HIV-2 Neutralisation: interactions between viral glycoproteins and cell surface receptors

Elaine Rhiannon Thomas

Submitted to University of London for the degree of Doctor of Philosophy

August 2002

Wohl Virion Centre
Department of Immunology and Molecular Pathology
Windeyer Institute of Medical Sciences
University College London
Abstract

Entry of HIV into target cells is the result of virus-cell fusion mediated by interactions between viral glycoproteins and cell surface receptors. Some HIV-2 strains can infect cells in the absence of the major receptor CD4, by direct interaction with a chemokine coreceptor, usually either CCR5 or CXCR4. ROD B is a highly CD4-independent variant of the prototype HIV-2 isolate ROD A. Investigation of neutralisation sensitivity of ROD A and ROD B to sera from HIV-2 infected individuals and to monoclonal antibodies showed ROD B is highly sensitive to neutralisation. CD4-dependent ROD A was relatively resistant to neutralisation. However, induction of ROD A infection of CD4 negative cells by soluble CD4 rendered the virus highly sensitive to neutralisation. Many clinically relevant primary isolates of HIV-2 are able to infect coreceptor positive cells in the absence of CD4. In contrast to the highly neutralisation resistant phenotype of primary isolates of HIV-1, primary isolates of HIV-2 were susceptible to neutralisation when utilising a CD4 independent route of infection. CD4-independent ROD B was also less sensitive to neutralisation when infecting some CD4 positive cells.

Antibody binding to viral envelope is often a predictor of neutralisation efficacy. Antibody binding to both monomeric and oligomeric envelope proteins of ROD A and ROD B assayed by several methods does not correlate with neutralisation sensitivity. However, further analysis of antibody binding to HIV-2 envelope using virion capture assays and Biacore technology did reveal subtle differences in antibody affinity that may contribute to the increased neutralisation sensitivity of CD4-independent ROD B. Therefore, both the affinity of the antibody for viral envelope and the use of cellular CD4 play a role in determining sensitivity to neutralisation.
Acknowledgments

Thanks first to my supervisor Áine McKnight for her help and encouragement throughout. Also to Paul Clapham and Robin Weiss for all their support and advice and the critical reading of this thesis.

Thanks to all in the Wohl Virion Centre, particularly the HIV group past and present, especially Graham Simmons, Jackie Reeves, Sam Hibbits and Sam Willey for their advice and support and making the cat 3 fun and a enjoyable place to work. Also thanks to Bushy, Stuart Neil and Keith Aubin for reading bits of this thesis and their support and encouragement during writing.

This thesis could not have been done without the financial support of AVERT and the MRC. Chris Shotton, David Hawkins and Jose Moniz-Pereira provided many valuable reagents for which I am very grateful.

Cheers to all the friends/crazy people in number 64, past and present. Also to Sandeep for being a fun and welcome distraction often at the other end of the phone.

Finally, heartfelt thanks to Richard Jenner for reading parts of this thesis without complaining too much and for all his support and encouragement - I can only hope that I can do as much for you.
Contents

Title page .......................................................................................................................1
Abstract .........................................................................................................................2
Acknowledgments.........................................................................................................3
Contents .........................................................................................................................4
Figures .........................................................................................................................11
Tables............................................................................................................................14
Abbreviations ..............................................................................................................15

Chapter 1
Introduction.................................................................................................................17
  1.1 Retroviruses ..............................................................................................................17
  1.2 Origins and diversity of HIV ...................................................................................17
  1.2 Genome organisation ...............................................................................................19
  1.3 Life cycle ...................................................................................................................20
    1.3.1 Entry ..................................................................................................................20
    1.3.2 Uncoating and Reverse Transcription .............................................................21
    1.3.3 Nuclear import ..................................................................................................24
    1.3.4 Integration ..........................................................................................................25
    1.3.5 Transcription and exit of RNA species from the nucleus ..............................25
    1.3.6 Particle formation and budding ........................................................................27
    1.3.7 Particle maturation ............................................................................................29
  1.4 Viral entry ................................................................................................................30
    1.4.1 HIV envelope .....................................................................................................30
      1.4.1.1 Glycosylation .............................................................................................30
      1.4.1.2 Proteolytic processing ...............................................................................31
      1.4.1.3 gp120 ..........................................................................................................31
      1.4.1.4 gp41 ............................................................................................................32
      1.4.1.5 Fusion .......................................................................................................33
    1.4.2 Cellular receptors ..............................................................................................34
      1.4.2.1 CD4 ............................................................................................................34
      1.4.2.2 Coreceptors ................................................................................................35
      1.4.2.3 Attachment receptors...................................................................................39
1.5 Course of HIV infection and pathogenesis of disease ..........................................40
  1.5.1 Epidemiology ....................................................................................................40
  1.5.2 Transmission .....................................................................................................40
    1.5.2.1 Protection from transmission ...................................................................41
  1.5.3 Primary HIV infection ......................................................................................42
  1.5.4 Chronic HIV infection ......................................................................................43
  1.5.5 AIDS ..................................................................................................................43
  1.5.6 Long term non-progressors ..............................................................................44
  1.5.7 CD4 cell death ...................................................................................................45
  1.5.8 Treatment ...........................................................................................................46

1.6 Immune responses to HIV .......................................................................................50
  1.6.1 Innate immune response ...................................................................................50
    1.6.1.1 Complement system ..................................................................................50
    1.6.1.2 Interferon ...................................................................................................51
    1.6.1.3 γδ T cells ....................................................................................................52
    1.6.1.4 Natural killer cells ...................................................................................52
  1.6.2 Cell mediated immune response ......................................................................53
    1.6.2.1 CD8 T-cells ................................................................................................53
    1.6.2.2 CD4 T cells ................................................................................................55
  1.6.3 Humoral immune response ..............................................................................56
    1.6.3.1 CD4 binding site epitopes .........................................................................58
    1.6.3.2 V3 epitopes ................................................................................................58
    1.6.3.3 V1/V2 epitopes ..........................................................................................59
    1.6.3.4 CD4 induced epitopes ...............................................................................59
    1.6.3.5 2G12 epitope ..............................................................................................59
    1.6.3.6 gp41 epitopes .............................................................................................60
    1.6.3.7 Antibody dependent enhancement ...........................................................60
    1.6.3.8 Recruitment of complement and cytotoxic cells .....................................61
  1.6.4 Immune evasion ................................................................................................61
  1.6.5 Vaccine development .......................................................................................63

1.7 Scope of this thesis ..................................................................................................66
Chapter 2

Materials and Methods........................................................................................................67

2.1 Buffers and solutions .................................................................................................67

2.2 Eukaryotic cell culture ..............................................................................................67

2.2.1 Cell lines ..............................................................................................................68

2.2.2 Passaging cells .....................................................................................................69

2.2.3 Freezing cells .......................................................................................................69

2.2.4 Thawing cells ......................................................................................................69

2.2.5 Preparation of peripheral blood mononuclear cells ..............................................69

2.2.6 Flow cytometry of cell surface receptors ............................................................70

2.2.7 Transfection of eukaryotic cells ..........................................................................70

2.3 HIV .........................................................................................................................71

2.3.1 Molecular clones of HIV-2 ..................................................................................71

2.3.2 HIV-1 and HIV-2 isolates ...................................................................................71

2.3.3 Pseudovirus production .......................................................................................72

2.4 Virus detection assays ...............................................................................................73

2.4.1 Immunostaining of HIV infected cells ...............................................................73

2.4.2 RT ELISA .............................................................................................................73

2.4.3 p24 ELISA ............................................................................................................74

2.4.4 Flow Cytometry .................................................................................................74

2.5 Infection assays .........................................................................................................75

2.5.1 Titration on adherent cell lines ..........................................................................75

2.5.2 Titration on PBMC, TCID₅₀ calculation ................................................................75

2.5.3 Neutralisation assay on adherent cell lines .......................................................76

2.5.4 Neutralisation assay on PBMC ..........................................................................76

2.5.5 Chemokine receptor blocking .............................................................................76

2.5.6 Cell-cell fusion ....................................................................................................77

2.5.7 Viral stability assay .............................................................................................77

2.6 Antibodies ................................................................................................................77

2.6.1 Rat monoclonal antibodies ................................................................................77

2.6.2 Isotyping of monoclonal antibodies ....................................................................78
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.6.3 Antibody mapping</td>
<td>78</td>
</tr>
<tr>
<td>2.6.3.1 Binding to infected cells</td>
<td>78</td>
</tr>
<tr>
<td>2.6.3.2 Binding to CD4</td>
<td>79</td>
</tr>
<tr>
<td>2.6.3.3 Construct mapping</td>
<td>79</td>
</tr>
<tr>
<td>2.6.3.4 Peptide mapping</td>
<td>80</td>
</tr>
<tr>
<td>2.6.4 Human monoclonal antibodies</td>
<td>81</td>
</tr>
<tr>
<td>2.6.5 Human sera</td>
<td>81</td>
</tr>
<tr>
<td>2.6.6 Purification of immunoglobulin from human sera</td>
<td>81</td>
</tr>
<tr>
<td>2.6.7 Protein quantitation</td>
<td>81</td>
</tr>
<tr>
<td>2.7 Envelope binding assays</td>
<td>82</td>
</tr>
<tr>
<td>2.7.1 Monomeric envelope binding</td>
<td>82</td>
</tr>
<tr>
<td>2.7.2 Competition of rat and human MAbs binding to ROD envelope</td>
<td>82</td>
</tr>
<tr>
<td>2.7.3 Capture assays</td>
<td>83</td>
</tr>
<tr>
<td>2.7.4 Binding to cell surface envelope by flow cytometry</td>
<td>84</td>
</tr>
<tr>
<td>2.8 DNA manipulation</td>
<td>84</td>
</tr>
<tr>
<td>2.8.1 Agarose gel electrophoresis</td>
<td>84</td>
</tr>
<tr>
<td>2.8.2 DNA extraction from agarose gels</td>
<td>85</td>
</tr>
<tr>
<td>2.8.3 Plasmid purification</td>
<td>85</td>
</tr>
<tr>
<td>2.8.4 DNA quantitation</td>
<td>86</td>
</tr>
<tr>
<td>2.8.5 Plasmids</td>
<td>86</td>
</tr>
<tr>
<td>2.8.6 Primers</td>
<td>86</td>
</tr>
<tr>
<td>2.8.7 Amplification of HIV-2 envelope</td>
<td>86</td>
</tr>
<tr>
<td>2.8.8 Cloning into pCR 3.1 uni</td>
<td>87</td>
</tr>
<tr>
<td>2.8.9 Transformation of competent bacteria</td>
<td>87</td>
</tr>
<tr>
<td>2.8.9 Colony screening</td>
<td>87</td>
</tr>
<tr>
<td>2.9 Biacore analysis</td>
<td>88</td>
</tr>
<tr>
<td>2.9.1 General procedures and maintenance</td>
<td>88</td>
</tr>
<tr>
<td>2.9.2 Pre-concentration</td>
<td>89</td>
</tr>
<tr>
<td>2.9.3 Regeneration</td>
<td>89</td>
</tr>
<tr>
<td>2.9.4 Antibody immobilisation</td>
<td>89</td>
</tr>
<tr>
<td>2.9.5 gp105 binding</td>
<td>90</td>
</tr>
<tr>
<td>2.9.6 Pseudovirus binding</td>
<td>91</td>
</tr>
</tbody>
</table>
Chapter 3
Neutralisation sensitivity of CD4-dependent and CD4-independent HIV-2 ....... 92

3.1 Introduction ..............................................................................................................92
3.2 Results .....................................................................................................................95
  3.2.1 Neutralisation of ROD A and CD4-independent variant ROD B by sera
       from HIV-2 positive individuals ...................................................................... 95
  3.2.2 Purification and neutralisation properties of immunoglobulin from
       HIV-2 positive serum ....................................................................................... 96
  3.2.3 Neutralisation of ROD A induced to infect CD4 negative cells ............... 99
  3.2.4 Neutralisation properties of human anti-HIV-2 MAbs ............................. 100
  3.2.5 Susceptibility of HIV-2 to neutralisation by 17b ...................................... 102
  3.2.6 Neutralisation of primary isolates of HIV-2 ............................................. 103
3.3 Discussion ...............................................................................................................107
  3.3.1 CD4-independent ROD B is more sensitive to neutralisation by HIV-2
       positive sera compared with ROD A ............................................................. 107
  3.3.2 sCD4 induction of ROD A renders it sensitive to neutralisation .......... 107
  3.3.3 Differences between ROD A and ROD B that may confer differential
       sensitivity to neutralisation ............................................................................. 108
  3.3.4 Infection of CD4 negative cells by primary isolates of HIV-2 is highly
       sensitive to neutralisation ............................................................................. 110
3.4 Summary .................................................................................................................112
Chapter 4
Production, characterisation and neutralisation properties of rat MAbs
that recognise different regions of the HIV-2 ROD envelope

4.1 Introduction

4.2 Results

4.2.1 Production of rat MAbs

4.2.2 MAb specificity

4.2.2.1 Envelope specific MAbs

4.2.2.2 CD4 specific MAbs

4.2.3 Mapping of envelope specific MAbs

4.2.3.1 Chimeric constructs

4.2.3.2 Peptide mapping

4.2.3.3 CD4 blocking

4.2.4 Neutralisation of ROD A and ROD B by rat MAbs

4.2.5 Neutralisation of ROD A induced to infect CD4 negative cells

4.2.6 Competition between rat and human Abs

4.2.7 Neutralisation sensitivity depending on target cell

4.2.8 Chemokine inhibition of ROD A and ROD B

4.2.9 CD4 mutants

4.2.10 Neutralisation on primary human cells

4.3 Discussion

4.3.1 MAbs of different specificities were generated by immunisation with gp105/sCD4

4.3.2 Neutralisation of ROD B is not epitope specific

4.3.3 Target cell type influences neutralisation sensitivity

4.4 Summary

113

115

115

115

116

116

117

117

118

119

121

122

123

126

130

131

132

134

134

135

137

139
Chapter 5

Exposure of neutralising epitopes on ROD A and the CD4-independent variant, ROD B

5.1 Introduction ................................................................. 140
5.2 Results ................................................................. 143
  5.2.1 Antibody binding to monomeric envelope ......................... 143
  5.2.2 Antibody binding to oligomeric envelope .......................... 145
  5.2.3 Cell to cell fusion .................................................. 147
  5.2.4 Virion capture ....................................................... 152
  5.2.7 Virus stability ....................................................... 154
  5.2.8 Biacore ................................................................. 155
    5.2.8.1 Development of Biacore assay ............................... 155
    5.2.8.2 Biacore analysis of gp105-77ow/2g interaction .......... 156
    5.2.8.3 Biacore analysis of gp105/sCD4-77ow/2g interaction .... 157
    5.2.8.4 Specificity ...................................................... 158
  5.2.9 Pseudovirus .......................................................... 158
    5.2.8.5 Production and characterisation of ROD A and ROD B pseudovirus 159
    5.2.8.6 Biacore analysis of pseudovirus ................................ 162
  5.3 Discussion ............................................................... 163
    5.3.1 Neutralisation sensitivity does not correlate with Ab binding to monomeric envelope .............................. 163
    5.3.2 Neutralisation sensitivity does not correlate with Ab binding to cell surface expressed envelope ................. 163
    5.3.3 Binding to virion associated envelope .......................... 164
    5.3.4 Viral stability ....................................................... 164
    5.3.5 Biacore analysis ................................................... 165
  5.4 Summary .................................................................. 167

Chapter 6

Summary ........................................................................... 168

References ........................................................................ 170
Figures

Chapter 1

Figure 1.1. Genomic maps of HIV-1 and HIV-2 .........................................................20
Figure 1.2. Life cycle of HIV ........................................................................................21
Figure 1.3. Reverse transcription ...................................................................................23
Figure 1.4. HIV particle .................................................................................................29
Figure 1.5. Crystal structure of gp120 ..........................................................................32
Figure 1.6. Domains of gp41 .........................................................................................33
Figure 1.7. Fusion of HIV with the cell membrane.......................................................34

Chapter 2

Figure 2.1. Production of envelope pseudotypes .........................................................72
Figure 2.2. HIV envelope chimeric constructs .............................................................80
Figure 2.3. Principles of Biacore ...................................................................................88
Figure 2.4. Sensorgram of 77ow/2g immobilisation ....................................................90

Chapter 3

Figure 3.1. Protein content, envelope binding and neutralisation of
immunoglobulin purified fraction of HIV-2 serum .................................................98
Figure 3.2. Neutralisation of sCD4-induced ROD A by HIV-2 positive serum ......99
Figure 3.3. Neutralisation of ROD A and ROD B by human MAbs ..........................102
Figure 3.4. Neutralisation of ROD by 17b ..................................................................103
Figure 3.5. Neutralisation of primary isolates of HIV-2 by HIV-2 positive sera ..105
Figure 3.6. Neutralisation of primary isolates of HIV-2 induced to infect CD4
negative cells .............................................................................................................106
Chapter 4

Figure 4.1. Binding of MAbs to soluble CD4. .............................................................117
Figure 4.2. Construct mapping of rat MAbs. .................................................................118
Figure 4.3. Binding of rat MAbs and sCD4 to ROD envelope. ......................................120
Figure 4.4. sCD4 induces neutralisation of ROD A ....................................................123
Figure 4.5. Competition analysis of human and rat MAbs. ..........................................125
Figure 4.6. Neutralisation of ROD A and ROD B infection of CD4 positive and
            negative CCC cells ....................................................................................127
Figure 4.7. Neutralisation sensitivity of ROD A and ROD B on different cell
            types ...........................................................................................................128
Figure 4.8. Flow cytometry of cell surface receptors ...................................................129
Figure 4.9. Chemokine blocking of ROD A and ROD B infection. ..............................130
Figure 4.10. Neutralisation of ROD A and ROD B infection of HeLa cells
              expressing mutant CD4 ............................................................................131
Figure 4.11. Receptor expression and ROD titre on HeLa cells expressing CD4
              mutants ......................................................................................................134
Figure 4.12. Neutralisation of ROD A and ROD B infection of PBMC. ......................133
Chapter 5

Figure 5.1. Binding of MAbs and HIV-2 positive sera to envelope protein of ROD A and ROD B. ......................................................... 144
Figure 5.2. Flow cytometry of transfected 293T cells. .................................................. 146
Figure 5.3. Antibody binding to ROD A and ROD B envelopes expressed on transfected 293T cells. ......................................................... 147
Figure 5.4. Fusion of 293T cells with HeLa/CD4.................................................... 149
Figure 5.5. Fusion of 293T cells with CD4 positive T cells. ...................................... 150
Figure 5.6. ROD transfected Cos-1 cells. ................................................................. 151
Figure 5.7. Capture of ROD A and ROD B detected by polyclonal HIV-2 serum. ............................................................................... 152
Figure 5.8. Capture ELISA of ROD A and ROD B detected by RT........................153
Figure 5.9. Stability of ROD A and ROD B compared with primary isolates of HIV-1 and HIV-2 .................................................................................... 154
Figure 5.10. Removal of background binding and reproducibility of Biacore...... 156
Figure 5.11. Biacore analysis of gp105 binding to 77ow/2g. ...................................... 157
Figure 5.12. Specificity of Biacore interaction. ............................................................. 158
Figure 5.13. ROD A and ROD B envelope cloning. .................................................... 159
Figure 5.14. Infectivity of pseudovirus................................................................. 160
Figure 5.15. Envelope, p24 and RT content of pseudovirus.................................... 160
Figure 5.16. Pseudovirus binding to 77ow/2g by Biacore. ........................................ 162
Tables

Chapter 1
Table 1.1. Coreceptors for HIV and SIV .................................................................36

Chapter 2
Table 2.1. Buffers and solutions ...........................................................................67
Table 2.2. Cell lines ...............................................................................................68
Table 2.3. Antibodies for flow cytometry analysis of cell surface receptors .......70
Table 2.4. Cell densities for infection assays .......................................................75
Table 2.5. Primers .................................................................................................86

Chapter 3
Table 3.1. Neutralisation titres of HIV-2 positive sera .......................................96
Table 3.2. Characteristics of human anti-HIV-2 MAb ......................................100

Chapter 4
Table 4.1. Characteristics of rat MAbs ...............................................................115
Table 4.2. Epitope mapping of rat MAbs ............................................................119
Table 4.3. Neutralisation titres of rat MAbs .......................................................122
Table 4.4. Competition between rat and human Abs .........................................124
Table 4.5. Neutralisation sensitivity of ROD A and ROD B depending on target
cell type. .............................................................................................................138

Chapter 5
Table 5.1. Half maximal binding of MAbs and HIV-2 positive serum to
envelope proteins ...............................................................................................144
Table 5.2. Affinity constants for gp150 binding to 77ow/2g ................................158
Table 5.3. Comparison of affinity constants .......................................................166
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody dependent cell cytotoxicity</td>
</tr>
<tr>
<td>ADE</td>
<td>Antibody dependent enhancement</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>ARP</td>
<td>AIDS Reagent Program</td>
</tr>
<tr>
<td>AZT</td>
<td>3’azido-3’-deoxythymidine</td>
</tr>
<tr>
<td>CA</td>
<td>Capsid</td>
</tr>
<tr>
<td>CAEV</td>
<td>Caprine arthritis encephalitis virus</td>
</tr>
<tr>
<td>CAF</td>
<td>CD8 T cell antiviral factor</td>
</tr>
<tr>
<td>CCR</td>
<td>CC chemokine receptor</td>
</tr>
<tr>
<td>CDK9</td>
<td>Cyclin dependent kinase 9</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CRF</td>
<td>Circulating recombinant forms</td>
</tr>
<tr>
<td>CRM1</td>
<td>Chromosome maintenance region protein</td>
</tr>
<tr>
<td>CR2</td>
<td>Complement receptor 2</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CXCR</td>
<td>CXC chemokine receptor</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>Dendritic cell-specific ICAM-3 grabbing non-integrin</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>eGFP</td>
<td>Emerald green fluorescent protein</td>
</tr>
<tr>
<td>EIAV</td>
<td>Equine infectious anaemia virus</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>Env</td>
<td>Envelope protein</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>EU</td>
<td>Exposed uninfected</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>ffu</td>
<td>Focus forming units</td>
</tr>
<tr>
<td>FIV</td>
<td>Feline immunodeficiency virus</td>
</tr>
<tr>
<td>GalC</td>
<td>Galactosyl ceramide</td>
</tr>
<tr>
<td>GNA</td>
<td><em>GALANTHUS NIVALIS</em> agglutinin (snowdrop bulb lectin)</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly active antiretroviral therapy</td>
</tr>
<tr>
<td>HAG</td>
<td>Heat aggregated gammaglobulin</td>
</tr>
<tr>
<td>HHV6</td>
<td>Human herpesvirus 6</td>
</tr>
<tr>
<td>HHV8</td>
<td>Human herpesvirus 8</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HSV-1</td>
<td>Herpes simplex virus-1</td>
</tr>
<tr>
<td>HRV</td>
<td>Human rhinovirus</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intracellular adhesion molecule-1</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IN</td>
<td>Integrase</td>
</tr>
<tr>
<td>IPC</td>
<td>Interferon producing cell</td>
</tr>
<tr>
<td>LTNP</td>
<td>Long term non-progressor</td>
</tr>
<tr>
<td>LTR</td>
<td>Long terminal repeat</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction

This thesis investigates neutralisation of human immunodeficiency virus type 2 (HIV-2). The biology of HIV will therefore be described. This is based primarily on research into human immunodeficiency virus type 1 (HIV-1). Generally HIV biology also applies to HIV-2 except in cases noted.

1.1 Retroviruses

The human immunodeficiency viruses are complex retroviruses. Retroviruses have diploid RNA genomes of positive polarity. They are characterised by the process of reverse transcription of the viral RNA genome into DNA prior to insertion into the host cell genome. The viral genome then mimics cellular DNA and is replicated with the host cell genome. There are 7 retrovirus subfamilies; alpharetroviruses, betaretroviruses, gammaretroviruses, deltaretroviruses, epsilonretroviruses, lentiviruses and spumaviruses. There are four human retroviruses; human T cell leukaemia virus types 1 and 2 (HTLV-1 and HTLV-2) and HIV-1 and 2. HTLV-1 is associated with Adult T cell leukaemia and HTLV-associated myelopathy or Tropical spastic paraparesis. HIV-1 and HIV-2 are the causative agents of acquired immune deficiency syndrome (AIDS), a diverse range of clinical symptoms including wasting, neurological disorders, malignancies and opportunistic infections. HIV-1 and HIV-2 are classed as lentiviruses within the retrovirus family, with a long clinically latent phase of infection. They are also characterised by the ability to infect non-dividing cells. Other mammals also harbour lentiviruses for example horses (Equine Infectious Anaemia Virus - EIAV), cats (Feline Immunodeficiency Virus – FIV), goats (Caprine Arthritis Encephalitis Virus - CAEV) and non-human primates (Simian Immunodeficiency Virus - SIV).

1.2 Origins and diversity of HIV

HIV was first isolated in 1983 from patients at risk from AIDS (Barre-Sinoussi et al., 1983). The two types of HIV, HIV-1 and HIV-2, both originate from cross species transmission of SIV found in African non-human primates (Hahn et al., 2000). The earliest HIV-1 positive sample, tested retrospectively, dates to 1959 (Nahmias et al., 1986; Zhu et al., 1998) and the first HIV-2 positive samples date to the 1960s
(Bryceson et al., 1988; Kawamura et al., 1989; Le Guenno, 1989). Molecular modelling techniques, based on the evolutionary rate of sequence changes, estimate the origin of HIV-1 group M in humans to around 1930 (Korber et al., 2000; Salemi et al., 2001).

HIV-1 is most closely related to SIV isolated from chimpanzees (Pan troglodytes troglodytes), SIV<sub>cpz</sub> (Gao et al., 1999; Hahn et al., 2000; Peeters et al., 1989). Based on sequence differences there are 3 groups of HIV-1, M (Main), O (Outlier) and N (New or Non-M, non-O), that probably arose by 3 separate zoonoses of SIV from chimpanzees. Group M viruses are responsible for the global HIV pandemic. The O group consists of a few divergent isolates, which are found mainly in Cameroon but some group O isolates have also been reported in Europe (Gurtler et al., 1994; Mauclere et al., 1997; Peeters et al., 1997; Vanden Haesevelde et al., 1994). Group N viruses are also found in Cameroon but are generally rare (Ayouba et al., 2000; Simon et al., 1998). The M group is further classified into subtypes, currently A-K, with no subtype E or I (Korber et al., 2001). The subtypes are distributed in distinct geographic clusters. Subtype B predominates in Europe, the Americas, Japan and Australia while subtypes A, C, D and AE are rapidly spreading in Africa and Asia. Recombinants between genomes of different subtypes have become established in the human population and are referred to as circulating recombinant forms (CRFs) (McCutchan, 2000; Peeters et al., 2000). The reverse transcriptase enzyme, RT, is highly recombinogenic due to the strand transfer during reverse transcription, and may account for the many recombinant forms now present within the human population (Goodrich et al., 1990; Hu et al., 1990). Inter-subtype recombinants are also causing local epidemics in West Africa, Russia and China (Peeters, 2000).

Geographical location (West Africa) and sequence similarity points to SIV of sooty mangabeys (Cercocebus atys), SIV<sub>sm</sub>, as the source of HIV-2 (Chakrabarti et al., 1987; Chen et al., 1997a; Chen et al., 1996b; Gao et al., 1994; Gao et al., 1992; Hirsch et al., 1989). HIV-2 was first suspected in healthy Senegalese individuals with strong antibody (Ab) responses to SIV (Barin et al., 1985) and subsequently identified in AIDS patients (Clavel et al., 1986). The 7 genetic subtypes of HIV-2, A-G, are thought to have arisen by separate introductions of diverse SIVs from sooty mangabeys (Chen et al., 1997a; Gao et al., 1994; Yamaguchi et al., 2000). A and B are the most widespread HIV-2 genotypes with two or fewer isolates reported for the
other subtypes. One HIV-2 recombinant genome, AB, has also been described (Takehisa et al., 1997).

Within the groups and subtypes of HIV there is additional diversity between individual HIV isolates. The viral RNA polymerase, Reverse Transcriptase (RT), lacks proofreading capabilities owing to the absence of 3'-5' exonuclease activity. Misincorporation, deletion, insertion, or duplication occurs with a frequency of $10^4$ to $10^5$, and this coupled with in vivo viral production rate of $10^9$ virions per day leads to high variability of viral sequences even within a single infected individual (Ho et al., 1995; Preston et al., 1988; Roberts et al., 1988; Wei et al., 1995).

HIV primarily infects CD4 positive T cells and cells of the monocyte/macrophage lineage. Viruses able to infect CD4 positive T-cell lines are described as T cell tropic (or T-tropic) and those infecting macrophages and primary T-lymphocytes as macrophage tropic (or M-tropic). Generally these phenotypes relate to the use of the coreceptors CXCR4 and CCR5 (see section 1.4.2.2) that are expressed preferentially on T-cell lines and macrophages respectively. HIV isolates are also classed as either primary isolates (PI) or T-cell line adapted (TCLA) depending on their passage history. PI have only been passaged through PBMC in vitro whilst TCLA have been passaged through T-cell lines in vitro. This adaptation influences coreceptor usage, cell tropism and sensitivity to neutralisation (see sections 1.4.2.2 and 1.6.3).

1.2 Genome organisation

All retroviruses have genes encoding Gag (group associated antigen), Pol (polymerase) and Env (envelope) polyproteins (Figure 1.1). Gag is synthesised as a precursor protein and subsequently cleaved by viral Protease during virion maturation into Matrix (MA, p17), Capsid (CA, p24), spacer peptides (p1 and p2), Nucleocapsid (NC, p7), and p6. A Gag-Pol polyprotein is also made due to a −1 ribosomal frameshift near the Gag-Pol junction. The Gag-Pol polyprotein is cleaved to give rise to the Gag proteins described above and the Pol proteins, Protease (PR, p10), Reverse Transcriptase (RT, p66 and p51) and Integrase (IN, p32). The Env precursor protein is cleaved to give surface (gp120) and transmembrane (gp41) proteins (see section 1.4.1.2). Lentiviruses are complex retroviruses that also have regulatory genes: Transactivator of transcription (tat) and Regulatory viral protein (rev). HIV also has accessory genes: Viral infectivity factor (vif), Negative factor (nef), Viral protein R
(vpr) and Viral protein U (vpu) (HIV-1 only) or Viral protein X (vpx) (HIV-2 and SIV only). The Long Terminal Repeat (LTR) regions at the 3 and 5 end of the genome contain repeat (R) and unique (U3 and U5) regions. The genomes of HIV-1 and HIV-2 were characterised in the mid 1980s (Clavel et al., 1986; Clavel et al., 1987; Franchini et al., 1987; Wain-Hobson et al., 1985). The genomic organisation of HIV-1 and HIV-2 is shown in Figure 1.1.

![Genomic maps of HIV-1 and HIV-2.](image)

LTRs are shaded in red, genes for the production of structural proteins in blue, regulatory proteins in green and accessory proteins in yellow (adapted from Levy, 1998).

### 1.3 Life cycle

HIV is an obligate intracellular parasite and thus requires a target cell in which to carry out the various life cycle stages. The life cycle of HIV is depicted in Figure 1.2. The stages (1-7) are described below.

#### 1.3.1 Entry

HIV-1, HIV-2 and SIV enter target cells by fusion with the lipid bilayer of the cell membrane. This is mediated by the viral envelope, which is made up of surface (SU), gp120, and transmembrane (TM), gp41, subunits (see section 1.4.1.3). To initiate fusion, gp120 interacts with specific cellular receptors, CD4 and a chemokine receptor, usually CCR5 or CXCR4 (see section 1.4.2). These interactions allow conformational changes in gp120 and gp41 resulting in fusion with the target cell membrane in a pH-independent manner (see section 1.4.1.4)(McClure et al., 1988; McClure et al., 1990; Stein et al., 1987). An endosomal entry route has also been described for certain isolates of HIV (Fackler et al., 2000; Schaeffer et al., 2001).
HIV enters target cells by interaction with the cell surface receptors CD4 and a chemokine receptor, and fuses with the cell membrane (stage ①). HIV core is released into the cell cytoplasm and, within the reverse transcription complex, the viral genomic RNA is reverse transcribed into double stranded DNA (stage ②). The preintegration complex containing viral cDNA is transported to the nuclear membrane, which it traverses by interaction of viral proteins of the preintegration complex with importins (stage ③). HIV DNA is inserted into the host cell chromosomal DNA by action of the Integrase enzyme and transcription is initiated from the Long Terminal Repeat (stage ④). Viral mRNAs are exported from the nucleus under the control of Rev and viral proteins are synthesised in the cell cytoplasm or endoplasmic reticulum (stage ⑤). Viral proteins accumulate at the cell membrane, encapsulate 2 copies of full length viral RNA and bud from the cell membrane (stage ⑥). Virion maturation occurs after budding from the cell membrane, whereby protease cleaves Gag to form mature virus capable of infecting a new target cell (stage ⑦).

1.3.2 Uncoating and Reverse Transcription

Subsequent to fusion with the target cell membrane the viral core is released into the cell cytoplasm and a structure called the reverse transcription complex (RTC) is formed (Karageorgos et al., 1993). The exact processes involved in uncoating are unclear but may involve contributions from the viral proteins Matrix, Nef, Vif and host cyclophilin A packaged in the incoming virion (Greene et al., 2002; Ohagen et al., 2000; Schaeffer et al., 2001). Within the RTC, the RNA genome is reverse
Reverse transcription is the conversion of single-stranded RNA viral genome into double-stranded DNA involving 2 priming events and 2 jumps. A schematic representation of reverse transcription is shown in Figure 1.3 (steps 1-7) and described below. Reverse Transcriptase (RT) is a heterodimer of p66 and p51 proteins that has reverse transcriptase enzymatic activity which catalyses both RNA and DNA dependent DNA polymerisation. Since reverse transcription of the viral RNA genome into DNA is essential for integration into the host cell genome RT is a target for drug therapy of HIV infection (see section 1.5.8).

Reverse transcription is initiated (step 1) by complementary base pairing between the primer binding site (PBS) of the viral genomic RNA and the 3' 18 amino acids of a tRNA$_{lys}$ primer that is packaged in the virion from the producer cell (Freund et al., 2001; Isel et al., 1995; Isel et al., 1996; Lanchy et al., 1996). This complex structure also involves interactions between the anti-codon loop of tRNA$_{lys}$ and the A rich loop upstream of the PBS in the viral RNA. Elongation to the 5' terminus then occurs to produce 'strong stop' negative strand DNA (step 2). The p66 domain of RT also functions as ribonuclease H (RNaseH) and degrades the RNA in this complex (Hansen et al., 1988) (step 2). The first jump then occurs, the newly formed negative sense single-stranded DNA (R-U5) anneals with complementary RNA (R) sequence at the 3' end of the same, or other copy, of the RNA genome. Negative strand DNA synthesis continues in a 3' direction from this new site (step 3). RNaseH degrades the RNA template in the complex except for 2 polypurine tract (ppt) regions, one central and one 3'. These RNA ppt regions act as the primers for synthesis of positive strand DNA (step 4). Following elongation from both ppt primers, RNaseH degrades the ppt regions and the original tRNA$_{lys}$ primer at the 5' end of the negative strand (step 5). A second jump then occurs as the newly synthesised positive strand PBS anneals to the negative strand PBS and elongation of both the positive and negative DNA strand continues in both directions (step 6). Positive strand synthesis continues through the central ppt region resulting in a 99bp overlap (DNA flap) in the full-length double stranded DNA (step 7). The resulting full-length DNA genome is longer than the template RNA due to an additional U region at either end of both strands of DNA in the LTR (see Figure 1.3).
Figure 1.3. Reverse transcription.

Reverse transcription is initiated by cellular tRNA_{tys} binding to the primer binding site (PBS) allowing elongation of the negative strand of DNA (step 1). Following production of strong stop DNA (step 2) the first jump occurs. Elongation of the negative strand then continues (step 3). RNaseH degrades the RNA in the complex except for 2 polypurine tracts that serve as primers for positive strand synthesis (step 4). Positive strand synthesis occurs from both polypurine primers (step 5). A second jump then occurs when the 3' positive strand jumps to the PBS at the 3' end of the negative strand (step 6). Synthesis of both strands then continues and a DNA flap is formed at the central polypurine tract (step 7). The red bar represents cellular tRNA_{tys}, pink bars represent RNA, dark blue represents negative strand DNA and light blue represents positive strand DNA.

The large nucleoprotein particle containing the reverse transcribed double stranded linear DNA, and several viral proteins, Matrix (MA), Nucleocapsid (NC), RT, Integrase (IN), Vpr and Vpx (for HIV-2 only) is referred to as the preintegration complex (PIC) (Bukrinsky et al., 1993b; Fletcher et al., 1996; Miller et al., 1997).
1.3.3 Nuclear import

HIV is able to infect non-dividing cells such as macrophages and thus the HIV PIC must traverse an intact nuclear membrane to gain access to the host cell nucleus. The nuclear pore complex (NPC) is a macromolar assembly of 50-100 polypeptides (reviewed in Mattaj et al., 1998) that spans the cellular nuclear membrane and creates an aqueous channel with a passive diffusion pore size of 9nm. Active transport allows bi-directional transport of host macromolecules up to 25nm (Vasu et al., 2001). This is controlled by importins and exportins that recognise nuclear localisation signals (NLS) and nuclear export signals (NES) respectively (Mattaj and Englmeier, 1998; Nakielny et al., 1999; Weis, 1998).

NLS classically comprise a short stretch of basic lysine residues or a bipartite basic NLS with 2 independent basic amino acid clusters and a spacer. These signals are recognised by importin-α that in turn binds to importin-β. This trimeric complex then interacts with nucleoporin components of the NPC and progressively moves across the pore in a series of sequential binding and release steps that require energy (Gorlich, 1997; Nakielny and Dreyfuss, 1999). Other proteins have signals that can bypass importin α and associate directly with importin-β. These NLS are rich in arginine rather than lysine (Palmeri et al., 1999).

Several HIV proteins that are within the PIC; IN, Vpr (and Vpx for HIV-2 and SIV) and MA, have been proposed to contain NLS motifs and have thus been implicated in facilitating nuclear import of the PIC. IN contains a short classical NLS sequence that has been implicated in PIC import into the nucleus (Bouyac-Bertoia et al., 2001; Gallay et al., 1997; Pluymers et al., 1999). HIV-1 Vpr has 2 distinct and novel NLS that may enhance importin α/β-dependent nuclear import and may be important for nuclear import in non-dividing cells such as macrophages (Heinzinger et al., 1994; Jenkins et al., 1998; Paxton et al., 1993; Popov et al., 1998a; Popov et al., 1998b; Vodicka et al., 1998). In the case of HIV-2 and SIV, Vpx, which shares sequence similarity with Vpr, may take on the role of nuclear import (Fletcher et al., 1996; Pancio et al., 2000; Sharp et al., 1996). Vpr may also induce herniations in the nuclear membrane that may facilitate nuclear entry, overcoming the limitation of pore size for the 56nm diameter PIC (de Noronha et al., 2001; Segura-Totten et al., 2001). MA is also present in PIC and has been proposed to contain a basic NLS that targets the PIC for nuclear import but this is disputed (Bukrinsky et al., 1993a; Fouchier et al., 1997; Freed et al., 1995a). Additionally, the central DNA flap that is formed during
reverse transcription has been reported to act as a nuclear import signal (Zennou et al., 2000). Maintenance of the central ppt that results in this DNA flap is critical for viral replication but this may be purely for its function as a primer for positive strand synthesis (see section 1.1.2.2) (Charneau et al., 1992).

