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The humoral immune response to HIV-1: Consequences for vaccine design

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Abstract

Some 42 million individuals worldwide are infected by the Human Immunodeficiency Virus (HIV) and no cure or vaccine is available. This thesis addresses approaches to humoral immunity to HIV-1.

In primary infection, the cytotoxic T lymphocyte (CTL) response is detected early and is thought to play a role in the viral decline. Neutralising antibodies (NAbs) are detected much later. However, non-neutralising anti-HIV-1 Env glycoprotein Abs (non-NAbs) are present concomitantly with the CTL response. The possible role of non-NAbs with complement was investigated using sequential sera and viruses expressing gp120 Env (gp120) glycoproteins amplified from blood samples from a cohort of newly HIV-1 infected patients. Autologous gp120 sequences were cloned and expressed into a replication-competent HIV-1 backbone. The autologous Ab pattern was studied. In the presence of complement, inactivation of autologous and heterologous HIV could be detected as early as day 9 post-onset of symptoms (POS). IgG were partly responsible for triggering the classical complement cascade.

In parallel, a new approach was investigated to generate a recombinant vaccine to HIV-1. Camelids synthesise IgG devoid of light chains. These IgG fragments (VHH) share the same characteristics as classical IgG but have unusually long CDR H3 regions that can adopt more flexible conformations. The possibility of generating VHH fragments that mimic the neutralising CD4 binding site (CD4BS) of HIV-1 was investigated. A llama was immunised with IgG1 b12 (b12), a potent cross-neutralising human NAb overlapping the CD4BS of HIV-1. The non-classical VHH repertoire was cloned, the resulting libraries were panned against b12 by phage display and five specific anti-b12 VHH fragments were isolated. Each of the five fragments was tested in animals for the induction of an anti-HIV-1 NAb response.

These studies are discussed with reference to the control of HIV-1 infection by drugs and vaccines.

DECLARATION

Dr Marlen Aasa-Chapman and Keith Aubin cloned and generated all the chimeric viruses used in Chapters 3 and 4, except for the viruses MM19 and MM22 that I generated myself.

Dr Marlen Aasa-Chapman performed the experiments described in Chapter 3 using viruses MM4, MM23 and MM28 as well as the development of the Ig response, except for the patient MM 22. I performed the experiments using all the other viruses described in Chapter 3 and I performed all the experiments described in Chapter 4.

Pim Hermans and Dr Hans de Haard amplified and cloned the anti-b12 VHH libraries. They contributed to the first round of selection in Chapter 5. They also expressed and purified VHH fragments without the Myc tag. Dr Peter Delves and his colleagues conjugated the VHH to KLH, immunised the animals and checked the immune response by ELISA. I performed the other experiments described in Chapter 5.

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Contents

Title Page 1
Abstract 2
Declaration 3
Acknowledgements 4
Contents 7
Figures 14
Tables and diagrams 17
Abbreviations 18
Chapter 1 Introduction to HIV 21
1.1 HIV history 21
1.2 Genome organisation 22
1.3 Classification and distribution of HIV-1 in the world 23
1.4 Origins of HIV 24
1.5 Replication cycle 26
1.5.1 Entry 27
1.5.2 Uncoating and reverse transcription 28
1.5.3 Nuclear import and integration 30
1.5.4 Transcription 32
1.5.5 Splicing and nuclear export of viral mRNAs 33
1.5.6 Protein assembly 33
1.5.7 Virus budding and maturation 34
1.6 Viral entry 36
1.6.1 Structure of HIV envelope 37
1.6.1.1 gp120 37
1.6.1.2 gp41 39
1.6.1.3 The gp120-gp41 interaction 41
1.6.2 Receptor and co-receptors and surface cellular structures involved in virus binding 41
1.6.2.1 CD4 41
1.6.2.2 The chemokine co-receptors 42
1.6.2.3 Surface cellular structures 47

1.6.3 Binding to CD4 and co-receptor and fusion..... 49
1.7 Cell targets..... 51
1.8 Transmission..... 51
1.9 Clinical symptoms of HIV and AIDS..... 52
1.9.1 Acute infection..... 52
1.9.2 Chronic infection and AIDS..... 54
1.9.3 Cell death and CD4 T cell dynamics..... 55
1.10 Viral reservoirs..... 56
1.11 Genetic factors and progression of HIV/AIDS..... 58
1.12 Innate non-immune responses to HIV..... 60
1.12.1 Post-entry restrictions targeting CA..... 60
1.12.2 APOBEC3G and Vif..... 61
1.12.3 The *murr1* gene..... 63
1.13 Immune responses to HIV and immune escape..... 63
1.13.1 The viral proteins Nef, Tat and Vpu..... 63
1.13.1.1 MHC Class I and Tat..... 64
1.13.1.2 MHC Class I and Vpu..... 64
1.13.1.3 Nef..... 64
1.13.2 The innate immune response..... 65
1.13.2.1 Natural killer cells..... 65
1.13.2.2 γδ T cells..... 67
1.13.2.3 Complement..... 67
1.13.3 The adaptive immune response..... 69
1.13.3.1 The CD4 T cell response..... 69
1.13.3.2 The CTL response..... 69
1.13.3.3 The humoral response..... 72
1.13.3.3.1 IgA..... 72
1.13.3.3.2 Evolution of the neutralising anti-HIV response in infected individuals..... 73
1.13.3.3.3 Role of neutralising antibodies *in vivo*..... 73
1.13.3.3.4 Neutralising epitopes..... 74
1.13.3.3.5 TCLA versus PI neutralisation sensitivity..... 74
1.13.3.3.6 Neutralising antibody escape..... 75

1.13.3.3.7 Cross-neutralising human antibodies.....77
1.13.3.3.8 Mechanisms of neutralisation.....78
1.13.3.3.9 Antibody-dependent enhancement.....78
1.14 Anti-HIV treatments.....79
1.14.1 HAART.....79
1.14.2 sCD4 and sCD4-IgG.....80
1.14.3 Chemokine receptor antagonists.....81
1.14.4 The anti-fusion peptide T20.....81
1.14.5 Microbicides and passive administration of neutralising antibodies.....82
1.14.6 Other drug targets.....83
1.15 Vaccines.....84
1.15.1 Envelope-based immunogens.....84
1.15.2 CTL vaccine.....84
1.16 Scope of this thesis.....87

Chapter 2 Materials and Methods.....88
2.1 Buffers and solutions.....88
2.2 Description of bacterial strains, mammalian cell lines and antibodies.....89
2.2.1 Bacterial strains.....89
2.2.2 Mammalian cell lines.....89
2.2.3 Antibodies and sera.....90
2.3 Mammalian cell culture.....91
2.3.1 Maintenance of cell lines.....91
2.3.2 Passage of cells.....91
2.3.3 Storage of cells.....91
2.3.4 Transfection of mammalian cells.....91
2.4 Primary cell culture.....92
2.4.1 Preparation of PBMCs from buffy coats.....92
2.4.2 Preparation of macrophages from buffy coats.....92
2.5 HIV strains.....93
2.5.1 Description of viral strains.....93
2.5.2 Growth of viral stocks.....93
2.5.2.1 From suspension cells.....93

2.5.2.2 From macrophages....94
2.5.3 Titration of viral stocks....94
2.5.4 Estimation of viral infection....94
2.6 Preparation and storage of complement....95
2.7 The Jenner patient cohort and the processing of samples....95
2.8 Neutralisation and CMI assays....96
2.8.1 Neutralisation assays....96
2.8.2 CMI assays....97
2.8.3 Inhibition of b12 neutralisation *in vitro*....97
2.8.4 Statistical analysis....98
2.9 DNA manipulation....98
2.9.1 Generation of chimeric HIV-1 viruses....98
2.9.1.1 Primers....99
2.9.1.2 Amplification of gp120 sequences....100
2.9.1.3 Cloning of gp120 sequences in pGEM® vector....100
2.9.1.4 Cloning of gp120 sequences in the HxB2 backbone virus....101
2.9.2 Preparation of VHH libraries....101
2.9.2.1 Llama immunisation and generation of VHH libraries....101
2.9.3 Bacterial transformation....102
2.9.3.1 Preparation of calcium chloride competent *E. coli*....102
2.9.3.2 DNA transformation....102
2.9.4 Colony screening....103
2.9.4.1 Screening by PCR and enzyme restriction....103
2.9.4.2 Plasmid purification and enzyme restriction....103
2.9.5 Sequencing reactions....103
2.10 Selections of the VHH libraries....103
2.10.1 Growth of libraries....103
2.10.2 Phage rescue....104
2.10.3 Phage precipitation....104
2.10.4 Selections....104
2.10.5 Clone screening....105
2.10.5.1 Master plate....105
2.10.5.2 Soluble expression and ELISA....105
2.11 Expression and purification of VHH fragments....106

2.11.1 Induction of protein expression..... 106
2.11.2 Periplasmic extraction and purification of VHH fragments..... 106
2.11.3 Protein gel electrophoresis..... 107
2.12 Characterisation of VHH fragments by ELISA..... 107
2.13 Detection of the anti-gp120 Ab response by ELISA..... 107
2.14 IgG purification..... 107
2.15 KLH conjugation to VHH fragments and to gp120..... 108
2.16 Immunisation regimes..... 108

Chapter 3 The role of anti-HIV-1 antibodies and complement during the course of infection

3.1 Introduction..... 110
3.2 Results..... 112
3.2.1 CMI of HIV-1 in the presence of heterologous cross-neutralising sera..... 112
3.2.1.1 T cell line adapted strains..... 112
3.2.1.2 Primary isolates..... 112
3.2.2 Neutralisation patterns of HIV gp120 from recently infected individuals..... 115
3.2.2.1 Description of the amplified gp120 115
3.2.2.2 Detection of the anti-gp120 Ab response..... 116
3.2.2.3 Autologous neutralisation assays..... 116
3.2.2.4 Heterologous neutralisation assays 118
3.2.3 CMI of viruses in the presence of autologous neutralising sera..... 119
3.2.4 CMI of viruses in the presence of autologous non-neutralising sera..... 119
3.2.5 CMI of autologous viruses in the early course of infection..... 123
3.2.6 CMI of viruses in the presence of heterologous non-neutralising sera..... 123
3.2.6.1 Virus MM4..... 123
3.2.6.2 Virus YU2 123
3.3 Discussion..... 127

Chapter 4 Characterisation of complement mediated-inactivation *in vitro*

4.1 Introduction.....129

4.2 Results.....132

4.2.1 Involvement of complement..... 132

4.2.1.1 Purified Guinea pig complement..... 132

4.2.1.2 EDTA/EGTA-treatment of complement..... 133

4.2.1.3 Serum from C1q- and C3-deficient individuals as a source of complement..... 133

4.2.2 Titration of complement..... 133

4.2.3 Antibody isotypes involved in CMI.....135

4.2.4 Monoclonal human antibodies and CMI.....135

4.2.5 Effect of complement in combination with sCD4-IgG2.....139

4.2.6 Influence of cell types in CMI..... 139

4.2.6.1 CMI comparison between macrophage-and PBMC-derived viruses..... 139

4.2.6.2 CMI comparison between H9- and PBMC-derived viruses..... 141

4.2.6.3 CMI comparison between 293T- and PBMC-derived viruses....141

4.3 Discussion.....143

Chapter 5 Isolation of llama non-classical VHH specific for b12, a potent neutralising anti-HIV-1 antibody: a vaccine approach

5.1 Introduction.....146

5.2 Results.....149

5.2.1 Generation of the anti-b12 libraries..... 149

5.2.2 Panning procedures of the libraries.....149

5.2.2.1 Panning of the libraries using b12, total HIV-1 lysate and gp140..... 149

5.2.2.1.1 First round of panning: non-specific elution.....151

5.2.2.1.2 Second round of panning: elution with virus lysate.....151

5.2.2.1.3 Second and third rounds of panning: elution with gp140..... 153

5.2.3 Isolation of specific anti-b12 VHH clones.....158

5.2.3.1 Screening by phage ELISA.....158

5.2.3.2 Restriction patterns..... 158

5.2.3.3 Sequencing of VHH fragments DNA..... 160

5.2.4 Characterisation of the anti-b12 VHH fragments..... 160
5.2.4.1 Expression of VHH fragments 160
5.2.4.2 Binding properties of purified VHH fragments..... 162
5.2.4.2.1 Binding properties against sCD4 and purified polyclonal human IgG..... 162
5.2.4.2.2 Binding properties against anti-HIV-1 monoclonal human antibodies..... 162
5.2.5 Inhibition properties of b12-mediated HIV neutralisation by VHH fragments..... 162
5.2.6 Anti-b12 VHH fragments used as immunogens for eliciting a neutralising antibody response against HIV-1..... 167
5.2.6.1 Sub-cloning and purification of anti-b12 VHH fragments..... 167
5.2.6.2 Neutralisation assays of HIV-1 with sera from the first boost of immunisation..... 167
5.2.6.2.1 In the absence of complement..... 168
5.2.6.2.2 In the presence of complement..... 170
5.2.6.3 CMI assays of HIV-1 with sera from the second boost of immunisation..... 170
5.2.6.4 CMI assays of HIV-1 with sera from the third boost of immunisation..... 170
5.2.6.5 Neutralisation assays with sera from animals boosted with monomeric gp120..... 173
5.3Discussion..... 174

Chapter 6 Summary and general discussion..... 177

References..... 178

Appendices..... 222

Figures

Chapter 1

Figure 1.1 DNA genomic organisation of HIV-1 and HIV-2.....23

Figure 1.2.A Geographic distribution of HIV clades in the world.....25

Figure 1.2.B Population estimated to be infected with HIV worldwide.....25

Figure 1.3 Replication cycle of HIV.....27

Figure 1.4 Reverse transcription of HIV RNA into genomic DNA.....29

Figure 1.5 Schematic representation of an infectious HIV virus particle.....36

Figure 1.6 Schematic representation of the monomeric gp120 backbone...38

Figure 1.7 Schematic representation of the domains of gp41.....40

Figure 1.8 Fusion between HIV and cell membranes.....50

Figure 1.9 Representation of the viral load, the CD4 T cell count, the emergence of the CTL response, the anti-HIV IgG and the development of anti-HIV Abs.....53

Chapter 3

Figure 3.1 CMI of IIIB, SF2 and RF with the heterologous neutralising QC serum 2.....113

Figure 3.2 CMI of IIIB and RF with the heterologous neutralising QC serum 5.....114

Figure 3.3 CMI of SF162 and SL2 with the heterologous neutralising QC sera 2 and QC 5.....114

Figure 3.4 Anti-gp120 Ab detection in HIV-1 infected patients.....117

Figure 3.5 CMI of chimeric viruses with autologous neutralising sera 120

Figure 3.6 CMI of chimeric viruses with autologous non-neutralising early sera 121-122

Figure 3.7 CMI of chimeric virus using sequential autologous sera 124

Figure 3.8 CMI of MM4 with sequential sera from recently infected patients.....125

Figure 3.9 CMI of YU2 with sequential sera from recently infected patients.....126

Chapter 4

Figure 4.1 Complement cascades..... 130
Figure 4.2 CMI assay using purified complement from Guinea pig..... 134
Figure 4.3 Inactivation of complement by EDTA/EGTA treatment..... 134
Figure 4.4 CMI assays using fresh serum from C1q- and C3-deficient individuals as sources of complement..... 136
Figure 4.5 Titration of complement in CMI..... 136
Figure 4.6 CMI of purified IgG and FT fractions of serial sera from patient MM19.....137
Figure 4.7 CMI using sCD4-IgG2..... 140
Figure 4.8 Comparison of CMI between macrophage- and PBMC-derived BaL.....140
Figure 4.9 Comparison of CMI between PBMC- and H9-derived 2044..... 142
Figure 4.10 Comparison of CMI between PBMC- and 293T-derived YU2....142

Chapter 5

Figure 5.1 Enrichment values obtained for the first round of panning..... 152
Figure 5.2 Enrichment values obtained for the second round of panning by specific elution with viral lysate..... 154
Figure 5.3 Enrichment values obtained for the second and third rounds of panning by specific elution with gp140 156
Figure 5.3.C Enrichment values for the second and third rounds of panning between b12 and polyclonal IgG..... 157
Figure 5.4 Screening of the VHH-encoded clones by phage ELISA.....159
Figure 5.5 ELISA assays of the purified anti-b12 VHH fragments..... 163
Figure 5.6 Inhibition of b12-mediated neutralisation of SF162 with anti-b12 VHH fragments.....165
Figure 5.7 Inhibition of b12-mediated neutralisation of IIIB with anti-b12 VHH fragments.....166
Figure 5.8 Neutralisation assays with sera from the first boost of immunisation..... 169
Figure 5.9 CMI assays with rabbit sera from the first boost of immunisation..... 171

Figure 5.10 CMI assays with rabbit sera from the second boost of immunisation 171
Figure 5.11 CMI with sera from the third boost of immunisation using human, Guinea pig and rabbit complement..... 172
Figure 5.12 Neutralisation assays with rabbit sera boosted with gp120-KLH..... 173

Tables and diagrams

Chapter 1

Table 1 Chemokine receptors that support HIV and SIV replication *in vitro*.....44

Chapter 2

Table 2.1 Description of bacterial strains.....89
Table 2.2 Cell lines.....89
Table 2.3 Antibodies.....90
Table 2.4 HIV isolates.....93
Table 2.5 Characteristics of the Jenner patients.....96
Table 2.6 Primers.....99
Table 2.7 Amount of antigen injected for immunisation of the rats.....109
Table 2.8 Amount of antigen injected for immunisation of the rabbits.....109

Chapter 3

Table 3.1 Viral titres of the chimeric HIV-1 viruses expressing the early gp120 from patients.....115
Table 3.2 Autologous neutralisation of chimeric viruses.....116
Table 3.3 Heterologous neutralisation of YU2.....118

Chapter 4

Table 4.1 CMI assays using human MAbs.....138

Chapter 5

Diagram 5.1 Panning strategies.....150
Table 5.1 Enrichment values obtained for the specific elution with gp140 for the second and third rounds of panning.....157
Table 5.2 Amino acid sequences of the anti-b12 VHH fragments isolated by phage display.....161
Table 5.3 References of the animals immunised with each VHH fragment.....168

Abbreviations

Ab	Antibody
ADCC	Antibody dependent cellular cytotoxicity
ADE	Antibody dependent enhancement
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
BBB	Blood brain barrier
CA	Capsid
CAF	CD8 anti-viral factor
CBP	CREB binding protein
CCR	CC chemokine receptor
CD4 BS	CD4BS
CDR	Complementary determining region
CMI	Complement mediated inactivation
CMV	Cytomegalovirus
CR	Complement receptor
CRF	Circulating recombinant form
Crm-1	Chromosome maintenance protein 1
CRP	Complement regulatory protein
CTD	C-terminal domain
CTL	Cytotoxic T cells
CXCR	CXC chemokine receptor
CypA	Cyclophilin A
DAF	Decay accelerating factor
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule grabbing non-integrin
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulphoxide
EDTA	Ethylenediamine-tetra-acetic acid
EGTA	Ethyleneglycol-tetra-acetic acid
EIAV	Equine infectious anaemia virus
ELISA	Enzyme linked immunosorbent assay
EM	Electron microscopy
Env	Envelope glycoproteins
ER	Endoplasmic reticulum
FCS	Foetal calf serum
FDC	Follicular dendritic cell
FFU	Focus forming unit
FT	Fall through
Glc	Glucose
HAART	Highly active anti-retroviral therapy
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HLE	Human leukocyte elastase
HR	Hydrophobic regions
HRP	Horse radish peroxidase
HS	Human serum

HTLV	Human T-cell leukaemia virus
IC	Integration complex
IFN	Interferon
Ig	Immunoglobulin
IgA	Immunoglobulin type A
IgG	Immunoglobulin type G
IgM	Immunoglobulin type M
IL	Interleukin
IN	Integrase
IPTG	Isopropyl-BD-thiogalactopyranoside
KLH	Keyhole limpet haemocyanin
LAV	Lymphadenopathy-associated virus
LC	Langerhans cell
LCMV	Lymphocytic choriomeningitis virus
LFA	Leukocyte function associated
LTNP	Long term non-progressor
LTR	Long terminal repeat
Lv	Lentivirus restriction
MA	Matrix
MAb	Monoclonal antibody
MAC	Membrane attack complex
MBL	Mannan binding lectin
MBV	Multivesicular bodies
MCP	Membrane co-factor protein
MHC	Major histocompatibility complex
MLV	Murine leukaemia virus
MVV	Maedi visna virus
NAb	Neutralising antibody
NC	Nucleocapsid
NES	Nuclear export signal
NHS	National health service
NIBSC	National institute for biological standards and control
NK	Natural killer
NLS	Nuclear localisation signal
NP	Normal progressor
NPC	Nuclear pore complex
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NSI	Non-syncytium inducing
OD	Optical density
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PBS	Primer binding site
PCR	Polymerase chain reaction
PHA	Phytoheamaglutinin
PI	Primary isolate
PIC	Pre-integration complex
POS	Post-onset of symptoms
PPT	Polypurine tract
PR	Protease

P-TEFb	Positive transcription elongation
RANTES	Regulated on activation normal T cell expressed and secreted
RNase H	Ribonuclease H
RPMI	Roswell Park Memorial Institute
RRE	Rev response element
RT	Reverse transcriptase
RT	Room temperature
RTC	Reverse transcription complex
RSV	Respiratory syncyticia virus
Ser	Serine
sCD4	soluble CD4
siRNA	Short interfering RNA
ssDNA	strong stop DNA
SDF	Stromal-derived factor
SI	Syncytium inducing
SIV	Simian immunodeficiency virus
SNP	Single nucleotide polymorphism
STI	Structured treatment interruptions
SU	Subunit
TAE	Tris-acetate EDTA
TAR	Transactivation response
TBS	Tris buffered saline
TCLA	T cell line adapted
TCR	T cell receptor
TGN	Trans Golgi network
TH	T helper
Thr	Threonine
TM	Transmembrane
TNF	Tumour necrosis factor
U	Unit

Chapter 1
Introduction to HIV

1.1 HIV history

In 1981, the Center for Disease Control reported cases of Kaposi's sarcoma and *Pneumocystis carinii* pneumonia among young homosexual men in New York and California (1981). These individuals were characterised by a virtual absence of their CD4 T cell count, had no lymphocyte proliferative responses to soluble antigens, and their responses to phytoheamaglutinin (PHA) were markedly reduced (Gottlieb et al., 1981).

An RNA virus, with a Mg^{2+} reverse transcriptase (RT) activity, was soon isolated and grown in peripheral blood mononuclear cells (PBMCs) from patients who developed acquired immunodeficiency syndrome (or AIDS), in France. The virus was called lymphadenopathy-associated virus (LAV) (Barre-Sinoussi et al., 1983). A similar virus, called HTLV-III, was later identified in the USA, from patients who presented signs of immuno-depression and from healthy individuals (Gallo et al., 1984). In parallel, Levy *et al* isolated similar viruses (that they called ARV), that serologically reacted with anti-LAV sera, from healthy and AIDS-stage homosexual men in San Francisco. The authors hypothesised that this virus could be transmitted sexually (Levy et al., 1984). Vilmer *et al* isolated LAV-viruses in two adolescent haemophiliac siblings and proposed that LAV could also be transmitted by transfusion of blood products (Vilmer et al., 1984). Cheingsong-Popov *et al* confirmed, in a large epidemiological study in the UK, that AIDS-associated viruses were exclusively found in homosexual men, intravenous drug users and haemophiliacs (Cheingsong-Popov et al., 1984).

Wain-Hobson *et al* discovered that LAV had the same open reading frames as the lentivirus meadi visna virus (MVV) and, consequently, demonstrated that LAV was a lentivirus and not an HTLV virus (Wain-Hobson et al., 1985). Ratner *et al* established that LAV, HTLV-III and ARV viruses were variants of the same virus (Ratner et al., 1985), suggested earlier by Cheingsong-Popov *et al* (Cheingsong-Popov et al., 1984), and the virus was later called human immunodeficiency virus or HIV (Coffin et al., 1986a; Coffin et al., 1986b).

Shortly after the isolation of HIV-1 (HTLV-III/LAV/ARV), a similar T-tropic retrovirus was isolated from four macaques and was called simian T-lymphotropic virus type III or SIV (Daniel et al., 1985). Another retrovirus was also isolated from two individuals originally from Guinea Bissau and Cape Verde, who presented with AIDS-like syndromes and a low CD4 T cell count. The viruses isolated from these two patients could be grown in primary PBMCs, had RT activity and a similar EM morphology to HIV-1. They reacted serologically with a serum directed against SIV_{mac} but failed to react with an anti-LAV (HIV-1) serum (Clavel et al., 1986). Similar viruses were found later in thirty African individuals who presented with AIDS syndromes. This new group of viruses was called HIV-2 (Clavel et al., 1987).

1.2 Genome organisation

All retrovirus genomes encode three polyproteins: Gag (group associated antigen), Pol (Polymerase) and Env (envelope). Gag is cleaved into Matrix (MA, p17), Capsid (CA, p24), spacer peptides (p1 and p2), Nucleocapsid (NC, p7) and p6. The Pol polyprotein is generated from Gag-Pol, by a ribosomal frameshift, and is cleaved into the Protease (PR, p10), the Reverse Transcriptase (RT, p66 and p51) and the Integrase (IN, p32) by PR. The Env polyprotein is cleaved into the surface protein (SU, gp120) and the transmembrane protein (TM, gp41).

HIV is a lentivirus and possesses regulatory genes: transactivator of transcription (*tat*) and regulatory viral protein (*rev*). The HIV genome also encodes accessory proteins that are not always necessary for the successful completion of the replication cycle in cell culture *in vitro*: Negative factor (Nef), Viral infectivity factor (Vif), Viral protein R (Vpr) and Viral protein U (Vpu). HIV-2 and SIV do not encode Vpu but the protein Vpx. The long terminal repeats (LTRs) delineate the coding sequences at the 5' and 3'ends. The schematic representations of HIV-1 and HIV-2 genomes are shown in figure 1.1.

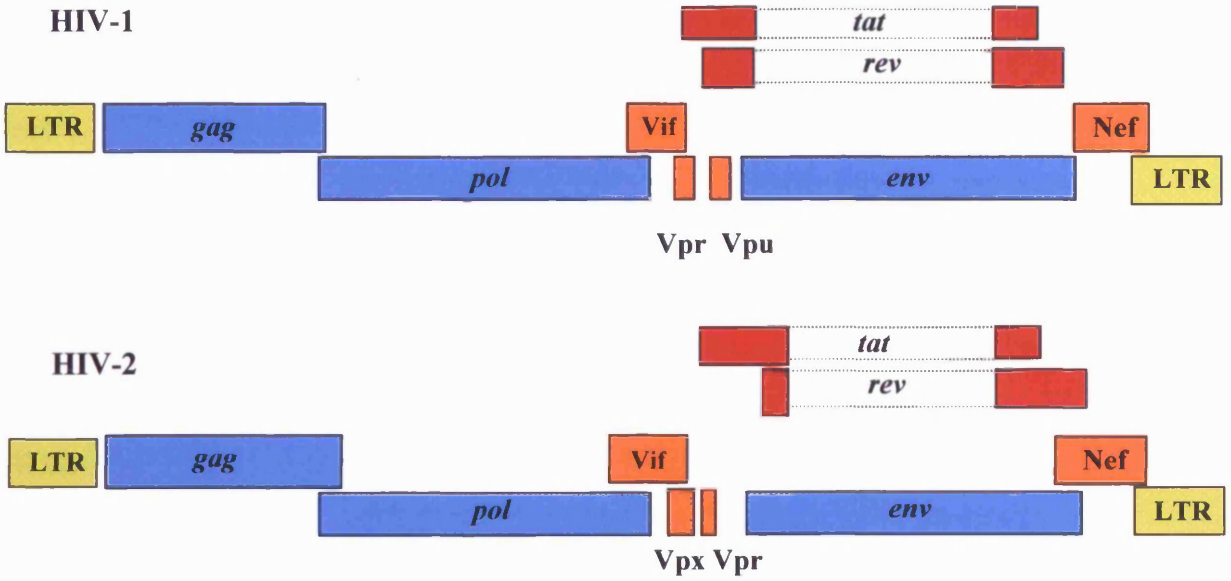


Figure 1.1 DNA Genomic organisation of HIV-1 and HIV-2
HIV encodes structural proteins, regulatory proteins (non-structural proteins that are necessary for the completion of the replication cycle) and accessory proteins (non-structural proteins that are not compulsory for the completion of the replication cycle in some cell lines). LTR regions are in shaded yellow, genes for the production of structural proteins in blue, regulatory proteins in red and accessory proteins in orange. The dotted lines show the introns and the solid lines represent the exons. (Levy, 1998)

1.3 Classification and distribution of HIV-1 in the world

HIV-1 and HIV-2 are the two lentiviruses causing AIDS currently found in humans. Like other retroviruses, the RT of HIV does not contain a proof-reading domain and, therefore, allows nucleotide substitution, which explains the diversity of the HIV genomes found all over the world. HIV-1 is classified into three groups: group M (for main), the group O (for outlier) and the group N (for new, non-M non-O). Group M represents over 95% of the HIV-1 isolates found in infected individuals worldwide and groups N and O are restricted to Central Africa. Group M is further divided into clades (or subtypes) A, B, C, D, E, F, G, H, J and K. Subtypes are genetically defined lineages that can be classified by phylogenetic analysis of the HIV-1 M group (Gaschen et al., 2002).

Based on their Env sequences, up to 35% divergence can be observed between two viruses from different clades, within the group M (Gaschen et al., 2002). The divergence is reduced to 20% between viruses from the same clade and up to 10% between viruses isolated from the same individual (Perrin et al., 2003). The clades, so far, are geographically restricted. Subtypes A and D are predominant in Central and

West Africa. Clade C represents the main clade in the world, being endemic in Southern Africa, the Horn of Africa, South America, China and India. Clade E viruses are found in South East Asia (UNAIDS). The clade B is predominant in Western Europe, Northern America, Japan, Australia and New Zealand but the number of non-subtype B viruses within those regions of the world is increasing, as reported in France (Couturier et al., 2000). Most of the non-clade B viruses found in developed countries are detected in immigrants who contracted HIV in zones where non-B subtypes are endemic. However, non-B subtypes have started to be transmitted in Cuba, where B clades viruses used to represent the main clade (Cuevas et al., 2002). The clade distribution in the world is shown in figure 1.2.A.

Within the same individual, recombination of viruses from different clades can occur, which leads to the rise of circulating recombinant forms (CRFs). To be classified as such, the same recombinant has to be found in three unrelated individuals. Fourteen CRFs have so far been identified in the world. As an example, CRF01AE is now predominant in heterosexual transmissions in Thailand (Gao et al., 1996). Without a drastic policy to stop transmission, the diversity of HIV is likely to become greater, which already poses a serious problem for a protective anti-HIV vaccine (Gaschen et al., 2002).

Between 38 and 42 million individuals are estimated to be infected with HIV, of whom 90% live in developing countries. HIV distribution is shown in figure 1.2.B.

1.4 Origins of HIV

HIV-2 is closely related to SIV_{sm} found naturally in sooty mangabeys *Cerbocerus atys* and probably arose from a zoonotic infection of SIV_{sm} in humans (Hirsch et al., 1989). HIV-1 seems to have evolved from SIV_{cpz} found in chimpanzees *Pan troglodytes troglodytes*, that live in Central Africa, where the three groups of HIV-1 are found and where HIV is the most diverse (Gao et al., 1999). The three groups of HIV-1 are thought to have arisen from three independent introductions of HIV_{cpz} in humans (Gao et al., 1999). Neither chimpanzees nor sooty mangabeys seem to develop an AIDS-like syndrome when infected with SIV_{cpz} and SIV_{sm} respectively.

The common ancestor of the HIV-1 group M is estimated to date back to the first half of the 20th century (1931±12 years), although this might not reflect the date of the actual zoonosis of this group (Korber et al., 2000). The timing of the ancestor of the

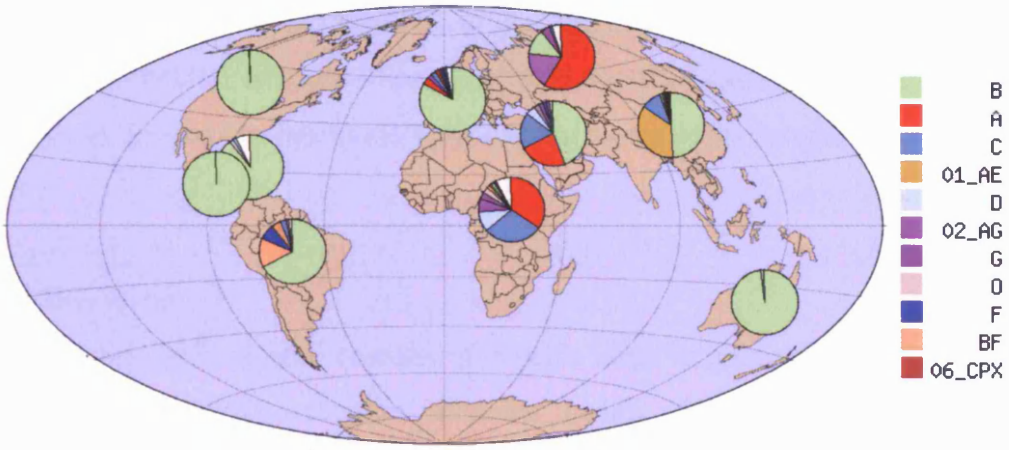


Figure 1.2.A Geographic distribution of HIV clades in the world
The clade B is predominant in developed countries (USA, Western Europe, Australia, New Zealand and Japan). The predominant clades in developing countries are clades A and C. The greatest HIV diversity is found in Central Africa, where the original zoonoses are thought to have occurred. From the Los Alamos web site

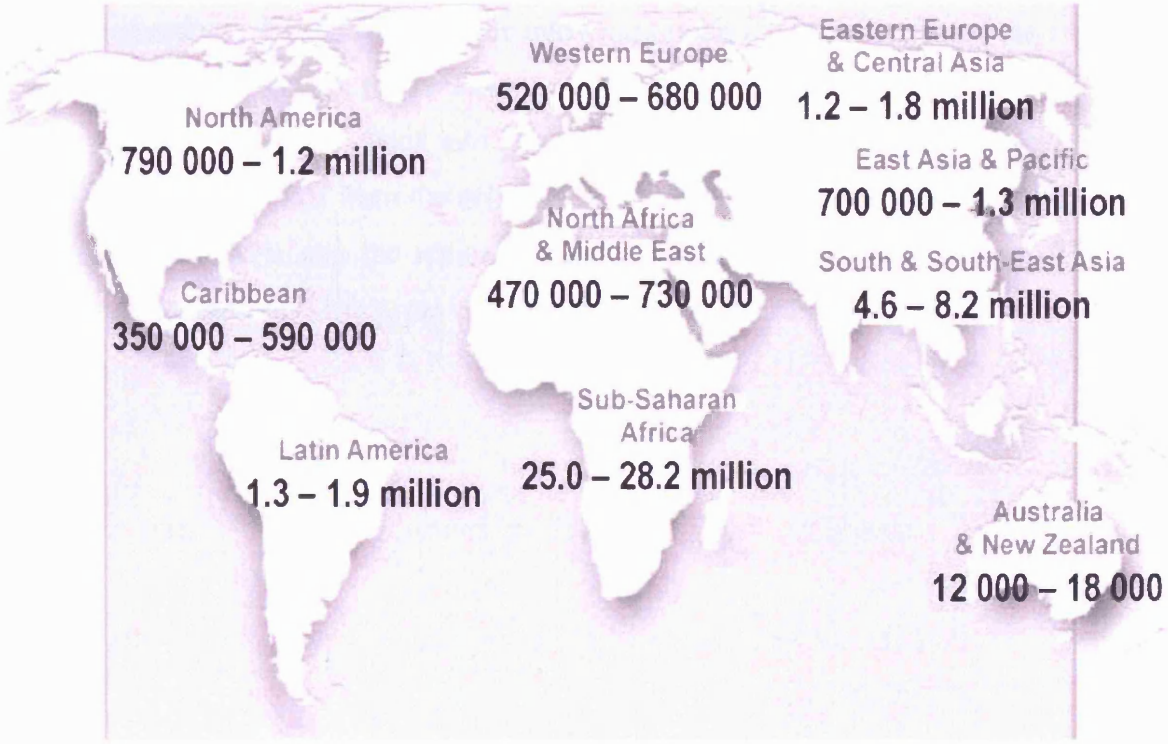


Figure 1.2.B Population estimated to be infected with HIV worldwide
Between 38 and 42 millions individuals are currently infected with HIV and 90% of HIV cases are reported from developing countries. From UNAIDS website

group M confirms that the HIV epidemic was not triggered (or favoured) by a possible SIV contamination of oral polio vaccine preparations used in Africa in the late 1950's (Berry et al., 2001; Blancou et al., 2001; Rambaut et al., 2001). The introduction of the HIV-1 subtype B into the US is thought to have occurred around 1968 (±1.4 year), around ten years before the earliest report of the infections (Robbins et al., 2003).

1.5 Replication cycle

The replication cycle of HIV consists of specific steps: binding of the virus onto the cell, entry via specific receptors allowing fusion of the viral and cellular membranes and release of the virus core into the cell. The HIV RNA genome is then reverse transcribed into double stranded DNA that subsequently reaches the nucleoplasm and integrates into the host genome. The HIV genome is then treated by the host transcription machinery as a cellular gene. By a complex mechanism of alternative splicing and nuclear export, proteins and polyproteins of HIV, as well as intact copies of its RNA, are translocated to the cytoplasm of the cell. The cellular machinery translates the RNA molecules into viral proteins. The assembly of the viral core occurs and two copies of the RNA genome, as well as several other viral and cellular proteins, are encapsulated into the cores. The core and Env of HIV associate and the virus particle buds from the cell. The maturation of the virus is then mediated by PR and this results into the typical conical shape of the mature HIV core. The representation of the viral life cycle is shown in figure 1.3.

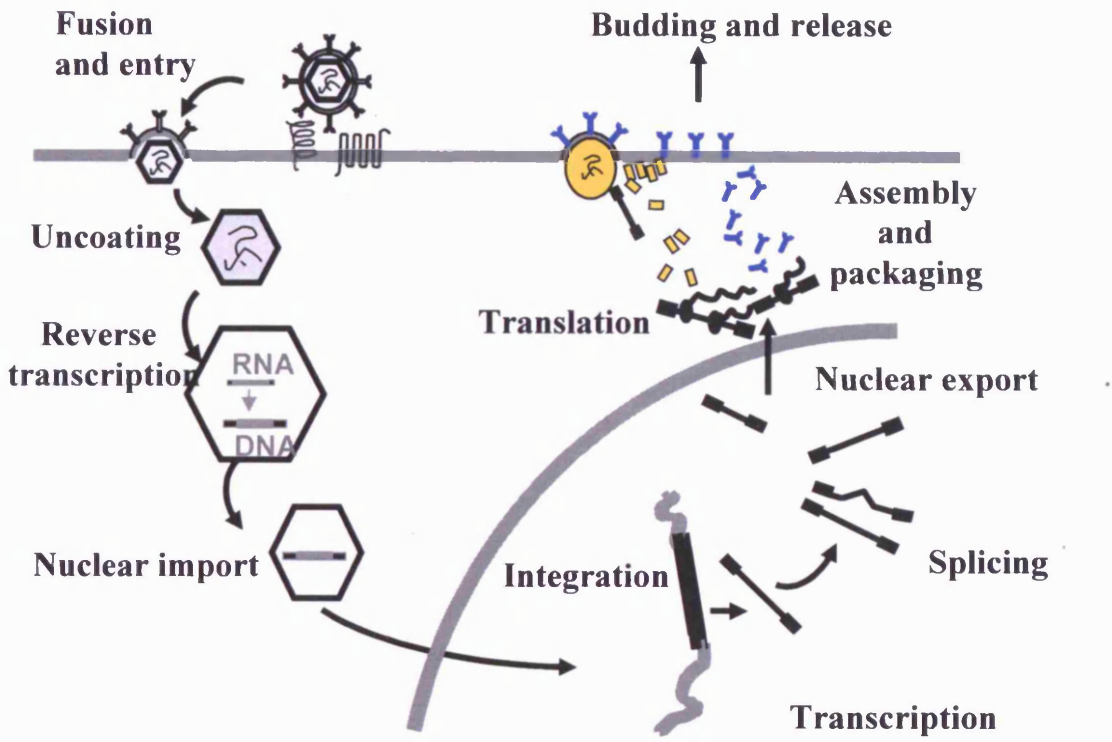


Figure 1.3 Replication cycle of HIV

HIV binds to CD4 and a chemokine co-receptor, typically CCR5 or CXCR4. The binding to the chemokine receptor triggers conformational changes within gp120 and gp41, revealing the fusion peptide of gp41 that mediates fusion between the viral and cellular membranes. The viral core is released into the cell, followed by uncoating that allows reverse transcription of the viral genomic RNA into a double stranded DNA form. The DNA genome is then transported into the nucleus where it integrates into the host genome catalysed by viral integrase. Transcription of viral RNA takes place, mediated by the cellular transcriptional machinery. Viral RNA is alternatively spliced and the resulting viral RNA molecules are transported into the cytoplasm via Rev. Viral core components (yellow) are synthesised in the cytoplasm and the Env glycoproteins in the endoplasmic reticulum. Viral core proteins accumulate at the cell membrane where they encapsulate two copies of full-length RNA and bud from the cell membrane. Viral protease cleaves Gag and the Env glycoproteins are incorporated. Mature virus particles are released and can infect new target cells

1.5.1 Entry

HIV entry is mediated by the interaction with the primary receptor CD4 (Dalgleish et al., 1984; Klatzmann et al., 1984), which is thought to change the conformation of gp120 that allows HIV to bind to a 7-Transmembrane (7-TM) protein that acts as a co-receptor (Alkhatib et al., 1996; Choe et al., 1996; Doranz et al., 1996; Dragic et al., 1996; Feng et al., 1996). The membrane of HIV then fuses with the

cellular membrane and the viral core, containing the viral genetic material, is released into the cytoplasm of the cell (see sections below for more details).

1.5.2 Uncoating and reverse transcription

Once inside the cell, uncoating takes place. It is still a poorly understood mechanism and is likely to involve the phosphorylation of MA, interaction with cyclophilin A (CypA), and actions from Nef and Vif (Greene and Peterlin, 2002). Successful uncoating generates the reverse transcription complex (RTC). The RTC consists of the RNA genome, $tRNA^{Lys3}$, RT, IN, NC, Vpr, host proteins and MA (Fassati and Goff, 2001; Greene and Peterlin, 2002). The RTC is liberated from the plasma membrane and the complex interacts with actin microfilaments (Greene and Peterlin, 2002). Reverse transcription then occurs.

Reverse transcription is mediated by the RT, which is an RNA-dependent, DNA-dependent DNA polymerase in 5' to 3' manner. The RT of HIV-1 is constituted of the heterodimer of p66 and p51. The p66 contains the RT sequence linked to the RNase H domain and p51 contains the RT sequence. The mechanism of reverse transcription is schematised in figure 1.4. Cellular $tRNA^{Lys3}$ binds to the complementary region, called the primer binding site (PBS) near the 5'end of the RNA (A) and the RT synthesises the DNA copy of the U5 and the R regions that constitute the strong stop negative DNA (ssDNA) strand (B). The RNase H of the RT degrades the corresponding RNA region. The first strand jump of reverse transcription then occurs (C) and the R region of the DNA strong stop anneals with the R region situated at the 3'end the RNA copy. The first jump can take place on the same RNA or on the second RNA copy. The strong stop acts as a primer for the polymerisation of the rest of the minus DNA strand. The RNase H degrades the RNA except for two regions rich in purines, called the polypurine tract at the 3'end of the RNA (PPT) and the central PPT (cPPT). The two PPT regions then act as primers to allow the polymerisation of the positive DNA strand (D). The PPT and the $tRNA^{Lys3}$ are degraded by the RNase H (E). The second jump of reverse transcription occurs. The positive DNA strand containing the PBS anneals at the 5'end of the minus DNA strand and elongation of both DNA strands is completed (F). The resulting double stranded DNA contains a 99 nucleotide flap situated on the positive strand of DNA within the cPPT.

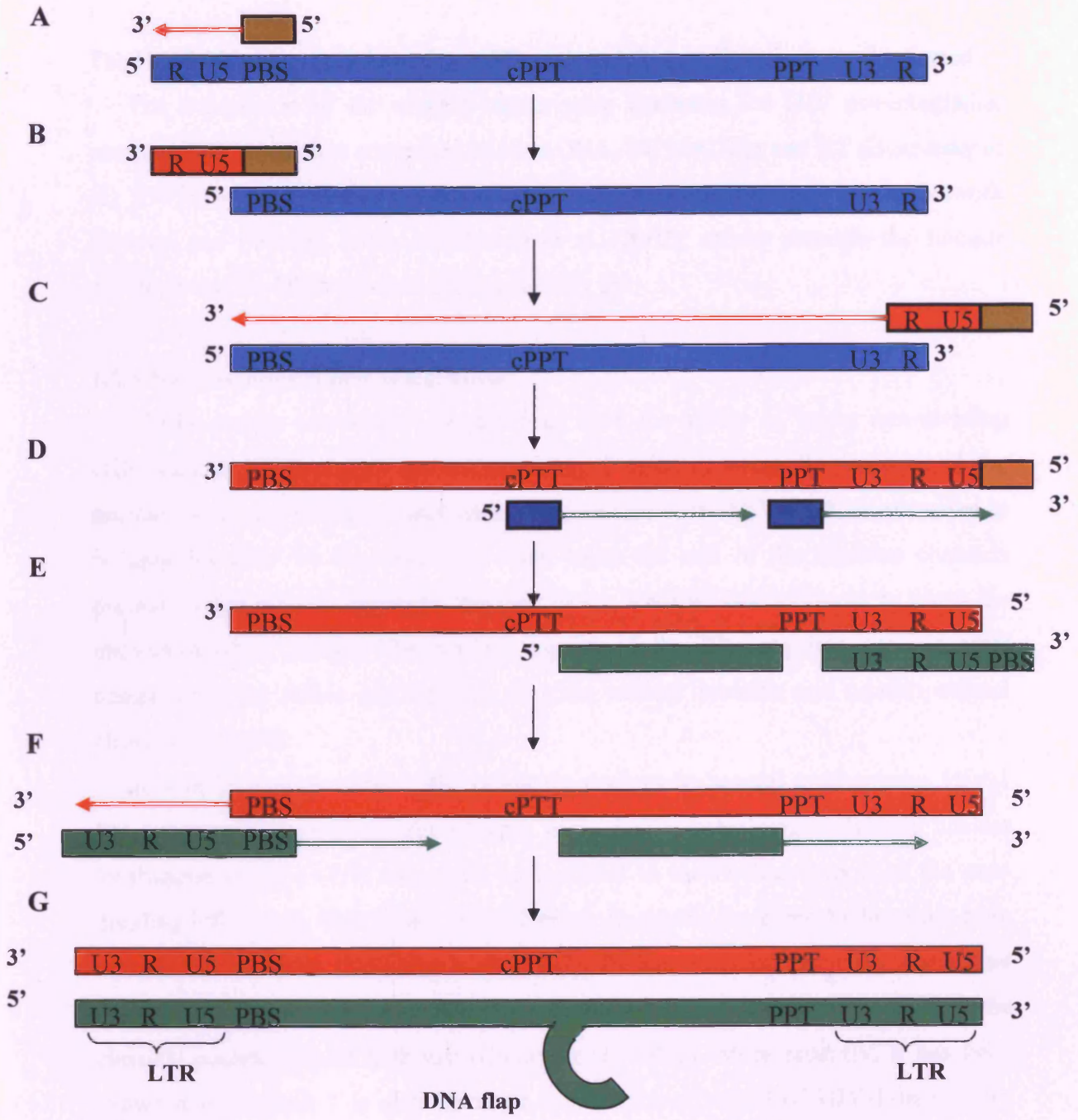


Figure 1.4 Reverse transcription of HIV RNA into genomic DNA
A. The binding of $tRNA^{Lys3}$ to the Primer Binding Site (PBS) starts the reverse transcription catalysed by the Reverse Transcriptase (RT). The DNA minus strand from 5' to 3' to the R region is first synthesised, creating a DNA species known as the strong stop. **B**. The RNAse H domain of the RT degrades the RNA copy of the nascent minus strand. **C**. The first jump of the reverse transcription occurs and allows the elongation of the minus DNA strand. **D**. The RNAse H degrades the RNA except the regions of the central Poly Purine tract (PPT) and the PPT. **E**. These 2 RNA regions then serve as primers for the synthesis of the positive strand of viral DNA. **F**. The second jump of reverse transcription occurs. The 3' end of the positive DNA strand binds to the homologous PBS region at the 3'end of the negative DNA. **G**. The synthesis of both DNA strands is completed and a 99 nucleotide DNA flap at the cPPT. The DNA genome of HIV is characterised by the duplication of the U3 and the U5 regions, at the extremities of the genome, creating the Long Terminal Repeats (LTR), necessary for the integration of the viral DNA and the initiation of transcription. The brown bar represents the cellular $tRNA^{Lys3}$, the blue bars the RNA, the red bars the minus DNA strand and the green bars the positive DNA strand

The HIV DNA genome is bound by LTRs, where U3 and U5 regions are duplicated.

The completion of the reverse transcription generates the HIV pre-integration complex (PIC), which is composed of viral DNA, IN, MA, Vpr and RT (Bukrinsky et al., 1993b). The PIC slides towards the nucleus using the microtubule network (Greene and Peterlin, 2002; McDonald et al., 2002), moves through the nuclear envelope and the DNA genome is integrated by IN.

1.5.3 Nuclear import and integration

Unlike simple retroviruses, lentiviruses have the ability to infect non-dividing cells, such as macrophages and naïve resting T cells, in which the integrity of the nuclear membrane remains intact. Moreover, the size of the HIV-1 PIC is estimated to be approximately 56 nm, which is about twice the size of the aqueous channels present in the nuclear envelope through which the PIC has to move to enter the nucleus (Cullen, 2001). The nuclear import of the PIC requires specific viral components, the active participation of some nuclear proteins and conformational changes of the PIC.

It is likely that the HIV-1 PIC enters the nucleus by several mechanisms. HIV-1 MA has been shown to contain a highly basic region resembling a classical nuclear localisation signal (NLS) and could be involved in the nuclear import of the non-dividing PtK-1 cells (Bukrinsky et al., 1993a). However, these results have not been reproduced by others (Fouchier et al., 1997). IN has a nuclear targeting signal that allows the interaction of the PIC with cellular importin α and importin β from the classical nuclear import pathway (Gallay et al., 1997). More recently, it has been shown that importin 7 is also involved in the nuclear import of HIV-1 in primary macrophages (Fassati et al., 2003).

The central DNA flap, formed during the reverse transcription from the PPT, has been reported to act as a nuclear import signal (Zennou et al., 2000). Mutations in the cPPT inhibiting the synthesis of the DNA flap lead to the formation of linear DNA accumulated in the cell cytoplasm at the vicinity of the nuclear membrane.

The auxiliary HIV-1 protein Vpr is a nucleocytoplasmic shuttling protein that contains two nuclear import signals (Sherman et al., 2001). It interacts with nucleoporin hCG1, one of the components of the nuclear pore complexes (NPC) (Le Rouzic et al., 2002). Vpr also binds importin α and increases its affinity for the NLS of MA (Popov et al., 1998). Then, importin α interacts with importin β. This trimeric

complex engages the nucleoporins and moves across the pore in a series of energy-dependent binding and releasing steps (Sherman and Greene, 2002). Vpr can also alter the integrity of the nuclear envelope by the formation of herniations, which rupture and permit the transit of the PIC (de Noronha et al., 2001). Vpr also arrests infected T cells in the G_2 phase of the cell cycle (Jowett et al., 1995; Re et al., 1995). However, the importance of the role of Vpr *in vivo* is still uncertain. Popov *et al* have shown that high concentrations of Vpr inhibit the PIC nuclear import and cytoplasmic lysate from Hela cells can rescue the nuclear import of Vpr-defective PICs in H9 cells (Popov et al., 1998). Vpr is not required for HIV replication in resting naïve CD4 T cells but seems to play an important role in replication in macrophages (Sherman and Greene, 2002). However, *vpr*-deleted plasmids can be successfully integrated in primary macrophages (Neil et al., 2001).

How Vpr, IN, MA, the DNA flap and the nuclear proteins act together *in vivo* is still unclear and the mechanisms of nuclear import could be dependent on the nature of the target cell.

The integration of the HIV DNA genome into the host cell chromosomal DNA is mediated by IN. IN catalyses the removal of the terminal dinucleotide from each 3'end of the viral cDNA and forms an integration site by the cleavage of the chromosomal DNA. IN then catalyses a strand transfer reaction to link the 3'end of the viral DNA to the host cell DNA (Bushman et al., 1990). Host proteins are also required for efficient integration but their roles remain unknown (Greene and Peterlin, 2002). The integration of HIV DNA is not always successful: the DNA can circularise and forms 1-LTR or 2-LTR circles (Greene and Peterlin, 2002).

Schröder *et al* showed that the insertion of HIV-1 does not seem to be random and active genes could be preferentially targeted *in vivo* (Schroder et al., 2002). They also identified hot spots of HIV integration where active genes are present. They suggested that integration could be promoted by increased chromatin accessibility in transcribed regions.

The integration of the HIV DNA genome is irreversible and creates permanent viral reservoirs, disseminated all over the body, which allows the generation of viruses that can escape suppressive treatments against HIV (see section 1. 14. 1 for details).

1.5.4 Transcription

Once integrated into the host DNA, the HIV genome is treated as a cellular gene by the cellular transcription machinery, using the 5'LTR as a promotor. The 5'LTR of HIV-1 contains regulatory domains: a TATA box, three Sp1 domains and two NF-κB sites. Multiple transcription factors, such as NF-κB, bind the 5'LTR (Marcello et al., 2004) and recruit the RNA polymerase II.

