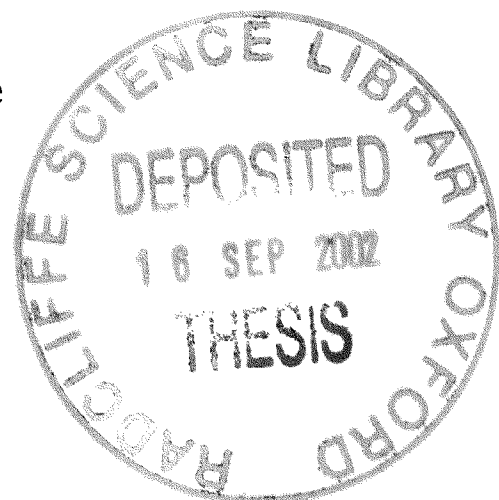


Macrophage-HIV interactions:
**Aptamers against the gp120 surface envelope
glycoprotein of the macrophage tropic strains of HIV-1**

**A thesis submitted for the degree of Doctor of Philosophy
in the University of Oxford
Hilary Term 2002**

Makobetsa Khati
Sir William Dunn School of Pathology
and
Wolfson College



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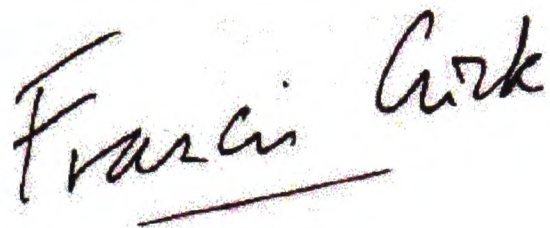
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University of Oxford, Hilary Term 2002**

Makobetsa Khati

Sir William Dunn School of Pathology and Wolfson College

"A neat job in a small space"(Crick, 1968)

Francis H. Crick (1916-Present)

Handwritten signature of Francis Crick in black ink. The name "Francis" is written in a cursive script, and "Crick" is written in a more stylized, bold cursive. A horizontal line is drawn under the word "Francis".

"The process of scientific discovery is, in effect, a continual flight from wonder."

Albert Einstein (1879-1955)

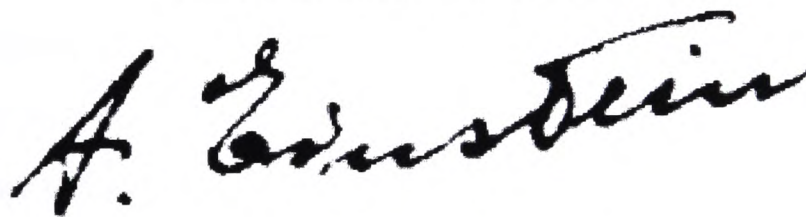
Handwritten signature of Albert Einstein in black ink. The signature is written in a highly stylized, cursive script, starting with a large "A." followed by "Einstein".

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Macrophage-HIV interactions: aptamers against the gp120 envelope glycoprotein of the macrophage tropic strains of HIV-1

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Submitted for the degree of Doctor of Philosophy, Hilary Term, 2002

Abstract

HIV-1 has evolved a number of strategies in response to current anti-retroviral drugs and the selection pressure of humoral and cellular immunity. In particular, R5 viral strains that are essential for AIDS pathogenesis are very resistant to neutralization by antibodies. Therefore, the aim of this thesis was to develop synthetic nucleic acid ligands, aptamers, against gp120 of an R5 strain of HIV-1, with a view of using aptamers as novel neutralization molecules and analytical tools to study HIV-1 entry into target cells. The central hypothesis of this thesis was that aptamers by virtue of their small size and slow dissociation rates, compared to antibodies, would easily access and bind occluded gp120 neutralization sites.

Using the SELEX protocol and SPR technology, I isolated 2'-Fluoro-pyrimidine-RNA aptamers against HIV-1_{Ba-L} monomeric gp120. Most of these aptamers not only bound gp120 with high affinities but also neutralized R5 primary isolates in human PBMC by 1,000 to 100,000-fold, truly unprecedented when compared with natural ligands such as antibodies. Some aptamers, like B4, defined a conserved site of gp120 that could not mutate to escape neutralization following stringent selection, *in vitro*, for breakthrough virus. This was consistent with subsequent findings that B4 aptatope (binding site) overlaps a poorly immunogenic but highly conserved CD4-induced epitope as determined by competition with 17b and 48d mAbs that map to this neutralization epitope on the gp120. This study was thus the first of its kind to describe neutralization of HIV-1 primary isolates by a ligand against the CD4-induced epitope. Most intriguing, although B4 potently neutralized HIV-1_{Ba-L} infection in PBMC, which is a mixed T cell and macrophage population, it modestly neutralized infection of the same virus in a purified culture of macrophages. These findings are intriguing in that they suggest that aptamers could be used to dissect unique sites on the virus that interact with target cell surface in ways that have not been revealed heretofore, and would help understand better HIV-1 entry pathways, especially in macrophages. Thus neutralizing aptamers such as these could be exploited to provide leads in developing alternative anti-HIV-1 drugs and a deeper understanding of the molecular interactions between the virus and its host cell.

Acknowledgements

I would like to thank my supervisors; William James and Siamon Gordon, for allowing me work in their laboratories, and I also thank respective members of their groups, past and present, for scientific inputs and making my stay an enjoyable one. I specially thank William James for his thoughtful insight and for critically reviewing this thesis. My special thanks also go to Siamon Gordon, my mentor, for his generous and unconditional support, especially during difficult times in my studies.

I am equally indebted to Herman Waldmann, in his capacity as head of the department, for giving me the opportunity to study at the Dunn School. My special gratitude goes to Christine Holt for her cheerful logistical assistance, Lance Tomlinson for photography, Alice Taylor for sequencing, John Marriott for IT support, Michael Hollinshead for fluorescent microscopy, Steve Clark and Michael Puklavec for ensuring smooth running of the HIV and tissue culture labs, respectively.

I thank George Gao for his generous gift of the recombinant gp120 construct. I also thank Ian Jones for his advice on expressing the gp120, and for kindly allowing me to set up the baculovirus expression experiments in his laboratory. I am also grateful to Quentin Sattentau for help with competition ELISA, and for willingly allowing me to stay in his lab to do the experiments. I would also like to acknowledge the AIDS Research and Reference Reagent Program, NIH, Bethesda, USA and the MRC AIDS Program, U.K. for providing HIV-1 isolates, antibodies and other necessary reagents required for this study.

For financial support I would like to thank the South African Medical Research Council, the Universities of Cape Town and Oxford, and Wolfson College.

Importantly, I owe it to my family and friends for their support, and for keeping me smiling. A special and a very big thank you to each and every one of you.

Pula nala!

Abbreviations

2'F-	2'fluoro-
ADCC	antibody dependent cellular cytotoxicity
AIDS	acquired immunodeficiency syndrome
APS	ammonium persulfate
AS	autologous serum
AZT	3'-azidothymidine
BSA	bovine serum albumin
c.p.e.	cytopathic effect
CA	capsid (protein)
CD4BS	CD4 binding site
CHO	Chinese hamster ovary (cells)
CRbs or CD4i	coreceptor binding site or CD4 induced site
CRFs	circulating recombinant forms
CTS	central termination signal
CypA	cyclophilin-A
DCs	dendritic cell
DC-SIGN	dendritic cell-specific ICAM-3 grabbing nonintegrin
DMEM	Dulbeco's modified Eagle's medium
DNA	deoxyribonucleic acid
DTT	dithioiteitol
EDC	N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme linked immunosorbant assay
ENV	envelope (protein)
FACS	fluorescence activated cell sorter
FcR	Fc receptor
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GalCer	galactosylceramide
gp	glycoprotein
GRID	gay-related immune deficiency
HAART	highly active anti-retroviral therapy
hGFP	humanized green fluorescent protein
HIV-1	human immunodeficiency virus type-1
HOS	human osteo-sarcoma (cells)
HR	heptad repeat domain
HS	heperan sulphate
hu-SCID	human severe combined immunodeficiency (mice)
ICAM-	intercellular adhesion molecule-
IL-	interleukin-
IMM	Institute of Molecular Medicine
IN	integrase (enzyme)
Kd	affinity constant
LFA-1	leukocyte function-associated antigen-1
LTR	long terminal repeats
m.o.i	multiplicity of infection

MA	matrix
mAb	monoclonal antibody
MDM	monocyte derived macrophage
MIP-	macrophage inflammatory protein
MΦ	macrophage
NIAID	National Institute of Allergy and Infectious Disease
NC	nucleocapsid protein
NF-κB	nuclear factor kappa B
NHS	N-hydroxysuccinimide
NIH	National Institute of Health
NP-40	Nonidet P-40
NSI	non-syncytium-inducing
OD	optical density
OI	opportunistic infection
PAGE	polyacrylamide gel electrophoresis
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells
PBs	primer-binding site
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethelyene glycol
PIC	preintegration complex
PIs	primary isolates (HIV-1)
PPT	polypurine tract
PR	protease
RANTES	regulated-upon activation, normal T-cell expressed and secreted
RNA	ribonucleic acid
RT	reverse transcriptase
RTCN	ratio to cell negative
SDF-1	stroma-derived factor-1
SDS	sodium dedecyl sulphate
SELEX	systematic evolution of ligands by exponential enrichment
SIV	simian immunodeficiency virus
SPR	surface plasmon resonance
SU	surface (protein)
SWDSOP	Sir William Dunn School of Pathology
TCID ₅₀	tissue culture infectious dose
TCLA	T cell line adapted
TEMED	N, N, N, N'-tetramethylethylenediamine
TM	transmembrane
TNF-	tumor necrosis factor-
UNAIDS	United Nations Program on HIV/AIDS
URL	uniform resource locator
u.v.	ultraviolet (light)
VEGF	vascular endothelial growth factor
WHO	World Health Organization

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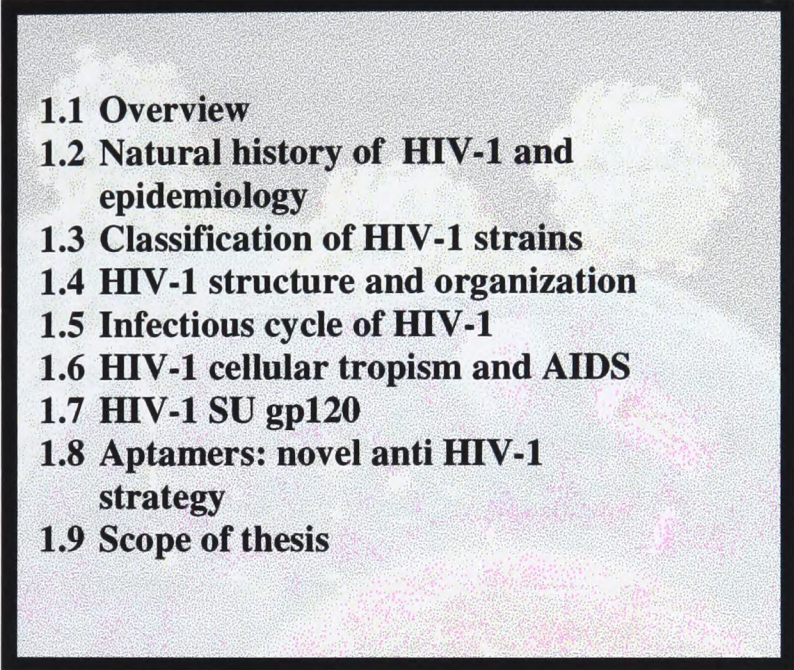
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Chapter One

Introduction and background

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 - 1.2 Natural history of HIV-1 and epidemiology**
 - 1.3 Classification of HIV-1 strains**
 - 1.4 HIV-1 structure and organization**
 - 1.5 Infectious cycle of HIV-1**
 - 1.6 HIV-1 cellular tropism and AIDS**
 - 1.7 HIV-1 SU gp120**
 - 1.8 Aptamers: novel anti HIV-1 strategy**
 - 1.9 Scope of thesis**

Background: The HIV-1 life cycle
(From the Russell Kightley Media, www.rkm.com.au)

1. Introduction and background

1.1 Chapter Overview

This chapter is a synthesis of our current understanding of human immunodeficiency virus type-1 (HIV-1) molecular and cellular biology in relation to AIDS (acquired immunodeficiency syndrome) pathogenesis, and strategies to combat the epidemic. First, to prime the reader, general perspectives on the natural history of HIV-1 and epidemiology are presented. Then, prevailing concepts and others' experimental findings are reviewed with a view to facilitate interpretation of data presented in subsequent chapters of this thesis. In particular, much attention is paid to the entry stage of the HIV-1 life cycle, from binding to fusion of the virus with host cells. These are putative targets of therapeutic interventions, and formed the basis of this thesis. Macrophages (MΦ) which are arguably the most essential cells of the immune system and primary targets of HIV-1, are discussed in the context of HIV-1 tropism and AIDS pathogenesis. HIV-1 gp120 surface (SU) glycoprotein, which is central to HIV-1 entry and function, is discussed in detail to provide a better understanding of HIV-1 infection and for the design of new efficacious therapeutic strategies. Finally, aptamers, which have been integral tools of this research, are discussed as novel and attractive therapeutic candidates against HIV-1.

1.2 Natural history of HIV-1 and epidemiology

HIV-1 originated from a primate reservoir, most probably chimpanzees. Three simian immunodeficiency virus (SIV) isolates called SIV_{cpz} that naturally infect common chimpanzees (*Pan troglodytes*) native to west equatorial Africa have striking sequence homology with HIV-1 (Gao et al., 1999). Since the three SIV_{cpz} isolates correspond to three distinct HIV-1 groups, M; N; and O (discussed in 1.3), it is suspected that there have been at least three independent cross-species (zoonotic) transmission events from the common chimpanzee to humans. A second type of HIV endemic to West Africa,

called HIV-2 was isolated subsequently (Clavel et al., 1986). The primate reservoir of HIV-2 has been clearly identified as the sooty mangabey (*Cercocebus atys*) (Chen et al., 1996; Gao et al., 1992; Hirsch et al., 1989), hence it has a different origin from HIV-1. HIV-2 is both less easily transmitted and pathogenic than HIV-1 (Pepin et al., 1991; Whittle et al., 1994), and unless otherwise stated this thesis is confined to HIV-1.

It was in 1983, two years after the appearance of the previously unknown disease, then called GRID (Gay-Related Immune Deficiency) (Brennan and Durack, 1981), that HIV-1 was isolated as the aetiological agent of what we now know as AIDS (Barre-Sinoussi et al., 1983; Gallo et al., 1984; Levy et al., 1984). HIV-1 is universally recognized as the cause of AIDS (Declaration, 2000), although this has since been disputed by some dissidents (Stewart, 2000). AIDS is characterized by a series of clinical, immunological and virological parameters and has been formally defined by the world health organization (WHO, 1993). The major indicators of AIDS are a CD4⁺ T cell count of less than 200/ μ l of blood, severe immunosuppression, opportunistic infections such as *Pneumocystis carinii* pneumonia (PCP) and Kaposi's sarcoma. The mean time for progression to AIDS is about 10 years in the absence of therapy.

Despite the introduction of several anti-retroviral drugs such as zidovudine and combination chemotherapy (Richman, 2001), HIV currently infects more than 36 million (1 in 162) people worldwide (Figure 1.1), making it one of the major global cause of mortality (Nicoll and Gill, 1999; Piot et al., 2001; UNAIDS, 2000). In addition to an estimated 36 million people living with HIV/AIDS, some 20 million people have already died, giving a cumulative total number of HIV infections of 56 million (UNAIDS, 2000). The worst of the epidemic, and the region where the HIV/AIDS reality is most devastating is sub-Saharan Africa, which accounted for 70% of all HIV/AIDS cases by December 2000 (UNAIDS, 2000). The high incidence of HIV/AIDS in this region could be attributed to a number of factors such as poor health care in general, and inaccessibility to current retroviral drugs because of their elevated costs, in particular. Other factors that generate vulnerability to HIV-1 infection and

AIDS are socio-economic, including lack/disregard of health education, promiscuity, poverty and migrant labour (Carael et al., 1997; Piot et al., 2001). More pertinently, the prevalence of reportedly more infectious and pathogenic HIV-1 strains (refer 1.3) also account for high HIV/AIDS incidences in the worst hit regions of the world (Kunanusont et al., 1995; Montano et al., 2000; Ping et al., 1999).

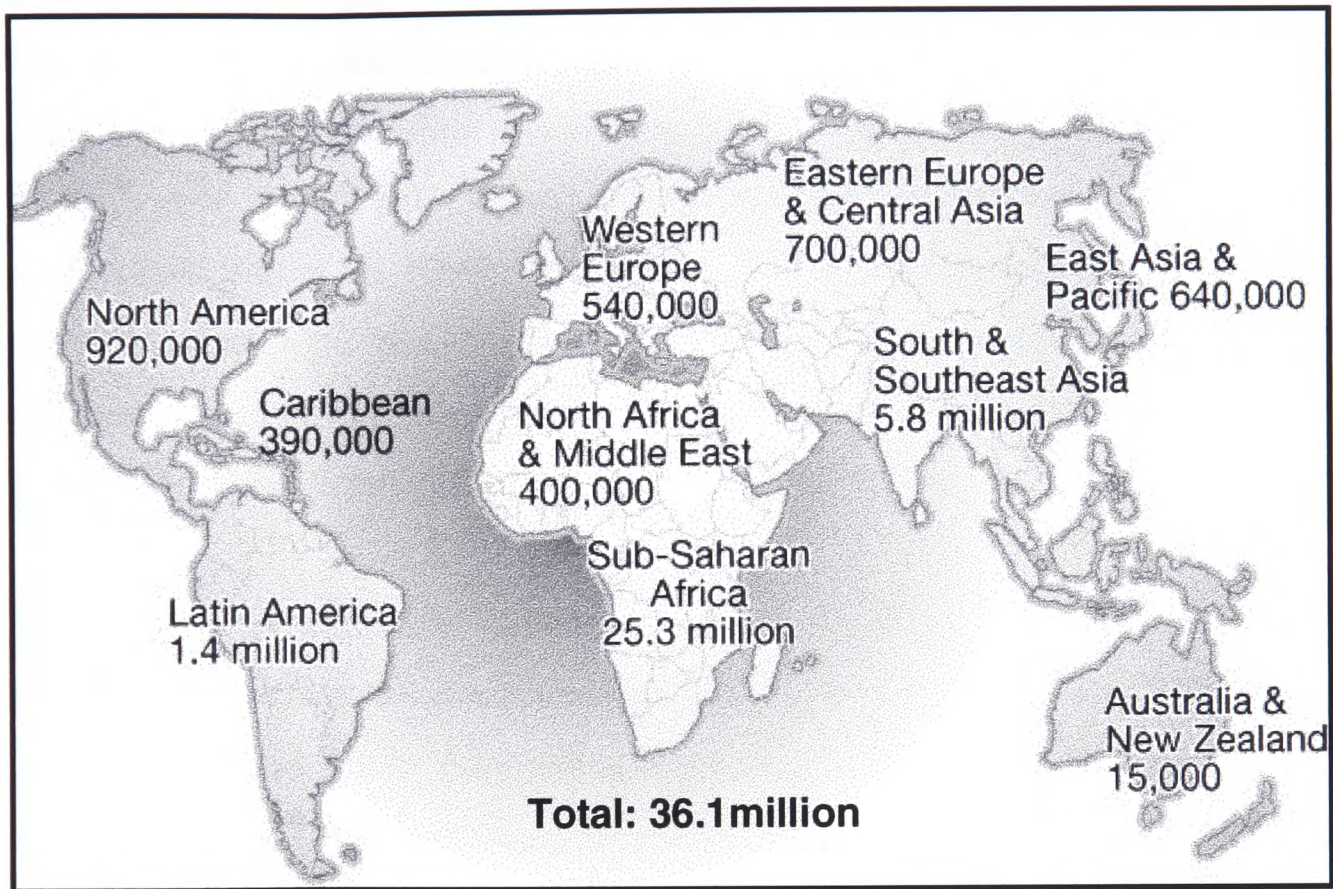


Figure 1.1: Worldwide numbers of adults and children living with HIV/AIDS at the end of 2000. (Source (UNAIDS, 2000))

1.3 Classification of HIV-1 strains: Implications in the global epidemic

HIV-1 is a member of the *Retroviridae* family, the members of which are RNA viruses that replicate through copying their DNA intermediate by a process called reverse transcription (refer 1.5). One of the distinguishing characteristics of HIV-1 is its extremely high genetic variability and rapid evolution. Extensive heterogeneity and rapid evolution of the virus result from the high error rate of reverse transcriptase (Preston et al., 1988), and the fast turnover of virions in HIV-infected individuals (Ho et al., 1995; Wei et al., 1995). In addition, the reverse transcriptase enzyme is highly recombinogenic (Hu and Temin, 1990a; Hu and Temin, 1990b), so that radically different genomic combinations may be generated in individuals infected by genetically diverse viruses (Peeters and Sharp, 2000).

Phylogenetic analyses of numerous strains of HIV-1, isolated from different geographic locations, have revealed that they can be subdivided into groups, subtypes, sub-subtypes and CRFs (circulating recombinant forms) (Robertson et al., 2000; Robertson et al., 1995; Vanden Haesevelde et al., 1994). HIV-1 is currently classified into three distinct groups, namely; M, N and O (Figure 1.2). The majority of HIV-1 strains prevalent globally and responsible for the pandemic belong to the group M (Major) lineage (Figure 1.2). Group O (Outlier), and the recently identified group N (for New, or non-M or non-O) are endemic to Cameroon in West Central Africa, and are represented by a limited number of isolates (Ayouba et al., 2000; Mauclore et al., 1997; Simon et al., 1998). Group M is subdivided into genetically distinct subtypes. Subtypes were proposed because most sequences of group M isolates fall into a limited number of discrete clades (Louwagie et al., 1993). The subtypes are approximately equidistantly related, and in order to be considered as a subtype, isolates should resemble each other, and no other existing subtype across the entire genome. According to this criterion, there are therefore presently nine clades within group M (Figure 1.2). These are clades A-D, F-H, J and K. All known representatives of what was initially described as subtype E, appear in fact to be recombinants of subtypes A

and E (Gao et al., 1996), and are now designated CRF01-AE (Robertson et al., 2000). Within some subtypes, further phylogenetic structures can be identified, leading to classification into sub-clades. For instance clade F is further subdivided into two sub-clades, F1 and F2 (Triques et al., 2000). It is also clear that clades B and D would be better designated as sub-clades of a single clade, but are considered as two independent subtypes of group M for historical reasons. Figure 1.2 also depicts the geographic distribution of HIV-1 subtypes and CRFs. The predominant viral forms in the global epidemic are clades A and C, followed by subtype B and the recombinants CRF01-AE and CRF02-AG (Peeters and Sharp, 2000).

Subtype designations have been powerful molecular epidemiological markers to track the course of HIV-1 transmission and the pandemic. It has been suggested that certain subtypes may be predominantly associated with specific modes of transmission. For example, subtype B with homosexual transmission and intravenous drug use (essentially via blood) and subtypes E (CRF01-AE) and C, with heterosexual/vertical (via a mucosal route) transmission (Kunanusont et al., 1995; Santiago et al., 1998; van Harmelen et al., 1997; Yoshino et al., 1998). It has also been indicated that some subtypes are easily transmissible and more virulent (Kunanusont et al., 1995; Peeters, 2000). In one study conducted in Thailand among 185 couples with one partner infected with subtypes E or B, it was found that the probability of both partners in a couple becoming infected was higher for subtype E (69%) than for subtype B (48%) (Kunanusont et al., 1995). This suggests that subtype E may be more easily transmissible. Recent studies on HIV-1 clade C infections (the subtype prevalent in regions with the highest HIV/AIDS incidences, refer to Figures 1.1 and 1.2), indicated that it displays a preferential use of the CCR5 coreceptor (discussed in 1.6), and therefore more pathogenic (Montano et al., 2000; Ping et al., 1999). It is however important to note that neither study was designed to control fully for multiple variables that may affect the risk of transmission and pathogenicity. There is much we do not yet understand about HIV-1 variation and it could be that the variation reflects stochastic founder effects (Weiss, 2001).

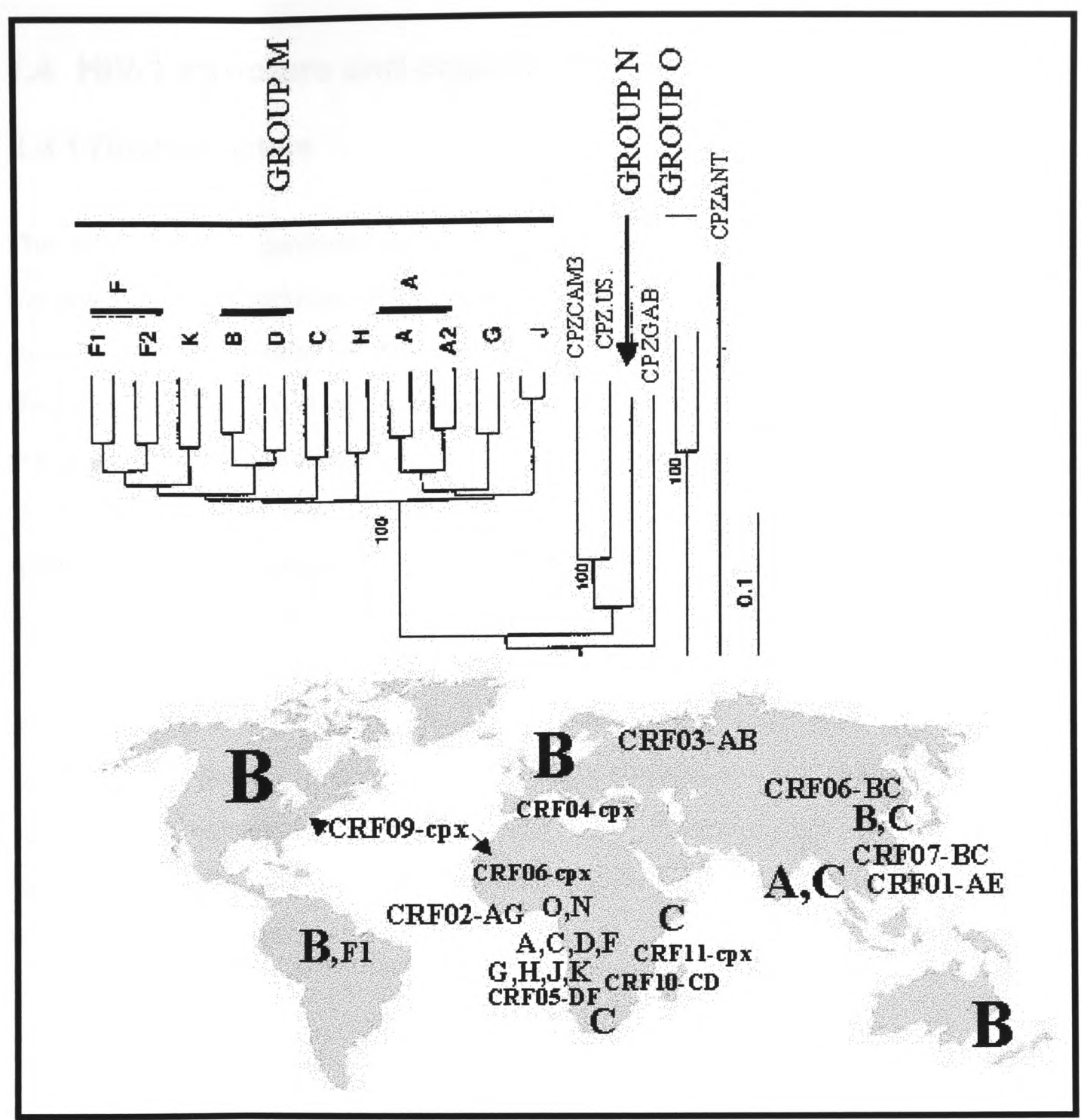


Figure 1.2: Evolutionary relationships among HIV-1 strains from the HIV-1/SIV_{cpz} lineage (upper panel), based on phylogenetic analysis of full-length genome sequences. The phylogenetic tree depicts the different HIV-1 groups, subtypes and sub-subtypes. The lower panel shows the geographic distribution of HIV-1 groups, clades and CRFs. Each of the internal branches defining a subtype or sub-subtype is supported by 100% bootstraps. (Source (Peeters, 2000)).

1.4 HIV-1 structure and organization

1.4.1 Virion structure

The HIV-1 virion conforms to a structural pattern described for all retroviruses (reviewed by (Gelderblom, 1991; Haseltine, 1991). It is enveloped, and the virion particle is about 100nm in diameter, and contains an electron dense conical core (Figure 1.3). The envelope displays two polypeptides derived from a gp160 single precursor; the transmembrane (TM) gp41 and the outer SU gp120. Each virion is estimated to contain about 216 copies of the envelope glycoprotein arranged in 72 spikes of three heterodimer each (Gelderblom, 1991). For a mature virion, on the inner leaflet of the envelope there are proteolytically processed Gag products: a myristoylated p17 matrix (MA) protein and a p24 capsid (CA) protein that form the core of the particle and an RNA-binding p7 nucleocapsid (NC) protein (Figure 1.3). Other proteins present in the core of the virion particle are derived from the *pol* gene and perform enzymatic functions (Table 1.1). The *pol* gene products, reverse transcriptase (RT) and integrase (IN) are involved in early stages of the HIV-1 life cycle, discussed in section 1.5.

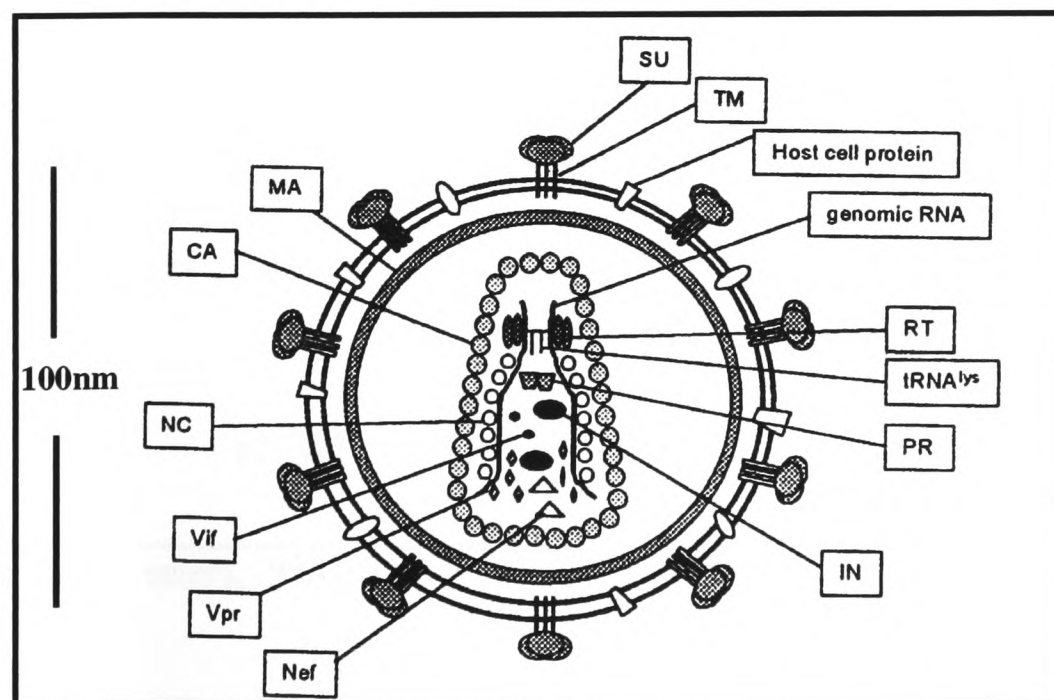


Figure 1.3: Typical structure of HIV-1 particle (see text and Table 1.1 for abbreviations)

Table 1.1: HIV-1 proteins, their size, functions and localization*

Name	Size	Function	Localization
Gag MA	p17	Membrane anchoring; env interaction; nuclear transport of viral core	Virion
Gag CA	p24	Core capsid	Virion
Gag NC	p7	Binds RNA	Virion
	p6	Binds Vpr	Virion
Protease (PR)	p15	gag/pol cleavage	Virion
Reverse Transcriptase (RT)	p66,p51	Reverse transcription	Virion
RNase H	(heterodimer)	RNase H activity	Virion
Integrase (IN)		DNA provirus integration	virion
Env	gp120/gp41	gp120 binds CD4 and chemokines, gp41 mediates fusion	Plasma membrane, virion envelope
Tat	p16/p14	Viral transcription activator	Nucleolus or nucleus
Rev	p19	RNA transport, stability and utilization factor (phosphoprotein)	Shuttling between nucleolus and cytoplasm
Vif	p23	Promotes virion maturation and infectivity	Cytoplasm, virion
Vpr	p10-15	Promotes nuclear localization of preintegration complex, inhibits cell division, arrest infected cells at G2/M	Virion nucleus
Vpu	p16	Promotes extracellular release of viral particles; degrades CD4 in the ER	Integral membrane protein
Nef	p27-p25	CD4 and class I downregulation	Plasma membrane, cytoplasm

* (Adapted from Peeters, 2000)

1.4.2 Organization of the genome

The genome of HIV-1, unlike other retroviruses is made up of a single RNA strand of 9.2 kb in length and is far more complex than those of other retroviruses. The genome of most retroviruses is diploid, being made up of two identical RNA strands with a + polarity, and all retroviruses have the *gag*, *env*, and *pol* genes, but HIV-1 has 6 additional regulatory and accessory genes (Figure 1.4 and Table 1.1). The genes of HIV-1 are located in the central region of the integrated form of HIV-1 (discussed in 1.5), also known as the provirus (Figure 1.4). The provirus is approximately 9.8 kb in length, and both ends of the provirus are flanked by a repeated sequence known as the long terminal repeats (LTRs). The LTRs are generated when RT synthesizes the proviral DNA, and although identical in sequence the two LTRs perform different functions; the 3' LTR provides the polyadenylation signal, while the 5' LTR promotes transcription as discussed in the next section.

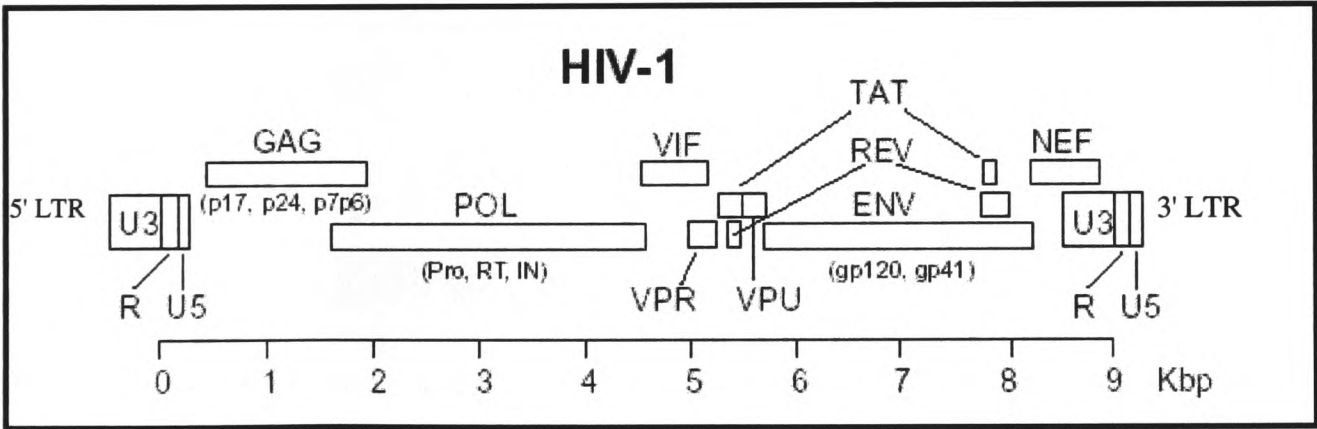


Figure 1.4: Organization of HIV-1 genome: In addition to the typical retroviral structural genes, *gag*, *pol* and *env* that encodes the shown proteins, HIV-1 has six regulatory and accessory genes, *nef*, *rev*, *tat*, *vif*, *vpr* and *vpu*. The sequences labeled U5 and U3 are respectively unique to the 5' and 3' of the viral RNA genome, and R is a short sequence repeated at each end of the viral genome. U5 and U3 are duplicated during synthesis of the proviral DNA so that each LTR in the provirus is composed of the U5, R and U3 regions. (Modified from://hiv-web.lanl.gov/content/hiv-db/)

1.5. Infectious cycle of HIV-1 and therapeutic interventions

1.5.1 Classic productive infectious pathway

HIV-1 primarily infects two principal target cells, the CD4⁺ T lymphocytes and macrophages, also CD4⁺. The infectious HIV-1 life cycle can be divided into an early and a late phase, and further into distinct phases that include: attachment and virus entry, reverse transcription, integration, gene expression, assembly, budding and maturation of nascent virus particles (Figure 1.5). A detailed knowledge of these events is required to develop effective antiviral strategies aimed at eliminating HIV-1 replication and the associated AIDS pathogenesis.

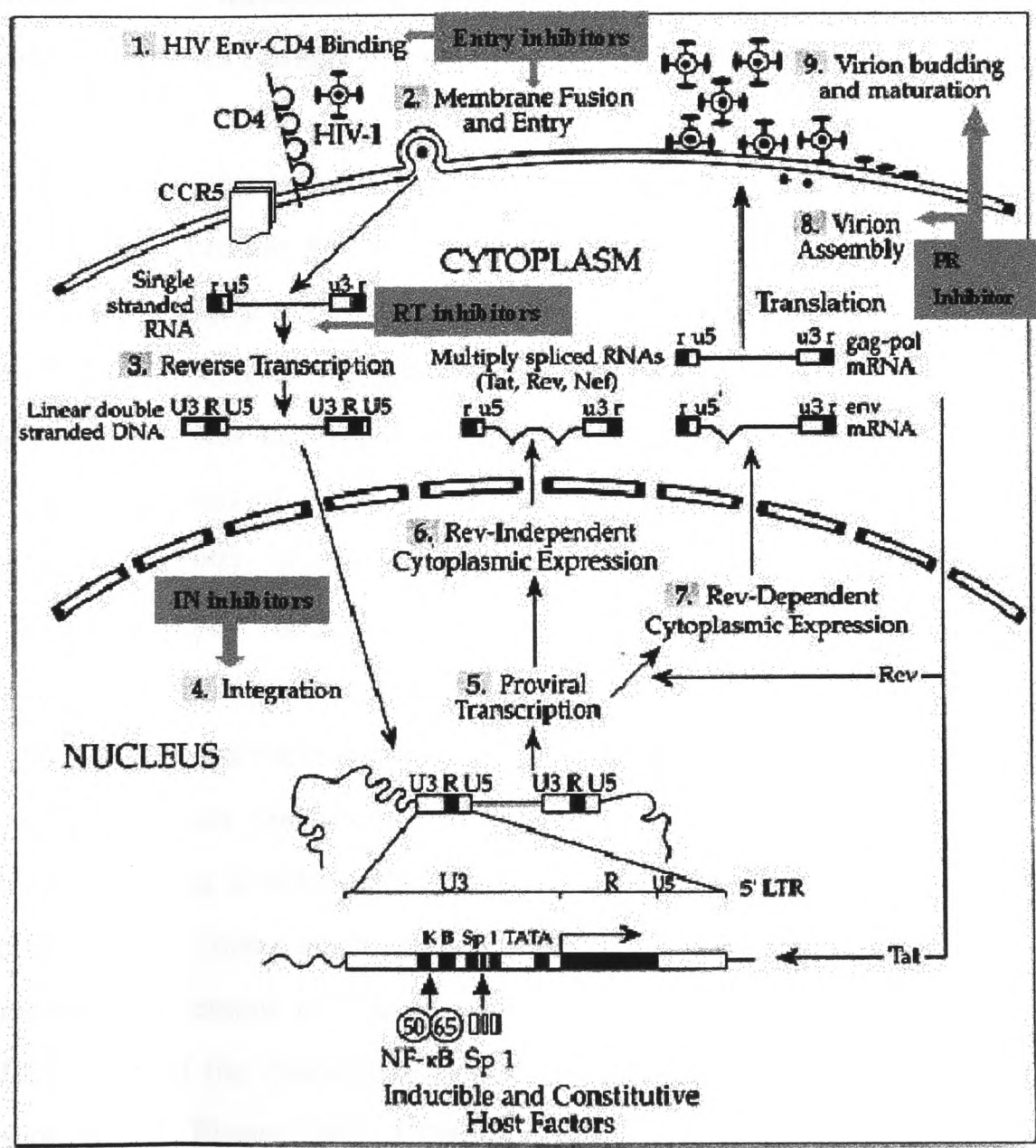


Figure 1.5: The HIV-1 life cycle showing steps available for attack by antiretroviral drugs (refer to text for details). Modified from: <http://hivinsite.ucsf.edu>.

a. Attachment and HIV-1 entry

The first and crucial step in HIV-1 replication cycle is the entry phase, which can be sub-divided into three stages: binding of the virus to its receptor, fusion of the membrane and uncoating or release of the viral nuclear material into the host cell cytoplasm (Figure 1.5, steps 1 and 2). Very little is understood about the uncoating process, as it is difficult to isolate experimentally from the fusion step, hence it will not be discussed further in this thesis. Different techniques are employed to assay HIV-1 entry, ranging from biochemical and ultrastructural studies, to more virological approaches involving infectious HIV-1 particles (Collin et al., 1993; Zack et al., 1990) and pseudotype viruses (Gregory et al., 1993).

The HIV-1 entry process is mediated by the high affinity interaction of the gp120 viral envelope SU glycoprotein with the first domain of host cell CD4 surface receptor (Figure 1.6). The affinity of CD4 for monomeric gp120, either dissociated from virions or the recombinant form of the protein is between 1 and 10nM (Layne et al., 1990). This does not, however necessarily reflect the affinity of oligomeric gp120 for CD4, which is several orders of magnitude lower (Brighty et al., 1991; Fouts et al., 1997; Ivey-Hoyle et al., 1991; Moore et al., 1992). Thus oligomerization of gp120 into a trimer of gp120-gp41 heterodimers appears to have dramatic effects on the accessibility of the CD4 binding site, reducing the affinity for soluble CD4 by as much as three orders of magnitude (Fouts et al., 1997; Moore et al., 1992). Binding to the CD4 receptor induces conformational changes in the gp120, some of which are detectable by changes in antibody binding (Sattentau et al., 1993; Sullivan et al., 1998; Wyatt et al., 1995; Zhang et al., 1999b). The conformational changes on the gp120 which include movement of variable loops (V1/V2 and V3) lead to the exposure and/or formation of the coreceptor-binding site (CRbs) between the inner and outer domains of gp120 (Figure 1.6). CD4 binding significantly reduces the flexibility of gp120, consistent with the formation of the ordered CRbs (Myszka et al., 2000).

b The fusion cascade

According to the prevailing model of Env-induced fusion cascade, the interaction between gp120 and the coreceptor induces further conformational changes in the gp120, resulting in gp41 activation that mediates membrane fusion in a pH-independent manner (reviewed by (Binley and Moore, 1997; LaBranche et al., 2001)). The native structure of gp41 and precise detail of gp41-mediated fusion is poorly understood, however several functional domains of gp41 have been identified (Chan et al., 1997; Tan et al., 1997; Weissenhorn et al., 1997). The amino terminus in the ectodomain of gp41 consists of a stretch of hydrophobic amino acids analogous to the fusion peptide of other fusogenic viruses, like the influenza hemagglutinin (Bosch et al., 1989; Gallaher, 1987). The external portion of gp41 also contains two heptad repeat regions near the amino terminus (HR1) and the carboxy-terminus (HR2) of the gp41 ectodomain. The three HR1 regions in the receptor-bound Env oligomer interact to form a coiled-coil structure and the three HR2 domains fold back like a hairpin into the hydrophobic grooves of the coiled-coil to form a thermodynamically stable six-helix bundle (Figure 1.6). Experimental evidence suggests that the HR1 coiled-coil is not formed prior to receptor binding because mutations in HR1 that abrogate coiled coil formation interfere with fusion, but not with the production and oligomerization of Env (Wild et al., 1994a). Likewise, formation of the six-helix bundle form of gp41 probably occurs subsequent to CD4 binding, as a peptide that mimics HR2 binds to CD4-triggered Env and prevents fusion (Furuta, 1998; Melikyan et al., 2000). Since the gp120-receptor interaction initiates the fusion cascades, it has been hypothesized that gp120 chaperones the energetically unstable form of gp41, and holds it in a native conformation (Doms and Moore, 2000; Weissenhorn et al., 1997). Coreceptor-induced modification of the gp120-gp41 interaction is sufficient to trigger gp41 activation, releasing gp41 to induce direct fusion between viral and cell membrane, thereby initiating viral entry (Doms and Moore, 2000).

