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# **Antibody-dependent enhancement of HIV-1 infection**

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for the degree of Doctor of Philosophy*

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## ABSTRACT

Antibody-dependent enhancement (ADE) is the process by which virus-specific antibodies enhance entry or replication of a virus, leading to increased infection and the potential to exacerbate disease progression or severity. In terms of HIV infection, ADE could make the difference between an ineffective vaccine and a dangerous one. There is also the possibility that ADE affects disease progression in natural infection. Here, one aspect of ADE, complement-mediated ADE (C'-ADE), was investigated in detail.

An assay was developed to study C'-ADE in both R5- and X4-tropic HIV-1 strains in the context of early, primary infection and vaccination. A CD4<sup>+</sup> T cell line was used for these studies which expressed the complement receptor 2 (CR2) and HIV-1 co-receptors CXCR4 and CCR5. C'-ADE in primary infection was investigated using serum samples collected longitudinally from infected individuals from as early as 12 days post onset of primary symptoms. When tested against autologous viruses isolated early after infection, C'-ADE was detected in 9 out of 10 patients. In some cases this was potent enough to produce increases in infection greater than 100-fold. Later virus isolates that evolved to escape antibody neutralisation were enhanced by sera that neutralised early virus. Competition studies carried out with neutralising and enhancing IgG showed neutralisation to be the dominant activity. Enhancing, but not neutralising, activity of patient sera was detected against heterologous primary isolates.

Post-vaccination C'-ADE was investigated using sera from volunteers vaccinated with a monomeric gp120 vaccine from a dual-tropic HIV-1 strain. Sera from vaccinees enhanced infection of the vaccine virus strain but did not neutralise it. Neutralisation was seen for an X4 virus strain, MN, and individuals that produced the most potent neutralisation against MN also produced the most potent enhancement of the vaccine strain.

CR2-mediated increased attachment of opsonised viruses to the target cell was shown to be the principle mechanism of C'-ADE. The use of a CR2 cytoplasmic tail mutant showed that receptor signalling was not necessary for C'-ADE.

Results from these studies show that antibodies that appear to be non-neutralising in neutralisation assay systems can actually have dramatic effects on virus infection in a different context. These new findings are considered in relation to existing knowledge on neutralisation and ADE of HIV.

I, Suzanne Willey, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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*Dedicated to*

*Mum and Dad*

*for inspiring, encouraging and believing in me  
every step of the way*

*and to the memory of  
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## ABBREVIATIONS

aa	amino acid
Ab	antibody
ADE	antibody-dependent enhancement
AIDS	acquired immune deficiency syndrome
AP	alkaline phosphatase
APC	antigen-presenting cell
ARP	AIDS Reagent Program
bp	base pair
C1qR	complement component 1q (C1q) receptor
C'	complement
C'-ADE	complement-mediated antibody-dependent enhancement
C'-MI	complement-mediated inactivation
C'-ML	complement-mediated lysis
C'-RN	complement-mediated rescue from neutralisation
CA	capsid
CAEV	caprine arthritis encephalitis virus
CCR	CC chemokine receptor
CDR	complementarity-determining region
CPE	cytopathic effect
CR	complement receptor
DAF	decay-accelerating factor (CD55)
DC	dendritic cell
DC-SIGN	dendritic cell-specific ICAM-3 grabbing non-integrin
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulphoxide
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
eGFP	emerald green fluorescent protein
EIAV	equine infectious anaemia virus
ELISA	enzyme linked immunosorbent assay
Env	envelope protein
FcR	Fc receptor
FCS	foetal calf serum
FDC	follicular dendritic cell
ffu	focus forming units
FIV	feline immunodeficiency virus
HIV	human immunodeficiency virus
iDC	immature dendritic cell
LC	Langerhans cell
LTR	long terminal repeat
MAb	monoclonal antibody
MAC	membrane attack complex
MBL	mannose-binding lectin
MCP	membrane co-factor protein (CD46)
min	minutes
MOI	multiplicity of infection
MPER	membrane-proximal external region

NAb	neutralising antibody
NHP	normal human plasma (or seronegative control plasma)
NHS	normal human serum (or seronegative control serum)
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PHA	phytohaemagglutinin
PHI	primary HIV illness
PI	primary isolate
RT	reverse transcriptase
RTC	reverse transcription complex
TCLA	T cell line-adapted
VSV	vesicular stomatitis virus
WNV	West Nile virus
7TM	seven transmembrane

# CHAPTER 1

## INTRODUCTION

### 1.1 HIV/AIDS IN CONTEXT

The human immunodeficiency virus (HIV) was first identified as the aetiological agent of acquired immunodeficiency syndrome (AIDS) in 1983 (Barré-Sinoussi et al., 1983). Now, almost a quarter of a century later, an estimated 40 million people worldwide are living with HIV, and 2.9 million people died of AIDS in 2006 alone (UNAIDS, 2006). Areas worst affected are mainly in sub-Saharan Africa, with this region accounting for two thirds of all adults and children living with HIV worldwide. Currently the outlook is bleak, with adult HIV prevalence as high as 30-40% in some countries in this region (UNAIDS, 2006). A cure or prophylactic vaccine remains elusive.

### 1.2 HIV PHYLOGENY AND EPIDEMIOLOGY

HIV-1 and HIV-2 belong to the *Lentivirus* genus of the family *Retroviridae*, along with the simian, feline and bovine immunodeficiency viruses (SIV, FIV and BIV respectively), Maedi/Visna virus (MVV), caprine arthritis encephalitis virus (CAEV) and equine infectious anaemia virus (EIAV; Coffin et al., 1997).

HIV-1 is most closely related to SIV<sub>cpz</sub>, which naturally infects chimpanzees, whilst HIV-2 is most closely related to SIV<sub>sm</sub>, which naturally infects sooty mangabeys. The origin of the HIV epidemic in humans has been attributed to multiple cross-species transmissions of these SIVs during the early part of the 20<sup>th</sup> century. In addition to chimpanzees and sooty mangabeys, many other African monkey species are natural hosts of SIVs, including baboons, mandrills and African green monkeys (Gordon et al., 2005). Despite high plasma viraemia, SIV infections of natural host animals are rarely pathogenic. In contrast, HIV-1 and HIV-2 infections of humans and SIV<sub>mac</sub> (experimentally derived from SIV<sub>sm</sub>) infection of rhesus macaques ultimately cause the chronic CD4<sup>+</sup> cell depletion and immunodeficiency characteristic of AIDS (Heeney et al., 2006). While a group of HIV-1 viruses have spread to cause the global

pandemic, HIV-2 is largely confined to West Africa, spreading more recently to Europe, in particular Portugal, and India.

Within HIV-1 are three distantly related groups of viruses: M, the main group, responsible for the global HIV pandemic; O, found in west central Africa; and N, a very rare group found in a small number of individuals in Cameroon. The distance between genome sequences of these three groups is large enough to imply three separate introductions from chimpanzees to humans, and the precursors of group M and N viruses have been identified in wild chimpanzees (Hahn et al., 2000; Keele et al., 2006). The earliest human sample containing HIV-1, dating back to 1959, was found in Kinshasa in what is now the Democratic Republic of the Congo (Zhu et al., 1998). Using sequences obtained post-1959, and assuming consistent evolutionary behaviour of the virus, the origin of the M group was extrapolated back to the 1930s (Korber et al., 2000).

The M group has been further subdivided into genetic clades, or subtypes, labelled A to K with no subtypes E or I. Due to rapid expansion of the pandemic, and evolution, divergence and recombination of the circulating viruses, the nomenclature system is frequently updated. Several of the subtypes have been divided into sub-subtypes, and circulating recombinant forms (CRFs) containing genome sections derived from different subtypes form part of the classification system (Leitner et al., 2005). Subtypes A and C account for the majority of current infections worldwide. Subtype C is common in southern Africa and India; A and D in central and eastern Africa, and B in Europe, the Americas and Australia.

Hereafter the use of HIV will refer specifically to HIV-1, unless making direct comparisons between HIV-1 and HIV-2.

### **1.3 HIV GENOME ORGANISATION AND PROTEIN PRODUCTS**

The 9.2-kb HIV RNA genome contains the genes *gag*, *pol* and *env*, which are common to all retroviruses (Coffin et al., 1997). Processing of the Gag (group specific antigen) polyprotein produces the matrix (MA, p17), capsid (CA, p24), nucleocapsid (NC, p7), and p6 proteins and the spacer peptides SP1 (p1) and SP2 (p2). The Pol (polymerase) polyprotein, produced as a Gag-Pol fusion polyprotein, is processed to produce protease (PR, p10), reverse transcriptase (RT, p66 and p51) and integrase (IN, p32). Processing of the Env (envelope) polyprotein produces the surface protein (SU, gp120) and transmembrane protein (TM, gp41). In addition, the HIV genome encodes

the accessory proteins Vif, Vpr, Nef, Tat, Rev and Vpu. The genome is flanked by two long terminal repeats (LTR), containing the regions R and U5 at the 5' end, and U3 and R at the 3' end. A schematic diagram of the genome organisation of HIV is shown in Figure 1.1.

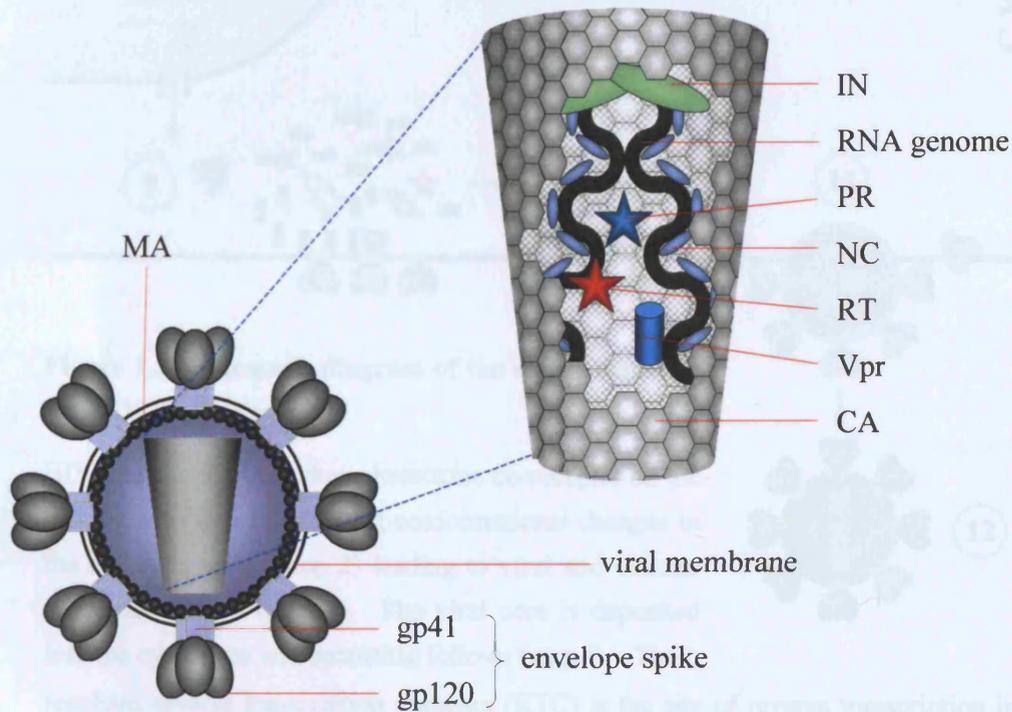
## **1.4 HIV REPLICATION CYCLE**

The four Gag proteins MA, CA, NC and p6, along with the two Env proteins gp120 and gp41, constitute the structural elements of the mature HIV virion (Coffin et al., 1997). Multiple hexameric CA proteins arranged in a lattice comprise the shell of the distinctive cone-shaped viral core, within which reside PR, RT, IN, p6, Vif, Vpr, Nef and the two copies of the RNA genome complexed with NC. MA forms a second, spherical shell immediately beneath the host-cell derived lipid bilayer, embedded within which are the Env spikes. A schematic diagram of the mature virion is shown in Figure 1.2. An overview of the replication cycle of HIV, beginning with the entry of the virus into the host cell and ending with the formation of a mature virus particle, is described in sections 1.4.1 to 1.4.6 and shown schematically in Figure 1.3.



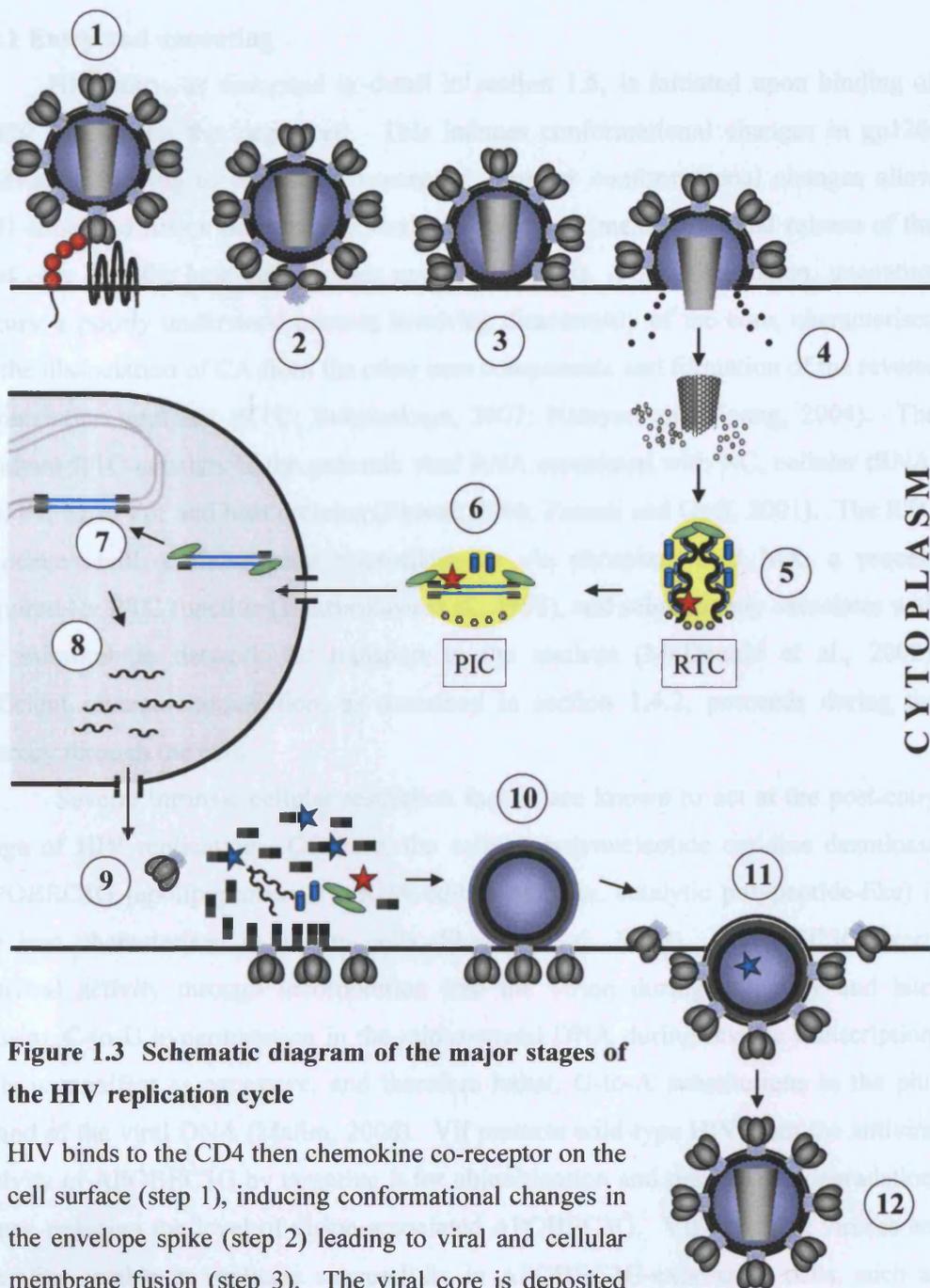
**Figure 1.1 Genome organisation of HIV-1**

The 5' and 3' LTRs are shown in grey; the major genes encoding the polyproteins Gag, Pol and Env are shown in turquoise; accessory genes are shown in red.



**Figure 1.2 Schematic diagram of an infectious HIV virion**

An overview of a mature HIV virion is shown on the left, with an enlarged view of the core on the right. Part of the CA shell has been cut away to show the viral proteins inside the core. (Note that the colours of the proteins are random and unrelated to the colours of the genome in Figure 1.1).



**Figure 1.3 Schematic diagram of the major stages of the HIV replication cycle**

HIV binds to the CD4 then chemokine co-receptor on the cell surface (step 1), inducing conformational changes in the envelope spike (step 2) leading to viral and cellular membrane fusion (step 3). The viral core is deposited into the cytoplasm and uncoating follows (step 4). The resultant reverse transcription complex (RTC) is the site of reverse transcription in the cytoplasm (step 5), and the dsDNA formed is the central component of the pre-integration complex (PIC; step 6). The PIC is imported into the nucleus and the viral DNA integrated into the host chromosomal DNA to form the provirus (step 7). Transcription and subsequent splicing results in the production of a variety of mRNA species (step 8) which are exported to the cytoplasm for translation into viral proteins (step 9). Assembly is mediated by Gag at the plasma membrane (step 10) and immature particles bud from the cell (step 11). Protease-mediated cleavage of the Gag and Gag-Pol structural components results in the formation of a mature infectious virus particle (step 12).

### 1.4.1 Entry and uncoating

HIV entry, as discussed in detail in section 1.5, is initiated upon binding of gp120 to CD4 on the target cell. This induces conformational changes in gp120, facilitating binding to the HIV co-receptor. Further conformational changes allow gp41-mediated fusion between the viral and target cell membranes and release of the viral core into the host cell (Gomez and Hope, 2005). Following fusion, uncoating occurs: a poorly understood process involving disassembly of the core, characterised by the dissociation of CA from the other core components and formation of the reverse transcription complex (RTC; Bukrinskaya, 2007; Narayan and Young, 2004). The resultant RTC consists of the genomic viral RNA associated with NC, cellular tRNA, RT, IN, MA, Vpr and host proteins (Fassati, 2006; Fassati and Goff, 2001). The RTC associates with cellular actin microfilaments via phosphorylated MA, a process required for RTC function (Bukrinskaya et al., 1998), and subsequently associates with the microtubule network for transport to the nucleus (McDonald et al., 2002). Efficient reverse transcription, as described in section 1.4.2, proceeds during the journey through the cell.

Several intrinsic cellular restriction factors are known to act at the post-entry stage of HIV replication. Of these, the cellular polynucleotide cytidine deaminase APOBEC3G (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like) is the best characterised in human cells (Sheehy et al., 2002). APOBEC3G exerts antiviral activity through incorporation into the virion during assembly and later causing C-to-U hypermutation in the minus-strand DNA during reverse transcription. This is manifest as excessive, and therefore lethal, G-to-A substitutions in the plus strand of the viral DNA (Malim, 2006). Vif protects wild-type HIV from the antiviral activity of APOBEC3G by targeting it for ubiquitination and proteasome degradation, hence reducing the level of virion-associated APOBEC3G. Vif-defective viruses are therefore unable to replicate successfully in APOBEC3G-expressing cells, such as macrophages and CD4+ T cells (Malim, 2006).

The tripartite interaction motif (TRIM) 5 $\alpha$  protein is important as a cross-species immunodeficiency virus restriction factor (Stremlau et al., 2004). TRIM5 $\alpha$  proteins from rhesus macaques, owl monkeys and African green monkeys, for example, block the ability of HIV-1 to replicate in these cells by targeting incoming capsid molecules through a mechanism that remains to be elucidated (Towers, 2005).

### 1.4.2 Reverse transcription

Initiation of reverse transcription appears to coincide with the onset of uncoating (Nisole and Saib, 2004); the trigger for initiation may simply be the exposure of the RTC to sufficient levels of deoxyribonucleotides (Freed and Martin, 2001). RT has three enzymatic activities: reverse transcription activity (production of DNA from an RNA template), ribonuclease H (RNase H) activity (degradation of the RNA strand of an RNA:DNA hybrid) and DNA polymerase activity (production of double-stranded DNA from a DNA template). Reverse transcription is initiated by a cellular transfer RNA (tRNA) primer annealed to the primer-binding site (PBS) on the RNA. The preferential tRNA for HIV is tRNA<sup>Lys3</sup>, and this is pre-packaged into the virion and associated with the RNA before entry to the cell. A diagram and detailed description of the reverse transcription process is given in Figure 1.4. Due to the lack of proofreading ability in RT, the process of reverse transcription is error-prone, with 0.2-2 mutations per genome per cycle (Drake, 1993).

#### **Figure 1.4 Reverse transcription**

RNA is shown in grey, minus-strand DNA in turquoise and plus-strand DNA in yellow. (1) DNA synthesis initiates from the 3'-OH of the tRNA annealed to the PBS. (2) The plus-strand RNA genome serves as a template, and a short section of minus-strand DNA is synthesised towards the 5' end of the RNA to generate U5 and R sequences. (3) RNase H degrades the RNA duplexed with the newly-formed DNA, forming the minus-strand strong-stop DNA. (4) The strong-stop DNA translocates from the 5' to the 3' end of the viral RNA. Jumps can occur *in cis* or *in trans*. (5) The minus-strand strong-stop DNA acts as a primer, and DNA synthesis proceeds to complete the minus strand. RNA:DNA hybrids formed render the RNA template susceptible to RNase H digestion. (6) A purine-rich region of the genome, the polypurine tract (ppt) is temporarily resistant to RNase H degradation and therefore serves as a primer for the initiation of the plus-strand DNA synthesis. Only then is the ppt degraded. (7) Plus-strand DNA synthesis continues until the end of the bound region of the tRNA. Part of the tRNA serves as a template for the production of a new PBS, and is then degraded by RNase H. The resulting intermediate is called the plus-strand strong-stop DNA. (8) The complementary plus- and minus-strand PBSs anneal to each other, and the circular intermediate formed allows completion of the plus- and minus-strands. (9) Minus-strand elongation leads to the displacement of the plus-strand and a linear dsDNA is formed.

### 1.4.3 Nuclear Import and Integration

The newly produced double-stranded HIV cDNA associated with NC, along with DA, MA, Vif, RT and host proteins comprise the pre-integration complex (PIC). The PIC components, MA, Vif and IN possess nuclear localization signals (NLSs) and are thought to participate in the active transport of the PIC into the nucleus (Fuselli,

2001). Nuclear import and integration is critical for infecting non-dividing cells such as macrophages and the central nervous system (CNS) where the target cells are long-lived and do not divide (Burkholder et al., 1992; Broderick et al., 1991). Once inside the nucleus, the viral DNA can be integrated into the host chromosomal DNA before transcription can occur (Fig. 1.4.3).

The first step, 3' processing, occurs in the cytoplasm. Removal of a pT<sub>1</sub> oligonucleotide from each 3' end of the LTRs, adjacent to the PBS, is required for integration. The first step, 3' processing, occurs

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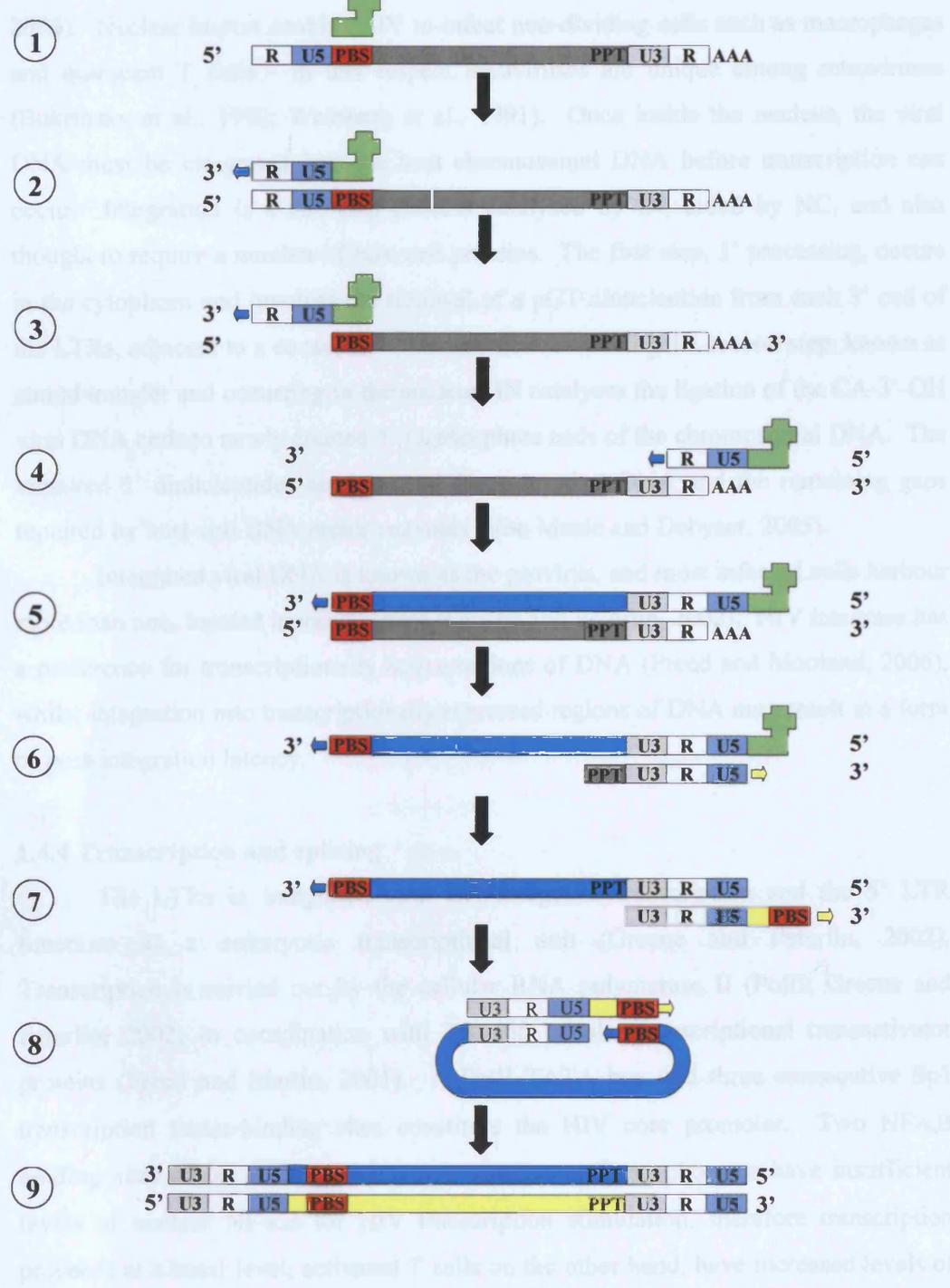
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### **1.4.3 Nuclear import and integration**

The newly produced double-stranded HIV cDNA associated with NC, along with IN, MA, Vpr, RT and host proteins comprise the pre-integration complex (PIC). The PIC components MA, Vpr and IN possess nuclear localisation signals (NLSs) and are thought to participate in the active transport of the PIC into the nucleus (Fassati, 2006). Nuclear import enables HIV to infect non-dividing cells such as macrophages and quiescent T cells - in this respect lentiviruses are unique among retroviruses (Bukrinsky et al., 1992; Weinberg et al., 1991). Once inside the nucleus, the viral DNA must be integrated into the host chromosomal DNA before transcription can occur. Integration is a two-step process catalysed by IN, aided by NC, and also thought to require a number of host cell proteins. The first step, 3' processing, occurs in the cytoplasm and involves the removal of a pGT dinucleotide from each 3' end of the LTRs, adjacent to a conserved CA dinucleotide. During the second step, known as strand transfer and occurring in the nucleus, IN catalyses the ligation of the CA-3'-OH viral DNA ends to newly created 5'-O-phosphate ends of the chromosomal DNA. The unpaired 5' dinucleotides are removed from the viral DNA and the remaining gaps repaired by host-cell DNA repair enzymes (Van Maele and Debysers, 2005).

Integrated viral DNA is known as the provirus, and most infected cells harbour more than one, located in various sites (Greene and Peterlin, 2002). HIV integrase has a preference for transcriptionally active regions of DNA (Freed and Mouland, 2006), whilst integration into transcriptionally repressed regions of DNA may result in a form of post-integration latency.

### **1.4.4 Transcription and splicing**

The LTRs in integrated viral DNA regulate transcription and the 5' LTR functions as a eukaryotic transcriptional unit (Greene and Peterlin, 2002). Transcription is carried out by the cellular RNA polymerase II (PolII; Greene and Peterlin, 2002) in coordination with Tat and cellular transcriptional transactivator proteins (Freed and Martin, 2001). A PolII TATA box and three consecutive Sp1 transcription factor-binding sites constitute the HIV core promoter. Two NF- $\kappa$ B binding sites act as enhancer elements. Quiescent T lymphocytes have insufficient levels of nuclear NF- $\kappa$ B for HIV transcription stimulation, therefore transcription proceeds at a basal level; activated T cells on the other hand, have increased levels of nuclear NF- $\kappa$ B and HIV transcription is upregulated (Freed and Martin, 2001). Tat

functions to enhance the processivity of PolII by binding to the TAR (transactivation response) element at the 5' terminal of all HIV RNAs. The mRNA transcripts produced are post-transcriptionally processed by cellular machinery. Due to sub-optimal splice sites in the 9.2 kbp primary HIV transcript, a variety of unspliced, partially spliced and multiply spliced viral mRNAs are produced, which are essential for the production of the various viral proteins. Completely spliced mRNAs, encoding Tat, Rev and Nef, are exported from the nucleus by the default cellular pathway. Unspliced and partially spliced viral mRNAs are escorted to the cytoplasm by Rev, which binds to the Rev response element (RRE) in these mRNAs and shuttles in and out of the nucleus. The unspliced viral RNA constitutes the genomic RNA incorporated into virions and the mRNA for Gag and Pol synthesis. Partially spliced mRNAs are translated into Vif, Vpr, Vpu and Env proteins (Freed and Martin, 2001). Translation and processing takes place in various locations in the cytoplasm, as described in the next two sections.

#### **1.4.5 Protein production and assembly**

The Gag and Gag-Pol polyproteins are translated from unspliced viral mRNA on cytoplasmic ribosomes (Freed and Martin, 2001). The Gag-Pol fusion polyprotein is produced by ribosomal frameshifting during Gag translation, and as such is produced at a rate of 5-10% that of Gag production. The Env polyprotein, translated on ribosomes of the ER and heavily glycosylated, is cleaved by cellular proteases in the Golgi then transported as non-covalently associated trimers of gp41 and gp120, to the plasma membrane for incorporation into virions.

The Gag polyprotein, hereafter referred to as Gag, is the major orchestrator of viral particle formation: Gag alone is sufficient for assembly and formation of non-infectious virus-like particles (Gheysen et al., 1989). Gag contains at least three functional assembly domains: M, the membrane binding domain (located at the N-terminal of MA); I, the Gag-Gag interaction domain (located within NC, CA and p2); and L, the late domain (located near the C terminus, within p6; Bukrinskaya, 2007; Resh, 2005). The M domain is comprised of an N-terminal cluster of basic residues and a co-translationally added myristyl group: together these function to target and bind Gag to lipid rafts in the plasma membrane (Ono and Freed, 2001; Ono et al., 2000b; Saad et al., 2006). This process requires the multimerisation of Gag, mediated by the I domain, in order to maximise the exposure of the myristyl group (Ono et al.,

2000a; Resh, 2004; Resh, 2005; Tang et al., 2004). An estimated 1500 to 5000 Gag molecules are required for virus assembly (Briggs et al., 2004). The multimerising Gag recruits viral RNA, Gag-Pol polyproteins, Env complexes, the tRNA primer and Vpr into the nascent virus particle. These processes are generally conducted by the I domain, with MA interacting with the gp41 cytoplasmic tail to incorporate Env trimers (Dorfman et al., 1994; Yu et al., 1992).

As with the early journey of the virus through the cytoplasm, the stages of virus assembly and production require HIV accessory proteins to adapt the cellular environment to the advantage of the virus. Vpr induces cell cycle arrest in the G2 phase, which favours efficient HIV transcription (Le Rouzic and Benichou, 2005). Vpu and Nef promote CD4 degradation and down-regulation in order to prevent inappropriate interaction between gp120 and CD4 (Frankel and Young, 1998). Nef also promotes the down-regulation of MHC class I on the cell surface, to evade immune system detection of the infected cells.

#### **1.4.6 Virus budding and particle maturation**

The late domain of Gag is essential for virus budding by virtue of the interaction of a PTAP motif in the L domain of p6 with components of the vacuolar protein sorting system (VPS). Binding of p6 to TSG101 causes recruitment of the ESCRT-1 complex (Gomez and Hope, 2005), which in turn is hijacked by the virus to mediate budding. Vpu is instrumental in virus release due to its promotion of CD4 degradation (Frankel and Young, 1998), and its putative repression of a late infection block (Bieniasz, 2003).

During or shortly after virus budding, PR-mediated cleavage of Gag and Gag-Pol takes place within the virion to precipitate maturation and render the virus infectious. Due to varying efficiencies among the PR target site sequences, protein products are released in a determined order. The products of Gag (in order of position from the N terminus) are MA, CA, NC, p6, and also two spacer peptides SP1, between CA and NC, and SP2, between NC and p6. SP2 is involved in virus budding as part of Gag, but the function of SP1 is unknown (Bukrinskaya, 2007). PR is first released from Gag-Pol, with cleavage occurring *in cis*, ending with the cleavage between CA and SP1, liberating CA for the formation of the core. In the immature virus particle, Gag forms a spherical shell beneath the viral membrane, with the N-terminal MA region interacting directly with the inner layer of the membrane (Fig 1.3 step 11). PR

processing of Gag causes a dramatic change in virion morphology, characterised by the rearrangement of CA proteins into the distinctive cone-shaped core of the infectious virion.

## **1.5 HIV ENTRY**

To gain entry into cells HIV requires two receptors: CD4 and a co-receptor. Details of the Env proteins that mediate these interactions (gp120 and gp41), the cellular receptors, and the entry process, are given below.

### **1.5.1 Envelope structure**

The *env* gene of HIV encodes a polyprotein precursor, gp160, that is translated and glycosylated in the ER (Gomez and Hope, 2005), then transported to the Golgi for extensive glycosylation and cleavage into two proteins, gp120 and gp41. Cleavage is mediated by a cellular furin-like protease and is necessary for infectivity (McCune et al., 1988). gp120 and gp41 remain non-covalently associated. The mature Env complex - the native spike - is a trimeric structure consisting of three gp120/gp41 heterodimers (Lu et al., 1995). gp120 mediates sequential interactions with the target cell receptors, inducing the conformational changes necessary to produce a fusion-competent Env spike; gp41 anchors the Env spike to the viral membrane and mediates fusion of viral and cellular membranes.

#### **1.5.1.1 gp120**

Sequence analyses from different HIV isolates have shown that gp120 is comprised of five conserved (C1 to C5) and five variable (V1 to V5) regions. The conserved regions form the core of the molecule, with conserved Cys residues forming disulphide bonds linking C1, C2, C3 and C4 and forming the base of the surface-exposed V1/V2, V3 and V4 loops (Leonard et al., 1990). gp120 is heavily glycosylated, with N-linked carbohydrates accounting for approximately half of its molecular weight (Poignard et al., 2001).

Important information regarding the structure of the gp120 molecule and the nature of its interactions with CD4, co-receptor and neutralising antibodies has been provided by X-ray crystallography studies (Huang et al., 2005; Kwong et al., 1998; Rizzuto et al., 1998; Wyatt et al., 1998; Zhou et al., 2007), in agreement with previous predictions made from biochemical, mutagenic and immunochemical data (McCune et

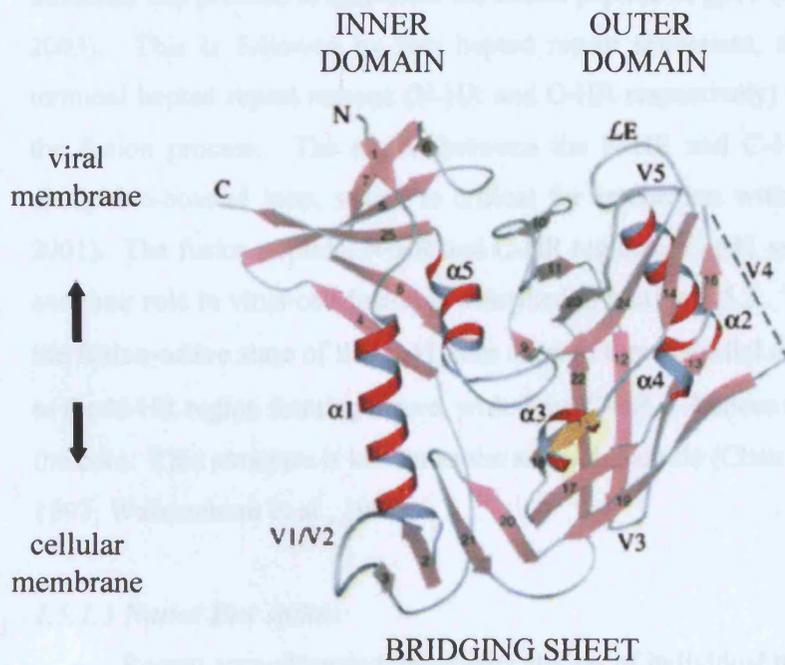
al., 1988). For stability reasons, the original crystal structure of the gp120 core, shown in Figure 1.5, was derived from a modified HXBc2 gp120 lacking the N and C termini, the V1/V2 and V3 loops, and stripped of over 90% of its carbohydrate content (Kwong et al., 1998; Wyatt et al., 1998). This version of the protein was crystallised in complex with a two-domain fragment of CD4 and the Fab fragment of the monoclonal antibody 17b (Kwong et al., 1998; Wyatt et al., 1998). 17b is representative of a group of antibodies that recognise an epitope on gp120 only revealed after conformational changes induced by CD4 binding – the CD4 induced (CD4i) epitope. It blocks co-receptor binding, and is therefore thought to recognise regions overlapping the co-receptor binding site. Subsequent X-ray crystallography reports detailed a V3-containing gp120 core (Huang et al., 2005) and a gp120 core stabilised in the CD4-bound state complexed with the neutralising antibody b12 (Zhou et al., 2007).

The core of gp120 is heart-shaped and comprised of two major domains: the inner and outer domains, linked by a bridging sheet, as shown in Figure 1.5 (Kwong et al., 1998; Wyatt et al., 1998). The inner domain is comprised of C1 and C5 and is the proposed gp41 contact interface (Kwong et al., 1998; Wyatt et al., 1998). The bridging sheet consists of four antiparallel  $\beta$  strands derived from the V1/V2 stem and C4 region. The outer domain is highly glycosylated and forms the putative exposed region of the trimeric envelope spike (Wyatt and Sodroski, 1998), with the bases of the V3, V4 and V5 loops anchored in this region (Kwong et al., 1998).

Receptor binding sites are conserved but shielded by highly glycosylated and variable regions. The CD4 binding site covers an extensive but recessed area located at the interface of the inner domain, bridging sheet and outer domain (Kwong et al., 1998; Wyatt et al., 1998). Direct interactions are formed between 22 CD4 amino acid residues and 26 gp120 residues, located on the  $\beta$  strands of the bridging sheet,  $\beta$  strands of the C3, C4 and C5 regions and a loop in the C3 region (the  $\beta$ 15- $\alpha$ 3 excursion or the CD4-binding loop). Two large cavities are present at the gp120-CD4 interface. The larger cavity is lined with hydrophilic residues that can tolerate considerable sequence variation due to the fact that many of them contact CD4 via main-chain atoms. The smaller cavity is highly conserved and known as the “Phe43” cavity, as into this deeply buried hydrophobic region extends the phenyl ring of the CD4 amino acid Phe43, a residue vital for CD4-gp120 interaction. Phe43 alone accounts for 23% of the direct interactions between CD4 residues and gp120 (Kwong et al., 1998).

The CD4 binding site of gp120 is more clearly defined than that of the co-receptor due to the availability of crystallised gp120-CD4 complexes. In the absence of a gp120-co-receptor complex, the nature of the interaction has been deduced from CD4i antibody binding sites and mutagenesis studies. The co-receptor binding site is highly conserved and consists of the bridging sheet and surrounding residues, and the V3 loop (Huang et al., 2005). It has been proposed, through analysis of the CD4-bound gp120 crystal structure containing the V3 loop, that the N terminus of the co-receptor binds to the bridging sheet and base of the V3 loop, while the second extracellular region (E2) of the co-receptor engages with the V3 tip (Huang et al., 2005). Despite designation as a variable region, the V3 loop is in fact a semi-conserved structure. Unlike the V2 region, for example, V3 is a constant 30-35 aa in length. It has a conserved disulphide bond at its base and a conserved type II turn at its tip with the sequence GPXR in subtype B isolates (Zolla-Pazner, 2004). The V3 loop also determines the co-receptor tropism of the virus: if the 11<sup>th</sup> and 25<sup>th</sup> amino acids of V3 are positively charged the virus will be X4 tropic; otherwise they will be R5 tropic (Resch et al., 2001).

gp120 dominates the surface of the Env spike, almost completely shielding the gp41 portion of the trimer from immune attack. The large surface of the inner domain faces the trimer axis, while the heavily glycosylated surface of the outer domain is exposed on the surface. Regions in gp120 responsible for gp41 interaction are located in the N and C termini of gp120, in the C1 and C5 regions (Kwong et al., 1998; Wyatt et al., 1998).



**Figure 1.5 Crystal structure of the gp120 core**

Ribbon diagram of the HXBc2 gp120 core structure. The structure is derived from the gp120 core in complex with a D1-D2 domain CD4 molecule, lacking the N and C termini, the V1/V2 and V3 loops and stripped of over 90% of its carbohydrate content. The orientation of the structure is indicated relative to the viral and cellular membranes. The bridging sheet, composed of four β strands, and the inner and outer domains are indicated. From Kwong et al. 1998.

### ***1.5.1.2 gp41***

gp41 anchors the envelope spike to the surface of the virion by its transmembrane region, and possesses a long cytoplasmic tail of approximately 150 amino acids. In the native spike, conserved regions of gp41 involved in membrane fusion are largely shielded from the immune system by gp120. A highly hydrophobic region at the N-terminus of gp41 is thought, by analogy to a similar domain in the influenza HA protein, to constitute the fusion peptide of gp41 (Colman and Lawrence, 2003). This is followed by two heptad repeat sequences, the N-terminal and C-terminal heptad repeat regions (N-HR and C-HR respectively) that are also central to the fusion process. The region between the N-HR and C-HR forms a conserved disulphide-bonded loop, which is critical for interaction with gp120 (Maerz et al., 2001). The fusion peptide, N-HR and C-HR regions of gp41 are shown in Figure 1.6, and their role in virus-cell fusion is described in section 1.5.3. The crystal structure of the fusion-active state of the gp41 core showed three parallel  $\alpha$ -helices corresponding to the N-HR region forming a core, with three C-HR  $\alpha$ -helices arranged antiparallel to the core. This structure is known as the six-helix bundle (Chan et al., 1997; Tan et al., 1997; Weissenhorn et al., 1997).

### ***1.5.1.3 Native Env spikes***

Recent cryo-electron tomography studies of individual trimeric SIV Env spikes have produced two models of the 3-dimensional spike. In the first (Zhu et al., 2006), the three gp41 molecules form a distinctive tripod structure, on top of which reside the three globular gp120 subunits. A cavity is evident between the legs of the spike, and each leg contacts the viral membrane and extends parallel to it for a short distance to form a “foot” structure (Zhu et al., 2006). In the second (Zanetti et al., 2006), the Env spike is better compared to a “mushroom” structure, with the three globular gp120 molecules forming a three-lobed structure on top of a stem formed by tightly-interacting gp41 molecules. A cavity exists at the junction between the gp41 and gp120 trimers (Zanetti et al., 2006). It is currently unclear whether methodological differences and limitations in the accuracy of the technique account for the two distinct structures presented, or whether the differences do in fact reflect variation of the spike structure (Subramaniam, 2006).

Use of the same technique has produced images of the surface of intact HIV virions, with interesting information about the distribution and number of trimeric Env

spikes (Zhu et al., 2006). In agreement with previous calculations (Zhu et al., 2003) (Chertova et al., 2002) a surprisingly low number of Env spikes appear to be present on the viral surface, averaging 14 per particle (ranging from 4 to 35). The Env spikes, occurring in clusters, were randomly dispersed rather than uniform, with closest spikes situated approximately 15nm from each other (Zhu et al., 2006).

The main limitation of the findings described above is the inability of electron microscopy techniques to distinguish between functional and non-functional Env spikes and, likewise, infectious and non-infectious viral particles. It is possible that only the virions with the highest number of spikes (up to 35 per particle), or those containing regions with particularly dense clusters of spikes, are infectious (Burton, 2006). The spikes visualised, however, showed distinct trimeric conformations and are therefore unlikely to include the monomeric or disintegrated spike variants thought to constitute the non-functional portion (Moore et al., 2006). Disassembly of the envelope spike, due to the weak non-covalent interactions between gp41 and gp120, occurs frequently (McKeating et al., 1991) and contributes to the formation of defective virions caused by the error-prone process of reverse transcription (Finzi et al., 2006).

### **1.5.2 HIV cellular receptors**

Sequential interactions between HIV envelope and cellular receptors lead to envelope rearrangements that induce fusion of the viral and cellular membranes. CD4 is the major HIV receptor, and the first to interact with envelope, followed by a seven transmembrane (7TM) chemokine co-receptor, as described below. Additional cell surface attachment receptors that may be exploited by HIV are also discussed.

#### **1.5.2.1 CD4**

The role of CD4 as the primary HIV receptor was first discovered in 1984 (Dalglish et al., 1984; Klatzmann et al., 1984). CD4 is a 55-kDa member of the immunoglobulin superfamily of proteins and is composed of four Ig-like extracellular domains, D1 to D4, a membrane spanning domain and a cytoplasmic tail (Maddon et al., 1987). The expression of CD4 defines a subset of T cells, the T-helper (T<sub>H</sub>) cells or CD4<sup>+</sup> T cells, which are the major targets for HIV infection *in vivo*, as described in section 1.6. Its natural function is as a ligand for MHC class II molecules. CD4 can also be expressed independently of the TCR on cells such as monocytes, macrophages

and DCs, also targets for HIV infection *in vivo*. The HIV binding sites on CD4 are located in the Ig-like domain furthest from the cell membrane, D1. A particularly protuberant Phe43 residue and an Arg59 residue of CD4 are critical for the CD4-gp120 interaction (Kwong et al., 1998).

#### 1.5.2.2 Co-receptors

Soon after the discovery of CD4 as the receptor for HIV, it became apparent that CD4 alone was insufficient for virus entry (Maddon et al., 1986); thus began the search for an HIV co-receptor. Viruses had been classified into two groups according to their biological properties: the non syncytium-inducing (NSI), macrophage tropic (M-tropic) strains, and the syncytium-inducing (SI), T-cell tropic (T-tropic) strains. These differences were later reconciled with different co-receptor usage of the viruses. The first HIV co-receptors to be identified were CXCR4 (Bleul et al., 1996; Feng et al., 1996) and CCR5 (Alkhatib et al., 1996; Deng et al., 1996; Dragic et al., 1996). These today remain the most relevant to natural HIV infection. The NSI/M-tropic viruses were re-classified as R5 viruses, and the SI/T-tropic viruses as X4 viruses (Berger et al., 1998). In addition, some viruses can use both CCR5 and CXCR4 and are termed dual tropic or R5X4 viruses. The use of the original terms SI and NSI, or T-tropic and M-tropic, is now considered misleading, as R5 viruses can infect primary T cells, some X4 viruses can infect macrophages, and both can cause syncytium induction depending on the cell type infected. In addition to CCR5 and CXCR4, at least a dozen other 7TM receptors can act as co-receptors for HIV, such as CCR3 and CCR8 (Clapham and McKnight, 2002), but whether these play a major role *in vivo*, for example in neuropathogenesis, remains to be conclusively shown.

The importance of CCR5 in natural HIV infection was highlighted by studies of individuals multiply exposed yet resistant to HIV infection. A 32-bp deletion in the coding region of the *CCR5* gene (*CCR5-Δ32*) introduces a premature stop codon and prevents cell-surface expression of CCR5 (Dean et al., 1996; Liu et al., 1996; Samson et al., 1996). Individuals homozygous for the *CCR5-Δ32* allele have a complete absence of CCR5, and are highly resistant to HIV infection; a small number of infected homozygotes identified in one study were shown to harbour X4 tropic viruses (Sheppard et al., 2002). Individuals heterozygous for the *CCR5-Δ32* allele, when infected, progressed to AIDS more slowly than wild-type individuals (Dean et al., 1996; Liu et al., 1996; Michael et al., 1997). *CCR5-Δ32* is at an average allele

frequency of approximately 10% across Europe, equating to a homozygote frequency of approximately 1% (Galvani and Novembre, 2005). It shows a distinct north-to-south gradient in Europe and western Asia and is virtually absent from Africans, East Asians and native Americans (Novembre et al., 2005).

The HIV co-receptors are 7TM G-protein coupled chemoattractant cytokine (chemokine) receptors. These consist of seven transmembrane domains arranged around a central pore, with four extracellular domains (the N terminus, and three loops, E1, E2 and E3) exposed on the cell surface (Clapham and McKnight, 2002). The natural ligands of CCR5 and CXCR4 are Mip-1 $\alpha$ , MIP-1 $\beta$  and RANTES, and SDF-1, respectively.

The extracellular domains of CCR5 and CXCR4 are modified by N- and O-linked glycosylation and tyrosine sulphation: CXCR4 has potential sites for N-linked glycosylation at the N terminus and E2; CCR5 has sites for O-linked glycosylation in the N terminus. Tyrosine-rich regions are present at the N terminus of both CXCR4 and CCR5 and may be sites of tyrosine sulphation (Zaitseva et al., 2003). Regions involved in HIV interaction are located on the N terminus and E2, and post-translational modifications at these regions may influence interactions with gp120 (Clapham and McKnight, 2002). Sulphated tyrosines at the N termini of CCR5 and CXCR4, along with acidic amino acids, would confer an overall negative charge on the co-receptor N terminus, which may facilitate interactions with positive amino acids in the bridging sheet of gp120. Likewise, negatively charged amino acids in the E2 region of CXCR4 may play an important role in interactions with the positively charged V3 loops of X4 viruses (reviewed by Clapham and McKnight, 2002; Huang et al., 2005).

There is evidence to suggest that co-receptors interact with CD4 in the absence of gp120 binding, probably by clustering in lipid rafts, with the avidity of CCR5 interaction with CD4 apparently higher than that of CXCR4 (reviewed by Zaitseva et al., 2003).

### ***1.5.2.3 Additional attachment receptors***

The initial step of the HIV entry process is the adsorption of HIV particles to the target cell surface. While the first “official” HIV-target cell interaction is between gp120 and CD4, it is thought that HIV exploits the presence of a variety of cell surface

molecules to mediate additional cell surface contacts, serving to increase the efficiency of the CD4 binding step.

The glycosphingolipid galactosyl ceramide (GalC; Kensinger et al., 2004), heparan sulphate proteoglycans (Mondor et al., 1998) and C-type lectin receptors (CLRs) such as DC-SIGN have been shown to bind directly to gp120 on HIV (Geijtenbeek et al., 2000; Lee et al., 2001). ICAM-1, incorporated into the virus membrane during budding from the host cell, can interact with LFA-1 on target cell membranes to enhance infection up to 10-fold (Fortin et al., 1997). None of these molecules is considered a HIV receptor *per se*, as none is absolutely required for viral entry. Furthermore, different attachment molecules are found on different cell types, and it is unclear which are important *in vivo*. Antibody-dependent enhancement (ADE) of HIV infection may be considered an extension of this scenario, with antibodies or antibodies-plus-complement bridging the viral envelope to Fc or complement receptors on the target cell. The mechanism of ADE, however, may differ from the simple increase in attachment outlined above. ADE is discussed in detail in section 1.11.

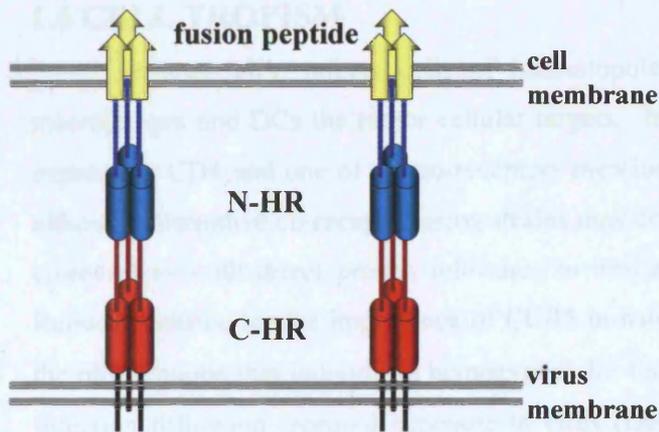
### 1.5.3 The entry process

The process of HIV entry into a target cell begins with gp120 binding to CD4 and ends with the fusion of viral and cellular membranes, resulting in a fusion pore large enough to accommodate the viral core. Many steps of the process remain incompletely resolved, including the exact conformational changes that take place within the Env spike, the number of Env spikes required for complete fusion, the fusion pore formation and widening process and the involvement of lipid rafts (Gallo et al., 2003). Therefore, the model described below serves only as an outline of the process, based on information from structural studies of the Env proteins, studies with entry inhibitors and analogies with other viral entry systems (Gallo et al., 2003).

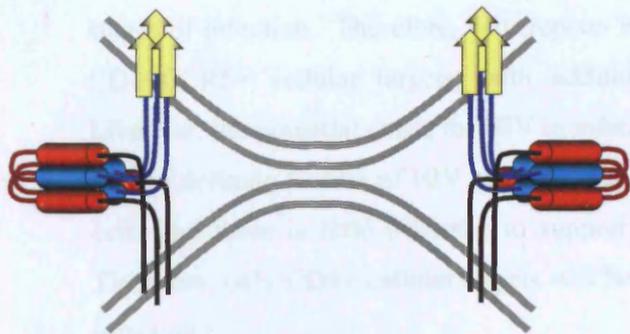
The first rate-limiting step of the entry process is the binding and engagement of CD4 by gp120 (O'Doherty et al., 2000; Ugolini et al., 1999). A time lag of 10-15 minutes has been observed during this step (Dimitrov et al., 1992; Frey et al., 1995; Weiss et al., 1996), but pre-incubation of virus and cells at 25°C (to prevent co-receptor binding steps) shortens the time lag (Melikyan et al., 2000). Resultant conformational changes occurring in gp120 include structural changes in the V3 loop (Sattentau and Moore, 1991), and the exposure or creation of the co-receptor binding

site, as determined by the CD4-bound gp120 crystal structure, encompassing the bridging sheet and surrounding regions (Kwong et al., 1998) and the base and tip of the V3 loop (Huang et al., 2005). The second rate-limiting step of entry is the engagement of the co-receptor, which can occur tens of minutes after CD4 binding (Gallo et al., 2003). The density of CCR5 on the target cell has been shown to affect the kinetics of the fusion process (Reeves et al., 2002).

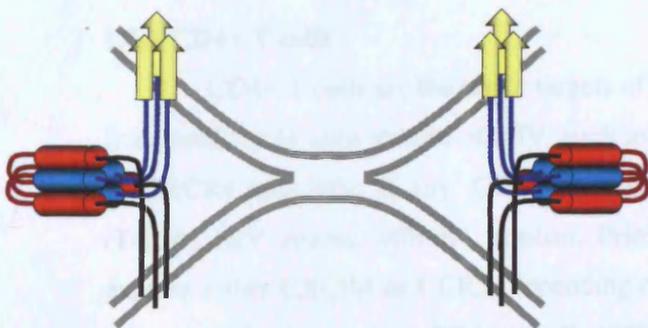
The result of co-receptor binding is the formation of a pre-hairpin structural intermediate, during which conformational changes in gp41 trigger the insertion of the highly hydrophobic gp41 fusion peptide into the target cell membrane (Doms and Moore, 2000; Freed et al., 1990). The entry-inhibitor sensitive, pre-hairpin intermediate is characterised by a triple-stranded coiled coil structure formed from the N-terminal helical regions (N-HRs) of each of the three gp41 molecules of the trimer (Moore and Doms, 2003). The formation of this structure rapidly leads to the formation of the six-helix bundle, during which the gp41 molecules fold back on themselves and the more C-terminal helical regions (C-HR) pack into the outer grooves of the triple-stranded N-HR coiled coil structure in an anti-parallel manner (Gallo et al., 2003; Moore and Doms, 2003). The formation of the six-helix bundle is predicted to bring the viral and cellular membranes into close proximity, leading to membrane curvature then mixing of the outer leaflets of the membranes and a hemifusion state (Clapham and McKnight, 2002). Hemifusion is rapidly followed by fusion of the inner layers of membrane, creating a fusion pore that rapidly expands (Melikyan et al., 2000). The fusion process is shown in Figure 1.6. The exact number of CD4 molecules, co-receptor molecules and Env spikes required for successful fusion is unclear, but is likely to be aided by co-localisation of CD4 and co-receptor clusters in lipid rafts on the cellular membrane (Viard et al., 2002), and clustering of Env spikes on the viral membrane (Burton, 2006; Zhu et al., 2006).



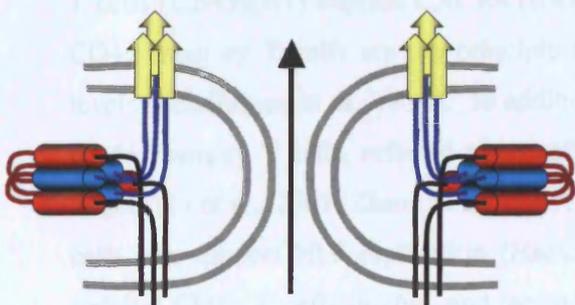
The N-terminal gp41 fusion peptide (yellow arrows) inserts into the target cell membrane.



The C-terminal helical regions (C-HRs; red cylinders) pack into the outer grooves of the triple-stranded N-HR coiled-coil structure (turquoise cylinders), forming the six-helix bundle and bringing the viral and cellular membranes into proximity.



Membrane curvature leads to the mixing of the outer layers of the viral and cellular membranes.....



.....leading to fusion of the inner layers of the membranes and the formation of a fusion pore. The viral core is inserted into the cell in the direction shown by the black arrow.

**Figure 1.6 The process of gp41-mediated viral and cellular membrane fusion**

Conformational changes resulting from gp120 binding to CD4 and a co-receptor lead to the insertion of the gp41 fusion peptide into the target cell membrane and the subsequent events outlined above.

## 1.6 CELL TROPISM

*In vivo*, HIV infects cells of haematopoietic origin, with CD4+ T cells, macrophages and DCs the major cellular targets. In theory, HIV can infect any cell expressing CD4 and one of the co-receptors mentioned in section 1.5.2.2. In reality, although alternative co-receptor-using strains may contribute to pathogenesis, the only co-receptors with direct proven relevance *in vivo* are, to date, CCR5 and CXCR4. Robust evidence for the importance of CCR5 in natural HIV infection is provided by the observations that individuals homozygous for the *CCR5-Δ32* allele are resistant to infection following repeated exposure to virus (Dean et al., 1996; Liu et al., 1996; Samson et al., 1996), and that R5 viruses predominate during early and asymptomatic stages of infection. Therefore, cell tropism is described below within the confines of CD4+CCR5+ cellular targets, with additional attention to CD4+CXCR4+ cells. Likewise, the potential exists for HIV to infect cells by CD4-independent mechanisms, yet the defining feature of HIV pathogenesis is the infection and destruction of CD4+ cells and there is little evidence to support direct infection of CD4- cells *in vivo*. Therefore, only CD4+ cellular targets will be discussed below in the context of direct infection.

### 1.6.1 CD4+ T cells

CD4+ T cells are the major targets of HIV infection. Continuous CD4+ T cell lines used for *in vitro* studies of HIV, such as H9 and MT-2 cells, express high levels of CXCR4 and little, if any, CCR5, causing the emergence of T cell line adapted (TCLA) HIV strains with X4 tropism. Primary CD4+ T cells, however, generally express either CXCR4 or CCR5, depending on their activation and maturation states. As a general rule, memory CD4+ T cells (CD45RO+) express CCR5, and naïve CD4+ T cells (CD45RA+) express CXCR4 (Bleul et al., 1997; Lee et al., 1999). Activated CD4+ memory T cells are the principle host cells in which HIV replicates to high levels (Schnittman et al., 1990). In addition, recently activated but apparently resting CD4+ memory T cells, referred to hereafter as resting CD4+ T cells, are also major targets (Li et al., 2005; Zhang et al., 1999; Zhang et al., 2004), and unlike truly resting cells can support HIV replication (Haase, 2005). HIV preferentially infects HIV-specific CD4+ T cells *in vivo*, and throughout all stages of infection higher levels of viral DNA were found in HIV-specific memory CD4+ T cells compared with other memory CD4+ T cells (Douek et al., 2002).

Compared with peripheral blood, where nearly all CD4<sup>+</sup> T cells are CXCR4<sup>+</sup>, the majority of CD4<sup>+</sup> T cells in the mucosal-associated lymphoid tissue (MALT) are CCR5<sup>+</sup> (Douek et al., 2003), and the gut-associated lymphoid tissue (GALT) in particular is a focus for high-level HIV replication (Brenchley et al., 2006; Mehandru et al., 2004). Local spread of HIV is therefore facilitated by the continuum of target cells in these tissues (Douek et al., 2003). In addition to local spread of cell-free virus, direct T cell-to-T cell spread of HIV is thought to be an important mechanism of propagation. The term “virological synapse” (VS) has been adopted to describe the events that occur at the point of contact between the uninfected (target) and infected (effector) cells, by analogy to the interactions that take place at the immunological synapse (Piguet and Sattentau, 2004). A model of the VS depicts clustering of CD4 and co-receptors on the target cell and HIV Env proteins on the effector cells, stabilised by the interactions of LFA-1 and ICAM-1 on the target and effector cells, respectively (Piguet and Sattentau, 2004). This stable site of contact facilitates transfer of HIV from the effector cell, through budding, to the target cell, through fusion. The signalling pathways and molecular mechanisms that further define the VS await elucidation.

### 1.6.2 Dendritic cells

DCs, including Langerhans cells (LCs) are key to the dissemination and propagation of HIV infection. DCs reside in the skin and mucosa as immature, resting cells equipped with receptors for recognition of pathogen-related antigens. DCs are activated upon pathogen recognition and uptake, triggering their concomitant differentiation into mature APCs and migration to local lymph nodes. Here they present antigen to T cells and stimulate the proliferation and activation of naïve and memory CD4<sup>+</sup> T cells (Banchereau et al., 2000). For this reason, DCs play an important role in HIV pathogenesis, as they are able to promote the direct infection and simultaneous activation of CD4<sup>+</sup> T cells, providing the ideal environment for HIV replication. Whilst DCs can be directly infected by HIV, they can also promote CD4<sup>+</sup> T cell infection without being productively infected by a mechanism known as *trans*-infection. The most thoroughly investigated mechanism of *trans*-infection by DCs involves the C-type lectin receptor (CLR) DC-SIGN (DC-specific ICAM-3-grabbing nonintegrin; CD209). DC-SIGN has been detected on immature DCs in the lamina propria of mucosal tissues from the rectum, uterus and cervix (Jameson et al., 2002) as

well as on DCs derived from blood monocytes *in vitro* (Geijtenbeek and van Kooyk, 2003). Uptake of HIV into early endosomes allows maintenance of HIV in an infectious form (Geijtenbeek et al., 2000; Kwon et al., 2002). DC-SIGN can also promote the infection of DCs *in cis*: co-expression of DC-SIGN with CD4 and co-receptor enhances HIV infection up to 100-fold (Lee et al., 2001). Other CLRs have been investigated and shown to be potentially as important for HIV capture as DC-SIGN. Langerhans cells, for example, bind gp120 via langerin (CD207), while the mannose receptor (CD206) plays a similar role on dermal DCs (Turville et al., 2002). The VS has been observed in DC-T cell interactions, but in the case of *trans*-infection is referred to as the infectious synapse (McDonald et al., 2003; Turville et al., 2004). Upon T cell contact with DCs, virions trapped within intracellular vesicles are recruited to the site of contact, where they are transferred to clustered CD4 and co-receptors on the T cell (McDonald et al., 2003; Turville et al., 2004).