The contribution of each of the proteins and signals in nuclear import of the PIC is still unclear and an active area of research and debate. Once within the nucleus some reverse transcribed cDNA molecules undergo recombination or end to end ligation to form one- and two-LTR circles. These are dead end products but can be used as an indicator of nuclear entry of double stranded cDNA (Sharkey et al., 2001).

1.3.4 Integration

Viral IN remains associated with the PIC as it translocates into the nucleus and acts to integrate the reverse transcribed viral cDNA into the host cell chromosomal DNA. IN catalyses the removal of the terminal dinucleotide from each 3' end of the viral cDNA and forms an integration site by cleavage of chromosomal DNA. IN then mediates a strand transfer reaction to link the 3' end of the viral cDNA to the cellular chromosomal DNA (Bushman et al., 1990). The site of integration into host DNA is thought to be unspecific, but nucleosomal DNA is favoured to allow easier access to transcription factors (Bushman, 1994; Pruss et al., 1994; Pryciak et al., 1992). Recently, preferential integration of HIV-1 into transcriptionally active genes has been reported (Schroder et al. 2002).

1.3.5 Transcription and exit of RNA species from the nucleus

Once integrated into the host cell chromosomal DNA, transcription of proviral DNA is carried out by cellular DNA-dependent RNA polymerase II. The transcription start site is located in the 5'LTR at the U3 R boundary. Initiation of transcription by RNA polymerase II is directed by cis-acting control elements. These include a TATA box, Sp1 and NFκB sites upstream of the start site (Nabel et al., 1987; Yang et al., 1997a; Cohen et al., 1990; Wang et al., 1995a). The TATA box recruits a series of transcription factors, firstly TFIID, followed by other basal transcription factors and assembly of RNA polymerase II. Cell type and differentiation state results in variability of activity of the transcription mechanism. During T-cell activation the NFκB inhibitor, IκB is degraded, releasing NFκB to bind to the LTR and enhance transcription. NFκB sites are conserved in all HIV-1 isolates (Nabel and Baltimore,
1987). HIV-2 LTR has slightly different transcription factor binding sites within the LTR; only a single NFκB site is present as compared to up to 3 in different HIV-1 subtypes. This may account for a lower activation of HIV-2 by NFκB (Arya, 1990; Hannibal et al., 1993; Markovitz et al., 1990; Montano et al., 2000; Tong-Starksen et al., 1990). Additional elements, found only within the HIV-2 LTR, may compensate (Clark et al., 1995; Fu et al., 1997; Hilfinger et al., 1993; Leiden et al., 1992; Markovitz et al., 1992).

The viral protein Transactivator of transcription (Tat) increases the processivity of RNA polymerase II. In the absence of Tat, transcription from the HIV-1 5’ LTR terminates within the first 60 ribonucleotides (Feinberg et al., 1991; Kao et al., 1987; Laspia et al., 1989; Marciniak et al., 1991). An arginine rich RNA-binding domain in Tat binds to a 3 nucleotide bulge of an RNA loop structure within the 5’ LTR, termed the trans-activation response (TAR) element (Berkhout et al., 1989; Garcia et al., 1989; Jakobovits et al., 1988; Selby et al., 1989). Cellular cyclin T1 and associated kinase, cyclin dependent kinase 9 (CDK9), are recruited to the TAR element by Tat of both HIV-1 and HIV-2 (Cullen, 1998; Emerman et al., 1998; Herrmann et al., 1993; Jones, 1997; Wei et al., 1998; Yang et al., 1997a; Yang et al., 1996b; Zhu et al., 1997). This complex phosphorylates the carboxy-terminal domain of the large subunit of cellular RNA polymerase II, which increases the elongation capacity of the transcription complex by countering negative factors (Chun et al., 1996; Marshall et al., 1996; Okamoto et al., 1996; Parada et al., 1996; Yang et al., 1996b). The HIV-1 LTR can also be transactivated by a number transcription factors encoded by other viruses including Epstein-Barr Virus (EBV) (Lin, 1993; Quinlivan et al., 1990; Romano et al., 1997), Herpes Simplex Virus 1 (HSV-1) (Albrecht et al., 1989; Chapman et al., 1991; Mosca et al., 1987), Human Herpesvirus 6 (HHV6) (Di Luca et al., 1991; Geng et al., 1992), Human Herpesvirus 8 (HHV8) (Caselli et al., 2001; Hyun et al., 2001) and Cytomegalovirus (CMV) (Davis et al., 1987). Cellular RNA processing machinery modifies each RNA product, a cap is added at the 5’ end and polyadenylated at the 3’ end. Generation of mRNAs for the different viral proteins is achieved by the differential use of splice donor and acceptor sites within the full length genomic RNA. At early time points completely spliced RNAs for Tat, Rev and Nef are exported into the cytoplasm via the standard cellular route. Accumulation of singly and unspliced viral RNA in the cytoplasm is dependent on the regulatory viral protein Rev (Feinberg et al., 1986; Felber et al., 1990; Sodroski
et al., 1986b). Rev is a product of spliced mRNA and has an arginine rich RNA binding domain that interacts with a complex RNA secondary structure called the Rev response element (RRE) (Heaphy et al., 1991; Kjems et al., 1992; Malim et al., 1989). It can regulate splicing of viral RNA by interaction with the cellular splicing machinery (Powell et al., 1997). Rev also contains both NLS and nuclear export signals (NES) to allow shuttling between the cytoplasm and nucleus. The arginine rich region also acts as a multimerisation dependent non-classical NLS that mediates nuclear localisation of Rev (Cochrane et al., 1990; Henderson et al., 1997; Malim et al., 1989; Szilvay et al., 1997; Truant et al., 1999). Binding of Rev to viral RNA RRE masks the NLS and exposes the NES. Rev interacts with the nuclear export pathway usually used by small nuclear RNAs and 5S RNA (Fischer et al., 1995). The leucine rich NES of Rev interacts with CRM1 (Chromosome Maintenance region protein) or exportin-1 that mediate export through the NPC (Fornerod et al., 1997; Fukuda et al., 1997; Ossareh-Nazari et al., 1997; Stade et al., 1997).

1.3.6 Particle formation and budding

HIV envelope glycoprotein precursor (gp160) is synthesised on ribosomes. A hydrophobic signal peptide within the leader sequence at the amino terminus of Env allows translocation of the precursor protein into the endoplasmic reticulum (ER). It remains embedded in the ER membrane due to the transmembrane domain in the gp41 portion of the polyprotein. Env is subsequently transported through the ER to the golgi network where it is subject to glycosylation and is cleaved by cellular proteases into surface, gp120, and transmembrane, gp41, subunits (see sections 1.4.1.1 and 1.4.1.2). The 2 subunits remain associated and form trimeric ‘spikes’. Env is then transported to the plasma membrane.

Gag and Gag-Pol precursor proteins are synthesised in the cytoplasm and are targeted to the plasma membrane by myristylation of MA (Hill et al., 1996). Multimerisation of Gag precursor protein drives particle formation. A region of Nucleocapsid (NC) mediates packaging of genomic RNA by recognition of a packaging signal (ψ) in the untranslated 5’mRNA via zinc finger domains (Dannull et al., 1994; Geigenmuller et al., 1996; Kaye et al., 1996; Lever et al., 1989; Poon et al., 1996; Schmalzbauer et al., 1996; South et al., 1993; Zeffman et al., 2000). Packaging of the HIV-2 genome is regulated in a slightly different way. Although all HIV-2 viral mRNAs, spliced and unspliced contain ψ, only full length genomic RNA is
incorporated into virus particles as newly translated Gag binds preferentially to its own full length template RNA (Griffin et al., 2001; Kaye et al., 1999). Along with 2 copies of viral genomic RNA, viral and cellular proteins are incorporated into the budding virion. There are conflicting reports on whether Vif is specifically incorporated into virions (Camaur et al., 1996; Dettenhofer et al., 1999; Khan et al., 2001; Sova et al., 2001). However, Vif enhances the viral infectivity of certain ‘non-permissive’ cells during viral production (Gabuzda et al., 1992). Recently, a human gene product, CEM15 has been shown to inhibit HIV infection and can be suppressed by the presence of Vif (Sheehy et al., 2002). Vpr (and Vpx for HIV-2) is also packaged into virions in quantities comparable to the structural Gag proteins, probably through an interaction with p6 region of p55 Gag precursor (Kondo et al., 1996; Lu et al., 1993; Paxton et al., 1993; Wu et al., 1994). The host cell protein cyclophilin A that is packaged in the virion by an interaction with Gag may play a role in disassembly of the core, but is not required for all primate lentiviruses (Billich et al., 1995; Braaten et al., 1996a; Braaten et al., 1996b; Franke et al., 1994; Luban, 1996; Luban et al., 1993; Thali et al., 1994a). Additionally, cellular tRNA\textsubscript{lys} that acts as the primer for reverse transcription (see section 1.3.2) is incorporated into the virus particle.

The late domain motif (PTAP) present within the p6 protein is important for budding (Huang et al., 1995; Strack et al., 2000; Vogt, 2000). The p6 domain of the Gag polyprotein interacts with Tumour suppressor gene 101 (TSG101) and proteins of the vacuolar protein sorting pathway (Garrus et al., 2001; Martin-Serrano et al., 2001; VerPlank et al., 2001). This couples the forming virion to cellular trafficking machinery to allow budding (Babst et al., 2000; Katzmann et al., 2001; Lemmon et al., 2000). Budding is thought to occur at specific cell membrane microdomains called lipid rafts (Aloia et al., 1993; Nguyen et al., 2000; Ono et al., 2001). As HIV buds from the cell membrane, host membrane proteins are also incorporated into the viral membrane. Molecules such as major histocompatibility (MHC) antigens and intercellular adhesion molecule-1 (ICAM-1) are present in the viral membrane and may play a role in attachment of virions to target cells (see section 1.4.2.3) (Fortin et al., 2000; Poon et al., 2000). Proteins that regulate the complement system, for example CD59, are also incorporated into the viral membrane and aid in viral evasion of the immune system (see section 1.6.4).
1.3.7 Particle maturation

After budding of the immature virus particle, Gag and Gag-Pol precursors are processed by the viral protease into 4 Gag proteins (MA, CA, p6, NC), Protease, RT and IN. The mature HIV particle is roughly spherical and approximately 100nm in diameter. A schematic representation of the structure of a mature HIV particle is shown in Figure 1.4.

Figure 1.4. HIV particle.
Schematic representation of the structure of HIV, indicating the location of the viral and cellular proteins and genomic RNA (adapted from Greene and Peterlin, 2002).
1.4 Viral entry

HIV entry into a target host cell is the first stage of the viral life cycle (see section 1.3.1 and Figure 1.2). Entry is mediated by interactions between the viral envelope glycoprotein and cell surface receptors resulting in fusion of the viral and cellular membranes.

1.4.1 HIV envelope

1.4.1.1 Glycosylation

HIV envelope glycoprotein precursor (gp160) is synthesised on ribosomes then translocated into the endoplasmic reticulum due to an N terminal signal peptide. It is then subject to extensive glycosylation. Approximately 50% of the molecular mass of gp160 is attributed to carbohydrate (Fenouillet et al., 1989; Fenouillet et al., 1990; Geyer et al., 1988; Leonard et al., 1990; Mizuochi et al., 1988). High mannose chains are linked to the side-chain NH$_2$ group of asparagine residues at Asn-X-Ser or Asn-X-Thr sites (N-linked glycosylation). These structures are further processed during transport through the golgi network (Kornfeld et al., 1985). Addition of sugars to the OH group of serine or threonine (O-linked glycosylation) also occurs in the golgi network (Bernstein et al., 1994). The degree of modifications of the terminal sugars depends on producer cell type (Liedtke et al., 1997; Willey et al., 1996). The exact number of glycosylation sites varies between HIV isolates but there are approximately 24 N-linked and 8 O-linked sites in gp120 and 4 N-linked sites within gp41. Many of these sites are highly conserved across isolates (Bernstein et al., 1994; Leonard et al., 1990; Myers et al., 1990). Addition of inhibitors of glycosylation to cells or mutation of glycosylation sites in envelope prevents subsequent cleavage of the envelope precursor protein (see section 1.4.1.2) due to retention within the ER (Dash et al., 1994; Dewar et al., 1989; Fenouillet et al., 1994; Fenouillet et al., 1993; Fenouillet et al., 1995; Gruters et al., 1987; Pal et al., 1988; Pal et al., 1989; Taylor et al., 1991; Walker et al., 1987b). However, virus producer cells with mutant glycosylation do not exhibit this phenotype (Fenouillet et al., 1996). The conservation of extensive glycosylation is probably related to a role in masking neutralisation epitopes (see section 1.6.4).
1.4.1.2 Proteolytic processing

HIV envelope is produced as a precursor, gp160 that is subsequently processed by host cell enzymes into gp120 (SU) and gp41 (TM) subunits. The subunits remain associated through non-covalent interactions (Freed et al., 1989; Helseth et al., 1991; Kowalski et al., 1987; McCune et al., 1988; Robey et al., 1985). Cleavage is carried out by cellular furin protease, or other members of the convertase family of enzymes, in the trans-golgi network (Decroly et al., 1997; Hallenberger et al., 1992; Hallenberger et al., 1997; Moulard et al., 2000a; Vollenweider et al., 1996). Following cleavage, heterodimeric envelope is trafficked via the host cell constitutive secretory pathway to the cell membrane for incorporation into the membrane of budding virions. In the native state on the viral membrane gp120/gp41 dimers form oligomers, spikes, most likely of trimers (Center et al., 2001; Kwong et al., 2000).

1.4.1.3 gp120

The surface subunit, gp120, is responsible for interactions with cell surface receptors (see section 1.4.2). Comparison of amino acid sequence of the gp120 subunits of different HIV-1 isolates revealed 5 variable regions (V1-V5) interspersed by 5 conserved regions (C1-C5) (Modrow et al., 1987; Starcich et al., 1986; Willey et al., 1986). The first 4 variable loops of HIV-1 are disulphide bonded at their base. HIV-2 and SIV envelope surface subunit is 30-40 amino acid longer than HIV-1 and predicted to have 2 additional disulphide bonds (Hoxie, 1991; Leonard et al., 1990). Crystallisation of a deglycosylated core domain lacking V loops of HIV-1 gp120 with CD4 and a human antibody, 17b (Figure 1.5), revealed 2 domains in gp120, the inner and outer, linked by a bridging sheet (Kwong et al., 1998; Wyatt et al., 1998a). A glycosylated structure with variable loops has been also been predicted (Zhu et al., 2000).

The peptide backbone of gp120 is involved in CD4 binding, allowing variation in the amino acid side chains between isolates without loss of CD4 recognition. The structure of gp120 prior to CD4 binding is still unclear. CD4 binding induces rearrangement of the V1/V2 loop (and probably also the V3 loop) and the core structure of gp120 (Myszka et al., 2000). These changes expose the bridging sheet, a four stranded anti-parallel β-sheet, involved in the coreceptor ligation (Kwong et al., 1998; Rizzuto et al., 1998; Thali et al., 1993; Wyatt et al., 1995). Interaction with a coreceptor leads to further conformational changes in gp41.
1.4.1.4 gp41

The transmembrane subunit, gp41, anchors envelope in the viral membrane and is responsible for pH independent fusion of viral and cellular membranes. gp41 comprises an extracellular (or ecto-) domain, a transmembrane domain and a cytoplasmic tail. A schematic representation of gp41 is shown in Figure 1.6. The cytoplasmic tail interacts with MA oligomers attached to the plasma membrane securing envelope in the membrane (Freed et al., 1995b; Freed et al., 1996; Hill et al., 1996; Mammano et al., 1995; Ono et al., 1997). Hydrophobic repeat regions in the ecto-domain associate gp41 into trimers (Caffrey et al., 1998; Center et al., 1997; Lu et al., 1995; Shu et al., 1999). Following CD4 and coreceptor binding by gp120 conformational changes are induced within gp41 resulting in insertion of the N terminal fusion peptide into the target cell membrane. The crystal structure derived from the gp41 ectodomain of HIV-1 and SIV revealed a thermodynamically stable 6-helix bundle of coiled coils formed by association of heptad repeats (Chan et al., 1997; Hunter, 1997; Tan et al., 1997; Weissenhorn et al., 1997).
Figure 1.6. Domains of gp41.

Functional domains of gp41. The N terminal fusion peptide inserts into the target cell membrane. The heptad repeat regions are involved in oligomerisation and formation of a coiled coil structure during fusion.

1.4.1.5 Fusion

The crystal structure of gp41 in a coiled coil formation is likely to represent an activated state of gp41 that brings the viral and cellular membranes into close proximity prior to fusion. How this structure facilitates formation of the fusion pore is still unclear. It has been suggested that the 6-helix bundle may form coincidently with or just prior to fusion (Furuta et al., 1998; Golding et al., 2002; Melikyan et al., 2000). A schematic representation of the current view of gp41 mediated membrane fusion is shown in Figure 1.7. It is similar to the fusion scheme for influenza virus in which the fusion peptide of haemagglutinin is projected into the target cell membrane and the membranes are drawn together by the formation of a thermodynamically stable coiled coil (Bullough et al., 1994). In contrast to influenza, fusion mediated by HIV gp41 is not dependent on acidic pH (McClure et al., 1988; Stein et al., 1987).
1.4.2 Cellular receptors

1.4.2.1 CD4

The ability of antibodies to CD4 to block HIV infection of permissive cells identified CD4 as the primary receptor for HIV (Dalgleish et al., 1984; Klatzmann et al., 1984). HIV-1, HIV-2 and SIV all utilise CD4 as a cellular receptor (Hoxie et al., 1988; Maddon et al., 1986; McDougal et al., 1986; Sattentau et al., 1988a; Sattentau et al., 1986). CD4 is a transmembrane protein of approximately 60kD and is a member of the immunoglobulin superfamily with 4 extracellular domains (D1-4) (Maddon et al., 1985). CD4 is expressed on a subset of T cells, T helper cells (Th), and cells of the monocyte/macrophage lineage. On T cells CD4 acts in conjunction with the T cell receptor to recognises peptides presented on major histocompatibility class (MHC) II molecules on antigen presenting cells. The N-terminal extracellular domain (D1) of CD4 interacts with a discontinuous structure on HIV envelope involving C4, that is conserved across isolates (Arthos et al., 1989; Lasky et al., 1987; Moore et al., 1994b; Olshevsky et al., 1990). The crystal structure of HIV-1 gp120 with 2 domains of CD4 and antibody 17b revealed amino acid 43 (phenylalanine) of CD4 recesses into a pocket in the groove between the inner and outer domains (Kwong et al., 1998; Wyatt et al., 1998a).

CD4 is down regulated from the surface of infected cells. High levels of cell surface CD4 may be detrimental to particle release and decrease infectivity by sequestration of envelope (Lama et al., 1999; Ross et al., 1999). CD4 is specifically
targeted for internalisation via clathrin coated pits and degradation in the lysosomal pathway (Aiken et al., 1994; Foti et al., 1997). The viral protein Nef interacts with the cytoplasmic tail of CD4 and couples to the endocytic machinery via a dileucine motif, Nef binding protein 1 (NBP-1) and adapters AP-1 and AP-2 (Bresnahan et al., 1999; Foti et al., 1997; Lock et al., 1999; Lu et al., 1998; Preusser et al., 2001). A dileucine signal in Nef acts as an internalisation signal (Bresnahan et al., 1998; Craig et al., 1998; Greenberg et al., 1998). HIV-2 and SIV Nef also down-modulate cell surface CD4 but may utilise a distinct motif (Hua et al., 1997).

Soluble CD4 (sCD4) can be a potent inhibitor of HIV infection. Infection can be blocked either by competition for the CD4 binding site or by the induction of dissociation of gp120 from gp41 (envelope shedding) (Moore et al., 1990; Orloff et al., 1993). However, it has become clear that whilst TCLA are highly sensitive to inactivation by sCD4, PI are relatively resistant (Ashkenazi et al., 1991; Daar et al., 1990; Moore et al., 1992; Moore et al., 1993a; Moore et al., 1995a; O'Brien et al., 1994). HIV-2 and SIV are also refractory to sCD4 inactivation (Sattentau et al., 1993). Factors influencing susceptibility to sCD4 inactivation include affinity for CD4 and the association between gp120 and gp41 (Moore, 1990; Moore et al., 1993a; Moore et al., 1992; O'Brien et al., 1994; Sattentau et al., 1993; Sullivan et al., 1995; Willey et al., 1994). sCD4 can also induce HIV-2, SIV and some primary HIV-1 isolates to infect CD4 negative cells (Clapham et al., 1992; Reeves et al., 1999; Schutten et al., 1995).

1.4.2.2 Coreceptors

Soon after the identification of CD4 as a receptor for HIV it was shown to be insufficient on its own to confer entry of HIV into a target cell. Not all human cells expressing CD4 could be infected and transfection of CD4 alone into non-primate cells did not allow infection of these cells by HIV (Clapham et al., 1991; Maddon et al., 1986). The identity of the HIV coreceptors was not elucidated until much later. The seven transmembrane G protein coupled receptor CXCR4 was the first HIV coreceptor identified and mediated infection of CD4 positive cells by T-tropic viruses (Feng et al., 1996). Physiologically, CXCR4 functions as a receptor for the CXC chemokine stromal derived factor (SDF)-1α (Bleul et al., 1996; Oberlin et al., 1996). Shortly after, a second receptor CCR5 was identified as the coreceptor for M-tropic viruses (Samson et al., 1996). CCR5 is the receptor for β chemokines; regulated upon
activation normal T cell expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1α and MIP-1β (Samson et al., 1996) and thus explained the earlier observation of inhibition of HIV-1 infection by β chemokines (Cocchi et al., 1995). Many other chemokine receptors and orphan seven transmembrane receptors have subsequently been identified as coreceptors with CD4 for HIV-1, HIV-2 and SIV entry into target cells in vitro (Table 1.1).

<table>
<thead>
<tr>
<th>Co-receptor</th>
<th>Ligand</th>
<th>Virus</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR1</td>
<td>MIP-1α, RANTES, MIPIF-1, MCP-3</td>
<td>HIV-2</td>
<td>(Edinger et al., 1999; Guillon et al., 1998; McKnight et al., 1998; Owen et al., 1998)</td>
</tr>
<tr>
<td>CCR2b</td>
<td>MCP-1, 2, 3</td>
<td>HIV-1, HIV-2</td>
<td>(Bron et al., 1997; Doranz et al., 1996; Guillon et al., 1998; McKnight et al., 1998; Owen et al., 1998)</td>
</tr>
<tr>
<td>CCR3</td>
<td>Eotaxin, Eotaxin-2, MCP-3, MCP-4, RANTES</td>
<td>HIV-1, HIV-2</td>
<td>(Bron et al., 1997; Choe et al., 1996; Doranz et al., 1996; Sol et al., 1997)</td>
</tr>
<tr>
<td>CCR4</td>
<td></td>
<td>HIV-2</td>
<td>(McKnight et al., 1998; Owen et al., 1998)</td>
</tr>
<tr>
<td>CCR5</td>
<td>MIP-1α, MIP-1β, RANTES, MCP-2</td>
<td>HIV-1, HIV-2, SIV</td>
<td>(Alkhatib et al., 1996; Bron et al., 1997; Chen et al., 1997b; Choe et al., 1996; Doranz et al., 1996; Dragic et al., 1996; Edinger et al., 1997a; Sol et al., 1997)</td>
</tr>
<tr>
<td>CCR8</td>
<td>I-309</td>
<td>HIV-1, HIV-2, SIV</td>
<td>(Jinno et al., 1998; Rucker et al., 1997; Simmons et al., 2000)</td>
</tr>
<tr>
<td>CCR9</td>
<td></td>
<td>HIV-1, HIV-2</td>
<td>(Choe et al., 1998)</td>
</tr>
<tr>
<td>CXCR4</td>
<td>TECK, SDF-1</td>
<td>HIV-1, HIV-2</td>
<td>(Bron et al., 1997; Feng et al., 1996; Schols et al., 1998; Sol et al., 1997)</td>
</tr>
<tr>
<td>CXCR5</td>
<td></td>
<td>HIV-2</td>
<td>(Kanbe et al., 1999)</td>
</tr>
<tr>
<td>CXCR6/STRL33/BONZO</td>
<td>CXCL13, CXCL16</td>
<td>HIV-1, HIV-2, SIV</td>
<td>(Alkhatib et al., 1997; Deng et al., 1997; Liao et al., 1997; Owen et al., 1998; Zhang et al., 2001c)</td>
</tr>
<tr>
<td>CX3CR1/V28</td>
<td>Fractalkine</td>
<td>HIV-2</td>
<td>(Reeves et al., 1997a; Rucker et al., 1997)</td>
</tr>
<tr>
<td>GPR1</td>
<td>?</td>
<td>HIV-1, HIV-2, SIV</td>
<td>(Farzan et al., 1997a; Liu et al., 2000; Shimizu et al., 1999)</td>
</tr>
<tr>
<td>GPR15/BOB</td>
<td>?</td>
<td>HIV-2, SIV</td>
<td>(Deng et al., 1997; Farzan et al., 1997a; Owen et al., 1998)</td>
</tr>
<tr>
<td>APJ</td>
<td>Apelin</td>
<td>HIV-1, HIV-2, SIV</td>
<td>(Choe et al., 1998; Edinger et al., 1998; Liu et al., 2000; Zhang et al., 1998)</td>
</tr>
<tr>
<td>ChemR23</td>
<td>?</td>
<td>HIV-1, SIV</td>
<td>(Samson et al., 1998)</td>
</tr>
<tr>
<td>RDC1</td>
<td>?</td>
<td>HIV-1, HIV-2, SIV</td>
<td>(Shimizu et al., 2000)</td>
</tr>
<tr>
<td>Leukotriene B4R</td>
<td>Leukotriene B4</td>
<td>HIV-1</td>
<td>(Owman et al., 1998)</td>
</tr>
</tbody>
</table>

Table 1.1. Coreceptors for HIV and SIV.

Coreceptors used by HIV-1, HIV-2 and SIV (adapted from Clapham et al., 2001).
All isolates of HIV can use either CCR5 and/or CXCR4, but the role of most of the other coreceptors for virus entry into target cells in vivo remains unclear (Clapham et al., 1999). The importance of CCR5 is demonstrated by a naturally occurring genetic polymorphism resulting in a deletion of CCR5 that confers protection from HIV transmission in homozygotes and a delay in disease progression in heterozygotes (Huang et al., 1996) (see section 1.5.2 and 1.5.6). Viruses isolated early after infection usually use CCR5 with a switch to CXCR4 usage associated with increased pathogenesis and disease progression in some individuals (Connor et al., 1997; Koot et al., 1996; Richman et al., 1994; Scarlatti et al., 1997; Schuitemaker et al., 1992; Shankarappa et al., 1998; Simmons et al., 1996). However, a switch to CXCR4 usage is not essential for progression to AIDS in humans particularly in infection with subtype C HIV-1 (Abebe et al., 1999; Ping et al., 1999). In addition, many pathogenic SIV isolates do not use CXCR4 yet cause AIDS in monkeys (de Roda Husman et al., 1999; Kimata et al., 1999; Li et al., 1999).

Coreceptor usage in conjunction with CD4 generally determines cell tropism. CCR5 is expressed on CD4 positive T cells and macrophages making these major targets for HIV in vivo. T cell lines cultured in vitro express CXCR4 and thus select for viruses using this coreceptor. Before the identification of chemokine receptors as coreceptors, HIV isolates were described as macrophage tropic (M-tropic) and T-cell tropic (T-tropic). Viruses can also be classified according to their ability to form syncytia in T-cells lines, either non-syncytium inducing (NSI) or syncytium inducing (SI). Generally NSI, M-tropic viruses use CCR5 as a coreceptor and SI, T-tropic viruses use CXCR4. The induction of syncytia in T-cell lines is due to cell to cell fusion mediated by viral envelope expressed on infected binding to CD4 and CXCR4 on adjacent cells. It should be noted that all isolates, M-tropic and T–tropic are able to replicate in primary T cells and some primary CXCR4 using SI isolates can replicate in macrophages (Simmons et al., 1998). CCR5 using viruses are now referred to as R5, CXCR4 using viruses as X4 and dual tropic viruses as R5X4 (Berger et al., 1998).

Isolates of HIV-2 can often use a broader range of chemokine receptors than HIV-1 (Bron et al., 1997; Deng et al., 1997; Guillon et al., 1998; Kanbe et al., 1999; McKnight et al., 1998; Rucker et al., 1997; Sol et al., 1997; Zhang et al., 2000). In addition, HIV-2 isolates are able to use chemokine receptors, again a broad range, for entry into cells in the absence of CD4 (Edinger et al., 1997a; Endres et al., 1996; Hoxie et al., 1998; Reeves et al., 1999). The contribution of promiscuous coreceptor
usage and CD4 independence to HIV-2 infection in vivo is unclear. However, HIV-2 isolates are able to infect CCR5 negative PBMC (Simmons et al., 2000; Sol et al., 1997; Zhang et al., 2000). In vitro, CD4 negative primary brain and testis derived cells can be infected with HIV-2 (Reeves et al., 1999, Willey et al. 2002 submitted). However, there is no evidence of HIV-2 infection of CD4 negative brain cells ex vivo (Morner et al., 2002).

The coreceptor binding site is not fully exposed on native gp120 until after CD4 has bound (Lapham et al., 1996; Trkola et al., 1996a; Wu et al., 1996). It involves the bridging sheet that lies between the inner and outer domains of gp120 (Figure 1.5), which is exposed following an undefined rearrangement of the V1/V2 loop (and probably also the V3 loop) and the core structure of gp120 (Kwong et al., 1998; Myszka et al., 2000; Rizzuto et al., 1998; Thali et al., 1993; Wyatt et al., 1995). In addition to the bridging sheet interaction with a coreceptor, the V3 loop of HIV-1 is a major determinant of coreceptor usage (Choe et al., 1996; Speck et al., 1997; Wang et al., 1999; Wang et al., 1998a; Xiao et al., 1998). The net charge of the amino acids in the V3 influences coreceptor usage. V3 loops of CXCR4 using isolates are highly positively charged which may promote interaction with negatively charged residues of CXCR4 (Fouchier et al., 1992; Kajumo et al., 2000; Wang et al., 1998b). V1/V2 sequence may also determine coreceptor usage (Cho et al., 1998). Both HIV-1 and HIV-2 are thought to interact with the N terminus and second extracellular loop of CXCR4 (Brelot et al., 1997; Potempa et al., 1997; Reeves et al., 1998). Similarly the N-terminus and second extracellular loop of CCR5 are thought to be important for its function as a HIV coreceptor (Atchison et al., 1996; Bieniasz et al., 1997; Farzan et al., 1997b; Farzan et al., 1998b; Lu et al., 1997; Picard et al., 1997).

Lipid rafts, microdomains of the plasma membrane enriched in cholesterol and sphingolipid, are important for viral entry (Liao et al., 2001; Popik et al., 2002). CD4 is localised in lipid raft microdomains whilst CXCR4 and CCR5 are thought to segregate into separate clusters (Kozak et al., 2002; Parolini et al., 1999; Singer et al., 2001). The movement of coreceptors to co-localise with CD4 upon ligation of HIV gp120 may disrupt the lipid environment and destabilise the membrane, aiding the fusion process (Kozak et al., 2002).
1.4.2.3 Attachment receptors

Other cellular receptors interact with HIV envelope and can aid attachment of the virus to the cell but do not activate the fusion mechanism. Heparan sulphate proteoglycans on the surface of non-lymphoid adherent cells can interact with HIV envelope in the absence of CD4 through association of the negatively charged sulphate groups with the positively charged V3 loop (and to some extent the V1/V2 loop) (Ibrahim et al., 1999; Mondor et al., 1998b; Moulard et al., 2000b; Patel et al., 1993; Roderiquez et al., 1995; Saphire et al., 2001a). Glycolipids such as galactosyl ceramide (GalC) may also aid attachment to, and infection of, a variety of cell types (Delezay et al., 1997; Fantini et al., 1993; Hammache et al., 1998; Harouse et al., 1991). HIV incorporates host cell proteins such as ICAM-1 and LFA-1 when budding from the cell membrane, which can aid in attachment to new target cells via interaction with their normal ligands (Hioe et al., 1998; Hioe et al., 2001). Interactions with attachment receptors is likely to concentrate virus particles at the cell surface increasing the chances of a productive interaction with CD4 and a chemokine receptor leading to fusion.

Attachment of HIV to dendritic cells (DCs) is mediated by interaction of sugar groups on gp120 with DC-SIGN (Dendritic cell-specific ICAM-3 grabbing non-integrin) (Geijtenbeek et al., 2000; Pohlmann et al., 2001a). HIV (and SIV) attached to the surface of DCs can be efficiently transmitted to CD4 and coreceptor positive target cells over a period of several days, even in the absence of viral replication in the DC (Cameron et al., 1992; Geijtenbeek et al., 2000; Granelli-Piperno et al., 1998; Pope et al., 1994; Weissman et al., 1995). Endocytosis of DC-SIGN captured virus into a low pH vesicle may be important for retention of infectivity (Frank et al., 2002; Kwon et al., 2002). A related molecule, DC-SIGNR, is expressed on the surface of endothelial cells and is also able to bind and transmit HIV to CD4 positive T cells (Pohlmann et al., 2001b).
1.5 Course of HIV infection and pathogenesis of disease

1.5.1 Epidemiology

AIDS is characterised by a diverse range of clinical symptoms and opportunistic infections. It was initially identified in homosexual men in North America (Brennan et al., 1981; Gottlieb et al., 1981; Masur et al., 1981; Siegal et al., 1981). HIV infection is now a worldwide pandemic, currently 40 million people are infected with 5 million new infections in the past year and 24 million people have died from HIV and AIDS (World Health Organisation/UNAIDS, 2002). The different subtypes of HIV-1 have differing prevalence throughout the world (see section 1.2). Despite HIV-2 infection evident in humans for a similar time period as HIV-1 (see section 1.2) HIV-2 prevalence is generally much lower. The highest prevalence is found in Western Africa and countries with socio-economic ties with the area, such as Portugal (Essex et al., 1997; Kanki et al., 1994; Markowitz, 1993; Nacler et al., 1991; Poulsen et al., 1989; Soriano et al., 2000; Wilkins et al., 1993). In contrast to rising HIV-1, the prevalence of HIV-2 is dropping (Bouckenooghe et al., 1999; De Cock et al., 1993). Currently there are 67 reported HIV-2 infected individuals and a further 13 dual HIV-1/2 infections in the UK compared with 25,000 HIV-1 infections (Public Health Laboratory Services, UK 2002).

1.5.2 Transmission

HIV is transmitted by sexual or parenteral route and also from mother to child. Sexual transmission is the most common. The likelihood of sexual transmission depends on many factors including the viral load of the infected partner and presence of another sexually transmitted infection (Fleming et al., 1999; Mastro et al., 1996; Vernazza et al., 1999). Early in the Western epidemic, homosexual transmission was the main route. However, currently heterosexual transmission is increasingly the main route of transmission worldwide (World Health Organisation/UNAIDS, 2002). Parenteral transmission occurs mainly due to shared needle usage between intravenous drug users. Screening of blood for transfusion and blood products (e.g. factor VIII) have minimised these as routes of infection. Prior to implementation of screening, haemophiliacs were infected by contaminated factor VIII (Ludlam et al., 1985). Mother to child transmission can occur in utero, at delivery and during breast-feeding (Datta et al., 1994; Nduati, 2000). No one subtype of HIV-1 is transmitted more or
less easily but it is clear that individuals with higher viral loads are more likely to transmit (Hu et al., 1999; Quinn et al., 2000). HIV-2 is less readily transmitted than HIV-1, perhaps due to the prolonged low viral load observed in HIV-2 infection (Kanki et al., 1994b).

1.5.2.1 Protection from transmission

Prevention of disease and reduction in transmission is the major goal of HIV vaccines. Cases of individuals naturally protected therefore are of great interest. Individuals exposed multiple times to HIV but who remain free of infection are referred to as exposed uninfected (EU). A major breakthrough in the understanding of the protection mechanism in some of these individuals was the discovery of a 32 base pair deletion (Δ32) in the gene encoding CCR5, a major coreceptor (see section 1.4.2.2). Individuals who are homozygous for Δ32 do not express CCR5 on the surface of cells and are therefore refractory to infection by CCR5 using strains of HIV (Dean et al., 1996; Huang et al., 1996; Liu et al., 1996; Samson et al., 1996; Xiao et al., 1999). However, a few Δ32 homozygotes have been infected with CXCR4 using strains (Balotta et al., 1997; Biti et al., 1997; Michael et al., 1998; Naif et al., 2002; O'Brien et al., 1997; Sheppard et al., 2002; Theodorou et al., 1997). The Δ32 mutation is common among European Caucasians (allele frequency 5–15%) but virtually absent from native African and East Asian ethnic groups (Anzala et al., 1998; Libert et al., 1998; Martinson et al., 1997; Martinson et al., 2000; McNicholl et al., 1997; Stephens et al., 1998). This mutation only accounts for some of the EU individuals identified. In the absence of any known CCR5 mutations PBMC from EU are often difficult to infect with HIV in vitro (Zhang et al., 1996). This can be associated with higher production of CC chemokines that bind to and downregulate CCR5, preventing its use by HIV (Cocchi et al., 1995; Paxton et al., 1998; Paxton et al., 1996). HIV-specific immune responses have also been detected in EU individuals exposed to HIV through a number of routes, including sexual and mother to child (Aldhous et al., 1994; Bernard et al., 1999; Cheynier et al., 1992; Goh et al., 1999; Kaul et al., 2000; Langlade-Demoyen et al., 1994; Rowland-Jones et al., 1998; Rowland-Jones et al., 1995b; Rowland-Jones et al., 1993).

Sexually transmitted infections may facilitate sexual transmission of HIV (Fleming and Wasserheit, 1999; Wasserheit, 1992). Interventions that aim to decrease STI also decrease the spread of HIV (Grosskurth et al., 1995). These strategies are
often problematic since tackling Sexually transmitted infections require behavioural and social changes. They include decreasing the number of sexual partners and increased condom use (Mayaud et al., 2001). There are several strategies that can be implemented to reduce mother to child transmission. Use of antiretroviral drugs for a short period in pregnancy or even in labour can reduce mother to child transmission, as well as obstetric management and the use of alternative feeding methods (Coutsoudis et al., 1999; Guay et al., 1999). There have also been reports of HIV-2 infection giving some protection from infection with HIV-1 (Greenberg, 2001; Greenberg et al., 1996; Kokkotou et al., 2000; Travers et al., 1995; Travers et al., 1998) however, others found no protection (Aaby et al., 1997; Ariyoshi et al., 1997; Norrgren et al., 1999; van der Loeff et al., 2001; Wiktor et al., 1999).