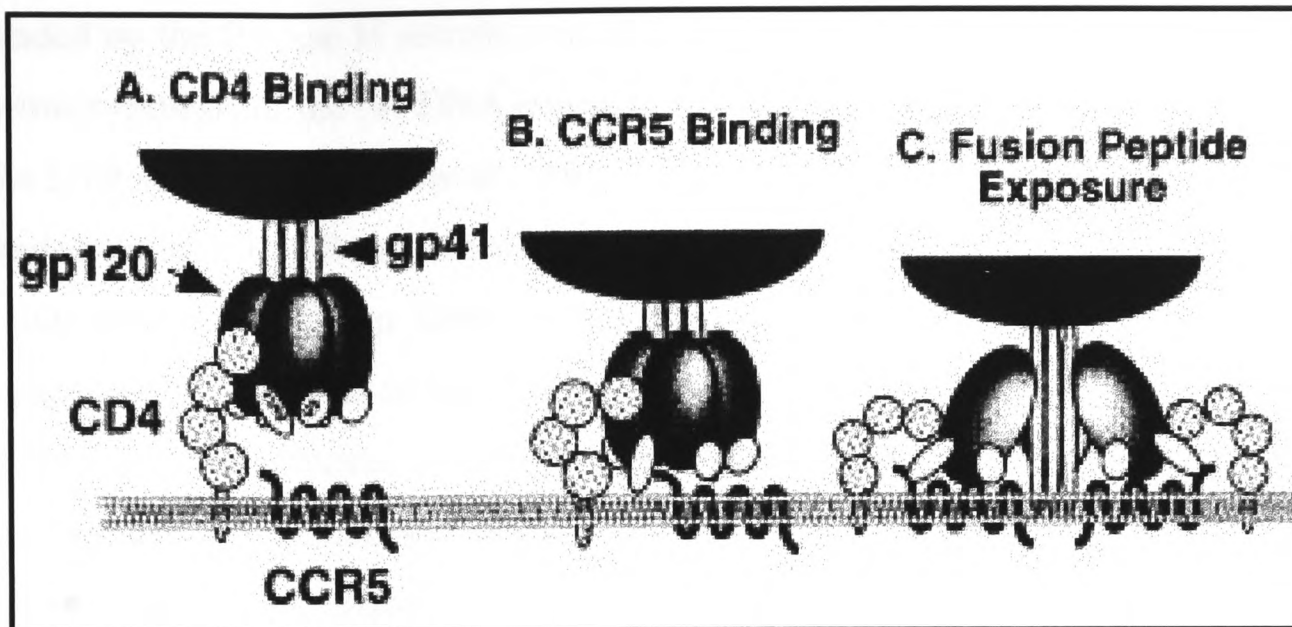


Figure 1.6: A model of Env-mediated, CD4 dependent HIV-1 entry. A gp120 oligomer binds domain 1 of CD4 (A), inducing conformational change on the gp120 that allows the coreceptor (CCR5) binding (B). The interaction then triggers the final conformational change in Env, including formation of the coiled-coil structure in gp41 that enables the fusion peptide to insert into the target bilayer (C). (Source Doms and Pieper, 1997)

c. Reverse transcription

After the viral entry, the nucleoprotein complex is released into the target cell cytoplasm where RT (Temin and Mizutani, 1970) orchestrates synthesis of a linear double-stranded DNA copy of a viral single-stranded RNA genome (Figure 1.5, step 3). HIV-1 RT is a heterodimer made up of two subunits, p66 and p51 (di Marzo Veronese et al., 1986) and its crystal structure has been solved (Arnold et al., 1992; Kohlstaedt et al., 1992). Reverse transcription takes place within a viral nucleoprotein complex and is initiated from the cellular tRNA^{Lys3} bound to the primer-binding site (PBs) near the 5' end of the viral template. The nascent DNA strand has a (-) polarity and is extended to the 5' end of the RNA genome, and is designated minus strand strong-stop DNA. The region of the RNA template used is degraded by an RNase H activity of RT, freeing the 3' end of the minus DNA for the first template jump. Thus, the exposed strong-stop DNA hybridizes (jumps) to the complementary sequence in R at the 3' end of the viral genome. Genomic RNA is then cleaved and completely

degraded by the RNase H activity, except for a polypurine tract (PPT) which is used to prime synthesis of the (+) DNA strand at a point corresponding exactly to the 5' end of the LTR (Charneau and Clavel, 1991; Smith et al., 1984). The plus DNA strand is extended through U3, R and U5 using a minus DNA strand as a template to produce the plus strand strong-stop DNA, which is an intermediate essential for the second template switch. The PBs in the plus strand is base paired with the 3' end of the minus strand by an intramolecular process (Panganiban and Fiore, 1988). After the second strand transfer, RT continues to elongate the plus strand DNA synthesis until it reaches the central termination signal (CTS) located immediately 3' to the central PPT. The plus strand initiated at the PPT is elongated to the end of the minus strand template, and the two strands are ligated to yield a linear double-stranded proviral DNA with an LTR at each end. The double stranded DNA remains associated with viral proteins and forms a large complex, called the preintegration complex (PIC).

d. Integration

After translocation into the host cell nucleus, the PIC serves as the machine that integrates proviral DNA into host-cell chromosomal DNA to form a provirus (Figure 1.5 step 4). This step is critically dependent on the activity of the viral IN protein and is essential for viral gene expression (Sakai et al., 1993). The crystal structure of the catalytic domain and the solution structure of the DNA binding domain of HIV-1 IN have been determined (Dyda et al., 1994; Lodi et al., 1995). Retroviral DNA can be integrated into the host genome at a large number of essentially random sites, although some target sites are preferred (Pryciak and Varmus, 1992; Shih et al., 1988). Integration requires that the IN protein recognizes linear viral DNA ends, called the *att* sites. Integrase protein catalyzes removal of two base pairs from the 3' ends of each viral DNA strand, exposing a CA dinucleotide that is highly conserved among all retroviruses and retrotransposons (Goff, 1992). These 3' processed or "recessed" viral DNA ends are then joined to the 5' ends of cleaved host genome target site DNA by a one-step transesterification reaction (Engelman et al., 1991). These steps can be reproduced *in vitro* by incubating synthetic oligonucleotides that correspond to viral

att sequences with purified IN protein. (Bushman and Craigie, 1991; Bushman et al., 1990). Cellular enzymes are thought to repair the junctions between integrated viral DNA and host-cell genomic DNA, generating the short, direct repeats that flank the viral sequences. Integration is completed by the final ligation step. Once integrated, the provirus forms a stable part of the host cell genome and directs expression of viral genes.

e. Viral gene expression

The expression of viral genes requires the collaborative activities of the host-cell transcription machinery. The transcriptional activity of HIV-1 is regulated by constitutive host-cell transcription factors (i.e., Sp1 and the TATA-binding factors), the activation-inducible members of the NF- κ B family of host transcription factors (i.e., p50 and p65), as well as the virally encoded regulatory Tat and Rev proteins (Figure 1.5, step 5). Following transcription of a full-length viral RNA, a complex array of alternatively spliced viral mRNAs is produced. The spliced viral mRNA transcripts give rise to structural Env and to the regulatory Tat, Rev, and Nef proteins. Unspliced transcripts encode the Gag precursor polyprotein, Pr55 Gag, and Pr160 Gag-pol fusion proteins, also serve as genomic RNA packaged into newly assembling virus particles. The HIV-1 Rev protein controls the differential expression of distinct species of viral mRNAs. The level of Rev present in an infected cell determines the preferential production of either the unspliced or the singly spliced mRNAs. In circumstances in which the amount of Rev present in uninfected cell is limiting, such as the early stages of viral infection or when the overall level of transcription is low, only the multiply spliced mRNA transcripts are available in the cytoplasm for the translation of viral proteins (Figure 1.5, step 6). Once a sufficient level of Rev accumulates, the singly spliced and unspliced HIV-1 mRNAs appear in the cytoplasm, and the synthesis of viral structural proteins can proceed (Figure 1.5, step 7). Once the Gag, Gag-pol and Env precursors are synthesized, they come together along with two copies of viral RNA and tRNA primers, at a common site on the cell membrane to assemble viral particles.

f. Assembly, budding and maturation of nascent HIV-1 particle

Assembly of HIV-1 particles takes place at the host cell surface (Figure 1.5, step 8), and is orchestrated by Pr55 Gag (reviewed by Gelderblom, 1991). Several Pr55 Gag functional domains play critical roles in assembly, including terminal cotranslational addition of myristic acid to viral MA proteins, required for membrane attachment of Gag and Gag-pol precursor proteins (Bryant and Ratner, 1990). Although MA contains the membrane binding (M) domain and can induce membrane budding, the incorporation of Gag and Gag-pol precursor proteins into functional viral particles requires the presence of interaction (I) domains of Gag and a late-acting L-domain of the p6 Gag protein (Parent et al., 1995). In retroviruses, viral genomic RNA is selectively taken up from the pool of cytoplasmic RNAs because the NC Gag protein recognizes specific cis-acting RNA packaging signals (Katz et al., 1986; Lever et al., 1989). Because the sequences required for HIV-1 RNA packaging are directly downstream of the major 5' splice donor of the virus, only unspliced genome-length viral transcripts can be packaged into viral cores (Lever et al., 1989). The packaging signal of HIV-1 forms part of the stable RNA secondary structure that is highly conserved between different viral isolates. (Harrison and Lever, 1992). Two cysteine/histidine motifs in NC, with the consensus sequence Cys-X2-Cys-X4-His-X4-Cys (in which X can be any amino acid), are critical for viral RNA packaging. (Aldovini and Young, 1990; Dupraz et al., 1990; Meric and Goff, 1989). Once packaged, viral nucleoprotein complex acquires viral Env proteins as they bud through the host-cell membrane to produce virion particles. Selected host cell proteins are also incorporated into mature virus particles, notably HLA-DR and β -2-microglobulin (Arthur et al., 1992), and the cytoplasmic protein cyclophilin-A (CypA) (Dorfman et al., 1997; Franke et al., 1994; Thali et al., 1994), including several adhesion molecules (reviewed in (Tremblay et al., 1998). The viral Gag and Gag-pol polyproteins are cleaved by viral protease during or shortly after budding, generating mature infectious virions (Figure 1.5, step 9). The minimum duration of the HIV-1 life cycle *in vivo* is 1.2 days on average (Perelson et al., 1996). In acutely infected individuals 1-10 billion HIV-1 particles are produced each day (Comar et al., 1996; Kaufmann et al., 1998;

Perelson et al., 1996; Young and Kuritzkes, 1999). These findings on viral dynamics provide not only a kinetic picture of HIV-1 pathogenesis, but also theoretical principles to guide the development of treatment strategies.

1.5.2 Chemokine receptors and HIV-1 infection

Although CD4 was unequivocally identified as the primary receptor for HIV-1, it was evident that an additional coreceptor was necessary for infection. CD4 had been found necessary but not sufficient for viral entry in model systems using an expression cloning strategy to confer an HIV-1 susceptible phenotype in nonpermissive cells expressing human CD4. For instance murine cells engineered to express human CD4 were shown to be refractory to HIV-1 infection (Weiner et al., 1991). Similarly not all CD4-expressing human cells are permissive to infection by all strains of HIV-1. Thus, HIV-1 coreceptors were hypothesized long before their identification. The 1996 identification of the CCR5 and CXCR4 chemokine receptors as the major HIV-1 coreceptors greatly advanced our understanding of HIV-1 entry and pathogenesis (Berger, 1997; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1997b; Dragic et al., 1996; Feng et al., 1996; Trkola et al., 1996a). The virus strains responsible for transmission, which are the predominant virus isolated from asymptomatic individuals, use CCR5 as a coreceptor, whereas viruses that emerge later in the course of infection use CXCR4 to gain entry into the cell, either instead of, or in addition to CCR5, thereby defining viral tropism (discussed in 1.6).

Progress has been made towards understanding how HIV-1 interacts with its coreceptors, although details remain to be resolved. The first clue as to the interactive mechanism was the observation that β -chemokines, natural ligands of CCR5, inhibited HIV-1 Env-mediated fusion by a competitive mechanism (Dragic et al., 1996), suggesting that an Env component was likely to bind directly to the CCR5 receptor. This was subsequently confirmed in two parallel studies (Trkola et al., 1996a; Wu et al., 1996) that used an MIP-1 β binding and competition assay to demonstrate that gp120 from macrophage (M-tropic) but not T-tropic HIV-1 strains (discussed in 1.6),

directly competed with MIP-1 β for CCR5, both on CCR5-transfected cells and on mitogen-activated CD4⁺ T cells. The gp120-CCR5 interaction was substantially enhanced by the binding of gp120 to CD4, but was inhibited by virus-neutralizing anti-gp120 antibodies (Trkola et al., 1996a; Wu et al., 1996). The former observation suggested that the binding of CD4 to gp120 unmasked the cryptic CCR5 epitope (refer back, Figure 1.6). The binding of gp120 from a T-tropic HIV-1 strain to a complex of CD4 and CXCR4 on CD4⁺ cells has also been reported, by the use of immunoprecipitation (Lapham et al., 1996). A natural ligand for CXCR4 has been identified as the α -chemokine stroma-derived factor 1 (SDF-1), which inhibits the infectivity of T-tropic, but not M-tropic HIV-1 strains (Bleul et al., 1996; Oberlin et al., 1996). The mechanism of interaction of T-tropic gp120 with CXCR4 is analogous to that of M-tropic gp120 with CCR5, and SDF-1 competitively inhibits the gp120-CXCR4 interaction (Hesselgesser et al., 1997).

In addition to elucidating the mechanism of interaction of HIV-1 with the chemokine receptors, progress has been made in identifying the regions on both CCR5 and CXCR4 that are involved in coreceptor function (reviewed by (Doms and Peiper, 1997)). The general approaches to identifying coreceptor domains required for Env-mediated HIV-1 entry include studying coreceptor homologues from other species, constructing chimaeric receptors between an HIV-1 coreceptor and a chemokine receptor that does not support HIV-1 entry, and site-directed mutagenesis. It should be clear that the gp120-chemokine receptor interactions are highly complex and conformational in nature with the participation of multiple regions in the viral Env protein and the coreceptor being involved. Moreover, interpretation of results is subject to several variables. For example, Env proteins derived from diverse HIV-1 strains can interact differently with CCR5 and CXCR4, receptor expression levels is another variable, and the cell type in which the coreceptor is expressed and the type of assay used can both influence whether a given Env-coreceptor combination results in a productive membrane fusion event or not. Thus, the conclusions reached about the relative importance of different chemokine receptor domains required for coreceptor function are strongly dependent upon the experimental context. Nevertheless, general

themes have emerged from studies published by several groups, and domains in both CCR5 and CXCR4 that are important in their function have been identified.

The CCR5 and the CXCR4 coreceptors share 31% amino acid identity and both contain 352 amino acids, have two N-linked glycosylation sites, at least one of which is utilized (Berson et al., 1996), and have four conserved cysteine residues in the extracellular domains that are predicted to form two disulfide bonds (Figure 1.7). Consequently, CCR5 and other chemokine receptors are apt to form compact structures, with all four extracellular domains in close proximity to one another (Horuk, 1994). It is therefore likely that mutations in one extracellular domain may indirectly affect the function of adjoining regions and that residues in all four extracellular domains are likely to participate in coreceptor function. Indeed analysis of chimaeric receptors based on human and murine CCR5 demonstrated that no single CCR5 domain was necessary and sufficient for coreceptor function (Atchison et al., 1996; Bieniasz et al., 1997). Rather, all four extracellular domains participate in coreceptor activity almost in concert, with the second extracellular loop being particularly crucial. The introduction of single point mutations into the human gene to the homologous mouse sequence, which has 82% similarity with human CCR5 (Atchison et al., 1996), failed to alter the coreceptor activity from that of the wild-type receptor, again suggesting that multiple residues and domains of CCR5 are important for virus entry and fusion (Doranz et al., 1997b). Receptor chimaeras based on CCR5 and other chemokine receptors, including CCR2b, CCR1, CXCR2, and CXCR4 have been analysed (Atchison et al., 1996; Doranz et al., 1997b; Edinger et al., 1997; Lu et al., 1997). Again, these independent studies showed the gp120-CCR5 interactions to be complex, with residues in all four extracellular domains participating in coreceptor function and with differential utilization of CCR5 evident, depending on the virus strain being studied.

In contrast to CCR5, it is apparent that some regions of CXCR4 are more important for coreceptor function. Studies with both human and rat CXCR4 have shown that the first and second extracellular loops of CXCR4 are important for coreceptor function (Brelot et al., 1997; Lu et al., 1997; Picard et al., 1997). CXCR4-CXCR2 receptor chimaeras containing the first and second extracellular loops of CXCR4 supported fusion by T tropic and dual-tropic HIV-1 (Lu et al., 1997). In fact, the second extracellular loop of CXCR4, but not other domains conferred receptor function on CXCR2 for most virus strains tested (Lu et al., 1997). Human CD4-transfected rat cells, which subsequently proved to express rat CXCR4, were shown to be fully susceptible to HIV-1_{IIIB} entry (Simon et al., 1993). Thus the rat CXCR4 homologue has been found to function as a coreceptor for some strains of HIV-1 (Brelot et al., 1997; Pleskoff et al., 1997), however, insertion of the second loop of human CXCR4 receptor into the rat homologue conferred receptor activity for additional viruses (Brelot et al., 1997). Furthermore, there is differential utilization of CXCR4 domains by different virus strains (Brelot et al., 1997; Lu et al., 1997). Whether differential CXCR4 utilization influences the efficiency of membrane fusion, correlates with subtle differences in tropism (discussed in 1.8), or simply reflects some degree of natural variation and plasticity remains to be determined.

The CCR5 and CXCR4 are clearly the coreceptors used *in vivo* for HIV-1 infection. However, a panoply of alternative coreceptors including CCR2b, CCR3, CCR8, and a growing list of known or putative chemokine receptors, and other attachment molecules have been postulated to support virus infection at least *in vitro* (Doranz et al., 1996; Geijtenbeek et al., 2000; Rucker et al., 1997).

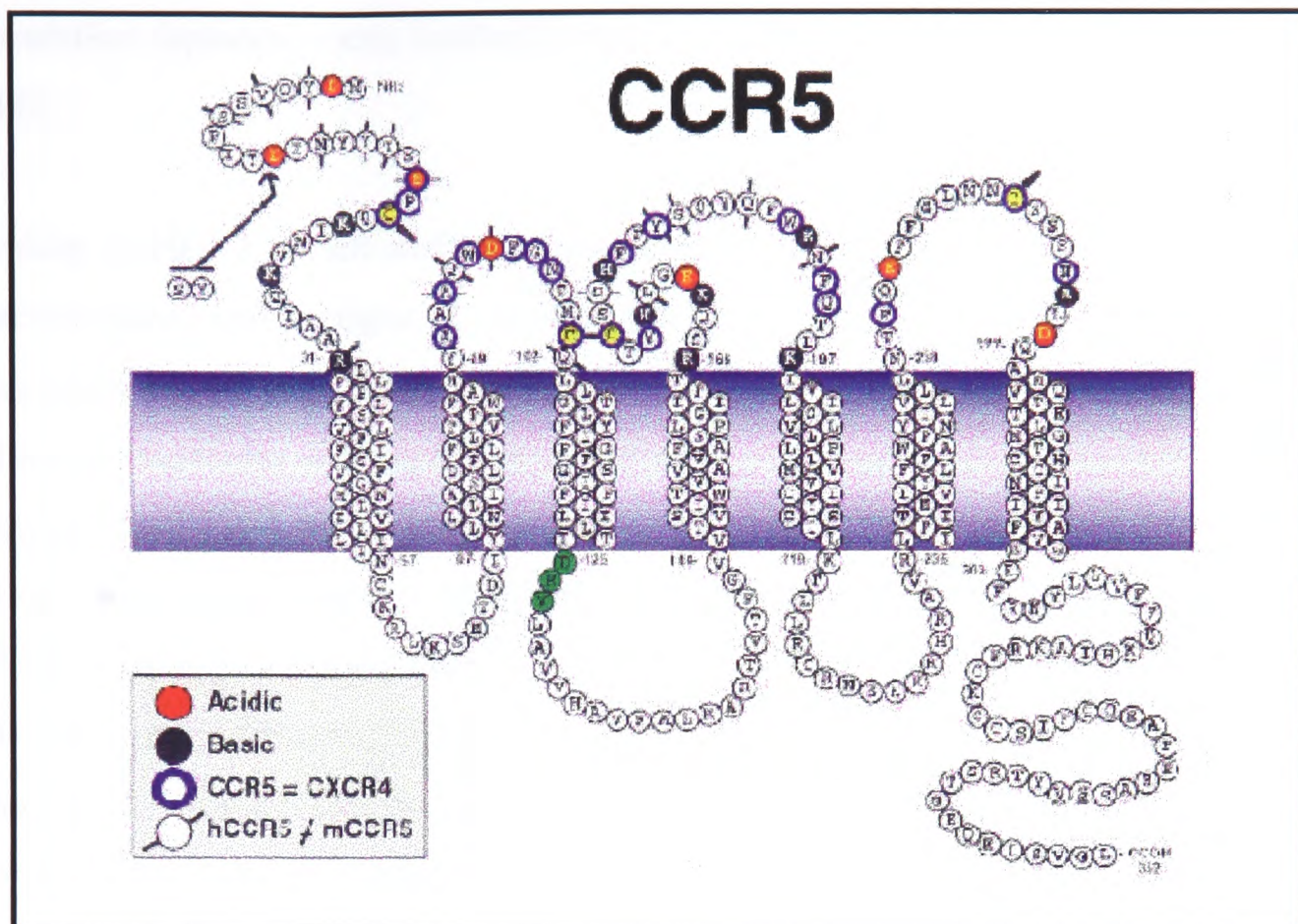


Figure 1.7: A schematic diagram of a serpentine 7-TM CCR5 HIV-1 coreceptor. Extracellular acidic residues are denoted in red, basic residues black, and residues shared with CXCR4 are bounded by a purple circle. The extracellular domain of CCR5 shares 20% amino acid identity with CXCR4. Extracellular residues that differ between human and murine CCR5 are indicated in slashes and the four extracellular cysteine residues are in yellow (source: Doms and Peiper., 1997).

1.5.3 The role of other attachment molecules and DC SIGN in HIV-1 infection

While engagement of CD4 and the chemokine receptors are prerequisite for efficient HIV-1 entry, simple attachment, and perhaps entry of the virus can occur via interactions with other cell surface molecules (reviewed in (Baribaud et al., 2001; Hioe et al., 1998; Ugolini et al., 1999). Attachment can be rate-limiting for viral infection as evidenced by the fact that procedures which concentrate the virus on the cell surface such as inclusion of polycations like polybrene in the virus inoculum, and centrifugal

inoculation (spinoculation) markedly improve infection efficiency (O'Doherty et al., 2000).

Binding of HIV-1 to cell-surface molecules such as adhesion proteins like leukocyte function-associated antigen 1 (LFA-1), ICAM-1 and -3 (intercellular adhesion molecule), CD44 or CD62L can enhance infection efficiency (Hioe et al., 1998). Adhesion molecules were first implicated in HIV-1 entry on the basis of experiments showing that LFA-1 monoclonal antibodies (mAbs) interfered with HIV-1 induced cell fusion/syncytium formation (Hildreth and Orentas, 1989). Subsequently, anti-adhesion mAbs, including those specific for ICAM-3 were shown to interfere with cell-free virus infection (Sommerfelt and Asjo, 1995). During budding from an infected cell (Figure 1.5), HIV-1 selectively incorporates molecules of cellular origin into its membrane, including several adhesion molecules (Figure 1.8). The type and quantity of adhesion molecules acquired by the virus depends on the producer cell type, which might in turn exert an influence on the type of cell targeted. For example, anti-CD44 mAbs were shown to inhibit macrophage and monocyte infection by M-tropic HIV-1 strain produced in cells overexpressing CD44 (Rivadeneira et al., 1995). In this way, recognition by virion-anchored adhesion molecules of their cognate receptors on the target cell surface enhances viral attachment in a cell-type specific fashion. The involvement of virion associated ICAM-1 in the entry process of HIV-1 has also been demonstrated with ICAM-1⁺ virion exhibiting enhanced infectivity relative to ICAM-1⁻ virus particles (Rizzuto and Sodroski, 1997). Conversely, LFA-1, a ligand for ICAM-1 can promote HIV-1 infection when expressed on target cells (Hioe et al., 2001). In most instances, these studies have used virus particles produced in cells that overexpress the protein of interest (Hioe et al., 1998; Hioe et al., 2001; Rivadeneira et al., 1995; Rizzuto and Sodroski, 1997). Therefore, whether incorporation of these adhesion molecules occurs to a significant extent *in vivo* is not known.

Cell surface glycosaminoglycans (Figure 1.8) such as heparan sulfate (HS) have also been found to enhance HIV-1 cell attachment and entry (Cladera et al., 2001; Patel et

al., 1993). Recently, a virus-incorporated host protein, CypA has been reported to attach exclusively to cell surface heparans (Saphire et al., 1999). However another group has also reported that its preliminary studies indicated that CypA interacts with CD147 on target cells (Pushkarsky et al., 2001). Nevertheless, this interaction between exposed CypA and cell surface heparans represents the initial step of HIV-1 attachment and a necessary precursor to gp120-CD4 interaction (Saphire et al., 1999). Cell surface HS has been previously reported to interact directly with HIV-1 Env glycoprotein (Callahan et al., 1991; Cladera et al., 2001; Roderiquez et al., 1995).

The mechanism of attachment of HIV-1 to HS is mediated by electrostatic interactions between basic structures on the surface of the Env glycoprotein and polyanionic sulphated proteoglycan side chains on the cell surface (reviewed in (Ugolini et al., 1999)). It is well established that the gp120 V3 loop of T-tropic viruses contributes to the HIV-1-HS interaction. HS and other polyanions such as dextran sulphate, interfere with the binding of V3-loop specific mAbs to T-tropic HIV-1_{IIIB} gp120 or synthetic V3 loop peptides, but interfere weakly if at all, with monomeric gp120-CD4 association (Callahan et al., 1991; Roderiquez et al., 1995). The gp41 fusion domain has also been reported to interact specifically with HS on the surface of T lymphocytes (Cladera et al., 2001). This interaction is blocked by interleukin-8 (IL-8) and abolished by pre-treating the cells with heparinase. The specificity of the reaction was assessed by observations that soluble HS competes with the cell membrane interaction whereas soluble heparin does not. Another region that has potential for interaction with polyanions is the basic and highly conserved CRbs on the gp120 (Rizzuto et al., 1998). The binding of gp120 to proteoglycans varies, depending on both the type and quantity of the sulphated side chains found on the different cell types and the gp120 charge. Thus, macrophages have little HS but significant amounts of chondroitin sulphate proteoglycans (Uhlen-Hansen and Kolset, 1988), which do not interact detectably with the HIV-1 gp120 (Patel et al., 1993).

Recently, a specific family of HS proteoglycans called syndecans was demonstrated to mediate binding and represents an abundant class of HIV-1 attachment receptors on

macrophages (Saphire et al., 2001). T-cell lines on the other hand have varying amounts of HS, whereas primary CD4⁺ T cells have little amounts of HS (Ohshiro et al., 1996). Interestingly, T-tropic HIV-1 strain binding and infectivity are strongly influenced by HS. This was demonstrated by experiments in which depletion of HS with heparinase from CD4⁺ T cells lines (Patel et al., 1993; Roderiquez et al., 1995) and HeLa cells (Mondor et al., 1998), resulted in a cell type dependent loss of virus binding, which translates into selective reduction in virus infectivity. Thus depletion of HS inhibited the infection of CD4⁺ T cell lines and HeLaT4 by X4 viruses but primary T cell infection was not inhibited (Ibrahim et al., 1999). In contrast, the attachment of M-tropic virus is much less influenced by HS than that of T-tropic viruses. Heparinase treatment of immortalized or primary CD4⁺ CCR5 T cells leads to little or no diminution in M-tropic HIV-1 syncytium formation or virus attachment (Ohshiro et al., 1996; Oravec et al., 1996). This independence of M-tropic virus on HS for attachment correlates with the weakly basic gp120 V3 loops, which vary generally from +2 to +5 (Fouchier et al., 1992). By contrast, the V3 loops of T-tropic and dual tropic viruses tend to be between +7 and +9 (Callahan et al., 1991). Supporting this concept at the molecular level is a study in which a panel of T- and dual-tropic viruses were found to have a motif of at least two basic amino acids in the V3 loop capable of forming strong divalent electrostatic interactions with disulphated sugar groups (Bhattacharyya et al., 1996). M-tropic viruses were found not to possess this structural motif.

The presence of attachment receptors for HIV-1 other than, or additional to, CD4 and chemokine receptors on target cells such as macrophages has not been adequately studied. However, the Fc receptor (FcR) of the immunoglobulin G (IgG) expressed on macrophages and other cells of the monocyte lineage enhances HIV-1 infection (Homsy et al., 1989; Kent et al., 1994; Takeda et al., 1988). To demonstrate that FcR mediates HIV-1 infection, studies were conducted in which the U937 cells (Takeda et al., 1988) and human peripheral blood macrophages (Homsy et al., 1989) were exposed to HIV-1 complexed with various concentrations of serum from HIV-1 antibody-positive individuals and monitored for HIV-1 replication. Serum samples

from antibody-negative normal individuals did not affect virus yields. High concentrations of antibody-positive sera showed virus-neutralizing activity; but sub-neutralizing concentrations enhanced virus replication by approximately 10-fold. This enhancement of infection was blocked by monoclonal antibodies against the FcR, but not Leu3a, a monoclonal antibody directed against the CD4 receptor, nor did soluble recombinant CD4 even at high concentrations prevent enhancement (Homsy et al., 1989).

Other molecules that are differentially expressed on various cell types might also be implicated in HIV-1 attachment. For example, a C-type lectin cloned from placental tissue binds T-tropic monomeric gp120 with high affinity (K_d 1-2nM), and is inhibited by mannan and D-mannose, suggesting it is a membrane-associated mannose binding lectin (Curtis et al., 1992). Inhibition of the mannose receptor has not directly been shown to inhibit HIV-1 infectivity. However by extrapolation, the mannose receptor found on macrophages and dendritic cells has been thought to be involved in binding HIV-1 in a CD4-independent manner via the high mannose carbohydrate groups found on gp120 (Curtis et al., 1992; Ugolini et al., 1999). Galactosylceramide (GalCer), a glycolipid expressed on gut epithelial cells and neural cells has also been shown to act as HIV-1 receptor for CD4 negative cells (Bhat et al., 1992; Cook et al., 1994; Harouse et al., 1991). GalCer binds to the gp120 V3 loop and is inhibited by GalCer soluble synthetic analogues (Cook et al., 1994; Fantini et al., 1997; Weber et al., 2000) and mAbs against GalCer (Harouse et al., 1991).

A recent and particularly striking example of how alternative and additional attachment receptors can have important consequences from HIV-1 infection came from studies with dendritic cells (DCs). Binding HIV-1 to DCs strongly enhances infection of cocultured, receptor-positive target cells such as the peripheral blood mononuclear cells (PBMC) even though the DCs themselves are either not infected or infected inefficiently (Cameron et al., 1992; Geijtenbeek et al., 2000; Granelli-Piperno et al., 1998). Using this type of experimental system, productive infection of PBMC occurs with amounts of virus that are not sufficient to initiate infection when applied

directly to activated T-cells (Baribaud et al., 2001). A protein expressed on the surface of DCs, called DC-SIGN for dendritic cell-specific ICAM-3 grabbing nonintegrin (reviewed in (Baribaud et al., 2001)), is largely responsible for the ability of DCs to efficiently transmit virus to other cell types (Bashirova et al., 2001; Geijtenbeek et al., 2000; Geijtenbeek et al., 2001). Although DC-SIGN is abundantly expressed on DCs, it does not function as a receptor for viral entry into DCs, but instead promotes efficient trans-infection of cells that express CD4 and chemokine receptors (Geijtenbeek et al., 2001)

DC-SIGN is a 404 amino acid long type II integral membrane protein (Figure 1.8), and mediates binding of all primary HIV-1 strains as well as HIV-2 and SIV strains to the cell surface, indicating that this molecule may function as a universal attachment factor for primate lentiviruses (Geijtenbeek et al., 2000; Pohlmann et al., 2001a). A closely related DC-SIGN homologue, termed DC-SIGNR, also binds and transmits primate lentiviruses (Bashirova et al., 2001; Pohlmann et al., 2001b). Virus binding to DC-SIGN is dependent upon the presence of the lectin domain (Figure 1.8), and can be abrogated by preincubation with a calcium chelator, ethyleneglycol-bis- (beta-aminoethylether)-N, N, N', N'-tetraacetic acid (EGTA) and mannan (Pohlmann et al., 2001a). These results indicated that recognition of carbohydrates in the viral envelope by the lectin domain is likely involved (Geijtenbeek et al., 2000; Pohlmann et al., 2001a). Thus far, DC-SIGN has been shown to bind to gp120 (Geijtenbeek et al., 2000), whether it binds to gp41 is not known (Baribaud et al., 2001). In addition, it is not clear, if DC-SIGN-gp120 interactions are solely the result of carbohydrate recognition, or if other structural features in Env make it a specific ligands for DC-SIGN.

DC-SIGN, is not only expressed on mature and immature DCs (Geijtenbeek et al., 2000), it is also expressed on some macrophages *in vivo*, including decidual macrophages and Hofbauer cells in the placenta (Elizabeth Soilleux, personal communication). It is however not clear whether the DC-SIGN expressed on macrophages can directly enhance gp120-mediate HIV-1 entry into macrophages. It will also be important to determine if DC-SIGN is expressed on other cell types *in vivo*, if its expression can be induced under certain conditions, and how expression on DCs and macrophages is regulated. DC-SIGN seems to protect cell-bound virus from inactivation since virus-pulsed DCs can efficiently transmit the virus even when receptor positive cells are added up to 5 days later (Geijtenbeek et al., 2000). Thus, virus bound to DCs via DC-SIGN remains in a transmissible form for a considerable length of time. Importantly, binding of HIV-1 to DC-SIGN does not obviate the need for CD4 and coreceptor binding in that transmission occurs only to cells that bear the relevant virus receptors (Geijtenbeek et al., 2000).

The presence of DC-SIGN on mucosal dendritic cells and its ability to bind and transmit virus efficiently could be important for sexual transmission of HIV-1 which accounts for over 80% of all HIV/AIDS cases. DCs are highly mobile cells, which migrate from peripheral mucosal tissues to the lymph node upon the encounter of antigen (reviewed in (Steinman and Inaba, 1999)). Since DCs are among the first cells encountered by HIV-1 during sexual transmission, it has been speculated that virus bound by DC-SIGN may ultimately be ferried to lymph nodes and gut associated lymphoid tissue as a consequence of the normal trafficking of DCs (Geijtenbeek et al., 2000). The potential role of DC-SIGN in virus transmission or AIDS pathogenesis might also be approached through correlative studies. Relatively high levels of DC-SIGN are needed for efficient virus binding and transmission (Pohlmann et al., 2001a). Thus, as for CCR5, differences in expression levels between individuals could have important consequences on HIV-1 transmission (Reynes et al., 2000; Wu et al., 1997). It will be interesting to determine if there is much variability in levels of DC-SIGN expression between individuals, and if so, to determine if there is a correlation between

DC-SIGN expression levels, virus tropism and transmission, disease progression and other clinical outcomes (Baribaud et al., 2001).

The impressive abilities of DC-SIGN and other attachment molecules to increase infection efficiency underscore the importance of virus attachment and entry as crucial steps in the HIV-1 life cycle. The discovery of these molecules also stresses the need to re-probe virus entry in general, and the functional characterization of the interaction of HIV-1 gp120 with these attachment molecules may serve as important targets for therapeutic interventions and vaccine developments.

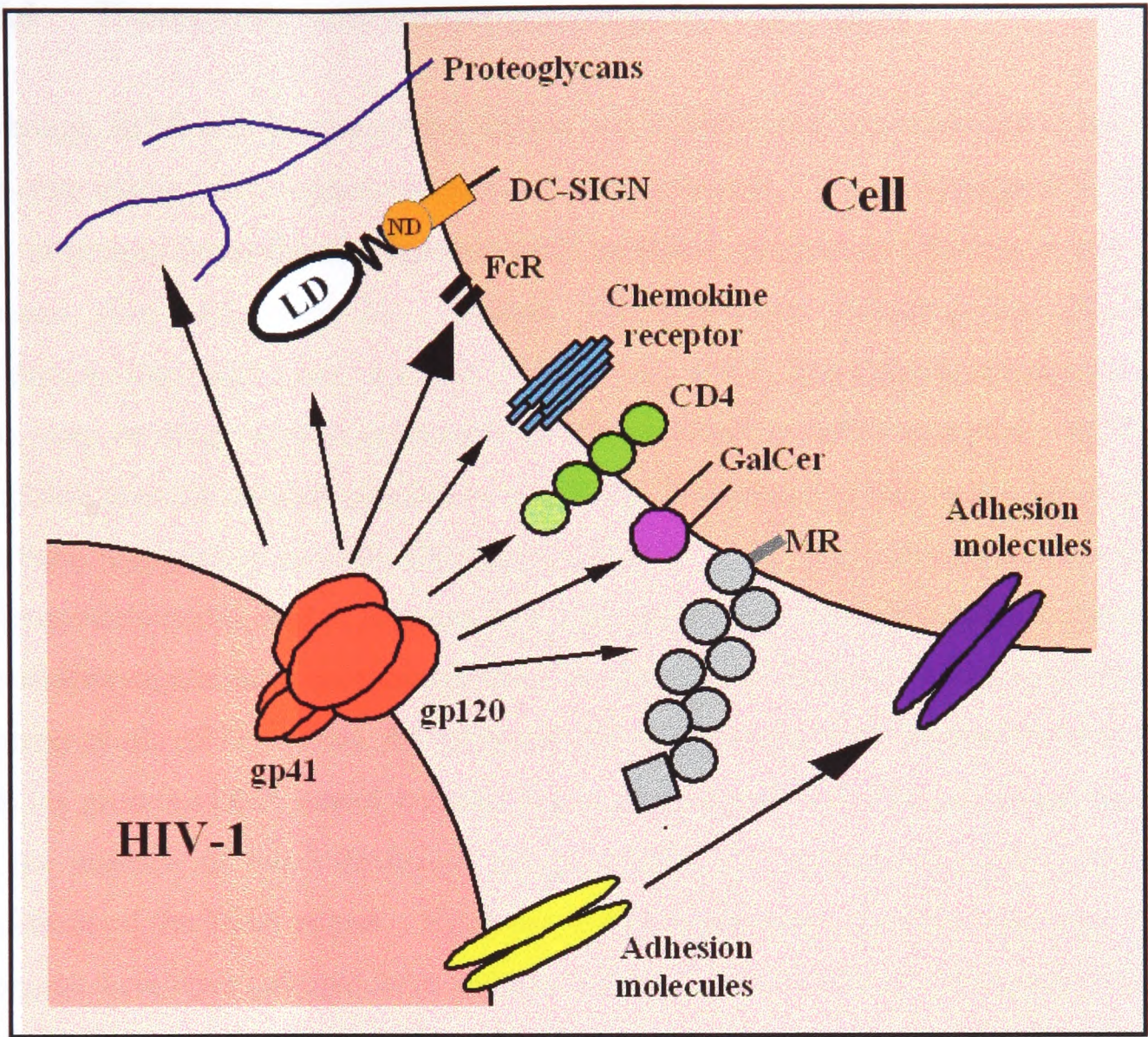


Figure 1.8: A composite diagram illustrating putative and potential attachment and entry receptors for HIV-1. These molecules are differentially expressed in various cell types. For instance, galactosylceramide (GalCer) is expressed on colon epithelial and neural cells, while DC-SIGN and mannose receptor (MR) are expressed on both DCs and macrophages. Although these different cell type attachment molecules are not drawn strictly to scale, some approximate differences in accessibility are highlighted. Thus, the sulphated side chains in proteoglycans and the calcium-independent cysteine rich domain of the MR (rhombus) are considerably more accessible for HIV-1 attachment than CD4, which in turn protrudes from the membrane to a much greater extent than do the chemokine receptors. The calcium-dependent lectin domain (LD) of DC-SIGN, which is connected to the neck domain (ND) by a repeat domain, is crucial for gp120 binding.

1.5.4 Therapeutic interventions during HIV-1 life cycle

Although AIDS was first described almost two decades ago, the challenge at hand for effective therapy development remains a formidable task. The knowledge that HIV-1 is a retrovirus immediately pinpointed RT synthesis of proviral DNA as a target for therapy (Fig. 1.5, step 3). Indeed, RT inhibitors including the prototype *azidothymidine* (AZT) were the first (Fischl et al., 1987), and remains among the only two types of drugs with demonstrable beneficial effect in AIDS treatment. AZT is a modified form of deoxythymidine in which the 3'-hydroxyl group on the sugar group has been replaced by an azido (N₃) group (Figure 1.9A). Such compounds are called dideoxynucleosides, and as suggested by their names they work in a manner similar to the dideoxynucleotides terminators used in the Sanger method of DNA sequencing (Sanger et al., 1977). Thus, for instance, RT incorporates AZT instead of thymidine into the growing DNA strand, culminating in abortive proviral DNA synthesis because of the absence of the 3'-hydroxyl group essential for making the sugar-phosphate backbone of the DNA strand. However, AZT-resistant viruses with mutations in the *pol* gene have been cultured from patients who have been treated for 6 months or more with AZT (Fitzgibbon et al., 1991). For this reason other dideoxynucleosides, such as *didanosine* (ddI); *lamivudine* (3TC); *stavudine* (d4T) and *zalcitibine* (ddC) have been developed and are in use as therapeutic agents to treat AIDS. Alternating regimes of one of the dideoxynucleosides with AZT is effective in reducing the chance of developing drug-resistant forms of HIV-1. In addition, because these drugs have different side effects, alternating treatment gives the patient a chance to recover from the effects of one drug while still being treated with a second.

The second type of drug used in the treatment of AIDS targets HIV-1 protease (PR). PR cuts the large polyprotein produced from the *gag* and *pol* genes into smaller proteins that include the four structural proteins of the viral coat (refer back, Figure 1.3), and the three viral enzymes, including RT and PR itself. This posttranslational processing is essential for virion assembly, and consequently productive HIV-1 replication. Defective virions are produced when the protease is inactivated by

mutations. HIV-1 PR is an aspartic protease, and synthetic inhibitors have been developed against this enzyme (Erickson et al., 1990; Hodge et al., 1996; Meek et al., 1990). PR inhibitors like *saquinavir* (Figure 1.9B) target virion assembly and maturation (Figure 1.5, steps 8 and 9), and are short peptides that are substrate for the enzyme, except that two amino acids on either side of the peptide bond cut by the enzyme are replaced and modified. The PR inhibitors bind to the active site of the enzyme, but cannot be cut, and by competitive inhibition prevent the true substrate from gaining access to the enzyme. Thus, PR inhibitors specifically antagonize the viral protease, preventing the maturation of nascent particles into infectious virions. PR inhibitors that include *ritonavir*, *nelfinavir* and *indinavir* are currently in clinical use, either alone or in combination with RT inhibitors.

By prevailing standards, state-of-the-art therapy against HIV-1, known as highly active antiretroviral therapy (HAART) simultaneously targets two different stages in the virus life cycle, namely RT and PR. Thus HAART consists of two or three RT inhibitors combined with at least one PR inhibitor. In many HIV-1 positive individuals, this combination antiretroviral therapy results in a dramatic reduction in viral load, decreasing the rate of CD4⁺ cell decline and the progression to AIDS (Detels et al., 1998; Hogg et al., 1998; Palella et al., 1998; Zhang et al., 1999a). However, the mutability of HIV-1 often leads to the emergence of drug resistant viruses, particularly in individuals who are unable to comply with the often complex treatment regimens (Blower et al., 2001; Hecht et al., 1998; Richman, 2001; Yerly et al., 1999). Additionally, current antiviral drugs produce prohibitive side effects in some individuals, which can further limit their utility (Richman, 2001; Richman et al., 1987).

Recently, the integrase (IN), the third enzyme of HIV-1 which is necessary for the integration process of proviral DNA into the host genome (Figure 1.5, step 4), has been identified as legitimate new drug target (d'Angelo et al., 2001). IN inhibitors are in pre-clinical development and testing. However, IN inhibitors and HAART (RT and PR inhibitors) act after HIV-1 infection of the target cell has occurred (Figure 1.5). Therefore recent development of novel classes of antiviral agents (reviewed in (LaBranche et al., 2001), targeting a different part of HIV-1 life cycle, namely entry (binding and fusion) have generated considerable enthusiasm that additional and possibly better tolerated drugs and drug combinations will be forthcoming. Importantly entry inhibitors (Figure 1.5, steps 1 and 2) intervene at a point before the virus infects the target cell.

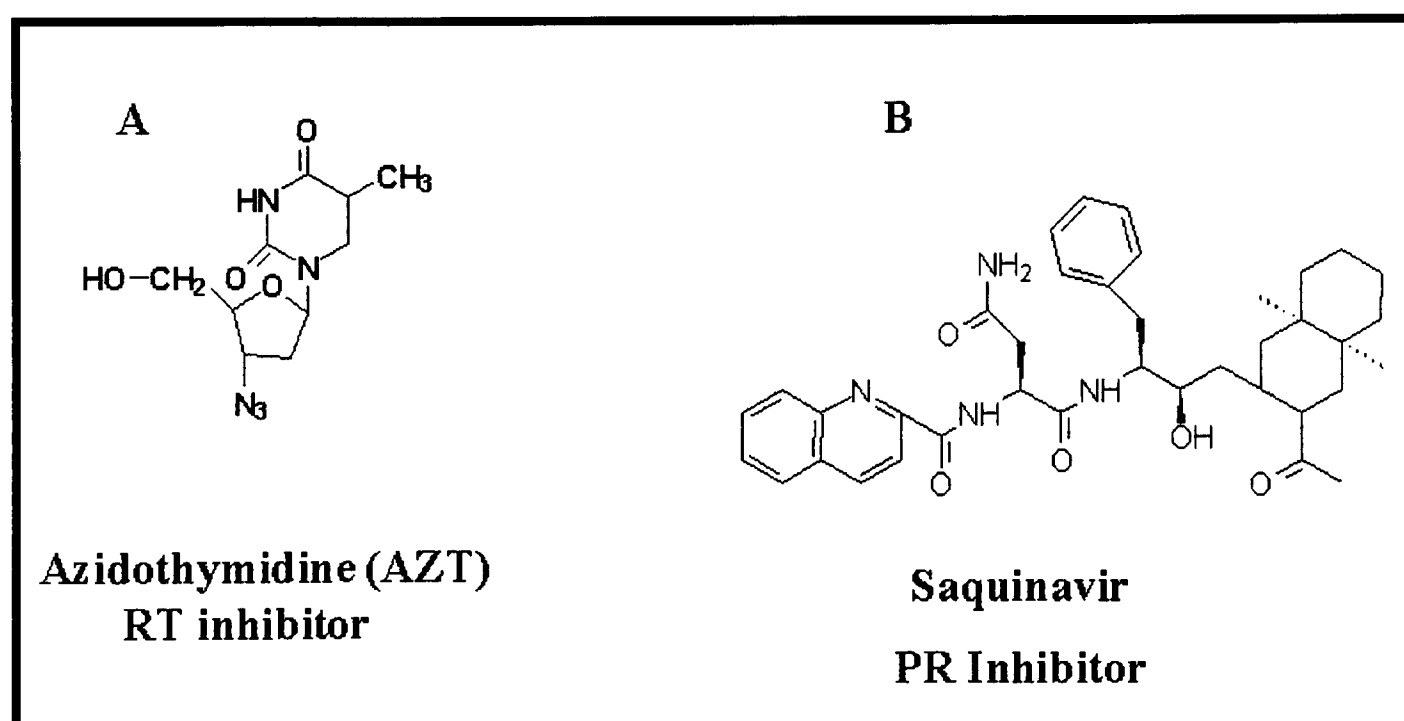


Figure 1.9: Some of the current anti-HIV-1 therapeutic agents. (A) AZT is a prototype dideoxynucleoside that inhibits RT. (B) Saquinavir is one of the common PR inhibitors. Both RT and PR inhibitors are used in combination therapy as part of HAART, and are the only classes of anti-HIV-1 drugs currently in clinical use.