The extent of direct DC infection *in vivo* remains unclear, due to conflicting reports regarding productive infection of DC and LC *in vitro* (reviewed by Wilflingseder et al., 2005). This may reflect the diversity of DC subsets found *in vivo*, and effects of culture conditions on susceptibility to infection. Immature DCs, in particular LCs, are susceptible to direct infection by HIV (Kawamura et al., 2003).

### 1.6.3 Macrophages and monocytes

As with DCs, the migratory potential of monocytes and macrophages, their interaction with CD4<sup>+</sup> T cells and apparent resistance to cytopathic effects of HIV infection make them important reservoirs and disseminators of virus (Verani et al., 2005). Furthermore, in the same way that DC-SIGN and other CLRs are important in virus binding to, *trans*-infection by and *cis*-infection of DCs, macrophage mannose receptor (MMR) may play a similar role on macrophages (Nguyen and Hildreth, 2003).

Original classification of HIV strains referred to R5 viruses as macrophage tropic and X4 viruses as T cell tropic. However, X4 is expressed on macrophages and although TCLA viruses do not efficiently infect macrophages, X4-using primary isolates may (Simmons et al., 1998; Verani et al., 1998).

#### **1.6.4 Other potential cellular targets for infection**

Evidence for direct infection of thymocytes *in vivo* has been shown, particularly in later stages of disease. As 90% of thymocytes express CD4 and 60-70% express CXCR4, X4 viruses are particularly destructive in the thymus (McCune, 1997; Moore et al., 2004). Double positive (CD4+ CD8+) populations express both CXCR4 and CCR5, while CCR5 is expressed at a low level on CD4 single positive cells (Moore et al., 2004). Intrathymic macrophages are also susceptible to infection (McCune, 1997). CD4+ CCR5+ macrophages and microglial cells are targets for HIV infection in the brain.

### **1.7 THE COURSE OF HIV INFECTION AND PATHOGENESIS**

The definitive feature of HIV pathogenesis is the depletion of CD4+ cells which, over a prolonged period, leads to immunodeficiency and fatal opportunistic infection (Adler, 2001). The course of disease, from initial infection to the clinical manifestations of AIDS, can be split into distinct phases, described below.

#### **1.7.1 Transmission and establishment of infection**

HIV can be transmitted sexually, vertically or intravenously. In terms of the global HIV epidemic, transmission occurs most commonly across reproductive or gastrointestinal mucosal surfaces. As addressed in section 1.6, R5 viruses are most important in natural transmission, despite the availability of CXCR4+ cellular targets. The reasons for this restriction are currently unclear, but are likely to be multiple and cumulative, together favouring the success of R5 viruses (Margolis and Shattock, 2006). Factors such as higher levels of R5 viruses in the transmitter at the time of transmission, unfavourable environment for X4 viruses in the genital tract, colon and rectum, the relative abundance of activated memory CD4+ CCR5+ T cells in mucosal tissue, early infection events involving macrophages, preferential early replication of R5 variants and a differentiating early immune response may all contribute (Margolis and Shattock, 2006).

The rate of successful transmission is highest during the peak viral load of acute infection; this has been estimated to be at least 10-fold higher than during chronic infection (Quinn et al., 2000; Wawer et al., 2005). It is unclear whether the virus transmitted is in the form of infected cells or cell-free virus, as both are present in genital secretions.

Macaque models intravaginally infected with high doses of SIV have been used to track early infection events (Miller et al., 1989; Miller et al., 2005), and human cervical explant models have been used to identify early targets of infection (Collins et al., 2000; Greenhead et al., 2000; Miller and Shattock, 2003). As most HIV infections occur across mucosal surfaces, these are considered highly relevant models yet, as information cannot be obtained directly from transmission in humans for practical and ethical reasons, knowledge of this is incomplete. It is unclear whether the majority of successful transmission events occur across intact genital or gastrointestinal epithelium, or whether trauma or microtrauma are necessary, and this has bearings on the identity of the original target cells.

Trauma or microtrauma in the cervicovaginal epithelium allows direct access to underlying DCs, macrophages and T cells in the lamina propria, many of which are CD4+CCR5+. In sexual transmission across the intact genital epithelium, LCs are considered an important primary target (Kawamura et al., 2005) as they reside in the epithelium and extend dendritic processes into the lumen. Immature LCs, expressing CD4 and CCR5, are found in vaginal, ectocervical and foreskin mucosal epithelium. Indeed, the removal of these target cells through circumcision has been credited with the high success rates of recent clinical trials designed to assess the effect of male circumcision on reduction of HIV acquisition rates (Weiss, 2007). Immature LCs have been shown to be susceptible to infection *ex-vivo* (Kawamura et al., 2000; Kawamura et al., 2003; Reece et al., 1998) and *in vivo* (Bhoopat et al., 2001; Zambruno et al., 1991). Memory CD4+ T cells have also been identified as the earliest targets of infection (Gupta et al., 2002).

Initial seeding of CD4+CCR5+ founder populations in the lamina propria, be it through direct access to the cells or transport on or in LCs and DCs, is a critical step in determining infection outcome. Infection must spread to other susceptible target cells in the mucosa and become self-propagating with a basic reproductive rate ( $R_0$ ) equal to or greater than 1, or it will be transient and abortive (Haase, 2005). This may account for the relatively low transmission rate seen for vaginal exposure, with estimates placed at approximately 1 in 200 (Piot et al., 2001) to 1 in 1000 (Gray et al., 2001) exposure events resulting in productive infection. In the macaque model, small foci of infected cells are detectable in cervicovaginal tissues within 3-4 days post exposure (Hu et al., 2000; Miller et al., 2005; Zhang et al., 1999). These foci expand to infect more cells in the lamina propria, probably through unbroken chains of resting CD4+ T

cells – a cell type in abundance in mucosal tissues (Haase, 2005; Zhang et al., 1999). Infection of activated CD4<sup>+</sup> T cells, which produce larger numbers of progeny virus than resting CD4<sup>+</sup> T cells but are fewer in number in mucosal tissue, serves to amplify virus production and transmit virus over greater distances (Haase, 2005).

The risk of transmission increases if the recipient or transmitter has a concomitant sexually transmitted disease (STD). Genital ulceration in the recipient greatly compromises the mucosal epithelial barrier and increases the chances of direct access to target cells. In addition, local immune activation results in an influx of immune cells, many of which are targets for HIV.

In the next stage of infection the virus disseminates to the lymphoid tissues. Local draining lymph nodes are the first target, progressing via the bloodstream to peripheral lymph nodes, the GALT and the spleen. This has been estimated to occur within approximately two weeks of vaginal exposure (Miller et al., 2005; Zhang et al., 1999), although low-level dissemination, not considered to constitute established systemic infection, may begin as early as 24 hours after exposure (Hu et al., 2000; Miller et al., 2005). DCs are the obvious candidates as vehicles for dissemination, given their role as sentinel cells: sampling and trafficking antigen to lymph nodes. In this way, DCs transport virus from entry sites to lymphoid tissues, and promote the infection of CD4<sup>+</sup> T cells, as described in section 1.6.2.

### **1.7.2 Acute infection**

The beginning of the acute phase is signalled by established infection in the lymphoid tissue, where CD4<sup>+</sup> CCR5<sup>+</sup> T cell targets are plentiful and in close proximity, favouring intensive viral replication. This is reflected in blood viral load measurements, which can peak at levels as high as 10<sup>8</sup> RNA copies per ml approximately 2 weeks post exposure, with levels of viral production detected in the order of 10<sup>12</sup> virions per day (Little et al., 1999); viral production during chronic infection was measured as 10<sup>10</sup> virions per day (Perelson et al., 1997). The GALT, containing at least 60% of total bodily T cells, is a major site of virus production. Recently it has been appreciated that a dramatic loss of CD4<sup>+</sup> T cells from the GALT is a major feature of acute infection (Brenchley et al., 2004; Li et al., 2005; Mattapallil et al., 2005; Mehandru et al., 2004; Veazey et al., 1998). These CD4<sup>+</sup> CCR5<sup>+</sup> memory T cells are underrepresented in the peripheral blood and lymph nodes, therefore this decimation has gone largely unnoticed through conventional sampling

methods. However, a temporary drop in peripheral blood CD4+ T cells can be seen at this time, and when individual populations of CD4+ T cells are examined, selective depletion of the CCR5+ memory subset can be seen in peripheral blood (Douek et al., 2003).

Peak viral load coincides with or precedes the emergence of a CTL response and seroconversion. Prior to this, infection cannot be detected by routine testing for antibody, despite high levels of replicating virus and high infectivity of the individual (Kahn and Walker, 1998); therefore, diagnosis of acute HIV infection relies on detection of RNA by PCR or p24 antigen by ELISA (Adler, 2001). Clinical manifestations often occur at this stage, known as primary HIV illness or infection (PHI), seroconversion illness, or acute HIV infection. Symptoms can include fever, headache, diarrhoea, fatigue and myalgia, probably resulting from the immune response to infection, and can last from several days to a few weeks (Kahn and Walker, 1998). Viral reservoirs are established, predominantly in the lymphoid tissues. The largest of these, the FDC-trapped viral reservoir (Pantaleo et al., 1993; Schacker et al., 2000), is established alongside the appearance of a humoral immune response to infection as a consequence of antibody and/or complement-mediated binding of virus to Fc and complement receptors. This causes a shift in focus, from T cell-associated virus being the major viral population, to FDC-associated virus dominating throughout infection (Haase, 1999; Haase et al., 1996). Other virus reservoirs are established in the form of latently infected cells.

Peak viral load is followed by a 100- to 1000-fold drop in RNA copies/ml, leading to the chronic stage of infection approximately 1 month after exposure, throughout which viraemia and the CD4 cell count remain relatively stable and the infection is largely asymptomatic. The reason for the precipitous drop in viral load is thought to be the emergence of a robust T cell response, as inferred by the temporal proximity of viral load decline and detection of a CTL response (Borrow et al., 1994; Borrow et al., 1997; Koup et al., 1994), although exhaustion of T cell targets in the GALT may also be a key contributing factor (Derdeyn and Silvestri, 2005; Haase, 2005).

### **1.7.3 Chronic infection**

The extent of damage inflicted on the CD4+ T cell population during the acute phase of infection has its consequences during chronic infection. Throughout chronic

infection the immune system struggles to compensate for vast losses suffered. In addition to this, chronic immune activation as a response to the constantly replicating virus has further detrimental effects on the immune system whilst providing more CD4+ cellular targets for HIV infection (Derdeyn and Silvestri, 2005). Generalised immune activation can impair the function of many parts of the immune system - CD4+ and CD8+ T cells in particular. This may contribute to an eventual lack of control of HIV replication, consequential increases in viral loads and further death of CD4+ cells.

At late stages of infection in some individuals, X4 virus strains start to emerge. This is known as the co-receptor switch and occurs alongside a sharp decline in CD4+ cell numbers and a rise in viral load. The use of the CXCR4 co-receptor broadens the tropism of HIV to include naïve T cells and thymocytes. It is uncertain whether the emergence of X4 strains precipitates the drop in CD4+ cells due to more aggressive replication or whether the deterioration of the immune system allows X4 strains to emerge (Weiss, 2003).

The chronic, asymptomatic stage of HIV infection lasts for several years before the CD4+ cell decline signalling the onset of AIDS. Infected individuals are classified as rapid, moderate, slow progressors, or long-term non-progressors (LTNP) according to the duration of the asymptomatic period. Factors proposed to influence the delay to onset of AIDS in LTNP are multiple, including more potent cellular or humoral immune responses, co-receptor polymorphisms and other genetic predispositions, and replication defects of the infecting virus, such as mutations in Nef (Crotti et al., 2006). The viral set-point, the steady state viral load as measured at the onset of chronic infection, is predictive of the rate of progression to AIDS: individuals with lowest set-points generally progress more slowly (Helbert and Breuer, 2000; Kahn and Walker, 1998).

#### **1.7.4 AIDS**

Prior to the onset of AIDS, individuals may present with persistent generalised lymphadenopathy, lasting several months (Adler, 2001). Symptoms of AIDS usually start to appear when the CD4+ cell count falls below 200 cells per  $\mu\text{l}$ . AIDS-defining illnesses cover a wide spectrum of manifestations, but many are due to opportunistic infection and malignancies resulting from immunosuppression, such as candidiasis, *Pneumocystis carinii* pneumonia and Kaposi's sarcoma (Adler, 2001).

In addition to immunodeficiency and opportunistic infection, HIV has direct pathogenic effects on other organ systems such as the central nervous system (CNS), lymph nodes and thymus. HIV can directly infect macrophages and microglial cells in the CNS. The virus enters the brain soon after systemic establishment of infection and is thought to replicate there at low levels throughout the course of infection. CNS disease is generally a late feature of infection. Clinical disorders include HIV-associated dementia (HAD), which is correlated with high plasma viral loads. Syncytia of microglia are an important component of CNS infection, especially in children.

Lymph node architecture deteriorates throughout infection, leading to atrophy by the end-stages of disease. This could be due to direct viral infection of cells in the lymph node or to cytokines produced in response to infection. The production of new T cells by the thymus is impaired early in infection and thymic function is progressively lost. X4 viruses are particularly aggressive in the thymus as they can infect the majority of developing T cell subtypes; therefore the co-receptor switch is likely to contribute to thymic damage (Moore et al., 2004).

## **1.8 THE ANTIBODY RESPONSE TO HIV**

As this thesis is primarily concerned with the effect of antibodies and complement on HIV infection, only these two aspects of the immune response to HIV will be described here. The complement system and the interactions of HIV and complement are addressed in section 1.9.

### **1.8.1 The antigenic nature of the Env spike**

Only antibodies that recognise intact virus particles have the potential to neutralise HIV, yet not all antibodies that bind to the infectious virion are neutralising. The prevailing model of HIV neutralisation depicts steric hindrance of envelope spikes through high-level antibody occupancy (Burton et al., 2001; Parren et al., 1998). In the case of HIV, the only two viral protein targets available for neutralising antibodies are gp120 and gp41. Neutralising antibodies are thought to recognise the native spike, whereas non-neutralising antibodies are elicited to envelope monomers or other non-functional forms of the envelope (Poignard et al., 2003) such as monomeric gp120-gp41 complexes and gp41 “stumps” (Moore et al., 2006).

Antigenically, gp120 consists of three faces: the silent, non-neutralising and neutralising faces (Moore and Sodroski, 1996; Wyatt and Sodroski, 1998). The silent face, located on the outer domain of gp120, is so-called because of its poor immunogenicity resulting from clustering of carbohydrate side chains in this region (Poignard et al., 2001). The non-neutralising face, located on the inner domain of gp120, is highly immunogenic and elicits a strong antibody response in infected individuals. However, due to its positioning within the trimeric envelope spike, antibodies to this region are thought to be incapable of binding to the native spike, and therefore do not neutralise functional virus (Wyatt and Sodroski, 1998). The abundance of antibodies to this region in infected individuals is probably due to the presence of shed monomeric gp120, or non-functional envelope monomers on the virus surface (Moore et al., 2006; Poignard et al., 2001). The neutralising face of gp120 incorporates regions from the outer and inner domains and the bridging sheet (Wyatt and Sodroski, 1998) and includes the receptor binding sites. Structures within this region important for functional activity of gp120 are conserved among diverse HIV strains, and as such are poorly available for antibody recognition (Wyatt and Sodroski, 1998). There are three immunodominant regions on gp41: cluster I (aa 579-604), II (aa 644-663) and III (aa 619-648; Gorny and Zolla-Pazner, 2003). As these regions are thought only to be exposed in the post-fusion or disassembled spike, antibodies to these regions are non-neutralising. A transitional epitope, the membrane-proximal external region (MPER), that is poorly exposed on the Env spike but thought to be accessible during the entry process, has given rise to neutralising antibodies, as described in section 1.8.2.

Multiple features of the envelope spike confer protection from neutralising antibodies:

- The sequential receptor binding process ensures that critical, conserved regions of the envelope, such as the co-receptor binding site and gp41 fusion protein, are not revealed until the moment of target cell receptor interaction. At this point proximity to the target membrane may sterically hinder antibody binding, and shield these regions from antibody attack. Some naturally occurring SIV strains, and HIV-2, along with some tissue-culture-adapted HIV-1 strains, are CD4-independent and can fuse with the target cell membrane following co-receptor interaction alone. It is therefore thought that early primate lentiviruses engaged directly with co-receptors, and that the CD4 binding step evolved later in HIV

strains as an additional immune protection mechanism. CD4 independent viruses are more sensitive to neutralisation, probably due to increased exposure of the co-receptor binding site (Bhattacharya et al., 2003).

- Receptor binding sites are shielded from antibody attack by the positioning of variable loops. The CD4 binding site is recessed and masked by the V1V2 loops; the co-receptor binding site is masked by V3. The variable loops are a major target for neutralising antibodies, but sequence variability and functional plasticity in these regions allows for rapid immune escape.
- The majority of the exposed face of gp120 in the Env spike is glycosylated. As the glycans are added to the protein during post-translational processing in the host cell, these carbohydrates are seen as “self” by the immune system, thus this face of gp120 is known as the silent face.
- Most of the surface region of gp41 is covered by gp120 or carbohydrate moieties (Sattentau et al., 1999).

Primary isolates (PIs) are often resistant to neutralisation. The native spike of PIs is thought to take on an energetically favourable “closed” conformation: tightly packed trimers with heavily glycosylated and variable exposed regions and occluded conserved neutralisation epitopes (Klasse and Moore, 1996; Sattentau et al., 1999). During the course of adaptation to growth in culture, TCLA strains take on a more “open” and fusogenic conformation, with the inner face of gp120 monomers more exposed within the trimers. This increases their efficiency of entry, but at the cost of increased sensitivity to neutralisation.

### **1.8.2 Neutralising antibodies**

A handful of monoclonal antibodies with broad neutralising activity against HIV have been described (reviewed by Burton et al., 2005; Pantophlet and Burton, 2006; Pognard et al., 2001; Zolla-Pazner, 2004). Any hint of vulnerability of HIV Env spike to neutralisation can be mapped to the epitopes for these monoclonal neutralising antibodies: the CD4-induced (CD4i), CD4 binding site, gp41 MPER, 2G12, and V3 loop epitopes. Several of these antibodies have unusual structural features, as described below.

The CD4i epitope overlaps with the gp120 co-receptor binding site and is exposed upon CD4 binding (Kwong et al., 1998) or in CD4 independent virus strains

(Decker et al., 2005). The CCR5 co-receptor binding site on gp120 is predominantly basic, and part of the region of CCR5 responsible for interaction with gp120, the N-terminal peptide, is postulated to interact with the co-receptor binding site via negatively-charged sulphated tyrosines (Farzan et al., 1999). Several characterised CD4i antibodies were found to have sulphated tyrosines in their long heavy chain third complementarity determining regions (CDR H3); others had a high proportion of acidic residues (Choe et al., 2003; Kwong et al., 1998). CD4i antibodies are broadly neutralising, with the MAb 17b showing activity against HIV-1 subtypes A, B, C, D, E and F (Salzwedel et al., 2000), and CD4i antibodies from HIV-1 infected individuals capable of neutralising HIV-2 strains (Decker et al., 2005). This cross-reactivity reflects the conserved nature of the CCR5 binding site and the gp120 residues that contact the CD4i antibodies (Wyatt et al., 1998). However, neutralisation is dependent upon pre-treatment of the viruses with sCD4; the antibodies alone are not potent inhibitors of infection due to the inaccessibility of the co-receptor binding site following cell-surface CD4-induced exposure. Potency is greatly increased when Fab or single-chain fragments are used (Labrijn et al., 2003; Moulard et al., 2002). It has been proposed that CD4i antibodies, which are produced to high titres in infected individuals, play a role in natural HIV infection by neutralising spontaneously generated CD4 independent variants and thus providing a selective pressure for continued CD4 tropism (Decker et al., 2005).

The prototype neutralising antibody to the CD4 binding site is IgG1b12 (Barbas et al., 1992), hereafter referred to as b12. b12 has been crystallised in complex with gp120 molecules constrained to remain in a CD4-bound conformation, revealing information about its interaction with gp120 (Zhou et al., 2007). Unusually, only the heavy chain of b12 interacts with gp120, with each of the three CDR Hs contributing to the interaction and providing a contact surface equivalent in size to typical antibody-protein interfaces (Zhou et al., 2007). Previously it had been predicted that the long (18 aa) CDR H3 region of b12 extended into the Phe43 binding pocket of the CD4 binding site, and this was the primary reason for the broad neutralising activity of b12 (Burton et al., 2005; Saphire et al., 2001). However, recent crystal structure analysis shows that this feature is not central to the gp120 binding properties of b12, and that in fact the CDR H3 binds peripherally to the Phe43 pocket (Zhou et al., 2007). Many other CD4 binding site antibodies are non-neutralising, but b12 has broadly neutralising activity against a range of primary isolates. This is thought to be because

b12 can bind to the CD4 binding site in both native spike and monomeric gp120 structures, whereas the non-neutralising antibodies only bind to the monomeric gp120 CD4bs (Burton et al., 2005).

A unique monoclonal antibody, 2G12, has been characterised as a glycan-binding antibody that recognises clustered  $\alpha 1 \rightarrow 2$ -linked mannose residues on the gp120 silent face (Hansen et al., 1990; Trkola et al., 1996). As most carbohydrate moieties appear as “self” to the immune system, and most of the gp120 silent face is covered with N-linked glycans, antibodies directed to this region are rarely identified. Structural analysis of this antibody revealed a domain-swapped configuration, in which the variable heavy chains ( $V_H$ ) from each Fab have exchanged places within the molecule, providing a novel interface between the two  $V_H$  chains in addition to the two  $V_H/V_L$  interfaces. This allows the antibody to interact with the specific cluster of oligomannose sugars over an extended contact region (Pantophlet and Burton, 2006).

The anti-V3 loop antibody 447-52D neutralises a range of isolates bearing the sequence GPGR on the tip of the V3 loop (Gorny et al., 1992). The importance of the GPGR region as a neutralising epitope is unequivocally more pronounced in TCLA strains and subtype B PIs, but as viruses from other subtypes do not possess the GPGR sequence on their V3 loops 447-52D has a limited breadth of neutralisation. Interestingly, main-chain atoms mediate most of the interactions between 447-52D and the GPGR sequence, with side-chain residues from the Pro and Arg contributing to the interaction. This allows for a degree of sequence variability in the epitope for this antibody.

Whilst the majority of antibodies to gp41 are non-neutralising, two broadly neutralising anti-gp41 monoclonal antibodies have been characterised – 2F5 and 4E10 (Purtscher et al., 1994; Trkola et al., 1995; Zwick et al., 2001b). The epitopes for these antibodies are located close to the transmembrane region of gp41; both are adjacent to the immunodominant cluster II, with the 2F5 epitope spanning aa 662-667 and the 4E10 epitope aa 671-677 (Zwick et al., 2001b). The region is exposed for only a brief period during the conformational changes involved in receptor binding. As peptides corresponding to these regions failed to elicit antibodies similar to 2F5 and 4E10, it is thought that the molecular context within which these epitopes are presented is important, probably requiring the presence of the viral membrane.

### 1.8.3 Non-neutralising antibodies

Immunodominant epitopes such as cluster I in the ectodomain of gp41, and regions in C5 of gp120 that interact with gp41, give rise to high titres of non-neutralising antibodies in infected individuals. Monoclonal antibodies to these regions, and many others that are not accessible on the functional spikes, are also non-neutralising. However, apart from neutralisation, antibodies may have other functional activities when acting in cooperation with other components of the immune system. These include complement-mediated inhibitory or enhancing activity and Fc $\gamma$ R-mediated effector activities.

Virus-inhibitory activity of antibodies may be increased when assayed on or in the presence of different cell types. Antibody-mediated inhibition of primary isolates was increased when PBMCs were used as target cells compared to purified CD4<sup>+</sup> T cells from the same donor. This was attributed to the presence of Fc $\gamma$ R-bearing NK cells, triggered to produce an antiviral response, partially mediated by  $\beta$ -chemokines, by antibody-opsonised virus engagement with Fc $\gamma$ Rs (Forthal et al., 2005).

A role for the *in cis* involvement of Fc $\gamma$ Rs on macrophages and immature DCs (iDCs) in increasing inhibitory activity of monoclonal antibodies was proposed by Holl and colleagues (Holl et al., 2006). Neutralising activities of several Nabs, including b12 and 2G12, against two subtype B PIs were increased 16- to 12,000-fold when assayed on macrophages or iDC target cells compared to PBMCs. In addition, MAbs that had no neutralising activity against BaL on PBMCs became neutralising when assayed on macrophages and iDCs. Similarly, several MAbs that had weak neutralising activity against a subtype C PI on PBMCs, showed an 8- to 2000-fold increase in activity on macrophages and iDCs. Furthermore, the inhibitory activity of sCD4 and the fusion inhibitor T20 were similar on the different target cells, suggesting that levels of CD4 or different efficiencies of fusion on the different target cells did not account for the results seen. Removal of antibody Fc portions, and subsequent antibody blocking of Fc $\gamma$ Rs, showed that increased inhibitory activity of the MAbs was Fc $\gamma$ R-mediated, and possibly as a result of endocytosis and degradation of the opsonised virus (Holl et al., 2006). Another study showed that, when using macrophages as target cells, autologous neutralising antibodies could be detected in infected individuals as early as 2 months after infection. In contrast, using the same sera on lymphocytes, neutralising activity was not detected until 10 to 14 months after

infection (Ruppach et al., 2000). Other studies have reported ADCC activity of both polyclonal non-neutralising antibodies from infected individuals and monoclonal antibodies (Forthal et al., 1995; McDougall et al., 1997; Subbramanian et al., 2002).

Complement-mediated mechanisms of virus inactivation in concert with non-neutralising antibodies have also been reported (Aasa-Chapman et al., 2005; Huber et al., 2006) and these are discussed in more detail in section 1.9.2.2. In contrast, the presence of non-neutralising antibodies may assist the virus, not only due to the direction of immune resources towards an ineffective response, but also through active subversion by HIV. The subject of this thesis, antibody-dependent enhancement of HIV infection, is an example of this situation and is discussed in detail in section 1.10, while other complement-mediated mechanisms that assist viral infection are described in section 1.9.2.3.

#### **1.8.4 The antibody response during the course of HIV infection**

The role of antibodies in controlling HIV infection is unclear. The drop in viral load during acute infection has been attributed to CD8<sup>+</sup> T cell-mediated control of infection (Borrow et al., 1994; Borrow et al., 1997; Koup et al., 1994) the exhaustion of large pools of cellular substrates, or certain activities of non-neutralising antibodies (see section 1.8.3) but not to viral neutralisation by antibody as this response is delayed relative to the decline in viral load.

Virus present during early stages of replication and acute infection is relatively homogeneous (Derdeyn et al., 2004; Zhu et al., 1993; Zhu et al., 1996), with gp120 sequence homogeneity approximating >99% in seroconvertors compared with approximately 90% in chronically infected individuals (Zhu et al., 1993). A study examining heterosexual subtype C virus transmission in HIV-discordant couples in Zambia found consistent differences in the viral sequences detected in the recipient compared with those in the donor. A bottleneck effect was observed upon transmission, wherein the viral sequences from the recipient formed a distinct subcluster within the viral sequences from the donor (Derdeyn et al., 2004). Likewise, other studies with subtype B viruses showed that the transmitted virus represented only a minor variant in the blood, and even semen, of the transmitter, indicating selective transmission or amplification (Zhu et al., 1993; Zhu et al., 1996). Observations that V3 sequences of virus in newly infected recipients remained predominantly homogeneous for at least 6 months favours the former explanation (Zhu et al., 1996). Interestingly,

phenotypic analysis of transmitted clade C viruses showed them to be particularly susceptible to neutralisation by plasma from the donor and also from unrelated clade C antibodies (Derdeyn et al., 2004). This was attributed to significantly shorter variable regions (V) 1-4. Increased exposure of the CD4 binding site through loss of N-linked glycosylation and modulation of the positioning of the variable loops was proposed as the mechanism for increased neutralisation sensitivity (Derdeyn et al., 2004). Shorter V1-4 regions in transmitted viruses were also observed for subtype A viruses, but not subtype B (Li et al., 2006). Thus, there is evidence to suggest some vulnerability of early viruses to neutralisation, yet uncertainty as to the potency of early autologous neutralising antibody responses.

Envelope-specific antibodies can be detected in the blood of infected individuals as early as 6 days after onset of symptoms of PHI (Aasa-Chapman et al., 2005), but neutralising antibodies are not thought to arise for one to several months; a figure that varies from report to report (Aasa-Chapman et al., 2004; Aasa-Chapman et al., 2005; Moog et al., 1997; Richman et al., 2003; Wei et al., 2003). Methodological differences between studies and the lack of a standard neutralisation assay make it difficult to amalgamate or even compare results from different laboratories (Moore and Burton, 2004). Likewise, the issue of whether a 50% or 90% reduction in infectivity constitutes neutralisation, and whether it is more accurate to present IC50 or IC90 titres, has bearings on the timing of an emerging neutralising response (Aasa-Chapman et al., 2004; Richman et al., 2003; Wei et al., 2003). Furthermore, even within studies, autologous neutralising responses vary enormously between individuals. For example, in a study by Richman and colleagues, in one group of individuals an autologous neutralising response was detected as early as 4-8 weeks post onset of PHI symptoms, in a second group 3-6 months, and in a third only negligible titres of autologous neutralising antibodies were detected throughout the 36 months of follow-up (Richman et al., 2003).

Thus, the earliest detection of autologous neutralising antibodies occurs at 1-2 months post onset of symptoms, and only in recent reports using the most sensitive of assays (Richman et al., 2003; Wei et al., 2003). Other reports generally quote a time frame of 3 months to a year, but always with some study subjects failing to produce detectable neutralising antibodies in this period (Aasa-Chapman et al., 2004; Aasa-Chapman et al., 2005; Li et al., 2006; Moog et al., 1997; Pilgrim et al., 1997).

Ongoing high-level replication throughout infection and in the face of immune pressure from neutralising antibody and CTL responses leads to the accumulation of genetic mutations, the production of viral quasispecies and rapid immune escape by the virus. Viral sequence variation within one chronically infected individual at a given time point is roughly equivalent to the global sequence variation of influenza virus for any given year (antigenic shift excluded; Korber et al., 2001). The situation of immune control of HIV infection is further frustrated by the pool of proviral DNA in latently infected cells, acting as a viral “archive” (Korber et al., 2001), and FDC-associated virus that is rendered apparently unaffected by otherwise neutralising antibodies (Burton et al., 2002; Heath et al., 1995).

Studies of HIV neutralisation escape have been made using sequential paired virus isolates and plasma samples from infected individuals. Successive antibody samples tested against a previous autologous virus isolate generally increase in neutralisation potency; successive virus isolates against a given autologous plasma or serum sample decrease in susceptibility to neutralisation. The rate at which these two processes occur is indicative of the rate at which neutralising antibody responses are mounted and the rate of phenotypic immune escape, respectively, and were shown to significantly correlate (Frost et al., 2005). Rates of neutralising antibody response and phenotypic escape were shown to vary between individuals, but this did not correlate with viral load set points (Frost et al., 2005). Most studies show weak or no neutralisation of autologous virus by contemporaneous plasma (Frost et al., 2005; Mascola, 2003; Moog et al., 1997; Richman et al., 2003; Wei et al., 2003), indicating that the neutralising antibody response remains a step behind the rapidly evolving virus. Either a pre-existing neutralisation-resistant minor viral variant is selected for dominance, or *de novo* mutations create a variant that is poorly recognised by the existing antibody response. A moving glycan “shield” has been proposed as one mechanism of immune escape by HIV, during which changes in the positions of N-linked glycosylation contributed to antibody escape but without compromising the protein structure of the underlying Env spike (Wei et al., 2003). Another study showed the continuous, ongoing accumulation of amino acid mutations spread across the gp120 sequence rather than focused on regions of N-linked glycosylation (Frost et al., 2005). Viruses that evolve to evade neutralisation in the host are not abnormally resistant to heterologous sera or neutralising monoclonal antibodies (Mascola, 2003; Richman et al., 2003).

The early neutralising response to infection is specific for the infecting strain; over time, the neutralising response broadens and becomes capable of heterologous neutralisation (Aasa-Chapman et al., 2004; McKnight et al., 1992; Moog et al., 1997; Richman et al., 2003). This was shown to be independent of rates of viral immune escape (Frost et al., 2005). The isolate-specific neutralising antibodies produced during the early neutralising response to HIV infection are commonly directed to the V3 loop, and less frequently the V2 loop (Wyatt and Sodroski, 1998; Zolla-Pazner, 2004). Rapid immune escape renders the efficacy of these antibodies short-lived. V3-specific neutralising antibodies with broad specificity may arise later in infection and these recognise conformational structures rather than linear epitopes (Zolla-Pazner, 2004). CD4 binding site antibodies are also credited with broad neutralisation later in the neutralising response (Wyatt and Sodroski, 1998). One study found CD4i antibodies in 178 out of 189 HIV-1 infected patients investigated, but these are thought mainly to contribute to the restriction of evolving virus to CD4 tropism rather than broad neutralising activity. The study of LTNPs and highly-exposed protected individuals (HEPS) has attributed a neutralising antibody response to the control or prevention of infection (Zhang et al., 1997), with CD4 binding site antibodies among those found in LTNPs and neutralising IgA found in HEPS (Devito et al., 2002; Humbert and Dietrich, 2006).

## **1.9 HIV AND COMPLEMENT**

### **1.9.1 The complement system**

The complement system consists of over 30 serum and cell-surface proteins involved in host defence against infection. Upon activation an enzyme cascade is generated, driven by the sequential proteolytic cleavage of complement components, under tight regulation by complement control proteins. This process culminates in the opsonisation and lysis of pathogens and the generation of an inflammatory response. Hepatocytes in the liver are the primary, but not sole, site of complement synthesis (Alper et al., 1969). Monocytes, macrophages, fibroblasts, epithelial cells, adipocytes, astrocytes and microglia are among the many cell types also capable of complement production (reviewed by Morgan and Gasque, 1997), either constitutively or under cytokine drive.

Here, the complement system is described within the framework of activation by, and consequences for, viral infection.

### ***1.9.1.1 Complement activation pathways***

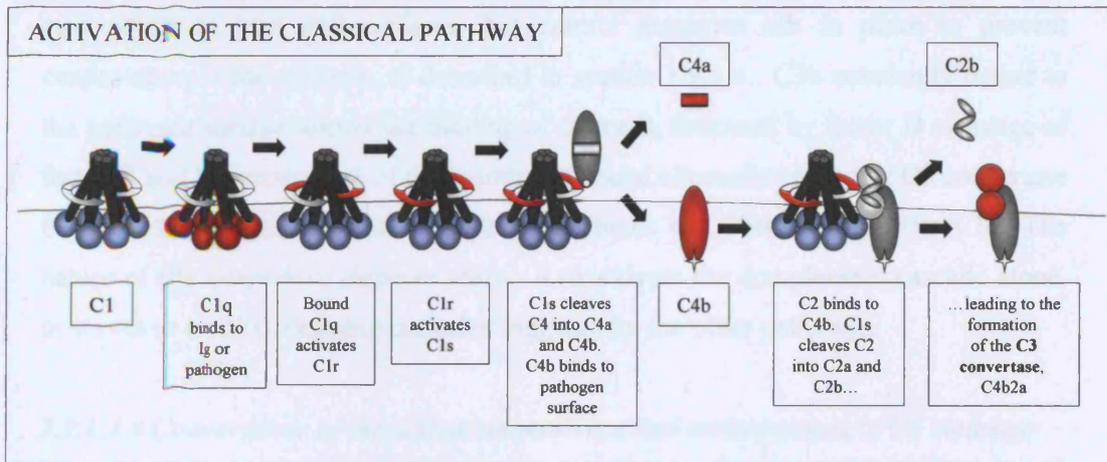
There are three pathways of complement activation: the classical, alternative and mannose-binding lectin (MBL) pathways. All three converge to cleave C3, the central component of complement, and ultimately trigger the terminal complement pathway if unimpeded.

#### ***1.9.1.1.1 The classical complement activation pathway***

The classical pathway is primarily activated by immune complexes, consisting of multiple IgG or single IgM molecules bound to antigen. IgM is the most powerful immunoglobulin activator of complement, followed by IgG. Of the IgG subclasses, IgG1 and IgG3 are the most potent activators of complement, followed by IgG2 and IgG4 (Spiegelberg, 1974). While IgA activates complement via the MBL pathway (Roos et al., 2001) and the alternative pathway (Hiemstra et al., 1987), complement activation is not as potent as that seen by IgM and IgG isotypes. An important feature of the classical pathway is that, by virtue of C1q activation by antibody, it forms a link between innate and adaptive immunity (reviewed by Barrington et al., 2001; Carroll, 2004; Ochsenbein and Zinkernagel, 2000).

Figure 1.7 shows the initial events of the classical complement activation pathway. C1, a multimolecular enzyme complex, is the first component of the classical complement pathway. It consists of one molecule of C1q, a collectin, and two molecules each of C1r and C1s, both serine proteases. The distinctive globular heads of C1q contain pathogen and Ig recognition motifs (reviewed by Kishore et al., 2002; Thielens et al., 2002). Activation by binding to immune complexes via Ig Fc regions, or directly to the pathogen, induces a conformational change in C1q, leading to the autoactivation of C1r and subsequent activation of C1s, reviewed by (Gal and Ambrus, 2001). Activated C1s then cleaves C4 into C4a and C4b, the latter of which covalently binds to the pathogen surface via an exposed thioester group. Covalently bound C4b, in turn, recruits C2, facilitating C1s-mediated cleavage of C2 into C2a and C2b\*. C2a remains bound to C4b in an active form, constituting the C3 convertase of the classical pathway, C4b2a.

\* A general rule in complement nomenclature is that larger products of a cleavage event are assigned the suffix “b”, and smaller ones “a” e.g. C4a and C4b. C2 is the exception to this rule, with C2a referring to the larger, active fragment of C2.



**Figure 1.7 Schematic diagram of early events in the classical complement pathway**

For each stage of the diagram, regions in red indicate the “active” components referred to in the text boxes immediately below. Inactive thioester bonds are shown as white discs; activated thioester bonds forming covalent bonds are shown as white stars. Note that the concept is the same for the MBL activation pathway, with the exception that C1q, r and s are substituted for MBL, MASP-1 and MASP-2 respectively.

#### 1.9.1.1.2 The MBL complement activation pathway

The activation of the MBL pathway results in the formation of the same C3 convertase as the classical pathway, yet the initial proteins of the pathway differ (Matsushita and Fujita, 1992). The MBL pathway is initiated upon binding of MBL, a collectin and structural homologue of C1q, to mannose and other residues on the pathogen surface (Stahl and Ezekowitz, 1998; Turner, 1996). This in turn activates MBL-associated serine protease (MASP) –1, a homologue of C1r, and then MASP-2, a homologue of C1s, with MASP-2 proceeding to cleave C4 and C2 (Matsushita et al., 2000; Thiel et al., 1997) to produce the C3 convertase C4b2a.

#### 1.9.1.1.3 The alternative complement activation pathway

The C3 convertase formed via the alternative pathway, C3bBb, differs from the classical and lectin pathway C3 convertase. The alternative pathway is activated by the spontaneous hydrolysis of C3. This induces a conformation change in C3, permitting

the binding of factor B, a homologue of C2. Factor B is then cleaved into Ba and Bb by factor D, and this forms a fluid-phase C3 convertase, C3(H<sub>2</sub>O)Bb. C3(H<sub>2</sub>O)Bb cleaves more molecules of C3 to C3a and C3b, and some of the C3b fragments may attach to a complement-activating surface, as described in section 1.9.1.2. Some may also attach to host cell surfaces, but control measures are in place to prevent continuation of the cascade, as described in section 1.9.1.4. C3b covalently bound to the pathogen surface allows the binding of factor B, followed by factor D cleavage of factor B and the production of the membrane bound alternative pathway C3 convertase C3bBb. Properdin, also known as factor P, binds to C3bBb and stabilises it. The nature of the alternative pathway allows it to activate the complement cascade alone, or serves to amplify ongoing cascades triggered by the other pathways.

#### *1.9.1.1.4 Convergence of the activation pathways and consequences of C3 cleavage*

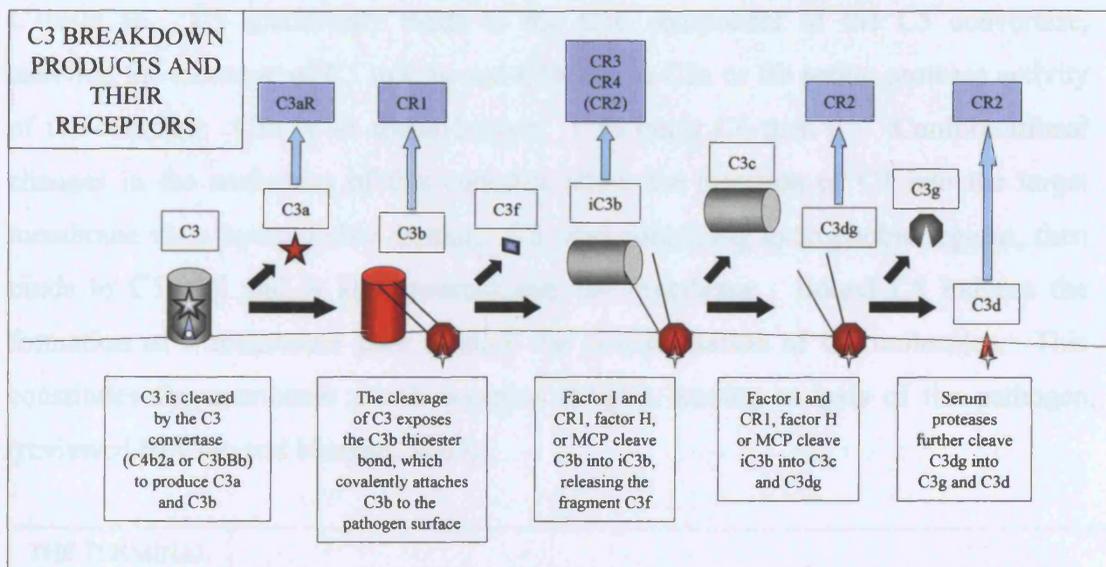
At the point of C3 convertase formation, the three activation pathways converge. The next step of the complement cascade is driven by C3 and the generation of its breakdown products. C3a is an anaphylotoxin and acts as a powerful inflammatory mediator, recruiting phagocytic cells and antibody to the site of infection. The formation of C3b has several consequences:

1. It acts as an opsonin, with the large quantities of C3b generated by C3 convertase activity coating the surface of the pathogen, signalling it for destruction via complement receptors on effector cells. This is discussed in more detail in section 1.9.1.2.
2. It binds to other complement components to form new multimolecular complexes that initiate the subsequent phase of the cascade, the terminal pathway. Binding of C3b to the classical and MBL pathway C3 convertase forms the C5 convertase C4b2a3b. Similarly, binding of C3b to the alternative pathway C3 convertase forms the C5 convertase C3bBbC3b.
3. The generation of C3b can further activate the alternative pathway, leading to amplification of complement activation and C3b production, and consequently increasing the occurrence of points one and two.

#### *1.9.1.2 Deposition of complement fragments on the surface of pathogens*

The cleavage of C3 to C3b, via any of the three activation pathways, induces a conformational change that reveals an active thioester group (Abdul Ajees et al., 2006;

Janssen et al., 2006; Wiesmann et al., 2006). This thioester reacts non-specifically with hydroxyl or amine groups to form a covalent bond between C3b and the pathogen surface (Law and Levine, 1977; Tack et al., 1980), or else is rapidly inactivated by hydrolysis. As many as 1000 C3b fragments can be generated in the vicinity of a C3 convertase, and these act as ligands for the complement receptor CR1. Furthermore, degradation products of C3b remain bound to the pathogen and also act as complement receptor ligands, as shown in Figure 1.8.



**Figure 1.8 Schematic diagram of C3 degradation products and their receptors**

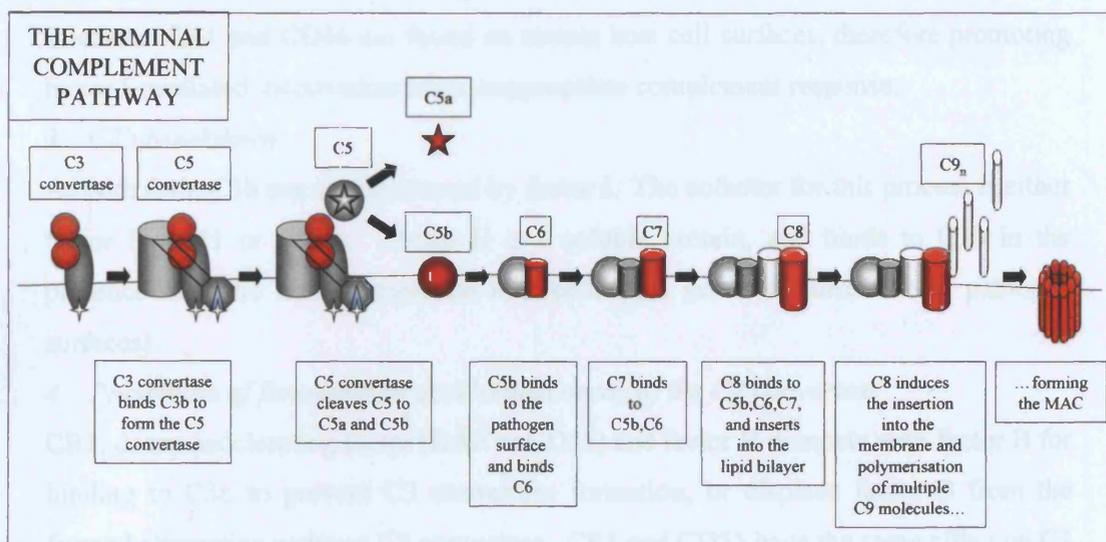
C3, upon contact with a C3 convertase on a pathogen surface, is cleaved to C3a and C3b, the latter becoming covalently attached to the pathogen surface. It is subsequently sequentially degraded into fragments, with each covalently bound fragment a ligand for complement receptors, shown in blue text boxes above their ligands. C3 structure is adapted from Janssen 2006 (Janssen et al., 2006).

Unless involved in the formation of other complement complexes, C3b is rapidly converted to its inactive form iC3b. This is mediated by factor I, and only occurs when C3b is associated with factor H, CR1 or membrane-cofactor protein (MCP or CD46). Factor H, a fluid phase protein, interacts with molecules, such as sialic acid, that are most commonly found on host cells, and in this way marks C3b molecules attached to self for inactivation. CR1 and CD46 are membrane-bound receptors found on host cells. iC3b is a ligand for CR3, CR4 and CR2. Factor I, again with CR1, CD46 or factor H acting as cofactors, further cleaves iC3b to produce the

soluble fragment C3c and membrane-bound C3dg. C3c has no known biological function. C3dg is a ligand for CR2, and can be further degraded to C3g and C3d (Lachmann et al., 1982), the latter of which is also a ligand for CR2.

### 1.9.1.3 The terminal complement pathway

The endpoint of the complement cascade is the formation of a lytic pore in the pathogen membrane. This occurs via the terminal complement pathway, shown in Figure 1.9, which is triggered by the formation of a C5 convertase, C4b2a3b or C3bBbC3b. C5 specifically binds to the C3b component of the C5 convertase, enabling the cleavage of C5 to C5a and C5b by the C2a or Bb serine protease activity of the complex. C5a is an anaphylotoxin. C5b binds C6 then C7. Conformational changes in the molecules of this complex allow the insertion of C7 into the target membrane via a hydrophobic domain. C8, also containing hydrophobic regions, then binds to C5b,6,7 and is also inserted into the membrane. Bound C8 induces the formation of a membrane pore through the polymerisation of C9 molecules. This constitutes the membrane attack complex (MAC), leading to lysis of the pathogen (reviewed by Cole and Morgan, 2003).



**Figure 1.9 Schematic diagram of the terminal complement pathway**

Active components, as referred to in the text boxes below each stage, are shown in red.

### 1.9.1.4 Complement system regulation

Given that the uncontrolled activation of the complement cascade could have catastrophic effects on the host, a number of regulatory measures exist to prevent

damage to self. Localised activation is an important control feature of the complement system: active proteases are retained on the surface of the pathogen to focus the cascade and prevent uncontrolled inflammation and tissue damage. For example, C4b is only able to bind C2 when it is covalently bound to the pathogen surface, and C2 is only susceptible to cleavage by C1s when it is bound to C4b. Accordingly, as C1s is bound to C1q, these reactions all occur in the vicinity of the C1q binding site. Rapid inactivation ensues if the activated fragments are not bound to the site of initial complement activation (Janeway, 2001). In addition, a number of membrane-bound and soluble factors act to contain and control the complement cascade, by dissociating complexes or catalysing the breakdown of active complement components (reviewed by Blue et al., 2004; Janeway, 2001; Morgan, 1999). These are described below in the order in which they intervene in the complement cascade:

*1. Inhibition of C1s protease activity:*

The C1 inhibitor, C1-inh, binds to the C1r-C1s complex and forces its displacement from C1q. It also prevents the spontaneous activation of C1 in solution.

*2. C4b breakdown.*

C4b can be inactivated by the protease factor I. This can occur before the formation of the C3 convertase, or after its dissociation. Factor I requires a cofactor to function, and this can take the form of C4 binding protein (C4bp), CR1 or CD46. C4bp is present in solution. CR1 and CD46 are found on certain host cell surfaces, therefore promoting factor I-mediated inactivation of an inappropriate complement response.

*3. C3b breakdown*

As with C4b, C3b can be inactivated by factor I. The cofactor for this process is either factor H, CR1 or CD46. Factor H is a soluble protein, and binds to C3b in the presence of sialic acids present on host cells (and generally absent from pathogen surfaces).

*4. Prevention of formation or accelerated decay of the C3 convertase*

CR1, decay accelerating factor (DAF or CD55) and factor H compete with factor B for binding to C3b to prevent C3 convertase formation, or displace factor B from the formed alternative pathway C3 convertase. CR1 and CD55 have the same effect on C2 binding to C4b in the classical or lectin pathway C3 convertase. CR1 and CD55 are found on host cell surfaces, and factor H has a higher affinity for C3b when it is bound to host cells.

5. *Inhibition of the MAC formation*

CD59, or protectin, is widely distributed on host cells and prevents the binding of C9 to the C5b,6,7,8 complex.

1.9.1.5 *Complement receptors*

There are five known receptors for fragments of the complement component C3 (Holers et al., 1992; Leslie, 2001; Roozendaal and Carroll, 2006): CR1, CR2, CR3, CR4 and the newly discovered CR1g (Helmy et al., 2006). The major characteristics of CR1-4, the best studied of the complement receptors, are shown in Table 1.1. An overview of the characteristics and function of CR1-4 is given below.

RECEPTOR	SIZE (kDa)	PROTEIN FAMILY	LIGANDS	CELLULAR EXPRESSION
CR1 (CD35)	290, 250 or 210	Regulators of complement activation	C3b, C4b, iC3b	Erythrocytes, monocytes/macrophages, neutrophils, eosinophils, basophils, NK cells, B cells, T cells, dendritic cells, FDCs, astrocytes, epithelial cells.
CR2 (CD21)	145	Regulators of complement activation	iC3b, C3dg, C3d EBV gp350/220 CD23 IFN- $\alpha$	B cells, FDCs, astrocytes, T cell subsets, epithelial cells.
CR3 (CD11b/CD18)	165/95	$\beta$ 2 integrin	iC3b, C3b, C3dg	Monocytes/macrophages, dendritic cells, neutrophils, eosinophils, basophils, NK cells, microglial cells, platelets, FDCs.
CR4 (CD11c/CD18)	150/95	$\beta$ 2 integrin	iC3b	Monocytes/macrophages, neutrophils, eosinophils, basophils, NK cells, microglial cells, platelets.

**Table 1.1 Major characteristics of the four C3 fragment receptors**

#### *1.9.1.5.1 Complement receptor 1*

CR1 is a type I transmembrane protein from the regulators of complement activation (RCA) protein family. It occurs in several allelic forms of 210, 250 and 290 kDa. The largest isoform of CR1 consists of 30 short consensus repeats (SCRs), each of 60-65 aa, a transmembrane region and a short cytoplasmic tail. CR1 is expressed on most blood cells, including erythrocytes, monocytes/macrophages, neutrophils, eosinophils, basophils, NK cells, B cells and some T cells (Stoiber et al., 2005). The highest affinity ligands for CR1 are C3b and C4b, with lower affinity binding of iC3b. CR1 can act as a receptor for antigen bound by these fragments, and through the action of CR1 erythrocytes play a major role in clearance of soluble immune complexes from the circulation. Furthermore, CR1 has cofactor activity for factor I-mediated degradation of C3b to iC3b, iC3b to C3c and C3dg, and C4b to C4c and C4d (Leslie, 2001; Speth et al., 1997) as described in section 1.9.1.4. CR1 also has decay-accelerating activity for the C3 convertases. It prevents the binding of factor B to, or accelerates the removal of Bb from, C3b in the alternative pathway; likewise, it prevents the binding of C2 to, or accelerates the removal of C2a from, C4b.

#### *1.9.1.5.2 Complement receptor 2*

Like CR1, CR2 is a type I transmembrane protein from the RCA protein family. Two isoforms of the protein exist, consisting of 15 or 16 short consensus repeats (SCRs) or complement control protein modules, a transmembrane domain and a short cytoplasmic tail. The SCRs are each 60-70 aa long and arranged as four homology groups of four, designated I-IV, ordered from the membrane distal end. In humans, CR2 is expressed mainly on mature B cells, but also on FDCs, thymocytes (Tsoukas and Lambris, 1988), subsets of CD4 and CD8 T cells (Fischer et al., 1991; Fischer et al., 1999; June et al., 1992; Masilamani et al., 2002; Sauvageau et al., 1990), basophils, mast cells, astrocytes (Gasque et al., 1996), keratinocytes and epithelial cells (Holers, 2005; Speth et al., 1997). The long (16 SCRs) and short (15 SCRs) isoforms of CR2 are selectively expressed on FDCs and B cells, respectively (Liu et al., 1997). The biological ligands of CR2 are C3dg, C3d, iC3b, CD23, IFN- $\alpha$  and the EBV envelope proteins gp350/220 (Holers, 2005). The binding site for iC3b, C3dg and C3d and gp350/220 of EBV is located in the most amino terminal two SCRs, in homology region I (Lowell et al., 1989). On B cells CR2 forms a co-receptor complex along with CD19 and CD81. When the B cell receptor (BCR) is co-ligated with the co-receptor,

most commonly by the binding of complement-opsonised antigen, B cell signalling is augmented by 1000- to 10,000-fold and lowers the B cell activation threshold. (Dempsey et al., 1996). On other cell types CR2 is expressed independently from the complex.

#### *1.9.1.5.3 Complement receptor 3*

The  $\beta 2$  integrin CR3 (CD11b/CD18, Mac-1) is a heterodimeric adhesion and recognition receptor. It consists of two glycoprotein subunits: the 165 kDa  $\alpha$  chain, CD11b, and the 95 kDa  $\beta$  chain, CD18, also shared by CR4 (CD11c/CD18) and LFA-1 (CD11a/CD18; Leslie, 2001; Speth et al., 1997). CR3 recognises a wide variety of ligands including LPS and ICAM-1, but in the context of the complement system its most important interaction is with iC3b (Ehlers, 2000). It also has low affinity for C3b and C3dg (Stoiber et al., 2005). Activation of CR3 on monocytes/macrophages and neutrophils results in phagocytosis and therefore clearance of iC3b-bound antigens. CR3 is the primary complement receptor on neutrophils and monocytes, and is also expressed on eosinophils, basophils, NK cells, platelets and FDCs.

#### *1.9.1.5.4 Complement receptor 4*

CR4, also a  $\beta 2$  integrin, is similar in structure and function to CR3. The  $\beta$  subunit of CR4, CD18, is shared with CR3, and its 150 kDa  $\alpha$  subunit, CD11c, shares 63% sequence homology with the  $\alpha$  subunit of CR3, CD11b. CR4 is found on the same cell types as CR3, with the exception of FDCs and DCs.

### **1.9.2 Interaction of HIV and complement**

Many viruses have evolved mechanisms to evade the deleterious effects of complement, and in some cases to further subvert the complement system to their own advantage (Blue et al., 2004). The mechanisms of interaction of HIV with complement and consequences thereof are described below.

#### *1.9.2.1 Complement activation by HIV*

HIV activates the complement cascade by both antibody-dependent and -independent pathways (Gregersen et al., 1990; Robinson et al., 1991; Spear et al., 1990; Spear et al., 1993). The antibody-independent pathway involves direct binding of C1q to several regions on gp41 (Ebenbichler et al., 1991; Marschang et al., 1997;

Stoiber et al., 1994; Thielens et al., 1993) and gp120 (Prohaszka et al., 1995; Susal et al., 1994; Susal et al., 1996), triggering the classical complement pathway, and the binding of MBL to carbohydrate moieties on gp120 (Haurum et al., 1993; Susal et al., 1994), triggering the MBL complement activation pathway. Direct activation of complement is important in early stages of infection prior to the generation of HIV-specific antibodies (Stoiber et al., 2005). During and following seroconversion, HIV-specific complement-activating antibodies are generated. Bound antibodies amplify the activation of complement and the deposition of complement fragments on the viral surface (Hidvegi et al., 1993; Saarloos et al., 1995; Spear et al., 1993; Stoiber et al., 2001; Thieblemont et al., 1993b). Antibody-dependent complement activation was shown to be significantly more efficacious than antibody-independent complement activation, as determined by a 10-20-fold increase in C3 deposition on HIV-infected cells (Saarloos et al., 1995). Specificities of anti-HIV antibodies capable of activating complement include the V3 loop of gp120 (Spear et al., 1994) and gp41 (Robinson et al., 1991).

#### ***1.9.2.2 Lysis of HIV by complement***

Several papers have documented the inactivation of HIV by antibodies and complement. Whether this leads to direct lysis of the virions remains to be definitively shown. A recent paper by Huber and colleagues, detailing a freeze-thaw assay for the measurement of complement-mediated lysis, showed that antibodies in the plasma of infected individuals could mediate lysis of autologous virus in concert with complement (Huber et al., 2006). Sullivan et al also showed direct complement-mediated lysis of 14-86% of HIV isolated from patient plasma (Sullivan et al., 1996). However, neither of these studies demonstrated a direct relationship between lysis of virions and a subsequent decrease in infectivity of these virus preparations, so it cannot be ruled out that lysed virions form part of the uninfected portion of HIV virus preparations. Aasa-Chapman et al addressed both issues: complement-mediated HIV inactivation and lysis, and subsequent infectivity, and found that, although complement increased the inactivation of patient-derived viruses by early autologous antibodies, lysis accounted for only a small proportion of this inactivation (Aasa-Chapman et al., 2005).

### ***1.9.2.3 HIV complement evasion strategies***

The cell type used to produce HIV has bearings on susceptibility of the virus to lysis. A large body of evidence suggests that primary isolates of HIV are intrinsically resistant to complement-mediated lysis through a variety of mechanisms (Gregersen et al., 1990; Saifuddin et al., 1997; Saifuddin et al., 1995; Stoiber et al., 1996; Stoiber et al., 2005). As the virus buds from the cell surface, host cell membrane proteins are incorporated into the viral membrane. Complement regulatory proteins are among these proteins, with CD55, CD59 and CD46 well characterised in their association with HIV virions (Marschang et al., 1995; Saifuddin et al., 1997; Saifuddin et al., 1995; Stoiber et al., 1996). Blocking CD55 and CD59 on HIV virions with specific antibodies increased the sensitivity of virions to complement-mediated lysis (Marschang et al., 1995; Saifuddin et al., 1995). In addition to this, the soluble protein factor H can bind directly to gp41 and gp120 (Pinter et al., 1995; Stoiber et al., 1995) and has been suggested to play a role in HIV resistance to C'-ML (Stoiber et al., 1996). This would also promote the degradation of C3b deposited on HIV to the inactive form iC3b via factor I, and likewise from iC3b to C3dg. MCP incorporated into the viral membrane can also act as a cofactor for factor I. These strategies mean that the terminal lysis activity of complement is for the most part inhibited, yet opsonisation still occurs – a process that many researchers have proposed may have benefits for HIV, as described in 1.9.2.4.

### ***1.9.2.4 Outcomes of HIV opsonisation by complement***

In the absence of lysis, the opsonisation of HIV and subsequent binding of HIV immune complexes to complement receptors can have several possible outcomes (Speth et al., 1997). It may be of benefit to the immune system by assisting viral clearance through phagocytosis and destruction of immune complexes. Alternatively, it could benefit the virus and facilitate infection, dissemination and establishment of viral reservoirs (reviewed by Banki et al., 2005b; Stoiber et al., 2001; Stoiber et al., 2005). Of particular importance is the role of complement in the trapping of viruses on the FDC network in lymph node germinal centres (GC; Banki et al., 2005a; Burton et al., 2002; Kacani et al., 2000). Binding of antibody and complement-opsonised HIV to CR2 is considered the major mechanism of FDC trapping (Joling et al., 1993; Kacani et al., 2000). Virus retained in this way, rather than being neutralised and subsequently destroyed, remains infectious for months and provides an infectious reservoir for GC-

resident T cells and other target cells passing through the GC. Complement and antibody-mediated binding of HIV to FDCs also appears to confer a significant degree of neutralisation resistance on the virus (Heath et al., 1995).

An extension to the scenario of CR-mediated trapping of HIV is CR-mediated *trans*-infection. Complement-opsonised HIV bound to cells such as DCs and B cells may promote the infection of susceptible target cells through cell-cell interactions. In this way, productive infection may be more efficient than cell-free virus alone. DCs express CR3 and CR4 (De Panfilis et al., 1989), and CR3 has been shown to contribute to the DC-SIGN-mediated mechanism of *trans*-infection (Bouhlal et al., 2007). B cells express CR1 and CR2 and have been shown to mediate *trans*-infection of T cells (Doepper et al., 2000; Dopfer et al., 2003; Jakubik et al., 1999; Moir et al., 2000). In addition, erythrocytes express CR1 and tightly bind HIV via complement in the presence and absence of antibody (Montefiori et al., 1994). They transport immune complexes to the liver and spleen for destruction by phagocytic cells, and may promote the infection of susceptible cells in these organs (Stoiber et al., 2005). B cells are also vulnerable to CR-mediated infection (Boyer et al., 1992; Gras et al., 1993; Legendre et al., 1996), a process that has been classified as a form of ADE, but it is unclear whether this makes a major contribution to pathogenesis *in vivo*.

In tissues, such as the brain, positive feedback loops drive complement production. Activation of complement and the binding of immune complexes to cells with complement receptors can lead to activation of these cells, and a further increase in local complement synthesis (Morgan and Gasque, 1997). This allows for complement-mediated entry of HIV into, or binding of HIV onto, cell types such as astrocytes in the brain, and also contributes to neurological damage (Depboylu et al., 2005; Speth et al., 2002; Speth et al., 2001). Microglial cells are the main cellular target for HIV infection in the brain and have been shown to express CR3 (Hassan et al., 1991) whereas astrocytes express CR1 and CR2 (Gasque et al., 1996).

Of all the forms of subversion of the complement system by HIV, C<sup>3</sup>-ADE of HIV infection is perhaps the most extreme. The presence of complement and antibody promote levels of infection higher than in the absence of opsonisation. This is discussed in detail in the next section.

