Identification of neutralising determinants in protection against HIV-1

Thesis

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Identification of Neutralising Determinants in Protection against HIV-1

A thesis submitted to the Open University in partial fulfilment for the degree of Doctor of Philosophy in Life Sciences.

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June 2010

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DATE OF SUBMISSION: 19 NOV 2009
DATE OF AWARD: 2 JUN 2010
Acknowledgements

I would like to wholeheartedly express my appreciation to everyone who has helped and supported me through the course of this project. For their time and patience I have only gratitude to repay them.

To Dr Neil Almond for his overall guidance for this work and for this thesis. To Dr Deborah Ferguson for her day to day supervision and direction through the practical aspects of this work, and to Dr Quentin Sattentau for his insight and critical reading of my thesis.

To all of my colleagues and friends at NIBSC who have helped, supported and cajoled me throughout the course of this PhD work. To all of the staff in BSD who undertook all of the immunisations and sample collections from my immunogenicity and monoclonal antibody production studies. To the members of Biostatistics for their advice and assistance in deciphering and understanding the significance of my results.

To all those who helped by collaborating with work; Dr Simon Jeffs, for the provision of a number of envelope glycoproteins, Dr Christiane Moog and Dr David Montefiori for the neutralisation work they undertook, and Dr Ian Jones for his structural epitope mapping work.

To my family, especially my parents for their love and support throughout my entire education and this work, I am where I am because of you. And finally to Laura, for not only helping me to review my writing but for being there, and her enduring patience, love and support throughout.
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Abstract

The main aim of this thesis is the characterisation and understanding of anti HIV-1 responses elicited by vaccination with HIV-1 envelope based vaccines. Initially, the specificity of anti HIV-1 envelope antibodies, elicited by vaccination with an HIV-1 envelope vaccine formulation that has been studied in Phase 1 clinical trials and has been demonstrated to be able to protect macaques against challenge, were determined. In particular, studies were focussed to understand whether the superior neutralising antibody responses that were detected following increasing numbers of vaccinations correlated with qualitative or purely quantitative changes in serological responses. The increase in neutralising ability was observed to be an overall maturation effect of the specificity and avidity of the response. The only correlate found for the incidence of viral breakthrough was a reduced specific V3 epitope response as measured by competition to a V3 crown loop peptide mAb. - 447-52D.

Subsequent topics of research were focussed on evaluation of serological responses generated by HIV-1 envelope immunogens of other clades. The goal of this work was to increase our understanding of HIV-1 isolates as serotypes rather than genotypes. It was found that with the select individual proteins from 5 clades utilised in this work, there were no matching serotypes that could be identified. Indeed the variance in responses from animals having received the same immunogen formula was in some cases quite marked, for both binding and neutralising specificity.

Initial work to investigate cross reactivity of binding antibody and neutralising responses was expanded to analyse serological responses to C clade immunogens by the production and characterisation of novel murine monoclonal antibodies. The successful production of novel anti C-clade monoclonal antibodies was somewhat tempered by the lack of induced neutralising ability of any of these. However these materials will still provide valuable reagents for further research into what is the most prominent clade of the virus worldwide.
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<tr>
<td>ADCC</td>
<td>Antibody dependant cell mediated cytotoxicity</td>
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<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
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<td>APS</td>
<td>Ammonium Persulphate</td>
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<td>ARV</td>
<td>Anti Retroviral Therapy</td>
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<td>AZT</td>
<td>Zidovudine</td>
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<td>CD</td>
<td>Cluster of Differentiation</td>
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<td>CFAR</td>
<td>Centralised Facility for AIDS Reagents</td>
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<td>CHO</td>
<td>Chinese Hamster Ovary</td>
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<td>cRPMI</td>
<td>Complete RPMI 1640 media</td>
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<td>Diaminobenzidine</td>
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<td>Dendritic Cell</td>
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<td>Distilled De-ionised Water</td>
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<td>Diethanolamine</td>
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<td>DMSO</td>
<td>DiMethyl Sulfoxide</td>
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<td>DNA</td>
<td>Deoxyribo Nucleic Acid</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
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<tr>
<td>Env</td>
<td>Envelope</td>
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<tr>
<td>ESCRT</td>
<td>Endosomal Sorting Complex Required for Transport</td>
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<tr>
<td>FCS</td>
<td>Foetal Calf Serum</td>
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<tr>
<td>GALT</td>
<td>Gut Associated Lymphoid Tissue</td>
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<td>G</td>
<td>Gravity</td>
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<td>gp</td>
<td>Glycoprotein</td>
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<td>GSK</td>
<td>GlaxoSmithKline</td>
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<tr>
<td>HAT</td>
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<td>Human Immunodeficiency Virus</td>
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<td>Horse Radish Peroxidase</td>
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<td>Hypoxine, Thymidine</td>
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XIII
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<tr>
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<td>IMS</td>
<td>Industrial Methylated Spirits</td>
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<td>Integrase</td>
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<td>KAC</td>
<td>Potassium acetate</td>
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<tr>
<td>kDa</td>
<td>Kilodalton</td>
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<td>LAV</td>
<td>Lympadenopathy Associated Virus</td>
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<td>Litre</td>
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<td>Luria Broth</td>
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<td>Long Term Non-Progressor</td>
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<td>Long Terminal Repeat</td>
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<td>mAb</td>
<td>Monoclonal Antibody</td>
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<td>MHC</td>
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<td>MIP</td>
<td>Macrophage Inhibitory Protein</td>
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<td>MVA</td>
<td>Modified Vaccinia Ankara Virus</td>
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<td>Nef</td>
<td>Negative Factor</td>
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<tr>
<td>ng</td>
<td>nanogram</td>
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<tr>
<td>NK</td>
<td>Natural Killer cell</td>
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<td>Peripheral Blood Lymphocyte</td>
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<td>Peripheral Blood Mononuclear Cell</td>
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<td>Phosphate Buffered Saline</td>
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<td>Polymerase Chain Reaction</td>
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<td>Plasmacytoid Dendritic Cell</td>
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<td>picogram</td>
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<td>Regulation on Activation normal T Expressed and secreted</td>
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<td>Rough Endoplasmic Reticulum</td>
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<td>Recombinant Glycoprotein</td>
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<td>Ribo Nuclie Acid</td>
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rpm  revolutions per minute
RT   Reverse Transcriptase
SCID Severe combined immunodeficiency
sfRPMI Serum free RPMI 1640 media
SDS-PAGE Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SHIV Simian/Human Immunodeficiency Virus
SIV  Simian Immunodeficiency Virus
SNF  Supernatant Fluid
STLV Simian T cell Lymphotrophic Virus
SU   Surface
TAE  Tris Acetateacid EDTA buffer
TAR  Transactivation Responsive RNA element
TAT  Transactivator of Transcription
TCID Tissue Culture Infectious Dose
TCLA Tissue Culture Adapted
TEMED Tetramethylethylenediamine
TM   Trans-membrane
TMB  3,3',5,5'-Tetramethylbenzidine
TNF  Tumour Necrosis Factor
TRIM Tri Partite Motif
TRIS tris(hydroxymethyl)aminomethane
TSG-101 Tumor susceptibility gene 101
VLP  Virus Like Particle
VPR  Viral Protein r
VPU  Viral Protein Unknown
VPX  Viral Protein X
UV   Ultra Violet
Chapter 1
Introduction

The current Human Immunodeficiency Virus (HIV) pandemic is having a devastating effect both socially and economically on the world and there is the urgent need for an effective vaccine to be produced. This race has been ongoing for 25 years and after early optimism it has been accepted that the fight will be long and arduous. This however does not mean that the future is bleak, and novel ideas to combat the virus are continually being explored.

In 2007 it was estimated that there were over 33 million people infected with HIV, with approximately 2.7 million new cases and over 2 million Acquired Immunodeficiency Syndrome (AIDS) related deaths per year (UNAIDS Global Report 2008). These statistics show that the HIV pandemic is causing an ever-expanding living population of infected individuals, which needs to be controlled and reduced. Many current strategies to reduce the rate of infection are in place from education, to anti retroviral therapy. However the greatest benefit would be from an efficacious vaccine that is affordable in the regions that are most in need.

The majority of infections with HIV are in developing countries; with sub-Saharan Africa accounting for approximately 67% of all current infections and 75% of related deaths (UNAIDS Global Report 2008). Although the clinical symptoms of AIDS were originally identified amongst homosexual men, it is now more frequently spread by heterosexual intercourse and the overall frequency of infection is approximately 50:50 for men to women (UNAIDS Global report 2008). However the current rate of infection is reported to be about three times higher in young women than in men. Due to these circumstances preventative measures need to be found to control this ongoing spread of infection. It is imperative that any means of control, such as vaccines that are developed, are affordable and easily distributed to those countries that are in dire need.
With the understanding that a prophylactic vaccine would be the ideal way to fight this pandemic, work has been ongoing to attempt to find the solution ever since the virus was first identified. The first Phase I vaccine trial was undertaken in 1987 and today over 35 candidate vaccines have been investigated in Phase I/II trials (Girard et al., 2006). However, only three Phase III clinical trials have been completed, two without success (Cohen J 2003; McCarthy M 2003), but with the most recent showing promising results (Rerks-Ngarm et al., 2009).

Origins of HIV

Clinicians in San Francisco and New York described the first cases of AIDS in 1981. They were seemingly isolated incidents causing many odd occurrences in those infected. People were being affected by generally unheard of causes in healthy adults, such as Kaposi’s sarcoma and Pneumocystis Carinii pneumonia, and it was not envisaged that this was the first description of a new pandemic disease. Two groups independently identified a virus as the causative agent of AIDS. In 1983 Luc Montagnier’s lab in France identified a new retrovirus and termed it Lympadenopathy Associated Virus (LAV) (Barre-Sinoussi, et al., 1983); and in 1984 Robert Gallo’s Lab in the US identified a retrovirus (Gallo, et al., 1984) as the causative agent of this condition termed AIDS. Robert Gallo named the retroviral agent HTLV-III, as it was believed to be related to HTLV-I and HTLV-II. This however was subsequently proved not to be the case and this virus was found to be a lentivirus with a much more complex genome, now known as HIV-1. Currently HIV-1 is categorised into three major groups termed M, N and O, with group M being further distinguished into 9 subtypes (or clades - A, B, C, D, F, G, H, J and K), and 15 recombinant forms (McCutcheon, F.E., 2000; Robertson et al., 2000). The related HIV type 2 (HIV-2) was discovered later in Western Africa (Calvel et al., 1986). Although very closely related to HIV-1, HIV-2 has never expanded into a pandemic.

As the name suggests HIV-1 and HIV-2 cause infected individuals to become immunocompromised and succumb to opportunistic infections. The progression of disease is
slow due to an inexorable reduction in CD4+ cells and ultimately leads to AIDS, at which point the immune system of the host has been effectively destroyed and death is usually caused by opportunistic infections that do not generally cause concerns in healthy individuals.

Although related, HIV-1 and HIV-2 have different disease profiles. HIV-1 produces a more vigorous infection that progresses faster and that leads to much greater immunosuppresion. Whilst HIV-2 is capable of causing a similar disease profile, a large proportion of individuals infected with HIV-2 live with the virus for many years, and the virus is accepted to be less virulent (Rowland-Jones and Whittle, 2007). HIV is a zoonotic infection (Hahn et al., 2000) and the two HIV types have been identified as relating to different original simian immunodeficiency viruses (SIV). It is believed that they crossed the species barrier to humans at some time approximately 70 years ago (Korber, et al., 2000). HIV-1 results from the cross species transmission of SIVcpz, a natural infection of chimpanzees - and the original distribution and focal point of the origins can be fairly well correlated to the regions in which SIVcpz is found (Gao et al., 1999). HIV-2 results from a similar transmission event of SIVsm, (Gao et al., 1992) which is a natural infection of sooty mangabey, distributed primarily in Western Africa.

An important difference between HIV and its Simian homologue is that natural infection of primates with SIV does not lead to immunodeficiency disease (Gueye et al., 2004; Muller-Trutwin et al., 1996; Pandrea et al., 2003; Rey-Cuille et al., 1998; Silvestri et al., 2003). A crucial issue is whether evolution to co-existence within the natural host leads to the apathogenic nature of infection, or whether they are simply exogenous retroviruses that become more pathogenic and vigorous when they cross the species barrier.

SIV in captive animals was initially observed in 1985 (Daniel et al., 1985), when an unexpected cluster of lymphomas was observed in a colony of captive rhesus macaques held at the California National Primate Research Centre in Davis (Benveniste et al., 1986; Mansfield et al., 1995). The virus isolated from these animals was originally termed STLV-IV and was discovered to be able to infect all Asian macaque species and produce symptoms that parallel those suffered by humans infected with HIV.
Genomic arrangement of retroviruses

HIV and SIV are members of the Lentivirus subgroup of Retroviruses. The name Lentivirus comes from the Latin meaning ‘slow virus’ distinguishing them from the oncogenic subgroup of Retroviruses. Retroviruses are diploid, single stranded, RNA positive viruses that infect mammals. These viruses are unique in that they contain an enzyme termed reverse transcriptase (RT), which gives them the ability to synthesise DNA from their RNA template.

The genome of more simple retroviruses encodes for viral structural proteins Gag, Pol and Env. The primate Lentiviruses – HIV and SIV - have more complex genomes and, from added genes, encode additional regulatory proteins. The HIV genome is 9KB, which is multiply spliced to encode for 9 genes (figure 1). These are gag, pol, env, tat, rev, nef, vpr and vpu (vpx in HIV-2 and most SIV isolates). Between them they are transcribed to produce the structural, regulatory and accessory proteins required by the virus.
Figure 1.1. The genomic arrangements of HIV-1, HIV-2 and the related SIV species. (adapted from Gordon et al., 2005).
Structural proteins

GAG: The \textit{gag} gene is expressed as a 55KDa polyprotein and is processed by the viral protease into 4 proteins. In the mature virion these structural proteins are the core/capsid (p24), the matrix (p17), the nucleocapsid (p7) and a protein termed p6. The capsid protein forms the core of the virion that contains the genome. For HIV-1, the core is also able to bind to cyclophilin A and enhance infectivity (Braaten et al., 2003; Franke et al., 1994). The viral matrix has a GlyGly motif that enables it to undergo myristoylation and thus target the Gag polyprotein to lipid rafts (Resh, M.D., 2005). This myristoylation of Gag is essential for viral replication. The Gag p17 protein is thought to interact with the envelope gp41 protein to restrict its diffusion in the envelope membrane and is also implicated in the nuclear import of the HIV pre-integration complex. The nucleocapsid p7 is a small protein that is required for assembly of virions, and for the budding of these from the cell membrane. It contains a zinc finger domain, which has been shown to be important for RNA packaging and infectivity (Aldovani, A., Young, R.A., 1990). The p6 interacts with the viral protein (vpr) and contains the late domain (PTAP) that binds the TSG101 component of the ESCRT-I pathway and participates in the terminal steps of virion budding (Garrus et al., 2001).

POL: The \textit{pol} gene is expressed as a 160KDa Gag-Pol precursor polyprotein that is processed by the viral protease and encodes the functional enzymes RT (p66), RNase H (p51), protease (p10) itself and integrase (p32). RT is an RNA dependant DNA polymerase, which is the enzyme that transcribes the viral RNA into DNA with the help of RNase H. The unique function of RT in mammalian cells makes it, along with protease, an attractive target for the development of anti-retroviral drugs. Integrase contains nuclear localisation signals, and is functionally essential for the integration of the virus genome into the cell DNA in the early phase of infection. Functional integrase cleaves bases from the 3’ prime end of synthesised viral DNA and ligates these cleaved ends into the cellular DNA (Reviewed in Brown, 1997).
Protease is translated as a Gag-Pol fusion product by ribosomal frameshifting and functions to cleave the Gag and Pol polyproteins into the functional protein components described above.

ENV: The \textit{env} gene encodes for a membrane bound, heavily glycosylated, envelope protein precursor (gp160) that is cleaved into the two envelope components of the virus (gp120 and gp41). For the virus to infect new cells the virion must fuse with the cellular membrane and transfer its genome into the cell’s nucleus. HIV utilises the envelope protein to selectively bind to the cellular receptor CD4 and a co-receptor on a cell’s surface, and subsequently fuse the two membranes. The synthesis of envelope starts in the Rough Endoplasmic Reticulum (RER) where a precursor of 845-870 amino acids is formed. The envelope precursor polypeptide gp160 is formed by the addition of Asparagine linked high mannose sugars. Gp160 is cleaved by cellular proteases in the Golgi to elicit the amino terminal, surface (SU), and carboxy terminal, transmembrane (TM), proteins gp120 and gp41 respectively (Earl et al., 1990; Moulard and Decroly, 2000). These two proteins form non-covalently linked trimeric heterodimers on the virion surface and are responsible for cell binding and cell-virion membrane fusion (Skehel and Wiley., 1998; Wyatt and Sodroski., 1998). There is suggested to be, on average, 14 functional trimers present on HIV virions, and 72 on SIV virions (Zhu et al., 2006).

Regulatory proteins

TAT: The \textit{tat} gene encodes for the transactivator of viral transcription (p14). This Tat protein binds to the transactivation responsive RNA element (TAR), and regulates viral transcription (Karn., 1999; Pumfery et al., 2003). In the presence of host cyclin II and CDK9 it enhances the RNA polymerase II elongation of the viral RNA template.

REV: The regulator of viral expression (Rev) protein, encoded by the \textit{rev} gene, is involved in sequence specific transport of unspliced and incompletely spliced viral mRNA from the nucleus.
to the cytoplasm. The protein functions by binding specifically to the Rev Responsive Element (RRE) target sequence (Daly et al., 1989; Malim et al., 1989, 1990). Through this, Rev regulates viral transport across the nuclear membrane and the change in splicing strategy from the early regulatory proteins to the late structural proteins.

**Accessory proteins**

**NEF:** The *nef* gene encodes a 35KDa myristoylated protein. It was originally termed NEgative Factor, as it was believed to down regulate virulence and pathogenesis, since disruption of the gene was associated with increased replication in T-cell lines *in vitro*. There is significant sequence variation between Nef proteins of HIV-1 and HIV-2, and in both viruses it has been discovered to be indispensable to the virus for pathogenesis. Patients infected with a Nef disrupted virus – such as those from the Sydney blood bank cohort - have frequently been found as long term non-progressors (Kirchoff et al 1995; Learmont et al., 1999). Nef has been associated with a diverse range of functions. It interferes with membrane protein sorting machinery, affects signal transduction pathways and enhances viral infectivity (Anderson and Hope., 2003; Greene and Peterlin., 2002). It also down regulates CD4 (Garcia, and Miller, 1991) and major histocompatibility complex (MHC) I expression (Schwarz et al., 1996).

**VIF:** The viral infectivity factor (Vif) protein is encoded by the *vif* gene and acts at the last steps of the virus life cycle. This protein is essential for replication of the virus in human CD4+ cells (Gabuzda et al., 1992; Sova et al., 1995; von Schwedler et al., 1993) and is also thought to play a role in the un-coating of the virus in cells. In addition, Vif works to prevent the action of the intracellular retroviral restriction factor APOBEC3G that causes base changes in the negative strand of synthesised viral DNA. Vif acts by binding APOBEC3G and transporting it to proteosomes for degradation (Sheehy et al., 2002). This reduces the hypermutation of viral DNA leading to increased fitness of produced quasi species.
VPR: Viral protein, encoded by the \textit{vpr} gene, acts early in the virus life cycle, to enable the entry of the pre-integration complex into the nucleus (Heinzinger et al., 1994; Popov et al., 1998). Vpr also alters the cell cycle progression of infected cells, arresting them in G2 phase, retarding proliferation and inducing apoptosis by inactivating the Cdk1/cyclin B1 complex (He et al., 1995; Re et al., 1995). Furthermore, Vpr acts as a transcriptional activator of the HIV-1 LTR as well as host cell genes (Cohen et al., 1990; Kino et al., 2002).

VPU (VPX in HIV-2 and SIV): The \textit{vpu} (or \textit{vpx}) gene encode for Viral Protein (Unknown). This protein connects CD4 to the protease degradation pathway, which facilitates the release of virus particles (Bour et al 1999). It also down regulates CD4 and MHC I (Hussain et al 2008; Kerkau et al 1997) and facilitates the infection of macrophages and enhances viral dissemination, by antagonising the action of tetherin (Neil et al., 2008).

Viral replication

The replication cycle of HIV can be divided into two phases, those which lead up to the integration of provirus, and those which occur subsequently resulting in the production of new virions. The stages of the life cycle are depicted in figure 2. The binding of HIV involves the envelope subunit gp120 binding to the cellular receptor CD4 and the subsequent binding to a co-receptor. The most widely utilised co-receptor is the chemokine co-receptor 5 (CCR5) during the early stages of infection, and some isolates utilise the chemokine co-receptor 4 (CXCR4) which is readily detected at late stages of virus infection, and indicates the imminent progression to AIDS. Other chemokine receptors that have been identified as co-receptors include CCR2b, CCR3 and CCR8, as well as the chemokine receptor like proteins BONZO (STRL33), BOB (GPR15), and APJ (Berger et al., 1999). Once gp120 has bound to CD4 it undergoes a conformational change, revealing the co-receptor binding domains and enabling the secondary binding to occur (Chapham and Weiss, 1997; Feng et al., 1996; Moore, 1997). These two binding events combined bring the gp41 fusion domain into proximity with the cellular
membrane. Initiation of fusion is brought about by the insertion of a six helical bundle of gp41 into the cellular membrane. Following fusion of the viral envelope with the cell membrane, the core of the virion moves through the cell cytoplasm on microtubule networks and interacts with host factors, which open up the core releasing the viral RNA and proteins. During this part of the replication cycle, the viral RNA is copied into double stranded DNA by RT. RT lacks an effective proof reading system and due to this there is a high rate of mutation in the virus during replication in a host. This leads to quasi species being present in a host at times of high replication (Goff, 1990; Whitcomb and Hughes, 1992).

After the completion of reverse transcription, viral DNA is transported into the nucleus in a pre-integration complex that appears to involve the matrix, integrase, RT, Vpr and association of cellular proteins (Miller et al., 1997). Vpr directs the nuclear localisation and then host nuclear import mechanisms transport the pre-integration complex into the nucleus (Fouchier et al., 1997; Nie et al., 1998), where the catalytic activity of integrase completes the integration of proviral DNA into transcriptionally active regions of the host genome (Mitchell et al., 2004; Scherdin et al., 1990; Turner and Summers, 1999).

The integrated proviral DNA is transcribed, driven by transcription factors and HIV Tat (Herrman and Rice, 1995; Reines et al., 1996). Initially, multiply spliced transcripts are translated to produce the regulatory and accessory proteins. The unspliced and incompletely spliced viral RNA is transported out of the nucleus, with the assistance of Rev for translation, or encapsulation (full length RNA) into budding virions. Env precursor protein is synthesised in the RER, and then post-translationally modified in the Golgi to produce the two subunits that are present in the mature envelope spikes. CD4 present in the Golgi is down regulated by Vpu to prevent trapping of Env gp160 precursor and ensure the translocation of envelope subunits to the surface membrane. The structural proteins of the virus, Gag and Gag-Pol accumulate on the inner side of the cytoplasmic membrane and get shuttled into a budding virion by the ESCRT pathway, along with two full length stands of viral RNA (Bieniasz, 2006; Martin-Serrano et al., 2003). Once formation of the virion particle occurs the particle undergoes budding and
maturation processes via the action of viral protease. Viral protease cleaves itself from the Gag-Pol fusion protein and subsequently cleaves the Gag and Pol polyproteins into the functional protein components to then assemble mature virions (Strack et al., 2003; von Schwedler et al., 2003).
Figure 1.2. Steps in HIV replication. The early phase of the life cycle (1-4) incorporates steps that lead up to the integration of the provirus into the host genome. The late phase (5-6) involves production and release of new virions. (Deciphering AIDS Vaccines; IAVI report.org., 2006).
Infection and the immune response

After primary infection there is a burst of viral replication that peaks 1-3 weeks after infection and lasts for up to 8 weeks (Clark et al., 1991; Daar et al., 1991). There then follows a down regulation of viraemia and detectable virus in peripheral cells of infected individuals (Clark et al., 1991; Daar et al., 1991). The focus of the primary viraemia is the Gut Associated Lymphoid Tissue (GALT). In animal model studies the infection is established in CD4+ cells in the GALT within 1 week and leads to a dramatic loss of these cells (Mattapallil et al., 1998; Veazey et al., 1998). This loss of cells has also been confirmed in HIV infected individuals (Brenchley et al., 2004; Mehandrou et al., 2004). As the course of primary viraemia progresses, the reduction in viral load is associated with a corresponding increase in the levels of CD4+ T-cells. An incomplete recovery of these cells occurs as the viraemia resolves to set point level. The magnitude of this level is an indicator of the rate of progression to disease (McMichael and Dorrel., 2005). The reduction of viraemia from peak to chronic phase has been attributed to CD8+ T-cells, but also may be due to the restriction of the target CD4+ T-cell population (Simon et al., 2006).

After primary infection, 70-80% of individuals progress typically, distinguished by a clinical latency of infection for approximately 6-8 years. During this time however the virus is not dormant, and there is a continual turnover and infection of CD4+ T-cells (Buchbinder et al., 1994; Embertson et al., 1993; Pantaleo et al., 1991, 1993; Piatak et al., 1993). Approximately 10-15% of infected individuals will become rapid progressors; who without intervention will advance to AIDS in 2-3 years (Phair, 1994). The faster progression in these individuals may be associated with an inability to control primary viraemia, or a delay in raising a controlling response (Pantaleo and Fauci 1996). Alongside these groups are individuals who may have a delay in the progression of disease for a period of time much longer than is typically observed. These Long term non-progressors (LTNPs) have been associated with disrupted or attenuated viruses, stable anti-HIV CD4+ and CD8+ specific T-cell counts, low viral loads, genetic factors,
high neutralising antibody titres and soluble anti-viral factors for this extended time period (Buchbinder et al., 1994; Cao et al., 1995; Chun et al., 2001; Deacon et al., 1995; Easterbrook P.J., 1994; Kirchoff et al., 1995; Levy J.A., 1993; Martinez et al., 2005; Pantaleo et al, 1995; Rinaldo et al., 1995; Schrager et al., 1994; Sheppard et al., 1993; Telenti and Bleiber 2006). Although most individuals will eventually progress to disease (Lefrere et al., 1997), the mechanisms of this “viral control” in LTNPs are of interest.

Viral control by the immune system plays a role in delay of disease progression, but is unable to clear infection. But as in all infections, HIV elicits a strong immune response, however unlike other responses it is not protective and is eventually suppressed. There are two forms of immune response in higher vertebrates - the innate (non-specific) and adaptive (specific) responses.

The innate immune system is comprised of a network of cellular, intracellular and extracellular factors that provide the first line of defence against invading pathogens. The role of the innate response in limiting viral replication and disease progression is the focus of much current research. Early innate responses also include activation of complement and secretion of pro-inflammatory chemokines and cytokines, such as interferons (IFNs) and tumour necrosis factor α and β (TNF-α/β). Cells involved include Dendritic Cells (DCs), Natural Killer (NK) cells, phagocytosing cells such as macrophages or granulocytes and γδ receptor expressing T cells. These responses limit viral infection and damage before acquired responses arise, but do not provide any immunological memory. However, they are important in inducing and modulating the adaptive cellular response. Innate immune cells possess broad specificity, pattern recognition receptors (PRRs), that recognise relatively conserved common pathogen associated molecular patterns (PAMPs), and on detecting these initiate rapid effector response (Iwasaki and Medzhitov., 2010). Toll like receptor (TLRs) are a family of PRRs on plasmacytoid dendritic cells (pDCs) that trigger the innate response, with HIV being found to specifically trigger TLRs 7 and 8, leading to high level release of IFN-α, TNF-α, IL-6 and IL-12 (Beignon et al 2005).
Type I IFNs (IFN-α/β) play a central role in immune responses against viral infection. Plasmacytoid DCs (pDCs), found in peripheral blood and T-cell rich areas of secondary lymphoid tissue, are the principal producers of these cytokines (Asselin-Paturel et al., 2005). Type I IFNs have a direct anti-HIV effect (Yamamoto et al., 1986), interfering with viral growth, as well as activating other cells such as NK cells (Newman et al., 2007). NK cells are large granular lymphocytes that secrete granzymes, which induce apoptosis and DNA fragmentation, and perforin, which inserts pores into membranes to induce osmotic lysis. This allows NK cells to destroy virus infected cells that downregulate MHC class I molecules (Campbell et al., 1996; Colonna et al., 1997). NK cells have also been shown to enhance HIV-1 neutralisation by interaction with the antibody Fc (Forthal et al., 2005).

Complement as an antiviral factor is an important part of the host defence. It can act either via the classical pathway (through the interaction with immunoglobulins) or alternative pathway (through direct interaction with microbes) to neutralise viral infectivity. Early in HIV infection the envelope protein activates antibody independent complement, either by gp120 or gp41 binding to MBL or C1q components of the complement pathways respectively (Ebenbichler et al., 1991; Spear et al., 1991; Susal et al., 1994). After seroconversion, virus specific antibodies bind viral proteins to form complexes which enhance complement activation leading to C3 cleavage product deposition on the viral surface (Stoiber et al., 1997). During pathogenesis, complement and HIV interact at all stages (Stoiber et al., 2008), but HIV has developed resistance against complement mediated lysis. The virus incorporates membrane proteins derived from host cells, which can down regulate the complement cascade (Frack et al., 1996; Schmitz et al 1995; Takefman et al., 1998).

An important innate component of mucosal tissues are gamma-delta (γδ) T-cells, which are more primitive than the acquired immune response CD8+ or CD4+ T-cells (Boismenu and Havran., 1997). They perform a number of functions, including the production of TH1 or TH2 type cytokines (Ferrick et al., 1995), which enhance cell mediated or humoral adaptive immune responses respectively. They also express extracellular anti-viral factors such as the
chemokines RANTES, MIP1α and MIP1 β, which in vitro have been shown to block and down regulate CCR5 expression preventing both HIV infection (Alkathib et al., 1996; Cocchi et al., 1995; Dragic et al., 1996; Paxton et al., 1996) and SIV infection (Lehner et al., 2005).

In recent years, two innate intracellular retroviral restriction factors - APOBEC3G and TRIM5α - have been identified that inhibit HIV by a number of routes. APOBEC3G is associated with T-lymphocytes, macrophages and monocytes. The main mechanism of APOBEC3G is the deamination of viral cytidines to uridine (Bishop et al., 2004; Sheehey et al., 2002). This requires APOBEC to be packaged into virions, and leads to inhibition of viral replication. There is believed to be a second mechanism by which APOBEC3G works, which is thought to take place either just prior or at the point of reverse transcription of viral RNA (Chiu et al., 2005). The viral Vif protein is utilised to bypass this factor. Vif prevents the incorporation of APOBEC3G into virions, and upregulates the ubiquitination and degradation of this factor (Sheehey et al., 2002). TRIM5α is a member of the TRIpartite motif family of proteins. Stremlau et al (2004) and Sayah et al (2004) showed that TRIM5α is required for HIV-1 restriction in primate cells. During infection it recognises viral capsid core structures and works to inhibit HIV-1 infection at a post-entry, pre-integration stage.