1.5.3 Primary HIV infection

The events occurring immediately after infection with HIV are difficult to study in humans but studies on SIV infection of macaques suggest that the virus is disseminated throughout the lymphatic system and bone marrow within 2 weeks of exposure (Zhang et al., 1999d). Viruses isolated early after HIV-1 infection in an individual seem to have nearly identical envelope genes and predominantly use CCR5 as a coreceptor (Richman and Bozzette, 1994; Roos et al., 1992; Schuitemaker et al., 1992; Wolinsky et al., 1996; Zhang et al., 1993; Zhu et al., 1993). The reason for the predominance of R5 strains is unclear but expression of SDF-1 in the mucosal layer may prevent X4 virus infection and dendritic cells may selectively transmit R5 strains (Agace et al., 2000; David et al., 2001b; Jameson et al., 2002; Pablos et al., 1999). Additionally primary epithelial cells express CCR5 and may preferentially transfer CCR5 using viruses to T cells (Meng et al., 2002).

Clinical presentation of primary HIV infection occurs within days or weeks of infection and can include a wide range of symptoms (Kahn et al., 1998). The rather non-specific symptoms often include a maculo-papular skin rash, fatigue, headache, fever and lymphadenopathy (Vanhems et al., 1999). During primary infection there is extensive viral replication with a peak in viraemia and a transient decline in CD4 T cell numbers occurring 6-15 days after onset of symptoms (Clark et al., 1991). The host immune response generally controls this primary burst of virus replication (see section 1.6.2.1) and plasma virus load falls. This time-point is referred to as seroconversion, and anti-HIV antibodies can be detected in the peripheral blood. The
level to which the viral load falls to is referred to as the 'set-point' and is indicative of the time to progression to AIDS (Hogervorst et al., 1995; Jurriaans et al., 1994; Mellors et al., 1995; Mellors et al., 1996; Vesanen et al., 1996).

1.5.4 Chronic HIV infection

Following acute HIV infection there is an asymptomatic period that varies in duration. There is continued HIV replication with approximately $10^9$ new virions produced each day (Ho et al., 1995; Wei et al., 1995). This drives an increased production rate of CD4 positive T cells in an attempt to retain CD4 levels in the face of high CD4 cell death (see section 1.5.7) (Fleury et al., 1998; Fleury et al., 2000; Hellerstein et al., 1999; Johnson, 2000; McCune et al., 2000). Ultimately the renewal of CD4 cells can not be sustained and CD4 cell numbers decrease.

1.5.5 AIDS

Progression from primary infection to symptoms indicative of AIDS generally takes a mean of 10 years (Chevret et al., 1992). Some individuals however progress to AIDS within a few years, rapid progressors (RP), whilst others can remain symptom free for many years, long term non progressors (LTNP) (Buchbinder et al., 1994). This may be due to differences in both host and virus genetics (see section 1.5.6). In contrast to R5 viruses isolated in primary infection, late stage viruses often have a SI phenotype and can use CXCR4 as a coreceptor but this is not essential for progression to AIDS (Connor et al., 1997; Koot et al., 1996; Richman and Bozzette, 1994; Scarlatti et al., 1997; Simmons et al., 1996).

The clinical manifestations of AIDS are diverse and include wasting, neurological disorders, opportunistic infections and onset of AIDS related malignancies such as Kaposi’s Sarcoma and non-Hodgkin’s lymphoma. Opportunistic infections associated with AIDS include Pneumocystis carinii and cytomegalovirus. Opportunistic infections often vary with the geography, fungal infections such as Cryptococcus neoformans are common in tropical climates (Heyderman et al., 1998). The clinical symptoms of HIV-2 AIDS are similar to HIV-1, although some differences have been noted. Encephalitis may be more common in HIV-2 infections and the incidence of Kaposi’s Sarcoma may be lower (Ariyoshi et al., 1998; Lucas et al., 1993).
1.5.6 Long term non-progressors

There are many factors, both viral and host that can influence the course of HIV infection (reviewed in Barker et al., 1998). LTNP are defined differently in different studies but generally are individuals who remain symptom free for many years in the absence of drug therapy with a CD4 count above 550 cells/ml. They have been reported to have stronger and persistent cytotoxic CD8, CD4 T helper cell and neutralising antibody responses (Klein et al., 1995; Cao et al., 1995; Carotenuto et al., 1998; Gea-Banacloche et al., 2000; Rosenberg et al., 1997; Zhang et al., 1997). Individuals heterozygous for a 32 base pair deletion in the gene encoding CCR5 have a reduced level of CCR5 expression on the cell surface (see section 1.4.2.2) (Benkirane et al., 1997; Moore, 1997; Wu et al., 1997). This may account for slower disease progression in individuals heterozygous for this mutation (Dean et al., 1996; Dean et al., 1999; Huang et al., 1996; Michael et al., 1997a; Michael et al., 1997b; Walli et al., 1998). Polymorphisms in other chemokines such as SDF-1α and chemokine receptor (e.g. CCR2) genes can also influence the rate of disease progression (Carrington et al., 1999a; Gonzalez et al., 1999; Ioannidis et al., 1998; Kostrikis et al., 1998; Martin et al., 1998; McDermott et al., 1998; Mummidi et al., 1998; Quillent et al., 1998; Rizzardi et al., 1998; Smith et al., 1997; van Rij et al., 1998; Winkler et al., 1998). However many of these associations may not translate to all ethnic groups (Ramaley et al., 2002). Certain MHC alleles are also associated with slower progression to AIDS, especially HLA-B27 and HLA-B57, while HLA B35 is associated with faster progression (Carrington et al., 1999b; Kaslow et al., 1996; Louie et al., 1991; Migueles et al., 2000). Homozygosity of HLA limits the diversity of the immune response and thus poorer control of viral replication and faster progression to disease (Carrington et al., 1999b; Tang et al., 1999). MHC Class II haplotypes are also associated with disease progression (Westby et al., 1996).

Viral factors that can influence disease progression include deletion or mutation of the nef gene as has been described for a cohort of patients in Australia who received HIV contaminated blood yet remained symptom free for many years (Alexander et al., 2000; Deacon et al., 1995; Greenough et al., 1999; Kirchhoff et al., 1995; Learmont et al., 1999; Tobiume et al., 2002). Often HIV-2 infected individuals have a longer clinically latent phase (Ancelle et al., 1987; Marlink et al., 1994; Poulsen et al., 1997; Whittle et al., 1994) but individuals do progress to AIDS.
Interestingly, HIV-2 isolates often have deletions in Nef (Switzer et al., 1998).

### 1.5.7 CD4 cell death

The central pathological feature of HIV infection and progression to AIDS is loss of CD4 T cells. Cells generally die by one of two mechanisms, apoptosis or necrosis. Apoptosis is characterised morphologically by condensation of the cytoplasm and nucleus and biochemically by activation of aspartate directed proteases called caspases. Phosphatidylserine, normally limited to the inside of the cell membrane, is present on the surface of apoptotic cells. This is recognised by macrophages and results in phagocytosis of apoptotic cells, preventing spilling of cell contents and generation of an inflammatory reaction (Fadok et al., 1992). In contrast, necrosis is characterised by loss of plasma membrane integrity, leakage of the cell contents and induction of an inflammatory response.

During HIV infection there is a high turnover of both virus and CD4 positive cells (Ho et al., 1995; Wei et al., 1995). There are several postulated mechanisms how HIV, directly and indirectly, kills CD4 positive cells. Disruption of cell membrane integrity normally leads to apoptosis. In vitro, syncytia formation can readily be observed in HIV infected cells although whether this occurs in vivo is uncertain but has been reported in brain tissue (Lifson et al., 1986a; Lifson et al., 1986b; Sharer, 1992; Sharer et al., 1985; Sodroski et al., 1986a). Cell membrane permeability can also be affected by gp41 (Cao et al., 1996; Miller et al., 1993). Death of HIV infected cells by apoptosis has been demonstrated in vitro (Gandhi et al., 1998; Laurent-Crawford et al., 1991; Terai et al., 1991; Zagury et al., 1986). Ex vivo, cells from HIV positive individuals exhibit increased apoptosis compared with controls (Groux et al., 1992; Meyaard et al., 1992; Oyaizu et al., 1993; Sarin et al., 1994). Additionally, features characteristic of apoptotic cell death have been observed in vivo (Muro-Cacho et al., 1995). However, this is not restricted to CD4 positive, HIV infected cells (Finkel et al., 1995; Lewis et al., 1994). This suggests killing of non-infected cells (bystander death) is also occurring (Chun et al., 1997a; Harper et al., 1986; Hellerstein et al., 1999; Mohri et al., 1998).

There is some controversy over the role of caspases and thus apoptosis in HIV induced cell death (Cao et al., 1996; Kolesnitchenko et al., 1997; Plymale et al., 1999). It may be that HIV induces cell death directly by a mechanism other than apoptosis,
which also results in an increase in susceptibility of neighbouring cells to apoptosis (Bolton et al., 2002; Lenardo et al., 2002).

The thymus is the main organ for T cell production. It involutes with age but in HIV infection the thymus can show some degree of regeneration in an attempt to produce new T cells to counter the massive destruction. There may be a defect in production of new T cells from the thymus as HIV disease progresses. The thymus itself can be infected resulting in thymocyte depletion, loss of corticomedullary demarcation and development of thymic medullary B cell follicles. Such destruction is mediated rapidly by X4 isolates (Berkowitz et al., 1998; Su et al., 1995) while R5 isolates cause slower destruction. Cells bearing TCR excision circles (TRECs), markers of recent intrathymic TCR rearrangement also decrease in frequency with age and as a function of HIV disease progression (Douek et al., 1998; Poulin et al., 1999; Zhang et al., 1999a). Peripheral lymphoid organs undergo marked alterations during HIV infection, with accumulation of virus in the follicular dendritic cell network leading to eventual destruction, decompartmentalisation and depletion of both CD4 and CD8 T cells (Haase, 1999; Rosenberg et al., 1999b). There is also suppression of multilineage and lineage specific haematopoiesis (Moses et al., 1998). Overall as HIV disease progresses there is a decrease in both the quantity and quality of CD4 T cells (Clerici et al., 1993; Gorochov et al., 1998; Haase, 1999; Rosok et al., 1996).

1.5.8 Treatment

Drug treatment of HIV infection has significantly reduced the morbidity and mortality of HIV disease in the western world (Cavert et al., 1997; Palella et al., 1998). There are currently three major classes of antiretroviral drugs used in HIV therapy that target two viral enzymes, RT and Protease. Initially single drug therapy was used but it has become clear that several drugs administered in combination are more effective, delaying the emergence of resistant strains. However, multidrug resistant viruses still do emerge and can be transmitted (Angarano et al., 1994; Conlon et al., 1994; Erice et al., 1993; Hecht et al., 1998; Imrie et al., 1997; Yerly et al., 1999).

Two classes of antiretroviral drugs target RT and thus block the conversion of viral RNA into cDNA for integration into the host cell genome. Nucleoside reverse transcriptase inhibitors (NRTIs) are nucleotide analogues that undergo anabolic phosphorylation by host cell enzymes to generate a 5' triphosphate. They lack 3'OH moieties and thus prevent formation of 3'-5' phosphodiester bonds between the
elongating DNA chain and incoming 5' nucleoside triphosphate, acting as chain terminators. Non-nucleoside reverse transcriptase inhibitors (NNRTIs) bind to RT near the polymerase catalytic domain and block DNA polymerase activity.

The third class of drug, protease inhibitors, mimic the viral peptide substrates but have non-cleavable structures in the scissile bond. A regimen of highly active antiretroviral therapy (HAART) generally consists of combined administration of 3-4 drugs from these 3 classes. HIV-1 infected patients who respond well to HAART experience a dramatic decrease in plasma viral RNA levels, improved CD4 T cell counts and partial restoration of immune function (Autran et al., 1997; Connick et al., 2000; Kelleher et al., 1996; Li et al., 1998; Pakker et al., 1999; Pakker et al., 1997). HIV-2 can also be treated with HAART but this is less well documented compared with the extensive studies on treatment of HIV-1 infection (Clark et al., 1998; Cox et al., 1994; Schutten et al., 2000; Smith et al., 2001b; Soriano et al., 2000). However, because HIV-2 RT contains sequences that confer drug resistance to HIV-1, NNRTIs are ineffective (Isaka et al., 2001; Ren et al., 2000).

In contrast to initial encouraging speculation, HAART does not eliminate HIV from an infected individual (Cavert et al., 1997; Chun et al., 1997b; Finzi et al., 1997; Furtado et al., 1999; Markowitz et al., 1999; Perelson et al., 1997; Wong et al., 1997; Zhang et al., 1999b). Cessation of therapy results in rapid rebound of viral load (Chun et al., 1999a; Chun et al., 2000; Davey et al., 1999a). This is probably in part due to continued low level replication of HIV during HAART and reservoirs of latent HIV infection (Chun et al., 1999c; Furtado et al., 1999; Lewin et al., 1999; Natarajan et al., 1999; Sharkey et al., 2000; Zhang et al., 1999b). Reservoirs are considered to be sites or cells of latent, long-lived HIV infection that can be reactivated. Several cell types and anatomical locations are thought to harbour reservoirs of HIV. CD4 T cells can be latently infected either pre or post integration (Chun et al., 1997a; Chun et al., 1995; Chun et al., 1997b; Finzi et al., 1997; Siliciano, 1999; Wong et al., 1997). Viral reservoirs are also thought to reside on the FDC network where anti-HIV antibody can capture virus onto Fc receptors (Burton et al., 1997; Grouard et al., 1997; Smith et al., 2001a; Smith-Franklin et al., 2002). Other lymphoid cells have also been postulated to contribute to the reservoir including macrophages/monocytes, B-cells and CD4 positive natural killer (NK) cells (Lambotte et al., 2000; Moir et al., 2000; Sonza et al., 2001; Valentin et al., 2002). Additionally, epithelial cells of the kidney may harbour latent virus (Marras et al., 2002).
Problems with toxic side effects of antiretroviral drugs, especially protease inhibitors, and development and transmission of drug resistant strains has encouraged generation of new therapies (Lucas et al., 1999; Yerly et al., 1999). New NRTIs, NNRTIs and protease inhibitors are being developed that are effective on the current multi-drug resistant strains. The targets for novel therapies include viral entry, Integrase, and viral assembly (reviewed in Condra et al., 2002). Derivatives of soluble CD4 to interfere with cellular CD4 binding have been developed but generally fail to yield successful results in the clinic. This is probably related to the observations in vitro that primary isolates are resistant to sCD4 inactivation (see section 1.4.2.1). A candidate agent currently in clinical trials is a tetravalent structure of 2 domains of CD4 fused to IgG, called PRO542 (Allaway et al., 1995; Jacobson et al., 2000; Shearer et al., 2000). The natural chemokine receptor ligands (see Table 1.1) and modified forms of them can block infection (Bleul et al., 1996; Cocchi et al., 1995; Heveker et al., 1998; Oberlin et al., 1996; Simmons et al., 1997). They compete with HIV envelope for binding and cause receptor downmodulation. However, in vitro both CC and CXC chemokines can, under some conditions, enhance infection leading to caution over their use in vivo (Kinter et al., 1998; Marechal et al., 1999; Trkola et al., 1999).

Small molecules that block chemokine receptors are therefore an active area of research. The bicyclam AMD3100 binds to CXCR4 and blocks virus entry in vitro but its use in vivo is limited due to poor clinical tolerability (De Clercq et al., 1994; Hendrix et al., 2000). Other CXCR4 inhibitors are in development but concerns over toxicity due to role of CXCR4 in normal embryonic development and haematopoiesis questions the use of these drugs in vivo (Kawabata et al., 1999; Ma et al., 1998; Ma et al., 1999; Nagasawa et al., 1996; Tachibana et al., 1998; Zou et al., 1998). Inhibitors of CCR5 such as TAK779 may be more promising (Baba et al., 1999a; Shiraishi et al., 2000) since CCR5 is not essential in development, individuals with a genetic deletion within CCR5 have no obvious defects (see section 1.5.2.1). A concern with the use of CCR5 inhibitors is potential acceleration of the appearance of pathogenic X4 strains. However, a report of HIV-1 escape from a small molecule CCR5 inhibitor showed no switch in coreceptor usage but rather persistent use of CCR5 even in the presence of the inhibitor (Trkola et al., 2002).

The final stage of entry, fusion of the viral and cellular membranes mediated by gp41 can be blocked by peptides (e.g. DP-107 and DP-178) that mimic one component
of the gp41 trimer (Jiang et al., 1993; Wild et al., 1992). T-20 is a similar peptide that has performed well clinically and is now in phase 3 clinical trials (Kilby et al., 1998; www.rocheusa.com). Integration of viral cDNA into the host cell chromosome is mediated by Integrase (see section 1.3.4). Analogues of 1,3, diketo acid that target this enzyme have been described [Hazuda, 2000 #1426; Wai, 2000 #1427.

Immunotherapy of HIV infection is a strategy that seeks to boost the immune response in conjunction with conventional drug therapy (reviewed in Emery et al., 1996; Pantaleo, 1997). Non-specific activators of the immune response, such as interleukin-2 (IL-2), activate T cells, leading to increased viral replication but can also result in an increased CD4 count (Davey et al., 1999b; David et al., 2001a; Kovacs et al., 1995; Kovacs et al., 1996). Immune stimulation of resting T cells may activate latent HIV, rendering it sensitive to antiretroviral drugs and helping reduce the latent reservoir (Chun et al., 1999b). A similar strategy is activation of HIV-specific immune responses by therapeutic vaccination with HIV antigens (reviewed in Hoff et al., 1999). This approach has had success in animal models (Hel et al., 2000). Approaches tried in humans include vaccination with HIV envelope, treatment with autologous dendritic cells pulsed with HIV peptides or DNA with the goal to boost the immune response to lessen the dependence on antiretroviral drugs and prolong survival (Dhodapkar et al., 2000; Dhodapkar et al., 1999; Lisziewicz et al., 2001). Remune is an envelope depleted inactivated HIV antigen and has been reported to increase immune responses in HIV infected individuals (Gotch et al., 1999; Moss et al., 1999) but others have reported no increases in response (Kahn et al., 2000).

An additional approach to augment functional anti-HIV immune responses in individuals on antiretroviral therapy is intervals without treatment, termed structured treatment interruptions (STI) (reviewed in Dybul, 2002; Lisziewicz et al., 2002). When treatment is withdrawn the virus load increases and is thought to boost the anti-HIV immune response to autologous virus (Rosenberg et al., 2000). Treatment can then be restarted before extensive damage occurs due to uncontrolled viral replication. After several rounds of treatment withdrawal and continuation the host immune response may be boosted sufficiently to control viral replication in the absence of therapy for a prolonged period. This is characterised by strong CD4 and CD8 T cell responses (Altfield et al., 2001b). Control of viral rebound following STI may be more successful in patients who were given antiretroviral therapy early after infection with preserved anti-HIV immune response, but chronically treated individuals may
also see a boosting of HIV-specific immune responses (Carcelain et al., 2001; Garcia et al., 2001; Ortiz et al., 1999; Ruiz et al., 2001; Ruiz et al., 2000). The number of therapy interruptions required and the success differs from patient to patient (Lori et al., 2000a; Lori et al., 2000b; Papasavvas et al., 2000). There are several disadvantages to STI including destruction of CD4 cells, potential selection of drug resistant variants, reseeding of viral reservoirs and symptoms similar to primary HIV infection due to the viral replication burst (Orenstein et al., 2000; Youle et al., 2000). An alternative strategy of treatment withdrawal is structured intermittent therapy (SIT) where treatment is stopped of a period of seven days and then continued for seven days even if no viral rebound is detected (Dybul et al., 2001). This is proposed to allow a boost in anti-HIV immune responses without the adverse effects of extensive viral replication.

1.6 Immune responses to HIV

The correlates of immune protection against HIV infection remain unclear. An immune response, innate and adaptive, is detectable in most infected individuals yet progression to disease still occurs.

1.6.1 Innate immune response

The innate immune system is an early response to infection by microorganisms. It consists of phagocytes, NK cells, γδ T cells, interferon producing cells and the complement system. Early in infection, before specific T and B lymphocyte responses are generated, the innate immune system may play an important role in controlling HIV infection and directing the development of the adaptive response.

1.6.1.1 Complement system

The complement system comprises about 30 proteins and includes active components and regulatory proteins. There are three activation pathways for the complement cascade: classical, alternate and lectin. The classical pathway is activated by antibody and is discussed in section 1.6.3.8. The lectin pathway is activated by mannose binding lectin (MBL) interacting with the surface of microbes. The alternate pathway is activated by a lack of regulatory factors on a target. Following activation, the complement cascade results in opsonisation of the microorganism, leading to the stimulation of phagocytosis or lysis. HIV can activate complement by all three
pathways (Lund et al., 1995; Saifuddin et al., 2000; Spear, 1993). High mannose glycans on the extensively glycosylated surface of gp120 may be important for activation of the lectin pathway by binding to MBL (Ezekowitz et al., 1989; Haurum et al., 1993). Activation of the classical pathway can occur in the absence of immunoglobulin by direct binding of HIV envelope glycoprotein to the complement component Clq (Marschang et al., 1997; Tacnet-Delorme et al., 1999). Complement depletion in animal models in primary infection suggests that complement may play a role in the control of primary HIV viraemia and subsequent progression to disease (Gauduin et al., 1998; Schmitz et al., 1999b). Components of the complement system also play a role in antibody effector mechanisms (see section 1.6.3.8). However, in vitro HIV is generally resistant to inactivation by complement due to incorporation of host regulatory proteins into the viral membrane (see section 1.6.4). In addition, HIV can utilise complement components attached to the viral surface to enhance viral spread by binding to cells expressing complement receptors, such as B cells and follicular dendritic cells (Jakubik et al., 1999; Jakubik et al., 2000; Kacani et al., 2000). Virus attached to cells via complement receptors remain infectious and can be transferred to CD4 positive T cells (Doepper et al., 2000; Olinger et al., 2000).

1.6.1.2 Interferon

The interferon (IFN) system is an innate antiviral response. There are two types of IFN, type I (IFNa and IFNb) and type II (IFNg). IFNg is mainly produced by T cells as part of an adaptive response (see section 1.6.2.1). A small subset of human leukocytes referred to as interferon producing cells (IPC) are specialized in the secretion of high levels of type I interferons in response to certain viruses (Ronnblom et al., 1983). These are now classified as a subset of dendritic cells, plasmacytoid dendritic cells (Cella et al., 1999; Siegal et al., 1999). Expression of Toll like receptors (TLR 7 and TLR9) on these cells allows recognition of various pathogens and stimulates production of type I IFN (Colonna et al., 2002). There is direct correlation between IPC number in peripheral blood, IFNa production, and clinical status in HIV-infected individuals, suggesting that these cells may play a role in HIV infection (Soumelis et al., 2001). Cells exposed to type I IFN initiate a signalling cascade resulting in the induction of many interferon inducible genes including 2'–S'oligoadenylate synthetase (Silverman et al., 1982), RNAse L (Silverman, 1994) and Protein kinase RNA activated (Meurs et al., 1990). As a result, type I IFN directs a
block in viral gene expression at multiple levels including inhibition of RNA transcription and translation and degradation of viral transcripts.

1.6.1.3 γδ T cells

γδ T cells expressing the γδ T cell receptor are a minor population in human peripheral blood but are found in larger numbers in the intestine, female genital tract, lymph nodes and spleen (Bucy et al., 1989; Christmas, 1991; Falini et al., 1989; Groh et al., 1989). T cells with αβ T cell receptors recognise peptides expressed in association with major histocompatibility complex (MHC) on the surface of antigen presenting cells and are the cellular component of the adaptive immune response (see section 1.6.2). In contrast, γδ T cells recognize various naturally occurring nonpeptide phosphoantigens expressed by a variety of pathogens in an MHC-unrestricted manner (Constant et al., 1994; Tanaka et al., 1995; Wallace et al., 1995). γδ T cells exert several antiviral effects including production of proinflammatory cytokines, for example IFNγ and tumour necrosis factor α (TNFα), and lyse or induce apoptosis of infected cells via perforin and granzymes (Gan et al., 1996). In addition, they can produce β chemokines, which block HIV infection by blocking and down modulating CCR5 (Poccia et al., 1999). γδ T cells from both HIV infected and uninfected individuals can lyse HIV infected targets in vitro (Boullier et al., 1997; Malkovsky et al., 1992; Wallace et al., 1996; Wallace et al., 1995). The importance of these cells in protection against HIV infection has been suggested by increased levels of γδ T cells in the rectal mucosa of macaques resistant to rectal mucosal SIV infection (Lehner et al., 2000).

1.6.1.4 Natural killer cells

Natural Killer (NK) cells contribute to innate defences against viral infections. Classical NK cells are regulated by opposing signals from activating and inhibitory receptors (Lanier, 1998). Their effector functions include target cell lysis, production of cytokines such as TNFα and IFNγ, and production of β-chemokines (Biron et al., 1999; Bluman et al., 1996; Fehniger et al., 1998; Oliva et al., 1998). An additional subset of NK cells, termed NK T cells, have a restricted T cell receptor repertoire and are activated by presentation of the non-peptide antigen, α-galactosylceramide by non-classical MHC, CD1a (Kawano et al., 1997). The role these cells may play in HIV infection is unclear (Biron et al., 2001).
1.6.2 Cell mediated immune response

Both CD4 and CD8 T-cell mediated immune responses are generated against HIV and play an important role in controlling HIV replication.

1.6.2.1 CD8 T-cells

CD8 positive T-cells recognise peptides of 8-10 amino acids in length presented in the groove of MHC class I and subsequently mediate cytotoxic and non-cytotoxic effector mechanisms (Yang et al., 1997b). The primary peak in viral replication during acute HIV infection declines coincidentally with the appearance of HIV-specific CD8 positive cytotoxic T lymphocytes (CTL) in peripheral blood. This implicates CTL in control of HIV replication at this early stage after infection (Borrow et al., 1994; Koup et al., 1994; Ogg et al., 1998; Pantaleo et al., 1994). HIV-specific CTLs can be directed against peptides of structural (Gag, Pol and Env), regulatory (Tat, Rev and Nef) and accessory proteins (Vpr and Vif but rarely Vpu) (Addo et al., 2001; Altfeld et al., 2001a; Brander et al., 1999; Walker et al., 1987a; Walker et al., 1988). During chronic HIV infection, HIV-specific CD8 T-cells persist at high frequencies, probably due to continued antigen stimulation (Altman et al., 1996; Gray et al., 1999; Kalams et al., 1999b; Ogg et al., 1999). Initially, in acute infection over 25% of PBMC can be HIV-specific CTL, this drops to approximately 1% of PBMC in the chronic phase of infection (Moss et al., 1995; Pantaleo et al., 1994).

Studies in SIV infected macaques have highlighted the role of CD8 T-cells in controlling viral replication in both acute and chronic infection. Elimination or depletion of CD8 cells in primary and chronic infection results in a rapid rise in viraemia and faster progression to disease (Jin et al., 1999; Schmitz et al., 1999a). Higher levels of HIV-specific CTLs are observed in LTNPs than individuals who progress to disease rapidly (Cao et al., 1995; Harrer et al., 1996; Musey et al., 1997). Additionally, several cohorts of EU individuals have detectable HIV-specific CD8 T-cell responses, implicating them in protection from infection (Fowke et al., 1996; Kaul et al., 2000; Pinto et al., 1995; Rowland-Jones et al., 1995a; Rowland-Jones et al., 1998; Rowland-Jones and McMichael, 1995b)

HIV-specific CTLs can lyse HIV infected cells by the release of perforin and granzymes (Shankar et al., 1999; Yang et al., 1996a). Increased expressed of Fas ligand on CTLs may also induce apoptosis of infected target cells that express Fas.
(Hadida et al., 1999). Non-cytolytic mechanisms of inhibition of HIV replication by CD8 T-cells include production of IFNγ, which restricts integration and transcription of viral genes (Emilie et al., 1992; Meylan et al., 1993). Production of the CC chemokines RANTES, MIP-1α and MIP-1β by CD8 T-cells can suppress HIV replication by competition for or downregulation of, CCR5 (Cocchi et al., 1995; Price et al., 1998; Wagner et al., 1998). Chemokines are contained within the same granules as the cytotoxic factors perforin and granzymes and are released in an antigen specific manner (Wagner et al., 1998). An undefined factor termed CD8 T-cell antiviral factor (CAF) that blocks HIV LTR mediated transcription in infected cells is also produced by CD8 cells (Copeland et al., 1995; Levy et al., 1996; Mackewicz et al., 1994; Mackewicz et al., 1994).

Detection of HIV-specific CTLs originally relied on labelling target cells with radioactive chromium, adding CD8 effector cells and measuring chromium release as an indicator of target cell lysis. HLA tetramers are now an important technique for the identification of antigen specific T-cells (Altman et al., 1996; Murali-Krishna et al., 1998). Tetramers are made up of purified HLA molecules folded round a specific antigen derived peptide and linked to streptavidin (reviewed in Klenerman et al., 2002). The use of tetramers has shown that the number of HIV-specific T cells peaks just after the viral load falls (Wilson et al., 2000b). However, tetramer analysis measures the number of antigen-specific CD8 T-cells and does not relay any information on functionality of the T cells. HIV-specific, tetramer positive CD8 T-cells have been reported to have functional defects, including poor cytolytic activity due to low perforin levels (Andersson et al., 1999; Appay et al., 2000; Champagne et al., 2001; Kalams et al., 1998; Lewis et al., 1994; Zajac et al., 1998). Additionally, HIV-specific CD8 T-cells detected by tetramers may be a poor representation of the immunodominant response in an infected individual (Betts et al., 2000). Techniques for CTL quantitation that also measure functionality include measurement of IFNγ or other cytokine production by ELISPOT or intracellular cytokine staining upon in vitro exposure to HIV antigens (reviewed in Imami et al., 2001; Kaul et al., 1999).

A robust CD4 response is required for good CTL responses. CD4 T helper cells recognise antigen on antigen presenting cells (APC) to then allow the APC to prime a CTL precursor that interacts with it (Ridge et al., 1998). Even though a strong CTL response is stimulated in HIV infected individuals there is frequent selection of viral mutants that escape CTL recognition (section 1.6.4).
1.6.2.2 CD4 T cells

CD4 T cells are the major target for HIV infection and it is their decline that is indicative of progression to AIDS (see section 1.5.7). CD4 T cells are a major component of the adaptive immune response. They produce a variety of cytokines and, through cell-cell interactions, can modulate the activity of other immune cells, including APCs and CTLs. Due to this role, they are referred to as T helper (Th) cells. CD4 T cell receptors recognise peptides of 13-18 amino acids presented on class II MHC expressed on APCs. Recognition of specific peptide coupled with an interaction between CD4 and MHC class II and costimulation by CD28/B7 ligation leads to the production of an array of cytokines and initiates cell proliferation. CD4 cells are classified into helper subsets depending on the cytokines produced. IL-2, IFNγ and IL-12 production and IL-18 receptor expression characterise Th1 cells, while Th2 cells produce IL-4, IL-5, IL-6 and IL-10 and express ST2L. Each set of cytokines down modulates the production of the opposing set. Generally Th1 cells activate macrophages to kill ingested microorganisms and Th2 cells prime for antibody production by B-cells. A switch in phenotype from Th1 to Th2 has been reported as disease progresses (Barcellini et al., 1994; Chan et al., 2001; Clerici et al., 1993; Klein et al., 1997) but others report no such switch (Fakoya et al., 1997; Graziosi et al., 1994). While it is clear that CD4 T cells are susceptible to infection with HIV, a CD4 T cell immune response is also generated to a variety of HIV peptides and epitopes, although these are generally less well defined than CD8 epitopes (Cosimi et al., 2000; Malhotra et al., 2001; Wahren et al., 1989).

CD4 T cell responses are measured by cytokine production and proliferation responses following addition of antigen in vitro. Cytokine production by CD4 T cells upon antigenic stimulation can be measured in individual cells by ELISpot and intracellular cytokine staining by flow cytometry (reviewed in Imami et al., 2001). There is a qualitative defect in HIV-specific CD4 function prior to reduction in CD4 T cell numbers with reduced IL-2 production and proliferative capability (Lane et al., 1985; McNeil et al., 2001; Murray et al., 1984; Musey et al., 1999; Pitcher et al., 1999b; Wilson et al., 2000a). CD4 responses to recall antigens are maintained but do decrease with progression to disease (Krowka et al., 1989; Pontesilli et al., 1995). It has recently been shown that HIV-specific CD4 cells are especially susceptible to HIV infection (Douek et al., 2002).
Anti-HIV specific CD4 T cell responses can be detected early in HIV infection, but often become undetectable within three months after infection (Clerici et al., 1989; Pitcher et al., 1999b; Rosenberg et al., 1997; Rosenberg et al., 1999a; Schwartz et al., 1994; Valentine et al., 1998). However, HIV-specific CD4 T cell responses are maintained in some LTNP and are inversely correlated with HIV plasma load (Kalams et al., 1999b; McMichael et al., 2001; Pitcher et al., 1999b; Rosenberg et al., 1997; Schrier et al., 1989; Schwartz et al., 1994). Additionally, HIV-specific CD4 responses have been detected in EU (Clerici et al., 1994). Strong CD4 T helper responses are also associated with strong CTL responses likely due to the interaction between CD4 cells, APC and CD8 cells required to generate a response (Kalams et al., 1999a; Lanzavecchia, 1998; Matloubian et al., 1994; Ostrowski et al., 2000; Rosenberg et al., 2000).

CD4 positive T cells able to lyse HIV infected cells have also been reported (Norris et al., 2001; Sethi et al., 1988; Siliciano et al., 1988). These have been described extensively in HIV-seronegative individuals vaccinated with recombinant gp160 (Hammond et al., 1992; Miskovsky et al., 1994; Orentas et al., 1990; Stanhope et al., 1993a; Stanhope et al., 1993b). Few have been identified in natural infection and their role in vivo in natural HIV infections is unclear (Appay et al., 2002; Littaua et al., 1992; Sethi et al., 1988).

1.6.3 Humoral immune response

Humoral, or antibody-mediated, immune responses are generated to a variety of HIV proteins. Antibodies (Abs) detected soon after infection recognise linear determinants in HIV structural proteins such as p24 and p17 in Gag (Niedrig et al., 1989). Thereafter Abs to Env and Pol epitopes appear. Detection of HIV-specific Abs by ELISA and Western blot is used in the laboratory diagnosis of HIV infection. Only envelope specific Abs are able to neutralise free virus as all other proteins are encased within the virus particle. Generally, anti-envelope Abs block infection by preventing attachment to target cells and interaction of HIV envelope with cell surface receptors and fusion of viral and cellular membranes (Trkola et al., 1996a; Ugolini et al., 1997).

The precise role of Abs in controlling HIV infection remains uncertain. The decline in primary viraemia occurs before neutralising Abs are detected (Koup et al., 1994). However, strong neutralising responses are found in some LTNP (Cao et al., 1995). Passive transfer of HIV-specific Abs in monkeys has been shown repeatedly to
confer protection from infection by a variety of routes (Baba et al., 2000; Emini et al., 1992; Hofmann-Lehmann et al., 2001; Mascola et al., 1999; Mascola et al., 2000; Parren et al., 2001; Prince et al., 1991; Putkonen et al., 1991; Ruprecht et al., 2001; Shibata et al., 1999; Xu et al., 2002). HIV-specific Abs have also been detected in exposed uninfected individuals (Broliden et al., 2001; Devito et al., 2002; Devito et al., 2000; Kaul et al., 2001).

The neutralisation properties of many anti-HIV Abs were originally tested using TCLA strains of HIV. It has now become clear that this is a poor representation of their ability to neutralise clinically relevant primary isolates. Primary isolates of HIV are generally less sensitive to Ab-mediated neutralisation than TCLA (Moore et al., 1995a; Moore et al., 1993b; Moore et al., 1995b; Sawyer et al., 1994; Wrin et al., 1995). The various genetic subtypes of HIV-1 do not generally translate to neutralisation serotypes (Kostrikis et al., 1996; Moore et al., 1996a; Nyambi et al., 1996; Weber et al., 1996). However, sera from some individuals, generally LTNP can neutralise diverse strains of HIV (Cao et al., 1995; Fenyo et al., 1996; Pilgrim et al., 1997; Zhang et al., 1997). Coreceptor usage does not affect neutralisation sensitivity (Cecilia et al., 1998; Trkola et al., 1998). Sera from HIV-2 infected individuals are more frequently neutralizing than HIV-1 sera (Bjorling et al., 1993; Fenyo and Putkonen, 1996). It has previously been reported that sera from HIV-2 infected individuals are more likely to cross neutralize HIV-1 than vice versa (Böttiger et al., 1990; Weiss et al., 1988).

Extensive studies of epitope exposure on the envelope of HIV-1 and X-ray crystallisation of HIV-1 gp120 (Figure 1.5) revealed 2 domains, inner and outer, on HIV envelope (Kwong et al., 1998; Kwong et al., 2000; Moore et al., 1994c; Moore et al., 1996b; Sattentau et al., 1995a; Wyatt et al., 1998a). The inner domain is likely to be buried within the interface between monomers forming the trimeric envelope spike. As a result the epitopes of the inner domain that are exposed on the monomer are hidden, and thus non-neutralising on the native oligomer. Epitopes of the outer domain are thought to be exposed in the oligomeric structure and thus compose the neutralising face. However the outer domain can be further divided into two domains, silent and immunogenic (Wyatt et al., 1998b). The silent face is exposed but epitopes within it are largely occluded by extensive glycosylation. The exposed immunogenic of the outer domain face therefore represents the major target for neutralising Abs.
1.6.3.1 CD4 binding site epitopes

Binding to CD4 is a critical step in the viral life cycle, allowing interaction with a coreceptor and fusion with the target cell membrane (see section 1.4). Therefore, the site responsible must be exposed on the surface of envelope trimers on the virus particle and be relatively conserved. However, the CD4 binding site is recessed into a groove so may be poorly accessible and may also be occluded by V1/V2 loop (Wyatt et al., 1995). In spite of this, many monoclonal Abs (MAbs) able to cross-neutralise a variety of isolates and neutralising Abs in human sera that target this site have been described (Burton et al., 1991; Burton et al., 1994; McKeating et al., 1993a; Moore et al., 1995a; Posner et al., 1991; Roben et al., 1994; Saphire et al., 2001b; Thali et al., 1992; Trkola et al., 1995).

A MAb generated from an HIV-1 infected individual, IgG1b12 (or b12), recognises the CD4 binding site and neutralises a broad range of primary isolates (Burton et al., 1991; Burton et al., 1994; Trkola et al., 1995). It is also sensitive to deletion of the V1/V2 loop although it is not clear if it contacts this structure (Binley et al., 1998; Roben et al., 1994). The crystal structure of b12 revealed its long complementarity determining region 3 that is likely to protrude into the groove of the CD4 binding site (Barbas et al., 1993; Saphire et al., 2001b). The strength of this MAb is demonstrated by its ability to protect from viral challenge in animal models when given alone (Gauduin et al., 1997; Parren et al., 2001).