## **1.10 ANTIBODY-DEPENDENT ENHANCEMENT OF VIRAL INFECTION**

### **1.10.1 History and significance**

Antibody-dependent enhancement (ADE) of virus infection (reviewed by Morens, 1994; Sullivan, 2001; Takada and Kawaoka, 2003; Tirado and Yoon, 2003) is a phenomenon in which virus-specific antibodies enhance the entry or replication of a virus, leading to increased infection and the potential to exacerbate disease progression or severity. It was first described in the 1960s (Hawkes, 1964), and was later given clinical relevance through epidemiological studies of dengue disease enhancement (Halstead et al., 1970; Halstead and O'Rourke, 1977a). Severe manifestations of dengue disease take the form of dengue haemorrhagic fever and dengue shock syndrome (DHF/DSS). Immune enhancement was investigated as a contributory factor to DHF/DSS as a result of two epidemiological observations. It was noted that DHF/DSS occurred mostly upon secondary infection with a viral serotype distinct from that of the primary infection (Russell et al., 1967), with the biggest risk factor being secondary infection with dengue serotype 2 (DEN-2; Halstead et al., 1970; Russell et al., 1967; Sangkawibha et al., 1984). More striking was the bimodal incidence curve in children. Peak incidences for DHF/DSS occurred first between 5 and 10 months and later between 3 and 5 years. Severe disease in the first age group coincides with the decline of maternally-acquired antibody titre (Halstead et al., 1969). Severe disease in the second age group coincides with the earliest age at which secondary infection can occur. Both situations pointed towards a role for low levels of anti-dengue antibodies in pathogenesis, and lead to the development of the ADE hypothesis (Halstead and O'Rourke, 1977a; Halstead and O'Rourke, 1977b; Halstead et al., 1977).

Aside from disease enhancement in natural infection, ADE can also be a risk factor for disease exacerbation and/or increased chance of infection following vaccination. ADE has been retrospectively implicated in vaccinations that resulted in enhanced disease severity (Morens, 1994), such as RSV (Chin et al., 1969; Kim et al., 1969; Prince et al., 1986), measles and even rabies (Morens, 1994), although experimental data directly supporting these claims is lacking. ADE has also been implicated in the enhanced disease states observed after lentiviral vaccine trials carried out on equine infectious anaemia virus (EIAV; Raabe et al., 1999; Wang et al., 1994), caprine arthritis encephalitis virus (Jolly et al., 1989; Jolly and Narayan, 1989) and

feline immunodeficiency virus (FIV; Siebelink et al., 1995); therefore it will be important to ascertain whether enveloped-based vaccines against HIV, also a lentivirus, might have a similar effect.

However, even when substantial evidence points towards ADE as a major factor in exacerbated disease *in vivo* (Halstead et al., 1970; Montefiori et al., 1990; Ponnuraj et al., 2003; Siebelink et al., 1995; Wang et al., 1994), it cannot be ruled out that other immunopathogenic phenomena are playing a role, such as profound T cell activation and death (Mongkolsapaya et al., 2003) or a bias towards a T<sub>H</sub>2 response (Openshaw et al., 2001). Clearly, enhanced disease is often a multifactorial process. Consequently, corroborating *in vivo* effect with evidence of *in vitro* enhancement is difficult. Reports have shown *in vitro* ADE of a wide variety of viruses; Ross River virus (RRV), Rift Valley fever virus (RVFV), simian immunodeficiency virus (SIV), ebola virus, feline infectious peritonitis virus (FIPV), rabies and coxsackie virus being but a few examples (reviewed by Morens, 1994; Sullivan, 2001; Takada and Kawaoka, 2003; Tirado and Yoon, 2003). ADE of HIV has been studied extensively but somewhat inconclusively due to conflicting observations in the literature, as discussed below.

### 1.10.2 Mechanisms of enhancement

In terms of virus-cell interactions, there are several ways in which enhancement could occur. The simplest model is the binding of enhancing antibody-opsonised, or enhancing antibody and complement-opsonised, virus to FcRs or CRs, increasing virus-cell contact and facilitating the engagement of envelope proteins with the primary virus receptors. With regards to HIV, as the CD4-gp120 interaction is considered the rate-limiting step of HIV entry (O'Doherty et al., 2000; Ugolini et al., 1999), it is thought that the virus makes use of a range of attachment receptors on the cell surface to increase concentrations of cell-associated virus and therefore increase the probability of CD4 engagement. ADE of HIV can achieve this aim, but may also have other effects on HIV entry and replication. Ligation of receptors by enhancing antibody could trigger endocytosis of the virus, or signal through the receptor to alter cellular conditions in favour of virus replication. In some cases ADE may occur independently of cellular receptors by altering the conformation of the viral entry proteins. The main mechanisms of ADE are described below, with additional information regarding the specifics of these mechanisms for HIV where relevant.

Further information on ADE of HIV is given in the introductions to Chapters 3, 4, 5 and 6.

#### ***1.10.2.1 FcR-ADE***

FcR-mediated ADE (FcR-ADE) was the first mechanism of ADE to be elucidated (Halstead and O'Rourke, 1977a; Halstead and O'Rourke, 1977b) and remains the most commonly observed form of ADE, having been demonstrated for dengue virus, West Nile virus (Peiris et al., 1981; Peiris and Porterfield, 1979), yellow fever virus (Schlesinger and Brandriss, 1981a; Schlesinger and Brandriss, 1981b) and measles virus (Iankov et al., 2006) among others. Involvement of FcR-ADE might be also inferred by observations of enhanced infection in the absence of complement in monocytes/macrophages, without directly showing the involvement of FcRs. Studies of flavivirus FcR-ADE represent the classic model of FcR-ADE and are performed on macrophages, monocytes or macrophage-like cell lines, such as human U937 cells and mouse P388D1 cells. Enhancement levels in these systems are generally in the range of 5- to 200-fold increases in infection compared to infection in the absence of virus-specific antibody (Halstead and O'Rourke, 1977a; Peiris et al., 1981; Peiris and Porterfield, 1979; Schlesinger and Brandriss, 1981a).

Receptor-blocking experiments have demonstrated the involvement of Fc $\gamma$ RI (Homsy et al., 1989; Jouault et al., 1991; Laurence et al., 1990; Perno et al., 1990; Takeda et al., 1990), Fc $\gamma$ RII (Laurence et al., 1990), and Fc $\gamma$ RIII (Homsy et al., 1989), and also the IgA receptor Fc $\alpha$ R (Kozlowski et al., 1995) in ADE of HIV infection.

#### ***1.10.2.2 C'-ADE***

Complement-mediated ADE (C'-ADE) is mediated by CRs on the target cell. As with FcR-ADE, it was first observed for a flavivirus, being described by Cardosa in 1983, who showed CR3-mediated C'-ADE of West Nile virus by IgM and complement (Cardosa et al., 1983). C'-ADE is usually characterised by the requirement of fresh, non-heat-inactivated supplementary serum in the assay, with generally lower levels of enhancement than those seen for classic FcR-ADE.

Most information regarding C'-ADE has come from studies of HIV, first observed by Robinson and colleagues (Robinson et al., 1988a) and subsequently investigated in detail by the same group (Robinson et al., 1991; Robinson et al., 1990a; Robinson et al., 1990b; Robinson and Mitchell, 1990; Robinson et al., 1989a;

Robinson et al., 1988b; Robinson et al., 1990c; Robinson et al., 1989b) and later by others (Delibrias et al., 1993; June et al., 1991; Lund et al., 1995; Prohaszka et al., 1997; Reisinger et al., 1990; Szabo et al., 1999). It was found that cells with a high level of CD4 and CR2 (MT-2 cells) supported C'-ADE, whereas cells with CD4 but low or undetectable CRs (H9, CEM, C3 and U937 cells) did not (Robinson et al., 1989a). The dependence of C'-ADE on CD4 and CR2 was subsequently confirmed with blocking antibodies to these receptors on MT-2 cells (Robinson et al., 1990c). CR1 (Delibrias et al., 1993; Delibrias et al., 1994; Gras and Dormont, 1991) and CR3 (Reisinger et al., 1990) have also been implicated in C'-ADE of HIV infection, and more recently C1qRs have been shown to play a role in C'-ADE of HIV (Prohaszka et al., 1997) and Ebola virus infection (Takada et al., 2003).

Besides T cells, C'-ADE has also been shown to occur in syncytiotrophoblasts (Toth et al., 1994), B cells (Boyer et al., 1992; Gras et al., 1993; Gras and Dormont, 1991; Legendre et al., 1996; Tremblay et al., 1990), thymocytes (Delibrias et al., 1994) and epithelial cells (Bouhlal et al., 2002). Importantly, C'-ADE of infection of primary cells has been demonstrated, with CR1 and CR3 mediating CD4-independent C'-ADE of monocytes (Thieblemont et al., 1993a) and CR3 mediating C'-ADE of DCs (Bajtay et al., 2004).

Existing evidence suggests that increased adhesion to the target cell is the principal mechanism of C'-ADE (June et al., 1991; Lund et al., 1995; Robinson, 2006) but this has not been exclusively proven and other mechanisms involving receptor signalling may play a role.

Enhancement of HIV can also be mediated by complement alone, in the absence of antibodies, although this is weaker than enhancement in the presence of antibodies (Sullivan, 2001). It is also possible that unopsonised HIV can interact directly with complement receptors as interactions between gp41 and CR3 have been demonstrated *in vitro* (Stoiber et al., 1997).

### ***1.10.2.3 ADE through alteration of cellular processes***

The alteration of cellular processes through ADE could be considered a subset of C'-ADE or FcR-ADE, as the same receptors are involved but the downstream mechanisms differ. Studies of enhanced RRV infection in macrophages delineated a novel ADE mechanism: FcR-mediated suppression of intracellular antiviral responses. Instead of the inhibition of RRV production in macrophages caused by LPS-induced

antiviral responses, enhanced viral production was seen in the presence of RRV-specific antibodies. This was shown to be due ADE-infection specific suppression of tumour necrosis factor (TNF)  $\alpha$  and inducible nitric oxide synthase (NOS2) genes (Lidbury and Mahalingam, 2000).

In HIV infection there is evidence to suggest that CR-mediated cell activation provides a favourable environment for HIV replication (Speth et al., 1997). Engagement of CRs on infected cells, or during the process of enhanced infection, may increase viral replication. This was shown for infected CD4+ T cells by CR1 ligation (Mouhoub et al., 1996) and monocytic cells via CR1 and CR3 ligation (Thieblemont et al., 1995). In the latter study, enhanced p24 antigen production was associated with NF- $\kappa$ B nuclear translocation, triggered by the activation of CR1 and CR3 (Thieblemont et al., 1995).

While these experiments used CR-specific monoclonal antibodies to stimulate the receptors, it is conceivable that antibody and complement-opsonised virus could mediate the same effect, either by signalling *in cis* during entry or signalling by cell-free virus in infected cells *in trans*. *In vivo*, a state of generalised immune activation characterises chronic HIV infection and this is thought to favour virus production through providing more activated target cells for infection. Ligation of CRs by HIV immune complexes may contribute to this, either directly via CRs on T cells, DCs and macrophages (Delibrias et al., 1992), or indirectly through cytokine production by activated B cells (Arvieux et al., 1988).

#### ***1.10.2.4 Receptor-independent ADE***

More recent studies of ADE of HIV have focused on enhanced infection via antibody-mediated modulation of the viral envelope conformation (non-FcR, non-C'-ADE) as determined by studies on cell lines lacking FcRs, in the absence of complement and also using Fab antibody fragments (Guillon et al., 2002; Schutten et al., 1997; Sullivan et al., 1995). This appears to be more dependent on viral strain than on specific antibody epitopes, and it is thought that the antibodies mediating the enhancement mimic the viral receptors, precipitating conformational changes and thereby increasing entry efficiency.

### ***1.10.2.5 Characteristics of enhancing antibodies***

The original model of ADE, developed for dengue virus enhancement, involves neutralising antibodies at sub-neutralising concentrations. This model applies to many examples of ADE, with neutralising antibody titration experiments showing the biphasic curves that have come to characterise classical dengue virus enhancement: neutralisation occurring at high concentrations of antibody and enhancement at low concentrations. Studies of monoclonal neutralising antibodies to WNV showed that neutralisation and ADE were functions of antibody occupancy; infection enhancement occurred when the virus was bound by antibody with a stoichiometry that was no longer sufficient for neutralisation (Oliphant et al., 2006; Pierson et al., 2007).

An alternative to sub-neutralising concentrations of neutralising antibodies involves high concentrations of antibodies that bind to non-neutralising epitopes. Again, with reference to dengue virus, this may apply to ADE by heterotypic non-neutralising serum, and in this case ADE can occur at high serum concentrations (Halstead and O'Rourke, 1977a; Halstead and O'Rourke, 1977b). This scenario could also lead to a biphasic titration curve when serum is used, reflecting the balance of neutralising and enhancing antibodies: neutralising antibodies dominate at high concentrations, but as these are diluted out, enhancing antibodies dominate.

Evidence exists to suggest that HIV-enhancing antibodies differ from neutralising antibodies. First indications of this were shown in 1988, when Robinson and colleagues showed that when HIV seropositive sera were applied to an affinity column containing a recombinant fragment of Env (the 20 C-terminal aa from gp120 and the N-terminal two thirds of gp41, produced in *E. coli*) the fraction that bound to the column was enhancing, while the flow-through neutralised (Robinson et al., 1988a). Subsequent experiments defined an antibody-enhancing epitope on gp41, located at aa579-613 of the immunodominant cluster I (Mitchell et al., 1998; Robinson et al., 1991; Robinson et al., 1990a; Robinson et al., 1990b). A second, minor antibody-enhancing epitope was mapped to aa 644-663 on gp41 (Robinson et al., 1991).

Other studies suggested that certain virus strains are more susceptible to enhancement than others. One study mapped the ability of V3-specific monoclonal antibodies to enhance infection of three strains of HIV: a difference of one amino acid in the antibody-binding epitope was sufficient to determine whether the virus was enhanced, neutralised or unaffected (Kliks et al., 1993). In a study investigating a

panel of fourteen primary isolates and twenty-four sera from the same cohort of HIV-infected individuals, the significant proportion of combinations resulting in enhancement of infection rather than neutralisation (28%) was associated more with certain viral isolates rather than certain sera (Kostrikis et al., 1996).

### 1.10.3 Measuring ADE

The choice of how to study ADE is more complicated than for neutralisation, as in addition to the virus-antibody interactions, the interaction of the immune complex with the target cell is a key determinant of assay outcome and adds a further layer of complexity. The issues associated with development of an enhancement assay are highlighted by Raabe and colleagues (Raabe et al., 1999). The target cell type, virus strain, antibody source and concentration, MOI, method of measuring infectivity levels, and the method of presenting enhancement data may all influence the outcome of the assay, and possibly account for differences seen between research groups (Raabe et al., 1999).

There are several ways in which results from enhancement assays may be presented:

1. Fold enhancement: the ratio of infection in the presence of virus-specific antibody to infection in the absence of virus-specific antibody.
2. Endpoint titre: the highest dilution of antibody at which enhancement is seen.
3. Peak titre: the dilution of antibody at which enhancement is greatest.

For most studies of classic flavivirus enhancement, the standard format for reporting results of enhancement experiments is fold enhancement, with additional information about antibody titres provided, as initiated by Halstead in 1977 (Halstead and O'Rourke, 1977a). For studies of HIV enhancement, the methodology of measuring enhancement of infection and presenting data varies between reports. For example, some measure CPE and define enhancement as anything greater than a 1 SD increase (Robinson et al., 1988a), or 50% increase, in CPE relative to the control culture and present results as endpoint titres (McDougall et al., 1997; Robinson et al., 1990c); others measure viral production, usually by RT ELISA, and present results either as fold enhancement (Banhegyi et al., 2003; Kozlowski et al., 1995; Lund et al., 1995; Montefiori et al., 1995; Prohaszka et al., 1997; Szabo et al., 1999) or as endpoint titres derived from a cut-off value of at least 2- or 3-fold increases in infection (Homsy, 1988; Homsy et al., 1990). Given that antibody titrations in enhancement

assays often result in a biphasic curve, with neutralisation occurring at low dilutions and enhancement occurring at high dilutions, and that increases in infection could be anything between 1.5- and 1000-fold, it seems logical to present both the fold enhancement and dilution of antibody at which this occurs (Montefiori et al., 1995). In addition, it may be more physiologically relevant to assay sera at low, rather than excessive, dilutions in order to determine the dominant antibody activity.

The most reproducible data to emerge on ADE of HIV has been carried out on cell lines such as MT-2, MT-4 and U937 cells. However, in the past this has restricted researchers to the study of X4-tropic TCLA strains of HIV, which are now known to bear less relevance to primary isolates than was previously thought. Several important studies have shown the capacity of primary cells to support enhanced infection, yet donor variability is a recognised complication associated with macrophage experiments, and the methods necessary for preparation of primary target cells do not make for a quick, reproducible assay for ADE.

### 1.11 Scope of this thesis

The drawback of the enhancement assays currently or previously used to study ADE of HIV is either that T cell lines are used, limiting study to TCLA X4 virus strains, or that primary cells are used, often incurring problems due to donor variability or time-consuming methods of primary cell preparation. This thesis describes the development of a reproducible, inexpensive and modern enhancement assay for the study of both R5 and X4 virus strains (Chapter 3).

The majority of studies investigating ADE of HIV infection have focused on sera from individuals at chronic or AIDS stages of disease. Few have addressed the potential for antibodies produced soon after infection with HIV to enhance infection, during which time the antibodies are non-neutralising. None have addressed the possibility that these early antibodies may enhance early autologous virus. In Chapter 4 of this thesis, the enhancement assay developed in Chapter 3 was used to investigate C'-ADE of early primary virus isolates by autologous sera. Sequential serum samples from individuals recently infected with HIV were tested against autologous viruses isolated at early and late time points after infection. The relationship between neutralisation and C'-ADE was investigated, with competition studies showing neutralisation to be the dominant response.

In Chapter 5, the enhancement assay was used to investigate C'-ADE in individuals vaccinated with an experimental gp120 subunit vaccine. C'-ADE, complement-mediated inactivation (C'-MI) and complement-mediated rescue from neutralisation (C'-RN) were also investigated, and it was shown that, depending on the assay and virus strain used, different antibody activities could be observed in the same vaccinee serum sample.

In Chapter 6 the mechanism of the C'-ADE and C'-RN shown in Chapters 3, 4 and 5 was investigated. CR2 was cloned from SupT1/R5 cells and stably expressed on NP2 cells, and conferred the capacity to support enhanced infection onto these cells. A CR2 cytoplasmic tail mutant tested in parallel showed that signalling ability of CR2 was not essential for enhanced infection, suggesting that the major mechanism of CR2-mediated C'-ADE is increased attachment to the target cell.

## **CHAPTER 2**

### **Materials and Methods**

#### **2.1 BUFFERS AND SOLUTIONS**

FACS	1% FCS (v/v); 0.1% azide in PBS
Formol saline	3.8% formaldehyde in PBS
LB broth	1% tryptone; 0.5% yeast extract; 0.5% NaCl
PBS	137 mM NaCl; 3 mM KCl; 10 mM Na <sub>2</sub> HPO <sub>4</sub> ; 2mM K <sub>2</sub> HPO <sub>4</sub> pH 7.4
PERM	1% saponin (w/v) in FACS buffer
SOC medium	2% tryptone; 0.5% yeast extract; 10 mM NaCl; 2.5 mM KCl; 10 mM MgCl <sub>2</sub> ; 10 mM MgSO <sub>4</sub> ; 20 mM glucose
TAE	40 mM Tris-HCl pH 7.8; 20mM sodium acetate; 1 mM EDTA
TBS	20 mM Tris-HCl pH 7.6; 120 mM NaCl
TBS-T	TBS containing 0.05% Tween 20
T/V	0.5% trypsin and 0.02% versene (v/v) in PBS

#### **2.2 CELL CULTURE**

##### **2.2.1 Human cell lines**

The cell lines used in this study are detailed in Table 2.1.

CELL LINE	ADHERENT or SUSPENSION	CELL TYPE	REFERENCE
H9	S	Human cutaneous T cell lymphoma	(Mann et al., 1989)
MT-2	S	Human T cell leukaemia (transformed with HTLV)	(Harada et al., 1985; Miyoshi et al., 1981)
MT-4	S	Human T cell leukaemia (transformed with HTLV)	(Harada et al., 1985; Miyoshi et al., 1981)
Molt-4	S	Human T cell leukaemia	(Kikukawa et al., 1986)
C8166	S	Human T cell leukaemia (transformed with HTLV)	(Salahuddin et al., 1983)
SupT1	S	Human T cell lymphoma (non-Hodgkin's)	(Smith et al., 1984)
SupT1/R5	S	As with SupT1 but transduced to express CCR5	From Dr James Hoxie
NP2/CD4/R5 NP2/CD4/X4	A	Human glioma (transduced to express CD4, CCR5 and/or CXCR5)	(Soda et al., 1999)
293T	A	Human embryonic kidney (expressing the SV40 T Ag)	(Graham et al., 1977)

**Table 2.1 Cell lines**

### 2.2.2 Maintenance of cell lines

Adherent cell lines were maintained in Dulbecco's modified Eagle medium (D-MEM; Invitrogen, UK) supplemented with 5% heat inactivated (56°C for 1 hour) foetal calf serum (FCS; Helena Biosciences). Suspension cell lines were maintained in

RPMI 1640 medium (Invitrogen, UK) containing 10% FCS. SupT1/R5 cultures were supplemented with 1 µg/ml puromycin (Sigma Biochemicals). All cells were incubated at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. Cell lines were split once every 3-5 days at ratios of 1:3 to 1:20 as required. For adherent cell lines, media was removed by aspiration and cells were washed once with 5 ml PBS before adding 1-2 ml of T/V and incubating at 37°C for 5 minutes or until the cells detached from the flask. 5 ml of fresh culture medium was added to inactivate the T/V, and the cells were diluted as necessary and replenished with fresh culture medium. Suspension cells were split as required, centrifuged at 325 g for 5 minutes to pellet, and resuspended in fresh culture medium.

### **2.2.3 Freezing cells**

Cells were pelleted by centrifugation for 5 min at 325 g and resuspended in FCS containing 10% dimethyl sulphoxide (DMSO; v/v; Sigma, UK) at a density of 10<sup>7</sup> cells/ml. 1 ml aliquots were immediately dispensed into cryotubes (Nunc), wrapped individually in paper, placed in a polystyrene box and slowly frozen at -80°C overnight before transferring to the vapour phase of liquid nitrogen for long-term storage.

### **2.2.4 Thawing cells**

Cryotubes containing cells were removed from liquid nitrogen and thawed in a 37°C water bath before adding 10 ml of fresh culture medium and pelleting at 325 g for 5 minutes. Cells were resuspended in 10 ml fresh culture medium, transferred to a 25 cm<sup>2</sup> tissue culture flask (Helena Biosciences) and maintained as in section 2.2.2 until required.

### **2.2.5 Transfection of 293T cells**

293T cells were used for the production of lentiviral vectors via three-plasmid transfections (section 2.11.1), and to ensure correct expression of CR2 and KHR constructs using single plasmid transfections using FuGENE 6 (Roche Diagnostics), according to the manufacturer's instructions. Cells were plated in a 6-well plate one day prior to transfection at a density of 4 x 10<sup>5</sup> cells per well, resulting in approximately 70-80% confluence on the day of transfection. Culture medium was changed before transfection. For each well of the 6-well plate, 6 µl FuGENE 6 was

added to 200  $\mu$ l serum-free Optimem (Invitrogen, UK) in a sterile tube, and gently mixed before adding 1  $\mu$ g total DNA. The transfection mixture was then incubated at room temperature for 15 minutes, with occasional gentle mixing, before adding it dropwise to cells. Medium was replaced after overnight incubation, and supernatant was harvested, or cells analysed by flow cytometry for receptor expression, 24 or 36 hours later.

### **2.2.6 Preparation of peripheral blood mononuclear cells**

PBMCs were prepared from fresh blood, processed within 2 hours of phlebotomy. Blood was drawn by myself or another qualified phlebotomist from healthy volunteers using S-Monovette collection tubes containing EDTA (Sarstedt, Germany). Anonymity of donors was maintained, according to ethical approval obtained from UCL Committee for the Ethics of non-NHS Human Research (project number 0335/001).

35 ml of blood, diluted 1:1 with PBS, was layered onto 15 ml of Lymphoprep (ficoll; Nycomed, Norway) in a 50 ml Falcon tube and centrifuged at 700 g (with the brake off) for 30 minutes at room temperature. The PBMCs formed a white layer at the interface between the Lymphoprep and plasma/PBS, and this was carefully harvested and washed once with PBS and once with RPMI 1640 at 325 g for 10 minutes. The pelleted cells were resuspended at a density of approximately  $1 \times 10^6$  cells/ml in RPMI containing 10% FCS and 0.5  $\mu$ g/ml phytohaemagglutinin (PHA; Biostat, UK) and grown for 3 days. Cells were then pelleted and resuspended at a density of approximately  $1 \times 10^6$  cells/ml in RPMI containing 10% FCS and 20 U/ml interleukin-2 (IL-2; Roche, Germany), and cultured for a further 2-3 days prior to infection.

### **2.2.7 Flow cytometry analysis of cell surface receptor expression**

For analysis of cell surface receptor expression,  $2 \times 10^5$  cells were tested per sample in 5-ml round-bottomed polystyrene tubes if analysing fewer than 10 samples; otherwise 96-well U-bottomed plates were used. Wash steps were carried out in 500 and 200  $\mu$ l volumes in the 5-ml tubes and 96-well plates respectively, with centrifugation at 325 g for 5 minutes. Antibodies were used at dilutions recommended by the manufacturers. All were specified as being suitable for flow cytometry. When primary antibodies directly conjugated to a fluorophore were used (see Table 2.2),

secondary antibody steps were not necessary. When primary antibodies were not directly conjugated to a fluorophore, polyclonal goat anti-mouse conjugated to phycoerythrin (PE; Dako, Denmark) IgG was used as a secondary antibody.

Adherent cells were detached from culture flasks by treatment with versene. Cells were washed once in PBS and once in FACS buffer, followed by incubation with 50  $\mu$ l of 10% goat serum in FACS buffer for 30 min at 4°C. 100  $\mu$ l of primary antibody was added to the cells and incubated for 30 min at 4°C. Cells were then washed three times in FACS buffer and incubated with 50  $\mu$ l of secondary antibody for 30 min at 4°C, before washing cells twice with FACS buffer, once with PBS and fixing in formol saline for 10 min at 4°C. Cells were washed with PBS and receptor expression analysed on a Becton Dickinson FACScan using Cellquest software (Beckton Dickinson, UK). 10,000 events were acquired per sample.

ANTIGEN	ANTIBODY TYPE	ISOTYPE	CLONE	MANUFACTURER
CR1 (CD35)	Monoclonal mouse anti-human	IgG1	To5	Dako, Denmark
CR2 (CD21)	Monoclonal mouse anti-human	IgG1	1F8	Dako, Denmark
CR3 (CD11b)	Monoclonal mouse anti-human	IgG1	2LPM19c	Dako, Denmark
FcγRI (CD64)	Monoclonal mouse anti-human	IgG1	10.1	Dako, Denmark
FcγRII (CD32)	Monoclonal mouse anti-human	IgG1	KB61	Dako, Denmark
FcγRIII (CD16)	Monoclonal mouse anti-human	IgG1	DJ130c	Dako, Denmark
Isotype control	Mouse	IgG1		Dako, Denmark
CD4	PE-conjugated anti-human monoclonal	IgG2a		BD Biosciences PharMingen
CCR5	PE-conjugated anti-human monoclonal	IgG2a	2D7	BD Biosciences PharMingen
CXCR4	PE-conjugated anti-human monoclonal	IgG2a		BD Biosciences PharMingen
Isotype control	Mouse	IgG2a	G155-178	BD Biosciences PharMingen
Isotype control	Mouse anti-rat-PE	IgG1	W3/25	Dako, Denmark

**Table 2.2 Antibodies used for cell surface receptor expression analysis by flow cytometry**

## **2.3 SOURCES OF CONTROL SERA AND PLASMA**

### **2.3.1 Complement source**

Fresh sera from 4 healthy, HIV-negative individuals were pooled and divided into 1-ml aliquots. Half of the aliquots were heat inactivated at 56°C for 1 hour and then stored at -80°C for use as the complement inactivated control (HIC') and the remaining aliquots were stored immediately at -80°C for use as a complement source (C'). Samples were stored for no longer than 3 months and freshly thawed immediately before use in each assay.

### **2.3.2 Seronegative control serum (NHS) and plasma (NHP)**

The same batch of seronegative control serum, also referred to as normal human serum (NHS; PAA Laboratories, Austria) was divided into 1-ml aliquots and stored at -80°C for use in all enhancement and neutralisation assays. Individual NHS samples, used for assessment of variation in enhancement assays, and individual NHP samples, used for the purification of control IgG and IgM, were collected from healthy individuals, heat inactivated for 1 hour at 56°C, divided into 1-ml aliquots in cryotubes colour-coded to denote separate donors, and stored at -80°C.

## **2.4 THE JENNER COHORT**

Blood samples were obtained from a cohort of individuals recently infected with HIV, the Jenner cohort, as previously reported (Aasa-Chapman et al., 2004; Aasa-Chapman et al., 2005). The subjects studied in this thesis were all men who have sex with men (MSM) presenting with primary HIV illness (PHI) following sexual exposure to HIV. All were found to be infected with HIV-1 subtype B. The study protocol was approved by the Camden and Islington NHS Trust Ethics Committee and written consent was obtained from all subjects. All samples used in this thesis were from treatment-naïve individuals.

Blood samples were obtained weekly for the first month, monthly for 3 months and subsequently every 3 months. Plasma, serum, and whole blood were divided into aliquots and stored at -80°C. PBMCs were isolated and stored in liquid nitrogen.

## **2.5 HIV**

### **2.5.1 Propagation of TCLA virus strains**

The TCLA virus isolates IIIB and MN were propagated in the T cell line H9. Cells were counted and approximately  $1 \times 10^6$  cells pelleted by centrifugation at 325 g for 10 minutes before resuspending in 0.5-1 ml of virus stock and incubating at 37°C for 3 hours. 5 ml of culture medium was added and cells were incubated in an upright 25 cm<sup>2</sup> flask for 3-4 days until syncytia were observed under the light microscope. Cells were then pelleted and co-cultivated with  $5 \times 10^6$  uninfected cells in 1 ml of culture medium for 3 hours at 37°C. 15 ml of fresh culture medium was added, and cells were incubated in an upright 75 cm<sup>2</sup> tissue culture flask for a further 3-4 days until large syncytia were observed under the light microscope. At this point virus was harvested by centrifuging the cells and aliquoting 1-ml volumes into cryovials for storage in vapour-phase liquid nitrogen.

### **2.5.2 Isolation and propagation of primary isolates**

All patient primary isolates and the vaccine virus strain W61D were propagated in PBMCs.  $1-2 \times 10^7$  PBMCs (prepared according to section 2.2.6) from two different donors were used for co-culture with patient PBMCs (prepared according to section 2.4) or inoculation with W61D virus (kindly provided by Anna Helander). Donor PBMCs were pelleted at 325 g for 10 minutes and resuspended in 1 ml of fresh culture medium (containing 5 U/ml IL-2). Patient PBMCs were thawed and washed in 5 ml of fresh culture medium and resuspended in 1 ml of fresh culture medium. Patient PBMCs or 1-ml of W61D virus stock were added to the donor PBMCs and incubated for 2 hrs at 37°C. 8 ml of fresh growth medium was added and the cells transferred to a 25 cm<sup>2</sup> flask and incubated for 7 days at 37°C. On day 8 post-inoculation, cultures were replenished with fresh PBMCs (prepared according to section 2.2.6) from two new donors. Infected PBMCs were pelleted and 8 ml of the supernatant stored in 500- $\mu$ l aliquots in liquid nitrogen for subsequent determination of virus content. The PBMCs were resuspended in the remaining 2 ml of supernatant and split into two separate tubes.  $3 \times 10^6$  PBMCs from the two new donors in 10 ml fresh culture medium were added to each tube, transferred to a 25 cm<sup>2</sup> culture flask and incubated for a further 7 days. This procedure was repeated on days 15, 22 and 29. Viral production was monitored using a commercial p24 ELISA (Biomerieux, France), and

virus titres from peak days of p24 production were determined on NP2 cells (see section 2.7.1.1).

## **2.6 VIRUS DETECTION ASSAYS**

### **2.6.1 *In situ* intracellular p24 staining**

*In situ* p24 staining was used to detect infection following NP2-based assays. Fixed cells were washed once with PBS containing 1% FCS, then incubated for 1 hour at room temperature with 200 µl per well of primary antibody: an equal mixture of 2 mouse anti-p24 monoclonal antibodies (ADP 365 and 366, AIDS Reagent Program, NIBSC, UK) diluted 1:40 in PBS containing 1% FCS. Cells were washed twice with PBS containing 1% FCS, then incubated for 1 hour at room temperature with 200 µl per well of secondary antibody: goat anti-mouse polyclonal Ig conjugated to β-galactosidase (Southern Biotechnology Associates Inc, USA) diluted 1:400 in PBS containing 1% FCS. Cells were then washed twice with PBS containing 1% FCS and once with PBS, and incubated at 37°C with 200 µl per well of β-galactosidase substrate (0.5 mg/ml 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-gal; Invitrogen, UK) in PBS containing 3 mM potassium ferrocyanide, 3mM potassium ferricyanide and 1mM MgCl<sub>2</sub>) overnight. Cells infected with HIV developed a blue colour. Foci of infection were counted by light microscopy.

### **2.6.2 RT ELISA**

A commercial lentivirus RT ELISA (Lenti-RT Activity Assay; Cavid Tech, Sweden) was used to determine RT activity in cell-free supernatants in order to quantify HIV in viral stocks or cell supernatant from T cell-based assays, according to the manufacturer's instructions. The RT in the sample synthesises a DNA strand from an immobilised template/primer construct, incorporating bromo-deoxyuridine triphosphate (BrdUTP), which is later quantified by a BrdUTP binding antibody conjugated to alkaline phosphatase (AP). The level of RT activity in the sample is proportional to the colour change of the AP substrate, measured by absorbance at 405 nm in a Lucy 1 luminometer (Anthos-Labtech, UK) and analysed using Stingray software (Dazdaq, East Sussex, UK).

### **2.6.3 Intracellular p24 stain and flow cytometry**

#### ***2.6.3.1 Intracellular p24 staining of T cell lines***

Intracellular p24 staining of T cell lines and subsequent analysis by flow cytometry was used to determine the percentage of infected cells following T cell line-based enhancement and neutralisation assays. Intracellular staining of T cell lines was carried out in 96-well U bottomed tissue culture plates. Wash steps were carried out in 200 µl volumes, with centrifugation at 325 g for 4 minutes. All steps were carried out on ice and all centrifugation steps at 4°C. All buffers were pre-cooled to 4°C before use. Cells were washed once with FACS buffer then fixed with 100 µl of formol saline for 10 minutes at 4°C. Cells were then washed once with FACS buffer and once with PERM buffer (1% saponin in FACS buffer) to permeabilise. Blocking was carried out with 50 µl of 10% goat serum in PERM buffer for 30 minutes at 4°C. 50 µl of primary antibody, diluted 1:20 in PERM containing 10% goat serum, was then added to each well and incubated for 1 hour at 4°C. Primary antibody was the same as that used for the NP2 cell assays: an equal mixture of two mouse monoclonal antibodies to two separate epitopes on p24 (ADP 365 and 366). Cells were then washed three times in PERM buffer then incubated for 1 hour at 4°C with 50 µl secondary antibody: goat anti-mouse polyclonal antibody conjugated to FITC (Dako, Denmark). Cells were washed twice with PERM buffer and once with FACS buffer, then fixed again with formol saline for 10 min at 4°C, then washed once with PBS before transferring to 2 ml polystyrene tubes for analysis on a FACScan flow cytometer (Beckton Dickinson, UK) using Cellquest software (Beckton Dickinson, UK).

#### ***2.6.3.2 Determination of percent infected cells by flow cytometry***

Using uninfected, stained cells as a control, cells were gated on the major (healthy) lymphocyte-like population on forward scatter (FSC) / side scatter (SSC) dot plots. Samples were analysed on SSC/FL-1 dot plots, and a second gate set up to count infected cells (represented by the level of p24 expression, as represented by FITC fluorescence measured on FL-1). 10,000 events were acquired in the healthy population per sample, in triplicate. Samples were “blanked” by averaging the number of events occurring in the infected gate for 6 uninfected samples (usually averaging <0.01%) and removing this from each test sample.

## 2.7 INFECTION ASSAYS

### 2.7.1 NP2 cell-based assays

#### 2.7.1.1 Titration of virus stocks

NP2/CD4/R5 and NP2/CD4/X4 cells were seeded in 48-well plates one day prior to infection at a density of  $2 \times 10^4$  cells/well. Ten-fold serial dilutions of viral stocks were made in fresh culture medium, and 100  $\mu$ l of each dilution were added to the cells in triplicate and incubated for 2 hours at 37°C. Cells were then gently washed with culture medium, overlaid with 500  $\mu$ l fresh culture medium and incubated for 72 hrs at 37°C. After 72 hours incubation, culture medium was removed and the cells fixed for 10 min at room temperature with 200  $\mu$ l per well of methanol/acetone (1:1 v/v), pre-cooled at -40°C. Cells were washed once with PBS then overlaid with PBS and stored at 4°C until *in situ* p24 staining, detailed in section 2.6.1.

#### 2.7.1.2 Neutralisation assay

Patient sera or plasma, or monoclonal antibodies, were two-fold serially diluted in NHS. Alternatively, longitudinal studies of neutralisation in sequentially collected autologous samples were performed on undiluted serum or plasma, resulting in a final concentration of 10% in the assay.

The prepared sera, plasma, monoclonal antibody, or NHS as a control, was incubated at a final concentration of 10% with 100 ffu of virus in 100  $\mu$ l fresh culture medium. This was incubated for 1 hour at 37°C before adding to the cells (prepared as in section 2.7.1.1) and incubating for 2 hours at 37°C. Cells were then gently washed with culture medium, overlaid with 500  $\mu$ l fresh culture medium and incubated for 48 or 72 hrs at 37°C. Cells were fixed as in section 2.7.1.1.

#### 2.7.1.3 C'-MI assay

C'-MI assays (Aasa-Chapman et al., 2005) were performed as per the neutralisation assays, with the following modifications:

C'-MI assays were performed with sera as an antibody source. Heat inactivated (HIC') or active (C') complement (see section 2.3.1) was added to the

virus/serum mix before the 1-hour incubation at 37°C. The final concentration of HIC' or C' was 10%; likewise, the final concentration of patient serum or NHS was 10%.

#### **2.7.1.4 Assays on NP2 cells transduced to express CR2, KHR or FcγRIIa**

Assays were performed as per the CMI assays, with the exception that NP2/CD4/R5/CR2 or -KHR cells were used in parallel with NP2/CD4/R5 cells as a control, or NP2/CD4/X4/CR2, -KHR or -FcγRIIa cells were used in parallel with NP2/CD4/X4 cells as a control. Two viral inputs of 10 and 100 ffu were used.

### **2.7.2 T cell line-based assays**

#### **2.7.2.1 SupT1/R5 enhancement assay**

The standard SupT1/R5 enhancement assay, developed in Chapter 3 of this thesis and used throughout Chapters 4, 5 and 6, is described below. Variations on the assay are given in section 2.7.2.2.

Virus (for virus quantity see section 2.7.2.2 below) was added to HIC' or C' and patient or vaccinee serum, or NHS as a control, in a total volume of 100 µl in serum-free culture medium and incubated for 1 hour at 37°C. Patient serum, vaccinee serum or NHS, and HIC' or C' as appropriate, were each used at a final concentration of 10% at the time of incubation.

Meanwhile, SupT1/R5 cells were seeded in U-bottomed 96-well tissue culture plates at 10<sup>5</sup> cells per well in 100 µl of fresh culture medium. After the 1-hour incubation, virus-serum mixtures were transferred to the cells, giving a final volume of 200 µl per well. Plates were incubated at 37°C. In order to monitor the RT output of the assay (section 2.6.2), and to provide fresh medium for the cells, on days 1 and 3 100 µl of cell supernatant was removed from each well and immediately stored at -40°C. Cell supernatant was replaced with 100 µl per well of fresh culture medium. On day 5 or 6 of the assay the entire 200 µl of cell supernatant was removed and stored at -40°C and the cells were stained for intracellular p24 expression (section 2.6.3). In some cases assays were stopped on day 5 rather than day 6 as cytopathic effect in the enhanced cultures was so advanced that it was feared the cells would not withstand intracellular staining.

### **2.7.2.2 Enhancement assay variations**

All viruses were titrated onto the relevant cell type in the presence of HIC' and NHS to determine their final output in the enhancement assay. For the standard enhancement assay on SupT1/R5 cells used in Chapter 4 onwards, an input that gave 0.1% infected cells in the presence of HIC' was used. In terms of ffu/ml, as determined by titration on NP2/CD4/R5 cells, this ranged from 100 to 500 ffu per well depending on the virus.

For the IIIB enhancement assays used in Chapter 3, on MT-2 and MT-4 cells, IIIB was used at an input of 100-300 ffu per well, that resulted in 1-2% infected cells in the presence of HIC' and NHS, which is equivalent to an output of 400-1000 pg/ml RT. RT output was determined by RT ELISA from culture supernatant obtained on day 5 for the MT2 cells and day 3 for the MT4 cells.

For the IIIB neutralisation assays used in Chapter 3 on H9, MT-2, MT-4 and SupT1/R5 cells, IIIB was used at an input that resulted in 2-10% infected cells in the presence of HIC'.

## **2.8 ANTIBODY PURIFICATION**

IgG and IgM were purified from patient plasma using a MabTrap protein G column (Amersham Biosciences, UK) and HiTrap IgM Purification (Amersham Biosciences, UK) kits, respectively, according to the manufacturer's instructions. 1 ml of plasma was used per purification, in parallel with control HIV seronegative plasma (NHP; see section 2.3.2). 500- $\mu$ l fractions were collected throughout the purification. All fractions collected were assayed for protein content using BioRad Protein Assay solution (BioRad, Germany). 10  $\mu$ l of each of the collected fractions were diluted 1:10 in PBS. Standards were prepared with known concentrations of human IgG. 10  $\mu$ l of each diluted fraction or standard was added to 70  $\mu$ l of PBS and 20  $\mu$ l of BioRad Protein Assay solution and mixed thoroughly. Absorbance was read in a microplate reader at 570 nm. Eluent fractions containing protein were pooled. If the pooled volume was greater than the original plasma input volume, samples were concentrated using Vivaspin protein concentrators (Vivascience, Germany).

## **2.9 ANTI-gp120 ANTIBODY ELISA**

96 well Maxisorb plates (Nalgene, NUNC International, Hereford) were coated with a sheep polyclonal anti-gp120 antibody (D7324; Aalto Bio Reagents, Dublin, Ireland) at 10 µg/ml PBS overnight at 4°C. Plates were washed 4 times with TBS-T and blocked with TBS containing 4% milk powder for 1 hour at room temperature. After one wash with TBS-T, plates were incubated with 50 µl per well of HIV IIIB gp120 (MRC AIDS Directive; product number EVA657) at a saturating concentration (0.2 ng/µl) in TBS containing 1% milk powder for 2 hours at room temperature. For each serum sample to be tested, a blank well excluding the gp120 was included. Plates were then washed 4 times with TBS-T then incubated with patient sera, serially ten-fold diluted in TBS-T containing 4% milk powder and 10% goat serum. An equal mix of QC sera 1, 2 and 4 (from individuals chronically infected with HIV (Aasa-Chapman et al., 2004)) was used as a positive control. Diluted patient sera and positive control sera were added to the plates in duplicate as well as to blank (no gp120) wells. After 1 hr of incubation at room temperature the plates were washed 4 times in TBS-T. 100 µl of alkaline phosphatase (AP)-conjugated goat anti-human Ig (Harlan SeraLab, Crawley Down, UK), diluted 1 in 2000 in TBS-T containing 4% milk powder and 10% goat serum, was added to each well and incubated for 1 hr at room temperature. Plates were washed 6 times in TBS-T before adding 100 µl per well of AP substrate (Lumi-Phos Plus, Aureon Biosystems, Vienna, Austria) and incubating in the dark at room temperature for 1 hr. Relative light units (RLU) were determined at 405 nm in a Lucy 1 luminometer (Anthos-Labtech, UK) and analysed using Stingray software (Dazdaq, East Sussex, UK).

## **2.10 NUCLEIC ACID PRODUCTION AND MANIPULATION**

The following procedures were used to clone CR2 (and produce a cytoplasmic tail mutant, KHR) from SupT1/R5 cells, for subsequent expression in NP2 cells.

### **2.10.1 Isolation of total cellular RNA**

5 x 10<sup>6</sup> SupT1/R5 cells were pelleted by centrifugation at 325 g for 5 min. Culture medium was removed by aspiration, and the cell pellet resuspended in 1 ml TRIzol (Invitrogen, UK) and incubated at room temperature for 10 min before adding 200 µl of chloroform. After thorough mixing and a further incubation at room

temperature for 3 min, cell debris was removed by centrifugation at 17,000 g for 10 min at 4°C, and the top, clear layer transferred to an RNase-free tube. 500 µl of isopropanol was added, mixed, incubated at room temperature for 10 min then centrifuged at 4°C for 10 min at 17,000 g to pellet RNA. The RNA pellet was then washed with 100 µl of 75% ethanol and left to air dry before resuspension in RNase-free water and treatment with DNase. Impurities were removed using the Qiagen PCR purification kit, according to the manufacturer's instructions.

### 2.10.2 cDNA synthesis

First-strand cDNA was synthesised from total cellular RNA using an Invitrogen SuperScript III kit (Invitrogen, UK), according to the manufacturer's instructions. Briefly, RNA was mixed with oligo(dT)<sub>20</sub> primers (specific for mRNA) and dNTPs at 65°C for 5 min, then cooled on ice for 1 min. cDNA synthesis mix, containing a version of M-MLV (Moloney murine leukaemia virus) RT, was added to the RNA/primer/dNTP mix and incubated at 50°C for 50 min for first-strand cDNA synthesis. The reaction was terminated by incubation at 85°C for 5 min, cooled and treated with RNase H to remove the RNA from the DNA:RNA hybrid. The resultant cDNA was stored at -20°C until use as a template for PCR.

### 2.10.3 PCR amplification of specific DNA

Primers to the coding sequence of CR2 were designed using the CR2 sequence available on Genbank, accession number NM\_001877 (*Homo sapiens* complement component (3d/Epstein Barr virus) receptor 2, transcript variant 2, mRNA) and publications detailing cytoplasmic tail mutants of CR2 (Barrault and Knight, 2004; Carel et al., 1990). The forward primer, containing the ATG start codon of CR2 (in bold type) was used for the amplification of both full-length and truncated (KHR) sequences, and had the sequence 5'-GCG CTG ATC AGC CAC **CAT** GGG CGC CGC GGG CC-3'. The reverse primer used to amplify the 3102-bp full-length CR2 contained the CR2 TGA stop codon (in bold type), and had the sequence 5'-GCG CTT CGA **ATC** AGC TGG CTG GGT TGT AT-3'. The cytoplasmic tail mutant KHR was constructed by inserting a stop codon after the first three amino acids, KHR, of the cytoplasmic tail sequence, resulting in a 21-aa truncation. The reverse primer used to produce the 3006-bp KHR CR2 sequence contained a TGA stop codon (in bold type),

and had the sequence 5'-GCG CTT CGA ATC ATC TGT GTT TTG ATA TCA CGT AT-3'.

PCR was performed using the AccuPrime *Taq* DNA Polymerase High Fidelity kit (Invitrogen, UK), which contains *taq* DNA polymerase and *GB-D* polymerase with proofreading activity. Reactions were set up containing 5 µl 10x AccuPrime PCR Buffer I (including dNTPs), 5 pmol each of the relevant forward and reverse primers, 1 µl of cDNA template (see section 2.10.2) and 0.2 µl AccuPrime *Taq* High Fidelity, and made up to 50 µl with autoclaved, distilled water. Reaction mixtures were incubated at 94°C for 3 minutes to activate the enzyme, followed by 30 cycles of 94°C for 30 seconds, 62°C for 30 seconds and 68°C for 3 min.

#### **2.10.4 Agarose gel electrophoresis of DNA**

Agarose gel electrophoresis was used to separate DNA fragments by size following restriction enzyme digestion (section 2.10.6), and to purify specific DNA products produced by PCR (2.10.3). Gels consisted of 0.8% agarose (w/v; Roche, UK) in 0.5x TAE buffer with 0.5 µg/ml ethidium bromide. A 1-kb GeneRuler DNA ladder (Fermentas Life Sciences, UK) was used as a molecular weight marker. DNA bands of specific size were excised from the agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen, UK) according to the manufacturer's instructions.

#### **2.10.5 Restriction enzyme digestion of DNA**

Restriction enzymes were obtained from Promega Life Sciences and used in the buffers provided according to manufacturer's instructions. Partial digests were performed by ten-fold serially diluting the restriction enzyme in appropriate buffer before adding to the restriction reaction mixture.

#### **2.10.6 Ligation of DNA fragments**

##### ***2.10.6.1 Ligation of purified PCR products into pCR2.1 TOPO***

Agarose gel-purified PCR products were ligated into the cloning vector pCR2.1 TOPO (Invitrogen, UK) according to the manufacturer's instructions. Ligation reactions were transformed directly into competent bacteria (section 2.10.7).

### **2.10.6.2 Sub-cloning of DNA fragments in pCSGW**

The restriction enzymes *Not* I and *Bam* HI were used to remove the CR2 and KHR coding fragments from pCR2.1-TOPO for ligation into CSGW. A partial restriction digest was performed with *Bam* HI due to an additional *Bam* HI recognition site within the CR2 coding sequence. The resultant 3-kb gel-purified fragments (see section 2.10.4) were ligated into *Bam* HI/*Not* I cut CSGW (with the eGFP-encoding gene removed) using Promega T4 DNA Ligase, according to the manufacturer's instructions, before transforming directly into competent bacteria (section 2.10.7).

### **2.10.6 Transformation of competent bacteria**

1  $\mu$ l of ligation mix was added to 80  $\mu$ l Dh5 $\alpha$  or Stbl 2 competent bacteria (Invitrogen, UK) and incubated on ice for 30 min, followed by heat-shock at 42°C for 45 seconds and a further 2 min incubation on ice. 250  $\mu$ l of SOC medium (supplied with the bacteria) was added and the bacteria were incubated at 30°C for 1 hour with shaking at 200 rpm. Cultures were then plated on L-agar plates containing 50  $\mu$ g/ml ampicillin and incubated for 24 hrs at 30°C.

### **2.10.7 Plasmid purification**

A single bacterial colony from an agar plate was inoculated into 3 ml of LB-broth containing 50  $\mu$ g/ml ampicillin and grown for 24 hrs at 30°C with shaking at 200 rpm. Bacteria were pelleted by centrifugation at 17,000 g for 5 min and used to prepare plasmid DNA using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions.

### **2.10.8 Screening plasmids for correct inserts**

Initial screening of pCR2.1-TOPO plasmids for correct CR2 and KHR inserts was carried out by restriction digestion. Plasmids showing the correct predicted digestion pattern were then sequenced (section 2.10.10).

### **2.10.9 DNA sequencing**

CR2 and KHR constructs in the cloning vector pCR2.1-TOPO were sequenced using the vector sequencing primers M13 reverse and M13(-20) forward, and internal sequencing primers designed using the NM\_001877 CR2 coding sequence. All sequencing was carried out by the DNA Sequencing Service at the Wolfson Institute

for Biomedical Research, UCL. Sequences were aligned with the NM\_001877 CR2 coding sequence, plasmids containing the correct inserts were selected, and full-length CR2 and KHR fragments were sub-cloned into CSGW.

## **2.11 STABLE EXPRESSION OF CR2 AND KHR IN NP2 CELLS**

### **2.11.1 Lentiviral vector production**

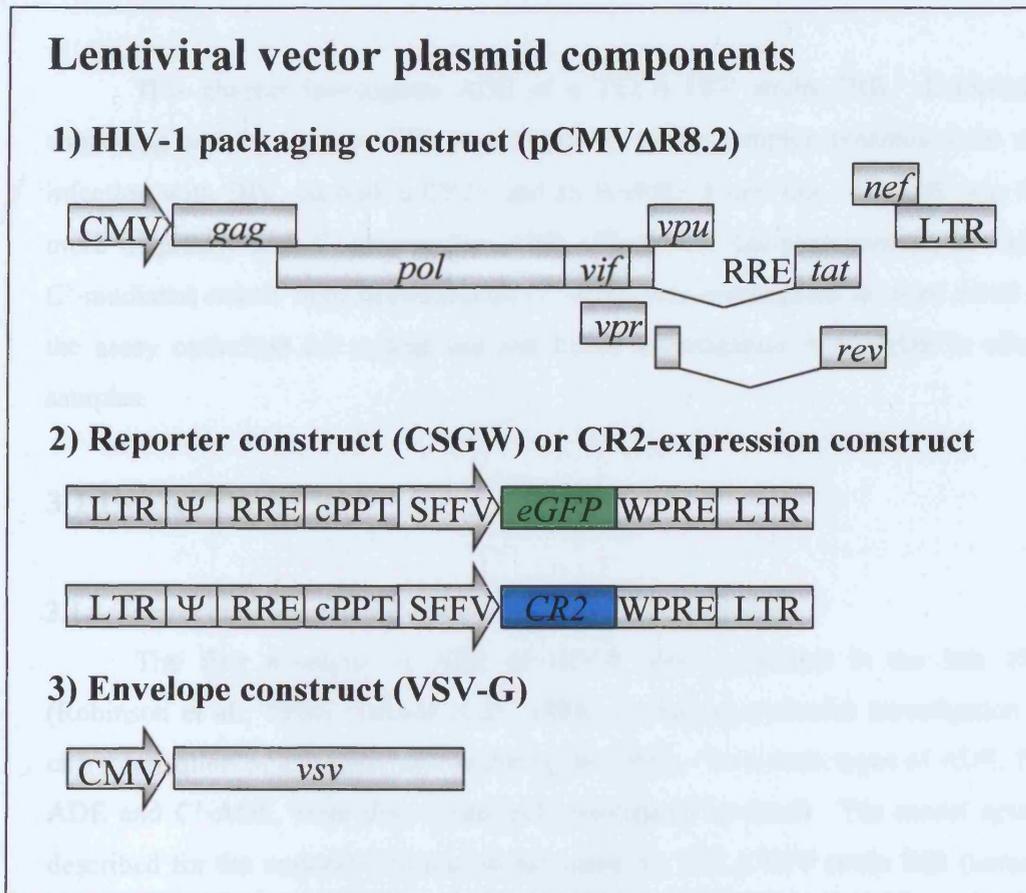
CSGW-CR2 and -KHR constructs were initially screened for expression of CR2 and KHR by single-plasmid transfection into 293T cells and staining for cell surface expression of CR2, as detailed in section 2.2.5 and 2.2.7. Lentiviral vectors for the stable expression of CR2, KHR, and GFP were produced in 293T cells by co-transfection of three plasmids (shown in Figure 2.1). The transfection procedure is described in section 2.5.5.

The packaging construct used was pCMV $\Delta$ R8.2 (Naldini et al., 1996), containing the genes *gag*, *pol*, *tat*, *rev*, *vif*, *vpr*, *vpu* and *nef* (see Figure 2.1). A CMV promoter drives the expression of the viral genes. The reporter construct, CSGW, encodes an eGFP reporter gene driven by a SFFV promoter, located in between two LTRs (Demaison et al., 2002). This allows for the integration of the construct into the cellular genome, resulting in stable and permanent expression of eGFP. CSGW-CR2 and CSGW-KHR are CSGW plasmids with CR2 or KHR in the place of eGFP, respectively. The envelope construct encodes VSV-G virus envelope, under control of a CMV promoter. All three constructs were within a pUC19 plasmid backbone. The three plasmids were transfected into 293T cells at an envelope: packaging: reporter (CSGW, CSGW-CR2 or CSGW-KHR) ratio of 3:2:1. The transfection procedure is described in section 2.2.5.

### **2.11.2 Stable expression of CR2 and KHR on NP2 cells**

GFP, CR2 and KHR lentivirus vectors produced according to section 2.11.1 were used to transduce NP2/CD4/X4 and NP2/CD4/R5. GFP lentiviral vectors were used in parallel with the CR2 and KHR lentiviral vectors in order to monitor protein expression by GFP intensity.  $1 \times 10^4$  NP2/CD4/R5 and NP2/CD4/X4 cells were seeded into each well of a 48-well plate. On day 1 following seeding, culture medium was removed, and 200  $\mu$ l of lentiviral vector incubated per well for 1 hour before removing and overlaying cells with 500  $\mu$ l fresh culture medium. This was repeated

on days 3 and 5. Cells were then grown up and analysed for cell surface receptor expression by flow cytometry (2.2.7).



**Figure 2.1 Schematic diagram of the constructs used to produce lentiviral vectors**

The constructs were contained within a pUC19 backbone, and used to produce lentiviral vectors via 3-plasmid transfections, as detailed in section 2.2.5.

## **CHAPTER 3**

### **Studies of antibody-dependent enhancement of HIV IIIB infection and enhancement assay development**

This chapter investigates ADE of a TCLA HIV strain, IIIB. Evidence of enhancing activity against IIIB was found in serum samples obtained soon after infection with HIV, on both a CR2+ and an FcγRII+ T cell line. C'-ADE was seen more frequently than C'-independent ADE. Therefore, this phenomenon, as well as C'-mediated rescue from neutralisation (C'-RN), was investigated in more detail and the assay optimised for routine use and future investigation of C'-ADE in clinical samples.

#### **3.1 INTRODUCTION**

##### **3.1.1 Target cells used in the study of ADE of HIV infection**

The first accounts of ADE of HIV-1 were published in the late 1980s (Robinson et al., 1988a; Takeda et al., 1988), prompting extensive investigation and characterisation of the phenomenon during the 1990s. Two main types of ADE, FcR-ADE and C'-ADE, were discovered and investigated in detail. The model systems described for the majority of these studies used the TCLA HIV strain IIIB (hereafter referred to as IIIB), with the cell lines U937 for FcR-ADE studies (Kozlowski et al., 1995; Laurence et al., 1990; Subbramanian et al., 2002; Takeda et al., 1992; Takeda et al., 1990; Takeda et al., 1988) and MT-2 for the C'-ADE studies (Lund et al., 1995; McDougall et al., 1997; Montefiori et al., 1991; Robinson et al., 1991; Robinson et al., 1990a; Robinson et al., 1990b; Robinson et al., 1989a; Robinson et al., 1988a; Robinson et al., 1990c; Robinson et al., 1989b; Subbramanian et al., 2002). A CR2+ cell line with similar characteristics to MT-2 cells, MT-4, was used in the case of one group (Banhegyi et al., 2003; Prohaszka et al., 1997; Szabo et al., 1999). FcR-ADE of primary isolates was also detected in PBMCs and macrophages (Homsy et al., 1990; Homsy et al., 1989; Jouault et al., 1991) and C'-ADE in PBMCs (Gras et al., 1988) and DCs (Bajtay et al., 2004). In this chapter, preliminary C'-ADE studies were carried out in a system similar to the MT-2 model system, to enable comparisons between my results and those previously published. A related cell line, MT-4,

expressing FcγRII (and therefore different in phenotype to those used by Banhegyi, Prohaszka, Szabo and colleagues mentioned above) was used in parallel for FcR-ADE studies.

### **3.1.2 Features of C'-ADE of HIV**

The receptor most frequently reported to mediate C'-ADE was CR2 (Prohaszka et al., 1997; Robinson et al., 1990c), although CR1 (Delibrias et al., 1993; Delibrias et al., 1994; Gras et al., 1993), CR3 (Reisinger et al., 1990) and later C1qR (Prohaszka et al., 1997) were also shown to mediate enhancement through this mechanism. Through studies with monoclonal antibodies and peptide mapping, "enhancement domains" were identified, the most clearly defined being within the primary immunodominant region on gp41, aa579-613 (Mitchell et al., 1998; Robinson et al., 1991; Robinson et al., 1990a; Robinson et al., 1990b). Part of this region is highly conserved and incorporates the disulphide loop responsible for interaction with gp120 (Gnann et al., 1987; Helseth et al., 1991; Maerz et al., 2001; York and Nunberg, 2004). Other regions on gp41 and gp120 have also been shown to elicit enhancing antibodies, including the V3 loop (Jiang and Neurath, 1992; Jiang et al., 1991). The prevalence of C'-ADE antibodies in serum samples from infected individuals differs between reports, probably due to variations in assay methods. In some cases C'-ADE is dose-dependent and most efficient at highest concentrations of sera (Jiang and Neurath, 1992; Jiang et al., 1991) while in others C'-ADE is only seen with extensive dilutions of neutralising sera (Montefiori et al., 1991; Subbramanian et al., 2002). Variation also applies to correlation of C'-ADE with disease: several studies have shown the positive correlation of C'-ADE and viral load (Szabo et al., 1999), or disease stage (Subbramanian et al., 2002), while others have found no correlation when comparing C'-ADE antibody titres with disease stage or pace of progression (Montefiori et al., 1991; Montefiori et al., 1996).

### **3.1.3 Features of FcR-ADE of HIV**

Previous reports of FcR-ADE have shown a role for the receptors FcγRI (Homsy et al., 1989; Jouault et al., 1991; Laurence et al., 1990; Matsuda et al., 1989; Perno et al., 1990; Takeda et al., 1990), FcγRII (Laurence et al., 1990), and FcγRIII (Homsy et al., 1989), and also the IgA receptor FcαR (Kozlowski et al., 1995). Enhancing epitopes for FcR-ADE have not been well defined, although some

monoclonal antibody studies implicated regions on gp120 (Takeda et al., 1992). FcR-ADE is generally associated with higher dilutions of sera (Matsuda et al., 1989), and in this respect resembles the classical dengue virus enhancement. Associations have been made between appearance of enhancing antibodies and progression to disease (Homsy et al., 1990).

#### **3.1.4 Studies of enhancing antibodies in seroconvertors**

While considerable investigation into the occurrence of enhancing antibodies and disease progression had been conducted on samples from late asymptomatic and AIDS stages of disease (Homsy et al., 1990; Montefiori et al., 1991; Montefiori et al., 1996; Subbramanian et al., 2002; Szabo et al., 1999), few researchers have conducted studies of serum obtained during and soon after seroconversion, probably due to difficulty of obtaining such samples. In one study that investigated this, C'-ADE activity to IIIB was detected in seroconversion samples from four individuals (Szabo et al., 1999). In this chapter the concept is explored further, with early serum samples from nine individuals (and later samples in some cases), tested for both C'-ADE and FcR-ADE activity.

#### **3.1.5 The effect of complement on neutralisation of HIV in CR2+ cell lines**

In many studies of C'-ADE on CR2+ cells it was noted that sera that were otherwise neutralising became less neutralising, or even non-neutralising, in the presence of complement (Lund et al., 1995; McDougall et al., 1997; Robinson et al., 1988a; Robinson et al., 1990c; Subbramanian et al., 2002; Szabo et al., 1999). While this is not enhancement *per se*, it has been assumed that the mechanisms of these phenomena are related. It also has important implications for vaccine and neutralisation studies. On the other hand, it has been shown that complement can increase the inactivation of HIV seen with antibody alone when assayed on CR-negative cells (Aasa-Chapman et al., 2004; Aasa-Chapman et al., 2005). Therefore, the effect of complement on neutralisation of HIV is investigated further in this chapter on CR2+ and CR2- T cell lines.

## 3.2 RESULTS

### 3.2.1 Cell lines for studies of ADE

Historically, studies of C'ADE have been carried out on MT-2 cells, therefore these were chosen as the target cell for initial studies of C'-ADE. C'-ADE in MT-2 cells was attributed to their high-level expression of CR2, since T cells such as H9 and CEM that lacked CR2 did not support enhanced infection (Robinson et al., 1989a). Several reports have indicated that high level CR2 expression on CD4+ T cell lines is not unique to MT-2 cells (Delibrias et al., 1992; Fingerroth et al., 1988; Prodinge et al., 1996; Sinha et al., 1993). Therefore six other T cell lines commonly used for *in vitro* studies of HIV (H9, MT-2, Molt-4, SupT1, SupT1/R5, C8166 and MT-4 cells) were tested for CR2 expression as potential future target cells for ADE experiments. As CR1 (Gras and Dormont, 1991) and CR3 (Reisinger et al., 1990) have also been implicated in C'-ADE, cells were also tested for these receptors. Results are shown in Figure 3.1. High-level expression of CR2 was found on four of the T cell lines: MT-2, Molt-4, SupT1, and SupT1/R5. C8166 cells expressed a lower but detectable level. H9 and MT-4 cells were the only cell lines on which CR2 could not be detected. H9, SupT1 and C8166 expressed a low level of CR1, but none of the cells expressed CR3.