In *tat*-deleted HIV genomes, transcription of HIV is initiated but elongation is severely impaired (Strebel, AIDS, 2003). Tat binds to the transactivation response region (TAR), a regulatory element situated between the positions +1 and +59 of the HIV RNA, downstream of the transcription elements (Feng and Holland, 1988). The association occurs between an arginine-rich region at the C-terminus of Tat and a U-rich region at the apex of the TAR element (Strebel, 2003). This association allows Tat to recruit cyclin T1 to the TAR element (Wei et al., 1998). Cyclin T1 associates with the protein kinase CDK9 of the P-TEFb (positive transcription elongation factor b) complex. CDK9 phosphorylates the C-terminal domain (CTD) of the RNA polymerase II (Okamoto et al., 1996; Strebel, 2003). Phosphorylation of the RNA polymerase II enhances its processivity, resulting in the elongation of the transcription and the generation of full-length HIV RNA molecules. The association of P-TEFb with the CTD could also release negative elongation factors from the RNA polymerase II (Garber and Jones, 1999).

Besides its indirect property of mediating the processivity of the RNA polymerase II, Tat has been shown to modulate chromatin remodelling (Marcello et al., 2001). Through specific interactions with histone acetylases such as CBP (CREB-binding protein), Tat promotes the acetylation of H3 and H4 histone proteins (Marcello et al., 2004), which results in activation of transcription via the recruitment of the transcription machinery to the HIV promoter. In parallel, Tat itself is acetylated, which leads to the dissociation of Tat from the TAR element (Kiernan et al., 1999), which could enhance the accessibility of the cellular transcription machinery to the HIV promoter (Strebel, 2003).

In addition to enhancing the synthesis of full-length RNA molecules of HIV, Tat was shown to repress the transcription of the major histocompatibility complex (MHC) class I genes (Weissman et al., 1998).

1.5.5 Splicing and nuclear export of viral mRNAs

The HIV genome is transcribed as a single RNA molecule. Each RNA molecule is modified at the 5' and the 3'ends by the addition of a CAP structure and polyadenylation respectively. The first molecules of RNA are totally spliced by cellular proteins, which leads to the generation of Nef, Rev and Tat. Tat enhances the synthesis of full-length RNA molecules. Unspliced copies of RNA have to be exported out of the nucleus to be incorporated into newly synthesised viral cores.

Splicing of HIV is inefficient (Cullen, 2001). Moreover, the regulatory viral protein Rev is a cargo transporting protein. It contains both a NLS and a nuclear export signal (NES). It exports unspliced RNA out of the nucleus into the cytoplasm. Shortly after its synthesis in the cytoplasm, Rev shuttles back into the nucleus via interactions between the NLS and importin β (Henderson and Percipalle, 1997). Once in the nucleus, the leucine-rich motif of Rev interacts with intact copies of RNA via the Rev responsive element (RRE) within the *env* sequence (Malim et al., 1989). This interaction leads to the multimerisation of Rev onto the RNA (Malim and Cullen, 1991), which masks the NLS and reveals the NES (Strebel, 2003). Rev then associates, in human cells, with Crm1 (chromosome maintenance protein 1), a protein that interacts with NES-containing proteins and with nuclear pore proteins (Neville et al., 1997). The stability of the association between RNA-Rev and Crm1 is thought to be regulated by Ran, (a cellular GTPase) involved in providing an energy gradient for nuclear import and export (Strebel, 2003). The RNA-Rev-Crm1-Ran complex then moves across the nuclear membrane. Once in the cytoplasm, Crm1 dissociates from Rev-RNA by hydrolysis of Ran-GTP (Neville et al., 1997) and, Gag, Gag-Pol and intact RNA molecules are released into the cytoplasm.

1.5.6 Protein assembly

HIV-1 Env is synthesised on ribosomes as an 845-870 amino acid long precursor called gp160. It is then translocated into the endoplasmic reticulum (ER), via a hydrophobic signal peptide situated at its N-terminus, where it is folded, oligomerised and glycosylated. Env is then transported to the Golgi apparatus where the carbohydrates moieties are modified and some are terminally sialylated (Fenouillet et al., 1989) and cellular furin-like proteases cleave Env into gp120 and gp41. gp120 and gp41 stay associated by non-covalent bonds and oligomerise to form trimeric spikes.

These structures are then transported to the plasma membrane via the secretory pathway.

The polyprotein Gag is translated by ribosomes as a single protein. The C-terminal domain of CA within Gag mediates the Gag-Gag interactions, which form the immature core of HIV-1. The binding of NC to two copies of viral RNA, via the ψ region at the 5'end of the RNA, enhances Gag assembly (Turner and Summers, 1999). Vif, RT, PR and IN are incorporated into the virion. The cellular components $tRNA^{Lys3}$, APOBEC3G (Cen et al., 2004) (see section 1. 12. 2) and CypA (Franke et al., 1994) are also incorporated into the virus particle through interactions with viral proteins: NC for APOBEC3G and CA for CypA. The myristoyl group present at the N-terminus of MA within Gag targets it to the plasma membrane in specialised microdomains termed lipid rafts (Scarlata and Carter, 2003).

1.5.7 Virus budding and maturation

HIV Gag polyproteins are synthesised and targeted to the plasma membrane where they take part in viral assembly and subsequent budding. Viral release relies on the late assembly domain (or L domain), which, in the case of HIV, consists of the PTAP motif found within the N-terminus of the protein $p6^{Gag}$ (Huang et al., 1995). The amino acid sequence of the L domain varies between viruses (Vogt, 2000). The L domain motifs are functionally interchangeable between HIV and Ebola virus (Martin-Serrano et al., 2001) but cannot be exchanged between HIV and Murine Leukaemia Virus (MLV) (Martin-Serrano et al., 2004).

For efficient budding, HIV recruits the whole multivesicular bodies (MVB) pathway, involved in endosomal sorting and endocytosis, via the interaction between the PTAP motif and Tsg101 (Greene and Peterlin, 2002; VerPlank et al., 2001), which is a part of the ESCRT-1 complex (for the description of the complete possible mechanism, see von Schwedler et al., 2003). Tsg101 has an E2-like domain in its N-terminus, found in the ubiquitination machinery (Freed, 2002; Vogt, 2000). The role of the ubiquitination of Gag and the mechanism by which HIV hijacks this complex protein degradation machinery to its advantage is currently unclear.

Simultaneously as budding occurs (or shortly after), PR cleaves Gag into MA, CA, NC, p1, p2 and p6. CA adopts a conical shape, characteristic of HIV and gp120-gp41 proteins, organised in trimeric spikes, associate with the viral core via an interaction of MA with the cytoplasmic tail of gp41. The successful cleavage of Gag

between CA and MA seems to be essential for new viruses to fuse with new target cells (Wyma et al., 2004).

Viral particles bud from lipid rafts, which are microdomains of the plasma membrane enriched in cholesterol and saturated lipids (Manes et al., 2003), and incorporate GPI-linked membrane proteins such as Thy-1, the lipid raft-specific ganglioside GM1 (Nguyen and Hildreth, 2000), complement regulatory proteins (CRPs) CD55, CD59, CD46 (Saifuddin et al., 1995), ICAM-1 and MHC class II (Cantin et al., 1997). Budding from lipid rafts seems to enhance the infectivity of HIV. It has been shown that Nef increases the synthesis and the transport of cholesterol to lipid rafts (Zheng et al., 2003). Full infectious HIV particles can then infect new cell targets and new cycles of replication can take place. A schematic representation of the HIV infectious particle is represented in figure 1.5.

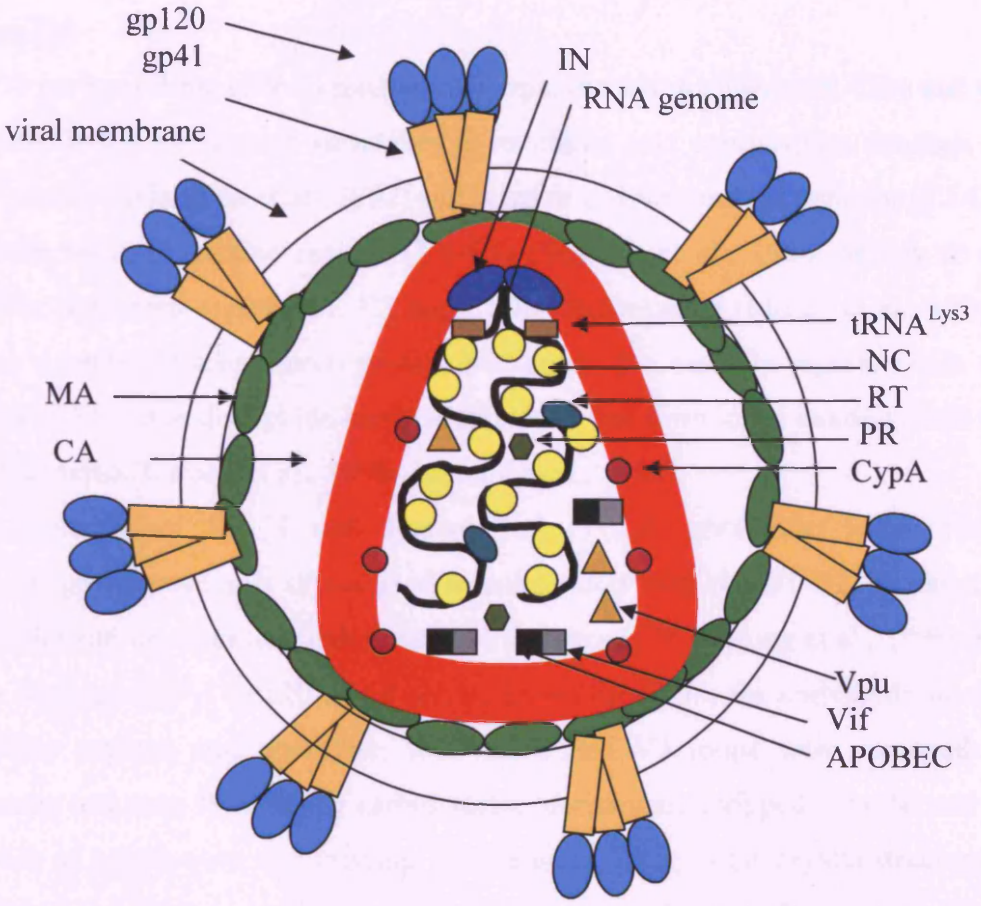


Figure 1.5 Schematic representation of an infectious HIV virus particle
The infectious mature particle of HIV has a viral membrane bearing the trimeric spikes constituted by the association of gp41 (TM) and gp120 (SU). Beneath the membrane, the core of the particle is formed by the polymerisation of MA interacting with the CA that forms a conical structure typical of HIV. Within CA, two copies of genomic RNA, covered by NC, interact with CA via IN. PR, RT and Vpu are also present inside the core. The cellular components $tRNA^{Lys3}$ and CypA are incorporated into the virus particle. Note that, in permissive cells, APOBEC is incorporated and in non-permissive cells, Vif is incorporated

1.6 Viral entry

The first step of the infection cycle of HIV consists of one or several specific interaction(s) with some cellular surface structures (see 1. 6. 2. 3 for details) and one or several receptors. In the case of HIV-1, gp120 binds to CD4 (Dalgleish et al., 1984; Klatzmann et al., 1984) and a chemokine co-receptor (Alkhatib et al., 1996; Choe et al., 1996; Dragic et al., 1996; Feng et al., 1996). The second step in the infection cycle is the fusion of the virus into the target cell. This step is mediated by gp41, which is involved in the fusion mechanisms between the viral and the cellular membranes.

1.6.1 Structure of HIV envelope

1.6.1.1 gp120

gp120 (or gp105 for HIV-2) mediates the specific interactions with CD4 and the co-receptor. It has the highest variability in its amino acid composition amongst all the HIV proteins (Gaschen et al., 2002) and consists of five constant domains (C1-C5) intercalated by five variable regions (V1-V5) (Starcich et al., 1986; Willey et al., 1986). The conserved regions C1, C2 and C5 interact together (Moore et al., 1994b) and form a central discontinuous protein backbone. The variable regions (with the exception of V5) have disulphide bonds at their base and form loops exuding from the protein backbone (Kwong et al., 1998; Leonard et al., 1990).

The structure of the T cell line-adapted (TCLA) molecular clone HxB2 monomeric gp120 core was crystallised in association with the D1-D2 fragment of CD4 and the anti-co-receptor binding site Fab fragment 17b (Kwong et al., 1998). For stability reasons, HIV-1 gp120 could not be crystallised with the carbohydrates and the variable regions and, therefore, the V1/V2 and V3 loops were removed by mutagenesis and over 90% of the carbohydrate motifs were stripped. The N- and C-extremities of gp120 were also missing. A schematic figure of the crystal structure of the resulting gp120 core is represented in figure 1.6. The overall structure of the gp120 core is heart-shaped and consists of two domains: the inner and the outer domain linked by a highly conserved β-sheet that forms the bridging sheet. The inner domain possesses helical features whereas the outer domain forms a barrel. The inner domain faces gp41 and the outer domain is more exposed on the surface of gp120 (Wyatt and Sodroski, 1998). The V1/V2 loop protrudes at the base of the inner domain. The variable loops V3, V4 and V5 protrude from the outer domain, at the base, at the external side and at the top respectively.

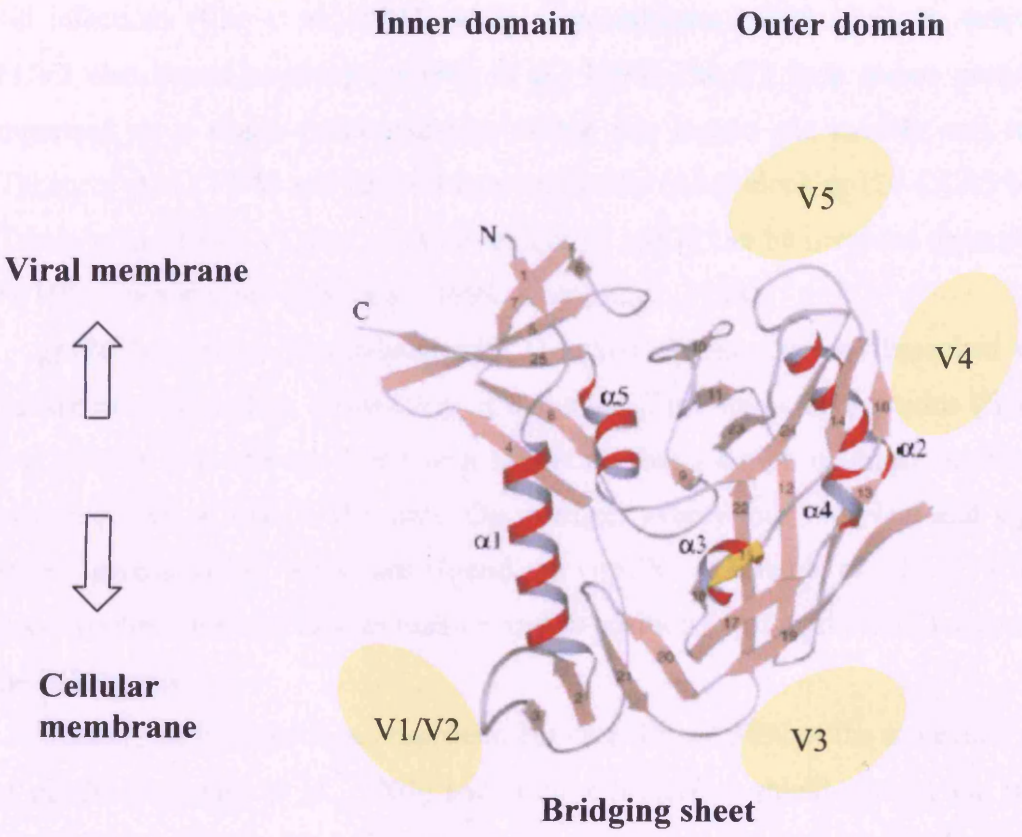


Figure 1.6 Schematic representation of the monomeric gp120 backbone
Crystal structure of the deglycosylated, V1/V2- and V3 loop-deleted gp120 from HIV-1 HxB2 clone in association with D1-D2 domains of CD4 and the MAb 17b. gp120 is organised into an inner and an outer domain. The positions of the variable loops (V) are indicated by the yellow circles. From Kwong et al, 1998

The CD4-binding site (CD4BS) on gp120 contains a hydrophobic cavity (Kwong et al., 1998) that is buried within the trimeric Env structure. The amino acid residues involved in the CD4BS seem to be scattered along the peptide backbone of gp120, particularly on C2, C3, C5 and V5 on the monomeric gp120 (Kwong et al., 1998; Levy, 1998). The conservation of some amino acid residues on gp120 is crucial for the interaction with CD4 (see section 1. 6. 3) but some degree of amino acid variation within gp120 is allowed, as some interactions are mediated through main-chain atoms (Kwong et al., 1998). The binding of CD4 is thought to induce large conformational changes at the CD4BS (Kwong et al., 1998; Myszka et al., 2000).

The binding of gp120 to CD4 reveals the co-receptor-binding site, characterised by a hydrophobic core surrounded by a positively charged periphery involving the bridging sheet and the V3 loop (Kwong et al., 1998). The role of the V1/V2 loop in the co-receptor interaction is less clear since an HIV-1 mutant with V1/V2 deleted was

still infectious (Cao et al., 1997) while a recombinant gp120 similarly deleted for V1/V2 also bound co-receptors (Wu et al., 1996) The V3 loop seems particularly important, as a single point mutation within this region can modify cell tropism (Takeuchi et al., 1991) and anti-V3 loop antibodies (Abs) block gp120-CCR5 binding (Trkola et al., 1996a). Other variable regions of gp120 can be involved depending on the HIV strain studied (Cho et al., 1998; Smyth et al., 1998).

gp120 is heavily glycosylated with O-linked oligosaccharides branched from a GalNac motif added to: serine (Ser) or threonine (Thr) amino acid residue (Bernstein et al., 1994). It is also modified with N-glycans that are high mannose motifs added on Asn-X-Ser or Asn-X-Thr sites. On average, twenty-four N-linked and eight O-linked glycosylation sites are found on gp120 (Leonard et al., 1990). The glycosylation sites can vary in number and in position (Wei et al., 2003) according to the HIV isolate.

Overall, the carbohydrates represent between 40 and 50% of the molecular weight of gp120 (Poignard et al., 2001) and form a protective shield against the immune system (see section 1. 13. 3. 3. 6 for details). Some carbohydrate motifs also modulate HIV infection. The overall deglycosylation of gp120 decreases the affinity of Env for CD4 but does not abrogate the interaction (Fenouillet et al., 1989). In SIV_{mac} some N-glycans appear to be crucial for viral infectivity (Ohgimoto et al., 1998). The absence of some other carbohydrates does not affect infectivity (Ohgimoto et al., 1998; Reitter and Desrosiers, 1998). Some glycosylation motifs could be involved in the modulation of interactions with the co-receptors (Hoffman et al., 1999; Pollakis et al., 2001; Polzer et al., 2002).

1.6.1.2 gp41

gp41 mediates the fusion between the viral and the cellular membranes. A schematic representation of gp41 is shown in figure 1.7. The N-terminus of gp41 consists of a stretch of hydrophobic amino acid residues resembling the fusion peptide of other fusogenic viruses, such as influenza (Colman and Lawrence, 2003), immediately followed by the ectodomain, composed of two hydrophobic repeat domains (HR1 and HR2), involved in the formation of a hairpin structure formed during the fusion (Colman and Lawrence, 2003). The ectodomain seems to be involved in the late steps of fusion, after the merging of the membranes (Bar and Alizon, 2004). The cytoplasmic tail of gp41 at the C-terminus, a stretch of

approximately 150 amino acids, interacts with MA during virus assembly (Freed and Martin, 1996). From the homology with other type I viral proteins, gp41 is thought to mediate the trimeric oligomerisation of gp120-gp41 through interactions between the HR regions.

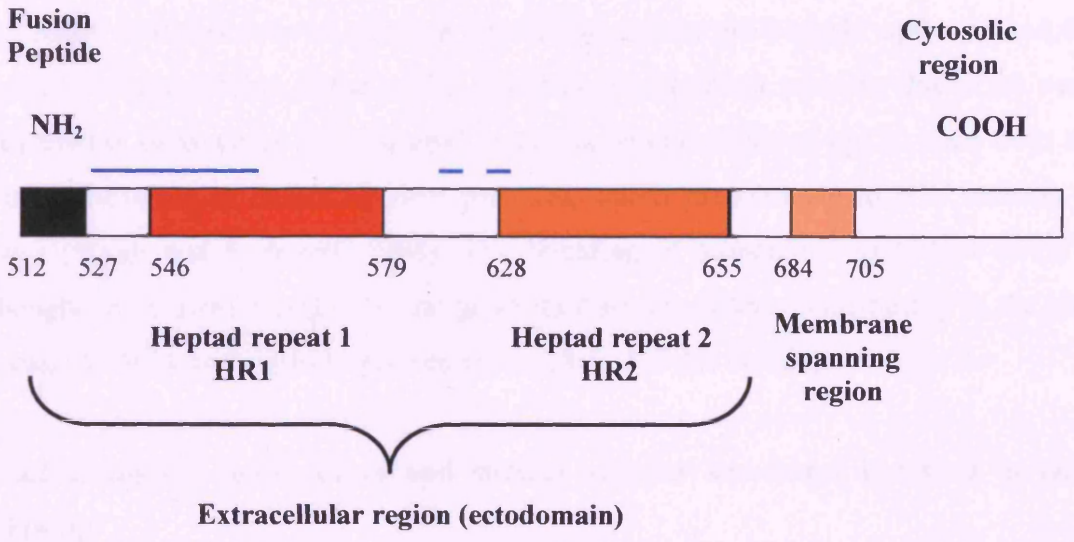


Figure 1.7 Schematic representation of the domains of gp41
The N-terminal fusion peptide (black square) inserts gp41 into the membrane of the target cell after the interaction with the chemokine receptor and prior to fusion. gp41 is characterised by the presence of two hydrophobic repeat domains (HR1 and HR2) that associate gp41 into trimers. The membrane-spanning domain that crosses the viral membrane and the cytosolic region, at the C-terminus of gp41, is found within the HIV particle and interacts with oligomerised MA. The residues are numbered according to their position in $gp160_{HxB2}$ (from Tan et al, 1997). The blue bars span the regions involved in the interaction with gp120 (from Cao et al, 1993).

gp41 is glycosylated and has five putative N-glycosylation sites, three of which have been shown to be glycosylated (Fenouillet et al., 1993). These sites are found within the HR2 region (Tan et al., 1997). They have been shown to be involved in the efficient cleavage of gp160, possibly by shaping gp160 into a suitable conformation (Cao et al., 1993; Fenouillet et al., 1993). Non-glycosylated gp41 seems to mediate the formation of syncytia less efficiently than the glycosylatyed form (Fenouillet et al., 1993) but these results are controversial (Lee et al., 1992).

6.1.3 The gp120-gp41 interaction

gp120 and gp41 form trimers through the non-covalent interactions of hydrophobic amino acid residues localised mainly in the C1 region, and less hydrophobic residues in the C2 region of gp120 (Helseth et al., 1991; Wyatt et al.,

1997). Hydrophobic amino acid residues within C3, C4 and C5 are also involved to a lesser extent (Helseth et al., 1991; Wyatt et al., 1997). gp41 interacts with gp120 via a region involving the amino acid residues 528 to 562, overlapping HR1, and amino acid residues at positions 608 and 628, the latter being found within HR2 (Cao et al., 1993).

Most of the structures exposed on the oligomeric gp120-gp41 spike are on the gp120. A large number of the gp120-gp41 trimers dissociate, possibly due to the weak association between gp120 and gp41, and monomeric forms of gp120 shed from the virions resulting in defective virus particles, which predominate in HIV cultures *in vitro* (Wyatt and Sodroski, 1998). The shedding of monomeric gp120 in serum is thought to be a main target for the generation of non-NAbs, contributing to the viral escape from immune attack (see section 1. 13. 3. 3. 6 for details).

1.6.2 Receptor, co-receptors and surface cellular structures involved in virus binding

1.6.2.1 CD4

CD4 was first identified as the receptor for HIV-1 (Dalgleish et al., 1984; Klatzmann et al., 1984) and also for HIV-2 and SIV (Hoxie et al., 1988; Sattentau et al., 1988). CD4 is a 55 kDa monomeric membrane glycoprotein that contains four extracellular immunoglobulin-like (Ig-like) domains (D1-D4), a transmembrane region (TM) and a cytoplasmic tail. CD4 is expressed on T helper cells (TH), a subset of natural killer cells (NK) and cells of the monocyte/macrophages lineage. It interacts with MHC class II molecules present on antigen presenting cells (APCs) (Janeway, 2001).

The interaction between CD4 and HIV is mediated by gp120. The primary interaction with gp120 occurs at the N-terminus of the D1 domain, that has a similar structure to the CDR H2 region of IgG (Arthos et al., 1989). The D4 domain appears to play a role in the post-binding events, possibly during fusion (Moir et al., 1996).

The affinity of gp120 for soluble CD4 (sCD4), lacking the transmembrane domain and the cytoplasmic tail, varies according the HIV strain (Ivey-Hoyle et al., 1991). Primary isolates (PIs) of HIV require larger amounts of sCD4 for efficient inhibition than TCLA strains (Kabat et al., 1994) and could have a lower affinity for CD4 than TCLA viruses (Daar et al., 1990).

CD4 is down-regulated from the cell surface by Nef in primary cells (Garcia and Miller, 1991). Nef mediates the endocytosis of CD4 via clathrin-coated pits (Foti et al., 1997). It interacts with CD4 via two leucine residues within its cytoplasmic tail (Peter, 1998) and dissociates CD4 from a lymphocyte-specific protein tyrosine kinase $p56^{lck}$, which leads to the internalisation of CD4 (Arora et al., 2002). Nef also interacts with one of the components involved in the formation of clathrin-coated pits, AP2 (Arora et al., 2002). By bringing CD4 and AP-2 together, Nef favours the endocytosis of CD4 and its degradation (Greenberg et al., 1997). Nef acts only on CD4 when it is expressed at the surface of the cell. The accessory protein Vpu contributes to CD4 degradation in the ER (Meusser and Sommer, 2004).

The degradation of CD4 by HIV has two purposes. The down-regulation of CD4 from the surface could prevent super-infection from occurring and prevent gp120 from associating with CD4 (Arora et al., 2002). The degradation of CD4 results in enhanced viral infectivity (Harris, 1999).

1.6.2.2 The chemokine co-receptors

After the discovery of the role of CD4 in HIV infection, Maddon *et al*, showed that the expression of human CD4 on murine cells was not sufficient to allow HIV infection (Maddon et al., 1986). Feng *et al* showed that TCLA HIV-1 strains, intensively passaged in immortalised T cells lines, used the chemokine receptor CXCR4 (previously known as Fusin, HUMSTSR, LCR-1 or LESTR) in addition to CD4 to allow their entry in mouse cells (Feng et al., 1996). CXCR4 also mediates the entry of HIV-2 CD4-independent strains (Endres et al., 1996). Shortly after, CCR5 and, to a lesser extent, CCR3 were found to be the main co-receptors for infection of HIV-1 PIs in primary PBMCs and macrophages (Alkhatib et al., 1996; Choe et al., 1996; Dragic et al., 1996). Chemokine receptors are 7-TM G-coupled proteins involved in signalling that are internalised by endocytosis via clathrin-coated pits when bound to their chemokines. CCR5 binds to a variety of chemokines: RANTES, MIP-1α, MIP-1β and MCP-2; whereas CXCR4 only binds to SDF-1.

PIs and TCLA strains of HIV-1, HIV-2 and SIV were found to use a wide range of chemokine receptors *in vitro* (see Clapham and McKnight, 2002 for details), HIV-2 being able to use a broader range of chemokine receptors than HIV-1 *in vitro* (McKnight et al., 1998). Table 1.1 lists the co-receptors used for HIV and SIV infection *in vitro*.

It became apparent that CCR5 and CXCR4 were the chemokine receptors that mediated HIV infection more efficiently *in vitro*. Before the identification of the co-receptors, HIV strains were described as macrophage-tropic (M-tropic) or T cell line-tropic (T-tropic) (Fenyo et al., 1988; Schwartz et al., 1989). They could also be classified by their ability to form synticia in T cells lines. Viruses that can form syncytia are called syncytium-inducing (or SI viruses) and the ones that are unable to form syncytia are called non-syncytium-inducing (or NSI viruses) (Tersmette et al., 1988). NSI viruses use CCR5 as their main co-receptor and SI viruses use CXCR4. There are exceptions to this rule (Simmons et al., 1998). Viruses showing predominant CXCR4 usage are called X4-tropic, viruses showing a predominant CCR5 usage are called R5-tropic and viruses showing a co-dominant usage of CCR5 and CXCR4 are called dual tropic X4R5 (Berger et al., 1998).

More importantly, CCR5 and CXCR4 seem to be the co-receptors relevant *in vivo*. Although some studies have shown that some HIV strains might enter primary cells via an alternative co-receptor. Lee *et al* showed that some X4-viruses could enter primary thymocytes isolated from children via CCR8 (Lee et al., 2000). Similarly, Willey *et al* reported that some HIV-1 and HIV-2 strains could infect PBMCs and astrocytes via an unidentified co-receptor (Willey et al., 2003). In these two cases, the alternative co-receptors mediated infection less efficiently than CXCR4 and CCR5 (Lee et al., 2000; Willey et al., 2003). Macrophages and dendritic cells (DCs) are mainly infected by R5-viruses, although some PIs infect macrophages via CXCR4 (Simmons et al., 1998). Zhang *et al* showed that a panel of adult and paediatric clinical isolates of HIV-1, SI and NSI, used CCR5 almost exclusively, although the tropism was broader for some SI isolates (Zhang et al., 1998). Individuals homozygous for a 32 base pair deletion in the CCR5 gene (CCR5 Δ32), which leads to the production of a non-functional receptor, are highly resistant to HIV infection (Dean et al., 1996; Deng et al., 1996; Samson et al., 1996). Moreover, infected individuals heterozygous for Δ32 CCR5 show a slower progression towards AIDS (Dean et al., 1996), which suggests that HIV infects their cell targets via CCR5 *in vivo*.

Co receptor	Ligand	Virus	Reference
CCR1	MIP-1α, MPIF-1, MCP-3, RANTES	HIV-2, SIV	(McKnight et al., 1998)
CCR2b	MCP-1, MCP-2, MCP-3	HIV-1, HIV-2, SIV	(Doranz et al., 1996)
CCR3	Eotaxin-1, eotaxin-2, MCP-3, MCP-4, RANTES	HIV-1, HIV-2, SIV	(Doranz et al., 1996)
CCR4	MDC, TARC, RANTES, MIP-1 α	HIV-2	(McKnight et al., 1998)
CCR5	MIP-1α, MIP-1β, RANTES, MCP-2	HIV-1, HIV-2, SIV	(Alkhatib et al., 1996; Deng et al., 1996; Dragic et al., 1996)
CCR8	I-309	HIV-1, HIV-2, SIV	(Rucker et al., 1997)
CCR9	TECK	HIV-1	(Choe et al., 1998)
CXCR2	IL8, NAP-2. ELR, CXCs	HIV-2	(Bron et al., 1997)
CXCR4	SDF-1	HIV-1, HIV-2, SIV	(Feng et al., 1996)
CX3CR1	Fractaline	HIV-1, HIV-2, SIV	(Garin et al., 2003; Reeves et al., 1997)
STRL/Bonzo	CXCL16	HIV-1, HIV-2, SIV	(Alkhatib et al., 1997)
GPR1	Unknown	HIV-1, HIV-2, SIV	(Farzan et al., 1997)
GPR15/Bob	Unknown	HIV-1, HIV-2, SIV	(Farzan et al., 1997)
APJ	Apelin	HIV-1, HIV-2, SIV	(Choe et al., 1998; Edinger et al., 1998)
Chem R23	Unknown	HIV-1, SIV	(Samson et al., 1998)
RDC1	Unknown	HIV-2, SIV	(Shimizu et al., 2000)

Table 1.1 Chemokine receptors that support HIV and SIV replication *in vitro*
The CXCR4 and CCR5 chemokine receptors are thought to be the most relevant co-receptors *in vivo*. Adapted from Clapham and McKnight, 2002

Viruses that harbour a predominant X4-tropism can be found in up to 50% of patients at the stage of AIDS development infected with subtype B viruses. R5- and

X4-viruses appear to infect different subsets of CD4 T cells *in vivo*. R5-viruses infect memory CD4 T cells whereas X4-viruses infect naïve CD4 T cells (Moore et al., 2004). X4-tropism *in vivo* is thought to be associated with a more rapid disease progression (Connor et al., 1997) but X4-tropism is not a prerequisite for AIDS development (de Roda Husman et al., 1999; Li et al., 1999). Macaques infected with SIV_{mac} usually die of AIDS-related syndromes without a switch to X4-tropism (Moore et al., 2004). Moreover, the R5-X4 switch could be a phenomenon observed predominantly for subtype B viruses, as X4-viruses from subtype C are more rarely isolated from infected individuals (Ping et al., 1999). Subtype C viruses, however, may not have the time to evolve into X4 as subtype C-infected individuals can die more rapidly, due to the enhanced cytopathy of subtype C viruses compared to subtype B or due to the precarious health care conditions in the countries where subtype C is endemic.

The possibility that HIV might use alternative co-receptors *in vivo* for infecting target cells in compartmentalised tissues, such as astrocytes in the brain, where the expression of CCR5 or CXCR4 is undetectable, is still unclear and cannot be ruled out (Willey et al., 2003).

The V3 loops of X4-viruses have an overall higher positive charge than the V3 loops of R5-tropic viruses.

X4- and R5-tropic viruses seem to interact with their co-receptors via different regions (Clapham and McKnight, 2002; Dragic, 2001). Chemokine receptors are proposed to form rods in the membrane with a central pore surrounded by the 7-TM regions. They have four domains exposed on the cell surface: the N-terminus and three extracellular loops: E1, E2 and E3 (Clapham and McKnight, 2002).

In the case of CCR5, the binding sites of the chemokines and gp120 seem to be overlapping but distinct (Atchison et al., 1996). The extracellular E2 loop is important for binding chemokines (Blanpain et al., 1999). HIV binding to CCR5 requires multiple interactions with several regions of CCR5 (Atchison et al., 1996; Bieniasz et al., 1997). The N-terminus tyrosine-rich region of CCR5 appears to be important for the binding of g120 (Farzan et al., 1998; Farzan et al., 1999) and the E2 loop seems more involved in the fusion and the entry of the virus (Clapham and McKnight, 2002). The N-terminus of CCR5 contains sulphated tyrosine residues that appear to interact directly and specifically with the V3 stem of gp120 (Cormier and Dragic, 2002). The overall negative charge of the N-terminus of CCR5, conferred by the

sulphated tyrosines, could interact with the positively charged V3 loop and facilitate the specific interactions between CCR5 and gp120, but the charges do not seem to drive the specificity of the interactions (Clapham and McKnight, 2002). The C4 domain of gp120, however, has been shown to directly interact with the N-terminus of CCR5, in a CD4-independent R5-tropic HIV-2 isolate (Lin et al., 2001), suggesting that the mechanisms of interaction with CCR5 might vary according to the strain (Rabut et al., 1998) or on the subtype studied (Thompson et al., 2002). Moreover, mutant strains of HIV-1, resistant to inhibition by CCR5 antagonist molecules, do not switch to X4-tropism *in vitro* (Trkola et al., 2002). In macaques, a transient and brief switch from R5 to X4 occurs but R5-tropism becomes again predominant (Wolinsky et al., 2004).

Unlike CCR5, mutations within CXCR4 do not clearly define a region that plays a crucial role in binding and entry of X4-tropic viruses (Dragic, 2001). The N-terminus of CXCR4 seems to be required for interacting with some HIV-1 strains but not all (Picard et al., 1997). However, the E2 loop seems to be more important for X4-tropic HIV-1 (Brelot et al., 1999; Clapham and McKnight, 2002) and X4-tropic, CD4-independent HIV-2 isolates (Reeves et al., 1998). The E2 loop has a negative charge, which could interact with the highly positively charged V3 loop of X4-tropic viruses. The major involvement of the E2 loop of CXCR4 at mediating the entry of X4-tropic HIV-1 viruses has been shown by the broad inhibitory effect of AMD3100 against HIV-1 (Labrosse et al., 1998). This molecule blocks HIV entry by binding residues on the E2 loop and the transmembrane domain TM4 of CXC4 (Labrosse et al., 1998).

Some strains of HIV-2 and SIV can infect cells without the requirement of CD4 (Borsetti et al., 2000; Reeves et al., 1998) but no HIV or SIV strain described so far is co-receptor independent (Farber and Berger, 2002). Moreover, the acquisition of CD4-dependence only requires a few changes on gp120 and gp41 in CD4-independent SIV_{mac} (Puffer et al., 2004). These observations have lead to the hypothesis that the chemokine co-receptor was the primary receptor of these lentiviruses; the requirement of CD4 for infection was acquired later in order to protect the virus from the attack of the immune system (LaBranche et al., 2001; Wyatt and Sodroski, 1998).

1.6.2.3 Surface cellular structures

The entry of HIV generally relies on the presence of CD4 and a chemokine co-receptor (Clapham and McKnight, 2002). Hiv also uses several molecular structures present on the cellular membrane to mediate attachment, accelerate fusion or promote dissemination.

Mondor *et al* showed that the attachment of HIV-1 to Hela cells relied on interactions between gp120, from an X4-virus, and heparan sulphate proteoglycans, independently of the interaction with CD4 (Mondor et al., 1998). Moreover, it appears that X4-tropic viruses interact directly with polyanions via their co-receptor binding site and their V3 loop *in vitro*. R5-viruses seem less dependent on interactions with heparan sulphates, probably due to the more neutral overall charge of their V3 loop (Moulard et al., 2000). The implication of the heparan sulphate for infecting CD4 T cells *in vivo* is probably less important, as they seem to express low levels of these motifs (Ibrahim et al., 1999). However, Bobardt *et al* recently showed that heparan sulphate and chondroitin sulphate could be exploited by HIV, regardless of the virus tropism, to enter and migrate through the blood brain barrier (BBB) (Bobardt et al., 2004). The removal of heparan sulphate proteoglycans also inhibits HIV infection in macrophages (Saphire et al., 2001a).

HIV seems to use molecular structures found in lipid rafts of the target cells to facilitate infection (Popik et al., 2002). Phospholipids, such as phosphatidylserine, have been shown to enhance HIV infection of macrophages (Callahan et al., 2003). Glycosphingolipids, such as sulphatide galactosyl ceramide (SGalCer), have also been shown to bind HIV virions, regardless of their co-receptor usage, (Kensinger et al., 2004) and could be important for the infection of HIV in neuronal cells (Bhat et al., 1991). Fusion between the cell and the HIV-1 membranes seem to be promoted by several ceramides (Alfsen and Bomsel, 2002; Puri et al., 1998) as well as the ganglioside GM3 (Hug et al., 2000), although its over-expression inhibits fusion (Rawat et al., 2004). The over-expression of human leukocyte elastase (HLE) on non-infectable cells expressing CD4 and CXCR4, has been shown to allow infection of the X4-tropic $NL_{4.3}$ by co-patching CD4 and HLE, suggesting that HLE could be required as a fusion receptor (Bristow et al., 2003). The marker LFA-1 (leukocyte function associated antigen type 1) can also promote viral attachment via its interaction with ICAM-1, acquired in the HIV membrane during budding from lipid rafts (Giguere and Tremblay, 2004).

In addition to docking and fusion, other cellular molecular structures, such as DC-SIGN and syndecans, can promote the transmission of HIV to other cells (*in trans*). DC-SIGN (dendritic cell-specific intercellular adhesion molecule grabbing non-integrin), or CD209, is a C-type lectin involved in the recognition of carbohydrate structures on self-antigens and pathogens (van Kooyk and Geijtenbeek, 2003). DC-SIGN is present on DCs and interacts with CD4 T cells via ICAM-3 (Geijtenbeek et al., 2000b). It is also expressed on monocytes and peripheral blood lymphocytes. HIV binds DC-SIGN, by specific N-glycans on gp120 (Hong et al., 2002; Kwon et al., 2002), independently of its interaction with CD4. Thus, DC-SIGN could enhance HIV infection of CD4 T cells (Geijtenbeek et al., 2000a; Kwon et al., 2002). DC-SIGN might be one of the motifs involved in the transmission of HIV from DCs to CD4 T cells *in trans* (Arrighi et al., 2004), as other C-lectins, such as langerin and mannose receptor, have been reported to bind HIV on DCs (Turville et al., 2002). DC-SIGN could mediate the enhancement of transmission by internalising HIV in DCs in a low pH endosomal compartment that can keep the virus fully infectious for a longer period than in a free state (Kwon et al., 2002). Turville *et al*, however, have shown that HIV could interact with other structures on DCs, such as the mannose receptor, and HIV internalisation could be medited by other structures (Turville et al., 2002).

DC-SIGN can also enhance HIV infection on the same cell (*in cis*) (Lee et al., 2001). DCs in mucosal tissues (such as the rectum and the cervix) express high levels of DC-SIGN (van Kooyk and Geijtenbeek, 2003). A DC-SIGN homologue expressed on endothelial cells, DC-SIGNR, has also been described to enhance HIV infection *in trans* (van Kooyk and Geijtenbeek, 2003). It has been shown that Nef from R5-tropic viruses up-regulates the expression of DC-SIGN in DCs (Sol-Foulon et al., 2002) and DC-SIGN could contribute to the dissemination of HIV after sexual transmission or blood contamination (van Kooyk and Geijtenbeek, 2003).

Syndecans, a class of proteoglycans, can enhance attachment and infection of HIV (produced in PBMCs) to target cells, by interacting with the V3 loop and the co-receptor binding site of gp120. HIV captured by non-infective cells expressing syndecans seems to retain infectivity longer than in a cell-free state (Bobardt et al., 2003). This finding could be of particular importance for HIV infection in the vascular system as the endothelium expresses high levels of syndecans (Bobardt et al., 2003; Saphire et al., 2001a).

Fc receptors, in association with anti-HIV Abs, have been involved in the enhancement of HIV infection (see section 1. 13. 3. 3. 9 for details).

Complement receptors CR1 (CD35), CR2 (CD21) and CR3 (CD11b/CD18) have been shown to enhance HIV infection of target cells (see section 1. 13. 2. 3 for details).

1.6.3 Binding to CD4 and co-receptor and fusion

HIV entry into target cells is mediated by gp120 and gp41, associated in trimeric spikes on infectious viruses. HIV has a type I fusion envelope, which means that Env is synthesised as a protein precursor that is cleaved by cellular proteases and the subsequent SU and TM proteins associate in trimers. Other viruses fitting into this classification are influenza, mumps and measles viruses (Colman and Lawrence, 2003). Unlike influenza, fusion and entry of HIV seems generally to be pH-independent (Colman and Lawrence, 2003).

The first step of entry generally requires the interaction of gp120 with CD4. gp120 interacts with CD4 via the amino acid residues of the N-terminus of D1. The amino acid residues Phe 43 and Arg 59 of CD4 interact with the residues Asp 368, Glu 370 and Trp 427 of gp120 and are crucial for the CD4-gp120 interaction (Kwong et al., 1998; Saphire et al., 2001b). Phe 43 accounts for 23% of the CD4-monomeric gp120 interaction. These amino acid residues are conserved in the SU proteins of all immunodeficiency lentiviruses (Kwong et al., 1998). Several trimeric spikes must interact with CD4 and at least two gp120 proteins per trimer need to be engaged with CD4 to continue entry (LaBranche et al., 2001).

The binding of CD4 triggers conformational changes, revealing the V3 loop (Sattentau and Moore, 1991). These modifications expose the co-receptor binding site on gp120 (Trkola et al., 1996a; Wu et al., 1996), composed of the V3 loops and the bridging sheet between the V1/V2 and the V3 loop (Kwong et al., 1998). Hoffman *et al* have shown that, in the CD4-independent HIV-1 strain IIIBx, the co-receptor binding site is more exposed and could be in a more stable conformation than on its CD4-dependent counterpart IIIB (Hoffman et al., 1999).

CD4 and CCR5 are associated within lipid rafts regardless of the presence of gp120 (Xiao et al., 1999). This clustering leads to a greater cooperativity between gp120, CD4 and the chemokine receptors, which could optimise the fusion,

particularly on cells expressing low levels of CD4 and co-receptors (Gallo et al., 2003; Popik et al., 2002).

The binding event with the chemokine receptor is a prerequisite for the initiation of fusion (LaBranche et al., 2001). Using a modified truncated gp140 Env, where extra cysteine residues were added to obtain disulfide-stabilised trimeric-like structures (Binley et al., 2000), it has been suggested that gp120 provides a clamp that holds gp41. The binding of gp120 onto the co-receptor then releases the clamp, allowing gp41 to modify its conformation to mediate fusion (Abrahamyan et al., 2003). The fusion peptide, at the N-terminus of gp41, is exposed and inserts into the membrane of the target cell. The three HR1 regions interact to form a coiled-coil structure and the three HR2 domains fold back in a hairpin-like structure into the hydrophobic regions of the coiled-coil to form a thermodynamically stable six-helix bundle (Tan et al., 1997). This brings the viral and the cellular membranes into close proximity, forming a hemifusion intermediate (Bar and Alizon, 2004). A fusion pore is then formed to allow the viral core to enter the cytoplasm of the cell. A schematic representation of HIV fusion with the cell membrane is shown in figure 1.8.

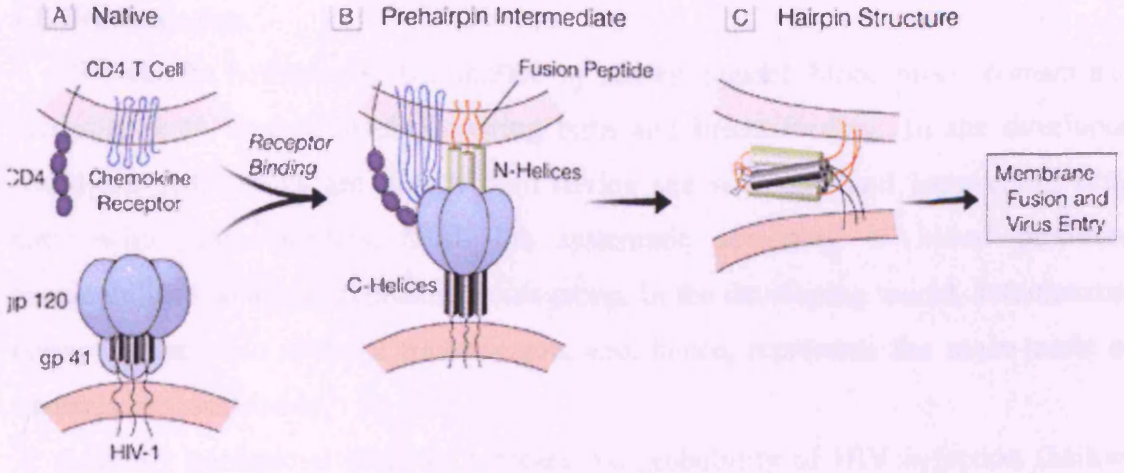


Figure 1.8 Fusion between HIV and cell membranes
The viral glycoprotein gp120 interacts with cellular receptors (CD4 and a chemokine receptor) and the envelope complex undergoes a conformational change (A). A pre-hairpin intermediate is formed in which the fusion peptide has inserted into the target membrane (B). The pre-hairpin intermediate resolves to the fusion-active hairpin structure when the C and N peptides interact, pulling the cell and viral membranes into close proximity and culminating in membrane fusion (C). From D'Souza et al., 2000

1.7 Cell targets

HIV potentially infects all human cells expressing CD4 and one of the co-receptors described in 1. 6. 2. 2. HIV infects CD4 T cells but not all HIV strains infect the same subset of these cells. R5-tropic viruses infect memory CD4 T cells ($CCR5^+$) whereas X4-tropic viruses infect naïve CD4 T cells ($CXCR4^+$). HIV also infects macrophages *in vitro* (Simmons et al., 1996) and *in vivo* (Miller and Shattock, 2003). The HIV life cycle in macrophages differs from the life cycle in CD4 T cells. In macrophages, viral components are assembled in late endosomes and, consequently, macrophage-derived HIV viruses express unique markers in their membranes (Pelchen-Matthews et al., 2003).

HIV-infected CD8 T cells, that express CD4, have been isolated from patients at the AIDS stage (Cochrane et al., 2004; Livingstone et al., 1996). Immature DCs have been infected *in vitro* by X4 and R5 isolates (MacDougall et al., 2002) but mature DCs are refractory to HIV infection (Miller and Shattock, 2003). Monocyte-derived Langerhans cells (LCs) are also susceptible to HIV infection (Kawamura et al., 2003). Astrocytes have been shown to be infected by HIV *in vitro* (Willey et al., 2003).

1.8 Transmission

HIV can be horizontally transmitted by sexual contact, blood-blood contact and vertically from mother to child during birth and breast-feeding. In the developed world, the risk groups are mainly men having sex with men and intravenous drug users who share needles. With the systematic screening of blood products, haemophiliacs no longer represent a risk group. In the developing world, heterosexual contact is the main route of transmission, and, hence, represents the main mode of transmission worldwide.

Sexually transmitted diseases increase the probability of HIV infection (Miller, 1998), which suggests that the presence of cells from the mucosal immune system mediates or enhances infection. Greenhead *et al* showed that HIV infection was enhanced in vaginal tissues incubated in the presence of PHA and interleukin 2 (IL2) (Greenhead et al., 2000). Moreover, HIV is not detected in vaginal epithelial cells and does not seem to be transcytosed (Greenhead et al., 2000). LCs and γδ T cells (see section 1. 13. 2. 2 for details) are found in the mucosal epithelium and macrophages while T cells reside in the lamina propria beneath the epithelial layers. The mechanisms of infection and successful dissemination in sexual transmission of HIV

are unclear. LCs are DC-SIGN negative and CD4, CXCR4 and CCR5 positive (Miller and Shattock, 2003). LCs and macrophages seem to be the prime targets during HIV transmission (Miller, 1998). LCs are infected by SIV *in vivo* (Miller, 1998). In the male genital system, LCs are mainly found in the foreskin. The removal of the foreskin has been shown to reduce the risk of HIV infection, which suggests that these cells are involved in transmission (Auvert et al., 2001; Miller, 1998). LCs could also transmit HIV to HIV-specific CD4 T cells (Sugaya et al., 2004).

In addition to LCs, DC-SIGN expressing DCs are found within the lamina propria of the vagina. Although the *in vivo* infection of DCs by HIV remains unclear, they could be involved in the early dissemination of HIV (Miller and Shattock, 2003).

It is still unclear if HIV transmission occurs by cell-free virus or infected cells (Levy, 1998; Miller, 1998). Cell-free HIV can infect target cells (see section 1. 6. 3) but can also be transmitted from cell to cell via the virological synapse (Jolly et al., 2004), which is a cytoskeleton-dependent, stable adhesive junction across which the viral core is transmitted by directed transfer, occurring between an infected cell and a permissive non-infected cell (Jolly and Sattentau, 2004).

1.9 Clinical symptoms of HIV and AIDS

HIV infection is characterised by two phases: the acute infection and the chronic infection, leading to AIDS. The course of HIV is represented in figure 1.9.

1.9.1 Acute infection

HIV acute infection is generally associated with flu-like symptoms one to four weeks after infection. The symptoms consist of headache, retro-orbital pain, muscle aches, sore throat, fever, swollen lymph nodes, rash affecting the trunk, weight loss, fatigue and night sweats. In some cases, oral and (or) genital *candidiasis*, nausea, vomiting, diarrhoea and central nervous system disorders, such as encephalitis, can occur (Kahn and Walker, 1998; Levy, 1998). Fever, fatigue and rash represent the most common symptoms observed in acute infection (Kahn and Walker, 1998). The symptoms of acute infection may last from a few days to more than ten weeks, but the duration is usually fourteen days (Kahn and Walker, 1998). The non-specific nature of the symptoms associated with HIV infection makes the diagnosis difficult to assess. Therefore, recent "risky" behaviour has to be known.