1.6.3.2 V3 epitopes

The V3 loop is a major determinant of coreceptor usage (see section 1.4.2.2). The V3 loop is also a major neutralisation domain of HIV-1 TLCA (Javaherian et al., 1989). Neutralisation by V3 Abs is generally highly isolate specific but broader neutralising Abs targeting this site have also been reported (Conley et al., 1994a; Gorny et al., 1992; Javaherian et al., 1990; Javaherian et al., 1989; Laman et al., 1992; LaRosa et al., 1990; Ohno et al., 1991). Their role in primary isolate neutralisation is thought to be insignificant perhaps as a result of the relative inaccessibility of V3 on PI compared with TCLA and may even result in enhancement of infection by some isolates (see section 1.6.3.7)(Bou-Habib et al., 1994; Kliks et al., 1993; Schonning et al., 1996; Spenlehauer et al., 1998; Vancott et al., 1995).
1.6.3.3 V1/V2 epitopes

The V1/V2 loop structure is also implicated in coreceptor usage. Several neutralising Abs to this region have been reported (Fung et al., 1992; Gorny et al., 1994; Ho et al., 1991a; McKeating et al., 1993b; Moore et al., 1993c; Sullivan et al., 1993; Warrier et al., 1994). High sequence variability and substantial length polymorphism of this region make it a very strain specific epitope (Wang et al., 1995b). However, neutralisation of primary isolates by V1/V2 Abs can occur, although the range of isolates is very limited (Gorny et al., 1994; Pinter et al., 1998; Vijh-Warrier et al., 1996). Additionally, in contrast to HIV-1, the principal neutralisation domain of SIV and HIV-2 may be V1/V2 as opposed to V3 (Kent and Bjorling, 1996; McKnight et al. 1996).

1.6.3.4 CD4 induced epitopes

Following ligation of CD4, conformational changes occur in HIV envelope to allow interaction with a coreceptor (Lapham et al., 1996; Trkola et al., 1996a; Wu et al., 1996). These conformational changes also form or expose the epitope for a group of Abs. The best characterised of these are 17b and 48d, human MAbs generated from infected individuals (Thali et al., 1993; Wyatt et al., 1995). Even though these MAbs recognise a conserved domain involved in coreceptor ligation (the bridging sheet) (Rizzuto et al., 1998), they only poorly neutralise TCLA, and especially, primary isolates unless sCD4 is added (D'Souza et al. 1995; Salzwedel et al. 2000). This may be due to the more 'closed' structure of primary isolate envelope oligomers, which only expose this site after binding to cellular CD4. In such proximity to the cell membrane these MAbs probably can not access their epitope (Poignard et al., 2001). MAb 17b has however been highly informative for delineating the coreceptor binding site and the generation of a crystal structure of gp120 (Kwong et al., 1998).

1.6.3.5 2G12 epitope

As described above, the silent face of HIV is occluded by carbohydrate. A single MAb (2G12) derived from an HIV-1 infected individual has been identified that depends on a carbohydrate moiety that is conserved across many isolates (D'Souza et al., 1997; Trkola et al., 1995). However, subtype C HIV-1 isolates are resistant to neutralisation by 2G12 (Bures et al., 2002). The 2G12 epitope is sensitive to deglycosylation and mutations that alter N-linked glycosylation sites (Trkola et al., 1996b). Recently, additional mapping of this epitope suggests it is dependent on N-
linked high mannose chains and probably has no direct involvement with the gp120 polypeptide surface (Sanders et al., 2002; Scanlan et al., 2002). Even though it does not target the receptor binding sites directly, 2G12 blocks binding to receptors and attachment to cells, probably due to steric hindrance of the bulk of Ab in the close proximity to the receptor binding sites (Trkola et al., 1996a; Ugolini et al., 1997).

1.6.3.6 gp41 epitopes

Fusion of viral and cellular membranes is mediated by gp41 (see section 1.4.1.5). The majority of gp41 is occluded in the trimeric envelope spike due to interactions with other gp41 molecules and association with gp120 to form a trimer of heterodimers (Sattentau et al., 1995b). Three major clusters of epitopes have been described on gp41, although Abs mapping to them are mostly non-neutralising (Binley et al., 1996). The most potent gp41 MAb described, 2F5, recognises an epitope containing the amino acid sequence Glu-Leu-Asp-Lys-Trp-Ala (ELDKWA) on the ectodomain of gp41, close to the transmembrane region (Muster et al., 1993; Parker et al., 2001). However, recognition of this epitope is dependent on an undefined fusion intermediate structure of gp41 (Gorny et al., 2000). 2F5 neutralises many isolates of HIV-1, including primary isolates (Conley et al., 1994b; D'Souza et al., 1997; Purtscher et al., 1994; Trkola et al., 1995). Two further anti-gp41 neutralising MAbs also mapped to the C terminal extracellular portion of gp41 (Zwick et al., 2001).

1.6.3.7 Antibody dependent enhancement

Antibody dependent enhancement (ADE) describes the situation where antibody binding to virus leads to enhanced infection. ADE of HIV is usually either via complement receptors or Fc receptors (reviewed by Fust, 1997; Sullivan, 2001). Fixation of complement components by antibody bound to the surface of HIV may allow interaction with complement receptors (Reisinger et al., 1990; Robinson et al., 1988; Robinson et al., 1990; Tacnet-Delorme et al., 1999). This can also occur in the absence of HIV specific antibody by direct activation of the complement cascade by HIV envelope (see section 1.6.1.1) (Boyer et al., 1991). Alternatively the Fc portion of an antibody molecule bound to HIV can interact with Fc receptors on target cells (Takeda et al., 1988). ADE is dependent on CD4 and coreceptor expression and is likely to be a result of enhanced attachment of HIV to target cells, increasing the opportunity to interact with specific entry receptors (June et al., 1991; Lund et al., 1995; Takeda et al., 1990). Abs able to mediate ADE are present in human sera from...
HIV infected individuals at both early and late stages of disease (Jolly et al., 2000; Montefiori et al., 1996). A major concern is that anti-HIV envelope Abs elicited by vaccination may exhibit enhancing rather than inhibitory properties (Bures et al., 2000; Mascola et al., 1993).

1.6.3.8 Recruitment of complement and cytotoxic cells

The classical pathway of complement is activated by binding of the complement component C1 to the Fc portion of an Ab. Similar to activation of the complement cascade by the alternate and lectin pathways (see section 1.6.1.1), antibody fixation of complement to HIV can result in opsonisation, stimulation of phagocytosis or lysis of the target. Complement can be recruited to HIV and HIV infected cells by both neutralising and non-neutralising Abs (Spear et al., 1990; Spear et al., 1993). Addition of complement can increase neutralisation titres of anti-HIV sera or Abs up to 10-fold (Spear et al., 1992).

Antibodies are generally not effective against virus infected cells but can recruit effector cells to lyse the target. This is termed antibody dependent cellular cytotoxicity (ADCC). NK cells and monocytes/macrophages bearing Fc receptors can recognise Ab bound to infected cells expressing viral proteins on the surface (Jewett et al., 1990). This results in the targeted release of granzymes and perforin by the effector cell. Induction of ADCC has been demonstrated with Abs recognising epitopes within both gp120 and gp41 (Evans et al., 1989; Koup et al., 1989; Ljunggren et al., 1989; Norley et al., 1990; Ojo-Amaize et al., 1987; Rook et al., 1987). Anti-HIV Abs present early in infection are able to recruit NK effector cells and may play a role in clearance of primary viraemia (Forthal et al., 2001).

1.6.4 Immune evasion

HIV has a great capacity to evade the immune response. Strategies for evasion from the innate immune response includes inhibition of the antiviral effects of IFNγ induced Protein Kinase RNA activated (see section 1.6.1.2), by high levels of TAR-containing RNA (Gunnery et al., 1990). Additionally, effects of the complement system are subverted by incorporation of complement control proteins into the viral membrane when budding from the cell surface (Marschang et al., 1995; Montefiori et al., 1994; Saifuddin et al., 1997; Stoiber et al., 1996; Sullivan et al., 1996; Takefman et al., 1998).
Evasion from the adaptive immune response occurs by changes in amino acid sequence as a result of rapid and error prone replication (Preston et al., 1988; Roberts et al., 1988). Escape from CTL recognition can occur rapidly (Borrow et al., 1997; Evans et al., 1999a; Price et al., 1997) and can also be associated with progression to AIDS (Goulder et al., 1997; Koenig et al., 1995). CTL escape often occurs via mutations of key anchor residues in peptides that reduce or eliminate MHC binding (Chen et al., 2000; Couillin et al., 1994; Evans et al., 1999a) or alter TCR recognition (Klenerman et al., 1994; Phillips et al., 1991). Variation in T helper epitopes can also diminish CD4 proliferative responses (Harcourt et al., 1998).

Downregulation of MHC class I from the infected cell surface can be mediated by Nef thus preventing recognition by CTL (Collins et al., 1998; Schwartz et al., 1996). Prevention of recognition by NK cells is achieved by restricted downregulation of to HLA-A and B leaving HLA-C and E on the cell surface, which inhibits NK cell killing (Arora et al., 2002; Cohen et al., 1999; Le Gall et al., 1998). Other HIV proteins such as Vpu have also been shown to down regulate expression of both MHC class I and II (Carroll et al., 1998; Kanazawa et al., 2000; Kerkau et al., 1997; Verhoef et al., 1998). Nef also upregulates Fas ligand expression on infected cells that may promote apoptosis in HIV specific CD8 and CD4 T cells expressing Fas ligand (Badley et al., 1996; Xu et al., 1999; Xu et al., 1997).

HIV Escape from antibody responses can also be rapid even when the infecting strain is highly sensitive to neutralisation (Albert et al., 1990; Beaumont et al., 2001; di Marzo Veronese et al., 1993). In infected individuals, the neutralising antibody response in sera is often active against previous isolates but inactive against viruses isolated at the same time as the serum sample (Tremblay et al., 1990; Tsang et al., 1994; Von Gegerfelt et al., 1991). Several structural aspects of the HIV envelope oligomer aid antibody evasion. The inner domain is shielded in the oligomeric structure whilst the exposed outer domain is largely occluded by carbohydrate (Back et al., 1994; Chackerian et al., 1997; Parren et al., 1999; Poignard et al., 2001; Reitter et al., 1998; Sattentau, 1998; Schonning et al., 1996; Wyatt et al., 1998a). The hypervariable loops also mask accessibility to critical epitopes. Additionally, many of the antibodies generated may be directed towards envelope debris that is not accessible on the oligomeric structure (Parren et al., 1997).
1.6.5 Vaccine development

A major goal of HIV research is the development of a vaccine to reduce transmission or lessen the pathogenesis of disease. It is unclear whether cellular or humoral immune responses are most important for protection from HIV infection and disease. It is therefore advantageous for a vaccine to elicit both arms of the immune response capable of targeting diverse primary isolates of HIV.

Animal models play a central role in vaccine assessment and elucidation of immune responses that may be important in HIV disease (reviewed in Geretti, 1999; Joag, 2000). HIV-1 does not infect small rodents such as mice and rats due to blocks in entry, proviral gene expression and particle assembly (Bieniasz et al., 2000; Mariani et al., 2000). Mice lacking immune cells (due to severe combined immune deficiency, SCID) can be reconstituted with human lymphocytes and lymphoid tissue and can be infected with HIV although an AIDS-like disease does not occur (Namikawa et al., 1988). The only true animal model for HIV-1 is infection of chimpanzees. However, patient isolates generally replicate poorly in chimpanzees and are a poor model for pathogenesis. This, in addition to ethical reasons and inclusion of Pan species in the CITES (Convention on International Trade in endangered species) conservation list has precluded widespread use of HIV-1 infection of chimpanzees as a model for human HIV infection. SIV does not usually cause AIDS-like disease in the natural host species (Hirsch et al., 1994). However, infection of some species of monkey, such as Asian macaques, with certain SIV strains can result in an AIDS like syndrome (Daniel et al., 1985; Desrosiers, 1988; Letvin et al., 1985; Letvin et al., 1990; Murphey-Corb et al., 1986). A further development of the SIV model was the production of chimeric viruses, usually SIV with the envelope of HIV-1 (SHIV), to allow evaluation of HIV-1 envelope specific vaccine strategies. Multiple passage of these chimeric viruses in monkeys gave rise to highly pathogenic isolates that cause rapid decline to AIDS (Reimann et al., 1996). Some monkey models may be more severe than HIV in humans and thus not be appropriate to test vaccines against, being too stringent a measure, whilst other strains may be less pathogenic so easier to protect against by vaccination (Feinberg et al., 2002; Hu, 1996).

Whole inactivated HIV was studied initially as a vaccine candidate and whilst these often gave protection it was likely due to a response against cellular antigens (Langlois et al., 1992; Murphey-Corb et al., 1989; Stott, 1991; Stott et al., 1990). Live attenuated HIV is not a realistic vaccine for use in humans due to concerns over
reversion to pathogenic wild type virus, although they do show protection in animal models (Baba et al., 1995; Baba et al., 1999b; Daniel et al., 1992; Mills et al., 2000; Whatmore et al., 1995; Wyand et al., 1999). Subunit vaccines containing monomeric HIV envelope protein have generally been found to be safe and immunogenic but they do not elicit Abs or CTLs able to neutralise heterologous primary isolates (Berman et al., 1990; Bures et al., 2000; Connor et al., 1998; Mascola et al., 1996; McCormack et al., 2000; Migasena et al., 2000; Nitayaphan et al., 2000). These studies suggested that monovalent HIV-1 envelope is a poor immunogen for generating cross-reactive neutralizing antibodies and that new strategies are required. Envelope subunits resembling the native trimer may be better at eliciting Abs (Sattentau et al., 1999; Yang et al., 2001). Immunogens resembling oligomeric envelope have been developed either by linking gp120 to the ectodomain of gp41 or removal of the cleavage site (Earl et al., 2001; Stamatatos et al., 2000; Yang et al., 2000a; Yang et al., 2000b). Further stabilisation of oligomeric structure has been achieved by the addition of cysteine bonds (Binley et al., 2000; Farzan et al., 1998a) and stable oligomeric structures are now being reported (Sanders et al., 2002; Schulke et al., 2002). Removal of glycosylation and variable loops that may mask important epitopes is also a strategy to induce better Abs (Barnett et al., 2001; Cherpelis et al., 2001). A vaccine thought to represent a ‘fusion competent’ structure gave promising results in a small animal model (LaCasse et al., 1999), although this has now been discounted (Nunberg, 2002).

Immunisation with DNA encoding HIV proteins augmented with the addition of cytokine genes such as IL-2 has resulted in control of viral replication in monkey models (Barouch et al., 2000; Egan et al., 2000). A newer strategy to generate HIV-specific CTLs is the use of bacterial or viral vehicles manipulated to be non-pathogenic and encoding HIV immunogens (Hanke, 2001). Modification of pox viruses such as vaccinia have been shown to elicits HIV-specific CTL and Abs in primates, including humans (Barouch et al., 2001; Cooney et al., 1993; Corey et al., 1998; el-Daher et al., 1993; Hammond et al., 1992; Hirsch et al., 1996; Hu et al., 1987; Meyer et al., 1991; Ourmanov et al., 2000; Seth et al., 2000; Shen et al., 1991). There are, however, some concerns over the use of vaccinia in immunocompromised individuals (Redfield et al., 1987). Other viruses and bacteria are therefore being investigated such as canarypox (Belshe et al., 1998; Belshe et al., 2001; Clements-Mann et al., 1998; Evans et al., 1999b; Ferrari et al., 1997), adenovirus (Shiver et al., 2002).
and the single strand RNA viruses Venezuelan equine encephalomyelitis virus and Semliki Forest Virus are also being pursued (Davis et al., 2000; Mossman et al., 1996). Mycobacterium bovis; Bacillus Calmette-Guerin-based candidate vaccine has been shown to elicit both CTL and Abs (Honda et al., 1995; Yasutomi et al., 1993). Combination of DNA, vehicle immunisation or protein subunits in a prime boost strategy seems to yield the best responses (Allen et al., 2000; Amara et al., 2001; Hanke et al., 1998; Hanke et al., 2000; Hanke et al., 1999; Kent et al., 1998; Letvin et al., 1997; Robinson et al., 1999). However eventual escape from vaccine elicited CTL has been reported in animals models after several years of infection (Barouch et al., 2002).
1.7 Scope of this thesis

CD4 is the main cellular receptor for HIV, yet many isolates of HIV-2 are able to infect CD4 negative cells (Endres et al., 1996; Reeves et al., 1999). The conformation of CD4-independent HIV-2 envelope presumably allows direct interaction with a chemokine receptor bypassing the need for CD4. The consequences of a CD4-independent envelope conformation and infection of CD4 negative cells for Ab-mediated neutralisation are the focus of this thesis. The related TCLA isolates, CD4-dependent ROD A and CD4-independent ROD B will primarily be used. Studies on HIV-1 TCLA reveal that they are highly sensitive to neutralisation compared with primary isolates. Unlike HIV-1 neutralisation of CD4-independent infection can also be studied using clinically relevant isolates of HIV-2 (Chapter 3).

A wide range of MAbs to a variety of epitopes on HIV-1 envelope have been instrumental for the current understanding of HIV-1 envelope structure and for the identification of neutralisation sensitive epitopes. Neutralisation with epitope specific MAbs can be used to determine if particular regions of viral envelope account for neutralisation sensitivity (Chapter 4).

Mechanisms of Ab-mediated neutralisation can vary, but all Abs must bind to an exposed region of the target to exert an effect. For HIV, factors such as envelope conformation and stability may also play a role in the sensitivity of a particular viral envelope to neutralisation. A variety of methods can be utilised to study binding interactions between Abs and HIV envelope proteins, which is the focus of Chapter 5.
Chapter 2
Materials and Methods

2.1 Buffers and solutions

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Constituents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coating buffer</td>
<td>100mM Na₂HCO₃ pH 8.5</td>
</tr>
<tr>
<td>Dntp</td>
<td>10mM dATP; 10mM dGTP; 10mM dTTP; 10mM dCTP</td>
</tr>
<tr>
<td>HBS-EP</td>
<td>0.01M HEPES pH 7.4; 0.15M NaCl; 3mM EDTA; 0.005% polyethylene glycol sorbitan monolaurate (Tween 20)</td>
</tr>
<tr>
<td>LB-Broth</td>
<td>1% Bacto Tryptone; 0.5% Bacto Yeast; 0.5% NaCl</td>
</tr>
<tr>
<td>PBS</td>
<td>137mM NaCl; 3mM KCl; 10mM Na₂HPO₄; 2mM K₂HPO₄ (pH 7.4)</td>
</tr>
<tr>
<td>TAE</td>
<td>40mM Tris-HCl pH 7.8; 20mM sodium acetate; 1mM EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>20mM Tris-HCl, pH 7.6; 120mM NaCl</td>
</tr>
<tr>
<td>TE</td>
<td>10mM Tris-HCl, pH 7.4; 1mM EDTA</td>
</tr>
<tr>
<td>TM</td>
<td>2% milk powder in TBS</td>
</tr>
<tr>
<td>TMTSS</td>
<td>4% milk powder; 0.1% Tween 20; 10% sheep serum in TBS</td>
</tr>
<tr>
<td>TMTGS</td>
<td>4% milk powder; 0.1% Tween 20; 10% goat serum in TBS</td>
</tr>
</tbody>
</table>

Table 2.1. Buffers and solutions.

2.2 Eukaryotic cell culture

In general, non-adherent cells were maintained in RPMI 1640 medium (Invitrogen, UK) supplemented with 10% foetal calf serum (FCS, Helena Biosciences, UK). Adherent cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, UK) supplemented with 5% FCS. Spodoptera frugiperda 9 (SF9) cells were grown in Grace's insect medium (Invitrogen, UK) supplemented with 10% FCS. All media was supplemented with penicillin (100 IU/ml) and streptomycin (100 μg/ml) (Invitrogen, UK). Mammalian cells were grown in a humidified atmosphere with 5% CO₂ at 37°C. SF9 cells were grown at 28°C with no CO₂. All cell lines used are listed in Table 2.2.
### 2.2.1 Cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Type/species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>293T</td>
<td>Human kidney</td>
<td>(Graham et al., 1977)</td>
</tr>
<tr>
<td>C8166</td>
<td>Human CD4+ T-cell</td>
<td>(Salahuddin et al., 1985)</td>
</tr>
<tr>
<td>CCC/CD4</td>
<td>Feline kidney fibroblast</td>
<td>(Clapham et al., 1991; Crandell et al., 1973)</td>
</tr>
<tr>
<td>CCC/CD4/X4</td>
<td>Feline kidney fibroblast</td>
<td>(Reeves et al., 1999)</td>
</tr>
<tr>
<td>CCC/X4</td>
<td>Feline kidney fibroblast</td>
<td>(Reeves et al., 1999)</td>
</tr>
<tr>
<td>Cos-1</td>
<td>African green monkey kidney fibroblast</td>
<td>(Gluzman, 1981)</td>
</tr>
<tr>
<td>H9</td>
<td>Human CD4+ T-cell</td>
<td>(Popovic et al., 1984)</td>
</tr>
<tr>
<td>HeLa</td>
<td>Human cervical carcinoma</td>
<td>(Gey et al., 1952; Maddon et al., 1986)</td>
</tr>
<tr>
<td>HeLa/CD4</td>
<td>Human cervical carcinoma</td>
<td>(Clapham et al., 1991)</td>
</tr>
<tr>
<td>HeLa/CD4 Cyt-</td>
<td>Mutated CD4</td>
<td>(Maddon et al., 1988; Pelchen-Matthews et al., 1991)</td>
</tr>
<tr>
<td>HeLa/CD4 H399</td>
<td>Mutated CD4</td>
<td>(Pitcher et al., 1999a)</td>
</tr>
<tr>
<td>Molt 4</td>
<td>Human CD4+ T-cell</td>
<td>(Ohta et al., 1988)</td>
</tr>
<tr>
<td>Mv-1-lu</td>
<td>Mink lung fibroblast</td>
<td>(Henderson et al., 1974)</td>
</tr>
<tr>
<td>Mv-1-lu/CD4</td>
<td>Mink lung fibroblast</td>
<td>(Clapham et al., 1991)</td>
</tr>
<tr>
<td>NP2/CD4</td>
<td>Human glioma</td>
<td>(Soda et al., 1999)</td>
</tr>
<tr>
<td>NP2/CD4/R5</td>
<td>Human glioma</td>
<td>(Soda et al., 1999)</td>
</tr>
<tr>
<td>NP2/R5</td>
<td>Human glioma</td>
<td>(Soda et al., 1999)</td>
</tr>
<tr>
<td>RD (TE671)</td>
<td>Human rhabdomyosarcoma</td>
<td>(McAllister et al., 1977; Stratton et al., 1989)</td>
</tr>
<tr>
<td>RD/CD4</td>
<td>Human rhabdomyosarcoma</td>
<td>(Clapham et al., 1991)</td>
</tr>
<tr>
<td>Spodoptera</td>
<td>Insect pupal ovarian</td>
<td>(Vaughn et al., 1977)</td>
</tr>
<tr>
<td>frugiperda 9 (SF9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ssCEM</td>
<td>Human CD4+ T-cell</td>
<td>(Foley et al., 1965)</td>
</tr>
<tr>
<td>SupT1</td>
<td>Human CD4+ T-cell</td>
<td>(Smith et al., 1984)</td>
</tr>
</tbody>
</table>

Table 2.2. Cell lines.
2.2.2 Passaging cells

Cells were passaged every 3-4 days. Adherent cells were washed once with trypsin/versene (0.5% trypsin in 0.02% versene, Imperial Cancer Research Fund, UK) and then incubated with trypsin/versene at 37°C for 5-10 minutes until cells became detached from the flask. Cells were split at 2 dilutions, 1:3-1:8, in fresh DMEM. Non-adherent cells were diluted, 1:3-1:8, as required in fresh RPMI 1640. SF9 cells were split every 3-4 days by gentle agitation to dislodge cells. Cells were then split at 2 dilutions 1:3-1:8 in fresh media (Grace's, 10% FCS).

2.2.3 Freezing cells

Cells were centrifuged at 325g for 5 minutes and resuspended at 5x10^6 cells/ml in cold media containing 20% FCS (DMEM for adherent cells, RPMI 1640 for non-adherent cells and Grace's for SF9 cells). An equal volume of media containing 20% FCS and 20% dimethyl sulphoxide (DMSO, Sigma, UK) was added dropwise. Cells were aliquoted into Nunc cryovials and placed in a polystyrene box at −80°C to freeze slowly (approximately 1°C per minute) overnight before transferring to vapour phase nitrogen.

2.2.4 Thawing cells

Frozen cells were removed from nitrogen storage and thawed rapidly at 37°C. Thawed cells were added to 10ml of fresh media and pelleted at 325g for 5 minutes. Cells were resuspended in 5ml of fresh media containing 20% FCS and transferred to a 25cm² tissue culture flask and passaged as required.

2.2.5 Preparation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats obtained from Brentwood Blood Transfusion Service, UK. Buffy coats were diluted 1:1 in cold PBS and 30ml was layered onto 15ml ficoll (Nycomed, Norway). After centrifugation for 30 minutes at 700g, the lymphocyte layer at the interface between ficoll and PBS/plasma was harvested. An equal volume of RPMI 1640 was added to the harvested cells, which were then centrifuged at 350g for 10 minutes. Cells were washed once more with RPMI 1640 and set up at 5x10^6 cells/ml in RPMI 1640 containing 20% FCS and 0.5μg/ml Phytoheamaglutinin (PHA, Murex, UK). After 2-3 days, cells were washed once in RPMI and resuspended at a density of 1x10^6 cells/ml.
in RPMI 1640 containing 10% FCS and 20U/ml IL-2 (Roche, Germany) for a further 2 days prior to infection.

2.2.6 Flow cytometry of cell surface receptors

Expression of CD4, CXCR4 and CCR5 were routinely tested on cell lines and PBMC. Adherent cells were detached from culture flasks by treatment with versene. Cells were washed once with PBS, 1% FCS, 0.001% sodium azide and resuspended at 5x10^6 cells/ml in PBS, 1% FCS, 0.001% sodium azide. Cells were diluted 1:1 in 5% heat aggregated gammaglobulin (HAG) and incubated for 30 minutes at 4°C to block non-specific Ab binding. 100μl of primary antibody (10μg/ml) was added to cells and incubated at room temperature for 30 minutes. Antibodies used for detection of cell surface receptors and isotype matched controls are shown in Table 2.3. Cells were washed twice with 500μl PBS, 1%FCS, 0.001% sodium azide, resuspended in 50μl anti-mouse Ig conjugated to FITC (Dako, UK) and incubated at room temperature for 30 minutes. Cells were then washed 3 times in PBS, 0.001% sodium azide and fixed in 10% Formol saline (10% paraformaldehyde in PBS). Ten thousand events were collected and analysed on a Becton Dickinson FACScan using Cellquest software (Becton Dickinson, UK).

<table>
<thead>
<tr>
<th>Cell surface receptor</th>
<th>Primary antibody</th>
<th>Isotype control</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td>Q4120 (ARP, NIBSC, UK)</td>
<td>IgG1 (Sigma, UK)</td>
</tr>
<tr>
<td>CXCR4</td>
<td>12G5 (ARP, NIBSC, UK)</td>
<td>IgG2a (Sigma, UK)</td>
</tr>
<tr>
<td>CCR5</td>
<td>2D7 (ARP, NIBSC, UK)</td>
<td>IgG1 (Sigma, UK)</td>
</tr>
</tbody>
</table>

Table 2.3. Antibodies for flow cytometry analysis of cell surface receptors.

2.2.7 Transfection of eukaryotic cells

Cells were transfected using Fugene (Roche, UK) as per manufacturer’s instructions. Two days before use cells for transfection were split 1:2. Cells were plated in 6 well tissue culture dishes at a density of 5x10^5 cells/well. After overnight incubation, to allow cells to reach approximately 80% confluence, culture media was replaced with fresh DMEM 5% FCS. For each well of a 6 well dish 6μl Fugene added to a tube containing 200μl OptiMem (Invitrogen, UK), making sure not to touch the sides of the tube. After gentle mixing, 2μg of DNA was added and incubated at room
temperature for 15 minutes. The transfection mixture was then added dropwise to cells. After overnight incubation transfection media was replaced with fresh DMEM containing 5% FCS.

2.3 HIV

2.3.1 Molecular clones of HIV-2

ROD A molecular clone pACR23 (Keller et al., 1993) and ROD B clone ROD B.14 (Reeves and Schultz, 1997b), were produced by transfected of 293T cells as described in section 2.2.7. Three days after transfection, virus containing supernatants were harvested, centrifuged at 700g for 5 minutes and supernatant passed through a 0.45μm filter (Sartorius Ltd, UK) to remove cell debris. Aliquots of 1ml were snap frozen in liquid nitrogen and stored in vapour phase nitrogen. Transfected 293T cells were cocultivated with ssCEM cells or PBMC to increase viral titers. Viral supernatants were harvested at peak of infection 5 - 7 days later as described above.

2.3.2 HIV-1 and HIV-2 isolates

Primary isolates of HIV-1 (2044) (Simmons et al., 1996) and HIV-2 (SAB, ALI and JAU) (Reeves et al., 1999) were prepared by passage through PBMC. Cell free, low passage virus stock was incubated with 5x10^6 PBMC in a small volume in a 37°C water bath for 2 hours. Cells were then resuspended in 5ml of RPMI 1640, 10% FCS, 20U/ml IL-2. Fresh media was added to cells every 2-3 days as required and cell density adjusted to 1x10^6 cells/ml. Supernatant was harvested 5-7 days later, centrifuged at 700g for 5 minutes and supernatant passed through a 0.45μm filter to remove cell debris. Aliquots of 1ml were snap frozen in liquid nitrogen and stored in vapour phase nitrogen. To increase virus titres infected PBMC were then cocultivated with a 4-fold quantity of uninfected PBMC in a small volume at 37°C for 2 hours before being adjusted to 1x10^6/ml in RPMI 1640, 10% FCS, 20U/ml IL-2. Virus was harvested as described above 5-7 days later.

The HIV-1 TCLA isolate LAI (Wain-Hobson et al., 1991) and HIV-2 non-clonal ROD isolate were prepared by passage through H9 cells. Cell free virus stock was incubated with 5x10^6 H9 cells in a small volume in a 37°C water bath for 2 hours and then resuspended in 5ml of RPMI 1640 10% FCS. Fresh media was added as required every 2-3 days and cell density adjusted to 1x10^6 cells/ml. Supernatant was harvested 5-7 days later centrifuged once at 700g for 5 minutes and passed through a
0.45μm filter to remove cell debris. Aliquots of 1ml were snap frozen in liquid nitrogen and stored in vapour phase nitrogen. To increase virus titres infected H9 cells were then cocultivated with 4-fold more uninfected H9 in a small volume at 37°C for 2 hours before being adjusted to 1x10⁶ cells/ml in RPMI 1640 10% FCS. Virus was harvested as described above 5-7 days later.

### 2.3.3 Production of envelope pseudotypes

Pseudovirus bearing cloned HIV-2 envelopes were produced by cotransfection of envelope expressing plasmids, a plasmid encoding HIV-1 structural genes (packaging) and a plasmid (vector) encoding emerald green fluorescent protein (GFP) containing a packaging signal, Ψ (see section 2.8.5). These constructs were transfected into 293T cells using Fugene (as described in section 2.2.7) at a ratio of 1:2:3 of envelope:packaging:vector to produce pseudovirus (Figure 2.1). Two days after transfection supernatant was harvested, centrifuged twice at 700g for 5 minutes and passed through a 0.45μm filter to removal any cell debris. Aliquots of 1ml were snap frozen in liquid nitrogen and stored in vapour phase nitrogen.

![Figure 2.1. Production of envelope pseudotypes](image)

**Figure 2.1. Production of envelope pseudotypes**

Pseudovirus bearing HIV-2 or VSV-G envelope were produced by cotransfection of 3 plasmids, packaging, envelope and eGFP, into 293T cells.
2.4 Virus detection assays

2.4.1 Immunostaining of HIV infected cells

HIV infected adherent cells in 48 well plates were fixed 3 days after infection with cold methanol:acetone (1:1) and washed with PBS containing 1% FCS. HIV-1 infection was detected with a 1:1 mixture of 2 mouse anti-p24 antibodies (ADP 365 and 366, AIDS reagent program (ARP), National Institute for Biological Standards and Control (NIBSC), UK) diluted 1:40 in PBS 1%FCS. HIV-2 infection was detected with HIV-2 positive serum (provided by D Hawkins, London, UK) diluted 1:4000 in PBS 1% FCS. Cells were incubated for one hour at room temperature with 100μl anti-HIV antibodies and then washed twice with PBS 1% FCS. Cells were then incubated for one hour with 100μl secondary antibody conjugated to β-galactosidase (1:400 dilution); goat anti mouse Ig for HIV-1 and goat anti-human Ig for HIV-2 (Southern Biotechnology Associates Inc., USA). Following 2 washes with PBS 1% FCS and a final wash with PBS alone, substrate (0.5mg of 5-bromo-4-chloro-3-β-D-galactopyranoside in PBS containing 3mM potassium ferrocyanide, 3mM potassium ferricyanide and 1mM magnesium chloride) was added. Positively stained cells appear blue after 1-2 hours incubation at 37°C and were then counted as focus forming units (ffu) using a light microscope.

2.4.2 RT ELISA

Reverse transcription activity in cell free virus supernatants was measured using a commercial lentivirus RT ELISA kit (Cavidi Tech, Sweden) following the manufacturer’s instructions. This assay detects incorporation of Bromo-deoxyuridine triphosphate (BrdUTP) into an immobilised primer template catalysed by viral RT. BrdUTP incorporation is detected by anti-BrdUTP antibody conjugated to alkaline phosphatase (AP). The amount of RT activity in the sample is proportional to the colour change of the AP substrate. Absorbance was measured in a Lucy 1 luminometer (Anthos-Labtech, UK) at 405nm and analysed using Stingray software (Dazdaq, UK)
2.4.3 p24 ELISA

Nunc maxisorb plates (Merck, UK) were coated overnight at room temperature with anti-p24 (D7320, Aalto bioreagents, Ireland) diluted 1:200 in coating buffer. Plates were washed twice with TBS before the addition of TM (Table 2.1) for 30 minutes at room temperature to block non-specific binding. Samples for analysis were centrifuged at 700g for 10 minutes to remove cell debris and 80μl was added to 20μl of TBS 5% Empigen (Surfachem, UK). Samples were then diluted 1:20 in TBS and 10-fold dilutions were made in TBS 0.05% empigen. Half log_{10} dilutions of p24 standard in 0.05% empigen were also carried out to give a standard ranging from 300 pg/ml to 100000 pg/ml. Plates were washed once with TBS 0.1% Tween 20 (Sigma, UK) before addition of 100μl of each sample and standard in duplicate. After incubation at room temperature for 4 hours, plates were washed twice with TBS 0.1% Tween 20. The secondary antibody, mouse anti-p24 conjugated to AP (EH12E1-AP, ARP, NIBSC, UK), diluted 1:400 in TMTSS was then added. Following incubation for 1 hour, plates were washed 6 times with TBS 0.1% Tween 20 and once with TBS alone before luminescent AP substrate (Labtech, UK) was added. After 1 hour incubation in darkness, relative light units (RLU) of each well was measured and analysed using a Lucy 1 luminometer and Stingray software.

2.4.4 Flow Cytometry

Infection of targets cells by HIV pseudovirus (section 2.3.3) was determined by eGFP expression. Three days after infection, cells were detached from tissue culture plates by incubation with versene. Cells were transferred to Falcon facs tubes (Beckton Dickinson, UK), washed once with PBS and fixed in 10% formol saline. Ten thousand events were collected on a Becton Dickinson FACScan and percentage eGFP positive cells determined using Cellquest software.
2.5 Infection assays

2.5.1 Titration on adherent cell lines

Cell lines were seeded in 48 well trays one day prior to infection at the density shown in Table 2.4. 100μl of ten-fold dilutions of virus were added to duplicate wells. After 2 hours, virus inoculum was replaced with 500μl of fresh media. Following three days incubation, cells were fixed and immunostained for HIV antigen as described in section 2.4.1. Titre was calculated as ffu per ml of virus stock.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Density a</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>1x10^4</td>
</tr>
<tr>
<td>CCC</td>
<td>4x10^4</td>
</tr>
<tr>
<td>NP2</td>
<td>1x10^4</td>
</tr>
<tr>
<td>RD</td>
<td>1x10^4</td>
</tr>
</tbody>
</table>

Table 2.4. Cell densities for infection assays.

a The number of cells seeded per well of 48 well tray one day prior to infection.

2.5.2 Titration on PBMC, TCID_{50} calculation

PBMC (prepared as described in section 2.2.5) were seeded in 96 well U-bottomed trays at 10^5 cells/well. Cells were centrifuged at 350g for 5 minutes and media removed. Cells were then exposed to half log_{10} dilutions of 100μl of virus for 2 hours. Each virus dilution was tested in four separate wells. Following removal of virus, cells were washed once with RPMI and 200μl of media (RPMI, 10%FCS, 20U/ml IL-2) was added. Supernatants were harvested after 7 days of infection and assayed for RT content by ELISA as described in section 2.4.2.

The 50% tissue culture infectious dose (TCID_{50}) was calculated based on samples positive for RT by ELISA. The dilution where 50% of wells were positive was calculated using the Karber method (McKnight et al., 1995), TCID_{50} = L-d(S-0.5). Where L is the highest dilution to give 100% of positives, d is the dilution factor (for half log dilutions this is 0.5) and S is the sum of the number of positive wells/total number of wells for all the dilutions to give any positives starting from L and going up to the highest dilution to contain a positive sample.
2.5.3 Neutralisation assay on adherent cell lines

100μl of virus stock containing 100 ffu of virus, 10μl antibody dilution and 10μl sCD4 (or control media) was mixed and incubated at 37°C for 1 hour. Antibody treated virus was then added to target cells seeded in 48 well trays the previous day (see Table 2.3 for cell densities). Cells were incubated with virus/antibody inoculum for 2 hours at 37°C before the mixture was replaced with 0.5ml fresh media. Following 2 days incubation at 37°C the cells were immunostained for HIV antigen as described in section 2.4.1.

2.5.4 Neutralisation assay on PBMC

100μl inoculum containing 100 TCID\textsubscript{50} of virus (calculated as section 2.5.2) and 10μl antibody dilution was incubated at 37°C for 1 hour. PBMC were plated in 96 well U bottom trays at a density of 10\textsuperscript{5} cells per well, centrifuged for 5 minutes at 350g, media removed and virus/antibody inoculum added. Following 2 hours incubation at 37°C virus was removed and cells washed once with RPMI before addition of 200μl RPMI, 20U/ml IL-2, 10% FCS. Fresh media was added to cells every 2-3 days as required. Supernatants for detection of RT content were harvested every 2-3 days and cultures terminated at day 9.

2.5.5 Chemokine receptor blocking

Inhibition of HIV infection by blocking chemokine receptors, target cells were pretreated for 30 minutes at 37°C with twice the final concentration of ligand. Three different blockers of CXCR4 were used, the natural ligand SDF-1α (provided by Ian Clark-Lewis), a small molecule inhibitor AMD3100 (provided by Eric De Clercq) and the mouse MAb 12G5 (ARP, NIBSC). As controls RANTES (provided by Amanda Proudfoot) and MAb 2D7 (ARP, NIBSC) that bind CCR5 were used. 75μl of chemokine or MAb was added to adherent cells in 48 well plates (see table 2.4). After 30 minutes, 75μl of virus stock containing 100 ffu was added and incubated for 2 hours at 37°C. Cells were then washed and fresh media was added. Cells were fixed 3 days after infection and immunostained as described in section 2.4.1.
2.5.6 Cell-cell fusion

Cell-cell fusion was assayed by coculture of cells expressing HIV envelope proteins and target cells expressing CD4 and CXCR4. 293T or Cos-1 cells were transfected with molecular clones of ROD A and ROD B as described in section 2.2.7. 3 days after transfection expression of cell surface envelope was tested by flow cytometry as described in section 2.7.4. Transfected cells were detached from tissue culture plates by incubation with versene for 10 minutes at 37°C. Cells were resuspended in DMEM containing 5% FCS and counted. 1x10^5 transfected 293T cells were mixed with 1x10^5 target cells in a 48 well tray and observed for syncytia by light microscopy. To allow better visualisation of syncytia, cells were fixed and stained with syncytial stain (1% methylene blue, 0.25 % Basic fuchsin in methanol) for 5 minutes before extensive but gentle washing with PBS to remove excess stain.