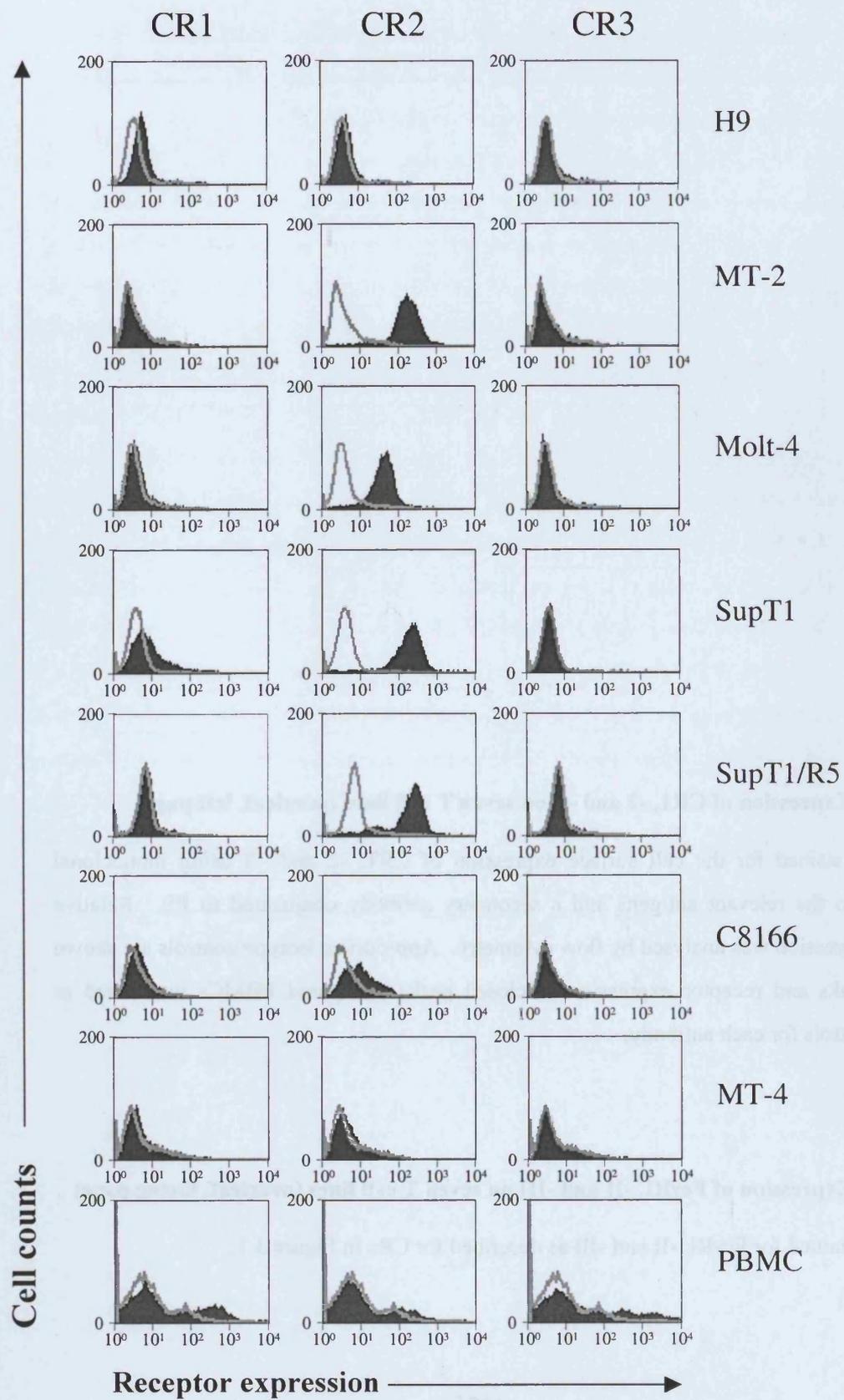
The T cell lines were also examined for the expression of FcγRI, -II and -III, in order to find a suitable T cell line for the study of FcR-ADE. Results are shown in Figure 3.2. MT-4 cells were found to express high levels of FcγRII and therefore deemed appropriate for studies of FcR-ADE. With the exception of C8166 and MT-2 cells, which expressed low levels of FcγRII, none of the other cell lines expressed detectable amounts of the FcRs tested.

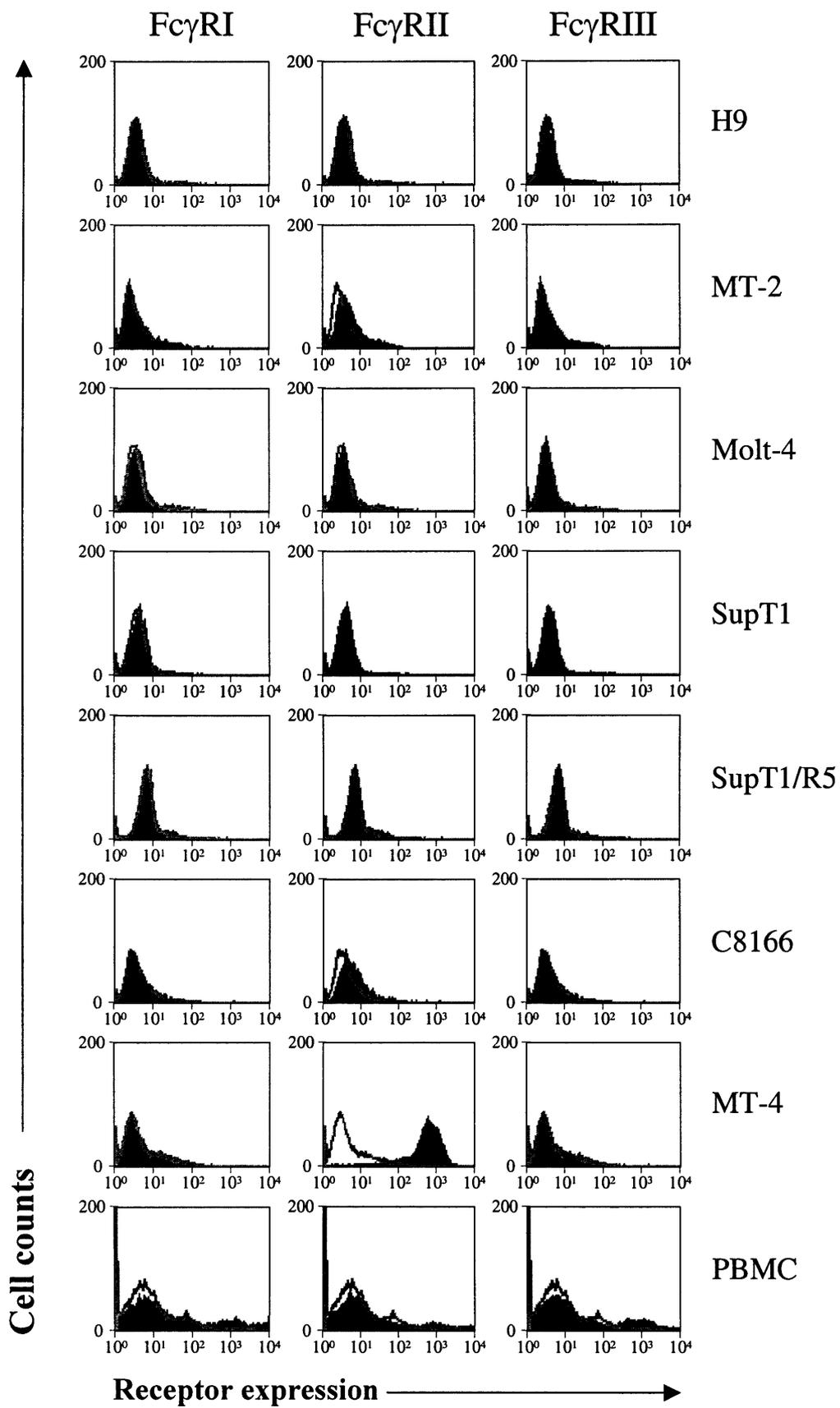
**Figure 3.1 Expression of CR1, -2 and -3 on seven T cell lines (overleaf, left page)**

Cells were stained for the cell surface expression of CR1, -2 and -3 using monoclonal antibodies to the relevant antigens and a secondary antibody conjugated to PE. Relative receptor expression was analysed by flow cytometry. Appropriate isotype controls are shown as open peaks and receptor expression as closed peaks. Ungated PBMCs were used as positive controls for each antibody.

**Figure 3.2 Expression of FcγRI, -II and -III on seven T cell lines (overleaf, facing page)**

Cells were stained for FcγRI, -II and -III as described for CRs in Figure 3.1.





Finally, cells were tested for the expression of the HIV receptors CD4, CXCR4 and CCR5. As expected, all of the cell lines expressed a high level of CD4 and CXCR4, with the exception of MT-4 cells, which expressed a lower level of CXCR4. Apart from the Molt-4 cells, which expressed detectable levels of CCR5, only the SupT1/R5 cells expressed a significant amount of CCR5 as these had been artificially transduced to stably express it. Results for expression of all receptors on all seven cell lines are summarised in Table 3.1.

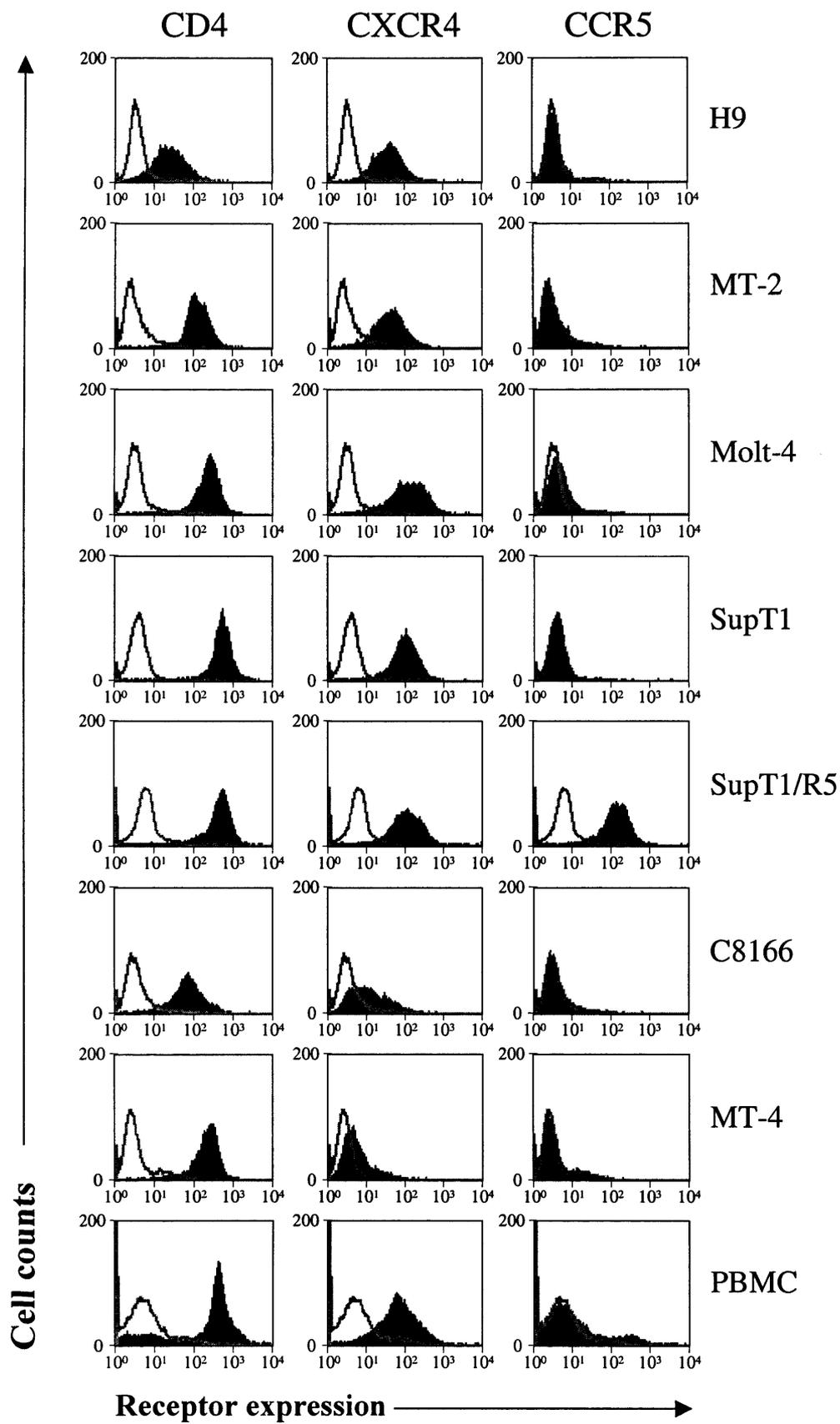
CELL TYPE	RECEPTOR								
	CD4	CXCR4	CCR5	CR1	CR2	CR3	FcγRI	FcγRII	FcγRIII
H9	++	++	-	+	-	-	-	-	-
MT2	+++	++	-	-	+++	-	-	(+)	-
Molt 4	+++	+++	(+)	-	++	-	-	-	-
SupT1	+++	+++	-	+	+++	-	-	-	-
SupT1/R5	+++	+++	+++	-	+++	-	-	-	-
C8166	++	+	-	(+)	+	-	-	(+)	-
MT4	+++	+	-	-	-	-	-	+++	-

**Table 3.1 Summary of CR, FcR and HIV receptor expression on seven T cell lines**

Cells were graded for expression of each receptor according to the results shown in Figures 3.1, 3.2 and 3.3. +++ represents the highest level of expression, followed by ++ and +, with (+) indicating expression levels just above the threshold of detection and – indicating no detectable expression of the relevant receptor.

**Figure 3.3 Expression of CD4, CXCR4 and CCR5 on seven T cell lines**

Cells were stained for the cell surface expression of CD4, CXCR4 and CCR5 using monoclonal antibodies directly conjugated to PE. Relative receptor expression was analysed by flow cytometry. Appropriate isotype controls are shown as open peaks and receptor expression as closed peaks. Ungated PBMCs were used as positive controls for each antibody.

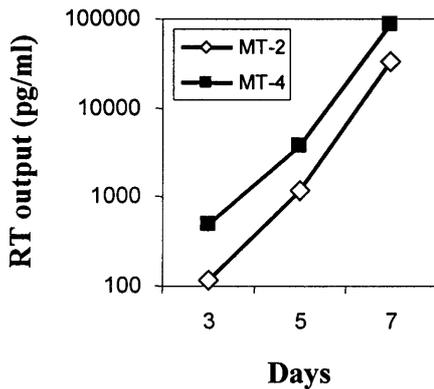


### **3.2.2 Studies of C'-ADE and FcR-ADE in sera from individuals with acute HIV infection**

MT-2 cells were used for preliminary serum screening assays to allow for direct comparisons between my results and those previously published. MT-4 cells were found to express a high level of FcγRII, and as they were the only T cells tested that expressed a high level of any of the FcRs, they were used for FcR-ADE screening experiments. This provided the opportunity to compare enhancement in two T cell lines, rather than in cells of unrelated lineage.

Serum samples obtained from individuals recently infected with HIV were used as only limited studies of ADE in early infection samples have been reported (Szabo et al., 1999). The samples from this patient cohort are studied in more detail in Chapter 4 and described in Chapter 2, Materials and Methods. Samples from as early as 6 days following the onset of PHI were tested for C'-ADE and FcR-ADE activity against IIIB. Pooled seronegative normal human serum (NHS) was used as a control.

As discussed in section 1.11.3 of the Introduction, there are several ways to present results from enhancement assays. Here I have chosen to present fold enhancement values, a measure of the scale of enhancement or neutralisation relative to infection in the absence of anti-HIV antibodies. Multiple serum samples were screened at one concentration, 10%, to enable direct comparisons and to avoid excessive dilutions in order to show the dominant antibody activity in the sera. Results were normalised to a control culture containing virus, NHS and complement (C'), or virus, NHS and heat inactivated complement (HIC') as appropriate. HIC' and C' were also used at a concentration of 10% in the assay. A value of 1 represents no effect; a value below 0.5 or 0.1 indicates >50% or >90% neutralisation, respectively; and a value of 2 represents a 2-fold increase in infection. The dynamics of IIIB infection on MT-2 and MT-4 cells differ (Harada et al., 1985), and see Figure 3.4. Therefore an MOI of 0.001 (100 ffu on 10<sup>5</sup> cells) was used for the MT-4 cells and 0.003 for the MT-2 cells. At the assayed time points (day 3 for the MT-4 cells and day 5 for the MT-2 cells) RT outputs in the control cultures (i.e. in the presence of NHS and HIC') were 250-1000 pg/ml.



**Figure 3.4 Dynamics of IIIB infection of MT-2 and MT-4 cells**

MT-2 cells were infected with IIIB preincubated with NHS and HIC' for 1 hr at 37°C. An MOI of 0.003 was used for MT-2 cells, and 0.001 for MT-4 cells. Cultures were monitored for RT output by RT ELISA. RT output is shown as a function of time.

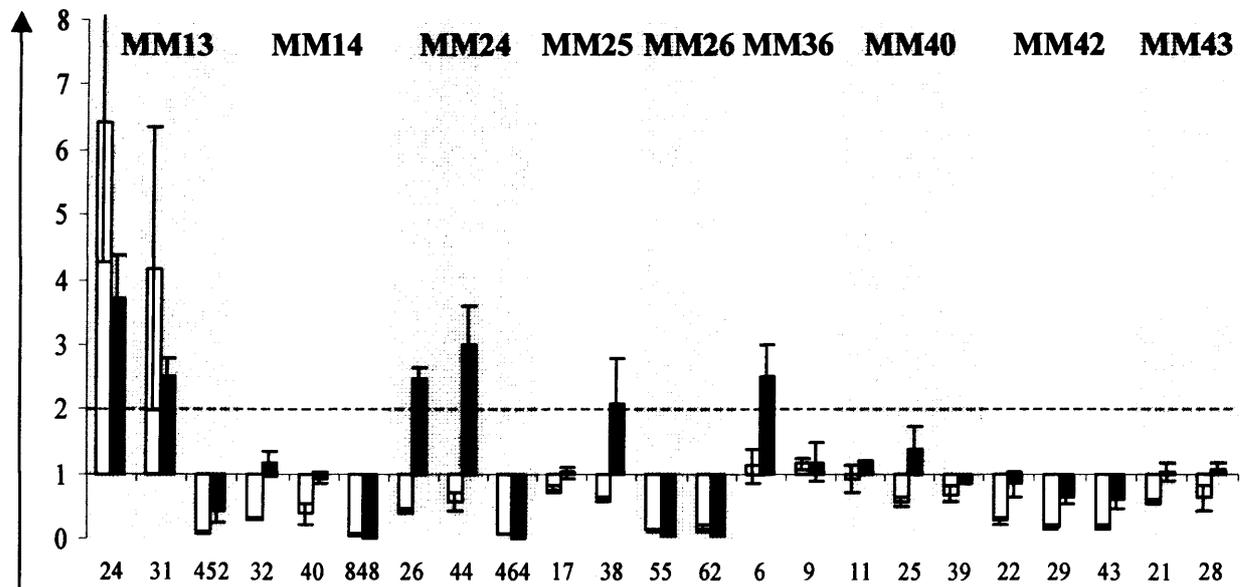
2-3 serum samples from 9 patients were tested for enhancing activity. Results are shown in Figure 3.5. On the MT-2 cells sera from 4 out of the 9 patients enhanced infection: MM13 days 24 and 31; MM24 days 26 and 44; MM25 day 38; and MM36 day 6. All of the enhancing serum samples were from early time points following infection; when a later sample, day 464, was assayed from MM24 it neutralised infection by >90%. Similarly, day 452 and 848 from MM13 and MM14 respectively, also neutralised infection. In the cases of MM24 and MM25, the serum samples that enhanced infection in the presence (C') of complement reduced viral infection in the absence (HIC') of active complement. For several serum samples neutralisation exceeded 50% but not 90% in the absence of C', yet when assayed with C' neutralisation is reduced or completely abrogated: MM14 days 32 and 40; MM42 days 22, 29 and 43. The day 24 and 31 serum samples from MM13 enhanced infection in both the presence and absence of C'.

On the MT-4 cells, serum samples from 2 out of the 9 patients enhanced infection: MM25 day 17 and MM40 day 11. Enhancement occurred only in the absence of C'. Again, the samples that enhanced infection were from early time points, although none of the samples that enhanced infection on the MT-2 cells enhanced infection on the MT-4 cells. The same samples that produced >90% neutralisation on the MT-2 cells, MM14 day 848 and MM24 day 464, did so on the MT-4 cells, with the exception of MM26 days 55 and 62. In several cases C' increased the inactivation of the virus: MM26 day 55; MM40 day 39; and MM42 day 29.

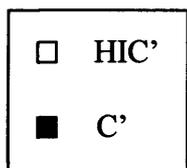
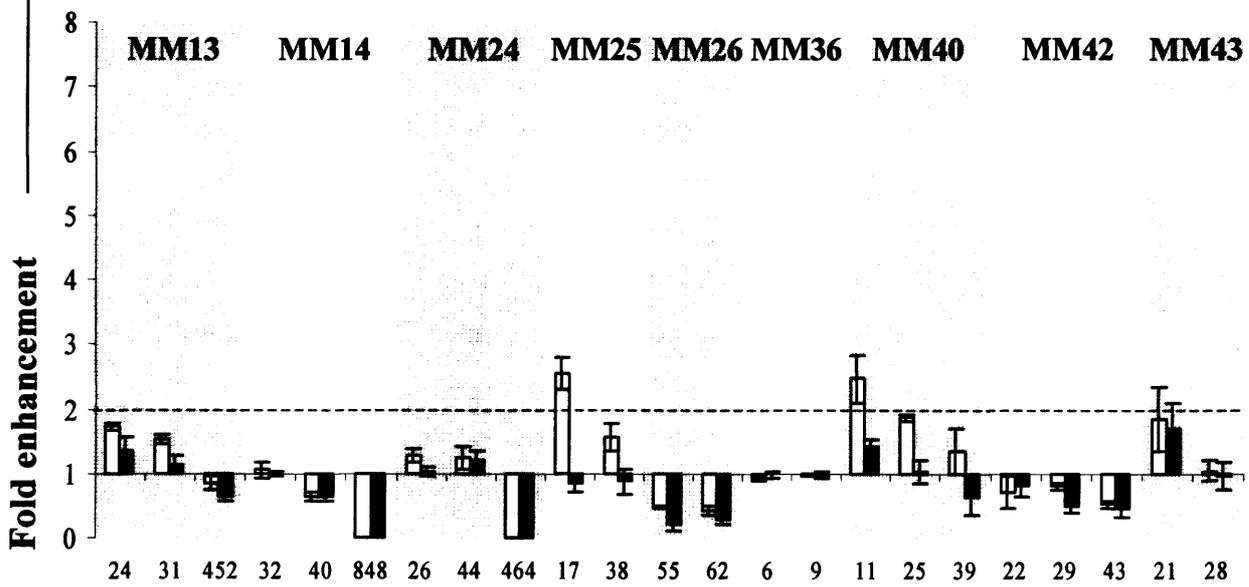
**Figure 3.5 Enhanced infection of IIB by sera isolated from patients at early time points after infection**

HIV IIB was incubated with patient serum or seronegative control serum (NHS) and complement (C') or heat inactivated complement (HIC') for 1 hour at 37°C, then added to MT-2 cells (top panel) or MT-4 cells (bottom panel). Levels of infection were determined by RT ELISA on day 5 after inoculation for MT-2 and day 3 for MT-4. Fold enhancement is the ratio of RT output in the presence of patient serum to RT output in the presence of NHS. Patient numbers (prefix MM) are shown above each serum set. The day of serum collection following onset of acute symptoms is shown below. Values above the dashed turquoise line indicate enhanced infection. Values below 1 indicate reduced infection, and below 0.1 neutralisation. Error bars represent SD from 3 experiments.

### MT-2 cells (CR2+)

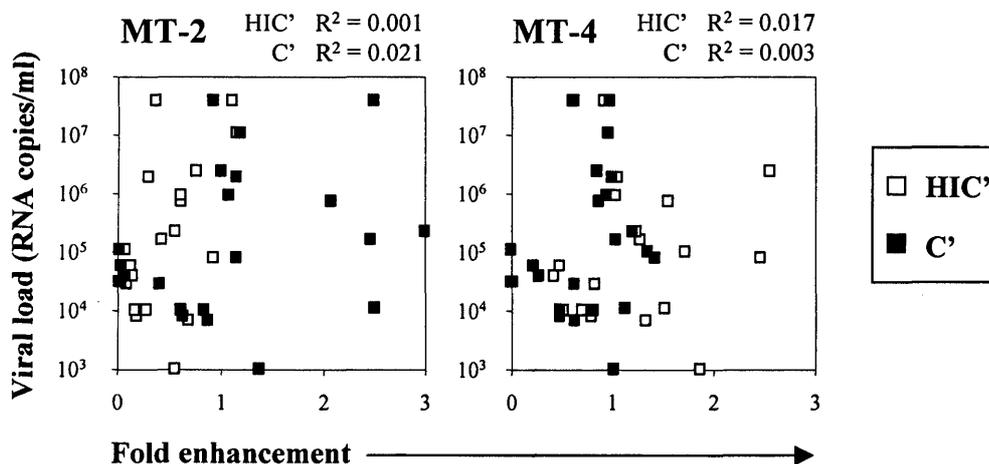


### MT-4 cells (FcγRII+)



Days following onset of symptoms

Only early serum samples (before day 100) enhanced IIIB infection of MT-2 and MT-4 cells, while later samples often neutralised infection. However, when fold enhancement values obtained for each serum sample were plotted against the patient viral loads on the day of serum collection, no correlation was seen, as shown in Figure 3.6.



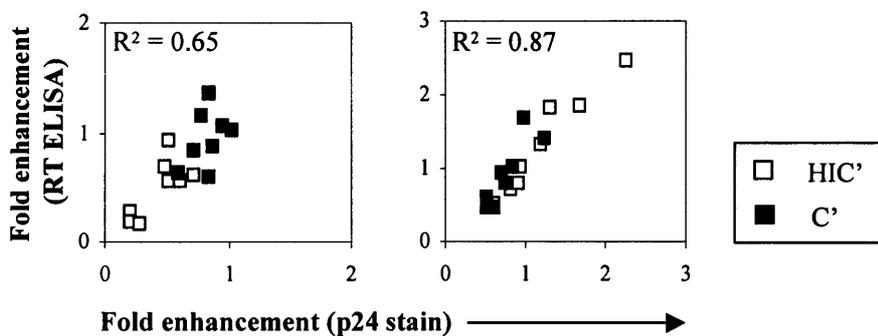
**Figure 3.6 Lack of correlation between viral load and fold enhancement on MT-2 and MT-4 cells**

Fold enhancement values obtained from the assays shown in Figure 3.4 were plotted against the patient viral load on the day of serum collection.

In summary, serum samples obtained at early time points following infection with HIV were able to enhance infection of IIIB on two T cell lines; on the MT-2 cells the enhancement was C'-dependent and on the MT-4 cells it was C'-independent. Enhancement was seen more frequently and to a greater magnitude on the MT-2 cells. In the presence of C', virus inactivation or neutralisation was often reduced on the MT-2 cells and increased on the MT-4 cells.

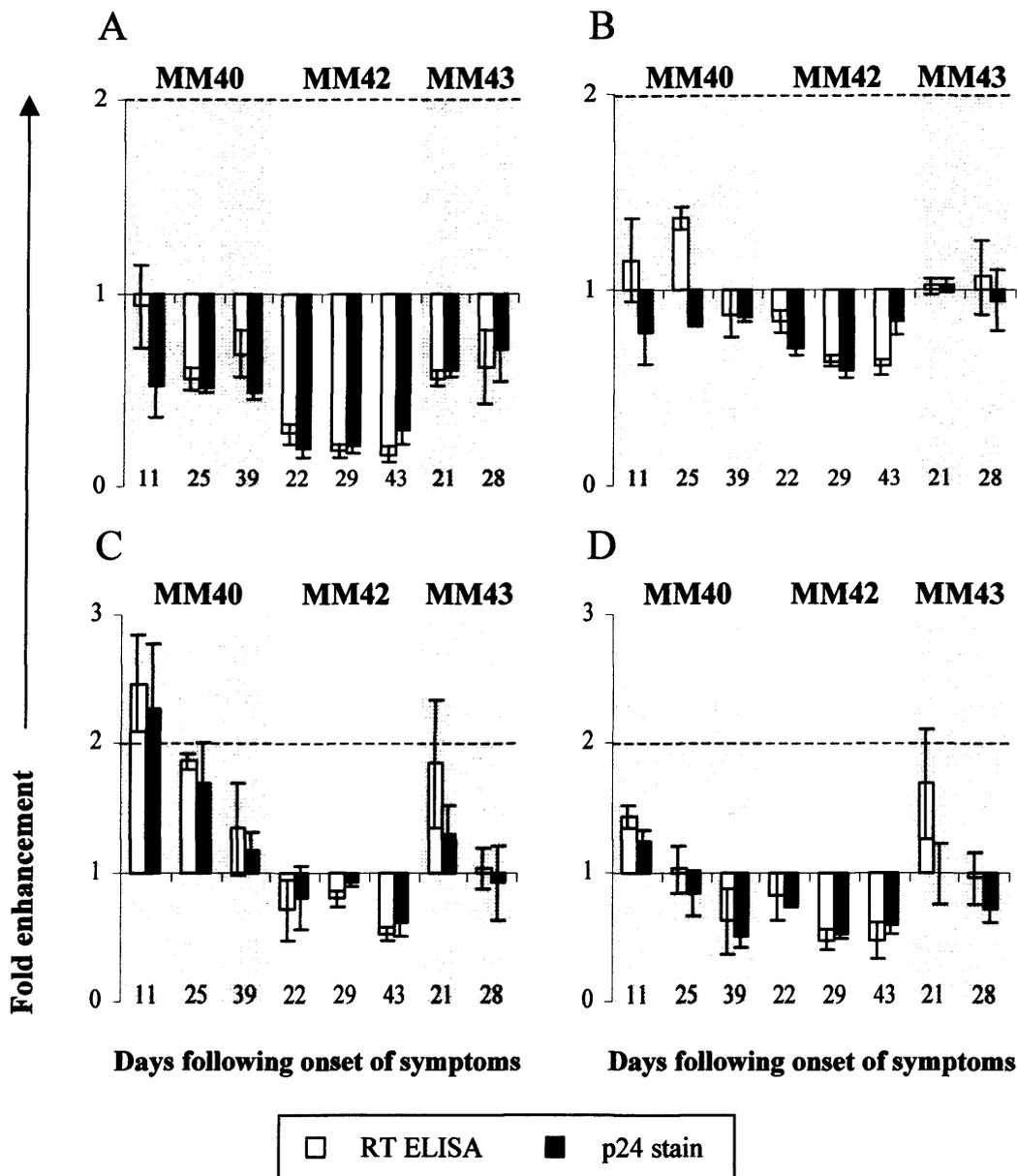
### 3.2.3 Evaluation of the use of percent infected cells and RT ELISA output as experimental parameters

The screening assays presented in Section 3.2.2 showed that enhancing activity could be detected in early serum samples from HIV positive individuals. However, for reasons of expense, measuring levels of infection by RT ELISA was not suitable for use in a routine enhancement assay. Therefore the use of intracellular immunofluorescent p24 staining, to give infection levels as the percentage of infected cells, was investigated as an alternative. Cell supernatant was collected and stored concurrently with each assay so that RT output levels could be determined as confirmation of results. Comparisons were made between RT output and percentage infected cells to ensure that both parameters gave a similar representation of enhancement and neutralisation, shown in Figure 3.7. Levels of infection correlated for both methods, as shown in Figure 3.8. On the MT-2 cells the  $R^2$  value was 0.65; on the MT-4 cells the  $R^2$  value was 0.87. Where neutralisation and enhancement occurred it was shown in both measurements. It was therefore deemed acceptable to measure the number of infected cells as the endpoint for future assays.



**Figure 3.8 Correlation between infection levels in enhancement assays measured by RT output and intracellular p24 staining**

Scatter graphs comparing fold enhancement levels measured by RT ELISA with fold enhancement levels measured by intracellular p24 staining, as shown in Figure 3.6. Enhancement assays were performed on MT-2 cells (left) and MT-4 cells (right).



**Figure 3.7 Comparison of infection levels measured by RT output and % infected cells**

IIIB was incubated with patient serum or NHS and complement (C') or heat inactivated complement (HIC') for 1 hour at 37°C, then added to MT-2 or MT-4 cells. Levels of infection were determined by RT ELISA and intracellular p24 staining on day 5 after inoculation for MT-2 and day 3 for MT-4: (A) HIC' on MT2 cells, (B) C' on MT2 cells, (C) HIC' on MT4 cells, and (D) C' on MT4 cells. Patient numbers (prefix MM) are shown above each serum set. RT output is measured by RT ELISA of cell supernatant and % infected cells is measured by intracellular p24 staining of the cells and then flow cytometric analysis. Fold enhancement is calculated relative to infection levels in the presence of seronegative control serum. Values above the dashed turquoise line indicate enhanced infection.

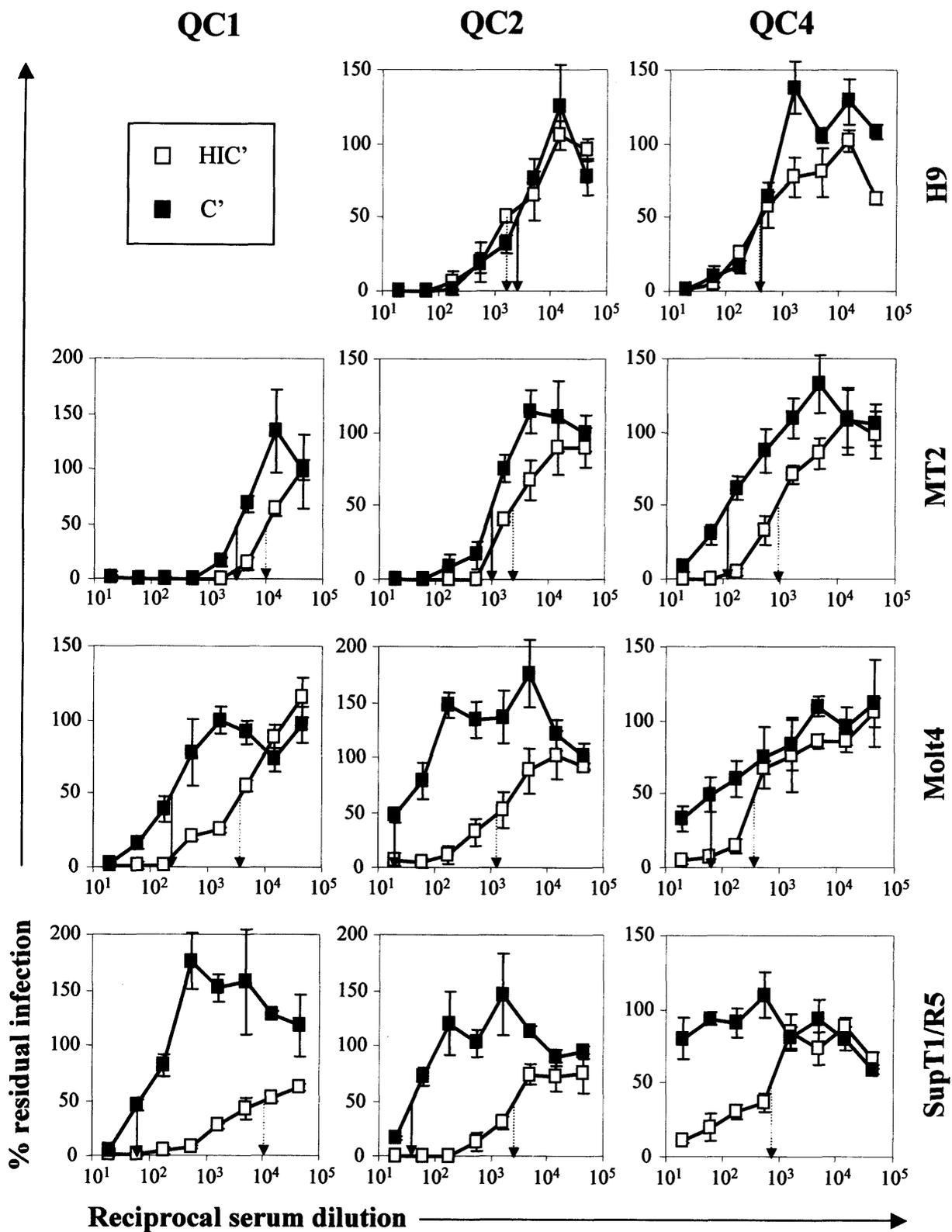
### 3.2.4 Neutralisation of IIIB on four T cell lines expressing different levels of CR2

The phenomenon of C'-mediated "rescue" from neutralisation (C'-RN) was observed in the MT2 C'-ADE studies (Figure 3.5: MM14 days 32 and 40; MM42 day 22) and has been observed by several other investigators (Lund et al., 1995; McDougall et al., 1997; Robinson et al., 1988a; Robinson et al., 1990c; Subbramanian et al., 2002). Also, C'-ADE has often been associated with high dilutions of neutralising antibody (Montefiori et al., 1991; Subbramanian et al., 2002). Therefore, in order to investigate both of these phenomena, four cell lines expressing different levels of CR2: H9 (undetectable CR2), MT2 (high CR2 but some FcγRII), Molt-4 (relatively high CR2) and SupT1/R5 (high CR2) were tested with IIIB and serial dilutions of three sera from chronically infected individuals with previously characterised neutralising activity (Aasa-Chapman et al., 2004). QC sera were used due to the limited availability of sera from recently infected individuals used in section 3.2.2. On NP2/CD4/X4 cells QC1 was the most potently neutralising serum, followed by QC2 and QC4. Results from the T cell assays are shown in Figure 3.9. While enhancement was not detected, four different patterns of neutralisation occurred in terms of differences in titre in the presence (C') and absence (HIC') of C'. These differences are summarised in Tables 3.2, 3.3 and 3.4.

In the absence of C', the sera had the same characteristics as those seen on NP2 cells, with neutralising activity being in the order QC1>QC2>QC4. The IC50 and IC90 values for each serum in the absence of C' were similar on all four cell lines, as emphasised in Figure 3.10 and Table 3.4. In the presence of C', however, the amount of serum required to neutralise IIIB was increased on the three CR2+ cell lines. This increase was greatest on the SupT1/R5 cells, which expressed the highest level of CR2, and smallest on the H9 cells, which express no detectable CR2.

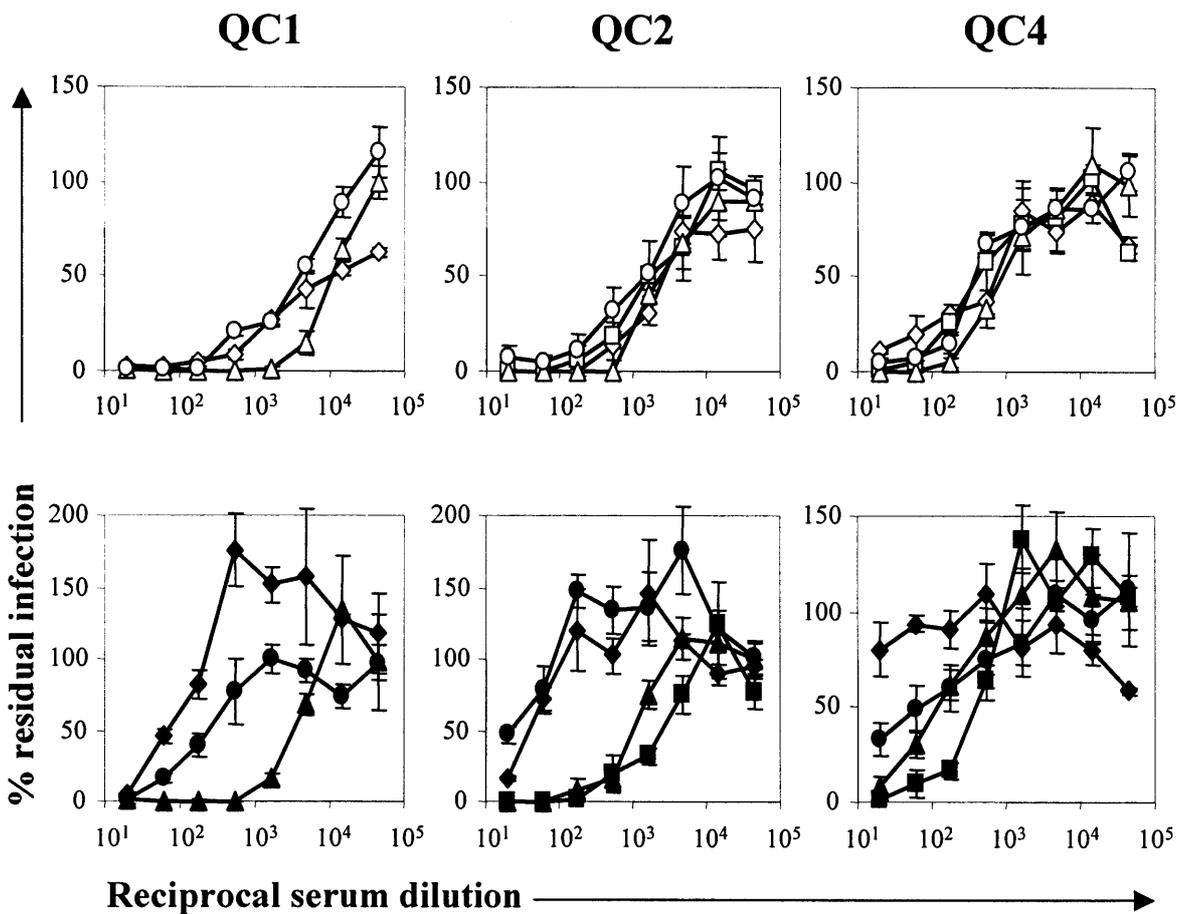
**Figure 3.9 Neutralisation of IIB on four different T cell lines**

QC sera 1, 2 and 4 were 3-fold serially diluted in control serum and incubated with HIC' or C' and IIB for 1 hour at 37°C, then added to H9 cells (top row), MT2 cells (second row), Molt 4 cells (third row), and SupT1/R5 cells (bottom row). Levels of infection were measured on day 5 following infection by intracellular p24 staining and flow cytometry analysis. % residual infection is relative to infection in the presence of control serum and HIC' or C' as appropriate. Red arrows show the serum IC50 in the presence of C' and dotted red arrows in the presence of HIC'. Error bars are SD from 3 experiments.



**Figure 3.10 Differences in IIB neutralisation curves in the presence of C' on cells that express CR2**

The results from Figure 3.9 are directly compared by superimposing the IIB neutralisation curves from all 4 cell types onto each other. H9 (■), MT2 (▲), Molt4 (●), and SupT1/R5 (◆). The top panel shows QC titration assays carried out in the absence of active C' (HIC'; open shapes); the bottom panel shows the difference that C' makes to neutralisation (C'; closed shapes).



IC50		SERUM					
		QC1		QC2		QC4	
		HIC'	C'	HIC'	C'	HIC'	C'
CELL LINE	H9	ND	ND	1620	1620	180	180
	MT-2	4860	1620	1620	540	540	60
	Molt-4	1620	180	540	20	180	60
	SupT1/R5	4860	60	1620	20	540	<20

Table 3.2 IIB 50% neutralisation titres (IC50) for three sera on four T cell lines

IC90		SERUM					
		QC1		QC2		QC4	
		HIC'	C'	HIC'	C'	HIC'	C'
CELL LINE	H9	ND	ND	180	180	60	60
	MT-2	1620	540	540	180	180	20
	Molt-4	180	20	60	<20	60	<20
	SupT1/R5	540	20	180	<20	20	<20

Table 3.3 IIB 90% neutralisation titres (IC90) for three sera on four T cell lines

HIC' and C' TITRE FOLD DIFFERENCES		SERUM					
		QC1		QC2		QC4	
		IC50	IC90	IC50	IC90	IC50	IC90
CELL LINE	H9	ND	ND	1	1	1	1
	MT-2	3	3	3	3	9	9
	Molt-4	9	9	27	>3	3	>3
	SupT1/R5	81	27	81	>9	>27	>1

Table 3.4 Fold differences in IC50 and IC90 values with and without C'

Fold decreases in IC50 and IC90 values in the presence of C' compared to in the presence of HIC', derived from data in Tables 3.2 and 3.3, are given for each sera on each cell type.

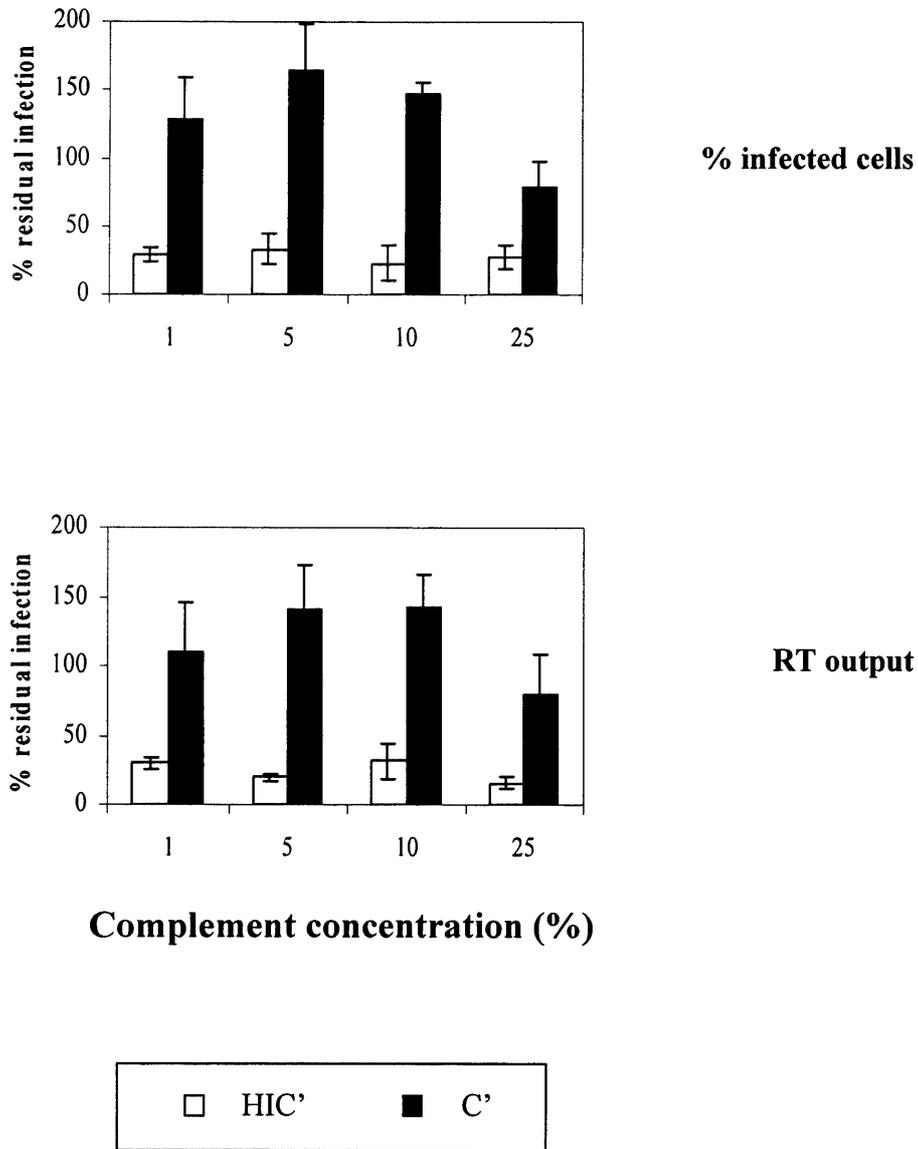
### **3.2.5 Evaluation of the use of SupT1/R5 cells for future C'-ADE assays**

Due to their expression of CCR5 (Figure 3.3), high-level expression of CR2 (Figure 3.1) and greatest C'-RN (Figures 3.9 and 3.10 and Table 3.4), SupT1/R5 cells were considered to be the optimal target cell for future studies of C'-ADE. Therefore, further investigation into the use of SupT1/R5 cells for future assays was conducted. First, as QC serum was in greater supply than the seroconversion serum samples, the SupT1/R5 cells were evaluated in the context of C'-RN. Next, neutralising and non-neutralising monoclonal antibodies were tested with IIIB in the presence and absence of C'. Finally, C'-ADE itself was investigated using the seroconversion serum samples that enhanced infection of IIIB on MT-2 cells.

#### ***3.2.5.1 Complement concentration***

Thus far all assays were carried out with a complement concentration of 10%. To ensure that this was the optimal concentration, several concentrations of complement were tested from serum concentrations of 1% to 25%. Levels of infection were determined by both intracellular p24 staining and RT output. Results are shown in Figure 3.11.

At fresh serum concentrations of 1% and 25% levels of C'-RN were reduced. 5% and 10% give similar levels of C'-RN, therefore a complement concentration of 10% was considered acceptable for future assays.



**Figure 3.11 Effect of the complement concentration on rescue from neutralisation**

Serum QC4 was used at a dilution of 1 in 540 (the last dilution that caused >50% neutralisation in the absence of C') and incubated with IIIB and various concentrations of complement (1%, 5%, 10% and 25%) for 1 hour at 37°C. This was then added to SupT1/R5 cells, and on Day 5 assayed for the % cells infected (top panel) and the RT output (bottom panel).

**3.2.5.2 The effect of neutralising and non-neutralising monoclonal antibodies on IIB replication on MT-2 and SupT1/R5 cells in the presence of C'**

Two monoclonal antibodies, known to neutralise IIB, were tested on MT-2 and SupT1/R5 cells. The antibody b12 is a broadly neutralising human monoclonal IgG1 antibody, and recognises an epitope on gp120 overlapping the CD4 binding site (Barbas et al., 1992; Zwick et al., 2001a; Zwick et al., 2003). 447-52D is a human monoclonal IgG3 antibody that recognises the GPGR epitope on the V3 loop and neutralises mainly clade B isolates (Conley et al., 1994; Gorny et al., 1992). Results are shown in Figure 3.12 and summarised in Tables 3.5 and 3.6.

IC50	447-52D (reciprocal dilution)			b12 (µg/ml)		
	HIC'	C'	DIFF <sup>1</sup>	HIC'	C'	DIFF <sup>2</sup>
MT-2	160	40	4	0.08	0.08	1
SupT1/R5	320	<10	>32	<0.0195	0.156	>8

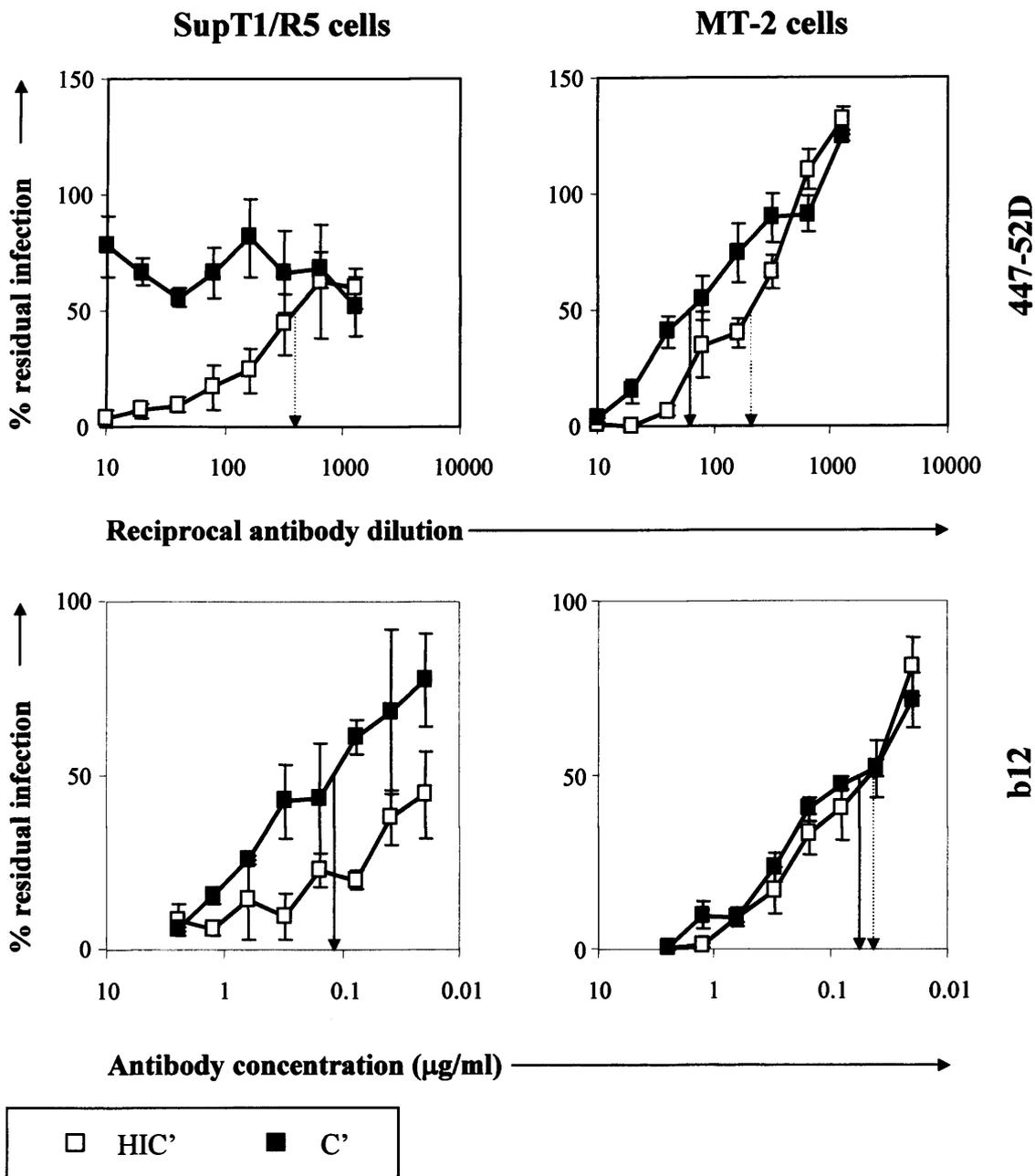
**Table 3.5 IIB IC50 values for two monoclonal antibodies on MT-2 and SupT1/R5 cells**

IC90	447-52D (reciprocal dilution)			b12 (ug/ml)		
	HIC'	C'	DIFF <sup>1</sup>	HIC'	C'	DIFF <sup>2</sup>
MT-2	40	10	4	0.625	0.625	1
SupT1/R5	40	<10	>4	1.25	2.5	2

**Table 3.6 IIB IC90 values for two monoclonal antibodies on MT-2 and SupT1/R5 cells**

<sup>1</sup> Represents the fold decrease in IC50 or IC90 value in the presence of C'

<sup>2</sup> Represents the fold increase in IC50 or IC90 value in the presence of C'

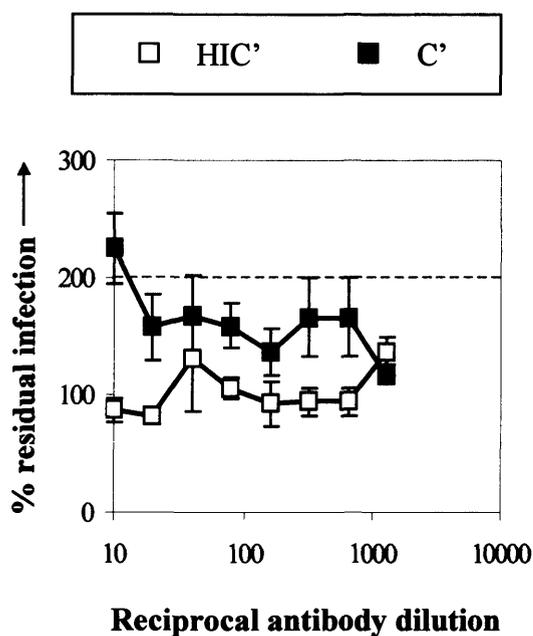


**Figure 3.12 Neutralisation of IIB by monoclonal antibodies 447-52D and b12 on MT-2 and SupT1/R5 cells**

Two known neutralising monoclonal antibodies, 447-52D and b12, were serially diluted and incubated with IIB and HIC' or C' for 1 hr at 37°C then transferred to MT-2 or SupT1/R5 cells. Cells were intracellularly stained for p24 on day 5 following infection, and % residual infection calculated relative to control cultures (NHS and HIC' or C' as appropriate). Solid arrows indicate IC<sub>50</sub> values in the presence of C'; dashed arrows in the presence of HIC'. For 447-52D the concentration of the antibody was unknown therefore antibody quantity is shown as reciprocal dilution of the hybridoma supernatant. Error bars represent SD from 3 experiments.

As with the QC serum, no enhancement was seen but C'-RN was evident. C'-RN was strongest on the SupT1/R5 cells, with IC50 values being more than 32-fold different in the presence and absence of active complement for 447-52D and more than 8-fold different for b12. 447-52D does not neutralise at all in the presence of C' on SupT1/R5 cells. The difference was less on the MT-2 cells, with 447-52D titres differing by 4-fold and b12 titres showing no difference.

As the SupT1/R5 cells supported considerable C'-RN, a non-neutralising antibody reported to enhance IIIB infection, 246-D (Forthal et al., 1995; Prohaszka et al., 1997) was tested for its effect on IIIB infection of SupT1/R5 cells. 246-D is a human monoclonal IgG1 antibody to the epitope 579-604 of gp41 (Gorny et al., 1989). Results are shown in Figure 3.13.



**Figure 3.13 Enhancement of IIIB infection of SupT1/R5 cells by monoclonal antibody 246D**

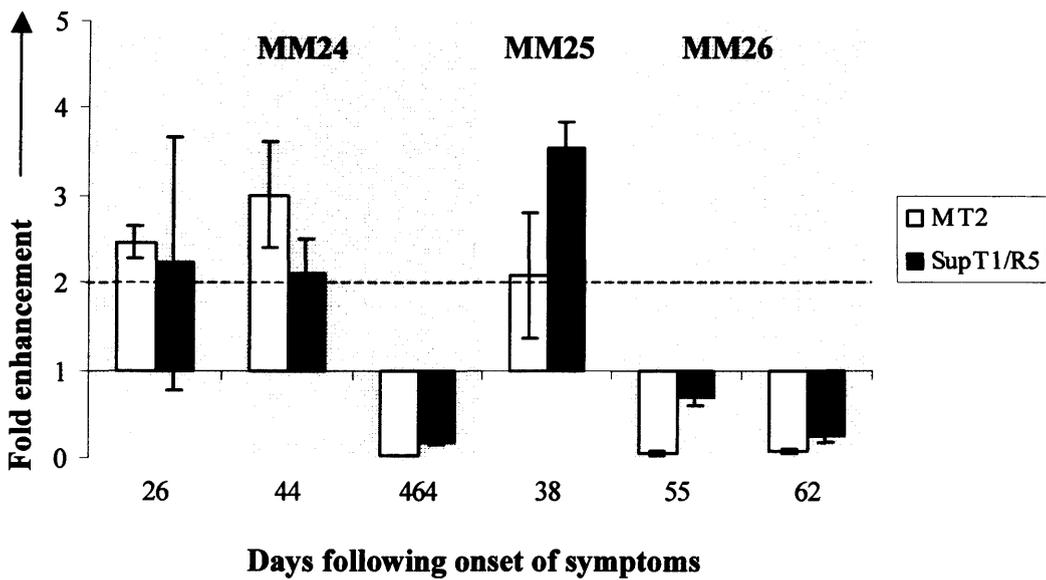
IIIB was incubated with serial dilutions of 246-D in the presence (C') and absence (HIC') of complement for 1 hour at 37°C, then added to SupT1/R5 cells. Levels of infection were determined by intracellular p24 staining on day 5 following inoculation. The dashed turquoise line represents the enhancement threshold. Error bars represent SD of 3 experiments.

In the absence of C' 246-D had no effect on IIIB infection of SupT1/R5 cells. In presence of C' the highest concentration of antibody enhanced infection, and this effect was rapidly abolished with increased dilution.

### ***3.2.5.3 Testing early sera that enhanced IIB infection on MT-2 cells on SupT1/R5 cells***

Several serum samples that enhanced IIB infection of MT-2 cells were tested on SupT1/R5 cells. Results are shown in Figure 3.14. The MM24 days 26 and 44, and MM25 day 38 sera enhanced infection of IIB on both MT-2 and SupT1/R5 cells. The level of neutralisation on MT-2 and SupT1/R5 cells also corresponded, with the exception of MM26 day 55.

In conclusion, where enhancement of IIB infection was seen on MT-2 cells it was also shown to occur on SupT1/R5 cells. C'-RN is more powerful on SupT1/R5 cells, for both neutralising sera and monoclonal antibodies.



**Figure 3.14 Comparison of C'-ADE of IIB on MT-2 and SupT1/R5 cells**

Enhancement assays were carried out in the presence of C' on MT-2 and SupT1/R5 cells with IIB and patient sera from MM24, 25 and 26. Fold enhancement is calculated relative to infection levels in the presence of seronegative control serum. Values above the dotted blue line indicate enhanced infection

## 3.3 DISCUSSION

### 3.3.1 ADE of HIV on T cell lines

#### 3.3.1.1 Magnitude of ADE

The aim of this chapter was to investigate the occurrence of ADE of the HIV strain IIIB, as reported in the literature, and to develop a suitable enhancement assay for further study of ADE in clinical HIV samples. From initial screening experiments with sera obtained at early stages after HIV infection, ADE was seen more frequently on a CR2<sup>+</sup> cell line, MT-2, than on an FcγRII<sup>+</sup> cell line, MT-4.

Levels of IIIB enhancement seen in the MT-2 system, between 2.08- and 6.4-fold, compared favourably with those reported previously, with several investigators quoting fold enhancement levels of 1.5-4 fold in similar experimental systems (Banhegyi et al., 2003; Lund et al., 1995; Prohaszka et al., 1997; Szabo et al., 1999). Others have reported higher enhancement levels. However, difficulties arise in attempting to directly compare my results with these particular studies, as most present enhancement measurements as antibody titres (Montefiori et al., 1991; Robinson et al., 1988a; Robinson et al., 1988b) or percent increase in cell death (McDougall et al., 1997; Robinson et al., 1991). But when quantitative methods, such as % IFA positive cells and RT release, were reported in some of these studies the fold enhancement levels ranged from 3.4 to 14-fold (Robinson et al., 1990a; Robinson et al., 1990b; Robinson et al., 1990c; Robinson et al., 1989b), and in another study levels of HIV cDNA and integrated provirus were increased 8- to 30-fold (Robinson, 2006). C'-independent ADE was also observed on the MT-2 system (MM13 days 24 and 31). This resembled previous observations of C'-independent ADE of HIV on FcR-negative cell lines, in which monoclonal antibodies were used to show enhancement of various HIV strains in the range of 3-10-fold (Guillon et al., 2002; Schutten et al., 1997).

ADE had not previously been investigated in FcγRII<sup>+</sup> MT-4 cells. One group reported the use of MT-4 cells in enhancement assays but their MT-4 cells had a different phenotype from the cells used here, being CR2<sup>+</sup> C1qR<sup>+</sup> CD4<sup>+</sup> T cells (Banhegyi et al., 2003; Prohaszka et al., 1997; Szabo et al., 1999). In systems using U937 cells, FcR-ADE was reported in the range of 1.5 to 3-fold (Laurence et al., 1990; Takeda et al., 1990; Takeda et al., 1988), and my results are in accordance with those.