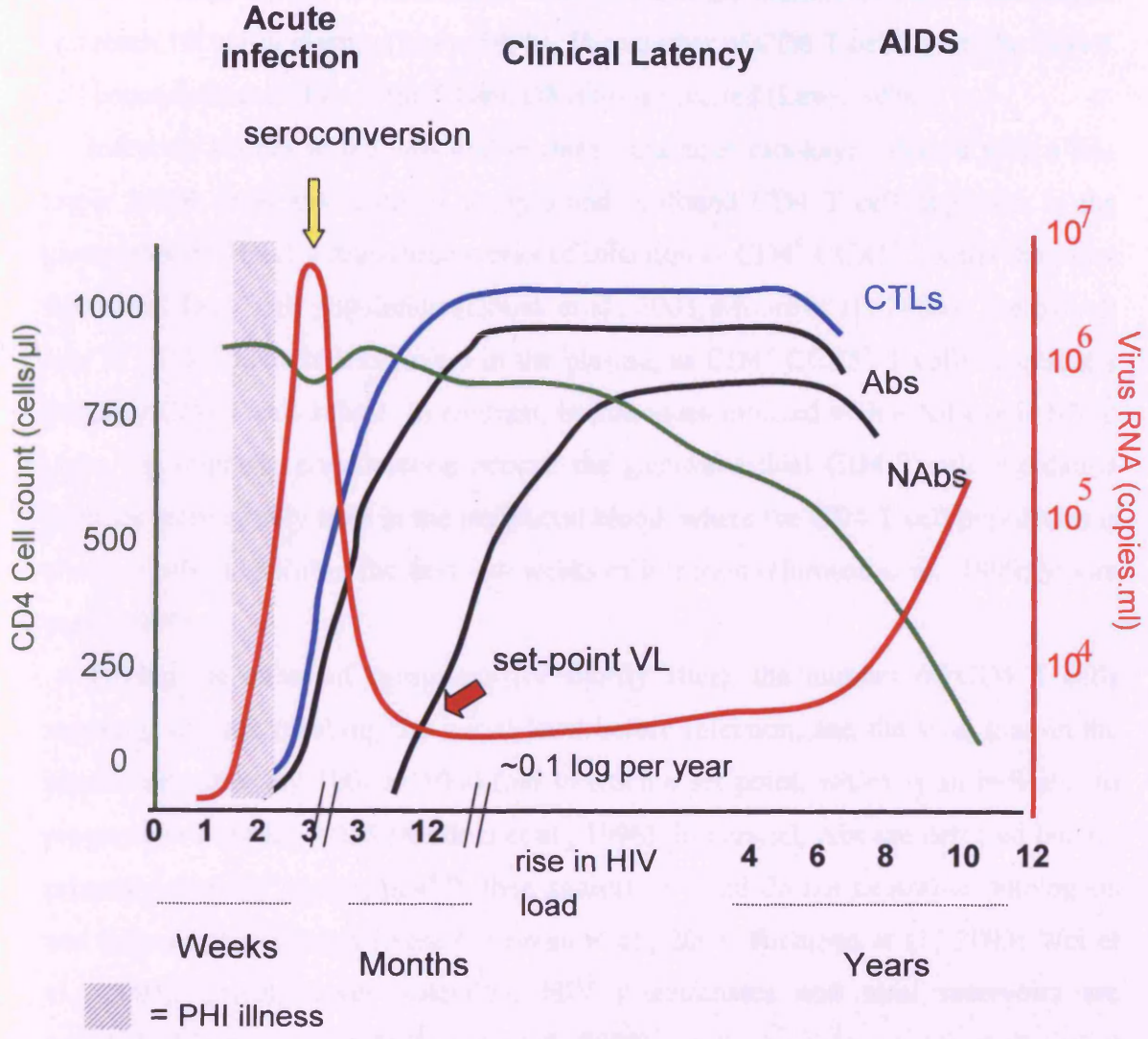


Figure 1.9 Representation of the viral load, the CD4 T cell count, the emergence of CTL response, the anti-HIV IgG and the development of neutralising anti-HIV Abs

After transmission, in acute infection, the HIV viral load (red line) arises to reach a peak concomitant with a decrease in the CD4 T cell count (green line), the detection of a specific CD8 T cell response (blue line) and anti-HIV Abs (black line). The HIV-infected individual usually presents with flu-like symptoms. The viral load then drops to a set point, detected between three and six months post-infection, and is predictive of the evolution of the disease. An increase in the CD4 T cell count occurs concomitantly with the drop in viral load. The HIV-infected individual becomes asymptomatic and HIV infection enters the chronic phase. NAbs are usually detected after the establishment of the viral set point. Despite the persistence of a specific immune response through the infection, the CD4 T cell count progressively decreases and the viral load increases. When the CD4 count is below 200 cells/µl, the individual enters the AIDS phase and is susceptible to opportunistic diseases. The AIDS stage occurs on average ten years after contracting the infection without any anti-viral treatment. The viral load re-augments and is comparable to levels reached before the set point. From Levy, 1998

Concomitantly, a peak of viraemia is observed and the number of HIV RNA copies can reach 10^8/ml in plasma (Levy, 1998). The number of CD8 T cells rises, the CD4 T cell count decreases, hence the CD4/CD8 ratio is inverted (Levy, 1998).

Infection studies in humans and in rhesus macaque monkeys infected with a R5-tropic SHIV show that there is a rapid and profound CD4 T cell depletion in the gastrointestinal tract within three weeks of infection as $CD4^+$ $CCR5^+$ T cells represent the main CD4 T cell population (Douek et al., 2003; Moore et al., 2004). The overall loss of CD4 T cells is less severe in the plasma, as $CD4^+$ $CCR5^+$ T cells represent a minority CD4 T cell subset. In contrast, in macaques infected with a X4-tropic SHIV virus, the opposite phenomenon occurs: the gastrointestinal CD4 T cell population declines more slowly than in the peripheral blood, where the CD4 T cell population is almost eradicated within the first few weeks of infection (Harouse et al., 1999; Moore et al., 2004).

During the onset of symptoms (or shortly after), the number of CD4 T cells recovers, without reaching the initial level before infection, and the viral load in the plasma decreases by 100- to 1000-fold to reach a set point, which is an indicator to progression towards AIDS (Mellors et al., 1996). In parallel, Abs are detected but are primarily directed against $p24^{Gag}$, then against Env and do not neutralise autologous and heterologous viruses (Aasa-Chapman et al., 2004; Richman et al., 2003; Wei et al., 2003). During acute infection, HIV disseminates and viral reservoirs are established (see section 1.9) (Douek et al., 2003).

1.9.2 Chronic infection and AIDS

Following acute infection, HIV infection enters an asymptomatic chronic phase that lasts on average ten years without any anti-viral treatment. NAbs are detected (Aasa-Chapman et al., 2004; Richman et al., 2003; Wei et al., 2003) as well as a CTL response but both branches of the immune system fail to clear the virus. The $CD4^+/CD8^+$ ratio continues to be inverted, the CD4 T cell population progressively declines, at a rate of 25 to 60 cells/µl per year, and viraemia eventually rises to levels similar to prior to seroconversion (Levy, 1998). In some cases, the decline of CD4 T cells occurs more rapidly and could be associated with a switch from R5 to X4-tropism (Connor et al., 1997).

When the CD4 T cell count is below 200 cells/µl in plasma, the infected individual enters the AIDS stage of the disease and opportunistic infections, such as

cytomegalovirus (CMV) and *Pneumocystis carinii* as well as AIDS-related malignancies, such as Kaposi Sarcoma, non-Hodgkin's lymphoma, anal and cervical cancers, usually appear (Levy, 1998). Neurological pathologies, such as dementia, can be detected in some patients. The emergence of opportunistic diseases is due to the exhaustion of the immune system, incapable of fighting pathogens.

1.9.3 Cell death and CD4 T cell dynamics

HIV infection is mainly characterised by the progressive depletion of CD4 T cells. Death of HIV-infected cells results from apoptosis or necrosis. Apoptosis is an active cell suicide mechanism, involved in the clearance of autoreactive T cells and the establishment of self-tolerance. Apoptosis requires the expression of cell markers, such as Fas receptor (CD95), p55 tumour necrosis factor (TNF) receptor and TRAIL receptors, which activate caspases, which in turn activate endonucleases involved in the fragmentation of the DNA genome (Alimonti et al., 2003; Janeway, 2001). Apoptosis is also characterised by mitochondrial leakage and the release of caspase activators (Alimonti et al., 2003). Apoptosis is regulated by the presence of anti-apoptotic regulatory molecules, belonging to the Bcl family, which act mainly on the caspases (Badley et al., 2000). Apoptotic cells do not trigger an inflammatory response, unlike necrotic cells. Necrosis is associated with swelling of cytoplasmic compartments, extensive vacuolisation and leakage of the cytoplasm.

HIV-infected CD4 T cells fail to proliferate and undergo apoptosis *in vitro* and *ex vivo* (Groux et al., 1992; Perfettini et al., 2004; Plymale et al., 1999) and apoptosis has been observed in neurons (Ryan et al., 2004). The half-life of infected CD4 T cells varies between twelve and thirty-six hours (Alimonti et al., 2003). Fas and FasL cell expression is up-regulated in HIV-infected T cells. The regulation of TNF receptors is also altered and TRAIL/APO 2 expression could be dysfunctional (Badley et al., 2000).

Viral proteins have been shown to have a direct apoptotic effect on infected and non-infected cells, (Alimonti et al., 2003; Badley et al., 2000). Tat, Env and Nef have been shown to induce apoptosis by upregulating the expression of Fas (Alimonti et al., 2003) but HIV could also induce direct killing by a Fas-independent mechanism (Gandhi et al., 1998). Vpu has been shown to induce apoptosis with an X4-tropic virus (Akari et al., 2001) but Vpu from PIs could have a protective role against apoptosis in PBMCs (Komoto et al., 2003). Vpr could have an effect on mitochondrial

permeability and PR could directly cleave and activate caspase 8 and the anti apoptotic Bcl2 (Badley et al., 2000). Soluble gp120 can cross-link CD4 and CXCR4 and induce apoptosis by enhanced susceptibility to Fas killing (Banda et al., 1992).

However apoptosis might not be the main cell death-inducer. Several studies suggest that necrosis is the main cell death mechanism in HIV-infected cells Bolton *et al* showed that cell death of laboratory HIV-infected cell lines was independent of caspases (Bolton et al., 2002), and appeared more necrotic (Lenardo et al., 2002). Similarly, a study by Plymale *et al* showed that laboratoty-adapted cell lines, infected with HIV, died more of necrosis than of apoptosis (Plymale et al., 1999).

HIV-infected cells are subject to killing by CD8 T cells (see section 1. 12. 3. 2) and macrophages. Non-infected T cells could be also killed by HIV-infected macrophages, contributing to the CD4 depletion (Badley et al., 2000).

During the chronic phase of infection, there is a high level of CD4 T cell activation (Douek et al., 2003), which could lead to the death of activated CD4 T cells in the periphery. The circulating CD4 T cells have a shorter half-life and, despite the CD4 activation, the number of circulating CD4 T cells is not increased (Hellerstein et al., 1999). As HIV infection progresses, a decline of quantity and the quality of the CD4 T cells occurs (Douek et al., 2003; Fleury et al., 2000). HIV also affects the thymus, the main organ for T cell production, in both children and adults and suppresses thymocyte proliferation (Dion et al., 2004). HIV also perturbs and inhibits the production of haematopoietic lineages other than CD4 T cells (Douek et al., 2003).

HIV infection of macrophages impairs their functions. Fcγ receptor signalling is impaired, resulting in the alteration of the mechanisms of phagocytosis and cytokine secretion (Verani et al., 2005). It also induces phenotypic and functional perturbations of B cells (Moir et al., 2001). Peripheral lymphoid organs are altered, the follicular dentritic cell (FDC) architecture is progressively destroyed and the lymphoid tissue is replaced by fibrotic tissue and fatty infiltration (Kacani et al., 2001).

1.10 Viral reservoirs

The initial infection is followed by the dissemination of HIV to various parts of the body, which form viral reservoirs. Blankson *et al* define a viral reservoir as a cell type or an anatomical site in which there is accumulation of replication-competent

viruses, characterised by slower turnover properties than actively replicating viruses (Blankson et al., 2002).

The main targets for HIV are CD4 T cells (Dalgleish et al., 1984; Klatzmann et al., 1984). Not all CD4 T cells are equally infected by HIV. The predominant population of infected T cells consists of memory CD4 T cells, with naïve CD4 T cells being more refractory to infection by R5-tropic viruses (Stebbing et al., 2004). HIV-specific memory CD4 T cells are more susceptible to HIV infection than other memory CD4 T cells, as they contain more HIV DNA at all stages of the infection (Douek et al., 2002). A small proportion of the infected cells can become quiescent and form a pool of resting cells, thus, forming a viral reservoir (Blankson et al., 2002; Stebbing et al., 2004). This reservoir is long-lived, with a half-life of 44 months, even after seven years of anti-viral drug suppression (Siliciano et al., 2003). The existence and the persistence of reservoirs in HIV infection makes the total eradication of HIV impossible, even in patients treated with anti-viral drugs (see section 1. 14. 1).

HIV also infects macrophages. Macrophage-derived viruses have also been reported to be less infectious (Dornadula et al., 1999). Post-mortem analysis of kidney, brain and liver tissues, isolated from AIDS patients, revealed that HIV was mostly found in infiltrated macrophages (van't Wout et al., 1998). Only a small proportion of macrophages is infected by HIV, but the percentage increases in patients suffering from opportunistic infections. Macrophages represent a viral reservoir as they may be involved in the second phase of decay observed during anti-viral therapy (see section 1. 14. 1).

Monocytes have been reported to be infected by HIV *in vitro* at low levels and could represent a viral reservoir in patients treated with anti-viral drugs (Sonza et al., 2001). Blankson *et al*, however, suggested that monocytes have a turnover too rapid to be truly classified as a viral reservoir (Blankson et al., 2002).

A subset of NK cells express CD4 and CCR5 and can be efficiently infected by HIV (Fleuridor et al., 2003) (see section 1. 13. 2. 1). Viral DNA can be detected in CD4 positive NK cells from patients on anti-retroviral therapy and replication-competent virus can be isolated (Valentin et al., 2002). This NK population could also represent a reservoir *in vivo* (Blankson et al., 2002; Stebbing et al., 2004).

Infection of activated CD8 T cells has been reported in patients with AIDS (Livingstone et al., 1996) as they can express CD4 *in vitro* (Cochrane et al., 2004). But the frequency of infected memory CD8 T cells is very low in patients (Stebbing et

al., 2004) and the contribution of infected CD8 T cells as a reservoir needs further investigation (Blankson et al., 2002).

Lymph nodes, where resting memory CD4 T cells can be detected in the same frequency as in the peripheral blood, are considered to be viral reservoirs (Blankson et al., 2002). FDCs are also associated with HIV and are thought to be a source of infectious viruses (Kacani et al., 2001). The thymus is also infected by HIV (Dion et al., 2004) but has yet to be characterised as a reservoir (Blankson et al., 2002; Stebbing et al., 2004).

1.11 Genetic factors and progression of HIV/AIDS

In the absence of anti-viral therapy, 90% of the individuals who have contracted HIV have died (or will die) of AIDS (O'Brien and Nelson, 2004). All individuals from risk groups, however, are not all equal regarding infection dynamics or in their progression towards AIDS. The evolution of HIV in a patient is, of course, modulated by their immune response but genetic factors also have to be taken into account.

Mutations affecting the level of chemokine receptor expression can have a potent effect on HIV infection. Based on studies comparing either infected versus non-infected individuals or normal, slow or rapid progressors, gene polymorphisms have been associated with prevention of infection, delay or acceleration towards AIDS *in vivo* (Liu et al., 2004; O'Brien and Nelson, 2004).

As mentioned in 1. 6. 2. 2, individuals who present a genetic mutation within the CCR5 gene (usually referred as Δ32), in both alleles, resulting in a truncated CCR5 receptor that is not expressed at the cell surface, are highly resistant to HIV infection (Dean et al., 1996). The homozygous mutation is found in 1% of the Caucasian population (O'Brien and Nelson, 2004). These individuals, however, are not entirely resistant to infection, as they can be infected by HIV viruses causing a rapid depletion of the CD4 T cells in their blood (O'Brien et al., 1997; Theodorou et al., 1997). The heterozygous mutation confers partial protection and the progression towards AIDS is delayed (O'Brien and Nelson, 2004). Recently, a study by Gonzalez *et al* reported that the number of copies of *CCL3L1*, the gene encoding the most potent chemokine ligand of CCR5 (RANTES), directly influences the susceptibility to HIV infection and disease progression (Gonzalez et al., 2005). The *CCR2* V64I variant is associated with a delay in AIDS progression (Smith et al., 1997). This mutated CCR2 receptor

could associate with CXCR4, which could prevent the R5-X4 switch (O'Brien and Nelson, 2004).

DC-SIGN is a prime target in the transmission and the dissemination of HIV. DC-SIGN interacts with mannose residues by its transmembrane domain. The transmembrane domain has of repeats of twenty-three amino acids residues (Liu et al., 2004). Polymorphisms of *DC-SIGN* exist but are rare and are characterised by the number of repeat motifs within the transmembrane domain. A study has shown that some exposed seronegative individuals expressed DC-SIGN with a decreased number of repeats (six repeats against seven or eight repeats) whereas all infected individuals synthesised the consensus number of the DC-SIGN sequence (seven repeats). This may suggest that some degree of protection could be associated with a decreased number of repeats within DC-SIGN (Liu et al., 2004). Considering that this polymorphism was found only in less than 1% of the cohort studied, these findings will have to be confirmed. Martin *et al* recently reported that a single nucleotide polymorphism (SNP), present within the promoter of DC-SIGN was slightly over represented only in parenterally exposed caucasian Americans (Martin et al., 2004). An SNP found within the coding sequence of *APOBEC3G* gene could be associated with rapid progression towards AIDS (An et al., 2004).

Polymorphisms within promoters modulating the synthesis level of chemokines such as RANTES, SDF-1, the ligands for CCR5 and CXCR4 respectively (see table 1. 1), have been linked to protection or disease progression (Martin et al., 1998). The polymorphisms could result in higher or lower levels of chemokine synthesis.

SNPs within the genes for MCP1, MCP2 and eotaxin, have been found more frequently in highly-exposed HIV-1 seronegative caucasian Americans (Modi et al., 2003). These SNPs were found mostly within non-coding regions of the genes. The mechanism of protection against HIV infection is not clear, and could be associated with a modulation in the recruitment of target cells at the site of inflammation (Modi et al., 2003).

The alleles of the MHC class I genes, known as the human leukocyte antigen (HLA) loci and controlling the CD8 T cell response, play a major role in the control of HIV viraemia and in the prevention of infection. Not all HLA types handle HIV in the same manner (for a complete review, see Nolan et al., 2004).

The HLA-B57 haplotype has been strongly associated with delay in HIV progression (Altfeld et al., 2003). Altfeld *et al* reported that this haplotype in

individuals presenting symptoms of HIV acute infection was under-represented compared to the general population. They showed in acute infection that HLA-B57 responses were dominant and had a broader and stronger effect than the responses restricted to all the other haplotypes combined (Altfeld et al., 2003). The other haplotypes linked to delay in disease progression are HLA-B27 and HLA-B18 (Nolan et al., 2004). The haplotypes HLA-B35, HLA-B22 and HLA-B8 have been linked to a more rapid disease progression (Nolan et al., 2004).

NK cells are lymphoid cells, involved in the innate immune response against viruses by releasing granules such as perforin and granzymes into infected cells (see section 1. 11. 2). The genotype of the receptors at their surface, called KIR, has been involved in the modulation of killing HIV-infected-CD4 T cells. Flores-Villanueva *et al* showed that the HLA-Bw4 locus ligand for the NK inhibitory receptor, was linked to protection from AIDS (Flores-Villanueva et al., 2001).

Polymorphisms found within the mannan binding lectin (MBL), one of the three pathways of complement (see section 1. 13. 2. 3), was associated with a slower (Maas et al., 1998) or a more rapid progression (Garred et al., 1997) towards AIDS.

Individuals, who possess one or more of the genetic polymorphisms associated with delay in disease progression, will see their life expectancy increased without the need for anti-viral treatments. Whether these genetic advantages can allow the infected individuals to live with HIV asymptomatically, without any CD4 T cell decline until they die of non HIV-related illnesses is premature to assess as the epidemic started twenty years ago.

1.12 Innate non-immune responses to HIV

Until recently, the immune system was thought to be the most potent arm of defence against the spreading of incoming mammalian retroviruses. However, the picture of innate complex intracellular networks capable of blocking the life cycle of some retroviruses *in vitro* (targeting pre- and post-RT steps) is now emerging (Bieniasz, 2004; Schmitz et al., 2004). This newly investigated field might lead to a better understanding of the early events of the retrovirus life cycle following entry and, more importantly, to the generation of new classes of anti-HIV drugs.

1.12.1 Post-entry restrictions targeting CA

Fv1 can block the replication of MLV type N or B, between the RT and the integration viral steps, due to the presence of the *fv1* alleles in some strains of laboratory inbred mice (Lee and KewalRamani, 2004). Fv1 sensitivity is determined by MLV CA at position 110. Similarly, a broader, saturable pre-RT blocking agent, termed Ref 1, was described for MLV-N and equine infectious anaemia virus (EIAV) in human cell lines. A comparable pattern of pre-RT restriction, also mediated by saturable factor(s) was also discovered against HIV-1 and HIV-2, in several mammalian cells, particularly in those from monkeys (Bieniasz, 2004). The potency of this restriction, named Lv1 (for Lentivirus restriction 1) seems to be dependent on the presence of CypA in the target cells (Towers et al., 2003). Both Ref1 and Lv1 also act on CA of HIV or SIV.

The protein TRIM5α, originally isolated from a rhesus monkey cDNA library (Stremlau et al., 2004), seems to be at the centre of the blockage(s) mediated by Lv1 and by Ref1 (Hatziioannou et al., 2004; Keckesova et al., 2004; Yap et al., 2004). The physiologic role of TRIM5α is unknown but it contains a RING domain characteristic of proteins with ubiquitin ligase activity (Stremlau et al., 2004). It is likely that TRIM5α constitutes only a piece in the restriction jigsaw. TRIM1 was also found to have a moderate effect on Ref1 (Yap et al., 2004). Moreover, Owens *et al* have mapped amino acid residues in two distinct regions of CA involved in the Lv1 restriction (Owens et al., 2004), which suggests that several cellular proteins might orchestrate in synergy to conduct the Lv1 restriction. Whether the same cellular machinery or the same cellular mechanisms act for both Ref1 and Lv1 restrictions remain to be clarified (Bieniasz, 2004; Lee and KewalRamani, 2004).

In parallel, Vyakarnam *et al*, reported a post-entry block to R5-tropic viruses in memory CD4 T cells (Vyakarnam et al., 2001). Schmitz *et al* also described a post-RT restriction, named Lv2, for an HIV-2 molecular clone on several human cell lines, involving Env and CA (Schmitz et al., 2004). The cellular components and the mechanism mediating this new restriction remain to be identified; although new data seem to infer that the Lv2 restriction might be linked to an endocytic pathway (Marchant *et al*, submitted).

1.12.2 APOBEC3G and Vif

Lv1, Lv2 and Ref1 restrictions have been described to (probably partly) explain why some retroviruses cannot replicate in some cell lines expressing their specific receptors (and co-receptors) and to explore new avenues in the requirements for a successful retroviral zoonosis. Some cellular proteins exist to counteract the infection of retroviruses in their natural host. It is the case for APOBEC3G, previously termed CEM15 (Sheehy et al., 2002), that specifically hinders the successful generation of infectious viruses. APOBEC3G belongs to a multi-gene family of cytidine deaminases. In non-permissive cell lines, APOBEC3G is incorporated into virions (Harris et al., 2003) by interacting with Gag (Cen et al., 2004) and targets the RT step of the second generation of HIV-1 by altering the viral sequences during the synthesis of the minus DNA strand by changing the C into a U. The complementary viral strand will therefore incorporate an A, instead of originally a G (Harris et al., 2003; Mangeat et al., 2003; Zhang et al., 2003). The introduction of U into DNA can direct the nascent DNA to degradation by endonucleases. APOBEC3G also inhibits the completion of the HIV cycle by targeting preferentially C/TCC sequences, which will be mutated into a stop codon, leading to the generation of inactive truncated proteins. APOBEC3G has also been shown to deaminate endogenous retroviruses, preventing their retrotranspositions (Esnault et al., 2005).

HIV-1 inhibits this anti-viral activity by the direct interaction of APOBEC3G with one of its accessory proteins, Vif (Sheehy et al., 2002). In non-permissive cells, Vif prevents the encapsidation of APOBEC3G (Mariani et al., 2003) and mediates the binding of APOBEC3G with an ubiquitin ligase complex, which subsequently ubiquitinates APOBEC3G and targets it to degradation by the proteasome (Mehle et al., 2004). Despite the presence of Vif in virions, G-A substitutions are frequently high in the HIV genome. This observation has let to the hypothesis that the anti-viral action of APOBEC3G is not totally inhibited by Vif and the consequent diversity of the HIV genome might aid escape from the adaptive immune response (Bieniasz, 2004).

Other cytidine deaminases also have anti-viral editing properties such as APOBEC3F (Liddament et al., 2004) and, perhaps more interestingly, AID (activation-induced cytidine deaminase) that is involved in the Ab-gene diversification (Gu and Sundquist, 2003). Bishop *et al* reported that APOBEC1 and APOBEC3B could inhibit HIV even in the presence of Vif (Bishop et al., 2004).

These proteins have also been shown to play a similar role with other viruses such as MLV, EIAV (Gu and Sundquist, 2003) and hepatitis B (Bieniasz, 2004).

1.12.3 The *murr1* gene

The *murr1* gene, involved in copper regulation, inhibits HIV replication in resting CD4 T cells (Ganesh et al., 2003). Murr1 is involved in the degradation of NF-κB, as inhibition of *murr1* by specific short interfering RNA (siRNA) up-regulates NF-κB activity in laboratory cell lines and enhances HIV replication in primary resting CD4 T cells. NF-κB is a transcription factor involved in HIV transcription by binding to one of the transcription boxes of the 5'LTR (see section 1. 5. 4). Murr1 does not directly down-regulate the synthesis of NF-κB but inhibits the degradation of IκB. When NF-κB is associated with IκB, NF-κB is sequestered in the cytoplasm and cannot translocate into the nucleus. IκB is ubiquitinated and is degraded by the proteasome, NK-κB is released and can translocate into the nucleus. Murr1 acts (directly or indirectly) on the proteosome complex pathway and prevents the degradation of IκB (Ganesh et al., 2003). By having an effect on the proteasome pathway, the authors speculated that *murr1* could also affect the budding of HIV in resting CD4 T cells, providing a second block in the replication cycle.

1.13 Immune responses to HIV and immune escape

When infecting an individual, HIV will trigger a potent adaptive immune response, mediated by both CTLs and Abs (see sections 1. 13. 3. 2 and 1. 13. 3. 3). HIV can actively escape immune control, by exerting a direct stress on the immune system itself, such as down-regulating key markers involved in the coordination of a potent and focused neutralising response (such as MHC class I and II molecules) via the direct action of the viral proteins, such as Nef (Piguet et al., 2000; Stumptner-Cuvelette et al., 2001), Tat (Howcroft et al., 1993), Vpu (Kerkau et al., 1997) and Rev (Bobbitt et al., 2003). HIV can also escape from the pressure of the adaptive immune response by nucleotide substitutions within its genome, resulting in the generation of escape mutants, (generally) without a severe cost to viral fitness.

1.13.1 The viral proteins Nef, Tat and Vpu

Antigen presentation to CD8 T cells triggers cytolytic attack against cells infected by microbes - an essential mechanism in fighting viruses - as they are intracellular

parasites. HIV has developed strategies to inhibit MHC Class I presentation to reduce CTL attack. HIV down-regulates MHC Class I by interfering with the transcription of the genes, via Tat (Carroll et al., 1998), by degrading the synthesis of the proteins in the ER, via Vpu (Kerkau et al., 1997) and by acting on the presence of the MHC Class I molecules on the surface of the infected cell, via Nef (Piguet et al., 2000; Stumptner-Cuvelette et al., 2001).

1.13.1.1 MHC Class I and Tat

Tat stimulates the synthesis of full-length RNA molecules of HIV (see section 1. 5. 4) but also influences the expression of some cellular genes and appears to repress the transcription of the MHC Class I genes (Howcroft et al., 1993), by modulating the acetylation of TFIID, involved in the transcription of MHC Class II genes (Kamp et al., 2000). Tat also represses the activity of the β_2 microglobulin promoter (Carroll et al., 1998).

1.13.1.2 MHC Class I and Vpu

Vpu has a role in degrading CD4 in the ER and has been shown to induce the loss of newly synthesised endogenous MHC Class I α-chains in the ER (Kerkau et al., 1997).

1.13.1.3 Nef

Nef is a protein that is not required for the efficient replication of HIV *in vitro* in constitutively activated cells (Arora et al., 2002). Nef, however, seems to play a crucial role for replication *in vivo* as viral replication in primary non-activated cells is enhanced by Nef (Arora et al., 2002). It has also been associated with HIV pathogenesis, as *nef*-truncated genomes of HIV have been found in long-term non-progressors (LTNPs) (Learmont et al., 1999).

As well as down-regulating CD4 (see section 1. 6. 2. 1), Nef contributes to the immune escape by down regulating MHC Class I (Schwartz et al., 1996). The down-regulation of MHC class I by Nef, partially protects infected primary cells from the CD8 T cell attack (Collins et al., 1998), providing HIV with a powerful tool for escaping the immune system. Nef down-regulates the MHC class I molecules by interacting with their cytoplasmic tail (Williams et al., 2002) and targeting them towards the trans Golgi network (TGN) involving an interaction with PACS-1, a

protein able to form vesicles for trafficking (Piguet et al., 2000), and the recruitment of other proteins involved in endocytosis (Blagoveshchenskaya et al., 2002). Not all MHC allotypes are down-regulated: the HLA-A and HLA-B seem to be the only target of Nef-mediated down-regulation, the expression of HLA-C and HLA-E on the cell surface does not seem to be affected by Nef (Cohen et al., 1999; Williams et al., 2002). The residual expression of MHC class I molecules on the surface of infected cells could be a sufficient disguise to (partially) escape the attack from the NK cells (see section 1. 13. 2. 1).

Nef also impairs the MHC Class II presentation (Stumptner-Cuvelette et al., 2001). Unlike MHC class I, MHC class II is only present on APCs, such as macrophages, B lymphocytes and DCs. Peptide presentation, within the context of MHC Class II, is therefore altered but Nef also up-regulates immature forms of MHC Class II (Stumptner-Cuvelette et al., 2001).

Nef has also been shown to down-regulate other surface molecules such as CD1a, on immature DCs (Shinya et al., 2004), involved in antigen presentation to CD8 T cells and NKs, and CD28, critical for the induction of CTL responses (Swigut et al., 2001).

1.13.2 The innate immune response

Despite the active involvement of HIV at escaping the immune system, barriers do exist *in vivo* against HIV infection. One of the hurdles that the virus can encounter is the innate immune response, which consists mainly of NK cells, γδ T cells and complement.

1.13.2.1 Natural killer cells

NK cells are large granular lymphocytes, which play an important role in the innate and adaptive immunity. They represent a subset of T cells expressing a very restricted, unmodified T cell receptor (TCR) specific for glycolipid antigens (Unutmaz, 2003). The TCR on NK cells recognizes the non-polymorphic MHC class I-like molecule CD1d, which presents non-peptide antigens (Unutmaz, 2003).

When activated, NK cells synthesise large amounts of cytokines (such as IFNγ, TNFα, IL1, IL5, IL8, IL10) and chemokines (MIP1α, MIP1β, RANTES). Hence, they can regulate immune function and haematopoiesis. They also destroy infected cells by releasing cytolytic substances such as perforin, serine-like proteases, granzyme A and

B and chondroitin sulphate proteoglycans (Jacobs et al., 2005). The action of NK cells can be triggered by IgG bound to the FcγRIII (CD16), a process called antibody-dependent cellular cytotoxicity (ADCC), in the context of adaptive immunity, or by the down-regulation of the MHC class I on APC, in the context of the innate immunity. NK cells are regulated by a complex network of stimulatory and inhibitory receptors, present on their surface.

The ability of NK cells to control HIV replication *in vivo* is unclear. Low NK cell numbers are associated with a more rapid disease progression (Jacobs et al., 2005; Kottilil et al., 2003) and some polymorphisms within some of the receptors have been shown to have an impact on the progression towards AIDS (see section 1. 11). NK cells could also represent a barrier against HIV infection. Some highly exposed HIV-seronegative drug users have NK cells with enhanced chemokine level and cytolytic properties compared to control seropositive drug users and seronegative individuals (Scott-Algara et al., 2003). These findings suggest that NK cells can be successful at fighting HIV.

Some other studies, however, suggest that HIV inhibits the anti-viral actions of NK cells. NK cells can be divided into two subsets: CD4 negative and CD4 positive cells. The CD4 positive subset also expresses large amounts of CCR5 and can be efficiently infected by R5-tropic strains of HIV-1 (Fleuridor et al., 2003; Motsinger et al., 2002; Valentin et al., 2002), which leads to the selective depletion of NK cells in infected individuals in the course of infection and seriously impairs innate immunity against HIV (Motsinger et al., 2002).

When infected with HIV, NK cells seem to have their cytolytic properties impaired and are unable to lyse MHC class I down-regulated HIV-infected CD4 T cells or DCs (Bonaparte and Barker, 2003; Tasca et al., 2003). This inhibition of the cytolytic properties could be mediated by Tat (Poggi et al., 2002; Tasca et al., 2003), which may inhibit a kinase involved in calcium flux, resulting in the blocking of granzymes release (Poggi et al., 2002). Tat is unlikely to be the only agent inhibiting the release of cytolytic substances, as the *in vivo* administration of IFNα can restore some cytolytic activity in HIV-infected NK (Portales et al., 2003).

Despite their inability to lyse MHC Class I down-regulated HIV-infected cells, NK cells could control HIV viraemia, via the release of CC chemokines. HIV-infected NK cells have a direct effect on viral replication by releasing RANTES, MIP1-α and

MIP1-β that bind to CD4 T cells from HIV-infected individuals *in vitro* and *ex vivo* (Fehniger et al., 1998; Kottilil et al., 2003).

ADCC is mediated when IgG binds to CD16 on NK cells. Forthal *et al* reported that HIV-negative NK cells could inhibit autologous and heterologous HIV strains in association with non-NAbs, during the episode of acute infection *in vitro* (Forthal et al., 2001). Moreover, ADCC and viral load could be correlated in some studies (Ahmad et al., 2001; Forthal et al., 2001), and ADCC was found to be prognostic in HIV infection (Ahmad et al., 2001).

1.13.2.2 γδ T cells

A small proportion of T cells express the γδ TCR receptor instead of the αβ TCR receptor. This particular subset represents a major T cell population in the skin, the intestinal epithelium and the pulmonary epithelium. Most of the γδ T cells consist of the Vδ2 chain in combination with the Vγ9 segment. A minor subset of γδ T cells expresses the Vδ1 chain (Boullier et al., 1997). Unlike αβ T cells, they do not appear to circulate extensively and remain fixed in the tissues. The ligand recognised by γδ T cells is unknown but these cells do not seem to be restricted by the MHC molecules and may bind to antigens like Ig. They might eliminate damaged cells as well as intruding pathogens in the mucosa (Janeway, 2001).

The γδ T cell subset is elevated in HIV-infected individuals and mainly consists of Vδ1, both in peripheral blood and in mucosa (Boullier et al., 1995; Poles et al., 2003). The Vδ1 subset, isolated from HIV-infected patients, can also produce anti-viral cytokines such as TNFα and IFNγ (Boullier et al., 1997; Lichterfeld et al., 2004). Vδ2 T cells from uninfected individuals, can also secrete MIP-1α, MIP-1β, RANTES and SDF-1 and can inhibit HIV infection *in vitro* (Poccia et al., 1999). But this subset might become anergic in HIV-infected individuals (Poccia et al., 1996). Macaques immunised with recombinant SIV gp120 and p27 then anally infected with SIV, however, have an increase in the γδ T cell subset in the rectal mucosa. Furthermore, γδ T cells, from immunised monkeys, inhibit HIV infection by secretion of CCR5 chemokines (Lehner et al., 2000).

1.13.2.3 Complement

Complement consists of more than 30 serum proteins organised in three cascades: the classical pathway, the alternative pathway and the MBL (for details, see the

introduction of Chapter 4). The triggering of the cascades leads to C3 deposition on pathogens, a process called opsonisation, and the generation of the membrane attack complex (MAC). By-products of the cascades, such as C3a, C4a and C5a, are called anaphylatoxins and are involved in inflammation, which results in the increase of smooth muscle contraction and vascular permeability. Complement also attracts phagocytic cells and neutrophils at the site of inflammation via interactions between anaphylatoxins and complement receptors CR1, CR2 and CR3. Complement is also involved in the clearance of immune complexes (Nash et al., 2001). Complement proteins are mainly synthesised by hepatocytes. Extra-hepatic sites of complement protein synthesis have been described, such as cells of the monocytic/macrophages lineage, fibroblasts, adipocytes, epithelial and endothelial cells. The brain is the second most important site of complement protein production (Stoiber et al., 2003).

HIV activates all three complement pathways. The classical pathway can be triggered by HIV (Senaldi et al., 1989) in the presence (Spear et al., 1993b) or the absence of Abs by the direct binding of C1q on gp41 (Ebenbichler et al., 1991; Susal et al., 1994). MBL is also activated by the presence of mannose residues on gp120 (Haurum et al., 1993). The alternative pathway has been also shown to be activated (Stoiber et al., 2001; Tacnet-Delorme et al., 1999).

Complement could have a dual role *in vivo* by inhibiting both HIV replication and by enhancing viral spread. Schmitz *et al* have shown that complement consumption, by cobra venom, in monkeys lead to an increase in viral load during primary viraemia (Schmitz et al., 1999b). Moreover, complement can enhance the protection mediated by the post-exposure administration of a NAb in mice (Gauduin et al., 1998b).

Complement-mediated enhancement of opsonised HIV has been reported *in vitro* in DCs via CR3 (Bajtay et al., 2004) as well as in epithelial cells (Bouhlal et al., 2002). Complement could also mediate the binding of HIV to erythrocytes (Horakova et al., 2004), playing a role in HIV dissemination. Complement also mediates binding of virions to B cells via CR2, which then may transmit HIV to CD4 T cells (Jakubik et al., 2000; Moir et al., 2000). FDCs, which constitute one of the main sources of infectious viruses during chronic infection (Schacker et al., 2000; Stoiber et al., 2001), can trap HIV virions, mainly via interactions between C3d and CR2 (Kacani et al., 2001).

Complement protein C3 synthesis is up-regulated in HIV-infected astrocytes (Speth et al., 2001) and could represent the source of increased levels of this protein in

the cerebrospinal fluid of HIV-infected patients (Stoiber et al., 2001). Complement, therefore, could be implicated in the neurodegeneration observed in some HIV-infected patients (Speth et al., 2001; Stoiber et al., 2001).

1.13.3 The adaptive immune response

1.13.3.1 The CD4 T cell response

CD4 T cells have a pivotal role in the induction of a specific adaptive immune response. They function as T Helper (TH) cells that recognise antigens in an MHC class II context, present on APCs, and become effector cells that secrete various cytokines important in the stimulation of CD8 T cells, B cells and macrophages. TH cells can be differentiated into TH1 cells or TH2 cells, based on the panel of secreted cytokines. TH1 cells secrete IL2, IFN-γ, TNF-β, GM-CSF and IL3 and stimulate a CTL response. TH2 cells secrete IL3, IL4, IL5, IL10 and IL13 and stimulate an Ab response (Janeway, 2001).

A specific CD4 response to HIV can be detected in acute infection (Pitcher et al., 1999). This CD4 response inhibits HIV *in vitro* by producing MIP-1α, MIP-1β and RANTES (Abdelwahab et al., 2003). This response, however, could be rapidly impaired as HIV preferentially infects HIV-specific CD4 T cells (Douek et al., 2002).

A robust CD4 response has been detected in LTNPs (Pitcher et al., 1999), and HIV-specific CD4 responses correlate inversely with the viral load (Kalams and Walker, 1998). This suggests that a functional CD4 response could be pivotal in the control of HIV *in vivo*. Pitcher *et al*, however, have shown that the CD4 T cell response against HIV could be detected in normal progressors (NPs), by IFN-γ production, which suggests that the protective role of CD4 T cells against HIV could be indirect and could influence the CTL or the Ab response (Pitcher et al., 1999).

1.13.3.2 The CTL response

CTLs are generated by the immune activation of cytotoxic T cells (T_c cells). They are MHC Class I restricted and, therefore, can recognise and eliminate almost any infected cell accessible to them. CTL proliferation results from the stimulation with IL2 synthesised by the CD4 positive TH1 cells that have been stimulated by APCs in a MHC Class II context. Contact between CTLs and cell targets are mediated by an interaction between the TCR on the CD8 T cells and the MHC Class I on the cell

targets. Following this initial contact, the integrin receptor LFA-1 on the CTLs interacts with adhesion molecules ICAM on the target cells. The cell-cell contact triggers the release of pore-forming proteins called perforin and serine proteases called granzymes into the cytoplasm of the target cell, which triggers DNA fragmentation. CTL cells express Fas ligand on their surface, and an interaction with Fas leads to the apoptosis of the target cell. After the death of the infected cells, the CTLs are released and can bind other target cells. CTLs can also control infections by releasing anti-viral cytokines such as IFNγ and TNFα (Janeway, 2001).

Anti-HIV CTLs are detected in almost all patients (Betts et al., 2001). The main CTL targets are mostly peptidic sequences from proteins Gag, Pol and Env, but CTLs also target Vif, Nef, Rev, Vpu and Tat (Addo et al., 2002; Altfeld et al., 2001; Borrow et al., 1994). The breadth of the CTL response varies among individuals and a broad and potent anti-HIV CD8 T cell activity is correlated with a low viral load and a slower progression towards AIDS (Borrow et al., 1994; Chouquet et al., 2002).

Anti-HIV CTLs can be detected in acute infection in humans (Borrow et al., 1994; Cao et al., 2003; Safrit et al., 1994) and in SIV_{mac}-infected macaques (Mothe et al., 2002). The CD8 T cell response against HIV thought to be the main immune effector in the viral decline observed during acute infection, preceding the detection of NAbs (Borrow et al., 1994; Cao et al., 2003). In macaques, the depletion of CD8 T cells leads to an uncontrolled viral load and a faster progression towards AIDS (Schmitz et al., 1999a). Moreover, an inverse correlation can be measured between plasma viral load and CTL activity, which suggests that CTLs play an important role in the containment of HIV *in vivo* (Ogg et al., 1998).

Anti-HIV CTL responses can be also detected in all stages of infection, even in the AIDS phase. In acute infection, up to 10% of the circulating CTLs are specific to HIV with the percentage decreasing to 1-2% in the chronic stage (Islam et al., 2001; McMichael and Rowland-Jones, 2001). The nature of the CTL response can differ in the acute and the chronic infection. Goulder *et al* showed, in a cohort of Caucasian patients, that the CTL response against a Gag-derived peptide was detected in almost all studied patients during the chronic phase of infection but was absent from the overall CTL response in the acute phase, which was directed against other peptides from other HIV proteins (Goulder et al., 2001a). The same observation was reported by Mothé *et al*, in macaques, where the CTL response was directed against Tat, Rev

and Nef in acute infection and against Gag, Pol and Env during the chronic stage (Mothe et al., 2002).

Although it is clear that CTLs are important to control HIV *in vivo*, the mechanism(s) responsible remain to be determined (McMichael and Rowland-Jones, 2001). The cytolytic properties of CD8 T cells, in peripheral blood as well as in lymphoid tissue, seem to be impaired in the acute and chronic phases of infection (Appay et al., 2000; Dagarag et al., 2003; Lieberman et al., 2001) due to low levels of perforin (Haridas et al., 2003; McMichael and Rowland-Jones, 2001). The circulating anti-HIV CTLs are $CD27^+$, are $CD45RA^-$ and therefore present the phenotype of immature CD8 T cells. The maturation of memory CD8 T cells specific to HIV is skewed (Champagne et al., 2001) and they are unable to lyse HIV-infected cells (Haridas et al., 2003; McMichael and Rowland-Jones, 2001).

CTLs could inhibit HIV by the release of cytokines such as IFNγ, and TNFβ (Appay et al., 2000; Jassoy et al., 1993), MIP1-α, MIP-1β (McMichael and Rowland-Jones, 2001), as well as by the production of RANTES, which competes with HIV for CCR5 binding and down-regulates CCR5 (McMichael and Rowland-Jones, 2001). RANTES also up-regulates Fas ligand expression on CD8 T cells and, thus, can enhance killing of HIV-infected cells (Hadida et al., 1999). CD8 T cells can inhibit HIV replication by secreting the CD8 anti-viral factor (CAF), that regulates the 5'LTR of HIV integrated genome by a Stat I-dependent mechanism and prevents the initiation of transcription (Chang et al., 2002; McMichael and Rowland-Jones, 2001).

Despite a strong induction of the CD8 T cell response in HIV infection, CTLs fail to eradicate infection. HIV and SIV can escape the CTL response by changing amino acid residues within peptides recognised by the CD8 T cells, during acute infection (Cao et al., 2003; O'Connor et al., 2002; Price et al., 1997), as one amino acid change within a peptide can lead to a complete loss of recognition by the CD8 T cells specific to the peptide (McMichael and Rowland-Jones, 2001). Draenert *et al* have shown that mutations flanking the Gag peptide recognised by CD8 T cells, in the HLA-B57 context, resulted in altered antigen presentation thus favouring CTL escape by HIV (Draenert et al., 2004). Furthermore, Bobbit *et al* have shown that some Rev mutants could decrease the export of Gag RNA from the nucleus, which would result in a reduction of Gag protein synthesis and and a reduction in the sensitivity against anti-Gag CTLs (Bobbitt et al., 2003). Moreover, they found similar Rev mutants in individuals who presented an HIV population that had escaped the CTL response,

suggesting that Rev could partially inhibit the anti-HIV CD8 T cell response *in vivo* (Bobbitt et al., 2003). The presence of a broad protective anti-HIV CTL response does not protect from superinfection (Altfeld et al., 2002).

CTLs need to be primed by CD4 T cells to undergo maturation. CD4 T cells are infected by HIV and their number drastically decreases as infection progresses. Therefore, in the context of HIV infection, the CTL response might be less and less efficient with time, as fewer and fewer CD4 T cells stimulate CD8 T cells (McMichael and Rowland-Jones, 2001). Nef and Vpu also down-regulate CD4, contributing to an altered collaboration between CD4 TH1 and CD8 T cells. They also initiate the degradation of MHC Class I, crucial in the initiation of CTLs.

Although CD8 T cells have an important role in controlling HIV infection, they also might favour the CD4 T cell depletion in HIV-infected individuals and could play a role in aggravating the disease (Douek et al., 2003). Moreover, Silvestri *et al* have shown that SIV infection in sooty mangabeys, its natural host, is characterised by an attenuated CD8 T cell response (Silvestri et al., 2003).

1.13.3.3 The humoral response

The first Ab response detected during primary infection is targeted against Gag^{p24}, the most abundant HIV protein. Abs against all other HIV proteins are detected later (Levy, 1998). The purpose of an Ab response *in vivo* is to prevent further dissemination of an infectious agent. In the case of HIV, protective Abs will interfere with receptor binding and fusion with the cell membrane, both mechanisms being mediated by gp120 and gp41. These Abs are called NAbs and are directed against gp120 and gp41. The paragraph 1. 13. 3. 3 will only describe NAbs, as they have been extensively studied.

1.13.3.3.1 IgA

IgA represent the predominant Ig in external secretions such as saliva, tears, breast milk and mucus of the bronchial, genito-urinary and digestive tracts. Specific IgA targeted against CCR5, capable of neutralising HIV-1 and HIV-2 *in vitro*, have been found in saliva and genital fluids from HIV-2 infected individuals (Lizeng et al., 2004) or from highly-exposed seronegative individuals (Barassi et al., 2004; Lizeng et al., 2004). IgA isotypes of 2F5 (see section 1. 13. 3. 3. 7) could also potently

neutralise PIs *in vitro* (Wolbank et al., 2003). IgA could therefore play a role in preventing infection in some exposed individuals.

1.13.3.3.2 Evolution of the neutralising anti-HIV response in infected individuals

Although the anti-gp120 IgG response develops during acute infection, between four and twenty-five days post onset of symptoms (POS) (Aasa-Chapman et al., 2004; Moore et al., 1994a), NAbs, that give 90% neutralisation, are usually detected between two and nine months of infection against autologous isolates (Aasa-Chapman et al., 2004; Moog et al., 1997; Pellegrin et al., 1996; Richman et al., 2003). The presence of an earlier NAb response has been occasionally reported (Lewis et al., 1998). Neutralisation may depend on the cell target as Ruppach *et al* have reported early autologous virus neutralisation in macrophages but not in lymphocytes (Ruppach et al., 2000). Neutralisation against heterologous isolates arises later (Aasa-Chapman et al., 2004; Moog et al., 1997; Pellegrin et al., 1996; Richman et al., 2003), is initially restricted to a few isolates, then broadens (McKnight et al., 1992) and is still detectable in sera from AIDS patients (Groopman et al., 1987; Weiss et al., 1985). Much of the Ab response, in humans and chimpanzees, is elicited against discontinuous, conformation-sensitive epitopes (Moore and Ho, 1993; Steimer et al., 1991), as the Ab binding is lost when sera are tested against denatured monomeric gp120 (Moore and Ho, 1993). The neutralisation potency of an Ab cannot be predicted by its binding property to monomeric gp120 (Moore et al., 1995). The first Abs that are detected during primary infection are mainly directed against the CD4BS and, later the V3 loop (Moore et al., 1994a).

1.13.3.3.3 Role of neutralising antibodies *in vivo*

The protective role of CD8 T cells against SHIV has been shown *in vivo* (Schmitz et al., 1999a), but the involvement of NAbs in the control of viraemia *in vivo* has yet to be fully demonstrated. Indirect evidence that NAbs can contribute to the containment of viral replication *in vivo* is, however, mounting.

In longitudinal studies, HIV variants from late time points are more resistant to neutralisation than viruses isolated from earlier time points. This suggests that NAbs are the selection pressure for the emergence of these variants (Albert et al., 1990; Richman et al., 2003). The neutralisation-sensitive TCLA IIIB isolate adopts a resistant phenotype to sCD4 and MAbs in an infected patient (Beaumont et al., 2001).

adults and children LTNPs have sera with higher neutralising titres and a broader cross-neutralisation pattern (Geffin et al., 2003; Pilgrim et al., 1997). Binley *et al* reported the existence of a strong NAb response in a treatment-naïve patient infected for at least sixteen years with undetectable viral loads and a weak CTL response (Binley et al., 1998). Moreover, Igarashi *et al* have shown that the half-life of SHIV was drastically reduced *in vivo* from 20 min (13-26 min) to 5.5 min (3.9-7.2 min) in monkeys that had previously developed a NAb response against the virus (Igarashi et al., 1999). Overall, these observations suggest that NAbs play a role in controlling viraemia and could delay the progression towards AIDS. However, Ab-mediated neutralisation is usually tested in the absence of complement and therefore, the protective role of non-NAbs in association with complement cannot be ruled out (see Chapters 3 and 4).

1.13.3.3.4 Neutralising epitopes

Numerous neutralising epitopes have been reported for HIV-1: on the CD4BS (Burton et al., 1991; McKeating et al., 1993; Trkola et al., 1995), the chemokine receptor binding site, that is CD4 binding-induced (Fouts et al., 2002; Moulard et al., 2002; Thali et al., 1993; Wyatt et al., 1995), motifs on the V3 loop (Gorny et al., 1992; Javaherian et al., 1989), on the V1/V2 structure (Fung et al., 1992; Gorny et al., 1994; McKeating et al., 1993; Warrier et al., 1994) and on the carbohydrate motif recognised by 2G12 (Trkola et al., 1996b). Neutralising epitopes are also present on the ectodomain of gp41 (Muster et al., 1993; Zwick et al., 2001b). Despite the description of various neutralising MAbs, PIs are weakly neutralised *in vitro* (Moore et al., 1995).

1.13.3.3.5 TCLA versus PI neutralisation sensitivity

It is now clear that the neutralisation sensitivity differs between TCLA and PI isolates. PIs are more refractory to sCD4 inactivation (Daar et al., 1990) and NAb-mediated neutralisation than TCLA viruses (Gauduin et al., 1996; Parren et al., 1998; Wu et al., 1996). Therefore, neutralisation studies using TCLA viruses do not reflect the neutralising potencies of Abs *in vivo*. The increased neutralisation sensitivity of TCLAs cannot be explained by the co-receptor usage (Montefiori et al., 1998; Trkola et al., 1998). The growth in immortalised cell lines could lead to the selection of viral genotypes with, possibly, altered Env conformations that allow more efficient entry

but at the expense of exposure to NAb neutralisation (Poignard et al., 1996b). PIs exhibit a two- to three-fold higher level of gp120 than TCLAs. PIs may have a higher Env spike density, which would have an influence on the inactivation by sCD4 and perhaps by NAbs (O'Brien et al., 1994). Karlsson *et al*, however, found no correlation between the amount of gp120 carried by various viruses from several clades and neutralisation (Karlsson et al., 1996).

1.13.3.3.6 Neutralising antibody escape

HIV infection can occur with a cell-free virus but also by cell-cell contact, using a virological synapse (Jolly et al., 2004), which could partially protect the virus from NAbs and contributing to escape mechanisms (Jolly and Sattentau, 2004).

HIV can also support amino acid variation, particularly within Env, due to the lack of proof-reading activity in the RT and, therefore, can adapt to the pressure exerted by the immune response. Poignard *et al* reported that a large amount of monoclonal NAbs did not control replication of two PIs (SF162 and JRFL) in SCID-huPBL mice, as escape mutants arose in the targeted epitope within a week after HIV infection (Poignard et al., 1999). Moreover, mutations within a particular region of Env can drastically influence the overall neutralisation sensitivity of a virus (Bouma et al., 2003; Park et al., 1998). Mutations within gp41 can also enhance or decrease the neutralising properties of NAbs directed against gp120 (Kalia et al., 2005). Furthermore, single point mutations can also modify the entire neutralisation profile of a virus (Watkins et al., 1996).

Neutralisation resistance could be due to Env conformations burying and hiding the neutralising epitopes (Kwong et al., 2002), as anti-co-receptor Fab fragments (such as 17b and X5) are more potent at neutralising HIV than whole IgG molecules (Darbha et al., 2004; Labrijn et al., 2003). Variable loops are the most divergent parts of gp120 and their presence could influence the neutralisation potency of NAbs. Deletion of the V1/V2 loop in HxB2 makes the virus more sensitive to NAbs (Cao et al., 1997). Similarly, immunisation of rabbits with the oligomeric SF162 Env deleted in the V2 region leads to the generation of a greater and a broader NAb response (Barnett et al., 2001), which also suggests that the V2 loop can protect conserved neutralising epitopes, such as the CD4BS and the V3 loop from NAbs (Krachmarov et al., 2005),. MAbs isolated from rats immunised with IIIB deleted for the V1, V2 and V3 loops neutralise a broader range of clade B and non-clade B isolates (Jeffs et al.,

2002). Moreover, the V1/V2 loop exchange between SF162 and JRFL leads to the swap of neutralisation sensitivity between these two isolates (Pinter et al., 2004; Stamatatos and Cheng-Mayer, 1998).

