the occasions when CTL activity was detected coincided with an increase in CD8 cells, an expansion of HLA-DR$^+$ CD8 cells and a loss of CD45RA$^+$ CD8 cells.

Keywords cytotoxic T lymphocytes CD8 subpopulations CD8 effector cells paediatric HIV infection clinical significance

INTRODUCTION

CD8$^+$ cytotoxic T lymphocytes (CTL) are thought to play a major role in the immune control of HIV infection. High levels of HIV-specific CTL have been demonstrated in asymptomatic infected adults, which, in some studies, have been shown to be lost with disease progression. Others have suggested that HIV-specific CTL may have a pathogenic or deleterious effect (reviewed in [1–3]).

Children infected by mother–child transmission are an important group of patients to study. Since they are newly infected, they may reveal the specificity of early cellular immune responses, which would be important in the design of vaccines. The clinical course of HIV disease in these children appears to follow one of two routes—fast progressors who progress rapidly and die within 1–2 years, and slow progressors who remain clinically stable for a number of years [4,5]. It is important to establish whether cellular, specifically CTL responses, have any role in favouring either of these clinical outcomes. While CTL responses to HIV have been reported in a number of studies of HIV-infected children [6–8], HIV-specific CTL have also been described in children who subsequently became antibody-negative [9], and it is important to determine whether these children have successfully cleared virus and become naturally immunized. In neonates, HIV infection, and the immune response to it, takes place in the context of a developing immune system, and the time at which infection occurs may be relevant in determining the ability of the child to resist infection, or to contain the virus.

A previous study in adults has suggested that it may be possible to indirectly identify CTL by means of CD8 subset markers in the cultured effector cells, in particular CD45RO, HLA-DR and S6F1 [10]. We and others have shown in HIV-infected children that CD8$^+$ lymphocytes and specific subpopulations are present in abnormally high proportions [11,12]. We have also previously shown that the loss of CTL activity in

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an uninfected child was accompanied by a normalization of CD8 subpopulations [9]. The aim of this study was to investigate CTL activity directed against a number of HIV gene products, CD8 subsets in both peripheral blood and cultured effector cells, and clinical status over 18 months in HIV-infected, and in exposed but presumed uninfected children.

MATERIALS AND METHODS

Subjects

Altogether, 20 children were studied. Seventeen of these are being monitored in Edinburgh, and are also included in the European Collaborative Study [13–15]. Three more are from Glasgow or Dundee. They are all seen at regular intervals, and venesection is performed as part of routine follow up.

The study group comprised nine HIV-infected children (mean age at start of study 43 months, range 3–88 months), and 11 children who have lost maternal HIV antibody, have no other indications of HIV infection, and so are presumed to be HIV uninfected (mean age at start of study 27 months, range 1–73 months). Eighteen of the children are known to be born to HIV-infected women; two were adopted, and as no maternal information is available the route of infection is unknown. The clinical classifications of the infected children were determined using criteria from the Centres of Disease Control (CDC) [16], and, together with details of clinical symptoms, are presented in Table 1.

Table 1. Clinical data of the children studied at each time of testing

Child	Age (months)	Clinical symptoms	CDC* stage
HIV infected			
1	79–93	Ear/throat infections/PGL/well	P2A
2	71–84	Well/well/PGL/well	P2C,F
3	53–59	Well	P1B
4	28–45	Well	P1B
5	21–31	Fever/chest infection/well	P2A
6	88–90	Well/chest infection	P2A
7	40–43	*Schistosoma haematobium*/chest/viral infections	P2A,C
8	4–6	Chest infection	P2A
9	3–6	Candidiasis/PGL	P2A
HIV uninfected			
10	73	Well	—
11	70	Well	—
12	36	Viral illness	—
13	30	Well	—
14	25	Diarrhoea and vomiting	—
15	15	Well	—
16	1	Candidiasis	—
17	10	Well	—
18	17	Well	—
19	6	Well	—
20	17–25	Tapeworms/Diarrhoea	—

*CDC classification for paediatric HIV.
PGL, Persistent generalized lymphadenopathy.

CTL assay

The method was based on that of Nixon *et al.* [17] using bulk cultured effector cells in a 4-h ^{51}Cr-release assay.

Effector cells. Peripheral blood lymphocytes (PBL) were isolated from heparinized blood, and stimulated with phytohaemagglutinin (PHA) (2·5 μg/ml; Wellcome, Beckenham, UK). For the HIV-infected samples, 10% of PBL were stimulated with PHA for 2 days, washed once, and then added back to the remaining cells in 24-well tissue culture plates. For the HIV-uninfected samples, all the cells were stimulated with PHA, this having been shown to give a better outgrowth of cells (unpublished observation). All cultures were maintained at 37°C in RPMI 1640 medium containing 10% fetal calf serum (FCS) for 7 days, and subsequently in medium containing IL-2 (5% Lymphocult, Biotest, Solihull, UK). Fifty percent of culture supernatant was changed every 3–4 days until assayed. Assays were carried out between day 9 and day 23 of culture, when there were deemed to be sufficient cells.

Target cells. Autologous Epstein–Barr virus (EBV)-transformed B cell lines were infected overnight with 10^6 PFU of either a vaccinia–HIV construct (*gag*, *tat*, *pol* or *env*), or a vaccinia control. The constructs used were: vaccinia control (Vac-Vsc8WR) and *vac-gag* (MRC, AIDS Directed Programme), *tat* (VVTG3196), *pol* (VVTG3167) and *env* (VVTG1132) (Transgene, Strasbourg, France). A tube containing EBV cells not infected with vaccinia constructs was set up as a 'medium' control.

Assay. Given the variable number of cells available, the effector cells were always used at the highest effector : target ratios possible, which was generally between 10 : 1 and 50 : 1. The per cent lysis of the target cells was measured after 4 h in culture, by release of ^{51}Cr into the supernatant, and calculated using:

$$\frac{\text{specific lysis} - \text{spontaneous lysis}}{\text{total lysis} - \text{spontaneous lysis}} \times 100$$

where total and spontaneous lysis were determined by incubating the target cells in 5% Triton-X and in medium, respectively.

Immunofluorescence analysis

Blood or effector cell phenotypes were analysed using standard dual-colour flow cytometry with the following MoAbs: anti-CD4(Leu-3a)-FITC, anti-CD8(Leu-2a)-PE, anti-HLA-DR-FITC, anti-CD45RA(Leu-18)-FITC (Becton Dickinson, Oxford, UK), anti-S6F1-RD1/anti-CD8-FITC, anti-CD29(4B4)-FITC (Coulter, Luton, UK) and anti-CD45RO(UCHL1)-FITC (Dako Ltd, High Wycombe, UK). All antibody mixes were diluted 1 : 2, except for S6F1 which was used at 1:16. Briefly, diluted blood or effector cells were added to the antibody pair and incubated for 15 min at room temperature. The erythrocytes in whole blood samples were lysed using Facslyse (Becton Dickinson). The samples were centrifuged, washed in saline, fixed in 1% formaldehyde/PBS and stored at 4°C until analysed on a FACScan (Becton Dickinson). Results are expressed as the percentage of CD8 cells co-expressing the antigen studied.

Analysis of results

CD8 lymphocyte subpopulation percentages in blood were compared with age-related normal ranges previously estab-

ished (M. C. Aldhous, submitted for publication). The percentages were converted to 'Z-scores', a measure of the number of standard deviations an individual is from the age-matched mean value. The Z-scores of children who showed CTL activity (CTL^+) were then compared with those who were CTL^-, using the two-sample *t*-test.

The percentage of CD8 effector cells co-expressing the subpopulations studied were similarly compared, but using the percentage values.

RESULTS

HIV-specific CTL activity

CTL activity against HIV-*gag*, *tat*, *pol* and *env* is shown in Figs 1 and 2 for HIV-infected (nos 1–9) and uninfected (nos 11–20) children, respectively. Results are presented as the per cent specific lysis net of medium and vaccinia controls, with values greater than 10% above controls being regarded as positive. Each infected child was tested between two and four times, and the uninfected children once, except for one child (no. 20) assayed twice.

HIV infected children. Six of the nine HIV-infected children showed CTL activity on at least one occasion. The most commonly recognized target was *pol*, followed by *tat* (six and four children respectively). *Gag* was recognized by only one infected child. In four cases the CTL activity was directed against a single target (children 1, 2, 3 and 4), and in two cases it was directed against two targets (*tat* and *pol* in children 5 and 9).

The specificity of the CTL response changed on successive tests. In the two youngest children who had CTL (children 9 and 5, aged 6 and 21–31 months, respectively) the response was against both *tat* and *pol*. In two older children (children 3 and 4, aged 53–59 and 28–45 months, respectively) the specificity of the response changed from *tat* to *pol*, and in the two oldest children, the specificity changed from *pol* to *gag* in child 1 (aged 79–93 months), and from *pol* to a weak *env* response in child 2 (aged 71–84 months).

Two children (children 2 and 4) lost CTL activity, and two children (nos 1 and 5) lost and regained CTL activity during the period of follow up. Three children (nos 6, 7 and 8) were CTL^- throughout the study.

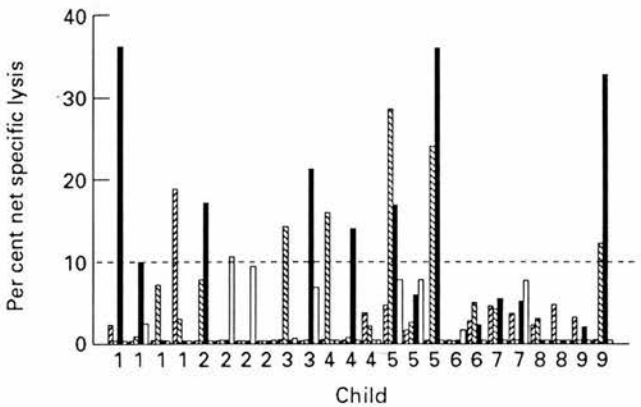

Fig. 1. HIV-specific cytotoxic T lymphocyte (CTL) activity on sequential samples in HIV-infected children. ▨, *gag*; ▧, *tat*; ■, *pol*; □, *env*.

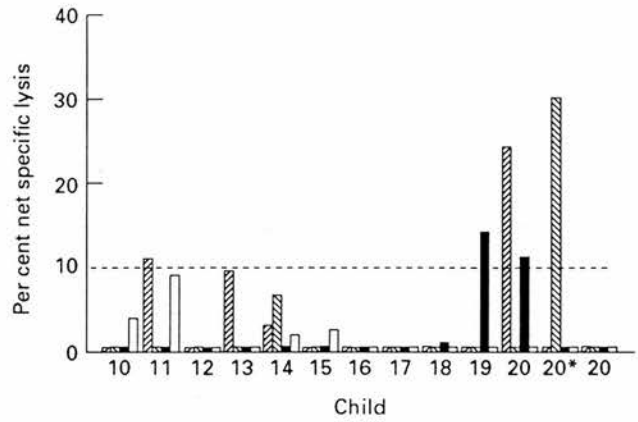

Fig. 2. HIV-specific cytotoxic T lymphocyte (CTL) activity in HIV-uninfected children. *Activity measured after day 11 and day 18 in culture. ▨, *gag*; ▧, *tat*; ■, *pol*; □, *env*.

HIV-uninfected children. CTL activity was detected in two HIV-uninfected children. A further two had borderline activity. Child 20 showed significant levels of anti-*gag*, anti-*tat* and weak anti-*pol* activity when first assayed at 17 months of age. On this occasion, the effector cells were used at two different time points, days 11 and 18 after stimulation, and the specificity was shown to change from *gag* and *pol* on day 11 to *tat* on day 18. At 25 months, this child showed high non-specific killing (data not shown), but no HIV-specific lysis. Child 19 showed anti-*pol* activity at 6 months of age. Children 11 and 13 had borderline (i.e. 10% lysis) anti-*gag* responses at 70 and 30 months, respectively.

CD8 subpopulations in whole blood

CD8 subpopulations were determined using the same blood samples as the CTL assay, and were compared with the age-related values determined for normal children (M. C. Aldhous, submitted for publication). Levels were regarded as being raised or decreased if they were above the 97th, or below the 3rd centiles, respectively.