Modalities to inhibit virtually every step in the HIV-1 entry pathway are being explored (Chan and Kim, 1998; De Clercq, 2000; Doms and Moore, 2000; D'Souza et al., 2000; Moore and Stevenson, 2000; Root et al., 2001; Tomaras and Greenberg, 2001). Conceptually, there are three steps in the HIV-1 entry process that could be inhibited: (a) binding of the viral envelope glycoprotein to the CD4 receptor; (b) binding of the envelope-CD4 complex to chemokine receptors; and (c) fusion of the viral and cell membranes.

a. CD4 attachment inhibitors

The initial interaction of gp120 on the virion with CD4 on the cell surface offers the highly conserved CD4 binding site on gp120 as a therapeutic target. Initial efforts to use soluble forms of human CD4 to interrupt this process were foiled by the rapid clearance of this molecule from blood and the lack of neutralization activity of CD4 against primary isolates (Daar and Ho, 1991; Daar et al., 1990). Also earlier efforts to combine sCD4 with the Fc portion of immunoglobulin (sCD4-Fc), in spite of a much longer half-life did not have any efficacy against HIV-1 *in vivo*. However, fusion of the gp120-binding portion of CD4 to the antigen-combining site of an immunoglobulin molecule has yielded a recombinant protein called PRO 542 from Progenics Pharmaceuticals (Mukhtar et al., 2000) with a long plasma half-life and anti-HIV-1 activity in phase I/II clinical trials (Jacobson et al., 2000; Shearer et al., 2000). PRO 542 a.k.a. CD4-IgG2 incorporates four copies of the D1D2 domains of CD4 into an antibody-like molecule (IgG2) and potently neutralizes primary HIV-1 isolates (Zhu et al., 2001). In phase I clinical trial with adult subjects, IV doses up to 10mg/ml of PRO 542 were well tolerated with no antibody reaction or dose-limiting toxicity, and showed plasma half-lives of 3-4 days (Jacobson et al., 2000). A similar phase I/II study in paediatric subjects showed PRO 542 tolerance, and acute decreases of >0.7 $\log_{10}$ copies/ml in serum HIV-1 RNA concentration were seen in 4 of the 6 children treated with 4 weekly 10 mg/kg PRO542 doses (Shearer et al., 2000). Cost-effective methods for the manufacture of PRO-542, by optimization of the production of the drug in the milk of transgenic dairy animals are underway to allow large-scale phase III clinical study (Mukhtar et al., 2000).

b. Coreceptor interaction inhibitors

The chemokine receptors that serve as coreceptors for HIV-1 entry have traditionally been good targets for therapeutic intervention, and several agents have been described that inhibit HIV-1 infection *in vitro* by binding to either CXCR4 or CCR5 (Arenzana-Seisdedos et al., 1996). CXCR4 interaction inhibitors include ALX40-4C, T22, and AMD3100, all of which are cationic compounds suggesting a charge-dependent binding and inhibition. ALX40-4C and T22 are peptides that bind to the negatively charged surface of the first and second extracellular loops of CXCR4 (Doranz et al., 1997a; Murakami et al., 1999), that are also involved in the interaction with HIV-1 gp120 (Chabot et al., 1999). AMD3100, on the other hand is a bicyclam compound that also binds a negatively charged second extracellular loop of CXCR4 (Labrosse et al., 1998; Schols et al., 1997). Of these three CXCR4-specific inhibitors, AMD3100 has gone through extensive pre-clinical development and testing (Blanco et al., 2000; Hatse et al., 2001), and is now in phase I/II clinical trials (reviewed in LaBranche et al., 2001).

The CCR5 is considered an attractive target for inhibition of HIV-1 infection because among others, individuals having a nonfunctional CCR5 receptor are apparently normal, but refractory to HIV-1 infection (refer to 1.6). CCR5 interaction inhibitors include small molecules, antibodies, chemokines and chemokine analogs. A low molecular weight compound called TAK-779 (Baba et al., 1999), inhibits productive interaction of the viral Env with CCR5 by inserting into a cavity formed between TM helices 1, 2, 3, and 7 near the extracellular surface of the receptor (Dragic et al., 2000). TAK-779 at its effective concentration of 10nM does not induce signaling via CCR5, however toxicity may limit its clinical use (Baba et al., 1999). Several mAbs to CCR5 inhibit infection of certain HIV-1 primary isolates (PIs). One of these, PRO 140 (originally described as PA14 (Olson et al., 1999)) broadly inhibited infection of PBMC by a panel of 25 subtype C South African M-tropic PIs (Trkola et al., 2001). The epitope on CCR5 recognized by PRO 140 appears to span multiple extracellular loops, explaining its broad antiviral activity against diverse HIV-1 strains (Olson et al.,

1999). PRO 140 does not induce signaling or down-regulation of CCR5 at effective concentrations, nor does it prevent the CC-chemokine signaling (Olson et al., 1999; Trkola et al., 2001). Although PRO 140 has not yet entered clinical trials, it is a desirable candidate for antibody therapy especially against CCR5-dependent HIV-1 strains.

c. Fusion inhibitors

The gp41-mediated fusion cascade also provides a variety of opportunities for pharmacological intervention of HIV-1 entry. It is a testimony to the innovative efforts applied to these opportunities that several fusion inhibitors are already in clinical trials. Inhibitors active at this stage include peptide mimetics of the gp41 HR2 region (T20 and T1249) that bind the trimeric N-terminal coiled-coil (Kilby et al., 1998; Wild et al., 1994b), the recently described five-helix protein that targets the HR2 region (Root et al., 2001), and the ADS-J1 cyclic compound that binds to the hydrophobic pocket in the HR1 coiled-coil (Chan et al., 1997; Ji et al., 1999). This group of inhibitors acts by a dominant negative mechanism of action, preventing formation of the six-helix bundle that is the driving force for the fusion process (discussed in 1.5.1b). Pre-clinical *in vitro* data indicated that T20 had a broad activity against laboratory adapted strains and PIs (Rimsky et al., 1998). In both the proof-of-principle and dose-escalation phase I/II clinical trials, treatment of HIV-1 infected individuals with T20 resulted in a 1.96 log₁₀ median decline in plasma viral load at the highest dose (Kilby et al., 1998). The dose-escalation trial showed that twice-daily sub-cutaneous injection of 100mg T20 was the most effective delivery regimen. Pharmacokinetic analysis showed median $t_{1/2}$ of T20 was 1.83 hours following sub-cutaneous administration, and plasma levels suggested that 60-70% of the administered compound was bioavailable. A concern with peptide-based antiviral therapy is the potential for eliciting an antibody response to the antiviral agent, rendering the agent ineffective. Animal studies involving cynomolgus monkeys showed that T20 was immunogenic, but that these T20-specific antibodies did not reduce the efficacy of the drug (Lawless et al., 1998). The same results were observed

in human trials where antibodies generated by T20 administration did not adversely affect the antiviral activity. Thus T20 administration in patients did not induce significant drug-related side effects (Kilby et al., 1998). However HIV-1 isolates have been characterized with reduced susceptibility to T-20, derived from serial *in vitro* passage in the presence of the compound (Rimsky et al., 1998). Larger, multi-center, randomized T20 trials are in development, and further clinical studies are underway to assess the potential for selection of variants resistant to T-20 *in vivo* as well as the possibility that anti-T20 antibody response could alter the efficacy of the compound (Kilby et al., 1998). Nonetheless, blocking HIV-1 entry in general establishes a new generation of antiviral therapy with promising potent activity against HIV-1 strains transmitted *in vivo* as defined by their cellular tropism and contribution to AIDS pathogenesis (discussed in the next section).

1.6 HIV-1 cellular tropism, transmission and AIDS pathogenesis

1.6.1 Co-receptors: determinant of HIV-1 tropism

Until recently, one of the less comprehensible behaviors of HIV-1 was its tropism. The phenotype of HIV-1 isolates was originally based on three classifications. The first classification defined primary isolates based on the cells in which they replicate *in vitro*, as M-, or T-tropic. M-tropic viruses are particularly those strains isolated early in infection that only infect macrophages and primary activated T lymphocytes. While T-tropic viruses are those strains that readily infect both primary activated T lymphocytes and transformed T-cell lines, but not macrophages. The second system categorized isolates as being either syncytium-inducing or non-syncytium-inducing (NSI) based on whether they form syncytia in the lymphocytic cell line, MT-2 (Cheng-Mayer et al., 1988; Fenyo et al., 1989; Schuitemaker et al., 1992). The third system defined viruses as either slow/low or rapid/high in culture (Fenyo et al., 1988). All the three classification schemes have been used interchangeably, but they were not synonymous and have been misleading.

The identification of CXCR4 and CCR5 chemokine receptors as having critical roles in the cellular entry of HIV-1 allowed development of a more precise system for defining the viral tropism. Prototype T-tropic strains use CXCR4, M-tropic strains use CCR5, and dual-tropic (D-tropic) strains use both chemokine receptors, culminating in the latest nomenclature X4, R5 and R5X4 tropic strains, respectively (Berger et al., 1998). While differential use of CXCR4 or CCR5 by HIV-1 strains has been observed, the striking differences seen appear to be in how R5 and R5X4 viruses utilize the CCR5 coreceptor. Generally, R5-tropic viruses are particularly well adapted for CCR5 use (Choe et al., 1996; Deng et al., 1996; Dragic et al., 1996), and can tolerate deletion or substitution of the CCR5 amino-terminal domain (Howard et al., 1999; Wang et al., 1999). R5X4 viruses, however, are much more sensitive to

mutations in CCR5, particularly in the amino terminal domain (Doranz et al., 1997b). Thus, there is a cost associated with the benefit to use dual chemokine receptors as exhibited by R5X4 tropic strains that have more stringent requirements for CCR5 usage (Doms and Peiper, 1997).

1.6.2 Tropism *in vivo*: MΦ tropism and AIDS pathogenesis

The phenotype of HIV-1 that is transmitted *in vivo* is R5, even when the contaminating virus is a mixture of R5, R5X4 and X4 (Figure 1.10). The reason for this selection of viral phenotype during transmission is not understood. The viraemia present during primary infection consists of oligoclonal R5 virus, and this virus type generally persists during the asymptomatic phase of the infection until the onset of AIDS (Figure 1.10). Viral transmission and disease progression, collectively called AIDS pathogenesis, corresponds to evolution of coreceptor phenotype. In about half of all HIV-1 infections there is viral evolution towards dual (R5X4) tropism and CXCR4 usage that is prognostic for accelerated progression to AIDS (Collman et al., 1992; Doranz et al., 1996). Thus when the phenotype switch occurs, there is an initial evolution from CCR5 using viruses to those that can use both CCR5 and CXCR4 (Connor et al., 1997).

To investigate the impact of coreceptor usage on HIV-1 pathogenesis, Penn *et al.* (Penn et al., 1999) and Schramm *et al.* (Schramm et al., 2000) exploited an *ex vivo* human lymphoid histoculture system, which recapitulates virus/host interaction integral to disease progression *in vivo* (Glushakova et al., 1995). Their data showed that coreceptor preference influences pathogenic potentials of HIV-1, with CXCR4 utilization strongly associated with aggressive depletion of CD4⁺ T cells. The same group (Goldsmith et al., 1999), using the lymphoid culture model also found that CCR5 dependent viruses efficiently infected both macrophages and CCR5 bearing T-cells (Bleul et al., 1997), thereby leading to robust replication within macrophages and preferential depletion of the CCR5⁺ T cells. Grivel and Margolis (Grivel and

Margolis, 1999) reported similar findings, that R5 isolates are highly cytopathic for their CCR5⁺ T-cell targets in lymphoid tissue *ex vivo*. The R5 viral isolates were also found to exhibit high levels of replication and to cause severe depletion of activated human CD4⁺ T cells in human peripheral blood lymphocyte SCID mice (hu-PBL-SCID mice) compared to X4 strains (Fais et al., 1999).

In an independent cross-sectional study, Li *et al.* (Li et al., 1999) examined the tropism and coreceptor usage of primary HIV-1 isolates collected from the blood of individuals at different stages of HIV-1 infection and pathogenesis, and from tissue-derived isolates. Their study indicated that in the late stage of AIDS pathogenesis, with increased viraemia and the viral quasispecies, CCR5 utilization and M-tropism persist in blood and tissues and the replicative ability increases in macrophages. These findings in general may explain why many individuals succumb to AIDS, with severe depletion of CD4⁺ T lymphocytes, without a switch from R5 to X4 tropic viruses (Scarlati et al., 1997; Tersmette et al., 1989). Grivel and Margolis (Grivel and Margolis, 1999) postulate that these individuals might have an unusually high proportion of CCR5⁺/CD4⁺ T cells, because of the effects of CCR5 promoter polymorphism (Martin et al., 1998; McDermott et al., 1998). These data also delineate specific interactions with coreceptors that influence target cell specificity, viral replication within macrophages, and disease progression within the host (Goldsmith et al., 1999; Li et al., 1999).

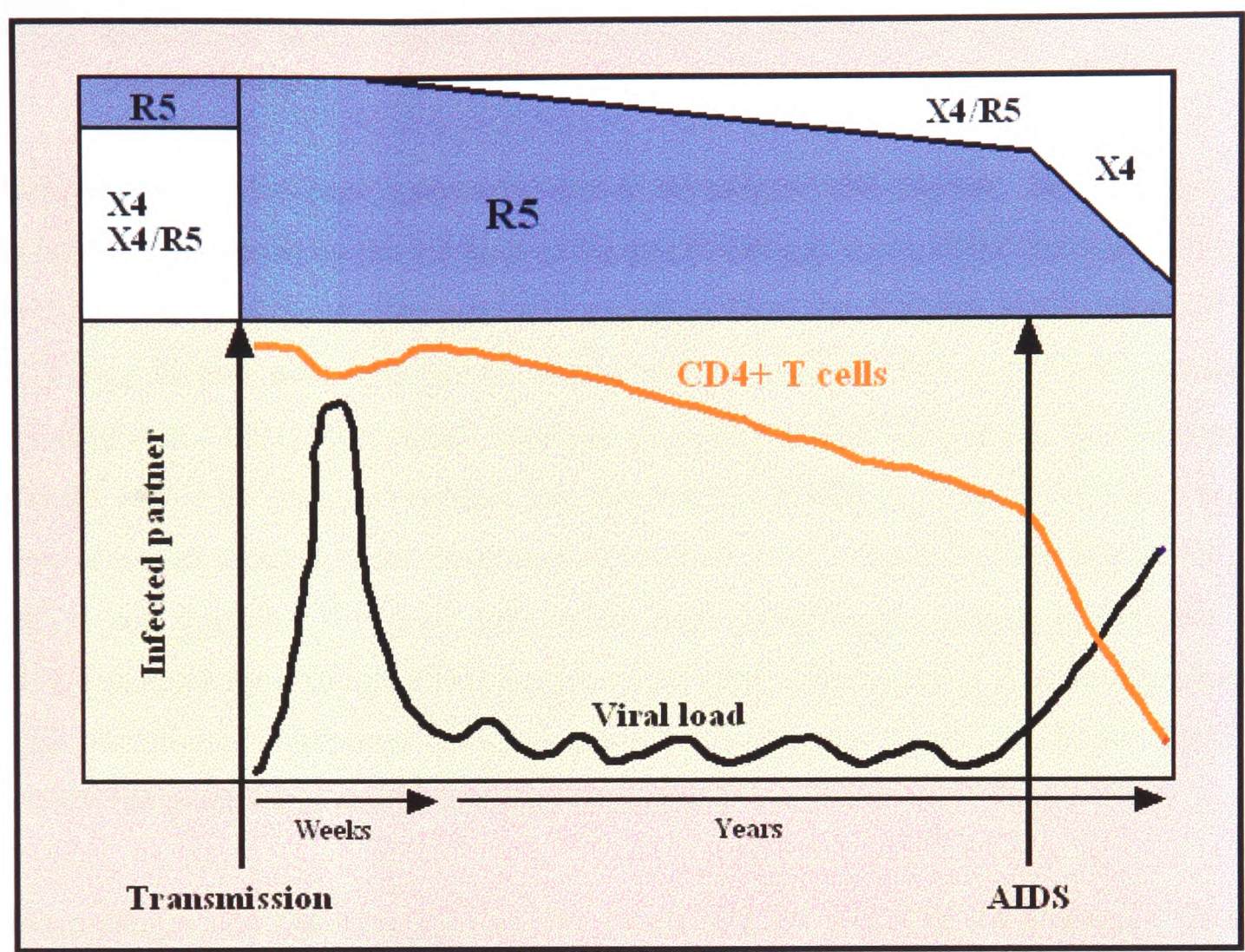


Figure 1.10: Temporal evolution of HIV-1 tropism during AIDS pathogenesis. HIV-1 transmission is restricted to R5 HIV-1 variants, which persist throughout the asymptomatic period until the onset of AIDS. In many, but not all individuals, viral tropism broadens to include X4 and R5X4 variants near the time when AIDS-defining symptoms are first observed.

1.6.3. Molecular and cellular determinants of MΦ tropism and transmission

Genetic analysis has been a powerful tool to investigate MΦ-tropism. HIV-1 tropism is largely determined by the V3 loop of the gp120 (Briggs et al., 2000; Gu et al., 1993; Hwang et al., 1991). A 20-amino acid sequence from the V3 loop of the classic R5 HIV-1_{Ba-L} isolate was sufficient to confer MΦ-tropism on HIV-1_{IIIB}, an X4 isolate (Hwang et al., 1991). This small sequence element is in the V3 loop tip, the envelope domain that is the principal neutralizing determinant of HIV-1. Thus, the V3 loop not only serves as a target of the host immune response, but is also pivotal in determining HIV-1 cell tropism. However, ambiguities arise regarding the tropism of individual HIV-1 isolates for different CD4⁺ human target cells. The situation is relatively clear with infection of continuous cell lines that express abundant levels of CXCR4 and/or CCR5, but much more complex for macrophages.

Macrophages constitutively express low levels of CD4 and CXCR4 whereas CCR5 expression is inducible, and R5 and R5X4 HIV-1 isolates readily infect macrophages. The extent to which primary X4 isolates can infect macrophages through the CXCR4 is highly contentious. Some studies suggest that all or most primary X4 isolates can infect macrophages, and that CXCR4 may be the sole macrophage coreceptor for these isolates (Schmidtayerova et al., 1998; Simmons et al., 1998; Verani et al., 1998). By contrast, other reports show little or no infection of macrophages by X4 isolates (Collman and Yi, 1999; Yi et al., 1999; Yi et al., 1998). Thus the CXCR4 receptor expressed on macrophages is thought to function as a coreceptor for entry by R5X4, but not X4 tropic HIV-1 isolates, and macrophage resistance to X4 tropic strains does not result from a lack of the CXCR4 coreceptor (Collman and Yi, 1999).

Contradictory reports on utilization of the CXCR4 on macrophages by X4 isolates reflect different methods of macrophage culture and levels of expression of CXCR4 (Edinger et al., 1999). Almost all *in vitro* studies have used monocyte derived macrophages (MDM), a macrophage model unlikely to represent all macrophage

populations *in vivo*. At least one study suggests that there are differences in post-entry events in MDM and alveolar macrophages that could influence the ability of X4 viruses to replicate in these cells (Huang et al., 1993). Although CXCR4 is also expressed on MDM, it is present in a functionally restricted form, hence CCR5 is preferentially utilized, and serves as the major coreceptor on macrophages for HIV-1 (Naif et al., 1998; Tuttle et al., 1998; Yi et al., 1998). However, CCR5 usage does not appear to be sufficient for macrophage infection, based on the isolation of HIV-1 strains that can use CCR5 yet fail to infect macrophages [Dittmar, 1997]. Thus, the intersection between viral phenotype, cell tropism and coreceptor utilization is both complex and beyond simple generalization.

Host genetic factors can also influence R5-tropism and transmission. Cohorts of HIV-1 infected individuals have been established based on specific clinical, virological and immunological criteria. One type of cohort contains individuals selected after frequent exposure to HIV-1 without seroconversion. A mutation in the CCR5 gene has been frequently found in this type of cohort (Zimmerman et al., 1997). This mutation is a deletion of 32 base pairs ($\Delta 32$), resulting in a defective CCR5 receptor. CD4⁺ T cells and macrophages carrying the $\Delta 32$ mutation are resistant to infection with R5 viruses. Homozygous individuals carrying the mutation on both alleles (1% of Caucasians) are almost completely refractory to infection with R5 HIV-1 strains (Samson et al., 1996; Zimmerman et al., 1997). Very rare examples of infection of these individuals have been reported (O'Brien et al., 1997; Theodorou et al., 1997); in these cases the transmitted R5 viruses appear to use CXCR4. Individuals heterozygous for the $\Delta 32$ mutation are not protected from HIV-1 infection, but have slower rate of progression to AIDS. This is because the mutated form of the protein acts as a dominant negative mutant and prevents most of the wild-type CCR5 protein from reaching the cell surface.

1.6.4 The Trojan Horse hypothesis: MΦ as reservoir and vehicle of HIV-1 transmission

Macrophages serve as an important reservoir of HIV-1 in different tissues (Gendelman et al., 1989; Levy, 1993; Phillips et al., 1998). Macrophages in the tissues are derived from blood monocytes, which migrate constitutively, or in response to inflammation. These cells have access to almost all organs in the body and are potential vehicles of virus dissemination (Gendelman et al., 1989; Meltzer et al., 1990). HIV-1 infected macrophages are found in the brain, lungs, lymph nodes, skin, bone marrow and blood of seropositive individuals (Cheng-Mayer et al., 1989; Gendelman et al., 1989). Although these are not only the systems in which the macrophage may contribute to viral replication and pathology, they epitomize the active role of macrophage within tissues in AIDS pathogenesis. HIV-1 harbored in macrophages may escape immune surveillance and anti-viral therapy.

Although HAART significantly suppresses viral replication, ongoing viral replication and dissemination have been noted during HAART, especially in macrophages compared with resting T-cells (Perelson et al., 1997). Thus, macrophages play an essential role in regulating the intensity and progression of disease even during therapy, thereby fulfilling the Trojan Horse hypothesis. The Trojan Horse hypothesis states that: upon entering the tissue with benign intent, the monocyte inadvertently imports latent virus that is activated when the cell differentiates. The infected macrophages then recruits other cells of the immune system and releases harmful products leading to tissue damage (Giulian et al., 1990). This hypothesis has lead to some suggestions that HIV-1 induced pathogenesis is a macrophage-centered immunopathology (Meyaard et al., 1993; Mosier and Sieburg, 1994).

1.6.5 Macrophage immune dysfunction and AIDS pathogenesis

Progression from acute infection to AIDS is often accompanied by macrophage-mediated immune dysregulation and susceptibility to opportunistic infections (OI). Infection of macrophages by HIV-1 impairs a number of effector functions normally carried out by these cells, resulting in defective phagocytosis and subsequent killing of opsonized pathogens, and dysregulation of cytokine production (reviewed in Khati et al., 2001). The underlying mechanism of waning immunity and function of HIV-1 infected macrophages are not restricted to OI, but include a number of factors associated with inflammation and cytokine dysregulation.

HIV-1 is capable of tipping cytokine production in its favor by either inhibiting the production of pro-inflammatory cytokines or augmenting the production of anti-inflammatory ones (Mosser and Karp, 1999). For instance, a pro-inflammatory cytokine, interleukin 12 (IL-12) that is central to the orchestration of both innate and adaptive immune response (reviewed in (Trinchieri, 1998)), is one of the cytokines dysregulated in HIV-1 infected macrophages. PBMC from HIV-1 infected individuals show attenuated IL-12 production in response to bacterial stimuli (Chehimi et al., 1994; Chougnet et al., 1996). Alveolar macrophages from HIV-1 patients also secrete less IL-12 compared with those from normal donors in response to *Staphylococcus aureus* (Denis and Ghadirian, 1994). The mechanisms responsible for HIV-1 related IL-12 suppression are not understood, but a number of hypotheses have been advanced and are reviewed in (Mosser and Karp, 1999).

Other properties of HIV-1 infected macrophages include an alteration of the antigen-presenting function and stimulation of interferon response in adjacent T cells (Knight and Macatonia, 1991). The HIV-1 accessory gene product Nef also results in macrophage-mediated immune dysregulation (Swingler et al., 1999). The Nef protein induces the production of MIP-1 α and MIP-1 β by HIV-1 infected macrophages, thereby mediating lymphocyte chemotaxis and activation, permitting productive HIV-1 infection.

Another macrophage-mediated immune dysregulation during HIV-1 infection includes apoptosis of CD8⁺ T cells (Herbein et al., 1998a; Herbein et al., 1998b). CD8⁺ T lymphocytes play an important role in the control of HIV-1 infection through their cytotoxic activity and by releasing soluble factors (Collman and Yi, 1999). Apoptosis of CD8⁺ T cells isolated from HIV-1 infected patients is mediated by macrophages through the interaction between tumor necrosis factor (TNF- α) bound to the membrane of macrophages (mbTNF) and a receptor on CD8⁺ T cells called TNF receptor II (Herbein et al., 1998a). The HIV-1 gp120 envelope protein activates CXCR4 on macrophages and CD8⁺ T cells, which increases the expression of two cell proteins, mbTNF and TNFII involved in apoptosis induction. Thus, the HIV-1 gp120 does not only mediate viral entry, but is also central in AIDS pathogenesis.

1.7 The HIV-1 gp120 SU envelope glycoprotein

1.7.1 Synthesis and assembly

The gp120 envelope glycoprotein is synthesized as an approximately 845 to 870 amino acid precursor in the rough endoplasmic reticulum. Env then migrates through the Golgi complex where it undergoes glycosylation with the addition of 25 to 30 complex N-linked high-mannose sugar side chains that are added at asparagine residues, to form the gp160 polypeptide precursor. The gp160 assembles into oligomers, and the preponderance of evidence suggests that these oligomers are trimers (Earl et al., 1990; Weissenhorn et al., 1997). Oligomerization has important structural and functional consequences. Studies on other membrane proteins have shown that assembly is contingent on correct initial folding in the endoplasmic reticulum and is a prerequisite for intracellular transport (Rose and Doms, 1988). Molecules that fail to assemble correctly are retained in the endoplasmic reticulum and are eventually degraded (Lippincott-Schwartz et al., 1988).

Prior to delivery to the plasma membrane, gp160 undergoes a single posttranslational cleavage, which creates two noncovalently associated subunits; gp120, which is external to the membrane, and gp41, which possesses both cytoplasmic and transmembrane domains. Posttranslational cleavage, like glycosylation is essential for infectivity, presumably because it generates the highly conserved and hydrophobic N-terminal domain of gp41 that plays a role in membrane fusion. When the gp120-gp41 complex reaches the infected cell surface, a fraction is incorporated into budding virus particles. A large number of complexes disassemble, releasing gp120 and exposing the previously buried gp41 ectodomain.

1.7.2 Structure and function of the gp120

The structure of the HIV-1 gp120 allows direct visualization of two overlapping sets of machinery. One, involved in entry, binds to both CD4 and chemokine receptor and transfers a signal to gp41 to initiate membrane fusion. The other uses loop variation, steric occlusion, conformational change and carbohydrate masking to evade the immune system. A comparison of the amino acid sequences of gp120 from different primate immunodeficiency viruses identified five variable regions (V1-V5) interspersed with five conserved (C1-C5) regions (Starcich et al., 1986). The variable region form loops on the surface of the protein, whereas the conserved regions form a central core.

The X-ray crystal structure has been solved at 2.5Å resolution of the gp120 core complexed with a two-domain fragment of human CD4 and an antigen-binding fragment (Fab) of the 17b neutralizing mAb (Kwong et al., 1998). The gp120 core forms a globular structure, and comprises 25 β -strands, 5 α -helices and 10 defined loop segments, all organized with a topology shown in Figure 1.11A. The structure folds to form an inner domain, containing the N and C termini, which in an intact Env would associate with gp41, and an outer domain, and a mini-domain called the bridging sheet that links the inner and outer domains. All the three domains are important for CD4 and coreceptor binding. Glycosylation is found over much of the surface of the protein, and particularly on the outer domain.

The CD4-binding surface comprises an area of 800 Å² and is located at the bottom of a depression formed at the interface of the outer domain with the inner domain and the bridging sheet of gp120 (Figure 1.11B). The CD4bs is devoid of carbohydrates and the surface is relatively acidic, complementing a basic patch on CD4. Although missing in the structure, experimental evidence suggests convincingly that the V1/V2 loops partially mask the CD4bs (Wyatt et al., 1998). This masking is more complete in clinically relevant HIV-1 primary isolates, as opposed to laboratory adapted strains, and is fully manifest only in the oligomeric functional form of gp120 (reviewed in

Poignard et al., 1996). Within the CD4 binding surface are two major cavities: a hydrophobic and a water-filled hydrophilic cavity. Residues within the hydrophobic cavity are highly conserved among primate immunodeficiency viruses, and the cavity is plugged by the Phe 43 residue of the CD4 that is critical for the CD4-gp120 interactions (Figure 1.11B). In contrast, the hydrophilic cavity is unoccupied by CD4 and is lined with variable residues. These two cavities probably reflect the stabilization of an energetically unfavorable gp120 conformation by CD4 (reviewed by Sattentau, 1998).

The conserved site of interaction with the chemokine receptor is overlapped in the structure by the gp120 interface with the 17b Fab fragment (Kwong et al., 1998). The interface between gp120 and the Fab fragment buries 450 Å² surface area composed mainly of basic and hydrophobic residues that are highly conserved between different gp120 molecules. The interface is centered on the residues of the bridging sheet and the stem of the V1/V2 (Rizzuto et al., 1998). The coreceptor-binding region is masked by the V2 and V3 loops in the intact, non-CD4 induced gp120 conformation (Wyatt et al., 1998). The V3 loop has been shown experimentally to become more exposed after CD4 binding, and this represents its movement away from the conserved segment of the CRbs, allowing efficient binding of neutralizing antibodies such as 17b (Sattentau and Moore, 1991).

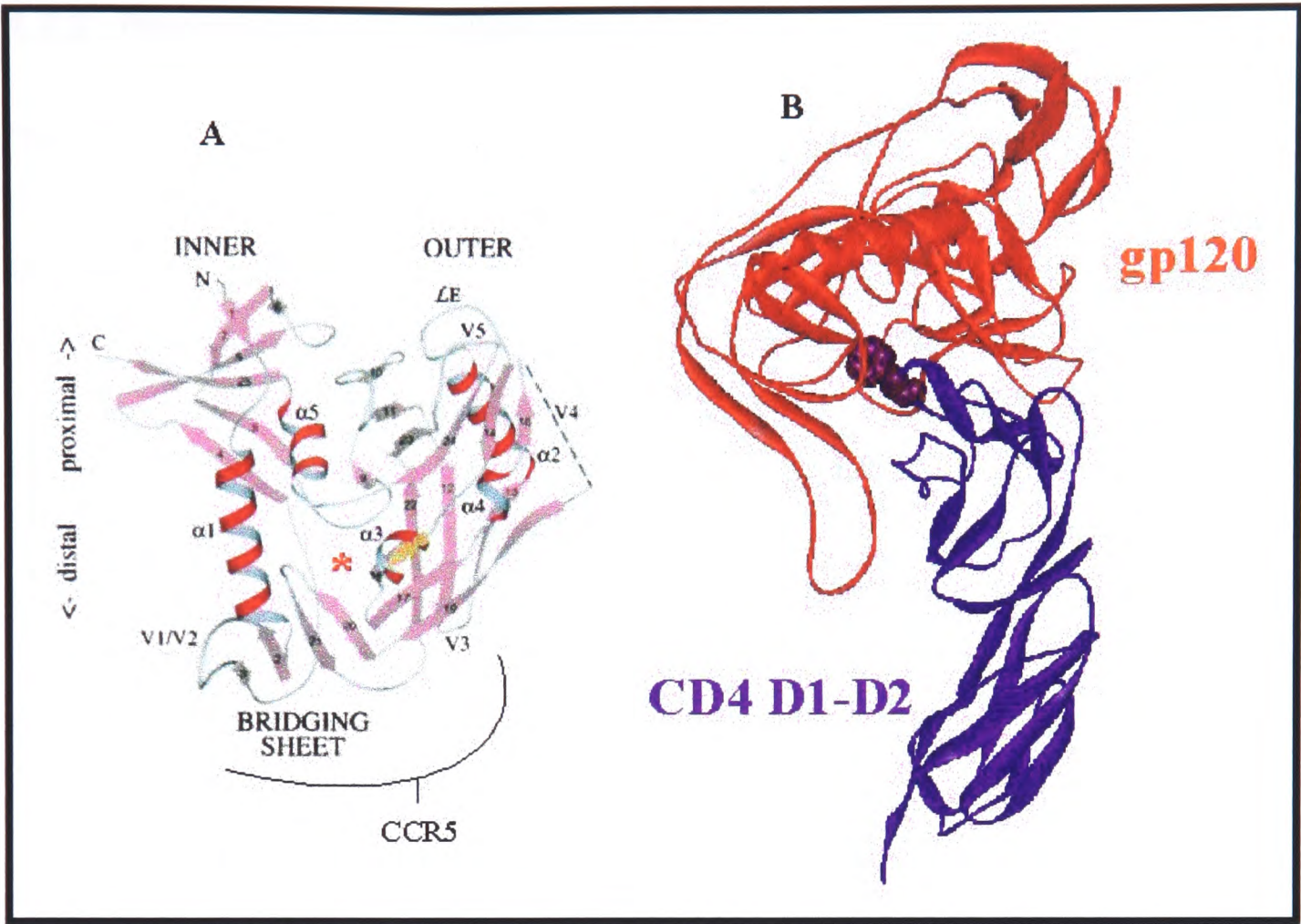


Figure 1.11: Ribbon diagram of the structure and orientation of the gp120 core. (A) α -Helices are depicted in red and β -strands in salmon-pink, except for strand β 15 (yellow) that makes an anti-parallel β -sheet with C' of CD4. Connections are shown in grey, except for the disordered V4 loop (dashed line), joining β 18 and β 19. The inner and outer domain linked by the bridging sheet and the base of the V1/V2 and V3 loops are labeled. Also labeled is the N and C termini of the truncated gp120 core, and the virus membrane proximal and distal faces. The position of the Phe 43 cavity involved in CD4 binding is indicated by an asterisk and the CD4 induced (CD4i) gp120 epitope implicated in binding to CCR5 chemokine receptor is shown. (B) The gp120 core (red) is seen from the perspective of CD4 (blue), and is oriented with the viral membrane at the top of the figure and the target cell membrane at the bottom. Residue Phe 43 of CD4 (space filling model) is also depicted reaching into the hydrophobic CD4 binding cavity of the gp120. The gp120 core was crystallized in a ternary complex with two-domain soluble CD4 and the Fab fragment of the 17b mAb (Reproduced from Kwong et al., 1998).

1.7.3 Neutralizing antibodies against gp120

The HIV-1 SU gp120 glycoprotein, which can be shed from the envelope complex, elicits both virus-neutralizing and non-neutralizing antibodies during natural infection. Antibodies that lack neutralizing activity are often directed against the gp120 regions that are occluded on the assembled trimer, and which are exposed only upon shedding (Moore and Sodroski, 1996). Neutralizing antibodies, by contrast must access the functional envelope glycoprotein complex and typically recognize conserved or variable epitopes near the receptor binding sites (Posner et al., 1991; Sattentau and Moore, 1995; Trkola et al., 1996a; Wu et al., 1996). The production and characterization of mAbs of human and rodent origin have allowed a precise determination of the neutralization epitopes present on the HIV-1 gp120 (Moore and Ho, 1995; Poignard et al., 1996).

Using epitope maps in conjunction with the X-ray crystal structure of gp120 complexed with CD4 and a neutralizing mAb (Kwong et al., 1998), spatial organization of conserved neutralization epitopes on gp120 was determined (Wyatt et al., 1998). More broadly neutralizing antibodies recognize discontinuous, conserved epitopes in three regions of the gp120 (Figure 1.12A and Table 1.2). In HIV-1 infected individuals, the most abundant of these are directed against the CD4 binding site, and block gp120-CD4 interaction (Ho et al., 1991; Posner et al., 1991). Less common are antibodies against epitopes induced or exposed upon CD4 binding (CD4i) (Thali et al., 1993). Both CD4i and V3 antibodies disrupt the binding of gp120-CD4 complexes to chemokine receptors (Trkola et al., 1996a; Wu et al., 1996). A third gp120 neutralization epitope is defined by a unique mAb, 2G12 (Trkola et al., 1996b), which does not efficiently block receptor binding (Trkola et al., 1996a). Although high-titer gp120 binding antibodies are sustained in infected individuals throughout the course of infection, these antibodies have very poor neutralizing activity against autologous as well as representative primary HIV-1 isolates (Montefiori et al., 1996; Moog et al., 1997). This observation suggests that neutralizing antibodies that arise in response to HIV-1 infection may not be critical in limiting viral replication.

Table 1.2: Conserved epitopes for neutralizing antibodies identified on the gp120 core

Competition group	Example of mAb	Mechanism of neutralization	Properties	Selected references
CD4 binding site(CD4bs)	F105, 15e, 21h, 1125h, 448D, 39.3, IgG1b12, 830D	Interfere with gp120-CD4 binding	CD4BS antibodies competes with CD4 and with antibodies against CD4i epitope	(Ho et al., 1991; Posner et al., 1991)
CD4 induced epitope (CD4i)	17b, 48d	Interfere with chemokine-receptor binding	CD4 binding increases exposure of the epitopes as a result of movement of the V2 loop	(Thali et al., 1993)
2G12	2g12	Unknown	Antibody binding is dependent upon proper N-linked glycosylation	(Trkola et al., 1996b)

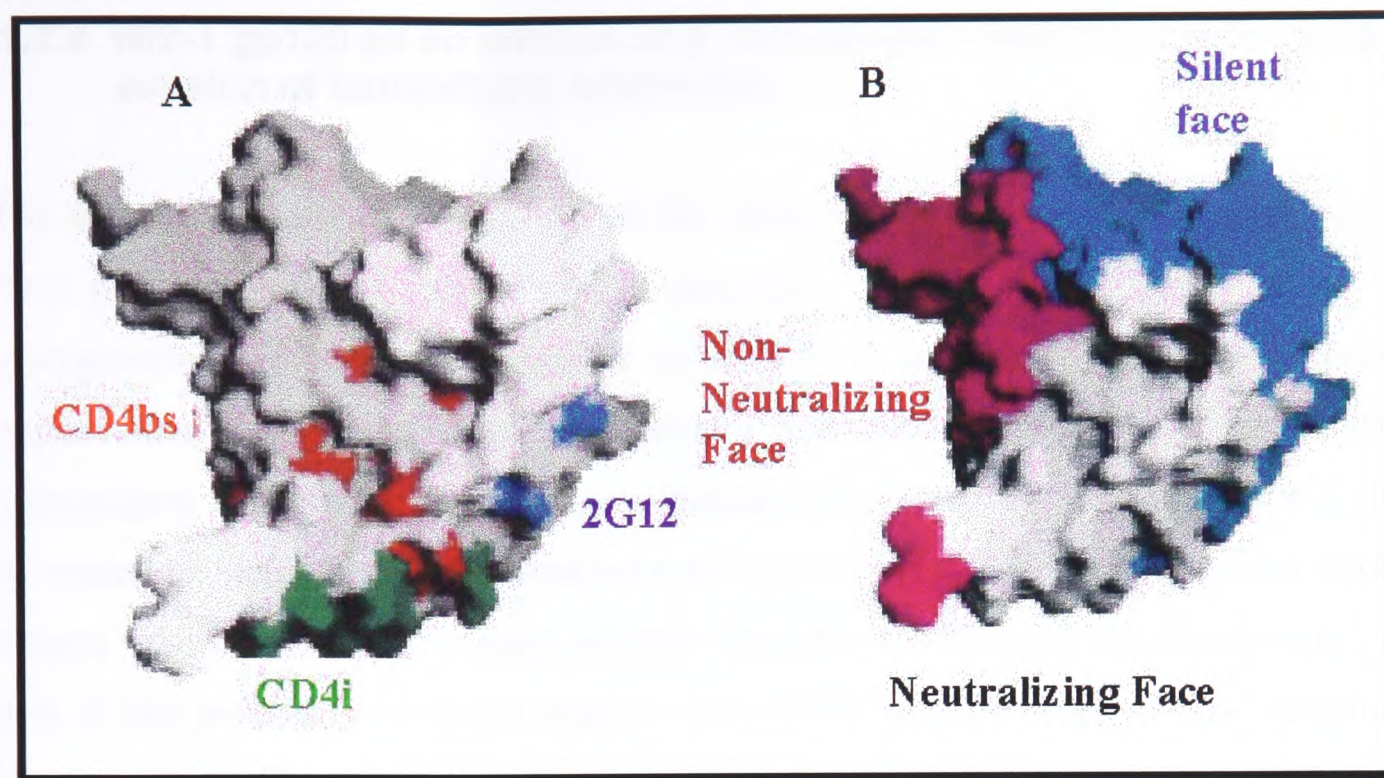


Figure 1.12: The spatial relationship of epitopes of the gp120 core. (A) The CD4bs and the CD4i coreceptor-binding surfaces as well as residues implicated in 2G12 binding (blue) are depicted. (B) Approximate locations of the faces of the gp120 core, defined by the interaction with antibodies. The molecular surface accessible to neutralizing ligands (CD4 and CD4bs, CD4i and 2G12 antibodies) is shown in white. (Reproduced from Wyatt et al., 1998)

1.7.4 HIV-1 gp120 as an antigen and immunogen: Implications for viral evasion of neutralizing antibodies

The *in vivo* exposure of Env gp120 on the surface of virions or infected cells makes them prime targets for antibodies that potentially block essential functions of this glycoprotein. However, the success of HIV-1 in achieving persistent infections implies that the viral envelope glycoprotein has evolved to be less than an ideal immunogen. The principal defence mechanism adopted by HIV-1 includes occlusion of conserved structures. Thus, structures on the viral gp120 that are conserved among diverse viral strains are, in general, poorly exposed to the humoral immune system. As part of this process of antibody evasion, the CD4bs has developed several important characteristics. First, the CD4bs is partially masked by the hypervariable V1/V2 loops and their associated glycosylation (Kwong et al., 1998; Wyatt et al., 1998; Wyatt et al., 1995). Secondly, despite being relatively well-conserved, the CD4bs contains clusters of residues that do not contact CD4 and are subject to mutations, such as those lining the hydrophilic cavity (Kwong et al., 1998). Finally the CD4bs is recessed to an extent that would impede direct access of antibody variable region, and residues flanking the site are variable or shielded by glycosylation.

Most carbohydrate moieties appear as "self" to the immune system, therefore extensive glycosylation may reduce the potential of a large portion of gp120 surface to serve as an immunogenic target. Thus in addition to the neutralizing and nonneutralizing faces of gp120 previously detected by antibody competition analysis (Moore and Sodroski, 1996), the crystal structure of the gp120 core reveals a third, immunologically silent face (Figure 1.12B). Despite the potential to exert potent antiviral effects, antibodies are not able to suppress virus replication completely in infected hosts. The efficacy of the humoral immune response in limiting the spread of HIV-1 *in vivo* is compromised by at least the relative resistance of PIs to neutralization, and the temporal pattern with which neutralizing antibodies are generated. A successful strategy may require solutions at several levels, such as using novel anti-viral agents with different modalities and properties from antibodies.

1.8 Aptamers: novel intervention strategy against HIV-1 gp120

1.8.1 The SELEX protocol: discovery and development

The use of synthetic oligonucleotide ligands called aptamers (*aptus* = *Latin*, fit) (Ellington and Szostak, 1990; Robertson and Joyce, 1990; Tuerk and Gold, 1990), shows great promise and has opened avenues for the development of analytical, diagnostic and therapeutic tools in areas that have been hitherto refractory (Osborne et al., 1997). Aptamers are generated *in vitro* using the SELEX (systematic evolution of ligands by exponential enrichment) protocol (reviewed in Fitzwater and Polisky, 1996; Gold et al., 1995). The SELEX combinatorial chemistry process is an iterative cycling of selection and amplification by which nucleic acid ligands with desired property are isolated against a molecular target of interest. The desired property is typically the ability to bind to a molecule of interest with high affinity and specificity. This property is dictated by the three-dimensional structure of the folded nucleic acid as determined by its sequence. The property is also a function of a combination of the folded nucleic acid's van der Waal's surface contacts, hydrogen bonds, stacking interactions and other non-covalent bonds that can form between the aptamer and its target (reviewed by James, 2000).

Since the first SELEX experiments, which were reported in 1990 (Ellington and Szostak, 1990; Robertson and Joyce, 1990; Tuerk and Gold, 1990), a large number of technical developments have been made that increased the power and utility of the technology (reviewed in Marshall and Ellington, 2000). An attempt has been made to put SELEX on a firm theoretical foundation by simulating the process with simple assumptions regarding the size of randomized region, the number of selection rounds, and the nature of the nucleotide (RNA v/s DNA). In designing a library, the objective is to produce sequences that can be amplified and transcribed efficiently. To achieve these, fixed, flanking regions are required for primer hybridization and *in vitro* transcription. The length of randomized domain in an oligonucleotide library is therefore limited by costs and the number of molecules that can be manipulated in a

standard molecular biology laboratory. Twenty-five fully randomized nucleotides ($4^{25} = 10^{15}$) is near the practical limit of saturation in a SELEX protocol (Gold et al., 1995). However, in order to produce a folded structure capable of specific interaction with a target molecule, the RNA needs to be large enough (where randomized region ≥ 35) to assume a complex tertiary structure (James, 2000). Thus use of enough randomized nucleotides is critical to get a high affinity ligand and probably a more stable folded structure.