2.5.7 Viral stability assay

The stability of viral titre over time was assayed in the presence of increasing concentrations of sCD4. Virus stock was incubated at 37°C for 0, 2, 4, 8 and 24 hours either alone or with 1, 2 or 5μg/ml of sCD4. At these time points an aliquot of virus was removed and ten-fold dilutions added to HeLa/CD4 cells seeded in 48 well trays. After 2 hours incubation at 37°C, virus inoculum was removed, cells washed and fresh media added. Cells were fixed 3 days after infection and immunostained as described in section 2.4.1

2.6 Antibodies

2.6.1 Rat monoclonal antibodies

Rat MAbs were kindly provided by Chris Shotton, Institute of Cancer Research, UK. Baculovirus derived HIV-2 ROD envelope protein gp105 (EVA621, ARP, NIBSC, UK) complexed with sCD4 (ARP 608/609, ARP, NIBSC, UK) was used as the immunogen. Rats (strain CBH/Cbi) were immunised 3 times at 21 day intervals via Peyers patches. 3 days after the last immunisation, rats were sacrificed and mesenteric lymph nodes removed and cells fused with the rat myeloma Y3-Ag1.2.3. Resultant hybridoma supernatants were screened by antigen capture ELISA with gp105/sCD4 by C. Shotton. Rat MAbs, 32/2f, 32/7g, which map to HIV-2 V3 loop and 8e, a conformational MAb, have been described previously (McKnight et al.,
1996). Rat MAb 8/38c specific for HIV-1 was used as a negative control (McKeating et al., 1992).

2.6.2 Isotyping of monoclonal antibodies

Mouse antibodies specific for rat immunoglobulin heavy chains were used to determine isotypes of the MAbs by radioimmunoassay. MAbs were tested for binding to wells coated with mouse Ab specific for each rat Ig isotype. Bound MAbs were detected with $^{125}$I-labelled rabbit anti-rat IgG (mouse-absorbed, Serotec, UK). This was kindly carried out by C. Shotton at the Institute of Cancer Research.

2.6.3 Antibody mapping

2.6.3.1 Binding to infected cells

HeLa/CD4 cells were infected with 100 ffu of non-molecular clone ROD and fixed three days later with 1:1 methanol:acetone and washed with PBS 1% FCS. 100 μl of each rat MAb (diluted to 10 μg/ml in PBS 1% FCS) was added to duplicate wells containing infected cells and incubated at room temperature for 1 hour. Following 2 washes with PBS, 1% FCS, anti-rat conjugated to β-galactosidase (1:400, Southern Biotechnologies Inc., USA) was added and incubated at room temperature. Following 2 washes with PBS 1% FCS and a final wash with PBS alone, substrate (0.5 mg of 5-bromo-4-chloro-3-β-D-galactopyranoside in PBS containing 3 mM potassium ferrocyanide, 3 mM potassium ferricyanide and 1 mM magnesium chloride) was added and the plates incubated at 37°C. Positively stained cells appear blue after 1-2 hours.

Molt4 and SupT1 cells chronically infected with ROD were used to test rat MAb binding to non-fixed envelope by flow cytometry. Cells were washed once with PBS, 1% FCS, 0.001% sodium azide and resuspended at 5 x 10⁶ cells/ml in PBS, 1% FCS, 0.001% sodium azide. 100 μl of each rat MAb (10 μg/ml) was added to cells and incubated at room temperature for 1 hour. MAb 8/38c, specific for HIV-1 was used as the isotype control. Cells were washed twice with 500 μl PBS, 1% FCS, 0.001% sodium azide, resuspended in 50 μl of anti-rat Ig conjugated to FITC (Dako, UK) and incubated at room temperature for 1 hour. Cells were then washed 3 times in PBS, 0.001% sodium azide and fixed in 10% formal saline. Ten thousand events were collected and analysed on a Becton Dickinson FACS® using Cellquest software.
2.6.3.2 Binding to CD4

Rat MAb were tested for binding to human CD4 stably expressed on the cell surface of Mv-l-lu and RD cells and compared with binding to the parental, CD4 negative cells. Binding was tested by flow cytometry as described above (section 2.6.3.1) for binding to chronically infected Molt4 and SupT1 cells.

Binding of rat MAb to sCD4 was tested by ELISA. A 96 well ELISA plate was coated with 50μl sCD4 (2μg/ml) in coating buffer (see Table 2.1) and incubated overnight at room temperature. Plates were washed twice with 200μl TBS before addition of TM to block non-specific binding by incubation for 30 minutes at room temperature. Following one wash with TBS, 100μl of the rat MAb were added to the plate at concentrations ranging from 100ug/ml to 0.003ug/ml. A mouse anti-CD4 MAb, L120 (ARP 359, ARP, NIBSC, UK) was used as a positive control. Following incubation for 1 hour at room temperature plates were washed 3 times with TBS 0.1% Tween 20. 100μl goat anti-rat conjugated to AP (Harlan Seralab, UK) diluted 1:2000 in TMTGS was added to the plate and incubated for 1 hour before washing 3 times with TBS 0.1% Tween 20 and 2 times in AMAPAK wash buffer (Dako, UK). 100μl per well of AP substrate (AMPAK, Dako, UK) was added and the plate incubated for 20 minutes at room temperature before amplifier was added and absorbance measured at 492nm in a microtitre plate reader (Anthos-Labtech, UK).

2.6.3.3 Construct mapping

SF9 cells were seeded at 4x10^5 cells/well in 24 well trays. After incubation overnight at 28 °C, baculoviruses encoding 25 chimeric HIV-1 LAI/HIV-2 ROD A envelope, produced by exchanges at conserved cysteine residues(Morikawa et al., 1992, provided by I. Jones, Oxford, UK, Figure 2.2) were used to infect SF9 cells. After 4 hours incubation, baculovirus inoculum was removed and fresh Grace’s media was added. After 3 days incubation at 28°C, without CO2, cells were fixed with 1:1 methanol:acetone and immunostained with rat MAb as described in section 2.6.3.1.
2.6.3.4 Peptide mapping

Peptides of 12 amino acid covering the whole of ROD A gp105 were made on a cellulose membrane using a SPOTs kit (Genosys Biotechnologies Inc., USA) as per the manufacturer’s instructions. Amino acids were linked together by a condensation reaction between the C-terminal COOH and N-terminal NH2 groups of two amino acids. The coupling reaction was monitored visually by staining the free amines after each coupling cycle with bromophenol blue. The resulting peptides are covalently attached to the membrane. Each 12mer peptide was synthesised to overlap the previous by 6 amino acids. Binding of rat MAbs to peptide spots was detected by the addition of anti-rat Ig conjugated to β-galactosidase. After addition of substrate positive binding was indicated by a blue spot on the membrane.
2.6.4 Human monoclonal antibodies

Human MAb (26C, 110C, 17A, B23, 23F, 211D, 15D, 34G) derived from B cells from HIV-2 infected individuals were kindly provided by J. Robinson (Cole et al., 2001; Martin et al., 1997; Robinson et al., 1998). The human anti-HIV-1 MAb 17b (Thali et al. 1993; Wyatt et al. 1995) was kindly provided by R. Wyatt.

2.6.5 Human sera

Serum samples from HIV-2 positive individuals were kindly provided by Dr. D Hawkins (Chelsea and Westminster Hospital, London, UK) and Dr. J Moniz-Pereria (Lisbon, Portugal). Sera from HIV-1 infected individuals, QC1 and QC5, have been described previously (McKeating et al., 1989) and were kindly proved by Prof. J. Weber. All serum samples were heat inactivated at 56°C for 20 minutes.

2.6.6 Purification of immunoglobulin from human sera

Immunoglobulin G (IgG) was purified from HIV-2 positive and control sera using a MabTrap protein G column (Amersham Biosciences, UK) as per manufacturer’s instructions. The column was washed with buffer prior to addition of 400μl sera diluted 1:1 in binding buffer. After further washes elution buffer was passed through the column. All fractions were collected for subsequent analysis.

2.6.7 Protein quantitation

The protein content of samples from the MabTrap column was measured using a Biorad protein quantitation kit (Biorad, UK). 400μl of the concentrate dye reagent was diluted with 1600μl of deionised water. Human IgG of known concentration was diluted in PBS (10μg/ml- 100μg/ml). 2μl of each sample and standard was added to a microtitre plate in duplicate. 100μl of diluted dye reagent was then added and mixed thoroughly. The plate was incubated at room temperature before the absorbance of each well was read in a microplate reader at 570nm (Anthos-Labtech, UK).
2.7 Envelope binding assays

2.7.1 Monomeric envelope binding

The inner wells of a 96 well ELISA plate (Immunolon II) were coated with 100μl *Galanthus nivalis* agglutinin (GNA, snowdrop bulb lectin, Sigma, UK, 10μg/ml) in coating buffer and incubated overnight at 4°C. Plates were blocked twice for 10 minutes with 200μl TM. 0.2μg/ml baculovirus derived envelope proteins from ROD A and ROD B (provided by J. Reeves) were captured onto the GNA coated plates by incubation for 2 hours at room temperature. Following 3 washes with TBS, 100μl of human sera (diluted 1:5 - 1:1000) or rat MAb (100μg/ml to 0.003μg/ml) was added to the plate. Following incubation for 1 hour at room temperature, plates were washed 3 times with TBS 0.1% Tween 20. 100μl goat anti-rat or anti-human IgG conjugated to AP (Harlan Seralab, UK) diluted 1:2000 in TMTGS was added to the plate and incubated for 1 hour before washing and development using the AMPAK system as described in section 2.6.3.2. Half maximal binding concentrations were calculated using FORCAST function in Microsoft Excel.

2.7.2 Competition of rat and human MAbs binding to HIV-2 envelope

The inner wells of a 96 well ELISA plate (Immunolon II) were coated with 100μl, GNA, 10μg/ml in coating buffer and incubated overnight at 4°C. Plates were blocked twice for 10 minutes with 200μl TM. 0.2μg/ml gp105 (EVA621, ARP, NIBSC, UK) was captured onto the GNA coated plates by incubation for 2 hours at room temperature. Following 3 washes with TBS, 100μl of human anti-HIV-2 MAb, or control HIV-1 MAb (10μg/ml) was added to the plate. Following incubation for 1 hour at room temperature plates were washed 3 times with TBS 0.1% Tween 20. Rat MAbs (10μg/ml) were then added to the plate and incubated for 1 hour at room temperature before 3 washes with TBS 0.1% Tween 20. 100μl goat anti-rat Ig conjugated to AP diluted 1:2000 in TMTGS was added to the plate and incubated for 1 hour before washing and development using the AMPAK system as described in section 2.6.3.2.
2.7.3 Capture assays

MAb capture of envelope incorporated or associated with viral particles was measured by ELISA. The inner wells of 96 well white ELISA plates (Maxisorb) were coated overnight at 4°C with 100μl MAb 77ow/2g, 64/4a or 8e (10μg/ml) in coating buffer (Table 2.1). Plates were blocked for 1 hour with 200μl TM. Viral stock supernatant undiluted or equalised for RT activity (measured as described in section 2.4.2) by dilution in TBS 0.05% Triton X-100 (Sigma, UK) or control baculovirus derived gp105 were captured onto the MAb coated plates by incubation for 2 hours at room temperature. Following 4 washes with TBS 0.1% Tween 20, 100μl of human sera (diluted 1:500 in TBS) was added. Following incubation for 1 hour at room temperature plates were washed 4 times with TBS. 100μl anti-human conjugated to AP diluted 1:2000 in TMTGS was added to the plate and incubated for 1 hour before washing 6 times with TBS 0.1% Tween 20. Luminescent AP substrate was then added and incubated for 1 hour in darkness. RLU of each well was measured and analysed using a Lucy 1 luminometer and Stingray software.

A second virion capture measured RT activity of Ab captured and non-captured virus. The inner wells of a 96 well Maxisorb plate were coated with 100μl MAb 77ow, 64/4a or 8e (10μg/ml) in coating buffer (Table 2.1) and incubated overnight at 4°C. Plates were blocked for 1 hour with 200μl TM. Viral stock supernatants were equalised for RT content (section 2.4.2) by dilution in TBS were captured onto MAb coated plates by incubation for 2 hours at room temperature. Virus samples were removed from the plate and the plates washed 6 times with TBS. 100μl sample dilution buffer from the RT ELISA kit (section 2.4.2) was added to the plate to solubilise captured virus. Samples removed from the plate prior to washing and captured virus samples were then assayed for RT activity as described in section 2.4.2.
2.7.4 Binding to cell surface envelope by flow cytometry

293T or Cos-1 cells were transfected with molecular clones pACR23 (ROD A) and ROD B.14 as described in section 2.2.7. Three days after transfection cells were detached from culture flasks by treatment with versene. Cells were washed once with PBS, 1% FCS, 0.001% sodium azide and resuspended at 5x10⁶ cells/ml in PBS, 1% FCS, 0.001% sodium azide. 100µl of primary antibody (rat MAb or human sera, diluted in PBS, 1% FCS, 0.001% sodium azide) was added to cells and incubated at room temperature or 37°C for 1 hour. Cells were washed twice with 500µl PBS, 1% FCS, 0.001% sodium azide and resuspended in 50µl of anti-rat or anti-human Ig conjugated to FITC (Dako, UK) and incubated at room temperature or 37°C for 1 hour. Cells were then washed 3 times in PBS, 0.001% sodium azide and fixed in 10% formal saline. Ten thousand events were collected and analysed on a Becton Dickinson FACScan using Cellquest software.

2.8 DNA manipulation

2.8.1 Agarose gel electrophoresis

DNA was fractioned according to mass using ultra pure agarose (Gibco BRL, UK). Gels were made with 0.8% w/v agarose in 0.5x TAE buffer (Table 2.1) and 0.1µg/ml ethidium bromide and submerged in 0.5xTAE buffer in a Mupid minigel tank (Eurogentec, UK). DNA was prepared in DNA loading buffer (40% glycerol, orange G) and 15µl loaded into each well and electrophoresed for approximately 20 minutes until adequate separation was achieved. Depending on the size of the DNA to be identified, the molecular weight standards used were a 100 base pair ladder, or 1 Kb ladder (Gibco BRL, UK). DNA was visualised by illumination with short wave (254 nm) ultraviolet light.
2.8.2 DNA extraction from agarose gels

DNA was extracted from agarose gels using the Qiaquick gel extraction kit (Qiagen, UK). The DNA band of the appropriate size was excised from a TAE gel using a clean scalpel and weighed. 300μl of buffer QG was added for each 100mg of gel. This was then incubated at 50°C for 10 minutes or until the gel slice had completely dissolved. Buffer QG contains a pH indicator that is yellow if the pH is 7.5 or below and orange/violet if pH is above 7.5. Appropriate conditions for DNA to bind to the silica membrane are pH below 7.5. Therefore if buffer QG was not yellow, 3M acetate pH 5 was added until the buffer colour returned to yellow. 100μl of isopropanol was then added for every 100mg of gel originally used. The sample was then added to a Qiaquick column and placed on a vacuum manifold. 0.5ml of buffer QG was added to the column and vacuum switched on. 0.75ml of buffer PE was then added to the column. The column was removed from the vacuum manifold and centrifuged for 1 minute to remove residual buffer PE. To elute the DNA from the column 50μl of buffer EB was added to the column and allowed to stand for 1 minute and then centrifuged for 1 minute.

2.8.3 Plasmid purification

4ml of LB-Broth (Table 2.1) containing 50μg/ml ampicillin was inoculated with a single bacterial colony from an agar plate (2.8.9) and grown overnight at 37°C with shaking. Cells were pelleted by centrifugation at 3000g for 15 minutes and resuspended in 250μl buffer P1 (containing RNase A) of a Qiagen Miniprep kit (Qiagen, UK) and transferred to a 1.5ml microcentrifuge tube. 250μl of buffer P2 was then added and the tube gently inverted 4-6 times. 350μl buffer N3 was added within 5 minutes and the tube gently inverted 4-6 times. The white precipitate formed was pelleted by centrifugation for 10 minutes. Plasmid DNA was purified from the supernatant on an anion exchange resin using a vacuum manifold. The resin was washed once with buffer PB and once with buffer PE using the vacuum manifold. The column was removed from the vacuum manifold and centrifuged for 1 minute to remove residual buffer PE. Plasmid DNA was eluted by the addition of 50μl TE.
2.8.4 DNA quantitation

The concentration of nucleic acids in aqueous solution was determined by spectrophotometry at 260 nm. An absorbance of 1.0 cm\(^{-1}\) was taken to be equivalent to 50\(\mu\)g/ml of DNA.

2.8.5 Plasmids

Rod A molecular clone pACR23 (Keller et al., 1993) and Rod B clone B.14 (Reeves and Schulz, 1997b) were used for the production of Rod A and Rod B virus stocks. For HIV envelope pseudotypes the following plasmids were used. The packaging plasmid pCMV\(\Delta\)R8.2 encodes HIV-1 gag, pol and regulatory genes tat, rev, vif, vpr, vpu and nef (Naldini et al., 1996). The vector plasmid pSincptcmveGFP contains a cytomegalovirus promoter (CMV)-driven emerald green fluorescent protein (eGFP) expression cassette (Follenzi et al., 2000). VSV-G envelope protein was in pMD-G (Naldini et al., 1996). Packaging, vector and VSV-G plasmids were kindly provided by M. Collins (UCL, UK). Plasmid (pCR3.1, Invitrogen, UK) containing Rod A and Rod B envelope were prepared as described in section 2.8.7.

2.8.6 Primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Env 5' (phosphorylated)</td>
<td>GTCTTCTGCATCAGACAAGTGAGTATG</td>
</tr>
<tr>
<td>Env 3'</td>
<td>CATCCCTTCCAGTCCCCCTTTTTTCTTTTA</td>
</tr>
<tr>
<td>V3 13418</td>
<td>GGTITGGCTTTAATGGCAGTAGAG</td>
</tr>
<tr>
<td>V3 13420</td>
<td>TTCTCCTCTGCAGTTAGTCCACAT</td>
</tr>
</tbody>
</table>

Table 2.5. Primers.

All primers were obtained from Oswell, UK.

2.8.7 Amplification of HIV-2 envelope

Full length envelope, gp160, of Rod A and Rod B was amplified based on the strategy for amplification of HIV-1 envelope clones (Gao et al., 1996). PCR amplifications were performed using primers Env 5' and Env 3' (see Table 2.5) and the Expand High Fidelity DNA polymerase system (HiFi, Roche, Germany). 25 \(\mu\)l reactions were run containing 5 pmol of each primer, 2.5 \(\mu\)l of HiFi reaction buffer, 0.4\(\mu\)l of HiFi and 0.5\(\mu\)l of 40mM dNTPs (Table 2.1), together with 0.25ng of DNA of pACR23 or Rod B.14. The PCR conditions were as follows: 20 cycles of 94°C for 45 seconds, 50°C for 45 seconds and 72°C for 4 minutes followed by 15 cycles of 94°C for 45 seconds, 50°C for 45 seconds and 72°C for 4 minutes 15 seconds. 5 \(\mu\)l of the
PCR was run out on a 0.8% agarose gel (see section 2.8.1), and reactions giving a band of the appropriate size (approximately 3kb) were ligated into vectors as described in section 2.8.8.

2.8.8 Cloning into pCR 3.1 uni

Due to the phosphorylation of the Env 5’ primer, envelope clones could be unidirectionally ligated into pCR3.1 (TA unidirectional cloning kit, Invitrogen, UK.). Approximately 150ng of PCR product together with 60ng of vector were ligated overnight at 15°C in a total of 10 μl using the T4 ligase and 10X ligase buffer (60mM Tris-HCl, pH 7.5, 60mM MgCl₂, 50mM NaCl, 1mg/ml bovine serum albumin (BSA), 10mM β-mercaptoethanol, 1mM ATP, 20mM dithiothreitol, 10mM spermidine).

2.8.9 Transformation of competent bacteria

2μl of a ligation reaction (section 2.8.8) or molecular clone DNA was added to 50μl competent Escherichia coli strain TOPF10 (Invitrogen, UK) and incubated on ice for 30 minutes. Cells were heat shocked for 45 seconds at 42°C and then incubated on ice for 5 minutes. 250μl of preheated SOC media from TA cloning kit was added and incubated with shaking at 37°C for 1 hour. Bacterial cells were then spread on agar plates containing ampicillin (100μg/ml) and incubated overnight at 37°C.

2.8.9 Colony screening

After overnight growth on agar plates colonies were picked and screened by PCR for V3 loop of HIV-2 envelope. A small amount of bacterial colony was transferred directly to a 50 μl PCR reaction containing 10pmol each of primers V3 13418 and V3 13420 (see Table 2.3). PCR was performed for 38 cycles at 96°C for 1 minute, 63°C for 1 minute and 72°C for 2 minutes resulting in a 381 nucleotide fragment. Clones that were positive by PCR for HIV-2 V3 loop were amplified and plasmid DNA purified (section 2.8.3). Plasmid DNA was digested using NheI and EcoRI sites that are present in the vector 5’ and 3’ of the insert respectively. 1μl of plasmid DNA was digested with 0.5μl of each enzyme (Promega, UK) by incubation for 1 hour at 37°C in Multicore buffer (Promega, UK). The reactions were then run out on a 0.8% agarose gel and a 3kb fragment was observed in positive clones.
2.9 **Biacore analysis**

2.9.1 **General procedures and maintenance**

Biacore surface plasmon resonance (SPR) technology allows interactions between biomolecules to be studied in real time. Biacore technology is based on the refractive index detected as SPR during the interaction between one molecule immobilised on a biosensor chip (the ligand) and another molecule passing over the chip in solution (the analyte). The interaction is displayed graphically as resonance units (RU) on a sensorgram. A schematic of general layout of a Biacore flow cell and detection apparatus is shown in Figure 2.4.

![Diagram of Biacore principles](image)

**Figure 2.4. Principles of Biacore.**

The ligand (Ab) is immobilised onto a biosensor chip and the analyte (●) in solution passages through the flow channel at a constant rate. The interaction between the two molecules is detected as a change in the refractive index by an optical detection unit.

A BiacoreX machine (Biacore, UK) was used. This machine allows simultaneous detection of interactions in two flow cells with manual injection of the analyte. All buffers and samples used were passed through a 0.2μm filter and degassed before use. All samples were equilibrated to 37°C before injection. Weekly and immediately prior to use the flow cell was cleaned using the Desorb and Sanitise programs within the Biacore software. Research grade CM5 sensor chips (Biacore, UK) that have a carboxymethylated dextran surface were used.
2.9.2 Pre-concentration

To determine the buffer to be used for immobilisation of MAb 77ow/2g a preconcentration procedure was carried out. For optimal immobilisation a buffer with a pH lower than the pI of the protein should be used. 0.5μg/ml 77ow/2g was made up in 5 different buffers: 5mM sodium malate pH 7, 5mM sodium malate pH 6, 10mM sodium acetate pH 4.8, 10mM sodium acetate pH 4.28, 10mM sodium acetate pH 3.98. 20μl of 77ow/2g in each buffer was run over an unactivated CM5 chip surface at a flow rate of 10μl/minute. 10mM acetate pH 4.28 gave the steepest pre-concentration curve and was used as the immobilisation buffer.

2.9.3 Regeneration

To enable the sensor chip to be reused the surface must be regenerated to remove bound analyte. Several different regeneration buffers were tested. 10mM glycine pH 2.5 gave complete regeneration of the chip, returning the sensorgram to base line. Regeneration did not significantly affect the chip surface as duplicate injections of the same concentration of the analyte gave overlaying sensorgrams (see section 5.2.8).

2.9.4 Antibody immobilisation

Antibody (rat MAb 77ow/2g) was coupled to the carboxymethylated dextran surface of a CM5 by amine coupling. Continuous buffer flow was set at 5μl/minute with HBS-EP (Table 2.1, Biacore, UK) as the running buffer. The carboxymethyl groups on the biosensor chip were activated to form active esters by injection of 30μl 0.5M hydroxysuccinimide (NHS) and 0.2M N-ethyl-N’-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). 50μl MAb 77ow/2g, diluted to 2 or 0.5μg/ml in 10mM acetate pH 4.28, was injected over the activated chip surface. Residual active esters on the chip surface were deactivated by injection of 30μl 1M ethanolamine-HCl, pH 8.0. 77ow/2g was immobilised onto two chips, one with approximately 6000 Response Units (RU) and another with 1000 RU (see Figure 2.4)
Figure 2.5. Sensorgram of 77ow/2g immobilisation using amine coupling.

1. Baseline
2. Activation of carboxymethyl groups by injection of EDC/NHS
3. Increase in baseline of approximately 150RU
4. Injection of 2μg/ml of 77ow/2g in 10mM acetate pH 4.28
5. Continuous flow of HBS-EP buffer removes loosely associated Ab
6. Deactivation of remaining active esters by injection of 1M ethanolamine-HCl
7. Difference in response units between point 7 and 3 reflects the amount of MAb immobilised (6000RU)

2.9.2 gp105 binding

Interactions for kinetic analysis were measured at 37°C. The running buffer was HBS-EP with an increased salt concentration of 500mM to reduce background binding of sCD4 to the sensor chip (see section 5.2.8.1). A dilution series of gp105 ranging from 50nM to 1000nM or 500nM gp120 was made in running buffer. A dilution series of gp105 ranging from 50nM to 500nM was also made with the addition of 500nM sCD4 and was incubated at 37°C for 1 hour before injection. 100μl of each concentration of gp105 was injected at a continuous flow rate of 50μl/minute and allowed to dissociate for 400 seconds. Binding was tested over a range of flow rates with no major change in initial binding rate indicating no mass transport affects occurring in the system. The sensor chip was regenerated by injection of 10μl of 10mM glycine pH 2.5. Sensorgrams were analysed and kinetics calculations performed using BIAEvulation 3.0 software (Biacore, UK).
2.9.6 Pseudovirus binding

HIV pseudovirus with HIV-2 envelope was prepared as described in section 2.3.3. Pseudotypes were equalised for RT content (measured as described in section 2.4.2) and resuspended in running buffer using an Amicon centrifugal filter with a 100kDa cut off (Millipore, UK). 100μl of pseudovirus was injected over the chip surface at a continuous flow rate of 50μl/min. Dissociation was allowed to continue over 400 seconds and the chip surface was regenerated by injection of 10μl of 10mM glycine pH 2.5. Sensorgrams were analysed using BIAEvaluation 3.0.
Chapter 3
Neutralisation sensitivity of CD4-dependent and CD4-independent HIV-2

3.1 Introduction

Individuals infected with HIV mount a response to the virus, including innate, cellular and humoral immunity (see section 1.6). Antibody responses to the HIV envelope can be detected in patient sera about 2-3 weeks postinfection (Moore et al., 1994a). However, such Abs are often low in affinity (Moog et al., 1997; Pellegrin et al., 1996). Over the course of infection, with constant exposure of the immune system to HIV antigens, the Ab response to envelope matures. This is characterised by an increase in Abs able to cross-react with diverse strains and progressively higher neutralisation titres (Cole et al., 1998; McKnight et al., 1992; Moog et al., 1997; Pilgrim et al., 1997; Wrin et al., 1994).

Besides T-cell immunity a major goal of an HIV vaccine is to induce production of Abs that neutralise a broad range of circulating HIV strains. HIV isolates serially passaged in T cell lines (TCLA) were used as targets for initial studies on neutralisation properties of sera from infected individuals (Karpas et al., 1988; Robert-Guroff et al., 1985; Weiss et al., 1985; Weiss et al., 1986). These gave encouraging results, with many isolates susceptible to neutralisation, however, it soon became clear that virus isolated from patients into PBMC (primary isolates) were relatively refractory to neutralisation. Subsequent studies have clearly demonstrated that primary patient isolates are refractory to neutralisation by a variety of Abs and sCD4 (Daar et al., 1990; Moore et al., 1993a; Moore et al., 1995a; Moore and Ho, 1995b; O'Brien et al., 1994; Sawyer et al., 1994; Wrin et al., 1995).

The major targets on the viral envelope for Abs in human sera are the V3 loop and the CD4 binding site. V3 loop reactivity of human sera has been based mainly on studies with TCLA viruses and is regarded as the principal neutralisation determinant on HIV-1 (see section 1.6.3.2) (Javaherian et al., 1989). The V3 loop is thought not to represent a major target in primary isolates (Spenlehauer et al., 1998; Vancott et al., 1995). Abs to the V3 loop are often highly type specific although broader anti-V3 Abs have also been described (Conley et al., 1994a; Javaherian et al., 1990; Ohno et al., 1991; Steimer et al., 1991). Abs to the CD4 binding site are also common in infected
individuals but again these are poorly reactive against primary isolates (Ho et al., 1991b; Moore and Ho, 1993b; Steimer et al., 1991). HIV undoubtedly employs various strategies to try to subvert the immune response from critical targets including masking by carbohydrate (see section 1.6.4).

Despite this, human MAbs generated from HIV-specific B cells from infected individuals can be potent and cross-reactive for many primary isolates (D'Souza et al., 1997). These MAbs tend to target conserved epitopes often involved in the critical processes of binding to cellular receptors and entry into target cells. IgG1b12 recognises the CD4 binding site and neutralises a variety of primary isolates (see section 1.6.3.1)(Burton et al., 1991; Burton et al., 1994; Trkola et al., 1995). 2F5 recognises an epitope on the ectodomain of gp41 and neutralises many isolates of HIV-1, including primary isolates (see section 1.6.3.6)(Conley et al., 1994b; D'Souza et al., 1997; Purtscher et al., 1994; Trkola et al., 1995). A third MAb, 2G12 targets a carbohydrate-dependent moiety on the envelope and also neutralises diverse primary isolates (see section 1.6.3.5)(Sanders et al., 2002; Scanlan et al., 2002; Trkola et al., 1995; Trkola et al., 1996b).

Such cross-reactive Abs are stimulated by natural infection with HIV-1 and are often found at higher titres in LTNP (can be identified in about 10% of people infected (Moore et al., 1996a). In contrast, Abs that neutralise TCLA are present in the sera of both LTNP and RP (Pilgrim et al., 1997). However, immunisation with monomeric envelope does not generate potent cross-reactive Abs in the sera of vaccinees (Richmond et al., 1998). Vaccine elicited Abs that neutralise TCLA viruses are abundant yet have weak or non-existent activity against primary isolates (see section 1.6.5). Attempts to generate a potent and broadly cross-reactive immune response by immunisation with HIV envelope have so far been largely unsuccessful. Further understanding of the targets for neutralisation in human sera may lead to the development novel antigens for immunisation. Many strategies are currently being investigated in an attempt to develop superior envelope structures to include in an HIV vaccine (see section 1.6.5).

HIV-2 differs antigenically from HIV-1. In particular, V3 loop does not appear to be the principal neutralisation determinant. The V1/V2 loops may have this role along with conformational regions (Kent and Björling, 1996; McKnight et al. 1996). A further defining feature of HIV-2 is its ability to infect cells in the absence of CD4 as well as resistance to sCD4 neutralisation (Clapham et al. 1992; Sattentau et al.,
Furthermore, primary isolates of HIV-2 are capable of CD4 independent infection \textit{in vitro} (Reeves \textit{et al.}, 1999). Efficiency of CD4 independent infection by primary HIV-2 strains is often 10-100 fold lower than infection of CD4 positive cells. However, infectivity titres on CD4 negative cells can be markedly increased by incubation with sCD4 (Reeves \textit{et al.}, 1999). Indeed sCD4 treatment can allow some HIV-2 primary isolates (SAB and MIL) to infect primary CD4 negative astrocytes (Reeves \textit{et al.}, 1999). The prototype HIV-2 TCLA strain, ROD A can also be induced to infect CD4 negative cells by incubation with sCD4. In contrast, ROD B, derived from ROD A, can infect CD4 negative and positive cells at an equal efficiency (Clapham \textit{et al.}, 1992; Reeves and Schulz, 1997b). This allows the neutralisation sensitivity of CD4-dependent and CD4-independent infection to be directly compared.

The aims of this chapter are

- to investigate the ability of sera from HIV-2 positive individuals to neutralise ROD A and the CD4-independent variant ROD B and determine whether CD4 independent infection differs in sensitivity to neutralisation by Abs present in human sera.

- to investigate neutralisation of primary isolates of HIV-2 capable of infecting CD4 negative cells, to establish if the route of infection affects neutralisation sensitivity of primary isolates.
3.2 Results

3.2.1 Neutralisation of ROD A and CD4-independent variant ROD B by sera from HIV-2 positive individuals

Serum samples obtained from HIV-2 infected individuals were used to investigate the neutralisation sensitivity of the two related HIV-2 strains, CD4-dependent ROD A and its CD4-independent derivative, ROD B. Of the thirteen HIV-2 serum samples obtained, twelve were from individuals in Portugal and one from an asymptomatic HIV-2 patient in London. The sensitivity of ROD A and ROD B to neutralisation by HIV-2 positive sera was compared to control serum from a non-infected individual. HeLa/CD4 cells were used as the target cell. Molecular clones of both ROD A and ROD B were also used to limit heterogeneity in virus stocks. 50% and 90% neutralisation titres were calculated as the dilution factor of HIV-2 positive serum required to reduce 50% or 90% of infection compared to infection in the presence of the control human sera. As shown in Table 3.1, all 13 HIV-2 sera preferentially neutralised infection by CD4-independent ROD B compared with ROD A, with 50% and 90% neutralisation achieved at higher dilution (lower concentration) of serum. The fold difference between titres for ROD A and ROD B were calculated (Table 3.1). For titres of <10, the difference was calculated as if the titre was 10 and thus probably underestimates in some cases. The magnitude of difference between neutralisation of ROD A and ROD B was over 3 log_{10} for some sera (sera 1, 50% titre difference 3.2 log_{10}) with an average difference in titre of 1.9 log_{10} for 50% and 2.4 log_{10} for 90% neutralisation. Serum 13 was the only serum that had a difference in 50% and 90% neutralisation titre for ROD A compared to ROD B of less than 1 log_{10}.

To compare the titres obtained for HIV-2 neutralisation with neutralisation of HIV-1 isolates, neutralisation of HIV-1 TCLA strain, LAI by 2 HIV-1 positive sera (QC 1 and QC5 McKeating et al., 1989) was tested in the same assay. A 50% neutralisation titre of 320 and 160 was observed for QC1 and QC5 respectively compared with titres of 320 for ROD A and up to 16000 for ROD B (see Table 3.1). This suggests that ROD A is comparable in neutralisation sensitivity to HIV-1 TCLA strains, such as LAI, in the assay used but that ROD B is highly sensitive to neutralisation by serum from HIV-2 positive individuals.
Table 3.1. Neutralisation titres of HIV-2 positive sera.

<table>
<thead>
<tr>
<th>Sera</th>
<th>50% Titre (^a)</th>
<th>Fold Difference (log (_{10}))</th>
<th>90% Titre</th>
<th>Fold Difference (log (_{10}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ROD A</td>
<td>ROD B</td>
<td>ROD A</td>
<td>ROD B</td>
</tr>
<tr>
<td>Normal human sera</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>16000</td>
<td>&lt;10</td>
<td>8000</td>
</tr>
<tr>
<td>2</td>
<td>320</td>
<td>16000</td>
<td>10</td>
<td>8000</td>
</tr>
<tr>
<td>3</td>
<td>80</td>
<td>8000</td>
<td>10</td>
<td>2000</td>
</tr>
<tr>
<td>4</td>
<td>80</td>
<td>12800</td>
<td>10</td>
<td>8000</td>
</tr>
<tr>
<td>5</td>
<td>320</td>
<td>6400</td>
<td>10</td>
<td>3200</td>
</tr>
<tr>
<td>6</td>
<td>160</td>
<td>6400</td>
<td>10</td>
<td>4000</td>
</tr>
<tr>
<td>7</td>
<td>320</td>
<td>12800</td>
<td>10</td>
<td>6400</td>
</tr>
<tr>
<td>8</td>
<td>320</td>
<td>12800</td>
<td>40</td>
<td>6400</td>
</tr>
<tr>
<td>9</td>
<td>80</td>
<td>9600</td>
<td>10</td>
<td>3200</td>
</tr>
<tr>
<td>10</td>
<td>320</td>
<td>16000</td>
<td>40</td>
<td>6400</td>
</tr>
<tr>
<td>11</td>
<td>80</td>
<td>6400</td>
<td>&lt;10</td>
<td>800</td>
</tr>
<tr>
<td>12</td>
<td>160</td>
<td>32000</td>
<td>&lt;10</td>
<td>1600</td>
</tr>
<tr>
<td>13</td>
<td>80</td>
<td>640</td>
<td>&lt;10</td>
<td>20</td>
</tr>
</tbody>
</table>

\(^a\) Titres are expressed as the dilution required to reduce infection of ROD A and ROD B by 50% and 90% compared to control (incubation with the same dilution of uninfected serum). Infection was assayed by \(\beta\)-gal immunostaining (see section 2.4.1) of infected HeLa/CD4 cells.

3.2.2 Purification and neutralisation properties of immunoglobulin from HIV-2 positive serum

Human serum contains factors other than immunoglobulin that can block HIV infection (reviewed in Spear, 1993). These include complement components (Sullivan \(et\ al.,\ 1996\)), mannose binding protein (Ezekowitz \(et\ al.,\ 1989\)), chemokines such as RANTES, MIP-1\(\alpha\), MIP1-\(\beta\) (Cocchi \(et\ al.,\ 1995\)), SDF (Bleul \(et\ al.,\ 1996\; Oberlin \(et\ al.,\ 1996\)) and other undefined factors produced by CD8 T cells (Mackewicz \(et\ al.,\ 1995\)). Indeed, HIV-2 infection may result in higher levels of \(\beta\) chemokine production by PBMC compared to HIV-1 infection in vitro (Akimoto \(et\ al.,\ 1998\; Kokkotou \(et\ al.,\ 2000\)). All serum samples used for neutralisation assays were heat treated at 56°C for 20 minutes. This was to inactivate complement components, thereby ruling out the effects of complement on neutralisation assays. To exclude neutralisation by other factors (e.g. chemokines) immunoglobulin was purified from HIV-2 positive sera.

IgG can be purified from human sera by binding to protein G, a cell surface protein of group G streptococci. Protein G binds to the Fc portion of IgG by a non-immune mechanism. It is similar to protein A of \(Staphylococcus\ aureus\), however protein G binds to all subclasses of human IgG (IgG 1-4) (Bjorck \(et\ al.,\ 1984\) whilst
IgG3 is not bound by protein A. IgG3 accounts for about 5-8% of total IgG in human sera, with IgG1 the most abundant Ig subclass (French, 1986). A commercial protein G column (see section 2.6.6) was used to purify IgG from HIV-2 serum (Table 3.1). The other sera were not available in sufficient quantities for this analysis. Fractions collected from the column were tested for protein content, binding to HIV-2 envelope protein and neutralisation of ROD B. As shown in Figure 3.2A (line), fractions 4, 5, 12, 13, and 14 contained detectable amounts of protein and fractions 12, 13 and 14 contained Ig able to bind HIV-2 envelope gp105 by ELISA (Figure 3.2 columns). Collected fractions were also tested for neutralisation of ROD B. Neutralising activity of ROD B was contained in fractions 12, 13 and 14 (Figure 3.1B). When the dilution factor during purification (400μl ⇒ 3 x 1ml, 7.5 fold) is taken into account, neutralisation titres of the positive fractions is equivalent to unfractionated HIV-2 sera. Thus the observed neutralisation is due to the Ab fraction and other non-immunoglobulin factors in human sera account for negligible neutralisation effects.
Figure 3.1. Protein content, envelope binding and neutralisation of immunoglobulin purified fraction of HIV-2 serum.