HIV-infected children. All nine had raised (>97th centile) levels of $S6F1^+$ CD8 cells, on both CTL^+ and CTL^- occasions. Eight had raised $HLA\text{-}DR^+$ CD8 cells, the one child (no. 6) with no raised HLA-DR levels being CTL^-. Six children (nos 1, 2, 3, 5, 8 and 9) had raised $CD45RO^+$ CD8 cells and correspondingly decreased (<3rd centile) $CD45RA^+$ CD8 cells. These were observed on both CTL^+ and CTL^- occasions in all except one child (no. 8), who was consistently CTL^- against the constructs used. Conversely, child 4, who was CTL^+ on 2/3 occasions tested, had normal CD45RO expression on CD8 cells. Children 5, 7 and 8 had raised $CD29^+$ CD8 cells, which corresponded to CTL activity in only one child (no. 5), and only on one occasion. Child 7 was the only child who showed raised $CD57^+$ CD8 cells, and this did not correspond to CTL activity. The S6F1 and CD45RO levels were raised persistently, whereas the HLA-DR, CD29 and CD57 levels were raised sporadically.

HIV-uninfected children. Children 13 and 20 had abnormal CD8 subpopulations in whole blood. In child 13, the abnormal phenotype levels corresponded to borderline CTL activity. The

next sample received showed lymphocyte markers within normal ranges for age (data not shown). In child 20, abnormally raised CD45RO$^+$, HLA-DR$^+$ and S6F1$^+$ CD8 cells in whole blood corresponded to CTL activity against HIV *gag*, *tat*, and *pol*; 8 months later no HIV-specific CTL activity was detected and the CD8 phenotypes had returned to normal. The anti-*pol* CTL activity of child 19 did not correspond to any raised CD8 subpopulations. All other uninfected children had CD8 subpopulations which were within the normal ranges for age.

CD8 subpopulations in effector cells

The percentages of the various CD8 subpopulations in the effector cell cultures were compared with those in the corresponding sample of blood. All children showed an increase in HLA-DR$^+$ CD8 cells and, apart from three infected children, who already had very high levels in blood, there was also an increase in CD45RO$^+$ CD8 cells. All samples showed a corresponding decrease in CD45RA$^+$ CD8 cells, findings which are consistent with immune activation. The percentage of CD29$^+$ CD8 cells decreased on effector cells, contrary to expectation as both CD29 and CD45RO are memory markers. S6F1 expression increased on the CD8 effector cells of uninfected children, and was maintained, but did not increase further, on the cells of the infected children, in whom expression was already very high (up to 80–90%).

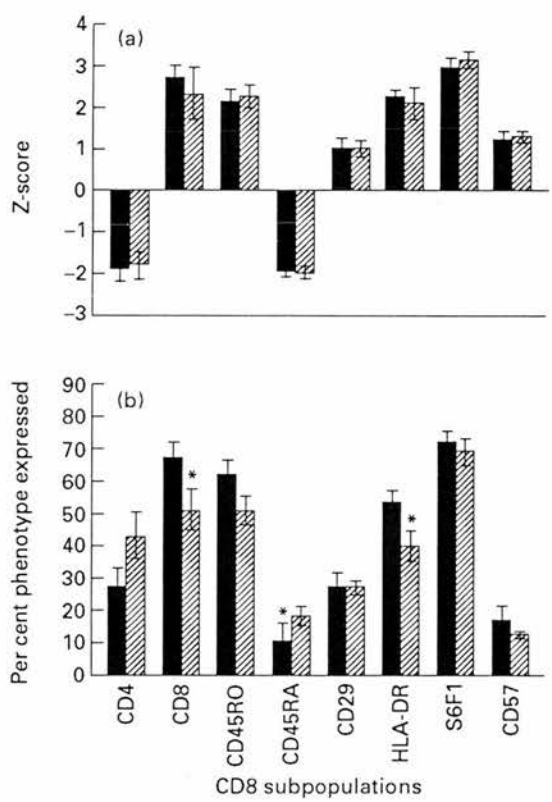

Fig. 3. CD8 subpopulations in children who changed cytotoxic T lymphocyte (CTL) activity. Comparison of CTL$^+$ (■) and CTL$^-$ (▨) occasions in (a) whole blood, (b) effector cells. Graphs are mean ± s.e.m. *Significant difference between CTL$^+$ and CTL$^-$ results ($P \leq 0{\cdot}05$).

Analysis of CD8 subpopulations in CTL$^+$ and CTL$^-$ children

Statistical comparisons of CD8 subpopulations in blood were made using Z-scores, and of effector cells using the percentage values. There were no significant differences between the levels of CD8 subpopulations in whole blood or effector cells between those children who had CTL activity on any occasion and those who did not.

We then analysed the results of the five children who changed CTL activity during the study period. CD8 subpopulation levels of blood (Fig. 3a) and effector cells (Fig. 3b) on the CTL$^+$ and CTL$^-$ occasions were compared. There was no difference in whole blood, but in the effector cells the percentage of CD8 cells was significantly increased ($P = 0{\cdot}05$) on the CTL$^+$ occasions, as was that of the HLA-DR$^+$ subpopulation ($P < 0{\cdot}05$). The increase in CD45RO$^+$ CD8 cells was not significant, but the percentage of CD45RA$^+$ CD8 cells was significantly decreased ($P < 0{\cdot}05$) on the CTL$^+$ occasions.

CTL activity and clinical status

The clinical details are shown in Table 1. Five of the six infected children with CTL activity were generally well (children 1–5) during the period of follow up. The sixth (child 9) was symptomatic throughout. The three CTL$^-$ children (nos 6, 7 and 8) all had infections during the study period.

DISCUSSION

There is increasing interest in the role of CD8 cells in suppressing HIV replication and in maintaining the asymptomatic phase of the disease. In particular cytotoxic T cells are believed to play an important part in the immune response to HIV [1–3]. CD8 subpopulations are increased in the peripheral circulation of HIV-infected individuals at all stages of infection [18–21], but their functional significance still needs clarification [22]. In this study we have investigated the CTL activity against HIV antigens in nine HIV-infected and 11 uninfected children, studying possible associations between CTL activity, CD8 subsets and clinical disease.

Because of the small samples of blood obtained (approximately 3 ml), all assays were performed using PHA-stimulated, bulk cultured lymphocytes as effector cells. The killing has been shown to be blocked by anti-CD8 antibody and to be MHC restricted (data not shown) [17]. The most commonly recognized antigen was *pol*, followed by *tat*, then *gag* and *env*. While six of the nine HIV-infected children showed CTL activity on at least one occasion, the specificity varied, both between children and within individual children with time, suggesting a possible sequential pattern of CTL specificity, with epitopes on *tat* and *pol* stimulating an early CTL response. This observation requires confirmation by further longitudinal data.

Our results are consistent with those of Luzuriaga *et al.* [23], who found anti-*gag* and -*env* activity in only a proportion of vertically infected children, but differ from those of Cheynier *et al.* [7], who found CTL activity to HIV-*gag*, -*env* and -*nef* in a high proportion of children tested, as did Buseyne *et al.* [8] against HIV-*gag*, -*env* and -*pol*. The reasons for these differences are unclear. Technical differences relating to alternative methods used to stimulate effector cells may be important—in this study, effector cells from one child (no. 20), maintained for

different lengths of time, had different specificities, which suggests that CTL precursors differentiated into separate dominant clones during culture. Different viral sequences used in the vaccinia constructs, or different strains of virus infecting the groups of children, may also account for inconsistencies seen between studies.

Apart from HIV-*nef*, the regulatory proteins have not generally been thought to be immunogenic [7,24–26]. Our results show that HIV-*tat* is strongly immunogenic and, together with *pol*, may stimulate important early responses, particularly in children. The *tat* gene product is one of the early regulatory proteins involved in the initiation of RNA transcription and elongation [27], and appears to be of great importance in up-regulating gene expression and HIV replication. Thus, *tat*-directed cytotoxicity may remove sites of high viral replication and limit the extent of further viral transmission, or potential for genomic variation. Increased expression of *pol* would be expected to occur during active intracellular viral replication [28], and cytotoxicity directed against these cells would also limit virus production and viral load. In cross-sectional studies of infected adults, CTL activity to both HIV-*tat* and *pol* has been detected (K. C. Watret, unpublished data) [26,29] but, for *tat*, less frequently compared with other HIV gene products [26]. Further longitudinal monitoring is needed to establish whether the pattern of response suggested by this group of children at different ages applies also to individual children, and whether other regulatory proteins such as HIV-*vif* and *rev* are also immunogenic. If it is confirmed that HIV-*tat* and *pol* induce an early CTL response, this may have implications for the design of vaccines for children.

Evidence of cellular immune responsiveness to HIV in uninfected individuals has been reported by a number of groups [7,9,30]. This study has shown two of 11 presumed uninfected children to have strong CTL activity on one occasion and a further two to have borderline levels. The intracellular processing and presentation of viral peptide to CTL is thought to require infection of the target cell, implying that these children have been exposed to live, though possibly non-replicating, virus. Recent evidence from vaccine trials has shown that CTL can be induced by non-replicating viral proteins if presented with appropriate adjuvants [31]. Of interest is the transient nature of the CTL response. Child 20, who was assayed twice, lost the response on the second occasion, suggesting that CTL precursors were no longer present in the circulation, and raising the question of whether this child is immunologically sensitized to HIV. Other laboratory tests, including HIV antibody, antigen culture and polymerase chain reaction (PCR) were consistently negative (data not shown), and this child was therefore diagnosed as uninfected. During the early stages of disease, HIV accumulates in the lymphoid organs, even when there is little or no evidence of infection in peripheral blood [32,33]. It may be possible that some virus is still present in the lymphoid tissues of these children, capable of emerging at a later date, as occurred in one child studied by Luzuriaga *et al.* [6]. PCR on lymphoid tissue would be necessary to determine whether the children are truly HIV-uninfected.

We examined whether any of the CD8 subpopulations, known to be raised in paediatric HIV infection, correlated with CTL activity, as phenotype monitoring of the numbers or proportions of the relevant effector or precursor cells would be a much easier method of following CD8 functional responses. We also wished to see whether any particular subpopulation was consistently different on comparing CTL$^+$ and CTL$^-$ occasions, which may correspond to a CTL precursor population. A previous study of a child who had transient CTL activity suggested that raised CD8 subpopulations correspond to CTL activity [9], an observation which was repeated in this study (child 20). However, the CTL activity without raised CD8 subpopulations in whole blood from one child (no 19), and the converse in another (child 8), indicate that this is not universal. Furthermore, all of the infected children had raised S6F1$^+$ CD8 cells, including those who were consistently CTL$^-$, and although six of the nine children had raised CD45RO$^+$ CD8 cells, these did not all correspond to the six who had CTL activity. There is no evidence, therefore, for a precursor phenotype in the whole blood for the populations studied here, even when the children who changed CTL activity during the study were analysed alone. However, it may be that a precursor phenotype is one that was not included in this study, or that those children who changed CTL activity still had CTL, but to an antigen not included, and did not truly lose the CTL activity altogether.

We have previously reported that loss of CTL activity in adults was associated with a failure to expand of the CD45RO$^+$, S6F1$^+$ and HLA-DR$^+$ subpopulations of CD8 cells in bulk cultured effector cells [10]. In this study, to allow for the possibility that the CTL$^-$ children may have CTL against target antigens not studied here, we limited the analysis of effector cell subpopulations to the five children who changed CTL activity during the study. In these children there was a rise in the proportion of HLA-DR$^+$ CD8 cells, and a decrease in the proportion of CD45RA$^+$ CD8 cells on the CTL$^+$ occasions, which is consistent with immune activation. However, there was no further expansion of S6F1$^+$ CD8 cells, probably due to the very high levels already present in the blood (a phenomenon not seen to the same extent in adults [10]), giving little room for further expansion. On the CTL$^-$ occasions, however, there was a lack of expansion of CD8 cells, and in particular a lack of increased expression of HLA-DR on CD8 cells, which was statistically significant. This study therefore confirms our previous findings that CTL activity is a consequence of the ability of particular CD8 subpopulations to expand in culture.

CD57$^+$ CD8 cells which suppress CTL activity have been described in peripheral blood and in alveolar fluid [34,35]. Here, there were no consistent changes in the expression of CD57 on CD8 cells, in either whole blood or effector cells, to explain the lack of CTL activity in any child, or to account for an increased lysis through natural killer function [36,37]. We were also unable to confirm the results of Sohen *et al.* [38], who defined CD8$^+$ cytotoxic T cells as CD29$^+$ S6F1$^+$ CD45RA$^-$. Our results showed a decrease in CD45RA$^+$ expression and the maintenance of S6F1$^+$ expression, but a decrease in the expression of CD29 on CD8 effector cells compared with corresponding blood samples.