The adaptive immune response is made up of two arms, the cellular and humoral responses. These responses are specific to particular antigens of invading pathogens, as opposed to the non-specific actions of the innate responses. These responses develop over time after infection (or vaccination) and give rise to memory responses, which lead to much faster responses following re-exposure to pathogens, and it is the target of vaccination to produce specific effective memory responses, able to mount defences soon enough to prevent infection. Cells involved in the adaptive immune response are CD4+ and CD8+ T lymphocytes and B cells. These cells mature through clonal selection and differentiation after infection leading to the specific cellular and humoral responses. Adaptive immune responses also serve to enhance innate immune responses. For example B cells produce antibodies that use effector mechanisms
of innate immunity, phagocytes and the complement system, to eliminate microbes, as well as mechanisms such as antibody dependant cellular cytotoxicity to destroy infected cells.

The cellular arm of the immune response, as it suggests, is the action of cells to prevent, isolate and destroy infections and pathogens. T cell responses are determined by the MHC molecules found on most cells. MHC I present foreign peptides produced by the processing of intercellular proteins through the endogenous pathway, whereas MHC II present extracellular proteins by internalisation utilising the exogenous antigen-processing pathway. CD8+ T cells are cytotoxic T-lymphoctes (CTL) and are involved in the killing of infected cells, and production of factors that can suppress viral replication and prevent infection of CD4+ T cells. CD4+ T cells are helper cells and recognise foreign proteins expressed in the exposed groove of MHC class II proteins on antigen presenting cells to elicit cytokines that help to regulate and maintain the functions of other cell types, such as CD8+ T cells (Altfield and Rosenberg, 2000), B cells (Clark and ledbetter, 1994) and macrophages (Brosterhus et al., 1999). CD8+ cells recognise small foreign antigen peptides in the exposed grove of MHC class I molecules on antigen presenting cells, to elicit the production of cytokines such as IFN-γ and chemokines such as MIP1-α and RANTES. The release by CD8+ T-cells of cytolytic factors such as perforins may also mediate specific lysis of cells (Shankar et al., 1998). A strong specific CD4+ response producing Interleukin-2 (IL-2) and IFN-γ has been observed in LTNPs and seems, along with a vigorous CD8+ T cell response and high neutralising antibody titre, to be highly important in controlling HIV. Studies in HIV and SIV have also shown the ability of cell mediated responses to control primary viraemia and viral burden in later stages of infection (Allen et al., 2002; Kaul et al., 2001; Stebbings et al., 2002).

Although strong anti-HIV T-cell responses have been reported, the virus has developed mechanisms to evade effective anti-HIV T-cell responses by down regulating the MHC class I proteins on the cell surface via the actions of Vpu and Nef (Hussain et al 2008; Kerkau et al 1997; Schwarz et al., 1996), a mechanism that may help infected cells evade cytotoxic T-cell recognition. Furthermore as the virus integrates into the host genome it can persist very
effectively from anti-viral cellular responses. In particular, if the virus is not replicating (or replicating at a very low and undetectable level) it can persist for many years in viral reservoirs before replication is triggered.

The humoral response is an antibody mediated response that blocks and prevents infection, as well as destroying some invading pathogens. Antibodies are produced by activated B-cells after they interact with MHC class II molecules and recognise their cognate viral peptides. Once activated in this way B cell clones further differentiate into plasma or memory cells, which all recognise the same antigen. Plasma B cells synthesise and secrete large amounts of antibody specific for the cognate antigen, whereas memory B cells are long lived cells that respond to re-exposure of the specific antigen. The production of memory cells as well as isotype switching of produced antibody is controlled by T cell cytokines. In most infections, antibodies bind to cell-free virus, contributing to the clearance of infection. In HIV infection however they are unable to clear the virus once infection has occurred.

Specific anti HIV antibodies have been detected around 2-4 weeks after infection, and can be identified before the primary burst of viraemia has subsided (Aasa-Chapman et al., 2004). Serum antibodies can be used diagnostically from this point (McMichael and Dorrel, 2005). The first antibodies produced include those to the structural protein Gag (McMichael and Dorrell, 2005), however, like the early anti-Env antibodies, these have no neutralising ability. The production of specific neutralising antibodies can be seen as early as 4 weeks after infection (Albert et al., 1990; Richman et al., 2003; Wei et al., 2003). Although, from this point onwards, neutralising antibodies are present throughout the course of infection, there are no clinical instances of clearance of the virus, even though continual high titres seem to correlate with long term non progression (Buchbinder et al., 1994; Cao et al., 1995; Levy J.A., 1993; Martinez et al., 2005; Pilgrim et al., 1997).

In animal model studies it has been demonstrated that specific anti-HIV antibodies can prevent the infection of cells by HIV-1 envelope expressing SIV chimeric virus (Baba, et al., 2000;
However the presence of virus neutralising activity in serum does not mean patients clear the virus. Early neutralising responses are not broadly cross reactive, but only capable of neutralising the infecting virus. Increasingly, multiple clonal responses to a number of epitopes on Env appear and can be seen in individuals with continual low viral loads (Scheid et al., 2009).

Humoral responses are hindered by a number of factors the virus utilises for survival. The Env protein has a number of mechanisms developed for immune evasion (which will be discussed later), and HIV has a number of pathogenic effects that damage B-cells (De Milito et al., 2001, 2004; Moir et al., 2004). During the course of infection, infected individuals eventually display B cell hyperplasia, increased levels of autoantibodies and hypergammaglobulinaemia (Nagase et al., 2001; Shirai et al., 1992; Yarchoan et al., 1986).

As there has been no observation of clinical clearance of the virus, the emphasis is placed on producing a vaccine that can prevent or reduce the actual primary infection. Immune responses have proved capable of defending against immunodeficiency virus challenge, with evidence of protection against HIV and SIV superinfection, and SIV and SHIV infection in animal models (Berman et al., 1990; Gonzales et al., 2003; Hu et al., 1992; Letvin et al., 1994). Antibody responses would hopefully be elicited together with good cellular responses to cope with clearing any virus that escaped the antibodies. The ultimate outcome would be sterilising immunity that would prevent infection.
Current therapy for HIV

It has been suggested that in HIV infection 99% of virus may be eradicated from the system, and in this state of undetectable viraemia, patients are outwardly healthy. This health can be helped with anti-retroviral (ARV) treatment, which helps to control infection, by the suppression of viral replication, and consequent increase in CD4+ cells. This has helped to reduce the rate of mortality associated with infection (Palella et al., 1998). Drug therapy to HIV has been moving forward since the first ARV treatment was licensed in 1987. This first drug AZT (Mitsuya et al., 1985) is an RT inhibitor. There are 6 groups of ARV’s to HIV currently licenced in Europe and the USA (table 1). These are nucleoside, and non-nucleoside RT inhibitors, protease inhibitors, integrase inhibitors, entry inhibitors, and fusion inhibitors. Inhibition of reverse transcription is utilised for drug therapy by two mechanisms, in the first nucleoside RT inhibitors act as chain terminating factors once incorporated into cDNA strands as they lack 3’-OH groups (Parker et al., 1991), whereas non-nucleoside inhibitors act via allosteric hindrance by binding to a pocket in RT that inactivates the enzyme (Esnouf et al., 1995). Protease inhibition prevents the processing of Gag and Gag-Pol precursor polyproteins leading to the production of non-infectious particles (Patick et al., 1998). Integrase inhibitors work by binding to a region of Integrase that is required to bind target DNA. This competition for binding then prevents the strand transfer from occurring (Espeseth et al., 2000). All of the above described treatments function in the cell after infection has occurred, however newer strategies are trying to prevent this. Entry inhibitors work on blocking the binding of gp120 to co-receptors by interacting with the CCR5 and preventing access, whereas fusion inhibitors bind to gp41 heptad repeat sequences and prevent the two cellular and viral membranes from fusing (DeClerq et al., 2002; LaBranche et al., 2001; Michael and Moore., 1999; Siebert et al., 2006). Along with the problems of drug resistance which require continual examination of treatment regimes, ARV can have unpleasant side effects and be very expensive, and due to this the importance of either being able to permanently control viraemia, or remove infection, continue to be the ultimate aims.
<table>
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<tr>
<th>Approved antiretroviral drugs for the treatment of HIV infection</th>
<th>Approval date</th>
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<tr>
<td><strong>Entry inhibitors</strong></td>
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<tr>
<td>Maraviroc (UK427,857, Selzentry®)</td>
<td>06 August 2007</td>
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<td><strong>Fusion inhibitors</strong></td>
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<td>Enfuvirtide (T20, Fuzeon®)</td>
<td>13 March 2003</td>
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<td><strong>Integrase inhibitors</strong></td>
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<td>Raltegravir (MK0518, Isentress®)</td>
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<td><strong>Reverse transcriptase inhibitors</strong></td>
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<td><strong>Nucleoside/nucleotide analogues</strong></td>
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<td>Zidovudine (AZT, Retrovir®)</td>
<td>19 March 1987</td>
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<td>Didanosine (ddl, Videx®)</td>
<td>09 October 1991</td>
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<tr>
<td>Zalcitabine (ddC, Hivid®)</td>
<td>19 June 1992</td>
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<td>Stavudine (d4T, Zerit®)</td>
<td>24 June 1994</td>
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<tr>
<td>Lamivudine (3TC, Epivir®)</td>
<td>17 November 1995</td>
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<tr>
<td>Abacavir (ABC, Ziaagen®)</td>
<td>17 December 1998</td>
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<tr>
<td>Tenofovir (DF, Viread®)</td>
<td>26 October 2001</td>
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<tr>
<td>Emtricitabine (FTC, Emtriva®)</td>
<td>02 July 2003</td>
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<tr>
<td><strong>Non-nucleoside inhibitors</strong></td>
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<tr>
<td>Nevirapine (NVP, Viramune®)</td>
<td>21 June 1996</td>
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<tr>
<td>Delavirdine (DLV, Rescriptor®)</td>
<td>04 April 1997</td>
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<tr>
<td>Efavirenz (EFV, Sustiva®)</td>
<td>17 September 1998</td>
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<tr>
<td>Etravirine (TMC125, Intelence®)</td>
<td>18 January 2008</td>
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<tr>
<td><strong>Protease inhibitors</strong></td>
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<tr>
<td>Ritonavir (RTV, Norvir®)</td>
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<tr>
<td>Indinavir (IDV, Crixivan®)</td>
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<tr>
<td>Nelfinavir (NFV, Viracept®)</td>
<td>14 March 1997</td>
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<tr>
<td>Saquinavir (SQV, Fortovase®, Invirase®)</td>
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<td>Amprenavir (AMP, Agenerase®)</td>
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<td>洛匹那韦 (ABT-378, Kaletra®)</td>
<td>15 September 2000</td>
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<tr>
<td>Atazanavir (ATZ, Rezatraz®)</td>
<td>20 June 2003</td>
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<tr>
<td>Fosamprenavir (GW-433908, Lexiva®)</td>
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<tr>
<td>Tipranavir (TPV, Aptivus®)</td>
<td>22 June 2005</td>
</tr>
<tr>
<td>Darunavir (TMC-114, Prezista®)</td>
<td>23 June 2006</td>
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Table 1.1. Single antiretroviral drugs currently approved in the USA and Europe for the treatment of HIV-1 infection (adapted from De Clerq., 2009).
The target for neutralising antibodies to HIV is the envelope spike that is essential for binding and fusion of target cells (Burton and Montefiori 1997). There are two components of this envelope spike; the surface gp120 and transmembrane gp41. Gp120 mediates primary binding of virus to target cells. For HIV-1, HIV-2 and SIV the primary receptor is CD4 (Dalgleish et al. 1984; Sattentau et al., 1988). The protein binds to CD4 on T-cell surfaces, allowing conformational changes to then occur which facilitate co-receptor binding. In the case of HIV-1, CCR5 and CXCR4 are the usual secondary receptors recognised by gp120 (Feng et al., 1996; Moore., 1997). This double binding brings the virus and cell membranes, together allowing the gp41 to introduce the fusion peptide. Gp120 consists of 5 variable regions (V1-V5), as indicated in figure 3, and 5 conserved regions (C1-C5) (Starcich et al., 1986). The first four variable regions form loops, held together with disulphide bonds (Leonard et al., 1990). The V1 and V2 loops shield the CD4 binding site and part of the V3 loop prior to conformational changes (Zolla-Pazner., 2004), and can induce neutralising antibodies.

The trimeric structure of gp120 forms three distinct faces - the neutralising, non-neutralising and silent faces. The silent face of the protein is very heavily glycosylated (Leonard et al., 1990) and therefore is very poorly immunogenic. This is most likely due to the glycan sugars being recognised as 'self' and therefore not inducing any antibody response. Reitter et al (1998) and Reynard et al (2007) have suggested that the removal of glycosylation sites on virus envelope leads to an increased neutralising response when compared to the wild type virus in rhesus macaques, and rabbits respectively. The non-neutralising face is the conserved inner core of the envelope trimer. It is therefore not accessible to antibodies when on the virion in the spike formation. However many antibodies to this region can be elicited by monomeric gp120 or gp160. This may be an immune evasion tactic of the virus, as gp120 can be shed as a monomer, and gp160 can be found in abundance in dead cells. These are highly immunogenic, and may produce an immunodominant response, which will be irrelevant for binding to native
virions, and as such will be ineffective in helping the immune system block cell binding. The neutralising face of the virus covers the CD4 binding site, and also CD4 induced antibody binding sites. This region also encompasses the V1/V2 and V3 regions of gp120 which mask the conserved sites. Antibodies to the conserved regions of Env bind to monomeric proteins preferentially over native Env, suggesting these regions are relatively inaccessible on virion spikes (Moore et al., 1994).

The V3 loop of HIV-1 has held the most interest for inducing responses. Not only is this an immunodominant region, it determines tropism and co-receptor usage of the virus (Chesebro et al., 1988; Fouchier et al., 1992). The V3 loop contains the greatest number of determinants for neutralisation in tissue culture laboratory adapted (TCLA) viruses and was originally proposed as an important immunogen component (Jahaverian et al., 1989; LaRosa et al., 1990; Rusche et al., 1988). However, the discovery that V3 antibodies do not neutralise primary isolates efficiently, reduced the focus on the V3 (Matthews et al., 1994). Primary isolates can sequester the loop and make it inaccessible (Bou-Habib et al., 1994), as opposed to the more accessible loop found in the ‘open’ confirmation that envelope spikes are held in when viruses have become adapted for tissue culture (Kwong et al., 2000). In addition, hindrance by glycans, sequence variation, and V1/V2 masking may also be reasons for the inability of most V3 monoclonal antibodies (mAbs) to neutralise primary isolates (Gram et al., 1994; Krachmarov et al., 2005, 2006; Pinter et al., 2004; Schonning et al., 1996). One of the most highly immunogenic regions of the virus is a ‘crown’ of 4 amino acids on the V3 loop. The majority of non B clade isolates of HIV carry a GPGQ motif, whereas in most B clade isolates this is GPGR (Gorny et al., 2002; Krachmarov et al., 2005). The variation in this motif can be seen to restrict neutralising ability of specific mAbs (Pantophlet et al., 2008), and anti-B clade antibodies tend to be ineffective against non B clade isolates. The majority of interest has been in the responses to B clade V3, and a switch to investigating non B clade responses may be beneficial to isolating broader neutralising characteristics, as it has been suggested that non B clade V3 mAbs have a more potent and broader neutralising ability (Gorny et al., 2006).
Figure 1.3. The primary structure of HIV-1 gp120 (A) Schematic of the protein structure indicating the location of the hyper variable regions of the sequence (V1-V5) and putative N-Linked glycosylation sites. The branched chains indicate sites containing high mannose and/or complex hybrid type oligosaccharide structures and the U shaped branches indicate sites containing complex type oligosaccharide structures (adapted from Leonard et al., 1990). (B) The approximate location of gp120 structures that are involved in protection from antibody responses is shown. Along with the variable loops V2, V3, V4 and V5 the N-linked glycosylation sites are shown in blue. (C) The relationship of the different surface faces of gp120 is depicted. The surface of gp120 that interacts with neutralising antibodies is shown in green. The surface of gp120 that interacts with non neutralising antibodies is depicted in red. The heavily glycosylated surface of the gp120 outer domain, which appears to be minimally immunogenic, is shown in yellow (adapted from Wyatt and Sodroski., 1998).
Gp41 (figure 4) is a transmembrane protein anchored into the virus lipid envelope. This protein is held in a coiled coil formation, until binding of gp120 with CD4 and secondary receptor (CCR5 or CXCR4) has occurred, then the protein opens up, which allows the amino terminal fusion peptide of gp41 to interact with the cell membrane. Fusion occurs whereby the gp41 protein ‘jacksnifes’ to produce a six-helix bundle of heptad repeats (HR). Three HR2 repeats form up in anti-parallel fashion in the hydrophobic grooves of three HR1 regions (Chan et al., 1997, Chan and Kim., 1998; Furuta et al., 1998; Weisenhorn et al., 1997). This draws the two membranes together and allows insertion of the fusion peptide into the cell membrane, and subsequently the insertion of the virus genome into the cell. It is the transition of HIV-1 into this six helix bundle that mediates fusion, and not the bundle itself (Melikyan et al., 2000).

Gp41 has low immunogenicity before fusion occurs. This is due to the majority of gp41 being masked by the gp120 when the spike is unbound. Once bound the gp41 fuses with the cell membrane. This process is fairly quick, and this coupled with the lack of space available makes antibody binding for neutralisation at this point very unlikely. However during this process, gp41 occupies an intermediate state known as the ‘prehairpin’ intermediate. In this state gp41 is in an α helical coiled coil (Chan et al., 1997; Tan et al., 1997; Weissenhorn et al., 1997), which is a target for fusion inhibitors, such as the licensed peptide Enfuvirtide.
Figure 1.4. The primary structure of HIV-1 gp41. (A) Schematic of the protein structure indicating the intracellular and extracellular domains as well as key structural and immunological regions. Possible glycosylation sites indicated by the branched structures. Solid circles indicate hydrophobic amino acids; unfilled circles indicate charged amino acids and heavy outlined circles represent neutral amino acids (adapted from Gallaher et al., 1989). (B) Formation of the hairpin structure leading to membrane fusion. The native trimer of gp120 (green)/gp41 (blue) binds with cellular receptors, the envelope complex undergoes a conformational change to the prehairpin intermediate, in which the fusion peptide (red lines) is inserted into the target membrane and the N peptide region (gray) is a trimeric coiled coil. The prehairpin intermediate resolves to the fusion-active hairpin structure when the C peptide region binds to the N peptide coiled coil and adopts a helical conformation. The interactions necessary for fusion are unknown, but may involve aggregation of gp41 trimers to form fusion pores. After fusion is completed, the fusion peptide and the transmembrane segment (purple lines) of gp41 lie within the same membrane (adapted from Chan and Kim., 1998).
Mechanisms of immune evasion by envelope proteins

There are a number of features of HIV-1 envelope that result in the virus being able to escape potent autologous neutralising responses (Albert et al., 1990; Richman et al., 2003; Wei et al., 2003), and these may be driven by the neutralising response of the host (Beaumont et al., 2001; Frost et al., 2005).

The gp120 can be shed as monomeric protein, which can lead to an antibody response that is targeted against irrelevant regions of the both the gp120 and exposed gp41. Immature gp160 protein can also be shed to elicit irrelevant responses to the protein, which are not exposed on the natural virus envelope spikes (Moore and Sodroski, 1996; Wyatt et al., 1998). Env is very highly glycosylated with nearly 50% of its molecular weight being accounted for by host derived asparagine linked carbohydrate (Leonard et al., 1990). This shields the ‘foreign’ protein backbone with ‘self’ derived high mannose sugars, leading to a proposed ‘glycan shield’ (Wei et al., 2003). The protein is highly folded, and this folding hides the regions that are highly conserved such as the CD4, and co-receptor binding regions (Kwong et al., 2000; Labrijn et al., 2003; Myszka et al., 2000; Wyatt and Sodroski, 1998). The main exposed regions of the protein are highly variable, and mutate rapidly, leaving the immune system trying to recognise a constantly shifting target (Pinter et al., 2004; Starcich et al., 1986). The virus also has the ability to spread directly from cell to cell utilising an induced virological synapse, caused when cellularly expressed gp120 binds to CD4 on a neighbouring cell (Jolly et al., 2004) thus avoiding antibody in the periphery.
The role of neutralising antibodies in protection against infection

Neutralising antibodies target the envelope protein, and neutralisation occurs by antibodies binding to and blocking the functional regions of envelope gp120/41 trimers (Fouts et al., 1997; Parren and Burton, 2001; Poignard et al., 1996). It is likely that any effective antibody based HIV vaccine will require an immunogen that elicits a broad neutralising response (Burton et al., 2004; Gallo, 2005; Garber et al., 2004). Neutralisation of viruses by antibody may be direct or indirect. Indirect utilises factors, such as complement or other cells (such as NK), to lyse or interact with the antibody-virus complex. Direct neutralisation is a physical blocking of virus attachment, fusion or even budding. However is the fact that the majority of antibodies produced during infection bind to non-functional regions of envelope and do not have neutralising activity (Herrera et al., 2003; Moore et al., 2006).

There is indirect evidence indicating that high levels of neutralising antibodies are associated with improved clinical prognosis, as many LTNP’s have high titres of neutralising antibodies. It has been demonstrated in-vitro that the neutralisation begins with a single antibody molecule binding to a trimer (Schonning et al., 1999; Yang et al., 2005,a,b), and progresses to complete neutralisation when trimers are saturated (Crooks et al., 2008). However, it is believed that it is impossible for antibodies to eradicate infection with HIV therapeutically because of the persistence of infection in virus-infected cells and the inability of antibodies to kill virus infected cells. Nonetheless, studies remain focussed on whether the pre-existence of potent neutralising antibodies produced by vaccines prior to virus exposure could have a protective effect against HIV-1. A practical challenge in the development of immunogens capable of eliciting a broadly effective neutralising response is the ability of the virus to change as a result of culturing in vitro. As part of the process of adaptation it appears that the virus changes its envelope conformation slightly, leading to the exposure of critical neutralising epitopes. As a result many antibodies can neutralise these TCLA viruses, but these same antibodies are ineffective against primary isolates. In particular, research indicates that anti-V3 specific
antibodies do not play any significant role in the neutralisation of primary HIV-1 isolates, but are able to readily neutralise a number of T-cell line adapted ones, frequently in a type specific manner (Matthews et al., 1994). Only a few mAbs that neutralise a broad range of HIV-1 isolates, including primary isolates, have been identified. These have been isolated from patients who were clinical LTNP’s. These antibodies termed b12, 2G12, 2F5 and 4E10 have been demonstrated to neutralise a broad range of isolates in vitro, and to protect in in-vivo passive transfer studies in macaques (Baba, et al., 2000; Hofman-Lehmann, et al., 2001; Mascola, et al., 1999, 2000; Parren, et al., 2001; Veazey, et al., 2003). However, passive administration of antibodies is not a feasible way to provide prophylactic protection except in a few specific situations, as the half-life of antibodies is approximately 4 weeks. Nevertheless, these observations provide evidence that prophylactic vaccines that elicit antibodies of an appropriate specificity could be beneficial. Unfortunately the potent and broadly neutralising antibodies used in these studies are all in some way unusual, and it has been theorised that individuals that harbour these and other broadly cross neutralising antibodies are infected with viruses which have unusually immunogenic Envs (Cham et al., 2006).

The antibody b12 recognises an epitope that overlaps the CD4 receptor binding site on gp120 (Burton et al., 1994, 2004; Roben et al., 1994). However X-ray crystallography has revealed that that the relatively high affinity of this antibody is achieved through the selection of an unusually long complementary determining region (CDR), which may allow the antibody to ‘dock’ into the hydrophobic binding pocket (Barbas et al., 1992).

2G12 recognises carbohydrate residues on gp120, and prevents binding to CD4. The carbohydrate dependant epitope involves the C2 and C3 regions around the base of the V3 loop, the V4 loop and the C5 region. The crystal structure of 2G12 indicates a very unusual immunoglobulin (Ig) structure in which the variable heavy and light chains of corresponding Fab fragments are swapped leading to an expanded binding groove as seen in Figure 1.5. (Trkola et al., 1995, 1996; Sanders et al., 2002; Scanlan et al., 2002).
Figure 1.5. The crystal structure of the neutralising mAb 2G12. The light chains are shown in Cyan, and the Heavy chains of each Fab in red and purple. The swapping of the variable domains between Fabs can clearly be seen. (adapted from Calarese et al., 2003).
2F5 binds to the ‘ELDKWAS’ region of gp41 (Muster et al., 1993; Purscher et al., 1994; Zwick et al., 2001) and probably prevents post CD4-binding fusion of viral and cellular membranes. Although this mAb has been mapped to a linear epitope, all in vitro attempts to recreate this mAb have failed (Liang et al., 1999), possibly due to the involvement of lipid membranes in the epitope conformation.

4E10 binds C terminally of the ELDKWAS region recognised by 2F5. The epitope is an ordered helical structure created by the peptide NWFDIT (Steigler et al., 2001; Zwick et al., 2001). It is very broadly effective at neutralising HIV-1 isolates in-vitro, and has been shown to neutralise representatives of Clades A, B, C, D and E. Interestingly in the first study, it was found that viruses resistant to 2F5 were susceptible to 4E10 and vice versa. No isolates tested in the first study were resistant to neutralisation by both mAbs. The original 4E10 antibody isolated was an IgG3, but it was re-cloned as an IgG1. This change was found to increase the neutralising capacity of the antibody, contradicting in vitro experiments suggesting that IgG3 has a higher neutralising potential than IgG1 (Schwarf et al., 2001) This function, however, may be dependent on the specificity of the antibody rather than the subtype.

The two most potent of these neutralising antibodies - 2F5 and 4E10 - have been shown to react with the host phospholipid cardiolipin (Haynes et al., 2005). This suggests the possibility that current immunogens to HIV-1 envelope will not be able to elicit antibodies of these specificities due to tolerance of self antigens and T-cell suppression rather than T-cell help against these epitopes.

A number of passive immunisation studies in primates have shown that combinations of these human derived mAbs can protect against challenge with a HIV/SIV chimeric virus, but they obviously do not protect in clinical infection. Whilst there is evidence that antibodies of this specificity can delay viral rebound in a prophylactic setting (Trkola et al., 2005), the challenge is to generate these antibodies by immunisation. It also reveals a probability that other broadly
cross reactive epitopes may exist on the HIV-1 envelope that could be the target of serological mediated protection, but which cannot be generated without effective vaccine platforms.

All of the current effective neutralising antibodies are of the IgG class. These are the most abundant form of antibody in the immune system. However the main route of transmission for HIV is via mucosal membranes, where IgA antibodies are also abundant. Within groups of people exposed to the virus, but who remain sero-negative, mucosal IgA has been shown to be present, and have neutralising ability to primary viruses (Devito et al 2000; Mazzoli et al 1999).

It may be that in order for a vaccine to be effective, a neutralising IgA response as well as a neutralising IgG response may be necessary.

Vaccine research

For most licensed viral vaccines, protection correlates with the generation of appropriate titres of virus neutralising antibodies, in an appropriate \textit{in vitro} neutralisation assay (Zinkernagel 2003). For HIV vaccines there are two challenges; are vaccines able to elicit antibodies above sufficient levels to protect, and what type of assay of anti-virus activity is appropriate to provide an \textit{in vitro} correlate of vaccine protection \textit{in vivo}. Indeed many believe that an effective HIV vaccine will need to elicit both cellular and humoral responses to protect. Whilst an antibody based anti-viral vaccine would only require envelope as its target antigen, a vaccine that is designed to generate both a cellular and a humoral responses against HIV would need many components, and be able to overcome the intrinsic properties of HIV that allow it to evade both cellular and neutralising responses (Feeney et al., 2005; Goulder et al., 2004; O’Conner et al., 2001; Richman et al., 2003; Wei et al., 2003).

The appearance of the AIDS epidemic coincided with the development of a range of novel recombinant vaccine platforms, and many successful human and veterinary vaccines based on this technology exist, such as Hepatitis B and Rabies. DNA immunisation, vectors (both viral
and bacterial) expressing specific proteins, and recombinant envelope proteins, have been evaluated for HIV vaccines.

Recombinant plasmid DNA has been shown to be immunogenic and has been used to deliver HIV Gag, Env and Pol antigens, which have shown the ability to produce both T-cell and antibody responses in mice. In pre-clinical studies these vaccines appear to have limited effectivity, even though protection against SHIV challenge in macaques (Barouch et al., 2000; Letvin et al., 1997) and HIV in chimpanzees (Boyer et al., 1997) has been observed. However when they are used in combination with other recombinant vectors the responses found have been markedly higher (Otten et al., 2005; Wang et al., 2005). Boosting has been tested with many vectors such as Modified Vaccinia Ankara (MVA) (Amara et al., 2001; 2002), and with recombinant proteins (Wang et al., 2006). MVA has also been evaluated as a vaccine candidate alone in clinical trials showing promising induction of responses (Cebere et al., 2006). Boosting with heterologous gp120 has been shown to improve the antibody response elicited after priming with canarypox and adenovirus vectors expressing gp160, with a greater ability to neutralise TCLA and primary isolates in clinical volunteers. (Clements-Mann et al., 1998) and chimpanzees (Zolla-Pazner et al., 1998).

Other studies with novel vectors include work in the SIV/macaque model, utilising recombinant Herpes Simplex Virus (HSV) expressing Env and Nef proteins (Murphy et al., 2000), which has shown promising protection. Vectors have also been shown to be capable of altering the bias of the immune response. Semliki forest virus vectors expressing Env have been shown to elicit comparable responses to that of recombinant glycoprotein (rgp)140, but elicited a TH1 bias response rather than a TH2 bias response, usually seen with recombinant proteins (Forsell et al., 2005). Most of the work done to date has focussed on B clade, and in some cases E clade, vectors. However the examination of C clade vaccine candidates, with NYVAC (a highly attenuated vaccinia virus strain) or MVA expressing Gag Pol and Nef have shown good immunogenicity and produced broad cellular and humoral responses in mice (Gomez et al., 2007).
Virus Like Particles (VLPs) have been produced in a number of ways, including from baculovirus, vaccinia, adenovirus and yeast expression systems (Girard, M., 2006). When HIV or SIV Gag and Env proteins are expressed in these systems they can form into pseudovirion structures (Girard et al., 2006). These VLP’s are non infectious as they only contain the envelope and core proteins, and as such are an attractive idea for human immunisations. Particles like these have been tested in small animal and non-human primate models, both alone and in conjunction with vector priming (Hammonds et al., 2003, 2005; Radaelli et al., 2003). Testing of VLPs as mucosal immunogens has been moderately successful and a hopeful strategy for producing mucosal protection (Kang et al., 2003). Various forms of VLPs have been shown to be able to produce cellular and neutralising antibody responses (Deml et al., 1997, 2005; Montefiori et al., 2001; Wagner et al., 1998).