A Protein content (line) of fractions of HIV-2 sera collected from a protein G column, at column elution stages indicated by the coloured bar, were measured using a Biorad protein assay. Production of a coloured precipitate was detected by reading the optical density at 570nm. Binding of collected fractions and whole sera to baculovirus derived monomeric HIV-2 envelope gp105 was measured by ELISA. Mean relative light units (RLU) of luminescent substrate for a 1/500 dilution of each fraction are shown (columns).

B Fractions collected and the original sera were tested for ability to inhibit ROD B infection of HeLa/CD4 cells (columns) at a 1/3200 dilution. % neutralisation was calculated as the reduction in ffu by β gal immunostaining compared to control (incubation with 1/3200 of normal human sera).
3.2.3 Neutralisation of ROD A induced to infect CD4 negative cells

While ROD B can infect cells without CD4, ROD A is dependent on CD4. However, infection of CD4 negative cells expressing an appropriate coreceptor by ROD A can be induced by incubation with sCD4 (Clapham et al., 1992). This induction of infectibility by sCD4 is in contrast to TCLA isolates of HIV-1 that are highly susceptible to neutralisation by incubation with sCD4 (see section 1.4.2.1). Presumably for ROD A, sCD4 acts similarly to membrane expressed CD4 in inducing conformational changes in the viral envelope to allow interaction with the chemokine receptor. ROD B has circumvented the requirement for CD4 for entry into target cells and thus presumably binds directly to the coreceptor in the absence of CD4 (Reeves and Schulz, 1997b). Since sCD4 treatment of ROD A may change the envelope conformation to resemble ROD B, I investigated if HIV-2 positive sera were potent neutralisers of sCD4-induced ROD A infection. CCC cells stably expressing CXCR4, either with or without CD4, were used as target cells for this assay to obtain sufficient titres for sCD4 induced infection by ROD A. Figure 3.2 shows that sCD4 induction rendered ROD A infection of CD4 negative cells substantially more sensitive to neutralisation. The magnitude of sensitivity of sCD4-induced ROD A is not as high as ROD B for these sera but is clearly increased compared to neutralisation than ROD A infection of CD4 positive cells. Serum 1 (from Table 3.1) has a 90% titre of <10 for ROD A, 8000 for ROD B and 160 for sCD4-induced ROD A. Serum 5 has a 90% titre <10 for ROD A, 3200 for ROD B and 40 for sCD4-induced ROD A.

![Figure 3.2. Neutralisation of sCD4-induced ROD A by HIV-2 positive serum.](image)

Neutralisation of ROD A infection of CCC/CD4/X4 cells (■) and sCD4 induced infection of CCC/X4 cells (□) by HIV-2 positive serum 1 (A) and serum 5 (B). Mean % residual infectivity compared to control incubation with the same dilution of non-infected control serum is shown.
3.2.4 Neutralisation properties of human anti-HIV-2 MAbs

MAbs derived from HIV-1 infected patients have proved invaluable in studying the structure, function of envelope and mechanism of neutralisation. The most potent and broadly cross-reactive MAbs for neutralisation of primary isolates of HIV-1 have all been generated from infected individuals, namely IgG1b12, 2F5 and 2G12 (see section 3.1). The availability of a panel of MAbs generated from HIV-2 infected individuals allowed further investigation of neutralising Abs in HIV-2 infected individuals. The panel of 8 MAbs (26C, 110C, 17A, B23, 23F, 211D, 15D, 34G) was generated from HIV-2 positive patients from the Ivory Coast by EBV transformation of peripheral B cells (provided by J. Robinson). Previous studies with these MAbs have been carried out to assess cross reactivity with SIV. Three of the MAbs (110C, 17A and B23) compete with each other for binding to SIV gp120 and also block SIV gp120 binding to CCR5 (Cole et al., 2001; Martin et al., 1997; Robinson et al., 1998).

A summary of the properties of these MAbs is shown in Table 3.2, limited information is available concerning MAbs 23F, 211D, 15D and 34G largely due to the lack of cross reactivity with SIV.

<table>
<thead>
<tr>
<th>MAb</th>
<th>Competition group&lt;sup&gt;a&lt;/sup&gt;</th>
<th>SIV strains neutralised&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Blocks SIV gp120 binding to CCR5&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Mapping&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>26C</td>
<td>I (N-term)</td>
<td>-</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>110C</td>
<td>VIa</td>
<td>17E-C1, B670</td>
<td>✓</td>
<td>Sensitive to deletions in V4, reactive with 45kDa fragment of SIV</td>
</tr>
<tr>
<td>17A</td>
<td>VIc</td>
<td>17E-C1, B670</td>
<td>✓</td>
<td>Reactive with 45kDa fragment of SIV</td>
</tr>
<tr>
<td>B23</td>
<td>VIA</td>
<td>17E-C1, B670</td>
<td>✓</td>
<td>Reactive with 45kDa fragment of SIV</td>
</tr>
<tr>
<td>23F</td>
<td>-</td>
<td>-</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>211D</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15D</td>
<td>-</td>
<td>-</td>
<td>X/✓</td>
<td>-</td>
</tr>
<tr>
<td>34G</td>
<td>-</td>
<td>-</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>7B2</td>
<td>Anti-HIV-1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.2. Characteristics of human anti-HIV-2 MAbs.

<sup>a</sup> from Cole et al., 2001.  
<sup>b</sup> from Robinson et al., 1998 and Cole et al., 2001.  
<sup>c</sup> from Martin et al., 1997.  
<sup>d</sup> mapping information is based on mutation studies in Robinson et al., 1998. The 45kDa fragment is the principal neutralising determinant of SIV Javaherian et al., 1992.  
X, no blocking; ✓, positive for blocking; -, no information available.
The panel of human MAbs were tested for recognition of HIV-2 ROD envelope by β-gal immunostaining on fixed infected cells. All of the MAbs stained infected cells to varying degrees, except 7B2, the HIV-1 human MAb used here as a control. The MAbs were tested for neutralisation of ROD A and ROD B. As shown in Figure 3.3 MAbs 26C, 15D and 34G all potently neutralised ROD B infection. 110C, 17A and B23 neutralised ROD B to a lesser extent with 90% neutralisation not being observed even at the highest concentration tested, 10μg/ml, while 23F and 211D neither reduced ROD A nor ROD B infectivity. None of the MAbs neutralised CD4-dependent ROD A infection (Figure 3.3). The three MAbs within the same competition group (110C, 17A and B23) also exhibited similar neutralisation of ROD B with 49.6%, 39.7% and 36.5% reduction in infectivity respectively. These three MAbs have previously been shown to neutralise SIV and block SIV envelope binding to CCR5 (Cole et al., 2001; Martin et al., 1997; Robinson et al., 1998). This suggests that they recognise a relatively conserved epitope or conformation between SIV and HIV-2 that may potentially comprise part of the coreceptor binding site. They have previously been mapped to a region of the SIV envelope, which contains the principal neutralising domain for SIV and relies on some degree of native conformation (Javaherian et al., 1992; Robinson et al., 1998).
3.2.5 Susceptibility of HIV-2 to neutralisation by 17b

The human MAb 17b recognises an epitope that overlaps with the bridging sheet/coreceptor binding site on HIV-1 (Thali et al., 1993; Wyatt et al., 1995) and therefore may be conserved across many HIV strains (see section 1.6.3.4). I investigated whether 17b could potentially recognise a coreceptor binding site conserved across HIV-2 as well and thus inhibit CD4 independent infection. Dilutions of 17b were incubated with ROD A, ROD B and HIV-1 strain LAI (as a positive control) and infection of HeLa/CD4 cells was assayed. As shown in Figure 3.4, at high concentrations (10-20μg/ml) the TCLA HIV-1 strain, LAI was neutralised by 17b. However even at the highest concentration tested 17b did not neutralise ROD A or CD4-independent ROD B. Thus while the 17b neutralisation epitope is conserved across many HIV-1 isolates it is not present on HIV-2 ROD.
3.2.3 Neutralisation of primary isolates of HIV-2

Primary isolates of HIV-1 are refractory to neutralisation by both Ab and sCD4 compared to TCLA strains (Ashkenazi et al., 1991; Daar et al., 1990; Mascola et al., 1994; Moore et al., 1993a; Moore et al., 1992; O'Brien et al., 1994; Sawyer et al., 1994). To study whether primary isolates of HIV-2 are intrinsically resistant to neutralisation, 3 primary HIV-2 isolates, SAB, ALI and JAU, were investigated. Many primary isolates of HIV-2 are unique in their ability to utilise chemokine receptors for entry into target cells in the absence of CD4 (Endres et al., 1996; Reeves et al., 1999). Therefore, it was also possible to investigate whether CD4 independent infection by primary isolates of HIV-2 is more sensitive to neutralisation. The primary HIV-2 isolate, SAB utilises CXCR4 both in the presence and absence of CD4. ALI utilises CCR5 in the presence and absence of CD4 and JAU uses CCR5 and CXCR4 in the presence of CD4 but only CCR5 in the absence of CD4 (Reeves et al., 1999). The infectivity titre of these primary viruses when infecting CD4 negative cells is approximately 10- to 100-fold less efficient compared to infection on CD4 positive cells. Infectious titres on CD4 negative cells however can be increased by incubation with sCD4 (Reeves et al., 1999). To test whether neutralisation sensitivity of these isolates was dependent on the route of infection, CD4-dependent, sCD4-induced and CD4-independent infection were tested following treatment of virus with HIV-2 positive sera. The neutralisation assay was adjusted to accommodate the lower titres of the primary viruses in the absence of CD4. CCC cells expressing CXCR4 with and without CD4 were used for assays of SAB and NP2 cells expressing CCR5 with and without CD4 were used for ALI and JAU. Two HIV-2 positive sera, at a constant

Figure 3.4. Neutralisation of ROD by 17b.

ROD A (■) and ROD B (♦) are not susceptible to neutralisation by 17b, whilst a TCLA HIV-1 isolate, LAI (○), is neutralised by increasing concentration of 17b. % Residual infectivity on HeLa/CD4 cells as compared to control Ab is shown.
dilution of 1/500, were tested for their ability to neutralise SAB, ALI and JAU on either CD4 positive or negative cells. As shown in Figure 3.5A, SAB (X4) infection of CD4 positive cells was relatively resistant to neutralisation by HIV-2 positive sera. However, when infecting CD4 negative cells or induced by sCD4 SAB was almost completely neutralised by a 1/500 dilution of HIV-2 positive serum. Similarly, infection of CD4 negative cells by the dual tropic isolate JAU is more susceptible to neutralisation (Figure 3.5C). Interestingly, ALI (R5) infection of CD4 positive cells was more susceptible to neutralisation than either SAB or JAU, with over 80% reduction in infectivity. However, ALI infection of CD4 negative cells showed increased neutralisation with up to 100% neutralisation at 1/500 dilution of HIV-2 serum (Figure 3.5B).
To investigate further the difference in neutralisation sensitivity of CD4-dependent and sCD4 induced infection primary isolates, serial dilutions of HIV-2 positive sera. CD4 independent infection could not be assayed in this way due to relatively low titres by this route of infection. As shown in Figure 3.6 primary X4 (SAB), R5 (ALl) and, to a lesser extent, dual tropic (JAU) isolates were all more sensitive to neutralisation when induced to infect CD4 negative cells by sCD4. A 1/640 dilution of sera was sufficient to neutralise 90% of infectivity for sCD4 induced SAB ALl and JAU. 90% neutralisation of infectivity of CD4 positive cells was only seen with ALl at 1/40 dilution with SAB and JAU infectivity reduced 75% and 30% respectively at the lowest dilution tested (1/20). Therefore primary isolates capable of infecting CD4 negative cells are substantially more susceptible to neutralisation when infecting cells in the absence of cell surface CD4.
Figure 3.6. Neutralisation of primary isolates of HIV-2 induced to infect CD4 negative cells.

Primary Isolates of HIV-2, SAB (top), ALI (middle) and JAU (bottom) were used to infect CD4 positive cells (■) or induced to infect CD4 negative cells (▲). Residual infectivity after incubation with dilutions of HIV-2 sera (1/20–1/640) was compared with incubation with the same dilution in non-infected control serum.
3.3 Discussion

3.3.1 CD4-independent ROD B is more sensitive to neutralisation by HIV-2 positive sera compared with ROD A

Many isolates of HIV-2, including primary isolates are able to infect chemokine receptor expressing cells independently of cell surface CD4 (Endres et al., 1996; Reeves et al., 1999). The isolates used primarily in this chapter are the prototypic HIV-2 lab adapted strain ROD A that requires CD4 for infection and an isolate derived from ROD A, ROD B, which efficiently infects cells without CD4 (Clapham et al., 1992). ROD B may therefore carry an envelope where the coreceptor binding site is more exposed. Such a conformation may also result in exposure of epitopes that can be targeted by neutralising Abs.

In this study serum samples from 13 HIV-2 infected individuals preferentially neutralised CD4-independent ROD B compared to ROD A. The titres obtained for 50% and 90% reduction in infectivity of ROD B were up to (and in one case over) 3 log₁₀ higher compared with neutralisation of ROD A. The titres for ROD B neutralisation are high, up to 32000 for 50% neutralisation and 90% reduction seen with up to 1/8000 dilution of some sera. In contrast, 90% neutralisation of ROD A was not observed for the majority (10 out of 13) of sera at the highest concentration tested (1/10 dilution). ROD B neutralisation titres are also much higher than is observed in the same assay for neutralisation a TCLA HIV-1 strain, LAI. These results therefore show that CD4-independent ROD B is highly sensitive to neutralisation and that the majority of HIV-2 infected individuals have high titres of Abs able to neutralise CD4 independent infection.

3.3.2 sCD4 induction of ROD A renders it sensitive to neutralisation

The prototype HIV-2 isolate ROD A is dependent on CD4 for infection. However, infection of CD4 negative cells can be induced by incubation with sCD4. This is in contrast to TCLA HIV-1 where incubation with sCD4 is neutralising (see section 1.4.2.1). The gp120-gp41 interaction in HIV-2 envelope may be more stable than HIV-1 in the presence of sCD4 (Sattentau et al., 1993). Yet the interaction still confers the necessary conformational changes to expose or create the coreceptor binding site in ROD A envelope to allow infection of CD4 negative cells. Induction of ROD A to infect CD4 negative cells by incubation with sCD4 dramatically increased
the neutralisation titre of HIV-2 positive sera to values similar to ROD B. This increase in neutralisation sensitivity concomitant with ability to bind and enter chemokine receptor expressing cells suggests that the regions exposed to allow interaction with the chemokine receptor are neutralisation sensitive. This is in agreement with studies using the HIV-1 MAb 17b which overlaps with the coreceptor binding site and preferentially neutralises HIV-1 in the presence of sCD4 (Thali et al., 1993). However this MAb did not neutralise ROD A or ROD B.

3.3.3 Differences between ROD A and ROD B that may confer differential sensitivity to neutralisation

There are 9 amino acid changes in CD4-independent ROD B compared with ROD A. Most are in SU (V1 T98I, V2 E138K and E171K, V3 Q310K, V4 L378F, K403R and R414I) and one close to the coiled coil domain in TM (A526T) (Reeves and Schulz, 1997b). A panel of human MAbs derived from HIV-2 infected individuals previously investigated for SIV neutralisation (Table 3.2) were tested for neutralisation of ROD A and ROD B. Six of the MAbs (26C, 110C, 17A, 15D, 34G and B23) preferentially neutralised ROD B. Three (110C, 17A and B23), which have been reported to block SIV envelope binding to CCR5 and are within the same competition group yet are thought to have separate specificities (Cole et al., 2001; Martin et al., 1997; Robinson et al., 1998). Blocking of CCR5 binding suggests that they overlap with the coreceptor binding site, similar to the anti-HIV-1 MAb 17b (Kwong et al., 1998; Thali et al., 1993). In this chapter I have shown that they also preferentially neutralise ROD B, suggesting that the chemokine receptor binding site on HIV-2 may be analogous to SIV and that it may be involved in the sensitivity of CD4 independent infection to neutralisation. MAbs 26C, 15D and 34G were more potent neutralisers than 110C, 17A and B23 for ROD B but there is no mapping data available on these MAbs mainly due to the lack or poor cross reactivity with SIV. This implies that they may target HIV-2 specific neutralisation sensitive epitopes not present on the SIV strains tested previously (Cole et al., 2001; Martin et al., 1997; Robinson et al., 1998). MAb 26C does bind to SIV envelope but was non-neutralising and it has been suggested to recognise an epitope in the N terminal portion of the envelope (Cole et al., 2001). The N terminus of HIV envelope is not usually reported as a neutralisation epitope but has been previously described for MAbs to SIV with some degree of neutralisation (Babas et al., 1997). Overall, due to limited mapping data on several of
the neutralising MAbs it is unclear whether a specific epitope confers ROD B with sensitivity to neutralisation.

Neutralisation sensitive CD4-independent isolates have recently also been described for HIV-1 (Hoffman et al., 1999; Kolchinsky et al., 2001b; Zhang et al., 2002) and SIV (Means et al., 2001; Puffer et al., 2002). Hoffman et al have described neutralisation of a CD4-independent derivative of the prototypic HIV-1 lab adapted isolate IIIB, IIIBx (Hoffman et al., 1999; Hoxie et al., 1998) with up to 100-fold increase in sensitivity to human sera neutralisation. Subsequently both CD4-independence and neutralisation sensitivity were mapped to a frameshift mutation resulting in a truncated cytoplasmic tail combined with mutations in either V4/C4 (N386K, I423V) or V3 (R298K, I320V) (Edwards et al., 2001; LaBranche et al., 1999). Truncation of the cytoplasmic tail of HIV-1 has often been associated with loss of infectivity (Chen et al., 1996a; Dubay et al., 1992; Shimizu et al., 1992) yet when the IIIBx frameshift was introduced into a range of HIV-1 envelopes, (HXB2, NL4.3, JRFL, ADA, 89.6) all were still able to function in a cell-cell fusion assay and were more sensitive to neutralisation by both MAbs and human sera (Edwards et al., 2002). In contrast to HIV-1, a shortened cytoplasmic tail is relatively common for cell line passaged isolates of HIV-2 and SIV (Hoxie et al., 1991; Kodama et al., 1989; Mulligan et al., 1992). However, both ROD A and ROD B have the same cytoplasmic tail length with a stop codon at amino acid 731 (Reeves and Schulz, 1997b). Cytoplasmic tail length therefore does not account for the difference in neutralisation sensitivity seen between ROD A and ROD B.

Kolchinsky et al. describe a variant of the HIV-1 R5 isolate ADA, which is CD4 independent and sensitive to neutralisation (Kolchinsky et al., 2001b; Kolchinsky et al., 1999). Changes conferring both CD4 independence and neutralisation sensitivity have been mapped to position 539/540 in gp41, C-terminal to the fusion peptide, and also changes in the C-terminal region of V2 resulting in loss of glycosylation. (Kolchinsky et al., 2001a; Kolchinsky et al., 2001b; Kolchinsky et al., 1999). Loss of glycosylation has previously been reported to increase neutralisation sensitivity and immunogenicity in CD4-dependent viruses (Benjouad et al., 1992; Ly et al., 2000; Reitter et al., 1998; Willey et al., 1996). Furthermore, the change in V4/C4 that enhance the CD4-independent phenotype of IIIBx also results in loss of a glycosylation site (Edwards et al., 2001). Another CD4-independent HIV-1 reported by Dumonceaux et al also requires loss of a glycosylation site along with an amino
acid change in the V3 loop to confer CD4 independence (Dumonceaux et al., 1999; Dumonceaux et al., 2001; Dumonceaux et al., 1998). However, ROD B is not predicted to have any changes in glycosylation compared with ROD A (Reeves and Schulz, 1997b).

SIV isolates, like HIV-2 are often able to infect cells independently of CD4 (Allan et al., 1990; Edinger et al., 1997b; Liu et al., 2000). Puffer et al. have recently shown that CD4-independent SIV envelopes (mac316, 1A11, 17E-Fr and 1100) are more sensitive to neutralisation by sera from SIVmac239 infected macaques and a range of MAbs (Puffer et al., 2002). Like ROD A, SIV can be induced to infect CD4 negative cells by sCD4 and this also induces neutralisation sensitivity (Allan et al., 1990; Puffer et al., 2002). As for some strains of HIV-1, CD4 independence of SIV could be achieved by elimination of glycosylation sites on gp120 (Puffer et al., 2002).

Thus previously reported CD4-independent, neutralisation sensitive viruses (HIV-1 and SIV) all have mutations resulting in either loss of glycosylation or truncation of the cytoplasmic tail. ROD B has neither a shorter cytoplasmic tail nor any predicted changes in glycosylation compared with ROD A that could account for enhanced sensitivity to neutralisation. Further investigation into epitopes that may account for ROD B neutralisation sensitivity will be presented in chapter 4.

### 3.3.4 Infection of CD4 negative cells by primary isolates of HIV-2 is highly sensitive to neutralisation

The dichotomy in neutralisation sensitivity between lab adapted and primary isolates of HIV-1 is clear and emphasises the need to study clinically relevant isolates (Ashkenazi et al., 1991; Daar et al., 1990; Mascola et al., 1994; Moore et al., 1993a; Moore et al., 1995a; Moore and Ho, 1995b; Moore et al., 1992; O'Brien et al., 1994; Sawyer et al., 1994; Wrin et al., 1995). CD4-independent viruses have been isolated directly from HIV-2 infected individuals but are rare in HIV-1 infection (Reeves et al., 1999). However, TCLA CD4-independent HIV-1 envelopes have been generated and are more susceptible to neutralisation by HIV-1 antisera as discussed above (Edwards et al., 2001; Kolchinsky et al., 2001b; LaBranche et al., 1999). Recently a CD4-independent envelope from a primary HIV-1 strain has been described that is more susceptible to neutralisation than other primary isolates but still required more MAb to achieve neutralisation equivalent to TCLA for all except one MAb (Zhang et al., 2002). The primary HIV-2 isolates SAB, ALI and JAU can infect cells in the absence
of CD4 and infection of CD4 negative cells can be enhanced by incubation with sCD4. When infecting CD4 positive cells SAB and ALI were resistant to neutralisation by human sera with modest (20-50%) neutralisation seen at 1/10 dilution of sera. JAU was more sensitive to neutralisation. When induced to infect CD4 negative cells however, all three viruses were highly susceptible to neutralisation with 90% titres of over 1/800. This is 80 fold greater than neutralisation of TCLA ROD A. Induction to infect CD4 negative cells by incubation with sCD4 presumably leads to conformational changes exposing, or stabilising the chemokine receptor binding site and also neutralisation targets. Thus, primary isolates of HIV-2 able to infect CD4 negative cells are not intrinsically neutralisation resistant but the route of entry plays a role in sensitivity to neutralisation. Entry via cell surface CD4 confers some degree of protection, presumably because interaction with cell surface CD4 does not lead to the exposure of epitopes on envelope that are induced by sCD4 (Salzwedel et al., 2000). Similar results have been described for primary SIV isolates able to infect CD4 negative cells, where use of cellular CD4 can protect from neutralisation (Puffer et al., 2002).

Why do CD4-independent envelopes frequently occur in HIV-2 infection whilst they are rare in HIV-1? It possible that immune pressure in the form of neutralising Ab preferentially neutralises CD4-independent HIV isolates and selects for a CD4-dependent phenotype in vivo. The increase in cellular targets afforded by the ability to infect cells that lack CD4 (Edinger et al., 1997b; Reeves et al., 1999) may only confer a replicative advantage in immune privileged sites with lower Ab levels, such as the central nervous system. However, in HIV-2 infected individuals there is evidence of infection only of CD4 positive macrophages and microglia in the brain (Morner et al., 2002). Thus while CD4-independent isolates can be isolated from HIV-2 infected individuals, evidence for infection of CD4 negative cells in vivo is lacking. The reason for maintenance of CD4 independence in primary HIV-2 isolates therefore remains unclear.
3.4 Summary

In this chapter I have shown that

- sera from HIV-2 infected individuals preferentially neutralise CD4-independent ROD B compared with CD4-dependent ROD A. Furthermore, incubation with sCD4 allows ROD A to infect CD4 negative cells and also renders ROD A highly sensitive to neutralisation.

- primary isolates of HIV-2 able to infect CD4 negative cells are sensitive to neutralisation by sera from HIV-2 positive individuals when using a CD4-independent route of infection.

These findings may help to explain the lack of infection of CD4 negative cells by HIV-2 in vivo. Investigation into epitopes that account for heightened neutralisation sensitivity of ROD B is presented in Chapter 4 while Ab binding as a contributor to differential sensitivity to Ab mediated neutralisation is presented in Chapter 5.
Chapter 4
Production, characterisation and neutralisation properties of rat MAbs that recognise different regions of the HIV-2 ROD envelope

4.1 Introduction

Entry of HIV into target host cells is mediated by the envelope glycoprotein gp120 interacting with cell surface receptors, CD4 and a chemokine coreceptor (section 1.4). After gp120 binding to CD4 the viral envelope undergoes conformational changes that enable it to interact with a coreceptor, usually either CCR5 or CXCR4 (Deng et al., 1996; Feng et al., 1996; Hill et al., 1997; Lapham et al., 1996; Trkola et al., 1996a; Wu et al., 1996). Coreceptor binding leads to further conformational changes in gp41 and fusion of viral and cell membranes (section 1.4). The binding sites for these cellular receptors are likely to be relatively conserved across HIV.

The complex topography of the HIV envelope allows receptor interactions and conformational rearrangements. Initial comparison of envelope sequences from different HIV-1 isolates yielded the identification of 5 variable (V1-5) and 5 conserved (C1-C5) regions (Modrow et al., 1987; Starcich et al., 1986). Further secondary structure was suggested by computer modelling (Gallaher et al., 1995). Additional characterisation by biochemical, mutagenesis and immunochemical methods generated a model of epitope exposure on the native envelope oligomer (Binley et al., 1996; Ditzel et al., 1997; Earl et al., 1997; Helseth et al., 1991; Leonard et al., 1990; Moore and Sodroski, 1996b; Olshevsky et al., 1990). Subsequent elucidation of the structure of gp120 co-crystallised with 2 domains of CD4 and 17b, a human MAb that overlaps with the coreceptor binding site confirmed and extended the previous findings (Kwong et al., 1998; Wyatt et al., 1998a) (see section 1.4). It became apparent that gp120 essentially has 2 domains, the inner and outer domains (see Figure 1.5). Also apparent was the bridging sheet thought to be a conserved region between the inner and outer domain that interacts with the coreceptor (Rizzuto et al., 2000; Rizzuto et al., 1998).

Despite the divergence of HIV-2 from HIV-1, HIV-2 also utilises CD4 as a primary receptor (Ottenen et al., 1993; Sattentau et al., 1988a; Sattentau et al., 1988b)
and can subsequently use a broad array of chemokine receptors to facilitate entry into target cells (Bron et al., 1997; Edinger et al., 1999; Hill et al., 1997; McKnight et al., 1998; Morner et al. 1999; Sol et al., 1997). However, there are several differences between HIV-1 and HIV-2 envelope not least the fact that many HIV-2 isolates are more promiscuous in coreceptor usage but are able to infect CD4 negative cells (Clapham et al., 1992; Endres et al., 1996; Hoxie et al., 1998; Liu et al., 2000; Reeves et al., 1999). Crystal structures for the envelope of HIV-2 and SIV have not been reported. The envelope of CD4-independent HIV-2 strains presumably represents a structure closer to that of HIV-1 envelopes subsequent to CD4 binding, with the bridging sheet exposed to allow direct contact with the coreceptor. A CD4-independent HIV-1 derived from IIIB (IIIBx) is thought to constitutively expose the coreceptor binding site as evidenced by direct binding to the MAb 17b (Hoffman et al., 1999). However the overall antigenic exposure of HIV-2 (and related SIV) envelope may be significantly different from HIV-1 (Kent and Bjorling, 1996; McKnight 1998).

In chapter 3, the CD4-independent HIV-2 strain ROD B was shown to be more susceptible to antibody-mediated neutralisation than CD4-dependent HIV-2, ROD A. For HIV-1 neutralisation sensitivity of certain isolates can be characterised by sensitivity to particular epitopes. For example, HIV-1 TCLA are sensitive to V3 MAbs (Javaherian et al., 1989) whilst the HIV-1 CD4-independent isolate IIIBx is more sensitive to MAbs overlapping with the coreceptor binding domain (Hoffman et al., 1999).

The aims of this chapter are

- to produce and characterise a panel of monoclonal antibodies to the HIV-2 envelope.
- to characterise neutralisation sensitivity of CD4-dependent and CD4-independent HIV-2 to monoclonal antibodies that recognise different epitopes on HIV-2 envelope.
4.2 Results

4.2.1 Production of rat MAbs

Antibodies were raised in rats to recombinant HIV-2 envelope glycoprotein, gp105 derived from the prototype HIV-2 strain ROD A, complexed to sCD4 (section 2.6.1). MAbs generated were primarily screened using ELISA to the immunogen (by C. Shotton). 15 MAbs were positive and designated for further study (Table 4.1).

4.2.2 MAb specificity

The immunogen was a complex of gp105 and sCD4 and thus potentially 3 populations of MAbs may be formed; (1) gp105 specific, (2) CD4 specific and (3) those which bind to the gp105/sCD4 complex. Each of these outcomes was tested.

<table>
<thead>
<tr>
<th>MAb</th>
<th>Isotype</th>
<th>gp105/sCD4 binding</th>
<th>Immunostaining</th>
<th>Flow cytometry</th>
<th>CD4+ cell binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a/5j</td>
<td>IgG1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>84b/3g</td>
<td>IgG2a</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>46/1a</td>
<td>IgG2a</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>34b/3d</td>
<td>IgG1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>71/1h</td>
<td>IgG1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>34a/4e</td>
<td>IgG1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>40a/8g</td>
<td>IgG1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>31b/3g</td>
<td>IgG2a</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>62/3k</td>
<td>nd</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>67a/2k</td>
<td>IgG2a</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>770w/2g</td>
<td>IgG1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>90ow/8k</td>
<td>IgG2a</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>70b/4g</td>
<td>IgG2b</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>64/4a</td>
<td>nd</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3b/1a</td>
<td>nd</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 4.1. Characteristics of rat MAbs.

*a Isotype of MAbs was determined by radioimmunoassay (section 2.6.2)
*b Binding to the immunogen gp105/sCD4 was tested by ELISA by C. Shotton
*c HeLa/CD4 cells infected for 3 days with ROD were fixed with methanol:acetone and then stained with the rat MAbs and detected with a β-glucosidase conjugate (section 2.6.3.1)
*d Binding to chronically infected Molt4 or SupT1 cells was assayed by flow cytometry (section 2.6.3.1)
*e Binding to CD4 positive uninfected cells was detected by flow cytometry (section 2.6.3.2)
*nd, not determined
4.2.2.1 Envelope specific MAbs

MAbs were first tested for binding to HIV-2 envelope. This was assayed by the ability of MAb to bind to ROD infected HeLa/CD4 cells by β-galactosidase immunostaining after methanol:acetone fixation. Additionally, MAb binding to unfixed chronically infected Molt4 and SupT1 cells was tested by flow cytometry. As shown in Table 4.1, 12 of the 15 MAbs (34b/3d, 71/lh, 34a/4e, 40a/8g, 31b/3g, 62/3k, 67a/2k, 77ow/2g, 90ow/8k, 70b/4g, 64/4a, 3b/la) bound to infected cells by both methods with no discordant results.

4.2.2.2 CD4 specific MAbs

MAb specificity for CD4 was also tested. Binding of MAbs to cell lines (RD, Mv-1-lu) stably transfected with human CD4 was compared to CD4 negative parental cells by flow cytometry. MAbs 84b/3g and 46/la bound to cells expressing CD4 but not to the parental control, all other MAbs were negative (Table 4.1). MAbs were also tested for binding sCD4 by ELISA. As shown in Figure 4.1 MAbs 1a/5j and 46/la bound to sCD4. Absorbance readings for the other MAbs were not above background, including 84b/3g, which recognises cell surface CD4. The half maximal binding for 1a/5j was calculated as 0.033μg/ml (using FORCAST function in Microsoft Excel). MAb 46/1a bound sCD4 but did not reach saturation at concentrations of up to 100μg/ml (Figure 4.1) thus, the half maximal binding could not be calculated. MAb 1a/5j did not bind to CD4 expressed on Mv-1-lu/CD4 or RD/CD4 cells yet it was positive for binding to sCD4 in ELISA. This implies that MAb 1a/5j recognises a region of CD4 that is exposed on sCD4 but may be obscured by membrane on cellular CD4. In contrast, MAb 84b/3g did not bind to sCD4 by ELISA yet was positive for binding to cell surface CD4.
Figure 4.1. Binding of MAbs to soluble CD4.

Binding of MAbs 1a/5j and 46/1a to sCD4 was determined by ELISA to MAb captured sCD4 (as described in section 2.6.3.2). O.D.(490nm); absorbance at 490nm. The mean +/- standard error of the mean is shown. For MAb 1a/5j the half maximal binding point is shown.

In summary, 15 MAbs were produced, 12 specific for HIV-2 envelope and 3 specific for CD4. No MAbs were identified that were dependent on both components of the immunogen, sCD4 and gp105 for binding.

4.2.3 Mapping of envelope specific MAbs

Antibodies can be mapped to specific regions and epitopes of an antigen by a variety of methods including binding to site directed mutants, competition with other MAbs of known specificity and binding to smaller components of the whole antigen. I employed 2 complementary methods to map the envelope specific rat MAbs: chimeric constructs and 12mer peptides covering the entire gp105.

4.2.3.1 Chimeric constructs

Envelope specific MAbs were mapped to specific regions of gp105 by examining their binding profiles to a panel of chimeras constructed from HIV-1 and HIV-2 envelopes (section 2.6.3.3 and Figure 2.2). Chimeric constructs were expressed in SF9 insect cells and MAb binding was determined by β-gal immunostaining. MAbs previously shown to bind to HIV-1 V3 loop (8/38c McKeating et al., 1992) or HIV-2 V3 loop (32/2f, McKnight et al., 1996) were used as positive controls. Assignment of MAbs to specific regions of the envelope was determined by examining the set of chimeras that were positive when immunostained with each MAb. For example, as shown in Figure 4.2 MAbs were assigned to V1/V2 if they bound to constructs specifically containing V1/V2 from HIV-2 and not constructs containing V1/V2 from
HIV-1. Similarly, MAbs were mapped to the V4/CD4 binding domain if they bound to cells expressing constructs containing V4/CD4 binding domain region from HIV-2 but not HIV-1.

Three MAbs, 84b/3, 1a/5j and 46/1a shown above to bind CD4, did not bind any of the HIV-1/HIV-2 envelope constructs. All other MAbs tested bound to SF9 cells expressing full length HIV-2 envelope and not to those expressing full length HIV-1 envelope apart from the positive control, 8/38c, which is specific for the HIV-1 V3 loop. Table 4.2 shows the region of HIV-2 envelope each MAb was mapped to by this method. Six MAbs (34b/3d, 71/1h, 34a/4e, 40a/8g, 31b/3g, 62/3k) mapped to V1/V2 of HIV-2 envelope. Two mapped to V4/CD4 binding domain (67a/2k and 77ow/2g). The remaining three MAbs (90ow/8k, 70b/4g and 3b/1a) could not be assigned envelope regions based on chimera binding profiles. These MAbs may therefore require conformational structure not configured by these constructs.

![Diagram of MAb Binding](image)

**Figure 4.2. Construct mapping of rat MAbs.**

Chimeric constructs of HIV-1 and HIV-2 envelope (full panel of constructs in Figure 2.1) in recombinant baculovirus were used to infect SF9 cells. Cells were then immunostained with rat MAbs. Binding domains were determined by the profile of constructs each MAb bound to

### 4.2.3.2 Peptide mapping

To further elucidate specific MAb epitopes, peptide mapping was carried out. A set of 12-mer peptides, each overlapping the previous one by six amino acids, was made covering the entire HIV-2 ROD envelope gp105 using a SPOTs kit (section 2.6.3.4). HIV-2 V3 MAb 32/2f was used as a control. Only MAbs 34b/3d, 71/1h, 34a/4e and 40a/8g, which had previously been localised to the V1/V2 region of the envelope (section 4.2.3.1) bound either one or two adjacent 12-mer peptides and thus recognised linear epitopes. Specific linear epitopes were identified for these four
MAbs, as shown in Table 4.2. The other MAbs did not bind to any of the 12mer peptides indicating that they may bind to linear peptides of longer than 12 amino acids, discontinuous epitopes, or that some local secondary structure or conformation is required for recognition of their specific epitope.

<table>
<thead>
<tr>
<th>MAb</th>
<th>Envelope domain</th>
<th>Epitope</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a/5j</td>
<td>-</td>
<td>nt</td>
</tr>
<tr>
<td>84b/3g</td>
<td>-</td>
<td>nt</td>
</tr>
<tr>
<td>46/1a</td>
<td>-</td>
<td>nt</td>
</tr>
<tr>
<td>8/38c</td>
<td>HIV-1 V3</td>
<td>nt</td>
</tr>
<tr>
<td>32/2f</td>
<td>V3</td>
<td>LMSGHVFHSYQ</td>
</tr>
<tr>
<td>34b/3d</td>
<td>V1/V2</td>
<td>TPCARADNC</td>
</tr>
<tr>
<td>71/1h</td>
<td>V1/V2</td>
<td>DTPCARADNCSG</td>
</tr>
<tr>
<td>34a/4e</td>
<td>V1/V2</td>
<td>DTPCARADNCSG</td>
</tr>
<tr>
<td>40a/8g</td>
<td>V1/V2</td>
<td>ARADNCSGLGEE</td>
</tr>
<tr>
<td>31b/3g</td>
<td>V1/V2</td>
<td>Conformational</td>
</tr>
<tr>
<td>62/3k</td>
<td>V1/V2</td>
<td>Conformational</td>
</tr>
<tr>
<td>67a/2k</td>
<td>V4/CD4</td>
<td>Conformational</td>
</tr>
<tr>
<td>77ow/2g</td>
<td>V4/CD4</td>
<td>Conformational</td>
</tr>
<tr>
<td>90ow/8k</td>
<td>Inconclusive</td>
<td>Conformational</td>
</tr>
<tr>
<td>70b/4g</td>
<td>Inconclusive</td>
<td>Conformational</td>
</tr>
<tr>
<td>64/4a</td>
<td>nt</td>
<td>Conformational</td>
</tr>
<tr>
<td>3b/1a</td>
<td>Inconclusive</td>
<td>Conformational</td>
</tr>
</tbody>
</table>

Table 4.2. Epitope mapping of rat MAbs.
Rat MAbs were mapped to specific envelope regions and linear epitopes by binding to a chimeric construct and b 12mer peptides. nt, not tested; - no binding; Inconclusive, no specific region could be assigned. Conformational, no binding was observed to any of the peptides.