The number of SELEX rounds required depends on the target's epitope chemistry, and the preponderance of "winners" in the starting library pool. For instance in the T4 DNA polymerase SELEX, two sequences out of 65,000 were found in 4 rounds (Tuerk and Gold, 1990), whereas in the R17 coat protein SELEX, roughly one sequence in 10^9 was selected in 11 rounds (Schneider et al., 1992). From these data and a number of other SELEX experiments, a reasonable rule for predetermining the number of rounds until the highest affinity ligands are found might be one round for 10-fold enrichment (Gold et al., 1995).

There are sparse data to support the use of DNA over RNA aptamers and *vice versa*, it is more of a personal choice. For the few cases in which proteins or small molecules like Adenosine Monophosphate (AMP) have been SELEX targets for both DNA and RNA libraries, the data suggest that binding affinity and specificity are similar for the aptamers from either library (Lin and Patel, 1997). However RNA-based aptamers, or nucleotides with RNA-like properties are capable of adopting a seemingly greater variety of structures ((reviewed in Ferre-D'Amare and Doudna, 1999; Patel et al, 1997). In addition, the RNA library pool can be "front-loaded" with modified nucleoside triphosphates such as 2'-fluoro-pyrimidines (Figure 1.13) or 2'-amino-pyrimidines resulting in oligonucleotide pools that are resistant to endonuclease digestion (Pieken et al., 1991). Other modification of RNA-based library include use of 5-iodo- or 5-bromo-UTP allowing a search for ligands potentially capable of covalently cross-linking the target (Willis et al., 1993). Thus, RNA aptamers are much more amenable to modifications and are desirable drug candidates.

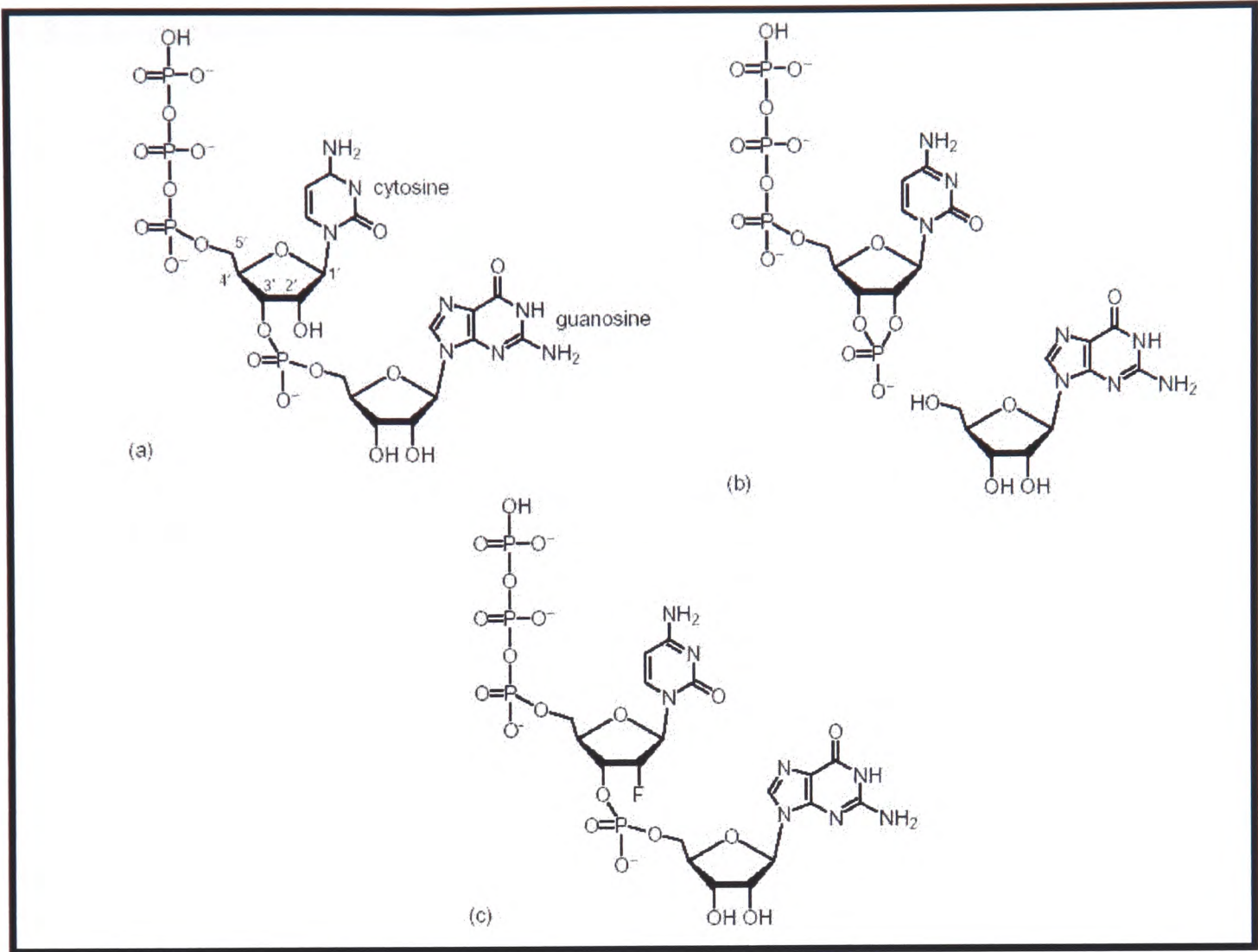


Figure 1.13: Representative RNA bases (a) A cytosine-guanosine dinucleotide, showing the numbering convention for carbons in the ribose moiety. (b) Phosphodiester bond hydrolysis at the reactive 2'-position of natural RNA, resulting in the 2', 3'-cyclic phosphate. (c) A 2'-fluoro modified cytosine that is resistant to endonuclease hydrolysis, yet compatible with *in vitro* transcription by T7 RNA polymerase.

1.8.2 Advantages of aptamers as drug candidates against HIV-1

The SELEX experiments have not only increased our understanding of nucleic acid biochemistry, but have also identified aptamers that can potentially act as useful therapeutic candidates. A number of RNA aptamers have been isolated that can bind to proteins encoded by HIV-1. For example, the HIV-1 Rev protein facilitates the transport of transcripts for the late genes of the virus. A subregion of the viral transcript called the Rev-binding element (RBE), which is the primary binding site for HIV-1 Rev, has been a target for several SELEX studies (Bartel et al., 1991; Giver et al., 1993a; Giver et al., 1993b; Tuerk and MacDougall-Waugh, 1993). The results of these studies have delineated what sequences contribute to the RBE and identified functional substructures. RNA aptamers for two other HIV-1 proteins, Tat and reverse transcriptase have been isolated (Tuerk and MacDougall-Waugh, 1993). These aptamers were also found specifically to inhibit the RNA-dependent DNA polymerase activity and the RNase H activity of their cognate enzymes.

The advantages of aptamers over alternative approaches include the relatively simple techniques and apparatus required for their isolation, their chemical simplicity, and the large number of combinatorial molecules (up to 10^{15}) that can be screened *en masse*. In addition, aptamers interact tightly with, and specifically disrupt the functions of their targets, at least *in vitro* (Conrad et al., 1994; Tuerk and MacDougall-Waugh, 1993). Some of the best aptamers form complexes that have dissociation constants in the 100pM range (Green et al., 1995; Jellinek et al., 1995; Jellinek et al., 1993), while many have dissociation constants that are similar to the antigen-binding fragment of antibodies (Gold et al., 1995). Aptamers range in size from approximately 6 to 40kDa. Their size and relatively large surface area is a decided advantage, in that they can form many more interactions with targets than can smaller molecules and can also discriminate between epitopes on related proteins. Paradoxically the high molecular mass of aptamers and the restricted range of suitable target sites (aptatopes) can be counter-productive. Nonetheless, the advantages emphasize the features of aptamers that warrant their consideration in real therapeutic circumstances.

1.8.3 Aptamers in real therapeutic circumstances and as potential AIDS prophylaxis

The therapeutic potential of aptamers hinges on many of the same issues that apply to more conventional pharmaceuticals. The main challenge to all these applications is the pharmacokinetics of aptamers *in vivo*. The pharmacokinetics of aptamers relate to the rate at which they are degraded by plasma nucleases; the rate at which they are sequestered by reticulo-endothelial cells and plasma proteins; the rate with which they are secreted by the kidneys; and the rate at which they redistribute from blood to the tissue or body fluid of interest (reviewed by James, 2000). While natural RNA molecules degrade rapidly in human sera or within cells, chemically modified RNA aptamers are resistant to nuclease hydrolysis. The stability in serum of the 2'-amino-modified anti-bFGF (basic fibroblast growth factor) aptamers was increased at least 1000-fold relative to unsubstituted RNA (Jellinek et al., 1995), while modified anti-VEGF (vascular endothelial growth factor) aptamer could survive for up to 17 hours in urine (Green et al., 1995). Solid-phase synthesis of a specific aptamer also allows post-SELEX modification such as addition of 2'-oxymethyl groups that are not compatible with *in vitro* transcription. The resulting aptamers are typically stable in human plasma for 15-24 hours at 37°C (White et al., 2000). Conjugation of aptamers to either lipids or polymers such as polyethylene glycol (PEG) improves their stability and distribution kinetics sufficient to produce therapeutic effects (Drolet et al., 2000; Floege et al., 1999; Tucker et al., 1999).

The development of stable aptamers with therapeutic effects now allows researchers to proceed to the more difficult problems of delivery and bioavailability. By virtue of their size, it is unlikely that aptamers will be orally available, and thus will have to be injected naked or administered parenterally packaged within liposomes (Osborne et al., 1997). Even if aptamers can be made bioavailable, their size and hence cost of production remains prohibitive. Fortunately, aptamers can form tight and specific interactions with their targets via domains of 30 to 40 nucleotides (Natalie Sayer and Kate Turner, unpublished data). While these 9 000 to 15 000 Dalton molecules are still orders of magnitude larger than a conventional drug (usually 200-800 Daltons), advances in solid state synthetic methods may soon allow their economic production.

1.9 Scope of thesis

The primary aim of this thesis was to isolate aptamers against gp120 of the R5 strain of human immunodeficiency virus type 1 with a view to:

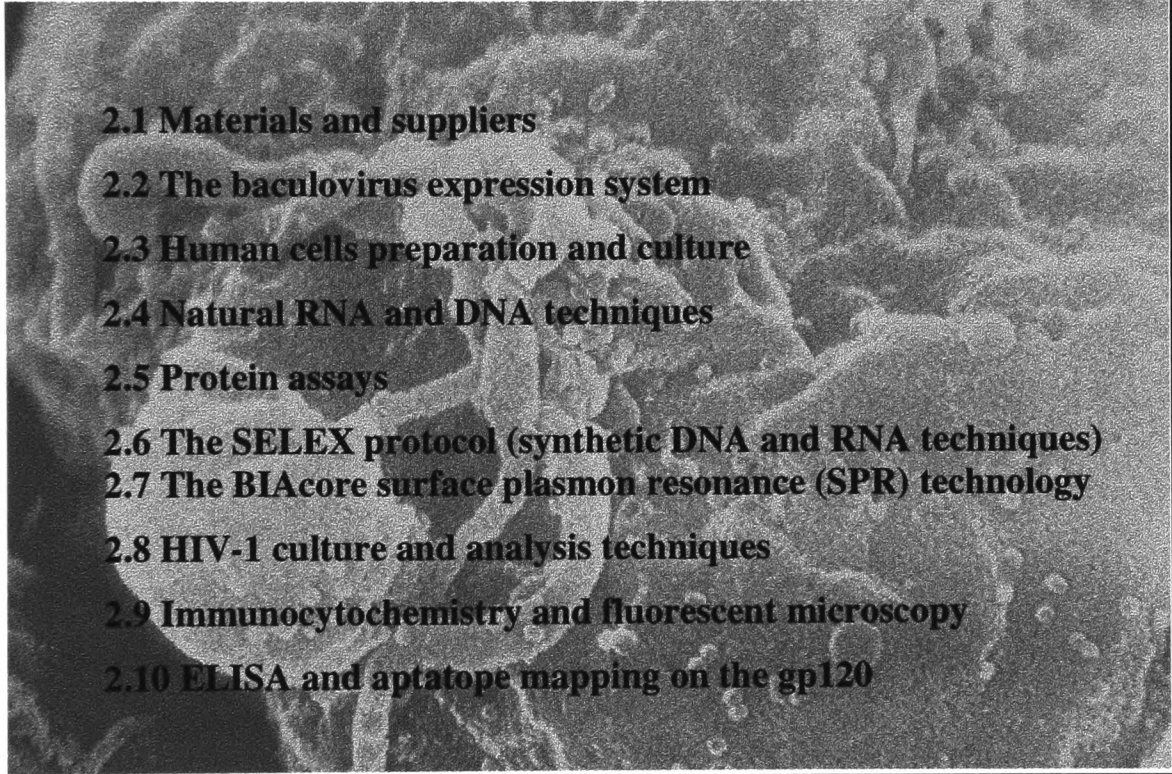
- (i). Understand better the molecular interactions between the R5 HIV-1 strains that are central in AIDS pathogenesis, and their natural host cells—the macrophages, and
- (ii). Produce and use anti-gp120 aptamers as novel diagnostic, analytical and potentially therapeutic tools that can be exploited by pharmaceutical industry and interested parties in the rational design and development of drugs useful in preventing or treating HIV-1 at the earliest stage of infection, namely entry.

Aptamers as generated by the SELEX technique were chosen because they epitomize and extend the undoubted power of combinatorial chemistry to generate diverse and synthetic ligands of biological macromolecules with potential therapeutic value. The R5 gp120 was used because it did not only present a novel target for aptamers, but it is also critical in HIV-1 entry and AIDS pathogenesis as discussed.

Chapter 2 outlines the general materials and methods used in this thesis, and experiments presented in Chapter 3 describe expression and purification of recombinant R5 gp120, a necessary prerequisite to isolate the aptamers. Chapter 3 also explores the effects of heparan sulfate on binding properties of the R5 gp120 to soluble human CD4. Chapter 4 focuses on optimization of the SELEX protocol using the BIAcore™ plasmon resonance technology (BIAselection), and the actual isolation and cloning of anti-gp120 aptamers. Chapter 5 presents aptamers' neutralization of R5 HIV-1 primary isolates using the PCR-based tissue culture infectious dose (TCID₅₀) assay. In chapter 6, the mechanism of neutralization by aptamers using binding studies and epitope-mapping in competition with anti-gp120 neutralizing mAbs is presented. The binding kinetics of selected neutralizing aptamers are also presented in chapter 6. Finally, chapter 7 summarizes the implications of this study in general, and relates them to the challenges ahead in winning the fight against HIV-1 and AIDS.

Chapter Two

General materials and methods

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- A scanning electron micrograph showing the surface of a cultured lymphocyte. The cell surface is covered with numerous small, spherical HIV-1 virions in various stages of budding. Some virions are clearly visible as distinct spheres, while others are integrated into the cell membrane, appearing as smaller protrusions. The overall texture of the cell surface is granular and irregular.
- 2.1 Materials and suppliers**
 - 2.2 The baculovirus expression system**
 - 2.3 Human cells preparation and culture**
 - 2.4 Natural RNA and DNA techniques**
 - 2.5 Protein assays**
 - 2.6 The SELEX protocol (synthetic DNA and RNA techniques)**
 - 2.7 The BIAcore surface plasmon resonance (SPR) technology**
 - 2.8 HIV-1 culture and analysis techniques**
 - 2.9 Immunocytochemistry and fluorescent microscopy**
 - 2.10 ELISA and aptatope mapping on the gp120**

Background: Scanning electron micrograph of HIV-1 budding from cultured lymphocyte (Source <http://www.med.sc.edu>)

2. General materials and methods

2.1. Materials

2.1.1. General Suppliers

- Aalto Bioreagents Plc. Dublin, Ireland
- Alpha Laboratories Ltd, Hampshire, UK
- Amersham Life Science International, Aylesbury, UK
- BDH Limited, Poole, UK
- Biogenesis Ltd, Poole, UK
- Bio-Whittaker, Poole, UK
- Boehringer Mannheim, Germany
- Calbiochem, Nottingham, UK
- DAKO, Cambridge, UK
- Difco laboratories, East Molesey, Surrey, UK
- Flowgen Instruments Ltd, Sittingbourne, UK
- Gibco BRL Life Technology, Paisely, Scotland, UK
- ICN Biomedicals Inc., Ohio, USA
- Invitrogen, Groningen, The Netherlands
- Jackson ImmunoResearch lab, West Groove, PA, USA
- NBS Biological, North Mymms, UK
- New England Biolabs, Beverly, MA, USA
- Pierce, Rockford, IL, USA
- Pharmacia LKB, Uppsala, Sweden
- Promega, Corporation, Madison, WI, USA
- Qiagen, West Sussex, UK
- R&D Systems, Abingdon, UK
- Roche Diagnostics GmbH, Mannheim, Germany
- Sigma Chemical Company, MO, USA
- TriLink BioTechnologies, Inc., San Diego, CA, USA
- USB Corporation, Cleveland, Ohio, USA
- Vector Laboratories Inc., CA, USA
- Wellcome Diagnostics, Dartford, UK

2.1.2. General chemicals and suppliers

• Agarose	Flowgen (LT) or Kramel Biotech (standard)
• Ammonium persulfate	Sigma
• AMV reverse transcriptase	USB
• Boric acid	BDH
• DNA molecular weight markers	Gibco BRL
• DNase I	Roche
• dNTPs (100 mM buffered aqueous)	Pharmacia
• DTT	BDH
• EDTA	BDH
• Ethidium bromide (solution)	Sigma
• Fluoro-uracil, and -cytosine.	TriLink BioTechnologies
• Glycine	BDH
• KCl	BDH
• MgCl ₂ (molecular biology grade)	Sigma
• Mineral oil (light)	Sigma
• NaCl	BDH
• NP-40	BDH
• Phenol/Chloroform	Sigma
• Protease inhibitor cocktail tablets	Boehringer
• Protein molecular weight marker	Amersham Life Science
• Proteinase K	BDH
• RNase-free DNase A	Sigma
• Sephadex G50	Pharmacia
• T7 RNA polymerase	New England Biolabs
• Taq DNA Polymerase	Promega
• TEMED	Sigma
• Tris base	ICN Biomedicals Inc
• Ultra Pure Water	Sigma
• Urea	BDH

2.1.3. General solutions and buffers

- 50 x TAE electrophoresis buffer: 40 mM Tris-acetate; 2 mM EDTA, pH 8.5 with acetic acid.
- 10 x Reverse transcriptase buffer: 500 mM Tris-HCl, pH 7.4, 600 mM NaCl, 60 mM magnesium acetate, 20 mM DTT
- 1 x PBS (Phosphate buffered saline): 137 mM NaCl; 2.7 mM KCl; 4.3 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$; 1.4 mM KH_2PO_4
- 1 x TE buffer 10 mM Tris-HCl, pH 7.4, 7.5 or 8.0; 1 mM EDTA
- 10 x TBE electrophoresis buffer: 890 mM Tris base; 890 mM boric acid; 20 mM EDTA, pH 8.0
- 10 x TBS: 1.44 M NaCl; 250 mM Tris-HCl, pH 7.5, 0.5% Tween-20
- Agarose DNA loading buffer: 0.25% (w/v) bromophenol blue; 0.25% (w/v) xylene cyanol; 25% (w/v) Ficoll in ddH₂O
- Blotting buffer 25 mM Tris; 90 mM Glycine 20% methanol
- Denaturing PAGE loading buffer: 95% (v/v) formamide; 0.25% (w/v) bromophenol blue; 0.25 % (w/v) xylene cyanol; 1 mM EDTA
- PAGE running buffer: 25mM Tris; 90 mM glycine; 0.1% SDS
- TCID₅₀ 10 x PCR master mix (20 x final concentration): 60 ng/μl each of U3₅₇₊ and U5₅₉₆⁻ primer pair, 4 mM dNTPs mix, 60 mM MgCl₂.
- Western blocking buffer: 0.1% (v/v) Tween-20; 5% (w/v) Marvel fat-free powder milk in PBS

2.1.4. Tissue culture reagents and media

BSA	Sigma
Ampicillin	Sigma
DMEM	Gibco-BRL
DMSO	Sigma
FCS ¹	Sigma
Ficoll-hypaque	Pharmacia
G418	Gibco BRL
Gelatin	Difco
Glucose	BDH
Glutamine	Sigma
Hygromycin	Sigma
IL-2 (purified)	Pharmacia
Lidocaine	Sigma
Lipofectin	Clontech
Luria-Bertani broth	SWDSOP
Neutral red	Sigma
PBS	Sigma
Penicillin/Streptomycin	Gibco BRL
Polybrene	SWDSOP
Puromycin	Sigma
RPMI-1640	Gibco BRL
SF 900 II serum-free insect medium	Gibco BRL
TC-100	Gibco BRL
X-VIVO 10	Bio-Whittaker

¹ FCS was heat inactivated at 56 °C for 30 min and filtered through a 0.22µm filter before use.

2.1.5. Monoclonal and other antibodies

Primary and other antibodies used in this study are listed in Table 2.1. The 17b and 48d mAbs to HIV-1 gp120 were obtained through the NIH AIDS Research and Reference Reagent Program (RRRP), Division of AIDS, NIAID, NIH, contributed by Dr James E. Robinson (Thali et al., 1993). The 2G12 mAb was contributed by Dr Hermann Katinger (Trkola et al., 1996), also through the NIH AIDS Research and Reference Reagent Program. Monoclonal antibody to HIV-1 gp120 (IgG1 b12) was from Dr Dennis Burton and Carlos Barbas (Burton et al., 1991; Burton et al., 1994; Roben et al., 1994), through the NIH AIDS Research and Reference Reagent Program. All other monoclonal antibodies to HIV-1 gp120 were from Quentin Sattentau who obtained them through the MRC AIDS Program (Table 2.1).

Table 2.1: Monoclonal and other antibodies used in this study

Name	Antigen	Species	Source or reference
α -human IgG biotin	Human IgG	Goat	Vector Laboratories
α -mouse IgG biotin	Mouse IgG	Goat	Vector Laboratories
17b,19b & 48d mAbs	HIV-1 gp120	Human	J. E. Robinson through the NIH AIDS RRRP
2G12 mAb	HIV-1 gp120	Human	H. Katinger (NIH AIDS RRRP)
Anti-FLAG M2 mAb	FLAG epitope	Mouse	Sigma
Anti-mouse IgG HRP mAb	Mouse IgG	Goat	Sigma
CG10	HIV-1 gp120	Mouse	Q. Sattentau from MRC AIDS
D7324	HIV gp120 COOH	Mouset	Aalto Bioreagents Plc.
HIV IgG & CD4IgG;	HIV gp120	Human	MRC AIDS (Q. Sattentau)
IgG1 b12 mAb	HIV-1 gp120	Human	D. Burton and C. Barbas (NIH AIDS RRRP)
Q4120	CD4	Human	Q. Sattentau through the MRC AIDS

2.2. Baculovirus expression system

2.2.1. Insect cells culture and maintenance

Insect cells were used for baculovirus expression of gp120 (Chapter 3). The key to obtaining good virus growth and expression when working with recombinant baculovirus is good insect culture. Two *Spodoptera frugiperda* (Sf) ovarian insect cell lines are widely used for baculovirus propagation, Sf9s and Sf21s. Laboratories vary in their belief that one line is better than another, but all the baculovirus expression done in this study has used Sf9s cells provided by Ian Jones (IVEM, Oxford).

Sf9s cells were cultured at 28°C, in SF 900 II serum-free insect medium (GibcoBRL) either in suspension culture in a simple stirred vessel (up to 1 liter culture in 3 liter flask) or as adherent cells and were switched between the two without any necessary adaptation. The culture density was kept below 1×10^6 cell/ml by regular passage to maintain the cells at a growing state. Cells doubled every 24 hours at 28°C in SF900 II medium and once the culture has reached saturation the cells lost viability. Sf9s cells were frozen when at exponential growth and at a high density (4×10^6 cells/ml) in 10% DMSO, 1% BSA in SF 900 II medium (filter sterilized) and stored over liquid nitrogen in jumbo tanks (SWDSOP).

2.2.2. Transfection of Sf9s cells

Sf9s cell were transfected with a mixture of transfer vector encoding HIV-1 gp120 and linearized baculovirus vector to generate recombinant virus following a protocol reviewed in the baculovirus expression system manual (King and Possee, 1992). Briefly, Sf9s cells were seeded in a 35 mm² dish at about 50% confluence (1×10^6 cells) and incubated at 28°C for 45 min. 500 ng of purified (Wizard DNA purification kit) transfer vector was mixed with linearized viral DNA in a total volume of 16 µl sterile water. 8 µl of 1mg/ml lipofectin stock (Clontech) was added to the mixture and allowed to stand at room temperature for 15 min. Sf9s cell monolayer was washed

once with 1 ml TC-100 medium (GibcoBRL) and overlaid with 1 ml TC-100. The lipofectin/DNA mixture was added to the TC-100 overlay dropwise, and swirled over the cells during addition. The cells were incubated at 28°C overnight, and 1 ml of TC-100 supplemented with 10% FCS was added, and the cells were incubated for a further two to three days. The transfection supernatant was harvested and stored at 4°C whilst the plaque assay was done (Chapter 3).

2.3. Human cells preparation and culture

2.3.1 Propagation and maintenance of GHOST cell-line transfectants

GHOST cells are a cell line that is derived from HOS (human osteo-sarcoma) cells and stably transduced with MV7neo-T4 retroviral vector and stably cotransfected with the HIV-2 LTR driving hGFP (humanized green fluorescent protein) construct. The GHOST (3) parental cell line engineered to express only the CD4, and GHOST (3) Hi-5 cells that express the CD4 and sorted for high CCR5 expression were obtained from Dr. Vineet KewalRamani and Dr Dan Littman (Morner et al., 1999) through the NIH AIDS Research and Reference Reagent Program. The cells were propagated and maintained in DMEM (Dulbecco's Modified Eagle's Medium), supplemented with heat inactivated 10% FCS (Sigma); 50IU/ml penicillin; 50µg/ml Streptomycin; 500µg/ml G418 and 100µg/ml hygromycin. For CCR5-encoding GHOST (3) Hi-5 cells 1µg/ml puromycin was also added to the culture medium. The cells were cultured at 37°C in a humidified incubator with 5% CO₂, and early passages (no more than 10–15 passages) were frozen because the phenotype of these cells changed over passaging.

For freezing and subculture, the monolayers were washed twice with cold PBS and harvested by treatment with 0.125% trypsin, 0.5 mM EDTA in PBS for 5 minutes at 37°C. Trypsinization was stopped by adding 5% FCS in DMEM and any cell clumps were disaggregated by pipetting gently several times. The cells were re-seeded at the required density in the supplemented culture medium, or frozen in 90% FCS, 10%

DMSO at a density of 1×10^7 cells/ml. For freezing, the cells were aliquoted into CryoTubes (Nunc Inc., IL, USA), and frozen gradually (-1°C per min until -70°C) and stored over liquid nitrogen (Cell Bank, SWDSOP). For retrieval from liquid nitrogen, the cells were thawed quickly in a water bath at 37°C , and transferred to 25ml prewarmed 5% FCS in DMEM in a sterile 50 ml Falcon tube and pelleted at $1000 \times g$ for 10 minutes. The cell pellet was resuspended in the propagation medium and seeded as required in a tissue culture flask.

2.3.2 Preparation and isolation of human PBMC

Human PBMC were isolated from heparinized buffy coats of normal, HIV-negative donors using density gradient centrifugation following a protocol outlined below. Buffy coats are the residual material after plasma, platelets, and red cell separation for patient use, and were supplied by the Bristol Hospital Services, through the Oxford National Blood Services. The general protocol used in this thesis included the following steps:

1. Endotoxin-free, tissue culture grade PBS (Sigma) was chilled on ice and Ficoll-Hypaque (Pharmacia) was used at room temperature.
2. 33ml of freshly-ordered buffy coat was slowly layered over 15ml of Ficoll-Hypaque in a 50ml Falcon tube and spun for 20 minutes at room temperature at $900 \times g$ (2 000 rpm), without applying brakes on the centrifuge so as not to disturb the PBMC interface layer.
3. After centrifugation, the plasma layer was removed by gentle aspiration and kept for autologous serum preparation. The autologous plasma-derived serum was prepared by heat inactivation at 56°C for 30 minutes, followed by cooling and spinning at 4 000 rpm at 4°C for 20 minutes. The supernatant was aliquoted and before use filtered through $0.45\mu\text{m}$ acrodysic syringe filters.

4. The PBMC sticking to the side of the falcon tube were dislodged, and the white cell interface was transferred in as small volume as possible (maximum 5ml) in a fresh 50ml Falcon tube.
5. The cells were resuspended in 5ml ice-cold PBS, followed by making up the volume to 50ml with the PBS. As an initial wash to remove any residual Ficoll, tubes were centrifuged at 1 500 rpm for 7 minutes, at 4°C, with half-brake. The supernatant was aspirated and the cell pellet was resuspended in cold PBS as previously described.
6. After the same washing protocol as in step 5, platelets were removed by 4–6 additional washes (1 000 rpm for 5 minutes at 4°C, full brake)
7. A cell pellet was resuspended in 5ml RPMI-1640 supplemented with 5% autologous serum (AS), and viable PBMCs counted by haemocytometer using 0.2% (v/v) trypan-blue (Sigma) exclusion stain. These steps produced PBMC that were free of platelets, from which relatively pure populations of monocytes could be isolated.

2.3.3. Isolation of monocyte-derived macrophages (MDM)

Adherence is commonly used in monocyte/macrophage isolation and was the manner in which MDM was isolated in this thesis. The predominant substrate used for isolation was gelatin-coated plastic following protocols developed by (Collin, 1992). The main advantage of this substrate is transient adherence of monocytes, which allows for initial isolation and re-plating upon detachment. Other surfaces such as tissue-culture and polystyrene (bacteriologic plastic) were used for initial adherence if no re-plating was required or for post-gelatin re-plating. The gelatin-coated plastic dishes were prepared by boiling 2% gelatin solution in deionized water, followed by filtering the solution under sterile condition. The filtered gelatin was dispensed as a thin coating on to plastic surfaces and left to air-dry horizontally at 37° C overnight.

Monocytes were isolated from prepared PBMC (2.3.2) by adherence either to gelatin-coated dishes, tissue culture plastic, or bacteriologic plastic at 37°C, 5% CO₂ for 90

minutes. Non-adherent lymphocytes were removed by gentle washing with warm RPMI-1640 until there were no free-floating cells upon examination with an inverted microscope. After the final wash, 5% autologous serum-RPMI-1640 was added and adherent monocytes incubated for a further 16 hours before re-plating.

Monocytes were harvested by placing gelatin-coated dishes at 4°C for 30 minutes to allow spontaneous detachment, followed by use of cell scraper to detach all remaining cells which were rounded in morphology and easily detachable. Monocytes adhered to bacteriological plastic were detached by incubation with 10 mM EDTA in PBS for 15 minutes at 37°C, followed by use of cell scraper. Once cells were scraped and detached, three PBS washes were done to maximise recovery. Harvested monocytes were spun at 1 000 rpm for 5 min. at 4°C and resuspended in X-VIVO-10 (Bio-Whittaker), supplemented with 2% plasma-derived autologous serum, and re-seeded into desired culture surface at cell densities shown in Table 2.2.

Table 2.2: Recommended plating density for human MDM*

Culture flask or plate	Total number of cells
175 cm ² flask	4-5 x 10 ⁷
75 cm ² flask	1-2 x 10 ⁷
25 cm ² flask	5 x 10 ⁶
6-well plate (706mm ²)	1.5 x 10 ⁶ /well
12-well plate (343mm ²)	8 x 10 ⁵ /well
24 well plate(177mm ²)	5 x 10 ⁵ /well
48-well plate (88mm ²)	2.5 x 10 ⁵ /well
96-well plate (28mm ²)	1 x 10 ⁵ /well

*Used 10 × more if plating for monocytes cultures starting from total PBMC

2.3.4. Culturing and maintenance of PBMC and MDM

There was considerable variation in both PBMC and MDM isolated from different donors in their viability and rate of differentiation when cultured in RPMI-1640. To improve uniformity of cultures, PBMC and MDM were cultured and maintained in the X-VIVO-10 medium supplemented with 2% plasma-derived autologous serum. X-VIVO-10 is a conditioned medium for optimal culture of PBMC and promoted MΦ differentiation and enhanced viability, especially when supplemented with autologous serum. Monocytes co-cultured with PBL as PBMC, or alone as pure monocyte cultures, were maintained in the 2%-AS-X-VIVO-10 medium for 5–7 days until they differentiated into macrophages and routinely assessed for purity using flow cytometry analysis (FACS).

2.3.5 Assessment of MDM purity by FACS®

FACS® analysis was used to assess MDM purity. The mouse-anti-human mAbs to the following cell surface markers supplied by DAKO, Denmark were used: T cell CD3; monocyte CD14; MΦ CD68, clone PG-M1; and MΦ MHCII clone CR3/43. A negative control without the primary antibody was also included, and FITC conjugated F(ab) goat-α-mouse secondary antibody was used. Day 7–10, viable monocyte-derived macrophages were detached using 4mg/ml lidocaine in 10 mM EDTA, and staining for FACS® analysis was performed as described in the FACS® staining protocol below.

2.3.6 FACS® protocol

The general single color standard FACS® (indirect detection) protocol was as follows:

1. Cells were washed twice with PBS and lifted by incubation with lidocaine (4mg/ml in 10 mM EDTA/PBS) at 37°C for 10–20 minutes.
2. Cells were washed in PBS and fixed with 4% paraformaldehyde in PBS for 20 min. at room temperature.
3. Cells were then washed twice with PBA (0.2% Triton X-100 in PBS supplemented with 0.1% v/v BSA) and incubated in PBA supplemented with 10% AS, 10% normal goat serum for 30 min. at room temperature to avoid Fc-dependent binding (Teppler et al., 1990).
4. Cells were centrifuged at $300 \times g$ for 10 min. and resuspended in PBA supplemented as before, followed by adding mouse primary mAbs at the recommended concentrations.
5. Cells were incubated for 45 min. at room temperature and washed twice with PBA.
6. Goat-anti-mouse F(ab')₂ specific FITC labeled IgG + IgM (H+L) secondary mAb (Jackson ImmunoResearch Laboratories, USA) was added at the recommended dilution in PBA.
7. Cells were incubated for 45 min at room temperature.
8. Cells were washed once with PBS, resuspended in 500µl PBA and analysed using a FACScan (Beckton Dickinson, CA, USA).

2.4. Natural RNA and DNA techniques

Manipulation of RNA and DNA followed general molecular biology procedures (Sambrook et al., 1989).

2.4.1 Methods for DNA purification and analysis *in vitro*

2.4.1.1 Agarose gel electrophoresis

DNA fragments were resolved by agarose gel electrophoresis. Agarose (Kramel Biotech) gels were prepared in 1 × TAE containing 1µg/ml ethidium bromide. The concentration of agarose was selected according to the size of DNA fragments to be resolved. A concentration of 0.7% (w/v) agarose was used for separation of DNA fragments between 800 bp and 12 kb, 1% for 500 bp to 10 kb, 1.5% for 200 bp to 3 kb, and 2% for 50 bp to 2 kb. DNA samples were mixed with 1/5 volume of 5 × DNA loading buffer, and electrophoresed at 120V in 1 × TAE buffer containing 0.5µg/ml ethidium bromide. One µg of 100 bp or 1 kb DNA ladder (GibcoBRL) was used as a reference for the size of DNA fragments in the sample. The DNA fragments were visualized with a UV transilluminator and photographed using a gel-documentation imager (Pharmacia).

2.4.1.2 Denaturing polyacrylamide gel electrophoresis

Denaturing (8 M urea) 12% polyacrylamide gel for analyses of DNA and RNA was prepared as follows:

Urea (BDH)	24.024 g
40% (w/v) acrylogel solution (BDH)	15 ml
10 x TBE	5 ml

The mixture was made up to 50 ml with dH₂O and stirred until dissolved, and polymerized by addition of 250µl 10% (w/v) APS (Sigma) and 50µl TEMED (Sigma) shortly before pouring into casting plates. Once polymerized the gel was pre-run for 20 minutes at 40W. The samples were mixed with the denaturing PAGE loading

buffer (2.1.3) and loaded onto the gel and run for 1 hour at 40 W, followed by ethidium bromide staining for visualization.

2.4.1.3 Isolation and purification of DNA/RNA fragments from agarose gels or denaturing PAGE

Resolved DNA fragment of interest was excised and purified from agarose gel using the QIAEX II gel extraction kit (Qiagen) following the supplier instructions. Salt and residual agarose was removed by washing beads in QX1 and PE buffers, respectively before air-drying. DNA was eluted from the beads in distilled water (dH₂O).

To purify DNA or RNA from denaturing PAGE, a resolved band of interest was also excised from the gel and the slice spun at 13 000 rpm for 5 min in an Eppendorf tube. The gel slice was snap-frozen in liquid nitrogen and crushed to powder. 400µl of the elution buffer (300 mM sodium acetate, 2 mM EDTA, pH 7.4) and an equal volume of phenol/chloroform was added to the crushed gel and extracted overnight rotating at room temperature. The sample was spun at 13 000 rpm for 5 min. and the extraction repeated three times, adding fresh elution buffer each time. The nucleic acid was precipitated as described in 2.4.1.5.

2.4.1.4 Phenol/chloroform extraction of DNA/RNA

Proteins were removed from DNA/RNA solutions by addition of an equal volume of Tris-buffered (pH 8) phenol/chloroform/isoamyl alcohol (v/v 25:24:1) followed by vigorous mixing using a vortex and centrifugation at 13 000 rpm for 5 min. The aqueous (upper) phase containing DNA/RNA was transferred to a clean Eppendorf tube, and the extraction was repeated. The aqueous phase was extracted with equal volume of chloroform and the DNA/RNA was precipitated with ethanol.

2.4.1.5 Ethanol precipitation of DNA and RNA

Both DNA and RNA were precipitated by addition of 0.1 vol. of 3 M sodium acetate (pH 5.2), and 2.5 vol. absolute ethanol cooled at -70°C or in the dry-ice/ethanol bath. In the case of RNA 0.5 μl of glycogen at 20 $\mu\text{g}/\mu\text{l}$ stock concentration was added to aid precipitation. After 15 min. in the dry ice/ethanol bath, or at -70°C , DNA or RNA was pelleted by centrifugation at 17 000 rpm (4°C) for 30 min., washed once with 3 vol. of 70% ethanol to remove residual salt, air-dried and resuspended in dH_2O .

2.4.1.6 Quantification of DNA and RNA

The concentration of the DNA and RNA were determined by the GeneQuant spectrophotometer (Pharmacia) measuring UV absorbance at 260nm (1 $\text{OD}_{260\text{nm}}$ = 50 $\mu\text{g}/\text{ml}$ for dsDNA or 40 $\mu\text{g}/\text{ml}$ RNA). The purity of the nucleic acids were automatically determined by the $\text{OD}_{260/280}$ ratio. Pure DNA and RNA have a value of about 1.5 and 2.0, respectively.

2.4.1.7 Restriction enzyme digestion of DNA

Restriction enzyme digestions were performed with at least 2 units of enzyme per μg of DNA for 2 hours, using the reaction temperature and buffer conditions recommended by the supplier (New England Biolab, Promega or Roche).

2.4.1.8 Dephosphorylation of DNA

The plasmid vector that had been linearized by restriction enzyme digestion was treated with bacterial alkaline phosphatase (Amersham) to remove the 5'-phosphate group and reduce self-ligation of the vector during ligation of DNA inserts. Dephosphorylation was carried out in 50 mM Tris, pH 8.5 and 0.1 mM EDTA with 0.05 units of bacterial alkaline phosphatase/pmole ends of the plasmid at 37°C for 15 min. followed by incubation at 70°C for 15 min. to inactivate the enzyme.

2.4.1.9 Ligation and TA cloning of DNA inserts into plasmid

For directional cloning, the molar ratio of insert: phosphorylated vector of 3:1 was used. The ligation was performed in a reaction volume of 10 µl containing insert and plasmid vector DNA, 1 × ligation buffer (66 mM Tris, 5 mM MgCl₂, 1 mM ATP, 1 mM dithiothreitol (DTT), pH 5 and 1 unit T4 DNA ligase (Roche) and the reaction mixture was incubated at 16°C overnight.

The eukaryote TA cloning[®] bidirectional kit (Invitrogen) was used to directly ligate a freshly *Taq* polymerase-amplified PCR product into the pCR[®] 3.1 vector (Figure 2.1) following the supplier's instruction manual. *Taq* polymerase has a nontemplate-dependent activity that adds a single deoxyadenosine (A) to the 3' ends of duplex molecule. The linearized vector supplied in the kit has single 3' deoxythymidine (T) residues that allows PCR inserts to ligate efficiently with the vector.

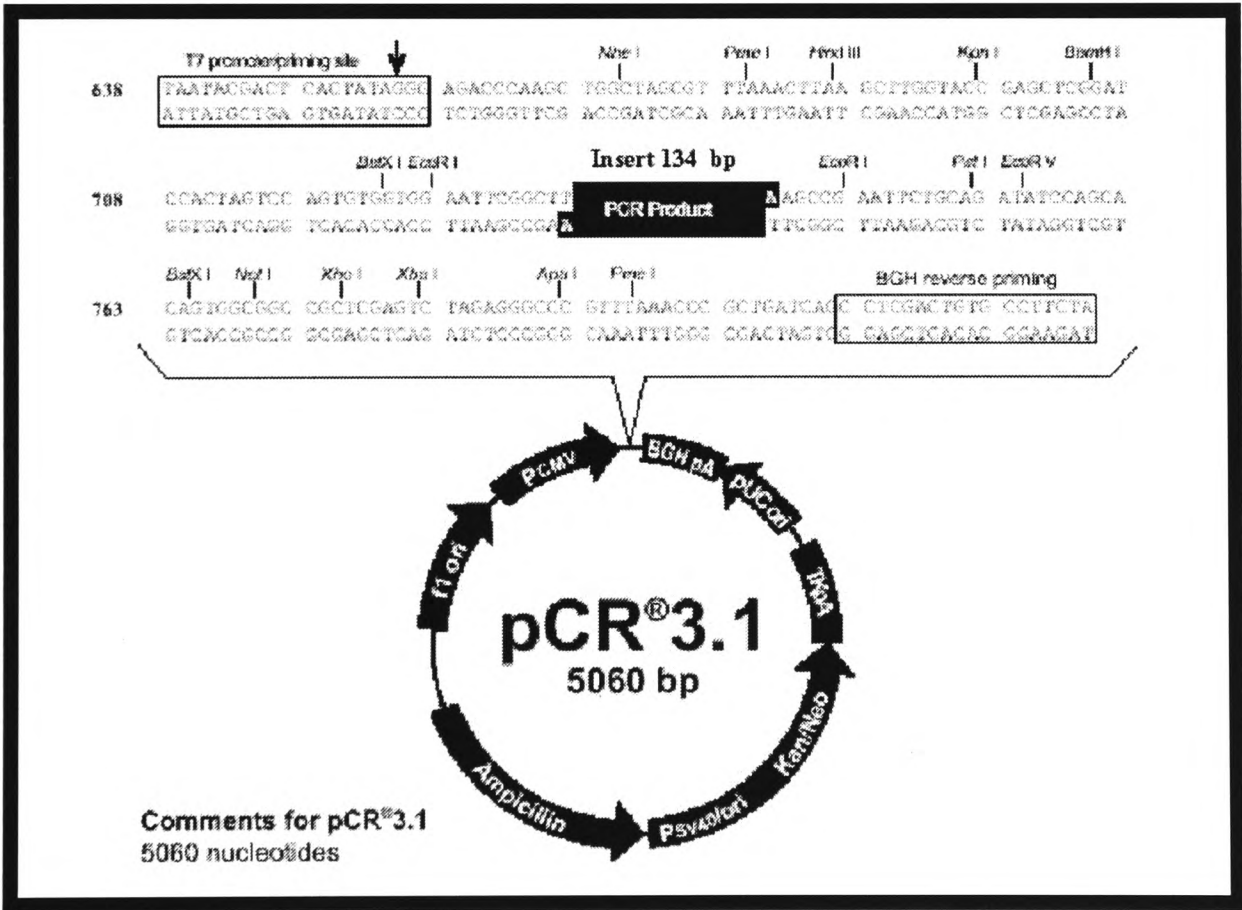


Figure 2.1 The TA cloning pCR[®] 3.1 vector map. Restriction sites are labeled and the TA Cloning[®] site is shown with the 134 pb PCR product insert. (Source: www.invitrogen.com)

2.4.2. Plasmid selection, amplification and transformation of bacterial cells

2.4.2.1 Preparation of competent cells

The DH5 α *E. coli* cells were made competent as described by (Groth et al., 1996). The DH5 α cells were streaked from a glycerol stock onto LB-agar plates and grown overnight at 37°C. A single colony was isolated from the overnight plate and inoculated into 5ml LB broth and grown shaking (225 rpm) for a further 16 hours at 37°C. 500 μ l of the overnight culture was diluted into 50ml LB (1:100 dilution) and grown at 37°C until the OD_{600nm} was 0.2. This ensured that the cells were at the logarithmic phase and that the number of viable cells did not exceed 10⁸ cells/ml to achieve optimal transfection efficiency. The cells were sedimented at 2 500 $\times$ g for 10 minutes at 4°C, then gently re-suspended in 5ml ice-cold transformation buffer (10% w/v PEG1500/30mM MgCl₂/LB + 5% DMSO). The cells were then stored on ice for 20 minutes to achieve competency. 50 μ l aliquots were added into pre-chilled Eppendorf tubes and stored at -70°C.

2.4.2.2 Transformation of bacterial competent cells

DH5 α competent cells (2.4.2.1) or One Shot[®] TOP10F' (Invitrogen) chemically competent cells were thawed on ice, and 2 μ l of the ligation reaction was directly added into 50 μ l competent cells and mixed gently by stirring with the pipette tip. The transformation mix was incubated on ice for 30 min., followed by heat shock for exactly 30 seconds in the 42°C water bath and immediately placed back on ice. 250 μ l of SOC medium (Invitrogen) equilibrated at room temperature was added to the transformation mix, and the vial was shaken (225 rpm) at 37°C for 1 hour. Different volumes (10-50 μ l) of transformed bacteria were plated on LB agar plates containing 50 μ g/ml ampicillin and incubated for 16 hours at 37°C.