Subunit vaccines consist of various forms of viral proteins, including Env, Gag, Pol, Rev, Tat and Nef. Although in vitro studies have shown these types of vaccines to produce good responses (Belsche et al., 1998; Berman et al., 1990; Mascola et al., 1996; Selvarajah et al., 2005), this hasn’t been replicated in clinical trials. Due to the fact that monomeric proteins have proved unsuccessful in clinical trials, work has focussed on developing enhanced Env immunogens to elicit better broader responses. Studies have moved to examining gp41 peptides (McGaughey et al., 2003; Purtscher et al., 1996) and trimeric envelope gp140, which elicit better neutralising responses (Beddows et al., 2007; Earl et al., 2001; Grundner et al., 2005; Li et al 2006; Srivastava et al., 2003). Many novel strategies involving Env components have also been examined. Multiple subunit vaccines have been trialled, including those with envelope from different clade viruses, to look at the production of broad type responses (Chakrabarti et al., 2005). Zhang et al (2009) have shown that fusing gp41 to the Fc region of IgG1 prolongs the half life of the immunogen and may enhance the generation of broadly effective neutralising antibodies. It may be that a combination of these innovative strategies is required to produce a truly effective Env-based vaccine.
The first phase I clinical trials were undertaken in 1987 with recombinant gp120 proteins. Since then, many Phase I and II, as well as a three subunit Phase III, vaccine trials have been undertaken. The first two Phase III trials involved bivalent monomeric gp120’s. The trials took place in the USA and Thailand with clade B/B and clade B/E bivalent formulations respectively. Unfortunately neither showed any evidence of protection (Cohen J 2003; McCarthy M 2003). The third trial was also undertaken in Thailand and involved priming with a recombinant canarypox vector vaccine expressing clade A/E gp120, and clade B Gag and protease (ALVAC-HIV vCP1521), followed by boosting with clade B/E envelope (AIDSVAX) (McNeil et al., 2004). Preliminary results from this study appear promising with a vaccine efficacy of 31.2% (Rerks-Ngarm et al., 2009).

A cautionary note about vaccine trials has been raised by the failure of the Merck STEP phase II trial. This trial utilised three recombinant adenovirus (rAD5) expressing either Gag, Pol or Nef, with an aim to elicit strong T-cell responses. However the trial was halted early due to evidence of no protection, and in fact a possibility that the incidence of HIV-1 infection was actually increased in individuals having pre-existing immune responses to adenovirus (Flynn et al., 2008)

One of the main problems for vaccine development is the difference in subtypes of the virus. Due to the lack of immunogenetic similarities in HIV-1 subtypes, vaccines produced may have to have regional specificity. With the current situation of not having any known correlates of protection, the best strategy is to combine basic and clinical research to look into multiple vaccine technologies and candidates. Much work is being done to try to discover the correlates for protection that can be utilised to efficiently examine potential vaccine candidates. The biggest stumbling block to this process is the lack of natural correlates of protection in clinical LTNPs. Although it appears that certain factors may correlate, such as enhanced neutralising responses in in-vitro studies using sera from these patients, this can’t truly be proved, as there is no case of spontaneous protection against HIV-1. Even LTNPs eventually succumb to AIDS and AIDS related conditions, and there is an argument as to whether the responses shown in
these people are actually cause or effect of the delayed progression of disease. Aiming to replicate this long-term non-progression is a secondary consideration for vaccine technology; however it may also be a useful one. Any vaccine that can reduce viral load to undetectable levels, whilst preventing the necessity for ARV therapy may have a place in the fight to reduce the global impact of HIV-1 infection.

It may well be that the future of vaccines lies not entirely with the production of a protective neutralising response. Although this has been a successful measure for other viral vaccines, the nature of HIV integration precludes the ability of clearing virus once infection has taken hold. Due to this, any vaccine response that is to be antibody driven would have to prevent infection at the source. Research suggests that a vaccine response would have to have a strong early antibody response, but also a cellular response allowing the immune system to destroy any infected cells before viral replication can take over and allow the virus to ‘hide’. T-cell vaccines may well need to be used in conjunction with vaccines producing neutralising antibodies.

Animal Models

A crucial block in the development and evaluation of HIV vaccines has been the lack of clarity over a suitable model system in which to test them. Conventionally rodents would be ideal to evaluate immunogenicity, however mice and rats do not succumb to HIV infection, and so in the absence of information on the types of vaccine responses needed to confer protection, their value is limited to very early experimentation. As a result it was recognised that there was a need to identify and apply appropriate challenge models. Early work suggested that HIV would not replicate in non-human cells, however it became apparent that non-human primate cells could at least be infected in-vitro.
Work on identifying viable animal models began shortly after the identification of HIV, and chimpanzees were originally shown to be susceptible when seroconversion was demonstrated, with some animals also developing lymphadenopathy (Alter et al., 1984; Francis et al., 1984). It was also proven that gibbons are susceptible to infection with the virus, when Lusso et al (1988) showed that HIV-1 infection led to persistence without development to an AIDS like disease. In addition, early work showed that baboon cells could be infected with HIV-1 in vitro, but not in vivo, and that they are susceptible to HIV-2 infection (Letvin et al., 1987; Nicol et al., 1989). These animals, however are endangered, and are therefore not viable models. The moral and ethical issues of using these animals, together with the fact that they are very expensive to keep, outweigh any benefit. With the discovery of SIV, work started to look at the use of African and Asian primates, especially macaques (Desrosiers et al., 1989; Johnson and Hirsch., 1992).

Currently studies focus on the use of rhesus and cynomolgus macaques, which can be infected with SIV or an HIV/SIV chimeric virus called SHIV and lead eventually to an AIDS like disease. Most SHIVs are SIV backbone viruses engineered to express HIV B clade Env, Tat, Nef and Vpu (Bogers et al., 1997; Harouse et al., 2001; Hsu et al., 2005; Li et al., 1995; Luciw et al., 1995) that can cause AIDS like progression in macaques. The production of C clade SHIVs (Song et al., 2006) is moving forward allowing this model to be utilised for examining vaccines against this most abundant clade. This provides the most accurate in-vivo model available for the study of disease pathology and vaccine efficacy.

SIV and SHIV models allow the evaluation and dissection of the immunogenicity of vaccine candidates, and comparison to infection (Derby et al., 2006). In particular, work with the SIV/Macaque model has consistently shown vaccine protection against SIV infection (Carlson et al., 1990; Desrosiers et al., 1989; Langlois et al., 1992; Murphy-Corb et al., 1989; Stott et al., 1990). Neutralising antibodies and recombinant protein vaccines have been shown to protect against infection in these models, but repeatedly this fails to be replicated in clinical trials. This
does not mean there is no value in these models; as any information that can be gleaned about how the animals can fight infection after vaccination will help in future work.

Using these models it is also possible to investigate attenuated vaccines that cannot ethically be used in clinical trials. Attenuated forms of SIV have been shown to be effective as vaccines (Cranage et al., 1997; Daniel et al., 1992; Whatmore et al., 1995; Wyand et al., 1999), and if the mechanisms of this protection can be fully fathomed, it may allow them to be replicated without the use of the virus itself, thus taking out any associated risk of reconversion to pathogenesis, as has been evidenced (Baba et al., 1999; Hofman-Lehmann et al., 2003).

In contrast to some of the earlier work on small animal models, macaques can be infected by all the routes of human infection – intravaginal, intravenous and intrarectal, and by vertical transmission (Cranage et al., 1992; Ferrantelli et al., 2004; Mascola et al., 1999, 2000; Parren et al., 2001; Shibata et al., 1999). This all combines to provide a model that has the potential to provide a wealth of data about not only the infection, but pathology, disease progression and possible vaccination strategies.

Attempts to produce either a rabbit or rodent model were made early on in the search. With rabbits it was discovered that infection could lead to seroconversion (Kulaga et al., 1989; Truckenmiller et al., 1989). This however was much more marked when the animals were dual infected alongside HTLV-1 (Kulaga et al., 1989). There was however little pathological evidence and no AIDS like disease occurred. A number of different mouse models have been examined. These include the use of transgenic mice (Leonard et al., 1988), nude mice (Puddu et al., 1991) and normal mice (Locardi et al., 1992). However no productive infection in-vivo was demonstrated and, as with rabbits, the virus has to be introduced into thioglycate stimulated peritoneal cavities, which is a very unnatural route of infection.

Other more successful mouse models include SCID-Hu mice and Hu-PBI-SCID mice. The SCID-Hu model consists of human foetal thymus and liver being surgically implanted under the
kidney capsule of SCID mice (Bonyhadi et al., 1997; McCune et al., 1988). This forms a conjoint organ that phenotypically and histologically resembles normal foetal thymus. This allows for a dynamic system that has *in-vivo* cells in varying stages of differentiation as opposed to *in-vitro* cultures of peripheral blood lymphocytes (PBL). The Hu-PBL-SCID mouse model involves transplantation of Human PBLs or cord blood lymphocytes into the peritoneal cavity of SCID mice (Boyle et al., 1995; Tary-Lehmann et al., 1995). As these mice lack mature B-cells and T-cells it allows the human cells to graft and infection is possible. HIV infection leads to CD4 cell depletion, and so could be used to assess viral pathogenic properties and passive immune therapies.

Other natural infection model systems have also been used to look at different areas of infection. Many have been used at to try to examine HIV infection of the central nervous system. These include the use Feline immunodeficiency virus (FIV) and Ungulate Lentiviruses such as, Equine Infectious Anemia Virus (EIAV), Maedi-visna Virus (MVV), Caprine Arthritis-Encephalitis Virus (CAEV) and Bovine Immunodeficiency Virus (BIV) (reviewed in Nesbit and Schwartz, 2002).

Animal models are of vital importance to the investigations into producing vaccines to HIV. From mice to non-human primates, all may provide insights into how the immune system tries to fight the virus. However the SIV/SHIV macaque model is currently the most beneficial in the investigation of vaccine candidates against HIV-1.
Aims

The target of neutralising antibodies is the HIV-1 envelope protein and currently there is a vast amount of work being done on possible vaccine candidates that are using an envelope protein as a component. The overall lack of success of these vaccine candidates so far means that there is a need for a better understanding of the responses required for efficacy, and how to elicit these responses. This project aims to determine whether broadly neutralising antibodies are crucial in the development of an effective HIV-1 envelope based vaccines and whether they can be elicited by active immunisation. This will hopefully assist in the design and development of improved AIDS vaccines. To address this topic these specific aims have been set:

- To characterise specific serological responses present in immune sera that can protect against viral challenge \textit{in vivo}.
- To assess how the subtype of an envelope vaccine may affect its ability to elicit broadly cross reactive protective antibody responses.
- To examine the use of an alternative immunisation schedule to produce neutralising monoclonal antibody reagents to C clade isolates.
Chapter 2

Materials and Methods

2.1. Enzyme linked immunosorbant assays (ELISA) used in this project

2.1.1. Direct binding assays

Immunosorb 96 well polystyrene microtitre plates (NUNC, Roskilda, Denmark) were coated with relevant proteins or peptides by overnight incubation at 4°C with each well containing 50µl of recombinant proteins (see specific ELISA for coating concentration) diluted in Phosphate-Buffered-Saline (PBS). After washing 5 times with PBS/0.01%tween-20 (v/v) (wash buffer), remaining protein binding capacity in each well was blocked with 200µl of PBS/0.01% tween-20 (v/v)/5%porcine serum (v/v) (blocking buffer) for 30 minutes. Plates were then washed 5 times in wash buffer and 50µl of blocking buffer added to all wells in rows B-H. 67.5µl of blocking buffer was added to the wells in row A. 7.5µl of antibody sample was added to the top row resulting in a 1 in 10 dilution. The samples were then titrated three fold down the plates (rows B-H), by removing 25µl from row A and transferring into Row B. This was repeated stepwise down the plates (row B to C, C to D etc) and the last 25µl from row H removed and discarded. The plates were incubated at room temperature for 1 hour. Plates were washed 5 times with wash buffer before the addition of 50µl of horseradish peroxidase (HRP) conjugated secondary antibodies (SIGMA, Poole, Dorset) diluted 1 in 2000 in blocking buffer and incubated at room temperature for 1 hour. Plates were washed 5 times, in wash buffer before 50µl of TMB/DMSO substrate (BIO-RAD, Hercules, California) was added. Reactions were stopped after 10 minutes incubation at room temperature by the addition of 25µl 2M H₂SO₄ per well. The absorbance in each well at 450nm was determined using a Biotek microquant plate reader (Biotek, Vermont).
2.1.2. Peptide screening assay for schedule 152.1 samples

Assays were carried out as described above with the exception that the plasma samples used were started at a dilution of 1 in 20 and titrated in 2 fold steps down the plate by transfer of 50µl. Briefly this was done by adding 50µl of block to all wells in rows B-H. 95µl of block was added to the wells in row A. 5µl of plasma from immunised or naïve macaques, was then added to each well in the row A. The assay was then completed as described above (2.1.1).

2.1.3. Cell lysate binding

Investigation of the binding to infected cell lysates by mAbs or polyclonal sera/plasma used a “dry coating” procedure. NP-40 lysates prepared from C8166 cells infected with HIV-1 or SHIV, or uninfected control cells, were obtained from in house stocks. They were diluted 1 in 50 in PBS and 50µl of diluted material was used to coat each well by drying overnight at 37°C. Plates were then washed 5 times in wash buffer and blocked with 200µl/well of blocking buffer and incubated at room temperature for 30 minutes. Plates were washed 5 times in wash buffer and 50µl of blocking buffer added to all the wells in rows B-H. Dilutions of each plasma sample were made up to 1 in 30 in blocking buffer. 75µl of each dilution was added in triplicate to wells in row A. Samples were titrated three fold down the plate by removing 25µl from row A and mixing into Row B. This was repeated down the plate, and 25µl discarded from row H. Plates were incubated at room temperature for 1 hour, and then washed 5 times in wash buffer before the addition of 50µl of HRP conjugated secondary antibodies diluted 1 in 2000 in blocking buffer for 1 hour. Plates were washed 5 times, and 50µl of TMB/DMSO substrate added. Reactions were stopped after 10 minutes incubation at room temperature by the addition of 25µl 2M H₂SO₄ per well, and the absorbance of each well at 450nm was determined using the Biotek uquant plate reader.
2.1.4. Avidity assay

To investigate the avidity of antibody present in the plasma samples (from Schedule 152.1), binding ELISA assays were carried out in the presence of 35mM of the chaotropic agent Diethylamine (DEA – Sigma-Aldrich, Poole Dorset).

ELISA plates were coated either with recombinant protein or cell lysates of infected or uninfected C8166 cells as previously described (2.1.1 and 2.1.3 respectively). After blocking the assay was carried out as described in section 2.1.1 or 2.1.3, but with the addition of 35mM DEA to the buffer used to dilute the primary antibody stage.

2.1.5. Competition assays

To examine the ability of one antibody to interact (either in competition or facilitation) with a sample, for binding to epitopes, a modified ELISA protocol may be employed. Antibody – usually a m – is used at a dilution that gives 50% of maximal binding, and the sample under investigation is titrated on the plate. If the two antibodies present interact the binding of the mAb antibody will be affected.

Immunosorb plates were coated with relevant proteins or peptides by overnight incubation at 4°C with each well containing 50µl of recombinant protein diluted in PBS to the appropriate concentration. After washing 5 times with wash buffer, remaining protein binding capacity in each well was blocked with 200µl of blocking buffer for 30 minutes. Plates were then washed 5 times and 25µl of blocking buffer added to all wells in rows B-H. 45µl of blocking buffer was added to the wells in row A. 5µl of antibody sample was added to the top row giving a dilution of 1 in 10. The samples were then titrated two fold down the plates (rows B-H), by removing 25µl from row A and transferring into Row B. This was repeated stepwise down the plates (row B to C, C to D etc) and the last 25µl from row H was discarded. After the antibody to be
characterised had been titrated the competitor mAb was added in a volume of 25μl to all wells. The final concentration of the competitor had been determined previously by titration to obtain the 50% maximal binding to the antigen. The plates were incubated at room temperature for 1 hour, and then plates were washed 5 times in wash buffer. The subsequent steps of the assay differed depending on whether the competing antibody samples were from the same or different species. For samples that were of a different species from the competitor, the detection of bound competitor mAb, was performed by addition of the appropriate HRP-conjugate antibody and substrate, as described in section 2.1.1. By contrast where the competing samples were derived from the same species (or if they originated from human and macaques, which are cross reactive), the competitor mAb had been labelled by biotinylation and detection was performed by the addition of 50μl of streptavidin conjugated to HRP (Sigma-Aldrich, Poole, Dorset) at a dilution of 1 in 1000 before the plates were incubated for 1 hour. This was followed by the addition of substrate as described previously (2.1.1).

2.1.6. Statistics for ELISA based assays.

Midpoint and endpoint titrations were calculated from lines of best fit using data derived from the straight line portion of graphs, plotting absorbance against log sample dilution, produced using the EXCEL package of Microsoft Office. Statistical significance for the competition assays were based on data where n=3 and one way analysis of variance (ANOVA) was used to test the null hypothesis that the means of all animals were the same (p<0.001). This was followed with Dunnett’s and Tukey’s testing to compare the relevance of results in reference to a known control sample of naive plasma or to each other, respectively (p<0.05).
2.2. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and coomassie staining

SDS-PAGE is a technique used to characterise and visualise proteins and glycoproteins. Proteins separate on a basis of molecular size with smaller molecules migrating rapidly through an acrylamide gel matrix. In the work described in this thesis it was used to determine the size of recombinant envelope glycoproteins and investigate the oligomeric state of protein samples. Non-specific staining such as coomassie blue may be used to visualise all proteins present. However for the analysis of poorly purified mixtures of proteins, electrophoresis may be followed by electro-transfer of the proteins onto nylon or nitrocellulose membrane to permit probing with antibodies of defined specificity. For example, it was used here to investigate the binding of antibodies with unknown specificity, to recombinant envelope proteins, homologous with those used to immunise mice.

Electrophoresis was undertaken for this thesis using the Bio-Rad mini protean II kit (Bio-Rad, Hercules, California). Prior to starting SDS-PAGE the glass plates and spacers were cleaned with 70% Industrial Methylated Spirit (IMS). To assemble, a spacer is placed on either side of a long glass plate and a short plate placed onto these. The whole apparatus is placed into a casting assembly, which is tightened, ensuring the plates are flush at the bottom. Once placed onto the casting stand, the space between the plates is filled with fresh gel mix to 1.5cm below the top of the short plate. To resolve samples of HIV-1 envelope a 10% (v/v) acrylamide gel was prepared. Table 2.1 shows how different percentage gels are produced sufficient to prepare two gels. Once prepared the running gel was overlaid with water saturated Butan-1-ol, and the gel was left to set for 30 minutes at room temperature. Once set the butan-1-ol was removed and the top of the gel flushed with running buffer, the remaining space between the glass plates was filled with the 6%(v/v) acrylamide stacking gel mix and a comb placed into the top to produce the stacking wells, and the gel left to set for 30 minutes at room temperature.
After the stacking gel had set it was possible to remove the comb, and clip the sandwich of plates and gel into place on the core of the apparatus. The core was placed into the electrophoresis tank and the inner space filled with running buffer to the top of the gel, and the outer space was filled to the level of the lower white screws on the gel casting stands. Once filled with buffer and just prior to loading of samples a drawn out pastette was used to flush the wells out (to remove any excess acrylamide).
<table>
<thead>
<tr>
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<th>Stacking gel 6%</th>
<th>Final gel percentage</th>
<th>Running gels</th>
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<tr>
<td></td>
<td></td>
<td>7%</td>
<td>8%</td>
</tr>
<tr>
<td>30% acrylamide/bisacrylamide (ml)</td>
<td>1</td>
<td>2.3</td>
<td>2.7</td>
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<tr>
<td>4X Tris/SDS pH 8.8 (ml)</td>
<td>1.25</td>
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<tr>
<td>(pH 6.8 for the stacking gel)</td>
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<tr>
<td>Distilled water (ml)</td>
<td>2.75</td>
<td>5.2</td>
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<tr>
<td>Ammonium Persulfate (μl)</td>
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<td>TEMED (μl)</td>
<td>7.5</td>
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Table 2.1. The make up of different percentage running gels, and stacking gels for SDS-PAGE. The volume these ratios make is 10ml for the running gels, and 5ml for the stacking gel. This is sufficient to produce two complete gels.
2.2.1. Running samples

To prepare the envelope samples for analysis by SDS-PAGE they were diluted in ddH₂O to a nominal concentration of 2µg/ml. The samples were then mixed with 2X loading buffer (Invitrogen, Paisley) to give a final concentration of 1µg/ml. Samples were then held in a heating block at 100 °C for 5 minutes, and once cooled were briefly vortexed and then spun at 2000g for 30 seconds. 25µl of protein sample was loaded into a well. For comparison to relative molecular mass, 10µl of molecular weight markers (Fermentas, Burlington, Ontario) were loaded in a separate lane. To complete the electric current the lid was placed on the tank and run at 150V for 90 minutes or until the dye front had run to the end of the gel. After switching off the power the casting apparatus was disassembled, the gels were removed and processed for either coomassie staining to reveal total protein or for electroblotting onto nitrocellulose membrane for western blot analysis.

2.2.2. Coomassie staining:

Once proteins have been run on gels, the presence of all proteins can be verified by visualisation using a staining method. Coomassie blue stain binds to all proteins without prejudice and therefore was utilised to identify all proteins present in samples of HIV-1 envelope.

The gel was placed into coomassie stain (50% v/v distilled water/40% v/v methanol/10% v/v acetic acid/0.05% (w/v) coomassie brilliant blue R-250) for 1 hour with gentle rotation of the staining tray on an orbital shaker. The stain was removed and replaced with coomassie destain (50% v/v distilled water/40% v/v methanol/10% v/v acetic acid) and incubated for 30 minutes with gentle rotation of the tray. Destain was renewed and the gel incubated for another 30 minutes. The destained gel was removed, photographed and if necessary the gels were preserved by drying under vacuum using a Bio-Rad gel vacuum dryer for 1 hour at 80 °C.
2.2.3. Electrophoretic transfer: using Bio-Rad mini protean kit

Once proteins have been transferred to nylon or nitrocellulose membrane, antibodies may be used to identify whether particular proteins are present in a sample, or conversely if antibodies of unknown specificity recognise specific proteins.

After electrophoresis, gels were washed in transfer buffer (25mM tris pH8.3/192mM glycine) for 5 minutes. Filter paper and nitrocellulose membranes (Amersham, Buckinghamshire) were cut to the size of the gel. These were moistened with transfer buffer and then a transfer sandwich prepared on a transfer cassette, in the order; 3 pieces of filter paper, gel, membrane and 3 pieces of filter paper, between two moistened fibre pads (figure 2.1). All air bubbles were removed from the sandwich and the cassettes clipped shut. The cassettes were placed into the electrode transfer apparatus with the membrane side towards the anode and the gel towards the cathode.

The electrode transfer apparatus was placed into the tank alongside an ice pack, and the tank filled with transfer buffer. The tank was placed onto a stirring plate with the addition of a magnetic stirrer, and the lid placed on. Electrophoretic transfer was performed for 1 hour at 100V with stirring at 500rpm. When complete the membranes were removed and rinsed in washing buffer for western blotting. After disassembling, the gels were stained with coomassie blue (section 2.2.2) to visualise any residual protein in the gel that may be evidence of incomplete transfer of samples to the nitrocellulose membrane.
Figure 2.1. Formulation of the 'gel sandwich' is prepared for electrophoretic transfer of samples onto nitrocellulose membrane.
2.3. Western blotting

After disassembly of the transfer sandwich, membranes were washed for 15 minutes in wash buffer (PBS-A/0.1% Tween-20) with gentle rotation and then residual protein binding sites on the nitrocellulose membranes were blocked for 1 hour in blocking buffer (Washing buffer + 5% w/v Marvel skimmed milk powder). After a further washing for 15 minutes the primary antibody was added (diluted 1 in 2000) in blocking buffer and the membranes incubated at room temperature for 1 hour with gentle rotation on an orbital shaker. After incubation the membranes were washed 3 times for 5 minutes each time in washing buffer. HRP conjugated secondary antibodies were diluted 1 in 2000 in blocking buffer and added to the membranes, and then they were incubated for 1 hour at room temperature with shaking. After washing 3 times the membranes were rinsed in ddH₂O. The membranes were viewed with the chromogenic Diaminobenzidine (DAB) Vectastain kit (Vector Laboratories, Peterborough) prepared following the manufacturer’s instructions (4 drops of buffer, 8 drops of colour reagent and 4 drops of hydrogen peroxide added to 10ml of water). 5ml of this visualisation reagent was added to each membrane and incubated for 5 minutes. The membranes were washed with ddH₂O to stop the reaction and then air-dried before being photographed.

2.3.1. Western blotting for multiple antibody screening

For multiple screening of mAbs to a single envelope protein a Bio-Rad multi screen kit (Bio-Rad, Hercules, California) was used. The SDS-PAGE gels were produced using combs that provided a single long well for the sample and a single thin well for molecular weight markers. 300μl of sample was prepared in the same manner as previously described (section 2.2.1.) and loaded into the large well. The remaining steps of the SDS-PAGE and electrophoretic transfer protocols were undertaken as described previously (section 2.2.). For the western blotting, the membranes after blocking were placed into the multi screen kit. This comprises a slotted face plate that can be clamped onto the nitrocellulose membrane, to create a series of water tight
troughs on the surface of the nitrocellulose membrane. The multi screen kit is able to analyse 20 different mAbs on each membrane. The primary mAb samples were diluted 1 in 2000 in blocking buffer, and 600μl of diluted primary antibody was added to individual channels. After incubation with the primary antibody the troughs were washed with 600μl of wash buffer before disassembling the apparatus and processing, as described previously (section 2.3.).

2.4. Preparation of monoclonal antibody stocks

2.4.1. Propagation of hybridomas and preparation of monoclonal antibodies

Hybridoma's were removed from long term storage in liquid nitrogen vapour phase and thawed quickly (10 minutes at 37°C). The contents were added to 50 ml of ice cold sterile RPMI1640 medium (Gibco), mixed and spun at 500g for 5 minutes. The supernatant was discarded and the pellet resuspended in 5ml of RPMI1640 containing 10% foetal calf serum (FCS) (v/v) and 100IU penicillin/100μg streptomycin per 500ml (hereafter called cRPMI), and placed into a T25 tissue culture flask (Becton Dickinson, Franklin Lakes, NJ). Cells were grown up and seeded into T175 tissue culture flasks. When cells had reached a density of 1x10^6 cells/ml and were at least 90% viable, they were washed, and then seeded (at a density of 1x10^7 cells/ml) into a high production cassette for the Integra Biosciences Tecnomouse. The Tecnomouse system is a hollow fibre bioreactor that utilises cassettes similar to dialysis cassettes to allow the growth of high density cell cultures. The cassettes growing space is enclosed in a semi-permeable membrane that allows waste products to diffuse out of the culture whilst nutrients can diffuse in. The membranes also contain any secreted product such as antibody in the growing space allowing for samples of highly concentrated antibodies to be taken. Each cassette was sampled twice weekly for 15 weeks. 10ml of medium containing cells and antibody were collected and replaced with 10ml of fresh cRPMI. The cells were enumerated and viability determined, before sedimenting them by centrifugation at 500g for 10 minutes. The supernatant was retained and stored at -20°C.
2.4.2. Ammonium sulphate precipitation of monoclonal antibodies

To discriminate between related antibodies that cannot be distinguished by a secondary antibody, one of the antibody preparations may be biotinylated so that they may be identified with streptavidin conjugated to an appropriate detection system. This was required for some competition assays undertaken for this thesis.

Cellular supernatant fluid (Snf) from Tecnomouse samples containing mAb was spun at 3000g for 30 minutes. The Snf was mixed with an equal volume of saturated ammonium sulphate solution. This was gently stirred overnight at 4°C. The precipitate was sedimented by centrifugation (3000g for 30 minutes). The pellet was resuspended in 3ml of PBS pH7.4 and dialysed (PERBIO slide-a-lyser cassettes, molecular weight cut-off of 30KDa, Pierce, Northumberland) in 3L of PBS pH7.4, changing the buffer on 3 sequential occasions 24 hours apart.

2.4.3. Biotinylation of monoclonal antibodies

Ammonium sulphate purified mAbs were adjusted to 3mg/ml using spin columns (Sigma-Aldrich, Poole, Dorset) with a molecular weight cut-off of 100KDa.

Samples were then dialysed 3 times against 0.1M sodium hydrogen carbonate at 4°C. The sample was spun at 10000g for 2 minutes, and mixed with 114μl of N-Hydroxysuccinimidobiotin (Sigma-Aldrich) at a concentration of 10.2mg/ml, in dimethylsulphoxide (DMSO). After rolling at room temperature for 4 hours in the dark, the sample was dialysed 3 times against PBS at 4°C, aliquoted and then frozen at -20°C.
2.5. Preparation and manipulation of plasmid DNA

2.5.1. Transfection of E.coli cells by electroporation

XI-1 Blue competent E.coli cells were retrieved from -70°C storage and thawed. 40μl of cells was placed into an electroporation cuvette and 1μg of plasmid DNA (1μg/μl) added to the competent cells. The cuvette was placed on ice for 1 hour. After 1 hour the cuvette was electroporated with settings of 2.5 Kilovolts, 129 Ohms resistance, 900 microFarad Capacaitance and a pulse length of 6 milliseconds.

Immediately after electroporation, 960μl of Luria Broth (LB) (Gibco) was added and the sample was transferred to a Sarstedt tube and incubated at 37°C for 1 hour.

2.5.2. Transfection of E.coli cells by heat shock

Super competent E.coli cells were retrieved from −70°C storage and a sample was thawed. Cells were aliquoted into two 100μl samples and 1μg of plasmid DNA was added to one aliquot and 2μg to the second. Mixed cells and DNA samples were held on ice for 30 minutes before transfer to a water bath at 42°C for 2 minutes. Samples were removed and allowed to cool. 10μl and 50μl samples were removed from the batches of cells incubated with 1μg and 2μg DNA respectively and plated onto LB agar plates containing ampicillin as the appropriate selection antibiotic.