4.2.3.3 CD4 blocking

Two MAbs, 67a/2k and 77ow/2g, mapped to the V4/CD4 binding domain region of HIV-2 envelope (Table 4.2). To determine if these MAbs overlap with the CD4 binding site it was tested whether they could block sCD4 binding to cell surface expressed ROD envelope. As shown in Figure 4.3, sCD4 binding to ROD envelope was not reduced by 67a/2k or 77ow/2g. Similarly, binding of MAbs was not diminished by simultaneous binding of sCD4 to envelope expressing cells. Therefore, although MAbs 67a/2k and 77ow/2g mapped to the V4/CD4 binding domain region of HIV-2 envelope they do not block CD4 binding to envelope.
Figure 4.3. Flow cytometry of rat MAb and sCD4 binding to ROD envelope

Binding of sCD4 (top panel, red histograms) to ROD envelope expressed on chronically infected SupT1 cells alone (No MAb) and in the presence of rat MAbs, 67a/2k and 77ow/2g, was assayed by flow cytometry (section 2.6.3.1). Binding of rat MAbs, 67a/2k and 77ow/2g to ROD envelope expressed on chronically infected SupT1 cell in the absence (middle panel, red histograms) and presence (bottom panel, red histograms) of sCD4 was detected by the addition of anti-rat conjugated to FITC and flow cytometry. Numbers in parenthesis indicated mean fluorescent intensity of 10000 events collected. Isotype controls are shown by black histograms.
4.2.4 Neutralisation of ROD A and ROD B by rat MAbs

In chapter 3 a panel of human sera from HIV-2 infected patients preferentially neutralised CD4-independent ROD B compared to CD4-dependent ROD A. To investigate whether the heightened neutralisation sensitivity of ROD B was epitope specific, neutralisation of ROD A and ROD B by the panel of rat MAbs described above (Table 4.1 and Table 4.2) and additional rat MAbs to V3 (32/2f and 32/7g) and 8e, conformational MAb (McKnight et al., 1996) were assayed. HeLa/CD4 cells were used as the target cell. 50% and 90% neutralisation titres were calculated as the concentration of MAb required to reduce 50% or 90% of infection compared to infection in the presence of the control MAb (8/38c McKeating et al., 1992). As shown in Table 4.3, all MAbs preferentially neutralised ROD B infection with 50% and 90% neutralisation achieved with lower concentration of MAb than for neutralisation of ROD A. For most of the MAbs even at the highest concentration tested, 10 or 20μg/ml, 50% neutralisation of ROD A was not observed (indicated by > in Table 4.3). However, at the highest concentration all but 2 MAbs (70b/4g and 3b/1a) could reduce ROD B infection by at least 50%. Three MAbs, 77ow/2g, 64/4a and 8e, results in highly increased neutralisation sensitivity of ROD B with concentrations as low as 0.02μg/ml, 5μg/ml and 0.3μg/ml respectively giving 90% reduction in infection.

In chapter 3 serum samples from HIV-2 positive individuals preferentially neutralised ROD B with a fold increase in titre of up to 3 log_{10}. MAbs 77ow/2g, 8e and 64/4a recapitulate this magnitude of difference with fold differences between ROD A and ROD B of 3.3, 1.7 and 0.9 log_{10} respectively for 50% neutralisation titres.
<table>
<thead>
<tr>
<th>MAb</th>
<th>Neutralisation Titre</th>
<th>50%</th>
<th>90%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ROD A</td>
<td>ROD B</td>
<td>ROD A</td>
</tr>
<tr>
<td>V1/V2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>34b/3d</td>
<td>&gt;10</td>
<td>1.25</td>
<td>&gt;10</td>
</tr>
<tr>
<td>71/1h</td>
<td>&gt;10</td>
<td>10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>34a/4e</td>
<td>&gt;20</td>
<td>5</td>
<td>&gt;20</td>
</tr>
<tr>
<td>40a/8g</td>
<td>&gt;20</td>
<td>20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>31b/3g</td>
<td>&gt;10</td>
<td>10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>62/3k</td>
<td>&gt;10</td>
<td>10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>V3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32/2f</td>
<td>&gt;10</td>
<td>5</td>
<td>&gt;10</td>
</tr>
<tr>
<td>32/7g</td>
<td>10</td>
<td>2.5</td>
<td>&gt;10</td>
</tr>
<tr>
<td>V4/CD4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>67a/2k</td>
<td>&gt;10</td>
<td>10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>77ow/2g</td>
<td>20</td>
<td>0.01</td>
<td>&gt;20</td>
</tr>
<tr>
<td>Conformational</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>90ow/8k</td>
<td>&gt;10</td>
<td>10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>70b/4g</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>64/4a</td>
<td>&gt;20</td>
<td>2.5</td>
<td>&gt;20</td>
</tr>
<tr>
<td>3b/1a</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>8e</td>
<td>&gt;10</td>
<td>0.2</td>
<td>&gt;10</td>
</tr>
</tbody>
</table>

Table 4.3. Neutralisation titres of rat MAb.

Titres are represented as the amount of each MAb (µg/ml) required to reduce 50% or 90% infection of ROD A or ROD B on HeLa/CD4 compared to control.

### 4.2.5 Neutralisation of ROD A induced to infect CD4 negative cells

ROD A is CD4 dependent but can be induced to infect CD4 negative cells by incubation with sCD4 (Clapham et al., 1992). In chapter 3 induction of ROD A by sCD4 to infect CD4 negative cells was found to be sensitive to neutralisation by HIV-2 positive sera (section 3.2.3). It was therefore tested whether the rat MAb were also able to preferentially neutralise sCD4 induced ROD A compared with ROD A infection of CD4 positive cells and whether this was demonstrated by MAb recognising specific epitopes. Rat MAb covering epitopes throughout HIV-2 envelope (34a/4e (V1/V2), 40/8g (V1/V2), 32/2f (V3), 32/7g (V3), 77ow/2g (V4/CD4), 64/4a (conf.) and 8e (conf.)) were assayed. CCC cells stably expressing CXCR4 either with or without CD4 were used as target cells for this assay to obtain
sufficient titres for sCD4 induced infection by ROD A. As shown in Figure 4.4 sCD4 induction of ROD A rendered ROD A as sensitive as ROD B to neutralisation by all the MAbs tested. Similar to ROD B, sCD4 induced ROD A was sensitive to neutralisation by MAbs mapping to epitopes across the entire envelope. However, MAbs 77ow/2g, 8e and to a lesser extent 64/4a that recognise V4/CD4bd and conformational epitopes emphasised the phenotype.

![Graphs showing neutralisation of ROD A infection](image)

**Figure 4.4.** sCD4 induces neutralisation of ROD A.

Neutralisation of ROD A infection of CCC/CD4/X4 cells (■) compared with neutralisation of infection of CCC/X4 cells by ROD B (●) and sCD4 induced ROD A (■). Mean residual infection as % of control (rat MAb to HIV-1) is shown.

### 4.2.6 Competition between rat and human Abs

In chapter 3 serum samples from HIV-2 infected individuals and MAbs derived from HIV-2 infected individuals preferentially neutralised CD4-independent ROD B compared with CD4-dependent ROD A. Mapping data for the human MAbs suggests that 110C, 17A and B23 may overlap with the coreceptor binding site on SIV envelope and 26C is reactive with the N terminus (see section 3.2.4 and Table 3.2) (Cole *et al.*, 2012).
2001; Martin et al., 1997; Robinson et al., 1998). To test whether the 3 most potent rat MAbs, 77ow/2g, 64/4a and 8e, compete with human MAbs 26C, 110c, 17A, B23 and HIV-2 positive human serum an ELISA for binding to HIV-2 gp105 was carried out. Microtitre plates coated with HIV-2 ROD gp105 were incubated with human MAbs or sera prior to washing and subsequent incubation with the rat MAbs. As shown in Figure 4.5A, human MAbs 26C, 110C, 17A, and B23 and HIV-2 positive serum bound to gp105 above the negative control, MAb 7B2 or normal human serum. Binding of rat MAbs to gp105 in the presence of human Ab was calculated as a percentage of control incubation with 7B2, a human anti-HIV-1 MAb or HIV-1 positive serum.

As shown in figure 4.5B and Table 4.4, gp105 binding by rat MAb 77ow/2g binding was reduced up to 30% by MAbs and HIV-2 serum. Greatest competition was with human MAb 26C that maps to the N-terminus yet this MAb previously mapped to V4/CD4 binding domain (Table 4.2). MAb 64/4a binding was reduced up to 64% by human MAbs and serum. Again competition was greatest with human Mab 26C. Therefore MAb 64/4a competes with the human MAbs and HIV-2 serum. The specific epitope for this MAb remains unclear as competition was observed with human MAbs in 2 different competition groups, coreceptor binding site (110C, 17A, B23) and N-terminus (26C). In contrast MAb 8e binding was not reduced by any of the human MAbs and only slightly by HIV-2 positive serum suggesting that Abs of this specificity may not be generated in HIV-2 infected individuals.

<table>
<thead>
<tr>
<th></th>
<th>7B2</th>
<th>N-term</th>
<th>Coreceptor binding domain</th>
<th>HIV-1 serum</th>
<th>HIV-2 serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>26C</td>
<td>110C</td>
<td>17A</td>
<td>B23</td>
</tr>
<tr>
<td>77ow/2g</td>
<td>100</td>
<td>70</td>
<td>87</td>
<td>82</td>
<td>81</td>
</tr>
<tr>
<td>64/4a</td>
<td>100</td>
<td>36</td>
<td>46</td>
<td>40</td>
<td>53</td>
</tr>
<tr>
<td>8e</td>
<td>100</td>
<td>107</td>
<td>106</td>
<td>102</td>
<td>102</td>
</tr>
</tbody>
</table>

Table 4.4. Competition between rat and human Abs.
Percentage binding of rat MAbs 77ow/2g, 64/4a and 8e to gp105 in the presence of human MAbs 26C, 110C, 17A, and B23 or HIV-2 positive serum compared to control binding in the presence of 7B2 or HIV-1 positive serum.
Figure 4.5. Competition analysis of human and rat MAbs.
A. Binding of human MAbs and HIV-2 positive serum to HIV-2 gp105 compared to control, MAb 7B2 or HIV-1 positive serum. Binding was measured at absorbance of 492nm.
B. Binding of rat MAbs, 77ow/2g, 64/4a and 8e to HIV-2 gp105 after binding of human MAbs or serum (X axis). Binding is shown as percentage of control incubation with 7B2 or HIV-1 positive serum.
4.2.7 Neutralisation sensitivity depending on target cell

CCC cells were used for neutralisation assays for CD4 independent infection by ROD B and sCD4 induced infection by ROD A, so CD4 positive CCC cells were used as a target cell for comparison. Surprisingly, in contrast to neutralisation sensitive ROD B infection of HeLa/CD4 and CCC/CXCR4 cells, infection of CD4 positive CCC cells by ROD B was highly resistant to MAb mediated neutralisation (Figure 4.6). 50% neutralisation of ROD A and ROD B infection of CD4 positive CCC cells was not achieved at the highest concentration of MAbs (20μg/ml). In contrast, 90% neutralisation of ROD A+sCD4 and ROD B infection of CD4 negative CCC cells can be achieved at concentrations as low as 0.15μg/ml (for 77ow/2g).

A further comparison of ROD A and ROD B neutralisation on different target cells was then carried out. Figure 4.7 shows neutralisation of ROD A and ROD B infection by rat MAbs, 77ow/2g, 64/4a and 8e, and HIV-2 positive serum on HeLa/CD4, RD/CD4, CCC/CD4/X4 and CCC/X4. ROD A infection of all CD4 positive cell lines tested was relatively resistant to neutralisation. However, as expected, ROD A was sensitive to neutralisation by all three MAbs and HIV-2 positive serum when induced to infect CD4 negative CCC cells by sCD4. ROD B was sensitive to neutralisation on CD4 positive HeLa and RD cells and CD4 negative CCC cells yet in contrast was resistant to neutralisation when CD4 positive CCC cells were used as targets. ROD B infection of RD/CD4 cells was also more sensitive to neutralisation than ROD A but not as sensitive as on HeLa/CD4 and CCC/X4 cells.

Therefore, there is cell type dependence to neutralisation sensitivity. CD4 expression on CCC cells prevents neutralisation of ROD A and ROD B while CD4 expression on HeLa cells only prevents neutralisation of ROD A but not ROD B.
Figure 4.6. Neutralisation of ROD A and ROD B infection of CD4 positive and negative CCC cells.
Neutralisation of ROD A (diamonds) and ROD B (squares) by rat MAbs, 34a/4e, 40a/8g, 77ow/2g, 64/4a and 8e. Percentage residual infection compared with control MAb on CD4 positive (black) and CD4 negative CCC cells is shown (sCD4 induced ROD A, ROD B).
Figure 4.7. Neutralisation sensitivity of ROD A and ROD B on different cell types.

Infectivity of ROD A (white bars) and ROD B (hashed bars) was tested on HeLa/CD4, RD/CD4, CCC/CD/X4 and CCC/X4 after incubation with 5µg/ml rat MAbs, 77ow/2g, 64/4a and 8e, and HIV-2 serum (1/1000 dilution). % residual infection compared to control incubation with anti-HIV-1 rat M Ab or normal human serum is shown.

Receptor expression levels may influence the infectivity and subsequently neutralisation efficiency (Wu et al., 1997). The expression level of CD4 and CXCR4 on the 4 cell types was therefore tested by flow cytometry. As shown in Figure 4.8 the expression level of CD4 and CXCR4 was similar on all cell types (apart from the lack of CD4 on CCC/X4 cells).
Figure 4.8. Flow cytometry of cell surface receptors.

Expression levels of CD4 (left panels, red histogram) and CXCR4 (right panel, red histogram) on cell lines was assayed by flow cytometry. Isotype controls are shown in black histograms and numbers in parenthesis show mean fluorescent intensity of 10000 events minus control histogram. For PBMC mean fluorescent intensity of CD4 positive cells (shown by marker) is shown.
4.2.8 Chemokine inhibition of ROD A and ROD B

Inhibition of HIV infection by blocking chemokine receptors is virus strain and cell type dependent (McKnight et al., 1997). Therefore it was next tested if blocking of ROD A and ROD B infection by chemokine inhibitors was also dependent on cell type and whether chemokine inhibition correlated with neutralisation sensitivity. Three different blockers of CXCR4 were used, the natural ligand SDF-1α, a small molecule inhibitor AMD3100 and mouse MAb I2G5. As shown in Figure 4.9, ROD A and ROD B were equally sensitive to chemokine blockers on each of the cell types except for SDF-1α inhibition on HeLa/CD4 and RD/CD4 cells. ROD A was more sensitive to SDF-1α than ROD B on both these cell types (Figure 4.9).

Figure 4.9. Chemokine blocking of ROD A and ROD B infection.

Infection of HeLa/CD4, CCC/CD4, CCC/X4 and RD/CD4 cells by ROD A (■) and ROD B (○) was assayed after preincubation of cells with dilutions of SDF-1α, AMD3100 and I2G5. Percentage residual infection is shown compared with infection in the presence of control, either RANTES or anti-CCR5 MAb 2D7.
4.2.9 CD4 mutants

CD4 undergoes endocytosis via clathrin coated pits yet this is not required for HIV infection (Maddon et al., 1988). To test whether internalisation of CD4 may play a role in the infectivity and neutralisation sensitivity of ROD A and ROD B, I used cell lines expressing two mutants of CD4 were used. CD4 H399 contains a stop mutation that results in a truncation at the junction of the transmembrane and cytoplasmic domain (Pitcher et al., 1999a). CD4 Cyto B has a stop codon at residue 402 and is thus 3 amino acids longer than the H399 construct (Maddon et al., 1988; Pelchen-Matthews et al., 1991). Both these proteins only undergo very slow bulk endocytosis. Both these constructs are stably expressed in HeLa cells (see Figure 4.11). ROD A and ROD B had infectivity titres on the CD4 mutant comparable to wild type HeLa/CD4. Neutralisation of ROD A and ROD B by rat MAbs and HIV-2 serum was tested on these target cells. As shown in Figure 4.10, ROD B was sensitive to neutralisation on cells expressing both mutant and wild type CD4 and ROD A was resistant.

![Graph](image)

**Figure 4.10.** Neutralisation of ROD A and ROD B infection of HeLa cells expressing mutant CD4. Infectivity of ROD A (white bars) and ROD B (hashed bars) was tested on HeLa/CD4, CytoB and H399 cells in the presence of 5μg/ml rat MAbs 77ow/2g, 64/4a, 8e and HIV-2 positive serum (1/1000 dilution). Percentage residual infectivity compared to control incubation with rat anti-HIV-1 MAab or normal human serum is shown.
Figure 4.11. Receptor expression and ROD titre on cells expressing CD4 mutants
CD4 (left) and CXCR (middle) expression on HeLa cells expressing wild type (top), CytoB (middle) or H399 (bottom) CD4 was measured by flow cytometry (red histograms). Infectious titre (ffu/ml) of ROD A and ROD B on these cell types is shown in the right panel.
4.2.10 Neutralisation on primary human cells

Due to the variability of ROD A and ROD B to neutralisation on several transformed cell lines a relevant *ex vivo* cells, PBMC were tested (see Figure 4.8 for CD4 and CXCR4 expression on PBMC). MAb neutralisation was determined by reduction in RT activity in supernatants of infected PBMC 7 days post-exposure to MAb treated ROD A or ROD B. As shown in Figure 4.11 ROD B was more sensitive than ROD A to neutralisation by MAbs 32/2f, 77ow/2g, 64/4a and 8e. MAb 34a/4e was non-neutralising for both ROD A and ROD B infection of PBMCs at concentrations up to 10μg/ml. All other MAbs (32/2f, 77ow/2g, 64/4a and 8e) gave up to 90% reduction in RT activity of ROD B infected cells but did not reduce ROD A RT production at up to 10μg/ml.

![Graph showing neutralisation of ROD A and ROD B infection of PBMC](image)

**Figure 4.11. Neutralisation of ROD A and ROD B infection of PBMC.**

Neutralisation of ROD A (■) and ROD B (●) infection of PBMC by rat MAbs 34a/4e, 32/2f, 77ow/2g, 64/4a and 8e. PBMC were infected with 100 TCID₅₀ of virus that had been incubated with dilutions of MAb. Infectivity was measured by ELISA for RT activity in cell free supernatant 7 days after infection.
4.3 Discussion

4.3.1 MAbs of different specificities were generated by immunisation with gp105/sCD4

To investigate neutralisation epitopes on HIV-2, a panel of MAbs was generated. HIV-2 envelope gp105 complexed to sCD4 was used as an immunogen. CD4 bound to envelope has been suggested to stabilise neutralising and cryptic epitopes for presentation to the immune system (Devico et al., 1996; DeVico et al., 1995; Gershoni et al., 1993; Kang et al., 1994; Kang et al., 1993; Thali et al., 1993). The gp105 was not irreversibly linked to sCD4, thus the separate components, gp105 and sCD4, could also act as independent immunogens. Indeed the majority of MAbs generated (12 /15) recognised gp105, without requirement for the presence of sCD4. Three MAbs were isolated that recognised CD4.

The envelope specific MAbs were further mapped to regions and specific epitopes of gp105. 6 MAbs mapped to either specific epitopes, or regions, within the V1/V2 loop (Table 4.2). The V1/V2 loop of HIV-2 and SIV is 30-40 amino acids longer than the corresponding loop of HIV-1 with two additional disulphide bonds predicted (Hoxie, 1991). Whether this influences the immunogenicity of this region is unknown but it has been described as the principal neutralising domain of SIV and is highly variable between HIV-2 isolates (Damond et al., 2001; Javaherian et al., 1992). V1/V2 of HIV-1 is also a neutralisation epitope (Ho et al., 1991a; McKeating et al., 1993b; Moore et al., 1993c; Sullivan et al., 1993).

No MAbs specific for the HIV-2 V3 loop were identified from this immunisation. The V3 loop is a highly immunogenic region for TCLA strains of HIV-1 and was considered the principal neutralising domain for these isolates (Javaherian et al., 1989; Spear et al., 1994). Previous studies have also reported failure to isolate HIV-2 V3 loop MAbs (Babas et al., 1994; Robert-Guroff et al., 1992; Skott et al., 1999). However, other studies have shown the V3 region of HIV-2 to contain important neutralising epitopes and V3 peptides to absorb neutralising activity of HIV-2 positive serum (Bjorling et al., 1991; Bjorling et al., 1994, McKnight et al. 1996). The V3 loop of HIV-2 is less variable than the corresponding region in HIV-1 which may suggest that less immune pressure is exerted on this region in vivo or that it is less able to escape from antibody attack by sequence changes (Damond et al., 2001; Parreira et al., 2000; Sankale et al., 1995).
Three MAbs specific for CD4 were generated from the immunisation. One, 46/1a, recognised cell surface CD4 on human T cells and sCD4 by ELISA. Two MAbs, 1a/5j and 84b/3g, recognise either sCD4 or cell membrane CD4 respectively. The reason for this differential binding is unclear. Lack of binding by 84b/3g to sCD4 in ELISA may be due to epitope competition with the capture antibody, Q4120, which recognises D1 of CD4 (Healey et al., 1990). Since MAb 1a/5j binds only sCD4 it may recognise an exposed region of CD4 adjacent to the transmembrane region normally occluded by cell membrane (Deen et al., 1988; Fisher et al., 1988; Hussey et al., 1988; Smith et al., 1987; Traunecker et al., 1988).

Even though the immunogen used was a complex of gp105 and sCD4, no MAbs were identified that required CD4 to bind HIV-2 envelope. A CD4 induced epitope of HIV-1 has been described that is recognised by MAbs generated from infected individuals such as 17b and 48d (Kang et al., 1993; Thali et al., 1993). Similar immunisations with gp120/CD4 complexes have generated conformational sensitive MAbs that are neutralising and cross-reactive (Devico et al., 1996; Kang et al., 1994) and sometimes elicit MAbs specific for the gp120/CD4 complex (DeVico et al., 1995).

### 4.3.2 Neutralisation of ROD B is not epitope specific

A panel of serum samples from HIV-2 infected individuals preferentially neutralised CD4-independent ROD B compared with CD4-dependent ROD A (chapter 3). I investigated whether a specific epitope exposed by adaptation to CD4-independence accounted for neutralisation sensitivity of ROD B. The panel of MAbs produced from immunisation with gp105/sCD4 were used since they recognised a range of different gp105 epitopes. Additional MAbs that mapped to the V3 loop and a further conformational MAb were included to test as wide a range of antibody specificities as possible. Overall ROD B was more sensitive to neutralisation by MAbs mapping to epitopes across the entire envelope including V1/V2, V3, V4/CD4 and some conformational sensitive epitopes (Table 4.3) indicating a global neutralisation sensitive phenotype. However, a conformational epitope(s) defined by MAbs 77ow/2g, 64/4a and 8e particularly accentuated the neutralisation sensitive phenotype of ROD B with 50% and 90% neutralisation titres up to 3 log_{10} lower than for equivalent neutralisation of ROD A.
There are 6 amino acid differences in surface envelope between the molecular clones of ROD A and ROD B used in this chapter: T98I in V1, E138K and E171K in V2, Q310K in V3, and L378F, K403R and R414I in V4 (Reeves and Schulz, 1997b). The epitope for the V1/V2 MAb 40a/8g encompasses glutamic acid at position 138 in ROD A within the V2 loop. In ROD B this amino acid is changed to a lysine. Similarly the epitopes of both the V3 MAbs overlap with a glutamine residue in ROD A at position 310 that is substituted for a lysine in ROD B. Both these changes introduce the basic amino acid lysine that may allow ionic interactions with residues in antibody paratope. Alternatively, the amino acid differences between ROD A to ROD B are located at the last or penultimate residue of the MAb epitope where alterations may be accepted without major disturbance in recognition. Neither the V3 MAbs nor 40a/8g have high neutralisation titres for ROD A or ROD B but neutralisation is consistently greater for ROD B indicating that the lysine substitutions within their epitopes does not prevent recognition. The 3 MAbs that show the greatest neutralisation differential between ROD A and ROD B (77ow/2g, 64/4a and 8e) are conformational. Therefore whether these MAbs map to regions that have sequence differences between ROD A and ROD B is unclear. 77ow/2g mapped to a conformational region within V4/CD4 binding domain. The 3 changes between ROD A and ROD B in V4 could account for lack of recognition by this MAb. This MAb potently neutralises ROD A infection of CD4 negative cells after sCD4 treatment suggesting that the epitope is present within the sequence of ROD A but it is cryptic or sequestered until after sCD4 binding.

A crucial amino acid change from ROD A to ROD B is located in gp41 (A526T) (Reeves and Schulz, 1997b). Alterations in gp41 sequence have been shown to influence the conformation and neutralisation sensitivity of HIV-1 (Edwards et al., 2001; Edwards et al., 2002; LaBranche et al., 1999; Park et al., 2000; Park et al., 1999; Park et al., 1998; Thali et al., 1994b; Watkins et al., 1996). Truncations in gp41 of HIV-2 and SIV are often associated with increased fusogenicity (Hoxie et al., 1991; Kodama et al., 1989; Mulligan et al., 1992; Ritter et al., 1993; Spies et al., 1994). However, both ROD A and ROD B have the same, truncated cytoplasmic tail length so can not account for the differential neutralisation sensitivity observed.

Similar to HIV-2 positive sera, ROD A could be sensitised to neutralisation by MAbs after induction to infect CD4 negative cells by incubation with sCD4. Again, MAbs 77ow/2g, 64/4a and 8e accentuated the phenotype. The ability of these MAbs
to neutralise ROD A only after incubation with sCD4 suggest they are dependent on a conformation that is formed or exposed on ROD A after binding to CD4. The HIV-1 MAbs 17b and 48d overlap with a CD4 induced epitope coreceptor binding site on HIV-1 envelope (Kang et al., 1993; Rizzuto et al., 1998; Thali et al., 1993; Wu et al., 1996). However, binding to monomeric ROD A envelope is not dependent on the presence of sCD4 (section 4.2.3 and chapter 5). Additionally there was only limited competition between MAbs 77ow/2g and 8e and human HIV-2 MAbs that block coreceptor binding to SIV envelope.

For other CD4-independent, neutralisation sensitive HIV-1 and SIV isolates, epitopes accounting for the neutralisation sensitivity have recently been described. The IIIB CD4-independent variant, IIIBx, is more sensitive to the CD4 induced epitope specific MAb 17b and 48d, consistent with exposure of the coreceptor binding site (Hoffman et al., 1999). The HIV-1 CD4-independent primary envelope described by Zhang et al. is susceptible to conformational V3 MAb (Zhang et al., 2002) probably as a result of specific mutation at the apex of the V3 loop in this envelope. Similarly the CD4-independent SIV mac316 is sensitive to V3 loop specific anti-sera (Means et al., 2001). A CD4 independent variant of HIV-1 ADA described by Kolchinsky et al. (Kolchinsky et al., 1999) was more sensitive to a variety of epitopes throughout envelope including the CD4 binding site (MAbs 15e, F105 and IgG1b12), V3 loop (19b), CD4 induced epitope (17b and 48d) and gp41 (2F5) (Kolchinsky et al., 2001b). Therefore the epitope specificity of neutralisation sensitivity of CD4-independent viruses seems to be isolate specific but epitopes within V3 and CD4 induced epitopes may be important.

4.3.3 Target cell type influences neutralisation sensitivity

ROD B can efficiently infect cells in the absence of CD4. However, it was noted that ROD B was relatively resistant to neutralisation when CCC/CD4/X4 were used as target cells. The various cell lines used and the sensitivity of ROD A and ROD B to neutralisation when infecting these cell types is summarised in Table 4.5. In general, infection of CD4 negative cells (CCC/X4) by both ROD A and ROD B was sensitive to neutralisation. ROD A was resistant to neutralisation on all CD4 positive cells tested. ROD B was sensitive to neutralisation on CD4 positive human HeLa cells and PBMC, partially sensitive on CD4 human RD/CD4 cells and resistant on cat CCC/CD4/X4 cells.
Different levels of expression of CD4 and CXCR4 on target cells can influence infectivity (Kabat et al., 1994; Kozak et al., 1999; Platt et al., 1998; Wu et al., 1997). The levels of CD4 and coreceptor expression were found to be similar on HeLa, CCC and RD cells, apart from the absence of CD4 from CCC/X4 cells. However the presence of CD4 on CCC cells seems to protect ROD B from neutralisation. This is similar to the observation in Chapter 3 that primary isolates of HIV-2 able to infect cells in the absence of CD4 were more resistant to neutralisation when CD4 positive cells were used as targets (CCC/CD4/X4 and NP2/CD4/R5). Increased affinity for CD4 following lab adaptation of HIV-1 (resulting from changes in C4 and gp41) results in a decrease in the amount of CD4 required for infection but also an increased sensitivity to sCD4 neutralisation (Kozak et al., 1997). Similarly, ROD B (which also has changes in C4 and gp41) has a slightly higher affinity for sCD4 than ROD A (~2 fold) (Reeves and Schulz, 1997b), which could similarly influence neutralisation sensitivity.

There have been previous reports of differential neutralisation depending on target cell, not only with MAbs but also chemokine receptor inhibitors (Martin et al., 2001; McKnight et al., 1997; Mondor et al., 1998a; Ruppach et al., 2000). However, no major difference was found between inhibition of ROD A and ROD B infection by inhibitors of CXCR4 on HeLa, RD, or CCC cells. CXCR4 is expressed endogenously in HeLa and RD cells and was stably transfected into CCC cells. Chemokine receptors

<table>
<thead>
<tr>
<th>Virus:</th>
<th>HeLa/CD4</th>
<th>CCC/X4</th>
<th>CCC/CD4/X4</th>
<th>RD/CD4</th>
<th>PBMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>77ow/2g</td>
<td>-+++</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>64/4a</td>
<td>-+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8e</td>
<td>-+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>serum</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

Table 4.5. Neutralisation sensitivity of ROD A and ROD B depending on target cell type.

+++ very sensitive: over 90% neutralisation with 10μg/ml of MAb or 1/8000 dilution of serum.
++ sensitive to neutralisation: 80% neutralisation with 1/8000 dilution of serum.
+ slightly sensitive to neutralisation: 50% neutralisation with 10μg/ml of MAb or 1/8000 dilution of serum.
- resistant to neutralisation: no reduction in infectivity with 10μg/ml of MAb or 1/8000 dilution of serum.
are associated with a plethora of intracellular signalling molecules that may not be properly coupled in transfected CCC/CD4 cells. However, CXCR4 is also stably transfected in CD4 negative CCC/X4 cells which, when used as targets, render ROD B (and ROD A induced by sCD4) susceptible to neutralisation. Additionally, others have reported chemokine receptors, both CCR5 (Doranz et al., 1997; Gosling et al., 1997) and CXCR4 (Lu et al., 1997) that are non-functional for signalling to be functional for use as coreceptors for HIV infection. Soda et al (Soda et al., 1999) have shown that calcium flux in CXCR4 and CCR5 transfected NP2 cells in response to the natural ligands, SDF-1β and MIP-1β respectively, is impaired. Primary isolates of HIV-2 have a similar reduction in neutralisation sensitivity as ROD B when infecting CD4 positive NP2 cells. Signalling by HIV envelope interaction with chemokine receptors may affect post entry steps in the virus life cycle (Davis et al., 1997; Weissman et al., 1997). However, whether this would impact on neutralisation at the cell surface is unknown. Signalling capability of CXCR4 expressed in CCC cells has not been tested with either natural ligands or HIV envelope.

Therefore, the reason for differential neutralisation resistance of ROD B depending on the target cell remains unclear. However similar reduced sensitivity to neutralisation has been reported for CD4-independent HIV-1 infection of CD4 positive Cf2Th (canine thymocyte) cells (Kolchinsky et al., 2001b) and SIV infection of CD4 positive 293T and Molt 4 cells (Allan et al., 1990; Puffer et al., 2002).

4.4 Summary

In summary in this chapter I have

- characterised a panel of rat MAbs specific for HIV-2 envelope.
- shown that MAbs directed to V1/V2, V3 and V4/CD4 binding domain and conformational domains of HIV-2 envelope preferentially neutralise CD4-independent ROD B compared with CD4-dependent ROD A. In addition, sCD4 induced ROD A infection of CD4 negative cells also showed increased neutralisation by all MAbs tested.
- shown that cellular CD4 on some cell types protects HIV-2 from neutralisation sensitivity.
Chapter 5

Exposure of neutralising epitopes on ROD A and the CD4-independent variant, ROD B.

5.1 Introduction

Antibodies can neutralise or inactivate a specific viral target by a variety of mechanisms. These include aggregation of viral particles, induction of structural changes, blocking receptor binding and inhibition of uncoating events (Klasse and Sattentau, 2001). Ab can also neutralise indirectly by recruitment of cytotoxic cells (antibody dependent cell cytotoxicity, ADCC) or activation of the complement cascade (section 1.6.3.8).

Aggregation of viral particles at certain Ab/virus ratios has been shown for certain Human Rhinovirus (HRV) 14 and poliovirus specific Abs (Brioen et al., 1983; Mosser et al., 1989; Thomas et al., 1985; Thomas et al., 1986) but this mechanism is sometimes excluded from the classical definition of neutralisation (Mandel, 1978). Both induction of structural changes and inhibition of uncoating due to stabilisation of the viral structure have also been suggested as mechanisms for neutralisation of HRV 14 (Mosser et al., 1989) and poliovirus (Emini et al., 1983; Mandel, 1976; Vrijsen et al., 1993; Wetz, 1993). A major mechanism of viral Ab mediated neutralisation is inhibition of receptor binding, the essential primary step in the viral lifecycle. This mechanism has been shown for a variety of viruses including HRV14 and 16 (Che et al., 1998; Smith et al., 1996) and Foot and Mouth Disease Virus (Verdaguer et al., 1997).

A major mechanism of HIV neutralisation is thought to be blocking interaction with cell surface receptors (Burton et al., 2001). Binding of HIV envelope to cellular CD4 is the primary step in the replication cycle of HIV-1 (see section 1.3.1). Abs able to block this interaction are present in sera from HIV infected individuals (Ho et al., 1991b; Moore and Ho, 1993b; Steimer et al., 1991) and many MAbs have been generated from both naturally infected individuals and immunised animals with epitopes that overlap with this site and block CD4 binding (Burton et al., 1991; Burton et al., 1994; McKeating et al., 1993a; Moore et al., 1995a; Posner et al., 1991; Roben et al., 1994; Saphire et al., 2001b; Thali et al., 1992; Trkola et al., 1995). After CD4 binding induced conformational changes there is a further interaction of the viral
envelope with a chemokine receptor that ultimately leads to fusion of the viral and cellular membranes. This coreceptor binding site is not easily accessible on native envelope but is formed or exposed upon CD4 binding (Kang et al., 1993; Thali et al., 1993; Wyatt et al., 1995). Several MAbs have been described that target this site (17b and 48D) and consequently bind only after CD4 ligation (Thali et al., 1993; Wyatt et al., 1995).

In contrast to blockade of receptor binding, other mechanisms of neutralisation may account for neutralisation by some HIV-specific MAbs. It has been suggested that b12, which overlaps with the CD4 binding site of HIV-1, may inhibit infection by post attachment and even a post fusion mechanism (McInerney et al., 1997). 2F5, a MAb that targets the coiled coil of gp41 blocks fusion whilst having no effect on virus binding to the target cell (Muster et al., 1993).

In vivo Abs are in constant interplay with other components of the immune system such as the complement system and killer cells able to mediate ADCC (see section 1.6.3.8). Therefore, attachment of Ab to the viral surface can induce viral lysis and killing by recruitment of these effector mechanisms. However, binding of Ab to a virus in the absence of direct neutralisation can also lead to antibody dependent enhancement of infection (see section 1.6.3.7) (Sullivan, 2001).

Irrespective of the actual mechanism of neutralisation, an Ab must first recognise and bind to the outer surface, or an exposed part of the virus particle. Thus for HIV the surface envelope is the main target for neutralising Abs. Immune evasion strategies to shield Ab binding sites on the envelope include burying of critical sites until necessary, such as the coreceptor binding site, and extensive glycosylation to occlude exposed sites (section 1.6.4). Primary isolates of HIV-1 are more refractory to neutralisation than cell line adapted isolates (Moore et al., 1995a; Moore and Ho, 1993b; Moore and Ho, 1995b; Sawyer et al., 1994; Wrin et al., 1995). A more 'closed' structure of the native trimer on primary viruses has been postulated as a mechanism for evasion of neutralisation (Poignard et al., 2001). Other factors such as density of envelope spikes, stability and fusogenicity of the envelope may also play a role in sensitivity of a particular virus to Ab-mediated neutralisation (Karlsson et al., 1996; Klasse and Moore, 1996).
In Chapters 3 and 4 CD4-independent ROD B was shown to be highly sensitive to neutralisation by HIV-2 positive sera and MAbs compared with the relative neutralisation resistant, CD4-dependent ROD A. The magnitude of differential sensitivity between ROD A and ROD B was up to 100-fold for some Abs. Increased neutralisation sensitivity of CD4 independent infection was also observed for primary isolates of HIV-2. A particular neutralisation epitope did not account for the increased sensitivity of ROD B. However, the presence of cell surface CD4 on some cell types may play a major role in determining neutralisation sensitivity.

The primary aim of this chapter is

- to investigate whether binding of HIV-2 specific antibodies to the envelope glycoproteins of ROD A and ROD B correlates quantitatively with neutralisation sensitivity.
5.2 Results

5.2.1 Antibody binding to monomeric envelope

The CD4-dependent HIV-2 isolate, ROD A, is less sensitive to neutralisation by HIV-2 positive sera and MAbs than the related CD4-independent isolate, ROD B (Chapters 3 and 4). I investigated whether neutralisation sensitivity of these viruses correlates with differential Ab binding. Firstly an ELISA was carried out to compare binding of HIV-2 positive sera and HIV-2 specific rat MAbs (77ow/2g, 64/4a and 8e, described in Table 4.2 and Table 4.3) to monomeric envelope protein of ROD A and ROD B. These MAbs and HIV-2 positive serum show up to 3 log\textsubscript{10} higher neutralisation titres for ROD B compared with ROD A (see Table 3.1 and Table 4.3). Ab binding was tested both in the presence and absence of sCD4 since sCD4 incubation induced neutralisation sensitivity of ROD A. Serial dilutions of HIV-2 positive serum or rat MAb (77ow/2g, 64/4a and 8e) were incubated with monomeric envelope proteins of ROD A and ROD B and binding measured by ELISA. As shown in Figure 5.1, binding curves for MAbs and HIV-2 positive serum were similar for ROD A and ROD B both in the presence and absence of sCD4.

Half maximal binding of Abs to monomeric envelope protein was calculated as the amount, µg/ml, of MAb, or dilution of serum, resulting in 50% of the maximal saturation of signal. Half maximal binding could not be calculated for MAb 64/4a since saturation was not achieved at the highest concentration of MAb tested (100µg/ml). As shown in Table 5.1 MAbs (77ow/2g and 8e) and HIV-2 serum bound with similar half max. to envelope of both ROD A and ROD B. This is despite ROD A being relatively resistant to neutralisation with neutralisation titres up to 3 log\textsubscript{10} higher for ROD B. The differences between the half max. values for binding to envelope of ROD A and ROD B did not reach statistical significance (p>0.01 for Student’s T-test) for 77ow, 8e or HIV-2 positive human serum. Furthermore there were no significant differences in MAb or serum antibody half max. binding for ROD A or ROD B envelope upon addition of sCD4.
Figure 5.1. Binding of MAbs and HIV-2 positive sera to envelope protein of ROD A and ROD B.