2.4.2.3 Small scale plasmid DNA preparation (mini-prep)

This protocol was used to prepare up to 20 µg of good quality plasmid DNA for general manipulations such as restriction enzyme digestion, sub-cloning and sequencing. Plasmids were amplified by growing an isolated transformed bacterial colony in 5 ml LB broth containing 100 µg/ml ampicillin in a shaking incubator (37°C) for 16 hours. Plasmid purification QIAprep Spin Miniprep Kit (Qiagen) was used following the supplier's instruction. Occasionally when the kit was not used, plasmid DNA was prepared by an alkaline lysis method originally described by (Birnboim and Doly, 1979) and later modified by (Zhou et al., 1990). Briefly, bacteria from the overnight culture (1.5 ml) was harvested by centrifugation (13 000 rpm, for 30 seconds) at 4°C. The cell pellet was resuspended in 100 µl of ice cold solution I (100 µg/ml RNase A; 50 mM glucose; 25 mM Tris; 10 mM EDTA, pH 8.0). After leaving the cells in solution I at room temperature for about 30 seconds, 200 µl of solution II (0.2 M NaOH; 1% SDS) was added into the cells and placed on ice for a minute. Within 3-5 minutes of adding solution II, 150 µl of solution III (KCH₃COOH) was added to lower the pH and facilitate reannealing of the plasmid. The mini-prep mix was micro-centrifuged for 5 min. at 4°C. The supernatant was transferred into fresh micro-tube, and extracted with phenol/chloroform, followed by ethanol precipitation. The DNA pellet was resuspended in dH₂O or TE buffer, and stored at -20°C.

2.4.3 The polymerase chain reaction (PCR)

The PCR (Mullis, 1990; Mullis and Faloona, 1987; Saiki et al., 1985) was generally used to amplify specific DNA sequences for transcription in the SELEX protocol, cloning, screening of positive clones in DNA transformation, or score cultures for HIV-1 infectivity. Oligonucleotide primer sequences were designed using fixed regions of the SELEX template, or highly conserved regions of HIV-1 nucleotide sequences published in (Kuiken et al., 1999). Primers specific for cloning vectors were also used, and all the primers were designated the nomenclature that described the position of the genome region to be amplified. Customized oligonucleotide

primers were synthesized and ordered from Gibco BRL Life Technologies and were shipped in a lyophilized form. Sequences of all the primers used in this thesis are listed in Table 2.3. PCR mixtures were routinely performed using autoclaved microtubes, barrier pipette tips, and dH₂O. A typical 50 µl PCR mixture contained 10 to 100ng template; 1× Mg free PCR buffer (Promega); 1-3 mM MgCl₂; 0.25 mM dNTPs; oligonucleotide primers (50 pmol each); and 2.5 units of *Taq* polymerase (Promega). A gradient thermal cycler (PTC-200, MJ Research) that enabled the simultaneous use of eight annealing temperatures and 96-well microtiter plate was routinely used. Cycling conditions varied according to experiment but the following general guidelines were observed. Template denaturing was performed at 93°C for 30–60 seconds, annealing temperature was as determined from the primer pair T_m (usually 55°C–72°C, for 30 seconds), a 72°C elongation period corresponding to 1 minute per kb of expected product, and a final extension at 72°C for 8 minutes. Plaque PCR was done by picking the plaque and dispensing it in 10 µl of 10 mM EDTA, pH 8.0. The tube was heated at 65°C for 10 min and 1µl of the lysate was used as a template for the PCR.

Table 2.3: Sequences and approximate T_m of oligonucleotide primers*

Name (Primer pair)	Sequence (5-3')	T _m (°C)	Product size (bp)
T3SELEX5' T7SELEX3'	AATTAACCCTCACTAAAGGGAACTGTTGTGAGTCTCATGTCGAA TAATACGACTCACTATAGGGAGACAAGACTAGACGCTCAA	61 60	134
Fp2Bac Rp2Bac	CCGGATTATTCATACCGTCCCACC GGGTCCAAGTTTCCCTGTAGAACTC	54 54	1600
Env ₆₃₀₉ ⁺ Env ₈₀₂₃ ⁻	AGCAGAAGACAGTGGC TAGTGCTTCCTGCTGCTCC	56 57	1714
U3 ₅₇ ⁺ U5 ₅₉₆ ⁻	CACACAAGGCTACTTCCCTGA GATCTCTAGTTACCAGAGTCAC	68 64	539
gbn ₁₆₁₄ ⁺ gbn ₁₈₅₁ ⁻	CCTTTGTTCCCTAAGTCCAA CCTCACCTTTCATGGAG	66 68	237

* Approximate T_m was calculated from the formula: T_m = 4 (G&C residues) + 2(A&T residues) °C.

2.4.4. DNA sequencing

DNA sequencing was performed by Alice Taylor (DNA sequencing service, SWDSOP) using the ABI PRISM™ BigDye™ Cycle Sequencing Ready Reaction kit (Perkin Elmer, Warrington, UK). Briefly, 600ng of plasmid template and 3.2 pmoles of primer in a total volume of 6 µl were mixed with 4 µl ready reaction mix to make up a total volume of 10 µl. The ready reaction mix contained AmpliTaq DNA Polymerase FS, the BigDye™ Terminators, dNTPs, MgCl₂ and the buffer. The sequencing reaction mix was denatured at 96°C for 30s, annealed at 50°C for 15s and elongated at 60°C for 4 minutes using a rapid thermal ramp for 25 cycles. The reaction product was precipitated, washed, dried, and resuspended in 5µl of deionised formamide/50 mM EDTA buffer, and analysed on an automated DNA sequencer (ABI 373A DNA sequencer Applied Biosystems). DNA sequence chromatogram was analysed using the program Seqed in GCG10 software package (Genetics Computer Group, Wisconsin-Madison, USA)

2.4.5. Total RNA isolation from human cells

RNase-free plastic-ware and reagents were used in the handling of RNA. Total RNA was isolated from human monocyte-derived macrophages using TRIZOL reagent (Gibco BRL) as described in (Chomczynski, 1993). TRIZOL reagent was added to the cell monolayer (1ml per 5×10^6 cells) and the cells were harvested using the scraper, and the lysate was transferred into a fresh Eppendorf tube. 200 µl of chloroform was added to the lysate, vortexed for 30s and left at room temperature for 5 min. The sample was centrifuged at 13 000 rpm for 15 min, at 4°C. The aqueous phase containing RNA was collected and re-extracted with 500 µl TRIZOL reagent and 200 µl chloroform, and precipitated with 500 µl isopropanol for 15 min at room temperature. The RNA was centrifuged at 13 000 rpm for 15 min at 4°C, and the pellet was washed with 1 ml 75% ice-cold ethanol. After centrifugation the pellet was

air-dried and dissolved in DEPC treated H₂O. The RNA was quantified by spectrophotometer (2.4.1.6) and analysed on a 1 % agarose gel.

2.5 Protein and peptide techniques

2.5.1 Immunoaffinity chromatography purification of gp120 and soluble human CD4

Expressed recombinant gp120 that was secreted into the medium was purified as soon as the supernatant was harvested. Anti-FLAG[®] M2 affinity gel (Sigma) packaged into HR16/5 empty column (Pharmacia) was used for immunoaffinity purification of the FLAG-fusion recombinant gp120. The packaged column was prepared and pre-equilibrated according to supplier's instructions, and mounted on automated HPLC machine (Pharmacia). Clarified supernatant was loaded at 0.5 ml/min through the column, followed by washing with five column volumes of TBS, pH 7.4. Bound FLAG fusion gp120 was eluted with 0.1 M glycine HCl, pH 3.5 into vials containing 25 µl of 1M Tris, pH 8.0. Thus 1 ml fractions were collected and peak of eluted protein was detected by the UV system of the HPLC machine. Peak fractions were also analysed by Western blot using the anti-FLAG[®] M2 mAb to confirm recovery of the gp120. Positive fractions were pooled and quantified (2.5.3) and purity of the protein was estimated by total gel Coomassie staining (2.5.4). Soluble human CD4 expressed in CHO cells (Neil Barclay, SWDSOP) was also purified using the RFT4-4BC (anti-sHuCD4) affinity chromatography following a similar protocol used for the gp120 purification.

2.5.2. Gel filtration

Immunoaffinity purified proteins which were to be used for BIAcore analyses (2.7) were further purified by FPLC (Pharmacia) using Superdex 200 HR10/30 column (Pharmacia) to exclude high order aggregates. The FPLC was operated following the instruction manual and gel filtration was performed using the automated bank method supplied with the machine.

2.5.3. Protein quantification

Proteins were quantified using bicinchoninic acid (BCA) protein assay kit (Pierce, Chester, UK) according to manufacturer's instructions, by comparison with a standard curve made from BSA standards. Samples were quantified in triplicates by reading the absorbance at 550 nm using an Anthos HTII (Dazdaq, East Sussex, UK) automated plate reader. Where accuracy was not crucial, protein concentrations were also estimated by measuring their OD at 280nm, and an extinction co-efficient of $5 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$ for an average protein (50kDa with normal aromatic amino content) was assumed if the true extinction co-efficient was unknown.

2.5.4. SDS-PAGE and Coomassie staining

Protein samples were analysed by SDS-polyacrylamide gel electrophoresis (PAGE) using Tris/glycine discontinuous buffer system ((Laemmli, 1970)) and a vertical electrophoresis unit (mini-PROTEAN II apparatus, Bio-RAD). Resolving gels were between 7.5 and 10% (v/v) acrylamide/bisacrylamide; 0.375 M Tris-HCL, pH 8.0; 0.1% (v/v) SDS; 0.05% (w/v) APS and 0.1% (v/v) TEMED. For non-reducing gels, samples were mixed with an equal volume of 2 × Laemmli loading buffer (0.5 M Tris-HCl, pH 6.8; 5% (w/v) SDS; 20% (v/v) glycerol and 0.005% (w/v) bromophenol blue) and for reducing gels 100 mM DTT was added to the Laemmli loading buffer. Samples were boiled for 5 min. before being loaded onto the gel, and were electrophoresed into the stacking gel (pH 6.8) at 100V and resolved by the separating gel at 30Vcm^{-1} in a PAGE running buffer (2.1.3). After electrophoresis, the gels were processed for Coomassie staining or Western blotting.

Polypeptide bands separated by SDS-PAGE were simultaneously fixed and stained with 40% (v/v) methanol; 10% (v/v) acetic acid, and 0.25% (w/v) Coomassie brilliant blue R-250 (BDH) for 1 hour at room temperature. The gel was destained in 20% methanol, 10% acetic acid solution over night and dried using the heat vacuum drier (Bio-RAD).

2.5.5. Western blotting

Resolved protein bands on SDS-polyacrylamide gel were electrophoretically transferred to Hybond C⁺ nitrocellulose membrane (Amersham) in the blotting buffer (2.1.3) for 1 hour at 250mA or overnight at 50mA following the modified protocol of (Towbin et al., 1979). After transfer, the membrane was rinsed once in TBS and incubated with blocking buffer (2.1.3) for 30 min at room temperature. After blocking for non-specific binding, the membrane was probed with appropriate primary mAb (Table 2.1) diluted in blocking buffer for 1 hour at room temperature. The membrane was washed 4 times, each 5 min, with TBS, and HRP-conjugated secondary mAb (Table 2.1) diluted in blocking buffer was added. After 1 hour, the membrane was washed as above and bound secondary mAb was developed with enhanced chemiluminescence (ECL, Amersham) HRP substrate for 1 min. The signal was detected by exposing the membrane to X-ray film which was developed (X-OMAT/LS, Kodak).

2.5.6. Peptide synthesis and purification

The FLAG peptide (ESGGDKDDDDKGGC) was synthesized by Maureen Pitkeathly (OCMS, Oxford University). The FLAG epitope (underlined) in the synthetic peptide was flanked by residues ESGG on the N-terminus that linked it to the gp120, and a cysteine residue on the C-terminus, which was used for thiol coupling onto the BIAcore chip (2.7.2). Briefly, the peptide was synthesized using Fmoc pioneer peptide synthesis system, where the functional group of the amino acid side chains were masked by attaching the Fmoc amino acid to insoluble support resin. After peptide synthesis, the resin bound peptide was deprotected and detached from the solid support via TFA (Trifluoro acetate) cleavage. The peptide quality was analysed using reverse phase HPLC, and lyophilized. TFA salt was removed by HCl exchange, i.e. the peptide pellet was resuspended in 500 µl of 50 mM HCl, and lyophilized. The process was repeated three times, with a final addition of 10 mM HCl. After the final lyophilization with 10 mM HCl, the pellet was resuspended in dH₂O.

2.6. The SELEX protocol (synthetic DNA and RNA techniques)

2.6.1. Template design

The SELEX library was designed by Abdou Tahiri-Alaoui (James lab, SWDSOP) following the methods of (Fitzwater and Polisky, 1996; Tuerk and Gold, 1990). The template DNA consisted of a variable sequence region flanked by defined 5' and 3' regions that allowed primer annealing sites for PCR, cDNA synthesis, and efficient T7 RNA polymerase transcription. The DNA library had a random region that consisted of 50 residues flanked by 44 and 40 residues on the 5' and 3', respectively, giving a full sequence of 134-mers. The 5'-fixed region was 5'-AAT TAA CCC TCA CTA AAG GGA ACT GTT GTG AGT CTC ATG TCG AA-3'. The 3' fixed region that contained the T7 promoter (underlined) was 5'-TAA TAC GAC TCA CTA TAG GG AGA CAA GAC TAG ACG CTC AA-3'.

2.6.2. Synthesis, amplification and purification of DNA library

The DNA library was produced by Genosys (Cambridge, U.K.), using automated solid-phase synthesis. During synthesis of the oligonucleotide template population, the random region was created by adding equimolar mixture of all deoxynucleoside phosphoramidites (A, C, G, and T) to effect library diversity as a function of 4^n , where n is the number of variable positions. The single-stranded synthesized DNA template was converted to double stranded DNA by PCR (2.4.3) using the T3SELEX5' and T7SELEX3' primers (Table 2.3). Pilot PCR (15pmol template) with varying numbers of cycles to produce dsDNA with few aberrant products was done to find conditions for scale-up of the reaction for PCR of 500pmol of input template. Six PCR cycles were the optimum number of cycles that produced the cleanest single band, and were used for large-scale PCR reaction that accommodated 500pmol input template. 1000pmol of PCR amplified dsDNA (about 88μg in total) was gel purified as previously described (2.4.1.3).

2.6.3. *In vitro* transcription

The dsDNA generated by PCR had a T7 RNA polymerase promoter at one end, and *in vitro* transcription with T7 RNA polymerase would result in the formation of a random pool of RNA molecules. Random sequence RNA with a diversity of at least 10^{14} was required for the first round of selection. To generate a representative RNA pool for the first round of the SELEX process, a 500 μ l reaction volume transcription was setup. From first principles, using Avogadro's constant and the molecular weight of dsDNA, 12 μ g (135 pmol) of the starting dsDNA library was sufficient to contain at least 10^{14} different molecules. However, for practical purposes, 20 μ g (225 pmol) was used. For a 500 μ l transcription, in addition to the DNA template (225 pmol), 1 mM (final concentration) each of the 2' fluoropyrimidine triphosphates (TriLink, USA) and unmodified purine nucleotides (Amersham), were used with an optimized fluoro-transcription buffer (40 mM Tris-Cl, pH 7.5, 6 mM $MgCl_2$, 5 mM DTT, 1 mM spermidine). 1000 units of T7 RNA polymerase (New England BioLabs) was added and the reaction mixture was incubated at 37°C, 2 hours later another 500 units of the enzyme was added and the mixture was incubated at 37°C overnight. Transcription was terminated by addition of 1 unit RNase-free Dnase I (sigma) per μ g of DNA template used, and the reaction was incubated for 15-30 min. at 37°C, followed by phenol/chloroform extraction. The 500 μ l transcript was concentrated down to 100 μ l using Centricon 10 (Amicon). The concentrated transcript was desalted and exchanged into dH_2O using a Sephadex-G50 nick spin column (Pharmacia) and quantified (2.4.1.6). The transcript was stored at -20°C to ensure that the RNA would fold into the most stable structures, until needed for the selection process (Chapter 4).

2.7. The BIAcore™ surface plasmon resonance technology

The BIAcore surface plasmon resonance (SPR) technology is a powerful tool for the real-time analysis of the interaction between biomolecules. This is particularly useful for low affinity interactions, which are difficult to study using conventional techniques (van der Merwe and Barclay, 1996). The BIAcore technology is based on the refractive index detected as SPR during the interaction between a molecule immobilized on the biosensor chip (the ligand) and another molecule in solution called the analyte (Figure 2.2).

2.7.1. Preparation of buffers and samples

An essential part of a BIAcore experiment is based on properly prepared buffers and solutions to avoid air bubbles, and to get good interpretable data. The HBS (10 mM HEPES, pH 7.4, 150 mM NaCl) continuous flow buffer used in all the BIAcore experiments was filtered (0.2 μ m) and degassed under vacuum at room temperature. All the samples which were injected onto the sensor chip were spun at 13 000 rpm for 10 min. and were pulsed for 30 seconds just before use to dislodge air-bubbles and to keep the meniscus horizontal. The running buffer and the samples were also equilibrated in a water bath set at 37°C just before use for the BIAselection, which was performed at 37°C.

2.7.2. Immobilization of ligands (amine and thiol coupling)

Ligands can be immobilized onto the biosensor chip either directly by chemical methods or indirect, through capture by covalently coupled molecule, e.g. antibody. In this study ligands were directly coupled to the carboxymethylated dextran surface of the CM5 biosensor chip (research grade) using either amine or thiol coupling that respectively utilize lysine or cysteine functional groups on the ligands.

A standard amine coupling protocol was followed (Karlsson et al., 1991). The BIAcore 2000 biosensor instrument (Uppsala, Sweden) flow rate was set at 5 μ l/min and the carboxymethyl groups on the chip was activated by injecting 50 μ l of 0.5 M N-hydroxysuccinimide (NHS), and 0.2 M N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). The protein (ligand) was diluted or buffer-exchanged into 10 mM sodium acetate, pH 5 to a final concentration of 50–100 μ g/ml and 35–100 μ l was injected onto the activated surface, thereby driving the chemical coupling reaction. The remaining activated carboxymethyl groups were blocked by injecting 70 μ l of 1 M ethanolamine-HCl, pH 8.0, which also helped elute non-covalently bound ligand. Optionally, 50 μ l of 100 mM glycine-HCl, pH 2.5, was injected to wash away non-specifically bound material that remained after ethanolamine treatment.

The thiol coupling method was used to immobilize the FLAG peptide (2.5.6) to the biosensor chip because the peptide had been engineered to specifically contain the cysteine residue at its C-terminus for this purpose. The thiol method was also used because it freed the amine groups in the peptide, essential for the integrity of the FLAG epitope. The thiol coupling protocol is similar to the amine coupling one, except for the use of 2-(2-pyridinyl-dithio) ethaneamine hydrochloride (PDEA) and cysteine-NaCl. Like in the amine coupling, 10 μ l of EDC:NHS (200 mM:500 mM final concentration) solution was injected onto the chip to activate the carboxymethyl group. This was followed by injection of 20 μ l of 80 mM PDEA (made up in freshly prepared 0.1 M borate buffer, pH 8.5) to introduce reactive disulfide groups onto activated carboxyl groups of the sensor chip matrix. 35 μ l of the FLAG peptide diluted 1/5 (v/v) in the HBS continuous flow buffer at 9 mg/ml was injected to saturate the chip. 20 μ l of 50 mM l-cysteine, 1 M NaCl (freshly prepared in 0.1 M borate buffer, pH 4.3) was injected to deactivate any remaining reactive disulfide groups.

2.7.3. Affinity measurements

Affinity measurements were performed at 37°C because it is a physiological core temperature, and many binding parameters are temperature dependent. 5 000–7500 RU of active ligand (gp120) was covalently immobilized on the chip to increase analyte (RNA) binding response. The concentration of the analyte was accurately measured, and dilution series of the analyte were prepared (5 nM–3 200 nM). Each concentration of the analyte (starting with the lowest) was injected at 5 µl/min and allowed to dissociate over 3 600 seconds. In cases where the analyte did not dissociate completely within the stipulated time, the ligand was regenerated by injecting 1–5 µl of freshly prepared 100 mM NaOH to hydrolyse the RNA that was still bound (injection of 1–5 µl of 100 mM NaOH did not affect gp120 activity). Regeneration of the ligand, where necessary, was done to ensure that the level of active ligand was the same for each concentration of analyte injected. Data was analysed using the BIAevaluation 3.0 (BIAcore) and GraphPad Prism 3.00 (GraphPad software Inc., USA) software and the affinity constant was calculated from the k_{on} and k_{off} .

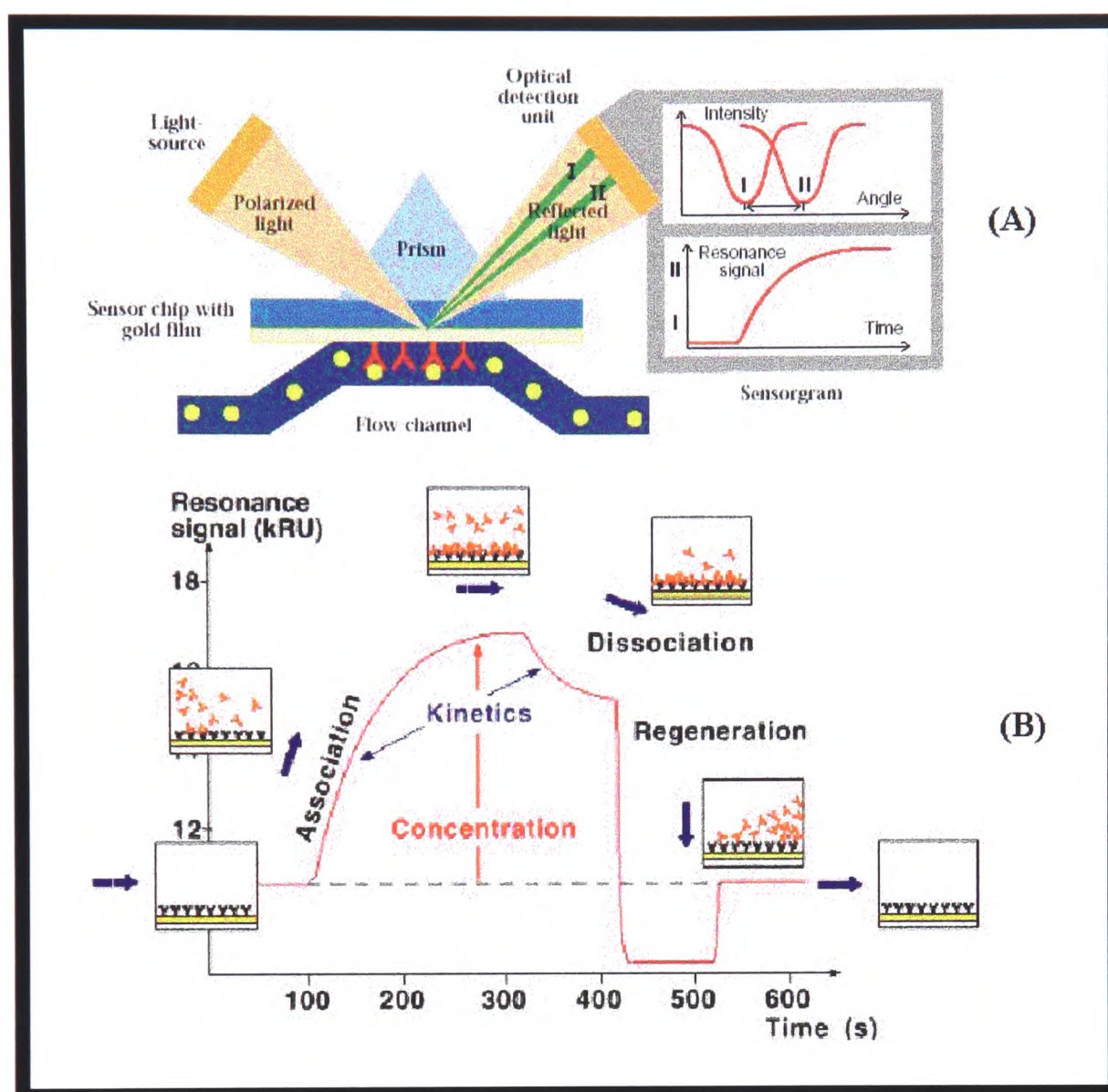


Figure 2.2: Principles of the BIAcore™ surface plasmon resonance technology. (A) The ligand is coupled to the surface of the biosensor chip and the analyte is passed over the chip into a flow cell. During binding, a change in refractive index is caused by varying the mass on the surface of the sensor chip due to interaction between the ligand and the analyte. A dip in the intensity of the reflected light occurs at a specific angle and is called the SPR signal. These changes in SPR signal are recorded continuously, and are expressed as response units (RU). The response units are plotted as a function of time (seconds) to form the sensogram. (B) A typical sensogram: during a binding event, the association between the analyte and ligand results in an increase in RU. An increase in the resonance signal reaches a maximum at equilibrium. At the end of injection, the analyte starts to dissociate when the injection is replaced with the continuous flow buffer, resulting in a decrease in RU. The ligand can be regenerated by injection of a stringent buffer and the signal returns to the base line. A response of 1 000 RU is equivalent to a change in surface concentration of 1 ng/mm². The figures are adopted from <http://www.biacore.com>)

2.8. HIV-1

2.8.1. Sources of HIV-1

All HIV-1 strains used in this study were obtained through the AIDS Research and Reference Reagent Program, NIAID, NIH, Bethesda, USA. HIV-1_{Ba-L} was contributed by S. Gartner, M Popovic and R. Gallo (Gartner et al., 1986), HIV-1_{ADA} by H. Gendelman (Gendelman et al., 1988), and HIV-1_{IIIB} by R. Gallo (Barre-Sinoussi et al., 1983).

2.8.2. Propagation of HIV-1 isolates

The type of cell chosen to grow a particular strain depends on the tropism of the virus. In this study only R5 strains were routinely used, and therefore they were expanded in PBMC or MDM. MDM were isolated and allowed to differentiate as previously described (2.3.3–2.3.4). Differentiated PBMC or MDM in a T75 flask were inoculated with 1 ml of HIV-1 stock, in a small volume of culture medium, sufficient to cover the cells, and were incubated overnight at 37°C. The culture was maintained in a low volume of medium, which was changed infrequently to encourage the spread of infection, until cytopathic effects were exhibited (usually 7 days post infection). At this point, about 20 ml of fresh medium per T75 flask was added, the aim being to achieve peak c.p.e. coincident with exhaustion of the medium. Rarely, if the appearance of c.p.e was slow, on day 14 post-infection, fresh monocytes were added. Conversely, if c.p.e. advanced very rapidly whilst the culture volume was still small, the supernatant was transferred to a new flask of differentiated cells. Although the growth of HIV-1 in different batches of PBMC and MDM cultures was unpredictable, when infection did occur, the formation of remarkably giant macrophage cells, was a reliable indication of peak replication, and at this point the virus was harvested.

2.8.3. Harvesting and titer of HIV-1

HIV-1 supernatants were harvested by centrifugation at 2 000 rpm for 10 minutes and filtration through 0.44 μm filters to clear cells and debris. Proviral DNA was removed by treating the filtered supernatant with 100 $\mu\text{g/ml}$ DNase I (Roche) in the presence of 5 mM MgCl_2 for 30 min at room temperature. HIV-1 supernatants were aliquoted into cryotubes (1 ml/tube) and stored at -70°C . HIV-1 titer was determined using the TCID_{50} (2.8.5) assay in human PBMC.

2.8.4. Infection protocol for GHOST HIV-1 indicator cells

GHOST cell transfectants (2.3.1) were used to evaluate HIV-1 sensitivity to aptamers isolated against the gp120 in this study. Infection was detected via induction of the hGFP gene within 24–48 hours post infection by FACS[®] or immunocytochemistry. GHOST (3) Hi-5 cells that express a high level of CCR5 receptor in addition to the CD4 receptor were seeded in a 12 well plate (5×10^4 cells/well) a day before infection and cultured as described (2.3.1). For immunocytochemistry, cells were seeded on 13 mm diameter ethanol-sterilized cover slips. A test monoclonal aptamer was sterilized, and unfolded at 95°C for 3 min and cooled to room temperature for 10 min. $5 \times \text{HBS}$ binding buffer (to $1 \times$ final concentration) was added to denatured aptamer to allow refolding. 500 μl of HIV-1 stock ($1 \times 10^{5-6}$ TCID_{50} IU/ml) was pre-incubated with 100 nM (final concentration) of aptamer at room temperature for 30 minutes, followed by infection of the cells. Thus about 500 μl of HIV-1 with or without the aptamer was used to infect one well of a 12 well plate, and the cultures were incubated overnight at 37°C . GHOST (3) parental cells were used as a negative control. The next day the inoculum was replaced with fresh culture medium and incubated for a further 24 hours. 48 hours post infection, the cultures were harvested and analysed as described (2.9).

2.8.5. TCID₅₀ assay: infection of human PBMCs with HIV-1 and neutralization with aptamers

PBMC (2.3.2) and macrophages (2.3.3) were isolated and cultured as described (2.3.4). Day 7 PBMC or MDM were infected with serially diluted virus that had been incubated with 100 nM of anti-gp120 monoclonal or control aptamer. Titration of infectivity was performed using log₁₀ dilution factor of virus strain with at least 8 replicate wells at each dilution to achieve statistically significant results. After 16 hours post infection the medium containing virus inoculum and aptamer was replaced with fresh culture medium. Infectivity assays were maintained for 14 days before preparing DNA for LTR-PCR.

Cell lysis, DNA preparation and PCR were carried out as described (Collin et al., 1991). Briefly, culture medium was aspirated and cells were washed once with 100 µl of PBS per well and lysed *in situ*. The lysis buffer contained 100 mM KCl, 20 mM Tris-HCl pH 8.4, 250 µg/ml proteinase K and 0.2% NP40. Plates were incubated at 60°C for 2 hours, and then heated at 95°C for 15 minutes. Infectivity in each well was assayed by PCR using a primer pair specific for the HIV-1 LTR: U3₅₇₊ and U5₅₉₆₋ (Table 2.3). Thus the 10 × PCR master mix (2.1.3) was diluted in dH₂O to 2 × final concentration, and 12.5 U of *Taq* polymerase per 500 µl reaction volume was added. The diluted 2 × PCR master solution was mixed 1:1 with the cell lysate (10 µl each). PCR cycling conditions were initial denaturation for 94°C for 60 sec, then 94°C for 30 sec., 60°C for 30 sec and 72°C for 60 sec for 30 cycles, followed by a final extension at 72°C for 8 min. PCR products were resolved on 2% agarose gel loaded eight times to produce a pattern of PCR bands corresponding to the wells of the assay plates. First and second order statistics of infectious titer and aptamer inhibitory doses were determined using the ID₅₀ 5.0 software developed by John Spouge and available at: (<http://www.ncbi.nlm.nih.gov/CBBresearch/Spouge/Virology/>).

2.8.6. Generation of escape mutants and analysis of gp120 sequences

The parental HIV-1_{Ba-L} (NIH request number 510) *env* gene region coding for gp120 from proviral DNA was amplified using the Env₆₃₀₉₊ and Env₈₀₂₃ primer pair (Table 2.1) specific for regions flanking the envelope protein. Expand™ high fidelity PCR system kit (Roche, Germany) was used to amplify the gp120 *env* gene. The PCR product was cloned into the TA vector and sequenced as described (2.4.4).

To generate aptamer B4 break-through variants, day 7 human PBMC ($1-2 \times 10^7$) were infected with parental HIV-1_{Ba-L} stock ($5 \log_{10}$ IU), incubated with aptamer B4 at 1nM final concentration in the first instance. Samples of cell supernatant were collected every other day, until cytopathic effects were exhibited. The virus titer of each sample was determined and the supernatant with the highest titer was amplified and used for subsequent rounds of infection ($\text{M.O.I} \geq 0.01$) of freshly differentiated day 7 human PBMC following the same protocol but with higher concentrations of aptamer B4 (i.e. 5; 15; 50 and 100 nM). Thus at intervals, after every dose of the aptamer, the infectious virus was expanded in the absence of aptamer to a titer of 10^5 TCID₅₀ IU/ml. At 100 nM aptamer concentration, HIV-1_{Ba-L} was cloned by limiting dilution with 12 replicate wells at each dilution to increase the chance of break-through events.

At 14 days post infection the culture plate was replicated by transferring the culture medium into another 96-well plate seeded with differentiated PBMC. The original plate was assayed by LTR-PCR to score wells for the presence of infection, and the *env* gene coding for gp120 in the positive wells was amplified by high fidelity PCR and sequenced for comparison with the gp120 of the parental virus. HIV-1_{Ba-L} clones from the corresponding positive wells in the replicate plates were rescued and propagated as described (2.8.2.), until they reached a titer of about $5 \log_{10}$ IU/ml. The expanded viral clones were re-evaluated for sensitivity to aptamer B4 at the highest concentration used, 100 nM, in PBMC as previously described (2.8.5).

2.9. Immunocytochemistry and fluorescent microscopy

2.9.1. Fixation and staining of cells

The culture medium was aspirated, and each well was washed with 1 ml of cold PBS. Cells were then fixed to the coverslips with 4% paraformaldehyde (PFA) in 250 mM HEPES, pH 7.4 for 30 min., followed by 8% PFA also in 250 mM HEPES, pH 7.4 for another 30 min., at room temperature. Cells were washed 3 times with 0.5 ml per well of cold PBS, for 5 min. each time. Free aldehyde groups were quenched with 50 mM NH_4Cl in PBS for 10 min, and cells were permeabilized with 0.2% (v/v) Triton X-100 in PBS for 5 min., followed by washing with cold PBS (2 × for 5 min each).

Diluted antibody drops (50–100 μl) were applied to the surface of Parafilm[®], and cells fixed on coverslips were stained by laying them (cell-side down) on antibody solution. Nuclear staining was done with Hoechst 33342 (Sigma) at 20ng/ml in PBS for 30 min at room temperature in the dark, followed by washing with PBS. Nonspecific binding of the primary antibody was blocked with 0.2% BSA in PBS for 2 min. and the rabbit-anti-hGFP mAb (Vector) diluted in the blocking buffer was applied for 45 min in the dark. Fixed cells on coverslips were washed 3 × with cold PBS for 5 min each time and anti-rabbit Texas red IgG (Vector) secondary antibody diluted 1/50 in blocking buffer was applied for 45 min. followed by washing with PBS. Coverslips were rinsed in dH_2O and mounted on slides with Vectorshield[™] medium (Vector).

2.9.2. Detection of hGFP using fluorescent microscopy

Cells were viewed for expression of hGFP using the Zeiss Axioplan 2 microscope under appropriate channels and filters. The images were captured using CCD camera and processed using Adobe Photoshop software.

2.10. ELISA

2.10.1 Titration of antibodies against gp120 for epitope mapping ELISA

HIV-1 neutralizing mAbs against the gp120 (2.1.5) were titrated against HIV-1_{Ba-L} gp120 to determine the appropriate antibody concentration to be used in epitope mapping enzyme-linked immunosorbent assay (ELISA). A 96-well ELISA plate was coated with 50 µl/well of D7324 anti-gp120 COOH peptide antiserum (Aalto Bioreagents Plc.) at 5 µg/ml in carbonate coating buffer, pH 8.5 at 4°C overnight. The plate was washed 3 × with PBS using automated Titertek plate washer, and 100 µl of blocking buffer (2% BSA in PBS) was added at room temperature for 1 hour. The plate was washed as previously described and 0.1 µg/ml of HIV-1_{Ba-L} gp120 in 50 µl blocking buffer was added for 1–2 hours at room temperature. The plate was washed and 50 µl of antibody at 1/3 dilutions in blocking buffer was added across the plate (3, 1/3, 1/9, 1/27, etc.), in triplicates for 1 hour at room temperature. Unbound primary antibody was washed 3 × in PBS, and 50 µl of anti-species IgG-conjugated to biotin (Vector) was added for 1 hour at room temperature. The plate was washed 3 × with PBS and 50 µl/well of freshly prepared Vectastain AB-HRP conjugate (ABC Elite amplification kit, Vector) was added for 30 min at room temperature. The plate was again washed 3 × with PBS and 50 µl/well of freshly prepared substrate (Sigma Fast HRP substrate kit) was added and the plate was left to develop in the dark for 10–20 min. The reaction was stopped with 50 µl/well of 1 N H₂SO₄.

The plate was read at 492nm using Labsystems microplate reader, and the data was analysed using Genesis software. Antibody dilution on the X-axis was plotted against OD at 492nm (Y-axis), and an antibody dilution at which the curve just begun to fall was used for epitope mapping ELISA.

Chapter Three

Expression and purification of R5 gp120

3.1. Summary

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Background: A space-filled molecular model of the interaction of heparin-binding domain (RP-135 peptide) of gp120 with a heparin octasaccharide (Source (Cardin et al., 1989)).

3. Expression and purification of HIV-1_{Ba-L} gp120 using recombinant baculovirus system

3.1. Summary

Expression and purification of HIV-1 gp120 in isolation of the viral environment is essential for a number of reasons. First, the expressed protein can be used as a target for the generation of aptamers and the affinity binding rate constants can be readily determined, as in this thesis. Second, the isolated gp120 can be manipulated by mutagenesis to determine the interplay of structure and function, and the function of the protein can be distinguished from that of the virion background. This chapter therefore describes expression and purification of full-length monomeric HIV-1_{Ba-L} gp120 using recombinant baculovirus system and immunoaffinity chromatography, respectively. The total yield of purified gp120 using this system was 1–2 mg per liter of 1×10^9 Sf9s cells and the protein had full biological activity as assayed by binding to human CD4 and relevant antibodies.

3.2. Introduction

Eukaryotic expression systems provide the opportunity for production of complex proteins that cannot be produced in native form in *E.coli* fusion systems. Glycosylation, phosphorylation, myristoylation and propeptide processing are some of the more common post-translational modifications that demand the use of eukaryotic expression systems. Eukaryotic systems offer a choice between expressing the protein in stably transected mammalian cell lines (e.g. CHO cells), or transiently during a viral infection cycle, like in the baculovirus expression system. Stable cell lines are more difficult and time-consuming to produce, but often allow for improvement of yield by gene amplification or manipulation of the growth conditions (e.g. fermentation albeit production of cytotoxic products). Transient baculovirus expression system is easy, faster and fairly sure of success, although the yield of any particular protein is difficult to predict. Thus, the baculovirus expression system is a method of choice for rapid

generation of sufficient protein that requires post-translational modifications, to enable functional and structural studies.

The baculovirus expression system has been widely used for the production of recombinant proteins (Smith et al., 1983). The methods for preparing recombinant baculovirus have improved dramatically over the years, but the basic concept remains the same. A non-essential, highly expressed polyhedrin gene is replaced by a foreign gene. Most recombinant baculoviruses, including the gp120 recombinant described in this chapter, make use of the polyhedrin gene transcription unit of *Autographica californica* nuclear polyhedrosis virus (AcMNPV) for expression of foreign protein. The genome of AcMNPV can accommodate large inserts of more than 10 kb with no apparent effect on packaging efficiency or virus stability. In this study, the full-length gp120 gene (about 1 600 bp) was incorporated into the virus genome by *in vivo* homologous recombination within Sf9s insect cells, such that it was transcribed late in the virus life cycle, by the polyhedrin promoter.

3.3. Recombinant baculovirus and transfection of Sf9s cells

The recombinant p2BaC-gp120 construct was made by George F. Gao (IMM, Oxford). Briefly, PCR amplified DNA encoding full-length monomeric HIV-1_{Ba-L} gp120 was cloned under the control of polyhedrin promoter by *Bam*HI/*Hind*III into the p2BaC (Invitrogen) transfer vector. The C-terminus of the gp120 was fused to a FLAG epitope, which could be recognized by the anti-FLAG M2 mAb to allow affinity purification. To generate recombinant baculovirus, recombinant p2Bac transfer vector was mixed with linearized pAcBAK6 viral DNA and used for transfection of Sf9s cells as described in the methods section (2.2.2), and outlined in Figure 3.1. As only circular viral DNA was infectious, DNA cleaved at the polyhedrin locus could not initiate infection unless it was rescued by re-circulation via recombination with the transfer vector (Figure 3.1). In practice, although the recombination rate remains the same when cells are transfected with linear or circular DNA, the wild-type background is reduced substantially when linearized DNA is used (Kitts et al., 1990). The

pAcBAK6 viral DNA was engineered to contain three restriction sites for the endonuclease enzyme *Bsu36I*. Therefore when *Bsu36I* was used to cut the viral DNA, almost no background virus was produced and recombinant virus selection was dependent solely on successful genetic modification of the transfer vector and good Sf9s cell culture. Within two to three days of Sf9s cells transfection, supernatant was harvested and the plaque assay was done (Figure 3.2).

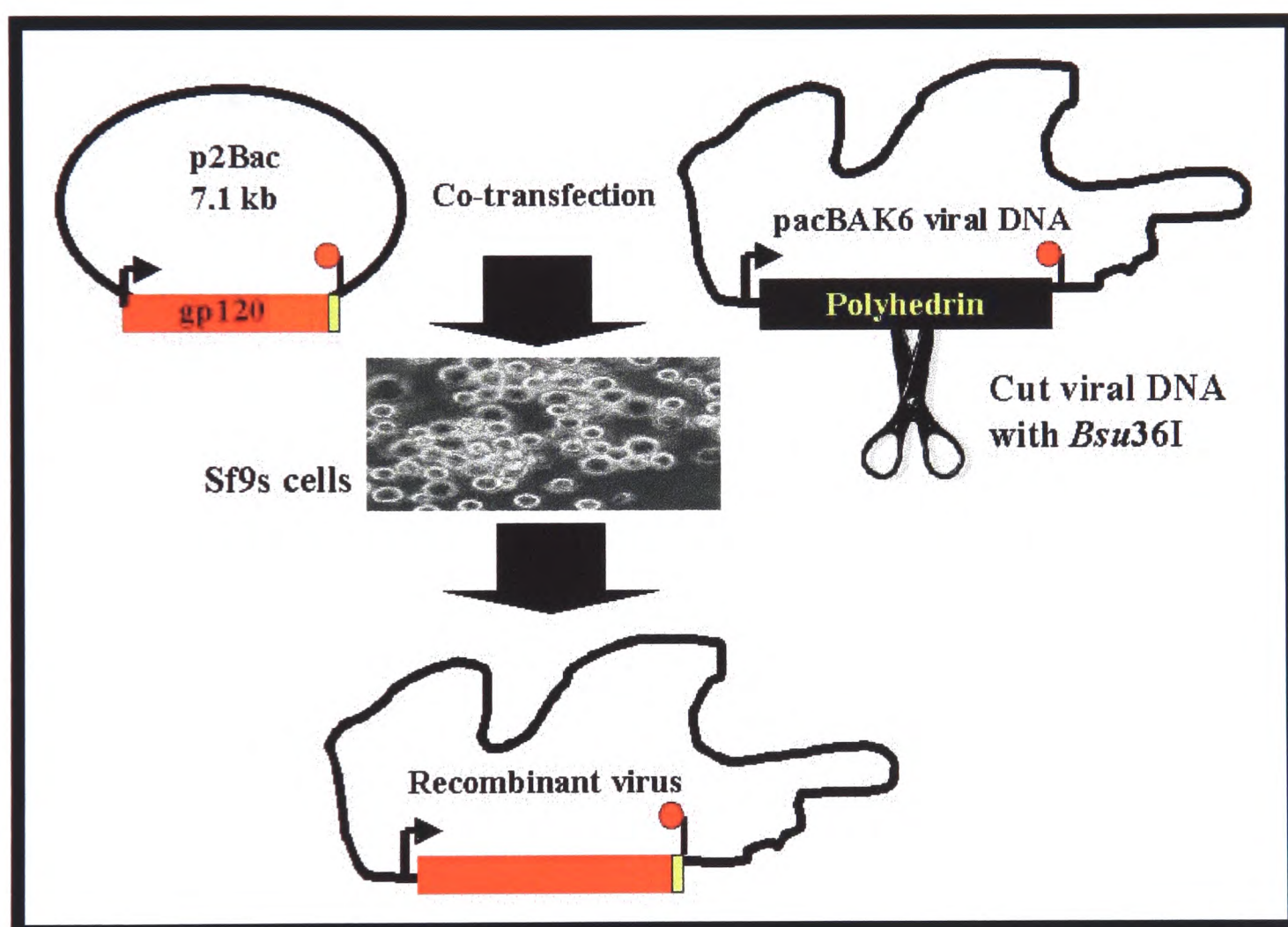


Figure 3.1: Schematic representation of the production of recombinant baculovirus encoding HIV-1 gp120. Both the p2Bac transfer vector and pacBAK6 viral DNA were flanked by polyhedrin promoter (arrow) and transcription termination signal (red circle) to allow homologous recombination after infection of the cells. Sf9s cells were co-transfected with the transfer vector and virus DNA using lipofection. Homologous recombination replaced the *polyhedrin* gene (black box) with the *gp120* gene (red box) having the FLAG tail (yellow box) in the recombinant baculovirus.

3.4. Plaque assay and analysis of recombinant virus

3.4.1. Plaque assay

Plaque assay was used to screen recombinant baculovirus and also to determine titer of the virus. Plaque assay was performed by setting down Sf9s cells in a 6 well tissue culture plate at a density of 1×10^6 cells/well, for 1 hour at 28°C. The transfection or viral supernatant was diluted to appropriate concentrations in TC-100 (GibcoBRL) supplemented with 10% FCS. The medium was aspirated from the wells and 200µl of virus dilution was added per well of cell monolayers and incubated for 1 hour at 28°C to allow virus absorption. After the absorption phase, all traces of virus inoculum was removed, and the cells were overlaid with 2 ml per well of solid overlay (2% low melting temperature tissue culture grade agarose in equal volume of TC-100 supplemented with 10% FCS) for 15 min. at room temperature. After the agarose has solidified, 1ml of TC-100 supplemented with 10% FCS was added per well, and the culture was incubated at 28°C for three days. On day 3, the cells were stained with 0.01% (w/v) neutral red in PBS (1 ml/well) for 2 hours at 28°C. After two hours, residual neutral red was removed and the plate was inverted in the dark for another 2 hours to allow dead cells to release the stain, hence allow plaques visualization (Figure 3.2). The yield of recombinant virus from the transfection was about 1×10^4 pfu/ml at three days post infection. Longer incubations gave higher virus yield but the proportion of recombinants did not change (Table 3.1).