Buried neutralising epitopes within gp120 can create an unfavourable energy requirement for the NAbs to bind Env (Kwong et al., 2002). The gp41-gp120 interaction is weak, and monomeric gp120 is the predominant antigen for B cell stimulation. Hence, the majority of Abs present in patients' sera will bind preferentially to monomeric gp120 rather than trimeric spikes on infectious viruses (Poignard et al., 1996b).

gp120 is organised into a neutralising face, a non-neutralising face and an immunologically silent face protected by a glycan shield. Both of the latter faces are exposed on the oligomeric spike protecting the neutralising face (Wyatt and Sodroski, 1998). The removal of glycans within the V1/V2 structure (Cole et al., 2004; Johnson et al., 2003; Quinones-Kochs et al., 2002) or within the V3 loop (Koch et al., 2003; McCaffrey et al., 2004; Polzer et al., 2002) increases the neutralisation of some viruses. Glycosylation could represent the main Ab escape mechanism *in vivo*. A study by Wei *et al* showed that Env escape mutants had changes primarily in their N-glycosylation patterns (Wei et al., 2003).

The acquisition of CD4 as a receptor has perhaps allowed HIV to increase its resistance against NAbs. Thomas *et al* have shown that HIV-2 CD4-independent isolates were much more sensitive than CD4-dependent HIV-2 strains and that the neutralisation sensitivity was epitope-independent (Thomas et al., 2003). Similarly, the artificially engineered CD4-independent strain of HIV-1 IIIBx is more sensitive to neutralisation by sera than its CD4-dependent counterpart (Edwards et al., 2001).

Although HIV can escape Ab-mediated neutralisation, a study by Derdeyn *et al* showed that viruses transmitted to a new person were sensitive to neutralisation by the sera of the transmitting partner. This may suggest that NAbs would exert a fitness cost on the virus (Derdeyn et al., 2004). Eliciting a sterilising Ab response by an immunogen will be a difficult challenge. However, eliciting a NAb response that will force the virus to escape at severe fitness cost might be sufficient to delay disease progression.

1.13.3.3.7 Cross-neutralising human antibodies

Although it is feasible to elicit a NAb response, it is usually isolate-specific and weakly cross-neutralising (Weiss et al., 1985). Nevertheless, some cross-clade neutralising MAbs (Binley et al., 2004), mapping to both gp120 and gp41, have been isolated from LNTPs.

The NAb IgG1 b12 (refered as b12 for the rest of this thesis) was isolated by screening by phage display an Ab library amplified from the bone marrow of an asymptomatic HIV-1 patient. It overlaps the CD4BS of HIV-1 and neutralises HIV-1 PIs from various clades (see introduction chapter 5 for details). The Ab IgG1 2G12 (refered as 2G12 for the rest of this thesis) binds to a discontinuous, purely mannose $\alpha1\rightarrow\alpha2$-dependent epitope, on the outer face of gp120, defined by the C2, C3, C4 domains and the V4 loop (Sanders et al., 2002; Scanlan et al., 2002; Trkola et al., 1996b). The Fab X5 was isolated by screening by phage display an Ab library from a seropositive patient with a high titre NAb serum, using a gp120-CD4-CCR5 complex (Moulard et al., 2002). It overlaps the binding site of 17b and binds to a highly conserved CD4-induced epitope, close to the CD4BS (Darbha et al., 2004; Moulard et al., 2002). It can neutralise cell-free virus entry as well as cell-cell fusion (Moulard et al., 2002).

The IgG1 2F5 and 4E10 NAbs (refered to as 2F5 and 4E10 for the rest of this thesis) bind to distinct epitopes on gp41 (Purtscher et al., 1996). The binding of 2F5 is correlated with the presence of the sequence ELDKWA, which is highly conserved amongst isolates from different clades. The epitope might also be conformation-dependent (Parker et al., 2001). 2F5 and 4E10 inhibit the fusion process of HIV with the cell membrane and their neutralisation potency is enhanced in a hydrophobic environment, which might present the neutralising epitope in the right conformation (Ofek et al., 2004). The actual neutralisation mechanisms are not fully understood. 2F5 seems to bind to Env in its native conformation and not to the pre-hairpin intermediate, prior to CD4 binding (Barbato et al., 2003).

It is interesting to note that 2F5, X5 and b12 have longer than average CDR H3 regions; 18 amino acid residues for b12 (Saphire et al., 2001b) and 22 amino acid residues for 2F5 and X5 (Ofek et al., 2004). The crystallisation of X5 has revealed that the CDR H3 region adopts a similar prominent structure to b12 (Darbha et al., 2004). Longer CDR H3 regions might result in more flexible loops that allow Abs to

bind tightly to structures, buried within gp120, that IgG with average CDR H3 regions fail to reach (Saphire et al., 2001b).

When these NAbs are used as cocktails, HIV neutralisation is enhanced *in vitro* (Binley et al., 2004; Zwick et al., 2001b) and can block virus transmission from DCs to T cells (Frankel et al., 1998). Although some promising strategies are currently designed to specifically elicit b12 as the single anti-CD4BS Ab (Pantophlet et al., 2003b; Pantophlet et al., 2004), it will be challenging to elaborate an immunogen that will exclusively trigger the synthesis of these MAbs. They, however, can be used in topical applications for preventing HIV infection (see section 1. 14. 5).

1.13.3.3.8 Mechanisms of neutralisation

Non-neutralising and NAbs cannot be distinguished in their ability to bind monomeric gp120 (Moore et al., 1995). However, it seems that only NAbs have the ability to bind oligomeric gp120 on virions with sufficient affinity (Poignard et al., 2003). Therefore, NAbs could neutralise HIV infectious virions irrespective of the nature of their epitopes by entirely coating the virion (Poignard et al., 2003). Studies, however, have revealed that some NAbs neutralise HIV by interacting in an epitope-specific manner. Neutralising anti-CD4 Abs against TCLA viruses favour the dissociation of gp120 from the virions, leading to the generation of non-infectious viruses (Poignard et al., 1996a). b12 competes with CD4 on the gp120 CD4BS for interacting with the crucial phenyalanine residue at the bottom of the CD4BS pocket of gp120 (Saphire et al., 2001b). Choe *et al* showed that some anti-CD4 induced Abs neutralised HIV by successfully competing with the sulphated regions of CCR5 involved in interactions with the virus (Farzan et al., 1998) via sulphate motifs on their CDR H3 regions (Choe et al., 2003). The crystal structure of 2G12 reveals a VH domain exchange. Hence, VH interacts with VL' and VH' interacts with VL. This domain exchange forms an extensive multivalent binding surface composed of the two conventional sites (VH/VL' and VH'/VL) and a homodimeric VH/VH' interface (Calarese et al., 2003).

1.13.3.3.9 Antibody-dependent enhancement

HIV is capable of escaping Ab neutralisation but also could use Abs to its own advantage to enhance its infectivity. Antibody-dependent enhancement (ADE) of HIV has been reported *in vitro* (Sullivan, 2001) but the *in vivo* relevance has yet to be

demonstrated. A recent report has shown that infected macaques, previously immunised with an attenuated strain of SIV, progressed to AIDS more rapidly than non-immunised animals. The sera from the immunised animals showed enhancement of HIV infection on MT2 cells (Staprans et al., 2004). The mechanisms for ADE are still unclear. The FcγRIII receptor could be involved in mediating HIV enhancement using sera from AIDS patients (Homsy et al., 1989). However, neutralisation studies using early non-neutralising sera, on macrophages which synthesise high levels of FcγRIII receptors, did not show enhancement of infection but showed increased neutralisation compared to PBMCs (Ruppart *et al*, J Virol, 2000). The binding of some Abs could also induce conformational changes in gp120 and activate the binding process (Sullivan, 2001).

1.14 Anti-HIV treatments

1.14.1 HAART

The currently available anti-viral drugs HIV target RT and PR. Nucleoside analogues lack 3'OH extremities and prematurely abort viral DNA elongation. Targets of RT include non-nucleoside reverse transcriptase inhibitors (NNRTIs) that bind at or close to the RT catalytic domain and block enzyme activity. The anti-PR drugs mimic viral peptide substrates but have non-cleavable structures that block the catalytic activity of the PR.

Highly active anti-retroviral therapy (HAART) includes several drugs. This treatment can reduce viral replication to undetectable RNA levels in plasma. HAART, however, does not eliminate viral reservoirs, as the treatment interruption is followed by viral rebound (Jubault et al., 1998). Furthermore, episomal intermediates can be detected in PBMCs from treated patients who show undetectable viral load in their plasma (Sharkey et al., 2000). Siliciano *et al* have estimated that over 70 years of consecutive therapy would be required to eradicate the memory CD4 T cell reservoir alone (Siliciano et al., 2003). The penetration rate of anti-viral drugs is weaker in semen than in blood (Taylor et al., 1999), which suggests that drug-resistant viruses might appear in the genital tract, independently of viral evolution in the peripheral blood. Drug-resistant viruses can be successfully transmitted to new individuals (Hecht et al., 1998), which could result in the emergence of the circulation of these variants in a population (Hecht et al., 1998).

The HAART regimen has serious side effects and can sometimes be lethal to some individuals who develop acute liver or renal failure months after the commencement of therapy (Clark et al., 2002; Murphy et al., 2003; Scherpbier et al., 2003) Structured treatment interruptions (STIs) could overcome this problem. STIs consist of alternating periods of medication and non-medication. The STI approach can lead to better control SIV and HIV replication, even months after the interruption of the treatment (Lori et al., 2000a; Lori et al., 2000b). This which could be due to the induction of a more protective immune response.

Despite the inability of HAART treatment to totally eradicate HIV infection, as well as the serious side effects, the wide prescription of these drugs to a population could seriously decrease HIV incidence and contribute to the control of the epidemic (Blower et al., 2000).

1.14.2 sCD4 and sCD4-IgG

sCD4 has been shown to inactivate TCLA isolates of HIV *in vitro* (Deen et al., 1988; Traunecker et al., 1988) by gp120 shedding (Moore et al., 1990) and could be a potential anti-HIV treatment. However, the half-life is sCD4 in humans is short, between 30 and 120 min (Capon et al., 1989), and sCD4 does not decrease viral loads of patients *in vivo* (Daar et al., 1990). PIs are also more resistant to sCD4 inactivation *in vitro* (Ashkenazi et al., 1991; Daar et al., 1990) and the difference in sensitivity between TCLA and PI seems to be due to CD4-gp120 affinity.

A hybrid molecule was designed, consisting of the Fc portion of an IgG and the D1+D2 domains of CD4 and was called immunoadhesin or sCD4-IgG. This molecule has a half-life extended by 200-fold in rabbit serum compared to sCD4, comparable to the half-life of human IgG (Capon et al., 1989). The intravenous injection of sCD4-IgG prevented infection with IIIB in chimpanzees (Ward et al., 1991) and sCD4-IgG was successfully transferred across the placenta (Byrn et al., 1990). Based on the same principle, a tetravalent form of D1-D2 of CD4 fused to the heavy and light chains of an IgG, called PRO 542, was designed (Allaway et al., 1995). This compound inhibits entry of X4- and R5-tropic HIV isolates from several clades *in vitro* (Trkola et al., 1995). Phase three clinical trials undertaken in children show that PRO 542 decreases viral loads *in vivo* by up to 7-fold and its administration does not cause serious side effects (Shearer et al., 2000). Another study, in HIV-infected adults, has shown that PRO 542 can decrease the viral load of patients up to 5-fold.

Moreover, the inhibitory effect of this molecule is stronger in patients with advanced-stage disease than with early-stage disease (Jacobson et al., 2004). Although HIV treatment by sCD4-IgG does not result in undetectable viral loads, it could be used as an alternative therapy.

1.14.3 Chemokine receptor antagonists

The relevant chemokine co-receptors for transmission *in vivo* are CXCR4 and CCR5 (see section 1. 6. 2. 2). Their respective ligands, SDF-1 and RANTES, compete with HIV for binding to the chemokine receptors. They down-regulate their chemokine receptors at the cell surface and, therefore, decrease the number of target cells for HIV. Some variants of RANTES are currently tested as HIV inhibitors (Shattock and Moore, 2003).

Several small molecule co-receptor antagonists have been described. The compounds AMD3100, T22 and ALX40-4C inhibit HIV entry on CXCR4 *in vitro* but their prescription *in vivo* may be toxic, as CXCR4 deletion (which might be provoked by CXCR4 downregulation) perturbs foetal development in mice and, therefore, the prescription of anti-CXCR4 compounds might lead to severe side effects (Moore and Stevenson, 2000). The anti-CCR5 compounds, such as TAK-779 (Dragic et al., 2000), AD101 (Tsamis et al., 2003) or CMPD 167, might not raise a toxicity problem, as individuals who not synthesise CCR5 do not suffer from obvious immunological defects. Recently, an antagonist molecule that inhibits X4- and R5-tropic viruses has been reported (Princen et al., 2004).

In the short-term, the treatment with small chemokine antagonists results in the inhibition of the replication of HIV *in vitro* and in the reduction of the viral load *in vivo* (Wolinsky et al., 2004). In the long-term, it results in the emergence of resistant viral mutants (Trkola et al., 2002) which retain the same tropism as the sensitive virus, even in the presence of the inhibitor *in vitro* (Farber and Berger, 2002; Trkola et al., 2002), and *in vivo*, although a brief and transient tropism switch is first observed (Wolinsky et al., 2004).

1.14.4 The anti-fusion peptide T20

T20, also known as DP-178 and enfuvirtide (Cervia and Smith, 2003), interferes with fusion between HIV and the cell membranes. T20 is a hydrophobic peptide corresponding to the 36 amino acid residues of the HR2 domain of gp41. T20 binds to

HR1 and locks gp41 into a structural intermediate that prevents gp41 from entering a fusion-active state. T20 is effective against HIV from all clades (Cervia and Smith, 2003). R5-tropic viruses are less sensitive to T20 than X4-tropic viruses (Derdeyn et al., 2000), due to a greater affinity for the co-receptor, which could increase the kinetics of fusion (Reeves et al., 2002). Phase three clinical trials have shown that T20 can reduce viral load between 0.8 and 1 log in patients failing HAART (Cervia and Smith, 2003). Unlike HAART, T20 is well tolerated. However, the hydrophobicity of T20 makes it difficult to inject. T20-resistant viruses emerge *in vivo* and mutations are found in gp41 (Lu et al., 2004; Wei et al., 2002) but T20-escape mutants seem to lose fitness (Lu et al., 2004). T20 is however very costly. The annual prescription of T20 would cost $20, 000 per patient, which makes this drug unavailable to developing countries.

1.14.5 Microbicides and passive administration of neutralising antibodies

In developing countries, the majority of HIV transmission occurs from man to woman. Condoms are the most reliable anti-HIV barriers but they require the consent of the male partner, which does not always happen. Therefore, cheap and reliable microbicides that can protect women from infection need to be developed (Shattock and Solomon, 2004). Microbicides are topical formulations that can block HIV infection when applied vaginally or rectally (Shattock and Solomon, 2004). Although an anti-HIV vaccine that will trigger a sterilising immune response is likely to still take many years, a topical microbicide that will prevent HIV infection altogether seems to be a more promising approach (Moore, 2005).

Non-specific microbicides have been tested to prevent HIV infection. The surfactant nonoxynol 9 disrupts viral particles and inhibits HIV *in vitro* (Greenhead et al., 2000). It also attacks the vaginal epithelium, which results in the increase of infection (Shattock and Solomon, 2004). Other non-specific microbicides, such as gramicidin (a peptide antibiotic with anti-viral activity) and PRO 2000 (a sulphonate polymer), have been shown to inhibit cervical X4-tropic HIV infection *in vitro* (Greenhead et al., 2000), possibly by modulating charge interactions between the V3 loop of gp120 and polyanions present on the target cells in cervical tissues (Shattock and Moore, 2003).

CXCR4 and CCR5 antagonists, sCD4-IgG and a modified version of the natural ligand for CCR5 (PSC-RANTES) can also inhibit HIV or SHIV infection of cervical tissue when used topically (Hu et al., 2004; Lederman et al., 2004).

The application of a single NAb or a cocktail of NAbs can prevent mucosal transmission of SHIV (Baba et al., 2000; Ferrantelli et al., 2004; Veazey et al., 2003a).

Preventing HIV infection by the use of topical application of anti-HIV compounds in monkeys is possible. Considering that microbicides should be primarily made available to poor countries, some compounds (such as RANTES), however, cannot be exploited, as they are too costly. The development of cheap molecules is now one of the focuses in anti-HIV treatments, along with the development of an anti-HIV vaccine (Moore, 2005).

1.14.6 Other drug targets

Anti-HIV treatments target viral entry, reverse transcription and the proteolytic maturation of the virus. Nevertheless, new insights into the mechanisms of the replication cycle, as well as the characterisation of cellular proteins that interact with HIV, have given new hopes for tackling HIV replication *in vivo*.

HIV infectivity seems to be dependent upon the interaction between CA and CypA during (or shortly after) uncoating (Franke et al., 1994). Cyclosporin A, which counteracts the action of CypA, has been shown to inhibit HIV replication *in vitro* (Billich et al., 1995). The problem is that cyclosporin A blocks CD4 T cell activation, is an immunosuppressive drug and cannot be prescribed to HIV-infected individuals. The development of non-immunosuppressive cyclosporin A analogues could be a promising avenue in anti-HIV treatments (Billich et al., 1995).

Zhou *et al* have reported a new compound capable of inhibiting HIV budding by delaying the cleavage between CA and p2, which impairs virus maturation (Zhou et al., 2004).

A new group of compounds that have anti-IN properties are new anti-HIV drugs in development. They contain a diketo acid moiety (Condra et al., 2002). They act by blocking strand transfer during the reverse transcription and by preventing HIV DNA integration (Hazuda et al., 2000).

1.15 Vaccines

Attenuated HIV strains can elicit a protective immune response but they can revert to a cytopathic state and causes an infection and AIDS in monkeys (Burton and Moore, 1998; Whitney and Ruprecht, 2004).

The classical approaches for vaccine design have been unsuccessful in triggering protection in immunised animals. Nevertheless, new approaches are now under investigation.

1.15.1 Envelope-based immunogens

Monomeric gp120 proteins from the clade B isolates MN and GNE8 have been used as immunogens in order to elicit NAbs that could inhibit HIV infection in clinical trials. Despite a strong anti-gp120 IgG response, the elicited Abs did not neutralise HIV in patients and no significant difference in the number of acquired infections was observed between the immunised and the placebo groups (Gilbert et al., 2005). Pantophlet *et al* reported the characterisation of the monomeric JRFL gp120, modified by extra N-glycosylation and mutated around the CD4BS by alanine substitutions that affect the binding of non-NAbs (Pantophlet et al., 2003a; Pantophlet et al., 2003b). This modified gp120 only binds b12 and not the non-neutralising anti-CD4 MAbs (Pantophlet et al., 2003b). This new monomeric Env could be used as a vaccine for specifically eliciting b12-like NAbs.

Oligomeric Envs, from clades A, B, and the subtype O have been generated and characterised (Jeffs et al., 2004) and could confer a better Ab protection than with the immunisation with monomeric gp120.

1.15.2 CTL vaccine

For some viruses, such as influenza, respiratory syncytia virus (RSV) and lymphocytic choriomeningitis virus (LCMV), CTLs alone can protect animals from death (McMichael et al., 2002a). Although it is not clear which branch(es) of the adaptive immune system will confer protection against HIV infection, several observations argue that an anti-HIV vaccine strategy based on CTLs alone might decrease HIV incidence. CTLs play an important role in controlling HIV infection *in vivo* (see section 1. 12. 3. 2 for details). Some highly-exposed non-infected sex workers have a strong and broad CTL response in the blood and genital mucosa (Rowland-Jones et al., 1998).

A CTL vaccine design consists in the immunisation with DNA encoding HIV proteins and administration of IL2. Canary pox virus (Belshe et al., 2001; Evans et al., 1999), vaccinia virus (Barouch et al., 2001), venezuelan equine encephalitis virus (Davis et al., 2002) and adenovirus (Shiver et al., 2002) genomes have been tested for induction of anti-SHIV CTL response *in vitro* or for protection in monkeys.

For example, the injection of DNA encoding the sequence of codon-optimised Gag, followed by boosts of adenovirus encoding the same Gag sequence in rhesus macaques elicited a strong CTL response that did not prevent from SHIV 89.6P infection but did control viraemia. A normal CD4 T cell count was maintained and the vaccinated monkeys did not show any signs of AIDS-related syndromes six months after virus challenge. Mock-vaccinated (or non-vaccinated) monkeys were not protected against SHIV infection. They had a weak CTL response, a dramatic decrease in their CD4 T cell count and rapidly showed health deterioration (Shiver et al., 2002).

Although the principle of an anti-HIV CTL vaccine approach seems promising, problems exist. CTL-induced vaccines do not prevent infection but control viral replication better. HIV superinfection can occur in HIV-infected individuals who have a broad CD8 T cell response (Altfeld et al., 2002). Thus, the same problem could occur in vaccinated individuals. Moreover, CTL viral escape has been reported in vaccinated animals, that initially controlled SIV infection (Barouch et al., 2002). Loss of viral replication control can occur by only one amino acid substitution within an epitope recognised by CD8 T cells (McMichael et al., 2002a) and can lead to uncontrolled viraemia, decline in the CD4 T cell count and onset of AIDS-related symptoms (Barouch et al., 2002). Furthermore, the Gag KK10 CTL escape mutant (in the HLA-B57 context) can be successfully transmitted by mother-child transmission (Goulder et al., 2001b). This could cause a problem in terms of protecting an overall population (Goulder and Watkins, 2004). From this observation, Goulder and Watkins suggest that all CTL responses could not be equally effective at controlling HIV viraemia *in vivo* and, like Abs, vaccines might trigger immuno-dominant CTLs at the cost of more protective responses (Goulder and Watkins, 2004). The aim of a protective anti-HIV CTL vaccine might not be simply to induce as many CTL responses as possible in vaccinated individuals (Goulder and Watkins, 2004). The issue of targeting the best peptides might be complicated by the co-existence of various HIV clades in countries where CTL vaccines are needed the most (McMichael

et al., 2002b). Developing a vaccine that, firstly, will target the peptides where mutations would occur at a severe fitness cost, and, secondly, that will contain the peptides found within all the HIV clades, predominant in a specific area, seems more problematic than originally planned.

1.16 Scope of this thesis

In HIV infection, NAbs are usually detected between two and nine months POS (Aasa-Chapman et al., 2004; Richman et al., 2003) and, therefore, are unlikely to play a role in the decline of viral load observed during acute infection. Non-neutralising anti-gp120 Abs, however, can be detected within days POS (Aasa-Chapman et al., 2004; Moore and Ho, 1993). I first investigated if early non-neutralising sera as well as neutralising sera could also inactivate autologous and heterologous viruses in association with complement (Chapter 3). In chapter 4, I investigated the mechanisms of CMI, the nature of the pathway, as well as the Ab isotype involved. I also tested CMI using anti-gp120 MAbs and sCD4-IgG and investigated the influence of the virus-producing cell line on CMI.

Finally, I re-examined the anti-idiotypic approach to generate an anti-HIV vaccine (Chapter 5). For this project, the cross-NAb b12 was used. A new anti-idiotypic strategy detailed in this thesis took advantage of a peculiarity of the camelid family (llamas) that naturally synthesise single chain non-classical IgG devoid of light chains (Hamers-Casterman et al., 1993). These non-classical IgG (referred to as VHH) have long CDR H regions that can adopt non-canonical conformations (Muyldermans, 2001) and therefore could potentially mimic the b12 binding site on HIV-1. Specific anti-b12 VHH fragments were isolated from a VHH library generated from a llama immunised with Fab fragments of b12. Five VHH fragments were tested for their specificity to b12 and were subsequently used as immunogens in rats and rabbits in order to determine if they could elicit a strong, b12-like NAb response.

Chapter 2
Material and Methods

2.1 Buffers and solutions

PEG/NaCl	20% polyethylene glycol 6000 (w/v), 2.5 M NaCl
PBS	137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM K_2HPO_4 pH 7.4
PBST	PBS, 0.05% Tween (v/v)
TBS	20 mM Tris-HCl, pH 7.6, 120 mM NaCl
TBST	TBS, 0.05% Tween (v/v)
TEA	100 mM Triethylamine
TMT/GS	TBST, 20% goat serum (v/v), 4% skimmed powder milk (w/v)
Extraction buffer	20% sucrose (w/v), 1 mg/ml lysozyme, 30 mM Tris pH 6.8
HRP substrate	1 mg/ml 3,3',5,5'-Tetramethylbenzidine (Sigma, UK) in 100 mM sodium acetate, 10 µl of hydrogen peroxide per 50 ml
SDS-PAGE 12%	for a 10 ml gel, 3 ml 40% acrylamide, 0.4 M Tris-HCl pH 8.8, 0.1% SDS (v/v), 0.05% ammonium persulphate (v/v), TEMED, 15µl
TAE	40 mM Tris-HCl pH 7.8, 20 mM sodium acetate, 1m M EDTA
Loading buffer 6X	0.25% bromophenol blue (w/v), 0.25% xylene cyanol FF (w/v), 30% glycerol (v/v)
SOC	2% Bacto-tryptone (w/v), 0.5% yeast extract (w/v), 10 mM NaCl 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose
2TY	1.6% Bacto-tryptone (w/v), 1% yeast extract (w/v), 0.5% NaCl (w/v)
2TYAmp	2TY, ampicillin 100 µg/ml
2TYAmpGlc	2TYAmp, 1% glucose (w/v)
2TYAmpKan	2TYAmp, kanamycin, 50 µg/ml
2TYAmpXgalIPTG	2TYAmp, 0.5 M IPTG, 80 µg/ml X gal

2.2 Description of bacterial strains, mammalian cell lines and antibodies

2.2.1 Bacterial strains

E.coli strain	Genotype
TG1	F'traD36 $lacI^q$ Δ(lacZ) M15pro$^+$B$^+$/supE Δ(hsdM-mcrB)5 (r_k-m_k-McrB$^-$) thi Δ(lac-proAB)
BL21	F-, ompT, $hsdS_\beta(r_\beta\text{-}m_\beta)$, dcm, gal, (DE3) ton A
TOP 10®	F- mcrA D(mrr-hsdRMS-mcrBC)f80lacZDM15 DlacX74 deoR recA1 araD139(ara-leu)7697 galU glK rpsL(Str^R) end A1 nupG

Table 2.1 Description of bacterial strains

2.2.2 Mammalian cell lines

Cell line	Description	Reference
293T	Human embryonic kidney	(Graham et al., 1977)
H9	Human CD4+ T cell	(Popovic et al., 1984a)
NP2/CD4/CXCR4	Human glioma/CD4/CXCR4	(Soda et al., 1999)
NP2/CD4/CCR5	Human glioma/CD4/CCR5	(Soda et al., 1999)

Table 2.2 Cell lines

2.2.3 Antibodies and sera

Antibody	*Mapping and properties*	*Reference*
447-52D	Human IgG3. Binds to GPXR on V3 loop of MN. Neutralises some TCLAs and PIs	(Gorny et al., 1992)
MN 215	Human IgG1. Binds to RIHIGPGRAFYTTKN on the V3 loop of MN. Neutralises some TCLAs	(Schutten et al., 1995)
268-D	Human IgG1. Binds to HIGPGR on V3 loop of MN. Neutralises some TCLAs	(Gorny et al., 1991)
GP 68	Human IgG1. Binds to CD4BS. Neutralises some TCLAs (IIIB)	(Schutten et al., 1993)
654-D	Human IgG1. Binds to CD4BS of LAI. Neutralises IIIB	(Laal et al., 1994)
b12	Human IgG1. Binds to CD4BS. Neutralises TCLAs and PIs from different clades	(Burton et al., 1991)
b6	Human IgG1. Binds to CD4BS of IIIB. Neutralises some TCLAs	(Parren and Burton, 1997)
A32	Human IgG1. Binds to C1-C4, to a CD4-induced epitope. Does not neutralise	(Wyatt et al., 1995)
17b	Human IgG1. Binds to a CD4-induced epitope. Neutralises some TCLAs, weakly some PIs. Is more potent as a Fab fragment	(Thali et al., 1993)
670-D	Human IgG1. Binds to PTKARR on C5. Does not neutralise	(Zolla-Pazner et al., 1995)
697-D	Human IgG1. Binds to ISTSIRGKVQKEYAFFYKLD on V2 loop of IIIB. Neutralises some PIs	(Gorny et al., 1994)
2G12	Human IgG1. Binds to a carbohydrate-dependent epitope. Neutralises some TCLAs and PIs	(Buchacher et al., 1994)

Table 2.3 Antibodies

The MAbs b12, b6, A32, 17b, 2G12 were kindly provided by Dr. R. Pantophlet and Prof. D. R. Burton (Scripps, USA). The others MAbs were provided by NIBSC (Potters Bar, UK).

The anti-HIV-1 QC sera 1, 2, 4, 5 and 6 were harvested from anonymous HIV-infected British patients. The sera were selected for the WHO programme on standardisation of neutralisation when described by McKeating *et al* (McKeating et al., 1989).

2.3 Mammalian cell culture

2.3.1 Maintenance of cell lines

Adherent cell lines were passaged in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, UK) supplemented with 5% (v/v) foetal calf serum (FCS) (Invitrogen, UK). Suspension cell lines were maintained with Roswell Park Memorial Institute (RPMI) (Invitrogen, UK) supplemented with 10% FCS (v/v). All cell lines were grown at 37°C in an humidified atmosphere with 5% CO_2 (v/v) except for 293T cells, which were grown in a humidified atmosphere with 10% CO_2.

2.3.2 Passage of cells

Every four or five days, adherent cells were washed with trypsin diluted in versene (0.5% trypsin v/v in 0.02% versene v/v) and left for 5 min at 37°C until the cells detached from the flask, $1/10^{th}$ of the trypsinised cells was diluted in DMEM-FCS and was seeded in a new flask. Non-adherent cells were diluted 1:5 in RPMI every four or five days.

2.3.3 Storage of cells

Exponentially-grown adherent cells were trypsinized as described in 2. 3. 2 and then centrifuged at 900 g for 10 min at RT. The cell pellet was resuspended in 90% FCS-10% DMSO (Sigma, UK) and the cells were aliquoted in cryovials. The vials were then carefully wrapped in paper and progressively frozen at -20°C between four and twelve hours, at -40°C and -80°C between four and twelve hours and finally stored in liquid nitrogen.

2.3.4 Transfection of mammalian cells

The day prior to transfection, 293T cells were split 1:2 or 1:3, depending on the confluence of the cells, and were plated in a 6-well tray at the density of $4x10^5$ cells/well. The following day, DMEM 10% FCS was replaced by fresh medium. For each well, 6 µl of Fugene® (Roche, UK) was premixed with 2 µg of total DNA in 200

µl of serum-free Optimem (Invitrogen, UK). The mix was incubated at RT for 20-30 min and was added dropwise to the cells. After an overnight incubation at 37°C, the medium was replaced and harvested 24-36 hours later.

2.4 Primary cell culture

2.4.1 Preparation of PBMCs from buffy coats

Buffy coats, provided by the blood Bank (Brentwood blood transfusion centre, London) were diluted 1:1 (v/v) in sterile PBS. 45 ml of diluted buffy coat were then carefully layered onto 15 ml of lymphoprep (Nycomed, Norway). The preparation was centrifuged at 1500 g for 30 min at RT (no brake). The layer containing the white blood cells was carefully harvested, washed with sterile PBS twice then recoverd by centrifugation at 900 g for 10 min at RT. The cells were incubated at RT for 10 min with 10 ml of red blood cell lysis buffer (Sigma, UK). After centrifuging at 900 g for 10 min at RT, the cells were diluted 1:100 in serum-free RPMI and counted using a haemocytometer. The cells were resuspended at the concentration of 10^6 cells/ml in RPMI-10% FCS containing PHA (Murex, UK) at the final concentration of 20 U/ml and grown for three days at 37°C in a humidified atmosphere with 5% CO_2. The cells were centrifuged at 900 g for 10 min at RT and then resuspended at the concentration of 10^6 cells/ml in RPMI-FCS supplemented with recombinant human IL2 (Roche, Germany) at the final concentration of 20 U/ml. Every three days, the cells were counted and adjusted at 10^6 cells/ml in RPMI-FCS-IL2.

2.4.2 Preparation of macrophages from buffy coats

The preparation of macrophages from buffy coats is the same as for PBMCs described in 2. 4. 1 until the incubation of the cells with red blood cell lysis buffer. After centrifugation, 10^8 cells, in RPMI-5% human serum (HS), were plated onto a 14 cm^2 tissue culture dish (Falcon, UK), previously coated for two hours with 5 ml of 2% bovine skin gelatine (Sigma, UK) at RT. The cells were incubated for two hours at 37°C to adhere to the plates and were washed gently once in their own medium, once in fresh medium and then left overnight in RPMI-10% HS at 37°C.

The following day, the medium was removed, the cells were washed with PBS and incubated with 5 mM EDTA in PBS for 10 min at 37°C. After another wash, the PBS/EDTA solution, containing the cells, was centrifuged at 900 g for 10 min at RT, the cell pellet was washed twice with PBS and the cells were resuspended in 20 ml of

RPMI. $5x10^6$ cells were seeded in a 25 cm^2 tissue culture flask in RPMI-10% HS for two hours at 37°C. The cells were washed with RPMI-10% HS and were incubated at 37°C until they differentiated into macrophages bearing the distinctive "fried egg" morphology (seven days).

2.5 HIV strains

2.5.1 Description of viral strains

Virus strain	History	Phenotype	Source	Clade	Status	References
IIIB	TCLA	SI	PBMC	B	AIDS	(Gallo et al., 1984)
RF	TCLA	SI	PBMC	B	AIDS	(Popovic et al., 1984b)
SF2	TCLA	SI	Brain	B	AIDS	(Levy et al., 1984)
BaL	PI	NSI	Brain	B	AIDS	(Gartner et al., 1986)
SF162	PI	NSI	Brain	B	AIDS	(Cheng-Mayer et al., 1989)
SL2	PI	NSI	PBMC	B	Asympt	(Simmons et al., 1996)
2044	PI	SI	PBMC	B	AIDS	(Simmons et al., 1998)

Table 2.4 HIV isolates

2.5.2 Growth of viral stocks

2.5.2.1 From suspension cells

Laboratory-adapted, X4-tropic strains of HIV were grown in the H9 cell line. A viral stock was thawed and incubated at 37°C for one to three hours with approximately 10^6 cells previously centrifuged at 900 g for 10 min at RT. The cells were then resuspended in 5 ml of RPMI-FCS for 3 or 4 days at 37°C in a humidified atmosphere with 5% CO_2. The infected cells were then centrifuged again and incubated at 37°C for between one and three hours with $5x10^6$ fresh non-infected

cells. The cells were resuspended in 12 ml of RPMI-FCS for further three or four days until syncytia were visible under the microscope. The cells were centrifuged and the supernatant, containing the viral particles, was aliquoted and snap frozen in liquid nitrogen. PIs of HIV were grown in PBMCs in the same manner.

2.5.2.2 From macrophages

Eight days after preparation of the macrophages (described in 2. 4. 2), the medium was removed from the cells and incubated with $8x10^5$ $TCID_{50}$ macrophage-derived stock of BaL (kind gift of Beatrice Kramer, UCL) for two hours at 37°C, after which the volume was increased to 6 ml. Seven days after the infection, half of the medium was harvested and replaced with fresh RPMI-10% HS every three days. The harvested medium was aliquoted and stored in liquid nitrogen.

2.5.3 Titration of viral stocks

All HIV titres were assessed on NP2/CD4/CXCR4 cells or NP2/CD4/CCR5 cells depending on the main co-receptor usage of the HIV strain.

The day previous to the experiment, cells were split (as described in 2. 3. 2) and 10^4 cells were seeded per well in a 48-well plate and kept at 37°C in a humidified atmosphere with 5% CO_2. The day of the experiment, a vial containing the viral stock was thawed and was serially diluted 1:10 (v/v) in DMEM-FCS. 200 µl of the serial dilutions of the virus were incubated onto the cells plated the day before for between one and four hours at 37°C in a humidified atmosphere with 5% CO_2. The medium was then discarded the cells were washed once with medium and incubated with 500 µl of DMEM-FCS per well. The plates were incubated for three days at 37°C in a humidified atmosphere with 5% CO_2.

2.5.4 Estimation of viral infection

Medium from the HIV-infected cells was discarded and the cells were incubated with an equal mix of ice-cold 100% methanol/acetone (v/v) for approximately 10 min at RT. The cells were then washed with PBS and incubated for one hour at RT with an equal mix of two anti-p24 monoclonal antibodies, ADP 365 and ADP 366 (AIDS Reagent Program, NIBSC, UK), diluted at 1:40 in PBS-1% FCS. After three washes with PBS-FCS, the cells were incubated with a goat anti-mouse polyclonal serum conjugated to β-galactosidase (Southern Biotechnology associates Inc, USA) diluted

in PBS-FCS at 1:400 for one hour at RT. After two washes in PBS-FCS and a final washing with PBS, the cells were incubated with β-galactosidase substrate (X-gal in PBS containing 3 mM potassium ferrocyanide, 3 mM potassium ferricyanide and 1 mM $MgCl_2$) for two or three hours at 37°C. The cells infected with HIV-1 developed a blue colour. Individual or grouped cells were counted as a focus-forming unit (ffu).

2.6 Extraction and storage of complement

50 ml of blood were harvested from individuals (who gave written consent) and was stored at 4°C until complete clotting. The tubes were then centrifuged at 900 g at 4°C and the upper phase containing the serum was carefully harvested, aliquoted and stored at -80°C until further use. Some aliquots of fresh serum, as well as all HIV-positive sera, were incubated at 56°C for two hours to completely inactivate complement. Alternatively, sterile solutions of EDTA or EGTA were added to fresh human serum at the final concentration of 2.5 mM for 5 min at RT.

2.7 The Jenner patient cohort and the processing of the samples

The study subject is detailed in Aasa-Chapman *et al* (Aasa-Chapman et al., 2004). The development of the antibody response was monitored in seven HIV-positive men who have sex with men (MM4, MM8, MM19, MM22, MM23, MM27 and MM28). All the subjects presented at the Mortimer Market clinic (UCL, London) with various symptoms. The characteristics of each patient are presented in table 2.5 below.

Patient	Age[a]	Acute infection symptoms[b]	Likely exposure[c]	Time point gp120[d*]	VL[e*] (copies/ml)	CD4 T cell count* (/μl)
MM4	41	F, L, ST, A, D, NS	- 35	17	160,000	780
MM8	33	F, L, NV, D, H, Ph	- 21	12	5,927,000	290
MM19	31	U[f]	- 4	13	5,678,900	400
MM22	U	U	- 21	14	8,311,000	360
MM23	27	F, R, NS, P	- 2	9	11,105,300	330
MM27	32	F, NV, D, P	- 20	28	437,800	570
MM28	30	NS, R, L	- 4	6	4,337,100	560

Table 2.5 Characteristics of the Jenner patients

[a] Age at which the patients were diagnosed with HIV-1 infection
[b] F, Fever. L, Lethargy. ST, Sore throat. A, Anorexia. D, Diarrhoea. NV, Nausea/vomiting. NS, Night sweats. H, Headaches. P, Pharyngitis. Ph, Photophobia. R, Rash
[c] Days prior to the onset of symptoms
[d] Days from the onset of symptoms at which gp120 sequences were amplified by PCR and cloned into the HxB2 MCS-Δ-*Env gp120* vector to generate infectious viruses.
[e] The viral load (VL) was determined by a Chiron 3.0 kit (Emeryville, USA)
[f] Unknown
* The VL, CD4 T cell count and the gp120 amplifications were performed from samples harvested on the same day

Acute HIV-1 infection was diagnosed by the detection of HIV genomes in the presence or absence of an evolving HIV-1 Ab profile which subsequently became fully positive. All subjects remained treatment naïve throughout the study with the exception of MM22, who commenced therapy 26 days after diagnosis. Blood samples were obtained weekly for the first month, monthly for three months and then at three months intervals. The study protocol was approved by the Camden and Islington NHS Trust Ethics Committee and written consent was obtained from all subjects.

Blood samples was centrifuged at 900 g for 10 min at 4°C, the supernatant, as well as the blood pellets, were stored at - 80°C until further use.

2.8 Neutralisation and CMI assays

2.8.1 Neutralisation assays

Virus (100 ffus) was incubated with two-fold serial dilutions of HIV-positive sera, HIV-negative sera as negative controls, (from 1:10 or 1:20 final concentration) or MAbs in 100 μl of DMEM-FCS. MAbs were used at the final concentration of 10 μg/ml or at the final dilution of 1:10 when the concentration was unknown. The mix

was placed for one hour at 37°C and was incubated on NP2/CD4/CCR5 or NP2/CD4/CXCR4 cells, plated in 48-well plates, for between one hour and four hours at 37°C. The cells were washed with DMEM-FCS and incubated with 500 µl of DMEM-FCS per well and the plates were incubated for three days at 37°C in a humidified atmosphere with 5% CO_2. The cells were fixed and the viral infections were estimated as described in 2. 5. 4. The neutralisation percentage was calculated with the formula:

$$100 \text{ x} \frac{(1 - (\text{average FFU with patient sera})}{(\text{average FFU with seronegative serum})}$$

2.8.2 CMI assays

Two-fold serial dilutions (from 1:10 or 1:20 final concentration) of heat-inactivated sera from HIV-1 infected individuals were incubated with 100 ffus of HIV and with human serum, source of complement, Guinea pig complement reconstituted in PBS (Sigma, Poole, UK) and EGTA- or EDTA-treated serum, at the final concentration of 10% (v/v) in a total volume of 100 µl. The mix was then pre-incubated for one hour at 37°C and then incubated on NP2/CD4/CCR5 or NP2/CD4CXCR4 for one to four hours at 37°C. The cells were washed once with DMEM and incubated with fresh medium for three days at 37°C. The cells were fixed and viral estimates were determined as described in 2. 5. 4. The percentage reduction of infection was calculated with the formula:

$$100 \text{ x} \frac{(1 - (\text{average FFU with patient sera})}{(\text{average FFU with seronegative serum})}$$

2.8.3 Inhibition of b12 neutralisation *in vitro*

The VHH fragments were diluted in PBS respectively at 10, 5, 1, 0.5, 0.1 and 0.01 fold in excess to b12 molecular ratio. b12 was diluted at the concentration of 50 µg/ml in PBS. 10 µl of VHH fragments were pre-mixed with 10 µl of b12 and the equivalent of 100 ffus of HIV-1 in a total volume of 200 µl. After one hour incubation at 37°C in 5% CO_2, the mix was plated on NP2/CD4/CCR5 or NP2/CD4/CXCR4 cells for between one and three hours at 37°C in 5% CO2. The cells were then washed with DMEM medium and incubated for three days.

2.8.4 Statistical analysis

The statistical analyses of the results of the neutralisation and the CMI assays were undergone using the Student's *t*-test. Wilcoxon test (a variant of the Student *t*-test) and the Mann-Whitney U test (a non parametric version of the Student *t*-test), ,as well as ANOVA test (a test comparing the variances) could also have been applied for the statistical analysis. However, both Wilcoxon and Mann-Whitney tests can be less sensitive than the Student t-test and the ANOVA test is only relevant when three groups or more can be compared. For each experiment described in this thesis, the comparison is between two groups (with compared to without complement and with or without Abs).Therefore, the Student *t*-test was chosen as it allows statistical analysis on small samples. The *t*-test compares the actual difference between two means (with or without Abs for neutralisation assays or with or without complement for CMI assays) in relation to the variation within the data (expressed as the standard error between experiments). The null hypothesis chosen for the tests was that the means of the two samples (inactivation data in the absence or the presence of complement or neutralisation with or without sera) were not significantly different from each other. The two samples to be compared are not randomly selected: the second sample is the same as the first after some treatment has been applied. So the populations are paired. An example of the *t*-test value is shown in appendix 1.

The number of replicates was six per experiment. The p value of $p<0.05$ shows that the probability that the two means are significantly different from each other (with a 95% certainty). For each comparison on each graph, the statistical significance is shown by a star.

2.9 DNA manipulation

2.9.1 Generation of chimeric HIV-1 viruses

gp120 sequences of HIV-1 Envs from the infected patients were first cloned in pGEM® vector and then sucloned into the backbone of the HIV-1 molecular clone HxB2, containing all the genetic elements of HIV-1 but lacking the sequence of gp120. The clones were then transfected in 293T cells and the resulting chimeric viruses were tested for their infectivity.

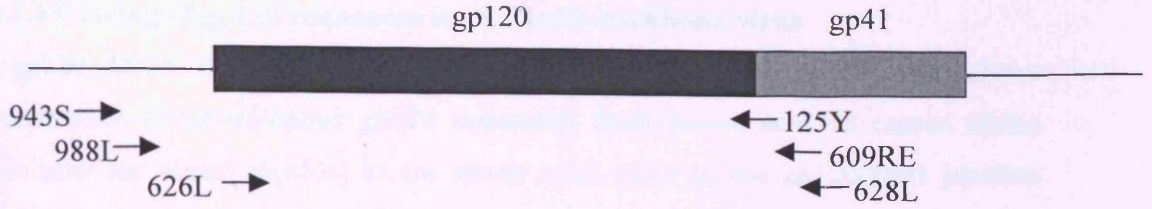
2.9.1.1 Primers

Primer	Position	Sequence
988L$^{+①}$	6089-6122[a]	5'GTA GCA TTA GCG GCC GCA ATA ATA ATA GCA ATA G
943S$^{+①}$	6084-6104[a]	5'CAA TAG (CT) AG CAT TAG TAG TAG
626L$^{d+①}$	6326-6347[a]	5'GTG GGT CAC CGT CTA TTA TGG G
125Y$^{d-①}$	7716-7742[v]	5'CAC CAC GCG TCT CTT TGC CTT GGT GGG
609RE$^{-①}$	7790-7816[a]	5'CCC ATA GTG CTT CCG GCC GCT CCC AAG
628L$^{-①}$	8065-8086[a]	5'TCA TCT AGA GAT TTA TTA CTC C
Sp6^{②}	pGEM®	5'GATTTAGGTGACACTATAG
T7^{②}	pGEM®	5'TAATACGACTCACTATAGGG
E80$^{+②}$	6564-6585[a]	5'CCA ATT CCC ATA CAT TAT TGT G
625L^{②}	3017-3027[a]	5'GGA TAT AAT CAG TTT ATG GG
A589$^{-②}$	6286-6307[a]	5'CAG AGT GGG GTT AAT TTT TAC AC
621^{②}	6651-6673[a]	5'GTA CAT TGT ACT GTG CTG ACA TT
E110^{②}	6708-6731[a]	5'CTG TTA AAT GGC AGT CTA GCA GAA
619^{②}	6716-6735[a]	5'TGG CAG TCT AGC AGA AGA AG
307d$^{-②}$	7021-7038[a]	5'CTG GGT CCC CTC CTG AGG
325h$^{-②}$	7222-7240[a]	5'GGG CAT ACA TTG CTT TTC C
015s$^{+②}$	6917-6936[a]	5'ACA TTG TAA CAT TAG TAG AG
MPE25^{③}	PUR4676	5'TTT CTG TAT GGG GTT TTG CTA
M13^{③}	PUR4676	5'GAG CGG ATA ACA ATT TCA CAC AGG A

Table 2.6 Primers

① Primers used for amplifying gp120
② Primers used for sequencing gp120
③ Primers used for sequencing VHH fragments
[a] The position is given according to HxB2 alignment

The primers annealed to regions of gp120 as shown below. For clarity, the primers used for gp120 sequencing are not indicated.



2.9.1.2 Amplification of gp120 sequences

Total DNA was extracted from the first blood sample available from each HIV-1 infected patient (see table 2.5) using the whole blood prep kit (Qiagen) according to the manufacturer's instructions. The gp120 DNA fragments were amplified by nested PCR as described by Aasa-Chapman *et al*, (Aasa-Chapman et al., 2004). For the first PCR, 1 pmol of primers $988L^{+}$, $943S^{+}$, $609RE^{-}$and $628L^{-}$, 2.5 units of proof-reading Long Expand DNA polymerase (Roche, Germany) in kit supplied PCR buffer 3 and 25 µM of each dNTPs were used. The DNA was amplified for 30 cycles using the following conditions: 92°C for 45 sec, 44°C for 45 sec and 68°C for 3 min 30 sec.

A second PCR was performed using 5 µl from the first PCR in the following 50 µl PCR mix: 1 pmol of primers $626L^{d+}$ and $125Y^{d-}$, 25 µM of each dNTPs and 2.5 U of Long Expand DNA polymerase (Roche, Germany) in PCR buffer 3. The gp120 sequences were amplified according to the same conditions described for the first PCR.

After completion of the second PCR, 15 µl of each PCR were mixed with 6X loading buffer and run on a 0.8% agarose gel in 1X TAE at 100 V for approximately 30 min. The gel was then visualised under UV light and the bands corresponding to 1.5 Kb fragments were excised and purified using the Qiaquick extraction kit (Qiagen, UK).

2.9.1.3 Cloning of gp120 sequences in pGEM vector®

The gp120 fragments were cloned into pGEM-T vector system I (Promega, UK) by mixing 10 µl of the purified gp120 DNA fragments with 1 µl of the vector and 5 U of ligase (Promega, UK) in ligation buffer, in accordance with the manufacturer's instructions. The ligation mix was incubated at RT for two hours. Half of the mix was used for transformation in Top10 *E.coli*.

2.9.1.4 Cloning of gp120 sequences in the HxB2 backbone virus

gp120 DNA fragments were cloned into MCS-Δ-*Env gp120* that allows incorporation of heterologous gp120 sequences from amino acid 38 (seven amino acids after the signal peptide) to six amino acids prior to the gp120/gp41 junction (McKeating et al., 1996).

The following day, individual white colonies (containing gp120 sequences cloned in pGEM®) were picked using sterile cocktail sticks and grown overnight in 2 ml of LBAmp (37°C, 250 rpm). The plasmids from each culture were extracted from the bacteria using the Qiagen miniprep kit (Qiagen, UK) according to the manufacturer's instructions and were eluted in 50 µl of elution buffer. Half of the purified plasmids and the vector containing the complete genome of HxB2 were then digested with 5 U of the restriction enzyme *MluI* (Roche, UK) for one hour at 37°C and then with 5 U of *BstEII* (Promega, UK) for one hour at 65°C. The digested plasmids and pHxB2-MCS-Δ-*Env gp120* were loaded onto a 0.8% agarose gel and the DNA fragments were purified again using the Qiaquick kit (Qiagen, UK). They were then eluted in 20 µl of elution buffer.

The ligation mix containing 3 µl of the vector HxB2 and 3 µl of the inserts were incubated with 5 U of ligase (Promega, UK) in ligation buffer for ½ hour at RT and overnight at 4°C. The mix was briefly frozen at -20°C to inactivate the ligase. Half of the ligation was used for transformation in Top10 bacteria (Invitrogen, UK).

2.9.2 Preparation of VHH libraries

2.9.2.1 Llama immunisation and generation of VHH libraries

This work was performed by Hans de Haard and colleagues in the Netherlands. Briefly, a llama was immunised six times with decreasing amounts of Fab b12. After verification of the anti-b12 Ab response by ELISA, 15 ml of blood were harvested, the B cells were extracted and the total RNA was isolated by acid guanidium thiocyanate extraction. Synthesis of cDNA was performed from total RNA, using random dNTP hexamers. VHH sequences were amplified as described by Dolk *et al* (Dolk et al., 2005). Long-hinge and short-hinge VHH fragments were amplified by PCR from the cDNA using *Taq* polymerase, primers LAM 07 ($^{5'}$AAC AGT TAA GCT TCC GCT TGC GGC CGC GGA GCT GGG GTC TTC GCT GTG GTG CG) and V_H-2B ($^{5'}$AGG TSM ARC TGC AGS AGT CWG G) where S is C or G, M is A or C, R is A or G and W is A or T) for the short-hinge fragments and LAM 08 (AAC

AGT TAA GCT TCC GCT TGC GGC CGC TGG TTG TGG TTT TGG TGT CTT GGG TT) and V_H-2B for the long-hinge fragments. The fragments of both long hinge and short hinge were then digested with *PstI* and *HindIII* (Roche, Germany) and cloned separately in phage display vector pUR 4676 encoding the sequences of a myc tag and a His-tag in order to fuse them at the 3' end of the VHH DNA. The two libraries were then cloned in TG1 *E.coli* by electroporation. The size of each library was estimated and subsequently stored at -80°C in 2TYAmpGlc 20% glycerol (v/v) until further use.

2.9.3 Bacterial transformation

2.9.3.1 Preparation of calcium chloride-competent *E.coli*

The calcium chloride-competent bacteria were prepared as described in Molecular Cloning (Sambrook et al., 1989). The day prior to the preparation, single colonies of the *BL21* strain of *E. coli*, grown on minimal medium plus agar, were resuspended in 5 ml of 2TY and grown overnight at 37°C and shaken at 200 rpm. An aliquot of the overnight culture was then resuspended in 50 ml of fresh 2TY medium at 200 rpm at 37°C until the bacterial culture reached the exponential growth (which is reached when the OD 600 nm is between 0.4 and 0.8). The bacteria were pelleted for 10 min at 3200 g at 4°C and incubated for 30 min on ice in cold sterile 500 mM $CaCl_2$ in half and then in 1/15th of the initial culture volume. The treated cells were then kept at 4°C between twelve and twenty-four hours before usage.