Serial dilutions of MAbs and HIV-2 positive serum were incubated with baculovirus derived monomeric envelope of ROD A (squares) or ROD B (circles) captured onto microtitre plates and detected with alkaline conjugated secondary antibody. Binding curves are shown for binding to envelope alone (black) or with addition of sCD4 (white).

<table>
<thead>
<tr>
<th></th>
<th>Half Maximal Binding a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>77ow/2g (µg/ml)</td>
</tr>
<tr>
<td>ROD A</td>
<td>1.20 +/- 0.26</td>
</tr>
<tr>
<td>ROD B</td>
<td>1.06 +/- 0.02</td>
</tr>
<tr>
<td>ROD A + sCD4</td>
<td>0.83 +/- 0.14</td>
</tr>
<tr>
<td>ROD B + sCD4</td>
<td>0.74 +/- 0.15</td>
</tr>
</tbody>
</table>

Table 5.1. Half maximal binding of MAbs and HIV-2 positive serum to envelope proteins.

a Half maximal binding was calculated as the amount of MAb (µg/ml) or HIV-2 positive serum dilution resulting in 50% of the maximal signal by ELISA.
5.2.2 Antibody binding to oligomeric envelope

In comparison to monomeric envelope, Ab binding to oligomeric envelope is thought to be a better predictor of HIV-1 neutralisation (Moore et al., 1995a; Parren et al., 1998; Sattentau and Moore, 1995a). Therefore, Ab binding to cells expressing envelope of ROD A and ROD B was tested using flow cytometry. 293T cells were transfected with full-length molecular clones of ROD A or ROD B. 293T cells do not express CD4 so any envelope shed from trimers on the cell surface should not become attached to cells in the culture as could occur with the use of infected PBMC or T cell lines to measure Ab binding (Parren et al., 1997; Watkins et al., 1996; Zolla-Pazner et al., 1995). Serial dilutions of MAbs (77ow/2g, 64/4a and 8e) and HIV-2 positive serum were tested for binding to cell surface expressed envelope by flow cytometry at 25°C and 37°C. None of the MAbs or HIV-2 positive serum showed above background binding to untransfected 293T cells (Figure 5.2A). As shown in Figure 5.3, binding curves for HIV-2 positive serum and MAbs were similar for ROD A and ROD B transfected cells. No difference was observed between the binding curves of MAbs and HIV-2 serum at 25°C or 37°C (Figure 5.3). Binding to cell surface envelope was also tested in the presence and absence of sCD4. Binding curves were unaltered by the addition of sCD4. sCD4 could be detected binding to ROD A and ROD B transfected 293T cells by flow cytometry (Figure 5.2B), although this may not be at a saturating level. Therefore, the differential neutralisation of ROD A compared to ROD B by MAbs and HIV-2 positive serum did not correlate with binding of Abs to envelope expressed on transfected 293T cells.
Figure 5.2. Flow cytometry of transfected 293T cells.

A. Binding of MAbs 77ow/2g, 64/4a, 8e and HIV-2 positive serum (red histograms) to untransfected 293T compared with controls (black histograms).

B. Binding of HIV-2 positive serum (left panels, red histograms) and sCD4 (right panels, red histograms) to untransfected (top), ROD A (middle) and ROD B (bottom) transfected 293T cells. Isotypes controls are shown in black histograms.

10000 events were collected for analyses
5.2.3 Cell to cell fusion

Since binding to cell surface expressed envelope did not correlate with neutralisation sensitivity of ROD A and ROD B, cell to cell fusion of transfected cells was also tested to assess the functionality of cell surface expressed envelope. As shown in Figure 5.2B, both ROD A and ROD B transfected cells bind sCD4. 293T cells transfected with either ROD A or ROD B and untransfected cells as control were incubated at 37°C with HeLa/CD4 target cells and observed by microscopy for syncytium formation. As shown in Figure 5.4, ROD B transfected 293T cells were highly fusogenic with large syncytia forming upon coculture with HeLa/CD4 cells after only a few hours. In contrast, ROD A transfected 293T cells cocultured with HeLa/CD4 target cells did not result in syncytia even after several days of incubation, similar to untransfected 293T cells (Figure 5.4). Addition of sCD4 often increases or induces fusion of HIV-2 (Clapham et al., 1992). However, ROD A transfected 293T
cells incubated with sCD4 cells still did not result in syncytia in cocultures with HeLa/CD4 cells (Figure 5.4). CD4 positive T cells lines were also tested as target cells for fusion. Cocultivation of ROD A transfected 293T cells with CD4 positive T cell lines, H9 and C8166, also did not result in syncytium formation (Figure 5.5). In contrast, large syncytia were quickly formed upon culture of ROD B transfected 293T cells with H9 or C8166 (Figure 5.5).

An alternative cell type transfected to express ROD A and ROD B envelope was also tested. Cos-1 cells transfected with molecular clones of ROD A and ROD B produce infectious virus and express envelope on the cell surface (Figure 5.6A and B). Similar to 293T cells, ROD A transfected Cos-1 did not form syncytia upon incubation with HeLa/CD4 cells, whilst ROD B transfected Cos-1 cells rapidly formed syncytia (Figure 5.6C).
Figure 5.4. Fusion of 293T cells with HeLa/CD4.
Coculture of untransfected (top), ROD A (middle) and ROD B (bottom) transfected 293T cells with HeLa/CD4 cells in the absence (left panels) and presence of sCD4 (right panels). Cells were fixed and stained with syncytial stain to visualise fused cells.
Figure 5.5. Fusion of 293T cells with CD4 positive T cells.
Coculture of untransfected (top), ROD A (middle) and ROD B (bottom) transfected 293T cells with H9 (left panels) and C8166 (right panels) cells.
Figure 5.6. ROD transfected Cos-1 cells.
A. Titre of ROD A and ROD B produced from transfected Cos-1 cells.
B. Binding of HIV-2 positive serum (red histograms) to untransfected, ROD A and ROD B transfected Cos-1 cells. 10000 events were collected for analysis.
C. Coculture of untransfected (left), ROD A (middle) and ROD B (right) transfected Cos-1 cells with HeLa/CD4 cells. Cells were fixed and stained with syncytial stain to visualise fused cells.
5.2.4 Virion capture

HIV envelope expressed on the surface of transfected cells may not truly represent envelope on the virion as evidenced by the lack of fusion of ROD A transfected 293T cells. Alternative assays based on antigen capture were therefore developed to test binding of MAbs to virion associated envelope. First, a capture assay with specific MAb capture of virions and subsequent detection by polyclonal anti-HIV-2 human serum was used. ROD A and ROD B viral stock, equalised for RT activity, were added to microtitre plates coated with HIV-2 specific rat MAbs, 34a/4e, 77ow/2g, 64/4a and 8e, in the presence and absence of sCD4. As shown in Figure 5.7, both ROD A and ROD B were captured similarly by MAbs, 34a/4e, 77ow/2g, 64/4a and 8e. Statistical analysis (Student’s T test) showed that significantly less ROD A was captured by MAb 64/4a in the absence of sCD4 than in its presence (p=0.033, n=4) or capture of ROD B (p=0.005, n=4). However, less ROD B (+ or -sCD4) was captured by 8e than ROD A (+ or -sCD4) (p=0.001). This is in contrast to the substantial increase in neutralisation sensitivity of ROD B to 8e compared to ROD A (Table 4.3). Therefore, there was no overall correlation between virus capture and neutralisation sensitivity except for MAb 64/4a. However, it should be noted that this assay potentially detects soluble as well as oligomeric virion associated envelope in the virus stock.

![Figure 5.7. Capture of ROD A and ROD B detected by polyclonal HIV-2 serum.](image)

Equal amounts (based on RT activity) of ROD A and ROD B were captured by MAbs in the presence or absence of 5μg/ml sCD4. Capture was detected by polyclonal anti-HIV-2 serum.
To eliminate interference by capture of soluble envelope, a second capture ELISA was carried out where RT activity of MAb captured virus was measured. MAb coated plates were incubated with equal amounts (based on RT activity) of ROD A and ROD B particles, samples were then removed from the plate. Virus captured in the plate was then detected by RT ELISA. The percentage of ROD A and ROD B not captured by MAb and removed from the ELISA plate during the first wash is shown in Figure 5.8A. The amount of ROD A or ROD B unbound was not reduced by incubation in specific MAb coated wells. RT activity of virus captured in the ELISA plates is shown in Figure 5.8B. Only very small amounts of RT activity were detected for both ROD A and ROD B (<10 pg/ml). Regardless, more ROD B was captured by MAbs 77ow/2g and 8e. Thus capture of virus using this method did correlate with neutralisation for MAbs 77ow/2g and 8e.

**Figure 5.8. Capture ELISA of ROD A and ROD B detected by RT.**

Equal amounts (based on RT activity) of ROD A (blue bars) and ROD B (red bars) were captured by MAbs. The amount unbound (A) and captured (B) was measured for RT activity by ELISA.
5.2.7 Virus stability

The stability of envelope oligomers is one of many factors that influence sensitivity to neutralisation (Karlsson et al., 1996; Klasse and Moore, 1996; Moore et al., 1993a; Willey et al., 1994). Therefore the stability of infectious ROD A and ROD B was tested by incubation over a 24-hour period at 37°C in the presence of increasing amounts of sCD4. For comparison X4 primary isolates of HIV-2 (SAB) and HIV-1 (2044) and a lab adapted HIV-1 isolate (LAI) were also tested. As shown in Figure 5.9 ROD B titre decreased upon prolonged incubation with sCD4, whilst ROD A was as resistant to prolonged incubation as primary isolates of HIV-1 and HIV-2. As expected lab adapted HIV-1 LAI was very sensitive to inactivation by incubation with sCD4 (Moore et al., 1990). Even though ROD B is less stable than ROD A in the presence of sCD4, in the absence of sCD4 ROD B titre is maintained. Therefore, decreased viral stability does not account for the enhanced neutralisation sensitivity of ROD B or sCD4 induced ROD A.

![Figure 5.9. Stability of ROD A and ROD B compared with primary isolates of HIV-1 and HIV-2.](image)

Infectious titre of ROD A and ROD B after 0, 2, 4, 8 and 24 hour incubation at 37°C was compared with a primary X4 isolate of HIV-2 (SAB) and a TCLA HIV-1 (LAI) and a primary X4 HIV-1 (2044). Titre was measured by infection of HeLa/CD4 cells after incubation in the absence (---) or presence of 1μg/ml (---), 2μg/ml (- - ) and 5μg/ml (---) of sCD4.
5.2.8 Biacore

Biacore is a system utilising surface plasmon resonance (SPR) change in the vicinity of a sensor chip surface. Biacore can be used for analysis of a variety of interactions between proteins and between nucleic acids. Interactions can be analysed in real time without the need for labelling of either reaction component. Several HIV and SIV envelope protein interactions with Ab or sCD4 have been analysed using Biacore (Hoffman et al., 2000; Hoffman et al., 1999; Kim et al., 2001; Myszka et al., 2000; VanCott et al., 1994; Zhang et al., 2001a; Zhang et al., 1999c; Zhang et al., 2001b). I used Biacore to further investigate Ab binding to HIV-2 envelope as it allows analysis of interaction occurring in real time with visualisation of kinetic on and off rates.

5.2.8.1 Development of Biacore assay

The instrumentation used for this study was a BiacoreX that allows interaction on 2 flow cells to be analysed simultaneously. MAb 77ow/2g was covalently immobilised onto flow cell 1 of a CM5 sensor chip using amine coupling chemistry (see section 2.9.4). This MAb was chosen as it gave the largest differential in neutralisation of ROD A and ROD B (Table 4.3). Flow cell 2 was used as a control. Prior to kinetic analysis, buffer and sCD4 controls run over the chip surface. As shown in Figure 5.10A sCD4 bound appreciably to the negative flow cell giving an inverted binding curve. To overcome non-specific binding, NaCl concentration in the buffer was increased from 150mM to 500mM. As shown in Figure 5.10A this abrogated non-specific binding of sCD4 to the chip surface. After each binding interaction the sensor chip surface was regenerated using a 10 second pulse of 10mM glycine pH 2.5. As shown in Figure 5.10B regeneration did not alter the binding curves from sequential injection of the same concentration of envelope protein gp105.
Figure 5.10. Removal of background binding and reproducibility of Biacore.

(A) Non-specific binding of sCD4 to flow cell 2 (—) was abrogated by increasing NaCl concentration in the running buffer from 150mM to 500mM (—). (B) Overlays of binding curves of 3 injections (—, — and —) of the same concentration of gp105 (20ug/ml) compared to buffer (—) carried out after injections of other concentrations of gp105 and multiple regenerations with 10mM glycine pH2.5.

5.2.8.2 Biacore analysis of gp105-77ow/2g interaction

Purified baculovirus derived ROD A monomeric envelope protein gp105 was run over the chip surface at 5 different concentrations (50nM - 1000nM) to allow kinetic analysis of the binding reaction. All binding assays were carried out at 37°C, with a continuous flow rate of 50µl/min. Equilibrium (curve plateau) was not reached with the highest concentration of gp105 (1000nM) but due to limited amounts of protein higher concentrations could not be tested. Kinetic rate constants were derived using BiaEvaluation software and the Langmuir 1:1 binding model. These calculations were based on first order kinetics where

\[ A + B = AB \]  \quad (A \text{ is ligand, MAb and } B \text{ is analyte, gp105})

The dissociation constant \( K_D \) is a ratio of association rate \( K_{on} \) and dissociation rate \( K_{off} \) such that \[ K_D = \frac{K_{off}}{K_{on}} \]

Overlays of experimental and fitted curves are shown in Figure 5.11. The 1:1 binding model fitted the experimental curves well as evidenced by the fitted curves overlaying the experimental curves and \( \chi^2 \) of <2. The on rate is \( 1.62 \times 10^4 M^{-1}s^{-1} \), the off rate \( 4.45 \times 10^{-4}s^{-1} \), giving a KD of \( 27.5 \times 10^{-9}M \) (Table 5.2).
5.2.8.3 Biacore analysis of gp105/sCD4 interaction

Since incubation with sCD4 rendered ROD A sensitive to neutralisation I next examined binding curves of ROD A gp105 incubated with sCD4. Dilutions of gp105 (50nM—500nM) were incubated with 2 fold molar excess of sCD4 for 1 hour at 37°C prior to binding analysis. Experimental and fitted curves are shown in Figure 5.11. The on rate for gp105 binding to 77ow/2g increased from 1.62 x10^4 M^-1 s^-1 to 3.61 x10^4 M^-1 s^-1 upon incubation with sCD4, a 2-fold increase (Table 5.2). The off rates are similar for gp105 in the absence and presence of sCD4 (4. 45 x10^-4 s^-1 and 4.19 x10^-4 s^-1), resulting in a lower dissociation constant for gp105+sCD4 of 11.6 x10^-9 M (Table 5.2). Unfortunately a sufficient quantity of purified ROD B monomeric envelope protein was not available to use in Biacore analysis for comparison with ROD A.

![Figure 5.11. Biacore analysis of gp105 binding to 77ow/2g.](image)

Association (A and C) and dissociation (B and D) curves are shown for gp105 (A and B) and gp105+sCD4 (C and D) binding to immobilised 77ow/2g. Model curves fitted by a Langmuir 1:1 model are shown in black. All experiments were carried out at a flow rate of 50μl/min at 37°C and analysis was carried out after subtraction of a buffer (A and B) or sCD4 (C and D) control from all curves using BiaEvaluation 3.0.
<table>
<thead>
<tr>
<th>Interaction</th>
<th>sCD4</th>
<th>$K_{on}$ ($\times 10^{4}$M$^{-1}$s$^{-1}$)</th>
<th>$K_{off}$ ($\times 10^{-4}$s$^{-1}$)</th>
<th>$K_D$ ($\times 10^{3}$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gp105 - 77ow/2g</td>
<td>-</td>
<td>1.62±0.30</td>
<td>4.45±0.26</td>
<td>27.5±6.38</td>
</tr>
<tr>
<td>gp105 - 77ow/2g</td>
<td>+</td>
<td>3.61±1.72</td>
<td>4.15±0.79</td>
<td>11.6±7.29</td>
</tr>
</tbody>
</table>

Table 5.2. Affinity constants for gp150 binding to 77ow/2g.

Association ($K_{on}$), dissociation ($K_{off}$) rate and dissociation ($K_D$) constant were derived from a Langmuir 1:1 binding model using BiaEvaluation 3.0.

5.2.8.4 Specificity

Specificity of the interaction between 77ow/2g and gp105 measured by Biacore was confirmed by injection of HIV-1 gp120 with and without sCD4 preincubation over the chip surface. As shown in Figure 5.12, 500nM of gp120 in the presence or absence of sCD4 did not exhibit binding above the buffer control.

![Graph showing specificity of Biacore interaction](image)

Figure 5.12. Specificity of Biacore interaction.

500nM gp120 in the presence (—) or absence (—) of sCD4 does not bind to immobilised 77ow/2g whilst 500nM gp105 (—) and gp105 +sCD4 (—) bind appreciably. All interactions were carried out at a flow rate of 50μl/min at 37°C

5.2.9 Pseudovirus

Monomeric envelope may not accurately represent envelope on the native oligomer, especially for Ab binding studies to predict neutralisation. Therefore, I attempted to develop a system for potentially analysing oligomeric envelope on the virion surface using Biacore. Due to the biological hazard status of HIV and lack of Biacore instrumentation within the category 3 laboratory a non-replication competent
HIV-based vector system was used where ROD A and ROD B envelopes were pseudotyped onto a HIV-1 core containing eGFP.

5.2.8.5 Production and characterisation of ROD A and ROD B pseudovirus

Full-length envelope (gp160) of ROD A and ROD B was PCR amplified from the molecular clones of these isolates. The forward primer was chemically phosphorylated to allow insertion of the product in the correct orientation into pCR3.1 uni vector (section 2.8.8). Seven clones each of ROD A and ROD B envelope were screened by PCR for the HIV-2 V3 fragment and presence of the envelope insert in the vector by digestion with enzyme *Nhel* and *EcoRI* (Figure 5.13).

![Figure 5.13. ROD A and ROD B envelope cloning.](image)

A PCR of full length envelope from molecular clones pACR23 and ROD B.14. B PCR for HIV-2 V3 loop from pCR3.1 clones with ROD A (A1-7) and ROD B (B1-7) inserts. C Restriction digest with *Nhel* and *EcoRI* of pCR3.1 with ROD A and ROD B envelope inserts
ROD A and ROD B envelopes were used to pseudotype an envelope defective HIV-1 vector containing eGFP by triple transfection of 293T cells as described in section 2.3.3. VSV-G envelope and no (delta) envelope pseudoviruses were also generated as controls. Infectivity of the pseudovirus was tested on CCC/CD4/X4, CCC/X4 and HeLa/CD4 cells by flow cytometry detection of eGFP expression in target cells (Figure 5.15). Neutralisation sensitivity to 77ow/2g was also tested on HeLa/CD4 cells (Figure 5.15). Similar to infectious virus, the ROD A pseudotype is dependent on CD4 while the ROD B pseudotype is CD4 independent. Similarly, ROD A pseudovirus was resistant to neutralisation by 77ow/2g whilst ROD B was sensitive. RT activity, p24 content and capture by 77ow/2g of pseudotypes was tested by ELISA (Figure 5.14)

A

![Graph A](image)

B

![Graph B](image)

C

![Graph C](image)

**Figure 5.14. Envelope, p24 and RT content of pseudovirus.**

A. HIV-2 envelope on pseudovirus with ROD A, ROD B, VSVG or no (delta) envelope was detected by capture ELISA with 77ow/2g and detection with polyclonal anti-HIV-2 serum.

B. HIV-1 p24 was detected by ELISA.

C. RT activity was detected using ELISA.
Figure 5.15. Infectivity of pseudovirus.
Pseudoviruses with ROD A, ROD B, VSV-G or no(delta) envelope were tested for infectivity on CCC/CD4/X4, CCC/X4 and HeLa/CD4 cells. Infectivity was detected by flow cytometry analysis of eGFP expressing cells. Infectivity of pseudovirus incubated with Mab 77ow/2g was also tested on HeLa/CD4 cells by analysis of eGFP expressing cells. Numbers in parenthesis indicate the percentage of eGFP positive cells based on 10000 events collected.
5.2.8.6 Biacore of pseudovirus

ROD A and ROD B envelope pseudoviruses were then tested for binding to 77ow/2g by Biacore. As shown in Figure 5.16, binding curves of equal amounts (based on RT activity) of ROD A and ROD B pseudotypes overlay with the control, delta envelope pseudovirus. Therefore, although both ROD A and ROD B envelope could be captured by MAb 77ow/2g by ELISA (Figure 5.14A) and ROD B pseudovirus was neutralised by 77ow/2g (Figure 5.15) no binding of pseudovirus to immobilised 77ow/2g was detected by Biacore.

![Figure 5.16. Pseudovirus binding to 77ow/2g by Biacore.](image)

ROD A (---), ROD B (—) and delta envelope (—) pseudovirus equalised for RT activity were injected over a 77ow/2g coated sensor chip at 50μl/min at 37°C.
5.3 Discussion

5.3.1 Neutralisation sensitivity does not correlate with Ab binding to monomeric envelope.

The CD4-independent HIV-2 isolate ROD B is highly sensitive to Ab mediated neutralisation compared with the related CD4-dependent ROD A (Chapters 3 and 4). I therefore investigated whether neutralisation sensitivity correlated with Ab binding to the surface envelope protein of ROD A and ROD B. HIV-2 positive human serum and HIV-2 specific MAbs bound similarly to monomeric baculovirus derived envelope proteins of ROD A and ROD B when assayed by ELISA. In addition, sCD4 did not alter the binding curves of Abs to monomeric envelope protein despite sensitising ROD A to neutralisation by these Abs. Epitopes for MAbs 77ow/2g, 64/4a and 8e are therefore exposed in monomeric ROD A envelope allowing Ab binding but may not be exposed on the native oligomer. Lack of correlation of Ab neutralisation with binding to monomeric envelope is in agreement with previous studies of HIV-1 positive serum and HIV-1 specific MAbs binding to monomeric envelope proteins of neutralisation sensitive and resistant HIV-1 isolates (Kostrikis et al., 1996; Moore et al., 1995a; Sullivan et al., 1995; Watkins et al., 1996).

5.3.2 Neutralisation sensitivity does not correlate with Ab binding to cell surface expressed envelope.

HIV envelope expressed on the cell surface may be a better representation of native trimers than monomeric protein. However, Ab binding to cell surface expressed envelope of ROD A and ROD B also did not correlate with neutralisation sensitivity of these isolates. For HIV-1, binding of MAbs to cell surface expressed envelope has been both correlated (Fouts et al., 1997; Park et al., 2000; Roben et al., 1994; Sattentau and Moore, 1995a; Sullivan et al., 1995; Watkins et al., 1996) and not correlated (Fouts et al. 1998; Nyambi et al., 1998; Nyambi et al., 2000; Stern et al., 1995; Wrin et al., 1995; York et al., 2001) with neutralisation sensitivity. The lack of correlation observed with ROD A and ROD B transfected cells may be due to the differential functionality of cell surface expressed envelopes. ROD B transfected cells readily formed large syncytia upon cocultivation with a variety of CD4 and CXCR4 positive target cells yet ROD A transfected cells did not form visible syncytia even with addition of sCD4. The lack of fusion suggests that the envelope expressed on the
surface of ROD A transfected 293T and Cos-1 cells may be uncleaved or insufficiently processed. However, uncleaved full-length gp160 is thought to be unstable and is degraded within the golgi network or targeted for lysosomal degradation (Kimura et al., 1996; Willey et al., 1988) so should not be present in large amounts on the cell surface. In contrast with other reports of non-functional cell surface expressed HIV-1 envelopes (Anderson et al., 1993; Bosch et al., 1990; Guo et al., 1990; Hallenberger et al., 1992; McCune et al., 1988), the envelope proteins of ROD A and ROD B produced from transfected cells are fusion competent when incorporated into budding virions as demonstrated by production of infectious virus. The reason for the lack of syncytia formation by ROD A transfected cells remains unclear.

5.3.3 Binding to virion associated envelope

Since Ab binding to monomeric envelope and cell surface expressed envelope did not correlate with neutralisation, assays to detect virion associated envelope were developed. Similar amounts of ROD A and ROD B were captured by HIV-2 specific MAbs when detected with polyclonal HIV-2 serum, except for MAb 64/4a that captured more ROD B than ROD A. However, a similar assay with detection of captured virus by RT activity did reveal greater capture of ROD B than ROD A by two out of three MAbs (77ow/2g and 8e but not 64/4a). Monomeric envelope may be detected by polyclonal HIV-2 serum whereas only envelope associated with RT activity and thus virus particles was detected by the second assay perhaps explaining the different results between the two assays. Therefore, Ab binding to native oligomers on RT active virus particles does correlate with neutralisation sensitivity for some Abs. Recently a similar assay, measuring p24 of captured virus, has been used to correlate binding of HIV-1 V3 MAbs to native virions with neutralisation sensitivity (Gorny et al. 2002).

5.3.4 Viral stability

Several factors other than Ab binding may influence the neutralisation sensitivity of a particular virus isolate (Klasse and Moore, 1996). The stability of ROD A and ROD B over time was investigated by incubation of viral stocks at 37°C in the presence of increasing amounts of sCD4. As previously reported for HIV-1, primary isolates of HIV-1 and HIV-2 were more stable, resistant to sCD4 neutralisation and retained titre over time compared with an HIV-1 TCLA isolate. However TCLA HIV-2, ROD A was just as stable as the primary isolates. ROD B
titre was reduced by prolonged incubation in the presence of sCD4 compared with ROD A but was as stable in the absence of sCD4. Loss of infectivity of ROD B may be due to envelope shedding thus reducing the number of functional envelope spikes, similar to HIV-1 lab adapted isolates (Gelderblom et al., 1987; Gelderblom et al., 1985; Hart et al., 1991; Kirsh et al., 1990; Layne et al., 1992; McKeating et al., 1991; Moore et al., 1991; Moore et al., 1990; Willey et al., 1994). The relative stability of ROD A may be as a result of a higher SU-TM interaction as has been described previously for primary isolates of HIV-1 and HIV-2 and isolates of SIV (Allan et al., 1992; Moore et al., 1993a; Moore et al., 1992; O'Brien et al., 1994; Sattentau et al., 1993; Sullivan et al., 1995; Willey et al., 1994). Lower levels of functional envelope may increase neutralisation sensitivity by reducing the number of spikes that would need to be bound by Ab to reach a neutralisation threshold. Thus, the higher levels of surface envelope on primary isolates of HIV-1 may account for resistance to neutralisation (Moore et al., 1993a; O'Brien et al., 1994). However, the envelope spike density may not fully explain resistance of all primary isolates to sCD4 and MAb neutralisation (Karlsson et al., 1996).

The stability of ROD B in the absence of sCD4 is similar to ROD A thus precluding this as a major contributor to its greater neutralisation sensitivity. In addition ROD A was as stable in the presence of sCD4 as in its absence yet is highly sensitive to Ab-mediated neutralisation in the presence of sCD4.

5.3.5 Biacore analysis

As demonstrated above, binding to monomeric envelope is a poor predictor of neutralisation when assayed by ELISA. However, Biacore analysis of some combinations of monomeric HIV-1 envelope and MAb have been shown to correlate with neutralisation sensitivity (Hoffman et al., 1999; Zhang et al., 2001b). Binding of monomeric ROD A envelope to MAb 77ow/2g was measured by Biacore in the presence and absence of sCD4. The addition of sCD4 increased the affinity of the interaction, by an increase in on rate. Thus, binding to monomeric protein assayed by Biacore correlates with the increase in neutralisation sensitivity of ROD A upon incubation with sCD4. An increase in binding affinity upon addition of sCD4 has also been described in Biacore analysis of the interaction between monomeric envelope of HIV-1 IIIIB and the CD4 induced MAb 17b (Table 5.3) (Hoffman et al., 1999; Zhang et al., 1999c; Zhang et al., 2001b). The increases in on rate for this interaction are 10-
fold and 37-fold compared with only 2 fold observed for ROD A-77ow/2g interaction (Table 5.3). Zhang et al also investigated the interaction of HIV-1 envelope from the JR-FL isolate and MAb 17b and similarly obtained a 20 fold increase in on rate in the presence of sCD4 (Zhang et al., 1999c). Hoffman et al. (Hoffman et al., 1999) also describe association and dissociation rates for the interaction of 17b with the CD4-independent HIV-1 envelope IIIB, derived from IIIB (Table 5.3). This interaction has a 10-fold increased on rate compared with IIIB which may account for a 6 fold increase in neutralisation titre of 17b for IIIB* compared with IIIB (Hoffman et al., 1999). In comparison, addition of sCD4 to ROD A increases 77ow/2g neutralisation over 100-fold yet only results in a 2 fold increase in on rate in MAb binding by Biacore.

<table>
<thead>
<tr>
<th>Interaction</th>
<th>sCD4</th>
<th>$K_{on}$ (x10^4 M^-1 s^-1)</th>
<th>$K_{off}$ (x10^-4 s^-1)</th>
<th>KD (x10^-9 M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROD A - 77ow/2g</td>
<td>-</td>
<td>1.62 ±0.30</td>
<td>4.45±0.26</td>
<td>27.5±6.38</td>
</tr>
<tr>
<td>ROD A - 77ow/2g</td>
<td>+</td>
<td>3.61±1.72</td>
<td>4.19±0.79</td>
<td>11.6±7.29</td>
</tr>
<tr>
<td>HXB2 - 17b</td>
<td>-</td>
<td>9.26±0.03</td>
<td>2.39±0.03</td>
<td>2.58±0.03</td>
</tr>
<tr>
<td>HXB2 - 17b</td>
<td>+</td>
<td>91.3±0.3</td>
<td>2.39±0.04</td>
<td>0.26±0.004</td>
</tr>
<tr>
<td>HXB2 - 17b</td>
<td>-</td>
<td>0.8</td>
<td>0.2</td>
<td>2.5</td>
</tr>
<tr>
<td>HXB2 - 17b</td>
<td>+</td>
<td>30</td>
<td>0.5</td>
<td>0.2</td>
</tr>
<tr>
<td>IIIB - 17b</td>
<td>-</td>
<td>10</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>IIIB - 17b</td>
<td>+</td>
<td>20</td>
<td>9</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Table 5.3. Comparison of affinity constants of HIV-1 and HIV-2.

*a Data from Zhang et al., 2001b  
*b Data from Hoffman et al., 1999

As discussed above, binding monomeric envelope may not represent oligomeric virion associated envelope. I therefore attempted to analyse the binding of MAb 77ow/2g to envelope proteins on virus particles by Biacore. An HIV-1 vector core was pseudotyped with ROD A and ROD B envelope proteins and pseudoviruses showed CD4 dependence and neutralisation sensitivity similar to the wild type viruses. Unfortunately neither ROD A nor ROD B pseudovirus exhibited detectable binding to 77ow/2g immobilised on a Biacore sensor chip. Both pseudoviruses, however, could be captured by 77ow/2g by ELISA. Previous Biacore studies of interactions of MAb with virus particles have been carried out for several viruses; influenza A, HRV and poliovirus (Casasnovas et al., 1994; Dubs et al., 1991; McDermott et al., 2000;
Pellequer et al., 1993; Schofield et al., 1996; Xing et al., 2000). However, all of these studies were conducted with virus immobilised onto the sensor chip surface. Low pH buffer is required for both amine coupling and regeneration of the chip surface. Immobilisation is therefore possible with viruses such as human rhinoviruses and poliovirus due to their stability in low pH. HIV envelope structure and particle integrity are likely to be disrupted by such conditions. The inbuilt instability of HIV may be the reason for lack of detection in this assay as the salt (NaCl) concentration of the running buffer was raised to 500mM to abrogate background binding of sCD4. This level of NaCl is approximately 5 times greater than present in tissue culture medium. An additional limitation may have been the titre of the pseudovirus not being sufficient for detection.

Overall, neither monomeric nor oligomeric envelope binding correlated well with neutralisation of ROD A and ROD B when assayed using standard techniques (ELISA and flow cytometry). Analysis of MAb binding to monomeric envelope of ROD A in a Biacore system suggests that Ab binding affinity may contribute to neutralisation differential of ROD A upon the addition of sCD4. A two-fold increase in Ab affinity for ROD A envelope by the addition of CD4 may not be sufficient alone to account for the increase in neutralisation sensitivity. Binding of an Ab to native, oligomeric envelope is obviously necessary for neutralisation yet envelope stability and stearic constraints of cellular CD4 may also contribute.

5.4 Summary

In summary I have shown that

- Ab binding monomeric envelope protein of ROD A and ROD B by ELISA does not correlate with neutralisation sensitivity.
- Ab binding to cell surface expressed envelope of ROD A and ROD B does not correlate with neutralisation sensitivity
- virion capture does correlate with neutralisation sensitivity.
- the affinity of the interaction between ROD A envelope and MAb 77ow/2g does correlate with neutralisation, with an increase in affinity upon addition of sCD4
- pseudovirus with envelope protein of ROD A and ROD B retain CD4 dependence and independence and differential sensitivity to MAb neutralisation.
Chapter 6

Summary

Unlike HIV-1, many isolates of HIV-2 are able to infect CD4 negative cells by direct interaction with a chemokine receptor. Within this thesis I have investigated the neutralisation sensitivity of CD4-dependent and CD4-independent isolates of HIV-2. I have shown that CD4 independent infection is more sensitive to Ab-mediated neutralisation than CD4-dependent infection. The factors associated with neutralisation sensitivity include increased affinity of Ab for CD4-independent envelopes and protection by cell surface receptor CD4.

I have been able to study neutralisation sensitivity of CD4 independent infection with clinically relevant viruses isolated from HIV-2 infected patients. HIV-1 primary isolates are generally refractive to neutralisation yet primary isolates of HIV-2 were highly susceptible to neutralisation by HIV-2 positive sera when using a CD4 independent route of infection. The results from TCLA isolates, CD4-dependent ROD A and CD4-independent ROD B, mirrored that of primary isolates in that ROD B was highly susceptible to neutralisation by HIV-2 positive sera, and both human and rat anti-HIV-2 MAbs. ROD A was resistant to Ab-mediated neutralisation when infecting CD4 positive cells but became sensitive when induced to infect CD4 negative cells by incubation with sCD4. This suggests that the use of cellular CD4 may protect HIV-2 from Ab-mediated neutralisation and may explain the lack of CD4 negative cells infected with HIV-2 in vivo and also the rarity of CD4-independent isolates of HIV-1. Whether the CD4 independence of primary isolates of HIV-2 impacts on the lower pathogenicity of HIV-2 is unclear but the lack of CD4 negative cell infection in vivo suggests that there is immune pressure preventing infection by this route. Despite this, viruses able to infect CD4 negative cells can be isolated from HIV-2 infected individuals.

The study of interactions between viral envelope and Ab is hindered by the difficulties in measuring binding to native virus particles. The importance of using functional envelope from virus particles was demonstrated by the lack of correlation between neutralisation and Ab binding measured using standard methods such as ELISA and flow cytometry. Using virus capture assays Ab binding does correlate with neutralisation for some Abs. In addition, Ab affinity for monomeric envelope measured by real time binding assays also correlates with Ab neutralisation. I
attempted to use the Biacore system to analyse further binding to viral particles. Unfortunately, due to technical limitations, a successful assay system for this was not accomplished.

Future directions for this work include investigation of neutralisation of autologous virus isolates by sera from HIV-2 positive individuals. Cloning of individual envelopes may allow identification of CD4-independent and CD4-dependent clones within the same individual, which could then be studied for neutralisation sensitivity with autologous Abs. Further development of real time binding assays for characterisation of Ab responses from vaccinated individuals may aid in vaccine design. It is clear that studying native conformations of envelope are important to relate binding studies to Ab neutralisation. Biacore analysis of viral particles or virus like particles interacting with Ab would be an exciting technology that so far has not been achieved for HIV. Use of newly developed stable, soluble envelope trimers (Sanders et al., 2002; Schulke et al., 2002), inactivated virus particles (Rossio et al., 1998) or beads coated with specific viral envelope (Grundner et al., 2002) may allow such a technique to be developed.
References


immunodeficiency virus Type 1 vaccine with or without gp120: a phase 2 study in higher- and lower-risk volunteers. J. Infect. Dis. 183: 1343 - 1352.


Dumonceaux, J., Goujon, C., Joliot, V., Briand, P. and Hazan, U. (2001). Determination of essential amino acids involved in the CD4-independent tropism of the X4 human immunodeficiency virus type 1
m7NDK isolate: role of potential N glycosylations in the C2 and V3 regions of gp120. J. Virol. 75: 5425 - 5428.


for Conformation-Sensitive Epitopes of V3 Neutralize Human Immunodeficiency Virus Type 1 Primary Isolates from Various Clades. J. Virol. 76: 9035 - 9045.


regimen consisting of consecutive priming with DNA and boosting with recombinant fowlpox virus. J. Virol. 72: 10180 - 10188.


Libert, F., Cochaux, P., Beckman, G., Samson, M., Aksenova, M., Cao, A., Czeizel, A., Claustres, M., de la Rua, C., Ferrari, M., Ferrec, C., Glover, G., Grinde, B., Guran, S., Kucinskas, V., Lavinha, J.,


Ly, A. and Stamatatos, L. (2000). V2 loop glycosylation of the human immunodeficiency virus type 1 SF162 envelope facilitates interaction of this protein with CD4 and CCR5 receptors and protects the virus from neutralization by anti-V3 loop and anti-CD4 binding site antibodies. J. Virol. 74: 6769 - 6776.


208


Matloubian, M., Concepcion, R. J. and Ahmed, R. (1994). CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. J. Virol. 68: 8056 - 8063.


Migasena, S., Suntharasamai, P., Pitisuttithum, P., Kitayaporn, D., Wasi, C., Huang, W., Vanichseni, S., Koompong, C., Kaewkungwal, J., Raktham, S., Ippolito, T., Hanson, C., Gregory, T., Heyward, W. L.,


Park, E. J. and Quinnan, G. V., Jr. (1999). Both neutralization resistance and high infectivity phenotypes are caused by mutations of interacting residues in the human immunodeficiency virus type 1 gp41 leucine zipper and the gp120 receptor- and coreceptor-binding domains. J. Virol. 73: 5707 - 5713.

immunodeficiency virus type 1 MN to antibodies directed at V3 and non-V3 epitopes. J. Virol. 72: 7099 - 7107.


220


Reeves, J. D., Hibbitts, S., Simmons, G., McKnight, A., Azevedo-Pereira, J. M., Moniz-Pereira, J. and Clapham, P. R. (1999). Primary human immunodeficiency virus type 2 (HIV-2) isolates infect CD4-
negative cells via CCR5 and CXCR4: comparison with HIV-1 and simian immunodeficiency virus and relevance to cell tropism in vivo. J. Virol. 73: 7795 - 7804.


immunodeficiency virus type 1 antibody 2G12 recognizes a cluster of alpha1-->2 mannose residues on the outer face of gp120. J. Virol. 76: 7306 - 7321.


Weissman, D., Li, Y., Orenstein, J. M. and Fauci, A. S. (1995). Both a precursor and a mature population of dendritic cells can bind HIV. However, only the mature population that expresses CD80 can pass infection to unstimulated CD4+ T cells. J. Immunol. 155: 4111 - 4117.