Table 3.1: Virus yield and proportion of recombinant plaques as a function of time (days)

Days post-transfection	Virus yield(pfu/ml± SEM)	Proportion of recombinants
2 days	$1.2 \times 10^4 \pm 1.6 \times 10^3$	9 out of 10
3 days	$1.6 \times 10^4 \pm 1.4 \times 10^3$	8 out of 10
5 days	$2.8 \times 10^4 \pm 2.5 \times 10^3$	8 out of 10

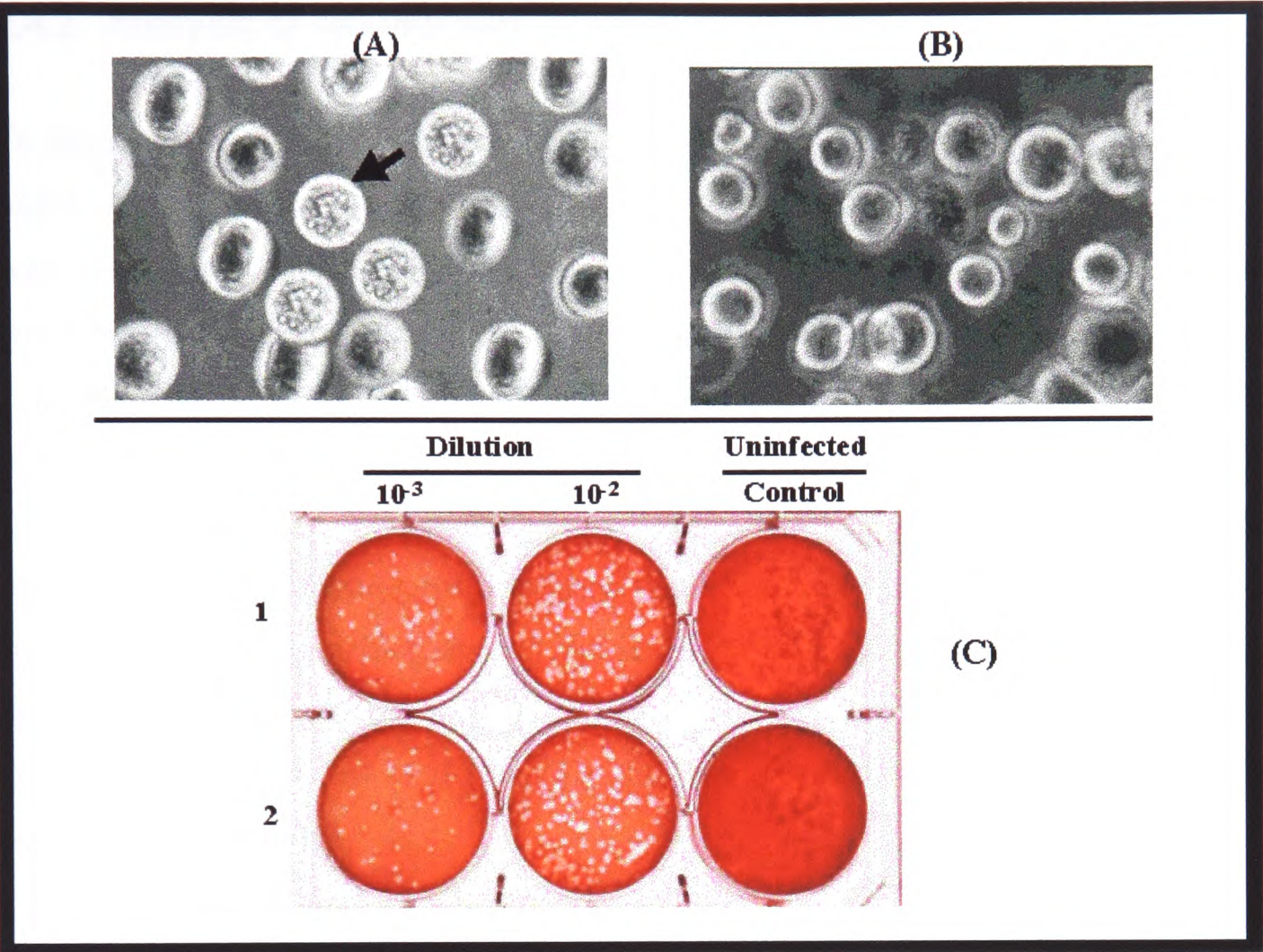


Figure 3.2: Sf9s insect cells infected with recombinant baculovirus. (A) Light micrograph of Sf9s cells infected with baculovirus (3 days p.i.). The cells floated in the medium and exhibited more rounded morphology and granular cytoplasm (arrow) characteristic of the c.p.e in this cell type, compared to uninfected controls (B), which remained as a homogeneous monolayer. (C) Sf9s cells stained with neutral red to visualize the plaques that appeared milky against the red background in infected wells. The number of plaques was proportional to virus dilution, i.e. more plaques in less diluted virus, and no plaques at all in uninfected control.

3.4.2. Analysis of recombinant plaques by PCR and western blot

To determine the ratio of recombinant to non-recombinant plaques, a PCR assay using p2BaC-cloning primers specific for gp120 (2.4.3) was performed on 10 plaques that were picked at random. On average 8 out of the 10 plaques picked at random were recombinant (Figure 3.3 and Table 3.1.). Expression of the full-length gp120 was confirmed by a Western blot. Insert positive plaques as assayed by PCR were individually inoculated into a 24-well plate, and at two days post-infection the cell lysate was probed for expression of the protein. Anti-FLAG[®] M2 mAb (Sigma) that detected the FLAG epitope that was fused to the C-terminus of the recombinant gp120 was used in a Western blot, and all the insert positive plaques expressed full-length gp120 (Figure 3.3). Taken together, these data showed that use of Bsu36I digested pacBAK6 as a source of viral DNA for transfection of Sf9s results in recombination frequency of over 80%.

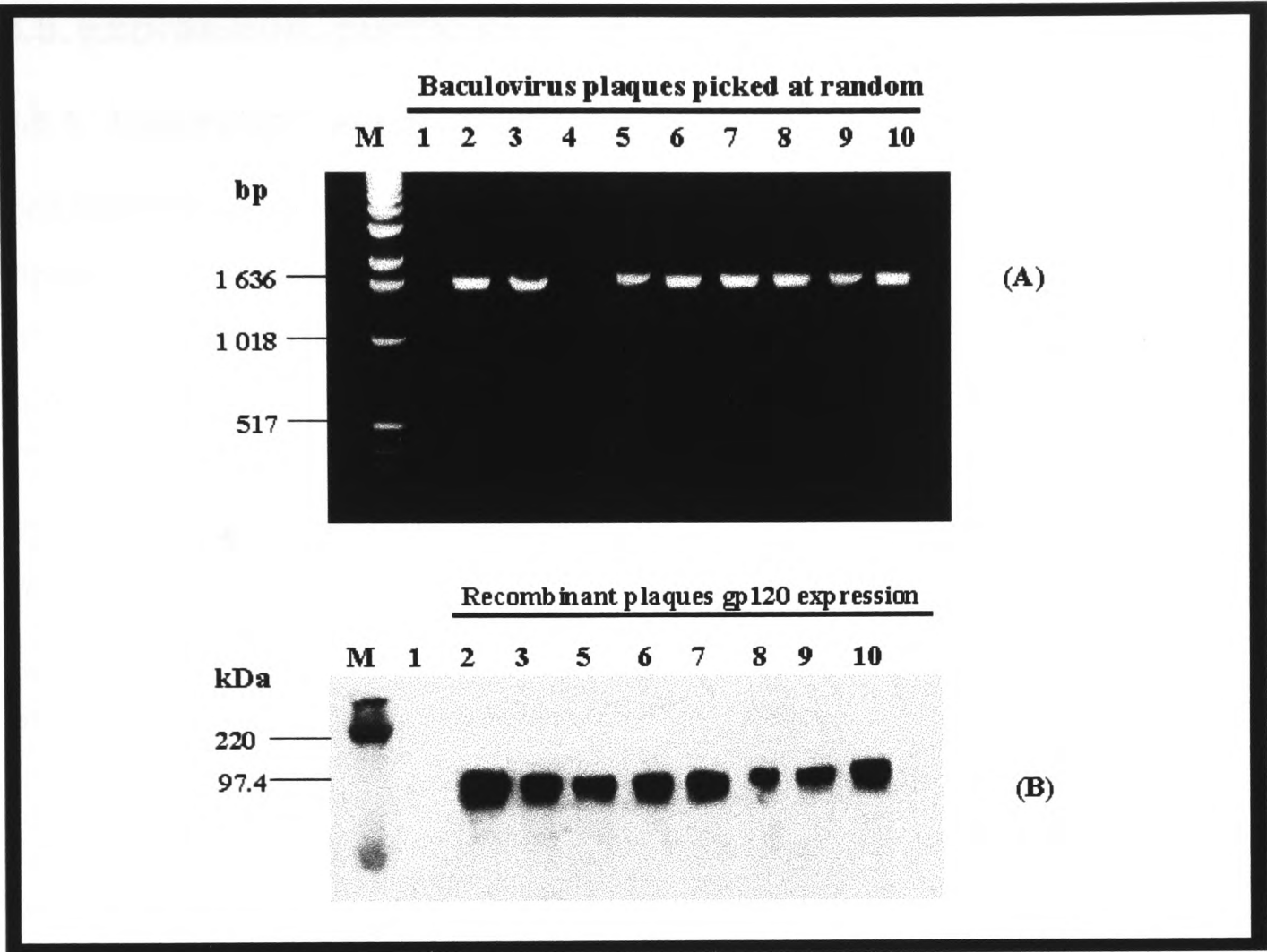


Figure 3.3: Recombinant gp120-baculovirus plaques. (A) 8 out of 10 of the randomly picked plaques assayed by PCR were recombinant (B) All the eight PCR positive plaques (lanes 2-10) assayed by anti-FLAG[®] M2 monoclonal antibody immunoblot expressed full-length gp120. Lane 1 is the negative control inoculated with a non-recombinant baculovirus plaque.

3.5. Expression, purification and biological activity of R5 gp120

3.5.1. Expression and purification of gp120

Cell lysates from recombinant baculovirus plaques that were positive for expression of the gp120 were pooled and expanded in Sf9s cell monolayers. Typically to expand the virus, Sf9s cells were infected with the recombinant virus at the multiplicity of infection (m.o.i) of less than 0.01, and grown in moderate volume (e.g. 10–20ml per T75 flask) to provide a high titer stock of virus. Infected cells were grown at 28°C until they exhibited cytopathic effect (c.p.e), which was characterized by rounded morphology and granular cytoplasm as compared to uninfected control cells (Figure 3.2). Once c.p.e was exhibited, usually on day 4 post-infection, the supernatant was harvested by centrifugation at 4 000 rpm for 20 minutes. The virus peak titer reached 10^7 to 10^8 pfu/ml as determined by plaque assay. Small aliquots (4×1 ml) of seed stock of the virus was stored at -70°C, and the bulk of the virus was stored at 4°C in the dark.

For expression of the recombinant gp120, only cells at the exponential growth phase that were almost 100% viable as determined by trypan blue exclusion stain were used because cells with less than 95% viability did not give the best yields. First, a small-scale time course of expression for the protein was done to determine the optimal time at which the protein was expressed (Figure 3.4). The time course of expression followed a well-observed pattern in which there was a slight expression of the gp120 at two days post infection. Abundant expression was at three days post infection and was maintained for a fourth day post infection (Figure 3.4). Cytolysis, which was accompanied by release of proteinases begun at 5 days post infection and the degradation of the gp120 product, became evident and progressed thenceforth. The results suggested that the highly glycosylated nature of gp120 required a long folding and secretion time. As a consequence of that, although expression was notable at two days post infection, the level of expression was higher on days 3 and 4 post infection, reflecting a lag in the secretion of this complex glycoprotein from Sf9s cells. From the

time-course of expression, the gp120 was best harvested on days 3 and 4 post infection.

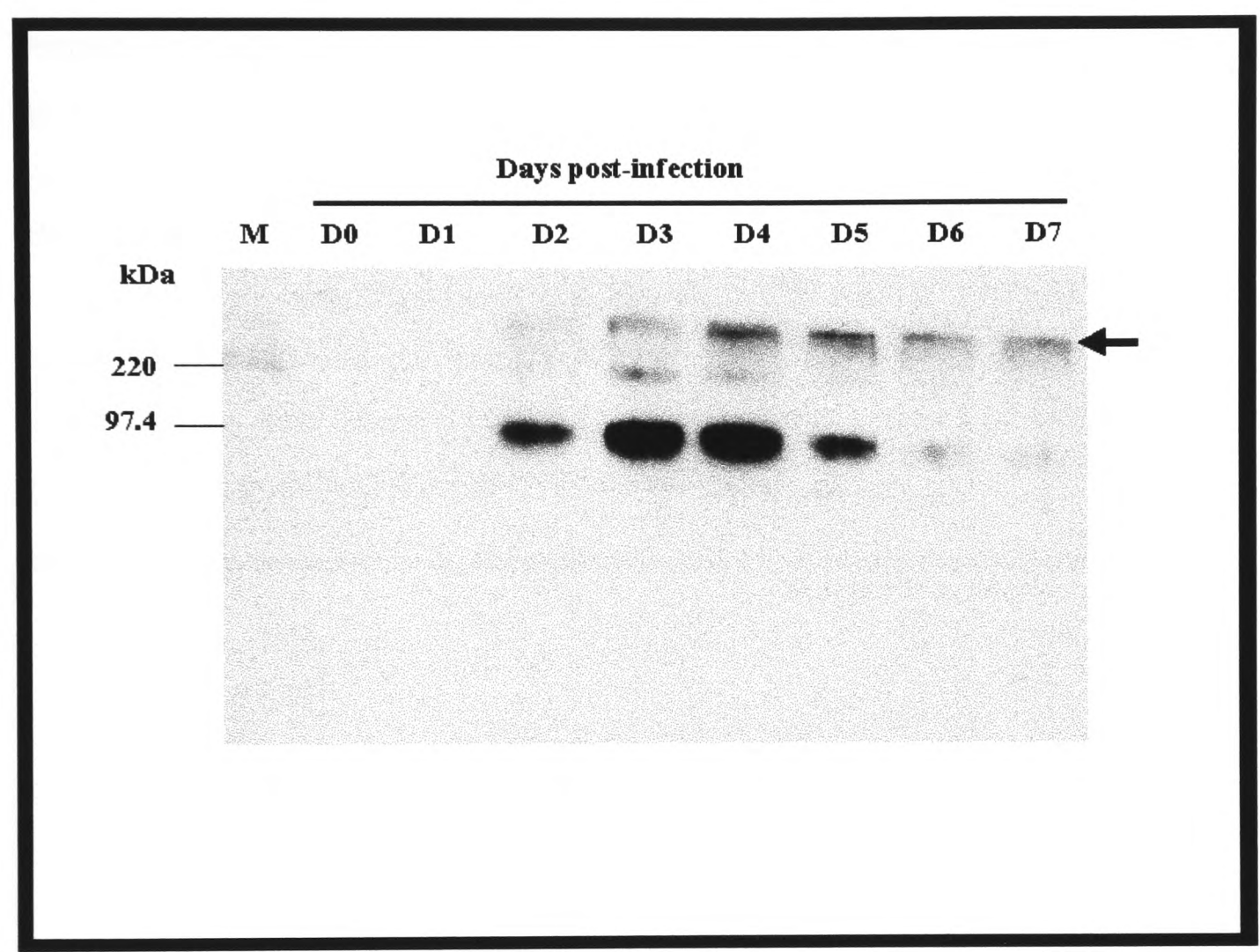


Figure 3.4: Western blot analysis of HIV-1_{Ba-L} gp120 expression time course by recombinant baculovirus. The protein was abundantly expressed on days 3 and 4 post infection, followed by cytolysis and degradation of the product on days 5 onwards. The arrow indicates what could be higher molecular weight aggregates or trimeric gp120. Equal amount of cell lysate was loaded on each lane and resolved by reducing SDS-PAGE.

Once conditions were established for optimal expression of the protein, a large-scale expression protocol was carried out. For large-scale expression of the gp120, 1×10^9 cells were seeded as monolayers in a Nunc Cell Factory (Nalge Nunc International, Denmark) in a total volume of 500 ml SF 900 II medium. The cells were infected with the high titer stock of recombinant virus ($m.o.i \geq 5$) to ensure that all cells were synchronously infected, and incubated at 28°C for 72 hours. On day three to four post infection, which corresponded with peak expression of the protein, the cell supernatant was harvested by centrifugation at 10 000 rpm for 30 min. at 4°C, and the secreted recombinant gp120 was purified by affinity chromatography as described in 2.5.1. Eluted fractions were detected by HPLC UV system and analysed by Western blot for presence of the protein as previously described (Figure 3.5).

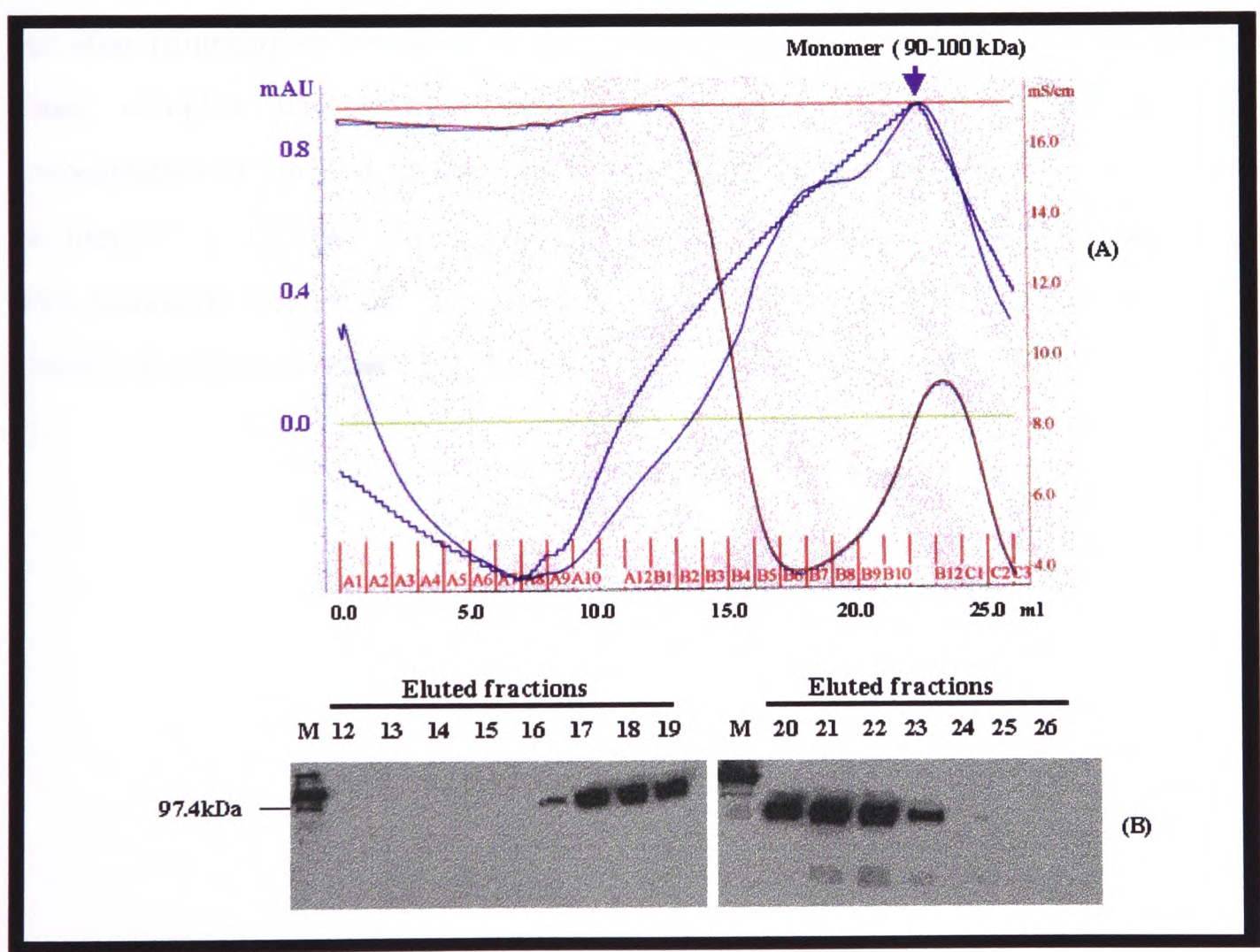


Figure 3.5: Elution profile of purified HIV-1_{Ba-L} gp120. (A) HPLC trace showing detection of the protein by the UV system from the collected fractions (blue line). (B) Western blot analysis of peak fractions to confirm recovery of the protein.

3.5.2. Quality and biological activity of gp120

The quality of purified gp120 was determined by reducing SDS-PAGE followed by total gel staining using Coomassie brilliant R-250 stain as described (2.5.4). Anti-FLAG[®] M2 affinity purified gp120 was 100% pure and had an apparent mobility of about 100 kDa on SDS-PAGE (Figure 3.6 A and B). The total carbohydrate load added to glycoproteins expressed in insect cells is marginally less than that added during secretion in mammalian cells, hence baculovirus expressed glycoproteins are correspondingly smaller than their mammalian counterparts (Luckow, 1993; Morikawa et al., 1990). Thus the glycosylation of proteins during their passage through the secretory pathway is the one secondary modification that is different in insect cells compared to mammalian cells. The early step of the glycosylation pathway are shared, but after trimming of precursor to the trimannose core, insect cells fail to assemble many complex carbohydrate structures (Wells and Compans, 1990). The concentration of purified protein was about 500 µg/ml and the total yield was 1–2 mg per liter of 1×10^9 Sf9s cells as quantified by the BCA assay (2.5.3) and comparison to BSA standards (Figure 3.6 B). The protein had full biological activity as assayed by binding to soluble human CD4 (Figure 3.6 C and D), and relevant antibodies (Chapter 6).

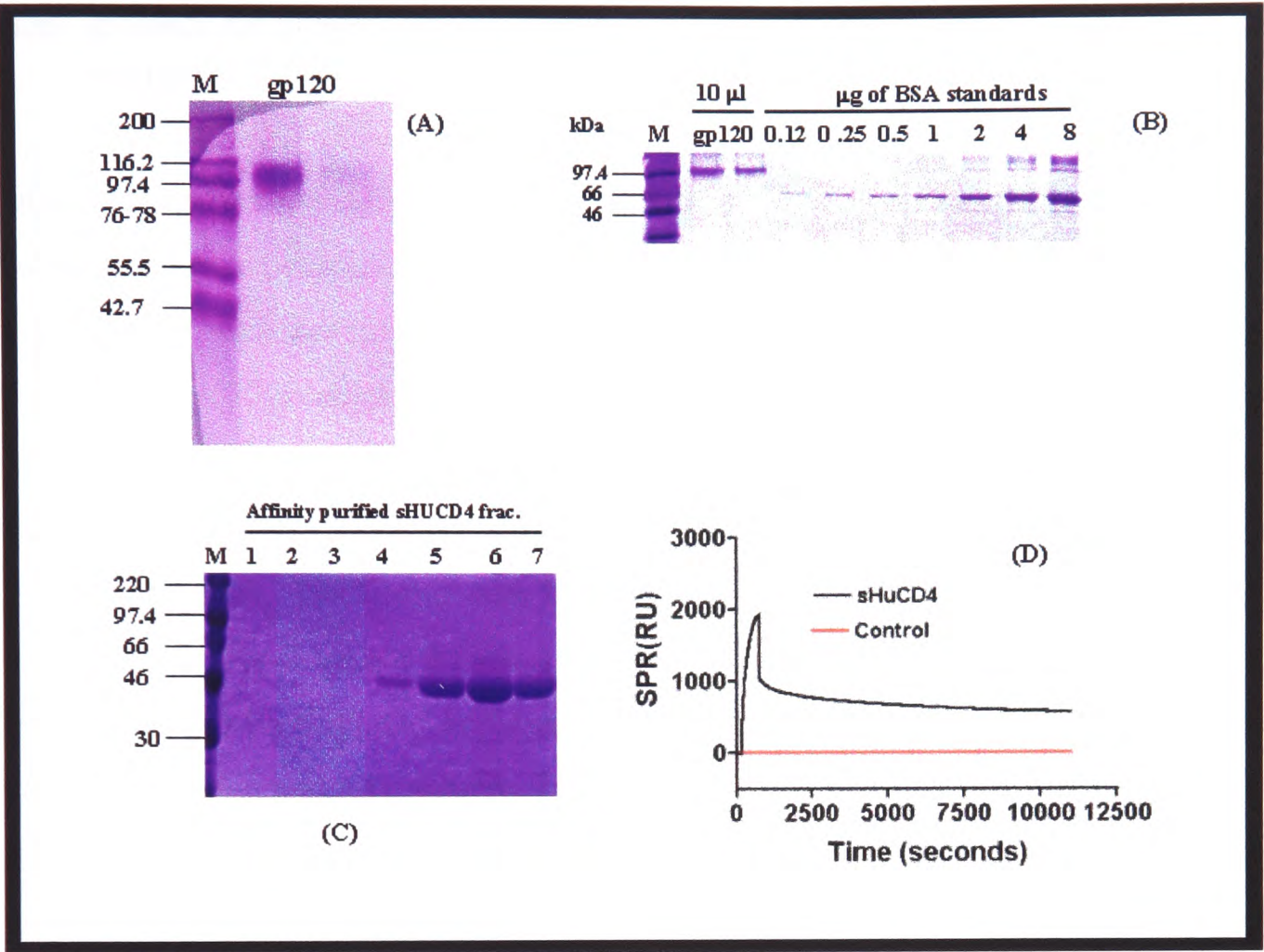


Figure 3.6: Quality and biological activity of purified HIV-1_{Ba-L} gp120. **(A and B)** Coomassie stained gp120 and quantification of purified protein by comparison to known amounts of BSA standards **(B)**. **(C)** Coomassie staining of affinity purified soluble human CD4. **(D)** Binding of purified HIV-1_{Ba-L} gp120 to soluble human CD4 as assayed by the BIAcore™ surface plasmon resonance technology.

3.6. Effect of heparan sulfate on R5 gp120 binding to soluble human CD4

Glycosaminoglycans (GAGs) such as heparan sulfate are believed to form part of the cellular receptor complex for HIV-1 by interacting with gp120 (discussed in 1.5.3). In this study it was therefore tested whether heparan sulfate could enhance binding of HIV-1_{Ba-L} gp120 to soluble human CD4 immobilised on the BIAcore biosensor chip. This experiment was carried out with a view to isolate aptamers against both gp120 and heparan sulfate. Heparan sulfate was biotinylated following the method described by (Foxall et al., 1995) and was indirectly coupled to a BIAcore chip coated with streptavidin. Three adjacent flow cells on a biosensor chip were respectively coupled as follows: (1) biotinylated heparan sulfate, (2) soluble human CD4 and biotinylated heparan sulfate, and (3) soluble human CD4. The fourth flow cell was left as a negative control. Anti-human CD4 mAb (Q4120) was injected through the flow cells to determine if equal amounts of active human CD4 were immobilised on the different flow cells (Figure 3.7 A).

Taking into consideration the relative amount of active human CD4 immobilised in the presence or absence of heparan sulfate, heparan sulfate did not enhance binding of HIV-1_{Ba-L} gp120 to human CD4. (Figure 3.7 B). Thus HIV-1_{Ba-L} gp120 did not bind to heparan sulfate alone, nor did heparan sulfate confer synergistic advantage for HIV-1_{Ba-L} gp120 binding to human CD4. The results were the same for T-tropic HIV-1_{IMB} gp120 which was used for comparison (Figure 3.7 C). These data showed that although heparan sulfate is thought of as an attractive candidate or adjunct receptor for HIV-1 (Ibrahim et al., 1999; Mondor et al., 1998; Patel et al., 1993; Roderiquez et al., 1995), this could not be demonstrated in a static BIAcore environment. The data therefore suggest that the interaction between gp120 and heparan sulfate could be more dynamic, and that monomeric gp120 probably interacts differently with heparan sulfate compared to virion associated oligomeric gp120. In retrospect, these data indirectly corroborated our earlier findings that heparan sulfate did not have a role in

the infection of peripheral blood lymphocytes by either HIV-1_{III_B} or HIV-1_{Ba-L} (Ibrahim et al., 1999).

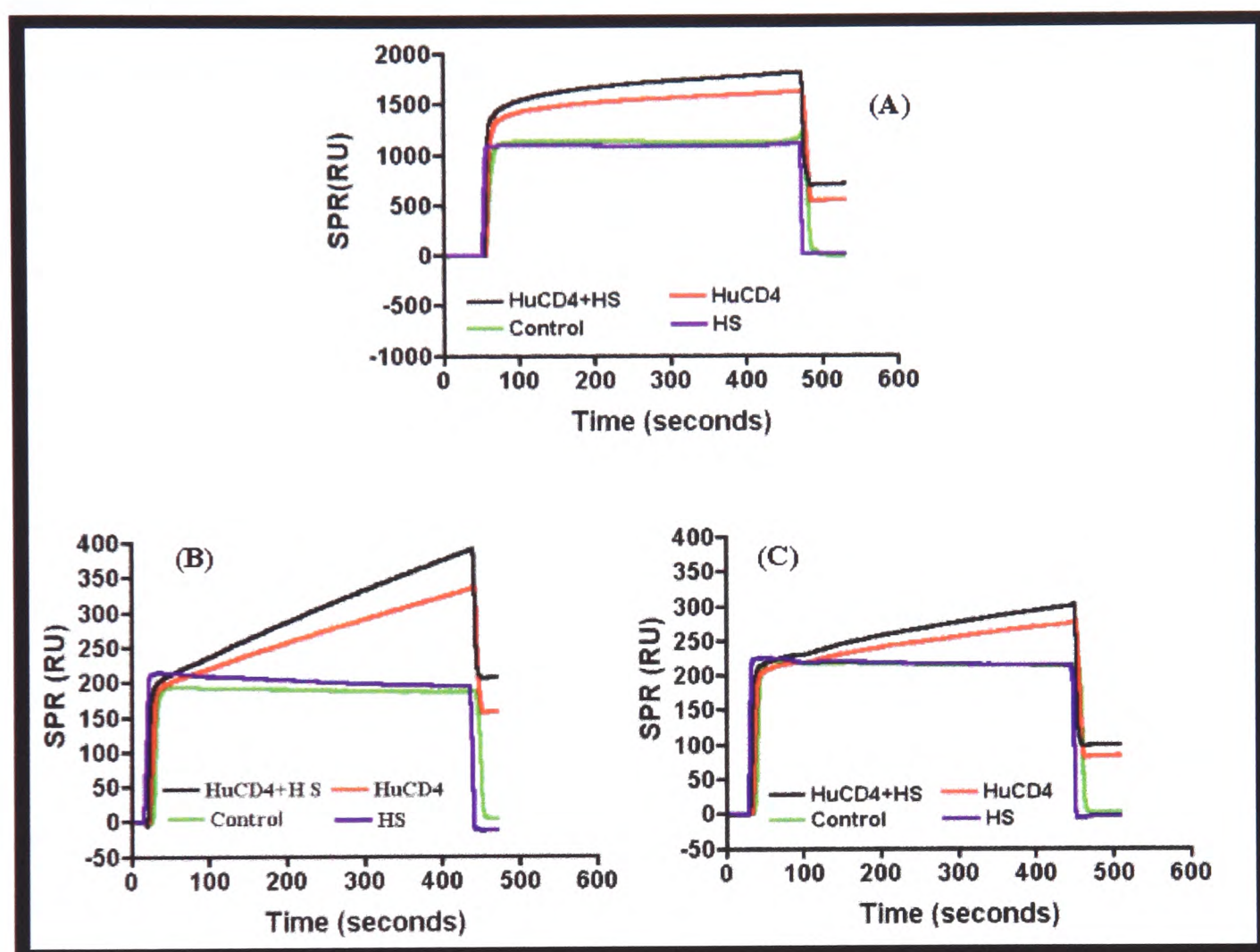


Figure 3.7: BIAcore™ analysis of the interaction between HIV-1 gp120 and soluble human CD4 in the presence or absence of heparan sulfate. **(A)** Analysis of the active amount of human CD4 immobilised on the different flow cells as determined by anti-human CD4 (Q4120). **(B and C)** Interaction of HIV-1_{Ba-L} and HIV_{III_B} gp120, respectively, with soluble human CD4 in the presence or absence of heparan sulfate. The gp120 was injected onto the three flow cells coupled with the shown ligands, and a control flow cell at a concentration of 20 µg/ml.

3.7. Discussion

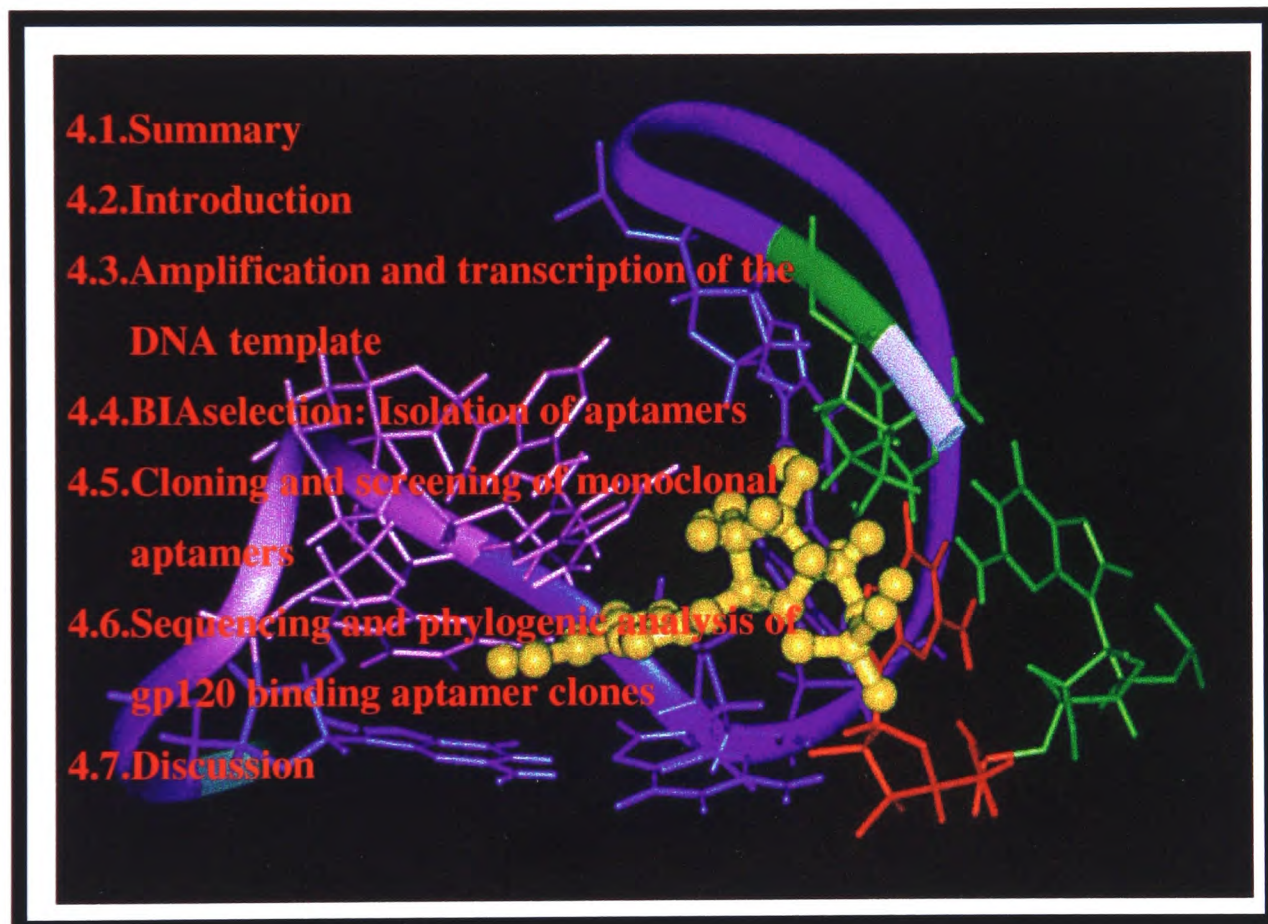
For many heterologous proteins expressed using the baculovirus system, cloning the gene of interest into one of the widely available standard baculovirus expression vectors results in recombinant protein production levels ranging from 50 to 500 µg/ml (Luckow, 1993). However, expression levels for secreted and glycosylated recombinant proteins are much lower, less than 1µg/ml (Luckow, 1991). In addition, expression of the gp120 derived from R5 strains, including HIV-1_{Ba-L} gp120 was inefficient in mammalian cells (Chapman et al., 1991). The results presented in this study showed that it is possible to express functional HIV-1_{Ba-L} gp120 using the baculovirus system. The data also demonstrate that the expression and secretion levels of this highly glycosylated secreted protein could be markedly improved to over 2 mg per liter by infecting highly viable Sf9s cells that are in the exponential phase using high titer virus stock, thereby synchronizing infection. Another study has however reported that the yield of the gp120 could be improved even to over 10 mg per liter by replacing the HIV-1 signal peptide with the one derived from two baculovirus proteins, egt and p67 (Murphy et al., 1993). The baculovirus signal peptides are derived from AcMNPV proteins and are thus thought to be more efficiently recognized by the host cell secretory pathway (Murphy et al., 1993). Whereas HIV-1 signal peptide could be associated with some toxicity in insect cells because there was a much faster decrease in cell viability infected with recombinant virus compared to when infected with the parental non-recombinant baculovirus. Nonetheless, for the purpose of this study the yield of the protein was sufficient to allow selection of anti-gp120 aptamers and associated experiments.

The secreted gp120 was readily isolated from the culture medium in one purification step. The purification process was simplified by the relatively high purity of HIV-1 gp120 in the starting medium because the SF 900 II culture medium was serum-free and was composed primarily of the secreted gp120. Importantly baculovirus expressed gp120 retained its biological activity and other Env proteins previously produced in this system such as the gp160 formed the basis for the HIV-1 vaccine trial (Watson, 1992), and was part of the successful protection experiments reported by (Hu

et al., 1992). It remains to be seen if this study that involved production of HIV-1_{Ba-L} gp120 using the baculovirus system will lead to therapeutically beneficial products. Nevertheless, the baculovirus expression system for study of HIV-1 protein structure and function does not seem in doubt.

Chapter Four

Isolation and cloning of anti-gp120 aptamers



*Background: Close-up of AMP binding aptamer core (Source: Donald H. Burke <http://bl-chem-ernie.chem.indiana.edu/~dhburke/index.html>. Used on the cover of *Nucleic Acids Research* Vol 25, number 10, May 15, 1997.)*

4. BIAselection: Isolation and cloning of 2'-fluoro modified RNA aptamers against R5 gp120

4.1 Summary

RNA aptamers have a penchant to home to functional sites on protein surfaces and to discriminate finely among these epitopes. This chapter describes isolation of 2'-fluoropyrimidine substituted RNA aptamers against the gp120 surface envelope glycoprotein of HIV-1. The gp120 derived from the R5; HIV-1_{Ba-L} strain was used as the target in the SELEX process. Aptamers were selected from a combinatorial library with a diversity of 10^{14} on the basis of their slow dissociation rates from the gp120 immobilized on the BIAcore sensor chip. After only five cycles of selection, high affinity aptamers were cloned and found to belong to at least 26 distinct sequence families. Roughly 1 in 10^8 random sequence RNA molecules folded in such a way as to create a specific binding site for HIV-1_{Ba-L} gp120. Although almost all the monoclonal aptamers selectively bound the HIV-1_{Ba-L} gp120, some like B19 and B36 also showed significant binding to the gp120 derived from HIV-1_{IIIB}, an X4 strain.

4.2. Introduction

The general principles for the SELEX protocol and isolation of aptamers and their applications have been reviewed in detail in chapter 1 (section 1.8). The main considerations in designing the SELEX library centers around the degree of randomness, the length of the random sequence tract, and the avoidance of amplification artifacts. Briefly, the method relies on mechanisms ascribed to the process of *in vitro* evolution, that is, variation, selection, and replication. A pool of RNA that is completely randomized at specific positions is subjected to selection for binding, in this case to the gp120 immobilized on the BIAcore sensor chip. The selected RNA is amplified as double-stranded DNA, which forms the component for subsequent *in vitro* transcription. The newly transcribed RNA is enriched for better binding sequences and is subjected to another round of selection. Multiple rounds of enrichment result in the exponential increase of the best binding RNA ligands called aptamers until they dominate the population of sequences.

In this study I utilize our knowledge of the gp120 structure and function (discussed in chapter 1, section 1.7) as a target for aptamers. Having previously developed aptamers against rat CD4 with useful functional properties in our laboratory (Kraus et al., 1998), I hypothesized that aptamers by virtue of their small sizes compared to antibodies, would access conserved regions of the gp120 such as the CD4 and CCR5 binding sites, that are protected from antibody attack. These aptamers would thereby block gp120-receptor interactions, and consequently preclude HIV-1 entry into the target cells. I also postulated that aptamers composed of RNA and not involving the use of animals or cells in their production would be unlikely to induce immunological reaction in therapeutic circumstances. Finally the 2'-fluoro substituted RNA aptamers form stable three-dimensional structure and are resistance to nucleases found in body fluids, making them suitable for diagnostic and/or therapeutic purposes.

4.3. Amplification and transcription of the DNA template

The first step in the SELEX experiments was to create a pool of variant sequences from which RNA ligands of relatively high affinity for gp120 could be selected. The template was designed and synthesized as described in the methods chapter (sections 2.6.1-2.6.2). A 134-mer DNA template randomized at 50 nucleotide positions (Figure 4.1) was amplified by PCR with varying number of cycles as described (2.6.2), to achieve at least 500 pmol of the template without aberrant products. The use of 50 randomized nucleotides, and a 134-mer library was critical for the production of a more stable folded RNA structure and high affinity ligands. Six PCR cycles yielded the cleanest single band of the expected size to allow large-scale amplification and transcription (Figure 4.1). Fewer cycles did not yield enough template, on the other hand, when I used additional cycles, the band of interest disappeared to be replaced by a diffuse band of apparently higher molecular weight (Figure 4.1). This probably resulted from undesirable opportunistic mispairing of individual strands within the variable region sequences between the members of the population. PCR-amplified dsDNA template was gel-purified and transcribed *in vitro* using 2'-fluoro-pyrimidines as described (2.6.3). The transcripts were used for the SELEX process, outlined in Figure 4.2.

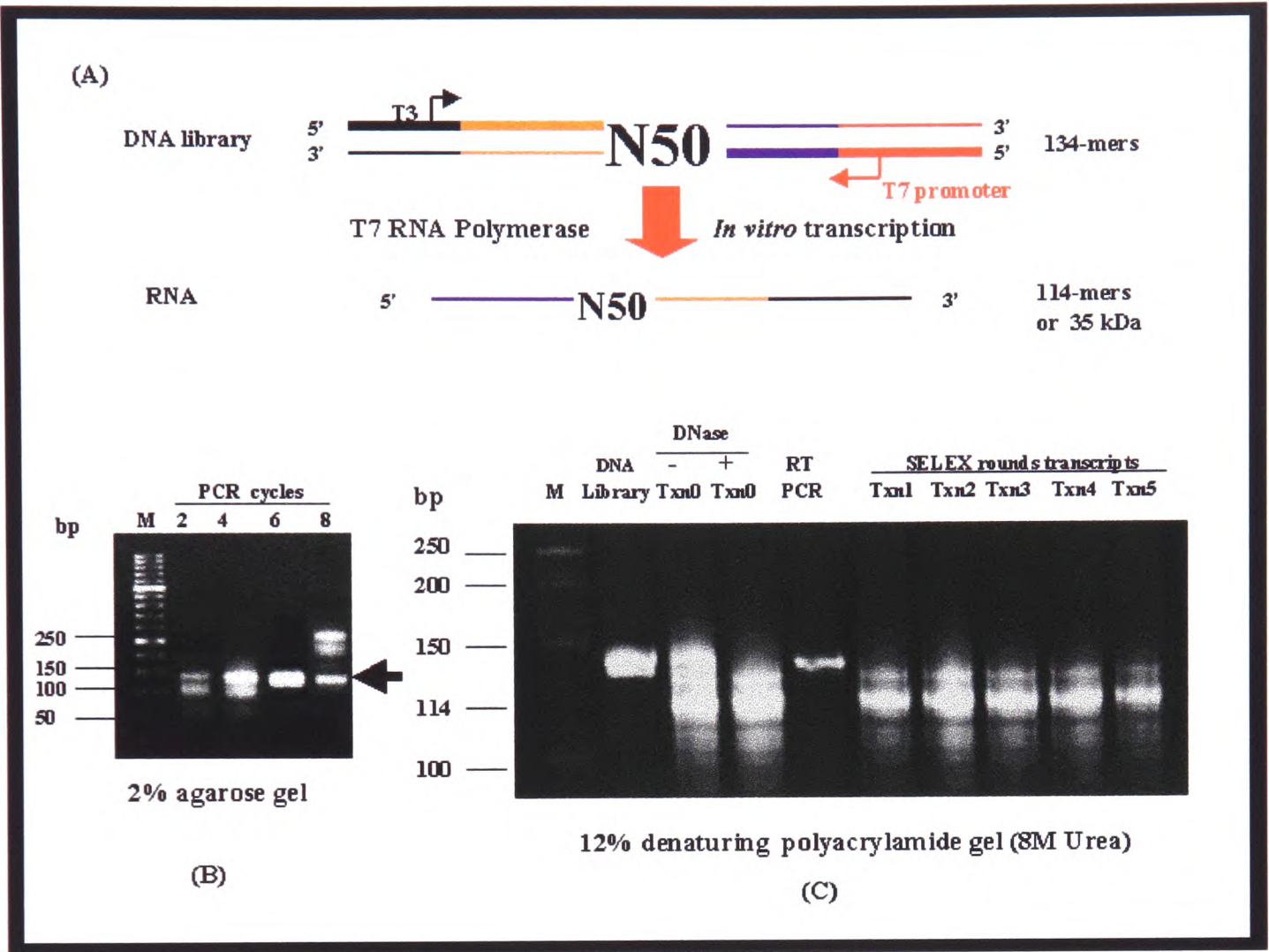


Figure 4.1: PCR amplification and transcription of the variant template. (A) The 134 nucleotide double-stranded DNA template was transcribed *in vitro* using the T7 RNA polymerase to give a 114 nucleotide 2'F-RNA molecule. Part of the T7 promoter that is used to initiate transcription is truncated in the final RNA product. (B) Agarose gel analysis of the PCR products yielded by varying numbers of cycles, showing the cleanest band of interest produced after 6 cycles (arrow). (C) Separation of the molecular weight marker (lane 1) against the DNA template (lane 2) and transcripts of each SELEX round (lanes 6–10) on denaturing urea gel. Lane 3 and 4 contained untreated and DNase-treated transcript respectively, while lane 5 contained RT-PCR product of binding RNA species eluted from the previous round. Typically, the polyclonal RNA pool did not yield the cleanest single band because of abortive transcription of some nucleotides.