It is still not clear whether CTL activity is universally beneficial to the individual infected with HIV. Loss of CTL activity in asymptomatic individuals occurs before clinical disease progression [34], suggesting that CTL activity is an important mechanism in viral control. However, the finding of CTL in bronchial lavage [39] and cerebrospinal fluid [40]

indicates that CTL may contribute to the pathological manifestations of HIV disease. In this study, CTL activity did not always correlate with clinically stable disease. Five of the six children who had CTL activity were, on the whole, clinically well, and the preliminary evidence that child 20 may have cleared HIV infection through CTL activity supports the theory that CTL activity is beneficial. No CTL activity was detected in three children who are still clinically stable. Conversely, a newborn child 9, who is a fast progressor, developed CTL activity at 6 months. All these children need to be monitored further, to determine whether any direct association between CTL activity and clinical disease can be shown.

In conclusion, our results show that CTL activity in children follows a sequential pattern of responses to different antigens. This needs to be confirmed in a larger cohort of children, and in newly infected adults, to determine whether a similar pattern of responses holds. CTL activity is not directly related to CD8 subpopulation in whole blood, but is associated with the ability of certain CD8 cell populations to expand in culture. CTL are not consistently associated with clinically stable disease, and further study in a larger cohort of children is required to show the relationship between CTL activity and disease activity.

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CD4 and CD8 Lymphocytes in Diagnosis and Disease Progression of Pediatric HIV Infection

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Abstract

Vertical infection with human immunodeficiency virus-1 (HIV-1) causes profound changes in the proportions of subpopulations of lymphocytes in the peripheral circulation. In this study the percentages in whole blood of CD4 and CD8 cells, and of immunologically important subpopulations, were measured in 19 HIV-infected children over periods of up to 4 years and compared to our recently published ranges for normal children of various ages.[1] The rate of CD4 decline and of CD8 increase differed between clinically fast and slow progressors. On CD8 cells, cytotoxic, memory ($CD11a^{bright}$ and CD45R0), and activation (HLA-DR) markers were raised soon after birth to levels outside the normal range, and compared favorably with HIV culture as a method for early diagnosis of HIV infection. Mean levels of naive (CD45RA) and memory (CD45R0, CD29) markers on CD4 cells became significantly altered after 48 months of age, suggesting that these are markers of more advanced disease. Despite different ages of enrollment into the study, in the cohort as a whole, the levels of the lymphocyte subpopulations studied changed consistently. Thus, their measurement could be useful both in the diagnosis and prognosis of HIV infection in individual children. This is the first report showing that lymphocyte subpopulation analysis can play a major role in the diagnosis of pediatric HIV infection.

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Introduction

Although much has been learned about the immune responses of adults following HIV infection, less is known about the ability of neonates or children to respond immunologically to HIV. A significant proportion of infants born to HIV-infected women is infected in utero or at birth, when the immune system is still immature.[2] Recent work with rhesus macaque monkeys has shown that neonates differ from adults in their susceptibility to infection with attenuated simian immunodeficiency virus (SIV),[3] suggesting that conclusions drawn from studies of adult patients cannot necessarily be extrapolated to neonates. Furthermore, different routes of infection can influence whether the immune response is predominantly T-helper type 1, leading to cell-mediated immunity mediated by interleukin 2 (IL-2) and gamma interferon, or T-helper type 2, leading to a predominantly humoral immune response mediated by release of IL-4, IL-6, and IL-10.[4] Thus both the route of infection and the stage of gestation at which it occurs may affect the type and degree of immune response that an infant makes to HIV.

Flow cytometric analysis of lymphocyte subpopulations has been widely used in clinical staging of adult HIV infection and to elucidate possible immunological responses.[5] Similar analysis in children is complicated by the substantial changes that take place as part of normal maturation. Recent studies by ourselves and others have established age-related normal ranges for CD4 and CD8 subsets, and for a number of immunologically important subpopulations.[1,6,7] In HIV-infected children, previous studies have shown changes in the levels of some of these subpopulations,[8–11] but to our knowledge no previous studies have related lymphocyte subpopulation analysis to diagnosis or to HIV disease progression. In this study we prospectively followed the levels of CD4 and CD8 cells, and of CD4 and CD8 cell subpopulations expressing activation,

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memory, and cytotoxicity markers in a cohort of infected children and compared them with our recently published ranges for normal children of different ages[1] and with clinical disease progression.

Methods

Subjects

Children born to HIV-infected women were enrolled in a perinatal transmission study and seen at regular intervals.[12,13] Venesection is performed as part of routine clinical follow-up. A diagnosis of HIV infection is made on the basis of two positive laboratory results indicating the presence of HIV, *viz* virus culture, HIV-specific polymerase chain reaction (PCR), and plasma p24 antigen. Clinical signs and symptoms were categorized according to the clinical staging system proposed by Tovo et al.[14] Children were classified as fast progressors on the basis of the appearance of the following signs and symptoms before the age of 1 year: specific secondary infectious diseases, severe bacterial infections, progressive neurological disease, anemia, fever, growth failure, persistent oral candidiasis, hepatitis, or cardiomyopathy.

Nineteen HIV-infected children aged from birth to 108 months were followed for a median of 27 months (range 2–55 months). Sixteen were vertically infected, one by blood transfusion, and the route of infection for the remaining two was unknown. Four children were defined as fast progressors, two of whom had been followed from birth, whereas the other two were enrolled at 10 and 13 months respectively. One of these children (no. 17) subsequently stabilized clinically. Of the fifteen slow progressors, four were followed from birth, whereas the remainder were enrolled between 23 and 80 months of age. Six children died during the study, but detailed lymphocyte subpopulation data were available on only two. Clinical details of the children studied are summarized in Table 1.

Age related normal ranges for lymphocyte subpopulations for children aged 0–110 months, were

Table 1. Clinical Details of Children

Child	Age (months) at venesection: First	Age (months) at venesection: Last	No. times tested	CDC stage[a] at start of study	HIV-related clinical events during study
Slow progressors[b]					
1	49	102	34	A2	Nonspecific signs and symptoms
2	38	93	34	B2	Thrombocytopenia
3	55	107	25	A1	Nonspecific signs and symptoms
4	29	77	21	A1	Nonspecific signs and symptoms
5	3	42	16	A1	Nonspecific signs and symptoms
6	23	59	17	A1	Recurrent respiratory infections, presumed lymphocytic interstitial pneumonia
7	24	53	11	A1	Nonspecific signs and symptoms
8	1	21	11	A1	Recurrent upper respiratory infections
10	80	107	18	B3	Salmonella meningitis and septicemia, failure to thrive, diarrhea
11	55	89	4	B2	Lymphocytic interstitial pneumonia
12	65	91	10	B3	Nonspecific symptoms, Ewing's sarcoma, died
15	66	82	9	B2	Nonspecific symptoms, cardiomyopathy, died
16	73	108	20	B3	Thrombocytopenia, HIV encephalopathy, disseminated herpes zoster, died
18	1	6	4	N1	Clinically well
19	1	4	2	N1	Clinically well
9	1	13	13	C1	HIV encephalopathy, cerebral toxoplasmosis, died
13	10	12	2	C1	Septicemia, failure to thrive, died
14	13	20	4	C3	Recurrent abscesses, failure to thrive, died
17	2	8	4	B2	Thrush, pneumonia, nonspecific symptoms

[a]The CDC stage was determined using the percentage of CD4 cells.[48]

[b]Slow and fast progressors were classified according to the system of Tovo et al.[14]

calculated from 168 control samples, analyzed concurrently with the patients, as previously described.[1]

Immunofluorescence Analysis

Cell surface phenotypes were analyzed in whole blood, by dual-color flow cytometry as previously described,[1] using monoclonal antibodies conjugated with fluoro-isothiocyanate (FITC), phyco-erythrin (PE) or rhodamine (RD1) as follows: anti-CD4(Leu 3a)-FITC, anti CD8(Leu2a)-PE, anti-HLA-DR-FITC, anti-CD45RA(Leu18)-FITC (Becton Dickinson, Oxford, UK), anti-CD11a(S6F1)-RD1/anti-CD8-FITC, anti-CD29(4B4)-FITC(Coulter, Luton, UK), and anti-CD45R0(UCHL1)-FITC (DAKO Ltd, High Wycombe, UK). Pairs of appropriately diluted antibodies were added to 100 μl of heparinized blood and incubated at room temperature for 15 min, before lysis, washing, and fixation in 1% formaldehyde/phosphate-buffered saline. The samples were analyzed on a FACScan (Becton-Dickinson) using Simulset software. Lymphocytes were labeled with anti-CD14/CD45 ("Leucogate," Becton-Dickinson) and gated using forward and side light scatter. Gating of total CD8 cells, and of the CD45RA and CD45R0 subpopulations, included both bright and dimly fluorescing cells as positive; gating of CD11a cells included only the brightly staining cells as positive ($CD11a^{bright}$). Expression of phenotyping results as percentages rather than absolute counts has been shown to be less variable because of the natural variation in blood lymphocyte counts,[15,16] and, in our experience, is more consistent longitudinally. The results are therefore expressed as the percentage of CD4 or CD8 cells coexpressing the antigen studied ($CD4^+CDX^+/CD4^+ \times 100$).

Table 2. Age Dependent Levels of Lymphocyte Subpopulations in HIV Infected Children

	Age (months)				
	0–24		25–48	49–60	61–110
Progression rate	Fast	Slow		Slow	
No. children	4	5	5	9	9
CD4	29 (−1.5**)	42 (−0.43)	29 (−1.30**)	25 (−2.19**)	18 (−3.06**)
CD8	45 (+2.34**)	27 (+0.47)	43 (+2.18**)	51 (+3.17**)	56 (+3.19**)
CD4 subpopulations					
CD45R0	15 (+0.22)	12 (−0.22)	26 (+0.29)	32 (+0.86**)	48 (+1.31**)
CD45RA	76 (+0.08)	78 (+0.26)	67 (−0.09)	57 (−1.08**)	46 (−1.27**)
CD29	16 (+0.16)	15 (+0.05)	24 (+0.05)	36 (+0.87**)	43 (+1.31**)
HLA-DR	7 (+1.07*)	5 (+0.63)	10 (+1.40**)	13 (+1.83**)	19 (+1.73**)
CD8 subpopulations					
CD45R0	28 (+1.67**)	25 (+1.28**)	37 (+1.68**)	46 (+2.31**)	61 (+2.68**)
CD45RA	57 (−2.22**)	67 (−1.48**)	53 (−1.77**)	47 (−1.91**)	35 (−1.94**)
CD29	54 (+2.12**)	41 (+1.26**)	54 (+1.19**)	55 (+0.98**)	53 (+0.66)
HLA-DR	30 (+2.47**)	21 (+1.67**)	32 (+2.17**)	38 (+2.80**)	42 (+3.30**)
CD57	18 (+1.27*)	6 (+0.10)	21 (+1.06**)	21 (+1.03**)	20 (+0.97**)
CD11a	63 (+2.68**)	45 (+2.06**)	74 (+2.90**)	81 (+3.21**)	79 (+3.56**)

Results are expressed as the mean percent (and age-standardized Z scores) for each marker, for each age band. * $p<0.05$, ** $p<0.01$: results significantly different from age matched normal children.