2.9.3.2 DNA transformation

3 µl of the ligation mix described in 2. 9. 1. 1 for the cloning of gp120 and 50 ng of plasmid DNA encoding the VHH fragments were added to 15 µl of commercially available chemically competent Top10 cells for the gp120 cloning or 100 µl of freshly made *BL21* cells for the VHH protein expression. The DNA-bacteria mixture was then incubated on ice for 30 min. The bacteria were then heat-shocked at 42°C for 90 sec and then immediately placed on ice. 100 µl of SOC medium was promptly added to the cells and incubated at 37°C for 30 min. 100 µl of the mix was plated on 2TYAmpXgalIPTG for cloning gp120 into pGEM vector®, 2TYAmp for gp120 cloning into HxB2 and 2TYAmpGlc for transformation of plasmids containing the VHH sequences. The plates were incubated at 37°C overnight for the VHH expression and at 30°C for the gp120 cloning.

2.9.4 Colony screening

2.9.4.1 Screening by PCR and enzyme restriction

For the VHH selection, single colonies were picked using sterile cocktail sticks and resuspended in a 50 µl PCR mix containing: 25 pmol of primers MPE 25 and M13, 1 µmol dNTPs, 2.5 mM $MgCl_2$ and 1 unit of *Taq* polymerase (Promega, UK) in 10X $MgCl_2$-free PCR buffer (Promega, UK). The PCR conditions were as follows: 1 min at 94°C, then 1 min at 94°C, 30 sec at 55°C, 1 min at 72°C for 30 cycles and a final step at 72°C for 1 min.

30 µl of each PCR reaction was digested for 1 hour at 37°C using 2 U of the frequent cutting restriction enzyme *HinfI* (New England Biolabs, USA). The restriction pattern of each clone was then checked on a 2.5% agarose gel.

2.9.4.2 Plasmid purification and enzyme restriction

From the same plates, colonies were picked using sterile cocktail sticks, resuspended in 5 ml of 2TYAmp for gp120 cloning and 2TYAmpGlc for VHH expression, and were grown overnight at 37°C for VHH or at 30°C for gp120 cloning and shaken at 200 rpm. The bacterial cultures were then centrifuged at 3200 g for 10 min at 4°C and the plasmid extraction was performed using Qiagen mini-prep kit (Qiagen, UK) according to the manufacturer's instructions. The plasmids were eluted in 50 µl of elution buffer and 10 µl of each PCR reaction were digested as described in 2. 9. 2. 1.

2.9.5 Sequencing reactions

For the VHH selections, 0.5 µg of plasmid DNA was sequenced on both strands using 0.6 nmol of MPE25 or M13. For the gp120 cloning, 1.2 µg of plasmid DNA was sequenced on both strands using 0.6 nmol of the primers described in 2. 9. 1. 1. Sequencing reactions were performed by the Windeyer Institute sequencing service using the Abi PRISM big dye terminator v 3.1 sequencing kit (Applied Biosystems, USA). The sequences were analysed using Sequencher software (Gene Codes corporation, Michigan).

2.10 Selections of the VHH libraries

2.10.1 Growth of the libraries

20 µl of the library glycerol stocks or 100 µl of an overnight grown TG1 culture, were inoculated in 5 ml of 2TYAmpGlc and were grown at 37°C and shaken at 200 rpm until log phase was reached, determined by the OD of the culture at 600 nm.

2.10.2 Phage rescue

The coding sequences of the VHH fragments are fused by their 3'end to the coat protein pVIII encoded by the filamentous phage M13. The pUR4676 plasmid has a filamentous phage backbone but the genes encoded for the phage arms have been deleted. To promote the production of full phage encoding the VHH fragments on their surface, helper phage (carrying the missing genes) was added to the log phase culture in a 20:1 ratio (helper phage/bacteria) and the culture was incubated for 30 min at 37°C (no shaking). The bacteria were then centrifuged at 3200 g for 15 min and the bacterial pellet was then resuspended in 50 ml of 2TYAmpKan. The culture was incubated at 37°C at 200 rpm and shaken overnight.

2.10.3 Phage precipitation

The overnight bacterial culture was centrifuged at 3200 g for 10 min at 4°C. The supernatant was incubated with 1/5th of the volume of the overnight culture of cold sterile PEG/NaCl for one hour on ice with occasional shaking. The phage were centrifuged at 3200 g for 30 min at 4°C. The supernatant was discarded and the phage pellet was carefully resuspended in 1 ml of cold sterile PBS. The phage were centrifuged for 5 min at 9000 g to spin down the cell debris. The phage-containing supernatant was precipitated again with PEG/NaCl and incubated for one hour on ice. The phage were pelleted and resuspended in 1 ml of sterile PBS. Phage were serially diluted 1:10 in PBS and phage diluted at 10^{-10} and 10^{-12} were used to infect an *E.coli* culture to determine the phage titre. For long-term storage, phage were kept in PBS-10% sterile glycerol at -20°C.

2.10.4 Selections

All selections were performed in 96-well Maxisorb plates (NUNC, UK). The purified NAb b12 (kind gift from Prof. D.R Burton), as well as purified polyclonal human IgG, were used at 5 and 10 µg/ml in PBS to coat a maxisorb plate overnight at 4°C. After blocking the plate with PBST-2% skimmed powder milk (w/v) at RT for

one hour, several phage inputs from each library (detailed in Chapter 5), diluted in PBST-2% skimmed powder milk, (pre-incubated with polyclonal human IgG from a HIV seronegative donor for 30 min at RT on a rotating wheel) were incubated on the plate for two hours at RT. After twenty washes with PBST and PBS, the phage were eluted either with virus lysate, gp140 (or PBS as a negative control) for 2 ½ hours at 37°C and (or) with 100 µl of TEA (100 mM triethylamine). The eluted phage were then neutralised with 50 µl of 1 M Tris buffer pH 7.5 for 10 min at RT. Half of the neutralised phage were used to infect 350 µl of an exponentially grown TG1 *E.coli* culture for 30 min at 37°C. Serial dilutions of the infected bacteria were then plated on 2TYAmpGlcAgar. The rest of the bacterial culture was pelleted and resuspended in 5 ml of 2TY AmpGlc and grown overnight shaking at 37°C. The bacterial culture was centrifuged, the pellet was resuspended in 2TYAmp/20% glycerol and stored at -80°C until further use.

2.10.5 Clone screening

2.10.5.1 Master plate

Enriched libraries obtained after successive rounds of selection were serially diluted and plated on 2TYAmpGlcAgar to have distinct clones. Colonies were randomly picked with sterile cocktail sticks into a 96 V-bottom plate (NUNC) in 2TYAmpGlc and were grown shaking at 100 rpm at 37°C until log phase was reached. Sterile glycerol was added to the wells at the final concentration of 20% and the master plate was stored at –80°C until further use.

2.10.5.2 Soluble expression and ELISA

5 µl from each well of the master plate were inoculated in 150 µl of 2TYAmpGlc in a 96 V-bottom plate. The plate was incubated at 37°C and shaken at 100 rpm until log phase was reached. It was then centrifuged at 900 g for 15 min, the medium was discarded, and the bacterial pellets were resuspended in 2TYAmpIPTG. The plate was incubated overnight at 37°C and shaken. After another centrifugation, 50 µl of each supernatant was tested for the detection of specific b12 binding by ELISA.

A maxisorb plate was coated overnight either with b12, polyclonal human IgG or BSA, diluted at a concentration of 5 µg/ml in PBS, overnight at 4°C. After washing with PBST, the plate was incubated for one hour at RT with PBST-2% skimmed

powder milk (w/v). After further washing with PBST, 50 µl of supernatant were mixed with 50 µl of PBST-2% skimmed powder milk and were incubated for two hours at RT. The plate was washed with PBST and an anti-myc Ab directly conjugated to HRP (Abcam, Cambridge, UK), diluted at 1:2000 in PBST-2% powder milk, was added and incubated for one hour at RT. The plate was washed with PBST then pure H_2O and 100 µl of HRP substrate were added. After the development of a blue color, the enzymatic reaction was stopped by the addition of 50 µl of 1 M HCl. The plate was read at 450 nm using a plate reader.

2.11 Expression and purification of VHH fragments

2.11.1 Induction of protein expression

15 µl of the glycerol stock of each clone were inoculated in 5 ml of 2TYAmpGlc and were grown overnight at 37°C shaking. 500 µl of the overnight culture were used to inoculate 50 ml of fresh 2TYAmp-0.1%glc and were incubated at 37°C until log phase was reached. The 50 ml culture was added to 450 ml of fresh 2TYAmp-0.1%glc complemented in IPTG (0.1 mM final concentration) and the cultures were then grown overnight at 37°C shaking. The cultures were centrifuged at 3200 g for 20 min, the supernatants discarded and the bacterial pellets were stored at -20°C.

2.11.2 Periplasmic extraction and purification of VHH fragments

The bacterial pellets were thawed and resuspended in 1/20th of the culture volume in the extraction buffer and incubated on ice for 30 min. The bacteria were centrifuged at 9000 g for 30 min and each supernatant was then applied onto 2 ml of Ni-NTA superflow column (Qiagen) previously equilibrated with PBS. Each supernatant was mixed with the column overnight at 4°C. Each column was washed at least five times with 15 ml of PBS-5 mM imidazole for one hour and the VHH fragments were eluted from the column with 2 ml of PBS-250 mM imidazole overnight at 4°C on a rotative wheel. Each eluant was then placed in a dialysis cassette (Pierce, UK) and was dialysed for 36 hours in PBS at 4°C with regular changes of PBS. After the dialysis, the VHH fragments were aliquoted in 100 µl volumes. BSA was added at the final concentration of 1 mg/ml and they were kept at -80°C until further use.

2.11.3 Protein gel electrophoresis

The production and the purification of the VHH fragments were checked by Coomassie staining on SDS-PAGE 12% gels and by Western blotting using the anti-His-HRP described in 2. 10. 5. 2. The concentration of the purified VHH fragments was determined by comparison with BSA on a Coomassie gel.

2.12 Characterisation of VHH fragments by ELISA

Purified VHH were tested against sCD4 and ant-HIV-1 Abs 17b and 2G12 (listed in table 2.5) by ELISA using the same protocol as described previously.

2.13 Detection of the anti-gp120 Ab response by ELISA

A 96-well plate was coated with the anti-gp120 Ab D7324 (Aalto Bioreagents Ltd, Ireland) at 10 µg/ml in PBS overnight at 4°C. The plate was washed four times with TBST and was blocked for one hour at RT with TBS-4% skimmed powder milk. After two washes with TBST, 50 µl of gp120, diluted at 0.2 ng/µl in TBS-1% skimmed powder milk (w/v), were incubated in each well for two hours at RT. For each tested serum, a blank was included omitting the gp120. The plate was washed four times with TBST. Sera, included a mix of QC sera 1, 2 and 4 used as a positive control and a seronegative serum used as a negative control, were serially diluted from a 1:100 dilution in TMT-GS and incubated on the plate for one hour at RT. The plate was washed four times with TBST and 100 µl of goat anti-human Ig conjugated to AP (Immunochemical direct, USA), diluted at 1:2000 in TMT-GS, was added in each well. After six washes with TBST, 100µl of luminescent AP substrate (Labtech, UK) was added and the plate was incubated in the dark for one hour at RT. Luminescence was measured in a Lucy 1 luminometer at 405 nm and analysed using Stingray software.

2.14 IgG purification

IgG from patients' samples were purified by binding to protein G using MAb trap kit (Amersham, UK) according to the manufacturer's instructions. Plasma samples were centrifuged for 1 min at 9000 g, diluted 1:1 with binding buffer (Amersham, UK) and applied onto the protein G column previously equilibrated with binding buffer. After washes, the IgG were eluted and renatured with neutralising buffer

(Amersham, UK) according to the manufacturer's intructions. All the collected fractions, fall through (FT) fractions, washes and eluants were then tested for the presence of protein with Biorad Protein Assay solution (Biorad, Germany). The collected fractions were diluted 1:10 in PBS, as well as serial dilutions of BSA of known concentration, and 40 µl of Biorad protein assay solution was added. Fractions containing proteins would develop a blue color, of which the intensity is proportional to the amount of proteins present in the fractions.

2.15 KLH conjugation to VHH fragments and to gp120

This work was performed by Dr Peter Delves and colleagues (UCL, London). KLH (Sigma, UK) was resuspended in 0.9 M NaCl to a final concentration of 40 µM. KLH was then mixed with purified the VHH fragments, diluted in 0.9 M NaCl, at a ratio VHH/KLH 40:1. Glutaraldehyde was then added to the VHH-KLH mix at the final concentration of 0.2% (v/v) and the mix was rotated on a wheel for one hour at 4°C. Glutaraldehyde was added again to reach a final concentration of 0.4% (v/v) and the mix was again rotated on a wheel for one hour at 4°C. Glutaraldehyde was inactivated by the addition of 50 µl of 0.2 M ethanolamine in 0.5 M bicarbonate pH 9.5 per ml of conjugate and the mix was rotated for further 30 min at 4°C.

The KLH-VHH fragments were then purified from ethanolamine and free glutaraldehyde by applying the mix onto 2x Hi Trap G25 columns (Pharmacia, UK) on an HPLC system previously equilibrated with 0.9 M NaCl in PBS. The first peak was collected. The protein concentration was estimated by BCA microplate protocol (Sigma, UK). The same protocol was used for the conjugation of gp120 to KLH.

2.16 Immunisation regimes

Four rats and one rabbit were used for the immunisation of each VHH fragment. The adjuvants used for the rabbits and the rats were MPL-TDN-CWS (Sigma, UK) and MPL-TDM (Sigma, UK) respectively. MPL is constituted of lipids from the bacterium *S. minnesota* and TDM is composed of an analogue of trehalose dimycolate from bacillus. CWS are cell wall skeleton from mycobacteria.

For each immunisation, the recommended dose was 25-125 µg in 500 µl per rat and 50-250 µg in 1 ml per rabbit, according to the manufacturer's instructions. For the rats, 200 µl of antigen mixed with adjuvant were injected at two sites subcutaneously and 100 µl intraperitoneally. For the rabbits, 50 µl of antigen mixed with adjuvant

were injected at six sites intradermally, 200 µl were injected intramuscularly into each hind leg, 100 µl were injected subcutaneously, in the neck region and 200 µl intraperitoneally. The amounts of antigen injected in each animal are detailed in tables 2. 7 for the rats and 2. 8 for the rabbits.

	Prime	First Boost
A3	26 µg	61 µg
B4	39 µg	42 µg
D3	52 µg	77 µg
D7	67 µg	61 µg
F10	10 µg	34 µg
OC	23 µg	65 µg

Table 2.7 Amount of antigen injected for immunisation of the rats

The rats were sacrificed after the first boost of antigen.

	Prime	First Boost	Second boost	gp120[a]
A3	104 µg	243 µg	ND	150 µg
B4	155 µg	170 µg	211 µg	150 µg
D3	210 µg	310 µg	Bled	-
D7	268 µg	243 µg	Bled	-
F10	39 µg	135 µg	135 µg	150 µg
OC	94 µg	260 µg	Bled	-

Table 2.8 Amount of antigen injected for immunisation of the rabbits

[a] The gp120 used for the boost of the recombinant monomeric gp120 from the X4-tropic isolate IIIB (NIBCS, UK).

ND: Not Done

- The rabbits immunised with the KLH-VHH D3, D7 and OC were sacrificed after the second boost of antigen.

Chapter 3

Role of anti-HIV-1 Antibodies and complement during the course of infection

3.1 Introduction

HIV infection in individuals is characterised by two phases; the acute and the chronic phases. During the acute phase, non-specific symptoms appear (see introduction, section 1. 9), the plasma viral load increases and the CD4 T cell count declines. Two to four weeks after the onset of symptoms, the CD4 T cell count increases without reaching the initial level prior to infection and the viral load drops to a set point around six months post-infection (Levy, 1998). The infection then becomes chronic and is characterised by a gradual depletion in CD4 T cells. When the CD4 T cell count is below 200/μl, the individual is declared to be at the AIDS stage (Levy, 1998).

The arm(s) of the immune system responsible for the drop in viral load remain(s) unclear. CTLs are probably implicated, as CTL activity is detected in acute infection (Borrow et al., 1994) and CD8 T cell depletion in monkeys leads to an uncontrolled viraemia (Schmitz et al., 1999a). Although some NAbs in the acute phase have been reported (Lewis et al., 1998), they are unlikely to be involved as they are generally detected after the drop in viral load (Aasa-Chapman et al., 2004; Richman et al., 2003). Anti-gp120 Abs, however, are detected concomitantly with CTLs, during primary infection by ELISA (Aasa-Chapman et al., 2004; Moore et al., 1994a).

Non-neutralising anti-gp120 Abs from the acute infection, could, however, play a role in viral decline as they can inactivate HIV with NK cells *in vitro* (Forthal et al., 2001). The isotype of the Ab response against HIV-1 proteins, including gp120, is restricted primarily to IgG1 (Khalife et al., 1988). IgG1 (IgG3, IgG4 and less potently IgG2) can activate the classical pathway of complement. The classical pathway has been shown to be activated by neutralising anti-HIV sera on laboratory-adapted viruses (Spear et al., 1992). Complement-mediated inactivation (CMI), however, has not been tested on viruses and sequential autologous sera isolated during the acute phase of infection, where Ab-neutralisation is not detected.

This chapter investigates whether non-neutralising anti-gp120 Abs, detected early during the acute phase, can inactivate viruses with autologous and heterologous gp120

in the presence of complement. CMI is higher in the presence of non-neutralising sera than in presence of neutralising sera.

Non-neutralising anti-gp120 Abs, in association with complement, could, therefore, play a role in the viral decline observed in acute infection.

3.2 Results

For each CMI assay, a Student *t*-test was used to evaluate if there was a significant difference between data obtained in the presence and in the absence of complement. The probability (p value) chosen was 0.05 in order to evaluate as accurately as possible small differences. When the p value was above 0.05, no statistically significant CMI was observed under the experimental conditions used.

3.2.1 CMI of HIV-1 in the presence of heterologous cross-neutralising sera

3.2.1.1 TCLA strains

TCLA X4-tropic strains IIIB, SF2 and RF were tested with QC serum 2 and IIIB and RF were tested with QC serum 5 in the absence or the presence of complement to determine CMI (see p 91 for details on the QC sera). In the experiments described in Chapters 3 and 4, CMI represents the inactivation of HIV in the presence of (neutralising or non-neutralising) sera and complement. The results are shown in figure 3.1 (QC serum 2) and in figure 3.2 (QC serum 5).

Serum QC 2 alone neutralised IIIB, SF2 and RF by 90% at the reciprocal dilutions of 80, 320 and 40 respectively. CMI increased the 90% neutralisation titre by 2-fold only for IIIB. CMI was only observed for reciprocal dilutions 80 and 160. For SF2 and RF, CMI did not increase the 90% neutralisation titres.

A similar result was observed for QC serum 5 with IIIB. CMI also increased the 90% reciprocal neutralisation titre by 2-fold and was 320. CMI was only observed for reciprocal dilutions 320 and 640. For RF, CMI did not increase the 90% neutralisation titre. CMI was also tested for PIs.

3.2.1.2 PIs

PIs R5-tropic strains SF162 and SL2 were tested for CMI with QC sera 2 and 5 in the absence and the presence of complement. The results are shown in figure 3.3.

Serum QC 2 alone neutralised SF162 by 90% at the reciprocal dilution of 80. For SL2, 90% neutralisation was not observed. CMI increased the neutralisation titre for SL2, at the reciprocal dilutions of 20, 40 and 80. CMI did not increase the neutralisation titre for SF162 except at the reciprocal serum dilution of 160.

Serum QC 5 alone neutralised by 90% SF162 and SL2 at the reciprocal dilutions of 160 and 20 respectively. CMI did not increase the 90% neutralisation for either SF162 or SL2.

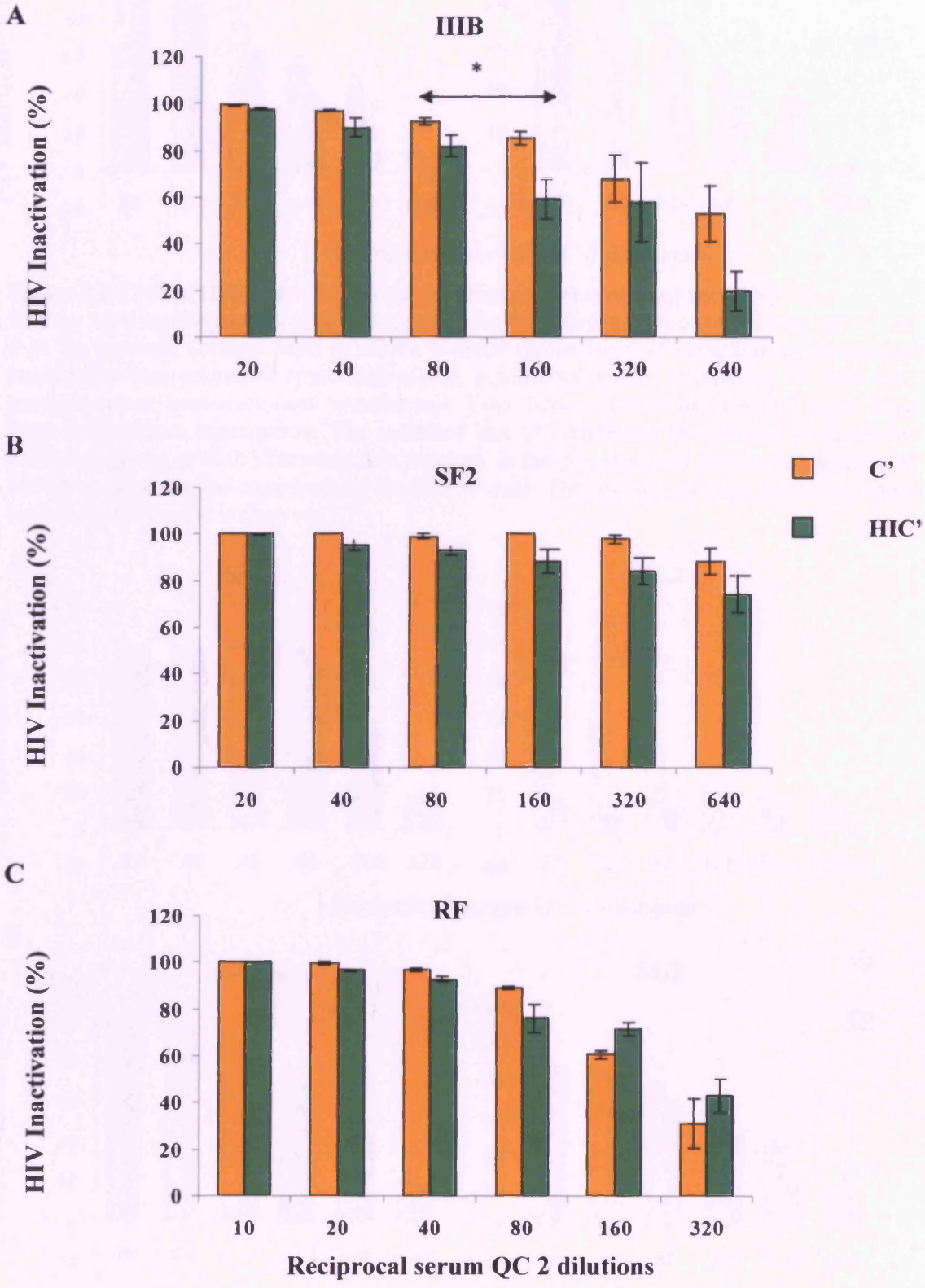


Figure 3.1 CMI of IIIB, SF2 and RF with the heterologous neutralising serum QC 2
Viruses were incubated with serial dilutions of the heterologous cross-neutralising serum QC 2 in the presence (orange bars) or the absence (green bars) of complement. Percentage inactivation was calculated from viral inputs, represented by the incubation of virus with complement or heat-inactivated complement. Error bars represent the standard error from three independent experiments. The indicated star (*) represents a statistically significant difference (*t*-test, $p<0.05$) between data obtained in the presence and in the absence of complement under the experimental conditions used. The arrows span the dilutions where significant difference is observed

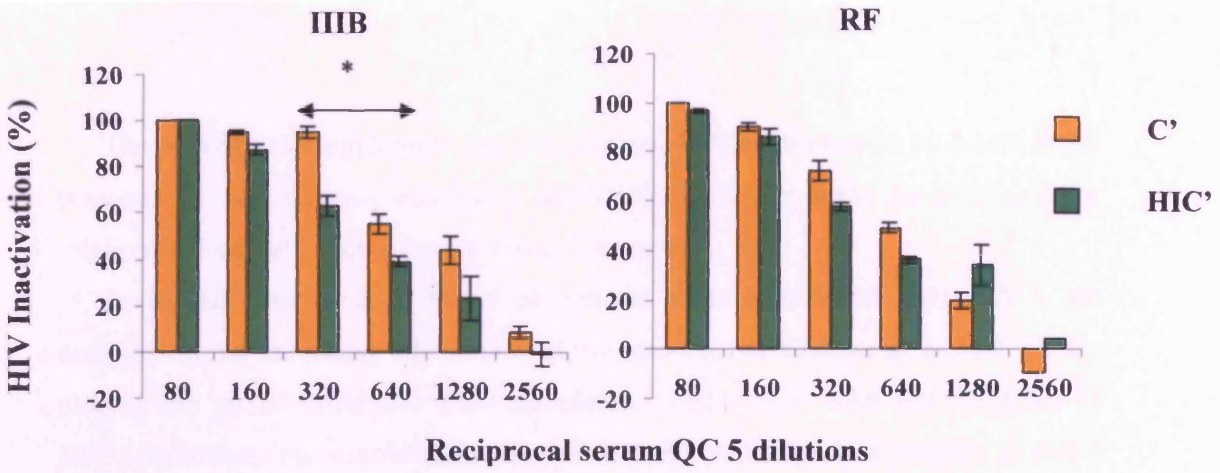


Figure 3.2 CMI of IIIB and RF with the heterologous neutralising serum QC 5
Viruses were incubated with serial dilutions of the heterologous cross-neutralising serum QC 5 in the presence (orange bars) or of the absence (green bars) of complement. Percentage inactivation was calculated from viral inputs, represented by the incubation of virus with complement or heat-inactivated complement. Error bars represent the standard error from three independent experiments. The indicated star (*) represents a statistically significant difference (*t*-test, $p<0.05$) between data obtained in the presence and data in the absence of complement under the experimental conditions used. The arrows span the dilutions where significant difference is observed

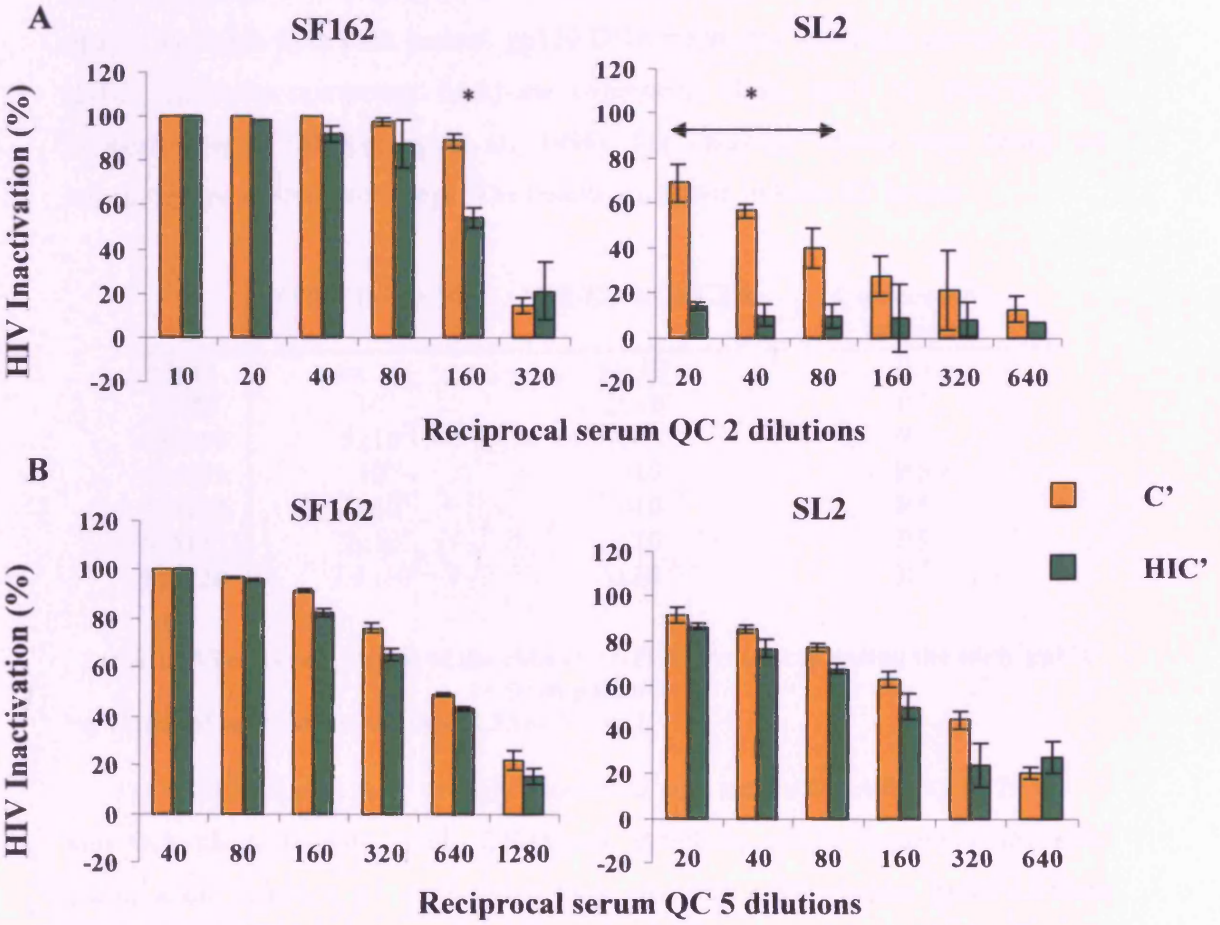


Figure 3.3 CMI of SF162 and SL2 with the heterologous sera QC 2 (A) and QC 5 (B)
Viruses were incubated with serial dilutions of the heterologous cross-neutralising sera QC 2 (A) and QC 5 (B) in the presence (orange bars) or the absence (green bars) of complement. Percentage inactivation was calculated from viral inputs, represented by the incubation of virus with complement or heat-inactivated complement. Error bars represent the standard error from three independent experiments. The indicated star (*) represents a statistically significant difference (*t*-test, $p<0.05$) between data obtained in the presence and in the absence of complement under the experimental conditions used. The arrows span the dilutions where significant difference is observed

The presence of complement can only enhance HIV neutralisation by 2-fold in the presence of some cross-neutralising anti-HIV-1 sera for some isolates and for dilutions where 90% neutralisation was not observed.

Sequential sera from a cohort of patients recently infected with HIV-1 are available to our laboratory (for details of the patients, see Chapter 2, table 2. 5). By cloning the gp120 sequences from samples, harvested soon after the diagnosis of HIV-1 infection, into a replication-competent HxB2 backbone, it is possible to detect autologous NAbs and determine CMI.

3.2.2 Neutralisation patterns of HIV gp120 from recently HIV-1 infected individuals

3.2.2.1 Description of the amplified gp120

Sequential sera from seven recently infected individuals were used for this study. gp120 sequences were amplified by nested PCR from genomic DNA of the first blood sample available from each patient. gp120 DNA fragments were then cloned into the HxB2 replication-competent backbone expressing HxB2 gp41 as described by McKeating *et al* (McKeating et al., 1996). The resulting viruses were tested for infectivity and co-receptor usage. The results are shown in table 3.1 below.

	NP2/CD4/CCR5	NP2/CD4/CXCR4	Co-receptor tropism
MM4	$5x\ 10^5$	$8x\ 10^2$	R5
MM8	10^6	$2x10^2$	R5
MM19	$5x10^5$	10^3	R5
MM22	10^4	<10	R5
MM23	$3x10^4$	<10	R5
MM27	$2x10^4$	<10	R5
MM28	$7.6x10^6$	$5x10^3$	R5

Table 3.1 Viral titres (ffu/ml) of the chimeric HIV-1 viruses expressing the early gp120 from patients
For details of each patient, see table 2.5 in Chapter 2

The CCR5 phenotype is strongly associated with the transmissibility of HIV-1 to new individuals (Moore et al., 2004) and, therefore, gp120 sequences amplified during acute infection are expected to have primarily a R5-tropism. Some of them could also use CXCR4 but the titres were between 2.2 and 3.5 logs lower than the titres obtained on NP2/CD4 expressing CCR5. Doms *et al* define dual tropic viruses

virus isolates that can equally use, within a log difference in infectivity, CCR5 and CXCR4 (Doms et al., 1998). Therefore, all viruses were categorised as R5-tropic.

3.2.2.2 Detection of the anti-gp120 Ab response

The detection of the specific anti-gp120 Ig response from each patient was determined by ELISA using the monomeric recombinant gp120 from IIIB. The results are shown in figure 3.4. For each patient, the detection of the anti-gp120 Ig response followed an exponential curve to reach a plateau at around 300 days POS. Anti-gp120 Abs could be detected at days 35, 49, 25, 25, 15, 28 and 19 days POS for the viruses MM4, MM8, MM19, MM22, MM23, MM27 and MM28 respectively.

3.2.2.3 Autologous neutralisation assays

The emergence of a specific anti-gp120 Ig does not correlate with the development of NAbs in the case of HIV (Aasa-Chapman et al., 2004; Moore et al., 1994a). Neutralisation assays were performed using heat-inactivated sequential sera from patients MM4, MM8, MM19, MM22, MM23, MM27 and MM28 using the chimeric HIV-1 virus expressing autologous gp120. The results are shown in table 3.2.

Patient sera	Days POS 90% neutralisation
MM4	206*
MM8	81*
MM19	519
MM22	NA
MM23	204
MM27	406
MM28	503

Table 3.2 Autologous neutralisation of chimeric viruses

The chimeric viruses were incubated with sequential autologous sera. The data represent the days POS, at which sera were harvested that neutralised autologous chimeric viruses by 90%. The percentage neutralisation was calculated from a viral input, represented by the incubation of the virus with HIV-negative serum, as described in 2.8.1

* from (Aasa-Chapman et al., 2004)

NA Not Applicable, as patient MM22 started anti-viral therapy 26 days POS

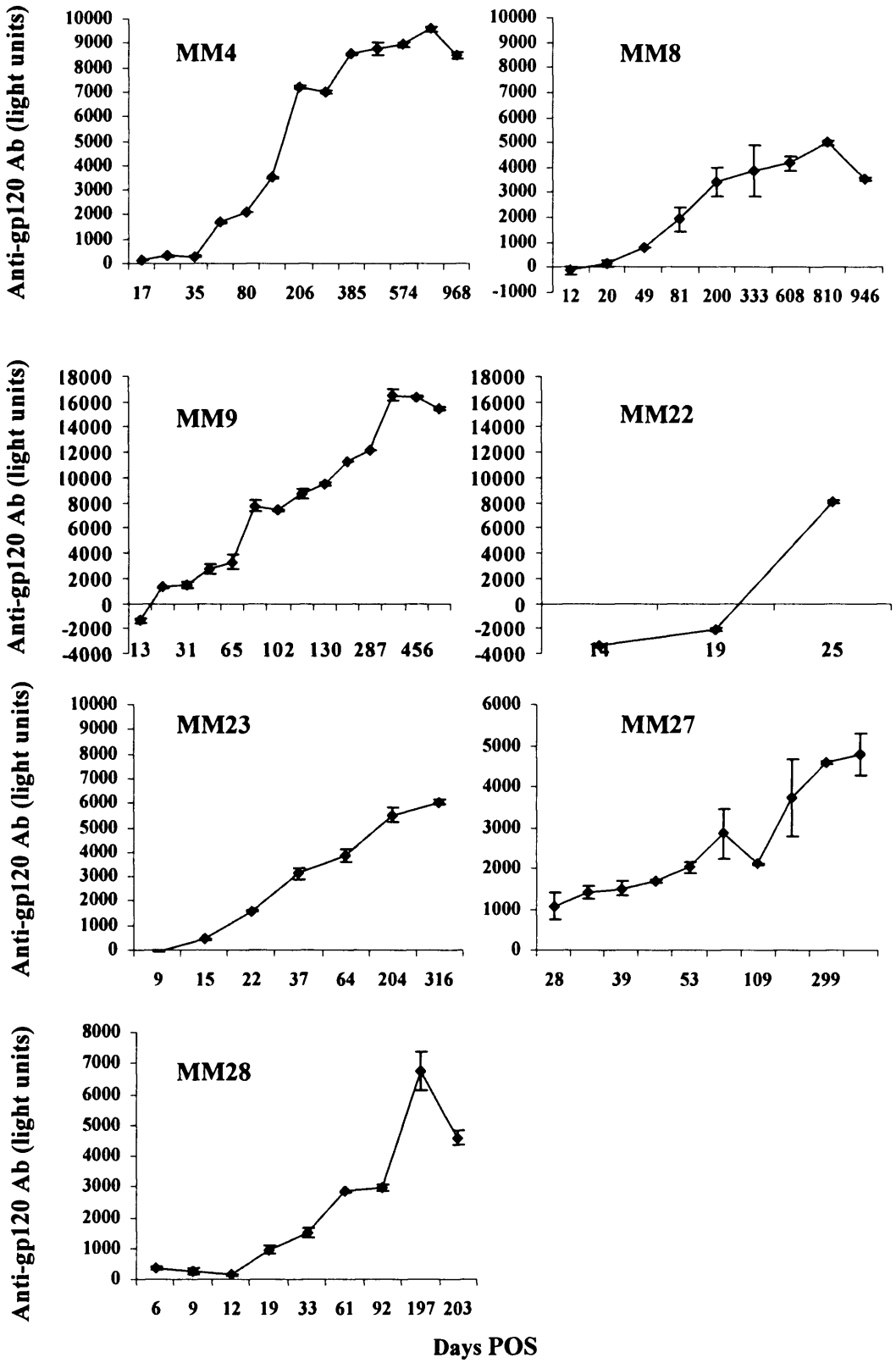


Figure 3.4 Anti-gp120 Ab detection in HIV-1 infected patients
Sequential sera on HIV-1 infected patients were diluted in 1/100 and were tested for the presence of anti-gp120 Abs by ELISA assay using monomeric recombinant gp120 from IIIB. The values for each time point were calculated from the background value given by HIV seronegative serum incubated on gp120 and by the absence of serum or gp120. Error bars represent the standard error from two experiments.

The detection of autologous NAbs varied from individual to individual. Three patterns of neutralisation could be distinguished. The first pattern was unique to chimeric virus MM8 that was neutralised after less than three months POS. The second pattern was seen with the chimeric viruses MM4 and MM23, where 90% neutralisation was observed between six and seven months POS. The third pattern contained MM19, MM27 and MM28, which were neutralised by 90% more than twelve months POS. For patient MM22, the NAb pattern could not be determined as this person underwent HAART treatment 26 days after onset of symptoms.

3.2.3.4 Heterologous neutralisation assays

The NAb pattern for each patient was determined using YU2 molecular clone. The results of the heterologous NAb patterns are shown in table 3.3.

Patient sera	Days POS 50% neutralisation	Days POS 90% neutralisation
MM4	ND[a]	ND[a]
MM8	333	608
MM19	889	>889[b]
MM22	NA	NA
MM23	498	>631[b]
MM27	>685[b]	>685[b]
MM28	>503[b]	>503[b]

Table 3.3. Heterologous neutralisation of YU2

YU2 was incubated with sequential sera. The data represent the days POS, at which the sera were harvested, which neutralised the virus by 50% or 90%. The percentage neutralisation was calculated from a viral input, represented by the incubation of the virus with HIV-negative serum as described in 2. 8. 1

[a] Not Done, not enough serum was available to perform the assay

[b] 50 or 90% neutralisation was not achieved with the latest serum available from patients

NA Not Applicable, as patient MM22 started anti-viral therapy 26 days POS

Neutralisation of YU2 was delayed compared to the autologous gp120- expressing virus as already observed (Aasa-Chapman et al., 2004). YU2 was only neutralised by 90% by sera from patient MM8, 20 months POS. None of the other sera neutralised YU2 by 90%. However, the virus could be neutralised by 50% by sera from patients MM19 and MM23, 30 and 16 months POS, respectively. The sera available from the patients MM27 and MM28 did not neutralise YU2 even by 50%.

Complement can enhance neutralisation of some laboratory-adapted strains of HIV-1 by a maximum of 2-fold using cross-neutralising sera in our study (see sections

3. 2. 1. 1). The effect of complement was next investigated using early neutralising sera (≥ 90 % neutralisation) and chimeric viruses expressing autologous gp120.

3.2.3 CMI of viruses in the presence of autologous neutralising sera

The chimeric viruses were incubated with autologous neutralising sera in the absence and in the presence of complement. The assay was not performed using MM22 as neutralising sera were not available. The results are shown in figure 3.5.

In the absence of complement, the reciprocal serum dilution for achieving 90% neutralisation for chimeric viruses MM4, MM8, MM19, MM23, MM27 and MM28 were 20, 40, 20, 160, 40 and below 10 respectively. CMI enhanced by 2-fold the 90% neutralisation titres for viruses MM4, MM8, MM19 and MM28 to 40, 80, 40 and 10. For these viruses, complement increased HIV neutralisation at Ab dilutions where 90% neutralisation was not achieved. For viruses MM23 and MM27, CMI was not observed.

CMI was then tested using early non-neutralising sera, harvested during the first month POS, in combination with autologous viruses.

3.2.4 CMI of viruses in the presence of autologous non-neutralising sera

Viruses were tested in CMI assays in the presence of non-neutralising sera, harvested during the early phase of infection. The figure 3.6 shows results for each patient using non-neutralising sera from two time points, positive for the presence of anti-gp120 Abs.

In the absence of complement, 90% neutralisation of the chimeric viruses was not achieved. The chimeric viruses MM4, MM8 and MM19 were neutralised by 50% or less. The other chimeric viruses, MM22, MM23, MM27 and MM28 were not neutralised.

In the presence of complement, three patterns of CMI were observed. High CMI (90%) was observed for viruses MM4, MM8 and MM19. A more modest increase (50-70%) was observed for viruses MM23 and MM28. For virus MM27, CMI was not detected in the presence of serum harvested at day 28 POS but was observed for day 39 POS.

For viruses MM14, MM19, MM23, MM27 and MM28, CMI was detected at low reciprocal serum dilutions, varying from 10 to 40. For MM8, however, CMI was observed for every serum dilution (up to 320). No CMI was observed for MM22.

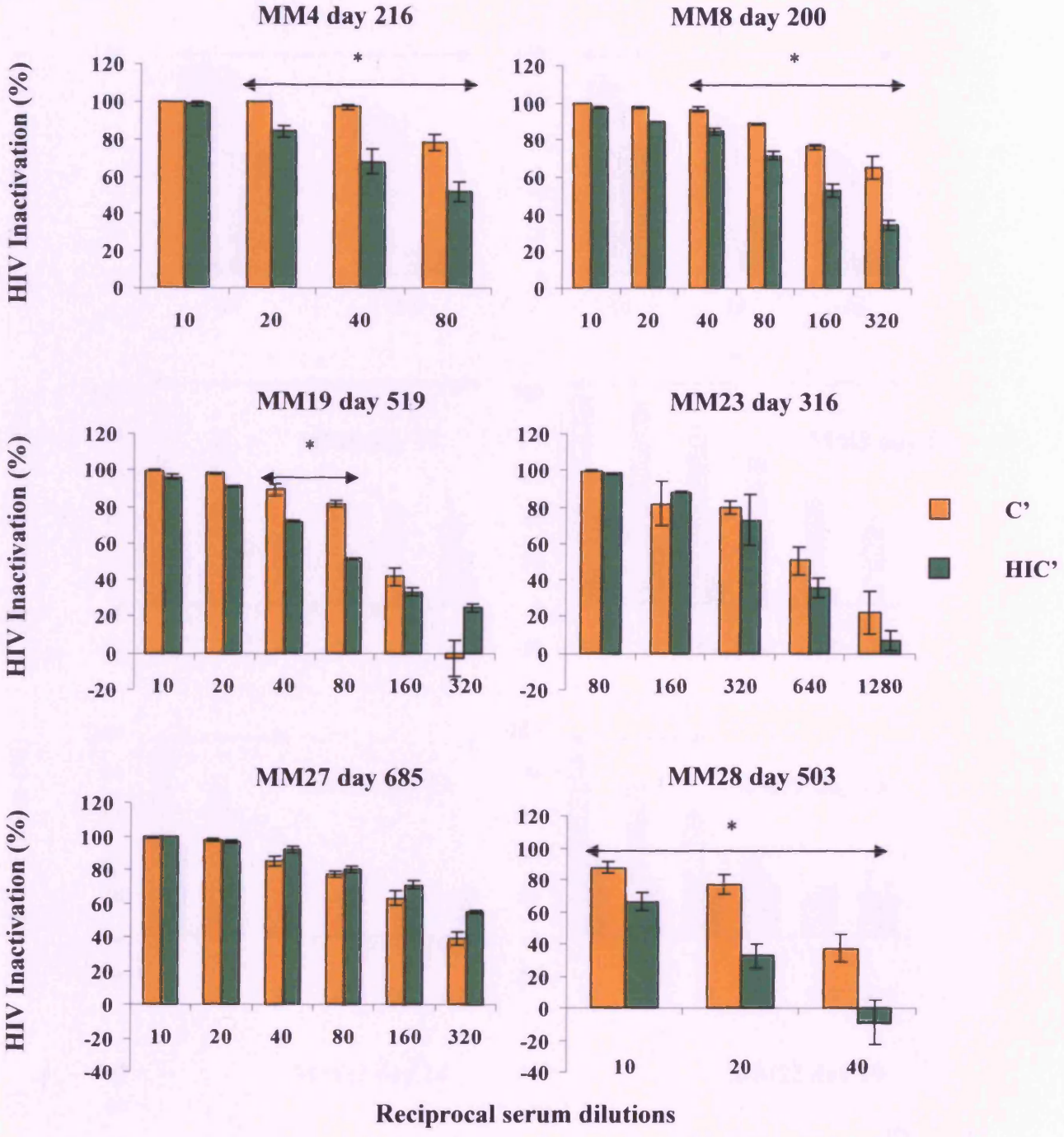
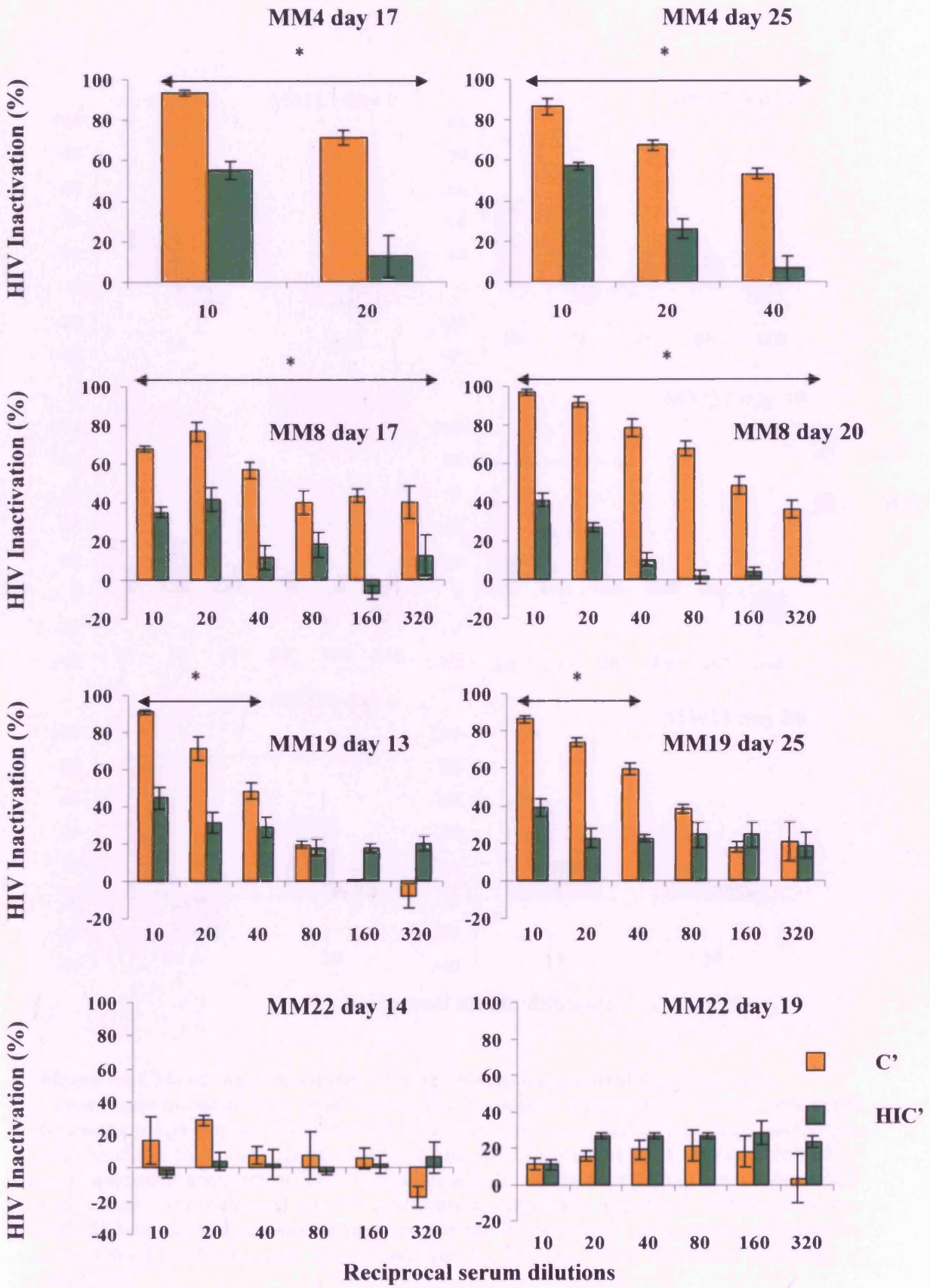


Figure 3.5 CMI of chimeric viruses with autologous neutralising sera
Viruses were incubated with serial dilutions of autologous neutralising sera in the presence (orange bars) or the absence (green bars) of complement. Percentage inactivation was calculated from viral inputs, represented by the incubation of virus with complement or heat-inactivated complement. Error bars represent the standard error from three independent experiments. The indicated star (*) represents a statistically significant difference (*t*-test, $p<0.05$) between data obtained in the presence and in the absence of complement under the experimental conditions used. The arrows span the dilutions where significant difference is observed


MM4 day 17
MM4 day 25
MM8 day 17
MM8 day 20
MM19 day 13
MM19 day 25
MM22 day 14
MM22 day 19
HIV Inactivation (%)
Reciprocal serum dilutions
C'
HIC'
*
100
80
60
40
20
0
-20
-40
10
20
40
80
160
320

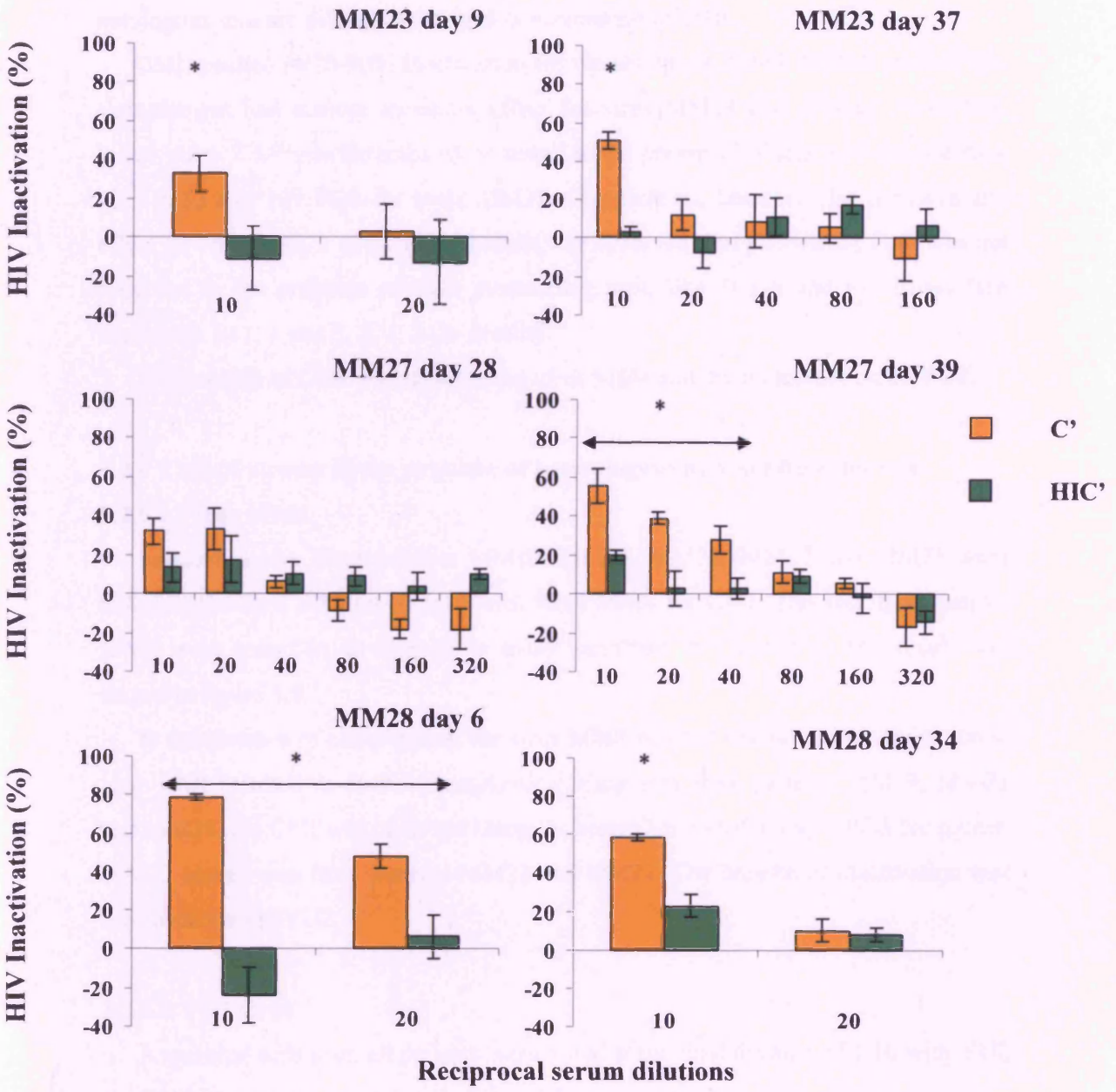


Figure 3.6 CMI of chimeric viruses with autologous non-neutralising early sera
Viruses were incubated with serial dilutions of autologous non-neutralising early sera in the presence (orange bars) or in the absence of complement (green bars). Percentage inactivation was calculated from viral inputs, represented by the incubation of virus with complement or heat-inactivated complement. Error bars represent the standard error from three independent experiments. The indicated star (*) represents a statistically significant difference (*t*-test, $p<0.05$) between data obtained in the presence and in the absence of complement under the experimental conditions used. The arrows span the dilutions where significant difference is observed

3.2.5 CMI of autologous viruses in the early course of infection

CMI assays were performed using autologous sera for each patient and for each time point. The results shown in figure 3.7 represent the inactivation curves when the autologous sera are diluted at the final concentration of 1/10.