4.4. BIAselection: Isolation of gp120 aptamers

The most important aspect of the *in vitro* selection process (Figure 4.2) was the method by which gp120-binding RNA species were physically separated from non-binding species. There are no hard and fast rules for choosing the selection procedure. However, selections that target small molecules such as peptides are routinely performed on affinity columns containing immobilized ligands (Ellington and Szostak, 1990). Selections that target proteins and utilize RNA pools are often carried out by isolation of protein-aptamer complex on nitrocellulose filters that bind proteins (Tuerk and Gold, 1990), or by immuno-precipitation of the RNA-protein complex (Tsai et al., 1991).

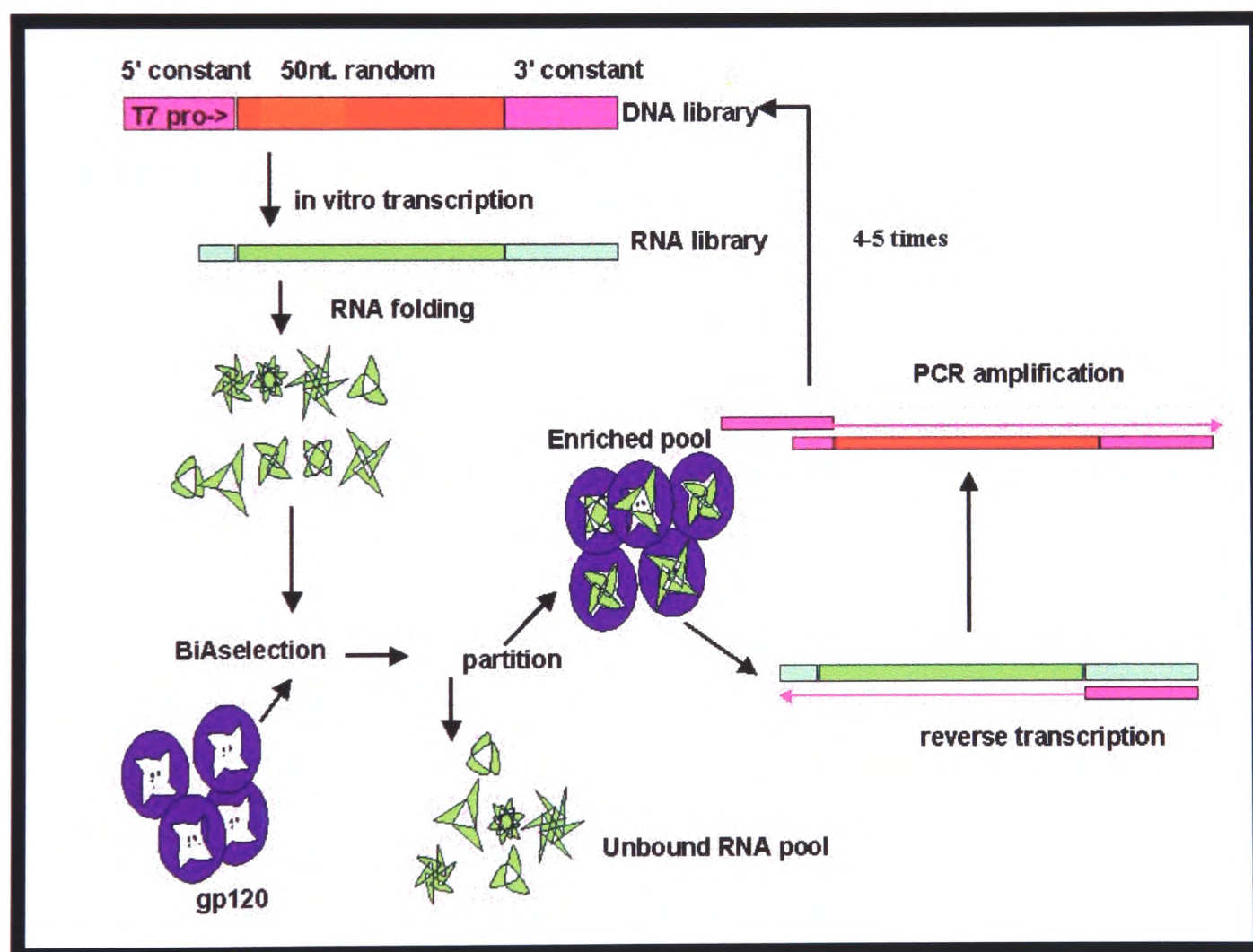


Figure 4.2: Schematic representation of the *in vitro* selection of RNA aptamers. Double stranded DNA consisted of 50 bases of random sequence flanked by defined regions. PCR-amplified DNA was transcribed into RNA, and RNA molecules of the desired property were selected. Bound RNA was eluted, converted to cDNA and amplified by PCR for the next SELEX cycle.

In this study a rapid BIAselection method, using the BIAcore™ surface plasmon resonance technology (discussed in section 2.7) was used for partitioning and isolation of gp120-binding aptamers. This method was used as an alternative, after I failed in 15 selection rounds to isolate any gp120-binding aptamers using the nitrocellulose partitioning method. In the first BIAselection round, 20 000 RU of HIV-1_{Ba-L} gp120 was immobilized by amine coupling to the CM5 (research grade) BIAcore sensor chip (BIAcore AB, Stevenage, UK). *In vitro* transcribed 2'F-RNA (section 2.6.3) was re-folded by heating at 95°C for 1–3 min in water, and cooled to room temperature for 10 min before being placed in the binding buffer as described in the methods. Partitioning was performed at 37°C, in HBS binding buffer (10mM HEPES, pH 7.4, 150mM NaCl, 1mM CaCl₂, 1mM MgCl₂, 2.7mM KCl). 20 µg of the RNA pool that represented a theoretical diversity of at least 10¹⁴ molecules was injected at 1 µl/min over the flow cell in which the gp120 was immobilized, to allow sufficient contact time between the ligand and analyte, during the first selection cycle. Nonspecifically bound RNA was removed by injecting 100 µl of the HBS buffer at 5 µl/min. Bound RNA was eluted with 100 µl of 7 M urea at 5 µl/min, and was deproteinized by extraction with phenol/chloroform and then precipitated with ethanol. Recovered RNA was reverse-transcribed to cDNA using 100 pmol of the T3SELEX5' primer, 1 × reverse transcriptase buffer, 0.5mM dNTPs, and 20 units AMV reverse transcriptase (USB) per 50 µl reaction volume. The reaction mixture was incubated at 37°C for 25 min. and 42°C for another 25 min.

The resulting cDNA was amplified using PCR under slightly mutagenic conditions (7.5mM MgCl₂, 1mM dNTPs, 1 000 pmol each of the primer pair, 1 × Mg free PCR buffer, and 6 units of *Taq* polymerase per 1000 µl reaction volume) to increase variation of the molecules and to give a new template for the next round of selection. The RNA: gp120 ratio was increased by a factor of about 4 each round to increase the stringency of selection. At every selection round the RNA was pre-cleared against at least two empty sensor chip flow cells to serve as controls, and also to reduce the inadvertent selection of aptamers that might bind the chip matrix. Immobilized gp120 initially bound less than 0.1% of the applied RNA, but after the fourth round of

selection, more than 70% of the applied RNA was bound (Table 4.1 and Figure 4.3 A). The proportion of RNA that bound the gp120 increased substantially at each selection round, and at round four, RNA aptamers that bound the gp120 with high affinity were isolated, and were enriched by a further selection round (Figure 4.3A and B). The cumulative amplification factor, which by definition is the inverse of the product of the proportion of bound RNA in each cycle (Ellington and Szostak, 1990), was about 1×10^6 after 5 BIAselection rounds (Table 4.1). Assuming that all of the molecules capable of specific binding to the gp120 were recovered in each cycle, dividing the estimated initial pool size by the cumulative amplification factor suggested that in the initial pool there were about 1 in 10^8 random RNA species that folded in such a way as to bind the gp120.

Table 4.1: The fraction of input RNA bound in each cycle ^a

Selection cycle	Fraction of input RNA bound
1	0.07%
2	1.5%
3	16%
4	60%
5	75%
Amplification factor ^b	1×10^6

^a The percentage of RNA retained per selection was calculated by dividing the amount of eluted RNA by the amount of RNA loaded. This was based on the assumption that the effective complexity of the starting RNA pool was 1×10^{14} and all the different RNA species capable of binding the protein were recovered in each cycle.

^b The 'amplification factor' is defined as the inverse of the product of the fraction bound in each cycle: $(\text{bound}_1/\text{total}_1 \times \text{bound}_2/\text{total}_2 \dots)^{-1}$

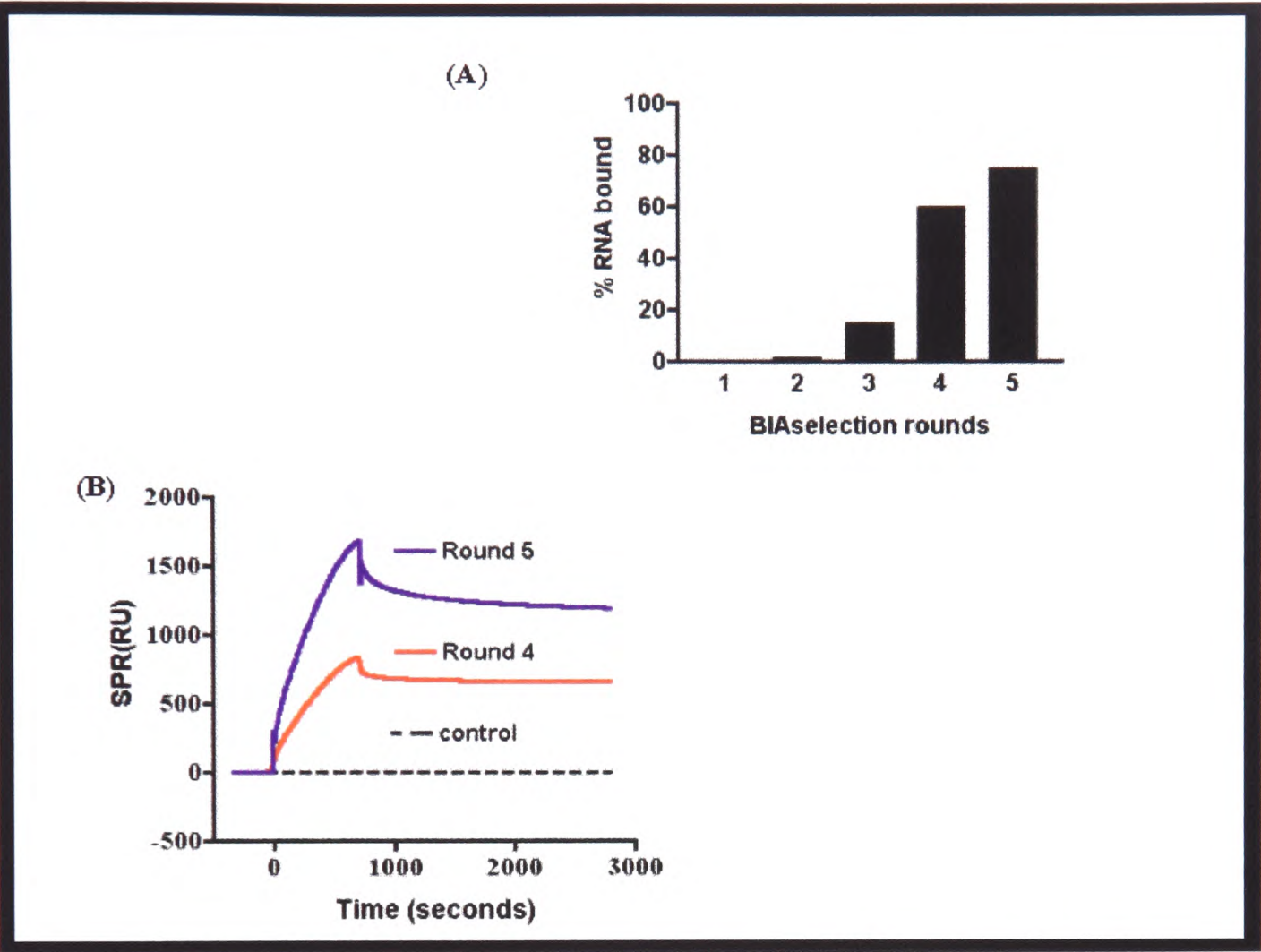


Figure 4.3: BIAselection of aptamers that bind the HIV-1_{Ba-L} gp120. (A) The fraction of RNA that bound the gp120 in each cycle. The percentage bound was calculated by dividing the amount of the RNA eluted by the amount originally injected. Multiple cycles of BIAselection and *in vitro* amplification resulted in the purification of binding species, until virtually the entire population was capable of binding the gp120. (B) The BIAcore™ sensorgram showing fourth and fifth round enriched 2'F-RNA aptamers against the gp120. The aptamers bound the gp120 with high affinities as reflected by their rapid on-rates and slow off-rates respectively.

4.5. Cloning and screening of monoclonal aptamers

4.5.1. PCR screening for insert positive clones

After the fifth round enrichment of binding species, the resulting PCR product was cloned using the TA cloning kit as described (section 2.4.1.9). The decision to clone after the fifth round of selection was made in order to strike a balance between diversity and high affinity aptamers. PCR conditions for cloning and subsequent screening procedures were altered to allow high fidelity amplification of the template and hence conserve the nucleotide sequence of the gp120-binding species. The final concentration of primers and dNTPs was reduced from 1 000 pmol and 1 mM to 50 pmol and 0.25 mM, respectively. The MgCl₂ final concentration was also reduced from 7.5mM to 3mM, and the annealing temperature was increased from 55°C to 72°C. No more than 10 PCR cycles, compared to 14 cycles in earlier round were required to yield a single band of interest for cloning under these stringent conditions, showing a decrease in the number of cycles in the subsequent round, and enrichment of the entire RNA pool. The TA cloned aptamers were used for bacterial transformation as described (2.4.2) and colony PCR using the T3SELEX5' and T7SELEX3' primer pair was performed to screen bacterial transformants for inserts. More than 85% of the 90 clones screened were insert positive (Figure 4.4). The results showed the simplicity and efficiency of the TA cloning kit.

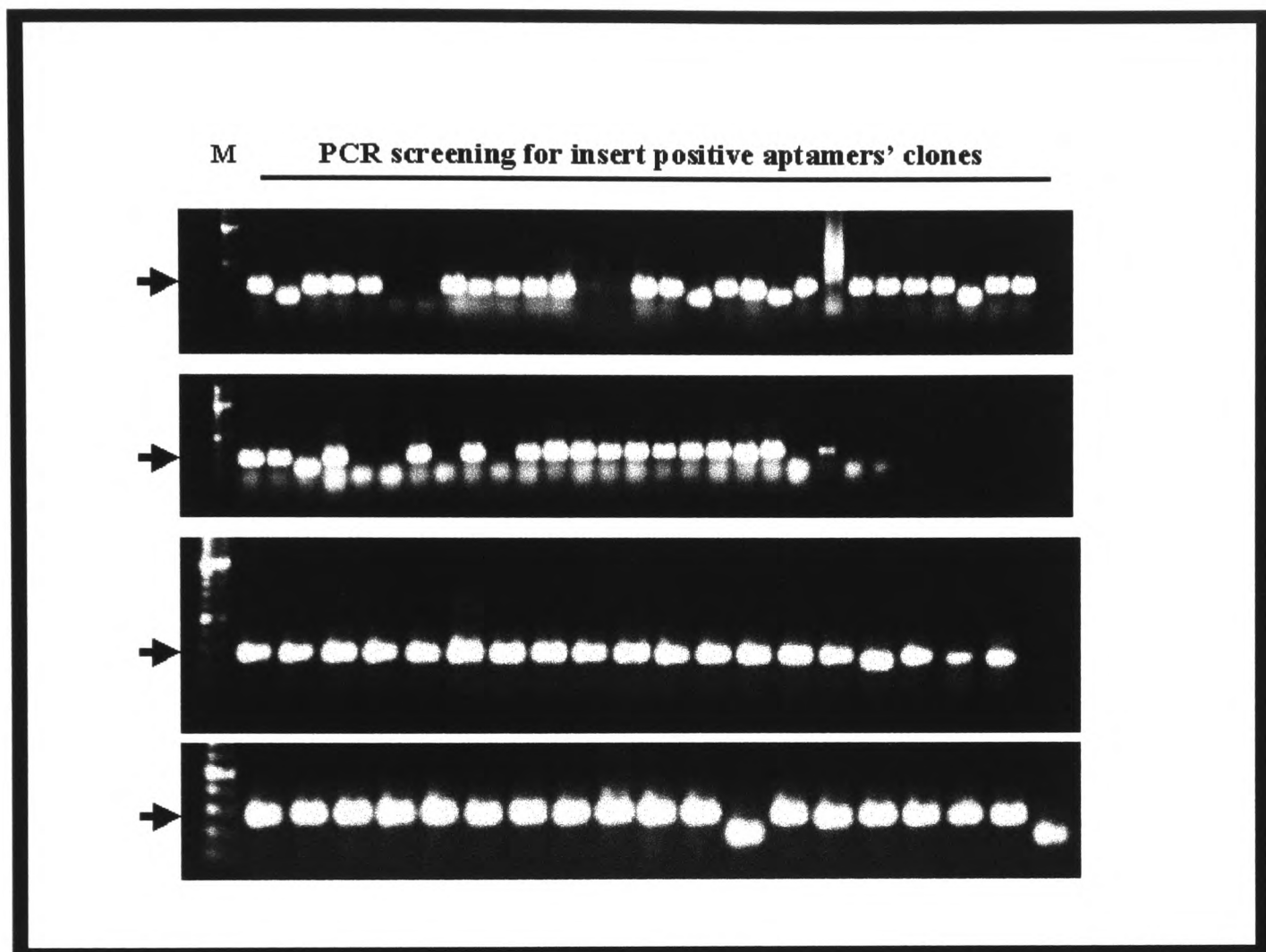


Figure 4.4: PCR screening for insert-positive clones that contain monoclonal aptamers. Arrows indicate the expected 134-mers double stranded DNA, which comprised over 85% of the 90 clones screened.

4.5.2. Activity of the synthetic FLAG peptide

Insert-positive bacterial colonies from replica plates were PCR-amplified and transcribed to allow screening of individual aptamer clones for binding to the gp120 and to the FLAG epitope, which was fused to the C-terminus of the recombinant protein. First, the activity of the synthetic FLAG peptide (2.5.6) was assayed. The FLAG peptide and HIV-1_{Ba-L} gp120 were immobilized by thiol and amine coupling, respectively (2.7.2) on different flow cells of a BIAcore sensor chip. Anti-FLAG M2 mAb (Sigma) diluted 1:1 000 (v/v) in the HBS BIAcore running buffer was injected over the coupled flow cells and the control flow cell. The antibody bound to both the FLAG peptide and the recombinant gp120 with similar association and dissociation

profiles, indicating that the synthetic peptide was in the native conformation and was able to be recognized by the anti-FLAG M2 monoclonal antibody (Figure 4.5).

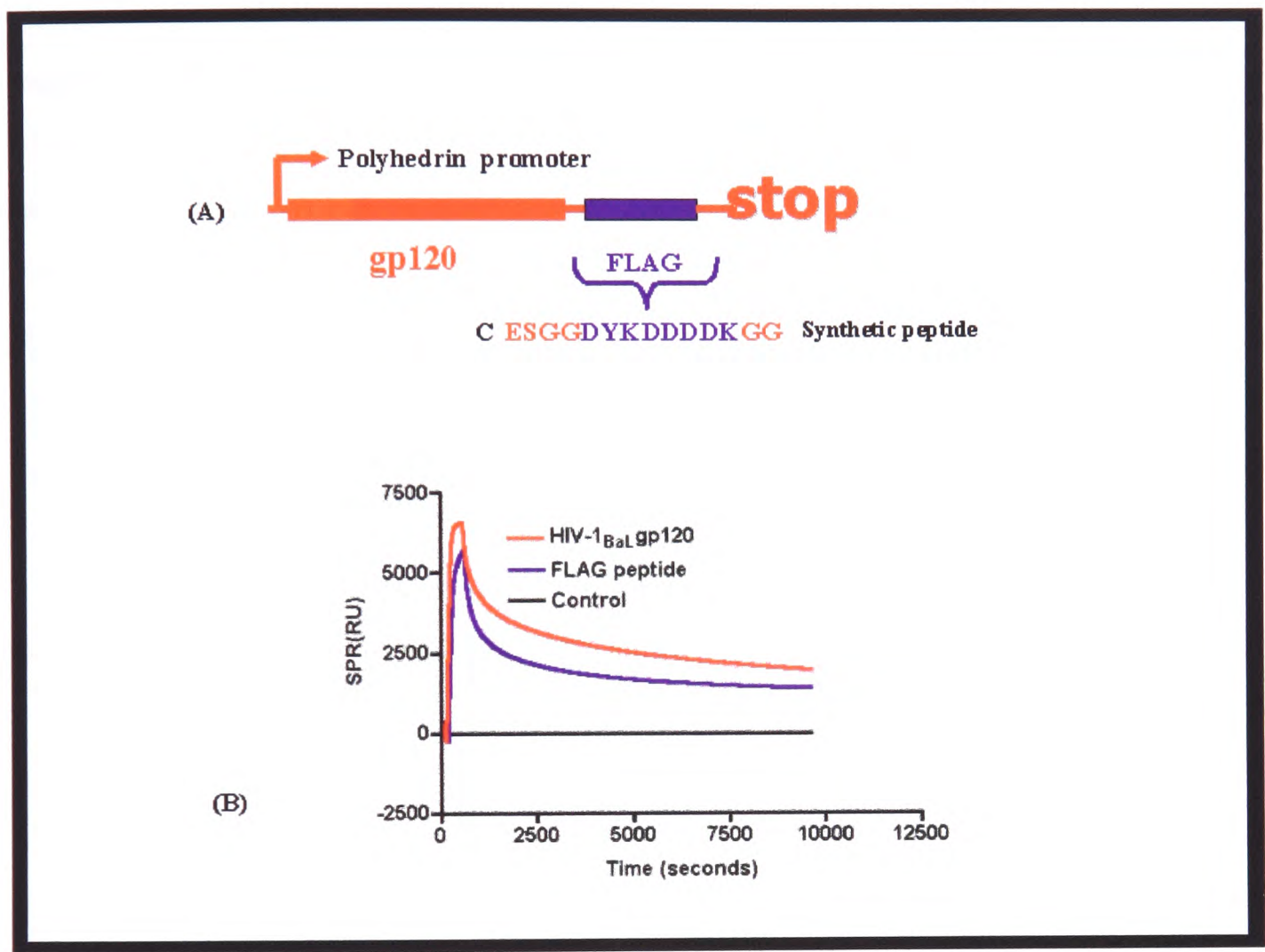


Figure 4.5: Activity of the synthetic FLAG peptide. (A) Recombinant gp120 construct cloned under the baculovirus polyhedrin promoter, with the FLAG peptide fused at the C-terminus. The FLAG recognition motif is shown in blue (DYKDDDDK), and the complete synthetic FLAG peptide had the motif flanked by ESGG and GG on the N- and C-terminus respectively, which also formed part of the recombinant gp120. The only additional residue not present in the recombinant protein was cysteine (C) that was included in the synthetic peptide to allow thiol coupling. (B) Binding of the anti-FLAG M2 monoclonal antibody to both the gp120 and the FLAG peptide as assayed by the BIAcore. A similar response was registered for both the peptide and the protein suggesting that the synthetic FLAG peptide was as active as the one expressed in the recombinant protein.

4.5.3. Screening of monoclonal aptamers binding to the gp120

Once activity of the synthetic FLAG peptide was established, individual aptamer clones were screened against the recombinant gp120 and the peptide. Immobilization matrix was also used, as a control. Screening of individual clones allowed elimination of any matrix-binding or FLAG-binding species from authentic gp120 binding aptamer species. Aptamer clones were arbitrarily numbered, with the prefix B, denoting Ba-L, the HIV-1 strain from which they were derived. All the screened individual aptamer clones selectively bound to the HIV-1_{Ba-L} gp120 (Figure 4.6). Thus none of the monoclonal aptamers bound the FLAG peptide or the immobilization matrix, suggesting specificity to the gp120. The results suggest that gp120-binding species were relatively abundant in the starting RNA pool and were preferentially isolated.

Although in most SELEX experiments, molecules that are amplified after each round of selection include a mixture of those that bound to a protein target and the immobilization matrix, this study indicated that the matrix-binding species were nonexistent. Pretreatment of the RNA pool, by passing it through the uncoupled biosensor chip matrix during BIAselection could have eliminated matrix-binding RNA molecules from the population. Interestingly, the results also indicated that the FLAG epitope that was fused to the recombinant gp120 did not compete with the protein for aptamers. It could be that FLAG epitope was not a good RNA aptamer target or the FLAG recognition motif was just too small relative to the gp120 epitopes that were recognized by the aptamers (aptatopes). Whatever the case the results were favorable in that the FLAG epitope did not interfere with interaction of aptamers with the gp120 nor did it compete for binding of aptamers.

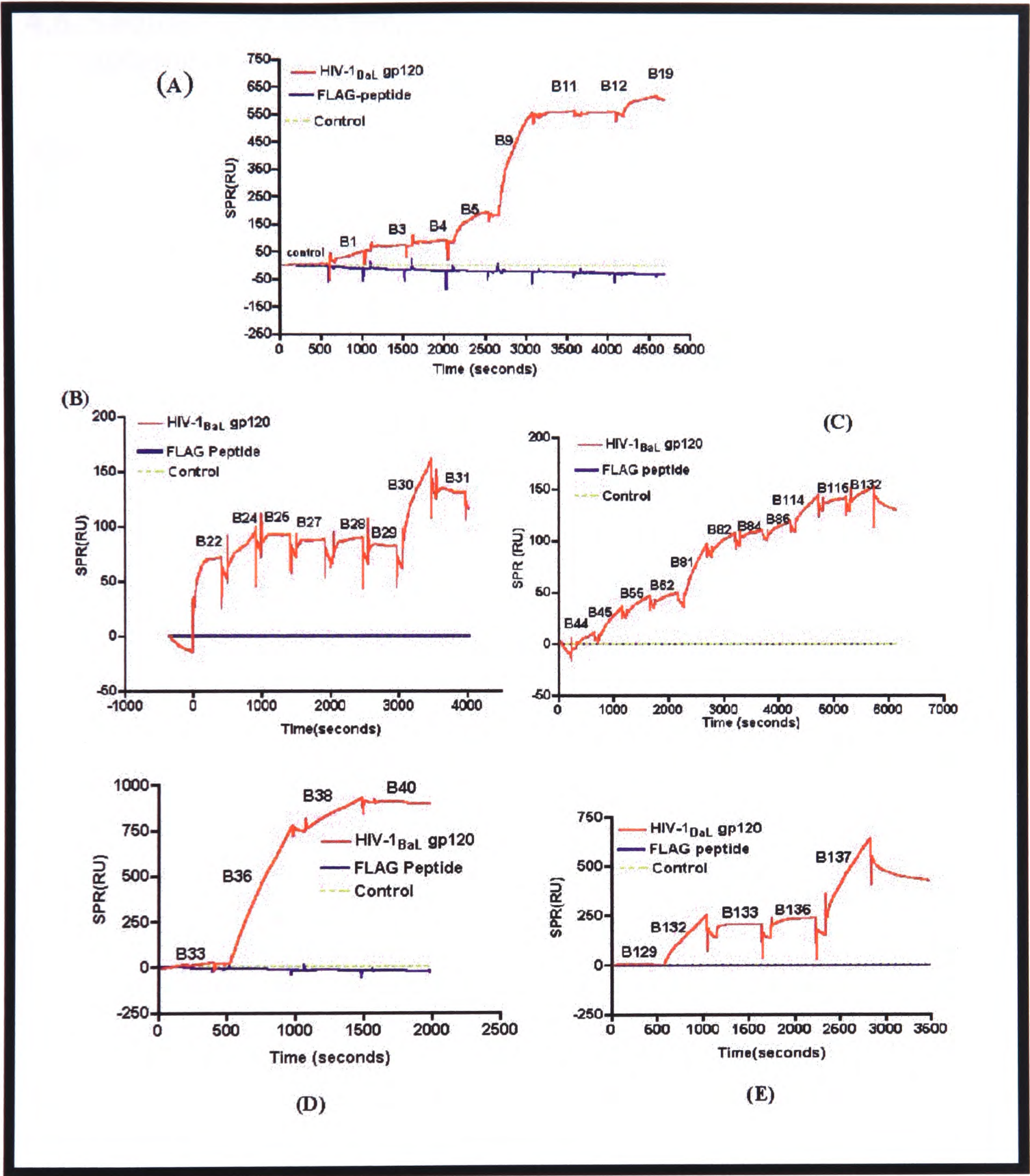


Figure 4.6: Screening of individual aptamer clones for binding to the gp120 (red) in relation to the FLAG epitope (blue) and the immobilization matrix, shown as control (golden broken line).

4.6. Sequencing and phylogenetic analysis of gp120 binding aptamer clones

Aptamer clones that selectively bound the HIV-1_{Ba-L} gp120 were sequenced as described (2.4.4) and compared to each other. Of the 27 clones that were sequenced, only two clones (B81 and B86) were identical, and the rest were unique, suggesting the gp120 binding aptamer species must have evolved independently from the random sequence ancestral library (Figure 3.7). Except for the two identical clones, all the other clones had no detectable similarity to any other clone, confirming the notion that there are many complex different ways of generating specific binding site for any given ligand. Nonetheless, careful sequence comparison and phylogenetic analysis revealed short regions of similarity between several pairs of aptamers, suggesting that those regions might be involved in gp120 binding (Figure 4.7).

Although almost all the monoclonal aptamers sequenced selectively bound to the HIV-1_{Ba-L} gp120, at least two clones namely B19 and B36 also showed significant binding to the gp120 derived from HIV-1_{IIIB}, an X4 virus, in addition to their cognate ligand (Figure 4.8). Thus despite aptamers being renowned to have high specificity and able to discriminate between enantiomers, these data implied that the two clones either could not discriminate between the two different Env glycoproteins, or they recognized epitopes on the gp120 that were structurally conserved between these two R5 and X4 strains. In addition there is also a limit to aptamers' specificity as could be expected. For instance aptamers against coenzyme A also recognized AMP (Burke and Hoffman, 1998), those against xanthine also recognized guanine (Kiga et al., 1998), and those against the disaccharide cellobiose also recognized cellulose which is a polysaccharide, but not the disaccharide maltose and lactose (Yang et al., 1998). Aptamer clones B19 and B36 therefore either define a structural conserved aptatope on HIV-1_{IIIB} and HIV-1_{Ba-L} Env glycoprotein or they also epitomize a limit to specificity by aptamers.

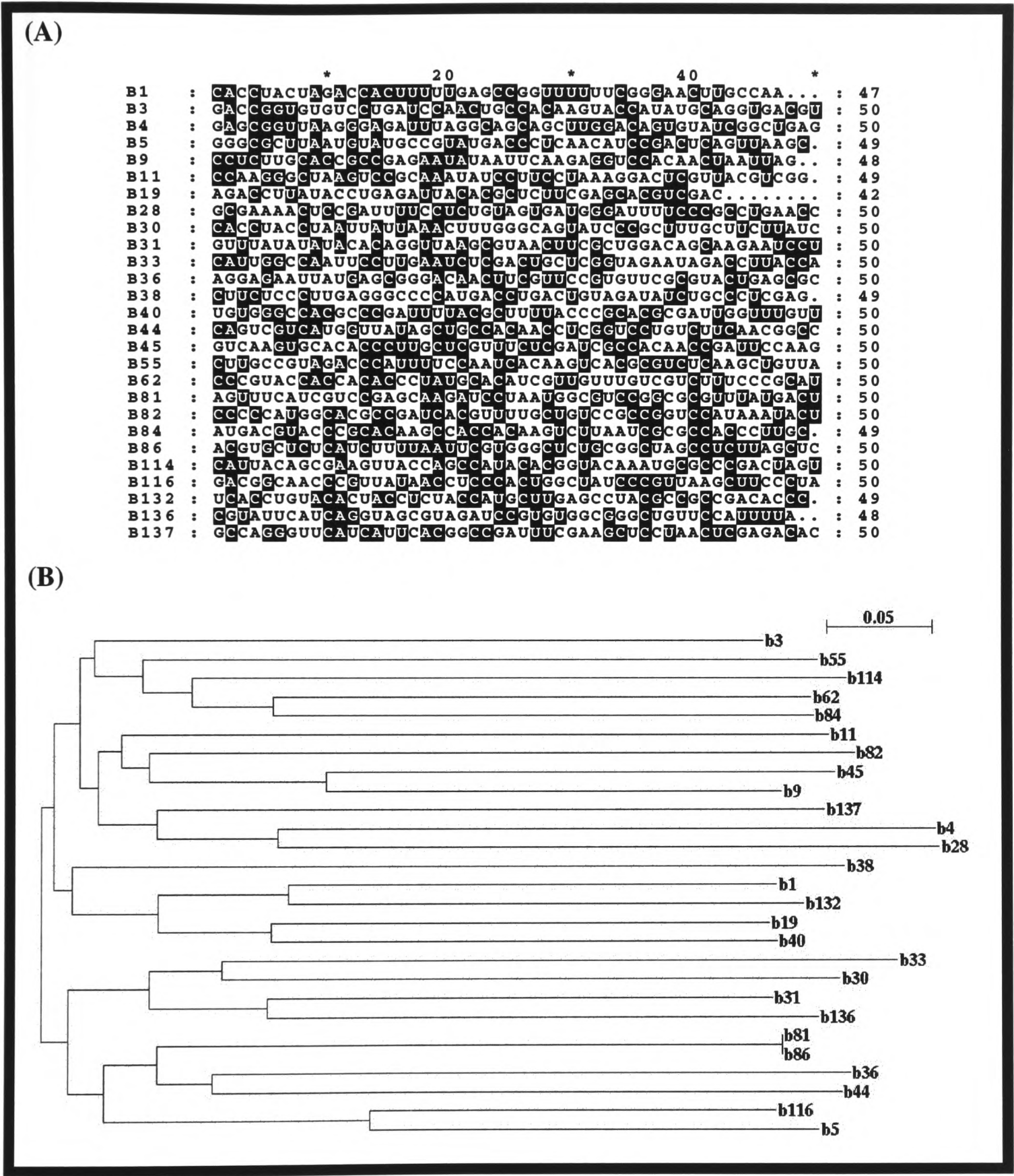


Figure 4.7: Sequence and phylogenetic analysis of the gp120 binding aptamer clones (A) Nucleotide sequence of the randomized region of the gp120 binding aptamer species. Shown in black are residues or short regions of similarity between several pairs of aptamers that might be involved in binding. (B) Phylogenetic tree of the gp120 aptamers clones, depicting identical (B81 and B86) and similar clones (e.g. B62 and b84). The lengths of the branches are drawn proportional to the number of sequence changes in the branch, and predict the evolutionary relationship.

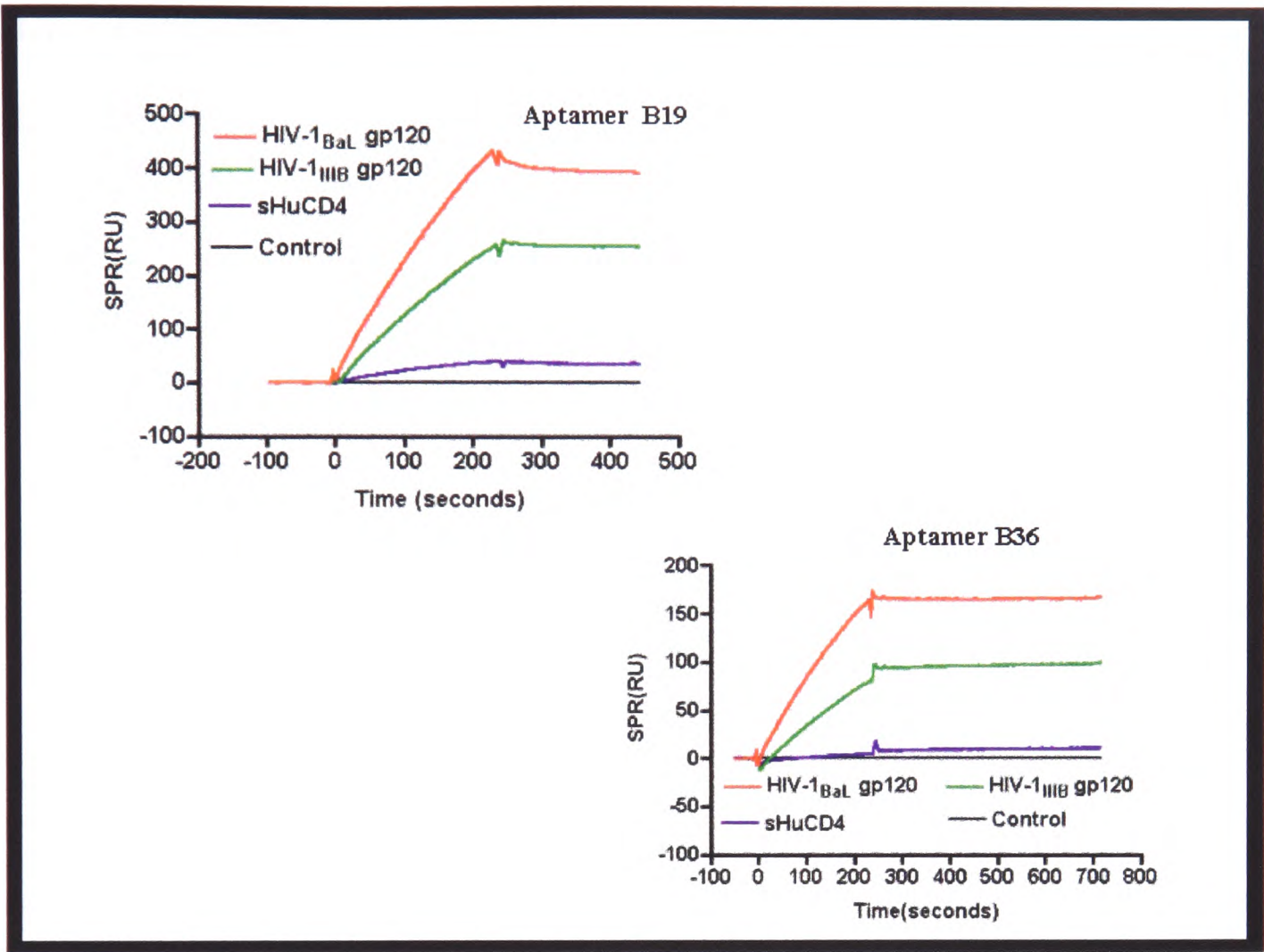


Figure 4.8: Cross-binding of B19 and B36 to HIV-1_{IIIB} in addition to HIV-1_{Ba-L}. Both clone B19 and B36 bound to the gp120 derived from HIV-1_{IIIB} (green) and HIV-1_{Ba-L} (red) with high affinities as shown by their rapid on-rates and very slow off-rates. The binding was specific to the two Env proteins, as there was no binding to soluble human CD4 (blue) or the immobilization matrix (black).

4.7. Discussion

4.7.1. BIAselection: a method of choice for finding a needle in the haystack

This chapter described the use of a novel partitioning method for isolation of the gp120 binding aptamers. The separation of desired gp120 binding RNA species from a diverse RNA pool (10^{14}) was analogous to finding a needle in the haystack, and as such constituted the critical part of this study. The ideal SELEX and partitioning protocol would be one that allowed the RNA pool and the target to interact freely, be able to monitor the progress of the interaction in real time, be able to retain and elute binding species efficiently, and be able to carry the entire SELEX process in short period. The BIAselection method used in this study largely fulfilled these criteria. Immobilization of the gp120 to the biosensor chip concentrated the protein to a confined chip surface, and facilitated the separation of bound from unbound RNA species, and also facilitated elution, all of which were monitored in real time. The relatively small size of the biosensor chips (70 nl) allowed use of modest amounts of the gp120, and also allowed generation of the ligand. Although conventional SELEX process usually requires several rounds (up to 16 cycles), and several months before binding species can be isolated, only 5 rounds using the BIAselection method were required and the entire process was reduced to a few weeks. As expected, there are drawbacks associated with the BIAselection method.

First, many protein immobilization regimes involve derivitization of the ϵ -amino groups of lysine, thereby spoiling a key feature of many aptotopes. Second immobilization reduces flexibility of the proteins, thereby could impede mixing of the protein with the RNA pool. Third, a progressive reduction in the molar ratio of target protein to RNA pool in successive cycles, in order to increase the stringency of selection, can be hard to achieve in immobilization systems. Also, the contact time between molecules screened and the target is significantly reduced in the BIAselection method, suggesting that mostly molecules with rapid on-rates and slow off-rates will be retained. Although these drawbacks are valid, at least in theory, they were not

much of a problem in isolating the aptamers against the gp120 using the BIAselection method.

In this study the RNA pool: gp120 ratios were adjusted based on the response unit of the immobilized target. In the first round of selection, a relatively low RNA pool to gp120 ratio (about 10:1) was used in order to amplify and fix binding species in the population prior to applying more stringent selection conditions. In subsequent rounds, the stringency of selection was increased by increasing the ratio of RNA pool to the gp120 by a factor of 4 each round, and these resulted in enrichment of binding species in each round. Higher RNA to gp120 ratios encouraged equilibrium competition between species and isolation of high affinity aptamers, whereas lower ratios allowed species with lower affinities to remain in the pool.

Buffer conditions under which partitioning was carried out was also crucial in the BIAselection process because it determined which species would be isolated. Higher concentrations of monovalent cations suppress nonspecific binding to a target, while higher concentrations of divalent cations such as magnesium, facilitate RNA folding and increase both specific and nonspecific binding to a target (Conrad et al., 1996). In this study a modified HBS binding buffer (10mM HEPES, pH 7.4, 150 mM NaCl, 1mM CaCl₂, 1 mM MgCl₂, 2.7 mM KCl) was used. The buffer contained relatively high concentrations of NaCl and KCl (monovalent cations) to suppress nonspecific binding of RNA to the gp120 target. The magnesium concentration was empirically determined to be optimal to allow folding and binding of RNA, and for selection of aptamers against almost all desired targets (Ellington and Szostak, 1990). A very important aspect about the binding buffer is that aptamers can only form binding complexes in the buffer in which they were originally selected. Alteration of the buffer conditions for selection leads to an alteration of aptamer conformation as epitomized by cyanocobalamin (vitamin B12) aptamers (Lorsch and Szostak, 1994).

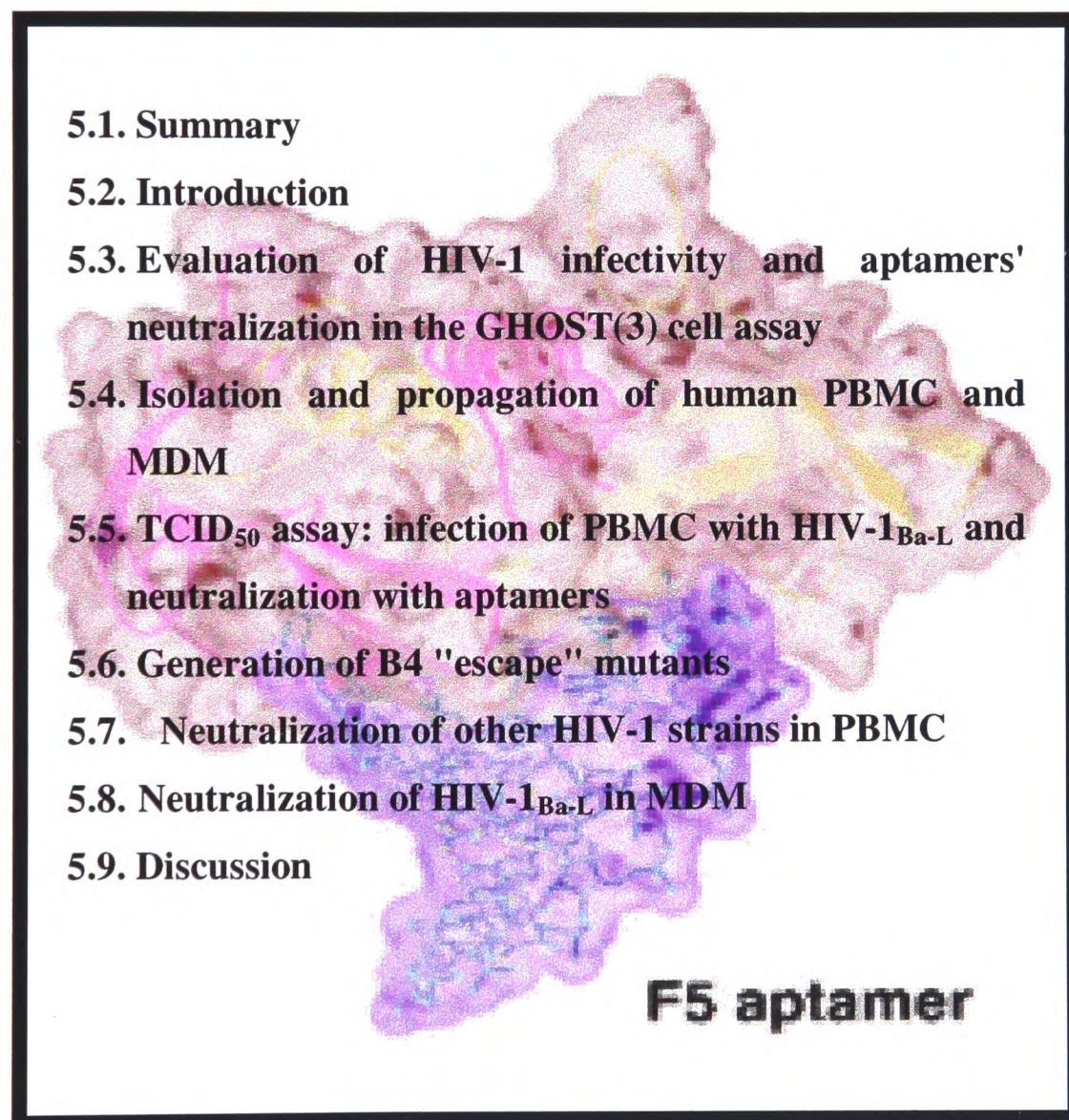
4.6.2. Implications

The probability that a given random sequence of RNA molecules will form a stable three-dimensional structure capable of binding to a molecule of interest like the gp120 is unknown, but is generally thought to be very low. A related problem, which has been equally difficult to address theoretically, is to estimate the number of fundamentally different families of RNA species capable of binding to a target molecule. In this chapter it has been estimated that an RNA pool with a theoretical diversity of 10^{14} molecules, of average length 50 nucleotides, potentially contains 10^6 different sequences capable of specific binding to the gp120. Because the total possible number of sequences 50 bases in length is 10^{30} , even a pool of 10^{14} molecules contains only a small fraction of all possible sequences (10^{-16}). Therefore the total theoretical number of different sequences that can bind to the gp120 must be around 10^{22} . So far, 26 unique sequences that selectively bind the gp120 have been identified.