Overall, levels of enhancement of IIIB are much lower than those reported for dengue virus enhancement, both in published reports and data presented in this chapter, with typical levels of dengue virus ADE ranging from 2 to 200 fold (Halstead and O'Rourke, 1977b).

### ***3.3.1.2 Correlations of ADE with disease***

Despite using similar methods, my results do not agree with two studies citing correlations between fold enhancement and viral loads (Banhegyi et al., 2003; Szabo et al., 1999). This could be because they studied patients at advanced stages of disease whereas my comparisons were made with sera from asymptomatic patients. At early stages of disease viral loads and antibody titres fluctuate more over a short period of time than during chronic and advanced stages of disease, and this may confuse the issue of correlations. Also, patient numbers were lower in my study although several samples were taken sequentially from each patient, which may not give the same representation as a cross-sectional study.

### **3.3.2 CR2 expression is common among T cell lines**

CR2 expression was frequently detected on the T cell lines tested, with MT-2, Molt-4, SupT1, SupT1/R5 and C8166 cells all expressing detectable levels. This is supported by publications showing high level CR2 expression on other CD4+ T cell lines including Jurkat (Braun et al., 1998; Fingeroth et al., 1988; Prodinger et al., 1996; Sinha et al., 1993), HBP-ALL (Delibrias et al., 1992; Fingeroth et al., 1988; Saifuddin et al., 1994), Molt-3 (Fingeroth et al., 1988), Molt-4 (Menezes et al., 1977), C91.PL (Prodinger et al., 1996), and SupT1 (Prodinger et al., 1996). Clearly, different clones of certain cell lines can exhibit different cell receptor expression patterns. Reports have shown an absence of CR2 expression on SupT1 cells (Romano et al., 1997), and MT-4 cells have been attributed with high-level expression of CR2 (Prohaszka et al., 1997), in contrast with results presented here. CR2 has also been detected on human peripheral blood T cells (Fischer et al., 1991; Fischer et al., 1999; June et al., 1992; Masilamani et al., 2002; Sauvageau et al., 1990) and thymocytes (Delibrias et al., 1994; Fischer et al., 1999; Tsoukas and Lambris, 1988). The function of CR2 on T cells has yet to be adequately explained. The fact that CR2 is readily detected on many T cell lines and also on primary T cells, however, could indicate an important role for this receptor that is yet to be appreciated.

### **3.3.3 Complement-mediated rescue of HIV from neutralisation**

The concept of complement reducing the power of neutralising antibodies is important, with implications for neutralisation assays and vaccine design. It is often described as a by-product of enhancement and has not been studied as a separate phenomenon. Here it is shown that the presence of complement can reduce the 50% neutralising titre of serum by up to 81 fold, and the corresponding 90% neutralising titre by at least 27 fold. In reports where the neutralising titre of serum on MT-2 cells is given in both the presence and absence of C', the fold difference in titre is in a similar range as the results of my study: 6-fold (Robinson et al., 1990c), 2-8-fold (Robinson et al., 1988a) and 3-81-fold (McDougall et al., 1997), compared with a range of 3-9-fold difference for the IC50 and IC90 values for the three neutralising sera tested in my assays. The same phenomenon has not been previously reported for Molt-4 or SupT1/R5 cells.

### **3.3.4 Mechanisms of ADE and C'-RN**

#### ***3.3.4.1 Mechanism of C'-RN***

From results shown in Figures 3.1, 3.2 and 3.9 it may be deduced that the C'-mediated rescue from neutralisation is mediated by the presence of CR2, as the only cell line that does not show this activity, H9, is the only cell line lacking CR2. However, this is not conclusive proof of the involvement of CR2, and will be addressed further in Chapter 6.

On the MT-2 cells the C'-RN was less than on the SupT1/R5 and Molt-4 cells, despite the fact that they express a high level of CR2. This could be due to low-level expression of FcγRII counteracting the effect of CR2, as in Section 3.2.2 an increase in neutralisation in the presence of C' is seen on the MT-4 cells, in contrast to the C'-RN seen on the MT-2 cells. Alternatively, receptors other than those investigated may contribute to differences seen with and without complement on the four cell lines. For example, C1q receptors or CR4 may be present and may account for the difference in C'-mediated rescue seen on SupT1/R5 compared with Molt4 cells which have apparently the same receptor expression.

It is unlikely that the sole mechanism of C'-RN is masking of neutralisation epitopes by complement as previously suggested (Hidvegi et al., 1993), as, if this were

the case it would be evident in the absence of CR2. However, it could be that complement binds to regions close to the neutralisation binding epitopes. It is possible that the mechanism of C'-mediated rescue is the same as that for C'-ADE, and that C'-RN is essentially a lower level of C'-ADE. C'-RN could occur because the balance of neutralising and enhancing antibodies results in net neutralisation, yet the presence of enhancing antibodies allows rescue from neutralisation in the presence of complement. Alternatively, when <90% saturation of the virus by neutralising antibodies occurs, C'-RN may provide the virus with increased attachment to the target cell and therefore greater chance of successful infection, that otherwise was not available in the absence of CR2.

If one were to consider C'-RN as a surrogate for assessing assays to measure C'-ADE, then in the direct comparison between H9, MT-2, Molt-4 and SupT1/R5 cells, the SupT1/R5 cells are the most appropriate cell line for the future studies of C'-ADE. More importantly, they provide the opportunity to study C'-ADE of autologous virus using patient virus isolates, most of which are R5-tropic.

#### ***3.3.4.2 Monoclonal antibodies***

The fact that the neutralising activity of two monoclonal antibodies with different epitopes – 447-52D to the V3 loop and b12 to the CD4 binding site – can be reduced on SupT1/R5 cells in the presence of C' indicates that the mechanism of rescue is a general rather than specific one. It is interesting that the fold rescue from neutralisation is different for the two monoclonal antibodies – potentially the b12 antibody is more potent on an individual molecule basis than the 447-52D antibody.

In this chapter, results with monoclonal antibodies suggest that neutralising antibodies have different specificities from enhancing antibodies, as proposed by other investigators. However, examining the effects of three monoclonal antibodies does not conclusively show different actions of neutralising and enhancing antibodies – for this to be the case a much greater number of monoclonal antibodies, of various specificities and isotypes, should be investigated. However, it is possible to say that in this case, two neutralising antibodies do not enhance, and one enhancing antibody does not neutralise, suggesting a difference in neutralisation and enhancement epitopes.

### **3.3.4.3 The net effect of complement – enhancement or inactivation?**

Results in this chapter have shown that complement can have various effects on viral infection depending on the target cell and serum used. C'-RN results presented in this chapter indicate that viruses assayed here are not irreversibly neutralised at all, nor are they lysed. As the virus was incubated with serum and complement for 1 hour before addition to target cells, lysis and neutralisation would occur at that stage, and addition to cells should make little difference. However, the fact that the viruses could be rescued from neutralisation indicates that they were not irreversibly inactivated by complement, thus are not completely lysed. Therefore I would argue that, when additional inactivation is seen in the presence of complement (i.e. in the absence of CR2+ cells), this is primarily due to opsonisation and not lysis.

The IIIB used for the experiments shown in this chapter was produced in H9 cells, which reportedly have a lower level of CD55 and CD59 on their surface compared with primary cells (Saifuddin et al., 1995). Thus this particular virus preparation would be expected to be relatively susceptible to lysis. However, as shown, this is not the case. Primary isolates grown in PBMCs, therefore, are likely to be even less susceptible to C'-MI and perhaps even more susceptible to C'-ADE, as investigated in Chapter 4.

### **3.3.5 Concluding remarks**

Although results presented here are in agreement with previous reports, levels of enhancement seen for IIIB are weak and in many cases variable. It became the model virus on which to study ADE because most work in the late 1980s focused on TCLA strains of HIV as these were easier to grow and characterise. Also, most T cell lines used for *in vitro* HIV studies express very little, if any, CCR5, and generally do not support infection by primary (predominantly R5-tropic) HIV strains, necessitating the use of an X4 tropic virus. Therefore, C'-ADE studies on IIIB set the precedent for later work. However, several studies have questioned the relevance of TCLA HIV strains to primary patient isolates (Moore and Ho, 1995), particularly in terms of neutralisation phenotypes. The same may be true of enhancement. In terms of clinical relevance it would be more informative to study enhancement of primary HIV isolates, particularly those relevant to currently circulating HIV strains.

One viewpoint is that the structure of the virus envelope is more important for enhancement than the epitope specificity of the antibodies themselves (Davis et al.,

2001; Kliks et al., 1993; Kostrikis et al., 1996) and that some viruses are more susceptible to enhancement than others. These considerations highlight the importance of studying enhancement of different HIV strains and, particularly, the enhancement of autologous primary isolates. This is investigated in Chapter 4.

## CHAPTER 4

### **Longitudinal studies of infection-enhancing antibodies from individuals infected with HIV**

This chapter investigates the occurrence of antibodies with C'-ADE activity in HIV infection using viruses isolated from patient samples and longitudinally collected autologous serum samples.

#### **4.1 INTRODUCTION**

##### **4.1.1 The need to study C'-ADE soon after infection with HIV**

Primary HIV infection is characterised by high levels of virus replication, a drop in CD4 cell counts and often concomitant clinical manifestation, PHI. Viral RNA levels in the plasma can exceed  $10^7$ - $10^8$  copies per ml. This usually precedes or coincides with seroconversion and is followed by a 100-10000 fold drop in viral load to reach a stable level known as the viral set point. Neutralising antibodies are generally not detected until several months after infection (Aasa-Chapman et al., 2004; Aasa-Chapman et al., 2005; Moog et al., 1997; Richman et al., 2003; Wei et al., 2003) and even then it is doubtful that they neutralise contemporaneous virus. This leaves a time frame in which non-neutralising antibodies are abundant, and the possibility that they are present throughout infection. Potential activities have been assigned to non-neutralising antibodies, including ADCC (Forthal et al., 1995; McDougall et al., 1997; Subbramanian et al., 2002) and FcR-mediated inhibition of viral replication (Forthal et al., 2005; Holl et al., 2006). Using the same patient cohort as the one studied in this chapter, Aasa-Chapman et al showed that early non-neutralising antibodies produced in response to HIV infection could inactivate autologous virus in the presence of complement (Aasa-Chapman et al., 2005). As inactivation was attributed principally to opsonisation and not lysis of HIV particles, it is also possible that these antibodies may enhance HIV infection and contribute to the initial burst of replication and establishment of viral reservoirs seen in early stages of disease.

Studies have indicated that C'-ADE can correlate with disease in infected individuals (Subbramanian et al., 2002; Szabo et al., 1999). Other studies have shown no correlation (Montefiori et al., 1991). However, all of these studies were carried out

on heterologous virus strains, most commonly IIIB, and concentrated on sera from late asymptomatic disease stages, or from AIDS patients. C'-ADE of SIV infection has been shown by early autologous antibody and results indicated a role for C'-ADE antibodies in disease progression (Montefiori et al., 1995). Early serum samples enhanced infection in the presence of complement, progressing to neutralisation by later samples. Such a study has not been done in humans, although samples from seroconversion panels have been shown to enhance heterologous virus (Szabo et al., 1999), with results similar to those shown in Chapter 3 of this thesis. Autologous studies of enhancement have been carried out on primary cells and showed enhancement to correlate with disease progression (Homsy et al., 1990), but these studies looked at later stages of disease and did not investigate a role for C' in this phenomenon.

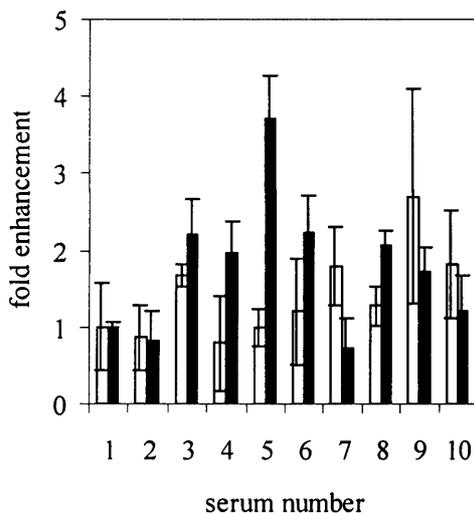
Therefore, the focus of this chapter is to address the issue of C'-ADE in early sequential serum samples against autologous virus, in order to assess whether there is a role for C'-ADE in early HIV infection and the development of this response over time. Using samples from a patient cohort, C'-ADE activity is investigated from as early as 12 days post onset of PHI symptoms to several years after infection.

#### **4.1.2 The enhancement assay**

This chapter contains results from experiments using an enhancement assay developed from the results in Chapter 3. SupT1/R5 cells were chosen as the optimal target cell for an enhancement assay because (a) they express a high level of CD4, CXCR4 and CCR5, allowing for the investigation of both X4 viruses and, importantly, R5-using primary isolates from patient samples, (b) they express a high level of CR2 and therefore provide a system in which to study C'-ADE, and (c) of all the CR2+ T cell lines tested in Chapter 3, they showed the most dramatic C'-mediated rescue from neutralisation, therefore they are the most sensitive cell line in which to find evidence of C'-ADE.

As in Chapter 3, for the assays that follow, results are shown as “fold enhancement”, meaning the ratio of infection in the presence of autologous serum to infection in the presence of pooled seronegative control serum (NHS), calculated separately for infection in the presence of HIC' and C'. This cancels out the effect of non-specific C' enhancement in the assays, which, for most viruses was 2-fold, and allows the data to be presented only in terms of the additional effect of the antibodies.

Infectivity was measured by the percentage of cells infected, by intracellular p24 staining and flow cytometry 6 days after infection. A cut-off point of 5 fold was set as the level of true enhancement, as normal human serum taken from 7 uninfected individuals enhanced infection up to 3.7 fold  $\pm$  0.57 (compared to pooled control serum, NHS) as shown in Figure 4.1.



**Figure 4.1 Background enhancement**

Virus was incubated with several different seronegative sera and added to SupT1/R5 cells. Serum number 1 is the control serum used for all enhancement assays and is commercially available pooled HIV antibody negative serum (NHS). Serum samples 2-10 were from 9 HIV negative individuals. Results were standardised to Serum 1. Results shown are from MM32 day 10 virus, but are representative of viruses from other patients as no more than 3.7-fold enhancement was seen in the presence of HIV antibody negative serum. Error bars represent standard deviation from 3 experiments. HIC' □ C' ■

The viral input was set at the low level of 0.1% infected cells (out of 10,000 cells acquired per replicate in the flow cytometry analysis) because the viruses isolated from patients were of low titre, and also as this leaves the greatest scope for enhancement. C', or the HIC' control, was used in the assay at a concentration of 10%, as was patient serum or NHS, unless otherwise stated.

#### 4.1.3 The patient cohort

As shown in Chapter 3, serum samples from early time points after infection showed C'-ADE activity against IIIB. It was therefore decided to test sequential serum sample sets from 10 patients for enhancing activity against autologous virus. Sequential samples were obtained from 10 individuals infected with clade B HIV-1. Samples were used from as early as 12 days post onset of symptoms up to 1000 days, as these were expected to cover the time frame of both non-neutralising and

neutralising antibody development. Patient primary isolates were propagated from patient PBMCs and the co-receptor tropism determined by titration on NP2/CD4/R5 and NP2/CD4/X4 cells. All patient primary isolates used in this study were R5 tropic. Further details of the patient cohort are given in Materials and Methods section 2.4 and Aasa Chapman et al 2005.

## **4.2 RESULTS**

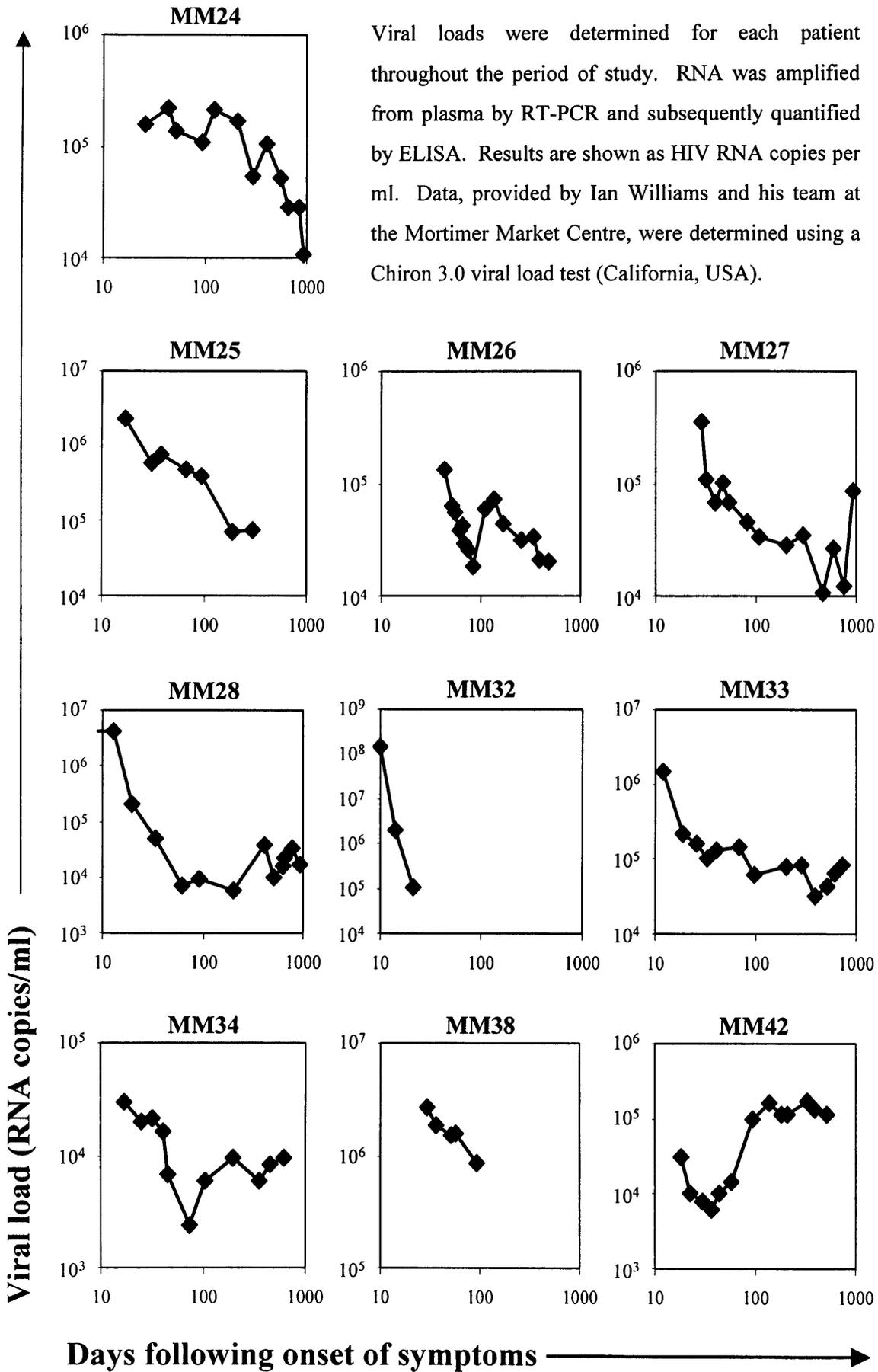
### **4.2.1 Characterisation of the patient cohort**

#### ***4.2.1.1 Patient viral loads***

Figure 4.2 shows plasma viral loads for the 10 patients investigated in this study over the time frame investigated (data provided by Ian Williams, Mortimer Market Clinic, UCL). Peak viral loads coincide with primary symptoms; therefore as the time of study is measured in days following the onset of symptoms we expect our study to begin during or after the decline in viral load signifying the beginning of the asymptomatic disease period. In some patients this precipitous drop in viral load was detectable (MM28, MM32), but in most the viral load was in gradual decline up until approximately day 100. After this the viral loads either reach a steady level, as in MM25 and MM33, or continue to fluctuate, as in MM26, MM34 and MM42. Table 4.1 shows the viral set-point for each patient, taken as the viral load at the time point closest to 6 months following onset of symptoms. Set-points were classified as high, medium or low according to thresholds stipulated by Mellors et al (Mellors et al., 1997).

**Figure 4.2 Patient viral loads**

Viral loads were determined for each patient throughout the period of study. RNA was amplified from plasma by RT-PCR and subsequently quantified by ELISA. Results are shown as HIV RNA copies per ml. Data, provided by Ian Williams and his team at the Mortimer Market Centre, were determined using a Chiron 3.0 viral load test (California, USA).



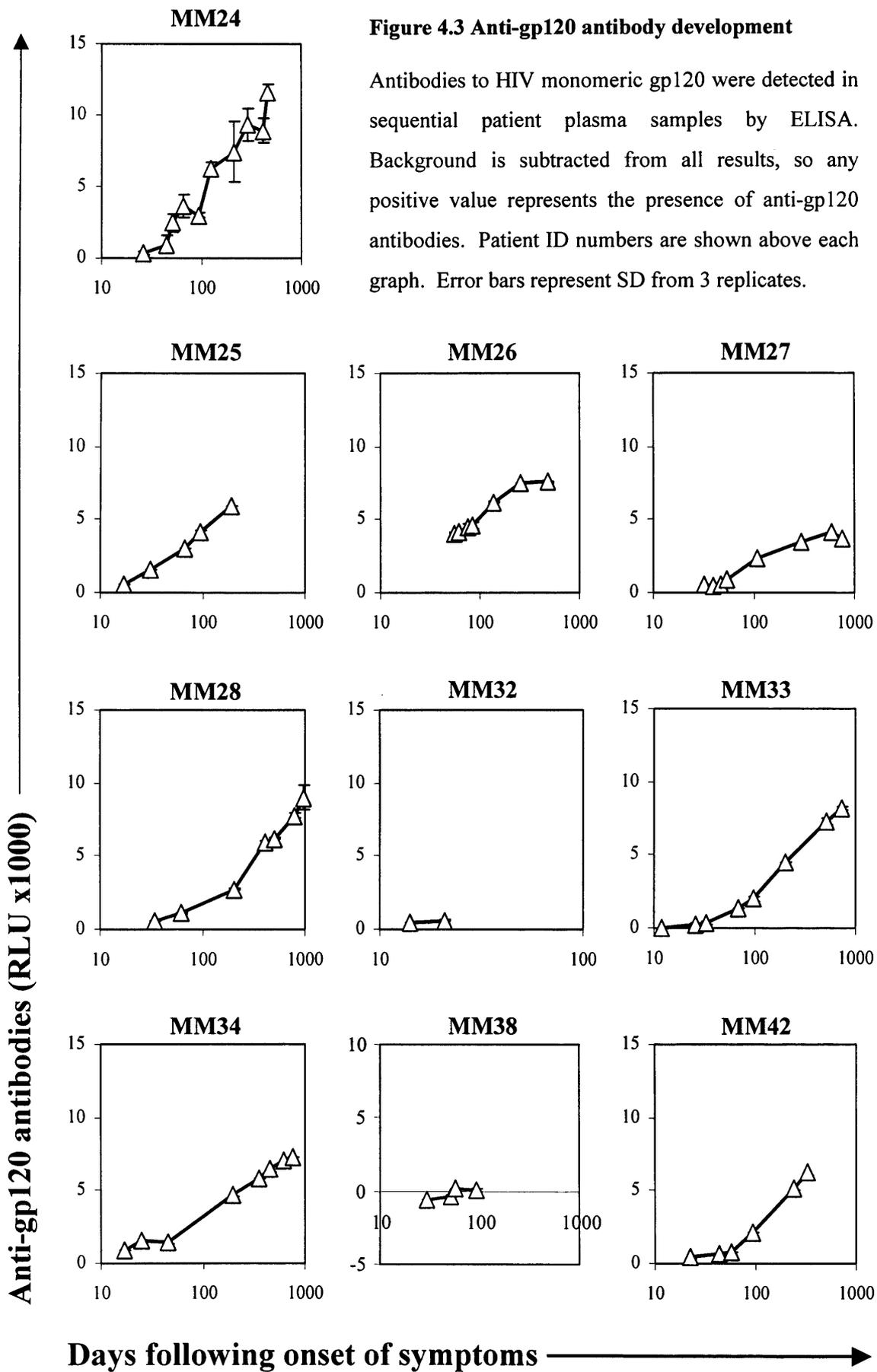
PATIENT	SET-POINT (copies/ml)	GROUP	PATIENT	SET-POINT (copies/ml)	GROUP
MM24	170,200	High	MM32	N/A	
MM25	71,100	High	MM33	80,000	High
MM26	44,600	High	MM34	9,600	Low
MM27	28,800	Medium	MM38	N/A	
MM28	5,600	Low	MM42	115,100	High

**Table 4.1 Patient viral load set-points**

The viral set-point for each patient is determined by the viral load at the time closest to 6 months. Patients are classified into 3 groups according to their set-points: low (<10,000), medium (10,000-30,000) and high (>30,000) according to thresholds determined by Mellors et al (Mellors et al., 1997). N/A: viral load data were not available.

#### ***4.2.1.2 The development of anti-HIV Env antibody responses***

The development of an anti-HIV antibody response was investigated in each patient by ELISA. Antibodies to monomeric gp120 from IIIB were detected in plasma samples collected sequentially throughout the period of study. Results are shown in Figure 4.3. Background antibody binding (measured in the absence of gp120) was subtracted for each sample therefore any value above 0 indicates the presence of anti-gp120 antibody. With the exception of MM38, all patients had detectable antibodies to HIV-1 gp120 from the earliest time point available, allowing the potential for ADE activity from the earliest samples used in the experiments that follow. The ELISA detects antibodies to monomeric HIV IIIB gp120, therefore it is possible that early antibodies to autologous native envelope were present but below the limit of detection of the assay. The assay also fails to detect antibodies to the gp41 portion of the envelope and possibly lower affinity antibodies such as IgM isotypes, so these should also be taken into account.



## 4.2.2 C'-ADE of early virus isolates by autologous sera

### 4.2.2.1 Strong C'-ADE activity is observed in early serum samples

Viruses isolated from early time points, between 6 and 62 days following onset of symptoms, were assayed in the presence of sequential autologous serum samples and C' or HIC'. Results from the autologous enhancement assays are shown in Figure 4.4. With the exception of MM27, all patients showed evidence of strong C'-ADE activity, with enhancement levels as high as 236-fold. The patients can be grouped according to the patterns of C'-ADE activity development over time:

#### *Group 1: MM24, MM25 and MM26*

C'-ADE was strongest at earliest time points then subsided to neutralisation. In both the presence (C') and absence (HIC') of active complement, greater than 90% neutralisation was seen at similar times for all three patients: on day 208 for MM24; day 185 for MM25 and day 253 for MM26. The phenomenon of C'-mediated rescue from neutralisation did not occur in these samples. Neutralisation is investigated in more detail in section 4.2.3.

While neutralisation occurred at a similar time and to the same degree in these patients, enhancement differed. The overall magnitude of enhancement was highest for MM24, peaking at 236-fold on day 44; then for MM26 at 35-fold on day 139; and lowest for MM25 at 8-fold on day 31. The peak enhancement was seen with sera from time points close to the day from which the virus was isolated (MM24 day 26 virus; MM26 day 31 virus; MM25 day 62 virus).

#### *Group 2: MM27*

C'-ADE was below the enhancement threshold and within 1 SD of infection in the presence of HIC'. It was highly variable, as shown by the large error bars seen in Figure 4.4. Neutralisation was not detected in this assay.

#### *Group 3: MM28, MM33, MM34 and MM42*

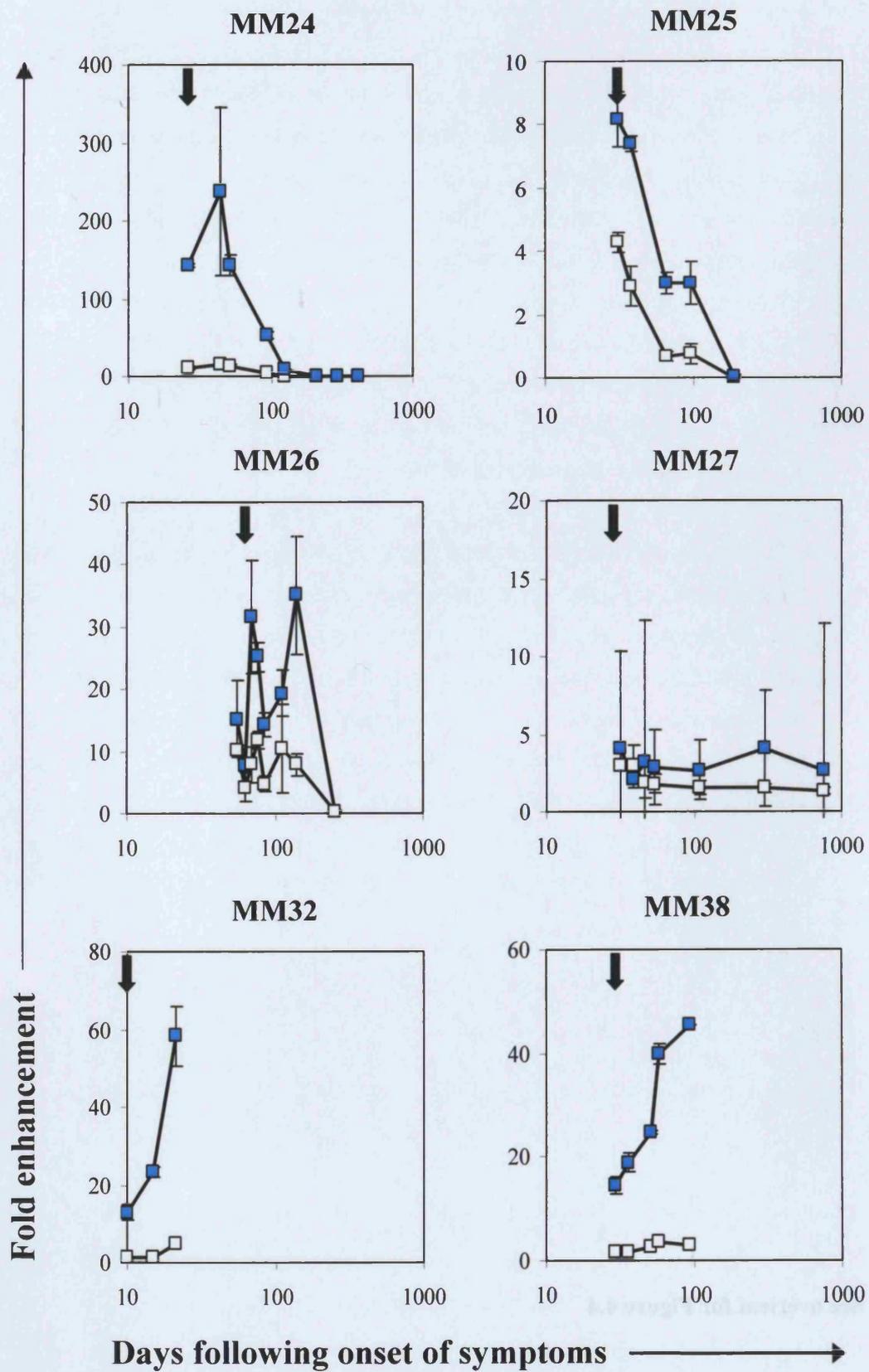
C'-ADE activity was weaker at earlier time points then steadily increased from day 100 onwards. In each patient there was a smaller early peak before the late peak of enhancement: for MM28 there was 29-fold enhancement on day 34 then 86-fold on

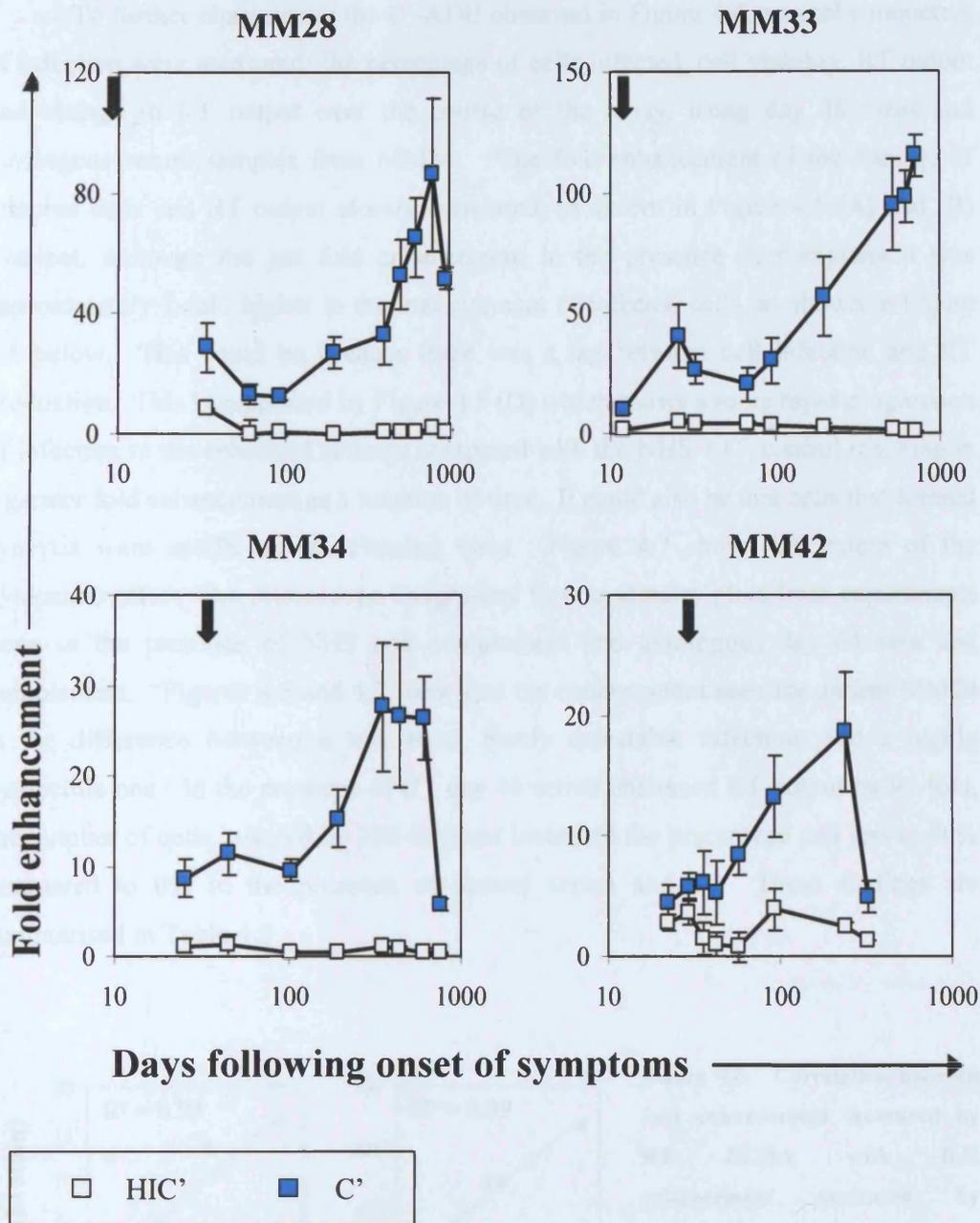
day 782; for MM33 41-fold on day 26 then 115-fold on day 719; for MM34 11-fold on day 45 then 28-fold on day 353; and for MM42 6-fold on day 36 then 19-fold on day 238. As with those seen in Group 1, the patterns in Group 3 were similar whilst the magnitudes differ. For all 4 patients in this group the second peak of enhancement was approximately 3 times greater than the first, yet peak enhancement ranges from 19-fold to 115-fold within patients and the length of time between the first and second peaks differed in all 4 patients. The first peak resembled that seen in the Group 1 patterns, occurring between days 26 and 45 with a magnitude of between 6- and 41-fold. It is the second, larger peak and the failure to neutralise the early virus that distinguishes the Group 3 patterns from those of Group 1. For MM33, the last of the samples assayed showed the greatest enhancement. For MM28, MM34 and MM42 the level of enhancement declined in the last sample; in the case of MM34 and MM42 this dropped to below the level seen in the early time points.

MM32 and MM38 were excluded from classification as the sample sets for these patients were incomplete: MM32 was lost to follow-up and MM38 commenced antiretroviral treatment. However, the time points assayed showed C'-ADE activity, with MM32 enhancement reaching 58-fold on day 21 and MM38 46-fold on day 93.

In all patients except MM27, the serum samples taken from the same time point that the virus was isolated enhanced infection. In several patients C'-independent ADE was detectable (MM24, MM26 and MM28), a phenomenon previously observed by Schutten and colleagues on SupT1 cells with monoclonal antibodies (Guillon et al., 2002; Schutten et al., 1997). However, the C'-independent ADE was of limited magnitude compared to the C'-ADE.

**See overleaf for Figure 4.4**



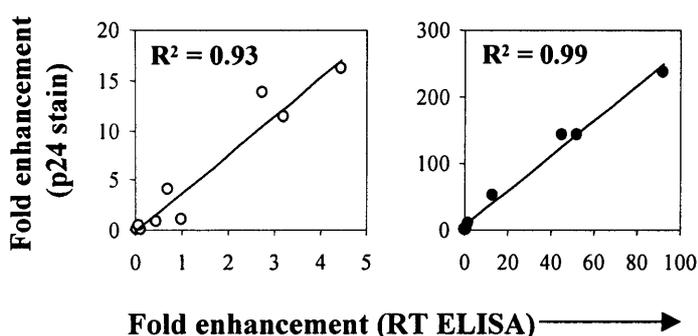


**Figure 4.4 C'-ADE of early viruses from 10 patients by autologous sequential sera**

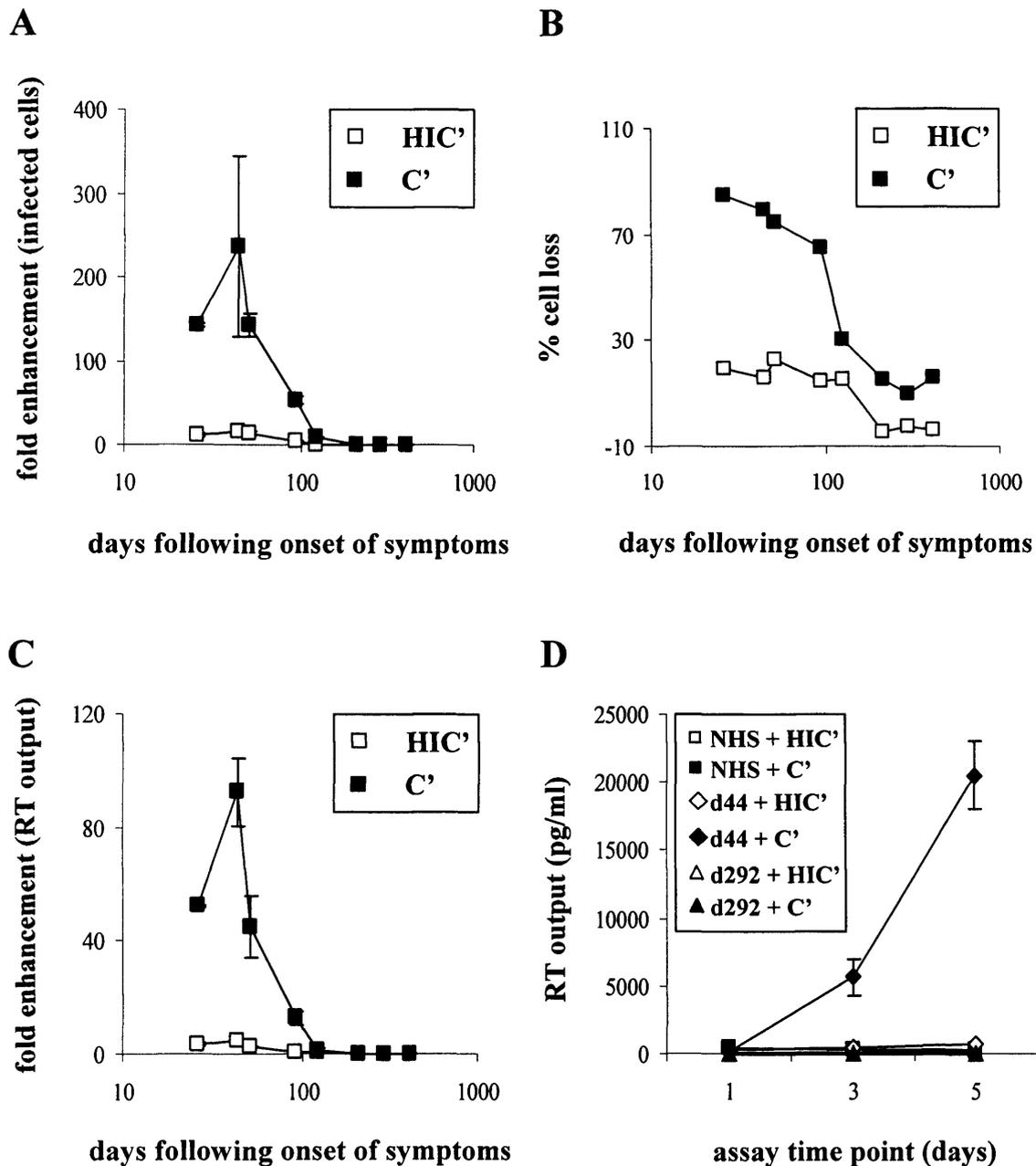
Serum sets from 10 patients were tested against autologous virus for C'-ADE activity. Virus was incubated with patient or control (NHS) serum in the presence of HIC' (□) or C' (■) for 1 hour at 37°C and then inoculated onto SupT1/R5 cells. After 6 days the percentage of cells infected is measured by intracellular p24 staining. Fold enhancement is calculated relative to infection levels in the presence of NHS, and HIC' or C' as appropriate. Patient numbers are given above each chart. Arrows indicate the time point from which virus was isolated. Error bars represent SD from 3 experiments.

#### 4.2.2.2 C'-ADE increases the number of cells infected, the RT output and cell death

To further characterise the C'-ADE observed in Figure 4.4, several parameters of infection were measured: the percentage of cells infected, cell viability, RT output and change in RT output over the course of the assay, using day 26 virus and autologous serum samples from MM24. The fold enhancement of the number of infected cells and RT output closely correlated, as shown in Figure 4.5 (A) and (B) overleaf, although the net fold enhancement in the presence of complement was approximately 2-fold higher in the measurement of infected cells, as shown in Figure 4.6 below. This could be because there was a lag between cell infection and RT production. This is supported by Figure 4.5 (D) which shows a more rapid progression of infection in the enhanced cultures compared with the NHS + C' control resulting in a greater fold enhancement as a function of time. It could also be that cells that formed syncytia were inefficient at releasing virus. Figure 4.7 shows the extent of the cytopathic effect with microscope images and flow cytometry plots from experiments done in the presence of NHS and complement and autologous day 44 sera and complement. Figures 4.5 and 4.7 show that the enhancement seen for patient MM24 is the difference between a low level, barely detectable infection, and a highly cytopathic one. In the presence of C', day 44 serum enhanced RT output by 92-fold, the number of cells infected by 236-fold and increased the percentage cell loss to 80% compared to 0% in the presence of control serum and C'. These findings are summarised in Table 4.2.

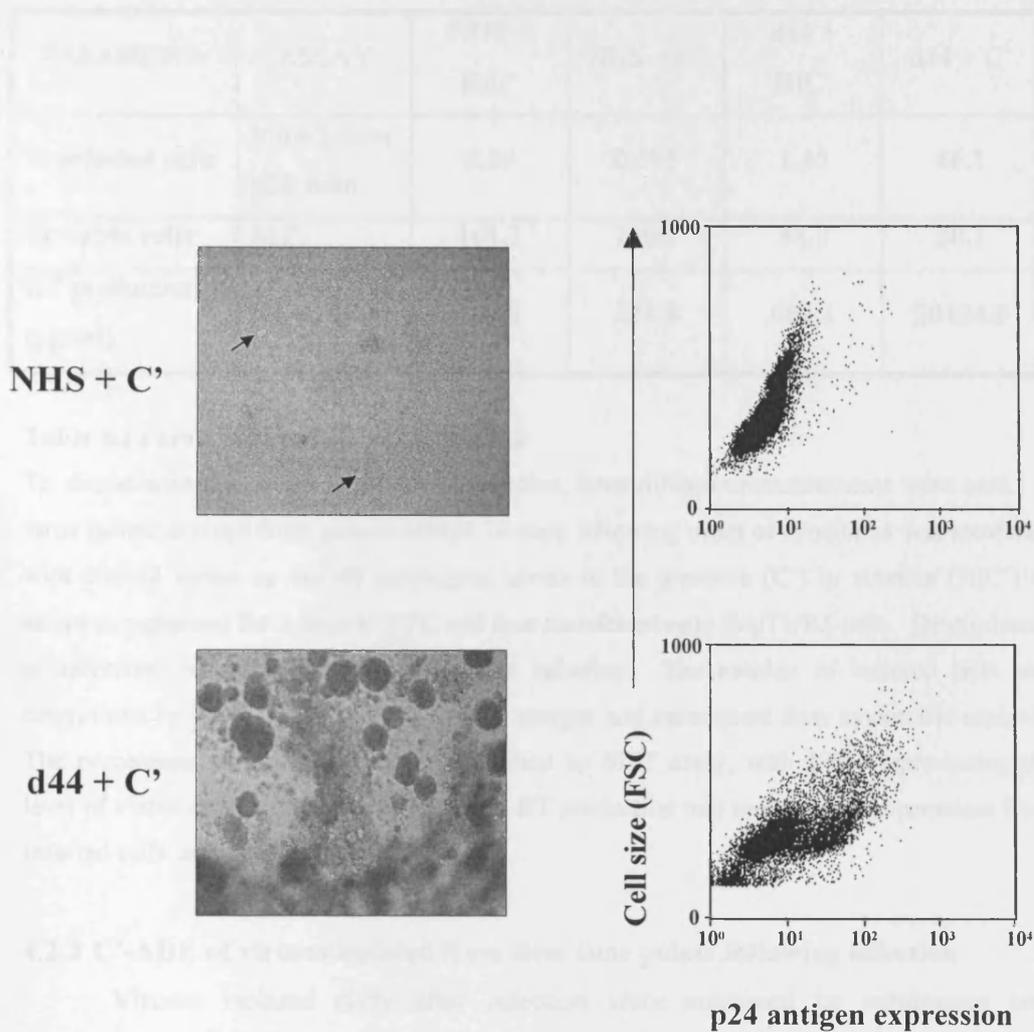


**Figure 4.6 Correlation between fold enhancement measured by RT ELISA with fold enhancement measured by intracellular p24 staining**  
Data are from Figure 4.5 A and C. Open circles indicate assays performed in the presence of HIC', closed circles with C'.



**Figure 4.5 RT output, % infected cells and % viable cells in enhanced infection**

Enhanced infection of virus isolated at day 26 following onset of symptoms from patient MM24. Assays were performed on SupT1/R5 cells with autologous sequential patient serum samples or an NHS control in the presence of HIC' (open squares) or C' (closed squares). Levels of infection are shown as (A) % infected cells measured by flow cytometry, (B) % cell loss measure by MTT assay, and (C) RT output measured by RT ELISA. Panel (D) shows the assay time course from (C). Fold enhancement and % viable cell loss are calculated relative to infection in the presence of NHS and HIC' or C' as appropriate. Error bars represent SD from 3 experiments.



**Figure 4.7 Microscopy images and flow cytometry plots from an enhancement assay**

Images show enhanced infection of virus isolated on day 26 (following onset of symptoms) from patient MM24. Assays were performed on SupT1/R5 cells with autologous virus in the presence of NHS and C' (top images) or day 44 serum and C' (bottom images). The left panels show microscopy images from Day 5 of the enhancement assay. Arrows in the top left image point to syncytia. On the same day the cells were intracellularly stained for p24 and analysed by flow cytometry (right panels). The fold enhancement seen for day 44 serum is calculated relative to infection in the presence of NHS and C' and in this case is 236-fold.

PARAMETER	ASSAY	NHS + HIC'	NHS + C'	d44 + HIC'	d44 + C'
% infected cells	Intracellular p24 stain	0.09	0.195	1.45	46.1
% viable cells	MTT	101.3	100.7	84.0	20.1
RT production (pg/ml)	RT ELISA	147.9	221.8	662.1	20494.8

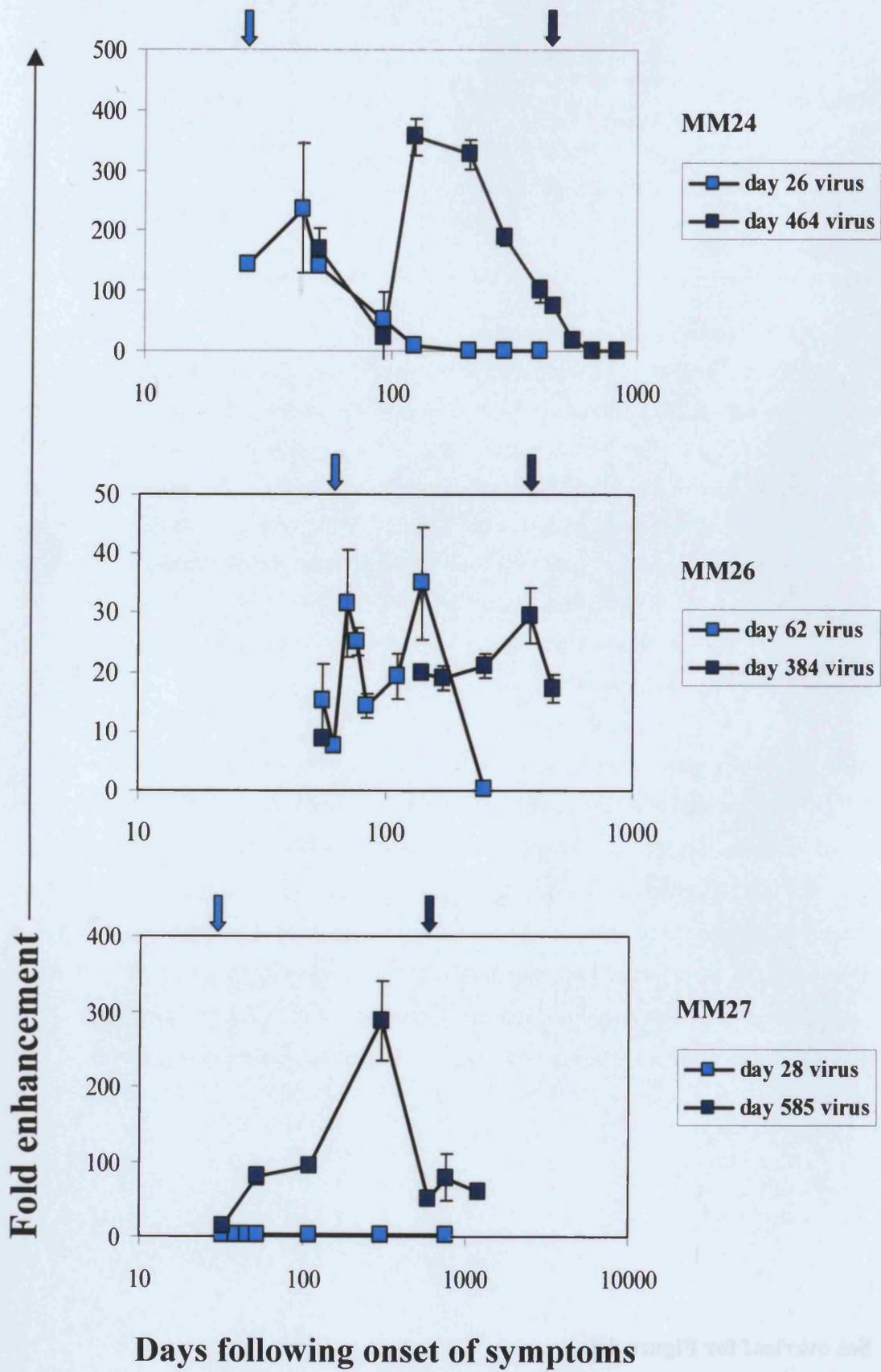
**Table 4.2 Parameters of enhanced infection**

To characterise the nature of enhanced infection, three different measurements were used. A virus isolate derived from patient MM24 26 days following onset of symptoms was incubated with control serum or day 44 autologous serum in the presence (C') or absence (HIC') of active complement for 1 hour at 37°C and then transferred onto SupT1/R5 cells. Determinants of infectivity were examined 5 days after infection. The number of infected cells was determined by intracellular staining for p24 antigen and subsequent flow cytometric analysis. The percentage of viable cells was determined by MTT assay, with 100% representing the level of viable cells in the absence of virus. RT production was measured in supernatant from infected cells using a commercial RT ELISA.

#### **4.2.3 C'-ADE of viruses isolated from later time points following infection**

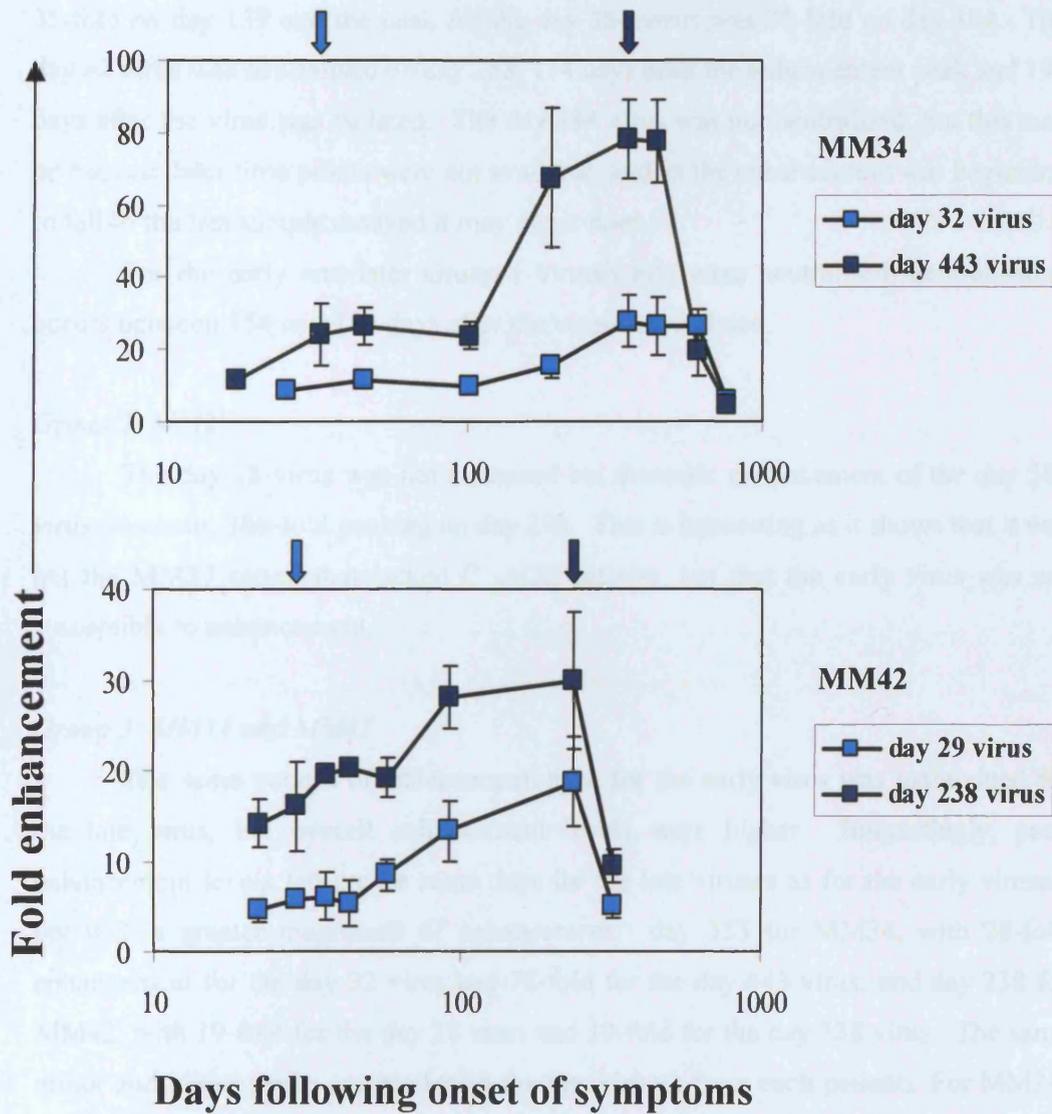
Viruses isolated early after infection were enhanced by autologous sera therefore it was decided to investigate enhanced infection of later virus isolates. This provided the opportunity to study enhancement in contemporary virus-serum pairs from two different time points. Also, the effect of serum time points taken before the virus was isolated could be examined to give information about the antibody environment in which the virus evolved. Representative patients from each of the patterns described in section 4.2.2 were used. Results are shown in Figure 4.8.

**See overleaf for Figure 4.8**



**Figure 4.8 C'-ADE of early and late viruses by autologous sera**

Viruses isolated at early and late time points following onset of symptoms were assayed on SupT1/R5 cells in the presence of C' and sequential autologous sera. Fold enhancement of infection of early viruses by autologous sera are shown as turquoise squares (■), and late virus as dark blue squares (■). The time at which the early virus was isolated is indicated with a turquoise arrow, and the late virus with a dark blue arrow. Error bars represent SD from 3 experiments.



For all patients except MM24, enhancement of later virus was seen to an equal or greater degree than with early virus. Again, the patients fall into the same three groups:

*Group 1: MM24 and MM26*

Sera that neutralised early virus enhanced later virus. Thus, virus that had escaped neutralisation maintained the capacity to be enhanced. For MM24 the peak of enhancement for the day 26 virus is 236-fold on day 44 and the peak for day 464 virus is 356-fold on day 124. The day 26 virus was neutralised at day 208, 164 days after the enhancement peak and 182 days after the virus was isolated. The day 464 virus was neutralised at day 660, 536 days after the enhancement peak and 196 days after the virus was isolated. For MM26 the peak of enhancement for the day 62 virus was 35-fold on day 139 and the peak for the day 384 virus was 30-fold on day 384. The day 62 virus was neutralised on day 253, 114 days after the enhancement peak and 191 days after the virus was isolated. The day 384 virus was not neutralised, but this may be because later time points were not available, and as the enhancement was beginning to fall in the last sample assayed it may occur later.

For the early and later Group 1 viruses that were neutralised, neutralisation occurs between 154 and 196 days after the virus was isolated

*Group 2: MM27*

The day 28 virus was not enhanced but dramatic enhancement of the day 585 virus was seen: 286-fold peaking on day 299. This is interesting as it shows that it was not the MM27 serum that lacked C'-ADE activity, but that the early virus was not susceptible to enhancement.

*Group 3: MM34 and MM42*

The same pattern of enhancement seen for the early virus was maintained for the late virus, but overall enhancement levels were higher. Interestingly, peak enhancement levels fell on the same days for the late viruses as for the early viruses, yet with a greater magnitude of enhancement: day 353 for MM34, with 28-fold enhancement for the day 32 virus and 78-fold for the day 443 virus; and day 238 for MM42, with 19-fold for the day 28 virus and 30-fold for the day 238 virus. The same minor and major peaks occurred with the late viruses from each patient. For MM34,

the first peak occurred on day 45 with a 26-fold enhancement, with the second occurring on day 353 with a 78-fold enhancement – again, the second peak was 3 times greater than the first, as with the early virus. For MM42 the difference between the peaks was not 3-fold, but a minor and major peak could still be distinguished: 20-fold on day 43 and 30-fold on day 238. These findings, along with data from the Group 3 early virus enhancement assays described in Section 4.2.2, are summarised in Table 4.3 below.

PATIENT	DAY OF VIRUS ISOLATION	1 <sup>ST</sup> (MINOR) PEAK		2 <sup>ND</sup> (MAJOR) PEAK	
		Day	Magnitude (fold)	Day	Magnitude (fold)
MM28	6	34	29	782	86
MM33	12	26	41	719	115
MM34	32	45	11	353	28
MM34	443	45	26	353	78
MM42	28	36	6	238	19
MM42	238	43	20	238	30

**Table 4.3 Details of the fold enhancement patterns found in Group 3**

With the exception of MM24, for all of the late viruses the peak of enhancement fell on or the time point before the day the virus was isolated. For MM24 the peak of enhancement for the late (day 464) virus was on day 124. The enhancement levels for the early and late viruses with contemporaneous sera is shown in Table 4.4. In all patients, except MM24, enhancement seen with contemporaneous virus-serum pairs was significantly greater for the later viruses.

PATIENT	DAY OF VIRUS ISOLATION	CONTEMPORANEOUS SERUM ENHANCEMENT <sup>1</sup>	P VALUE*
MM24	26	143	N/A
	464	76	
MM26	62	8	0.02
	384	32	
MM27	28	4	0.008
	585	50	
MM34	32	7	<0.001
	443	78	
MM42	29	6	<0.001
	238	30	

**Table 4.4 Comparison of C'-ADE levels in contemporaneous virus-serum pairs for early and late viruses**

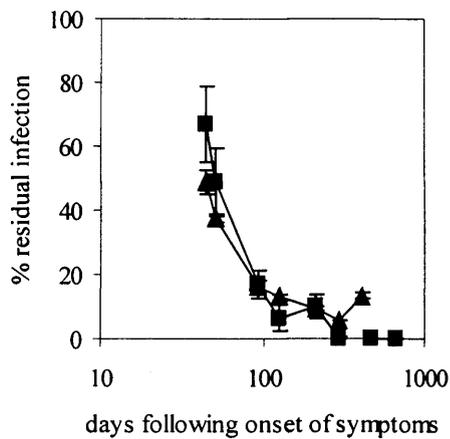
<sup>1</sup>Fold enhancement (in the presence of C') is shown for contemporaneous serum with early and late viruses. \*P values are derived from a two-tailed Student's T test assuming equal variance, based on the null hypothesis that the two means are equal.

#### **4.2.4 The relationship between C'-ADE and neutralisation**

While studies of neutralisation were possible in the enhancement assays, these assays were specifically developed to study enhancement and so the initial viral input was low. Therefore it was appropriate to confirm neutralisation data from the enhancement assay with a separate neutralisation assay routinely used in our laboratory. The neutralisation assay is described fully in Materials and Methods. Briefly, patient plasma, at a concentration of 10%, was incubated with autologous virus for 1 hour at 37°C, then inoculated onto NP2/CD4/R5 cells. After 2 hours cells were washed, then incubated for 2 days before fixing, staining for p24 and counting foci of infection.

Neutralisation in the enhancement assay was determined by the RT ELISA readings, as these are more sensitive at a low level of infection than the % infected cell measurements. As cells were left for 2 days after infection in the neutralisation assays, data were taken from the day 3 RT ELISA time points in the enhancement assay.

Figure 4.9 shows a comparison of neutralisation data taken from day 3 of an enhancement assay compared with data from a neutralisation assay. Results from other patients are comparable. Levels and timing of neutralisation were the same for data derived from both assays. This is important as it shows that the neutralising activity of the patient samples, whether derived from sera or plasma, was the same when assayed on different cell types as long as assays were carried out in the absence of active complement.



**Figure 4.9 Comparison of neutralisation seen in enhancement and neutralisation assays.**

Assays were performed using MM24 virus from day 26. Enhancement assays (triangles) were performed on SupT1/R5 cells with patient serum in the absence of C', and neutralisation assays (squares) on NP2/CD4/R5 cells with patient plasma. Error bars represent standard deviation from three experiments.

Some C'-independent ADE was detected in the enhancement assays, as described in Section 4.2.2. Whilst samples giving greater than 50% neutralisation remained comparable in the neutralisation and HIC' results of the enhancement assays, early non-neutralising samples enhanced infection even in the absence of complement on day 6 of the enhancing assay.