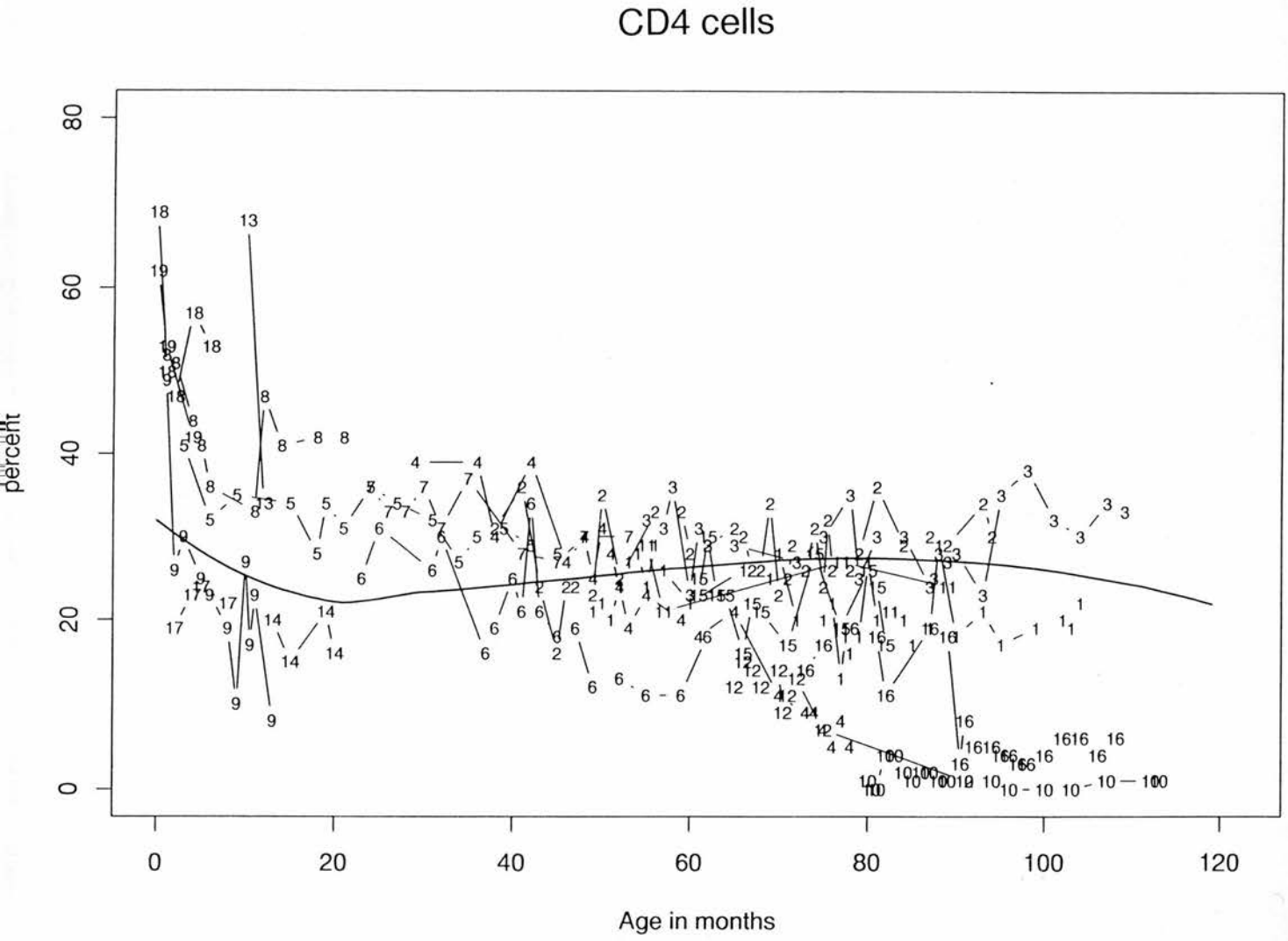

Figure 1. (A) The percentage of $CD4^+$ lymphocytes in individual patients.

Statistical Analysis

Because both the absolute levels and proportions of lymphocyte subpopulations change with age in normal children, the individual results for each patient were converted to age-standardized z-scores, or standard deviation units, relative to the age-related mean for normal children.[1] These were calculated by subtracting from each individual measurement the corresponding specific mean for normal children, and dividing by the age-specific standard deviation for no children. Thus, the z-scores for normal children would have a mean value of zero and a standard deviation of 1. The z-scores for the HIV-infected children were compared with those of normal children in 24-month age bands between 0–71 months, and a 36-month band between 72–108 months, as follows: within each age band, the mean of the individual results for each child was calculated (the child-specific mean z-score). The means of the child-specific mean z-scores were then calculated for each age band. Children in the first 24-month age band were subdivided into fast and slow progressors. Differences between the mean z-scores for HIV-infected children and the normal reference range were assessed by testing for a difference from a normal distribution with mean 0 and standard deviation of 1.

Results

The results, expressed both as the mean percentage and the mean z-score for each lymphocyte subpopulation, in each age band are shown in Table 2. For those subpopulations where patient results fall substantially outside the normal range, the results from individual children are shown, plotted against age (Figs. 1 and 2).

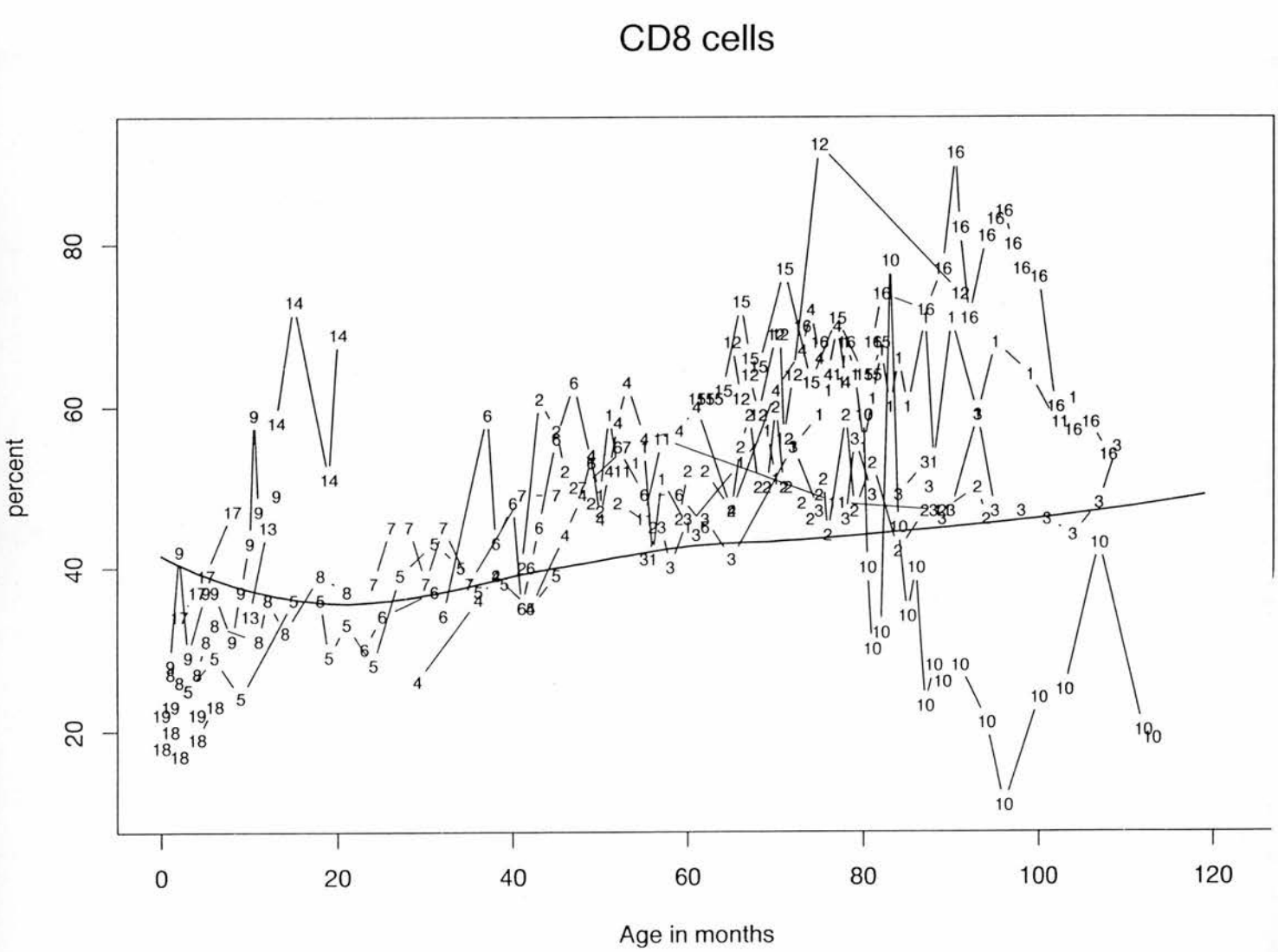

Figure 1. (B) The percentage of CD8$^+$ lymphocytes in individual patients. The numbers refer to patients 1–19 as in Table 1. The solid lines show the 3rd and 97th percentiles respectively of the normal ranges for CD4 and CD8 cells.

CD4 and CD8 Cells

The composite results show a fall over time in the percentage of CD4 cells and a rise in the percentage of CD8 cells. The rise in CD8 cells occurred faster and was more pronounced than the fall in CD4 cells, indicating that this is not simply a reciprocal of the CD4 percentage fall (Fig. 1 and z-scores in Table 2). Apart from one child whose level fell after 80 months, CD8 cell levels were at or above the 97th percentile for age-matched normal children by 45 months (Fig. 1B), whereas CD4 cell levels in several children remained between the 3rd and 10th percentile (Fig. 1A). Three of the four progressors (child 9, 14, and 17) showed a more rapid fall in CD4 cell loss, and all four (nos. 9, 13, 14, and 17) showed a more rapid rise in CD8 cells than the slow progressors. The differences between the infected and uninfected children reached statistical significance in the first age band (0–23 months) for fast progressors and in the second age band (24–47 months) for the slow progressors (Table 2). In two slow progressors clinical disease progression to CDC stage B3 was accompanied by a fall in both CD4 and CD8 cells (nos. 10 and 16, Fig. 1).

CD4 Subpopulations

Apart from HLA-DR+ CD4 cells, the mean percentages of all the CD4 cell subpopulations studied became significantly different from age matched uninfected children in the 48–71-month age group ($p < 0.01$, Table 2). HLA-DR+ CD4 cell numbers were elevated by 24 months in the fast progressors and by 48 months in the slow progressors (Table 2).

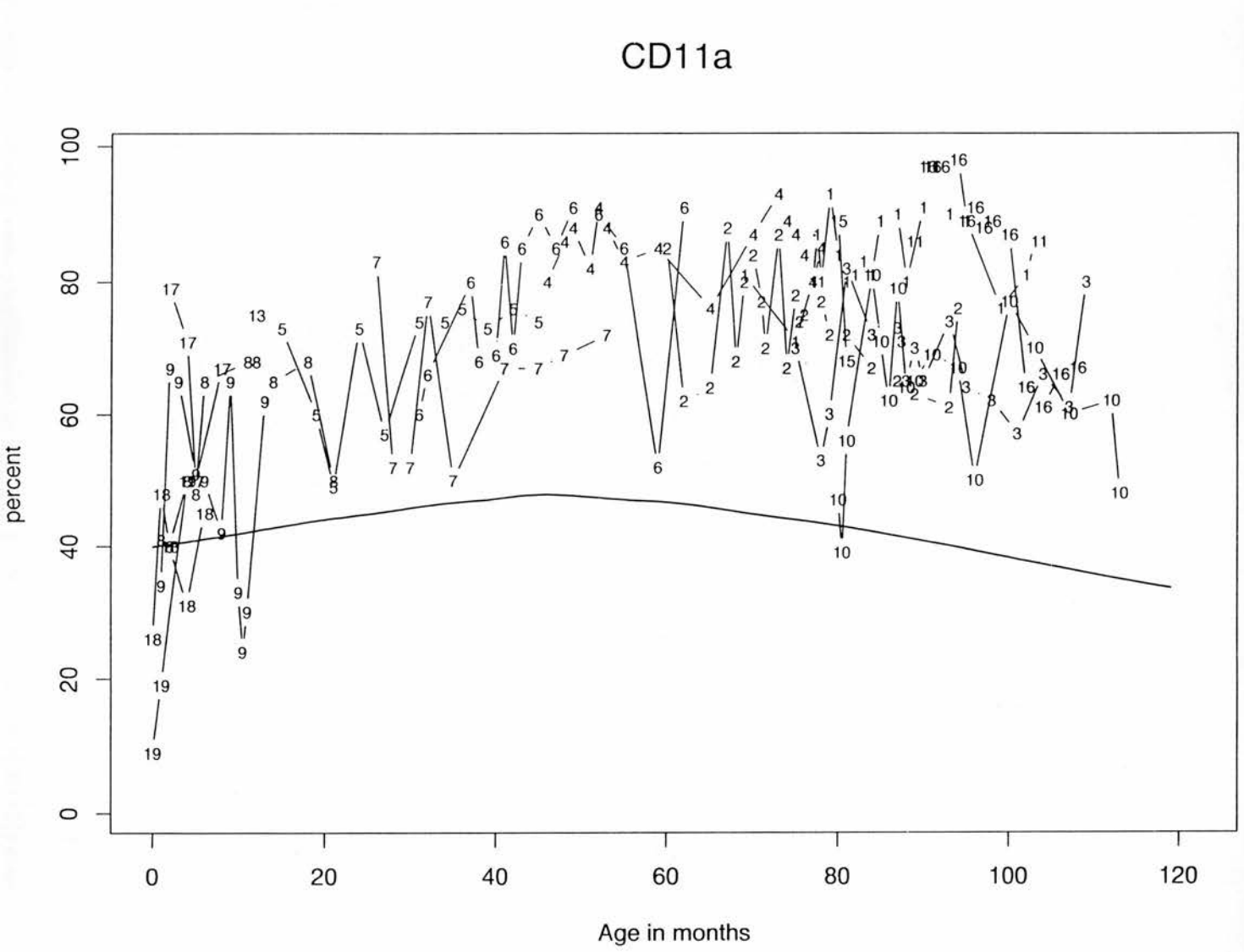

Figure 2A. Percentages of CD8 cell subpopulations in individual patients: CD11a+. Line numbers are as for Fig. 1. The solid lines show the 97th percentiles of each normal range.