CMI resulted in 70-90% inactivation for viruses MM4, MM8, MM19 and MM28. Complement had a more moderate effect for virus MM23 and resulted in 40-50% inactivation. CMI was detected when tested in the presence of sera harvested at days 32, 39, 53 and 109 POS for virus MM27. Complement, however, did not have any effect on virus MM22 and no inactivation was observed. For all viruses, CMI was not observed in the presence of 90% neutralising sera, like TCLA and PI viruses (see sections 3. 2. 1. 1 and 3. 2. 1. 2 for details).

The breadth of CMI was tested using virus MM4 and the molecular clone YU2.

3.2.6 CMI of viruses in the presence of heterologous non-neutralising sera

3.2.6.1 Virus MM4

Sequential sera from patients MM19, MM22, MM23, MM27 and MM28 were tested at the final dilution of 1/10 with virus MM4 for CMI. The sera from patient MM8 were tested in an alternative assay described in Chapter 4. The results are shown in figure 3.8.

In the absence of complement, the virus MM4 was not neutralised by the patients' sera. CMI resulted in 50-90% inactivation using sera from patients MM19, MM23 and MM28. No CMI was observed using the serum harvested at day 9 POS for patient MM23 and all sera from patients MM22 and MM27. The breadth of inactivation was then tested using YU2.

3.2.6.2 YU2 virus

Sequential sera from all patients were tested at the final dilution of 1/10 with YU2 for CMI. The results are shown in figure 3.9.

In the absence of complement, YU2 was 90% neutralised by none of the sera except sera from day 385 POS from patient MM4. CMI resulted in 65-95% inactivation for all patients except for patient MM27. CMI was not observed in the presence of neutralising sera from patient MM4 from day 385 POS.

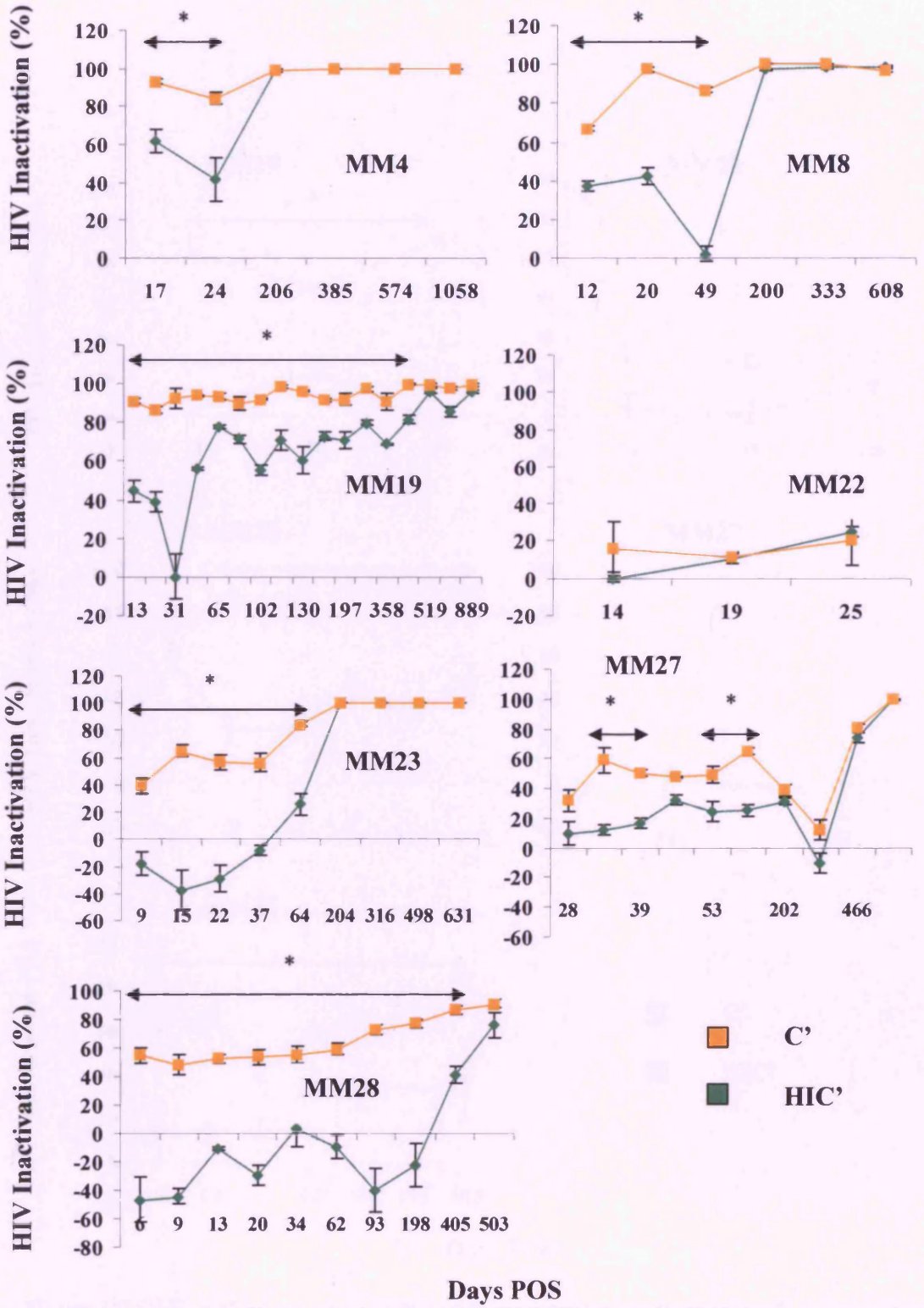


Figure 3.7 CMI of chimeric virus using sequential autologous sera.
Viruses were incubated with sequential autologous sera (at the final dilution of 1/10) in the presence (orange bars) or the absence (green bars) of complement. Percentage inactivation was calculated from viral inputs, represented by the incubation of virus with complement or heat-inactivated complement. Error bars represent the standard error from three independent experiments. The indicated star (*) represents a statistically significant difference (*t*-test, $p<0.05$) between data obtained in the presence and in the absence of complement under the experimental conditions used. The arrows span the time points where significant difference is observed

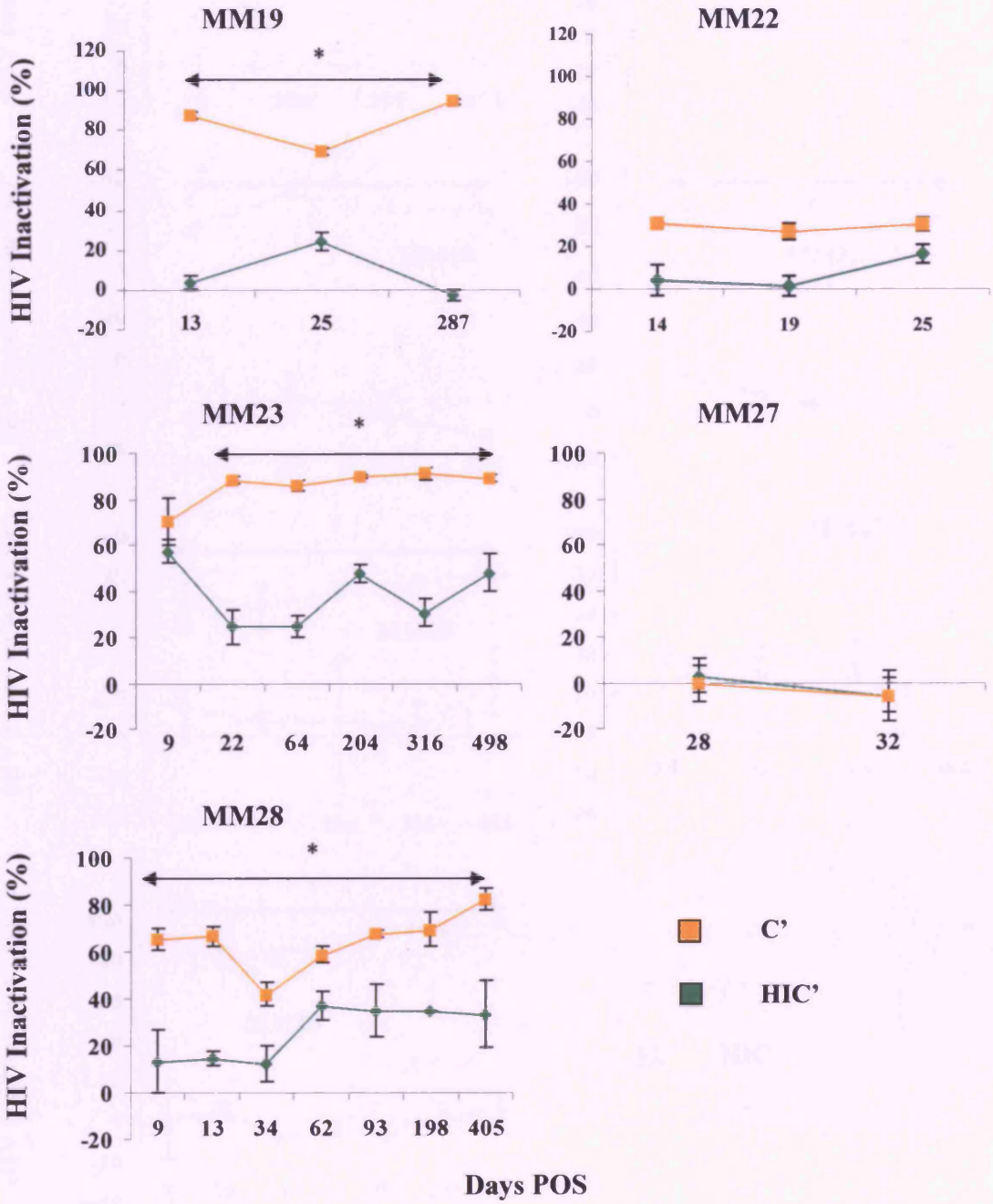


Figure 3.8 CMI of chimeric virus MM4 with sequential sera from recently infected patients
Virus MM4 was incubated with non-neutralising sera (at the final dilution of 1/10) in the presence (orange bars) or the absence (green bars) of complement. Percentage inactivation was calculated from viral inputs, represented by the incubation of virus with complement or heat-inactivated complement. Error bars represent the standard error from three independent experiments. The indicated star (*) represents a statistically significant difference (*t*-test, $p<0.05$) between data obtained in the presence and in the absence of complement under the experimental conditions used. The arrows span the time points where significant difference is observed

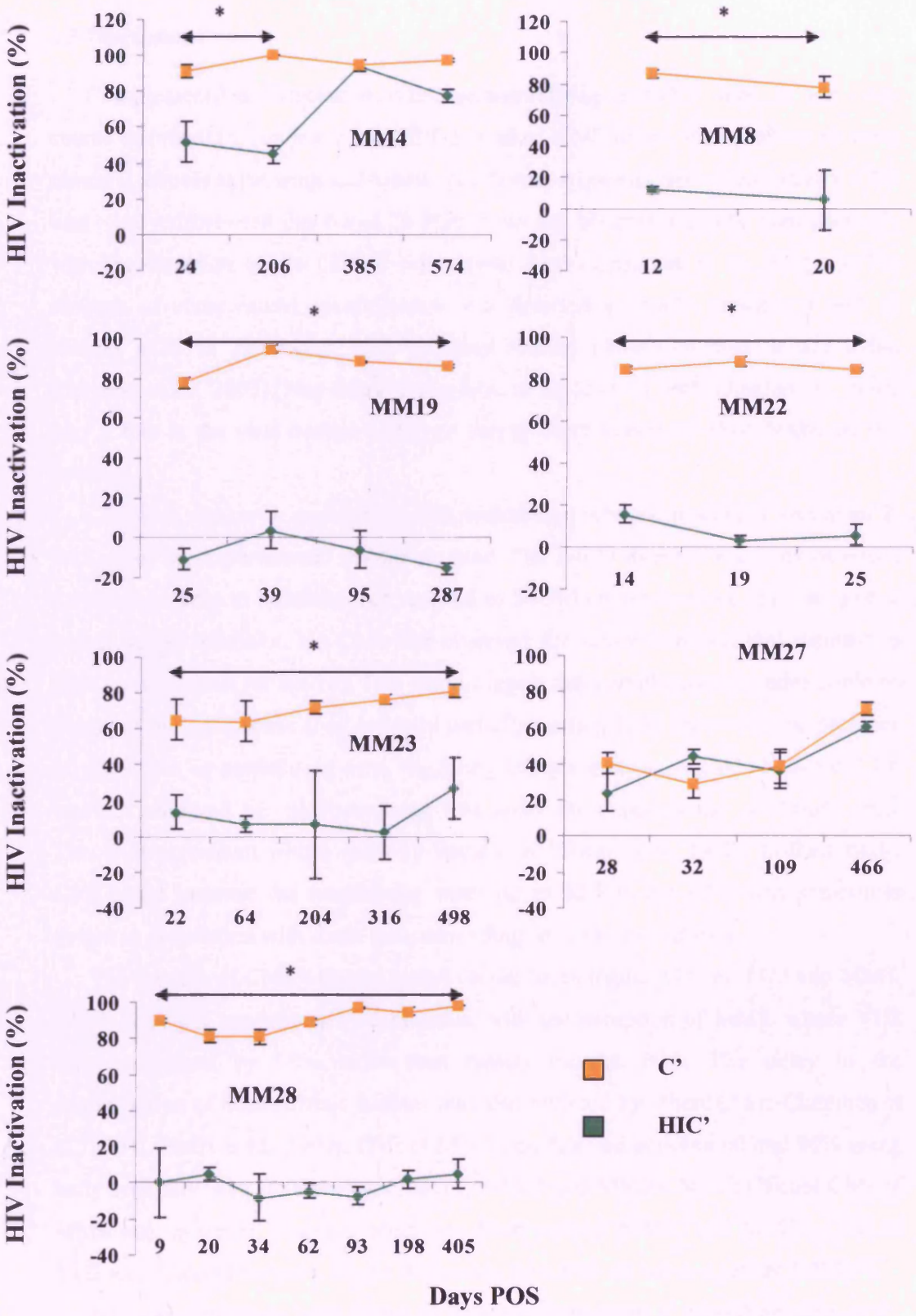


Figure 3.9 CMI of YU2 with sequential sera from recently infected patients
YU2 was incubated with non-neutralising sera (at the final dilution of 1/10) in the presence (orange bars) or the absence (green bars) of complement. Percentage inactivation was calculated from viral inputs, represented by the incubation of virus with complement or heat-inactivated complement. Error bars represent the standard error from three independent experiments. The indicated star (*) represents a statistically significant difference (*t*-test, $p<0.05$) between data obtained in the presence and in the absence of complement under the experimental conditions used. The arrows span the time points where a significant difference is observed

3.3 Discussion

Complement, in association with non-neutralising anti-HIV sera, in the early course of infection, can inactivate HIV-1 *in vitro*. CMI assays were performed using chimeric viruses expressing autologous gp120 with sequential sera from patients. CMI was observed between day 6 and 28 POS in six out of seven patients, concomitantly with the detection of the CD8 T cell activity (Aasa-Chapman et al., 2005). In the absence of complement, neutralisation was detected generally between 6 and 12 months POS in agreement with previous studies (Aasa-Chapman et al., 2004; Richman et al., 2003). Non-neutralising Abs, in association with complement, could play a role in the viral decline observed during acute infection, when NAbs are not detected.

CMI was weaker in association with neutralising sera and reached a maximum 2-fold under the experimental conditions used. The 2-fold increase was only observed for some isolates at dilutions that resulted in 50-70% neutralisation, and was lost at higher serum dilutions. No CMI was observed for serum dilutions that resulted in 90% neutralisation (or above). This could suggest that complement cascades could be triggered by NAbs when they are only partially coating HIV. However, the presence of non-NAbs in neutralising sera, triggering complement, cannot be ruled out. CMI was not observed for all neutralising sera under the experimental conditions used. This is in agreement with a study by Spear *et al* (Spear et al., 1992). In their study, CMI could increase the neutralising titres up to 32-fold but CMI was sometimes absent in association with some sera, depending on the source of Abs.

The breadth of CMI was also tested on the heterologous viruses YU2 and MM4, which were not neutralised by sera alone, with the exception of MM8, where YU2 was neutralised by 90% more than twenty months POS. The delay in the neutralisation of heterologous isolates was also reported by others (Aasa-Chapman et al., 2004; Albert et al., 1990). CMI of MM4 was detected between 60 and 90% using early sera harvested from patients MM19, MM23 and MM28. No significant CMI of MM4 was, however, detected using sera from patients MM22 and MM27. CMI of YU2 was detected between 70 and 90% using sera from all but one patient (MM27).

HIV has previously been shown to activate complement *in vitro* (Saifuddin et al., 2000; Spear et al., 1991b; Tacnet-Delorme et al., 1999) in the presence and the absence of anti-HIV Abs (Spear et al., 1992; Tacnet-Delorme et al., 1999). The Ab-

independent complement activation against HIV might be triggered by natural IgM, present in all sera (Saarloos et al., 1995). In this study, all CMI values were calculated from viral inputs, determined after the incubation of the virus with fresh human serum containing natural Abs, as the complement source. CMI mediated by natural Abs was therefore excluded from the calculations.

Ab-dependent CMI can be triggered by Abs directed to cell protein complexes, particularly MHC class I and II (Spear et al., 2001; Spruth et al., 1999). Moreover, CMI against TCLA viruses was observed using sera from macaques containing anti-cellular Abs (Spear et al., 1993a). For patient MM22, CMI was not observed against chimeric autologous virus but was detected at 90% against YU2. For patient MM27, CMI was observed against the autologous virus (50% inactivation with some sera) but not against YU2. Since these two viruses are produced in the same cell line (293T) they express the same cellular protein complexes, it is therefore unlikely that CMI was triggered by Abs against MHC class I and II proteins, which could be present in the HIV-1 positive sera used in this study. Thus, CMI observed was likely to be due to the presence of anti-HIV Env Abs in the sera of patients.

Envs of the chimeric viruses, described in 3. 2. 2, consisted of gp41 from the TCLA strain HxB2 and gp120 amplified from patient DNA. Anti-gp41 Abs are unlikely to be the main Abs involved in CMI, as sera from patient MM27 inactivated the autologous chimeric virus but not YU2. Therefore, it is likely that CMI was triggered by anti-gp120 Abs.

Complement can activate the alternative pathway (Tacnet-Delorme et al., 1999), the MBL (Ying et al., 2004) and the classical pathway (Spear et al., 1992). As CMI, described in this chapter, was triggered by anti-gp120 Abs, the classical pathway is likely to be the branch of complement involved. IgM and IgG can equally trigger complement and, therefore, the isotype of anti-gp120 Abs should be determined. CMI could be dependent on the presence of epitope-specific Abs in sera. To address this issue, CMI will be tested with MAbs mapping to various regions of gp120 and with sCD4-IgG2. The influence of the cell line in the modulation of CMI has to be studied. All these issued are addressed in Chapter 4.

Chapter 4

Characterisation of complement-mediated inactivation of HIV-1 *in vitro*

4.1 Introduction

Complement consists of more than 30 serum proteins and cell surface receptors, organised in three cascades: the classical pathway, the alternative pathway and the MBL pathway. The three complement cascades are schematised in figure 4.1. The serum proteins of complement are zymogens that are pro-enzymes requiring proteolytic cleavage to become active. All three cascades lead to the formation of the central component C3 and its cleavage triggers the late steps of complement activation, by the formation of the MAC.

The classical pathway (the first historically described) is triggered by Abs (IgM, IgG1, IgG3 and, less potently IgG2), by the interaction of C1q with the CH2 domain of IgG and the CH3 domain of IgM in a crab-like configuration. This subsequently leads to the rearrangement of C1q, which reveals interactions sites with two molecules of C1r. C1r then interacts with two molecules of C1s and C1q, C1r and C1s form the C1 complex. The C1 complex then cleaves C4 into C4a and C4b via a Ca^{2+}-dependent-mechanism. C4b then cleaves C2 into C2b and C2a and $\overline{C4b2a}$ form the C3 convertase that cleaves C3 into C3a and C3b, leading to the triggering of the late steps of complement cascades. $\overline{C4b2a3b}$ constitutes the C5 convertase and cleaves C5 into C5a and C5b. C5b binds to C6 and C7 and forms $\overline{C5b67}$ that has hydrophobic properties and inserts into membranes. C8 subsequently inserts into the membrane, followed by polymerised C9 that generates the MAC.

The MBL pathway is activated by the recognition of Pathogen-Associated-Molecular-Patterns (PAMPs) characteristically repeats of Mannose and N-acetyl glucosamine or GlcNac (Carrol, Nat Immunol, 2004). MBL is structurally similar to C1q and associates with MASP1 and MASP2, which resemble C1r and C1s. The MBL cascade is identical to the classical pathway.

Unlike the classical and the MBL pathways, the alternative pathway is constitutively activated and is characterised by the spontaneous cleavage of an unstable thioester bond within C3 into C3a and C3b. C3b can bind to surface antigens. When bound on a surface, C3b binds to factor B that is subsequently cleaved by factor

D and C3bBb is generated. This complex has a C3 convertase activity and can cleave more C3, amplifying the alternative pathway and triggering the late steps of complement described above.

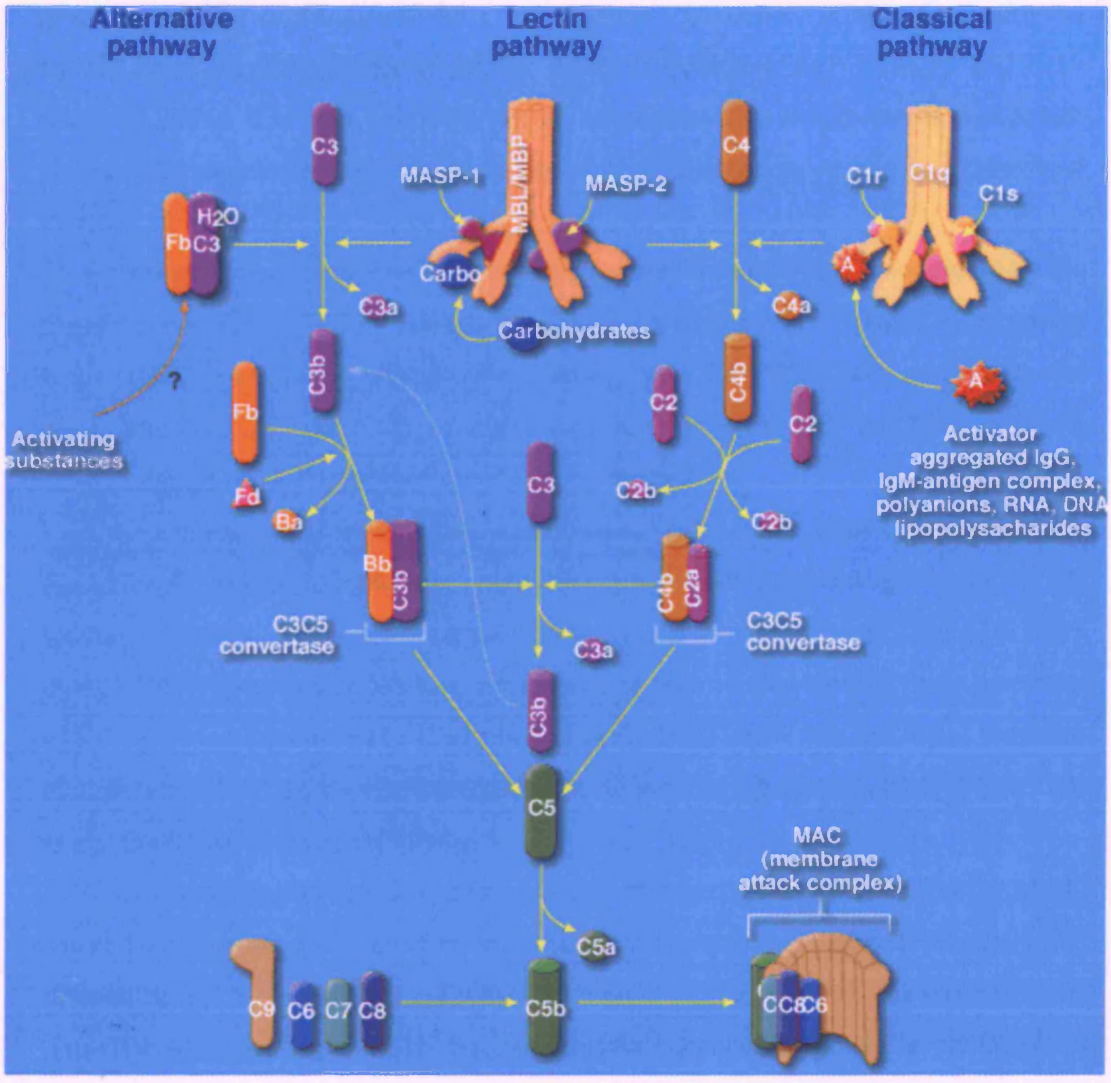


Figure 4.1 Complement cascades
The classical pathway is triggered by IgG and IgM. The MBL pathway is triggered by mannose residues on pathogens and the alternative pathway is spontaneously activated and is mainly regulated by the presence of complement regulatory proteins (CRP) at the surface of the cells. Adapted from biocarta.com

Complement is directly involved in fighting pathogens by the formation and the insertion of the MAC into membranes, which leads to lysis. C3 also entirely coats the pathogens (known as opsonisation), which, sometimes, is sufficient for inactivation by steric hindrence (Spear et al., 2001). Opsonised antigens are presented to B cells via interactions with C3 degradation products, such as iC3b, C3d, C3dg, C3b and C4b,

and CD35 (CR1) and CD21 (CR2) expressed on B cells and FDCs. These interactions lower the threshold for B cell stimulation and the uptake of antigens by FDCs. The maintenance of antigen presentation to FDCs results in the selection of high affinity antibody-secreting B cells (Boes, 2000; Carroll, 2004). Complement is also thought to play a role in the regulation of the T cell response, via uptake of opsonised antigens by APC and the modulation of release of some cytokines such as IL12 and IL10 (Carroll, 2004). C3a and C5a also act as anaphylotoxins, which increase vascular permeability and recruit other components of the inflammatory response to the site of infection.

Complement can be potentially harmful to the self so it is tightly regulated by the expression of CRPs. CD59 inhibits the formation of the MAC. Decay accelarating factor (DAF or CD55) inhibits the binding of C2 to C4b (or accelerates the dissociation of C2a from C4b). Membrane co-factor protein (MCP or CD46) acts as a co-factor to promote the catabolism of C4b by factor I.

Complement is activated by many pathogens, like bacteria, fungi and also viruses. For example, human complement inactivates non-primate mammalian viruses via the binding of natural Abs against α-galactosidase motifs found on viral Envs (Takeuchi et al., 1996). Complement has also been shown to be activated by HIV in the presence of anti-HIV sera (Spear et al., 1992; Stoiber et al., 2001). HIV has also been shown to incorporate CRPs into its membrane, in order to prevent total inactivation (Montefiori et al., 1994; Saifuddin et al., 1994).

This chapter explores the mechanism of CMI described in Chapter 3. First of all, the pathway involved was elucidated, as HIV has been show to activate the classical, alternative and MBL pathways (Tacnet-Delorme et al., 1999). Then, the isotype of the anti-HIV Abs involved in CMI was investigated, particularly in the acute phase of infection, where anti-HIV IgG are barely detectable. CMI was tested using neutralising and non-neutralising MAbs, mapping to various regions of gp120. CMI against HIV is modulated by the incorporation of CRPs in the viral membrane and therefore could depend on the HIV producing-cell line (Saifuddin et al., 1994). HIV viruses were grown in several cell lines and CMI was investigated.

4.2 Results

For each CMI assay, a Student *t*-test was used to evaluate if there was a significant difference between data obtained in the presence and in the absence of complement. For the complement titration experiment described in 4. 2. 2, the same test was used to evaluate if there was a significant difference between data obtained with 10% and 20% of complement. The probability (p value) chosen was 0.05 in order to evaluate small differences as accurately as possible. Therefore, when the p value was above 0.05, no statistically significant CMI (or no difference between 10 and 20% complement) was observed under the experimental conditions used.

For comparing the neutralisation profiles between the same isolate grown in two different cell types, the same statistical test was used. There was a significant difference between neutralisation data obtained in one cell type and the other cell type when the p value was below 0.05 under the experimental conditions used.

4.2.1 Involvement of complement

Burrer *et al* have reported that soluble factors in serum can inhibit HIV growth *in vitro* (Burrer et al., 2001). To formally involve complement in CMI described in Chapter 3, inactivation assays were carried out using Guinea pig purified complement.

4.2.1.1 Purified Guinea pig complement

Guinea pig complement has been shown to be activated by HIV (Spear et al., 1991b). The chimeric virus MM4 was incubated with serum from day 49 POS from patient MM8 and purified Guinea pig complement. The results are shown in figure 4.2.

In the presence of heat-inactivated Guinea pig complement, MM4 was not neutralised. Purified Guinea pig complement inactivated the chimeric virus by 70% and 40% at the reciprocal dilutions of 10 and 20, respectively. This supports that the inactivation observed in Chapter 3, where whole seronegative human serum was used as a source of complement, is mainly due to the complement itself rather than some other heat labile compounds found in the serum.

The identity of the complement pathway involved in HIV inactivation was then investigated.

4.2.1.2 EDTA/EGTA-treatment of complement

Complement consists of cascades of proteases. The first step of the classical pathway is the formation of the C1 complex, which requires Ca^{2+} ions. EGTA preferentially chelates Ca^{2+} ions whereas EDTA chelates all bivalent ions (Spear et al., 1991a). Therefore, EGTA inactivates the classical pathway alone whereas EDTA inactivates the three different pathways.

MM19 was tested for CMI using complement, heat-inactivated complement, EDTA- and EGTA-treated fresh human serum with the autologous from day 65 POS. The results are shown in figure 4.3.

In the presence of EDTA-treated serum and EGTA-treated serum, no CMI was observed with MM19 as observed in the presence of heat-inactivated complement. These results showed the involvement of the classical pathway in CMI.

Fresh snap-frozen sera from C1q- and C3- deficient individuals, kindly provided by Dr. Marina Botto from the Hammersmith hospital in London, were used for CMI assays to further confirm the role of the classical pathway.

4.2.1.3 Serum from C1q- and C3-deficient individuals as source of complement

CMI assays were performed using chimeric viruses MM8 and MM28 with autologous sera from day 12 and 6 POS in the presence of C1q- and C3-deficient sera. The results are shown in figure 4.4.

In the presence of C3- and C1q-deficient sera, no CMI was observed for either virus. The addition of recombinant C3, at a final concentration of 1.3 mg/ml, restored the inactivation of HIV for both viruses at 50 and 70%.

Results from 4.2.1 demonstrate that the classical pathway of complement, in association with anti-HIV Abs, is involved in CMI described in sections 3. 2. 5 and 3. 2. 6.

4.2.2 Titration of complement

All assays performed in Chapter 3 and in 4. 2. 1 used complement at the final concentration of 10% as already described (Saifuddin et al., 1995). CMI assays were repeated using complement diluted at 20% to test whether an increased concentration of human complement could enhance CMI with neutralising and non-neutralising sera. The chimeric virus MM19 was tested with early and late autologous sera. The results are shown in figure 4.5.

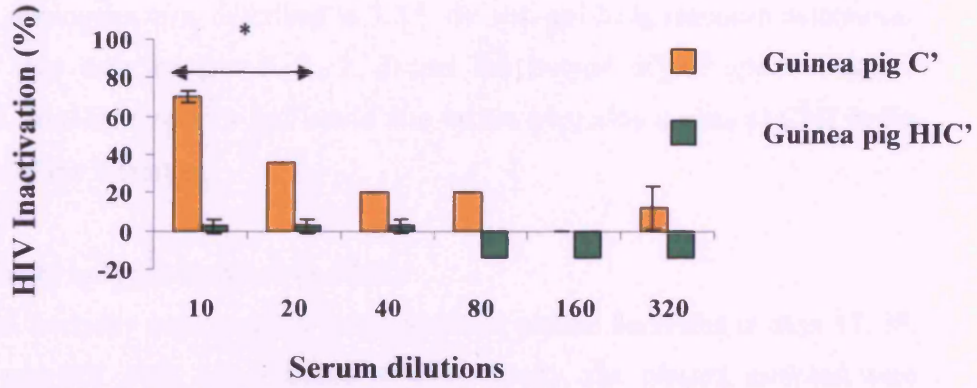


Figure 4.2 CMI assay using purified complement from Guinea pig
MM4 was incubated with serial dilutions of the heterologous serum from day 49 POS from patient MM19 in the presence (orange bars) or the absence (green bars) of Guinea pig complement. Percentage inactivation was calculated from viral inputs, represented by the incubation of virus with complement or heat-inactivated complement. Error bars represent the standard error from three experiments. The indicated star (*) represents a statistically significant difference (*t*-test, $p<0.05$) between data obtained in the presence and the absence of complement under the experimental conditions used. The arrows span the dilutions where significant difference is observed

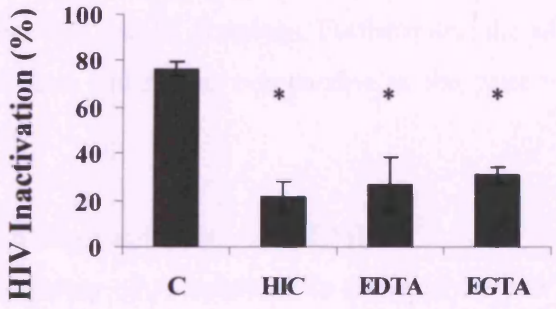


Figure 4.3 Inactivation of complement by EDTA/EGTA treatment
MM19 was incubated with the autologous serum from day 65 POS (at the final dilution of 1/10), in the presence of complement C, heat-inactivated human serum, fresh human serum treated with EDTA or EGTA at the final concentration of 2.5mM. Percentage inactivation was calculated from viral inputs, represented by the incubation of virus with complement, heat-inactivated complement, EDTA-treated or EGTA-treated complement. Error bars represent the standard error from three experiments. The indicated star (*) represents a statistically significant difference (*t*-test, $p<0.05$) between data obtained in the presence of complement and with inactivated complement under the experimental conditions used

Complement inactivated the chimeric virus as efficiently regardless of the final concentration of complement. The amount of complement proteins in 10% serum is sufficient to mediate maximum CMI.

The classical pathway is activated by IgM or IgG. In the longitudinal study using sequential autologous sera, described in 3.2.5, the anti-gp120 Ig response determined by ELISA was detected (see 3. 2. 2. 2) and the isotype of anti-gp120 was not determined. Anti-HIV IgM or IgG could also be the triggering agents of CMI in the early phase of the infection.

4.2.3 Antibody isotypes involved in CMI

The IgG fractions were purified from sequential plasma harvested at days 13, 39, 130, 519 and 701 POS from patient MM19. Briefly, the plasma samples were centrifuged and supernatants were applied onto a protein G column. The Fall Through (FT) fractions were kept and, after washing, the IgG were eluted. CMI assays were performed using purified IgG and FT fractions. The results are shown in figure 4.6.

For the earliest time point, CMI was observed in the FT fraction at days 13 and 39 POS (figure 4.6.A). With later time points, CMI was lost in FT fractions and was undetectable in time points 519 and 701. In parallel, no CMI was observed with the purified IgG from the earliest time point (day 13 POS) but developed with later time points (days 39 and 130 POS). At late time points (days 519 and 701 POS), no CMI was detected in both IgG and FT fractions. Furthermore, the additive potency of both fractions, for each time point, was comparable to the pattern obtained with whole serum (figure 4.6.B).

4.2.4 Monoclonal human antibodies and CMI

The classical pathway of complement is activated by one molecule of IgM in a "crab-like" conformation or by the agglomeration of IgG3, IgG1 and, more weakly, IgG2. The IgG isotype in the anti-HIV Ab response is primarily constituted of IgG1 (Khalife et al., 1988).

To identify if particular HIV epitopes were involved in CMI, a series of human anti-gp120 MAbs (all with an IgG1 isotype except IgG3 442-57D), mapped to the V3 loop, the CD4BS, the V2 loop, the C5 region and to CD4-induced epitopes (binding to the co-receptor region) were tested individually for their properties in CMI of the

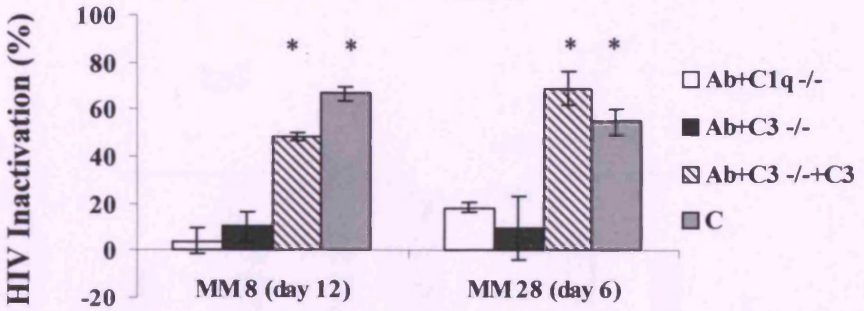


Figure 4.4 CMI assays using fresh serum from C1q- and C3-deficient individuals as sources of complement

MM8 and MM28 were incubated with autologous sera from days 12 and 6 POS (at the final dilution of 1/10) in the presence of serum from C1q- and C3-deficient individuals. As a control, recombinant C3 was added at 1.3 mg/ml. Percentage inactivation was calculated from viral inputs, represented by the incubation of the virus with complement, heat-inactivated complement, C1-deficient serum, C3-deficient serum and C3-deficient serum supplemented with C3. The indicated star (*) represents a statistically significant difference (*t*-test, $p<0.05$) between data obtained in the absence and in the presence of complement or in the presence of C3-deficient serum and C3-deficient serum where C3 was added under the experimental conditions used

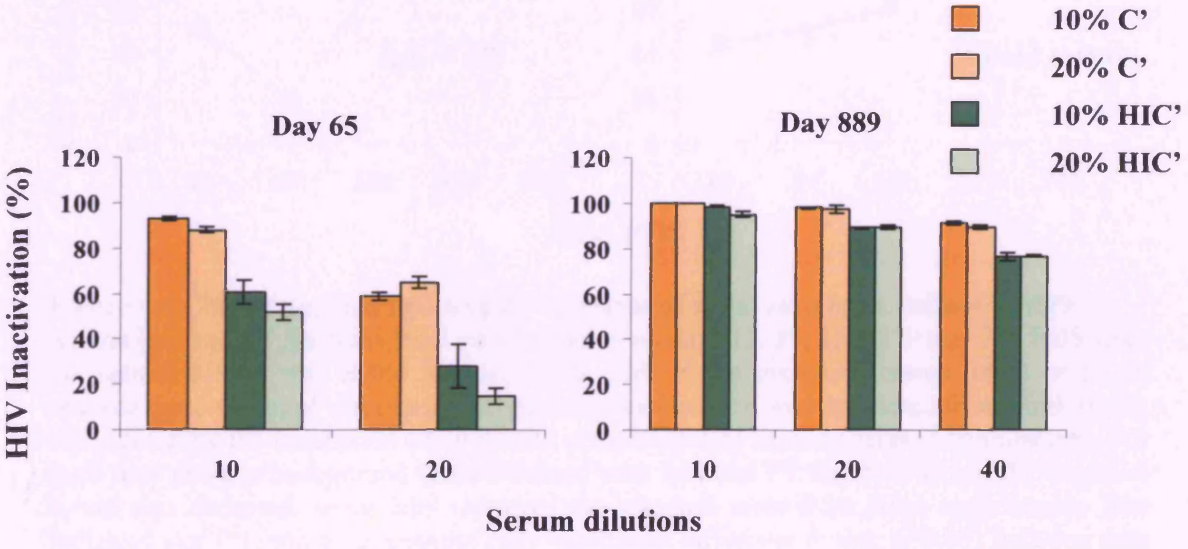


Figure 4.5 Titration of complement in CMI

Virus MM19 was incubated with autologous sera from day 65 and day 889 POS (at the final dilution of 1/10) in the presence of 10 and 20% of complement or heat-inactivated complement. Percentage inactivation was calculated from viral inputs, represented by the incubation of virus with complement or heat-inactivated complement. The *t*-test statistical analysis (*t*-test, $p<0.05$) showed that there was no significant difference observed between CMI performed with 10% complement and 20% complement under the experimental conditions used

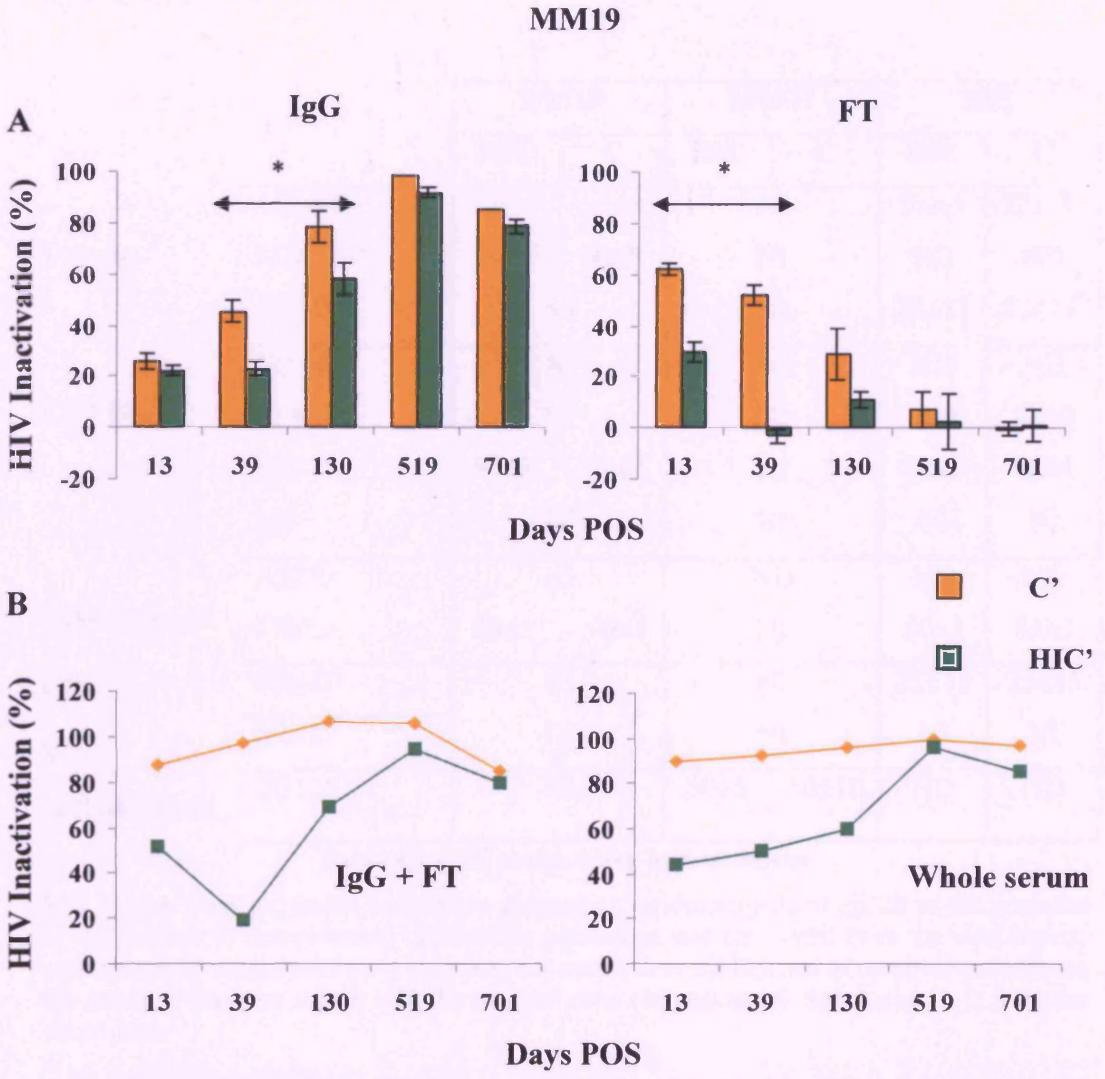


Figure 4.6 CMI of purified IgG and FT fractions of serial sera from patient MM19
A. The IgG and FT fractions from sera harvested on days 13, 39, 130, 519 and 701 POS from the patient MM19 was tested for autologous CMI in the presence (orange bars) or in the absence (green bars) of complement. Percentage inactivation was calculated from viral inputs, represented by the incubation of virus with complement or heat-inactivated complement. For each time point, a background value obtained with IgG and FT fractions from HIV-negative serum was deducted. Error bars represent the standard error from three experiments. The indicated star (*) represents a statistically significant difference (*t*-test, $p<0.05$) between data obtained in the absence and in the presence of complement under the experimental conditions used. The arrows span the time points where significant difference is observed
B. The inactivation values (obtained for the IgG and the FT fractions of sequential sera from patient MM19 in the presence or the absence of complement) were added and compared to the inactivation profile obtained with the whole sera as described in 3.2.6

TCLA strain SF2 and the chimeric viruses MM19 and MM27. The results are shown in table 4.1.

		MM19		MM27		SF2	
		HIC	C	HIC	C	HIC	C
V3-loop	442-57D[§]	40±5	55±5	NI		54±3	72±5[¶]
	MN 215[§]	80±5	90±3	NI		ND	ND
	268-D[§]	NI		NI		28±11	53±11[¶]
CD4 BS	GP 68[§]	NI		NI		ND	ND
	654-D[§]	NI		NI		76±6	80±9
	b12	90±5	90±5	NI		85±2	86±4
	b6*	NI		NI		NI	NI
CD4-induced	A32*	NI		ND		ND	ND
	17b*	90±5	90±2	NI		80±3	83±3
C5	670-D[§]	NI		NI		22±10	25±15
V2	697-D[§]	NI		NI		NI	NI
carbohydrate	2G12*	NI		50±5	50±10	ND	ND

Table 4.1 CMI assays using human MAbs

HIV viruses were incubated with MAbs mapped to various regions of gp120 in the presence or the absence of complement. Inactivation percentage was calculated from the viral inputs, represented by the incubation of viruses in the presence or the absence of complement without the MAbs. Values are shown with the standard error (values±error). See section 2. 2. 3 for the description

[NI] No Inactivation (<10%)
[ND] Not Done
[§] The supernatant of the hybridoma was tested and the Ab concentration was unknown. The supernatant was then tested at the final dilution of 1/10.
* The MAb was tested at the final concentration of 20 μg/ml
[¶] Significant difference of inactivation between complement and heat-inactivated complement (*t*-test, $p<0.05$)

CMI was detectable only in the presence of neutralising anti-V3 loops Abs with SF2. CMI was not detectable in the presence of any of the MAbs tested with MM19 and MM27. CMI was not detectable with neutralising MAbs that mapped to other regions on gp120 or with non-neutralising MAbs.

4.2.5 Effect of complement in combination with sCD4-IgG2

sCD4-IgG2 (or immunoadhesin) is a hybrid molecule consisting of four copies of the D1-D2 immunoglobulin super family domains of CD4 fused to the Fc region of IgG2 (Allaway et al., 1995). sCD4-IgG2 neutralises isolates of HIV *in vitro*, including PIs (Trkola et al., 1995), also protects hu-PBL SCID mice (Gauduin et al., 1998a) *in vivo* and reduces viral loads of infected patients in clinical trials (Jacobson et al., 2004; Shearer et al., 2000).

A previous form of immunoadhesin, where the N-terminal region of sCD4 was fused to the Fc region of IgG1, was tested for its effect in association with complement and the effect was dependent on the strain used (Spear et al., 1994a). The effect of complement on sCD4-IgG2 was investigated here by using SF162 and the chimeric virus MM19. The results are shown in figure 4.7.

CMI enhanced inactivation of SF162 by 2-fold when sCD4-IgG2 was used at the concentration of 50 and 25 μg/ml, whereas no CMI was observed for MM19, despite both viruses being neutralised.

4.2.6 Influence of cell lines in CMI

As described in Chapter 3, there was little difference observed in CMI of H9- and PBMCs-derived viruses in the presence of cross-neutralising sera. To assess if the producing cell has a major influence on CMI, the same HIV isolate was grown in two different cell types and CMI assays were performed.

PBMCs and macrophages were first compared, as they are likely to represent the main targets of HIV infection *in vivo*.

4.2.6.1 CMI comparison between macrophage- and PBMC-derived viruses

PBMCs and macrophages are the main targets of HIV infection and virus production. Viruses produced in macrophages acquire markers characteristic of late endosomal compartments (such as Lysosome-associated antigens LAMP-1 and CD63) and, do not seem to incorporate the CRP protein CD55 (Pelchen-Matthews et al., 2003) unlike PBMCs-derived viruses (Saifuddin et al., 1995). The virus BaL is a R5-tropic virus, that is particularly efficient at infecting primary macrophages (Simmons et al., 1996). The sensitivity to CMI of BaL was compared between BaL

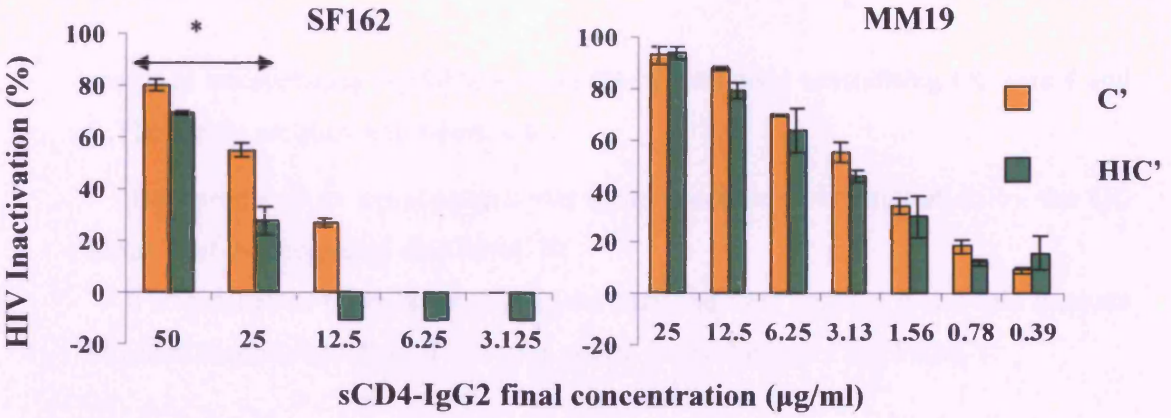


Figure 4.7 CMI using sCD4-IgG2
SF162 and MM19 were incubated with serial concentrations of sCD4-IgG2 in the absence (green bars) or the presence (orange bars) of complement. Percentage inactivation was calculated from viral inputs, represented by the incubation of virus with complement or with heat-inactivated complement. Error bars represent the standard error of three independent experiments. The indicated star (*) represents a statistically significant difference (*t*-test, $p<0.05$) between data obtained in the presence of complement and data obtained in the absence of complement. The arrows span the concentrations where significant difference is observed under the experimental conditions used

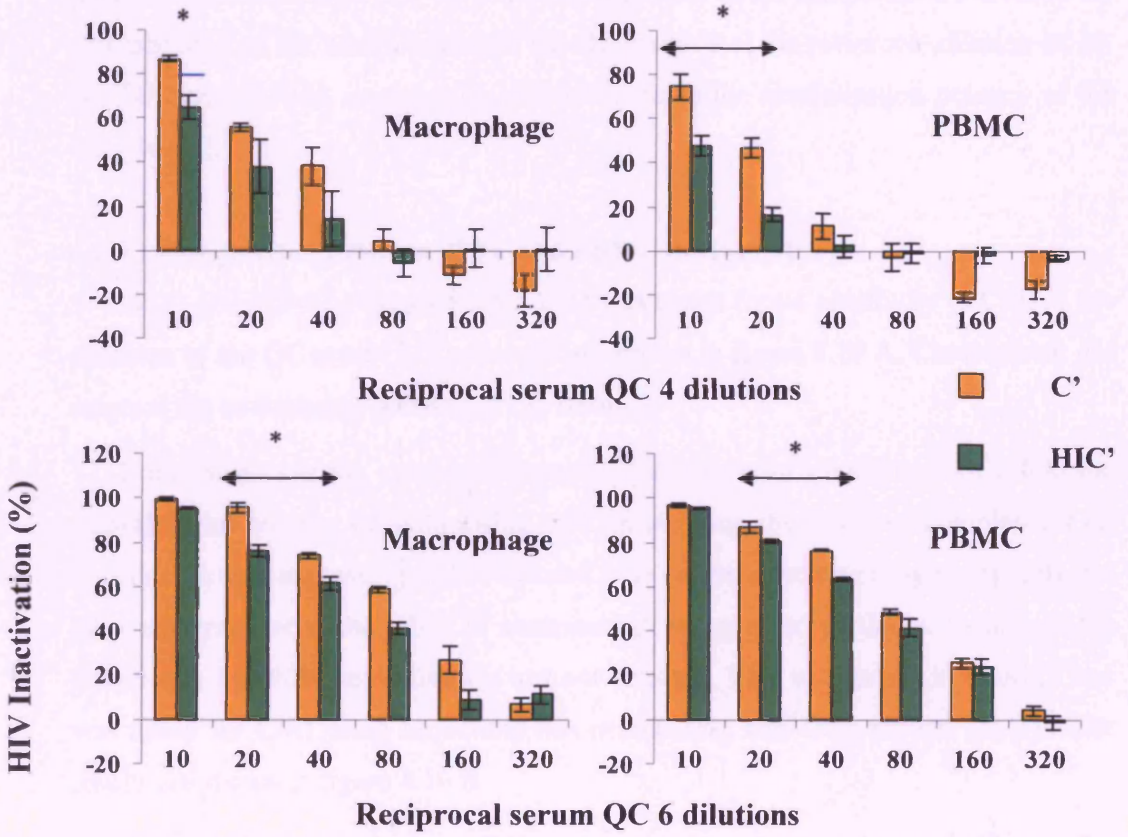


Figure 4.8 Comparison of CMI between macrophage- and PBMC-derived BaL
Macrophage- or PBMC-derived BaL was incubated with serial dilutions of the heterologous neutralising sera QC 4 (A) and QC 6 (B) in the presence (orange bars) or the absence (green bars) of complement. Percentage inactivation was calculated from viral inputs, represented by the incubation of virus with complement or heat-inactivated complement. The indicated star (*) represents a statistically significant difference (*t*-test, $p<0.05$) between data obtained in the presence and in the absence of complement. The arrows span the dilutions where significant difference is observed under the experimental conditions used. The symbol (—) represents a statistical difference between neutralisation under the experimental conditions used

grown in macrophages or PBMCs using the heterologous neutralising QC sera 4 and 6. The results are shown in figure 4.8.