Figure 4.10 compares neutralisation by plasma on NP2/CD4/R5 cells with C'-ADE by serum on SupT1/R5 cells for viruses from 6 different patients. Unlike in previous neutralisation results shown in this thesis, graphs here are shown as % neutralisation rather than % residual infection in order to emphasise the divergence of neutralisation and enhancement levels. Figure 4.10 shows that after the appearance of antibodies capable of neutralising infection by greater than 90% a drop in enhancement levels was seen. Broken lines on the graphs show both the 90% neutralisation and the 5-fold enhancement thresholds. For patients MM24, MM25, MM26 and MM34, enhancement levels either dropped dramatically or disappeared completely at the point

when neutralisation exceeded 90%. In samples that neutralised to a level less than 90%, enhancement appeared to parallel the neutralisation pattern. This was shown most clearly for MM28, MM33 and MM34. In MM28 and MM33 neutralisation did not exceed 90% and the change in enhancement levels closely mirrored that of the neutralisation. For MM34, neutralisation and enhancement levels paralleled, until day 759 when >90% neutralisation occurred and enhancement dropped to just above the 5-fold threshold.

Table 4.5 below summarises the enhancement data for early viruses and the occurrence of neutralisation for all patient sera tested. The peak levels of enhancement obtained for each patient did not appear to correlate with the classification of the viral set-point shown in Table 4.1.

PATIENT NUMBER	VIRAL SET POINT	VIRUS <sup>1</sup>	PEAK (FOLD) <sup>2</sup>	DAY OF PEAK <sup>3</sup>	90% NEUT <sup>4</sup> (50%)
MM24	high	26	236	44	124 (51)
MM25	high	31	8	31	185 (66)
MM26	high	62	35	139	253 (76)
MM27	medium	28	3	299	- (46)
MM28	low	6	86	782	- (503)
MM32	N/A	10	58	21	- (-)
MM33	high	12	115	719	- (69)
MM34	low	32	28	353	759 (443)
MM38	N/A	29	46	93	N/D
MM42	high	28	19	238	N/D

**Table 4.5 Peak C'-ADE and appearance of neutralisation**

Summary of the magnitude and timing of the peak fold enhancement for each patient shown in Figure 4.5 and the appearance of neutralising antibodies shown in Figure 4.10. <sup>1</sup> The time, in days, following onset of symptoms at which the virus used in the assay was isolated. <sup>2</sup> The peak fold enhancement seen within a given serum set for each virus and <sup>3</sup> the sample day on which this occurs. <sup>4</sup> The day, following onset of symptoms, on which >90% neutralisation is seen and, in parentheses, >50% neutralisation is seen. N/A = not applicable; N/D = not done.

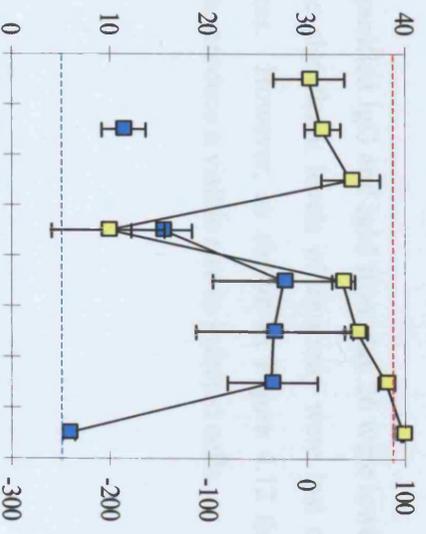
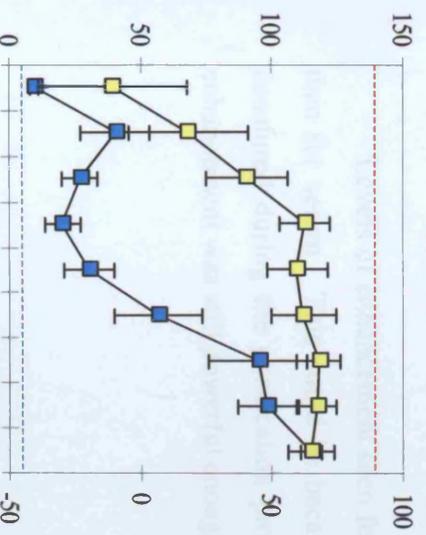
**Figure 4.10 Neutralisation and enhancement of HIV infectivity by autologous sequential plasma and serum samples.**

Neutralisation assays (yellow squares) were performed on NP2/CD4/R5 cells with heat inactivated patient plasma. Results are shown as % neutralisation relative to control plasma. Enhancement assays (turquoise squares) were performed on SupT1/R5 cells in the presence of patient sera and complement. Results are shown as fold enhancement of infection relative to control serum. Autologous early virus isolates are used for all experiments. The dashed yellow line indicates the 90% neutralisation threshold. The dashed turquoise line represents the 5-fold enhancement threshold. Error bars represent standard deviations from 3 experiments.

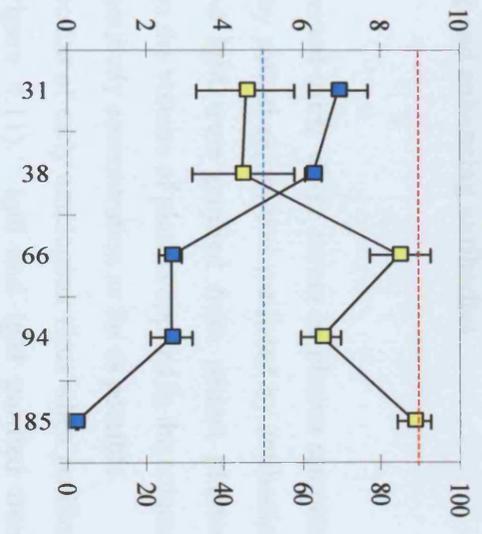
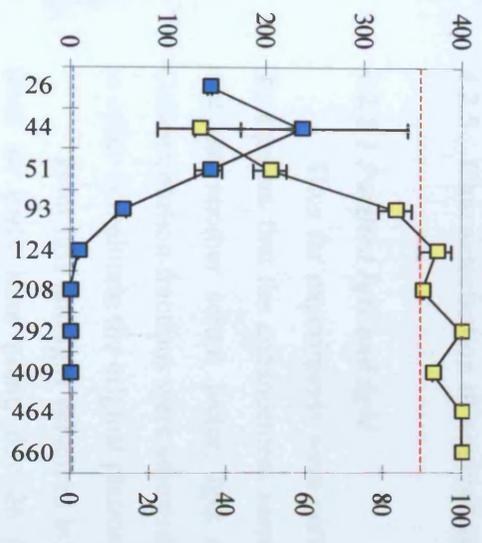
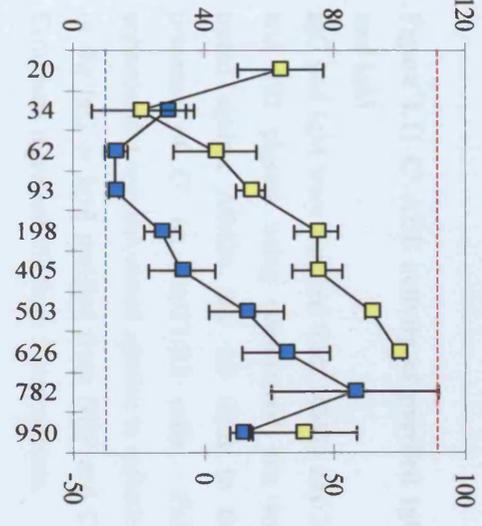
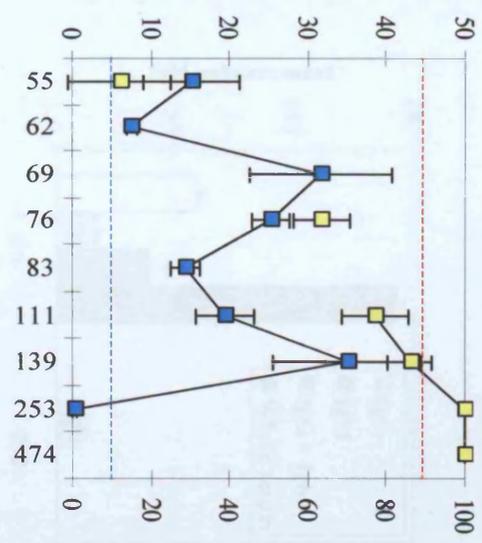
Days following onset of symptoms

154

Fold enhancement ■



% neutralisation ■

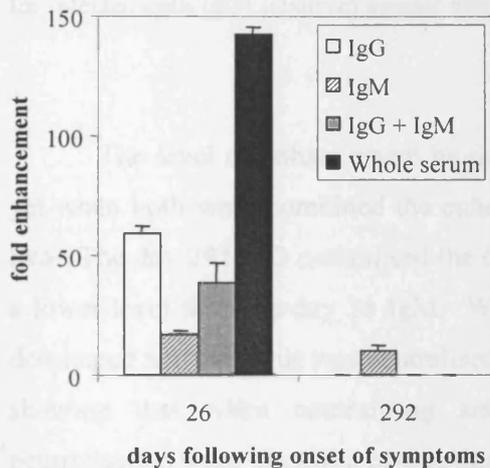


## 4.2.5 Characterisation of neutralising and enhancing antibodies

### 4.2.5.1 Purified IgG and IgM

Thus far experiments were performed using whole serum or plasma samples. To confirm that the enhancement seen by patient serum was mediated by antibodies and not another serum factor, IgG and IgM were purified from patient plasma. Column elution fractions were adjusted to the volume of plasma applied to the column in order to maintain the original plasma antibody concentration as far as possible.

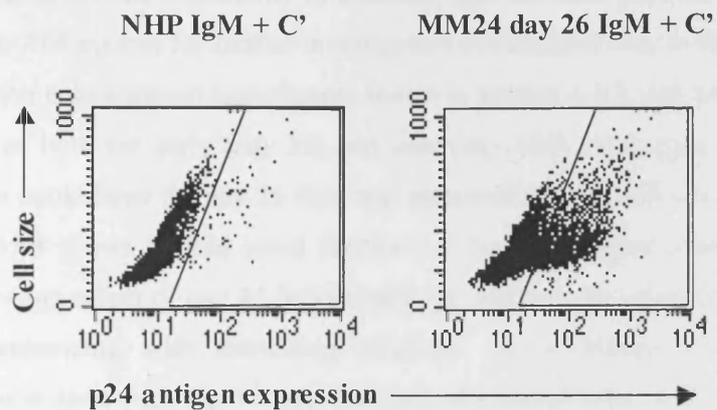
Both IgG and IgM enhanced infection of early autologous virus when purified from an early time point, day 26 (Figure 4.11). IgG and IgM purified from seronegative human plasma (NHP) were used as controls.



**Figure 4.11 C'-ADE activity of purified IgG and IgM**

IgG and IgM were purified from MM24 day 26 and 292 plasma using commercial kits and tested against MM24 day 26 virus in the presence of C' on SupT1/R5 cells. Fold enhancement was calculated relative to infection in the IgG or IgM purified from NHP and C'. Error bars represent SD from 3 experiments.

Levels of enhancement seen for purified IgG and IgM from day 26 were lower than for serum. This could be because significant levels of antibody were lost or denatured during the purification process. However, as shown in Figure 4.12 the enhancement was still powerful enough to produce a visible rise in infected cells.



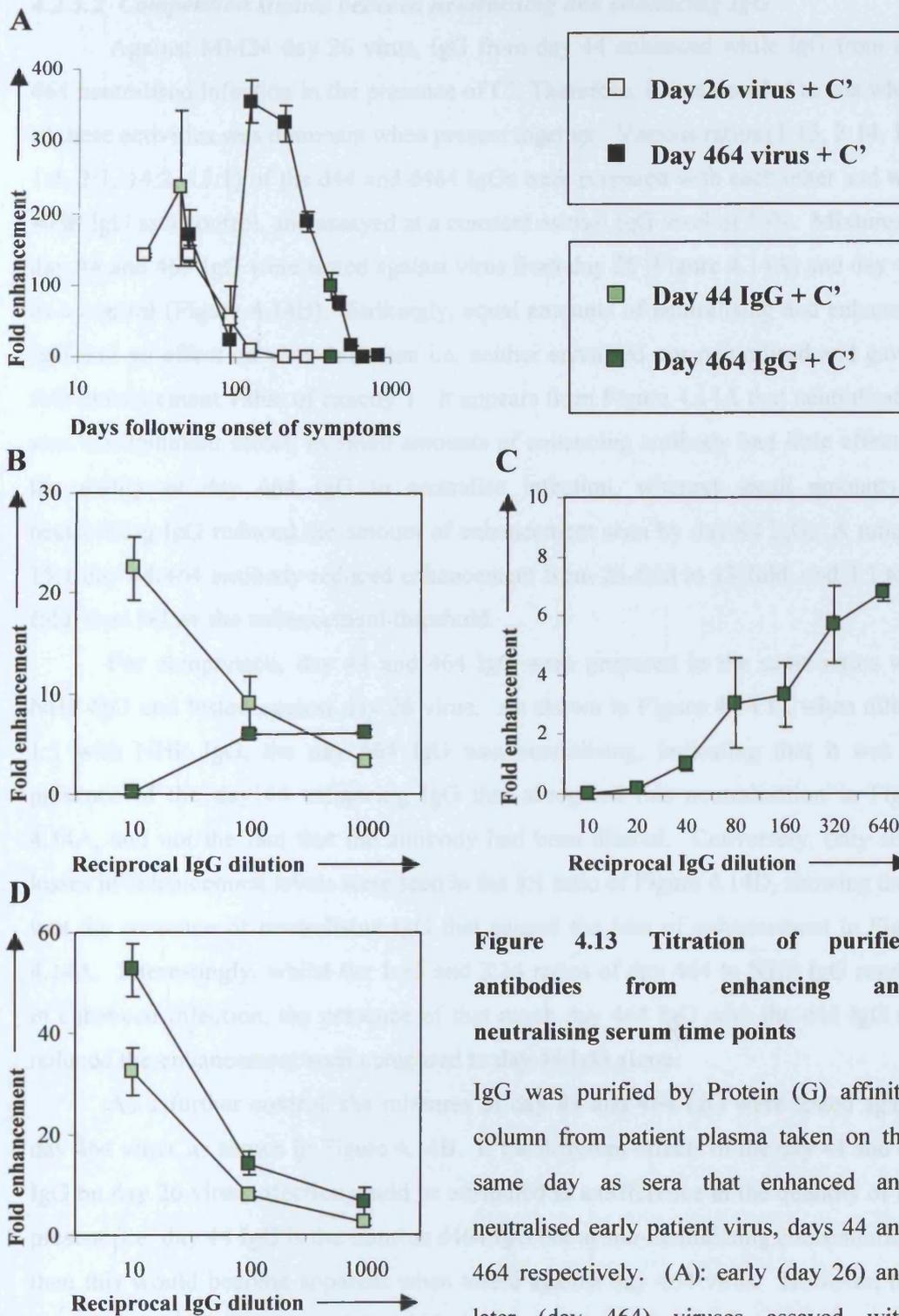
**Figure 4.12** Flow cytometry plots of MM24 day 26 virus infection in the presence and absence of day 26 IgM

SupT1/R5 cells were infected with MM24 day 26 virus, preincubated with IgM from NHP and C' (control infection; left), or IgM from MM24 day 26 plasma and C' (right). Cells were gated for infected cells (p24 positive) against uninfected cells incubated with C'.

The level of enhancement by day 26 IgM was lower than that for day 26 IgG, yet when both were combined the enhancement reached an intermediate between the two. The day 292 IgG neutralised the day 26 virus, whilst the IgM enhanced, albeit at a lower level than the day 26 IgM. When combined, however, the effect of the IgG dominated and the virus was neutralised. This supports data presented in Section 4.2.4 showing that when neutralising antibodies capable of achieving >90% virus neutralisation were present a co-existing enhancing response was suppressed.

Due to limited availability of material, IgG was later purified from MM24 day 44 and day 464 plasma for further investigation and assayed only in the presence of C'. In the serum enhancement experiments shown in section 4.2.3, day 44 serum enhanced infection of both the early (day 26) and later (day 464) autologous virus, whilst day 464 serum neutralised the day 26 virus and enhanced the day 464 virus (Figure 4.13A). Figure 4.13B shows 10-fold serial dilutions of the IgG assayed against day 26 virus. The enhancing effect of day 44 IgG titrated out, whereas the neutralising day 464 IgG became enhancing with increasing dilution. At a dilution of 1:100 the fold enhancement seen with day 44 and 464 IgG was comparable, and at 1:1000 the day 464 IgG was still enhancing while the day 44 IgG had dropped below the 5-fold enhancement threshold. Figure 4.13C shows serial 2-fold dilutions of the day 464 IgG to focus in on the point at which neutralisation became enhancement. This process was gradual rather than occurring at a critical dilution point.

The IgG was then tested against the day 464 virus at 10-fold serial dilutions (Figure 4.13D). As expected, both the day 44 and 464 IgG enhanced the later virus and this effect could be diluted out in the same way as against the early virus.



**Figure 4.13 Titration of purified antibodies from enhancing and neutralising serum time points**

IgG was purified by Protein (G) affinity column from patient plasma taken on the same day as sera that enhanced and neutralised early patient virus, days 44 and 464 respectively. (A): early (day 26) and later (day 464) viruses assayed with sequential sera. Light and dark green squares show the time points, days 44 and 464 respectively, from which IgG was isolated. (B): 10-fold serial dilutions of IgG tested against day 26 virus. (C): Further 2-fold dilutions of day 464 IgG tested against day 26 virus to show the point at which neutralisation becomes enhancement. (D): 10-fold serial dilutions of IgG tested against day 464 virus.

#### 4.2.5.2 Competition studies between neutralising and enhancing IgG

Against MM24 day 26 virus, IgG from day 44 enhanced while IgG from day 464 neutralised infection in the presence of C'. Therefore, it was decided to test which of these activities was dominant when present together. Various ratios (1:15, 2:14, 1:3, 1:1, 3:1, 14:2, 15:1) of the d44 and d464 IgGs were prepared with each other and with NHP IgG as a control, and assayed at a constant overall IgG level of 10%. Mixtures of day 44 and 464 IgG were tested against virus from day 26 (Figure 4.14A) and day 464 as a control (Figure 4.14B). Strikingly, equal amounts of neutralising and enhancing IgG had no effect on virus infection i.e. neither enhanced nor neutralised and gave a fold enhancement value of exactly 1. It appears from Figure 4.14A that neutralisation was the dominant effect, as small amounts of enhancing antibody had little effect on the ability of day 464 IgG to neutralise infection, whereas small amounts of neutralising IgG reduced the amount of enhancement seen by day 44 IgG. A ratio of 15:1 day 44:464 antibody reduced enhancement from 23-fold to 13-fold, and 3:1 to 4-fold, thus below the enhancement threshold.

For comparison, day 44 and 464 IgG were prepared in the same ratios with NHP IgG and tested against day 26 virus. As shown in Figure 4.14 C, when diluted 1:1 with NHP IgG, the day 464 IgG was neutralising, indicating that it was the presence of the day 44 enhancing IgG that abrogated this neutralisation in Figure 4.14A, and not the fact that the antibody had been diluted. Conversely, only slight losses in enhancement levels were seen in the 1:1 ratio of Figure 4.14D, showing that it was the presence of neutralising IgG that caused the loss of enhancement in Figure 4.14A. Interestingly, whilst the 1:15 and 2:14 ratios of day 464 to NHP IgG resulted in enhanced infection, the presence of that much day 464 IgG with the d44 IgG still reduced the enhancement seen compared to day 44 IgG alone.

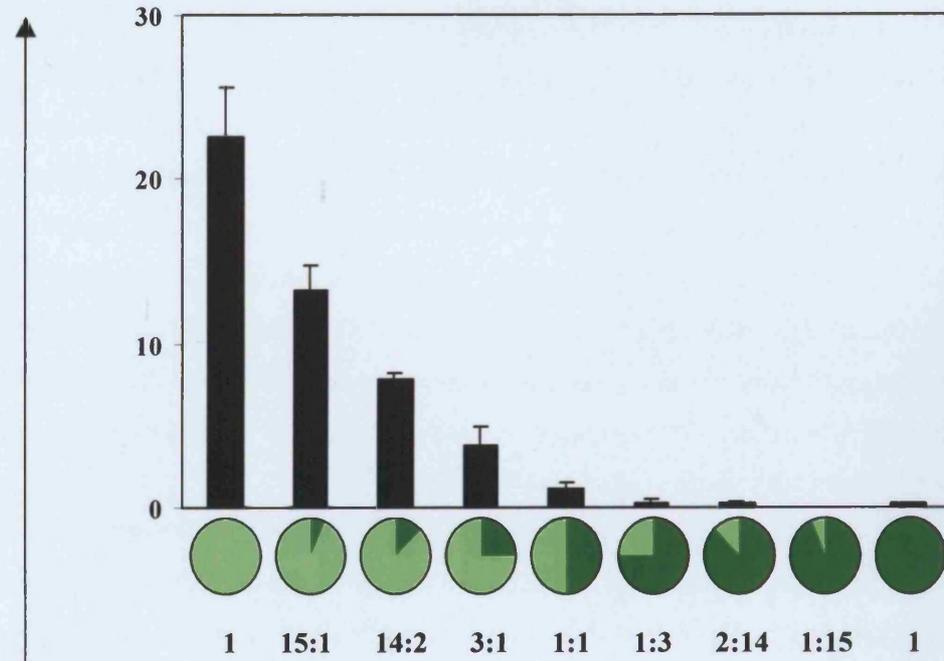
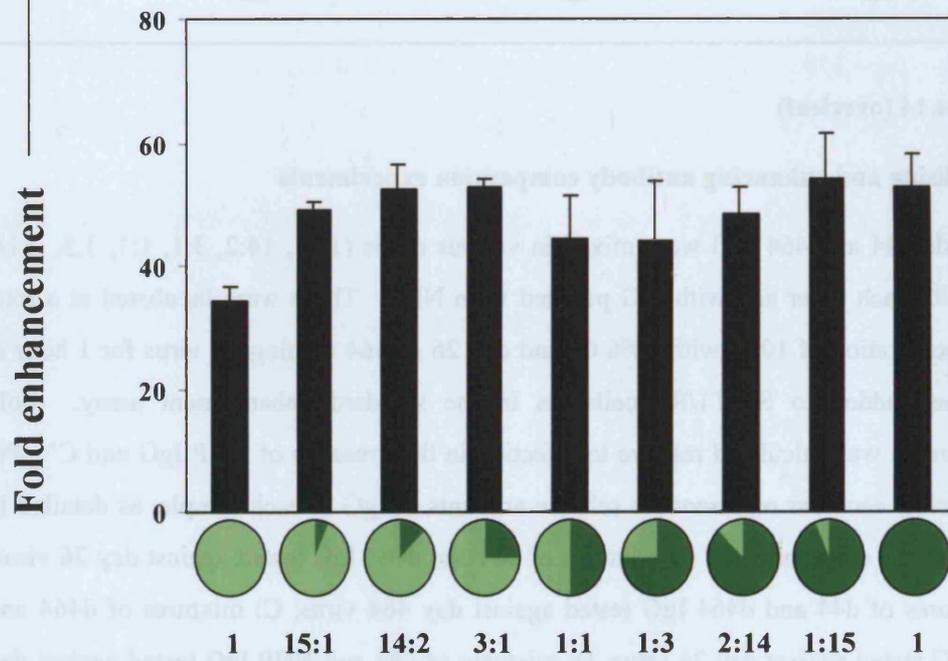
As a further control, the mixtures of day 44 and 464 IgG were tested against day 464 virus, as shown in Figure 4.14B. If the different effects of the day 44 and 464 IgG on day 26 virus infection could be attributed to a difference in the quantity of IgG present (i.e. day 44 IgG is the same as d464 IgG but at sub-neutralising concentrations) then this would become apparent when tested against day 464 virus. However, both day 44 and 464 IgG enhanced day 464 virus to a high level, and mixing various ratios of the two antibody preparations had no effect.



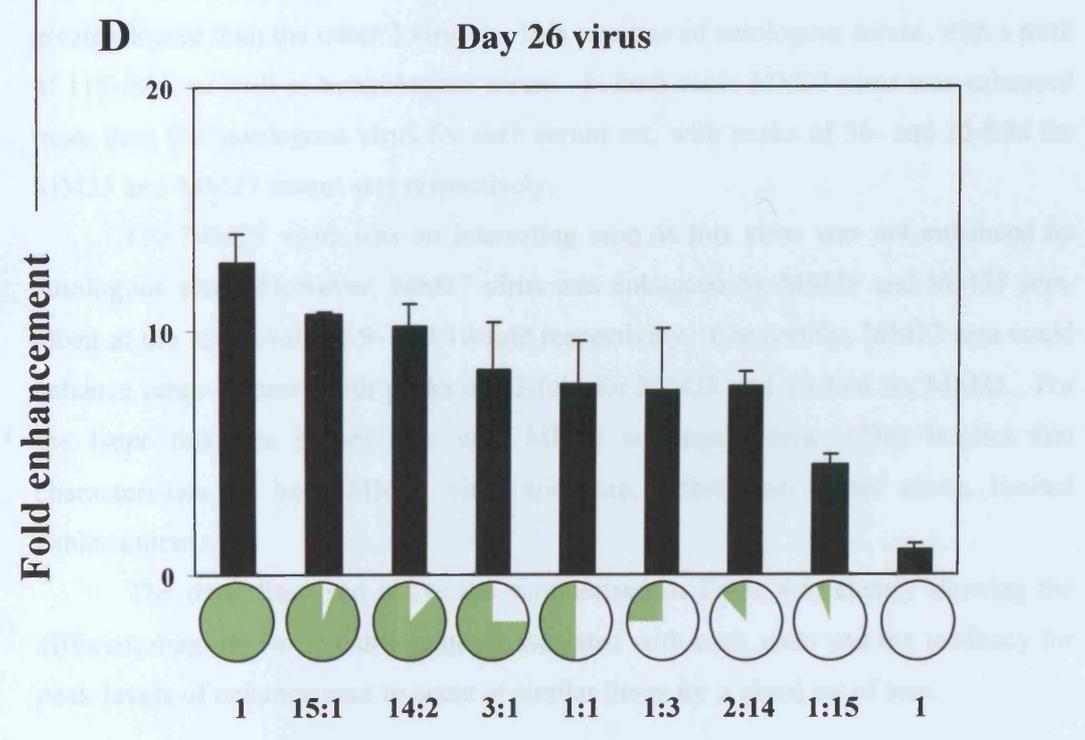
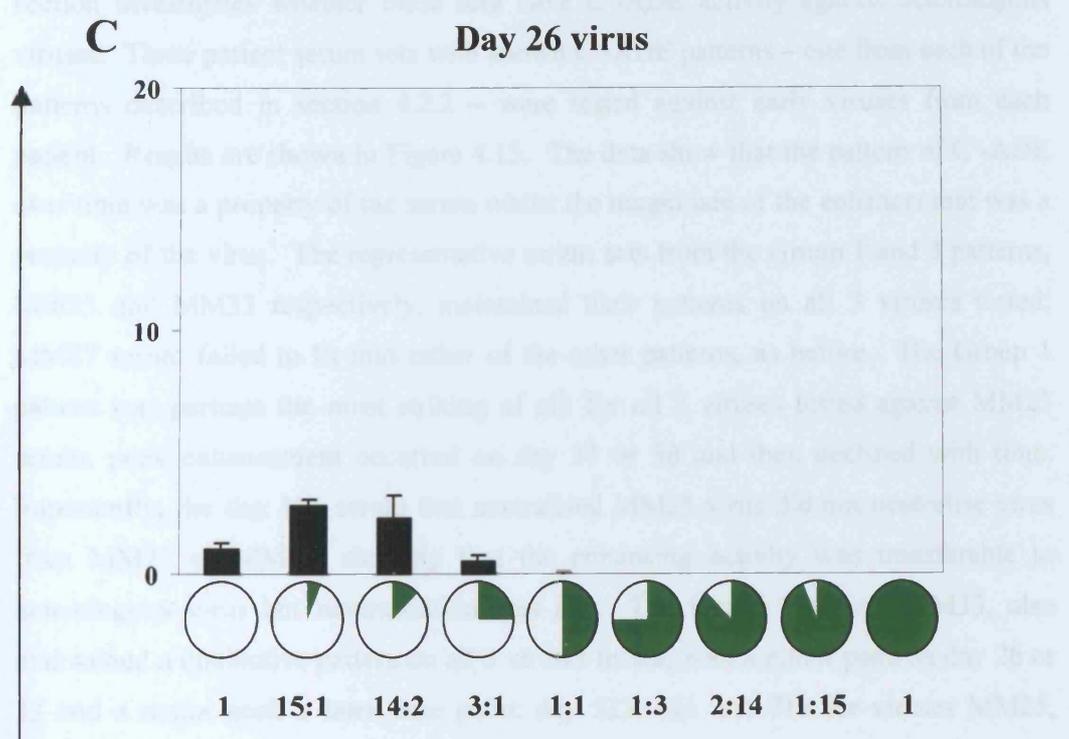
**Figure 4.14 (overleaf)**

**Neutralising and enhancing antibody competition experiments**

MM24 day 44 and 464 IgG were mixed in various ratios (15:1, 14:2, 3:1, 1:1, 1:3, 2:14, 1:15) with each other and with IgG purified from NHP. These were incubated at a total IgG concentration of 10% ,with 10% C' and day 26 or 464 autologous virus for 1 hour at 37°C then added to SupT1/R5 cells, as in the standard enhancement assay. Fold enhancement was calculated relative to infection in the presence of NHP IgG and C'. Pie charts below each bar represent the relative amounts of IgG in each sample, as detailed in the key on the adjacent page. A: mixtures of d44 and d464 IgG tested against day 26 virus; B: mixtures of d44 and d464 IgG tested against day 464 virus; C: mixtures of d464 and NHP IgG tested against day 26 virus; D: mixtures of d44 and NHP IgG tested against day 26 virus. Error bars represent SD from three replicates.

**A****Day 26 virus****B****Day 464 virus**

Fold enhancement



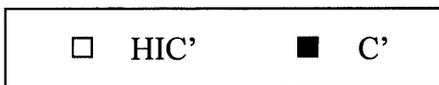
#### 4.2.6 C'-ADE of heterologous viruses

Results presented so far addressed C'-ADE in an autologous system. This section investigates whether these sera have C'-ADE activity against heterologous viruses. Three patient serum sets with known C'-ADE patterns – one from each of the patterns described in section 4.2.2 – were tested against early viruses from each patient. Results are shown in Figure 4.15. The data show that the pattern of C'-ADE over time was a property of the serum whilst the magnitude of the enhancement was a property of the virus. The representative serum sets from the Group 1 and 3 patterns, MM25 and MM33 respectively, maintained their patterns on all 3 viruses tested; MM27 serum failed to fit into either of the other patterns, as before. The Group 1 pattern was perhaps the most striking of all: for all 3 viruses tested against MM25 serum, peak enhancement occurred on day 31 or 38 and then declined with time. Importantly, the day 185 serum that neutralised MM25 virus did not neutralise virus from MM27 or MM33, showing that the enhancing activity was transferable to heterologous virus but neutralisation was not. The Group 3 serum, MM33, also maintained a distinctive pattern on all 3 viruses tested, with a minor peak on day 26 or 33 and a major peak a later time point: day 523, 621 and 719 for viruses MM25, MM27 and MM33 respectively.

In terms of magnitude of enhancement, virus from MM33 was enhanced to a greater degree than the other 2 viruses. This was true of autologous serum, with a peak of 115-fold, as well as heterologous serum. In both cases MM33 virus was enhanced more than the autologous virus for each serum set, with peaks of 36- and 25-fold for MM25 and MM27 serum sets respectively.

The MM27 virus was an interesting case as this virus was not enhanced by autologous sera. However, MM27 virus was enhanced by MM25 and MM33 sera, albeit at the low levels of 9- and 10-fold respectively. Conversely, MM27 sera could enhance other viruses, with peaks of 33-fold for MM33 and 10-fold for MM25. For the latter this was higher than with MM25 autologous sera. This implies that characteristics of both MM27 virus and sera, rather than either alone, limited enhancement.

The data discussed above are summarised in Table 4.6, clearly showing the different magnitudes of enhancement associated with each virus and the tendency for peak levels of enhancement to occur at similar times for a given set of sera.



**Figure 4.15 (overleaf)**

**C'-ADE by heterologous serum sets**

Early virus and sequential serum sets from 3 patients were tested against each other to investigate heterologous C'-ADE. Representatives were chosen from each of the 3 groups described in Section 4.2.2. Virus was incubated with patient serum or NHS in the presence of HIC' (white squares) or C' (black squares) for 1 hour then transferred to SupT1/R5 cells and incubated for 6 days. Fold enhancement is calculated relative to infection in the presence of NHS. The patient from which virus was derived is indicated on the far left in yellow, and serum is indicated at the top in red. Results from autologous virus-serum sets are edged in green. Error bars represent SD from 3 experiments.

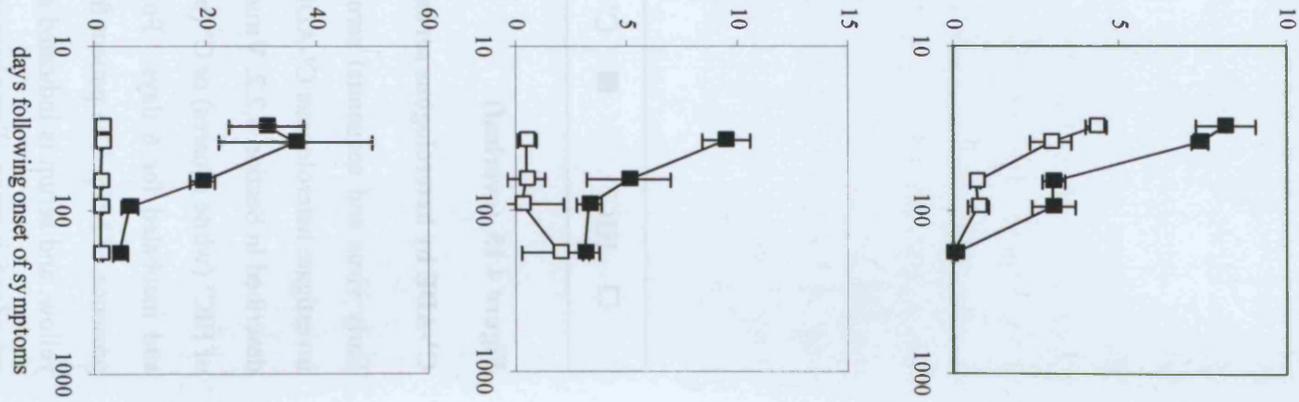
# VIRUS

MM33

MM27

MM25

Fold enhancement

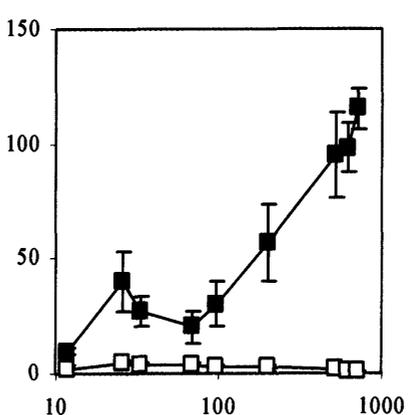
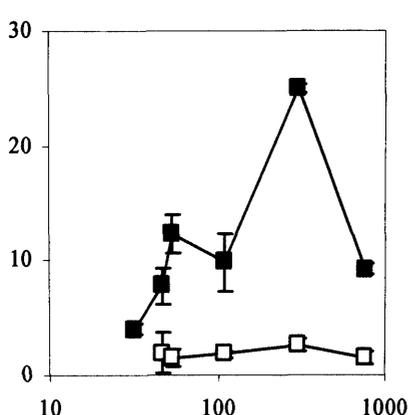
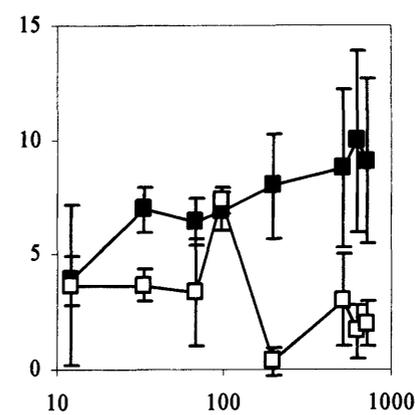
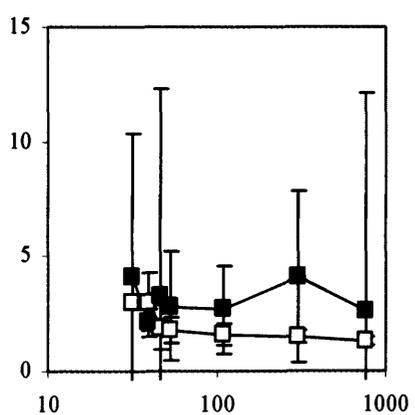
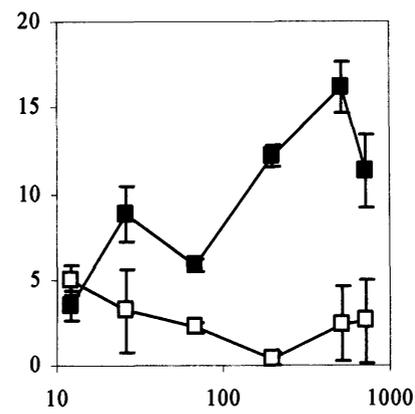
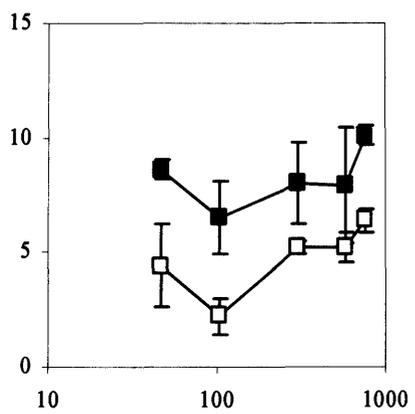


MM25

# SERUM

**MM27**

**MM33**



days following onset of symptoms

days following onset of symptoms

		SERUM					
		MM25		MM27		MM33	
		Peak <sup>1</sup>	Day <sup>2</sup>	Peak	Day	Peak	Day
VIRUS	MM25	8	31	10	755	16	523
	MM27	9	38	4	<b>299</b>	10	621
	MM33	36	38	25	299	<b>115</b>	<b>719</b>

**Table 4.6 C'-ADE in heterologous virus-serum sets**

Enhancement assays on SupT1/R5 cells were carried out with combinations of viruses and serum sets to investigate C'-ADE by heterologous sera. Results were summarised from Figure 4.15. Serum sets from 3 patients, MM25, MM27 and MM33, were tested against early viruses from each patient. Results are shown as <sup>1</sup>the peak fold enhancement seen for a given serum set and <sup>2</sup>the serum sample day on which this peak occurs. Numbers in bold type are the results for autologous virus-serum sets.

## 4.3 DISCUSSION

### 4.3.1 C'-ADE of early viruses in comparison to previous reports

The frequency and magnitude of C'-ADE shown in this chapter are surprising. Recent literature on C'-ADE of HIV showed levels of enhancement to be closer to those shown in Chapter 3, with 2- or 3-fold increases in infection routinely reported (Banhegyi et al., 2003; Lund et al., 1995; Prohaszka et al., 1997; Szabo et al., 1999). As HIV is a chronic infection, proceeding over several years, these enhancement levels are considered important as a 2-3-fold increase in infection carried over numerous cycles of infection would have a significant effect on overall virus production. Therefore it had previously been accepted that levels of HIV enhancement were not as dramatic as those of dengue. Here this is challenged, with levels of enhancement reaching almost 350-fold, well within the range of dengue virus enhancement, which is commonly reported to increase levels of infection by 2-200 fold. (Halstead and O'Rourke, 1977a; Halstead and O'Rourke, 1977b) The high levels of enhancement

were consistent when measured as the percentage of cells infected, the percentage of remaining viable cells, and the level of RT production.

The use of a CR2<sup>+</sup> T cell line expressing CCR5 may account for the high enhancement level. As other T cell lines used for studies of C'-ADE lack CCR5, investigation has been limited to X4 tropic viruses such as the TCLA strain IIIB. As shown in Chapter 3, in my experience enhancement of this virus is weak, often variable, and observed far less frequently than for the patient-derived R5 tropic viruses. To exclude the possibility that the high enhancement levels were artefacts of using SupT1/R5 cells, NP2/CD4/R5 cells transduced to stably express CR2 were used for enhancement assays in Chapter 6.

Aspects of my study reflect results from SIV infection of macaques presented by Montefiori and colleagues (Montefiori et al., 1995). In their study, macaques were inoculated with the pathogenic SIV<sub>mac251</sub>, and sequential early serum samples tested for C'-ADE against the inoculum virus. Peak powers of enhancement were seen in early sera, most commonly at the lowest serum dilution tested. Powers of enhancement decreased over time as neutralisation emerged. These results resemble my results from the Group 1 patient early virus experiments (Section 4.2.2). However, the power of enhancement decreased over time as neutralisation emerged, yet no studies were made of later virus isolated from the macaques. As later viruses isolated from Group 1 patients in my study were enhanced by sera that neutralised early virus, the same may occur in the macaques if later isolates were investigated. Therefore their results are realistically only semi-autologous, and are only likely to represent the very early events following infection with SIV<sub>mac251</sub> before significant diversification of the virus has occurred. To a certain extent my results have the same limitations, as each serum sample tested was not matched by a contemporaneous virus isolate, but tested against an isolate from a fixed early or late time point.

#### **4.3.2 Reconciliation of current neutralisation models with C'-ADE results**

No evidence of neutralisation of autologous virus by contemporaneous sera was found. It is therefore possible that enhancing antibodies are the dominant antibody response throughout infection, due to rapid virus escape from neutralisation. Competition studies showed that, when present, neutralisation is a more powerful response than enhancement.

The current neutralisation model for HIV states that neutralising antibodies bind to functional Env spikes and inhibit viral entry via steric hindrance at a critical level of occupancy. Meanwhile, non-functional Env spikes elicit non-neutralising antibodies, constituting an immunogenic “decoy” to direct the immune response towards epitopes considered non-essential for virus infection. Linking my results in with this model, there are two interpretations of the enhancement data:

1. Neutralising antibodies bind functional spikes while enhancing antibodies bind non-functional spikes, such as gp41 stumps.
2. Enhancing antibodies are NABs at sub-neutralising concentrations.

I favour the first interpretation, for several reasons. Firstly, neutralisation can often be observed in the absence of C', yet when complement is added the neutralisation is abrogated. This indicates that the virus is coated by antibody, and therefore it is not a question of low levels of neutralising antibodies binding to virus to enhance infection. Model 1 fits my data and previous literature of C'-ADE better. It would explain the discovery of a gp41 “enhancing domain”, which happens to overlap the gp41-gp120 interacting region and therefore would only be exposed after shedding of gp120.

#### **4.3.3 The nature of enhancing antibodies – clues from studies of heterologous virus and sera.**

Further clues about the differences between enhancing and neutralising antibodies are provided by data on heterologous enhancement. The fact that heterologous sera can enhance viruses shows that there is cross-reactivity, yet only in terms of enhancement and not neutralisation. This could suggest that neutralising antibodies must bind specific epitopes in order to neutralise, whereas enhancing antibodies must simply be able to bind to the virus. Alternatively, it could be that the enhancing antibodies recognise a more conserved epitope, such as in gp41, whilst the neutralising antibodies recognise more variable epitopes, probably as a result of the fact that these regions are targeted by neutralising antibodies and are constantly evolving.

#### **4.3.4 C'-ADE and C'-ML - is there scope for both to occur?**

As discussed in Chapter 3, the producing cell type can have an effect on whether or not virus is susceptible to lysis by complement. Perhaps the preparation differences between the IIB used in Chapter 3 and the primary isolates used in this

chapter contributes to the different levels of enhancement seen. Yet, as production of virus in PBMCs is more physiologically relevant than in the T cell line H9, results presented in this chapter are more representative of what might occur *in vivo* than those in Chapter 3.

Other studies have reported that inactivation (C'-MI) of early autologous virus occurs in the presence of early autologous sera and complement (Aasa-Chapman et al., 2005; Huber et al., 2006). There may be several explanations for the differences seen between these and my studies. Firstly, it is possible that C'-ADE occurs on CR2+ cellular targets, while C'-MI occurs on CR2- cellular targets as reported by Aasa-Chapman and colleagues (Aasa-Chapman et al., 2005). This is investigated in more detail in Chapter 5. Therefore the cell type first encountered by the virus might determine the outcome of the opsonisation, provided that lysis does not occur in the majority of viruses. This interpretation does not agree with results presented by (Huber et al., 2006), who used a freeze-thaw assay system to show lysis of primary patient HIV isolates by early autologous sera and complement. However, while lysis is inferred, the involvement of the MAC was not formally demonstrated. In addition, the proportion of lysis in each virus preparation was not directly correlated with infectivity of each strain following opsonisation, so it is possible that a proportion of the viruses are lysed, yet these viruses are in the portion that appear uninfected. In theory, if a given viral stock has an infectious particle-to-particle ratio of 1:1000, then 50% of these virions could be lysed while the remaining particles could account for a maximum of 500-fold enhancement. Alternatively, increased lysis in the freeze-thaw assay may be a measure of complement-opsonisation of the virus rather than MAC-mediated lysis. Virions with higher membrane protein contents may be more susceptible to rupture by freeze-thaw cycles than those with less surface-associated protein. Another possibility is that lysis does occur in the enhanced viruses, but instead of rendering the viruses uninfected it facilitates virus-cell fusion. This could be investigated using C5-deficient serum as a complement source for enhancement assays. To investigate the occurrence of both C'-mediated lysis and C'-ADE, parallel assays on autologous virus-serum pairs should be undertaken and compared directly with the infectivity of the autologous virus.

#### 4.3.5 Evidence for the occurrence of C'-ADE *in vivo*

Unlike in Chapter 3, where the same virus, IIIB, was tested with each serum sample, drawing direct correlations between C'-ADE and viral loads in this chapter was complicated by the fact that different viruses were used for each patient, and sometimes two different viruses, early and late, were used per patient. As mentioned in section 4.3.1, the most representative method of comparing C'-ADE longitudinally with viral load would be to analyse sequential contemporaneous virus-serum pairs. This approach was not feasible due to the labour-intensive and technically demanding method of virus propagation, therefore only early and late viruses from each patient were studied. Neutralising antibodies from later sera therefore interfere with the C'-ADE profile by neutralising early virus.

However, conclusions may be drawn from the fact that all viruses tested were enhanced by contemporary autologous serum, meaning that enhancement is possible *in vivo*. With the exception of MM24, later isolates were enhanced significantly more by autologous contemporary sera than early isolates. Therefore, later viruses that have escaped neutralisation maintained their capacity for enhancement, and in most cases were enhanced to a greater magnitude than early isolates, suggesting an evolutionary advantage of increasing the capacity to be enhanced. The peak enhancement time points for all late viruses tested occurred on or before the virus isolation day, adding weight to the suggestion that these viruses have evolved due to their capacity for enhancement.

Several studies have shown the existence of CR2<sup>+</sup> CD4<sup>+</sup> T cells in peripheral blood, which provides the possibility that C'-ADE could occur *in vivo* (Fischer et al., 1991; Fischer et al., 1999; June et al., 1992; Masilamani et al., 2002). One study showed that the proportion of CR2<sup>+</sup> CD4<sup>+</sup> T cells was lower in HIV-infected individuals compared with uninfected individuals, implying destruction of these cells by HIV (June et al., 1992). Alternatively, C'-ADE could be an *in vitro* surrogate marker for the important process of FDC trapping of opsonised virus. This would represent an equally important pathogenic activity of C'-ADE antibodies, and ought to be investigated in more detail, perhaps using an *in vitro* germinal centre model as previously described (Heath et al., 1995).

#### **4.3.6 Concluding remarks**

This chapter shows the successful application of the enhancement assay developed in Chapter 3 to the investigation of C'-ADE in natural infection with HIV. For the first time, sequential serum samples from early to chronic infection were tested with autologous early virus and produced levels of enhancement up to 236-fold. When later virus isolates from the same individuals were tested with the same serum samples, enhancement levels were equivalent to or greater than those seen with early virus, with enhancement levels reaching 356-fold.

The importance of C'-ADE in natural infection remains to be seen. The high levels of C'-ADE, the fact that C'-ADE occurs in contemporaneous virus-serum pairs, and the fact that later isolates from the same patients were enhanced to an equal or greater degree than early isolates, sometimes having escaped neutralisation, all suggest that C'-ADE may play a role in HIV infection and should be further investigated.

## **CHAPTER 5**

### **Infection-enhancing antibodies from individuals vaccinated with an envelope subunit vaccine**

This chapter investigates the appearance of antibodies with C'-ADE and C'-mediated inactivation (C'-MI) activity after immunisation with a HIV gp120 subunit vaccine. Sequentially collected serum samples taken from individuals following vaccination were tested against the TCLA strain MN and the vaccine virus strain W61D for enhancement, neutralisation and C'-MI. Finally, to investigate the potential for antibodies elicited by vaccination to enhance infection of currently circulating virus strains, sera were tested against a primary virus isolate from the patient cohort investigated in Chapter 4.

#### **5.1 INTRODUCTION**

##### **5.1.1 The importance of studying enhancement in the context of vaccination**

Elicitation of enhancing antibodies by vaccination could lead to an increased risk of infection or exacerbated disease symptoms. There have been several cases of increased disease severity in human vaccine recipients (Morens, 1994). Whether this is linked to enhancing antibodies has not been conclusively proven due to the possibility of alternative mechanisms of immunopathology. In animal studies, however, the evidence linking enhancing antibodies and vaccine-related disease exacerbation is more convincing. This is mainly due to results from experimental infections following vaccination or passive antibody transfer of the target animals. Studies of feline infectious peritonitis virus (FIPV), a coronavirus infection of cats that is predominantly subclinical, have shown that experimental infection of cats passively immunised with antibodies to FIPV resulted in a rapid and exacerbated disease state compared to seronegative cats (Weiss and Scott, 1981). Likewise, attempted immunisation with an avirulent FIPV strain increased the chances of infection with challenge virus (Pedersen and Black, 1983).

Disease enhancement occurred in ponies upon challenge after an EIAV envelope subunit vaccination, with vaccinated ponies developing higher plasma viraemia and succumbing earlier to a more severe form of acute disease than

unvaccinated ponies (Raabe et al., 1998; Wang et al., 1994). Nonetheless, EIAV enhancement assays tested to date are not conclusively predictive of vaccine outcome, suggesting the disease enhancement has an added level of complexity (Raabe et al., 1999). Enhanced FIV disease has been observed in cats upon challenge following FIV immunisations of various strategies: DNA vaccination (Richardson et al., 1997), envelope subunit vaccination (Siebelink et al., 1995) and cell-based vaccination (Giannecchini et al., 2002).

Of the above examples, EIAV and FIV are lentiviruses, making the investigation of HIV vaccine-related disease exacerbation particularly pertinent. SIV vaccine studies in macaques, developed as a model for HIV vaccination and challenge, have also produced enhancing antibodies. In a thorough study of C'-ADE following immunisation of macaques with SIV rgp160, no clear relationship was found between titres and power of C'-ADE and vaccine outcome; despite high levels of C'-ADE antibodies some animals were protected and some infected following challenge with a homologous virus strain (Montefiori et al., 1995). However, it should be borne in mind that these studies were conducted with challenge viruses identical or closely resembling the vaccine strain, therefore in many cases a degree of protection was observed – a situation that is unlikely to occur in field settings of HIV vaccine trials. In a study in which macaques were not protected from virulent virus challenge following DNA vaccination, C'-ADE was implicated in vaccine failure, but again, no clear relationship between enhancing antibodies and vaccine outcome was determined (Lu et al., 1996).

A recent VZV/SIV vaccine trial in macaques resulted in enhanced disease severity, with vaccinated macaques exhibiting higher viral loads and progressing to AIDS dramatically faster than the unvaccinated control animals (Staprans et al., 2004). Enhancing antibodies were not detected and the accelerated disease progression was attributed to CD4 cell priming and increased activation of CD4 T cells upon virus challenge (Staprans et al., 2004). A lack of enhancing antibodies in one particular assay, however, does not rule out their involvement entirely, and the examples cited above highlight the difficulties involved in linking cause and effect of vaccine-related disease enhancement.

A prophylactic vaccine against HIV infection remains an elusive target and vaccine trials in various stages are being undertaken throughout the world. It is accepted that a neutralising antibody response will form an important component of an

effective vaccine (Moore and Burton, 2004). Many initial attempts at eliciting protective antibody responses through vaccination focused on the use of recombinant HIV envelope proteins: gp160 or gp120. A high-profile vaccine trial involving a recombinant envelope subunit vaccine (VaxGen/AIDS VAX) was conducted recently (Billich, 2004). This trial attracted controversy due to the belief of many researchers that this strategy had been previously tested and proven ineffective (Johnston, 2003; Watanabe, 2003). Yet, to date it is the only vaccine candidate to reach phase III clinical trials. Disease enhancement has not been seen following HIV vaccination trials in humans. But the risk remains, as many HIV vaccine trials are in their infancy and it is possible that ADE may still occur. Particular concerns have been raised about the use of envelope subunit vaccines against HIV (Weiss, 2001) as monomeric envelope subunits are known to elicit non-neutralising antibodies, and have yet to show any evidence of inducing a protective neutralising response. These antibodies may therefore be enhancing in certain situations.

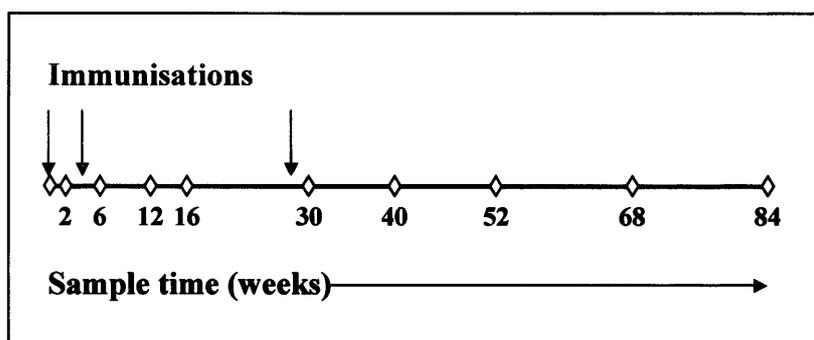
It is unclear from *in vitro* studies whether HIV vaccines can elicit enhancing antibodies. One study that investigated FcR-ADE found no evidence of enhancement following vaccination with an rgp160 subunit vaccine derived from NL4-3, a strain similar to IIIB (Haubrich et al., 1992). Another study, investigating C'-ADE in samples from a similar vaccine trial, found antibodies with C'-ADE activity in 6 out of 24 subjects tested; neutralising activity was found in only 5 of the 24 subjects (Dolin et al., 1991). Both of these studies were conducted using IIIB. To date, no studies of C'-ADE have been conducted on vaccinee sera using clinically relevant HIV strains or HIV vaccine strains other than IIIB.

Given the evidence of *in vitro* ADE of HIV, combined with reports of disease enhancement in vaccine trials of other lentiviruses, further information on the risks and mechanisms of HIV ADE is warranted. On the other hand, it is possible that vaccine-elicited non-neutralising antibodies could have a protective effect through mechanisms such as C'-MI. The aims of this chapter, therefore, are to investigate the functional activities of the antibodies produced following vaccination with an envelope subunit vaccine. This also provides the opportunity to investigate the co-existence of C'-MI, using the assay presented by Aasa-Chapman and colleagues (Aasa-Chapman et al., 2005) and C'-ADE, using the enhancement assay described in Chapter 4, in serum samples that have been previously thoroughly characterised for neutralising and binding antibody activity (Beddows et al., 1999; McCormack et al., 2000). This will

allow direct comparisons between these two different C'-mediated activities of antibodies. The use of the enhancement assay allows the investigation of a range of viruses, to add to the limited repertoire of post-vaccination ADE studies.

### 5.1.2 The vaccine trial

The serum samples used in this chapter are from participants of a Phase I clinical trial, MRC V001 (McCormack et al., 2000). Healthy HIV seronegative volunteers were immunised with monomeric recombinant gp120 (rgp120) derived from a R5X4 subtype B HIV-1 strain, W61D. W61D is derived from a molecular clone of a Dutch clinical isolate (Groenink et al., 1991). Individuals were vaccinated on weeks 0, 4 and 28; serum samples were obtained on weeks 0, 2, 6, 12 or 16, 28, 30, 40, 52, 68 and 84, as shown in the vaccine regime diagram in Figure 5.1.



**Figure 5.1 Timeline of the vaccine regime**

Red arrows indicate immunisations, carried out on weeks 0, 4 and 28. Sample time points are shown as diamonds with the time in weeks below. Note that in the case of some individuals, samples were taken on week 16 instead of week 12, and week 32 instead of week 30.

### 5.1.3 Overview of the virus strains, serum samples and assays used for the characterisation of the antibody response in vaccinees

Three virus strains were used to characterise the antibody response to the vaccine: MN, a well characterised X4 TCLA HIV strain susceptible to neutralisation by a wide range of sera and used for initial characterisation of the neutralising response following vaccination (McCormack et al., 2000); W61D, an R5X4 dual tropic virus strain from which the vaccine was derived; and MM32 d10 virus, an R5 primary isolate from the recent patient cohort investigated in Chapter 4. Enhancement and C'-MI assays were used to investigate the nature of the antibodies elicited by vaccination.

The C'-MI assay, using NP2/CD4/X4 or -R5 cells, is essentially an NP2-based neutralisation assay adapted to assess the additional effect of complement on virus inactivation, as published previously (Aasa-Chapman et al., 2005). Note that neutralisation refers to the inactivation of virus by antibody alone, as seen in the absence of active complement (HIC'), whereas C'-MI refers to any additional inactivation by antibody seen in the presence of C'. Results from C'-MI assays are shown in yellow and orange throughout this chapter. The enhancement assay, using SupT1/R5 cells, was described in section 4.1.2. Results from enhancement assays are shown in blue and white throughout this chapter, as in Chapter 4. All serum samples were tested at 10% in the enhancement and C'-MI assays to enable direct comparisons between and within assays.

## **5.2 RESULTS**

### **5.2.1 Characterisation of the neutralising antibody responses in vaccine recipients**

According to previous reports characterising the antibody response in recipients of this vaccine, neutralising antibody responses were weak (Beddows et al., 1999; McCormack et al., 2000). Of several viruses tested, MN was most susceptible to neutralisation by vaccinee sera (McCormack et al., 2000), therefore it was used as a reference strain for neutralisation assays presented in this chapter. Throughout this chapter, the twelve vaccine recipient sera sets are divided into two groups according to neutralisation and antibody binding data presented by McCormack and colleagues (McCormack et al., 2000) and personal communication from Sheena McCormack, as summarised in Table 5.1. JUL008, JUJ009, JUX011, JUF061, JUB063 and JUX065 showed highest 50% neutralising antibody titres against MN and overall highest binding antibody titres by ELISA, therefore these will be referred to as Group A. JUA001, JUV012, JUR014, JUZ064, JUV066 and JUT067 showed lower or no neutralising antibody titres to MN and lowest antibody binding titres by ELISA, therefore these will be referred to as Group B. JUZ064 is the exception to this rule, as week 30 samples from this vaccinee showed high MN 50% neutralisation and MN V3 loop binding antibody titres, yet had low W61D gp120 and W61D V3 loop binding antibody titres. On the basis of this finding, and MN neutralisation data presented in section 5.2.1.1, this subject was placed in Group B.

GROUP A	MN 50% <sup>1</sup>	MN V3 <sup>2</sup>	W61D V3 <sup>3</sup>	W61D gp120 <sup>4</sup>	GROUP B	MN 50% <sup>1</sup>	MN V3 <sup>2</sup>	W61D V3 <sup>3</sup>	W61D gp120 <sup>4</sup>
JUL008 <sup>y</sup>	160	800	1600	25600	JUA001 <sup>x</sup>	20	400	200	6400
JUJ009 <sup>z</sup>	320	6400	6400	102400	JUV012 <sup>y</sup>	40	400	400	12800
JUX011 <sup>z</sup>	160	1600	3200	102400	JUR014 <sup>y</sup>	40	200	200	6400
JUF061 <sup>z</sup>	320	1600	3200	102400	JUZ064 <sup>x</sup>	320	1600	800	12800
JUB063 <sup>y</sup>	160	1600	1600	51200	JUV066 <sup>y</sup>	40	400	400	12800
JUX065 <sup>y</sup>	320	6400	1600	51200	JUT067 <sup>z</sup>	80	400	800	12800
<b>MEAN</b>	<b>240</b>	<b>3067</b>	<b>2933</b>	<b>64000</b>	<b>MEAN</b>	<b>97</b>	<b>567</b>	<b>467</b>	<b>10667</b>
<b>RANGE</b>	160- 320	800- 6400	1600- 6400	25600- 102400	<b>RANGE</b>	20- 320	200- 1600	200- 800	6400- 12800

**Table 5.1 Characteristics of the twelve week 30\* vaccinee serum samples.**

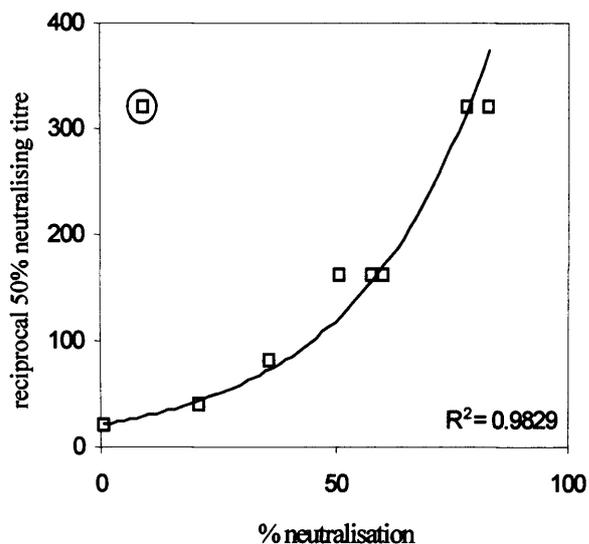
For comparison, values were used from the week 30 samples (2 weeks following the third immunisation) from each individual (\*week 32 results are shown for JUV066). <sup>1</sup> MN neutralisation data, shown as the reciprocal of the last serum dilution at which 50% neutralisation occurs. <sup>2</sup> MN V3 loop peptide, <sup>3</sup> W61D V3 loop peptide, and <sup>4</sup> recombinant W61D gp120 binding antibody titres, determined by ELISA and shown as the last reciprocal dilutions showing positive substrate binding. Adjuvants used are indicated <sup>x</sup> Alum, <sup>y</sup> SBAS-1, <sup>z</sup> SBAS-2. Data were kindly provided by Sheena McCormack.

Due to the limited volume of sera available, a selection of vaccine recipient sera were tested in each assay; only in the enhancing assays presented in section 5.2.2.2 were all twelve sets tested. Certain time points were unavailable for two of the individuals: day 30 or 32 serum was not available for JUX065, and samples after day 30 were not available for JUB063. In the case of JUV066 a week 32 sample was used instead of week 30.

#### **5.2.1.1 Neutralisation of the TCLA strain MN**

Sequential serum samples from nine immunised individuals were tested for neutralisation and C'-MI activity against MN. Results are shown in Figure 5.2. As no sera attained more than 90% neutralisation, the level of 50% neutralisation is marked on each graph as a broken red line. At time points immediately following the third immunisation (in most cases week 30), those from Group A neutralised MN by up to

81%. Sera from all individuals from this group neutralised MN by at least 50%. None of the sera from Group B subjects showed more than 50% neutralisation, yet the sequential samples show a similar, but weaker, pattern as the Group A subjects, with peaks of neutralisation activity occurring on week 30. The neutralisation assay used in our laboratory is known to be more stringent than others, in that IC50 and IC90 values are lower for a given serum than results from other assays (Aasa-Chapman et al., 2004), therefore overall levels of neutralisation are lower than those published by McCormack and colleagues (McCormack et al., 2000). However, relative levels of neutralising antibodies agree with published levels, with Group A subjects showing highest levels of neutralisation against MN, whereas Group B subjects showed lowest levels of neutralisation. For clarity, a comparison between my MN neutralisation data and those presented in McCormack 2000 is shown in Figure 5.3.