2.5.3. DNA maxipreps

A single colony of E.coli, obtained by "streaking out" transfected cells on LB agar plates containing the appropriate selection media, was removed and used to inoculate 5ml LB medium.
This was incubated at 37°C overnight with shaking. 5ml of this culture was added to 50ml of fresh LB medium containing ampicillin, and incubated overnight at 37°C with shaking. Cultures were grown for 24 hours and then the culture harvested and spun at 6000g for 15 minutes. Qiagen maxiprep kits (Qiagen, Crawley, West Sussex) were used to isolate plasmid DNA from the cell pellet using the method recommended by the manufacturers. Briefly, the supernatant was discarded and the cell pellet resuspended into 10ml of buffer P1 (100μg/ml RNASE A, 50mM TRIS-HCL, 10mM EDTA pH8), by repeated pipetting. 10ml of buffer P2 (200mM NaOH, 1%SDS w/v) was added and the sample inverted to mix. After 5 minutes incubation at room temperature, 10ml of chilled buffer P3 (3M KAc pH5.5) was added and the tubes placed on ice for 20 minutes. Samples were then centrifuged at 20,000g for 30 minutes at 4°C. The supernatant was removed and the centrifugation repeated. Qiagen-tip 500 columns were equilibrated with 10ml of buffer QBT (750mM NaCl, 50mM MOPS pH7, 15% isopropanol v/v, 0.15% Triton X-100 v/v), and the sample supernatant added to the column. The supernatant was allowed to run through the column by gravity flow, and the column was then washed with 10ml of buffer QC (1M NaCl, 50mM MOPS pH7, 15% Isopropanol v/v). DNA was eluted from the column by the addition of 15ml of buffer QF (1.25M NaCl, 50mM TRIS-HCL pH 8.5, 15% Isopropanol v/v). The eluted DNA was precipitated by the addition of 10.5ml of isopropanol, and pelleted by centrifugation at 15,000g for 30 minutes. Supernatant was removed carefully with a pastette and the remaining pellet washed with 70% ethanol, then centrifuged as before. The supernatant was removed and the pellet allowed to air dry for 10-15 minutes. The DNA was resuspended in 500μl of distilled water.

2.5.4. Restriction enzyme digests of plasmids

2μl of samples containing between 1 and 5ng DNA were mixed with 1μl of Eco R1 and Hind 3 restriction endonucleases (Gibco). 1μl of ‘Buffer B’ (Invitrogen, Paisley) and 5μl of distilled water was added. Samples were incubated at 37°C for 1 hour. Analysis of restriction was by
flat bed agarose gel electrophoresis. 10µl of sample was mixed with 10µl loading buffer (Invitrogen) and loaded into the well of 1% (w/v) agarose gel.

2.5.5. Agarose gel Electrophoresis

1% (w/v) agarose gels were prepared by adding 1g of agarose to 100ml of Tris acetate EDTA (TAE) buffer. The mixture was heated in a microwave for 30 seconds until all granules of agarose were dissolved. Gels were cast; a comb was inserted and the gel left to cool down. Once cool the gel was immersed in a tank filled with TAE buffer, the comb removed and then Plasmid DNA mixed 1:1 with loading dye was loaded into wells alongside φλ rDNA ladders (Gibco). The gels were run at 100V for 1 hour. The DNA fragments were visualised by staining with ethidium bromide (diluted 1 in 10,000) for 10 minutes and washing in distilled water for 10 minutes before UV transillumination, when gels could be photographed.

2.5.6. Plasmid DNA quantification

Samples were quantified by dot blotting against known concentrations of DNA. DNA standards of known concentrations of 50/25/12.5/6.25/3.125/1.56/0.78/0 ng/2.5µl were prepared. The plasmid samples for quantification were serially diluted 1in 2 from a neat stock solution. 2.5µl of standards were taken and mixed with 2.5µl of ethidium bromide (diluted 1 in 10,000). Samples containing unknown concentrations of DNA were prepared in a similar manner. The 5µl mixtures were dotted onto Saran wrap and then viewed by UV transillumination and photographed. The concentrations of the plasmid samples were then calculated by comparison of the intensity of the samples with the standards containing known amounts of DNA.

The calculation for quantifying DNA in this manner is:

\[
\text{Dilution factor of sample} \times \text{Standard concentration (ng)} = \text{Concentration (ng/µl)}
\]

2.5
2.5.7. Ethanol precipitation of plasmid DNA

The samples were mixed with a 1 in 10 volume of 3M sodium acetate pH5.2, and 2.5 volumes of 100% ethanol. The sample was incubated for 15 minutes at -20°C, thawed if necessary and the precipitate pelleted by centrifugation in a microfuge at 10000g for 10 minutes. The supernatant was discarded and the tubes inverted to air dry. Once dry the samples were resuspended in 500μl of sterile ddH₂O.

2.6. Fusion of splenocytes and myeloma cells.

mAbs were produced by the immortalisation of activated B cells by fusion with a myeloma cell line NS-0 that does not synthesise immunoglobulin.

2.6.1 Immunisation protocols and splenocyte fusion.

6 month old female Balb/C mice were immunized on 4 occasions by intra muscular injection of 50μg of plasmid DNA designed to express a relevant HIV-1 gp140 envelope glycoprotein, on weeks 0, 4, 8 and 12. Mice were bled by superficial tail vein puncture at week 14, and serological responses against the antigen of interest detected by ELISA (section 2.1.1). The animal with the highest titre of anti envelope seroreactivity was then boosted by intra-peritoneal immunisation with 50μg of recombinant HIV-1 gp140 envelope protein on three consecutive days. After a rest day, the boosted mouse was killed humanely and the spleen removed.

The whole spleen was rinsed in serum free cRPMI (sfRPMI) media. The spleen cells were disaggregated by teasing with forceps and aspiration with a pipette. Cells were washed in sfRPMI 3 times, resuspended and centrifuged at 500g for 5 minutes, then resuspended in sfRPMI. The NS-0 cells were washed in a similar manner. Spleen cells were counted and mixed with NS-0 cells at a 4 to 1 (NS-0 to spleen cell) ratio and the cells sedimented by
centrifugation at 500g for 5 minutes. The supernatant was discarded and the cell pellet air dried for 5 minutes. 1ml of 50% polyethylene glycol 1500 (Hybrimax PEG obtained from Sigma-Aldrich) was added drop wise over 1 minute. 1ml of sfRPMI was added dropwise over 1 minute, and then 10ml of sfRPMI over 10 minutes. Cells were spun at 500g for 5 minutes and the cell pellet was resuspended in cRPMI/ with the addition of 100IU/hypoxanthine, aminopterin, thymidine (HAT) (Sigma-Aldrich) and recombinant human IL-6 at 80pg/ml (obtained from Dr S.Poole, NIBSC). Cells were dispensed into sterile 96 well tissue culture plates (200μl per well) and incubated in a humidified atmosphere at 37°C 5%CO₂.
IMMUNISATION

50ug DNA immunisations on weeks 0, 4, 8 and 12.

Immunisation boost with 50ug recombinant protein on 3 consecutive days

Fusion and growth of cells in selective media

Screening by ELISA

Cloning by limiting dilution and screening at intervals to isolate specific mAb producing hybridoma colonies.

Selection and proliferation of positive hybridoma lines

Figure 2.2. Flow diagram of novel DNA prime/protein boost protocols used to immunise mice for the production of mAbs.
2.6.2. Screening of colonies

The production of relevant antibodies by hybridoma colonies was assessed by screening tissue culture supernatant from individual colonies by ELISA.

After approximately 10 days culture, wells containing colonies of HAT resistant cells become acidic (yellow), and the supernatant collected from wells exhibiting at least 50% confluence was analysed for specific antibody production by ELISA. Neat supernatant was added to wells of an NUNC immunosorb microtitre plates coated with an appropriate antigen prepared as previously described (section 2.1.1). Specific binding was assessed by the sequential addition of HRP conjugated anti mouse antibody, and ELISA substrate, with relevant washing steps (see section 2.1.1).

Colonies selected for further expansion and analysis were transferred sequentially to 24 well plates and T25 flasks in cRPMI/ with the addition of 100IU/hypoxanthine, thymidine (HT) (Sigma-Aldrich). Cloning of cells was carried out by limiting dilution in 96 well plates (see section 2.7 below). Recombinant human IL-6 was added to support the growth of individual cells.

2.7. Cloning of hybridoma cell lines.

Two methods of limiting dilution were undertaken during this project. The first was endpoint cloning, which was used for the production of hybridoma lines expressing antibodies specific for 97CN001 envelope. The second was mini cloning which was utilised for the hybridoma lines specific for 96ZM651 envelope.
2.7.1. Endpoint cloning

Hybridoma colonies to be cloned were counted using a neu-bauer haemocytometer, and diluted in cRPMI containing 10% FCS/20pg/ml IL-6, to a concentration of 1000 cells/ml. The diluted hybridoma cells were dispensed (200μl/well) into the 8 wells that formed the first column of a 96 well tissue culture plate. 100μl of cRPMI was added to the remaining, and cells were double diluted across the plate by transfer of 100μl of cells from the preceding well, mixing and transfer to the next well. The last 100μl from column 12 was discarded. 100μl of cRPMI was added to each well of the plate and the plates were covered and incubated at 37°C/5%CO₂. 7-10 days after plating, the colonies that had grown were covering approximately 1/4 of the wells, and the media had acidified. The columns of 8 wells that contained cell growth derived from, on average, a single cell, were determined by a Poisson distribution calculation which states that any column with 1/3 or fewer wells containing growing colonies should be clonal. Those wells that were statistically clonal were screened by ELISA for specific antibody production as described above (2.1.1.).

2.7.2. Mini-cloning

Hybridoma colonies to be cloned were counted using a bauer Haemocytometer, and diluted in cRPMI containing 10%FCS/20pg/mlIL-6, to achieve a cell density of 200 cells/ml. Diluted hybridomas were plated (200μl/well) into triplicate wells of row A of a 96 well tissue culture plate. This allowed four hybridoma lines to be cloned per plate. The remaining wells had 100μl of cRPMI added, and then the hybridomas were double diluted down the plate. 100μl of cRPMI was added to each well of the plate and the plates were covered and incubated at 37°C in 5%CO₂. 7-10 days after plating, statistically clonal wells, selected as described above, were then screened by ELISA for specific antibody production.
2.8. Neutralisation assays

2.8.1. PBMC based assay to measure neutralisation of primary isolate

Peripheral blood mononuclear Cells (PBMCs) were obtained from CFAR (NIBSC, Herts). These were resuscitated from frozen storage as for normal cell culture methods. Once growing, the cells were cultured for 4 days until the start of the assay. The cells were stimulated for 2 days by the addition of 5μg/ml PHA-P (Sigma-Aldrich) to cRPMI. The day prior to starting the assay, cells were then changed into media supplemented with 10IU/ml IL-2 (CFAR, NIBSC, Herts) and 2μg/ml polybrene (Sigma-Aldrich). Two different cultures of PBMC’s were utilised for each assay. The two cell cultures were mixed prior to the assay. The virus stocks had been previously titrated to give 50 x tissue culture infectious doses (TCID<sub>50</sub>) values. The virus was diluted appropriately to give TCID50 values when mixed with the antibodies for neutralisation. mAb and virus were mixed 1 to 1 (final volume 50μl) in wells of a 96 well plate. mAbs were pre diluted as required so that the final concentration in each well was 20μg/ml. A titration of the stock virus was also performed, in which 10 wells contained virus at TCID50 concentration and these were titrated in two steps to give a 1 in 10 and 1 in 100 dilution of this. Plates were then incubated for 1 hour at room temperature.

After one hour 200μl of cells (10<sup>5</sup> cells/ml) were added to all wells and the plates sealed with plastic covers. Plates were incubated at 37°C/5%CO<sub>2</sub>. After 3 days the plates were examined and the media changed. To change the media 150μl of media was removed from each well without disturbing the cells. Then 150μl of fresh cRPMI was added and the plates sealed again. Plates were incubated again at 37°C/5%CO<sub>2</sub>. 7 days after setting up the plates 50μl of NP-40/Trypan blue was added to each well to inactivate any virus, and left over night at 4°C. Samples were then harvested in order to measure P24 Gag levels using a commercial kit (section 2.8.2).
2.8.2. Innotest HIV-1 p24 kit

To test for viral growth by the presence of Gag, an Innotest HIV antigen mAb kit (Innogenetics) was used. The reagents were prepared as directed and the assay was carried out as described in the directions supplied with the kit. Briefly, 100μl of sample was mixed with 100μl of biotinylated murine anti p24 mAb conjugate in wells of strips provided in the kit. The strips were sealed with plastic covers and samples were incubated for 60 minutes at 37°C. Strips were washed with provided wash buffer and 200μl of peroxidise conjugated streptavidin reagent added to each well. The strips were sealed and incubated for 60 minutes at 37°C. After washing 5 times with wash buffer, 200μl of TMB substrate was added to each well and the reaction left to develop for 30 minutes. After this time the reaction was stopped with the addition of 50μl of 2M H₂SO₄ per well. After 5 minutes incubation the absorbance was recorded at 450nm using the Biotek μquant Plate reader.

2.8.3. C8166 cell based assay to measure neutralisation of SHIV W61D

C8166 cells were grown to confluence using standard tissue culture techniques. The virus stocks had been previously titrated to give TCID50 values.

The virus was diluted appropriately to give TCID50 values when mixed with the antibodies for neutralisation. mAb and virus were mixed 1 to 1 (final volume 50μl) in wells of a 96 well plate. Each mAb was tested at 4 concentrations, of 20μg/ml, 10μg/ml, 5μg/ml and 1μg/ml. A virus titration was also performed, in which 10 wells contained virus at TCID50 concentration and these were titrated in two steps to give a 1 in 10 and 1 in 100 dilution of this. Plates were then incubated for 1 hour at room temperature.

After one hour 200μl of cells were added to each of the wells of all plates and the plates sealed with plastic covers. Plates were placed into a 37°C incubator. After 3 days the plates were
examined and the media changed. To change the media 150\mu l of media was removed from each well without disturbing the cells. Then 150\mu l of fresh complete media was added and the plates sealed again. Plates were incubated again at 37°C. 7 days after setting up the plates 50\mu l of NP-40/Trypan blue was added to each well to inactivate any virus, and left over night. Samples were then harvested to measure the p17/p27 gag levels by using an in-house SIV Gag antigen capture assay (2.8.4).

2.8.4. In-house P17/P27 ELISA

There is an in-house assay available at NIBSC that has been utilised for a number of years to measure SIV/SHIV growth by the presence of Gag protein in cell cultures.

ELISA plates (Greiner, Stonehouse, Gloucestershire) were coated with 50\mu l per well of anti SIV Gag p17, and p27 monoclonal capture antibodies KK59/62 (5\mu g/ml each) in distilled water and dried overnight at 37°C. Plates were washed in standard ELISA wash buffer five times, and 200\mu l per well of blocking buffer added. After incubation at room temperature for 30 minutes plates were washed 5 times in wash buffer. 50\mu l of sample from each well of the neutralisation assay was transferred to the prepared ELISA plates. Plates were incubated at room temperature for 60 minutes before washing 5 times with wash buffer. 50\mu l of secondary detector antibody hyperimmune macaque serum E3520 (isolated from a previous macaque schedule), diluted 1 in 160 in blocking buffer, was added to each well. Plates were incubated at room temperature for 60 minutes, and then washed 5 times in wash buffer. 50\mu l of anti-human HRP conjugate, diluted 1 in 200 in PBS + 5% porcine serum, was added per well. Plates were incubated and washed as above. 50\mu l of TMB-DMSO substrate was added to each well, and the assay was allowed to develop for 5 minutes. The reaction was halted by the addition of 25\mu l of 2M H2SO4 to each well. Absorbance was detected at 450nm using a Biotek µquant plate reader.
2.9. IgG subtyping

To determine the IgG isotype of mAbs, a murine antibody isotyping strip kit (Roche, Welwyn Garden City, Hertfordshire) was used. Briefly mAb supernatant was diluted 1 in 100 and 100μl added to tubes containing coloured beads with bound anti-mouse antibodies. A strip coated with anti mouse specific antibodies (IgG1, IgG2a, IgG2b, IgG3, IgM, and Kappa and Lambda light chain), was inserted into the supernatant and bead mixture, and then left at room temperature for up to 10 minutes. Once the positive control bands appeared the subtype of IgG could be determined by the presence of a coloured band corresponding to one of 4 IgG subtypes. The strip also indicates whether the light chains of the mAbs were either Kappa or Lambda. The assay kit is also able to identify murine IgM isotype antibodies, but these are not produced by hybridoma lines formulated using the methods described.
Chapter 3
Examination of immune responses from macaques vaccinated with HIV-1 W61D rgp120.

3.1. Introduction

In the quest for an effective vaccine against HIV many avenues have been explored. In the mid 1980’s, and building on the success at that time of the recently introduced recombinant protein based vaccine against Hepatitis B virus, it was believed that vaccines based upon recombinant proteins from the virus, especially envelope glycoproteins, would be the basis of a successful anti HIV vaccine. As a result, various regions of the env gene of HIV-1 were cloned and expressed in recombinant expression systems. In order to express soluble recombinant proteins that were recognised strongly by immune serum, the surface region (gp120), or the extracellular region of the transmembrane protein (gp41) were expressed in eukaryotic cells as glycosylated proteins (Barrett, et al., 1989; Chakrabati, et al., 1986). Early studies in the HIV-1/chimp challenge model demonstrated that immunisation with recombinant gp120 (rgp120), or gp160 (rgp160) of a TCLA isolate of HIV-1, was able to protect against challenge with a genetically closely related strain of HIV-1 (Berman, et al., 1990; Bruck et al., 1994). This data led eventually to the evaluation of bivalent formulations of the extra-cellular domain gp120 mixed with Alum as adjuvant in Phase III clinical trials. However, both of these studies failed to provide any evidence of protection following vaccination (Cohen, J., 2003; McCarthy, M., 2003). The failure of these clinical trials was very disappointing. Retrospective analyses of samples from these and earlier clinical studies revealed that the TCLA virus derived envelope based vaccine formulations had failed to elicit antibodies in patients capable of neutralising field viruses, i.e. viruses being transmitted within the cohorts of vaccine volunteers under study.

Theoretically, vaccines that elicit antibodies capable of neutralising field isolates of HIV are the most likely approach of achieving the “gold standard” of sterilising immunity, i.e. preventing
any detectable infection with HIV-1. Due to the high mutability of HIV, there are some who believe that only a vaccine that achieves sterilising immunity will prevent the emergence of escape variants that exploit loopholes in protection elicited by vaccination and eventually overwhelm the immune system. The challenge for envelope based vaccine design is to formulate antigens and adjuvants capable of generating high titre antibody responses focussed towards the most conserved regions of envelope that would be likely to cross react between HIV-1 isolates and thus provide broad protection.

Whilst there is only limited data in the SIV/macaque model that envelope based vaccines can confer anything apart from partial protection, even against homologous virus challenge (Ahmad et al., 1994; Almond et al., 1999; Daniel et al., 1994), studies using the SIV/HIV-1 chimaeric virus challenge (SHIV) have been more promising. Using this model, it has been demonstrated that broadly cross neutralising anti HIV-1 envelope monoclonal antibodies can be protective when passively transferred (Baba, et al., 2000; Hofman-Lehmann, et al., 2001; Mascola, et al., 1999, 2000; Parren, et al., 2001; Veazey, et al., 2003). Furthermore it has been shown that vaccination with monomeric rgp120 envelope protein formulated with more potent adjuvants can protect against homologous SHIV challenge (Mooij, et al., 1998). However this vaccine formulation has also been shown to be unable to provide protection against a heterologous SHIV challenge (Stott et al., 1998). This did not formally demonstrate that the protection was mediated by anti-envelope antibodies alone. Recent reports however are suggesting that the protection against homologous and heterologous virus challenge is associated with the eliciting of neutralising antibodies (Barnett et al., 2008; Rasmussen et al., 2007).

At NIBSC, a number of macaque vaccination and challenge studies have been undertaken, and these have confirmed that immunisation with recombinant Env-based immunogens can protect against detectable infection with SHIV expressing CXCR4(X4) or X4/CCR5(R5) utilising envelope proteins homologous with the envelope protein in the vaccine. Furthermore, it has been demonstrated that this protection against homologous virus to the vaccine can be
transferred to naïve macaques by the transfer of immune serum alone (Almond et al in preparation). The demonstration that immune serum elicited in response to envelope based vaccination alone can prevent virus infection in the macaque model provides an opportunity to characterise the anti-viral antibodies that protect in this model.

Plasma samples from a vaccine/challenge study in the SHIV/macaque model undertaken at NIBSC were characterised in detail with regards to epitope mapping and avidity, to determine whether these features could provide an in vitro correlate for a protective serological response. For this study, samples were derived from SHIV/macaque animal schedule 152.1 at NIBSC. Recombinant gp120 HIV-1\textsubscript{W61D} derived from the Dutch, B clade isolate HIV-1\textsubscript{ACH20.3.1} (Groenink et al., 1992) supplied by GSK and formulated in the adjuvant AS02A had been used to immunise groups of 4 macaques on 3 or 8 occasions before intravenous challenge with SHIV\textsubscript{W61D} (Ranjbar et al., 1997) that expressed the homologous envelope protein. It had been observed that 2/4 animals that had received 3 immunisations were protected (T143 and T146) against infection and all 4 that had received 8 vaccinations were also protected against detectable infection. Previous analyses of serological responses had not identified marked differences between the 2 groups of vaccinated macaques in titres of binding antibodies against rgp120, measured by ELISA. However, titres of virus neutralising activity revealed a >6 fold difference between the two groups and furthermore one of the two macaques (T145) that were originally observed to become infected after challenge had the lowest titre of virus neutralising activity just prior to virus challenge (Table 3.1, unpublished data). During the course of this work it was determined that animal T144 in fact had not however become infected. The detailed analysis of the serological responses described in this chapter reveal that this rgp120 vaccine formulated in AS02A adjuvant elicits responses targeted against epitopes in the V3, V2 and, to a lesser extent, CD4 binding regions. Comparative analysis of responses of macaques receiving 3 or 8 immunisations identified significant group specific differences only in serological reactivity targeting the V2 region. Overall, the serological responses in the vaccinated macaque (animal T145) where virus breakthrough was reported were the poorest by all criteria examined.
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<tr>
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<td>Mean</td>
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Table 3.1. Comparison of the serological responses against HIV-1\textsubscript{W01D} envelope determined by ELISA and in virus neutralisation assays for individual vaccinated animals in Group A that received 3 immunisations and Group B that received 8 immunisations of HIV-1 envelope formulated in AS02A as part of Schedule 152.1
3.2. Methods

3.2.1. Peptide mapping of responses

An ELISA based assay was established to detect and quantify binding antibodies, against linear peptide epitopes, in the plasma of macaques that had been immunised with HIV-1_W61D rgp120 administered in AS02A (a proprietary adjuvant from GSK that contains QS21, Monophostatidyl A and an oil in water emulsion). Due to the limited volumes of plasma available and previous reports indicating that limited responses were likely to be elicited to many of the linear peptides derived from the HIV-1_W61D gp120 following immunisation (Beddows et al, 1999), attempts were made to pool groups of peptides selectively.

The ELISA assay was optimised to determine the greatest number of peptides that could be pooled without affecting the sensitivity. Pools of non V3 peptides were spiked with a V3 peptide (CFAR catalogue ARP7035.29) known to be highly reactive, and the anti V3 response was compared between wells with and without non-reactive peptides. The ELISA was performed as in methods section 2.1.2. Briefly, plates were coated with pools of 2, 4, 6, or 9 irrelevant peptides, spiked with the reactive V3 peptide (the final concentration of each individual peptide was 1μg/ml irrespective of the number of peptides in the pool). After blocking plates, an anti-W61D mAb - TH1 directed against V3 peptide ARP7035.29 - was diluted 1 in 1000 and titrated in 2-fold steps, followed by detection with HRP-conjugated secondary antibody and substrate.

For testing of plasma samples from schedule 152.1, plates were coated with either pools of 10 peptides, at a total concentration of 10μg/ml (1μg/ml of each peptide), or individual peptides at 1μg/ml. Plasma samples were diluted 1 in 20 and titrated down the plate in 2-fold steps. A pre challenge plasma pool from control animals was included as a negative control, and selected wells coated with HIV-1_W61D rgp120 included on each plate as a positive control.
3.2.2. Infected cell lysate binding

Neutral detergent lysates of virus infected cells were prepared and used as antigen in selected ELISA assays. C8166 cell cultures infected with HIV-1 \textsubscript{W61D} were harvested at a point after infection when high levels of cytopathic effects could be observed by light microscopy. Infected cells were sedimented by centrifugation at 400g for 30 minutes, the supernatant discarded and the cells resuspended in 10 volumes of 50% NP-40 mixed gently and then used as antigen for coating microtitre plates. Coating of antigen diluted 1 in 50 in PBS was performed overnight at 37°C. Once coated, the plates were washed and blocked and the ELISA was carried out as previously described (see section 2.1.3).

3.2.3. Competition to HIV-1 neutralising reagents

A competitive ELISA assay was developed. Briefly, human mAbs and soluble CD4 (sCD4) were biotinylated using Pierce EZ-link biotinylation kits, following manufacturer’s instructions. The mid-point (50% of maximal binding) titre of each biotinylated mAb and sCD4 was established by ELISA on plates coated with HIV-1 \textsubscript{W61D} rgp120 or HIV-1 IIIB rgp120 at 1 \mu g/ml (CFAR catalogue numbers EVA648 and EVA607 respectively). Subsequently, serial dilutions of plasma collected from immunised or naive macaques were prepared on microtitre plates coated with HIV-1 rgp120 antigen (1 \mu g/ml), followed by addition of biotinylated mAb or sCD4 competitor, at a final concentration of the calculated mid-point titre as described in 2.1.5.

Statistical analysis of competition shown was undertaken using ANOVA with Tukey’s and Dunnett’s testing, to examine relevance of competition between test samples and a naive control, and between each test sample.
3.2.4. Avidity of responses

To assess the avidity of serological responses, binding ELISA assays were performed in the presence of Diethylamine (DEA) following the method previously described by Stott et al. (1998). In brief, the standard ELISA protocol was adapted so that the binding of serum or plasma samples to antigen coated plates was performed in the presence of buffers that contained 35mM DEA (see section 2.1.4). The avidity index was calculated by determining the relative end point titres obtained by ELISA from assays performed with the chaotropic agent as a percentage of titres without (Hedman and Rousseau., 1989).

3.2.5. IgG subtype profiles of responses

Anti-human IgG sub-class reagents (Calbiochem, San Diego, CA) were used in the ELISA protocol (section 2.1.1.) to replace the HRP conjugated anti human antibody in order to detect levels of specific IgG subtypes in macaque plasma binding to HIV-1
\[ \text{W61D} \] recombinant envelope protein.

3.2.6. Gnaan peptide recognition to identify productive infection

Binding to the immunodominant SIV equivalent of the Gnaan peptide in gp41 was assessed by ELISA. The Gnaan peptide (CFAR, catalogue number ARP7022,) was coated as antigen onto 96 well microtitre plates overnight at 4°C at a concentration of 1μg/ml diluted in PBS. The subsequent steps of the ELISA were performed as described in section 2.1.1.
3.3. Results

3.3.1. Characterisation of anti HIV-1\textsubscript{W61D\rgp120} serological responses using homologous linear peptides

3.3.1a. Development of assay

A series of overlapping peptide antigens of HIV-1\textsubscript{W61D\rgp120} were used to coat ELISA plates for assays designed to measure and characterise serological responses elicited against HIV-1\textsubscript{W61D\rgp120} vaccine. The impact of pooling peptide antigens on the ELISA sensitivity was modelled by investigating the effect of adding multiple irrelevant peptides, on the binding of an anti-W61D mAb TH1 to its cognate receptor, ARP7035.29 (a V3 peptide of gp120). Wells of a microtitre plate were coated with V3 peptide alone or when mixed with pools of 2, 4, 6 or 9 additional irrelevant peptides. The presence of 2 or 4 additional irrelevant peptides did not detectably modify the binding of TH1 to its cognate peptide (data not shown). In the absence of its cognate peptide, pools of 2 or 4 peptides also showed no detectable increase in background binding (data not shown). The impact of mixing the cognate peptide with 6 or 9 irrelevant peptides is presented in Figure 3.1. Only when microtitre wells were coated with a pool of 10 peptides was a significant increase in background absorbance detected. Likewise whilst the presence of 6 or 9 irrelevant peptides reduced the peak absorbance obtained, this would be unlikely to result in the failure to detect significant reactivity to a specific peptide. Since plasmas analysed in the clinical trial, using an identical vaccine formulation, failed to detect reactivity against 30 of the overlapping homologous peptide series (Beddows et al 1999), the incorporation of these peptides into 3 pools each comprising 10 peptides would make the overall analysis more economical with plasma and yet the assay would remain capable of detecting anything apart from low level sero-reactivity.
Figure 3.1. A comparative evaluation of the ability of mAb TH1 to bind to cognate peptide ARP7035.29 when the peptide is coated onto the microtitre plate alone or mixed with 6 or 9 irrelevant peptides. The binding of monoclonal antibody TH1 that binds specifically to a HIV-1_W6D gp120 V3 peptide (ARP 7035.29) was titrated on microtitre plates coated with the cognate peptide alone at 1μg/ml (—) or mixed with 6 (—) or 9 (—) irrelevant peptides each present at 1μg/ml. The background reactivity of 7 (—) or 10 (—) irrelevant peptides is also presented (n=3, +/- 3sd).
3.3.1b. Peptide scanning analysis of Schedule 152.1

Peptide scanning ELISA’s were used to screen for reactivity against the series of overlapping peptides derived from gp120 region of HIV-1<sub>w61D</sub> (CFAR catalogue ARP 7035.1-47) in the plasma from immunised and challenge control animals from the day of challenge with SHIV<sub>w61D</sub> in schedule 152.1. Figures 3.2 and 3.3 show the responses against individual peptides and pools of peptides in plasma from macaques in groups A and B that had received 3 or 8 immunisations respectively prior to challenge. Reactivity was detected against peptide 8 located in the V1 region, peptide 12 located on the V1/V2 boundary, peptide 15 located in V2, peptides 28 and 29 located in the V3 loop and peptide 47 in the C5 terminal peptide. Whilst no significant reactivity was detected against pool 2 in either group, high absorbance (>1.0) was obtained with plasmas from animals T143, and T144 against pool 1 and T149 to pool 3. On the day of challenge, subject T145, that became infected first after challenge, had the lowest reactivity against peptides 12 and 29 of all 8 immunised subjects and was also the only animal not to recognise pool 3. Animals in group A following 3 immunisations had higher responses to pool 1 peptides and also to peptide 14 that those in group B following 8 immunisations. 12 weeks after infection, the challenge control animals had responses detectable to only selected peptides, primarily peptides 14, 15 (V2) and 28, 29 (V3) (see appendix). Intriguingly, the responses against the V3 region peptides in control infected animals were higher against peptide 28 than for 29, whereas in the vaccinated animals the responses were higher against peptide 29. At 12 weeks post challenge, the main responses for the vaccinated animals were against peptides 8 (V1), 12(V1/V2), and 28, 29 (V3) (see appendix).