BaL produced in macrophages was more sensitive to neutralisation by the QC serum 4, at the reciprocal dilution of 10.

The potency of CMI, however, did not vary and CMI resulted in a 2-fold increase of neutralisation regardless if BaL was grown in macrophages and PBMCs.

4.2.6.2 Comparison between H9- and PBMC-derived viruses

The PI macrophage-tropic 2044 uses CXCR4 as its main co-receptor (Simmons et al., 1996) and equally infects PBMCs and H9 cells. The 2044 strain, previously passaged in PBMCs, was grown twice in H9 cells. The two viruses were then tested for CMI with the QC serum 2. The results are shown in figure 4.9.

H9-derived 2044 was more sensitive to neutralisation by the QC serum 2, at the reciprocal dilution of 20 and 40. For PBMC-derived 2044, complement increased the neutralisation of the neutralisation of the QC serum 2 at the reciprocal dilution of 20. For H9-derived 2044, complement did not increase the neutralisation potency of the QC serum 2.

4.2.6.3 Comparison between 293T-and PBMC derived viruses

293T- and PBMC-produced virus YU2 was tested for its sensitivity to CMI in the presence of the QC serum 2. The results are shown in figure 4.10 A. Complement did enhance the neutralising potency of QC serum 2.

Thus, in our system, complement can only enhance by a maximum of 2-fold the neutralisation potency of neutralising sera. In studying the effect of complement on chimeric viruses expressing gp120 isolated from patients (or heterologous gp120) and their sequential sera, the effect of complement was greater, particularly early in the infection, when 90% neutralisation was not detected. YU2 was grown in PBMCs and was tested for CMI using sequential non-neutralising sera from patient MM19. The results are shown in figure 4.10 B.

Surprisingly, the producing cell line seemed to have a more profound effect on the CMI using these early sera and YU2. In the presence of complement, 293T- derived YU2 was inactivated between 85 and 90%. When expressed in PBMCs, CMI was only observed in the presence of serum day 39 POS.

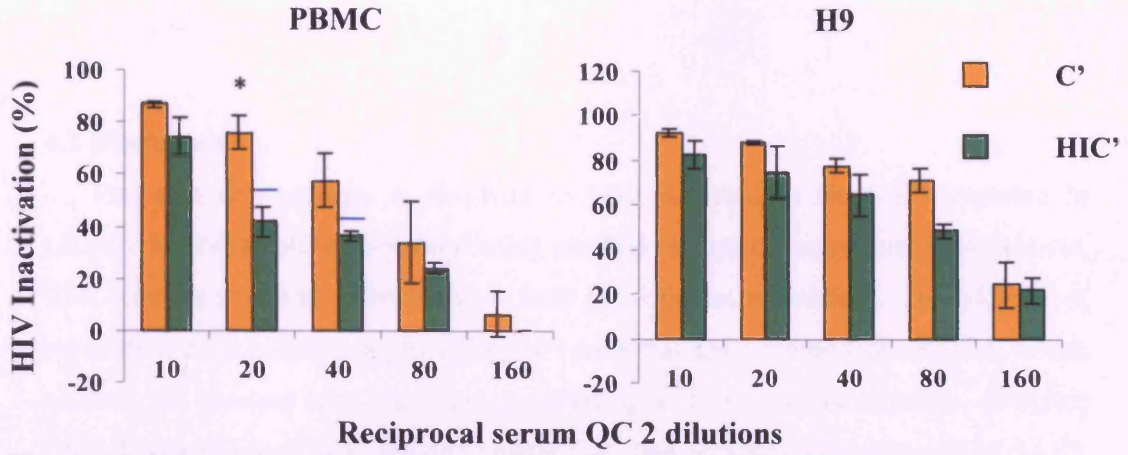


Figure 4.9 Comparison of CMI between PBMC- and H9-derived 2044

H9- or PBMC-derived 2044 virus was incubated with serial dilutions of the heterologous neutralising serum QC 2 in the presence (orange bars) or the absence (green bars) of complement. Percentage inactivation was calculated from viral inputs, represented by the incubation of virus with complement or heat-inactivated complement. The indicated star (*) represents a statistically significant difference (*t*-test, $p<0.05$) between data obtained in the presence and in the absence of complement. The arrows span the dilutions where significant difference is observed under the experimental conditions used. The symbol (—) represents statistical difference between neutralisation

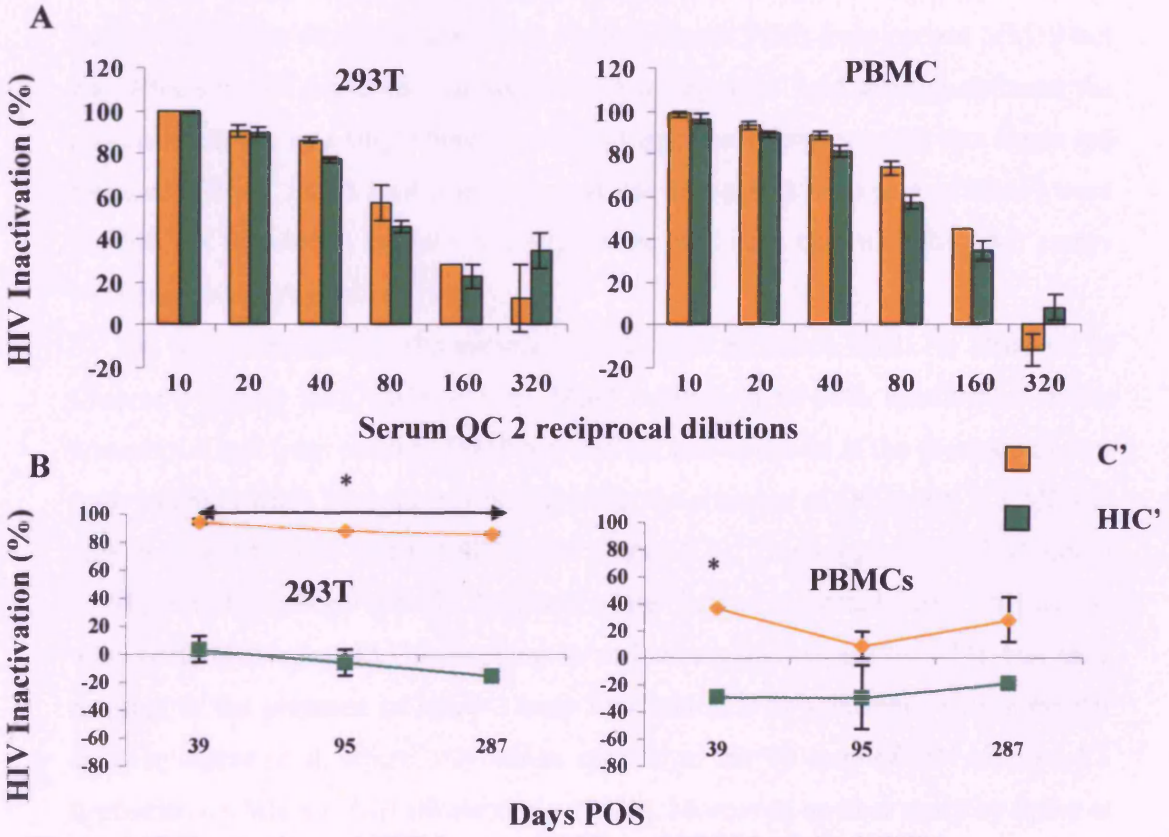


Figure 4.10 Comparison of CMI between 293T- and PBMC-derived YU2

A. 293T- or PBMC-derived YU2 was incubated with serial dilutions of the heterologous neutralising serum QC 2 in the presence (orange bars) or the absence (green bars) of complement

B. 293T- or PBMC-derived YU2 was incubated with non-neutralising sera (at the final dilution of 1/10) from patient MM19 in the presence (orange bars) or the absence (green bars) of complement. Virus inactivation was calculated from viral inputs, represented by the incubation of virus in the presence of complement or heat-inactivated complement. The indicated star (*) represents a statistically significant difference (*t*-test, $p<0.05$) between data obtained in the presence and in the absence of complement under the experimental conditions used. The arrows span the dilutions where significant difference is observed

4.3 Discussion

The classical pathway is involved in CMI detected in the study reported in Chapter 3. CMI could be observed using purified Guinea pig complement. Moreover, CMI was not seen using fresh serum from C3-deficient individuals. The addition of recombinant C3 proteins at physiological concentrations restored CMI. EDTA, which chelates all bivalent ions, therefore inactivating all complement cascades, inhibited CMI. Furthermore EGTA, which chelates Ca^{2+} and prevents the formation of the C1 complex, totally inhibited CMI, similarly observed when serum from C1q-deficient individuals was used as a source of complement. These results suggest that the classical pathway is the main pathway involved in CMI observed in both Chapters 3 and 4.

CMI, however, could be influenced by multiple factors.

The Ab isotype is involved in modulating CMI. CMI was not observed with purified IgG from the early sera (from the first month POS) from patient MM19 but was detected in FT fractions, that are likely to contain IgM. IgM strongly activates the classical pathway as a single bound IgM can trigger complement and lyse a single red blood cell (Boes, 2000). IgM from early and late time points from patient MM19 were purified but the elution buffer was toxic for the NP2 cells on which the CMI assays were performed (data not shown).

The specificity of the Abs present in sera could influence CMI. As observed in Chapter 3 (figure 3.8), CMI of virus MM4 resulted in 80-90% inactivation in the presence of sera from patient MM19 but no CMI was observed in the presence of sera from patient MM27. Moreover, when tested in the presence of QC serum 2, CMI was only observed for IIIB and not SF2 or RF (figure 3.1). This suggests that CMI could be triggered by epitope-specific Abs, which was further supported when CMI assays were performed using MAbs mapping to various regions on gp120. CMI was only detected in the presence of anti-V3 loop Abs, which is in agreement with a similar study by Spear *et al*, where only MAbs specific to the V3 loop of MN induced C3 deposition on MN and IIIB (Spear et al., 1993b). Moreover, another study by Spear *et al* showed that CMI observed with MN and sera from HIV-infected patients could be abrogated by incubating the V3 loop peptide of MN with the tested sera (Spear et al., 1994b). Therefore, the difference in CMI observed with MM4 could be due to the presence (or the absence) of anti-V3 loop Abs in the tested sera. However, the presence of Abs, capable of triggering CMI that do not bind to the V3 loop is also

possible. A study by Trkola *et al* showed that 2G12 could also activate complement and reduce syncytia formation in MN-infected cells (Trkola et al., 1996b). In this study, CMI triggered by 2G12 was not observed. The effect of CMI on syncytia formation was not investigated. To verify if anti-V3 loop Abs are involved in CMI in this study, CMI could be performed in the presence of sera previously incubated with autologous and heterologous V3 loop peptides.

CMI could also be influenced by the modulation of CRP incorporation in the viral membrane. HIV incorporates CD55 and CD59 when produced in cell lines, such as H9 (Montefiori et al., 1994; Saifuddin et al., 1995) and in cell lines transfected for CD55, CD59 and CD46 (Saifuddin et al., 1997). CMI was observed for PBMC-derived 2044 in the presence of the QC serum 2 at the reciprocal dilution of 20 but not for H9-derived 2044. This in agreement with a previous study by Saifuddin *et al* that suggests that CMI could depend on the producing cell line, due to the possible difference in the incorporation of CRPs in the viral membrane (Saifuddin et al., 1994). To formally assess the effect of CRP in CMI, incorporation CMI assays could be performed in the presence of anti-CRP Abs that interfere with the inhibitory action of the CRPs against the MAC formation as described by Montefiori *et al* (Montefiori et al., 1994).

CMI could also be influenced by the nature of the virus. In the presence of non-neutralising sera, CMI is detected for 293T-derived YU2 but not for PBMC-derived YU2. This could be due to fewer functional Env spikes on 293T-expressed YU2. Similarly, Ying *et al* recently described that MBL alone could inactivate some TCLA isolates but not PIs (Ying et al., 2004). They hypothesised, based on Poignard's report (Poignard et al., 2003), that PIs tested contained more functional spikes than TCLA viruses (Poignard et al., 2003). Alternatively, PBMC-derived YU2 could be due a higher density of the glycosylation shield present on gp120 (Wyatt et al., 1998).

The triggering of complement cascades can result in opsonisation or in lysis. Understanding the mechanism of CMI is important as it might determine the *in vivo* implications of complement in association with non-NAbs. Opsonised virions could enhance HIV infection of target cells. Studies have reported *in vitro* enhancement of HIV infection by complement on primary cells expressing CRs (Bajtay et al., 2004; Bouhlal et al., 2002; Kacani et al., 2001). Moreover, immune complexes containing HIV can bind to B cells and be transmitted to T cells via CR2 (Jakubik et al., 2000; Moir et al., 2000). Alternatively, complement could contribute to the viral clearance

during primary viraemia. Complement enhanced the neutralising activity of an anti-HIV Ab in mice *in vivo* (Gauduin et al., 1998b). Sullivan *et al* have shown that HIV virions taken directly from plasma only incorporated CD59 and were more susceptible to lysis than viruses from producing cell lines (Sullivan et al., 1996). Schmitz *et al* have shown that complement depletion, by cobra venom, in monkeys lead to an increase in viral load during primary viraemia (Schmitz et al., 1999b). As CMI observed in this study could lead to lysis (Aasa-Chapman et al., 2005), CMI might play a role of viral decline, particularly in the acute phase, concomitantly with the CTL response (Borrow et al., 1994).

Chapter 5

Isolation of llama non-classical VHH specific for b12, a potent neutralising anti-HIV-1 antibody: a vaccine approach

5.1 Introduction

The CTL and IgG responses against HIV are inefficient at eradicating the virus *in vivo* and no cure is currently available. Anti-HIV compounds are, however, available but they are not dispensed to patients in developing countries, where over 90% of the HIV cases are found. Moreover, they can be highly toxic when prescribed in the long term and they do not eradicate the infection. There is currently no sign of a decline in the number of transmissions worldwide. The high mutation rate of HIV facilitates the generation of escape mutants from both drugs and the anti-viral immune response. Thus an effective vaccine that protects against infection is urgently required.

Classical vaccine approaches against HIV have been, so far, unsuccessful and confer very little protection. Attenuated strains of SIV in monkeys can revert into their virulent state and, therefore, are not safe for clinical trials (Burton and Moore, 1998). Alternative vaccine strategies have not lead to great success. Immunisation with recombinant monomeric gp120 has failed to confer protection in clinical trials (Gilbert et al., 2005). An anti-HIV vaccine approach based on CTL, has so far failed to induce full protection, as escape mutants emerge rapidly (Barouch et al., 2002). The passive administration of NAbs can prevent HIV infection in macaques (Mascola et al., 2000; Veazey et al., 2003b). Therefore, it is likely that an efficient anti-HIV vaccine will involve the induction of cross-neutralising NAbs (Burton and Moore, 1998).

NAbs target gp120 and gp41 of HIV and inhibit the events of viral entry into host cells. Numerous neutralising epitopes have been identified on gp120 including the CD4BS (Burton et al., 1991; McKeating et al., 1993; Trkola et al., 1995), the chemokine receptor binding site that is CD4 binding-induced (Fouts et al., 2002; Moulard et al., 2002; Thali et al., 1993; Wyatt et al., 1995), motifs on the V3 loop (Gorny et al., 1992; Javaherian et al., 1989), on the V1/V2 structure (Fung et al., 1992; Gorny et al., 1994; Warrier et al., 1994) and on the carbohydrate motif recognised by 2G12 (Trkola et al., 1996b). Neutralising epitopes are also present on

gp41, localised on the ectodomain (Muster et al., 1993; Zwick et al., 2001b). Although it is possible to generate a high titre neutralising Ab response against a particular virus strain, the neutralising response is generally weak against other isolates and does not confer protection (Parren et al., 1998; Weiss et al., 1985). HIV can escape the Ab pressure by several means. The virus can accommodate mutations within gp120 and gp41 and, as a result, some Abs fail to recognise their targets. A glycosylation shield, present on the silent face of gp120, prevents NAb access to the neutralising epitopes (Wyatt et al., 1998). The Abs might have a higher affinity *in vivo* for the shed monomeric gp120 or uncleaved gp160 rather than for the trimeric structures (Burton and Moore, 1998).

Despite the strain-specific nature of most anti-HIV NAbs, cross-neutralising NAbs have been elicited *in vivo* in some infected individuals. One of them is b12, which has been isolated by phage display from the IgG repertoire of an HIV-1 positive man, asymptomatic for six years, using gp120 of IIIB as a target (Burton et al., 1994). Its target epitope overlaps the CD4BS of HIV-1 and neutralises a broad range of isolates from different clades (Binley et al., 2004; Parren et al., 1998). It has an unusually long CDR H3 region (18 amino acid residues against 12 in general). The crystal structure of b12 bound to gp120 reveals that the unique ability of b12 to bind directly to the deep CD4BS of gp120, via the Trp residue at the apex of CDR H3 region, and to interact with the Phe residue present at the bottom of the pocket which is highly conserved among HIV isolates (Saphire et al., 2001b).

One possible strategy to trigger a protective NAb response *in vivo* could be to activate B cells to produce b12-like Abs by stimulating them against a mimic of the binding site of b12 on HIV-1. In other words, generating an anti-idiotype of b12, which will bind to b12 in the exact manner as b12 binds to gp120, would mimic an important HIV-1 neutralising epitope and its use for immunisation could lead to the generation of a high NAb response *in vivo*.

The anti-idiotype approach has been investigated before (Beverley et al., 1989). Previous attempts also included anti-CD4BS Abs. Although these Abs neutralised TCLA viruses, they did not cross-neutralise PIs and, very probably, did not target conserved residues within the CD4 binding pocket, like b12. Furthermore, mouse Abs were used for the anti-idiotypic approach. Mice do not make Abs with long CDR regions, the average length for the CDR H3 is 8 amino acid residues, which could fail to mimic the deep pocket of the CD4BS of HIV-1.

The approach described in this chapter involved non-classical llama IgG for mimicking conserved regions of CD4BS and b12, a cross-neutralising Ab.

Llamas belong to the camelid family, which also includes camels, dromedaries and vicuñas (small llamas). These animals have the unique characteristic of synthesising both classical and non-classical IgG (referred as VHH) (Hamers-Casterman et al., 1993). VHH are formed by the association of two heavy chains. They lack the CH1 domain, which is replaced by a hinge, and do not interact with light chains. The length of the hinge can vary and defines two sets of VHH: the long-hinge VHH and the short hinge VHH. The antigen-binding site of the VHH only consists of the CDR regions of the heavy chains, which show hydrophilic properties unlike classical IgG. The VHH CDR regions are generally longer than the CDR regions of the heavy chains of classical IgG, particularly the CDR region 3, which can contain up to 22 amino acid residues compared to 12 amino acid residues in classical IgG. The unusual length of the CDR regions is thought to compensate for the loss of the light chain in the diversity of the Ab repertoire (for a review, see Muyldermans, 2001). As a result, the antigen binding sites of VHH display, besides the classical canonical conformation, more flexible conformations (Decanniere et al., 1999), ideal for anti-idiotypic approaches (Muyldermans and Lauwereys, 1999).

VHH fragments, therefore, could be used for isolating anti-idiotypes of b12, as in this particular case, the anti-idiotypic mimic may need to have flexible loops to adopt the conformation of the crevice formed by the CD4BS of HIV-1 that is recognised by b12.

In this chapter, the isolation of five VHH anti-idiotypes of b12 is described. They were isolated from VHH libraries of a llama immunised with the Fab fragment of b12. These five fragments inhibited the neutralising properties of b12 against SF162 and IIIB *in vitro*. The VHH fragments were conjugated to KLH and were injected into rats and rabbits. The sera of the immunised animals were assessed for their neutralising properties against HIV-1 *in vitro*.

5.2 Results

5.2.1 Generation of the anti-b12 libraries

This work was performed by Pim Hermans (Unilever, The Netherlands). The protocol followed was described by Dolk *et al* (Dolk et al., 2005). Purified Fab fragments of b12, provided by Prof. D. R. Burton (Scripps, USA), was used to immunise a llama. After several boosts and verification of the specific immune response against b12, 15 ml of blood was harvested, the PBMCs were isolated by ficoll gradient, the total RNA was extracted and cDNA was generated. Short- and long-hinge VHH encoding DNA was specifically amplified by PCR and cloned into a phagemid and transformed *E. coli* generating the long- and short-hinge VHH libraries. After estimation of the size of each library, the panning selections were undertaken.

5.2.2 Panning procedures of the libraries

In order to optimise the selection of VHH fragments anti-idiotypic for b12, purified phage were pre-incubated with total purified IgG fractions from a commercially available HIV-1 and HIV-2 seronegative human serum for 30 min. The phage encoding VHH fragments specific for conserved regions of Fab regions of IgG1 would, therefore, bind to seronegative human IgG and not to b12. The pre-incubation step was performed for each round of panning described below in 5. 2. 2. 1 and 5. 2. 2. 2.

5.2.2.1 Panning of the libraries using b12, total HIV-1 lysate and gp140

The screening of both short- (07) and long-hinge (08) VHH encoding libraries was undertaken using b12 as a target for selection followed by non-specific TEA elution of the phage for the first round of panning in order to elute all the VHH fragments binding to b12. For the second and third rounds of panning, purified total viral lysate or purified gp140 from IIIB were used to elute by competition VHH fragments that interact with the epitope of b12 involved in the binding of HIV. The panning selections are represented in diagram 5.1. Pim Hermans (Unilever, Vlaardingen, The Netherlands) collaborated with me for the first round of panning (5. 2. 2. 1. A) and I performed all the other selections described below at the Wohl Virion Centre.

PANNING DIAGRAMS

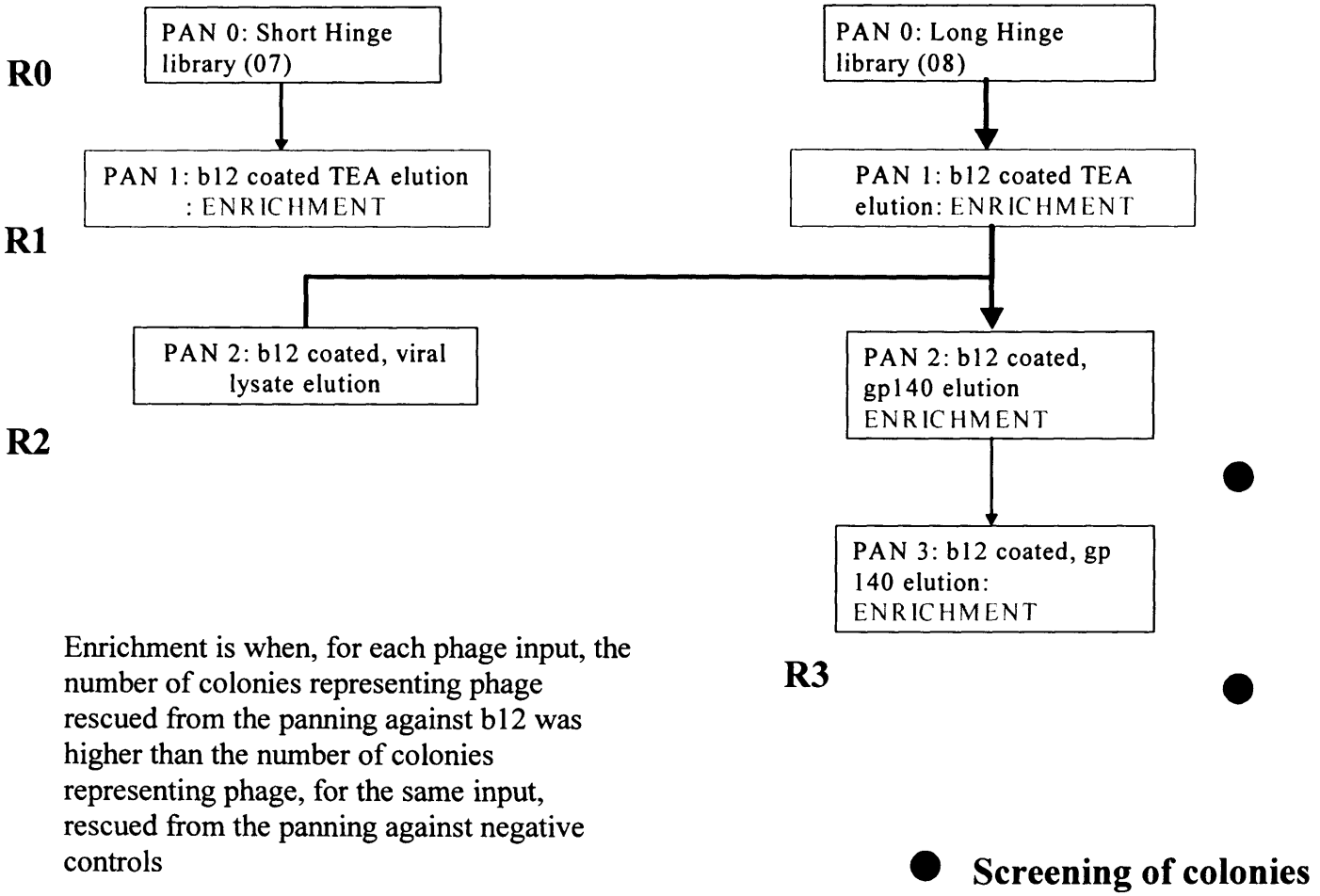


Diagram 5.1 Panning diagrams for the selection of anti-b12 VHH fragments

5.2.2.1.1 First round of panning: non-specific elution

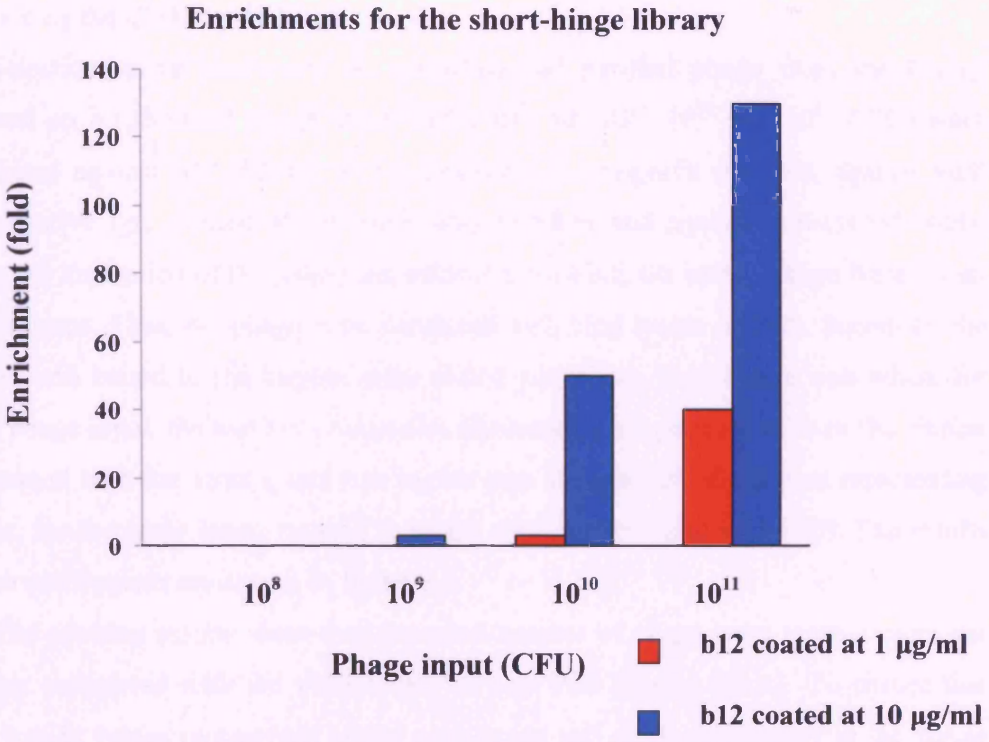
Both 07 and 08 libraries were used for the first round of selection. For each library, several phage inputs (10^7, 10^8, 10^9, 10^{10} and 10^{11} colony forming units or CFUs) were incubated against b12 diluted at 10 and 1 μg/ml and against non-coated wells as negative controls. After incubation of the phage and extensive washes, the phage bound to b12 and to the non-coated wells were eluted with TEA, which increases the pH and disrupts the bonds between the target and the VHH expressed on the phage. The eluted phage were inoculated into an exponentially growing *E. coli* culture, serial dilutions of the bacterial culture were plated and colonies were counted. There was enrichment when, for each phage input, number of colonies representing phage rescued from the panning against b12 was higher than the number of colonies representing phage, for the same input, rescued from the panning against non-coated wells. The results of the enrichments are shown in figure 5.1.

The selections performed for this first round of panning gave consistent enrichments compared to the negative controls and compared to the concentration of b12 coated onto the plate. For example, the enrichment for each library for the panning against b12 coated at 10 μg/ml is approximately ten times higher than the enrichment observed with the panning against b12 coated at 1 μg/ml for the same phage input. These results suggest that the enrichments observed are highly specific, in accordance with the very low background of phage rescued from non-coated plates (data not shown). To obtain highly specific anti-b12 VHH fragments, the phage input that yielded the highest enrichment was re-amplified in *E. coli* and used for further selections. The long-hinge library, amplified after the screening performed with the initial input of 10^{10} CFU/ml on b12 coated at 1 μg/ml (designed as R1) was used for the second round of selection.

5.2.2.1.2 Second round of panning: specific elution with virus lysate

For the second round of panning, the stringency of the selections was increased in order to optimise the probability of eluting specific VHH fragments mimicking the binding site of b12 onto HIV-1 Env. In a first attempt to achieve this goal, selections for the second round of panning were performed on b12 and elutions were performed using a purified virus lysate containing gp120 and gp140. The strain from which the lysate was obtained is a clone of IIIB. The virus lysate contains Env proteins,

A



B

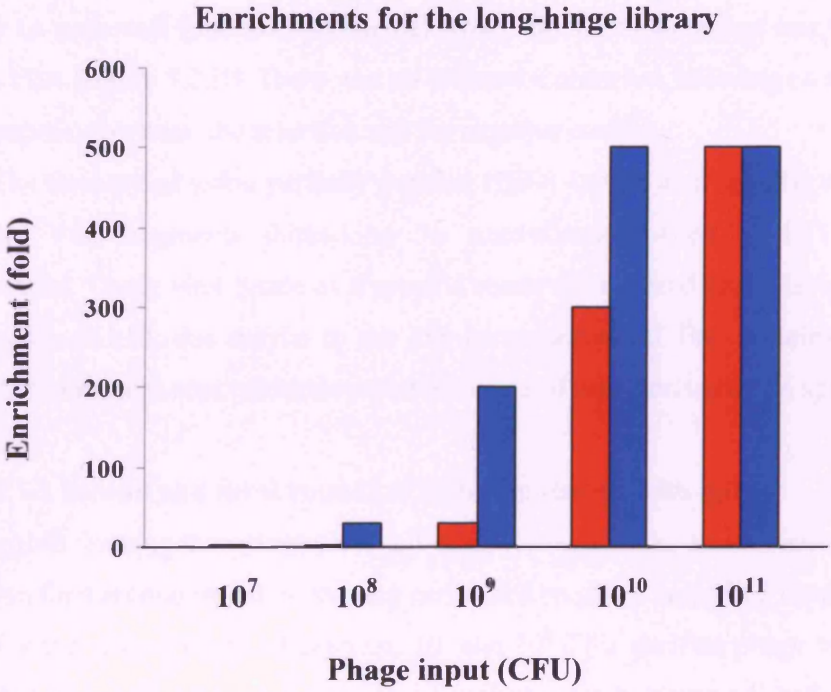


Figure 5. 1 Enrichment values obtained for the first round of panning
A. Enrichment values for the short-hinge library. Enrichment values of several inputs (10^8, 10^9, 10^{10} and 10^{11} Colony Forming Units or CFU) of purified phage from the non-selected short-hinge library (07) against b12 and eluted non-specifically with TEA. The enrichment values (in fold) are calculated from the number of phage rescued panned against non-coated wells performed with the same inputs of phage
B. Enrichment values for the long-hinge library Enrichment values of several inputs (10^7, 10^8 10^9, 10^{10} and 10^{11} CFU) of purified phage the non-selected long-hinge (08) library against b12 and eluted with TEA. The enrichment values are calculated as described for **A**

which would specifically compete for the binding site of b12 with the VHH fragments mimicking the CD4BS of b12.

Selections were performed on amplified and purified phage from the library rescued on R1. Several phage inputs (10^6, 10^7, 10^8, 10^9, 10^{10} and 10^{11} CFU) were incubated against b12 diluted at 5 μg/ml and, for negative controls, against HIV seronegative IgG diluted at the same concentration and against non-coated wells. After the incubation of the phage and extensive washing, the bound phage were eluted in two steps. First, the phage were incubated with viral lysate or PBS. Secondly, the phage, still bound to the targets, were eluted with TEA. Enrichment was when, for each phage input, the number of colonies representing phage rescued from the elution performed with the virus lysate was higher than the number of colonies representing phage, for the same input, rescued from the elution performed with PBS. The results of the enrichments are shown in figure 5.2.

The panning results show that the same number of phage were rescued with the elution performed with the virus lysate or with PBS (figure 5.2.A). To ensure that enrichment values represented actual enrichment and not inconsistency in the phage input on each well (due to experimental error), the rest of the phage was totally eluted with TEA (figure 5.2.B). There was no difference observed, showing no evidence of a discrepancy between the selection and the negative control.

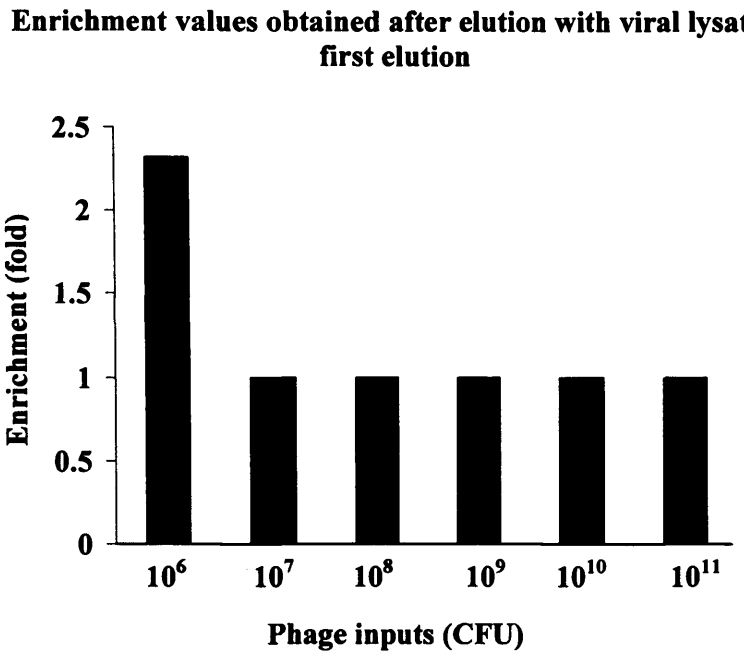
The strategy of using partially purified HIV-1 lystate as a specific competitor to isolate VHH fragments mimicking the neutralising epitope of HIV-1 was not successful. Using viral lysate as a specific eluate did not lead to the isolation of anti-idiotypes of b12, due maybe to the low concentration of Env proteins in the mix. Purified and more concentrated recombinant gp140 was then used as a specific eluant.

5.2.2.1.3 Second and third rounds of panning: elution with gp140

gp140, lacking the cytoplasmic tail of gp41,was used as an alternative method of elution for a second round of panning performed on phage amplified from R1.

For the second round of panning, 10^7 and 10^8 CFU purified phage from R1 were incubated on b12 or human IgG, as an indicator for background, both diluted at 5 μg/ml. The elution was performed in two steps as described in 5. 2. 2. 1. 2 using gp140 and TEA. The amount of gp140 used for elution was used in excess compared to b12 (around 10 molecules of gp140 for 1 molecule of b12) to maximise competition

A



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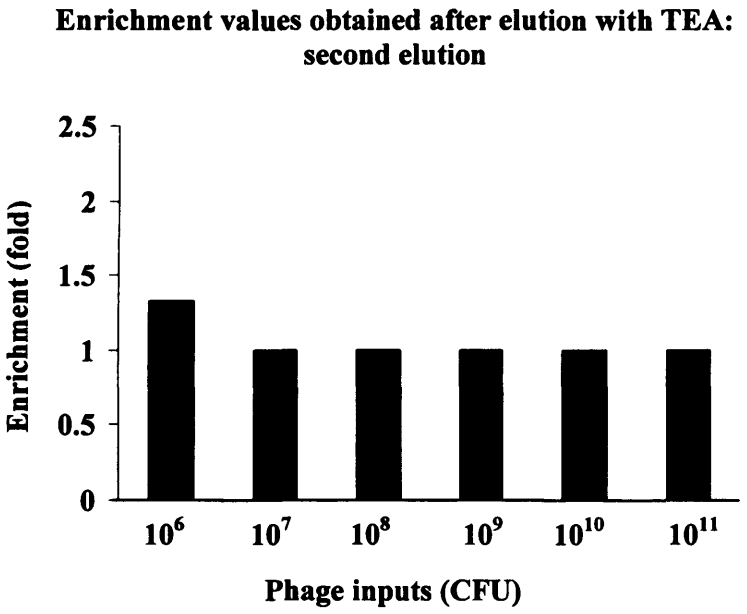


Figure 5.2 Enrichment values obtained for the second round of panning by specific elution with viral lysate

A. First step of elution: viral lysate. Enrichment values of several inputs (10^7, 10^8, 10^9, 10^{10} and 10^{11} CFU) purified phage from R1 and selected against b12 and eluted with 100µl of non-purified virus lysate. The enrichment values, in fold, are calculated from a non-specific elution with PBS performed with the same inputs of phage

B. Second step of elution: TEA. Enrichment values of the same phage inputs from R1 selected against b12, previously eluted with viral lysate, and non-specifically eluted with TEA. The enrichment values are calculated from the number of phage eluted with TEA after the non-specific elution with PBS performed with the same inputs of phage

between gp140 and the VHH fragments expressed on the phage. The enrichments were calculated by the number of phage incubated on b12 and eluted with gp140 compared to the number of phage incubated on b12 and eluted with PBS. The enrichments were also calculated for the TEA elution based on the same principle. The enrichments of the second round of panning are shown in figure 5.3.A.

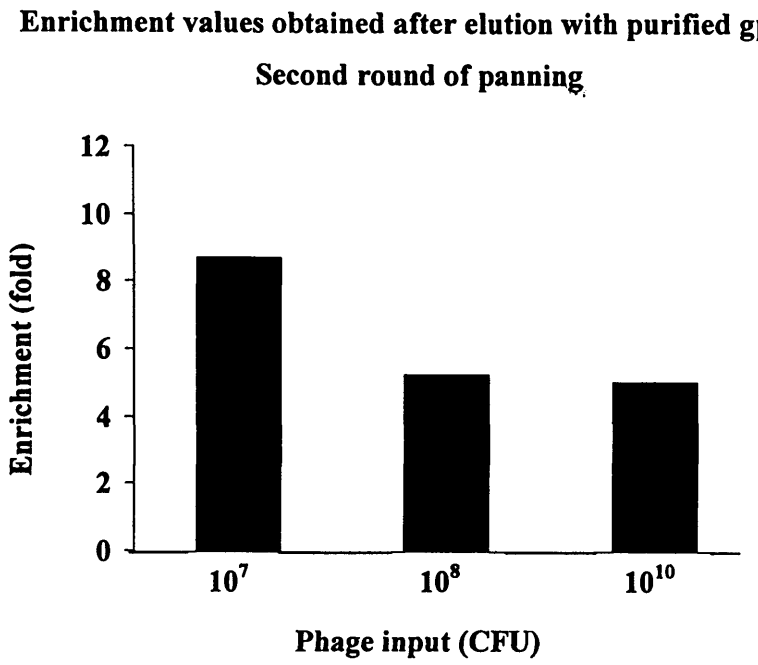
The selections performed using gp140 as a specific eluant show low enrichment values, between five and nine times. However, they are consistent, which could suggest some specific elution of anti-idiotypes of b12.

If the enrichment values represent only background values, they are likely to be lost in a third round of panning using the same selection strategy. If they represent the elution of phage encoding VHH fragments mimicking the CD4 neutralising site of HIV-1, then a third round of panning will allow the amplification of those clones, which will result in an increase in the enrichment values. A third round of selection was undertaken to confirm the relevance of the enrichments. The library, amplified after the screening performed with the initial input of 10^8 CFU on b12 and eluted with gp140 (designed as R2), was used for the third round of selection.

For the third round of panning, b12 and human IgG were used at a final concentration of 3 μg/ml. The selections were performed as described as for the second round of panning. The enrichment values were calculated in the same manner and are shown in figure 5.3.B.

The selections show low values of enrichment, similar to the ones observed in the second round of panning between four and ten times. These values are low and could still represent background values. However, they could also reflect the elution of a small pool of phage encoding anti-idiotypes of b12. Moreover, the selections performed against b12 show high enrichment values compared to human IgG. The enrichment values of the selections against b12 compared to IgG for the second and third rounds of panning using gp140 as a specific eluant are shown in figure 5.3.C. Moreover, the elution performed with TEA after the elution with gp140 showed negative enrichments, suggesting that more phage were eluted for gp140 than with PBS and that the total number of incubated phage was equal for the selections and the negative controls, as shown in table 5.1.

A



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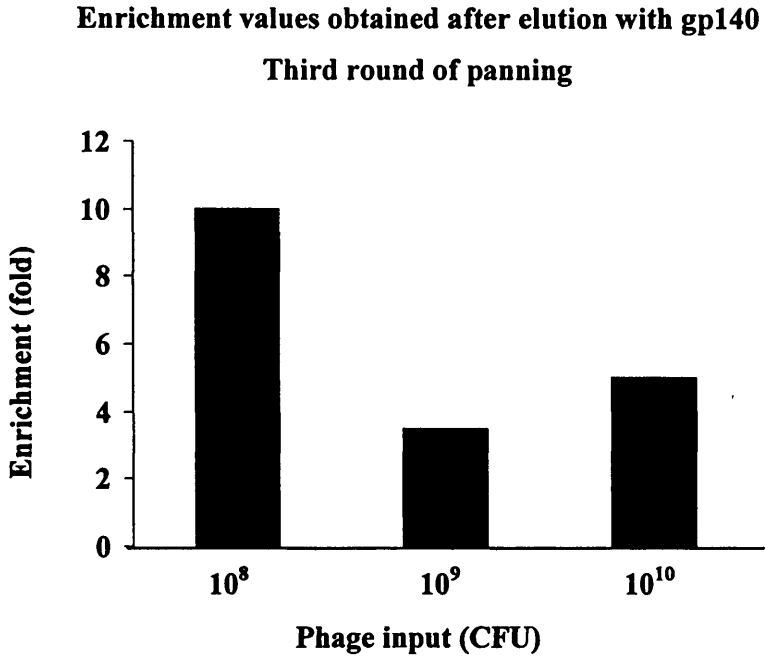


Figure 5.3 Enrichment values obtained for the second and third rounds of panning using gp140

A. Second round of panning: elution with gp140. Enrichment values of several inputs (10^7, 10^8 and 10^{10} CFU) purified phage from R1 selected against b12 and eluted with an excess of gp140. The enrichment values, in fold, are calculated from a non-specific elution with PBS performed with the same inputs of phage

B. Third round of panning: elution with gp140. Enrichment values of several inputs (10^8, 10^9 and 10^{10} CFU) purified phage from R2 selected against b12 and eluted with an excess of gp140. The enrichment values are calculated as described for the second round of panning

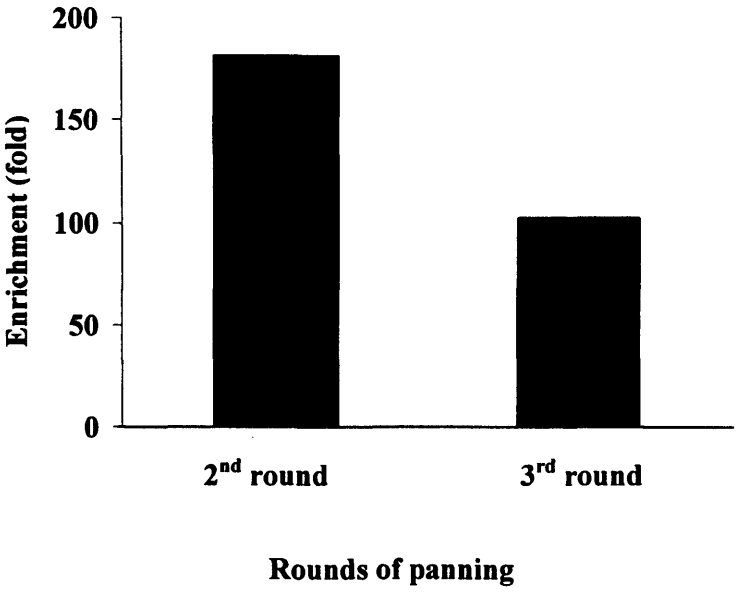


Figure 5.3.C Enrichment values for the second and third rounds of panning between b12 and polyclonal IgG

Phage input	Enrichment (fold) after TEA elution for the second round of panning performed with gp140	Enrichment (fold) after TEA elution for the third round of panning performed with gp140
10^7	-7	ND
10^8	-5	-8
10^9	ND	-4
10^{10}	-6	-4

Table 5.1 Enrichment values obtained for the specific elution with gp140 for the second and the third rounds of panning
Enrichment values of the same phage inputs from R1 and R2 selected against b12, previously eluted with gp140, and non-specifically eluted with TEA. The enrichment values are calculated from the number of phage eluted with TEA after the non-specific elution with PBS performed with the same inputs of phage
ND. Not Done

Since the enrichment values calculated reflected the elution of anti-idiotypes, the isolation of specific VHH fragments was undertaken.

5.2.3 Isolation of specific anti-b12 VHH clones

Libraries rescued from the second and third round of panning, using gp140 as a specific eluant, were diluted in medium and plated to obtain separate colonies. Ninety-six clones were then picked and were grown in a 96-well V-bottom plate, which constituted the master plate. The colonies were screened for their properties to specifically bind to b12.

5.2.3.1 Screening by phage ELISA

From the master plate, a duplicate plate was generated in which the phage production was induced. Phage ELISA was performed by testing the supernatant of these "mini-cultures" against b12, human IgG and BSA, all diluted at 5 μg/ml. The detection was performed using an anti-p8 Ab (the major coat protein of the phage) directly conjugated to HRP. The results of the screening are shown in figure 5.4. All the tested clones bound strongly to b12 and give background values on human IgG and BSA, suggesting that all the clones tested were anti-idiotypes of b12. However, the picked clones were likely to show redundancy in their sequences. The restriction profile of the sequence of each of the 96 clones was then performed.

5.2.3.2 Restriction patterns

All the clones were tested for the coding sequence of the VHH fragment. The primers used for the amplification annealed within the P*lac* promotor for the 3' primer (M13REV) and the coding sequence of the gene III for the 5' primer (MPE25). The expected size of the amplified fragment is 600 base pairs in length. The PCR fragments were then digested using the restriction enzyme *HinfI*. The restrictions were then loaded on agarose gel to identify the different profiles.

The restriction pattern shows some redundancy amongst the clones. The clones that had a different restriction pattern were further selected for sequencing.

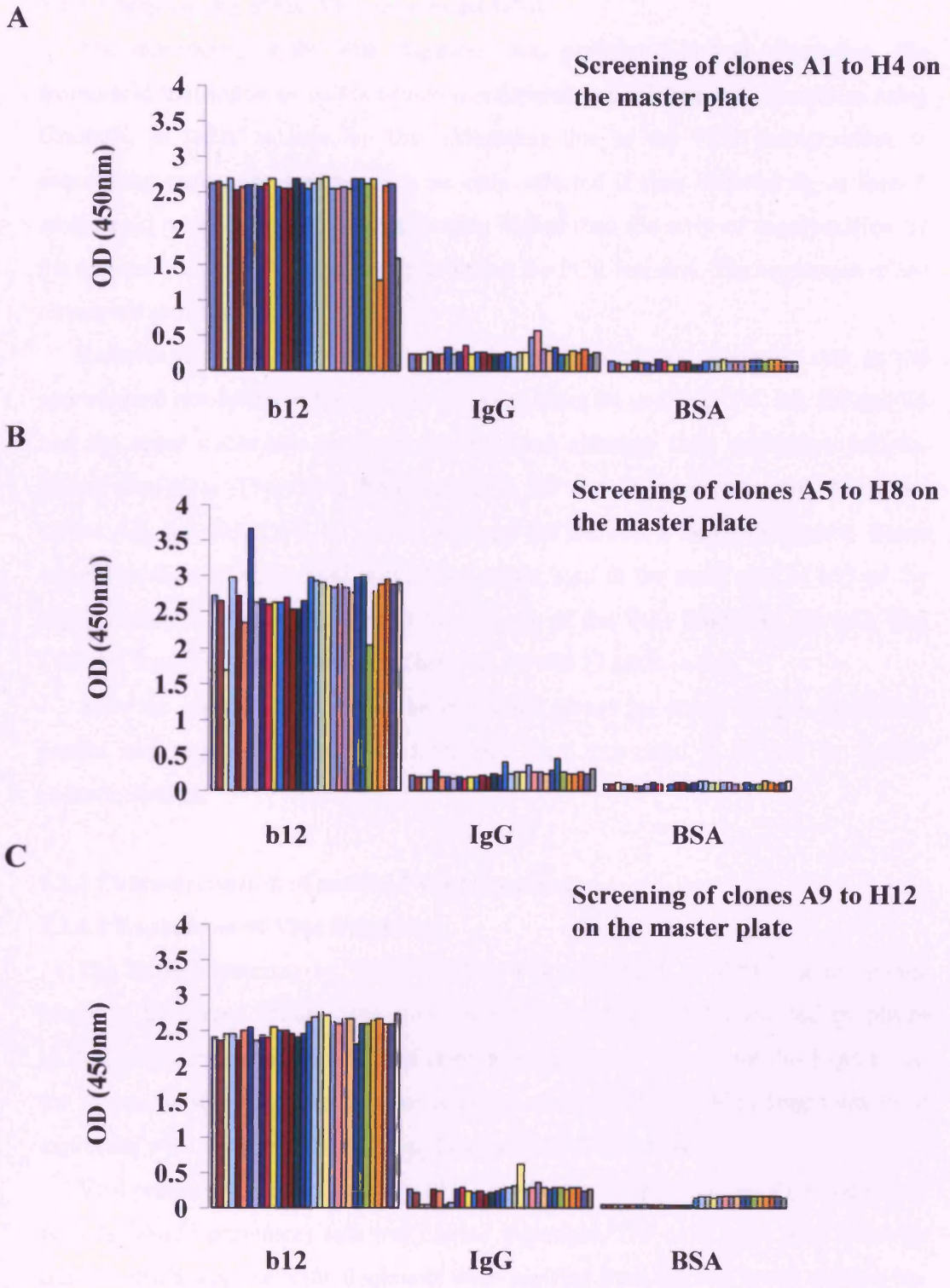


Figure 5.4 Screening of VHH-encoded clones by phage ELISA
96 clones were randomly picked and screening by phage ELISA for their binding properties on b12, human IgG and BSA. **A** represents the screening of the first 32 clones (from A1 to H4 from the master plate), **B** represents the screening of the clones 33 to 64 (from A5 to H8) and **C** represents the screening of the clones 65 to 96 (from A9 to H12)

5.2.3.3 Sequencing of the VHH fragments DNA

The sequencing of the VHH fragments was performed in both directions. The amino acid translation of each clone was compared and aligned with the others using ClustalX. In order to rule out the differences due to the PCR incorporation or sequencing errors, the sequences were only selected if they differed by at least 3 amino acid residues, which is significantly higher than the error of incorporation by the non-proof-reading *Taq* polymerase during the PCR reaction. The sequences of the clones are shown in table 5.2.