**Figure 5.3 Comparison of MN neutralisation**

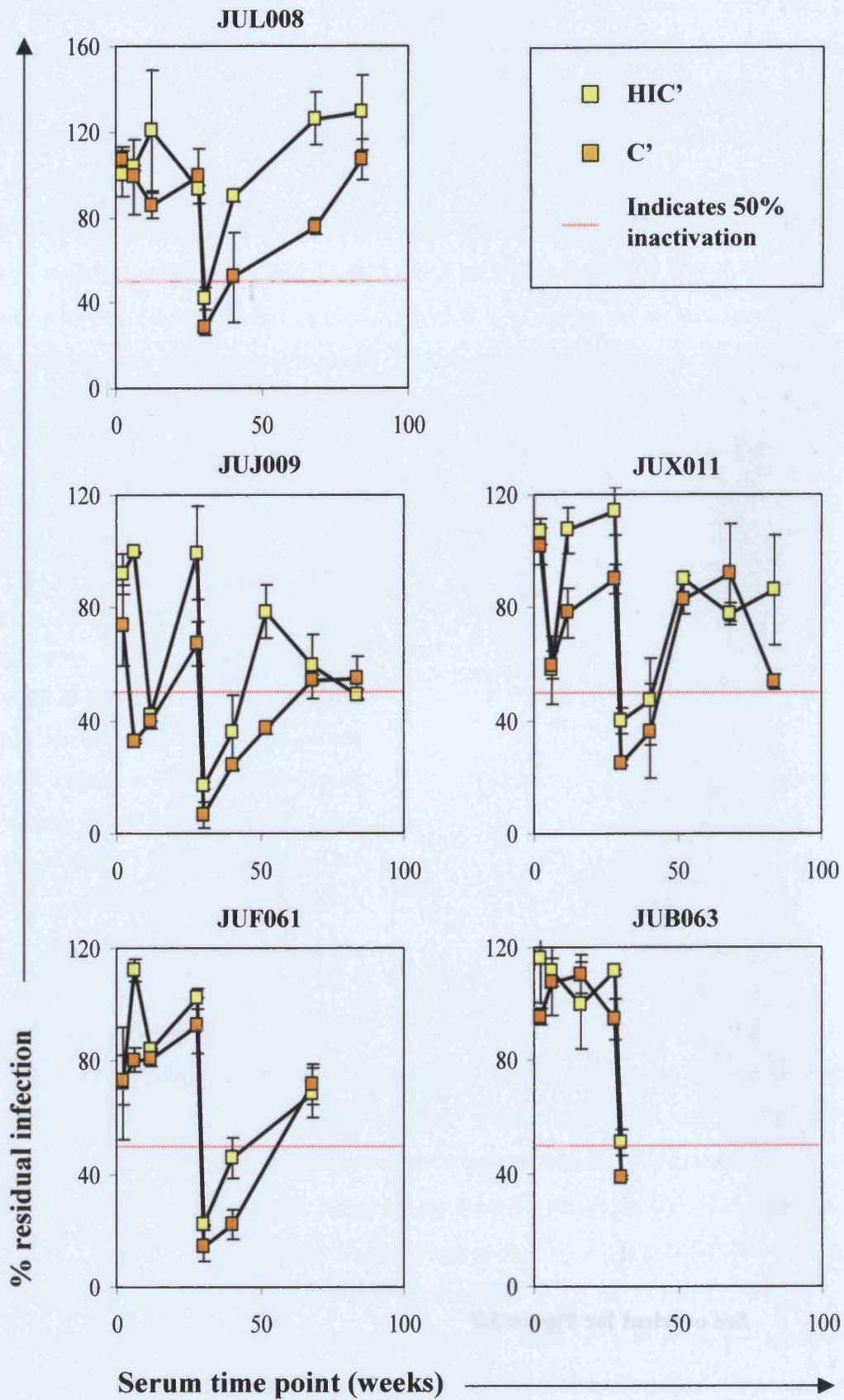
Data from McCormack 2000, shown as 50% neutralising titres, were plotted against data from my assays in Figure 5.2, shown as percent neutralisation. Results from week 30 sera from the nine individuals were used for the comparisons. One outlier, JUZ064, ringed in red, was excluded from the correlation.

C'MI is detectable in both groups. For Group A, due to the relatively high level of neutralisation seen for day 30 samples, C'-MI is not significant for any of the day 30 samples. However, several samples taken before and after day 30 for each subject show weak or no neutralising activity augmented by C'-MI. Significant C'-MI is seen for JUL008 week 68; JUJ009 weeks 6 and 52; and JUF061 week 6 ( $p < 0.05$  using a two-tailed student's T test assuming equal variance).

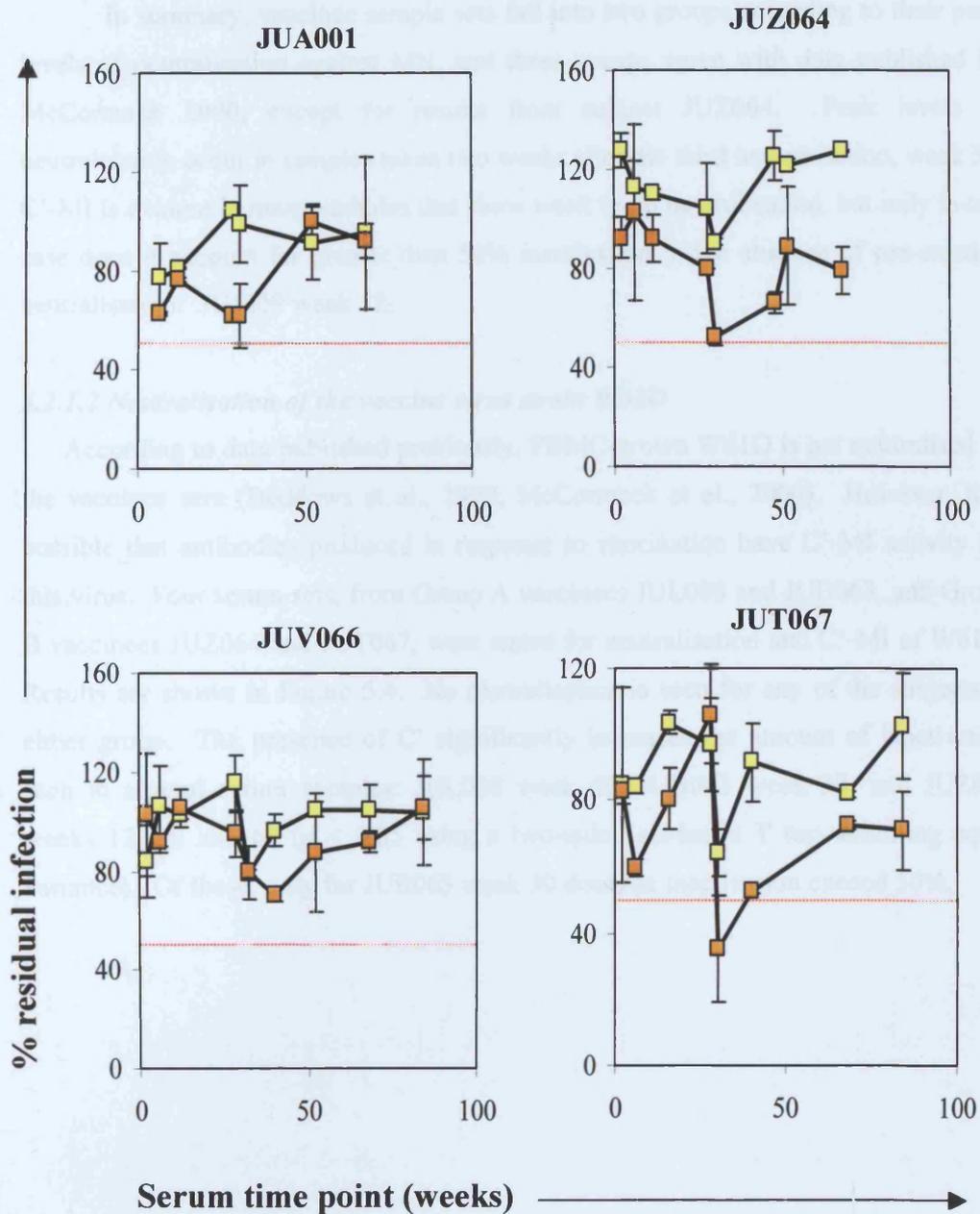
For Group B, in which neutralisation levels are lower overall, C'-MI is more frequent, again with post-week 30 samples showing the greatest difference in the presence of C'. Significant C'-MI is seen for JUZ064 weeks 2, 30, 40 and 68; JUV066 week 40; and JUT067 weeks 6, 40 and 68.

**See overleaf for Figure 5.2**

# GROUP A



## GROUP B



**Figure 5.2 Neutralisation and C'-MI of MN by sequential serum samples from nine vaccinated individuals**

C'-MI assays were carried out on NP2/CD4/X4 cells with MN and sequential vaccinee serum samples. % residual infection is calculated relative to the Week 0 control samples for each individual, in the presence (C') and absence (HIC') of active complement as appropriate. Points that fall below the dashed red line indicate >50% inactivation. Error bars represent SD from 3 experiments.

In summary, vaccinee sample sets fall into two groups according to their peak levels of neutralisation against MN, and these groups agree with data published by McCormack 2000, except for results from subject JUZ064. Peak levels of neutralisation occur in samples taken two weeks after the third immunisation, week 30. C'-MI is evident in many samples that show weak or no neutralisation, but only in one case does it account for greater than 50% inactivation in the absence of pre-existing neutralisation: JUJ009 week 52.

### 5.2.1.2 Neutralisation of the vaccine virus strain W61D

According to data published previously, PBMC-grown W61D is not neutralised by the vaccinee sera (Beddows et al., 1999; McCormack et al., 2000). However, it is possible that antibodies produced in response to vaccination have C'-MI activity for this virus. Four serum sets, from Group A vaccinees JUL008 and JUB063, and Group B vaccinees JUZ064 and JUT067, were tested for neutralisation and C'-MI of W61D. Results are shown in Figure 5.4. No neutralisation is seen for any of the subjects in either group. The presence of C' significantly increases the amount of inactivation seen in several serum samples: JUL008 week 40; JUB063 week 30; and JUZ064 weeks 12, 30 and 68 ( $p < 0.05$  using a two-tailed student's T test assuming equal variance). Of these, only for JUB063 week 30 does the inactivation exceed 50%.

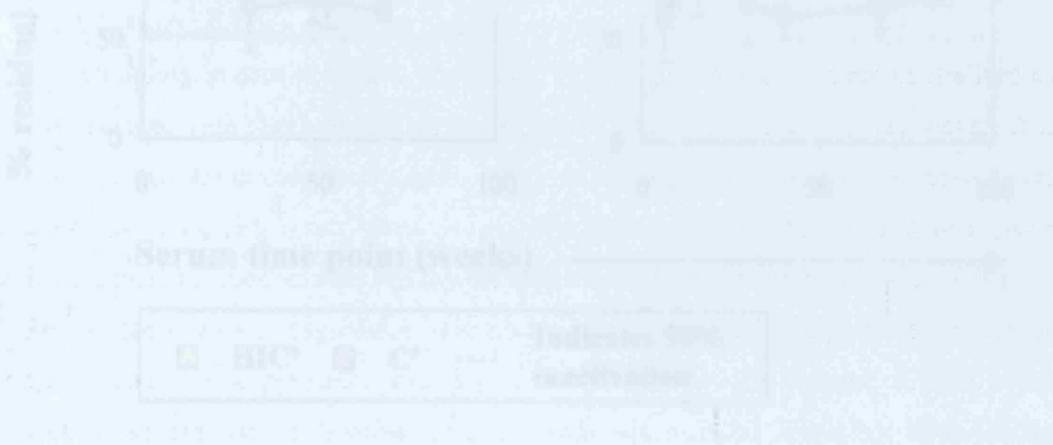
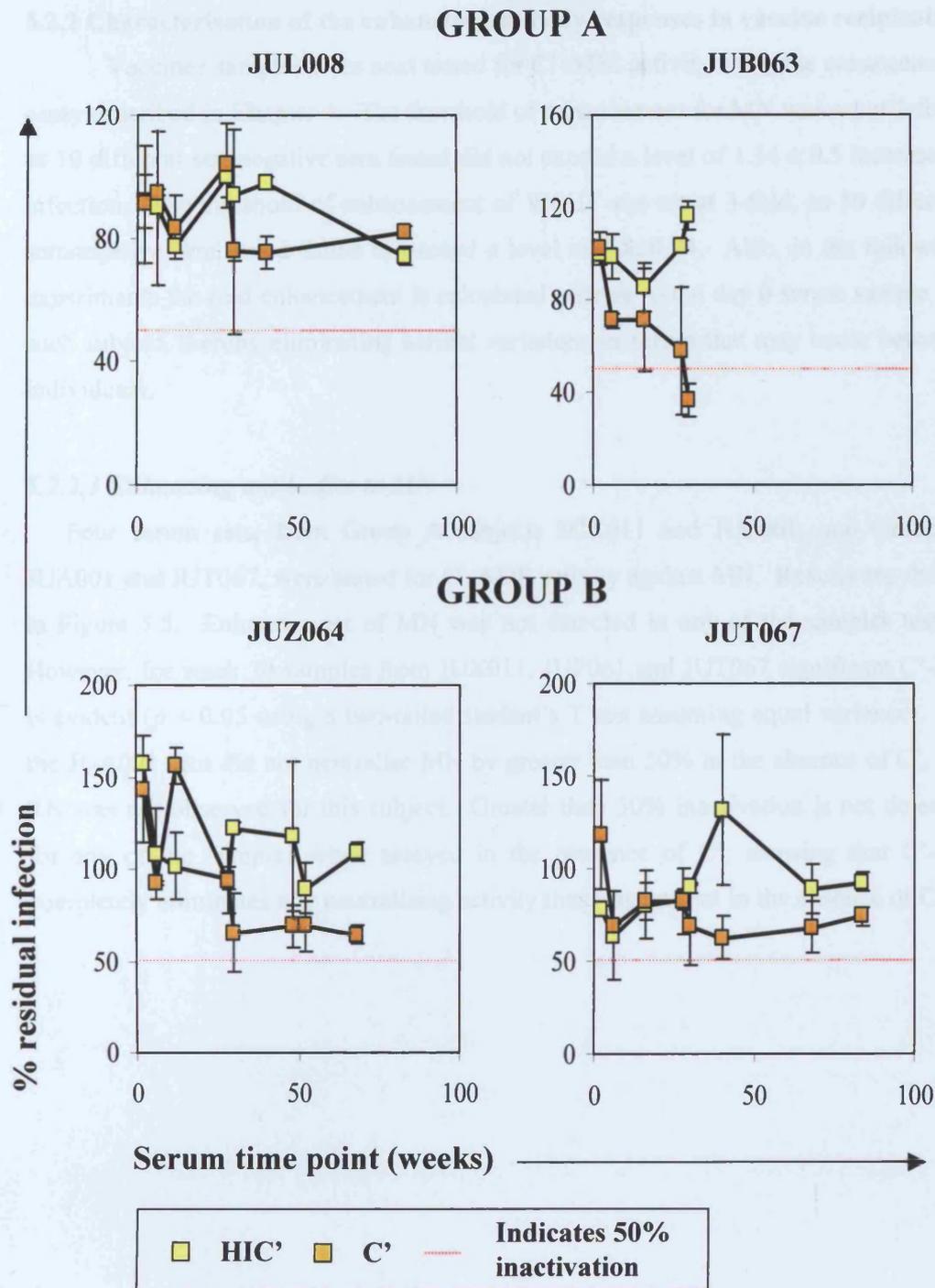


Figure 5.4 Neutralisation and C'-MI of W61D vaccine virus strain using sequential serum samples from four vaccinated individuals.

C'-MI assays were carried out on PBMC-grown W61D cells with serially diluted serum samples. % residual infection is calculated when the optical density of control samples for each individual, at the previous time point, are subtracted from the complemented suspension. Points that fall above the horizontal line indicate 50% inactivation. Error bars represent 95% confidence intervals.



**Figure 5.4 Neutralisation and C'-MI of W61D on NP2/CD4/R5 cells by sequential serum samples from four vaccinated individuals**

C'-MI assays were carried out on NP2/CD4/R5 cells with W61D and sequential vaccinee serum samples. % residual infection is calculated relative to the Week 0 control samples for each individual, in the presence (C') and absence (HIC') of active complement as appropriate. Points that fall below the dashed red line indicate >50% inactivation. Error bars represent SD from 3 experiments.

## 5.2.2 Characterisation of the enhancing antibody responses in vaccine recipients

Vaccinee samples were next tested for C'-ADE activity using the enhancement assay described in Chapter 4. The threshold of enhancement for MN was set at 2-fold, as 10 different seronegative sera tested did not exceed a level of  $1.14 \pm 0.5$  increase in infection. The threshold of enhancement of W61D was set at 3-fold, as 10 different seronegative sera tested failed to exceed a level of  $1.8 \pm 0.24$ . Also, in the following experiments the fold enhancement is calculated relative to the day 0 serum sample for each subject, thereby eliminating natural variations in serum that may occur between individuals.

### 5.2.2.1 Enhancing antibodies to MN

Four serum sets, from Group A subjects JUX011 and JUF061, and Group B JUA001 and JUT067, were tested for C'-ADE activity against MN. Results are shown in Figure 5.5. Enhancement of MN was not detected in any of the samples tested. However, for week 30 samples from JUX011, JUF061 and JUT067 significant C'-RN is evident ( $p < 0.05$  using a two-tailed student's T test assuming equal variance). As the JUA001 sera did not neutralise MN by greater than 50% in the absence of C', C'-RN was not observed for this subject. Greater than 50% inactivation is not detected for any of the samples when assayed in the presence of C', showing that C'-RN completely eliminates any neutralising activity that was present in the absence of C'.

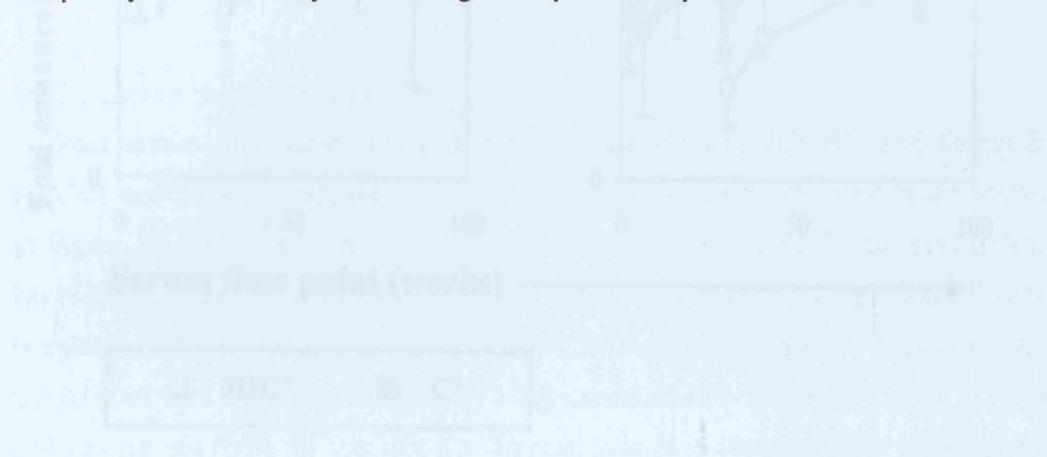
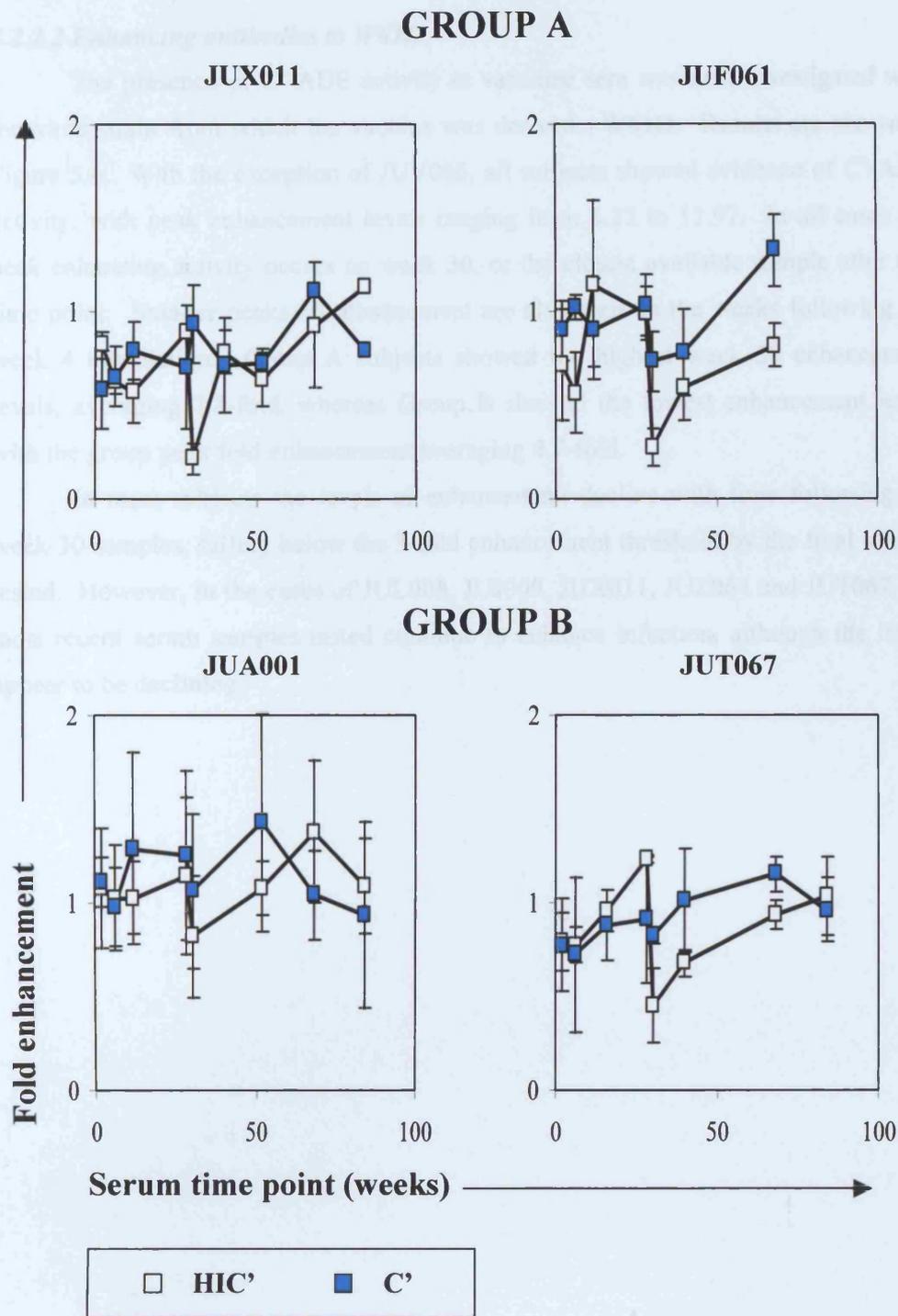


Figure 5.5 Lack of C'-ADE of MN in SeptiVAC trial by sequential vaccinee serum samples. The fold enhancement of MN infection was determined by sequential enhancement assays were carried out on the 293T-23 cells with MN and sequential vaccinee serum samples. Fold enhancement was calculated relative to infection in the presence of pre-circulation (Day 0) serum for each individual in the presence (C'+) and absence (C'-) of active complement. The enhancement threshold for MN is 2-fold. Error bars represent SD from 5 experiments.



**Figure 5.5 Lack of C'-ADE of MN on SupT1/R5 cells by sequential vaccinee serum samples**

Enhancement assays were carried out on SupT1/R5 cells with MN and sequential vaccinee serum samples. Fold enhancement was calculated relative to infection in the presence of pre-vaccination (Day 0) serum for each individual, in the presence (C') and absence (HIC') of active complement. The enhancement threshold for MN is 2-fold. Error bars represent SD from 3 experiments.

### 5.2.2.2 Enhancing antibodies to W61D

The presence of C'-ADE activity in vaccinee sera was next investigated with the virus strain from which the vaccine was derived: W61D. Results are shown in Figure 5.6. With the exception of JUV066, all subjects showed evidence of C'-ADE activity, with peak enhancement levels ranging from 4.32 to 11.97. In all cases the peak enhancing activity occurs on week 30, or the closest available sample after this time point. Smaller peaks of enhancement are also seen on the weeks following the week 4 inoculation. Group A subjects showed the highest week 30 enhancement levels, averaging 7.3-fold, whereas Group B showed the lowest enhancement level, with the group peak fold enhancement averaging 4.7-fold.

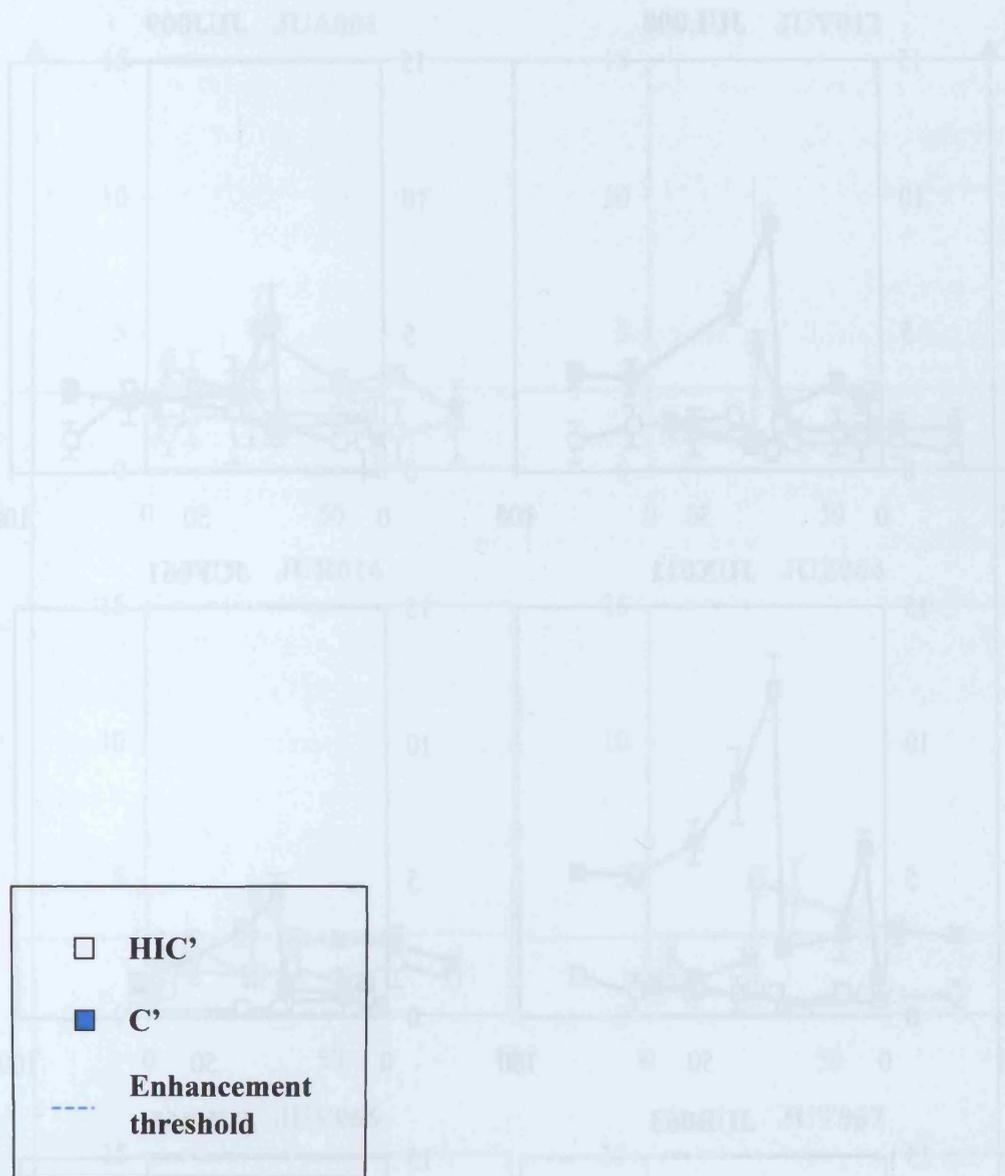
In most subjects the levels of enhancement decline with time following the week 30 samples, falling below the 3-fold enhancement threshold by the final sample tested. However, in the cases of JUL008, JUJ009, JUX011, JUZ064 and JUT067, the most recent serum samples tested continue to enhance infection, although the levels appear to be declining.



Figure 5.6 (continued)

C'-ADE of W61D on Hep-2 cells by sequential vaccinee serum samples

Enhancement data were derived from Hep-2 cells with the vaccine virus strain W61D and sequential vaccinee serum samples. Fold enhancement was calculated relative to infection in 50 percent of representative (Day 0) serum for each individual, in the presence (C') and absence (C'') of added complement. Volunteers were vaccinated on weeks 0, 4 and 20. Points that fall above the dashed reference line indicate enhancing infection. Data have standard SD from 3 experiments.

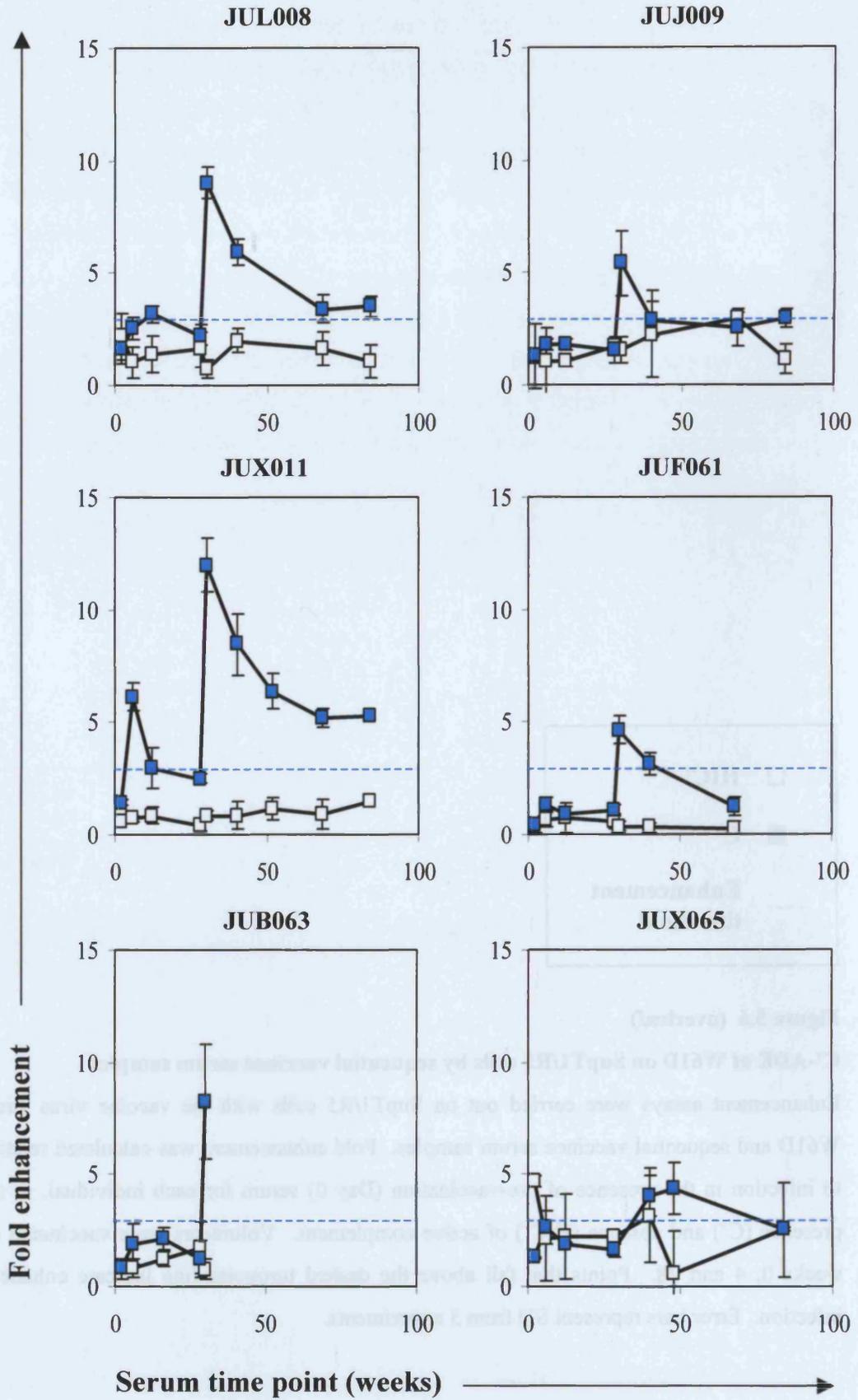


**Figure 5.6 (overleaf)**

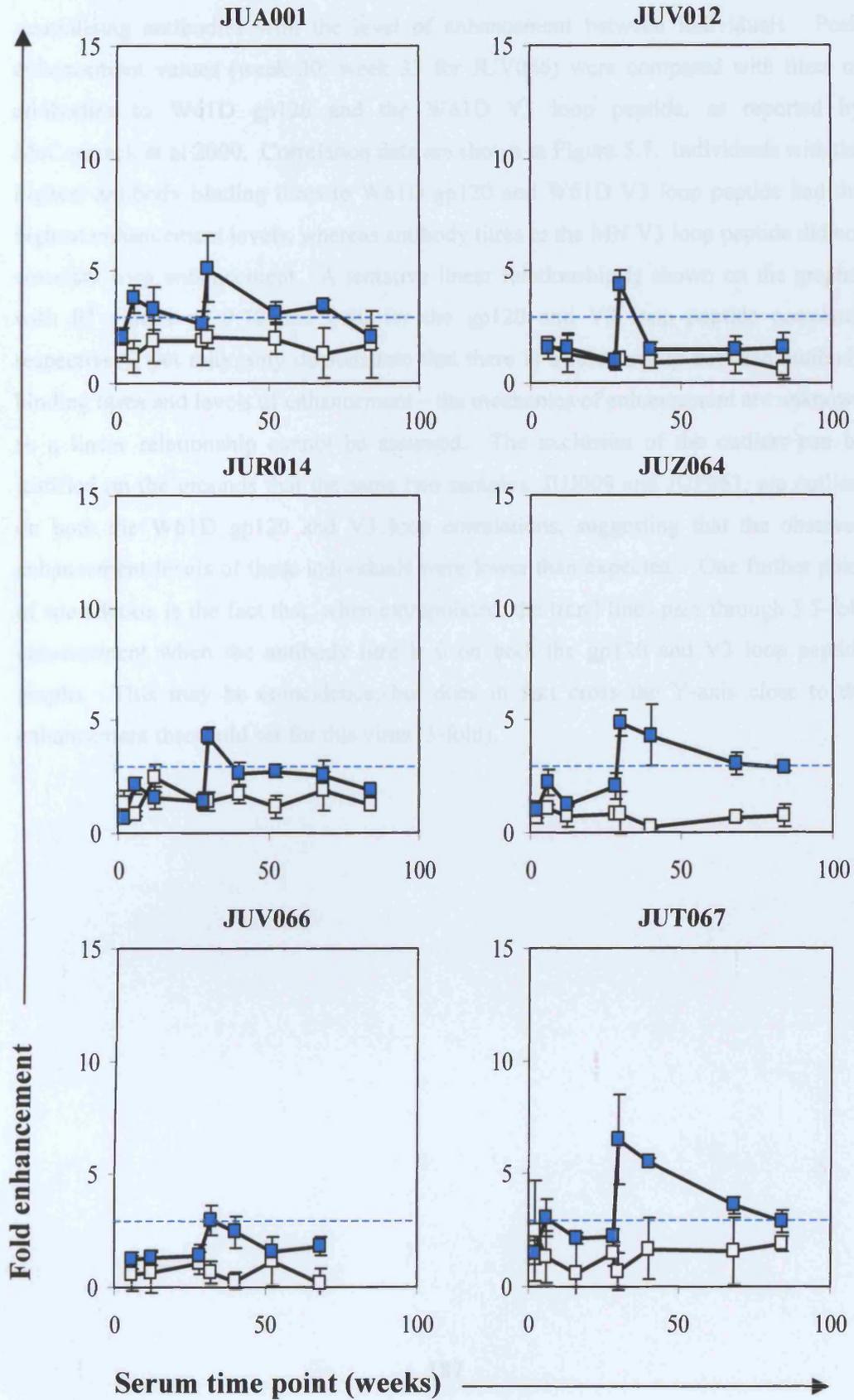
**C'-ADE of W61D on SupT1/R5 cells by sequential vaccinee serum samples**

Enhancement assays were carried out on SupT1/R5 cells with the vaccine virus strain W61D and sequential vaccinee serum samples. Fold enhancement was calculated relative to infection in the presence of pre-vaccination (Day 0) serum for each individual, in the presence (C') and absence (HIC') of active complement. Volunteers were vaccinated on weeks 0, 4 and 28. Points that fall above the dashed turquoise line indicate enhanced infection. Error bars represent SD from 3 experiments.

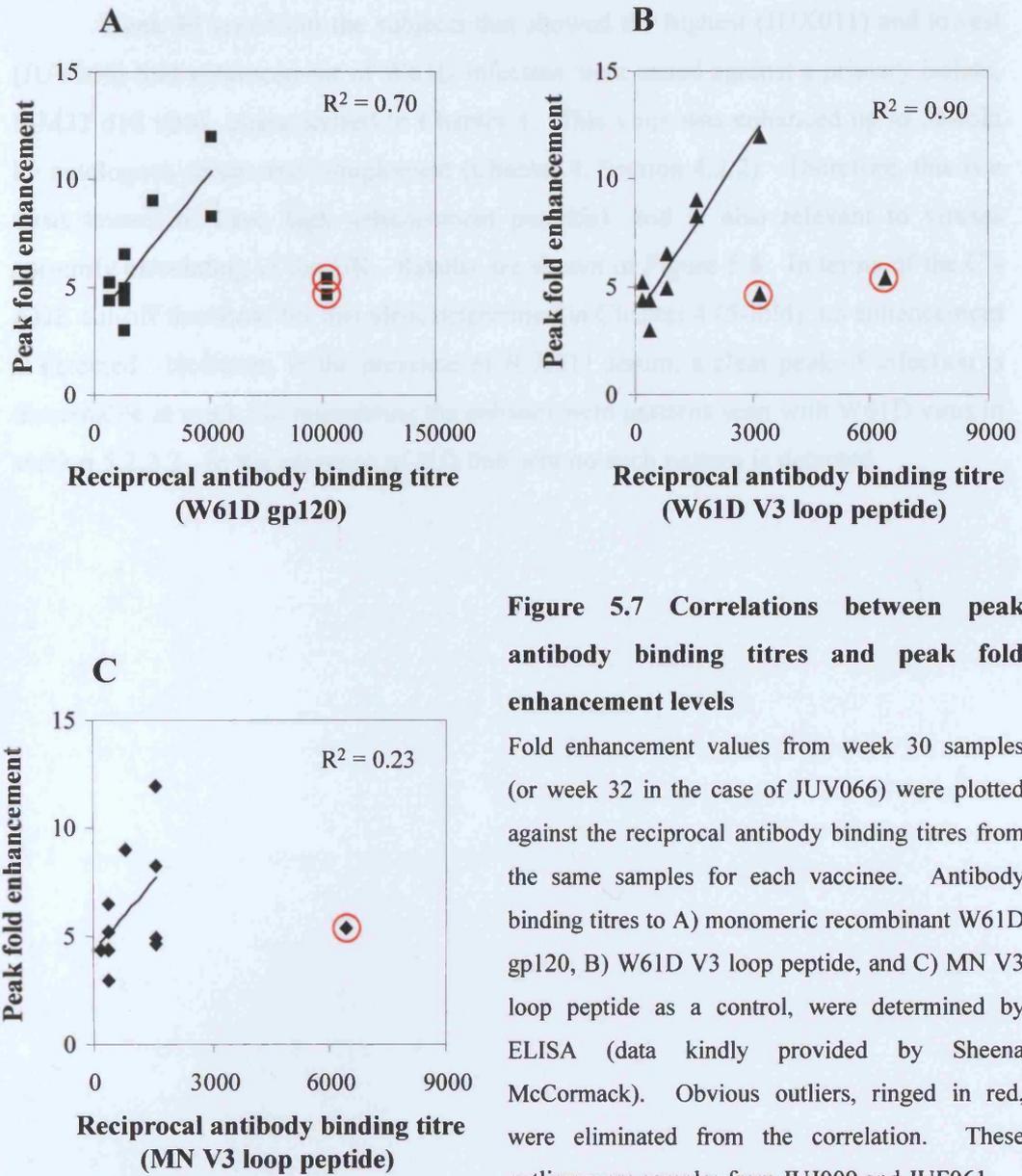
# GROUP A



## GROUP B



The fact that the antibodies in these individuals were elicited by the same immunogen, W61D gp120, and that none of these individuals produced detectable neutralising antibodies to W61D, provides the opportunity to compare the level of non-neutralising antibodies with the level of enhancement between individuals. Peak enhancement values (week 30; week 32 for JUV066) were compared with titres of antibodies to W61D gp120 and the W61D V3 loop peptide, as reported by McCormack et al 2000. Correlation data are shown in Figure 5.7. Individuals with the highest antibody binding titres to W61D gp120 and W61D V3 loop peptide had the highest enhancement levels, whereas antibody titres to the MN V3 loop peptide did not correlate with enhancement. A tentative linear relationship is shown on the graphs, with  $R^2$  values of 0.70 and 0.90 for the gp120 and V3 loop peptide correlates respectively, yet may only demonstrate that there is a relationship between antibody binding titres and levels of enhancement – the mechanics of enhancement are unknown so a linear relationship cannot be assumed. The exclusion of the outliers can be justified on the grounds that the same two samples, JUJ009 and JUF061, are outliers on both the W61D gp120 and V3 loop correlations, suggesting that the observed enhancement levels of these individuals were lower than expected. One further point of speculation is the fact that, when extrapolated, the trend lines pass through 3.5-fold enhancement when the antibody titre is 0 on both the gp120 and V3 loop peptide graphs. This may be coincidence, but does in fact cross the Y-axis close to the enhancement threshold set for this virus (3-fold).



**Figure 5.7 Correlations between peak antibody binding titres and peak fold enhancement levels**

Fold enhancement values from week 30 samples (or week 32 in the case of JUV066) were plotted against the reciprocal antibody binding titres from the same samples for each vaccinee. Antibody binding titres to A) monomeric recombinant W61D gp120, B) W61D V3 loop peptide, and C) MN V3 loop peptide as a control, were determined by ELISA (data kindly provided by Sheena McCormack). Obvious outliers, ringed in red, were eliminated from the correlation. These outliers were samples from JUJ009 and JUF061.

### 5.2.2.3 Enhancing antibodies to a primary clinical isolate

Week 30 sera from the subjects that showed the highest (JUX011) and lowest (JUV066) fold enhancement of W61D infection were tested against a primary isolate, MM32 d10 virus, characterised in Chapter 4. This virus was enhanced up to 58-fold by autologous serum and complement (Chapter 4, Section 4.2.2). Therefore, this is a virus known to have high enhancement potential, and is also relevant to viruses currently circulating in the UK. Results are shown in Figure 5.8. In terms of the C'-ADE cut-off threshold for this virus determined in Chapter 4 (5-fold), no enhancement is detected. However, in the presence of JUX011 serum, a clear peak of infection is discernable at week 30, resembling the enhancement patterns seen with W61D virus in section 5.2.2.2. In the presence of JUV066 sera no such pattern is detected.

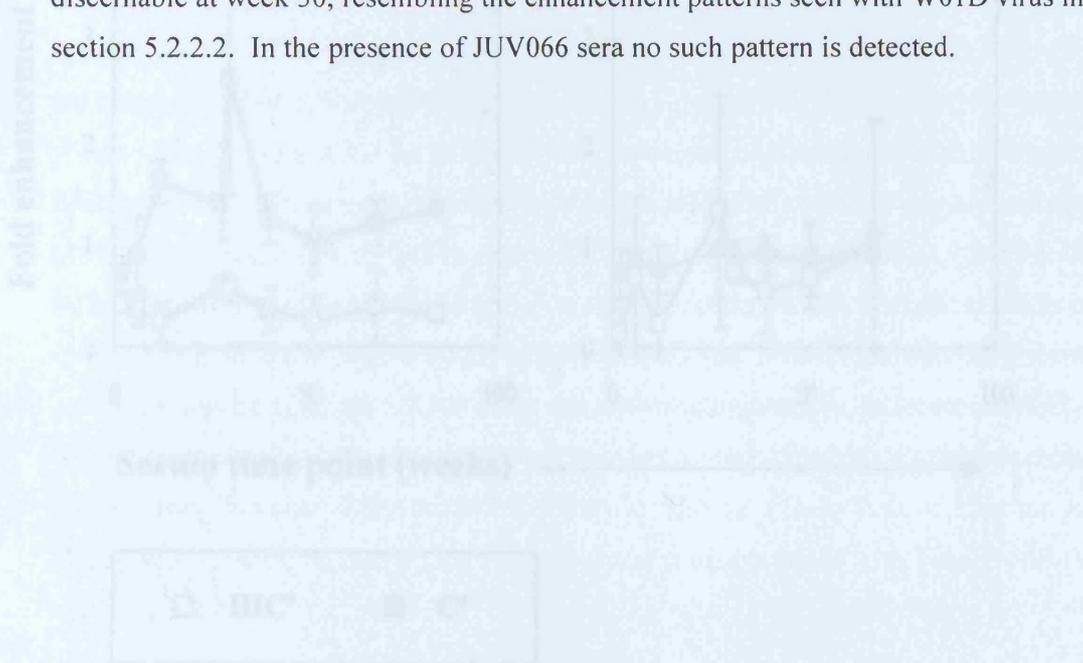
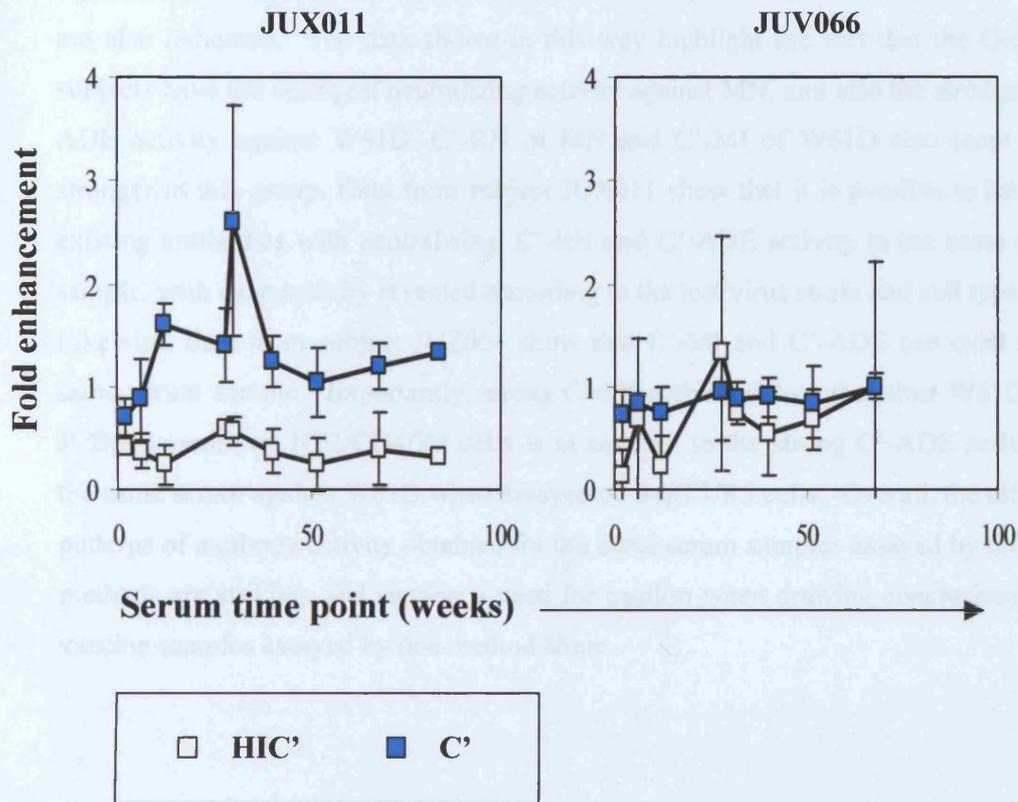


Figure 5.8 Lack of C'-ADE activity in serum samples from two volunteers against the primary patient isolate MM32 d10.

A clinical primary isolate MM32 d10, was tested in an enhancement assay with sequential serum samples from volunteers JUX011 and JUV066. Enhancement results are not shown as these were pre-determined by JUX011 and JUV066 to be low, which falls outside the scale used on the graph. Error bars represent SD (n=3 replicates).



**Figure 5.8 Lack of C'-ADE activity in serum samples from two vaccinees against the primary patient isolate MM32 d10.**

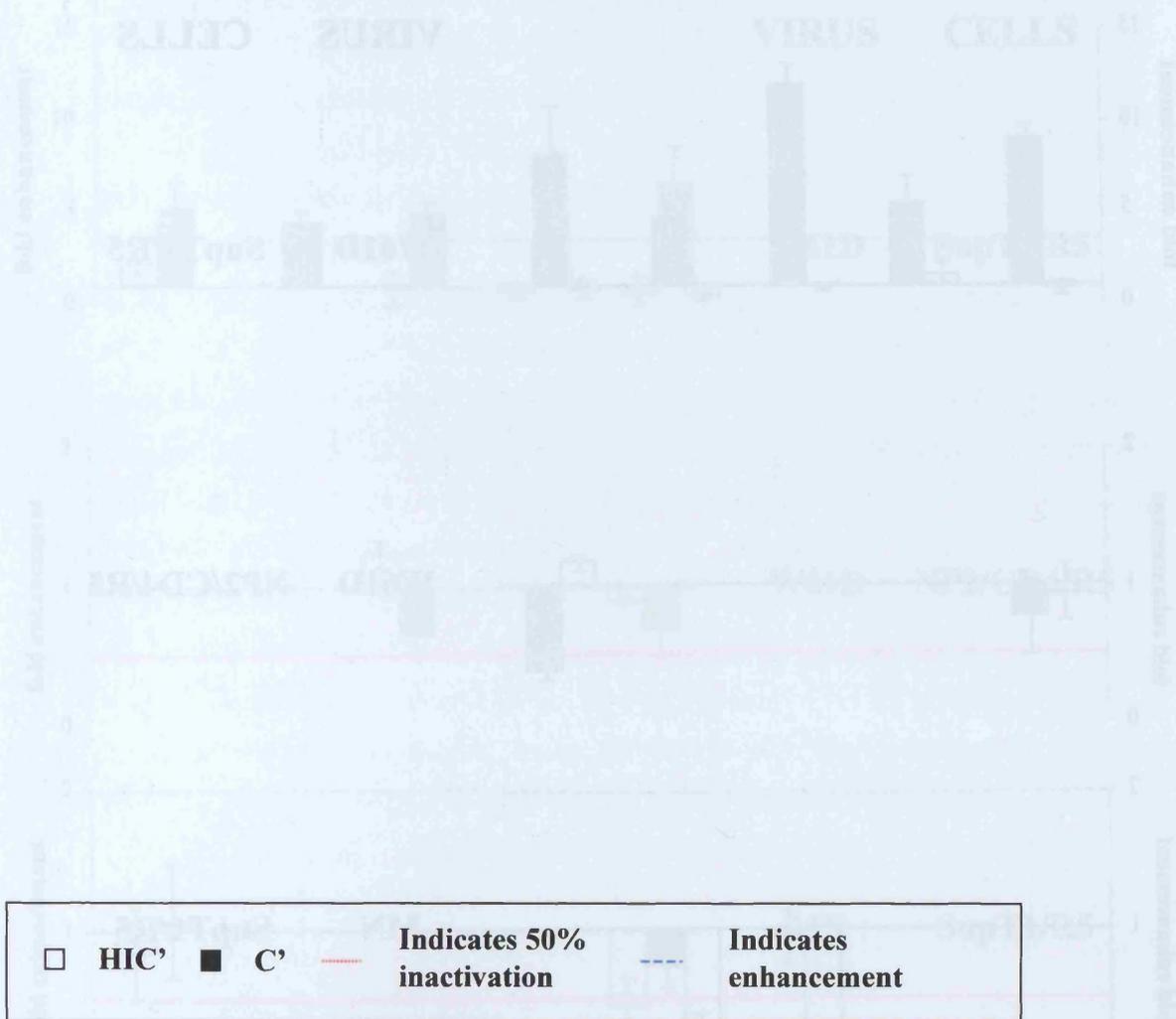
A clinical primary isolate, MM32 d10, was tested in an enhancement assay with sequential serum samples from vaccinees JUX011 and JUV066. Enhancement thresholds are not shown as these were pre-determined for MM32 d10 virus at 5-fold, which falls outside the scale used for the graphs. Error bars represent SD from 2 experiments.

### 5.2.3 Summary of antibody activity found in vaccinee samples

Data presented in this chapter, with the exception of those shown in section 5.2.2.3, are summarised in Figure 5.9 using week 30 or 32 sample results from each volunteer as these showed peak neutralisation and enhancement levels in all sequential serum sets tested. Infection levels on each chart, including those from C'-MI assays, are shown as "fold enhancement" for ease of comparison between assays. The 50% inactivation thresholds, and enhancement thresholds (2-fold for MN, 3-fold for W61D) are also indicated. The data shown in this way highlight the fact that the Group A subjects have the strongest neutralizing activity against MN, and also the strongest C'-ADE activity against W61D. C'-RN of MN and C'-MI of W61D also seem to be stronger in this group. Data from subject JUX011 show that it is possible to have co-existing antibodies with neutralising, C'-RN and C'-ADE activity in the same serum sample, with each activity revealed according to the test virus strain and cell type used. Likewise, data from subject JUZ064 show that C'-MI and C'-ADE can exist in the same serum sample. Importantly, strong C'-MI activity detected against W61D with JUB063 serum on NP2/CD4/R5 cells is in contrast to the strong C'-ADE activity of the same serum against W61D when assayed on SupT1/R5 cells. Overall, the different patterns of antibody activity obtained for the same serum samples assayed by different methods are striking, and support a need for caution when drawing conclusions from vaccine samples assayed by one method alone.



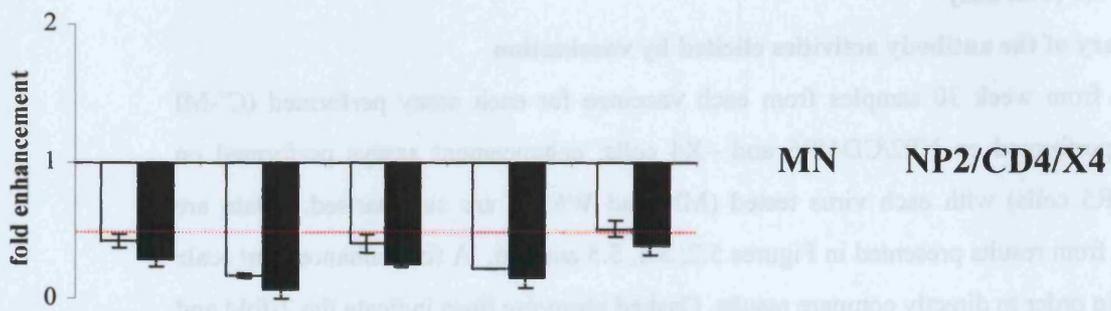
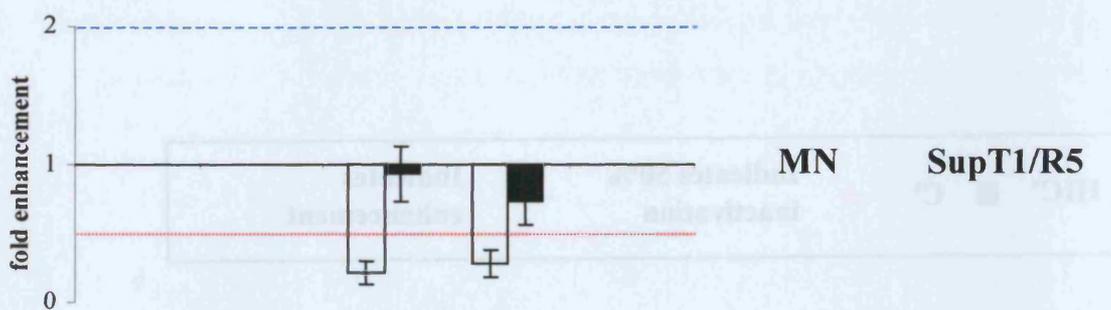
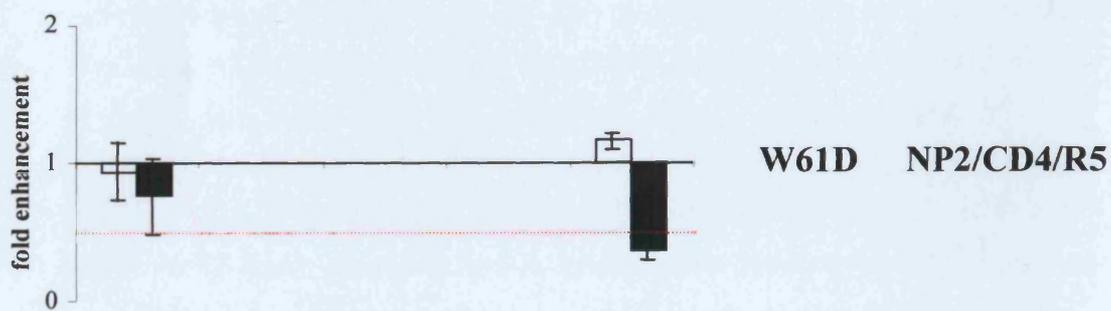
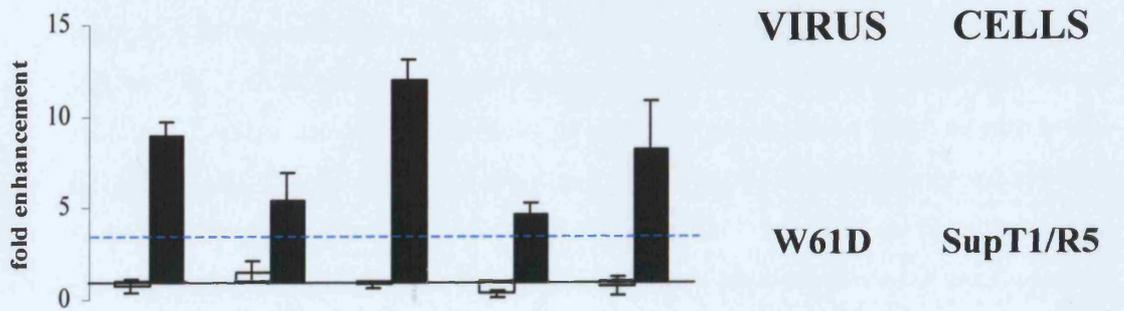
Figure 5.9 (overleaf)  
Summary of the antibody activities against MN and W61D. Results from week 30 sample sets. Assays performed on all cell types (NP2/CD4/R5 and SupT1/R5 cells) with each virus strain. The data is derived from results presented in Figure 5.8. A 50% inactivation threshold is indicated for neutralisation assays and 2-fold enhancement threshold for C'-MI and C'-ADE assays. The data is derived from results presented in Figure 5.8. A 50% inactivation threshold is indicated for neutralisation assays and 2-fold enhancement threshold for C'-MI and C'-ADE assays.



**Figure 5.9 (overleaf)**

**Summary of the antibody activities elicited by vaccination**

Results from week 30 samples from each vaccinee for each assay performed (C'-MI assays performed on NP2/CD4/R5 and -X4 cells; enhancement assays performed on SupT1/R5 cells) with each virus tested (MN and W61D) are summarised. Data are derived from results presented in Figures 5.2, 5.4, 5.5 and 5.6. A fold enhancement scale is used in order to directly compare results. Dashed turquoise lines indicate the 2-fold and 3-fold enhancement threshold for MN and W61D enhancement assays respectively; dashed red lines indicate the 50% inactivation threshold; therefore, samples above the dashed turquoise line indicate enhanced infection while samples below the dashed red line indicate inactivation. Error bars represent SD for 3 experiments.

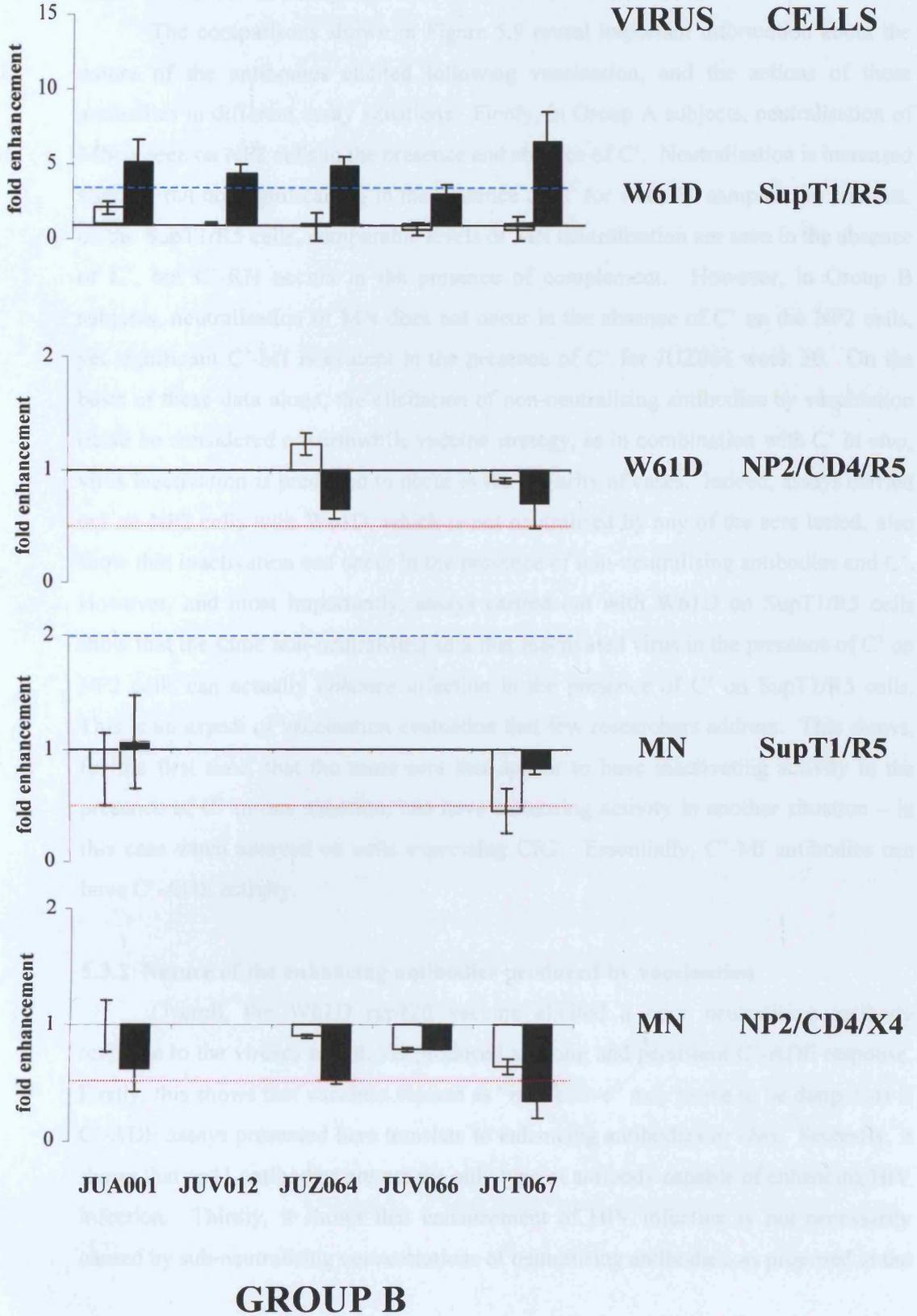


JUL008    JUL009    JUX011    JUF061    JUB063

**GROUP A**

### 5.3 DISCUSSION

#### 5.3.1 Different antibody activities can be detected after immunisation



## 5.3 DISCUSSION

### 5.3.1 Different antibody activities can be detected after immunisation

The comparisons shown in Figure 5.9 reveal important information about the nature of the antibodies elicited following vaccination, and the actions of these antibodies in different assay situations. Firstly, in Group A subjects, neutralisation of MN is seen on NP2 cells in the presence and absence of C'. Neutralisation is increased slightly, but not significantly, in the presence of C' for week 30 samples. In contrast, on the SupT1/R5 cells, comparable levels of MN neutralisation are seen in the absence of C', but C'-RN occurs in the presence of complement. However, in Group B subjects, neutralisation of MN does not occur in the absence of C' on the NP2 cells, yet significant C'-MI is evident in the presence of C' for JUZ064 week 30. On the basis of these data alone, the elicitation of non-neutralising antibodies by vaccination could be considered a worthwhile vaccine strategy, as in combination with C' *in vivo*, virus inactivation is predicted to occur in the majority of cases. Indeed, assays carried out on NP2 cells with W61D, which is not neutralised by any of the sera tested, also show that inactivation can occur in the presence of non-neutralising antibodies and C'. However, and most importantly, assays carried out with W61D on SupT1/R5 cells show that the same non-neutralising sera that inactivated virus in the presence of C' on NP2 cells can actually *enhance* infection in the presence of C' on SupT1/R5 cells. This is an aspect of vaccination evaluation that few researchers address. This shows, for the first time, that the same sera that appear to have inactivating activity in the presence of C' in one situation, can have enhancing activity in another situation – in this case when assayed on cells expressing CR2. Essentially, C'-MI antibodies can have C'-ADE activity.