Figure 3.4 summarises the pattern of binding against the individual peptides compared between animals and groups. There does not appear to be any patterns that distinguish protected or infected macaques or groups that received 3 or 8 immunisations. Nevertheless it highlights that strong responses detectable with peptides are mostly directed against V1, V2 and V3 regions of the envelope and the C terminus. The failure to identify a specific vaccine response, detectable
using linear peptides that distinguished vaccinated and infected or protected macaques led us to investigate whether responses against epitopes that relied on envelope protein conformation are more relevant.
Figure 3.2. Ability of plasma collected on the day of challenge, from macaques that had received 3 immunisations, to bind to peptides derived from HIV-1 

Data shows absorbance from wells in row A of ELISA plates, containing the highest concentration of plasma, normalised against naive macaque plasma (n=3, +/- 3sd).
Figure 3.3. Ability of plasma collected on the day of challenge, from macaques that had received 8 immunisations, to bind to peptides derived from HIV-1\textsubscript{W91D} rgp120.

Data shows absorbance from wells in row A of ELISA plates, containing the highest concentration of plasma, normalised against naive macaque plasma (n=3, +/- 3sd).
Figure 3.4. Patterns of linear peptides, across the variable and constant regions of recombinant HIV\textsubscript{W61D} rgp120, recognised by plasma samples from schedule 152.1. (Strength of binding is defined by absorbance values measured at 450nM. Strong >0.5; Weak 0.1-0.5; None <0.1).
3.3.2. Binding to infected cell lysates

A detergent (NP-40) treated virus infected cell preparation was obtained from previously prepared stocks to be evaluated as antigen for an ELISA based assay for detecting and quantifying humoral responses against “native” HIV$_{W61D}$ envelope. NP-40 lysates of C8166 cells infected with either HIV-1$_{W61D}$ or SHIV-1$_{W61D}$ were used as antigen to examine responses in immunised macaques on the day of challenge. Figure 3.5 presents the binding curves of plasma, collected prior to challenge from subjects in Group A (panel A) that received 3 immunisations and group B (panel B) that received 8 immunisations against HIV-1$_{W61D}$ (SHIV-1$_{W61D}$ data not shown). Peak absorbance levels appear lower than that normally observed when coating with peptide antigens. Also, although end point titres for Group A and B are similar, it would appear that there is limiting amount of envelope on the plate, as in Group B there is evidence of a plateau of the absorbance recorded at low dilutions. Furthermore, if the mid-point titre is calculated from the same data, then the mean mid-point titre for Group B is significantly higher than that for Group A and moreover, the midpoint titre for T145 (which became infected after virus challenge) is markedly lower than the rest of this group.

Table 3.2 shows the endpoint binding titres of individual animals and the group means. This data is comparable to that for the binding to rgp120 envelope as seen in table 3.1. The responses to the ‘more native form’ of envelope are slightly increased, but show the same reduced level of binding by animal T145 compared to the others. The use of a recombinant envelope immunogen statistically increases the binding to more native HIV-1 envelope protein after a greater number of immunisations (group B over group A binding $P=0.034$) however not to the more native SHIV envelope protein ($P=0.118$). Which suggests that the epitopes eliciting the majority of measurable responses from rgp120 may be more similar to those expressed on SHIV envelope than HIV envelope, and it is the continual exposure that brings about a change in the recognition. are more exposed on the native form of the envelope.
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</tbody>
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Table 3.2. The Log\textsubscript{10} endpoint titres and group means of immunised macaque plasma samples from schedule 152.1 binding to HIV-1\textsubscript{W6ID} or SHIV\textsubscript{W6ID} infected C8166 cell lysates as determined by ELISA. For HIV-1 envelope binding by group B samples is significantly higher than group A (P=0.034 by t-test,) however for SHIV envelope it is no different (P=0.118.).
Figure 3.5. The titration of immune plasma from subjects immunised on a) 3 or b) 8 occasions with HIV-l_{w61D} rgp120 formulated with AS02A as adjuvant and evaluated by ELISA using NP-40 detergent treated HIV-l_{w61D} infected C8166 cells as antigen (n=3, +/- 3sd).
3.3.3. Competition assays to known neutralising epitopes

To determine whether antibodies with specificity for selected neutralising epitopes on HIV-1 envelope were present in the serum of HIV-1_W6ID rgp120 immunised macaques, plasma collected on the day of virus challenge was tested in a competitive ELISA against a number of known neutralising mAbs, and sCD4. The mAbs selected had specificity for the V2 and V3 regions, the CD4 binding site and the complex neutralisation sensitive carbohydrate residues recognised by 2G12. In addition competition with sCD4 was used to assess binding to the CD4 binding site.

A positive control was included in all assays, in which competition against the biotinylated neutralising mAb was demonstrated by addition of a self unbiotinylated antibody (data not shown). Resulting competition was normalised against naive macaque sera included in each assay.

3.3.3a. Competition to a neutralising antibody mapped to carbohydrates on gp120

The mAb 2G12 is a broadly neutralising antibody that was isolated from an HIV infected individual and recognises a carbohydrate epitope on HIV envelope (Trkola, et al., 1996b; Scanlan et al., 2002). Figure 3.6 presents the competition observed between this mAb and immune plasma collected after 3 or 8 immunisations with HIV-1_W6ID rgp120 on plates coated with homologous or heterologous gp120. These data failed to demonstrate any evidence of competition between the polyclonal immune serum and the mAb 2G12.
Figure 3.6. Competition ELISA against 2G12. Immune plasma collected from macaques 4 weeks after their final of 3 (T143-T146) or 8 (T147-T150) immunisations with HIV-1<sub>ws1D</sub> formulated in AS02A were titrated in microtitre plates coated with HIV-1<sub>ws1D</sub> rgp120 in the presence of biotinylated mAb 2G12 (n=3, +/- 3sd).
3.3.3b. Competition to a neutralising antibody mapped to the V3 region

To determine the presence of antibodies that bind to the V3 region in the plasma of macaques immunised with HIV-1$_{W61D}$ rgp120, a competition ELISA was set up using the more broadly cross-neutralising anti V3 mAb 447-52D (Gorny et al., 1993). Figure 3.7 presents the data from competition of immune plasma to the broadly neutralising mAb 447-52D when either the homologous W61D gp120 (panel A), or the heterologous gp120 from IIIB (panel B) was used as antigen. When the homologous HIV-1$_{W61D}$ rgp120 was used as antigen, competition was observed by all 8 immune plasmas on the day of challenge, as indicated by a decrease in binding of the biotinylated mAb. The most potent competition was observed with plasma from T149 and the least potent for T145. By contrast, only limited competition was observed at the lowest dilution of plasma.

Statistical analysis by ANOVA of the relative degree of competition by plasma from T145 compared with the other plasma samples from immunised macaques on the day of challenge confirmed that the difference was statistically significant (p<0.001). When HIV-1$_{IIIB}$ rgp120 was used as antigen only limited competition was observed between the immune plasmas from immunised macaques and mAb 447-52D.
Figure 3.7. Competition ELISA against 447-52D. Immune plasma collected from macaques 4 weeks after their final of 3 (T143-T146) or 8 (T147-T150) immunisations with HIV-1_W61D formulated in AS02A were titrated in microtiter plates coated with a) Homologous W61D rgp120 and b) Heterologous IIIB rgp120 in the presence of biotinylated mAb 447-52D (n=3, +/- 3sd).
3.3.3c. Competition to a neutralising antibody mapped to the V2 region

To determine the presence of antibodies with specificity for the V2 region in the serum of macaques immunised with HIV-1 \textsubscript{W61D} formulated with AS02A, a competitive ELISA was developed using the V2 specific mAb 697-D (Gorny et al., 1994). In this assay either the homologous HIV-1 W61D rgpl20 or the heterologous HIV-1 \textsubscript{IIIIB} rgp120 was used as antigen to coat the microtitre plate. The selection of the 697D mAb was a result of published data showing that it binds to an epitope that has been mapped to the V2 region, and moreover it has been shown to be able to neutralise heterologous isolates within the B clade. Plasma samples collected on the day of virus challenge from immunised macaques from schedule 152.1 were able to compete with 697D for binding to both homologous HIV-1 \textsubscript{W61D} rgp120 (figure 3.8a) and to a lesser extent heterologous HIV-1 \textsubscript{IIIIB} rgp120 (figure 3.8b). Against both the homologous and heterologous envelope proteins there was great variability between the degrees of competition between the individual plasma samples. For example, plasma samples from macaques T143, T145 and T146 exhibited no significant competition with 697D against HIV-1 \textsubscript{IIIIB} rgp120. Against the homologous envelope protein the competition detected by serum from macaques that received 8 immunisations was significantly greater than that from macaques that had received 3 immunisations (Dunnett’s testing \( p<0.05 \)).
Figure 3.8. Competition ELISA against 697-D. Immune plasma collected from macaques 4 weeks after their final of 3 (T143-T146) or 8 (T147-T150) immunisations with HIV-1 W61D formulated in AS02A were titrated in microtitre plates coated with a) Homologous W61D rgp120 and b) Heterologous IIIB rgp120 in the presence of biotinylated mAb 697-D (n=3, +/- 3sd).
3.3.3d. Competition to a neutralising antibody mapped to the CD4 binding site.

The CD4 binding site is a complex region of HIV-1 gp120 that expresses conformational dependent epitopes that are capable of neutralising the virus. The mAb b12 has been shown to be capable of neutralising a number of primary isolates of HIV-1 (Barbas et al., 1992). Figure 3.9 shows the competition between immune serum collected on the day of challenge from schedule 152.1 with mAb b12 when either homologous HIV-1\textsubscript{w61D} rgp120 (Panel A) or heterologous HIV-1\textsubscript{mB} rgp120 (Panel B) was used as antigen. Using the homologous antigen, competition was observed in all serum samples. However the ability of this assay to discriminate between samples was limited due to the relatively low binding of b12 to the HIV-1\textsubscript{w61D} antigen. Better binding was observed between b12 and HIV-1\textsubscript{mB} rgp120. In general, greater competition with b12 was detected in serum from macaques immunised 8 times compared with those immunised 3 times although this was not statistically significant.
Figure 3.9. Competition ELISA against b12. Immune plasma collected from macaques 4 weeks after their final of 3 (T143-T146) or 8 (T147-T150) immunisations with HIV-1 
\textsubscript{W61D} formulated in AS02A were titrated in microtitre plates coated with a) Homologous W61D rgp120 and b) Heterologous IIIB rgp120 in the presence of biotinylated mAb b12 (n=3, +/- 3sd).
3.3.3e. Competition against sCD4 protein

Soluble CD4 is able to neutralise HIV-1 infection by TCLA virus in vitro by its ability to bind to the CD4 binding site on envelope and thus preventing the virus from binding CD4 on the surface of cells. The presence of antibodies in immune serum able to bind to envelope and interfere with the rgpl20/CD4 interaction would be indicative that these antibodies could neutralise virus infectivity. Figure 3.9 presents the result of this competitive ELISA to determine whether plasma samples collected from macaques immunised with HIV-1 W61D rgpl20 competes with sCD4 for the binding of homologous monomeric HIV-1W61D rgpl20 (Panel A) or with heterologous monomeric HIV-1IIIb (Panel B).

In a manner similar to that of binding of mAb b12, CD4 bound to HIV-1IIIb rgpl20 more strongly than to HIV-1W61D rgpl20 resulting in higher recorded absorbance levels in the absence of immune serum. Furthermore there appeared to be greater variability between replicates in the binding of CD4 to HIV-1W61D rgpl20 compared with HIV-1IIIb rgpl20. Nevertheless, immune plasma from macaques that had been immunised either 3 or 8 occasions, were able to effectively compete with sCD4 binding. Intriguingly, when the homologous HIV-1W61D rgpl20 was used as antigen, serum from T145 that became infected after virus challenge showed the lowest degree of competition, this was not the case when heterologous HIV-1IIIb was used as antigen (Dunnett’s testing p<0.05).
Figure 3.10. Competition ELISA against sCD4. Immune plasma collected from macaques 4 weeks after their final of 3 (T143-T146) or 8 (T147-T150) immunisations with HIV-1W61D formulated in AS02A were titrated in microtitre plates coated with a) Homologous W61D rgpl20 and b) Heterologous IIIB rgpl20 in the presence of biotinylated mAb sCD4 (n=3, +/- 3sd).
3.3.4. Examination of the avidity of the responses to homologous recombinant protein.

The analysis of the specificity of anti-envelope antibodies present in serum of macaques immunised on 3 or 8 occasions did not identify marked differences in the specificities of serological responses between the 2 groups. It is possible that the superior titres of neutralising antibodies in macaques T147-T150 are a result of antibody responses of higher avidity, as a result of the affinity maturation of antibody responses following additional immunisations. In order to investigate this further, an ELISA was established using HIV-1w6ID rgp120 as antigen but performed in the presence of the chaotropic agent DEA. Table 3.3 compares the avidity indices of plasma collected on the day of challenge of schedule 152.1 after 3 (T143-T146 Group A) or 8 (T147-T150 Group B) immunisations with HIV-1w6ID rgp120 formulated in AS02A adjuvant. The mean avidity index of immune plasma for HIV-1w6ID rgp120 from macaques immunised on 8 occasions was significantly greater than that of macaques immunised on 3 occasions. Intriguingly the lowest avidity index was obtained in plasma from macaque T145 that was the macaque where breakthrough of virus was detected after challenge. The table presents the avidity indices for each immunised animal in schedule 152.1. The group mean avidity index for Group B is significantly greater than that for Group A (ANOVA p=0.012).
<table>
<thead>
<tr>
<th>Group</th>
<th>Animal number</th>
<th>Avidity index</th>
<th>Mean avidity index (+/- standard deviation)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>T143</td>
<td>0.74</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T144</td>
<td>0.75</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T145</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T146</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>0.745 +/- 0.045</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>T147</td>
<td>0.82</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T'48</td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T149</td>
<td>0.82</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T150</td>
<td>0.83</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>0.828 +/- 0.010</strong></td>
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Table 3.3. The avidity of immune plasma collected from macaques in schedule 152.1 that had been immunised with HIV-1\textsubscript{W06D} gp120 formulated in AS02A as adjuvant on 3 (group A) or 8 (group B) occasions. The avidity index was calculated for plasma collected just prior to virus challenge by determining the relative end point titres obtained by ELISA from assays performed with the chaotropic agent DEA expressed as a percentage of titres without (Hedman and Rousseau., 1989).
3.3.5. Recognition of the Gnaan peptide

Previous work from the group at NIBSC has demonstrated that transfer of immune serum collected from macaques in group B was sufficient to protect naïve recipients against infection when challenged with a homologous SHIV. This would indicate that serological responses elicited by vaccination would be sufficient to protect. However, the detailed characterisation of the specificity and avidity of serological responses amongst vaccinated macaques in group A above distinguished the responses in the macaques (T144 and T145) - for which there was virological evidence of infection. The responses for T144 were similar to those for T143 and T146 which appeared by all criteria to be protected against detectable infection. Since the crucial diagnostic assay to determine the outcome of challenge with SHIV is PCR analysis of DNA isolated from blood and lymphoid tissue, an alternative assay was needed to confirm independently the outcome of challenge. Many serological based diagnostic assays for the detection of HIV include a highly immunogenic peptide located in gp41 called the Gnaan peptide. An equivalent highly immunogenic peptide has been identified in an equivalent region of SIV and HIV-2. Since none of the anti-envelope responses elicited by vaccination with rgp120 would be directed to epitopes in gp41, the detection of serological responses directed against this peptide would indicate evidence of virus replication.

An ELISA based assay was established to determine whether plasma collected from immunised and naïve challenged controls 12 weeks after inoculation with the homologous SHIV were able to bind to the Gnaan equivalent peptide in gp41. Compared with naïve challenge controls, none of the macaques in group B showed any significant sero-reactivity (Figure 3.11 b), whereas distinct sero-reactivity was detected in the sample from T145, but not from T144 or the remaining uninfected animals in group A animals (Figure 3.11a). Plasma taken 3 months after challenge from one of the challenge animals (T225) was included as a positive control as these animals all became productively infected. The response in T145 is lower than that of the challenge animal suggesting some abrogation of infection due to the vaccination.
Figure 3.11. The detection of serological responses to the immuno-dominant Gnann peptide by ELISA in serum collected 12 weeks after homologous virus challenge from macaques immunised on 3 (panel A) or 8 (panel B) occasions with HIV-1_W6D rgp120 (n=3, +/- 3sd).
3.3.6. Breakdown of IgG subtypes present in responses of vaccinated macaques

Humans possess four subclasses of IgG. Each sub-type has different properties and is present in different proportions in serum. Furthermore, IgG responses to different antigens are known to comprise IgG sub-types in differing proportions. Using anti human IgG subtype specific antibodies, it has been demonstrated that there are 3 subtypes in rhesus macaques which appear to correlate with human IgG1, IgG2 and IgG4 homologues (Shearer et al., 1999). Using the same reagents from the previously published work, the breakdown of the subtype response to the vaccinating rgp120 was investigated. The outcome of ELISA based assays using the human anti-IgG subtype reagents are presented in figure 3.12. These data indicate that the only two antibody subtypes, antigenically equivalent with IgG1 and IgG4 in humans, were detectable as anti-envelope specific antibodies in plasma. The ratio of absorbance obtained with anti IgG4 and anti IgG1 reagents did not differ markedly between the 2 groups of macaques immunised 3 or 8 times nor were there differences between macaque T145 that was patently infected and the remaining immunised macaques that did not become infected following virus challenge (Table 3.4).
Figure 3.12. The detection of anti HIV-1\textsubscript{w61D} rgp120 reactivity detectable by ELISA, using anti human IgG sub-type specific reagents, in plasma collected 4 weeks after 3 or 8 immunisations with homologous envelope protein formulated in AS02A. Data shows absorbance from wells in row A of ELISA plates, containing the highest concentration of plasma, normalised against naive macaque plasma. Only anti-human IgG1 and anti human IgG4 specific reagents detected any anti-envelope reactivity in the plasma samples tested (n=3, +/- 3sd).
<table>
<thead>
<tr>
<th>Animal</th>
<th>Proportion of IgG4 to IgG1</th>
</tr>
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<tbody>
<tr>
<td>T143</td>
<td>0.186</td>
</tr>
<tr>
<td>T144</td>
<td>0.222</td>
</tr>
<tr>
<td>T145</td>
<td>0.141</td>
</tr>
<tr>
<td>T146</td>
<td>0.136</td>
</tr>
<tr>
<td>T147</td>
<td>0.197</td>
</tr>
<tr>
<td>T148</td>
<td>0.161</td>
</tr>
<tr>
<td>T149</td>
<td>0.225</td>
</tr>
<tr>
<td>T150</td>
<td>0.189</td>
</tr>
</tbody>
</table>

Table 3.4. The proportional levels of IgG4 compared with IgG1 present in plasma samples from vaccinated animals on the day of challenge.
3.4. Discussion

The vaccine strategy that was selected for this study involved recombinant monomeric HIV-1 envelope (HIV-1 rgp120) formulated in a potent adjuvant. The envelope protein antigen was a recombinant gp120 derived from a Dutch HIV-1 isolate (ACH 320.3.1) that is reported to be dual tropic (Trkola et al. 1996a). This was formulated with an adjuvant from GSK (AS02A) which is identical to 1 of 2 formulations used in clinical trials (Mc Cormack et al., 2000; Beddows et al., 1999). The clinical trial showed that, although the vaccine failed to elicit antibodies capable of neutralising a range of primary isolates, some individuals did produce serological responses capable of neutralising selected TCLA HIV-1 strains (McCormack et al., 2000). Using the SHIV/macaque model, studies at NIBSC have shown that this same candidate HIV-1 rgp120 envelope based vaccine can confer apparent sterilising immunity when macaques are challenged with a SHIV that expresses the homologous envelope (Almond et al., in preparation). These protection data allow us to examine the responses these vaccines elicit, and to investigate which of these responses correlate with the observed protection. As a result this information will provide a guide for the development of improved vaccines.

In the study, summarised above, it was shown that 8 vaccinations with a recombinant gp120 in adjuvant provided protection against detectable infection (Almond et al in preparation). This would, of course, be the ideal outcome from a clinical trial. This protection was effective against homologous and heterologous SHIV challenges (Page et al in preparation). Furthermore, it was shown that serological responses were central to protection against homologous but not heterologous SHIV’s, since the protection was transferable to naïve macaques by the transfer of immune serum alone, but only when challenged with homologous SHIV (Almond et al in preparation, Page et al in preparation). There was further evidence that serological responses are central to vaccine protection in this model. The titres of neutralising antibodies in the group of macaques that received 3 vaccinations were lower than titres amongst those that received 8 immunisations. In addition the immunised macaque with the lowest titre
of neutralising antibodies on the day of challenge was the only immunised individual where evidence of virus breakthrough was observed. The observed differences between the 2 groups provided the opportunity to define, in greater detail, the serological responses elicited by vaccination with the hope of identifying the specific component responses that correlate with the observed protection. The hope would be that this information may assist in the evaluation of current clinical trials using envelope based vaccine candidates and lead to the development of improved envelope immunogens.

Initially, it was investigated whether there were differences between the binding of plasma samples collected prior to virus challenge to linear peptides derived from the same envelope sequence as the immunogen. Furthermore, it was studied whether any differences in the binding to peptides could predictably differentiate, between macaques that had received 3 or 8 immunisations or between the macaque that became infected after challenge from those that were protected. Strong responses to selected peptides were detected in all animals, with similar focus on the V1, V2, V3 and C5 regions to that seen by Beddows and colleagues (Beddows et al., 1999). However, no distinct patterns of binding were identified by this peptide scanning analysis, which differentiated macaques that had 3 or 8 immunisations or between the immunised macaque that got infected after challenge and the remaining immunised macaques where no evidence of challenge virus was detected. Although the assays undertaken using peptides were not truly quantitative, there was no evidence from absorbance values at a single dilution, to indicate that the size of serological responses to these linear peptides contributed significantly to the neutralising antibody response. These are not completely surprising observations since Beddows et al (1999) performed a similar type of study using the same set of peptides and serum collected from clinical volunteers that had received the same vaccine formulation (Beddows et al 1999). Although there were some differences in the detail of peptides recognised by immunised humans and immunised macaques, for example the low level sero-reactivity detected in macaque plasma to pools of peptides that were assessed to be non reactive in humans, overall a very similar pattern was obtained. More promising perhaps from the view of the vaccine was the indication that a significant proportion of the sero-reactivity
detected in immunised macaques was directed against conformational dependent determinants, as it is believed that the majority of potent anti-viral neutralising activity is directed against non-linear epitopes (Bou-Habib et al., 1994; Burton and Montefiori., 1997; Parren et al., 1999). This is especially the case for neutralisation of primary virus isolates, which are much more resistant than TCLA viruses, especially to laboratory produced monoclonal antibodies that map to linear peptides (Mascola et al., 1996; Moore et al., 1995; Sawyer et al., 1994; Vancott et al., 1995; Wrin et al., 1995). Since it is possible that the immunogenetics of the immunised macaques could have influenced epitopes recognised by antibodies between the two groups of Mauritian derived macaques (Stebbings et al personal communication), plasma samples were analysed from animals in Group B after 3 immunisations and compared with samples collected after 8 immunisations. This analysis did not reveal that there had been a qualitative maturation of serological responses directed against linear epitopes of envelope (data not shown). Since the analysis of sero-reactivity against linear epitopes did not appear promising, further analyses focussed on the comparative analysis of responses to conformation dependent epitopes on rgp120.

It was considered probable that the use of a neutral detergent NP40 would be unlikely to result in marked denaturation of the virion envelope or disruption of the trimeric structure, therefore providing a more native conformation of envelope. As a result it was anticipated that antibodies elicited by immunisation with monomeric rgp120 but that bind to the non-neutralising face of gp120 which interfaces with the other subunits in the timer would not bind to this preparation of HIV-1 \text{W61D} antigen, thus allowing antibodies against the neutralising face to be investigated. Whilst this study demonstrated that T145, which was the only immunised macaque where breakthrough virus was detected, had the lowest levels of binding, there was no evidence that the levels of binding to this antigen in an ELISA based assay correlated with virus neutralising activity (Table 3.1).

The failure to detect evidence of correlation between virus neutralisation and antibody binding to multiple epitopes on a complex antigen, led us to consider whether neutralising antibodies,
elicited by vaccination with rgp120, could be mapped to epitopes recognised by known broadly cross reactive neutralising monoclonal antibodies that bind to HIV-1 W61D envelope. The establishment of competitive ELISA where the presence of antibodies with defined specificity were detected by their ability to prevent binding of selected neutralising mAbs, and other proteins such as soluble sCD4 to bind to homologous and/or heterologous HIV-1 rgp120, revealed interesting observations about the specificity of responses following vaccination with rgp120. It was not surprising that no detectable competition was observed with the mAb 2G12 (figure 3.6). This mAb recognises a complex epitope comprising primarily carbohydrate residues (Trkola, et al., 1996b; Scanlan et al., 2002) and an unusual cross-over conformation of immunoglobulin heavy chains to create its binding site. As a result this would be a highly unusual antibody specificity to be detected in anti-rgp120 immune serum. Interestingly, only relatively limited reactivity against the CD4 binding domain was detected when either sCD4, or the CD4 binding mAb b12 were used as the competitor in the assay. Intriguingly, the ability of plasma from the vaccinated individuals to compete with the binding of mAb b12 or to soluble CD4 protein appeared greater when a heterologous envelope HIV-1IIIb was used as antigen rather than homologous HIV-1 W61D. This may reflect the fact that the level of binding of both b12 and soluble CD4 were greater to the envelope of the tissue culture adapted HIV-1IIIb. These data would be consistent with the belief that HIV-1IIIb rgp120 has a more open conformation around the CD4 binding region as a result of adaptation to rapid replication in tissue culture.

Competition with the broadly cross reactive anti V3 mAb 447-52D and the anti V2 mAb 697-D appeared to indicate that these regions are likely to be significant components of the protective serological responses elicited by this vaccine formulation. The anti-V3 competition ELISA using homologous and heterologous HIV-1 rgp120 as antigen demonstrated that the anti V3 response elicited by HIV-1 W61D was highly specific, since there was only limited or no cross reactivity detectable when HIV-1IIIb rgp120 was used as antigen. The 447-52D epitope is on the crown of the V3 loop (Gorny et al., 2002) which is present in both homologous and heterologous HIV-1 rgp120; therefore this data would suggest that the anti V3 responses raised by immunisation do not bind directly to the 447-52D epitope. The likely importance of anti V3 neutralising antibodies in protection was highlighted by the observation that plasma from T145,
that was the vaccinated macaque where there was evidence of virus breakthrough, exhibited a lower levels of competition against this anti V3 neutralising monoclonal antibody compared with the rest of the immunised animals in both group A and group B (fig 3.7). This could highlight a specific “gap” in the serological response of this individual that correlates with the lower neutralising titre observed for this individual. The relatively poor anti V3 response may also have resulted in the notably lower avidity index for this specific member of Group A (Table 3.2). By contrast with the individual differences in levels of competition between immunised macaques that were protected and the individual that became infected, competition between immune plasma and the anti V2 monoclonal 697-D appears to provide a correlate between the neutralisation titres obtained in serum from macaques that had been immunised on 3 or 8 occasions (fig 3.8). These data suggest that the quantitative difference in neutralisation titre between macaques immunised on 3 and 8 occasions may be due to the titre of anti V2 specific neutralising antibodies. It is intriguing the anti V2 responses of macaque T145 are not significantly different from the rest of Group A. It would be interesting to establish whether this difference in response reflects a difference in the MHC Class II of this individual from the rest of the vaccine recipients. The distinct clustering of competition against this V2 epitope after 3 or 8 immunisations may indicate a region of envelope where multiple immunisations specifically enhances responses.

The failure of the competitive ELISA to distinguish absolutely between the anti rgp120 responses of vaccinated macaques that appeared protected by virus co-culture and PCR based assays, led to a consideration as to whether these types of virological assays were unable to detect low levels of infection. As a result, sero-reactivity against the valuable sero-diagnostic Gnann epitope was evaluated. Although this epitope is located in envelope, it is located in the transmembrane region that was not present in the vaccine. Thus the detection of responses to this epitope would provide evidence of infection with the SHIV. Whilst diagnostic assays based upon the detection of anti-viral antibody responses have the benefit of using host responses to amplify the signal this assay confirmed that only T145 was detectably infected with SHIV. By contrast, the response from T144 was not significantly different from that of the 6 other serum
samples collected from macaques T143 and T146 to T150 where there was no virological
evidence of infection. This provides evidence contrary to the original observation that T144
was infected. This animal has been conclusively proved to not be infected by immunogenetic
analysis of the original PCR positive samples.

It was possible that the key feature contributing to the neutralisation titre, and more important to
protection in vivo, was not mediated by the specificity of the antibody response but by other
factors such as the immunoglobulin subclasses or the avidity of the response opposed to the
specificity.

Scharf and colleagues have shown that IgG3 from human sera is more efficient at blocking
fusion, and neutralising X4, R5 and X4/R5 than either IgG1 or IgG2 (Scharf et al., 2001). This
work was suggestive that the hinge region in IgG3 which is longer and more flexible was
crucial to the enhanced function. To examine if any differences in the IgG subclass distribution
either between protected or infected animals, or after a greater number of immunisations, a
limited study was performed. There was no indication that IgG subclass distribution was a key
factor in determining the differential protective capacity of the serological response to
vaccination. However, interpretation of this study is difficult since a complete analysis of the
IgG sub-classes expressed by Mauritian derived cynomolgus macaques is lacking and the cross
reactivity of anti human IgG subclass reagents against cynomolgus macaques has not been
undertaken. Since this sub-species of cynomolgus macaque is widely used in bio-medical
research as a pre-clinical model, it would be valuable if this information was determined. One
such study has identified in rhesus macaques four IgG subclasses that correspond by sequence
similarity to the four human IgG subclasses (Scinicariello et al., 2004). Whereas a previous
study had shown only 3 IgG subclasses detectable in macaques, with the missing subclass
suggested to be an equivalent to human IgG3 (Shearer et al., 1999). Therefore it may be of
benefit if these subclasses can be formally identified and reagents made available for all species
utilised in studies.
Previous studies utilising the same vaccine components as here, measured avidity after 3 and 5 vaccinations (Mooij et al., 1998; Stott et al., 1998). Avidity levels in this study correspond well with those, with the difference in avidity after an increased number of vaccinations being shown, and are in agreement with data from Richmond et al (1998), who reported that avidity responses in rabbits immunised with envelope matured over 6 months after DNA priming and protein boosting. Although there is no correlation between the avidity of response and protection, correlation is present between higher avidity and higher neutralising titres between groups. Recent studies have shown that higher avidity of antibody responses correlates with a greater breadth of neutralising ability in sera from HIV positive anti retroviral naive subjects (Sather et al., 2009), and inversely correlates with peak postchallenge viraemia in rhesus macaques (Zhao et al., 2009). This evidence promotes avidity as being one of the factors associated with a stronger neutralising ability.