CD8 Subpopulations

The mean percentages of all the CD8 cell subpopulations studied were significantly altered by 24 months of age, except for CD57+CD8 cells, which were increased by 48 months ($p < 0.01$, Table 2). The CD11a+^{bright} and HLA-DR+ subpopulations showed the largest increases (z-scores: 2.68 and 2.47, respectively, by 24 months in the fast progressors, Table 2). Individual levels of CD11a+^{bright}, CD45R0+, and HLA-DR+ CD8 cells were outside the normal range for most of the children tested (Fig. 2). In particular the individual levels of CD11a+^{bright} CD8 cells were above the 97th percentile in all children by 12 months of age. The increase in CD29+ CD8 cells, seen in the first 24 months, lessened over time, and levels returned to normal after 72 months of age.

Relationship between Lymphocyte Subpopulations and Virus Culture

The ages at which CD11a+^{bright}, CD45R0+, and HLA-DR+ CD8 subpopulations became elevated above the 97th percentile were compared with the ages at which virus culture was first positive in six children followed from birth. The results are shown in Table 3. Two children who were virus-culture positive in the neonatal period also had raised levels of these CD8 subpopulations at the time of their initial lymphocyte analysis at 3 weeks and 2 months of age respectively. Two additional children had elevated CD8 subpopulations at the time that they were first virus-culture positive, at 2 and 3 months respectively. Both had previously been virus-culture negative, and one had previously had normal CD8 subpopulation levels. A fifth child had elevated CD8

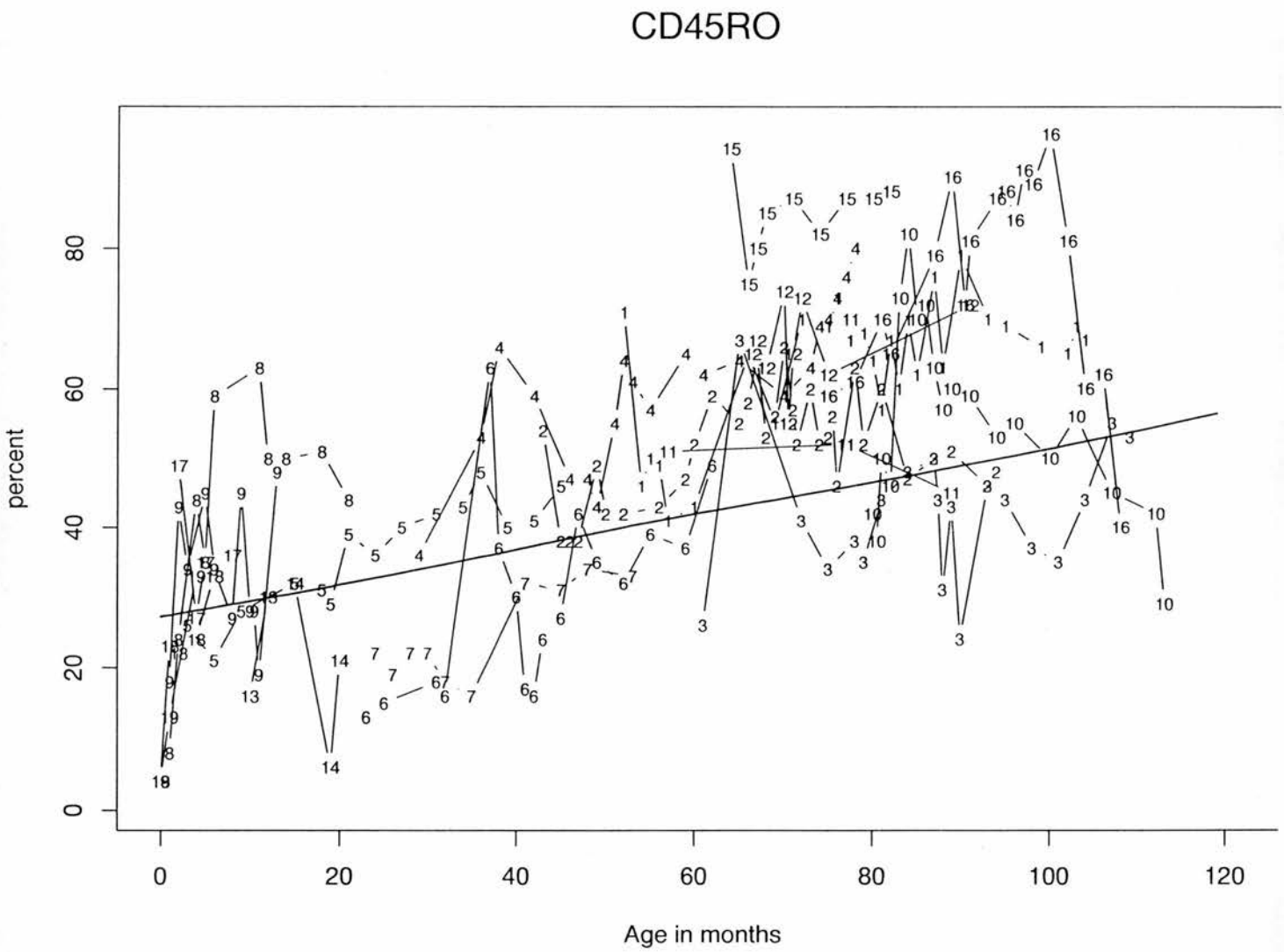

Figure 2B. Percentages of CD8 cell subpopulations in individual patients: CD45R0+. Line numbers are as for Fig. 1. The solid lines show the 97th percentiles of each normal range.

subpopulations at 1 month of age, 5 months prior to positive-virus culture, and a sixth child was virus-culture negative at 1 week, culture positive at 1 month, but did not show elevated CD8 subpopulations until the subsequent testing, 3 months later.

Discussion

This study has shown substantial and consistent differences in the expression of CD4 and CD8 cell markers in HIV-infected children compared to age-matched uninfected children and to HIV-infected adults. By expressing the results as z-scores, a derived statistic expressing units of standard deviation, we were able to analyze differences of expression over and above the changes due to normal maturation. The most surprising results were the rapid rate of increase of total CD8 cells in the four fast progressors, compared to slow progressors, and the levels in all the children of CD8 subpopulations expressing CD11a^{bright}, CD45R0, and HLA-DR markers.

The loss of CD4 cells and the differential rate o loss in the fast and slow progressors has been described previously in studies by Plaeger-Marshall et al.[11] and Shearer et al.[17] The European Collaborativ Study[18] has shown that the rate of fall of CD4 numbers is a better predictor of *Pneumocystis carini* pneumonia (PCP) than the absolute count; this i consistent with our results. The Italian pediatric co hort study also reported an increased rate of loss i CD4 cells, but did not show an increase in CD cells.[19] Our study has shown that an increase in the percentage of CD8 cells occurs, which is more rapic than the loss of CD4 cells and of greater magnitude.

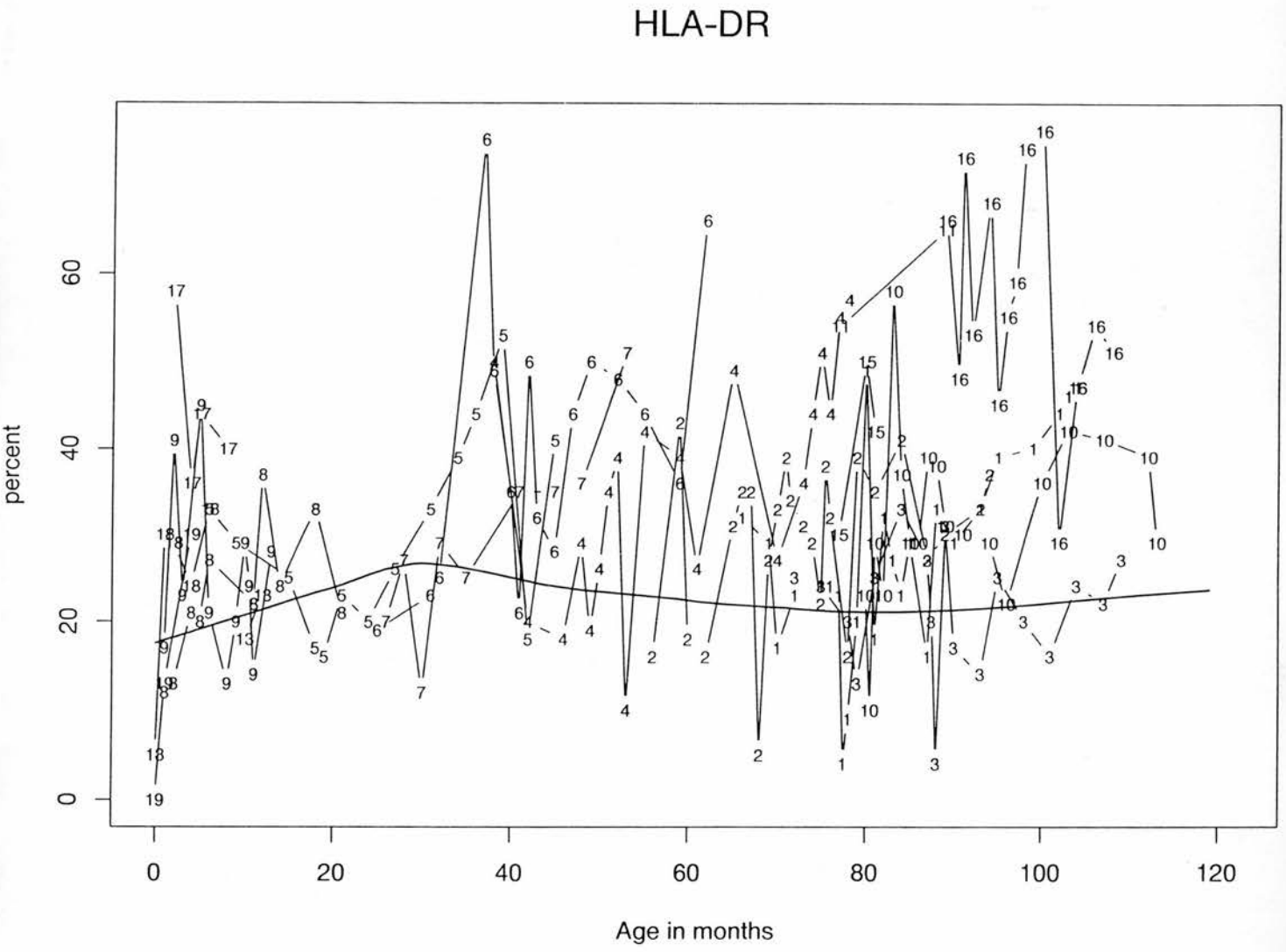

Figure 2C. Percentages of CD8 cell subpopulations in individual patients: HLA-DR+. Line numbers are as for Fig. 1. The solid lines show the 97th percentiles of each normal range.

The absolute values of the z-scores were consistently greater for the CD8% than for the CD4%, indicating that the CD8% increase was not simply the reciprocal of the CD4% decrease. Because of the relatively small number of subjects, statistical analysis was carried out only at 24 months; however, it is likely that with increased numbers, the changes in CD4 and CD8 subsets in fast progressors will be shown to be significant earlier than 24 months. An increase in CD8 cells is also seen in HIV-infected adults, particularly at seroconversion,[20] and may indicate an active immune response.[21] We and others have shown that HIV infected children have effective CD8 mediated immunity against HIV,[9,21–25] although in our studies, an elevated number of CD8 cells is not always associated with HIV-specific cytotoxicity.[22] The more rapid rate of increase of CD8 cells in the fast progressors may be a consequence of their having been infected in utero and, therefore, exposed to HIV for longer than the children who were infected at birth.[2] A third possibility is that the level of CD8 cells may be indicative of active disease, or perhaps higher viral burden, and may not necessarily represent an effective immune response to HIV.

There was no overall trend in CD4 or CD8 cells associated with progression to symptomatic disease or death. Of the two children who showed substantial loss of both CD4 and CD8 cells (no. 10 and 16), one has died and one has progressed to AIDS. However, similar changes were not seen in the other five children who died during the study. Larger cohort studies will be required to determine whether changes in lymphocyte populations can predict clinical progression.