### 5.3.2 Nature of the enhancing antibodies produced by vaccination

Overall, the W61D rgp120 vaccine elicited a poor neutralising antibody response to the viruses tested, yet produced a strong and persistent C'-ADE response. Firstly, this shows that vaccines classed as “ineffective” may prove to be dangerous if C'-ADE assays presented here translate to enhancing antibodies *in vivo*. Secondly, it shows that gp41 antibodies are not the only type of antibody capable of enhancing HIV infection. Thirdly, it shows that enhancement of HIV infection is not necessarily caused by sub-neutralising concentrations of neutralising antibodies, as proposed in the

“classical” dengue enhancement model, but by high concentrations of non-neutralising antibodies, as suggested in Chapter 4.

As a result of HIV enhancement studies published in the early 1990s showing that regions on gp41 contained the major enhancing epitopes (Mitchell et al., 1995; Robinson et al., 1991; Robinson et al., 1990a; Robinson et al., 1990b) and that gp120 was the major target for neutralising antibodies, subsequent envelope-based vaccines focused on the use of gp120. As the only characterised enhancement epitopes reside on gp41 of HIV, the results presented in this chapter suggest that, either enhancing epitopes exist on gp120, or specific epitopes are unimportant for enhancement. However, levels of enhancement presented in this chapter are lower than those seen in Chapter 4, and the lack of gp41 antibodies may account for this. Interestingly, a formally protective vaccine, derived from SIV recombinant vaccinia virus expressing SIV gp160 (VVrpg160), was rendered ineffective after boosting with a peptide fragment corresponding to the immunodominant (i.e. infection-enhancing) domain of gp41 (Mitchell et al., 1995), implying contribution of these antibodies to an enhancing effect. High titres of C'-ADE antibodies were detected in those macaques that progressed rapidly to disease, whereas macaques boosted with an irrelevant peptide were protected and produced low titres of C'-ADE antibodies (Mitchell et al., 1995). Alternatively, low levels of enhancement in my results could also be explained by the virus used for the enhancement assays, W61D, which may be less susceptible to enhancement than the primary isolates tested in Chapter 4, or the overall titre of HIV-binding antibodies produced, which is lower than those seen in infected individuals (Beddows et al., 1999; McCormack et al., 2000).

C'-ADE is strongest at peak binding antibody titres, which may translate to a specific risk period for vaccinated individuals. The duration of the C'-ADE response against W61D appears to be longer than the neutralising response against MN – the only other definite antibody activity observed in these samples. As shown in section 5.2.2.2, final samples from JUL008, JUJ009, JUZ064, JUX011 and JUT067 continue to enhance W61D infection, indicating the existence of this response past the time of follow-up. Not only do enhancement levels correlate with peak antibody titres within individuals, but enhancement levels also correlate with antibody binding titres compared between individuals. Those individuals that produced the highest level of antibodies capable of binding to W61D gp120 or V3 peptide showed the highest enhancement.

On no occasion is MN enhanced by the sera tested, despite the fact that the majority of these sera enhanced infection of W61D. This supports the suggestions made in Chapter 3, that C'-ADE could be a property of the virus, and that C'-RN is a phenomenon that occurs in the absence of true enhancement, perhaps reflecting the balance between enhancement and neutralisation.

Results of enhancement assays carried out with MM32 d10 virus indicated that antibodies produced by vaccination may not be capable of enhancing the infection of clinically relevant primary isolates. This may be because the antibodies elicited by the W61D gp120 vaccine do not have a high affinity for the gp120 of MM32 d10 virus. Perhaps vaccination with a strain more closely related to MM32 d10, or use of other primary isolates in the assay, would result in enhanced infection. In assays presented in this chapter the fold enhancement is calculated relative to the Week 0 samples for each subject, therefore the serum donor variation that exists when comparing serum from patient viruses with NHS should not exist. It is therefore possible that the peak of infection seen in the presence of week 30 serum from JUX011 and C' represents a very low level of enhancement. Further investigation ought to be carried out on the capacity for vaccine-induced antibodies to enhance infection of other clinically relevant primary isolates.

### **5.3.3 Do enhancing antibodies put vaccinated individuals at risk?**

With regards to HIV, vaccine-induced enhancement of disease could reveal itself in several forms. The most obvious is an increased rate of infection among vaccine recipients. While this is the main indicator of vaccine success or failure, there may also be more subtle outcomes. The time period between infection and onset of AIDS may be reduced in the presence of enhancing antibodies. This would be the equivalent of an exacerbated disease state of other viruses, such as atypical measles. Alternatively, the vaccine may have different risks according to an individual's genotype for certain cellular receptors, for example, Fc polymorphisms or CR density may vary among individuals.

Many of these "adverse" effects would only become apparent after the period of follow-up. This becomes important for recent vaccine trials, which, relative to the long course of HIV infection, are still in their infancy. Take, for example, the case of dengue disease enhancement in infants born to immune mothers: peak age risk for ADE-induced DSS or DHF occurs at approximately 6 months, and this is for

maternally-acquired antibodies which are passively acquired. Vaccination induces active antibody production and these antibodies can still be detected years after vaccination. Also, as mentioned in section 5.3.2, there may be specific risk periods following vaccination during which infection becomes more likely. Re-analysis of breakthrough infections from larger clinical trials of HIV envelope subunit vaccines may reveal such patterns.

#### **5.3.4 Concluding remarks**

Overall, given that this vaccination strategy failed to elicit antibodies capable of greater than 90% neutralisation of any of the viruses tested in the presence or absence of complement, even at the peak of antibody production, it is unlikely that this vaccine would be protective against circulating clinical HIV strains. Indeed, this was the conclusion of the researchers involved in the trial (Beddows et al., 1999). This argument is further strengthened by the detection of enhancing antibodies against the vaccine strain, providing the potential for this vaccine to have harmful, rather than beneficial, effects on its recipients if infected with HIV. However, as suggested by SIV studies in macaques (Montefiori et al., 1995) and animal models for other viruses (Raabe et al., 1999), detection of enhancing antibodies may not correlate directly with vaccine outcome, therefore further study is warranted. This may only be possible through studies of breakthrough infection following subunit vaccination. As results presented here are from a phase I clinical trial, the recipients are unlikely to become infected with HIV and are therefore followed-up for a limited period. A similar trial that reached phase III clinical trials, the Vaxgen trial, did result in infected individuals. Retrospective studies of the antibody profiles in individuals that did become infected with HIV, compared with those that did not, would provide valuable information on the relevance of enhancing antibodies to vaccine outcome.

## CHAPTER 6

### **Further investigations into antibody-dependent enhancement mechanisms and proposals for future work**

This chapter details investigation into the mechanism of the C'-ADE and C'-RN seen in Chapters 3, 4 and 5. Co-receptor usage, infectious virus to particle ratios, the involvement of CR2 and the requirement of the CR2 signalling domain were all investigated. Finally, in the context of results presented in this chapter, directions for future work are proposed.

#### **6.1 INTRODUCTION**

This chapter further investigates aspects of C'-ADE of HIV seen in Chapters 3, 4 and 5. Separate experiments, involving the investigation of co-receptor usage in enhancement, the bearing of infectious particle-to-particle ratios of individual virus isolates on capacity for enhanced infection, and the involvement of CR2 in experiments presented so far, are presented. Together they provide insight into the mechanism of C'-ADE in terms of receptor involvement and viral characteristics.

As high levels of enhancement shown in this thesis were obtained with R5-using primary isolates (Chapter 4) and an R5X4-using strain W61D (Chapter 5), but not with two X4-using TCLA strains, MN and IIIB (Chapters 3 and 5), it is possible that the co-receptor usage of these viruses has some influence on the capacity to become enhanced and that enhancement is inefficient through the X4 route of infection. Alternatively, it is possible that certain virus strains are not efficiently enhanced, regardless of co-receptor usage, by the sera tested thus far in this thesis. To determine whether enhancement can occur through the X4 route of infection, the R5X4 vaccine strain W61D, investigated in Chapter 5, was assayed on SupT1 cells lacking the CCR5 co-receptor but naturally expressing CXCR4. Levels of enhancement were compared with those seen on SupT1/R5 cells.

To gain further information about the nature of viruses enhanced by antibody and complement, the relative infectivity levels of nine HIV primary isolates studied in Chapter 4 were determined. Virus produced *in vivo* and virus preparations produced *in vitro* are known to consist of a high proportion of apparently noninfectious or defective

particles (Burton, 2006; Finzi et al., 2006) due to the high error rate of RT, low level of Env trimers on the viral surface and to a certain extent due to the shedding of gp120 leaving behind non-functional spikes (McKeating et al., 1991). Infectious particle-to-particle ratios have been estimated to be as high as 1:100 to 1:60,000 (Layne et al., 1992; Piatak et al., 1993), and can vary according to viral strain and culture conditions (McKeating et al., 1991). Given the high level of enhancement seen for the primary isolates investigated in Chapter 4, it is possible that increased infection seen through C'-ADE draws on this so-called "noninfectious" pool of virions, allowing infection that otherwise would not be seen in the absence of enhancing antibody and complement, as opposed to increasing the infectivity or replication of already infectious virions. Therefore, ratios of infectious titre to total viral content were calculated and compared with peak fold enhancement levels observed for eight of the primary isolates studied in Chapter 4.

Limited investigation has been carried out on the mechanisms of C'-ADE occurring through CR2, and the ability of CR2 to mediate these effects on various cell types. Potential ways in which C'-ADE could occur through CR2 are:

1. Increased attachment of the virus to the target cell, resulting in increased efficiency of entry.
2. Signalling through the receptor resulting in endocytosis of the virus and subsequent infection via an alternative pathway.
3. Signalling through the receptor to suppress intracellular antiviral activity.
4. Signalling through the receptor to increase viral replication.
5. Partial lysis of the virion resulting in increased efficiency of virus-cell membrane fusion.

The mechanism of C'-ADE of HIV has yet to be formally shown. Several papers have proposed that increased attachment to the target cell, resulting in enhanced viral entry, is the principal mechanism through mathematical modelling (Lund et al., 1995) and quantitative PCR (Robinson, 2006). Furthermore, the involvement of CR2 in C'-ADE on T cell lines has been shown using blocking antibodies (Prohaszka et al., 1997; Robinson et al., 1990c), but never using a molecular approach. It has not been shown if expression of CR2 in a cell type other than a T cell line confers the ability to support enhanced infection on the target cell.

A role for the cytoplasmic tail of CR2 in antigen internalisation and presentation on B cells has been proposed. Studies have shown CR2-dependent endocytosis of C3dg-coated antigens by antigen-non-specific B cells (Hess et al., 2000). Efficiency of B cell receptor (BCR)-mediated antigen internalisation and presentation is enhanced when cross-linked to CR2, but not when cross-linked to a CR2 mutant lacking a cytoplasmic tail (Barrault and Knight, 2004). Furthermore, while the cytoplasmic tail of CR2 is not required for binding of C3dg or EBV, it is required for internalisation of C3dg and for EBV infection (Carel et al., 1990). CR2 has also been shown to signal independently of the BCR and B cell co-receptor (CD19 and CD81) complex through two different pathways. In the first, ligation of CR2 leads to the activation of the Src tyrosine kinase pp60src. This in turn phosphorylates tyrosine on nucleolin, which then interacts with SH2 domains of phosphatidylinositol 3'-kinase (PI 3-kinase). The activated PI 3-kinase subsequently phosphorylates Akt on serine and threonine residues, activating Akt and inducing the serine phosphorylation of GSK-3, resulting in the down-regulation of GSK-3 activity (Barel et al., 2003; Baribaud et al., 2001). While the events downstream of this pathway await resolution, one potential outcome of the down-regulation of GSK3 activity is inhibition of apoptosis (Franke et al., 1995). In a second elucidated pathway, tyrosine phosphorylation of Cbl under control of c-Src kinase lead to, among other signalling events, the release of Vav from Cbl. Vav proteins represent a group of signal transduction molecules that may play a role in oncogenesis and development (Bustelo, 2001).

Overall, the potential for ligand-bound CR2 to interact with and activate signalling proteins independently of the B cell receptor complex means that CR2-triggered signalling cascades could occur in any cell type, and have the potential to mediate the signalling-induced C'-ADE mechanisms outlined above. In this chapter, cloned full-length and cytoplasmic tail-truncated CR2 molecules are expressed in NP2 cells to investigate, firstly, whether CR2 mediates the C'-ADE shown in this thesis, and secondly, whether CR2-activated intracellular signalling is required for this process.

Given time restraints, the results presented in this chapter are not intended to represent a full investigation into the mechanism of C'-ADE, but contribute interesting insights into the process. Therefore, they could form the basis of further investigation

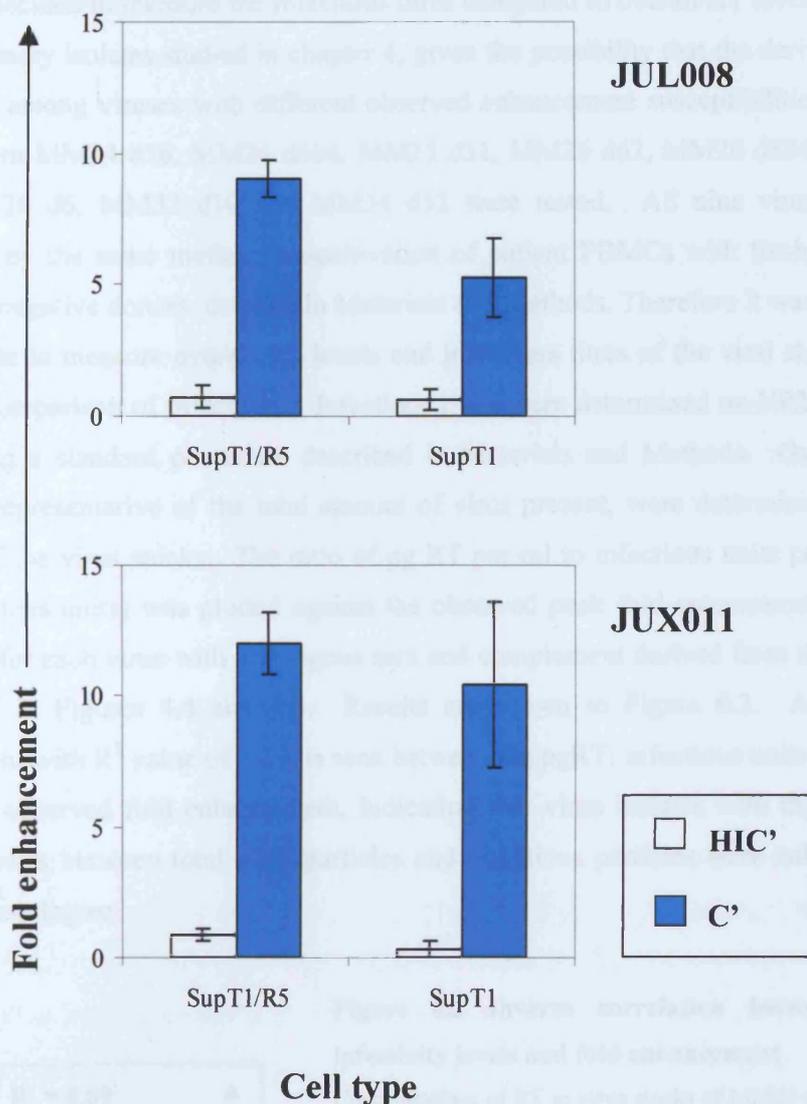
into the mechanism of C'-ADE of HIV, and as such proposals for future work are incorporated into the discussion.

## 6.2 RESULTS

### 6.2.1 C'-ADE can occur via the CXCR4 route of infection.

As shown in Section 3.2.1, SupT1 cells do not express detectable levels of CCR5, and are not infected by R5-only using isolates. Therefore virus infection of these cells is most likely to occur via the CXCR4 co-receptor. The vaccine virus strain studied in Chapter 5, W61D, is dual tropic and therefore provides the opportunity to investigate C'-ADE in both SupT1 and SupT1/R5 cells. If C'-ADE via CCR5 were more efficient, then this would be apparent upon comparison of enhanced infection of SupT1 and SupT1/R5 cells.

Parallel enhancement assays were carried out on SupT1 and SupT1/R5 cells using W61D virus and week 0 and 30 sera from JUL008 and JUX011 in the presence (C') and absence (HIC') of active complement. As with the results presented in Chapter 5, fold enhancement was calculated relative to infection levels in the presence of week 0 serum for each subject. Results are shown in Figure 6.1. There was no significant difference between enhancement levels seen on SupT1 and SupT1/R5 cells for JUX011 ( $p > 0.05$  using a two-tailed student's T test assuming equal variance), although the enhancement seen with JUL008 serum was significantly higher on the SupT1/R5 cells, 9-fold, compared with 5-fold on the SupT1 cells ( $p < 0.05$ ).

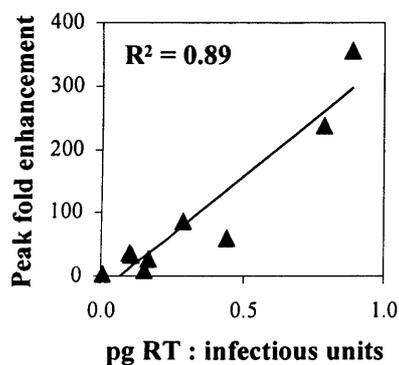


**Figure 6.1 C'-ADE of W61D on SupT1/R5 and SupT1 cells**

The R5X4-tropic vaccine virus strain, W61D, was tested with known enhancing sera from two vaccine recipients on SupT1/R5 and SupT1 cells. Virus was incubated with week 0 or 30 sera and HIC' or C' for 1 hour at 37°C and transferred to either SupT1/R5 or SupT1 cells. Levels of infection were determined by intracellular p24 staining on day 6 following inoculation. Fold enhancement was calculated relative to the week 0 control cultures. The top panel shows experiments using JUL008 week 30 serum, and the bottom panel JUX011 week 30 serum. Error bars represent SD from 3 experiments.

### 6.2.2 Ratios of infectious particles to particles in virus preparations susceptible to enhancement

I decided to measure the infectious titres compared to overall RT level of eight of the primary isolates studied in chapter 4, given the possibility that the derived ratio may vary among viruses with different observed enhancement susceptibilities. Viral stocks from MM24 d26, MM24 d464, MM25 d31, MM26 d62, MM26 d834, MM27 d28, MM28 d6, MM32 d10 and MM34 d32 were tested. All nine viruses were produced by the same method: co-cultivation of patient PBMCs with fresh PBMCs from seronegative donors, detailed in Materials and Methods. Therefore it was deemed appropriate to measure overall RT levels and infectious titres of the viral stocks as a relative comparison of infectivity. Infectious titres were determined on NP2/CD4/R5 cells using a standard procedure described in Materials and Methods. Overall RT activity, representative of the total amount of virus present, were determined by RT ELISA of the virus stocks. The ratio of pg RT per ml to infectious units per ml (pg RT:infectious units) was plotted against the observed peak fold enhancement values obtained for each virus with autologous sera and complement derived from the results presented in Figures 4.4 and 4.8. Results are shown in Figure 6.2. A positive correlation, with  $R^2$  value of 0.89, is seen between the pgRT: infectious units ratio and the peak observed fold enhancement, indicating that virus isolates with the greatest discrepancies between total virus particles and infectious particles were enhanced to the greatest degree.



**Figure 6.2 Inverse correlation between viral infectivity levels and fold enhancement**

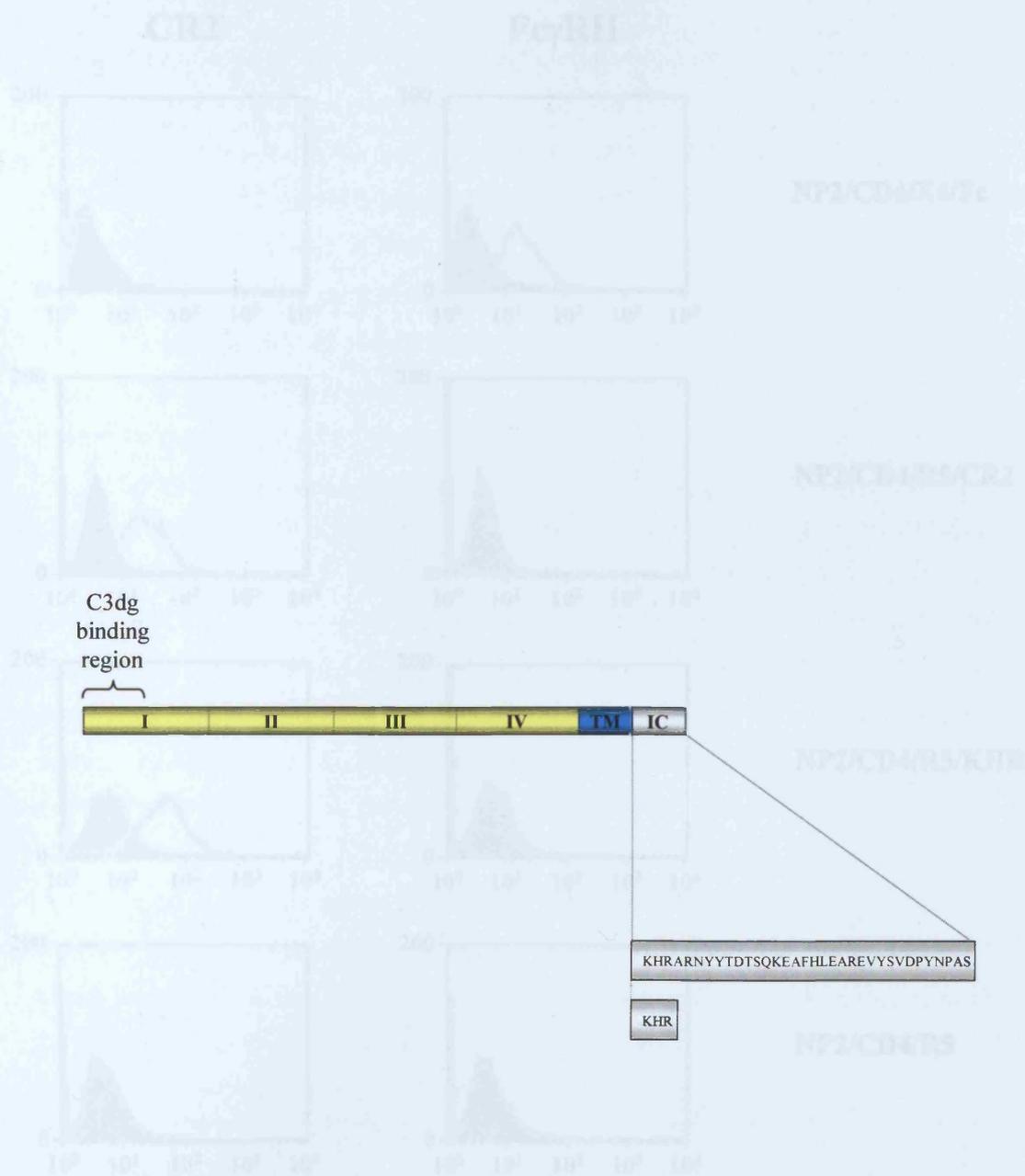
Concentrations of RT in virus stocks of MM24 d26, MM24 d464, MM25 d31, MM26 d62, MM26 d384, MM27 d28, MM28 d6, MM32 d10 and MM34 d32, were determined by RT ELISA. Infectious viral titres were determined by titration on NP2/CD4/R5 cells. Ratios of pg RT to infectious units were determined as a relative measure of the proportions of infectious viral particles in each virus preparation. These values were plotted against the peak fold enhancement seen with autologous serum for each virus.

## 6.2.4 Mechanism of CR2-mediated C'-RN and C'-ADE

### 6.2.4.1 Construction of cell lines expressing CR2, mutant CR2 and FcγRIIa

Although a role for CR2 in the C'-ADE and C'-RN results presented so far in this thesis has been presumed due to the use of a CR2+ T cell line (lacking other CRs and FcRs) and deductions from previous reports in the literature, it has not been formally proven. Also, the exceptionally high levels of enhancement of clinical HIV isolates could be SupT1/R5-cell type specific. To address both of these issues, CR2 was cloned from SupT1 cells and stably expressed in NP2/CD4/R5 and NP2/CD4/X4 cells.

To further probe the mechanism of C'-ADE, a mutant form of CR2, lacking all but 3 amino acids of the cytoplasmic tail, was constructed. The cytoplasmic tail mutant, based on constructs used in previous reports investigating a role for the cytoplasmic tail in CR2 function (Barrault and Knight, 2004; Carel et al., 1990), consisted of the entire extracellular and transmembrane portions of CR2, but only the amino acids K, H and R of the cytoplasmic tail, and is therefore referred to as KHR. The C3dg binding site is located in the two most membrane-distal SCRs of CR2 (Lowell et al., 1989); therefore removal of the majority of the cytoplasmic tail was not expected to affect ligand binding. A schematic diagram of CR2 and the mutant KHR are shown in Figure 6.3. Further details of the CR2 and KHR constructs are given in Materials and Methods. CR2 and KHR constructs were subcloned into a lentiviral vector system for stable expression in NP2/CD4/R5 and -X4 cells. For further investigation of the C'-RN results presented in Chapter 3, NP2/CD4/X4 cells were also transduced to stably express FcγRIIa (kindly provided by Dr. Francesca Gennari and Prof. Mary Collins). Cell surface expression of CR2, KHR and FcγRIIa are shown in Figure 6.4. NP2 cells were negative for the expression of other complement and Fc receptors, and CD19.



**Figure 6.3 Schematic diagram of full-length CR2 and the KHR mutant**

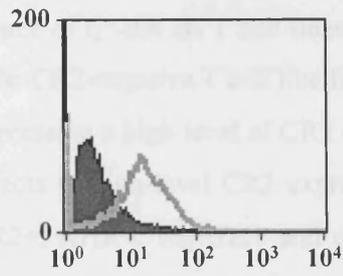
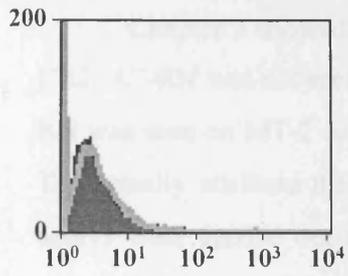
Full-length CR2 consists of a 34 aa cytoplasmic tail, a 28 aa transmembrane region and a long extracellular domain of consisting of 15 or 16 short consensus repeats (SCRs) of 60-70 aa each. These are divided into four homology domains, designated I-IV as shown, each consisting of four SCRs. In the short CR2 isoform homology domain III contains only 3 SCRs. The C3dg binding region is located in SCRs 1 and 2 of homology domain I. A cytoplasmic tail mutant was produced by inserting a stop codon after the first three amino acids of the cytoplasmic tail, and is consequently referred to as KHR.

**Figure 6.4 Expression of CR2, KHR and FcγRII on NP2 cells**

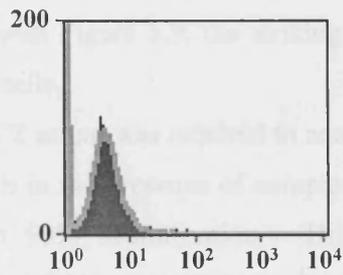
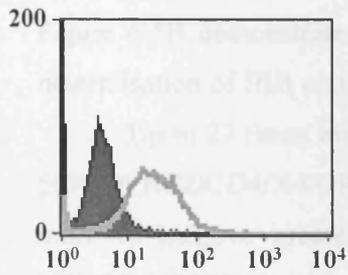
FcγRIIa was stably expressed on NP2/CD4/X4 cells (top row). CR2 and the cytoplasmic tail mutant CR2, KHR, were stably expressed on NP2/CD4/R5 cells (2<sup>nd</sup> and 3<sup>rd</sup> row respectively). NP2/CD4/R5 and -/X4 cells were tested for CR2 and FcγRII expression as controls. NP2/CD4/X4/CR2 and -/KHR cells expressed equivalent levels of CR2 and KHR to the NP2/CD4/R5/CR2 and -/KHR cells, data not shown.

### CR2

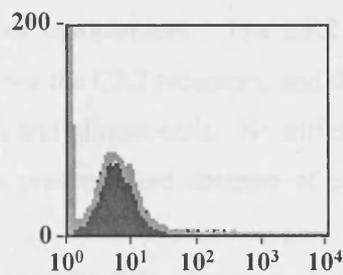
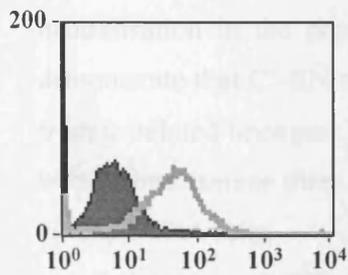
### FcγRII



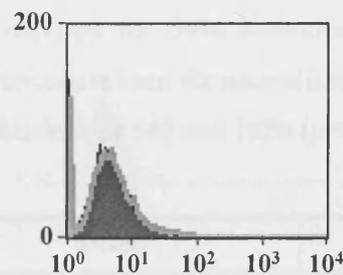
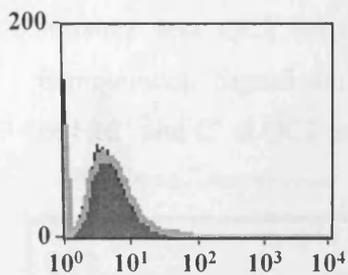
NP2/CD4/X4/Fc



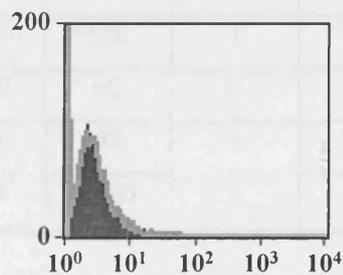
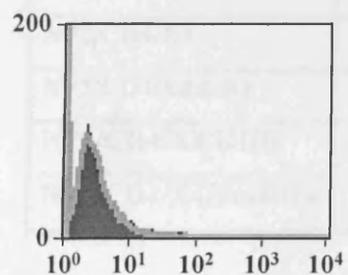
NP2/CD4/R5/CR2



NP2/CD4/R5/KHR



NP2/CD4/R5



NP2/CD4/X4

#### 6.2.4.2 The effect of CR2, KHR and FcγRIIa on neutralisation of IIIB

Chapter 3 showed evidence of C'-RN on T cell lines expressing high levels of CR2. C'-RN was not seen on the CR2-negative T cell line H9, and lower levels of C'-RN was seen on MT-2 cells expressing a high level of CR2 and a low level of FcγRII. To formally attribute these effects to high-level CR2 expression, IIIB neutralisation assays were carried out on CR2+, KHR+, FcγRIIa+ and control NP2/CD4/X4 cells with QC2 serum. Results are shown in Figure 6.5A and summarised in Table 6.1. Figure 6.5B demonstrates, as with Figure 3.9, the striking effect of complement on neutralisation of IIIB on CR2+ cells.

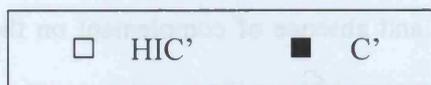
Up to 27 times more QC2 serum was required to neutralise IIIB to greater than 50% on NP2/CD4/X4/CR2 cells in the presence of complement, and at least 9 times more to achieve greater than 90% neutralisation. This was slightly lower on NP2/CD4/X4/KHR cells, with 9-fold more QC2 required for both 50% and 90% neutralisation in the presence of complement. The CR2 and KHR cells therefore demonstrate that C'-RN occurs via the CR2 receptors, and that this is possible on cells from unrelated lineages: T cells and glioma cells. No difference was seen for 50% or 90% neutralisation titres in the presence and absence of complement on the control NP2/CD4/X4 cells.

Interestingly, the presence of FcγRIIa slightly alters the IC50 values, but in this instance less QC2 serum is required for 50% neutralisation in the presence of complement. Significant differences are seen for neutralisation of IIIB in the presence of HIC' and C' at QC2 serum dilutions of 540 and 1620 ( $p < 0.05$ ).

QC2	IC50			IC90		
	HIC'	C'	DIFF <sup>1</sup>	HIC'	C'	DIFF <sup>1</sup>
NP2/CD4/X4	540	540	1	180	180	1
NP2/CD4/X4/CR2	1620	60	27	180	<20	>9
NP2/CD4/X4/KHR	540	60	9	180	<20	>9
NP2/CD4/X4/FcγRIIa	540	1620	3	180	180	1

**Table 6.1 IIIB 50% (IC50) and 90% (IC90) neutralisation titres for QC2 on NP2/CD4/X4 cells expressing CR2, KHR or FcγRIIa**

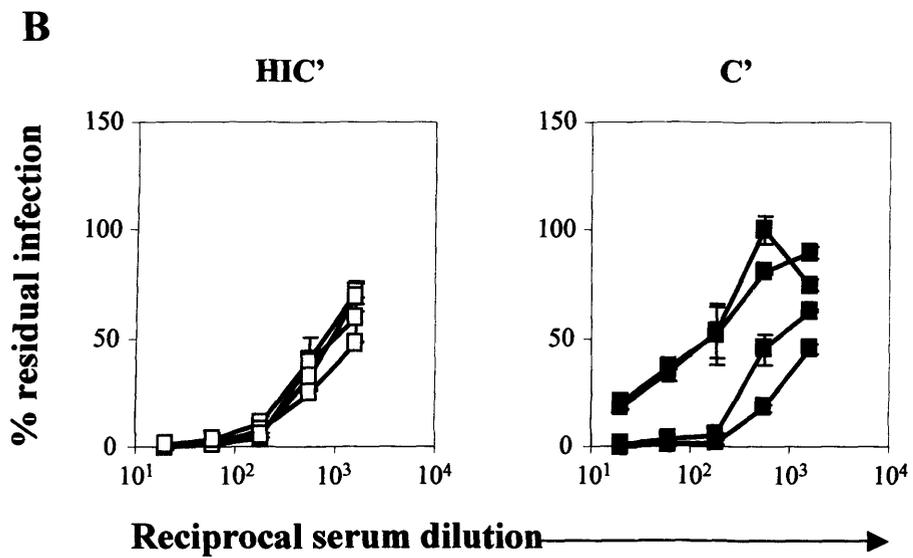
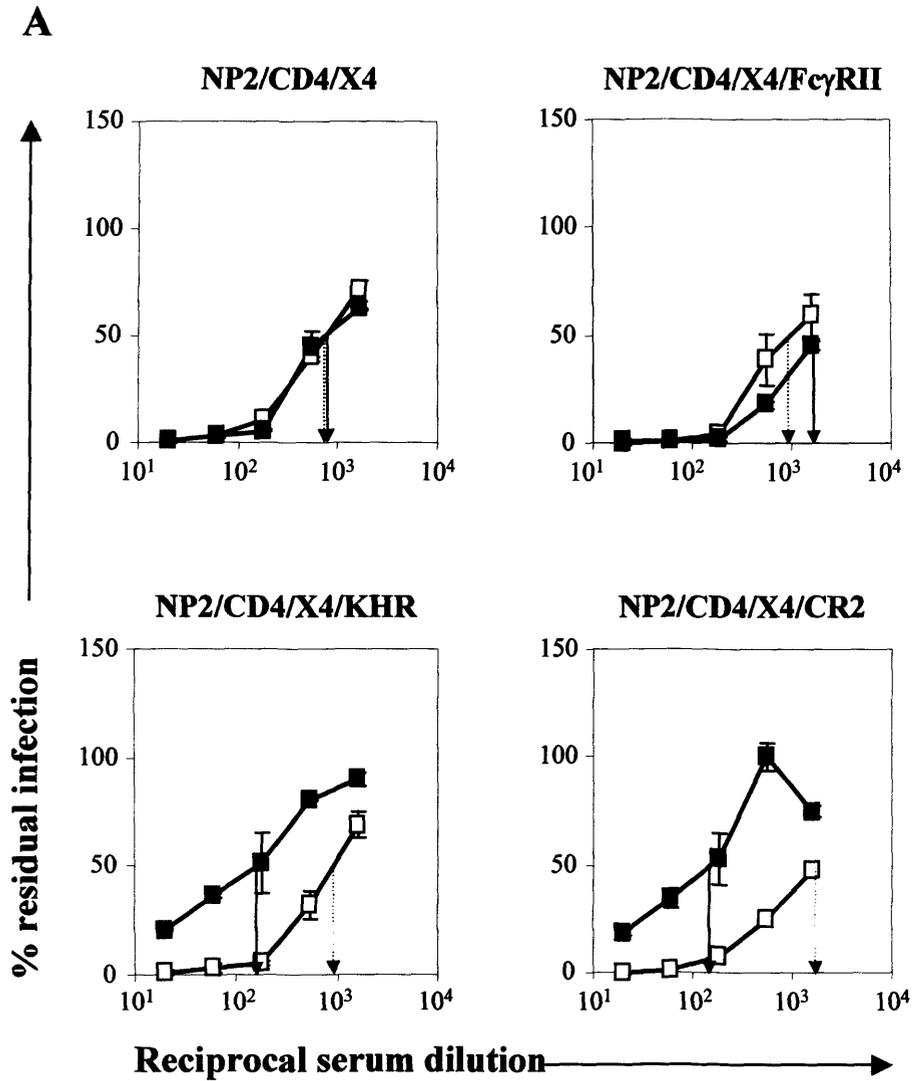
IC50 and IC90 values, derived from Figure 6.5, are summarised. <sup>1</sup>Fold differences between IC50 and IC90 values in the presence and absence of active complement.



**Figure 6.5 Neutralisation of IIIB on NP2/CD4/X4 cells expressing CR2, KHR or FcγRIIa**

(A) Neutralisation of IIIB by QC2 serum was assayed on NP2/CD4/X4 cells, and NP2/CD4/X4/CR2, -/KHR and -/FcγRIIa cells in the presence (C') and absence (HIC') of active complement. IIIB was incubated with serial dilutions of QC2 diluted in NHS, or NHS as a control, and HIC' or C' for 1 hr at 37°C then added to each of the 4 different cell lines. Cells were incubated for 2 hrs at 37°C then washed and fresh media added. After a further incubation of 48 hrs at 37°C cells were fixed and stained for p24 expression. Ffu per well were counted, and % residual infection levels determined relative to the NHS control. Solid and dashed red arrows indicate IC50 values in the presence (C') and absence (HIC') of active complement respectively. Error bars represent SD from 3 experiments.

(B) Results from Figure 6.6A from all 4 cell lines shown as infection in the presence (right) and absence (left) of C', to emphasise the effect that complement has on neutralisation on cell lines with and without CR2.

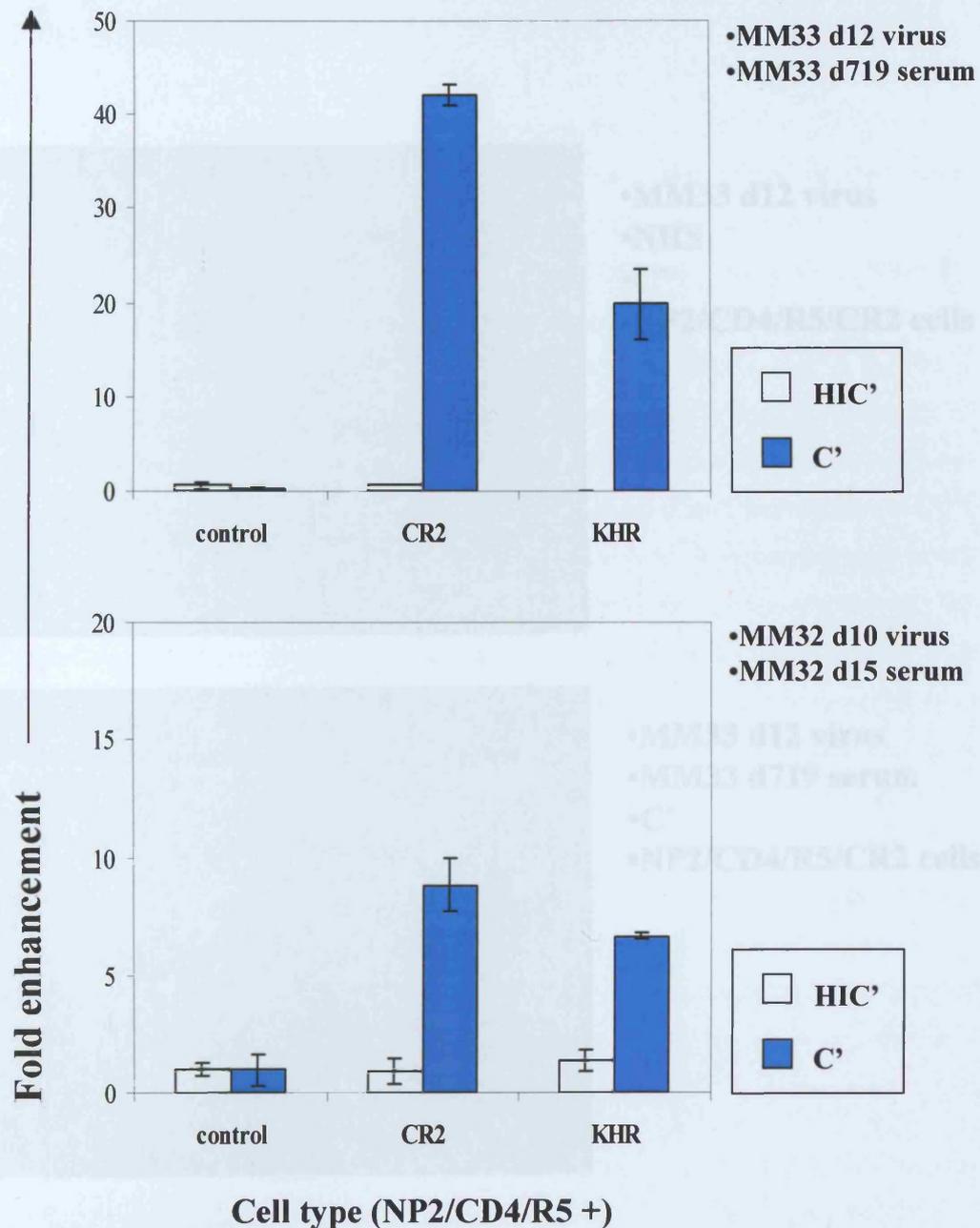


#### ***6.2.4.3 The ability of CR2 and KHR to mediate enhancement of early patient viruses by autologous sera***

Chapter 4 showed high-level enhancement of patient primary isolates, obtained soon after infection with HIV, by autologous sera and complement on SupT1/R5 cells. Two primary isolates were tested with known autologous enhancing sera, in the presence (C') and absence (HIC') of active complement, to investigate whether these results can be re-created on NP2/CD4/R5 cells expressing CR2 or KHR. Viruses from two patients were used: MM33 d12 virus, which was enhanced on SupT1/R5 cells by late autologous sera (peak d719, 115-fold), and MM32 d10 virus, which was enhanced on SupT1/R5 cells by early autologous sera (peak d21, 58-fold). Autologous serum from d719 was used with the MM33 virus, which enhanced to a level of 115-fold, and d15 autologous serum was used with MM32, which enhanced to a level of 24-fold on SupT1/R5 cells in the presence of C'.

Results are shown in Figure 6.6. Fold enhancement is calculated relative to infection in the presence of NHS and HIC' or C', as appropriate, on each cell type in exactly the same way as experiments carried out on SupT1/R5 cells in Chapter 4. On the NP2/CD4/R5 control cells, no enhancement of infection is seen for either of the viruses. In contrast, on the NP2/CD4/R5/CR2 and -KHR cells, C'-ADE of both viruses is detected. For the MM33 d12 virus, d719 serum enhanced infection 42-fold on the NP2/CD4/R5/CR2 cells and 20-fold on the NP2/CD4/KHR cells. For the MM32 d10 virus, d15 serum enhanced infection 9-fold on the NP2/CD4/R5/CR2 cells and 7-fold on the NP2/CD4/KHR cells. No enhancement is seen in the absence of complement.

Two inputs of each virus were used, but only the lower input was used to calculate fold enhancement as the higher input resulted in too many ffu to count in the enhanced wells. Pictures from the higher input enhanced wells for MM33 are shown in Figure 6.7, highlighting the dramatic effect of enhancing antibodies and complement on the infectivity of these primary isolates. The only difference between the two assays in the photo is the presence of NHS (top panel) or d719 autologous serum (bottom panel).

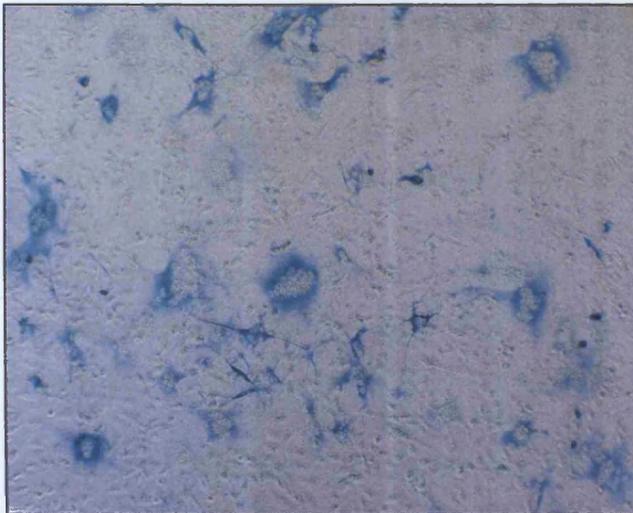


**Figure 6.6 C'-ADE of primary patient isolates with autologous sera on NP2/CD4/R5 cells expressing CR2 and KHR**

Autologous patient sera, shown to enhance infection of primary patient isolates in enhancement assays detailed in Chapter 4, were tested for enhancement on NP2/CD4/R5/CR2 and -/KHR cells, and control NP2/CD4/R5 cells. Virus was incubated with autologous patient serum, or NHS as a control, and C' or HIC' for 1 hour at 37°C, then transferred to NP2/CD4/R5 (control), NP2/CD4/R5/CR2 (CR2) or -/KHR (KHR) cells. After incubation for 48 hrs at 37°C cells were fixed and stained for HIV infection. Fold enhancement was calculated relative to the NHS + C' control on each cell type.



- MM33 d12 virus
- NHS
- C'
- NP2/CD4/R5/CR2 cells



- MM33 d12 virus
- MM33 d719 serum
- C'
- NP2/CD4/R5/CR2 cells

Magnification x 100

**Figure 6.7 C'-ADE of MM33 d12 virus by autologous serum on NP2/CD4/R5/CR2 cells**

Photographs of infected NP2/CD4/R5/CR2 cells. MM33 d12 virus was incubated with NHS or MM33 d719 serum , and C' for 1 hour at 37°C then added to cells. After 2 hours virus was removed and cells washed, then incubated for 48 hours at 37°C. After fixing, cells were stained for HIV infection with anti-p24 primary antibody, followed by  $\beta$ -gal substrate, resulting in blue staining of infected cells and syncytia. The top panel shows infection in the presence of C' and control serum, NHS. The bottom panel shows enhanced infection, in the presence of C' and d719 autologous serum.

## **6.3 DISCUSSION**

### **6.3.1 Infectious units and the nature of the viral envelope**

Laboratory preparations of HIV typically have low infectious particle-to-particle ratios (Burton, 2006; Finzi et al., 2006; Layne et al., 1992; Piatak et al., 1993), making growth of and experimentation with primary isolates troublesome. Furthermore, in the absence of appropriate target cells, infectious particles rapidly lose infectivity due to the disintegration of the envelope spike (McKeating et al., 1991). C'-ADE has the potential to alter this situation, both by retaining the infectivity of infectious particles for prolonged periods and by rendering previously uninfected or inefficient virions infectious, for example due to receptors with lower affinity for CD4, or due to lower numbers of Env spikes on the viral surface. A result important in this context is the observation that viruses that had the greatest discrepancy between RT content and infectivity also showed the greatest enhancement, indicating that the least infectious viruses were most enhanced. At this stage it is impossible to say whether the correlation is because the viruses with fewer infectious particles are enhanced more because C'-ADE is a strategy used by these viruses for infectivity, or if the viruses with the most infectious particles are enhanced less because there are insufficient residual viral particles to allow greater enhancement. Future work stemming from this observation would be to compare the nature of the envelopes. Do the most enhanced viruses have fewer or more envelope spikes? Are they more susceptible to gp120 shedding? Do their affinities for CD4 or co-receptor differ?

### **6.3.2 Co-receptor usage in C'-ADE**

Equivalent levels of C'-ADE of the R5X4 tropic virus strain W61D were seen on SupT1 cells in both the presence and absence of CCR5. This implies that C'-ADE can occur both through the CCR5 and CXCR4 routes of infection. For the JUL008 serum, however, the fold enhancement level on the SupT1/R5 cells was almost twice as high as that seen on the SupT1 cells. It is possible that the co-receptor usage accounts for this difference. To investigate this further, enhancement experiments using a wide range of X4-, R5- and R5X4 viruses should be carried out on the NP2/CD4/R5/CR2 and NP2/CD4/X4/CR2 cells. It would be interesting to determine whether R5 viruses are generally more susceptible to enhancement than X4 viruses, and whether X4 TCLA strains such as IIIB and MN are poorly adapted for high-level

enhancement. An interesting investigation in this context would be to isolate X4 viruses from individuals at late-stage of HIV infection, following the co-receptor switch, and investigate their potential for enhancement compared to R5 viruses from the same individual.

### **6.3.3 Mechanisms of CR2-mediated C'-ADE**

Here it is shown for the first time that HIV-permissive cells transduced to express CR2 are able to support enhanced infection. Previously, the involvement of CR2 in C'-ADE had only been shown through blocking antibody experiments on the MT-2 and MT-4 cell lines (Prohaszka et al., 1997; Robinson et al., 1990c). This finding eliminates the possibility that C'-ADE is a unique feature of SupT1 and SupT1/R5 cells, or T cell lines in general, as NP2 cells are a glioma cell line conferred with susceptibility to HIV infection through the artificial expression of CD4 and CCR5 or CXCR4. Results with the CR2+ NP2 cells also confirm that C'-ADE and C'-RN are mediated through the same mechanism, as both phenomena were observed on CR2+ and KHR+ NP2 cells.

The results presented in this chapter strongly suggest that increased attachment of the virus to the target cell surface is the primary mechanism of C'-ADE, as enhancement is achieved with a CR2 receptor lacking a cytoplasmic tail and therefore lacking signalling ability. However, while increased attachment may account for the majority of enhancement seen, results from the MM33 d12 virus suggest that enhancement is approximately two-fold more efficient on the cells with CR2 compared with cells with KHR. This is only seen for MM33 d12 virus, which is enhanced 40-fold on the CR2+ cells compared with 20-fold on the KHR+ cells, whereas MM32 d10 virus is enhanced 9-fold on the CR2+ cells and 7-fold on the KHR+ cells. It is possible that the discrepancy seen in the higher-level enhancement of MM33 d12 virus is accounted for by signalling-dependent mechanisms, and this warrants further work. The use of molecules that block certain signalling pathways may reveal the nature of the difference in enhancement on the two cell lines – specifically blocking a signalling pathway on the CR2+ cells may reduce the level of enhancement to that seen on the KHR+ cells. Alternatively, the difference could be accounted for by the nature of the expression of CR2 on the cell surface. For example, cytoplasmic tail truncation may affect localisation of the receptor in the membrane.

The possibility that partial complement-mediated lysis of the virions may be the mechanism of C'-ADE, by increasing the efficiency of fusion between virus and CR2+ cells, could be investigated by using C5-deficient serum as the complement source in enhancement assays. However, the fact that C'-MI does not occur in the presence of complement on the CR2-negative cells (see Figure 6.6, control cells) suggests that this is not the case, as the occurrence of C'-ML would be expected to result in decreased infectivity of the viruses on CR2-negative cells.

The contribution of the CD4 and CCR5 receptors to enhancement should also be investigated. Can CR2-mediated C'-ADE permit infection through CD4-independent mechanisms? Can higher levels of CR2 compensate for lower levels of CD4 or CCR5 expressed on the cells? To extend the results further, probing the abilities of other complement receptors, and potentially Fc receptors, to support enhanced infection could be investigated, and this would have further relevance to enhanced infection of HIV cellular targets *in vivo*.

## CHAPTER 7

### DISCUSSION

#### 7.1 SUMMARY OF RESULTS

In Chapter 3, MT-2 and MT-4 cells were used to investigate ADE of the TCLA virus strain IIIB, as previously performed by others. Low-level C'-ADE and ADE activity were seen in serum samples obtained at early time points following infection with HIV. Later serum samples neutralised IIIB infection. C'-ADE on the MT-2 cells was seen more frequently and to a greater magnitude than ADE on the MT-4 cells. C'-ADE was therefore investigated further. Studies of neutralisation in the presence and absence of complement were undertaken on T cell lines expressing various levels of CR2. Complement-mediated rescue from neutralisation (C'-RN) was observed on T cells expressing CR2, but not on H9 cells that were CR2-negative. C'-ADE, however, was not observed with neutralising sera from chronically-infected individuals. C'-RN was used as a surrogate for C'-ADE in order to optimise the enhancement assay for the future investigation of C'-ADE of clinical primary isolates. SupT1/R5 cells were chosen as the target cell for future enhancement assays due to their high-level expression of CR2, CD4, CXCR4 and CCR5, allowing for the study of C'-ADE of both primary isolates and TCLA virus strains, and due to the fact that C'-RN was greatest on the SupT1/R5 cells.

In Chapter 4, the enhancement assay developed in Chapter 3 was used to investigate C'-ADE of primary virus isolates by autologous sequential sera. Using viruses isolated at early time points following infection, C'-ADE was seen to a high level, up to 236-fold, in 9 out of 10 patients investigated. When later virus isolates were investigated from 5 of these patients, including the one that failed to produce C'-ADE activity against the early isolate, C'-ADE was also seen to a high level, up to 356-fold. Furthermore, sera that neutralised early viruses enhanced later viruses. When neutralising and enhancing sera were tested in competition studies against early virus, neutralisation was the dominant activity. C'-ADE was detected against heterologous primary isolates, but neutralisation was not.

In Chapter 5, the enhancement assay was used to investigate serum samples obtained from individuals participating in a monomeric HIV gp120 vaccine trial. A C'-MI assay was used in parallel to investigate C'-MI activity in the same vaccinee

serum samples on CR2-negative NP2 cells. Some neutralisation was detected in the vaccinee samples, but only against the TCLA strain MN. No neutralising activity was detected against the vaccine virus strain, W61D. C'-ADE activity against W61D was detected in all vaccinees, and in some could still be detected in the last sample taken, indicating that this response may endure past the period of follow-up. Individuals that showed the strongest neutralising response to MN showed the highest levels of C'-ADE of W61D. C'-MI was also detected against W61D, and this was often seen in serum samples that enhanced W61D infection of SupT1/R5 cells. It was demonstrated that, depending on the virus strain and assay system used, C'-MI, C'-ADE, C'-RN and neutralisation could be detected in the same serum samples.

In Chapter 6, the mechanism of the C'-ADE and C'-RN reported in Chapters 3, 4 and 5 was investigated. It was shown that the viruses enhanced to the greater degree by autologous sera in Chapter 4 had the greatest discrepancies between infectious titres and overall level of virus particles present in the virus stocks. C'-RN and C'-ADE were shown to be mediated by CR2, as demonstrated by the stable expression of CR2 in NP2 cells. A cytoplasmic tail mutant of CR2, KHR, was also able to mediate C'-ADE, indicating that the principle mechanism of C'-ADE was increased attachment of opsonised viruses to the target cell.

## 7.2 CONCLUDING REMARKS

The interplay between HIV, antibodies, complement and target cells is complex, and the outcome of such interactions varies according to the assay system used: neutralisation and lysis, as well as opsonisation leading to viral inhibition or clearance mechanisms, *trans*-infection and enhancement of infection have all been reported. The overall balance of the antibody effector mechanisms involved will determine which outcomes dominate *in vivo*, and whether the virus or the immune system wins the final battle. The results presented in this thesis point towards a favourable effect of antibodies and complement on HIV infection; at least when the target cells express CR2. CR2 expression has been detected on CD4+ T cells *in vivo* (Fischer et al., 1991; Fischer et al., 1999; June et al., 1992; Masilamani et al., 2002), with some studies placing the proportion of normal peripheral blood T cells expressing CR2 as high as 40% (Fischer et al., 1991). Further investigation and characterisation of CR2+ T cells may reveal a role for them in HIV infection.

Here I have investigated only one aspect of enhancement in detail, C'-ADE via CR2, yet it is possible that other forms of ADE may be relevant to HIV infection. This is touched upon briefly in Chapter 3, where enhancement of IIIB by certain serum samples seen on the CR2+ T cell line MT-2, was not seen by the same serum samples on the FcγRII+ T cell line MT-4. There is a possibility that even where C'-ADE is undetectable, other forms of ADE are active. C'-ADE has been shown to occur via CR1 and CR3 (Delibrias et al., 1993; Delibrias et al., 1994; Gras et al., 1993; Reisinger et al., 1990), and in one study CR3-mediated C'-ADE occurred in primary DCs (Bajtay et al., 2004). The use of CR1 as a binding receptor for HIV dissemination throughout the body has also been proposed (Stoiber et al., 2005). CR2 has also been shown to mediate *trans*-infection (Doepper et al., 2000; Dopper et al., 2003; Jakubik et al., 1999), and other CRs and FcRs may play a similar role. As shown in Table 7.1, FcRs and CRs are commonplace on cells infected by HIV. Furthermore, in the absence of CR+ or FcR+ target cells, other cells in the vicinity of infection are almost certain to express at least one type of CR or FcR. Overall, this provides the potential for C'-ME, C'-ADE, FcR-ADE, *trans*-infection, or a combination of all of these, to occur throughout the course of HIV infection.

CELL TYPE	COMPLEMENT RECEPTOR	Fc RECEPTORS
CD4+ T lymphocytes	CR1, CR2	FcαR, FcμRI
Monocytes/macrophages	CR1, CR3, CR4	FcγRI, FcγRII, FcγRIII, FcαR
Dendritic cells	CR1, CR3	FcγRI, FcγRII

**Table 7.1 The potential for HIV target cells to express complement and Fc receptors.** Note that not all subsets of the listed cell types express all receptors listed. Adapted from Speth 1997 and Sullivan 2001 (Speth et al., 1997; Sullivan, 2001).

The influence of C' on neutralisation or inactivation of HIV is often ignored in favour of simplified, high-throughput assays designed to screen a wide range of antibodies against a wide range of viral variants. The target cells used for these studies are uniform and are engineered to express only the receptors essential for HIV entry –

CD4 and CCR5 or CXCR4. Furthermore, viral assays are usually carried out in the absence of complement *in vitro*, yet, *in vivo*, the majority of virus is likely to be opsonised with antibodies and complement. Therefore, while these systems are invaluable for studies of HIV neutralisation, they are not always fully representative of the situation *in vivo*. Results presented in Chapter 5 highlight the fact that a single serum sample can cause C'-ADE, C'-MI, and C'-RN depending on the virus strain tested and the target cell used.

Aside from investigations of receptor expression patterns of HIV target cells, one might look to the virus for clues about its interactions with antibodies and complement. Features of gp120 ensure protection of conserved receptor-binding sites from neutralisation while maintaining high levels of sequence variability to enable rapid escape from neutralising antibodies. The disassembly of functional spikes and the presence of non-functional spikes on the viral surface ensure the production of a barrage of non-neutralising antibodies by the host immune system. Regions of gp41, for example, that are only exposed on non-functional, disassembled or post-fusion spikes are immunodominant. In addition, intact Env spikes on the viral surface are few in number and arranged in clusters (Zhu et al., 2006), and the efficiency of interaction with CD4 for primary isolates is low (Ugolini et al., 1999). Mechanisms such as increased binding to the target cell via C-type lectin receptors (Geijtenbeek et al., 2000; Lee et al., 2001), heparin sulphate proteoglycans (Mondor et al., 1998), GalC (Kensinger et al., 2004) and host proteins incorporated into the viral membrane, such as ICAM-1 (Fortin et al., 1997) may all contribute to increasing the efficiency of HIV interaction with the target cell. It is unknown how efficient these attachment mechanisms are in the presence of antibody and complement. Strategies such as cell-to-cell transfer at the viral synapse (Piguet and Sattentau, 2004) and *trans* infection by B cells, DCs, FDCs and RBCs (Banki et al., 2005a; Stoiber et al., 2005) also serve to protect the virus from neutralisation while promoting infection. C'-ADE may be as efficient as, if not more efficient than, these mechanisms at promoting infection of CD4+ target cells. ADE would work particularly well as it combines a decoy strategy - directing the immune response towards non-functional spikes or enhancing domains - with an attachment strategy. Indeed, in Chapter 4, autologous viruses that had evolved to evade a neutralising response maintained the capacity for enhanced infection, to a level equal to or greater than earlier viruses. This suggests that, not only do viruses

evolve to escape a neutralising response, they may also evolve to maintain an enhancing response.

The role of ADE in HIV transmission and establishment of infection has not been thoroughly investigated. A shift in focus from blood to mucosae due to recent reports of high-level destructive infection of the MALT provides the opportunity to study a new set of cellular targets, albeit difficult to access, for CR and FcR expression. IgA may be more efficient at mediating ADE than IgG or IgM, as proposed by Kozlowski and colleagues (Kozlowski et al., 1995). Therefore studies of Fc $\alpha$ R and CRs on T cells of the MALT may prove particularly interesting.

To take the data presented in this thesis to their final conclusions, one might propose that antibodies and complement in HIV infection are harmful. Yet, numerous situations suggest otherwise, not least the fact that the virus rapidly evolves to evade neutralisation (Frost et al., 2005; Richman et al., 2003; Wei et al., 2003), and that cobra-venom treated (Schmitz et al., 1999) and B-cell depleted (Miller et al., 2007) monkeys with SIV have higher viral loads and progress rapidly to disease and death, compared to monkeys with intact complement and antibody responses. Therefore, involvement of C'-ADE may serve only to select for certain viral variants, i.e. those that are resistant to neutralisation and enhanced to the greatest degree, or C'-ADE may only function to establish virus reservoirs on FDCs, for example. Alternatively, ADE described in this thesis may correlate with other phenomena occurring *in vivo*, such as increased viral clearance.

While the role of C'-ADE in natural infection is likely to be complicated and determined by many different factors, the role of C'-ADE following vaccination may be more straightforward for the major reason that most subunit vaccines fail to produce neutralising antibodies. Thorough analyses of breakthrough infections following vaccine trials ought to be undertaken from the perspective of potentially enhanced infection. If it emerges that there is a timeframe in which vaccinated individuals are more likely to become infected than placebo controls, or if individuals with detectable enhancing antibodies at the time of infection have higher viral load set points than those without enhancing antibodies, then there might be cause to be concerned about enhancing antibodies.



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