Thus, the overall conclusion from this detailed comparative analysis of serological responses between the vaccinated individuals in this study are, that there were no specific antibody responses missing in macaque T145 that succumbed to infection after challenge. Rather that the responses elicited by this individual animal were of insufficient titre and avidity to provide a protective neutralising response in vivo. Similarly, the benefit of 8 immunisations over 3 immunisations is not that qualitatively new responses were elicited, but that the magnitude of produced responses increased, in particular to the V2 region.

Whilst the competition of immune serum with selected mAbs have helped to characterise the specificity of response elicited by immunising with this monomeric rgp120 vaccine, it has unfortunately revealed that these responses are most likely focused against the V2 and V3 regions of envelope which, as the designation indicates, are highly variable. This was clearly demonstrated by the failure of plasma to compete with an anti V3 mAb binding to a heterologous envelope antigen and to a lesser extent with the anti V2 mAb. An important feature for improving envelope based vaccines is to attempt to focus responses against more conserved regions of envelope which are sufficiently accessible to antibodies on the native
virion. The use of oligomeric envelope as antigen is one approach to reduce exposure of the non neutralising face of the gp120 molecule. An engineered envelope protein where the V2 region has been removed is a potentially complementary approach that has proved advantageous in pre-clinical studies (Barnett et al., 2001; Derby et al., 2006; Sharma et al., 2006; Xu et al., 2006a; Xu et al., 2006b). Clearly a challenge for formulating envelope protein in vaccines that generate potent serological responses is the selection of adjuvant that should be used. Many adjuvants denature proteins or at least would disrupt interactions between non-covalently linked proteins in an oligomer. Alternative approaches to generate potent neutralising mAbs against envelope are needed. This is explored more in a later chapter.
Chapter 4
Cross clade binding and neutralising capability of polyclonal sera elicited by recombinant gp120/140 immunisation

4.1 Introduction

Recombinant proteins are powerful tools for generating immunological responses. They have been used successfully on numerous occasions to immunise animals for the production of polyclonal sera as well as monoclonal antibodies. In this way they have been helpful in the production of useful reagents for further study of the envelope proteins of HIV-1/2 and SIV. Recombinant protein antigens to the envelope of Hepatitis B virus are used successfully as vaccines (Plotkin, S., 2005), and there is hope that recombinant protein antigens of HIV envelope could be used in the same way. Responses elicited by HIV envelope have shown these proteins to be possible immunogens and vaccine candidates in both animal models and in human clinical trials (Binley at al., 2000; Haigwood et al., 1992; Mascola et al., 1996; Pancera et al., 2005; Srivastava et al., 2003). However, it is not known how cross reactive the elicited vaccine responses to HIV recombinant proteins could be. Although HIV-1 can be classified into a number of genotypes, these do not match any of the serotyping work done so far, and so cannot be readily classified into serotypes. Without a complete understanding of cross strain or clade reactivity it is unknown how or indeed if, the genotypes can be grouped into distinct serotypes or whether there is inter- as well as intra-clade variation in possible serotypes and in the recognition of different genotypically-classified clades of virus.

Due to the extensive variation in the envelope glycoproteins between isolates of HIV-1, the production of a monovalent vaccine antigen that will recognise multiple viral clades may well be unrealistic (Wei et al., 2003). Monomeric recombinant envelope proteins generate serological responses with limited capacity to neutralise the virus, especially viruses from
heterologous clades to the original protein (Beddows et al., 1999; Bures et al., 2000). It may be that vaccines that are eventually produced may have to be geographically specific.

Research shows that there is a much more sensitivity to neutralisation for TCLA viruses than primary isolates (Mascola et al., 1996; Moore et al., 1995; Vancott et al., 1995). To this end, it is of paramount importance to try to identify any serotypes of primary HIV-1 that can be identified in terms of vaccine responses. Although work on V3 serotypes has been undertaken and it has been discovered that within this region there are certain apparent immunogroups, it is not known if these groups are specific across the whole of the envelope region. These groups are not fully characterised and therefore do not provide a full picture (Vancott et al., 1994). There is so much variability in Env that it may be that serotypes will have to be identified for polyclonal vaccine responses rather than relying upon the surrogate of mAb binding.

Many groups have attempted neutralising serotyping of HIV-1, using not only monoclonal antibodies, but also sera from patients infected with different strains of HIV-1, but have not identified any relationship between clades of virus and serotypes (Bures et al., 2002; Kong et al., 2003; Kostrikis et al., 1996; Moore et al., 1996; Nyambi et al., 1996, 2000; Verrier et al., 2000; Weber et al., 1996). The sera used have all been able to neutralise the virus to some degree. Although in some cases certain serotypes do seem to have been identified, it cannot be stated that they are distinct (Barin et al., 1996). Due to the intra and inter-clade variation in HIV-1 isolates, current work does not suggest that individual isolates from the same clade will be able to be grouped. It is possible that some isolates are far enough removed that they will not be immunologically recognised as the same clade as others.

Work in the previous chapter showed that in the animal model system it is possible to show serological mediated protection elicited by monomeric envelope protein vaccines. However, oligomeric proteins, which may be more similar in structure to the native envelope protein, may be better at focussing the response. A number of groups have produced soluble trimers formed from gp120 covalently linked to gp41 in an effort to elicit more functional antibodies (Binley et
al., 2000; Iyer et al., 2007; Kim et al., 2005; Lu et al., 1995a; Sanders et al., 2002; Srivastava et al., 2003; Yang et al., 2001). It has been shown that these trimeric recombinant envelope proteins elicit neutralising antibodies against heterologous isolates more effectively than monomeric ones (Barnett et al., 2001; Beddows et al., 2005; Earl et al., 2001; Grundner et al., 2005; Kim et al., 2005; Yang et al., 2001). This then leads to asking whether these proteins can give up serotyping information and what role the genotype of the virus plays in any possible serotypes?

To examine these questions, a number of polyclonal guinea pig sera raised against recombinant envelope glycoproteins from a range of different clades of HIV-1 have been produced. Both rgp120 and rgp140 recombinant envelope proteins were used. Recombinant gp140 is formed by removal of the cleavage site between gp120 and gp41 of the gp160 precursor, by site directed mutagenesis (Broder et al., 1994; Earl et al., 1991, 1994, 2001). This allows rgp140 to form stable trimers. Rather than just look at the binding of these sera to distinguish serotypes it will be of interest to examine the ability of these sera raised to recognise heterologous envelope proteins across a range of different clade viruses, and perhaps compare to any neutralising ability present in these sera. Included in this work are both rgp120, and rgp140 envelope derived sera. This will show if there are any possible groups of serotypes that can be distinguished by neutralising responses to the whole of the envelope region rather than specific epitope regions, and also compare the responses elicited by gp120 and gp140.
4.2 Methods

4.2.1 Immunisation of Guinea pigs

Nine Hartley strain female guinea pigs (from an outbred population) were immunised with 1 of 6 different preparations of recombinant HIV-1 envelope proteins, representing 5 isolates of clades (A, B, C, E and F); A (UG37 rgp140 – 2 animals), B (Han2 rgpl40 – 1 animal), C (CN54 rgp120 – 2 animals; CN54 rgp140 – 2 animals), E (93TH975.15 rgp120 – 1 animal), and F (BR29 rgp140 – 1 animal). The recombinant proteins were supplied by Dr S Jeffs (formerly CFAR, NIBSC, now Imperial College, London) with the exception of 93TH975.15 rgp120 that was supplied by CFAR, NIBSC. Animals were immunised at weeks 0, 4 and 8, and bled under terminal sedation 2 weeks after the final immunisation. For each immunisation the animals received 30μg of recombinant protein in 2% Quil A adjuvant, administered sub-cutaneously in the loose skin at the back of the neck.

4.2.2 Binding titres to heterologous and homologous recombinant proteins

The binding titres of sera obtained from these animals were tested by ELISA to a range of HIV-1 envelope proteins (W61Drgp120, IIIBrgp120, CN54rgp140, CN54rgp140, ZM96rgp140 and 93TH975.15rgp120) as described in section 2.1.1. All proteins used were coated at 1μg/ml.

4.2.3 Neutralisation of SHIV\textsubscript{W61D}

The ability of sera to neutralise SHIV\textsubscript{W61D} was evaluated by mixing virus with a dilution series of a serum sample for 1 hour before adding to C8166 cells and culturing for 7 days as described in section 2.8.3. Evidence of virus replication was assessed by determining the presence of SIV
Gag protein in cultures as described in section 2.8.4 and calculating the reduction in virus titre in the presence of sera.

4.2.4 Competition ELISA using broadly neutralising and W61D specific monoclonal antibodies

Competition assays were carried out as described previously (section 2.1.5). Serum collected from animal T149 after 8 immunisations with HIV-1\textsubscript{W61D} formulated in AS02A from schedule 152.1 was used as a positive control. 4 mAbs (TH1, TH3, TH7 and TH8), raised against W61D envelope were used. The anti-W61D monoclonal antibodies tissue culture supernatants were produced using the Tecnomouse (Integra Biosciences) hollow fibre culture system (2.4.1). In addition, 3 broadly cross reactive, neutralising, human mAbs used in the previous chapter (b12, 697-D and 447-52D) recognising CD4 binding domain, V2 and V3 regions of envelope respectively, were also employed.

4.2.5 Binding to HIV-1\textsubscript{W61D} specific recombinant peptides

Binding assays to the specific peptides that mAbs TH1 and TH3 are mapped to were performed on the sera. The peptides ARP 7035.14 and 7035.29 map to TH3 and TH1 respectively, and were coated at 1\(\mu\)g/ml and the assays carried out as for the standard binding ELISA (2.1.1). TH1 and TH3 mAb supernatants were included as positive controls and TH7 and TH8 mAb supernatants were tested as negative controls.
4.3 Results

4.3.1. Endpoint titrations of Guinea pig sera

Guinea pigs were immunised with recombinant HIV-1 envelope protein derived from representative isolates of differing clades and produced in eukaryotic expression systems. Antiserum was collected at the terminal bleed and the end point binding titres of each of the anti-sera to a panel of homologous and heterologous recombinant HIV-1 envelope proteins were determined by ELISA. These titres are presented in Table 4.1. All antiserum bound to all available recombinant proteins evaluated with differing titres. Unfortunately at the time of testing not all of the homologous recombinant proteins used for immunising the animals were available for investigation. Intriguingly the end point titres were not always the highest against the homologous protein used to immunise, or to recombinant proteins of the same clade. The highest titres against the range of envelope proteins tested were raised by CN54 derived rgpl20 and rgp140 envelopes. The most antigenic envelope protein was CN54 rgp120, that frequently yielded the highest end point titre in most anti-sera tested.

The serum raised against HAN2 protein had generally the lowest titres against all the protein antigens tested. Titres ranged from $2.6 \log_{10}$ for CN54rgp140 to $4.5 \log_{10}$ for CN54rgp120. Anti- BR29 sera had titres greater than $3.5 \log_{10}$ against every protein tested, and are all except for CN54rgp140 approximately $4 \log_{10}$. The anti-sera raised using UG37 as immunogen had titres ranging between $3.5$ to $5 \log_{10}$ of binding. Where more than one guinea pig was immunised, there was little variation between the titres of the two anti sera. Sera raised to an E clade envelope has high titres to all the proteins, but along with the HAN2 and BR29 sera does not reach $4 \log_{10}$ of binding to CN54rgp140. Between the same clade proteins there are also apparent differences in the recognition. Of the two B clade proteins, the titres to IIIB are markedly higher with sera raised to clades A, B and E, in some cases over half a $\log_{10}$ more. In contrast to this however, responses from sera raised to clades A and B for W61D are markedly
lower than the others, suggesting it is less well recognised by sera raised to recombinant envelope proteins. The sera raised to CN54rgp120 and CN54rgp140 also appear to have different binding specificities, with CN54rgp120 producing higher titres for every serum sample than the CN54rgp140. For sera raised to A,B and E clade proteins there is between 1 and 2 \( \log_{10} \) higher responses to CN54rgp120 than rgp140, and interestingly the sera raised to CN54rgp140 has almost \( \log_{10} \) higher response to the CN54gp120 than to the immunising protein. This suggests that the monomeric rgp120 has more exposed binding sites than the trimeric rgp140.
<table>
<thead>
<tr>
<th>Immunogen used to raise sera</th>
<th>Clade</th>
<th>Endpoint titre (Log_{10}) against recombinant protein</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>W61D rgp120</td>
</tr>
<tr>
<td>UG37rgp140 (1)</td>
<td>A</td>
<td>3.77</td>
</tr>
<tr>
<td>UG37rgp140 (2)</td>
<td>A</td>
<td>3.55</td>
</tr>
<tr>
<td>Han2rgp140</td>
<td>B</td>
<td>3.88</td>
</tr>
<tr>
<td>CN54rgp120 (1)</td>
<td>C</td>
<td>4.2</td>
</tr>
<tr>
<td>CN54rgp120 (2)</td>
<td>C</td>
<td>4.3</td>
</tr>
<tr>
<td>CN54rgp140 (1)</td>
<td>C</td>
<td>4.5</td>
</tr>
<tr>
<td>CN54rgp140 (2)</td>
<td>C</td>
<td>4.25</td>
</tr>
<tr>
<td>93TH975.15rgp120</td>
<td>A/E</td>
<td>4.34</td>
</tr>
<tr>
<td>BR29rgp140</td>
<td>F</td>
<td>4.21</td>
</tr>
</tbody>
</table>

Table 4.1. Log_{10} endpoint titres of guinea pig sera against recombinant proteins, showing endpoint binding titres to a number of different recombinant HIV-1 Env proteins. Titres calculated from the linear portion of binding curves (r^2 > 0.9).
4.3.2 Neutralisation of SHIV\textsubscript{W61D}

Since all of the antisera elicited from the immunised guinea pigs had binding titres of greater than 3.5 log\textsubscript{10} to HIV-1\textsubscript{W61D}\textsubscript{rgp120} detectable by ELISA, these guinea pig sera were tested for their ability to neutralise SHIV\textsubscript{W61D}. The outcome of these analyses is presented in Figure 4.1.

Neither antisera raised to HIV-1\textsubscript{CN54}\textsubscript{rgp140} exhibited detectable anti-viral neutralising activity against this heterologous SHIV defined by the usual criteria, i.e. a 75% reduction in detectable virus replication at a 1 in 10 dilution of serum or plasma (Kent et al 1991), with reduction in Gag only reaching 30-40% at the lowest dilution. Furthermore, no detectable neutralisation was observed in immune sera elicited by immunisation with HIV-1\textsubscript{BR29} \textsubscript{rgp140}, HIV-1\textsubscript{93TH973.15} \textsubscript{rgp120} and one of the animals immunised with HIV-1\textsubscript{CN54} \textsubscript{rgp120}. However, sera from the second guinea pig immunised with HIV-1\textsubscript{CN54}\textsubscript{rgp120}, the guinea pig immunised with HIV-1\textsubscript{Haicgp140} and also both guinea pigs immunised with HIV-1\textsubscript{UG37}\textsubscript{rgp140} exhibited greater than 80% neutralisation of SHIV\textsubscript{W61D} when mixed neat with virus. This apparent neutralising ability to SHIV\textsubscript{W61D} in these sera is very quickly titrated out, suggesting a fairly low affinity response.

Clearly these data indicate that simple binding titres to a recombinant envelope protein by ELISA do not correlate with the ability of the same antisera to neutralise viruses expressing the homologous envelope. It may have been informative to look at homologous neutralising ability, however none of the corresponding HIV/SHIV isolates were available for the sera, and the cognate W61D peptide was not chosen to immunise any animals. These assays were performed with heat inactivated sera samples, and perhaps the addition of complement to that assay would have enhanced the little neutralisation seen.

Further studies were undertaken to investigate the epitopes on HIV-1\textsubscript{W61D} recognised by neutralising and non-neutralising immune serum.
Figure 4.1 Neutralising capabilities of heat inactivated antisera raised in guinea pigs to HIV-1 recombinant Env against SHIV W61D. The neutralisation is calculated as a percentage decrease in the amount of SIV Gag present after incubation of virus with sera samples, and growth on C8166 cells. The horizontal lines indicate 0 and 100% neutralisation, and it can be seen that 6 of the serum samples show neutralising capacity. This ranges for undiluted samples between 30% for sera specific for CN54rgp140 to approximately 95% for UG37, and CN54rgp120-specific sera (n=3, +/- 3sd).
4.3.3. Competition between guinea pig sera and TH monoclonal antibodies

A competition ELISA was established using HIV-1\textsubscript{worldgp120} and 4 mAbs that had been generated from mice immunised with this homologous envelope (T Hollett unpublished work). The specificity of these mAbs have been mapped previously to be TH1 (V3 region), TH2 (V2 region) and for TH7 and TH8 to conformational regions of envelope. The outcome of the competition ELISAs using biotinylated mAb to compete with the immune sera are presented in figure 4.2 (TH1), figure 4.3 (TH3), figure 4.4 (TH7) and figure 4.5 (TH8). Whilst in each assay unlabelled monoclonal was able to compete with its cognate labelled monoclonal (data not shown), there was no evidence that any of the anti-sera raised in guinea pigs was able to compete with any of the TH mAbs.
Figure 4.2 Competition ELISA against mAb TH1. Data shows the ability of guinea pig sera collected before (naive) or after immunisation with recombinant HIV-1 envelope protein, formulated with Quil A as adjuvant, to compete with biotinylated mAb TH1 that binds an epitope in the V3 region of recombinant HIV-1<sub>W61D</sub> gp120 (n=3). Plates were coated with HIV-1<sub>W61D</sub> gp120 at 1 µg/ml.
Figure 4.3 Competition ELISA against mAb TH3. Data shows the ability of guinea pig sera collected before (naive) or after immunisation with recombinant HIV-1 envelope protein, formulated with Quil A as adjuvant, to compete with biotinylated mAb TH3 that binds an epitope in the V2 region of recombinant HIV-1\textsubscript{W6ID} gp120 (n=3). Plates were coated with HIV-1\textsubscript{W6ID} gp120 at 1\mu g/ml.
Figure 4.4 Competition ELISA against mAb TH7. Data shows the ability of guinea pig sera collected before (naive) or after immunisation with recombinant HIV-1 envelope protein, formulated with Quil A as adjuvant, to compete with mAb TH7 that binds to an unmapped conformational epitope of recombinant HIV-1 6D gp120 (n=3). Plates were coated with HIV-1 6D gp120 at 1μg/ml.
Figure 4.5. Competition ELISA against mAb TH8. Data shows the ability of guinea pig sera collected before (naive) or after immunisation with recombinant HIV-1 envelope protein, formulated with Quil A as adjuvant, to compete with mAb TH8 that binds to an unmapped conformational epitope of recombinant HIV-1_{w61D} gp120 (n=3). Plates were coated with HIV-1_{w61D} gp120 at 1μg/ml.
4.3.4 W61D peptide recognition of guinea pig sera

It was unexpected that immunisation with recombinant HIV-1 envelope protein was unable to elicit anti-V3 or anti-V2 specific antibody responses detectable by competition ELISA. Therefore peptide ELISA’s were established using the recombinant cognate V3 (amino acids 311-330) and V2 (amino acids 161-180) peptides recognised by monoclonal antibodies TH1 and TH3 respectively. These peptide ELISAs were used to investigate whether HIV-1W61D rgp120 specific anti-V3 or anti-V2 cross reactive responses were present at detectable levels in undiluted polyclonal guinea pig anti-envelope anti-sera.

The results of the peptide ELISA’s are presented in figure 4.6. No significant anti-peptide reactivity was observed in polyclonal immune sera with the exception of HIV-1CN54rgp120 and HIV-1CN54rgp140. Both sera elicited by immunisation with HIV-1CN54rgp120 exhibited stronger binding to the V2 peptide, whereas the two sera elicited by immunisation with HIV-1CN54rgp140 exhibited similar binding reactivities to both peptides. The binding of sera raised against CN54 gp120 and gp140 suggests that there should be competition. However the binding is not very strong for sera diluted only 1 in 10 and therefore may not be able to outcompete the specific mAb to a measurable level.
Figure 4.6. The binding of guinea pig sera to the linear peptides, peptide 29 and peptide 14 that are recognised by mAbs TH1 and TH3 respectively. Antibody supernatants are included as controls. Data shows absorbance from wells in row A of ELISA plates, containing the highest concentration of sample (1 in 10), normalised against naive guinea pig serum.
4.3.5 Competition of guinea pig sera with neutralising monoclonal antibody

The failure to epitope map anti HIV-1 envelope, cross reactive responses, using HIV-1 \( \text{W61D} \) specific reagents may have reflected the relative affinity of the antibody reagents for cognate and cross reactive epitopes. In the previous chapter, it was demonstrated that it was possible to map specific epitope reactivities in polyclonal sera using biotinylated neutralising monoclonals that recognised a broad range of HIV-1 envelope proteins. To investigate this, competition ELISA’s were performed involving the polyclonal guinea pig antisera and unlabelled human mAbs 697-D, 447-52D and b12 that are specific for epitopes in V2, V3 and CD4 binding site regions of HIV-1 envelope.

The Figures 4.7, 4.8 and 4.9 present the outcome of competition ELISA’s between guinea pig sera, and mAbs 447-52-D, 697-D and b12 respectively. Detectable competition was observed between all of the polyclonal serum samples and all three monoclonal antibodies (ANOVA \( P<0.01 \)). However, the relative degree of competition observed for each antisera varied depending upon the mAb competitor used.

The mAb b12 appeared to have only a limited capacity to bind to HIV-1 \( \text{W61D} \) rgp120, with low and erratic binding observed in this assay. All immune sera competed with b12 for binding to HIV-1 \( \text{W61D} \) rgp120 to varying degrees with the most consistent and marked being obtained with HIV-1CN54 rgp120 and rgp140 immunogens (Figure 4.9). The inhibition of b12 binding at the highest concentration of immune serum was significant when analysed by ANOVA and confirmed by Dunnett’s test (\( P<0.05 \)). Monoclonal antibodies 697-D and 447-52D bind to HIV-1\text{W61D} better than b12, and provide clearer evidence of competition (Figures 4.7 and 4.8).

In addition, more marked competition was observed when the immune guinea pig sera were titrated in these ELISA’s particularly against the V3 region binding mAb (Figures 4.7). The ability of each immune serum to compete with each of the antibodies appeared independently variable as demonstrated in Table 4.2. Furthermore, where more than one guinea pig was
immunised with the same immunogen differences between the epitope specificity of the immune sera was observed for example the #1 guinea pig immunised with HIV-1<sub>CN54</sub> rgp120 exhibited the greatest competition against mAb 697D, and sera from the #2 guinea pig immunised with HIV-1<sub>CN54</sub> rgp120 exhibited the greatest competition against mAb 447-52D. However it is only the difference in responses between guinea pigs immunised with HIV-1<sub>CN54</sub> rgp120 competing with 447-52D and 697-D, and sera from guinea pigs immunised with HIV-1<sub>UG37</sub> rgp140 competing to 697-D which were significantly different (Dunnett’s test P<0.05).

Dunnett’s statistical analysis of competition to the V2 mAb 697-D shows that results for all immune sera are significant compared to the naïve sera. Comparison of all the groups to one another by Tukey’s testing (P<0.05) shows that the sera that were raised to 93TH975.15tgpl20 had significantly higher levels of competition than that raised to CN54rgp140 (1), BR29rgp140 and UG37rgp140 (1). The sera raised to UG37rgp140 (2) showed significantly more ability to compete than those raised to UG37rgp140 (1), BR29rgp140, HAN2rgp120 and CN54rgp140 (1).

Even though all sera showed significant competition to the mAbs, the differences seen are not just between sera raised to different envelope proteins, with the CN54gp140 (2) sera showing significantly more competition than CN54gp140 (1). Similar differences are not seen however in the competition to mAb b12, for which only the two UG37gp140 sera are statistically different to the BR29gp140 sera. Aside from this, all sera can be seen to be competing significantly compared to the naïve sera by Dunnett’s testing (p<0.05).
Figure 4.7 Competition assay against mAb 447-52D. Data shows the ability of sera from guinea pigs immunised with recombinant HIV-1 envelope protein, or HIV-1w61D as a positive control (sample T149), to compete with mAb 447-52D for binding to W61Drgp120 (n=3, +/- 3sd).
Figure 4.8 Competition assay against mAb 697-D. Data shows the ability of sera from guinea pigs immunised with recombinant HIV-1 envelope protein, or HIV-1 \text{W61D} as a positive control (sample T149), to compete with mAb 697-D for binding to W61D\text{rgp120} (n=3, +/- 3sd).
Figure 4.9 Competition assay against mAb b12. Data shows the ability of sera from guinea pigs immunised with recombinant HIV-1 envelope protein, or HIV-1_W61D as a positive control (sample T149), to compete with mAb b12 for binding to W61D_{rgp120} (n=3, +/- 3sd).
<table>
<thead>
<tr>
<th>Sera</th>
<th>Percent reduction in 50% maximal binding against mAb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>447-52D</td>
</tr>
<tr>
<td>T149</td>
<td>64.1</td>
</tr>
<tr>
<td>CN54rgp120 (1)</td>
<td>63.4</td>
</tr>
<tr>
<td>CN54rgp120 (2)</td>
<td>84.6</td>
</tr>
<tr>
<td>CN54rgp140 (1)</td>
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<td>BR29rgp140</td>
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<td>68.1</td>
</tr>
<tr>
<td>93TH975.15rgp120</td>
<td>54.1</td>
</tr>
</tbody>
</table>

Table 4.2 showing the percentage reduction in the 50% maximal binding for mAb binding when competing with guinea pig sera at a dilution of 1.5 log_{10}.
4.4 Discussion

Genetic analyses of a large number of HIV-1 isolates from a variety of geographic locations have divided HIV-1 into a number of clades, based upon the overall level of genetic diversity (McCutchon, F.E., 2000; Robertson et al., 2000). However, it is not known what the relevance of clade means in terms of the likely breadth of protection that could be achieved with a monotypic HIV-1 vaccine. As a result there has been an effort to make reagents and vaccines that correspond to the widest range of clades possible, and evidence suggests that combinations of Env from different clades could be used to expand the breadth of neutralisation (Chakrabarti et al., 2005). However although Env immunogens have been able to elicit high titre neutralising antibodies, these are only capable of neutralising small groups of homologous or highly neutralising sensitive viruses (Beddows et al., 1999; Bures et al., 2000).

The goal of these studies is to determine the minimum number of antigenic variants required in a vaccine to confer protection against a broad range of HIV-1 variants circulating in the field. Presently the exact make up required for an effective anti HIV-1 vaccine is unknown, but it is likely that the virus envelope would be included. At NIBSC, within the context of the Centralised Facility for AIDS Reagents (CFAR) a range of recombinant envelope proteins have been produced that have been expressed in mammalian cell lines (Jeffs et al., 2004). Purified rgp120 and rgp140 were used as immunogens and were characterised by SDS polyacrylamide gel electrophoresis under denaturing conditions (data not shown). Whereas rgp120 was monomeric in nature, the rgp140 envelopes were identified as being oligomeric. The goal of this study was to probe the immune responses generated against this range of proteins.

As described in the previous chapter, studies performed at NIBSC have demonstrated that a recombinant Env-based vaccine was able to protect macaques against detectable infection in the SHIV macaque model (Almond et al manuscript in preparation). Furthermore it was shown that this protection could be transferred with immune serum collected from immunised macaques.
prior to challenge. However, this antibody mediated protection was restricted to homologous virus challenge. In the previous chapter, the serological responses of macaques to vaccination with the monomeric HIV-1 envelope were probed. In particular a competition ELISA identified that protected macaques elicited more potent responses to V2 and V3 regions than an immunised macaque where virus breakthrough was observed. Clearly the V3 region of envelope is highly variable, and whilst TCLA viruses are susceptible to neutralisation by anti V3 antibodies, field isolates of HIV-1 appear more resistant to neutralisation through this epitope (Hanson, 1994; Matthews, 1994). The purpose of this project was to evaluate and compare the epitope specificity and breadth of cross reactivity in serum generated following immunisation with HIV-1 envelope (rgp 120 and rgp 140) derived from isolates from different clades.

The envelope proteins were formulated with the same Quil A adjuvant that is a component of the AS02A adjuvant used in the macaques study described in Chapter 3. Guinea pigs were immunised with recombinant HIV-1 envelope based vaccines, since they yield a reasonable volume of immune serum to allow characterisation of responses. The immune sera generated in the guinea pigs were first characterised for binding endpoint titres to recombinant envelope from different clades of HIV-1. These ranged between log$_{10}$ 2.6 observed for anti HIV-1$_{Han-2}$ rgp140 binding to HIV-1$_{CN54}$ rgp140, to log$_{10}$ 5.6 for anti HIV-1$_{CN54}$ rgp140 binding to the rgp120 homologue.

It is recognised that not all envelope binding antibodies have the same properties, such as the ability to neutralise virus (Belshe et al., 1993; Parren et al., 1997), and not all antigens elicit the same results. For example, in the previous chapter macaques that had been immunised on 3 or 8 occasions had very similar titres of binding antibodies (approx log$_{10}$ 4.0) against recombinant HIV-1$_{W61D}$ rgp120 by ELISA. However there was a significant difference in the binding titres of the two groups to HIV-1 infected cell lysates ($P=0.034$), but not SHIV infected cell lysates ($P=0.118$). Alongside this there was approximately a 6 fold difference in the group mean titres.
of neutralising antibodies in sera collected from each group. A similar observation was made in this study. Comparison between the titres of anti-HIV-1_W61D binding antibodies detected by ELISA and the titre of SHIV_W61D neutralisation in guinea pig anti-sera were markedly different. Titres up to log\textsubscript{10} 4.3 were detectable by ELISA. None of the antisera exhibited a titre of greater than 1 \textlog{10} for 75% neutralisation of SHIV_W61D. Although only limited neutralisation of SHIV_W61D was observed some intriguing observations were noted. No detectable neutralisation of SHIV_W61D was observed in anti serum to the F clade HIV-1_BR39 and E clade HIV-1_93TH975.15 envelope proteins. Marginal neutralisation by anti sera raised against HIV-1_CN54 rgp140 and the best neutralisation activity was observed in antisera raised to the B clade HIV-1_Han-2 rgp120, and A clade HIV-1_UG37 rgp140. Although there have been possible patterns of antigenicity relating to clades (Moore et al., 1996; Nyambi et al., 1996; Weber et al., 1996), the failure so far to isolate specific neutralising serotypes relating to clades is a stumbling block in eliciting broadly effective responses (Bures et al., 2002; Kong et al., 2003; Kostrikis et al., 1996; Moore et al., 1996; Nyambi et al., 1996, 2000; Verrier et al., 2000; Weber et al., 1996).