Despite the different ages of recruitment, the changes in CD4 and CD8 subpopulations were re-

Table 3. CD8 Subpopulations and HIV Culture in the Diagnosis of HIV Infection in Children[a]

		Age when tested								
		Neonatal (wks)				(months)				
Child		cord	1	2	3	1	2	3	4	6
5	CD8 subpops							↑		↑
	virus culture			neg				**pos**		**pos**
8	CD8 subpops	norm			norm		↑		↑	↑
	virus culture				neg		**pos**		**pos**	**pos**
9*	CD8 subpops				↑		↑	↑		↑
	virus culture				**pos**		**pos**	**pos**		**pos**
17*	CD8 subpops						↑		↑	
	virus culture				**pos**		**pos**		**pos**	
18	CD8 subpops		norm			↑	↑		↑	↑
	virus culture					neg	neg		neg	**pos**
19	CD8 subpops		norm			norm			↑	
	virus culture		neg			**pos**			**pos**	

[a]*, clinically fast progressor; neg, negative virus culture after 4 weeks; **pos**, positive virus culture after 4 weeks; norm, all CD8 subpopulations in normal ranges; ↑, one or more CD8 subpopulation (CD11a^{bright}, CD45R0, or HLA-DR) outside the normal range.

markably consistent, with the levels of three of the CD8 subpopulations showing particularly rapid and sustained increases. The levels of HLA-DR+, CD45R0+, and CD11a+^{bright} CD8 cells increased soon after birth, with most individual patient's results crossing, and remaining above the 97th percentile. This observation suggested to us that these measurements may be useful in diagnosis. As HIV polymerase chain reaction (PCR) was not performed routinely during this study, the results were compared with the time that diagnosis was confirmed by a positive virus culture. In five out of six children followed from birth, HLA-DR+, CD45R0+, and CD11a+^{bright} CD8 cell subpopulations were elevated above the 97th percentile at the same time as or before they were virus-culture positive. We reported previously that the levels of these three CD8 subpopulations vary independently.[1] Thus, in a child born to an HIV-infected woman, an increase of one or more of these subpopulations, especially CD11a+^{bright} CD8 cells and one of the other two, is suggestive of HIV infection. CD45R0 and HLA-DR expression have been shown to be elevated in other infections, including intrauterine infection, and are not specific to HIV.[26,27] Our unpublished studies, however, suggest that high levels of CD8 subpopulations are transitory in other viral infections, whereas they persist in HIV infection. Further studies are required, however, to distinguish the changes in CD8 subpopulations in HIV from other pediatric viral infections, and to determine their validity as a diagnostic marker.

The immunological functions associated with these three CD8 subpopulations have been explored in a number of studies. CD11a+^{bright} and CD45R0+ CD8 cells have both been associated with both cytotoxic and memory cell functions.[28,30] In our studies, however, we found a predominance of CD8 cells with both of these markers in both cytotoxic-positive and -negative cultures;[22] thus there appears not to be a simple association between surface marker expression and immune function. More recently, CD11a+^{bright} CD8 cells have been associated with increased spontaneous Ig production in HIV-infected adults.[31] Polyclonal hypergammaglobulinemia is almost invariably seen in children with HIV,[32] which would be consistent with increased CD11a^{bright} expression. In a recent study, Okumura et al. showed that memory cell activity in the CD8 population resides in both CD45R0+ cells and in a subset of CD45RA cells expressing the CD11a^{bright} marker.[33] It has been suggested that elimination of the stimulating antigen allows CD45R0 cells to revert to CD45RA cells, while retaining memory for the antigen.[34] If this is the case, the continuously elevated CD45R0 expression seen in this study may be an indication of continuous restimulation by HIV antigens. CD8+CD57+ cells have been shown to suppress MHC-restricted cytotoxic T cells (CTLs).[35,36] CTLs are thought to be an important means of suppressing HIV replication in vivo and maintaining

asymptomatic disease.[37] In this study the fast progressors had higher levels of CD8+CD57+ cells than the slow progressors, which would be consistent with the view that increased CD8+CD57+ cells inhibit the beneficial effect of HIV-specific CTLs, and permit more rapid disease progression.

Changes in the CD4 subpopulations studied were also consistent but showed a different pattern than the CD8 subpopulations. Differences in the levels of the putative memory and naive markers, CD45R0 CD29 and CD45RA, on CD4 cells became statistically significant after 48 months, and were not as marked as in the CD8 subpopulations. This is in contrast to studies in HIV-infected adults, which have shown CD45R0+ and CD29+ CD4 cells to be either decreased[38,39] or unchanged.[40] Occurring later, these changes may be a result of altered immune function, reflecting later stage disease, rather than a response to virus expression as suggested for the CD8 subpopulations. It has been suggested that CD45R0+CD4+ cells in neonates[41] or CD45R0+ CD45RA+ double staining CD4 cells in cord blood[42] may represent an embryonic population of T cells that are functionally anergic. We do not believe that this accounts for the increase of CD45R0+CD4+ cells seen here, because the percentages at birth were similar in the HIV-infected and normal children. In the infected children, changes induced by HIV and CD4 cell regeneration as described recently by Ho et al. and others,[43–45] superimposed on changes due to natural maturation, will all contribute to the altered levels. Further studies are required to determine whether the increase of the CD45R0 marker on CD4 cells is a consequence of accelerated maturation due to HIV infection, or whether it is related to HIV pathogenesis, and therefore useful as a marker of disease progression. The activation marker, HLA-DR, increased earlier on CD4 cells, becomes significantly elevated in the fast progressors by 24 months and in the slow progressors by 48 months. Thus, increased numbers of activated CD4 cells may be more indicative of active disease than either the CD45RA or CD45R0 cell markers.

In conclusion, we have shown that children infected vertically with HIV show progressive and consistent changes in their levels of CD4 and CD8 lymphocytes, and in various subpopulations, which exceed the changes taking place as part of normal maturation. The rates at which some of these changes occur distinguish clinically slow and fast progressors. The degree of change in the CD8 subpopulations is such that these measurements could be used to aid the diagnosis of HIV infection in individual children, and may be particularly useful as a screening test where virus or DNA-based assays are not available. Changes in CD8 subpopulations may also identify children who have made an immune response to HIV without becoming infected.[46] Changes in the levels of some of the subpopulations studied are associated with early, active disease and others with later disease. Recent work using three-color staining suggests that more detailed surface marker analysis may further segregate different clinical groups (M. Aldhous, unpublished results and [47]). Longitudinal studies with larger cohorts are required to determine possible associations between changes in subpopulation levels and disease progression. Also, the associations between surface markers, immune function, and the immune mechanisms that determine clinical progression need to be further explored.

Acknowledgments

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Cytotoxic T Lymphocyte Activity in Children Infected with HIV

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ABSTRACT

Of the Edinburgh cohort of approximately 130 children born to HIV-infected women, 9 are infected and alive. This article describes results from the first 18 months of a natural history study of seven of these, and two adopted children, studying the CD8 T cell-mediated cytotoxicity against HIV proteins (Gag, Tat, Pol, and Env), over time, and relating it to clinical progression and viral activity. Autologous EBV cell lines infected with vaccinia–HIV constructs were used as target cells, and bulk-cultured peripheral blood mononuclear cells as effector cells. The children ranged in age from 0 to 93 months, with six of the nine showing CTL activity to one or more HIV proteins. The specificity of the response was directed against Tat in the younger children, switching to Pol, then Gag or Env. Preliminary analysis of virological data showed no association between CTL and virus activity. The children with CTLs tended to be well clinically, but the cohort needs to be studied longer before conclusions can be made about CTL activity and HIV disease progression. Cytotoxic T lymphocyte activity has also been observed in two children diagnosed as HIV uninfected. These results show the importance of looking at CTL specificity, and may have implications in vaccine design.

INTRODUCTION

CYTOTOXIC T LYMPHOCYTES (CTLs) are thought to be an important part of the immune response against HIV in adults.[1] HIV-specific CTLs develop after the burst of viral replication associated with acute infection, and are thought to suppress disease progression by killing infected cells. In children born to infected women, CTLs have been less well characterized. Vertical infection can take place *in utero* before the immune system is mature and able to distinguish self from nonself, at birth,[2] in association with an infusion of maternal IgG, which includes anti-HIV antibody, or after birth by the ingestion of infected breast milk.[3] Thus both the primary target cell for infection, and the nature of the immune response, could differ, depending on the timing and route of infection.

Scotland has the same incidence of HIV infection per head of population as the United Kingdom, but has two major differences. First, half of the 2000 Scottish cases, reported on September 30, 1993, are in the Edinburgh region, although the region has only one-tenth of the Scottish population. Thus, with 1000 cases, the city has a disproportionate concentration of HIV-infected people. Second, a much higher proportion (27%, or 270) of cases are women, who have been infected either through intravenous drug injection or through sex (Scottish Centre for Infection and Environmental Health, Glasgow, UK, personal communication, 1994). A further difference in Edinburgh is that the vertical transmission rate of HIV, at approximately 10%, is one of the lowest global rates reported.[4] The Edinburgh Paediatric HIV Clinic follows all children who are born to HIV-infected women in the area from birth, plus any children who are referred because of clinical symptoms. To date this numbers approximately 130, of whom 9 are infected and still alive. Seven of these, and two adopted children, in whom the route of infection is unknown, are included in this study. The children range in age from birth to 93 months. As most were above 2 years of age at enrollment they were selected for "slow" clinical progression.

In preliminary experiments, we found an absence of HIV Gag-specific CTL activity in children, despite a high incidence in adults from the same cohort.[5] In Edinburgh most of the HIV-infected drug-taking community are thought to have been infected by recognizably similar viruses[6] and we therefore rea-

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soned that the lack of a Gag response in the children was unlikely to be due to nonrecognition of the viral sequence used in the target construct, but may reflect a difference in the nature of the immune response. The present study was undertaken to determine whether HIV-infected children had CTL activity to HIV antigens other than Gag and, if so, how these related to viral activity and disease progression. In this article we report the preliminary results of CTL activity against HIV *tat*, *pol*, *gag*, and *env* gene products in 9 infected and 11 uninfected children, tested over an 18-month period.

MATERIALS AND METHODS

Patients

The study group comprised 9 HIV-infected children (mean age at start of study, 43 months; range, 3–88 months), and 11 children who had lost maternal HIV antibody, had no other indications of HIV infection, and so were presumed to be HIV uninfected (mean age at start of study, 27 months; range, 1–73 months). Eighteen of the children were known to be born to HIV-infected women; 2 were adopted and, as no maternal information was available, the route of infection was unknown. The clinical classifications of the infected children were determined using criteria from the Centers of Disease Control (CDC; Atlanta, GA),[7] and, together with details of clinical symptoms, treatment, and laboratory results, are presented in Table 1.

Cytotoxic T lymphocyte assay

The method was based on that of Nixon *et al.*,[8] and used bulk-cultured effector cells in a 4-hr ^{51}Cr-release assay. The majority of effector cells were phenotypically CD45R0- and CD11a-expressing CD8 cells and killing was completely blocked by anti-CD8 antibody[5] (M.C. Aldhous, unpublished observation, 1994). Effector cells were peripheral blood mononuclear cells (PBMCs), isolated from heparinized blood, and stimulated with phytohemagglutinin (PHA, 2.5 μg/ml; Wellcome, Research Triangle Park, NC). All cultures were maintained at 37°C in RPMI-1640 medium supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), 1 mM sodium pyruvate, 0.5 mM L-glutamine, and 10% fetal calf serum (FCS) ("RPMI medium") for 7 days, and subsequently in medium containing 5% interleukin 2 (IL-2) (Lymphocult, Biotest, Solihull, United Kingdom) replacing 50% of the culture supernatant every 3–4 days. Assays were carried out after 9–23 days of culture, when there were deemed to be sufficient cells. Target cells were autologous Epstein–Barr virus (EBV)-transformed B cell lines, infected overnight with 10^6 plaque-forming units (PFU) of either a vaccinia–HIV construct (*gag*, *tat*, *pol*, or *env*), or a vaccinia control. The constructs used were as follows: a vaccinia control (Vac-Vsc8WR) and vac-*gag* (from HIV-IIIB; MRC, AIDS Directed Programme, National Institute for Biological Standards and Control, Hertfordshire, United Kingdom), vac-*tat* (VVTG3196), vac-*pol* (VVTG3167), and vac-*env* (VVTG1132) (from HIV-LAI (Transgene, Strasbourg, France). A culture tube containing EBV cells not

TABLE 1. CLINICAL AND LABORATORY DATA OF CHILDREN STUDIED AT EACH TIME OF TESTING

Child	*Age (months)*	*Clinical symptoms*	*Treatment*	*CDC*[a]	*CTL*	*Virus culture*	*PCR*
1	79	Ear infection	Amoxil	P2A	Pol	–	–
	82	Mild throat infection	—		±	+	+
	87				–	–	–
	93	Well	—		Gag	–	+
2	71	Well	—	P2C, F	Pol	+	–
	75	Well	—		±	–	–
	78				±	nd	–
	84	Well	—		–	–	–
3	53	Well	–	P1B	Tat	+	+
	59	Well	—		Pol	+	+
4	28	Well	—	P1B	Tat	–	–
	35	Well	–		Pol	–	–
	45	Well	—		–	–	–
5	21	Pyrexia	Erythromycin	P2A	Tat/Pol	+	–
	24	Chest infection	Augmentin		–	+	–
	31				Tat/Pol	–	–
6	88	Well	—	P2A	–	–	–
	90	URTI	—		–	–	–
7	40	*Schistosoma haematobium*	—	P2A, C	–	+	+
	43	URTI, herpes simplex	Acyclovir		–	+	+
8	4	Chest infection	Amoxil	P2A	–	+	–
	6	Mild URTI	—		–	+	–
9	3	Candidiasis	—	P2A	–	+	–
	6	PGL	—		Tat/Pol	+	+

[a]CDC classification for pediatric HIV.
URTI, Upper respiratory tract infection; PGL, Persistent generalized lymphadenopathy.

infected with vaccinia constructs was set up as a "medium" control. Given the variable number of cells available, the effector cells were used at the highest effector-to-target ratios possible, generally between 10:1 and 50:1. The percent lysis of the target cells was measured after 4 hr in culture, by release of ^{51}Cr into the supernatant. Total and spontaneous lysis were determined by incubating target cells in 5% Triton X-100 and in medium respectively.