Redundancy was observed amongst the selected clones. Probably due to the approximate resolution of the agarose gel, the clones B4 and B12, C4, D9, D7 and E6 had the same nucleotide sequence were picked although their restriction patterns looked dissimilar. Therefore, the clones B12, D9 and E6 were discarded. The other clones A3, A5, D3, D10, E11, F10, G6 and G9 showed a unique sequence. Some sequences showed some similarity. They might bind to the same part of b12 or the slight variation might be related to the affinity of the VHH fragments for b12. The CDR H3 length of the clones varies between 15 and 17 amino acids.

After the identification of all the individual clones by phage ELISA, restriction pattern and sequencing, the VHH fragments were expressed in *E. coli* for further characterisation.

5.2.4 Characterisation of anti-b12 VHH fragments

5.2.4.1 Expression of VHH fragments

The initial screening by phage ELISA was performed by detecting the phage bound to the target. Phage have sticky properties and the signal detected by phage ELISA might lead to the isolation of false positive clones. To rule out this hypothesis, the fragments were expressed in the bacterial strain *BL21*. The VHH fragments were expressed with a 6-His and a Myc tag fused at their C-terminus.

VHH production was induced by IPTG (as the transcription of the VHH sequences is under $Plac^{Iq}$ promotor) and was carried overnight. The cells were then lysed by osmotic shock and the VHH fragments were purified from the cell lysate using a Ni-NTA super low column via interaction with the 6-His tag. An irrelevant VHH

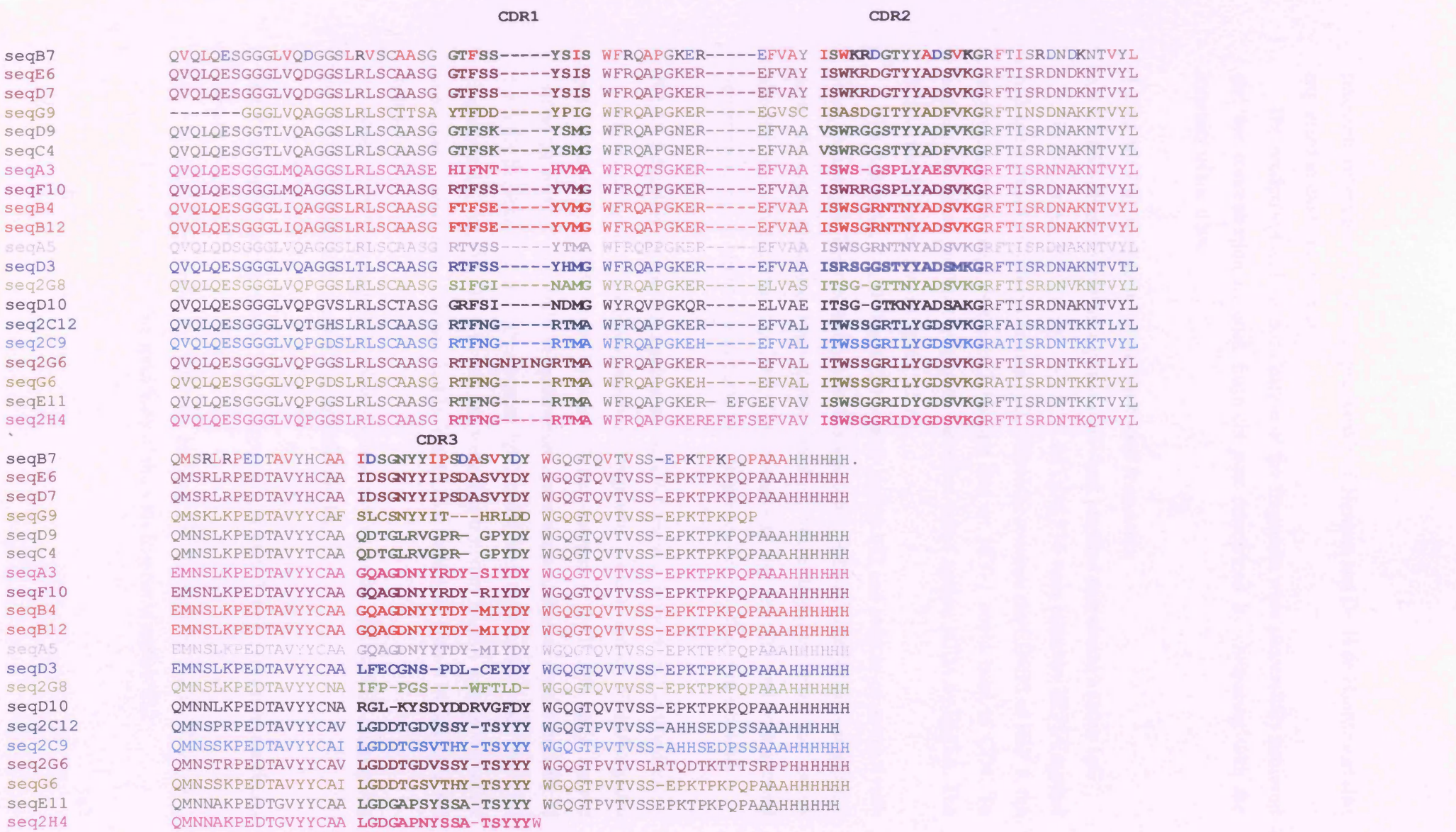


Table 5.2 Amino acid sequences of the anti-b12 VHH fragments isolated by phage display.
The sequences are aligned by Clustal X and distinct sequences are highlighted in different colours. The CDR regions of each VHH are in bold characters.

fragment, referred as OC (kindly provided by P Herman and Dr. H de Haard) was also expressed in the same manner.

The production and the purification of the fragments were successfully achieved and the concentration of each fragment was determined by comparing with the intensity of the BSA.

5.2.4.2 Binding properties of purified VHH fragments

5.2.4.2.1 Binding properties against sCD4 and purified polyclonal human IgG

The clones A3, A5, C4, D3, D7, D10, E11 and F10 were tested by ELISA against b12 and purified polyclonal human IgG. Since b12 overlaps the CD4BS of HIV-1, the anti-idiotype that mimics the b12 binding site on HIV-1 could bind to CD4. To address this point, the VHH fragments were also tested against sCD4 by ELISA. The ELISA results are shown in figure 5.5.A.

The clones A1, A5, D10 and E11 weakly bind to b12 and seem to cross-react with other human IgG. They probably bind to some conserved structures of human IgG and were therefore discarded from further studies. Interestingly, none of the clones showed relevant binding to sCD4. The clones A3, B4, D3, D7 and F10 showed specific and strong binding to b12 and their properties were further characterised.

5.2.4.2.2 Binding properties against anti-HIV-1 human monoclonal antibodies

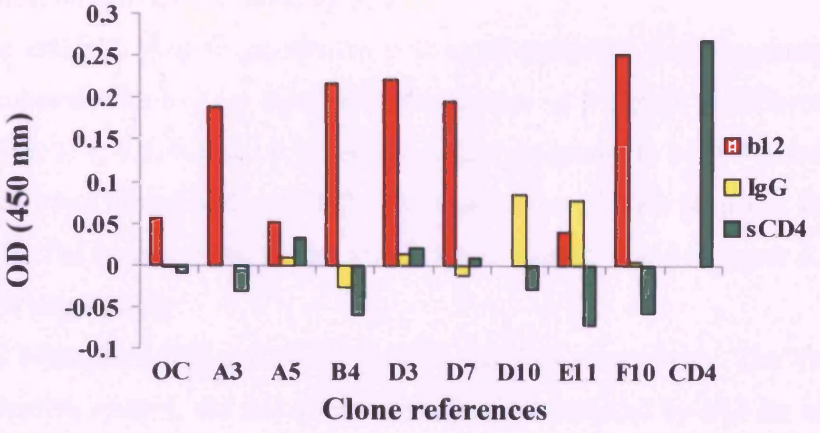
b12 is an IgG1. Previously, the VHH fragments were tested by ELISA against polyclonal human IgG, which includes all the isotypes of IgG. The IgG1 isotype within the pool of IgG could be under-represented and any sign of cross-reactivity of the VHH between b12 and other human IgG1 could have been undetected. The VHH fragments A3, B4, D3, D7, F10 and OC were then tested by ELISA against two other monoclonal IgG1 Abs (17b and 2G12). The ELISA results are shown in figures 5.5.B and 5.5.C.

None of the VHH fragments showed cross-reactivity with 17b or 2G12, suggesting that all the isolated clones are highly specific to b12.

5.2.5 Inhibition properties of VHH fragments in HIV-1 neutralisation mediated by b12

b12 neutralises strains of HIV by binding the CD4BS via its CDR regions, particularly the CDR H3. The specificity of the VHH fragments against b12

A



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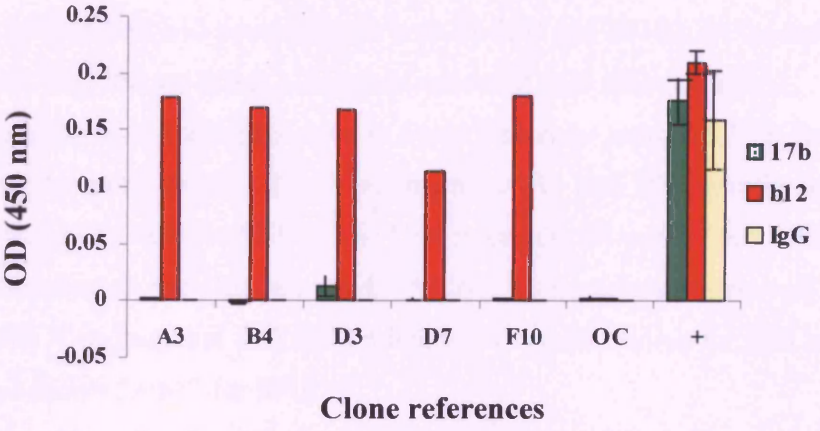

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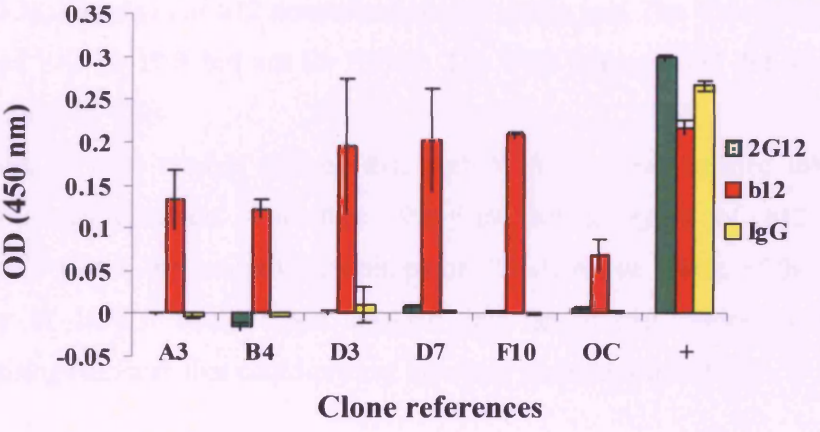


Figure 5.5 ELISA assays of the purified anti-b12 VHH fragments
A. ELISA assay of anti-b12 VHH fragments against b12, human IgG and sCD4
B. ELISA assay of anti-b12 VHH fragments against b12, human IgG and 17b
C. ELISA assay of anti-b12 VHH fragments against b12, human IgG and 2G12
For all assays, the proteins were coated at the concentration of 5 µg/ml. The detection of the VHH fragments was performed using an anti-HIS conjugated to HRP. Soluble CD4 was detected by using a rabbit anti-CD4 antibody and by an anti-rabbit IgG-HRP. Human Mabs were detected by using an anti-human IgG conjugated to HRP

was tested in an *in vitro* assay. True anti-idiotypes of b12 should totally inhibit all neutralisation activity mediated by b12.

The anti-b12 VHH fragments, as well as an irrelevant VHH fragment (OC) were pre-incubated with b12 (at the final concentration of 5 μg/ml) at different molecular ratios (10, 3, 1, 0.5, 0.1 and 0.01 fold in excess compared to b12). Neutralisation was assessed when mixed with anti-b12 VHH fragment or theVHH fragment OC on SF162 and IIIB. The results of the assays are shown in figure 5.6 and in figure 5.7 for SF162 and IIIB respectively.

b12 neutralised IIIB and SF162 by 95% and 70% respectively. The VHH OC, used as a negative control, did not affect neutralisation mediated by b12 for either IIIB or SF162. In contrast, the five anti-b12 VHH fragments, at 10 and 3 times in excess, totally inhibited all b12 neutralisation both for IIIB and SF162. At 0.1 and 0.01 times in excess, they did not affect b12 neutralisation for both IIIB and SF162.

When the VHH fragments were in equal molecular ratio, the VHH fragments did not give the same results. The VHH fragments A3 and D3 partially inhibited b12 neutralisation for IIIB for SF162. The VHH fragments B4 and D7 still totally inhibited b12 neutralisation for IIIB but not for SF162, where neutralisation was restored by 40%. The VHH fragment F10 did not inhibit b12 neutralisation for IIIB but lost most of its inhibition for b12 for SF162.

When one molecule of VHH A3, D3 or F10 was present for two molecules of b12 (ratio 0.5), inhibition of b12 neutralisation was totally lost. The VHH fragment B4 still inhibited b12 for IIIB but not for SF162. The VHH fragment D7 did inhibit b12 for IIIB but not for IIIB.

These *in vitro* studies showed that each VHH fragment isolated inhibited b12-mediated neutralisation. Therefore, they are anti-idiotypes of b12 and could potentially mimic the neutralising epitope of HIV-1. A true mimic of the neutralising epitope of HIV-1 could when injected into an animal, produce an anti-HIV neutralising response that could present the same properties than b12.

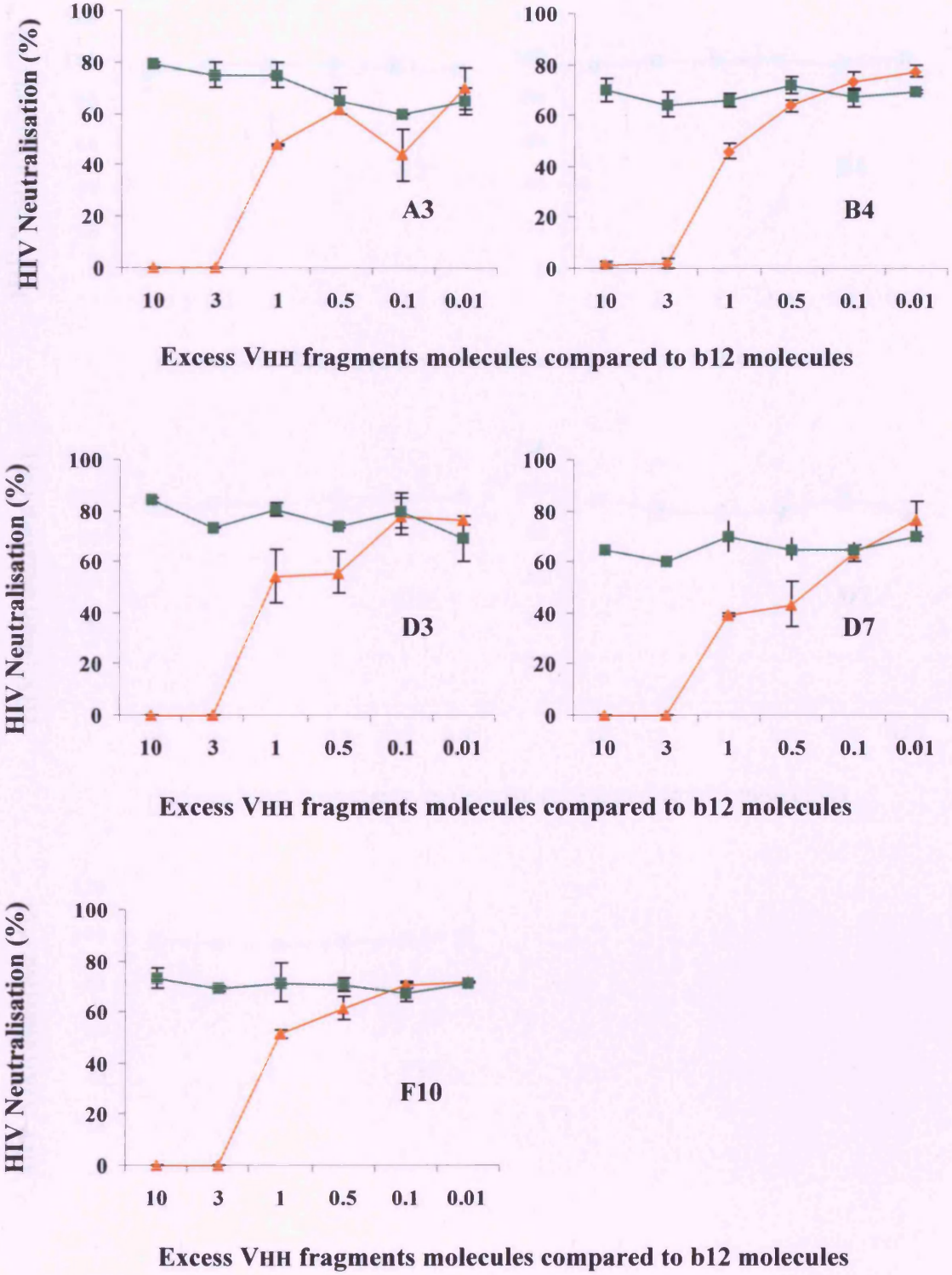
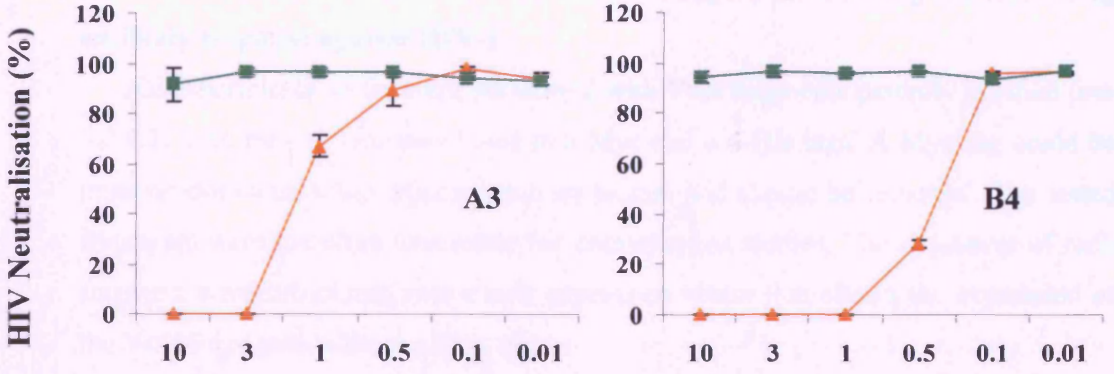
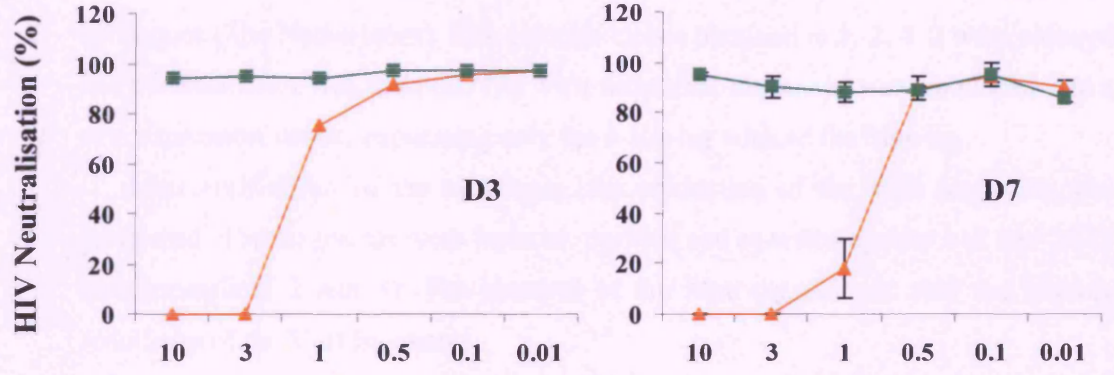


Figure 5.6 Inhibition of b12-mediated neutralisation of SF162 with anti-b12 VHH fragments

Neutralisation of HIV-1 strain SF162 by b12 is inhibited with specific anti-b12 VHH fragments A3, B4, D3, D7 and F10 (-) but not with a non-specific VHH fragment OC (-). The inhibition effect is dependent on the amount of VHH fragments incubated with b12. All the experiments were carried on IIIB with b12 diluted at the final concentration of 5 μg/ml





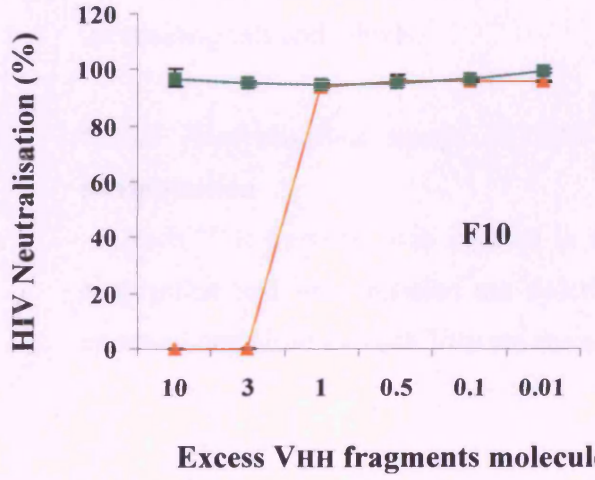


Figure 5.7 Inhibition of b12-mediated neutralisation of IIIB with anti-b12 VHH fragments

Neutralisation of HIV-1 strain IIIB by b12 is inhibited with specific anti-b12 VHH fragments A3, B4, D3, D7 and F10 (-) but not with a non-specific VHH fragment OC (-). The inhibition effect is dependant on the amount of VHH fragments incubated with b12. All the experiments were carried on IIIB with b12 diluted at the concentration of 5 µg/ml.

5.2.6 Anti-b12 VHH fragments used as immunogens for eliciting a neutralising antibody response against HIV-1

All experiments so far were performed with VHH fragments partially purified (see 5.2.4.1) with their C-terminus fused to a Myc and a 6-His tags. A Myc tag could be immuno-dominant when injected into an animal and should be removed. The tested fragments were therefore unsuitable for immunisation studies. The sequences of each fragment were sub-cloned into a new expression vector that allows the expression of the VHH fragments without a Myc tag.

5.2.6.1 Sub-cloning and purification of the anti-b12 VHH fragments

The work described below in 5. 2. 6. 1 was carried out by Dr Hans de Haard and colleagues (The Netherlands). The glycerol clones obtained in 5. 2. 4. 2 were cultured and plasmid DNA was isolated. The VHH fragments sequences were re-cloned into a new expression vector, expressing only the 6-His tag without the Myc tag.

After verification of the sequences, the production of the VHH fragments was performed. The fragments were isolated, purified and re-tested against b12 and 2G12 (see appendices 2 and 3). The removal of the Myc tag did not alter the binding specificity of the VHH fragments.

After purification, the five anti-b12 VHH fragments and the negative control were conjugated to KLH by Peter Delves and colleagues (UCL, London). For details of the KLH conjugation, refer to Chapter 2, section 2.15. The VHH were then used for immunising rats and rabbits.

5.2.6.2 Neutralisation assays of HIV-1 with sera from the first boost of immunisation

Each VHH fragment was injected in four rats and one rabbit. The protocols of conjugation and immunisation are described in Chapter 2. The references of the animals immunised for each VHH are shown in table 5.3.

	Rats	Rabbits
A3	5, 6, 7, 8	1
B4	17, 18, 19, 20	3
D3	21, 22, 23, 24	3
D7	9, 10, 11, 12	4
F10	13, 14, 15, 16	5
OC	1, 2,3, 4	6

Table 5.3 References of the animals immunised with each VHH fragment

Each animal was immunised and then boosted with the same VHH. After the first boost, blood samples were harvested from each animal, serum was extracted and tested for neutralisation against HIV *in vitro*.

For each neutralisation assay, a Student *t*-test was used to evaluate if there was a significant difference between data obtained with sera from immunised animals and pre-bleed sera from the same animals. Similarly, for each CMI assay, a Student *t*-test was used to evaluate if there was a significant difference between data obtained in the presence of complement and in the absence of complement. The probability (p value) chosen was 0.05 in order to evaluate as accurately as possible small differences.

Therefore, when the p value was above 0.05, no neutralisation or CMI was observed under the experimental conditions used.

5.2.6.2.1 In the absence of complement

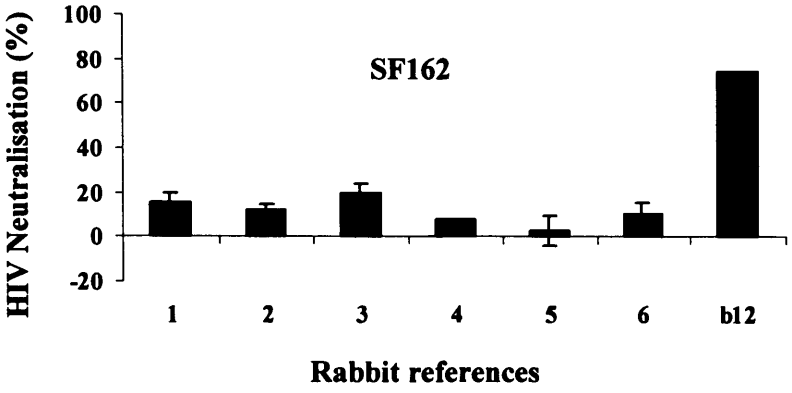
Each animal gave an immune response against the VHH with which they were immunised (Peter Delves, personal communication). The sera from rabbit 2 gave a positive signal against gp120 and gp105 by ELISA (Peter Delves, personal communication).

The serum from each animal, as well as a pre-bleed serum, were tested for its neutralising properties on SF162 and IIIB. The neutralisation results for both IIIB and SF162 using sera from rats and rabbits are shown in figure 5.8.

None of the rabbit or the rat sera neutralised SF162 or IIIB compared to the pre-bleed sera. The rat sera showed similar neutralising patterns.

The sera were collected and tested after the first boost of immunisation. b12-like specific Abs could be present in the animal sera and could coat the virus particles but their affinity might be too weak to neutralise the virus alone. However, the coating of

A



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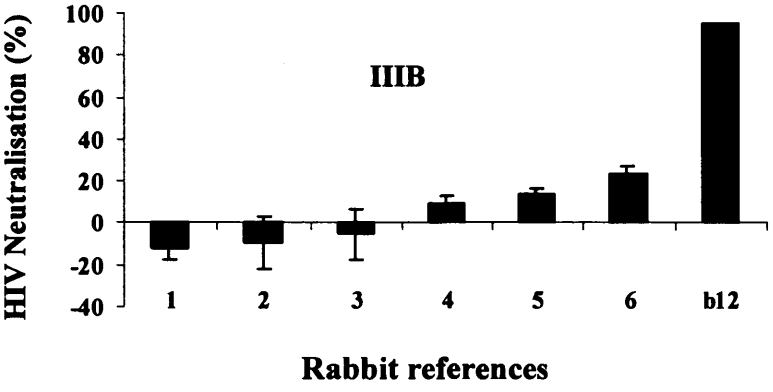

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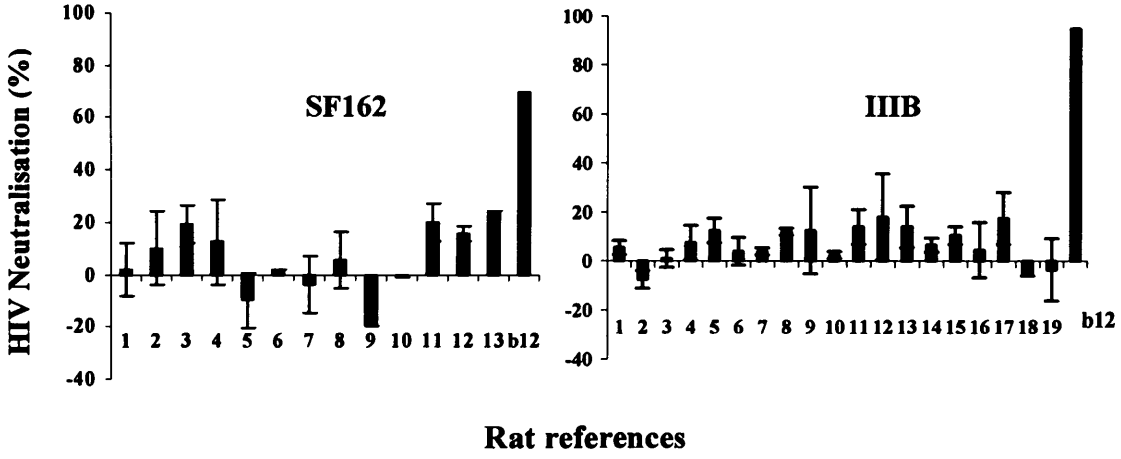

Figure 5.8 Neutralisation assays with sera from the first boost of immunisation
Neutralisation assays of SF162 (**A**) and IIIB (**B**) with the sera from the 6 immunised rabbits. **C.** Neutralisation assays of SF162 and IIIB with the sera from the 19 immunised rats.
All assays were performed with sera diluted at 1/20. Neutralisation percentages were calculated from data obtained with pre-bleed sera from the same animal for each assay. For the decoding of each animal, please refer to table 5.1. For all experiments, a neutralisation assay with b12 diluted in a rat or rabbit serum at the final concentration of 5 μg/ml was included as a positive control. The error bars represent the standard error from three experiments. The *t*-test statistical analysis (*t*-test, $p<0.05$) showed that there was no significant neutralisation by any of the tested sera compared to the pre-bleed under the experimental conditions used

the virus with the Ab might be sufficient to trigger the classical cascade of complement and inactivation of the virus might be observed as described in Chapters 3 and 4.

5.2.6.2.2 In the presence of complement

The sera from the immunised rabbits were tested for neutralising IIIB and SF162 in the absence or the presence of human complement. Too little sera from rats were available to perform the experiments. Sera were tested with 10% of snap-frozen human serum containing complement or with 10% heat-inactivated human serum. The inactivation data were calculated from the pre-bleed of the same animal in the presence or the absence of complement. The results are shown in figure 5.9. No CMI was observed with any of the sera.

The immunisation by anti-b12 VHH fragments did not lead to a potent neutralising anti-HIV-1 Ab response in rats or rabbits. This could be due to a weak affinity of the immune response against the VHH fragments. The rabbits were boosted again and the rats were sacrificed.

5.2.6.3 CMI assays of HIV-1 with sera from the second boost of immunisation

After the second boost with VHH fragments, the sera from the rabbits were tested again for neutralising IIIB. The neutralisation assay was set in the same conditions as described in 5. 2. 6. 2. b. The results are shown in figure 5.10.

No CMI was observed with IIIB in the presence of the rabbit sera. Rabbits 2 and 5 were boosted again with their respective VHH fragment.

5.2.6.4 CMI assays of HIV-1 with sera from the third boost immunisation

The sera of the rabbits 2 and 5 were tested again for CMI activity against IIIB in the absence or the presence of human complement, Guinea pig complement and rabbit complement. The results are shown in figure 5.11.

No CMI was observed with the sera. Immunising with gp120, linked to KLH, could lead to the secretion of b12-like Abs.

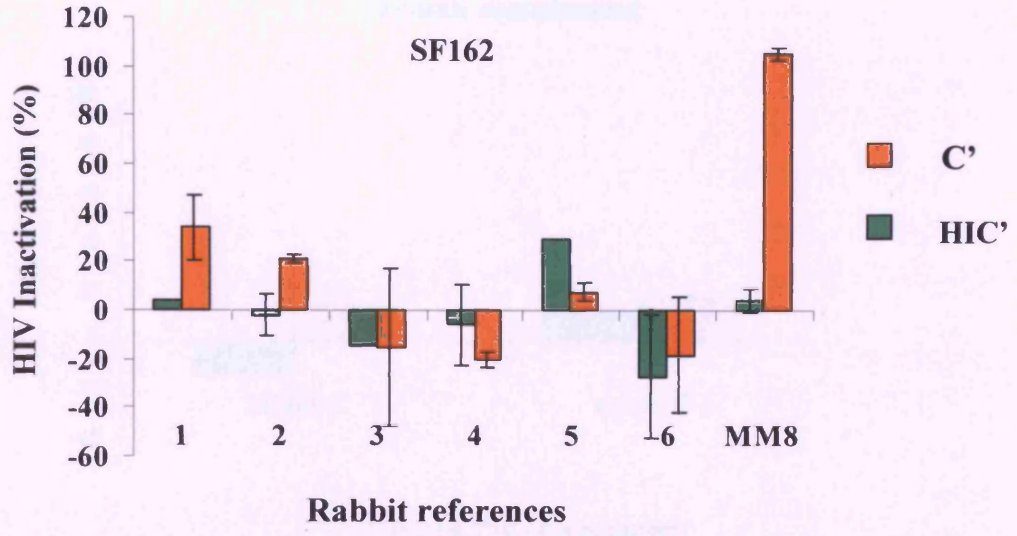

Figure 5.9 CMI assays with rabbit sera from the first boost of immunisation
All assays were performed with sera diluted at 1/20. Inactivation percentages were calculated from the data obtained with pre-bleed sera from the same animal for each assay in the presence (orange bars) or in the absence (green bars) of complement. The positive control for complement was performed using MM8 with serum day 49 POS in the presence of the same source of complement. Error bars represent the standard error from three experiments. The *t*-test statistical analysis (*t*-test, $p<0.05$) showed that there was no significant difference observed between data obtained in the presence and the absence of complement under the experimental conditions used

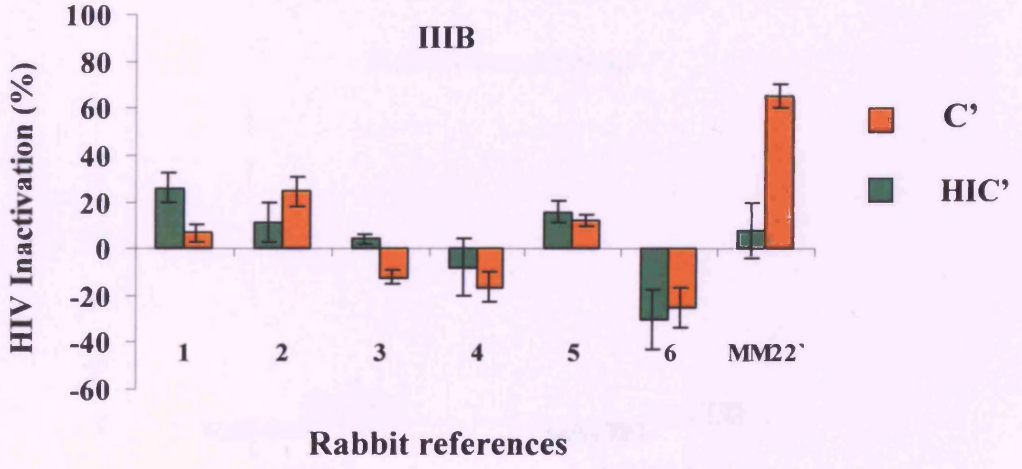

Figure 5.10 CMI assays with rabbit sera from the second boost of immunisation
All assays were performed with sera diluted at 1/20. Inactivation percentages were calculated from the data obtained with pre-bleed sera from the same animal for each assay in the presence (orange bars) or in the absence (green bars) of complement. The positive control for complement was performed using MM22 with serum day 31 POS in the presence of the same source of complement. Error bars represent the standard error from three experiments. The *t*-test statistical analysis (*t*-test, $p<0.05$) showed that there was no significant difference observed between data obtained in the presence and the absence of complement under the experimental conditions used

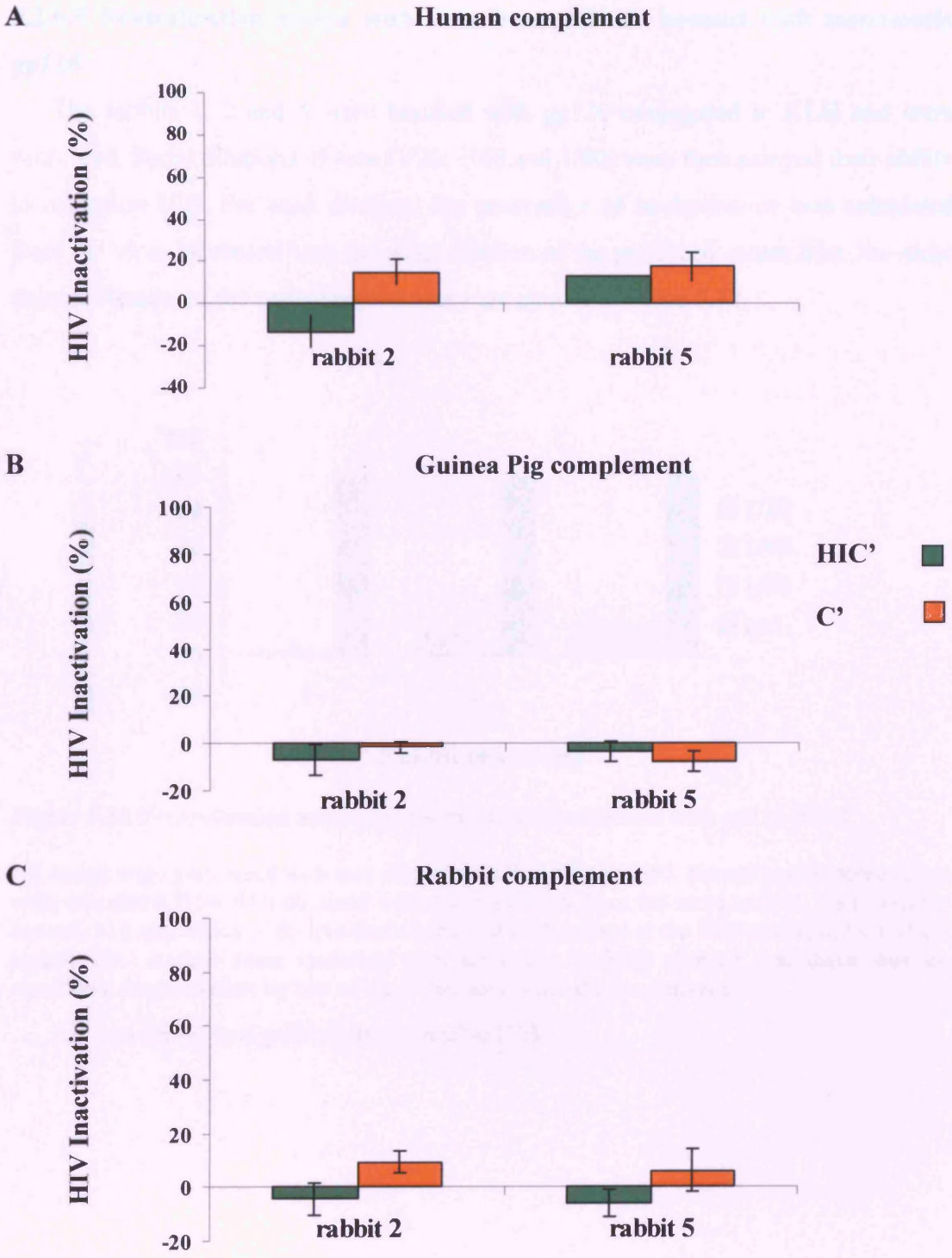

Figure 5.11 CMI with sera from the third boost of immunisation using human, Guinea pig and rabbit complement

All assays were performed with sera diluted at 1/20. Inactivation percentages were calculated from data obtained with pre-bleed sera from the same animal for each assay in the presence (green bars) or the absence (green bars) of complement from human (A), guinea pig (B) and rabbit (C) serum. Error bars represent the standard error from three experiments. The *t*-test statistical analysis (*t*-test, $p<0.05$) showed that there was no significant difference observed between data obtained in the presence and the absence of complement under the experimental conditions used

5.2.6.5 Neutralisation assays with sera from animals boosted with monomeric gp120

The rabbits 1, 2 and 5 were boosted with gp120 conjugated to KLH and were sacrificed. Serial dilutions of sera (1/20, 1/40 and 1/80) were then assayed their ability to neutralise IIIB. For each dilution, the percentage of neutralisation was calculated from the virus incubated with the same dilution of the pre-bleed serum from the same animal. Results of the neutralisation assay are shown in figure 5.12.

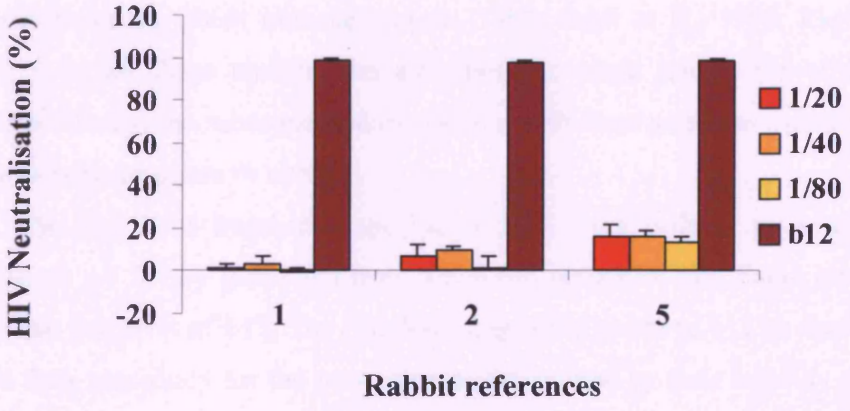

Figure 5.12 Neutralisation assays with sera of rabbits boosted with gp120-KLH

All assays were performed with sera diluted at 1/20, 1/40 and 1/80. Neutralisation percentages were calculated from data obtained with pre-bleed sera from the same animal. As a positive control, b12 was added in the pre-bleed serum of each animal at the final concentration of 2.5 µg/ml. The Student *t*-test statistical analysis (*t*-test $p<0.05$) showed that there was no significant neutralisation by any of the tested sera under the conditions used.

All sera failed to significantly neutralise IIIB.

5.3 Discussion

This chapter describes the isolation of five non-classical llama VHH fragments (A3, B4, D3, D7 and F10) that are anti-idiotypic for b12, a potent cross-neutralising anti-HIV-1 Ab. b12 neutralises HIV-1 by binding to the CD4 pocket through interactions with its CDR regions, particularly the CDR H3 region (Saphire et al., 2001b).

Anti-idiotypes have been generated in an attempt to mimic the antigen binding sites of NAbs against enterovirus 70 (Wiley et al., 1992), bovine herpesvirus 1 (Hariharan et al., 1991), reovirus 3 (Bruck et al., 1986), hepatitis B (Thanavala et al., 1986) and HIV-1 (Boudet et al., 1994; Dalgleish et al., 1987; Denisova et al., 1995; Denisova et al., 2000; Hohmann et al., 1993; Sutor et al., 1992; Zaghouani et al., 1991). In all these studies, the anti-idiotypes could inhibit the antibody-antigen interaction and the subsequent immunisation with these anti-idiotypes induced a weak neutralising response *in vitro*.

The five VHH fragments, specific to b12, were isolated by screening a non-classical Ab library generated from the B cell repertoire of a llama immunised with the Fab fragment of b12. The five VHH fragments bound to b12 as shown by ELISA and their specificity for the paratope was determined by their inability to bind MAbs of the same isotype, namely 17b and 2G12. Furthermore, all five VHH fragments inhibited HIV neutralisation mediated by b12 in a dose-dependent manner. They are therefore anti-idiotypes of b12.

The five anti-idiotypes of b12 were used to immunise rats and rabbits in an attempt to generate a b12-like NAb response against HIV-1. After an initial immunisation and a further boost with VHH conjugated to KLH, none of the sera from any of the rabbits or rats significantly neutralised IIIB or SF162, which are sensitive to b12. We and others have reported that complement can be triggered by the binding of anti-HIV Abs on viruses (Aasa-Chapman et al., 2005; Spear et al., 1992). However, complement, in association with rabbit sera did not inactivate HIV. Further boosts of immunisations performed in rabbits did not lead to the generation of neutralising sera, even in the presence of Guinea pig or rabbit complement. Rabbits were challenged with gp120 conjugated to KLH. The sera were subsequently tested with IIIB and did not neutralise this isolate under the experimental conditions used.

Other reports have shown the isolation of anti-idiotypes that induced a weak neutralising response against HIV-1. To generate an anti-idiotypic response, they used

Abs for TCLA viruses such as IIIIB (Denisova et al., 1995; Sutor et al., 1992; Zaghouani et al., 1991), MN (Boudet et al., 1994; Kang et al., 1992), RF and SF2 (Dalgleish et al., 1987)]. Such NAbs target the V3 loop (Denisova et al., 1995) the CD4BS (Kang et al., 1992) or CD4 itself (Dalgleish et al., 1987; Sutor et al., 1992). In these studies, the anti-HIV Abs were apparently type-specific, neutralising the specific strain recognised by the immunogen and were not tested for PI neutralisation. Because b12 cross-neutralises a wide range of TCLA and PI isolates (Parren et al., 1998), it represented a good candidate to generate a mimic of a conserved neutralising epitope of HIV Env. Anti-idiotypic peptides for b12 have been previously isolated (Zwick et al., 2001a) but their inoculation into mice and rabbits did not elicit NAbs. Muyldermans suggested that, because of the extra flexibility of the CDR regions, VHH fragments would be ideal for anti-idiotypic approaches (Muyldermans and Lauwereys, 1999). VHH fragments were therefore chosen to generate anti-idiotypes to b12. Despite their flexible nature, the immunisation of the anti-b12 VHH fragments in animals did not elicit a b12-like NAb response, nor did the boost with gp120 conjugated to KLH. The experiments in this chapter did not determine whether the anti-VHH Abs bound to HIV. To check if they bind to HIV, cells expressing trimeric Envs on their surface could be incubated with the purified anti-VHH IgG (and purified IgG from a non-immunised rabbit) and then analysed by FACS. However, the detection of binding does not always correlate with neutralisation (Poignard et al., 2003).

The lack of induction of neutralisation could be explained in several ways. Firstly, the internal image of b12 might have been isolated but the conjugation to KLH could have altered its conformation. However, this compound has been used successfully for the generation of other anti-idiotypes (Hohmann et al., 1993; Zaghouani et al., 1991). Conjugation of anti-idiotypes to ovalbumin has previously been used to generate Abs that bound to the V3 loop (Boudet et al., 1994). Perhaps using ovalbumin to conjugate the anti-b12 VHH fragments could lead to the induction of a NAb response in immunised animals. Further studies are needed to address this issue.

Secondly, the generation of a neutralising anti-HIV-1 Ab response would require the induction of the internal image of the b12 antigen binding site. Anti-idiotypes can be categorised into four groups: anti-Idα, anti-Idβ, anti-Idγ and anti-Idε (Thanavala and Pride, 1994). Anti-Idγ consist of all the anti-idiotypes that recognise epitopes overlapping the CDR regions whereas anti-Idβ are internal images (Thanavala and

Pride, 1994). Anti-Idγ and anti-Idβ could inhibit b12 neutralisation *in vitro*, but only immunisation with anti-Idβ will trigger a neutralising response (Thanavala and Pride, 1994). In this study, the five VHH fragments might be anti-Idγ and not anti-Idβ and, therefore, might have failed to elicit any b12-like Abs. The crystal structure of each VHH fragment in association with b12 could detail the interactions and reveal if any of the VHH fragment has the characteristics of the internal image of b12.

Thirdly, the antigen binding site of b12 might not be immunogenic in llamas and anti-Idβ were not generated. The induction of classical IgG mimics of the CD4BS never occurs or does so very rarely (Beverley et al., 1989; Davis et al., 1992; Sattentau et al., 1989) and this also could be the case for VHH fragments.

Finally, the isolation of a unique anti-Id that exactly mimics a conserved epitope of HIV-1 might be too technically difficult. The internal image of b12 could have been under-represented in the library against which the selections were performed. The library has a normal size for isolations of anti-b12 VHH fragments (10^9 CFU/ml, Dr. Hans de Haard, personal communication) but could have been too small to successfully isolate the anti-Idβ of b12.

Regardless, the five VHH fragments generated in this study could be useful for screening sera from patients to evaluate the presence of b12-like NAbs.

Chapter 6
Summary and general discussion

The overall focus of this thesis was to analyse humoral immunity to HIV-1.

In the first part of this thesis, the role of complement in HIV-1 inactivation using sera from patients with acute infection was investigated. In the absence of complement, NAbs were detected between 3 and 9 months POS. In the presence of complement, CMI was detected as soon as 9 days POS, for both autologous and heterologous viruses, concomitantly with the detection of CTL. Characterisation showed that the classical pathway, triggered by anti-gp120 IgG and possibly anti-gp120 IgM, was involved. Although CMI was tested with some MAbs, the nature of the epitopes involved in CMI has not been thoroughly investigated. If non-NAbs can inactivate HIV with complement *in vivo*, either directly or by stimulating phagocytes, the early humoral response could represent another immune pressure from which HIV has to escape. Hence, triggering an immune response against epitopes involved in CMI (if they are specific) could provide an alternative strategy to vaccine design against HIV. It will be equally important to rule out *in vitro* the possibility that non-NAbs with complement enhance HIV infection of FDCs or of phagocytes such as macrophages which express complement receptors at their surface. *In vitro* studies exploring the hypothesis of complement-mediated antibody-dependent enhancement of HIV early in infection might clarify if such phenomena could happen *in vivo*. Early non-NAbs, in association with complement, could play a more important role in HIV infection than initially thought.

The second part of this thesis explored the possibility of eliciting anti-HIV-1, b12-like NAbs based on an anti-idiotypic approach. Non-classical llama VHH fragments were selected by phage display against b12 in order to isolate a mimic of the b12 epitope on HIV-1. The anti-b12 VHH fragments isolated were specific to b12 as shown by ELISA and inhibited b12 neutralisation *in vitro* but the immunisation of rabbits and rats with VHH conjugated to KLH did not lead to the generation of a cross-neutralising b12-like Ab response against HIV-1. These anti-b12 VHH fragments could be useful for screening of anti-HIV sera to investigate the presence and the occurence of b12-like Abs against HIV *in vivo*.

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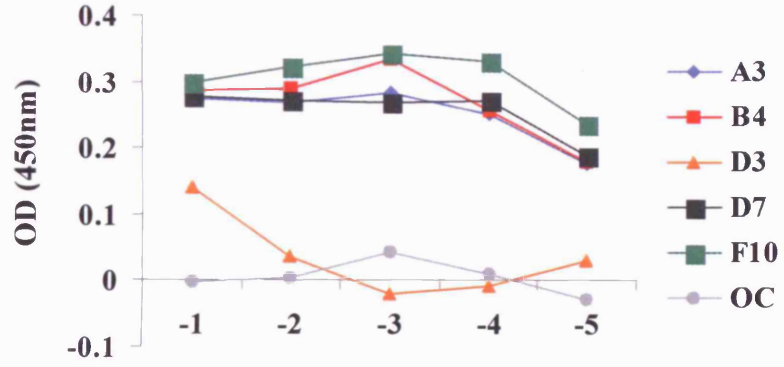
APPENDICES

	C'	HIC'
Replicate 1	90	72
Replicate 2	70	96
Replicate 3	65	92
Replicate 4	90	60
Replicate 5	90	72
Replicate 6	73	70
Σx	478	460
n	6	6
$\overline{x}$	80	76
Σx^2	38 654	36 548
$(\Sigma x)^2$	230 400	211 600
$(\Sigma x)^2/n$	38 400	35 267
$\Sigma d^2 = \Sigma x^2 - ((\Sigma x)^2/n)$	254	1281
$\sigma^2 = \Sigma d^2 / n-1$	50.85	256.2
$\sigma d^2 = \sigma_1^2/n1 + \sigma_2^2/n2$	51.16	
σd	7.15	
$t = (\overline{x_1} + \overline{x_2})/ \sigma d$	0.559	

The t value for 6 degrees of freedom is 2.45 for a p value of 0.05. The t value found is 0.559, below 2.45. Therefore, for this example, there is no significant difference between the data obtained in the presence (C) and the absence of complement (HIC).

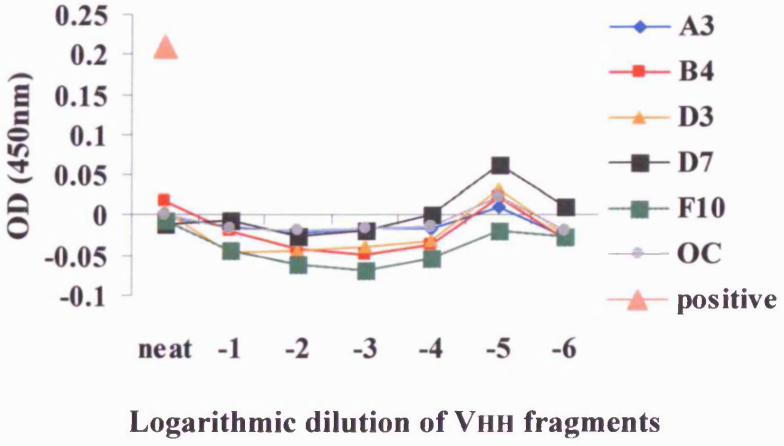
Appendix 1. Example of the *t*- test calculations used for used for statistical analysis.

The data were obtained for patient MM27 in association with autologous serum at day 28 POS



Appendix 2. Titration of purified VHH fragments against b12 by ELISA.

Purified VHH fragments, lacking the Myc tag at their C-terminus, were serially diluted in PBS and were tested for binding to b12 by ELISA. The VHH fragments were tested in uniplicate.



Appendix 3. Titration of purified VHH fragments against 2G12 by ELISA

Purified VHH, lacking the Myg tag at their C-terminus, were serially diluted in PBS and were tested for binding to 2G12 by ELISA. The VHH fragments were tested in uniplicate. 2G12 was detected by an anti-human IgG conjugated to HRP (positive control)