Whilst the immunogen is important in determining the neutralising activity in the serum, host factors also play a role. This is exemplified by the marked difference in neutralising titres of 2 guinea pigs immunised with the same HIV-1_CN54 rgp120 vaccine. Whilst only a minimal difference in titre was observed in binding titres observed by ELISA, no detectable neutralising activity was observed in serum from guinea pig #1 whereas some of the highest neutralising activity was observed in serum from guinea pig #2.

These sets of distinct binding and virus neutralising activity provided an opportunity to probe the specificity of the antibodies that contributed to the cross neutralising activity in the guinea pig antisera. Due to the value of competitive ELISA in the dissection of anti-envelope antibody responses described in the previous chapter, this approach was applied again. Initially, the mAbs used as competitors in the ELISA had been raised against the homologous HIV-1_W61D envelope protein. Two of these mAbs have been mapped to bind peptides derived from the V2 and V3 regions of the HIV-1_W61D. Although the remaining two have not been mapped, it was
known that they did not compete with each other and thus they did not bind to overlapping epitopes on the HIV-1 envelope. However, none of the polyclonal immune sera were able to compete detectably with these monoclonals. By contrast, when alternative mAbs, that also bind to V2 and V3 epitopes, but are broadly cross reactive between a number of envelope variants, were introduced in this assay, competition was observed. Whilst these apparent contradictory observations may suggest that the guinea pig polyclonal sera recognise epitopes that are similar to those recognised by the human but not the mouse immune system, it is probably more likely to be accountable by the relative high affinity for the HIV-1 envelope by the mouse mAbs raised by immunisation with the cognate protein, and the lower affinity of antibodies raised to heterologous envelope proteins and which cross react with epitopes on the HIV-1 envelope. Whereas the human mAbs are neutralising, the mouse mAbs are not neutralising, which exemplifies the fact that the affinity of binding to HIV-1 envelope alone is not sufficient to enable the antibody to neutralise a virion expressing this envelope. Likewise the guinea pig sera obviously had the ability to bind W61D close to epitopes to which neutralising antibodies may bind, but there are subtle differences which mean that these sera may not necessarily neutralise the homologous SHIV.

The role of the relative affinity of polyclonal sera and monoclonal competitor to bind to an epitope in order for evidence of competition to be observed was reinforced by a direct binding ELISA to the peptide epitopes recognised by the anti-V2 and anti-V3 HIV-1 envelope mAbs. The antisera raised in guinea pigs immunised with heterologous HIV-1 envelopes bound to these peptides very poorly compared with the mAbs, with only the CN54-raised sera having significant responses to both peptides. The three best neutralising sera do not have strong responses, and interestingly not to the V3 loop peptide that has been shown to be a potent neutralising epitope of viruses (Binley et al., 2004; Chakrabati et al., 2005; Conley et al., 1994; Gorny et al., 2004; Hioe et al., 1997; Krachmarov et al., 2001, 2005; Liao et al., 2000; Yang et al., 2004). In contrast to the epitopes examined here, Derby et al., (2007) found that a trimeric HIV-1SF162gp140 derived immunogen elicited neutralising responses that preferentially targeted
the V1 loop. Currently there is no broadly neutralising mAb that is specific for the V1, but it would be of interest to examine the responses here for V1, compared to V2 and V3.

One of the key difficulties for interpreting the results of this study using guinea pigs is the apparent variability in the results obtained when 2 animals of the Hartley strain were immunised with the same immunogen. For example, in the competition analysis of these polyclonal antisera with the anti-V3 monoclonal 447-52D, anti-sera from one of the two guinea pigs (#2) inoculated with HIV-1\textsubscript{CN54} rgp120 competed significantly better than the other (#1), and even competed better than the antisera from macaque T149 that was used as part of the pool that protected naive macaques against SHIV\textsubscript{W61D}. By contrast, in competition with the anti V2 monoclonal 697-D, the antiserum from guinea pig #1 inoculated with the HIV-1\textsubscript{CN54} rgp120 competed markedly better than the antiserum from animal #2. These differences apparent by competition ELISA were associated with differences in the ability of these antisera to neutralise SHIV\textsubscript{W61D}. Since the immunisation of both guinea pigs was undertaken sequentially using material prepared and shared between the 2 individuals, the most likely factor accounting for these marked differences would be immunogenetic differences between the 2 individuals.

The complexity of evaluating responses elicited by HIV envelope proteins was demonstrated in a subsequent study using 4 of the guinea pig sera described above, undertaken by the group of David Montefiori at the University of North Carolina. Analysis of the pairs of antisera raised against HIV-1\textsubscript{CN54} rgp120 and HIV-1\textsubscript{UG37} rgp140 and their ability to neutralise a panel of HIV-1 isolates failed to provide a clear pattern of cross neutralisation based upon the clade of the challenge virus or even comparable relative neutralising titres for both anti-sera generated by the same immunogen (Table 4.3). The differences are starker between the two UG37 raised sera, where sample 1 had ID50 titres of over 20 for all 9 viruses tested, whereas sample 2 only had measurable titres for 5 out of 14. Both of the CN54 sera tested had neutralising titres for all viruses, however they also neutralised the two control viruses (MLV, and SIVmac) with similar titres to the HIV isolates. This suggests that not only are animal to animal variations affecting
the neutralising ability against HIV-1, but some of the sera possess an intrinsic non-specific neutralising capacity.

One of the goals of this study was to ascertain the relationship between genotype and serotype for HIV-1 envelope protein, in order to assist in the likely requirements for an HIV-1 envelope based vaccine that can elicit broad cross-neutralising antibody reactivity. Whilst the data generated may not provide a complete answer, it does demonstrate that genotypic variation does not directly relate to serotypic differences. Furthermore, evaluating potential envelope proteins to be used in vaccinations is complicated. Antigenic characterisation may provide part of the evaluation, however currently there is a limited understanding of the immunogenic potential of different preparations and of the variability of responses that may be encountered by individuals in an outbred population.

It is important to determine the key neutralising epitopes of the SHIV's used in macaque challenge studies. For them to provide a relevant pre-clinical challenge model for vaccines that elicit potentially neutralising antibodies, then the pattern of neutralising and non-neutralising epitopes on the SHIV must mimic the pattern on primary isolates and field strains of HIV-1. Dissecting the component reactivities in polyclonal antisera is also challenging. These challenges can be overcome if in-bred animals are used and polyclonal responses are dissected through the generation of monoclonal antibodies in response to candidate HIV-1 envelope protein immunogens.

Currently many different immunogens, are being examined alone and in combination. One of these is the use of DNA to prime the immune response before boosting. This is suggested to be able to elicit much better neutralising antibody as well as cellular responses. The ability of the use of DNA immunisation followed by protein boosting to produce novel neutralising antibodies will be examined in a later chapter.
<table>
<thead>
<tr>
<th>Virus</th>
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<th>Neutralisation titres (ID50) in TZM-bl cells</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>CN54rgp140-1</td>
</tr>
<tr>
<td>Q23.17</td>
<td>A</td>
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<td>A</td>
<td>45</td>
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<tr>
<td>Q168.a2</td>
<td>A</td>
<td>149</td>
</tr>
<tr>
<td>Q461.e2</td>
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<td>159</td>
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<td>SIVmac239CS.23</td>
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<td>106</td>
</tr>
</tbody>
</table>

Table 4.3 Neutralising ability of selected guinea pig sera against a panel of Env-pseudotyped viruses. (Data adapted from D, Montefiori Duke University). MLV used as a control virus to assay for non-specific neutralising ability (nt = not tested).
Chapter 5

Production of monoclonal antibodies to C clade isolates

5.1 Introduction

Monoclonal antibodies are extremely useful reagents in biomedical research and since the publication of a method for producing cell lines producing a single antibody of defined specificity (Kohler and Milstein., 1975), they have been of incalculable value for research. Of particular value is their use as tools, not only to confirm the identity of molecules, but also to characterise and define sub-molecular regions and epitopes. mAbs have also proved valuable research reagents for the specific purification of macromolecules from complex mixtures without the need for potentially denaturing conditions and there is excitement as to their use and future further potential as therapeutic clinical treatments for chronic non infectious disease treatments. Since 1986, a number of mAb therapies have been approved for treatment of autoimmune conditions such as rheumatoid arthritis, in cases of allograft rejection, and against some cancers (Waldman. T.A., 2003).

AIDS research has benefited from the availability of mAbs, both in the antigenic characterisation of the virus and of surface markers of cells infected with virus, or which are involved in the pathology of disease. In terms of AIDS vaccine development, mAbs have been essential tools to explore and characterise the issue of virus neutralisation.

Neutralisation by antibody is the key measure by which the efficacy of most current vaccines to infectious diseases is measured, and convalescent serum collected from patients after recovery has significant virus neutralising titres. For HIV this is not the case, since infected individuals have never been reported to fully clear infection, there is no such thing as convalescent serum to analyse. Using the macaque model, it has been reported that passive infusion of potent neutralising mAbs is able to protect naïve animals against infection (Baba, et al., 2000; Hofman-

However, antibodies with the ability to neutralise a broad range of HIV isolates are very rare and, so far, difficult to generate by immunisation. If a vaccine against HIV-1 is to be developed that protects by eliciting anti-viral neutralising antibodies that work across a range of genotypes, then we need to understand more about the antigenic differences that accompany this genetic variation.

Currently, there is a vast range of mAbs and other reagents to HIV-1 envelope. These reagents are important to determine the structure of envelope, to define functional regions and so to inform vaccine development. However the majority of mAbs have been derived from immunisation with or infection by, HIV-1 isolates of the B clade. To understand the impact of genetic variation of clades in vaccine design, further antibody reagents are needed that have been generated following immunisation with non-B clade envelope protein. In particular, there is a lack of reagents to HIV-1 C clade isolates, which would be valuable since it is the most abundant clade worldwide (UNAIDS Global Report 2008). Up to 60% of all worldwide infections are attributed to this clade alone, and it is especially abundant in third world areas where it is estimated that up to 60% of HIV-1 cases in sub-Saharan African and South Asia are clade C (Hemelar et al., 2006; UNAIDS Global Report 2008). Clade C viruses present a unique challenge for vaccine studies due to a number of characteristics that differentiate them from a large range of highly studied B clade isolates. These include; high levels of intra clade variation (van-Harmelen., 2001; Novitsky et al., 1999); higher viral loads (Neilson et al., 1999); CCR5 preference (Adebe et al., 1999; Tscherning et al., 1998) and unique genome features (Novitsky et al., 1999; Rodenberg et al., 2001).

In the previous chapter I described studies designed to characterise and compare the antigenic structure of recombinant envelope proteins cloned from representative isolates of the major clades using polyclonal antibodies. Whilst some differences were identified, they frequently required competition with mAb reagents to be able to dissect the fine discrimination between the different proteins at key epitopes. In order to assist with the antigenic mapping of HIV-1 C
clade isolates, it is clear that new mAb reagents are required, preferably, produced following immunisation with HIV-1 C clade envelope proteins that are being used in vaccine development.

In Europe, there are 2 major vaccine research programs that include HIV-1 C clade envelope as a component immunogen. These programs are investigating a Chinese clone termed CN54 derived from the HIV-1 isolate 97CN001, and an African isolate, termed ZM96, derived from the HIV-1 isolate 96ZM651-8 respectively. To support these vaccine programmes, this part of the project set out to elicit novel, characterised mAbs that react against these two envelopes and investigate their value as reagents with defined specificities. In the process of producing these mAbs a strategy utilising a prime boost approach, avoiding the need to sub-clone the envelope protein into multiple vectors was developed. Figure 2.2 in methods section 2.6.1 sets out the proposed schedule for antibody production and characterisation.

DNA immunisation with Env expressing plasmids has been shown to elicit anti-Env antibody responses (Lu et al., 1995b, 1996, 1998). Furthermore these responses can be improved by boosting with Env proteins after DNA priming (Barnett et al., 1997; Richmond et al., 1998), and improve the production of neutralising antibodies (Vaine et al., 2008; Wang et al., 2006). Due to the increase in neutralising activity observed in studies compared to Env protein immunisation alone, this DNA prime/protein boosting method was applied to the production of useful C clade mAb reagents.
5.2 Methods

5.2.1 Production of plasmid DNA

An HIV-1 97CN001 envelope recombinant gp140 subclone –hereby known as CN54 – in the pEE14tpa vector was provided by Dr S Jeffs (Imperial College, London). The vector was transfected into XL-1 electrocompetent cells, and a single colony was selected and grown up in Luria Broth. DNA was prepared from cultures using Qiagen Maxiprep kits following the manufacturer’s instructions and the resulting purified plasmid DNA was checked for the presence of the recombinant envelope insert by restriction endonuclease digestion and subsequent fragment analysis by agarose gel electrophoresis (see methods section 2.5 for details).

An HIV-1 96ZM651.8 envelope recombinant gp140 sub-clone– hereby known as ZM96 – expressed in the pEE14tpa vector was supplied by Dr S Jeffs and prepared in a similar manner with the exception that the vector was transfected into super competent CaCl treated E.coli cells by heat shock. This was due to the failure to transform XL-1 cells with ZM96 DNA by electroporation.

5.2.2 Production of hybridoma cell lines

Groups of 5 Balb/C mice were primed at weeks 0, 4, 8 and 12, by intra-muscular inoculation with 50µg plasmid DNA expressing either CN54 or ZM96 envelope produced at 500µg/ml in ddH2O. Two weeks after the 4th immunisation a test bleed was performed to identify the mice with the strongest detectable serological anti-envelope responses, as determined by a direct ELISA. These mice were then boosted on 3 consecutive days, by subcutaneous inoculation with the corresponding homologous recombinant protein expressed in Chinese Hamster Ovary (CHO) cells and immuno-purified by Dr S Jeffs as described previously (2004).
Immunisation protocols were later optimised and adapted so that after priming at weeks 0, 4, 8 and 12, mice were boosted once with the recombinant protein formulated with adjuvant Quil A, and a week later boosted twice more (on consecutive days) with the recombinant protein alone.

One day after final boosting of the serological response by inoculation with recombinant envelope protein, the fusion of splenocytes was performed as described in section 2.6.1. Growth of colonies in the presence of selective medium was identified by acidification of culture medium and microscopically. Acidified media samples were screened for production of antibodies specific for the homologous C clade envelope protein by ELISA as described in section 2.6.2. Selected B cell colonies were cloned by limiting dilution, before further screening of growing colonies that would be expected to statistically be derived from a single cell. The cloning procedure was repeated twice.

### 5.2.3 Characterisation of anti-envelope reactivity

B cell lines and clones were expanded in tissue culture and the antibody reactivity of tissue culture supernatant was characterised. ELISA and western blotting using homologous and heterologous envelope proteins and epitope mapping against a series of overlapping peptides covering the whole of ZM96 rgp140 were applied. All proteins and peptides used in ELISA in the characterisation of these mAbs were coated as described in section 2.1.1 at 1μg/ml. Western blotting was performed as described in section 2.3.1.

### 5.2.4 IgG subtyping of selected monoclonal antibodies

The isotypes of each of the mAbs was determined using Roche Mouse IgG isotyping strip kits as described in section 2.9.
5.2.5 Competition analysis

Competitive ELISA's were performed between the various mAbs produced, as described in 2.1.5. This required the partial purification, by ammonium sulphate precipitation, and biotinylation of samples of each monoclonal antibody as described in 2.4.2 and 2.4.3 respectively.

5.2.6 Neutralisation of HIV-1\textsubscript{ZM96} and SHIV\textsubscript{W61D}

The ability of these mAbs to neutralise the homologous and heterologous viruses was examined. For mAbs raised against CN54 envelope this was performed by Dr C Moog (Strasbourg) and for anti-ZM96 mAbs it was performed as described in 2.8.1. mAbs with immuno-reactivity against HIV-1\textsubscript{W61D} envelope protein were evaluated for neutralisation of SHIV\textsubscript{W61D} as described in section 2.8.3. The replication of virus was monitored by determining the amount of gag protein in tissue culture supernatant. This was determined using either the Innotest Kit (section 2.8.2) for HIV or an in-House anti-SIV gag assay for SHIV\textsubscript{W61D} (section 2.8.4).
5.3 Results

5.3.1 Plasmid DNA quantification

Samples of purified DNA were checked for the presence of the plasmid insert by restriction digest and agarose gel electrophoresis. Samples were then quantified by comparison against known concentrations of stock DNA samples (data not shown). Figure 5.1 shows the restriction digest for prepared samples of CN54 DNA. The plasmid can be seen to be present in all 6 samples produced in E.coli cultures (ZM96 data not shown).
Figure 5.1. Restriction endonuclease analysis of pEE14tpa[CN54gp140] with HindIII and EcoR1, analysed on a 1% (w/v) agarose gel by electrophoresis. Lanes 1 and 8 are φλ running ladders. Lanes 2-7 are separate preparations of plasmid DNA purified from E.coli cultures.
5.3.2 Production of C clade hybridoma lines

The anti-CN54 rgp 140 reactivity in the serum of mice at week 14, after priming with pEE14tpa[HIV-1_{CN54} rgp140] is shown in figure 5.3. The mouse with the strongest detectable serological anti-envelope response was selected and boosted at week 16, on 3 consecutive days, with rgp140. Subsequently, primed mice were boosted on 3 consecutive days, as required. Two fusions were performed with spleen cells from mice 1 and 4. No stable hybridomas eliciting anti-envelope antibodies were produced that were able to survive in the absence of feeder cells and they died off after approximately two weeks of growth. As a result recombinant human IL-6 (20pg/ml) was added to culture medium of all subsequent studies. Consequently, in subsequent fusions between approximately 30% and 60% of wells contained stable cell lines that were screened for the production of specific antibodies. The third fusion (mouse 5) produced no cell lines or clones that had titres of specific anti-CN54 antibodies so were not characterised any further.

The failure to produce clones producing a detectable anti-envelope response suggested that a more potent immunological boost was required prior to fusion. Accordingly, another primed mouse was boosted with HIV-1_{CN54}rgp140 formulated with Quil A as adjuvant for the first boost, and this was followed with two further boosts with envelope in the absence of adjuvant 7 and 8 days later. A comparison of the immunisation protocols are presented in figure 5.2, and table 5.1 summarises the impact of changing protocols on the outcome of the fusion procedure.
Figure 5.2 The two immunisation protocols used in this project. Protocol b had changes to the boosting procedure that led to the successful production of anti C clade envelope specific mAbs.
Table 5.1 The outcome of 5 fusions undertaken to produce mAbs. Fusions 1 and 2 were undertaken without the use of IL-6, and along with fusion 3, did not have adjuvant included with the first recombinant protein boost.
The fourth fusion of splenocytes (mouse 3) yielded stable B cell lines producing envelope specific antibodies that bound the homologous recombinant HIV-1 envelope protein in ELISA. Primary analysis of tissue culture supernatant gave high absorbance readings indicating either high level production of antibody and/or antibodies with high affinity. Five of these cell lines were selected and cloned twice by limiting dilution, producing 8 anti-CN54 hybridoma lines (MH9-MH16). The specificity of each of the 8 clones isolated was characterised by ELISA (figure 5.4) using rgp140 and rgp120 proteins, Western blotting (figure 5.5) against rgp140 and rgp120 proteins and neutralisation of HIV-1\textsubscript{CN54} (undertaken by C. Moog at the University of Strasbourg). These data are summarised in table 5.2.

Although the immunisation protocol involved a DNA vector expressing rgp140 boosted with recombinant gp140 envelope protein, it was possible to select and clone antibodies with apparent specificity to bind to rgp120 as well as rgp140 (see figure 5.4). Antibodies MH9 and MH10 were mapped to the V3 region by Prof Ian Jones (Reading University - personal communication) using a binding analysis of different peptide loops of gp120. The mAb MH16 was mapped to the V3 by competition studies with MH10. The remaining competition groups were presumably reactive to discordant epitopes that were created by the folding of the polypeptide chain to form the protein. Intriguingly, antibodies from only 1 of the 2 competition groups that were mapped to V3 were able to bind to homologous recombinant envelope protein in a Western blot (MH10 and MH16, see figure 5.5).
Figure 5.3 Titration of anti-CN54 rgp140 reactivity, determined by ELISA, in the serum of mice at week 14, that had been immunised with pEE14tpa[HIV-1CN54 rgp140] at weeks 0, 4, 8 and 12.
<table>
<thead>
<tr>
<th>mAb</th>
<th>Original Colony</th>
<th>Immunogen</th>
<th>Specificity</th>
<th>Isotype</th>
<th>Competition Group</th>
<th>Elisa</th>
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<td>1C4</td>
<td>CN54 gp140</td>
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</table>

* Some neutralising activity against HIV-1 Bal in Macrophage cell based assays.

Table 5.2. Summary table of the characteristics of anti-CN54 antibodies produced from stable hybridoma cell lines.
Figure 5.4 The relative ability of anti-HIV-1_{CN54} mAbs to bind to gp120 or gp140 as determined by ELISA. The binding of mAb CA13 that binds to a highly conserved motif in the C1 region of HIV-1 envelope isolates of a number of clades is included as a control. Recombinant CN54gp120 was coated onto plates at 1μg/ml and the response of culture supernatants diluted 1 in 10 are shown.
Figure 5.5 Western blot of a 10% SDS-PAGE gel showing the reactivity of anti-CN54 mAbs to CN54gp140 in denaturing gel. Lanes 1) MH9; 2) MH10; 3) MH11; 4) MH12; 5) MH13; 6) MH14; 7) MH15; 8) MH16. Two mAbs - MH10 and MH16 - are shown to be recognising gp140, but no other bands. Envelope is known to cleave through the V3 region when run on denaturing gels. This gives two gp70 fragments for gp140, but neither of these bands appears to be recognised.
Following the successful production of hybridoma cell lines producing mAbs with specificity against CN54, a very similar immunisation protocol was undertaken using HIV-1 _ZM96_ rgp140 immunogens. A group of 5 mice were primed with pEE14tpa[HIV-1 _ZM96_ rgp140] on 4 occasions. Log_{10} titres of anti-ZM96 antibodies 2 weeks after the final immunisation varied between 2.6 and 4.2 (figure 5.6). The mouse with the highest titre of anti-HIV-1 _ZM96_ seroreactivity was selected and boosted with HIV-1 _ZM96_ rgp140 protein formulated with Quil A as adjuvant, and 7 and 8 days later boosted with protein without adjuvant. This was due to be used for the fusion, but it died prematurely. As a result the fusion was performed using splenocytes from animal number 2 that had the next highest binding reactivity. Tissue culture supernatants from B cell lines, elicited antibodies giving absorbance readings of up to A_{450} 2.6 when measured by ELISA against homologous envelope proteins obtained from different sources. These rgp140 proteins were obtained from S Jeffs and Polymun Scientific (Vienna, Austria) and were obtained from the same stable CHO cell line, but with the protein purified by differing methods. Cell lines were selected from this fusion and cloned twice by limiting dilution, producing 10 anti-ZM96 hybridoma lines (MH17-MH26). The binding specificity of tissue culture supernatant from the 10 clones isolated from the original 10 cell lines was characterised by ELISA (fig 5.7) and Western blotting (fig 5.8). Neutralisation of the parental virus 96ZM651-8 on PBMC’s was also examined and all these data are summarised in table 5.3. They demonstrate that, of the original ten cell lines selected, there are at least 8 different specificities.

The isotyping of these antibodies was undertaken to see if there were any different IgG subtypes of mAbs produced using this DNA prime/protein boost. The results in tables 5.2 and 5.3 show that all the mAbs selected in this work are IgG1, and bar the one exception of MH21 are kappa light chains.
Figure 5.6 Titration of anti-ZM96 reactivity, determined by ELISA, in the serum of mice that had been immunised with pEE14tpa[HIV-1zm56 rgp140] at weeks 0, 4, 8 and 12.
<table>
<thead>
<tr>
<th>mAb</th>
<th>Original colony</th>
<th>Immunogen</th>
<th>Specificity</th>
<th>Isotype</th>
<th>Competition group</th>
<th>Elisa</th>
<th>Western blotting</th>
<th>Neutralisation</th>
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<tr>
<td>MH17</td>
<td>1A8</td>
<td>ZM96 gp140</td>
<td>ND</td>
<td>IgG1κ</td>
<td>1</td>
<td>+</td>
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<td>—</td>
</tr>
<tr>
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<td>ZM96 gp140</td>
<td>ARP 7104.79</td>
<td>IgG1κ</td>
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<tr>
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<td>ARP 7104.79</td>
<td>IgG1κ</td>
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<td>+</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
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</tr>
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<td>ZM96 gp140</td>
<td>V3</td>
<td>IgG1λ</td>
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<td>+</td>
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</tr>
<tr>
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<td>ZM96 gp140</td>
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<td>4</td>
<td>+</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
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<td>+</td>
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</tr>
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<td>IgG1κ</td>
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<td>+</td>
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<td>—</td>
</tr>
<tr>
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<td>IgG1κ</td>
<td>7</td>
<td>+</td>
<td>—</td>
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</tr>
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</table>

sup Some reactivity to peptide ARP 7104.80 (CFAR) - these two peptides cover the V3 loop crown

Table 5.3. Summarises the characterised anti-ZM96 antibodies produced from stable hybridoma cell lines.
Figure 5.7 The relative ability of anti-HIV-1_{ZMPK} mAbs to bind to two different samples of ZM96gp140; the first was obtained from Dr S Jeffs, and was the one used to boost the mice. The second was produced by Polymun and obtained through the CFAR programme at NIBSC. The binding of mAb CA13, that binds to a highly conserved motif in the Cl region of HIV-1 envelope isolates of a number of clades is included as a control. Recombinant proteins were coated at 1μg/ml on plates, with the response of hybricoma supernatant diluted 1 in 10 shown.
Four mAbs - MH17, MH18, MH19 AND MH23 - specifically recognise HIV-l\text{ZM96} envelope protein as monomeric rgp120 and rgp140 as well as rgp140 multimers. Envelope is known to be cleaved at a point in the V3 region that yield two fragments (2 gp70 fragments from rgp140, and a gp70 and gp50 fragment from rgp120), when run on denaturing gels. Neither of these products is recognised.
5.3.3 Epitope mapping

A definitive method for determining the specificity of antibody reactivity is to determine whether they bind to synthetic peptides based upon the sequence of the immunogen. Unfortunately a series of peptides based upon the HIV-1\textsubscript{CN54} gp140 was not available. However, a series based upon HIV-1\textsubscript{ZMB6} gp140 was available (CFAR, ARP 7104.1-169). Interestingly only 2 of the 10 monoclonals (MH18, MH19) bound to linear peptides. These antibodies both bound to V3 region.

5.3.4 Competition analysis

In the absence of definitive data regarding the specificity of the mAbs raised against HIV-1\textsubscript{CN54} and HIV-1\textsubscript{ZMB6} envelope, information can be obtained through the application of competitive ELISA’s. Amongst the anti-HIV-1\textsubscript{CN54} mAbs, 5 distinct binding specificities were identified (competition groups 1-5, see table 5.2). Each of these specificities may be related back to the original colony selected after fusion. Within each competition group, marked competition is observed preventing the binding of biotinylated antibody. Intriguingly, amongst the antibodies that had been identified to bind to the V3 region, two competition groups were defined which did not appear to cross compete detectably.

Amongst the monoclonals that were produced following immunisation with HIV-1\textsubscript{ZMB6} gp140, 7 distinct competition groups were identified (see table 5.3). Unlike the anti-HIV-1\textsubscript{CN54} gp140 generated monoclonals a complex inter-relationship of cross competition was detected that was not associated to the original colonies selected. In some situations, eg MH18, MH19 and MH21 it was possible to obtain complete and reciprocal competition between the antibodies. MH17 and MH23 competed against each other, but in this case the competition is not completely reciprocal. Furthermore there is some very low level of competition between MH17 and MH18,
MH19, MH20 and MH21. Whilst MH18 and MH19 are mapped to the same linear epitope in V3, the other monoclonals have not been mapped for binding to a linear peptide. The monoclonal antibody MH21 was observed to be able to compete with a broad range of the anti-HIV-1Zm96 mAbs. By contrast MH24, MH25 and MH26 represented apparently unique specificities and did not compete with any of the other anti-HIV-1Zm96 rgp140 monoclonals.
Table 5.4  Competition between mAbs raised against CN54gp140. Values are the maximum percentage inhibition of binding to CN54 gp140 by biotinylated antibodies in the presence of titrated unlabelled competitor antibodies.
Table 5.5 Competition between mAbs raised against ZM96gp140. Values are the maximum percentage inhibition of binding to ZM96gp140 by biotinylated antibodies in the presence of titrated unlabelled competitor antibodies.
5.3.5 Binding analysis using homologous and heterologous recombinant envelope proteins

The ability of the mAbs, produced in response to immunisation with HIV-1 C clade envelope protein, to bind to a number of recombinant HIV-1 envelope proteins was evaluated by ELISA. The results are presented in figure 5.9. The antigens used were B clade envelope proteins HIV-1_W61D gp120 and HIV-1_IIB gp120, and C clade envelope proteins HIV-1_C54 gp120, HIV-1_C54 gp140 and HIV-1_ZM96 gp140. The HIV-1_W61D, HIV-1_C54 gp140 and HIV-1_ZM96 gp140 proteins were CHO cell derived, whereas the HIV-1_IIB and HIV-1_C54 gp120 were produced in a baculovirus expression system. The binding of the mAbs produced against HIV-1 C clade immunisation was compared with selected mAbs raised against non C clade antigens, but known to be broadly cross reactive against a variety of envelope proteins. These were CA13 (a C1 region cross reactive antibody raised against vaccinia virus expressing an A clade UG35 gp160 protein), 447-52D (a V3 specific antibody), 697-D (a V2 specific monoclonal), and b12 (a broadly cross neutralising human mAb cloned from an HIV infected individual).

The non C clade antibodies exhibited a greater ability to bind to the HIV-1 B clade envelope proteins HIV-1_IIB gp120 and HIV-1_W61D gp120, however, they also bound to the C clade proteins. All of these control antibodies except CA13 bound well to the baculovirus expressed CN54gp120. By comparison with the control antibodies, the mAbs produced by immunisation with HIV-1 C clade immunogens, bound better to the C clade rather than the B clade envelope proteins, as expected. Indeed only MH 17, MH20, MH23, MH24 and MH25 exhibited any significant binding to the B clade envelope proteins (figure 5.9), with no antibodies produced against HIV-1_C54 gp120 showing detectable binding. Antibodies MH11, MH12 and MH15 demonstrated significant binding to HIV-1_C54 envelope proteins, whereas the remainder (MH9, MH10, MH13 and MH16) exhibited cross reactivity against HIV-1_ZM96 envelope. For the antibodies produced by immunisation with HIV-1_ZM96 envelope, greater cross reactivity was observed. Intriguingly, for some of the antibodies (MH17, MH18, MH19, MH24, and in
particular MH21 and MH26) there appears to be a differential recognition of the HIV-1\textsubscript{ZM96} envelope proteins. Indeed, for some of these there is superior binding to heterologous HIV-1\textsubscript{CN54} envelope proteins than to homologous HIV-1\textsubscript{ZM96} envelope. For MH18 and MH19, these antibodies recognise a peptide mapping to the V3 region of the envelope, which should be present in both HIV-1\textsubscript{ZM96} envelope proteins.
Figure 5.9 Binding of MH mAbs, compared to previously isolated mAbs, to B clade (W61D and IIIB) and C clade (CN54 gp120, CN54 gp140, ZM96 (S. Jeffs) and ZM96 (Polymun) recombinant envelope proteins in ELISA. Binding, represented by absorbance at 450nm, is of cell supernatant samples at 1:10 dilution except for purified b12 which is at 1μg/ml, normalised against an irrelevant, negative control, antibody.
To investigate the epitopes on HIV-1 envelope recognised by cross reactive antibodies further, antibody MH23 was used to probe a Western blot analysis of the 6 envelope preparations used in the ELISA previously.