Virus isolation by culture

Peripheral blood mononuclear cells were isolated from ethylenediaminetetraacetic acid (EDTA)-anti-coagulated blood by separation over Ficoll-Hypaque (Nyegaard, Oslo, Norway) at $800 \times g$ for 20–25 min. The lymphocyte fraction was washed and resuspended in 10 ml of RPMI medium supplemented with amphotericin B (1.25 μg/ml; GIBCO, Paisley, United Kingdom), (10 IU/ml; MRC AIDS Directed Programme National Institute for Biological Standards and Control, Hertfordshire, UK), PHA (3 μg/ml), and containing 2×10^6 PHA-stimulated PBMCs from uninfected donors. Virus production was assayed, using a p24 antigen kit (Coulter, Hialeah, FL), every 7 days for 28 days. At each time of antigen testing, 1×10^6 uninfected donor PBMCs were added to the culture.

Polymerase chain reaction

A two-stage (nested) polymerase chain reaction (PCR) was performed using the method of Simmonds *et al.*[9] An outer primer pair complementary to the *pol* region of the HIV genome was used in the first PCR reaction. An aliquot of the product from this reaction was reamplified using an inner primer pair complementary to a region within the first PCR product. The product from the second PCR reaction was visualized by electrophoresis on a 2% agarose gel containing ethidium bromide.

RESULTS

Cytotoxic T lymphocyte activity against HIV Gag, Tat, Pol, and Env is shown in Figs. 1 and 2 for the HIV-infected and uninfected children, respectively. The results are presented as the percent specific lysis, net of medium and vaccinia controls, with values greater than 10% above controls being regarded as positive. Each infected child was assayed from two to four times, and the uninfected children once, except for one child (No. 20) who was assayed twice.

HIV-infected children

Six of the nine HIV-infected children showed CTL activity on at least one occasion (Fig. 1). The most commonly recognized target was Pol, followed by Tat (six and four children, respectively), with Gag being recognized by only one infected child. In four cases the CTL activity was directed against a single target (children Nos. 1, 2, 3, and 4) and in two cases it was directed against two targets (Tat and Pol in children Nos. 5 and 9). The specificity of the CTL response changed on successive tests. In the two youngest children who had CTLs (children Nos. 9 and 5, aged 6 and 21–31 months, respectively) the re-

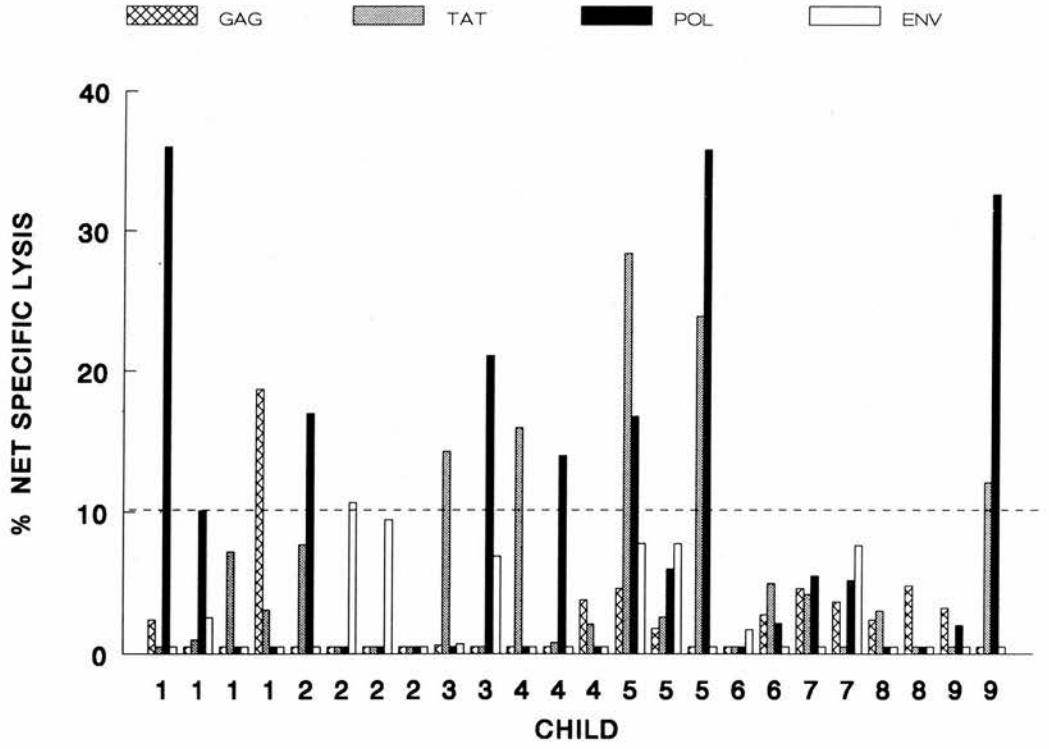

FIG. 1. Cytotoxic T lymphocyte activity against constructs of vaccinia and HIV *gag*, *tat*, *pol*, and *env* of sequential samples from HIV-infected children. The ages of the children at the time of testing are shown in Table 1.

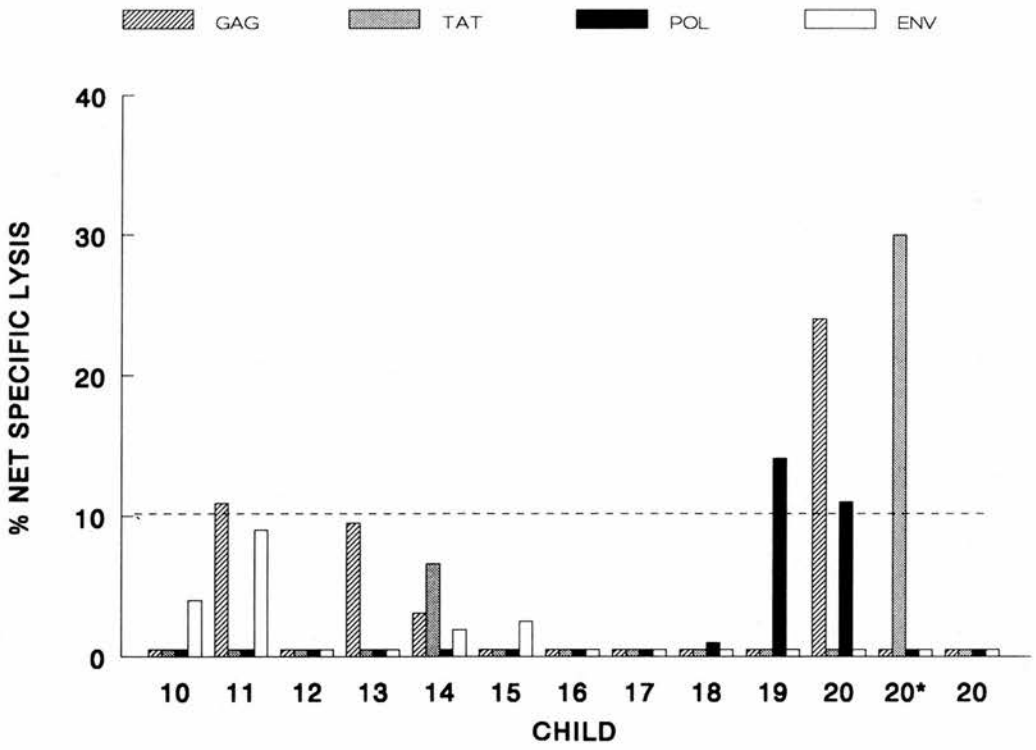

FIG. 2. Cytotoxic T lymphocyte activity against constructs of vaccinia and HIV *gag*, *tat*, *pol*, and *env* of HIV-uninfected children. *, Denotes activity measured after 11 and 18 days in culture.

sponse was against both Tat and Pol. Two children in the middle of the age range (children Nos. 3 and 4, aged 53–59 and 28–45 months, respectively) lost their response to Tat, but retained that to Pol; in the two oldest children, the specificity changed from Pol to a Gag in one (child No. 1, aged 79–93 months), and from Pol to a weak Env response in the second (child No. 2, aged 71–84 months). Two children (Nos. 2 and 4) lost CTL activity, and two children (Nos. 1 and 5) lost and regained activity during the study period. Three children (Nos. 6, 7, and 8) were CTL negative throughout the study.

HIV-uninfected children

Strongly positive CTL activity was detected in two HIV-uninfected children, and a further two had borderline activity. Child No. 20 showed significant levels of anti-Gag, anti-Tat, and weak anti-Pol activity when first assayed at 17 months of age. On this occasion, the effector cells were used at two different time points, days 11 and 18 after stimulation, and the specificity was shown to change from Tat and Pol on day 11 to Gag on day 18. At 25 months, this child showed high nonspecific killing, but no HIV-specific lysis (data not shown). Child No. 19 showed anti-Pol activity at 6 months of age. Children Nos. 11 and 13 had borderline (i.e., 10% lysis) anti-Gag responses.

Cytotoxic T lymphocyte activity and clinical progression

The clinical records of the infected children were examined for evidence of intercurrent infection and for other indications of HIV disease progression, at the times they were tested for CTL activity. Eight of the nine children are "slow" progressors with regard to HIV disease, and showed no clinical progression during the study period. The results, summarized in Table 1, show that five of the six children with CTL activity remained well during the period of follow-up. The sixth (child No. 9) was clinically a fast progressor and, despite developing CTLs at 6 months, had persistent generalized lymphadenopathy. This child lost CTL activity at 9 months, and developed toxoplasmosis at 10 months (data not shown). The three children in whom no CTL activity was detected tended to have intercurrent infections during the study period.

Cytotoxic T lymphocyte activity and viral activity

The results of virus culture and plasma PCR assays carried out during the study period were compared with the CTL responses (Table 1). There was no relationship between CTL activity and the ability to recover virus from either cells or plasma.

DISCUSSION

Cellular immune mechanisms, in particular HIV-specific CTL activity, are thought to be important means of immune-mediated suppression of HIV, and stimulation of the CTL response is increasingly being used as a measure of the efficacy of experimental vaccines. In this study we have asked which HIV antigens stimulate CTLs in vertically infected children, and have begun to address the question of whether CTL activity correlates with clinical stability and virus activity.

The main result of the study is the different specificity of CTL target recognition of the children compared with adults from the same cohort. The predominant HIV antigens recognized by this group of infected children were Pol and Tat. Gag and Env were recognized by only one infected child each. The second observation was a pattern of recognition in the infected children that was related to age, and therefore to time after infection. Although the numbers are small, the children come from a relatively homogeneous cohort, and it is possible that similar changes in CTL target recognition take place in the individual children. If this is confirmed, it may be important for infants and young children that anti-HIV immune therapy be directed against the epitopes recognized early in the course of infection, that is, Pol and Tat, rather than, or in addition to, Gag and Env sequences.