Figure 5.10 shows a coomassie stained gel of the proteins run under denaturing conditions. It shows that there are 4 bands in the first ZM96 rgp140 sample as opposed to one weak band for the second Polymun sample. The calculated molecular mass is consistent with trimeric rgp140, monomeric rgp140 and a rgp70 cleaved product. The band of around 110kDa may well be rgp120 that has cleaved of the rgp41 portion of the original gp140. The 70kDa product is due to the cleavage that can occur in V3 when HIV-1 rgp120/140 is run under denaturing conditions. CN54rgp120 shows two bands of tri, monomer and cleavage product. CN54rgp140 also shows two clear bands for trimer and monomer but no 70kDa cleavage product is observed only a weak possibly rgp120 band. IIIB and W61D only show monomeric bands present. It can noted that proteins of the same monomeric/oligimeric state run differently depending on the HIV-1 strain and expression system the envelope proteins were obtained from. This reflects differences in size and glycosolation of the envelope proteins.

When screened with MH23 there are marked differences in the bands that can be seen (figure 5.11). When looking at the two sample of ZM96 used, the Polymun sample produces one very strong band of monomeric rgp140 and a weaker monomer of rgp120, whereas the first ZM96 sample appears only with one weak band of the cleavage product. Both of the CN54rgp120 bands in the gel are recognised by MH23 along with a weak monomeric band of the CN54rgp140. No band appears for W61D, which is expected as MH23 did not bind to this antigen in ELISA, and the IIIB monomer band is strongly presented. This data not only confirms the ELISA data, but also shows the differences that can be present in samples of envelope proteins.
Figure 5.10 Coomassie stained acrylamide gel of recombinant proteins used for heterologous ELISA screening of MH mAbs.

Lanes – 1)Markers, 2)ZM96rgp140, 3)ZM96rgp140 from Polymun, 4)CN54rgp120, 5)CN54rgp140, 6)W61Drgp120 and 7)IIIBrgp120. All rgp140’s and W61Dgp120 were produced in CHO cell lines, and the other Gp120’s produced in baculovirus.
Figure 5.11 Western blot of equivalent gel to figure 5.10 after transfer to nitrocellulose membrane. The mAb MH23 was used to blot membrane.

Lanes – 1) Markers, 2) ZM96rgp140, 3) ZM96rgp140 from Polymun, 4) CN54rgp120, 5) CN54rgp140, 6) W61rgp120 and 7) IIIBrgp120.
5.3.6 Neutralisation of homologous primary virus

Anti-CN54 envelope specific mAbs were sent to Dr C. Moog (Strasbourg) to be tested as this was currently the only place that had a reliable functioning neutralisation assay to CN54 running. Dr Moog has two different single round neutralisation assays running for testing against CN54. The first of these was a conventional PBMC based assay, and showed that none of the mAbs actually neutralised the virus. The second assay was a macrophage based assay, which is reported to be more sensitive with greater involvement of FcγR I (CD64) than PBMC based assays (Holl, et al., 2004) and this showed two of the mAbs, MH10 and MH11, to have a low level of neutralising ability. (Dr C Moog, personal communication).

The anti-ZM96 envelope specific mAbs were tested for neutralisation against 30TCID₅₀ ZM96 virus infecting PBMC’s. The mAbs showed no neutralising ability in two separate assays to the virus, as indicated by no significant decrease in Gag production compared with virus alone. However a caveat to this is that as no known neutralising mAb to ZM96 is available, a combination of mAbs containing b12, 2G12 and 2F5 (Tri mAb) was used to test the set up of the neutralisation assay. The Tri mAb also failed to neutralise the virus, which could suggest that the assay is not functioning. This data is presented in figure 5.12.
Figure 5.12. Neutralising ability of anti-ZM96 MH mAbs as shown by absorbance measured in anti-HIV-1 Gag ELISA (n=3, +/- 3sd).
5.3.7 Neutralisation of SHIV<sub>W61D</sub>

As there was no apparent neutralisation of the homologous HIV-1<sub>ZM96</sub> virus by the anti-ZM96 MH mAbs, the two that recognise W61D (MH20 and MH24) were examined for neutralising ability against the SHIV<sub>W61D</sub>. This assay is run routinely in-house and should therefore give a definitive answer, unlike the previous neutralisation assay.

Figure 5.13 shows the neutralising ability of MH20 and MH24 compared to CA13. Although CA13 has a stronger recognition of W61D, it is known that it is not a neutralising antibody and this is shown in the assay. MH24 is also shown to have no neutralising ability either. However MH20 can neutralise SHIV<sub>W61D</sub> by up to 30% at a concentration of 20μg/ml. This reduces as the amount of antibody decreases, but even at 1μg there is approximately 5-10% neutralisation of the SHIV.
Figure 5.13 Neutralising ability against SHIV<sub>W61D</sub> of two anti-ZM96 MH mAbs that recognise W61Drgp120. Neutralisation is calculated as the percentage inhibition of virus growth in the presence of mAbs compared to absence. CA13 is a highly cross reactive mAb that is known to not neutralise HIV or SHIV (n=3, +/- 3sd).
5.4 Discussion

The aim of this part of the project was to produce novel anti C clade specific mAbs, and to examine whether they would be neutralising. It has been shown that using DNA priming followed by boosting with either recombinant proteins or vectors expressing proteins, can increase both humoral and cellular responses, and to provide control of viraemia in pre-clinical studies (Amara et al., 2001; Barnett et al., 1997; Barouch et al., 2000; Cherpelis et al., 2001; Doria-Rose et al., 2003; Leung et al., 2004; Richmond et al., 1997; Shiver et al., 2002). To this end the use of a DNA prime and boost regime was utilised to produce novel mAbs that could be useful in future studies of C clade viruses or animal model trials. These mAbs were characterised to try to identify epitope specificity and neutralising ability.

Groups of mice were primed with either pEE14[CN54 gp140] or pEE14[ZM96 gp140]. Fusions were performed with splenocytes from animals that had responded well to the DNA priming as determined by ELISA, and following boosting of responses. Unfortunately it was found that the stability of the cells from the first two fusions was poor at low cell density, and the cells were lost. Historically, irradiated peritoneal mouse cells were used as feeder cells, but as an alternative to the use of these cells, recombinant human IL-6 was tested as a way to increase the stability of the fused cells. Recombinant human IL-6 was added to the medium at 20pg/ml to support B-cell growth in the absence of feeder cells, and this led to much higher stability of colonies of cells that could be grown up to a level for screening. After the fusions were stabilised it was discovered that there were very few, if any, hybridomas produced that were eliciting envelope specific mAbs. Although the DNA priming was shown to be able to elicit responses in the animals in ELISA, the possibility remains that the protein boost did not actually produce a boosting effect on those memory B cells that had been formerly produced to the DNA exposure. Therefore this was boosted by the addition of an adjuvant (2% Quil A) to the first recombinant protein boost, which led to a large increase in the number of hybridomas producing envelope specific mAbs. Many new adjuvants have been formulated to try to
improve either arm of the immune system, or possibly both, in vaccinations and this data suggests that utilising adjuvants at either priming or boosting stages may increase the production of neutralising antibodies.

After the fusions were stabilised, hybridoma cell lines that were positive in ELISA screening to the recombinant proteins were cloned by limiting dilution. The limiting dilution was carried out twice to ensure monoclonality, but after characterisation it appeared the original selected colonies were producing only mAbs with a single specificity. This may be due to the inclusion of the IL-6 that allows the fusion to be plated out at a very low density and still be stable for growth. For HIV-1CN54, 5 original colonies were chosen and after cloning a second time, multiple colonies from each original colony were pursued. Clones from the same original colony were found to produce antibodies with identical specificities (data not shown), suggesting that there was no benefit of pursuing multiple clones from the same original colony. To increase the likely range of specificities against HIV-1ZM96gp140 only a single colony was selected and pursued from each original colony. This appeared to lead to the production of more exclusively specific mAbs.

The anti-CN54 mAbs were screened against both CN54gp120 and CN54gp140, and this showed differences in the binding specificities of the antibodies. The two proteins that the antibodies were screened against were produced in different systems, the gp120 being baculovirus derived and the gp140 CHO cell derived. It is known that the different expression systems produce differing patterns of glycosylation on synthesised proteins, which can account for a sometimes smaller size band of baculovirus derived protein appearing on gels. However, this does probably not provide reasoning for the different binding as the glycosylation of HIV-1 is a major factor in immune evasion and does not generally produce an antibody response. The glycosylation may have a slight affect on some of the conformation of the protein which could be playing a role in these differences observed. The majority of these differences may well be down to the oligomeric state of the envelope however, as gp120 is a monomer whereas gp140 is more commonly found in a trimeric form. The ability of the gp140 to form stable trimers is due...
to the removal of the cleavage site between gp120 and gp41 of the gp160 precursor. This is done by altering the cleavage site REKR to REKS by site directed mutagenesis (Broder et al., 1994; Earl et al., 1991, 1994, 2001).

From the epitope mapping data for the anti-ZM96 mAbs it appears that only two (MH18 and MH19) recognise linear epitopes on the envelope, which are from the crown of the V3 loop. Competition analysis showed competition between MH18, MH19 and MH21, suggesting MH21 also recognises the V3 crown but, not a linear epitope. Three of the anti-CN54 mAbs have been mapped to the V3 region as well. MH9 and MH10 were identified by work done by Ian Jones (personal communication) and MH16 by competition analysis to MH10. There appear to be 5 distinct anti-CN54 mAbs, as seen by the lack of competition between mAbs from different original colonies. Of these antibodies only MH10 and MH16 recognise rgp140 in western blotting on denatured gels. This suggests the other anti-CN54 antibodies are recognising more discontinuous epitopes that may be altered in the denaturation process.

The anti-ZM96 mAbs seem to have less distinct epitopes than the anti-CN54 ones. There is more competition at low levels between the different anti-ZM96 antibodies (from different original colonies). The three V3 crown specific mAbs (MH18, MH19 and MH21) compete at low levels with a number of the others, and this suggests that many of the epitopes recognised by these mAbs are in and around the V3 region. They may be conformational and only encompass a slight contact with the V3 crown. Some of the competition observed may also be down to steric hindrance due to proximity of epitopes, as opposed to competition for the same epitopes. The lack of linear epitope responses shown here is in agreement with previous work, showing that oligomers are more effective than monomers at inducing responses to conformational epitopes (Earl et al., 1994; VanCott et al., 1995; Yang et al., 2001).

The binding data of mAbs to various B and C clade envelope proteins, shows a number of different epitope specific antibodies have been produced in this project, which should be of benefit to work with these C clade isolates. The majority of current antibodies have been
produced against B clade, and the heterologous screening work here shows how much better these C clade antibodies recognise C clade envelope compared to those antibodies raised against heterologous clade envelope. It can also be seen that the majority of these antibodies are more specific to the C clades than B-clades. There are some that have a cross reactive nature, but these have not been mapped. It can be assumed that they recognise epitopes that are more conserved between certainly B and C clades, and maybe other clades, which would be something to test for the future. The mapping of these may not have been successful, but the region of binding may well be able to be identified by looking at binding to envelope fragments, or those with C or V region deletion. There are two mAbs, MH20 and MH24, that recognise all the envelope proteins tested, and are the only two to recognise W61D, and it would be of interest to see the range of B clade isolates these can recognise.

Interestingly there is only one mAb that does not appear to recognise any rgp120 used in this study. This is MH15, which only appears to recognise CN54gp140. In the original screening process this was picked as a weak binding antibody, in order to determine if different binding abilities affected specificity and characterisation. MH15 is a weak binding mAb and as such cannot be described definitively as a gp41 specific mAb, as suggested by its ability to bind to rgp140 which contains a region of gp41, but not to rgp20, picked out in this study. The lack of induced gp41 specific mAbs is in contrast to work done by Derby et al (2007). Using rgp140 or V2 deleted (ΔV2) rgp140 immunogens either alone or in concert with DNA prime, they isolated a number of gp41 as well as gp120 specific mAbs. The gp120 mAbs were predominantly produced by ΔV2rgp140 and were were focussed to the V1 and as here the V3 regions. But in contrast to the lack of neutralisation found in this work, the V1 mAbs had homologous neutralising ability and the V3 limited heterologous neutralising ability. Whereas three gp41 mAbs where produced by DNA prime/rgp140 boost, 1 by rgp140 alone, and 1 by DNA prime/ΔV2rgp140 boost, and did not have any neutralising ability. Also, the rgp120 specific mAbs they produced

The lack of antibodies specific only for rgp140 and not recognising rgp120 suggests that
although the DNA priming and boosting used gp140, that should be trimeric, it did not elicit antibodies specific to epitopes only exposed in the trimeric form of the envelope protein. It may be that the trimers break apart into monomers in the time it is exposed to the immune system. Alternatively, it may be that there were mAbs with trimer specificity, which were not picked out in this work. It would be interesting to know whether the sera from the mice after immunisation is neutralising. This would also provide evidence of whether neutralising antibodies could be picked out this way, as none of those selected appear to have any major ability to neutralise.

Antibodies MH10 and MH11 were found to have a low level of neutralising ability when tested in a macrophage based assay by C Moog in Strasbourg. Along with MH10 and MH11, MH20 was found to have some neutralising ability. When tested in a T cell line assay with a B clade SHIV derived from W61D (which MH20 and MH24 recognise) there appears to be up to 20% neutralisation of the SHIV. This is compared to the second mAb – MH24 – that recognises W61D, which shows no neutralising ability. How relevant this neutralisation is in terms of the current thoughts is not known. The accepted view on neutralisation assays is to use primary PBMC’s and primary virus isolates as this provides a much less sensitive assay than ones using T cell lines or molecular clones of the virus. It is known that primary isolates are much less sensitive to neutralisation than TCLA viruses, and therefore using primary isolates and primary cells may provides an assay which is much more relevant. Using this form of assay suggests that none of the mAbs produced here neutralise the homologous virus.

The lack of production of neutralising antibodies produced by this work is not indicative of the current literature on the form of immunisation used. It has been shown that DNA priming and protein boosting does elicit strong neutralising and cellular responses in small animals and non human primate models, and sterilising immunity against a non-pathogenic SHIV (Cristillo et al., 2006; Pal et al., 2005, 2006). Conversely this work has not elicited any apparent neutralising response; however this does not mean none were produced, and it would be highly surprising if there were to be no neutralising ability of the sera collected from immunised mice. The main factor in the lack of neutralisation may be due to the methods used to screen and therefore select
these mAbs. One idea may be to use a more native envelope to screen fusions, and see if any mAbs which may be more biologically active could be picked out. An attempt was made to see if any of the anti-ZM96 mAbs produced could recognise ZM96 infected cell lysates as a possible future method for screening (data not shown). Unfortunately this was unsuccessful, and no positive control was identified that could suggest whether there was any binding that could be picked up. In future, if good lysates could be identified with levels of envelope that could be recognised well, then this may be an alternative method for screening. The neutralisation assay used to look at ZM96 in PBMCs is also not conclusive here. The mAb used as a positive was the Tri mAb sample, currently being used in the Neutnet studies, that is highly cross reactive and neutralising, but unfortunately did not appear to neutralise ZM96. This means that although there appears to be no neutralisation in this assay, it cannot be conclusively said this is the case as the assay may not have been functioning, as it should have done. Further to this, work has shown that the HIV-1ZM96 used in this assay was not growing in vitro, and the Gag levels examined were a result of the initial inoculums rather than productive infection (data not shown). If consistent and sensitive neutralisation assays to ZM96 and CN54 could be developed, they would also provide good methods to screen the original fusions specifically for neutralising antibodies. It may be that utilising all three of these forms of screening would give a panel of mAbs that would give greater coverage of all antibody specificities produced, and potentially increase the number of useful reagents that could be obtained from immunising this way.

Since this work has been completed it has been discovered that the two individual proteins utilised may not have actually been distinct. HIV-1ZM96gp140, obtained from Polymun through CFAR and used to boost primed mice has been identified by sequencing to actually be the HIV-1 CN54 used for boosting the first group of mice. If the original DNA plasmid used to immunise ZM96 mice was indeed expressing ZM96, then this effective multiple immunisation, may account for the greater cross reactivity of the second group of mAbs produced in this study. Perhaps this broadening of the response could be investigated, by looking further at the effect of multiple single clade immunisations. However it does not explain the variation in appearance of
the proteins when examined with SDS-PAGE, and perhaps identifying the point at which the proteins were mixed up would be of benefit. Knowing the sequence of the envelope insert in the plasmids used in this study will provide the necessary information to know if the second group of mAbs produced here were actually a product of mixed immunisation. Previous work has shown that multiple clade envelope DNA prime/protein boosting or DNA prime/rAD5 boosting provides a broad cellular and neutralising antibody response in human volunteers (Wang et al., 2006) and enhanced neutralising capacity in macaques (Seaman et al., 2007). But the breadth of responses that can be produced by multiple intra clade envelopes has not been examined.
Chapter 6

General Discussion

With the current worldwide situation of HIV infection a massive concern, the need for treatments and ultimately safe and effective vaccine programmes is of paramount importance. Prophylactic vaccination has been an aim since the discovery of HIV-1 and as the pandemic has progressed the need has grown more urgent. Much of the work on vaccination has been focussed on envelope based vaccines, and for this work there is a continuing need to be able to unravel the complexities of protection that has been shown in the animal model systems available. The lack of natural protection or clearance of virus has meant there are no correlates of protection that can be identified. This has led to a need to identify in vitro correlates of protection that would be able to be applied to vaccine candidates to provide early assurances of effectiveness. For this work new reagents are constantly required that will help to unravel the mystery of protection against HIV-1. I hope that this work that will go some way to aiding in the provision of knowledge required to investigate and to ultimately provide protection against HIV.

The neutralisation of HIV is a massive target for protective vaccines and as such it needs to be fully understood what responses need to be elicited to be effective, and ultimately how it would be possible to produce these responses with an economically viable and efficacious vaccine. Work with other viruses has shown how effective vaccines can be at eliciting protective neutralising responses. The problem with HIV is that the neutralisation generally seems to be clade specific. This can be seen with sera obtained from clinically infected patients that are very effective at neutralising homologous virus but not heterologous isolates. These sera come mainly from long term non-progressors, and help to control infection and dramatically slow the progression towards AIDS. These responses however are not able to clear infection and are probably only so strong for such a long period of time due to prolonged stimulation by a low level of infection. To utilise responses such as these for protection with current vaccine
technology is a real challenge due to the levels of protective antibodies required. To keep a stimulated response, to the level of protection, involves regular vaccination that is just not a feasible concept currently, especially for those regions most in need of protection. There are two possible methods to counteract this problem, either to produce vaccines that elicit long lasting effective antibody responses by continual stimulation such as slow release mechanisms or slow replicating vectors, or to produce a more targeted response, so that it would be effective at a lower level than is currently needed for protection in animal models. The most likely way this could be accomplished is by identifying novel vaccination targets and by gaining a greater understanding of what responses are the most protective so that vaccines can be targeted to these.

The aims of this thesis were to examine responses elicited by HIV-1 envelope protein immunogens. Neutralisation was a key element to this and the ability of envelope induced responses to protect in macaque models, produce broadly reactive and neutralising response, and to use them to elicit useful non B clade reagents for studies.

Work done at NIBSC using the SHIV/Macaque model provides a unique forum from which to examine possible vaccine strategies. The SHIV/Macaque schedule 152.1 allows the examination of protective antibody responses that have been elicited by simple vaccines, and also the examination of differences that can be elicited by vaccination schedules of different lengths. From this schedule, 1 out of 4 animals in the group receiving 3 immunisations showed evidence of virus breakthrough after challenge. It was therefore hoped that a detailed examination of the serological responses elicited in this group would show differences between protected and non-protected animals. This is vital in terms of identifying in vitro correlates of protection that might eventually be able to be transferred to predicting efficacy of vaccines in the clinic.

This work was only able to identify a single simple in-vitro correlate of protection for this study. The reduced V3 competition presented by the infected animal T145 is the only evidence that
could posthumously be used to suggest whether this macaque would have been protected or not prior to challenge. This apparent reduction in V3 response may be playing an important role in the lack of protection seen in this animal, but it alone does not correlate with the increased neutralising ability of animals in the longer immunisation study. V3 responses are known to be able to neutralise sensitive TCLA isolates and this may be the reason the remaining 7 animals were protected against homologous SHIV challenge but not heterologous challenge. A later repeat of this schedule has shown this immunogen can protect against one heterologous SHIV after 8 immunisations, but not a second (Page et al., in preparation). However this is not able to provide a definitive correlate of protection, as V3 responses are not protective against broad primary isolates. Also, ideally a correlate of protection would be conserved. In addition, TCLA viruses are more sensitive to neutralisation than native isolates and therefore the antibody response against the challenge virus may not be representative.

However, the characterisation of these responses has elicited much information about epitopes recognised by antibodies produced with current protocols. Differences that were identified in groups receiving 3 or 8 immunisations seem to suggest that there is a certain maturation or alteration of the immune response of the animals after receiving an increased number of vaccinations. The responses from animals vaccinated with shorter or longer schedules are not different in their binding ability, but neutralising ability is increased in the group receiving more immunisations. The differences identified in this work were an increase in antibody avidity and an increase in antibodies directed against the V2 region of envelope. This maturation may correlate to the enhanced homologous neutralising responses here, but V2 responses overall have not been found to be broad and strongly neutralising and the effect of deleting the V2 loop of immunogens provides for better neutralising responses (Srivastava et al., 2003; Stamatatos et al., 1998; Pinter et al., 2004). The increase in avidity is in agreement with previous work (Richmond et al., 1998) and correlates with enhanced neutralising responses (Sather et al., 2009). Evidence of the maturation of the responses examined in this work agrees with the premise that broadly neutralising responses are multi factor, and the complexity of these involves a number of particular factors (Zhao et al., 2009). Results from this work has helped to
reiterate the ideas that more novel vaccination strategies and immunogens are required to further the work.

The lack of broad heterologous neutralisation in this work is indicative of one of the major problems of AIDS vaccine research. The genetic variability of HIV presents a huge obstacle to the effectiveness of a vaccine. The progress of effective HIV vaccines is partly measured by the in vitro ability of responses to neutralise a broad range of clade isolates (Mascola et al., 2005), and as such serotyping HIV is an important issue. The lack of defined serotypes in the small number of clade immunogens examined in guinea pig immunisations shows this. This work shows the ability of Env rgp immunogens to elicit responses, however there is no pattern of binding or neutralisation, or even epitope specificity as examined here. These two areas of work highlight the intrinsic problems with current immunogens of differing individual responses. The single macaque that became infected may have immunogenetic specifics that are distinct from the others, thus not producing the same protective response. The variance in responses from guinea pigs immunised with the same immunogens has shown this too. Even though the binding abilities of sera were comparable, the neutralising abilities, and specific epitope recognitions were different. Production of trimeric rgp140 immunogens have helped to produce better neutralising responses (Binley et al., 2000; Kim et al., 2005; Sanders et al., 2002; Srivastava et al., 2003; Yang et al., 2000, 2002). Evidence found here shows neutralising ability can vary enormously between individuals. The rgp120 immunogens utilised in this study did not produce any measurably different responses to the rgp140 immunogens. Indeed the monomeric CN54gp120 elicited a response in one animal that outcompeted all others for the binding of the broadly neutralising antibody 447-52D. This work only utilised single clade immunisations and the future for env immunogens to produce broader cross clade responses lies with multi clade immunisations. A number of groups have looked at multiple clade immunisations, and have found a greater breadth of neutralisation elicited. Even so the route of administration may well be important.
The need for improved cellular as well as humoral responses is pushing the production of novel immunogens. Such improvements have been seen by the use of DNA, vectors and VLPs as priming and boosting immunogens and some of these have given promising results.

The use of DNA has been reported to enhance neutralising responses in small animals, and macaque models. This idea along with trimeric rgp140 boosting was looked at for the important area of reagent production for continued research into HIV prophylactic vaccines. This proved capable with adjuvanted protein boosting to elicit monoclonal antibodies, both clade specific and broadly responsive. Although these were found to not be strongly neutralising in assays utilised here, previous work done that shows DNA can produce neutralising responses suggests that more in depth screening may be able to pick out neutralising specificities. To improve the selection original screening could be undertaken with native envelopes, neutralisation assays and competition assays to known neutralising antibodies. The antibodies produced here have however been shown to be generally recognising conformational epitopes on envelope, and also the emphasis of the response is not as great towards the V3 regions as monomeric immunogens tend to elicit. Hopefully these antibodies will be of benefit to the broader research field and have been made available to researchers through the CFAR programme at NIBSC.

The usefulness of the unique SHIV/macaque model is clear, and the future for this allows for the in depth examination of further responses elicited to envelope immunogens. Utilising this model gives a unique insight into the protection that can be conferred. Although this protection has never been duplicated in clinical studies the number of cases of protection seen using varying forms of immunogens in the animal model gives hope. One of the most promising avenues is the ability of live attenuated vaccines to provide very adequate protection. Although this is not a feasible pathway for human vaccination due to the inherent risks, it allows us to examine the way that this protection is conferred. If it is possible to identify the major important factors of protection in these models then the future may lie in trying to reproduce them with other safer vaccines. The protection that has been identified in animal models is at the moment not applicable to clinics, partly due to the immunogens, but also to the controls
allowed in the models. The failure of three phase III trials to elicit protection has shown the
difficulty in transferring the principles of animal model studies. Work shows that the protection
in these studies may last up to 6 months or more, but realistically, human vaccines would have
much longer lasting protection and this must be investigated. This length of protection is one of
many factors that cannot be controlled with clinical studies. The others are such factors as the
infecting isolate, the route of infection and the infectious dose. All of which are unknown in
clinical trials. Utilising animal models allows complete control of all of these factors, although
within the models, the infectious dose is many times higher that would be the case for natural
infections. Due to this the investigation of more natural challenge doses may help to understand
the lower limits of responses required for protection (McDermott et al., 2004).

The recent success of a Phase III trial in Thailand promises much for the future of HIV
vaccines. This study in which individuals receiving the ALVAC-HIV recombinant canary pox
vectors, and AIDSVAX B/E rgp120 booster found that there was a 31% reduction in infection
compared to the placebo group (Rerks-Ngarm et al., 2009). This is the first instance of a
clinically significant reduction in the infection, and shows that envelope based vaccines are a
route to follow. The detailed analysis of this study will be of great importance and interest as it
is dissected in the future. If the protective elements of responses in those uninfected individuals
can be examined they may provide further information on what responses should be focussed on
and how to elicit such responses with tailored immunogens.

To further expand this work, larger panels of viruses such as those looked at by Brown and
colleagues (2005) would need to be examined for neutralising ability to continue the
investigation of possible serotypes. Further examination of priming and boosting regimens
would be of benefit to try to produce more neutralising antibodies. This could perhaps involve
the use of multiple clade protocols for producing monoclonal antibodies. It also serves to
highlight how important collaboration has become to effectively research all avenues. There is
such a large expanse of work to be covered, and evidence of neutralisation being differentially
detectable in alternative neutralisation assays (Binley et al., 2004; Brown et al., 2005; Choudhry
et al., 2007; Polonis et al., 2008) shows that standardisation of neutralising assay protocols, reagents and viruses is required. Such a program is currently underway and Neutnet which is a joint working initiative of 14 groups to co-ordinate the development of standardised methods for measuring mAb neutralisation of HIV-1 for use in clinical trials of candidate HIV vaccines has identified key differences in currently used assays (Fenyo, et al., 2009). The availability of reagents too is of paramount importance to be able to examine possibilities between experimental programmes. Resources such as the NIH and CFAR are providing services to allow comparison of multiple reagents and viruses to keep the field of HIV vaccine research moving towards the ultimate goal of a safe prophylactic vaccine available where it is most urgently required.

Collectively this data demonstrates

- That a strong serological response can be elicited in two different animal models after immunisation with either monomeric or oligomeric proteins.
- That binding titre does not correlate with neutralising ability.
- That the immunogen is not the only factor to determine what response will be elicited, and the importance of host factors cannot be ignored.
- The differences in the breadth of response that can be produced by protected and non-protected animals.
- The lack of a relationship between genotype and serotype.
- The need to determine key neutralising epitopes in SHIVs and to compare them with primary isolates and field strains with the aim to provide a relevant pre-clinical challenge model for vaccines.
- That eliciting potentially neutralising antibodies and the pattern of neutralising and non-neutralising epitopes on the SHIV should mimic the pattern on primary isolates and field strains of HIV-1.
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### Appendix

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Table A.1. Peptide sequences for the series ARP 7035.1-46 which covers the whole of HIV-1$_{W6D}$ envelope gp120.

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Table A.2. Make up of peptide pools used in scanning assay.
Figure A.3. Ability of plasma collected 12 weeks post challenge, from control macaques that had been infected with SHIV-1w61D virus, to bind to peptides derived from HIV-1w61D rgp120. Data shows absorbance from wells in row A of ELISA plates, containing the highest concentration of plasma, normalised against naive macaque plasma (n=3, +/- 3sd).
Figure A.4. Ability of plasma collected 12 weeks post challenge, from macaques that had received 3 immunisations, to bind to peptides derived from HIV-1\textsubscript{WAD} rgp120. Data shows absorbance from wells in row A of ELISA plates, containing the highest concentration of plasma, normalised against naive macaque plasma (n=3, +/- 3sd).
Figure A.5. Ability of plasma collected after 3 immunisations from macaques in group B that would eventually receive 8 immunisations, to bind to peptides derived from HIV-1\textsubscript{W6D,rgp120}. Data shows absorbance from wells in row A of ELISA plates, containing the highest concentration of plasma, normalised against naive macaque plasma ($n=3$, +/- 3sd).
Figure A.6. Ability of plasma collected 12 weeks post challenge, from macaques that had received 3 immunisations, to bind to peptides derived from HIV-1 \textsubscript{w610} rgp120. Data shows absorbance from wells in row A of ELISA plates, containing the highest concentration of plasma, normalised against naive macaque plasma (n=3, +/- 3sd).