Our results are consistent with those of Luzuriaga *et al.*,[10] who found anti-Gag and -Env activity in only a proportion of vertically infected children, but differ from those of Cheynier *et al.*[11] and Bruseyne *et al.*,[12] who found CTL activity to HIV Gag, Env, Pol and Nef in a high proportion of children tested. There are a number of possible explanations for these differences. The possibility that the results are due to technical or methodological differences cannot yet be ruled out. The effector cells are grown from comparatively small blood samples, and may be kept in culture for longer periods than is required for adult samples. Bulk-cultured effector cells, in one experiment, which were tested at two separate time points, showed differing target specificities, suggesting that different CTL clones may expand at different rates and become dominant at different times after stimulation. Differences may also result from variations in virus strain or sequences used for the vaccinia constructs. The causes of these differences need to be clarified if CTL activity is to become a valid measure of vaccine effectiveness.

To address the question of whether CTLs are effective *in vivo* we examined the case notes for these children for evidence of HIV disease progression and of intercurrent infections. During the period of the study, eight of the nine children, that is, five with CTL activity and the three without, were stable with regard to HIV disease progression. Of the two children followed from birth, the child who made a transient CTL response deteriorated clinically, whereas the second, who was CTL negative, was clinically stable. All three CTL-negative children had recorded intercurrent infections, compared with three of the six CTL-positive children. The data, although preliminary, suggest that CTLs may be associated with fewer intercurrent infections, although it is too early to draw any conclusions about long-term HIV disease prognosis.

In a cross-sectional comparison of CTL activity, virus coculture, and HIV PCR results, we found no relationship. The virus test results, however, were qualitative, and this comparison may mask quantitative changes. Further longitudinal, quantitative comparisons are in progress.

The second main result of the study is the relatively high proportion of exposed but presumed uninfected children that have HIV-specific CTLs. Two of 11 presumed uninfected children had strong CTL activity on 1 occasion and a further 2 had borderline levels (Fig. 2). Evidence of cellular immune responsiveness to HIV in uninfected individuals has been reported by a number of groups.[11,13,14] The intracellular processing and presentation of viral peptide to CTLs is thought to require infection of the target cell, implying that these children have been exposed to live, although possibly nonreplicating, virus. Of interest is the transient nature of the CTL response. Child No. 20, who was assayed twice, lost the response on the second occasion, suggesting that CTL precursors were no longer present in the circulation and raising the question of whether this child has retained immunological sensitivity to HIV. Other laboratory tests, including HIV antibody, antigen culture, and PCR, were consistently negative (data not shown), and the child was diagnosed as uninfected. It is possible, however, that some virus is still present in the lymphoid tissues of the children with CTLs,[15,16] capable of emerging at a later date, as occurred in one child studied by Luzuriaga *et al.*[10]

It is still not confirmed that CTL activity is universally beneficial to the individual infected with HIV. Although studies have shown that loss of CTL activity in asymptomatic individuals occurs before clinical disease progression, the finding of CTLs in bronchial lavage[17] and cerebrospinal fluid[18] indicates an inflammatory response that may contribute to the pathological manifestations of HIV disease. Early infection with HIV (*in utero,* rather than at delivery) may lead to a degree of tolerance and therefore a lack of cytotoxicity, resulting in more rapid progression, as the infection has been able to proceed for a longer time in the absence of a competent immune system. It is important to establish whether slow clinical progression is due to the effectiveness of the immune response, and if CTLs contribute to this.

In conclusion, our results show a vigorous CTL response in the majority of infected children, and in a significant proportion of uninfected children, with target specificities primarily against HIV Tat and Pol, but changing as the children get older. The study needs to be continued until the infected children reach a clinical end point, to determine the *in vivo* anti-HIV effectiveness of CTLs with different specificities. The study also needs to be repeated in other cohorts of children, and in newly infected adults, to determine whether the pattern of responses relates to time after infection, or whether HIV antigen presentation and the immune response in children differ from that in adults.

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HIV-specific cytotoxic T-cell activity in an HIV-exposed but uninfected infant

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The factors necessary for protective immunity against HIV-1 are unknown. Important information about these factors should come from study of people at high risk of HIV infection who have not apparently become infected. Among these are the estimated 60–85% of children who may be exposed in utero or perinatally to HIV-1 but do not become infected. We observed the transient appearance of HIV-specific cytotoxic T-lymphocyte (CTL) activity in a baby born to HIV-1-infected parents, in whom all standard markers of infection remained negative. These findings suggest that HIV-specific CTLs may be a marker for recently exposed, but uninfected, individuals.

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An important advance in the rational design of a prophylactic vaccine against human immunodeficiency virus (HIV) would be identification of people who have been exposed to HIV and generated an immune response, but not become persistently infected. A characteristic feature of the immune response to HIV is presence in healthy seropositive subjects of vigorous HIV-specific cytotoxic T-lymphocyte (CTL) activity. Cytotoxic T cells appear early in the immune response to most viral infections and are thought to have an important role in the eradication of virus, or in the control of viral replication in persistent infections.[1] The specific CTL response to HIV is of much greater magnitude than that observed in almost any other viral infection, and is thought to be one of the main factors contributing to the long symptom-free period.[2] HIV-specific CTLs can readily be detected in seropositive patients but are not normally observed in uninfected subjects. We describe the finding of HIV-specific CTL activity in a child born to an HIV-infected mother; all standard markers of HIV infection in the child were negative by the age of 13 months.

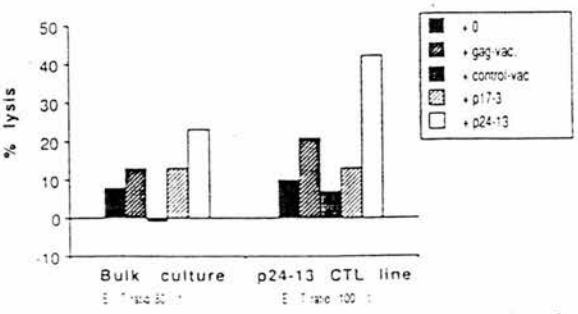
The wife of an HIV-infected haemophiliac was first diagnosed as anti-HIV-positive in January, 1989. Two years later she became pregnant and gave birth to a healthy boy by spontaneous vaginal delivery. During the pregnancy she had symptoms that were probably related to HIV infection, including diarrhoea, weight loss, and oral candidiasis. Her absolute CD4 count ranged from 0·17 to $0·3 \times 10^9$/L and p24 antigen was consistently positive.

At delivery, cord blood from the baby was anti-HIV-positive (Behring Enzygnost 1/2 enzyme immunoassay [EIA]) (presumed maternal antibody) and negative for p24 antigen (Abbott). Cord blood mononuclear cells enriched on a Ficoll-Hypaque gradient, co-cultured with phytohaemagglutinin-stimulated lymphoblasts from an anti-HIV-negative donor, and assayed weekly for p24 antigen in the culture supernatant showed no evidence of HIV infection. HIV-specific CTL activity against *gag* and *pol* antigens could not be detected either from freshly isolated cord blood mononuclear cells or with cells cultured with autologous phytohaemagglutinin-stimulated lymphoblasts in the presence of interleukin 2 (phytohaemagglutinin increases expression of autologous viral antigens in HIV-infected cells and these antigens would be expected to stimulate proliferation of HIV-specific CTL in culture[3]).

At the age of 5 months, the child was thriving and developing normally. His blood was still seropositive for maternal HIV antibody but negative on all other tests for HIV infection, including p24 antigen, viral co-culture, and polymerase chain reaction with HIV-*gag*-specific primers. However, HIV-*gag*-specific CTL activity could be detected with peripheral blood mononuclear cells expanded in vitro, as outlined above (figure). Target cells were an Epstein-Barr-virus-transformed B-cell line from the baby, which were infected with recombinant vaccinia viruses expressing HIV *gag* or a control protein from influenza, and labelled with ^{51}chromium. Specific lysis was also observed when target cells were incubated with a synthetic peptide from HIV *gag* containing a CTL epitope restricted by HLA-B8, which the child shares with his father. We have described recognition of this peptide epitope, p24–13, by CTLs from the father.[4] It was possible to grow a CTL line from the baby specific for the p24–13 peptide with irradiated peptide-pulsed autologous B cells as stimulators.

Studies that used the antibody UCHL-1 to measure surface expression of the CD45RO antigen associated with "memory" antigen-specific T cells, showed abnormally high proportions of CD8 lymphocytes expressing this marker—ie, 42% of CD8 T cells expressing CD45RO at 5 months old (normal for this age 0–15%). Increased concentrations of circulating CD8 T cells that stain with UCHL-1 antibody are strongly associated with HIV infection in children born to infected mothers.[5]

3 months later, the baby still had HIV antibody, although concentrations had declined, but viral culture for HIV was again negative and there was no evidence of HIV RNA or DNA by polymerase chain reaction with nested *gag* and *pol* primers. Immunoglobulins and the CD4/CD8 ratio were normal. At the age of 13 months, the child was healthy and anti-HIV (Behring Enzygnost HIV1/2 EIA, Enzygnost micro-HIV-1 competitive EIA) negative for the



CTLs from the baby recognise HLA-B8-restricted antigen in HIV *gag*.

See text for details of assay. +0 = no added peptide. E:T ratio = effector cell to target cell ratio.

first time. HIV antigen testing (Organon Vironostika) was negative and polymerase chain reaction analysis showed no evidence of HIV RNA or DNA. HIV-specific CTL activity could no longer be detected and the proportion of CD45RO CD8 lymphocytes was normal—ie, 20% of normal (range at 13 months 4–20%). At 18 months, the child is well and continues to grow and develop normally.

Although diagnosis of paediatric HIV infection may be difficult, it is generally agreed that a seronegative child with no clinical symptoms or immunological or virological evidence of infection after a year can be regarded as uninfected.[6] Some of the large cohort studies of children born to infected mothers[7,8] have identified a small population (2·5–4·7%) of seronegative children in whom viral markers have been or continue to be positive. The longterm outlook of such children is unknown, but studies suggest that they have a good prognosis. Exhaustive studies in the child reported have shown no evidence of active HIV infection. Only the transient appearance of HIV-specific CTL activity at the age of 5 months, in parallel with an increase in a marker on CD8 lymphocytes thought previously to indicate HIV infection, suggests that the child was exposed to HIV-1. These signs returned to normal and by all currently accepted criteria, the child is not infected.

However, if the child has never been infected it is difficult to explain the finding of HIV-specific CTL activity, which normally requires actively replicating virus for stimulation. It is possible that transfer across the placenta of maternal p24 antigen or defective virus incapable of replication might have stimulated production of HIV-*gag*-specific CTLs. Transfer of maternal CTLs is unlikely because the baby's CTLs responded to the same peptide epitope as CTLs from his father, presented by the HLA molecule B8, which the mother does not share. Similarly, antibody-dependent cellular cytotoxicity, mediated by transferred maternal antibody, could not account for the specificity of CTL lines grown for some weeks in culture from the child.

It is possible, therefore, that the child had encountered HIV but subsequently controlled or even cleared the virus, and that his CTL activity may have contributed to this process. In 20 children born to HIV-1-infected mothers,[9] a strong CTL response correlated with a better clinical course. This study also noted HIV-specific CTL activity in 3 children who later became seronegative; viral studies in these children were also negative, although 1 had presented with symptoms of HIV-1 infection at age 5 months.

There has been much interest in markers of immunity in HIV-exposed but seronegative individuals: such patients include haemophiliacs who are known to have received contaminated factor VIII and the sexual partners of HIV-infected people who continue high-risk behaviour. A study[10] of helper T-cell reactivity, measured by interleukin 2 production from peripheral blood mononuclear cells in response to synthetic HIV-envelope peptides, found evidence of HIV-specific activity in 5 anti-HIV-negative homosexual men at continued high-risk of HIV infection, in whom other markers of viral infection were negative; CTL activity was not measured in these men. 1 man subsequently seroconverted, but the others remain apparently uninfected, suggesting that there may have been exposure to HIV at a level sufficient to prime T-cell immunity but insufficient to induce antibody production.

Further studies of exposed but uninfected people are essential to provide information on the components of protective immunity to HIV, which might then form the basis of an effective prophylactic vaccine.

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Transmural myocardial infarction with sumatriptan

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For sumatriptan, tightness in the chest caused by an unknown mechanism has been reported in 3–5% of users. We describe a 47-year-old woman with an acute myocardial infarction after administration of sumatriptan 6 mg subcutaneously for cluster headache. The patient had no history of underlying ischaemic heart disease or Prinzmetal's angina. She recovered without complications.

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Sumatriptan is a serotonin-1 agonist used to treat acute attacks of migraine and cluster headache.[1] In trials, sensations of pressure and tightness in the chest occurred in 3% of patients treated with 100–300 mg orally and in 5% of