The question is therefore how many of these sequences represent truly independent species, with different three-dimensional structural conformations, and different binding sites on the gp120. Previous studies have shown that when the aptamer-binding sites on large target proteins are mapped, it has usually been found that they are coincident, even if aptamers fall into unrelated sequence families. For example four distinct aptamers against HIV-1 reverse transcriptase bound exactly the same region of the enzyme (Burke et al., 1996). Also, in our laboratory, when five different sequence classes of aptamers against rat CD4 were analyzed, all mapped to a single region of one of the four immunoglobulin-like domains (Kraus et al., 1998). Therefore, it could be that although most of the aptamers isolated in this study have distinct sequences, they might bind the same region of the gp120. Besides, the HIV-1 neutralization studies using these aptamers have shown that more than 60% of the aptamers neutralize the virus by the same magnitude (Chapter 5), consistent with the suggestion that they might bind to the same aptatope on the gp120. The apparent complexity of the selected gp120 binding aptamer species implies that there are many independent "sequence" solutions to the gp120 problem which is crucial in HIV-1 entry and AIDS pathogenesis.

Chapter Five

Evaluation of aptamers in functional assays: neutralization of HIV-1



*Background: structure of the F5 RNA aptamer complexed with bacteriophage MS2 coat protein showing van der Waals surfaces of protein (yellow) and RNA (blue).
(Source: (Parrott et al., 2000))*

5. Evaluation of aptamers in functional assays: neutralization of HIV-1

5.1. Summary

HIV-1 has evolved impressive adaptive strategies in response to the anti-retroviral drugs and selection pressures of the humoral arm of the immune system. The structure of the envelope glycoprotein in particular has evolved to mediate virus entry while evading the neutralizing antibody response. In order to penetrate these antibody-selected evolutionary adaptations, I isolated aptamers against gp120 (chapter 4) that neutralized the infectivity of the homologous virus by up to 100 000-fold in PBMC. One aptamer, called B4, defined a conserved site of the gp120 that could not mutate to escape neutralization following stringent selection for break-through virus. Most intriguing, although B4 completely (100 000-fold) neutralized infection in PBMC grown as a mixed T cell/macrophage population, it modestly (≤ 10 -fold) neutralized infection in purified macrophages cultured and differentiated alone. These results indicate that B4 in particular has the potential to provide a unique insight into the function of sites on the gp120 that interact with the susceptible cell surface in ways that have not been revealed heretofore. In general the results suggest that HIV-1 neutralizing aptamers such as those described in this chapter could be exploited to provide leads in developing alternative anti-retroviral drugs, and a deeper understanding of the molecular interactions between the virus and its host cell at entry.

5.2. Introduction

The current generation of anti-retroviral drugs such as HAART represents a milestone in the battle against AIDS. However, while these drugs have prolonged the quality of life for many HIV-positive individuals, they alone will not be sufficient for HIV-1 eradication (Finzi et al., 1999; Tomaras and Greenberg, 2001). Even in wealthy, industrialized countries where current anti-retroviral therapeutics are readily available, poor toleration of the drugs and the emergence of resistant viruses make long-term responses to antiviral treatment far from universal. Containment of the virus requires a much deeper understanding of the entry mechanism and new classes of antiviral drugs such as entry inhibitors, in addition to those that inhibit HIV-1 reverse transcriptase and protease.

Binding of the viral gp120 surface envelope glycoprotein to target cell-surface receptors (discussed in 1.5.1.–1.5.3) is a crucial step in receptor-dependent infection by HIV-1. Blocking the receptor-binding sites on some or all gp120 molecules on the virion should conceptually inhibit infection. Consequently, competitive gp120-targeted inhibitors such as soluble CD4, synthetic CD4 peptides and lately recombinant proteins like CD4-IgG2 (reviewed in 1.5.4) have been investigated *in vitro* and *in vivo*, and the molecular mechanism and clinical efficacy of some of these inhibitors are being explored further. As an innovative approach, in this study I quantitatively examined blocking HIV-1 entry into human PBMC and monocyte-derived macrophages by anti-gp120 aptamers with the view of dissecting further the complex interaction between the virus and macrophages at entry, and using the aptamers as structural leads for AIDS prophylaxis.

5.3. Evaluation of HIV-1 infectivity and its neutralization by aptamers in the GHOST (3) cell assay

Virus neutralization is defined as the reduction of infectivity *in vitro* by antibodies or by molecules that bind the gp120 like the aptamers, in the absence of other inactivating factors such as the complement or antibody-dependent cellular cytotoxicity (ADCC) accessory cells. Quantitative evaluation of HIV-1 infectivity was fundamental to this study. Infectivity is generally measured by allowing single infectious units of virus to replicate until their progeny reach a detectable level. These are assayed by biochemical or immunological tests or by formation of plaques, which allows the titer of viral preparation to be calculated directly. However many standard techniques of virological analysis are restricted with HIV-1. For instance, single step growth curves and infectivity assays are difficult to perform, owing to the slow growth and fragility of HIV-1. HIV-1 is also not readily amenable to plaque assay and does not produce sufficiently clear cytopathic effects because of its slow growth rate and a restricted host range. There have however been attempts to develop a plaque assay for HIV-1, as plaque assays are more convenient and accurate. These have used continuous cell lines and monitor focus formation in a variety of ways. Some depend on the appearance of c.p.e. or syncytia (Harada, Koyanagi, and Yamamoto, 1985; Nara et al., 1987) while others use more sensitive quantitative focal immunoassay (Chesebro and Wehrly, 1988) or staining (Matsui et al., 1987) to detect noncytopathic isolates. Pseudotype assay (Gregory et al., 1993) and transfection of a *gpt* resistance gene and transactivation of β -galactosidase (Kimpton and Emerman, 1992; Page, Landau, and Littman, 1990) have also been used to detect viral infection. In spite of these ingenious methods, all are limited by their dependence on continuous cell lines, which precludes the measurement of most primary isolates and R5-tropic viruses.

In this study, I attempted to exploit the utility of the GHOST (3) indicator cells as a rapid and simple way to quantitatively measure HIV-1 infectivity, and to evaluate the ability of aptamers to inhibit or reduce the infection. GHOST (3) cells are derived from the human osteosarcoma cell line, HOS, and have been engineered to stably

express CD4 and one of several chemokine receptors, rendering them permissive to infection by all primary isolates and strains of HIV-1, HIV-2, SIV, or pseudotype virus (Cecilia et al., 1998; Morner et al., 1999). GHOST (3) cells carries the HIV-2 LTR-driven GFP gene, which becomes activated upon infection with either HIV-1 or HIV-2. The GHOST (3) parental and GHOST (3) Hi-5 cells were propagated and maintained in DMEM supplemented with 10% FCS and antibiotics as described (section 2.3.1). One day before infection 12-well plate were seeded with 5×10^4 cells/well in 2 ml medium. For immunocytochemistry, cells were seeded as described on 13 mm diameter coverslips. Cells were infected with 500 μ l/well of HIV-1 stock (at m.o.i. of 0.5) as described (2.8.4) and incubated overnight for a single round of infection. After an overnight incubation, cells were washed once with 1ml PBS, and 1 ml of fresh medium was added and cells were incubated for a further 24 hours. 48 hours post infection cultures were harvested and prepared for fluorescent microscopy as described (2.9). Thus in the GHOST (3) cell assay, GFP becomes activated upon synthesis of Tat in infected cells, and since this is the early event in the virus replication cycle, infected cells were readily detected 48-72 hours post infection. The simplicity and speed of the readout are the main advantage of the GHOST (3) cell assay, and conceptually make it particularly suitable for screening a large number of HIV-1 isolates. Qualitative data were obtained by simple fluorescent microscopic observation, 48 hours post infection. The observed mean GFP fluorescent intensity of the positively infected cells was about 10-fold greater than mock infected cells, and the observed proportion of the GFP fluorescence-positive cells was 2-5% per well (Figure 5.1).

A more quantitative data was obtained by instrumental measurement using a flow cytometer. GHOST (3) cells infected with HIV-1_{Ba-L} in parallel with those observed by fluorescent microscopy were analysed on a FACS[®] machine 48 hours post infection. The sensitivity of HIV-1_{Ba-L} to a panel of four monoclonal aptamers was also evaluated in the GHOST (3) Hi-5 cell assay using FACS[®] as the quantitative readout system. The GFP fluorescence intensity of 10^4 GHOST (3) cell population selected on the side scatter-forward diagram was measured using the FACScan

(Beckton Dickinson, USA), and compared to controls. Controls were mock-infected cultures from the corresponding coreceptor-expressing GHOST (3) cell lines. To obtain a quantitative way of evaluating the efficiency of infection I took into consideration both the percentage of GFP fluorescence-positive cells (%) and the mean fluorescence intensity (FI) measured for each sample. The two parameters were multiplied for each sample and the difference from control cells calculated as $RTCN = (\% \times FI)_{HIV} / (\% \times FI)_{control}$, where RTCN (ratio to cell negative) gave a quantitative measure of infection. The FACS[®] histogram statistics as evaluated by the Cell Quest software, resulted in the RTCN value of 1.5 for the control, in which the GHOST (3) Hi-5 cells were infected with HIV-1_{Ba-L} only. (Figure 5.2). The data simply indicated that there was no difference in the percentage of GFP fluorescence-positive cells and the mean fluorescence intensity between mock-infected and HIV-1_{Ba-L} infected GHOST (3) Hi-5 cells. Thus the constitutively expressed background GFP in mock-infected GHOST (3) Hi-5 indicator cells was not different from the HIV-1_{Ba-L} induced GFP expression as measured by FACS[®]. This result invalidated data obtained using aptamers to evaluate sensitivity of HIV-1_{Ba-L} (Figure 5.2). RTCN value of less than 5 in the control was considered negative and therefore the GHOST (3) cell assay failed as a reliable quantitative method to measure HIV-1 infectivity and sensitivity to aptamers. Thus although others reported success with the GHOST (3) indicator cell assay as a quantitative way to measure HIV-1 infectivity and evaluation of coreceptor use (Vodros et al., 2001), the assay proved not reliable in my hands, for one reason or another.

A major disadvantage of the GHOST (3) Hi-5 cell line used in this study is that they constitutively expressed detectable levels of endogenous GFP, thereby adversely affecting the readout system. Another apparent disadvantage of the system is that the infection efficiency was low, about 2-5% as observed by fluorescent microscopy and measured by FACS[®]. The infection protocol supplied with the cells or as generally used (Cecilia et al., 1998; Morner et al., 1999; Vodros et al., 2001) called for use of 20 µg/ml polybrene to enhance infection efficiency, however I elected not to use polybrene in order to keep the conditions as natural as possible. Thus as the eventual

intention was to study the interaction of virus with host cell receptors and their inhibition by novel ligands, I felt that the use of an agent that modulates the efficiency of infection by providing non-natural infection between virus and cell would be ill-advised. Following unsatisfactory results using the artificial GHOST (3) cell assay I opted for the more physiological cell system, primary human PBMC.

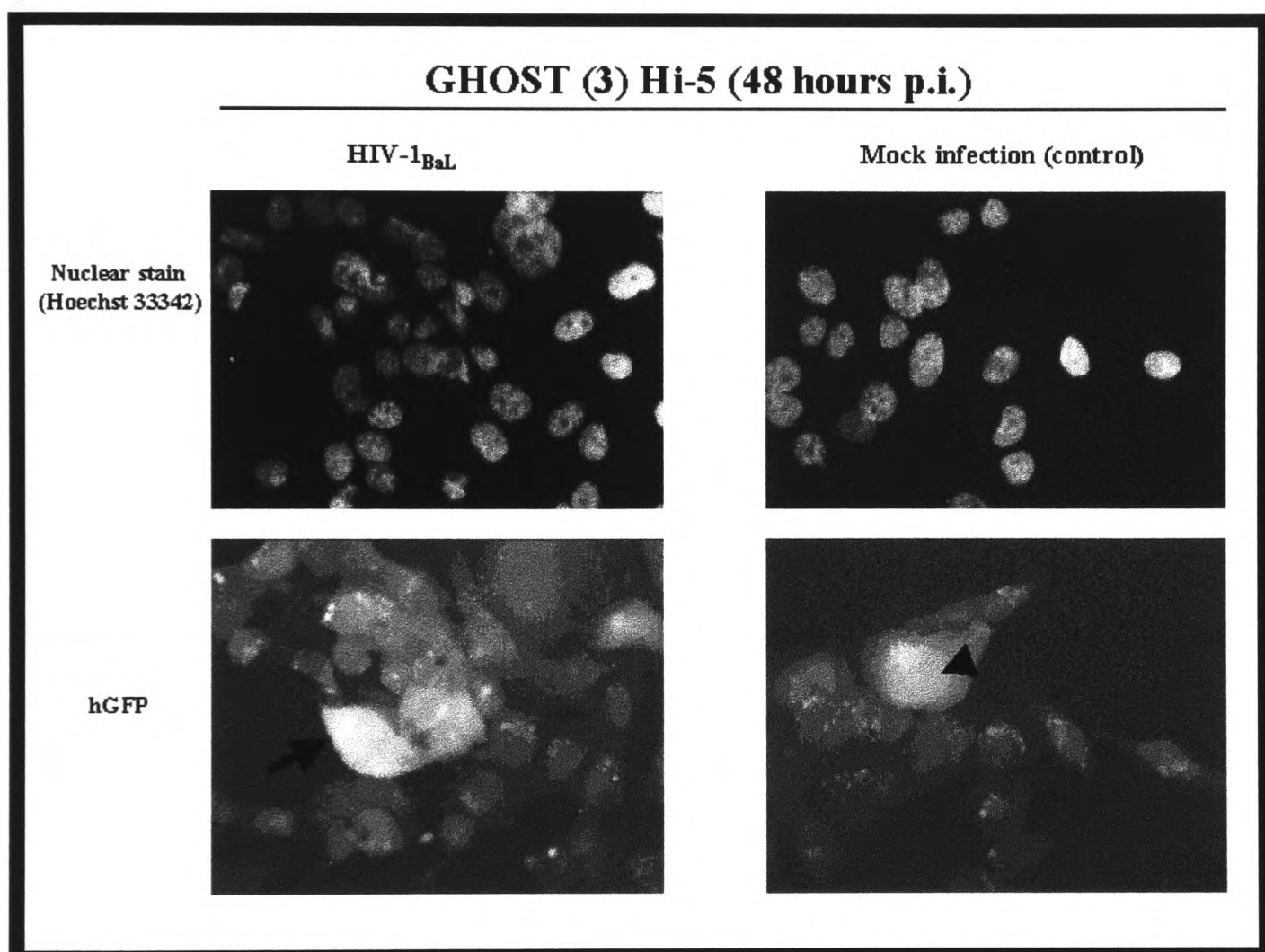


Figure 5.1: Fluorescent microscopic evaluation of GHOST (3) Hi-5 cells infection with HIV-1_{BaL}. Infection and viral entry was followed by GFP expression (arrow). Mock-infected cells constitutively expressed low levels of GFP (arrowhead), about 10 × less than virus infected cells. Hoechst 33342 nuclear stain enabled accurate enumeration of the cells. The mean proportion of GFP fluorescence-positive cells was 2-5% per well of infected cells.

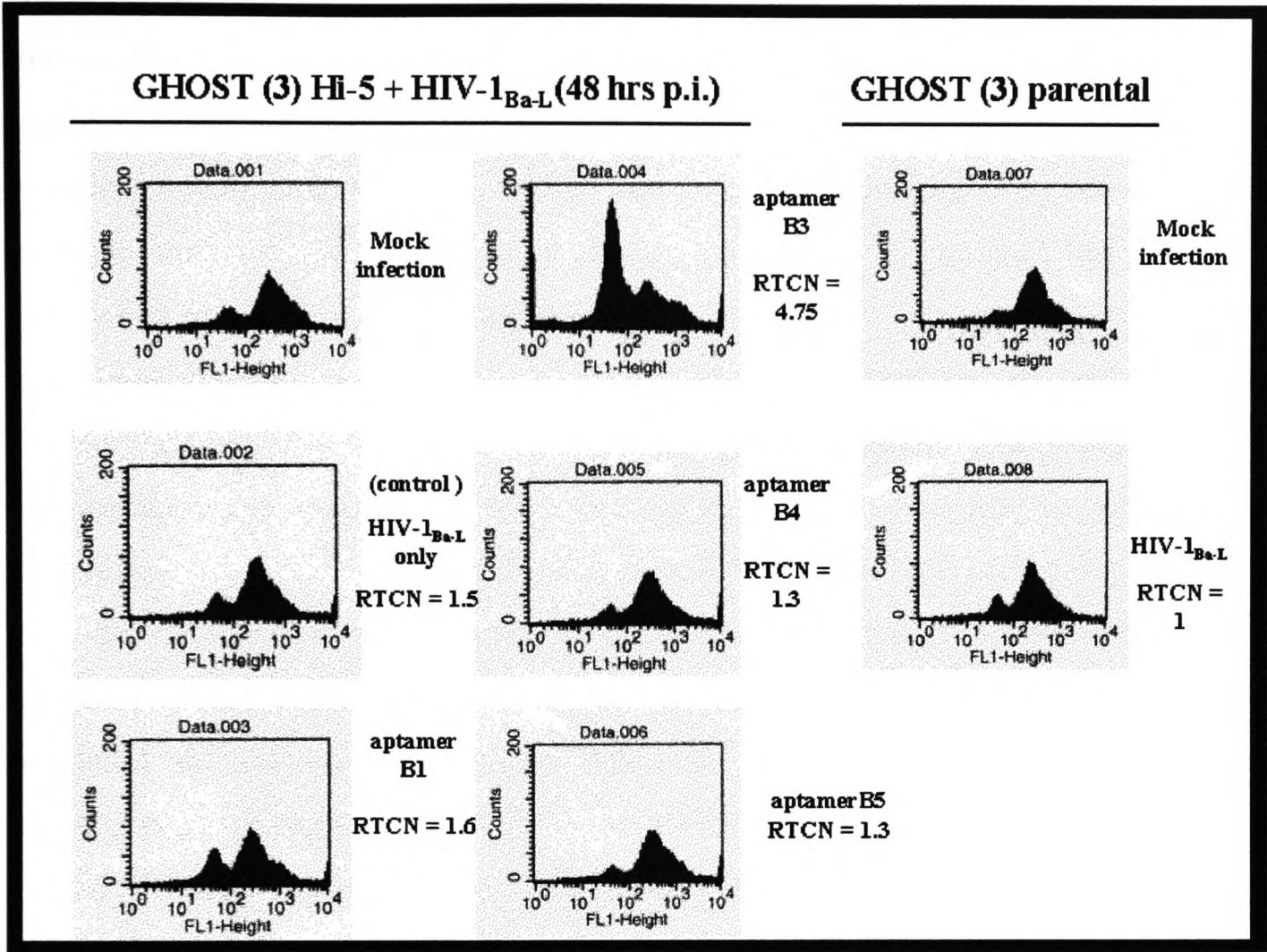


Figure 5.2: Quantitative evaluation of HIV-1_{Ba-L} infectivity as measured by GFP expression and sensitivity to aptamers using the GHOST (3) indicator cell assay. The ratio to cell negative (RTCN) value for the control is close to unity (1.5), indicating negative results and rendering the evaluation of data using aptamers as invalid. RTCN is a function of the proportion of the product of GFP-fluorescence intensity and the percentage of fluorescence-positive cells in virus infected and mock infected cells. Thus there was no difference in the percentage of GFP fluorescence-positive cells and the intensity of fluorescence in HIV-1_{Ba-L} infected and mock-infected GHOST (3) Hi-5 cells. Control GHOST (3) parental cells did not show the difference in GFP expression as expected.

5.4. Isolation and propagation of human PBMC and MDM

Human PBMC were isolated from buffy coats of HIV-negative donors and cultured as described in the materials and methods (2.3.2 – 2.3.5). Pure populations of monocytes suitable for work with R5-tropic HIV-1 were isolated from PBMC by adherence to a substrate and the nonadherent cells (mostly PBL) were removed. The degree of enrichment and subsequent detachment of the monocytes depended on the substrate and culture medium used. Optimization was difficult because purity and yield were opposing factors, the purest preparations being those mostly firmly adherent. In addition there was variation in attachment, viability and differentiation between cells from different donors, especially if cultured in RPMI-1640. The uniformity of cultures from different donors was improved by culturing the cells in X-VIVO 10 medium supplemented with 2% autologous serum as described (2.3.4). Gelatin-coated dish compared to tissue culture plastic provided a better substrate for isolation of monocytes by adherence, although monocytes left longer than 24 hours without replating detached spontaneously. Preparing monocytes on this surface is well documented (Collman et al., 1989; Hassan, Campbell, and Douglas, 1986; Hassan, Cutilli, and Douglas, 1990; Jones et al., 1989), but there are many variables, the method as performed in this study is described (2.3.3.).

On average, a yield of 2 to 6×10^6 monocytes/ml of buffy coat was harvested, subject to donor variability. Differentiation of adherent monocytes into monocyte-derived macrophages was associated with increased cell size and a more heterogeneous cell population (Figure 5.3A). Differentiated MDM cell population was characterized with mouse mAbs against surface antigens, principally to assess the contamination of macrophages by $CD4^+$ T cells, which are permissive to all isolates of HIV-1. Staining of MDM for flow cytometry was performed as described (2.3.5). Purity of MDM as assessed by cell specific markers and FACS[®] analysis was over 95% (Figure 5.3B). The FACS[®] histogram statistics showed that only 2.6% of the 10^4 cells analysed were positive for contaminating $CD3^+$ T lymphocytes. No cells stained positive with the mouse-anti-human mAb CD14, suggesting that CD14; a monocyte specific marker

was downregulated as monocytes differentiate into macrophages (Figure 5.3B). Almost 97% of day 7 MDM stained positive for the MHCII marker, although the histogram was broad, indicating that MDM was a heterogeneous cell population (Figure 5.3B). Using the described technique for MDM isolation, cell purity of over 95% was standard. The experience I acquired from routine isolation of MDM enabled me to assess the purity of the cells just by phase contrast microscopy. A further biological control for purity of MDM assessed by phase contrast microscopy was reinforced by the consistent inability of X4 viruses adapted to T cell lines to productively infect these purified cultures.

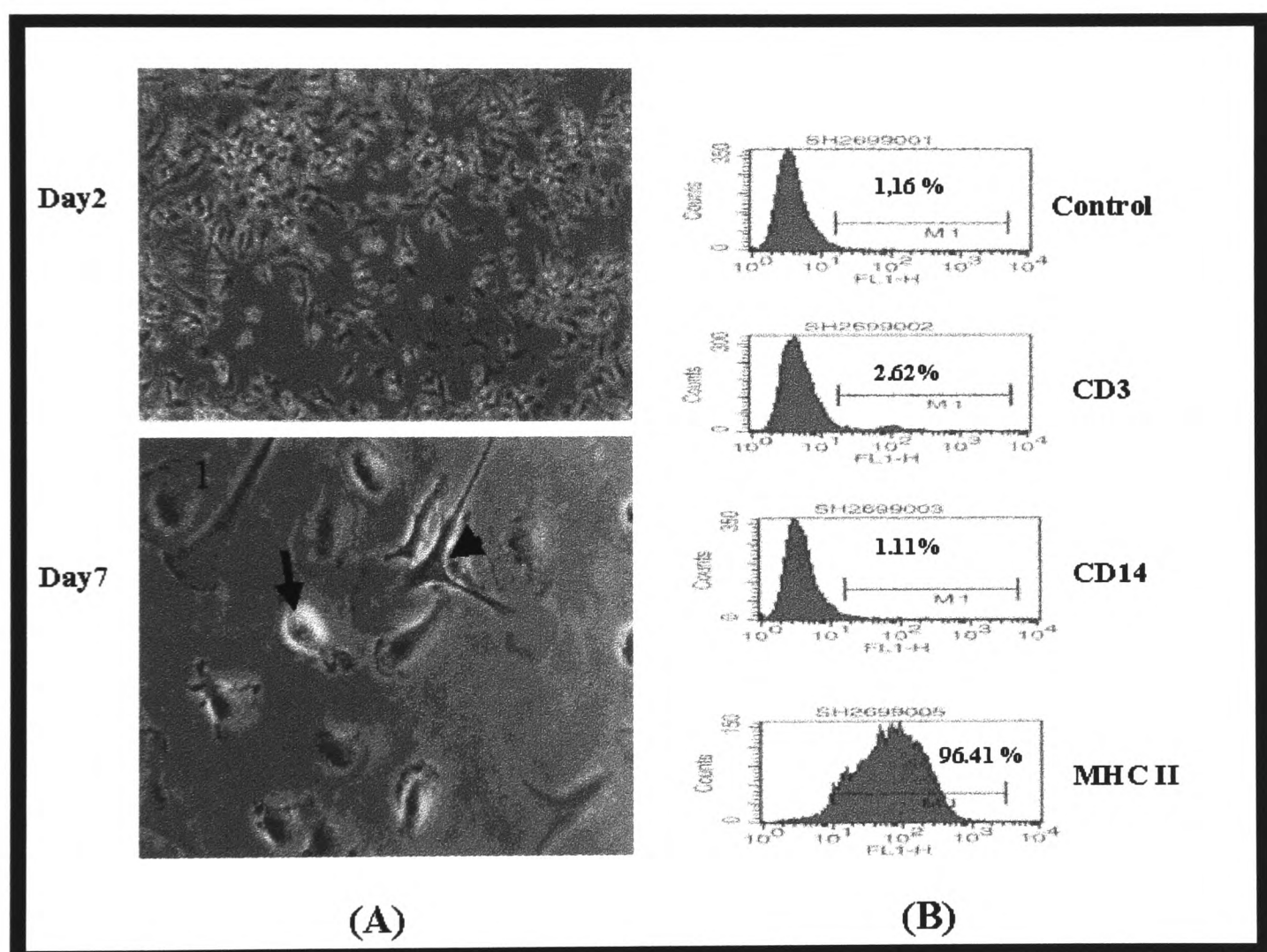


Figure 5.3: Morphology and purity of monocyte-derived macrophages. (A) Phase-contrast morphology of MDM on day 2 (before differentiation) and day 7 (after differentiation). Day 2 MDM are almost homogenous while day 7 MDM are a heterogeneous population with cell that can be grossly characterized as fried-egg like (1), fibroblast-like (arrow-head), and adherent lymphocyte-like (arrow). (B) FACS profile of day 7 MDM stained for surface markers with mouse-anti-human mAbs shown.

5.5. TCID₅₀ assay: infection of human PBMC with HIV-1_{Ba-L} and neutralization with aptamers

Primary human PBMC provided an ideal *ex vivo* model to study HIV-1 infectivity and evaluate aptamer sensitivity, as these cell types are natural targets of HIV-1 and mimic the *in vivo* scenario closely compared to other cell types. The drawback is that these cells do not lend themselves to focus-formation, owing to their heterogeneity, lack of adherence and variable susceptibility to cytopathic effects. Measurement of infectivity in this cell type therefore required endpoint dilution, the tissue culture infectious dose (TCID₅₀) assay (Collin, 1992; Collin, James, and Gordon, 1991). In this method, day 7 PBMC seeded in 96 well plate were infected with serially diluted HIV-1_{Ba-L} that had been incubated with 100 nM of anti-gp120 monoclonal aptamer or control aptamer as described (2.8.4 – 2.8.5). TCID₅₀ assay was performed using 10-fold dilution of virus with 8 replicate at each dilution. On day 14 post-infection, cultures were lysed *in situ* and DNA prepared for LTR-PCR as described (2.8.5). Cultures were scored for infection and the endpoint determined as the dilution of virus where 50% of the replicate wells became infected (Figure 5.4). The concentration of median tissue culture infectious dose (TCID₅₀) present in the original suspension of virus was the reciprocal of the endpoint dilution.

Three of the five monoclonal aptamers tested in the initial pilot study (B1, B4 and B9) reduced HIV-1_{Ba-L} infection of the PBMC by at least 1000-fold as scored by LTR-PCR (Figure 5.4). Aptamer B3 and B5 tested in this study did not seem to neutralize the virus. These results were reproduced in a comprehensive study in which all the 27 monoclonal aptamers were evaluated in neutralization of HIV-1_{Ba-L}. 19 of the 27 aptamers' clones assayed, neutralized homologous HIV-1_{Ba-L} in PBMC by $\geq 3 \log_{10}$, 6 by 1-2 $\log_{10}$ and 2 aptamers, namely B3 and B5 as in the pilot study did not neutralize the virus (Figure 5.5). Most remarkably, two of the aptamers, B4 and B84 neutralized HIV-1_{Ba-L} by more than 4 $\log_{10}$, tantamount to complete neutralization (Figure 5.5). Taken together these data suggest that neutralizing aptamers bound at or near

functional epitopes of the gp120 essential for interaction with the receptors on PBMC surface.

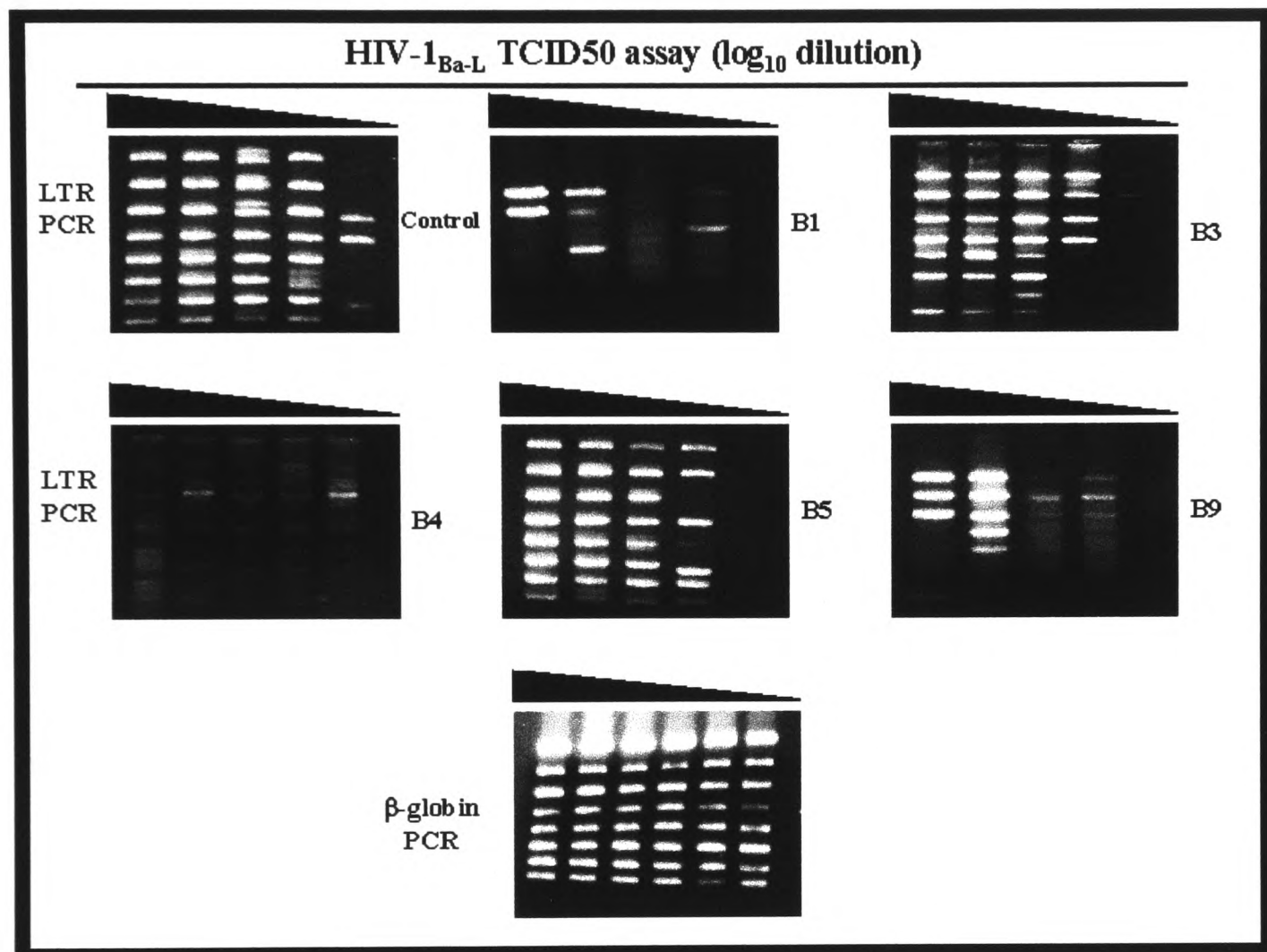


Figure 5.4: Titration and neutralization of HIV-1_{Ba-L} infectivity of PBMC by anti-gp120 monoclonal aptamers. Endpoint dilution was used to measure infectivity and LTR-PCR was used to score cultures for the presence of infection. Three of the aptamers B1, B4 and B9 clearly neutralized the virus, while B3 and B5 did not seem to. In the control, the virus was incubated with 100 nM of a streptavidin aptamer derived from the same original library as the anti-gp120 aptamers. Control β -globin PCR is representative of all the samples and was performed to monitor the uniformity of DNA preparation.

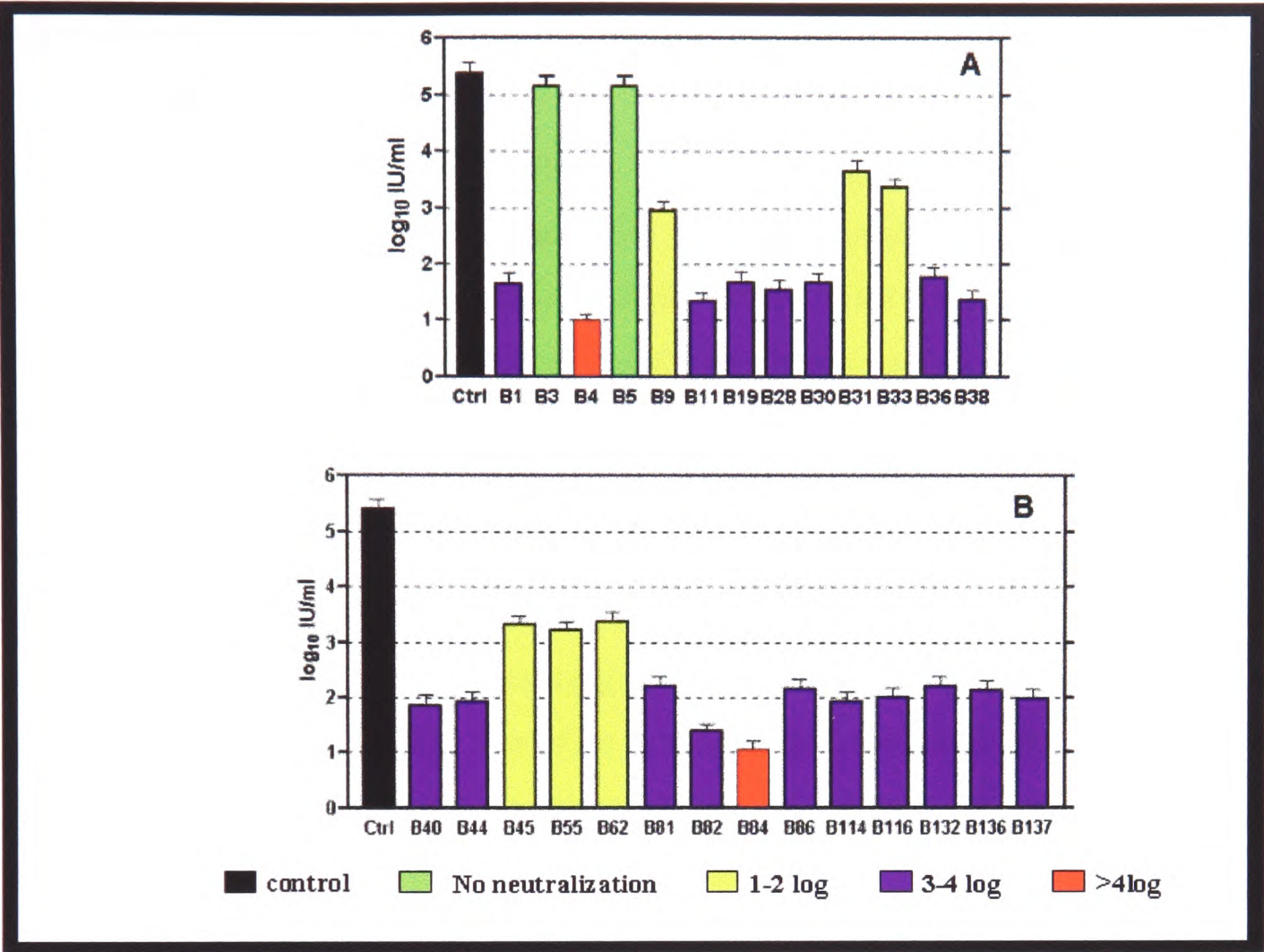


Figure 5.5: (A and B) Neutralization of HIV-1_{Ba-L} by monoclonal aptamers in PBMC. Seventeen aptamers neutralized the virus by 3–4 $\log_{10}$ infectious units per millimeter (IU/ml) of the TCID₅₀ assay, while B4 and B84 neutralized HIV-1_{Ba-L} by more than 4 $\log_{10}$ IU/ml, equivalent to complete neutralization of the input infectious viral titer (5 $\log_{10}$ IU/ml). Endpoint dilution cultures were scored for the presence of infection by LTR-PCR as in Figure 5.4. Then, first and second order statistics of infectious viral titer and aptamer inhibitory doses were determined using the ID₅₀ software developed by John Spouge and available electronically from the following URL: <http://www.ncbi.nlm.nih.gov/CBBresearch/Spouge/Virology/>

5.6. Generation of HIV-1_{Ba-L} "escape" mutants and sensitivity to aptamer B4

HIV-1 rapidly mutates during infection *in vivo* in response to negative selection pressures, thereby propagating variants that can escape immune recognition and also resistant to anti-retroviral drugs (Borrow et al., 1997). Thus the main challenge in the clinical development of any compound that shows activity against HIV-1 *in vitro* is the common problem of rapid drug resistance both *in vitro* and *in vivo*. For this reason there was concern that inhibitors that target the CCR5 coreceptor might for example, select for new and more pathogenic HIV-1 variants that preferentially use CXCR4 (Michael and Moore, 1999). Recently, R5 primary isolate (CC1/85) escape mutant with more than 20, 000-fold resistance to CCR5-specific entry inhibitor, AD101 was reported (Trkola et al., 2002). Therefore in this study I circumspectly tested whether HIV-1_{Ba-L} could mutate to escape neutralization by aptamer B4, one of its potent inhibitor in PBMC (section 5.5).

To generate aptamer B4 break-through mutants, HIV-1_{Ba-L} was serially passaged in human PBMC in the presence of increasing doses of B4 (i.e. 1, 5, 15, 50 and 100 nM) as described (2.8.6.). At each passage, the viral titer was monitored by TCID₅₀ assay to ensure that virus replication was occurring and to determine the extent of inhibition by B4 at each concentration (Figure 5.6). At intervals, the virus was expanded in PBMC without the aptamer until it reached a titer of about 10⁵ TCID₅₀ IU/ml, before adding the next dose of the aptamer. At the highest concentration of B4 used, 100nM, where HIV-1_{Ba-L} was neutralized by more than 99.99% (Figure 5.6), the virus was cloned by limiting dilution with 12 replicate wells at each concentration to increase the chance of break-through events. Break-through clones were rescued and the *env* gene coding for the gp120 in each clone was amplified by Expand™ high fidelity PCR system kit (Roche, Germany) and sequenced for comparison with the gp120 sequence of the parental HIV-1_{Ba-L} as described (2.8.6). All the four break-through clones had several amino acid substitutions in the gp120, including the putative principal neutralization epitope at the V3 loop (Table 5.1). I then tested whether these mutations had conferred resistance to B4 by propagating the rescued break-through

clones (as described in 2.8.2), until they reached the maximum titer ($5\log_{10}$ IU/ml). Propagated clonal HIV-1_{Ba-L}-derived stock was re-challenged with 100 nM of B4 and evaluated for sensitivity in human PBMC as described (5.5). Aptamer B4 completely neutralized all the break-through clones (Figure 5.7), implying that the mutations did not confer any selective advantage to HIV-1_{Ba-L}. These data suggested that aptamer B4 could be binding to a region of the gp120 that is critical to HIV-1_{Ba-L} function (chapter 6), and therefore not free to mutate without overall efficiency loss to the virus in infecting PBMC

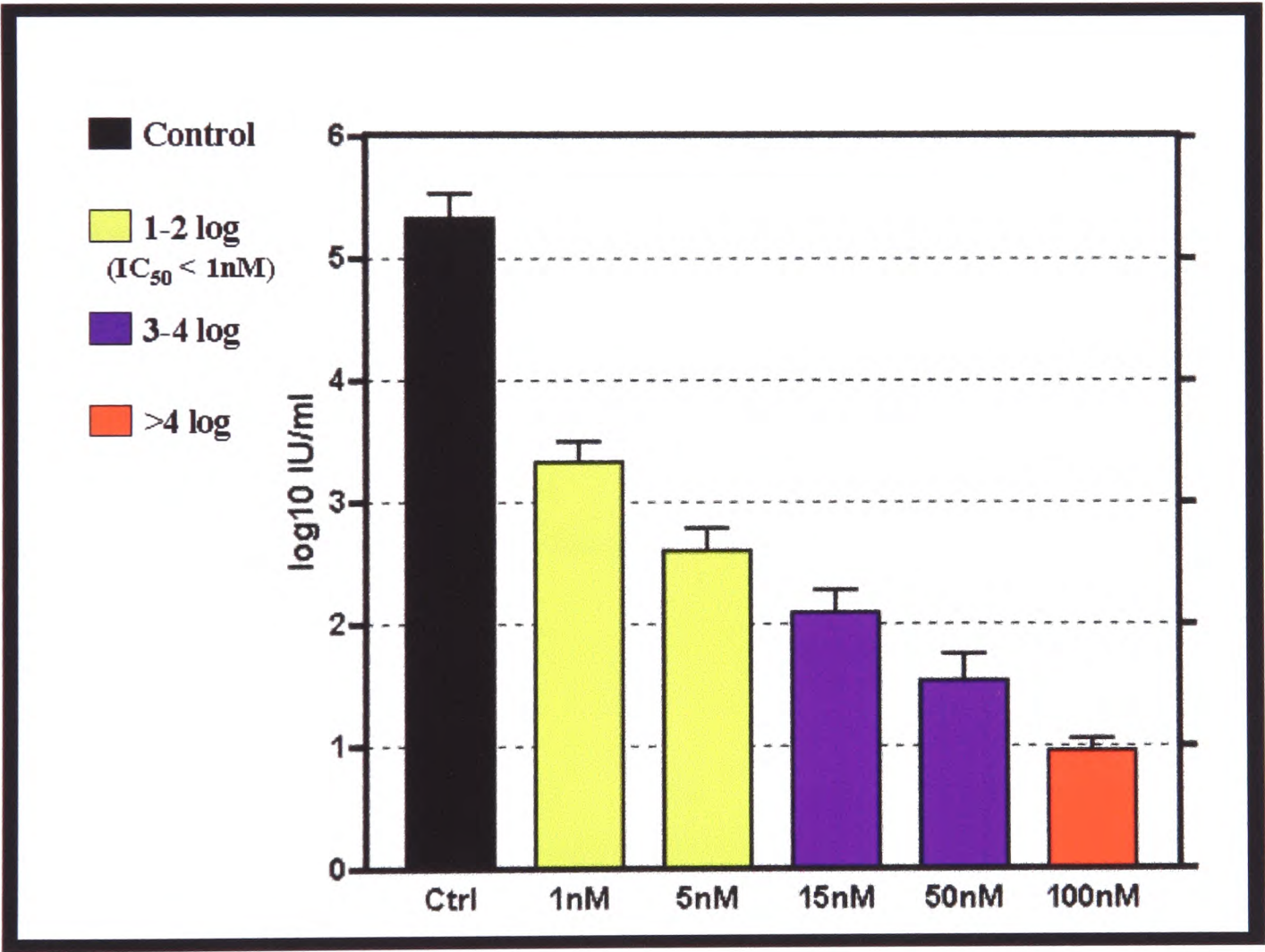


Figure 5.6: Concentration-dependent neutralization of HIV-1_{Ba-L} in human PBMC by aptamer B4. Aptamer B4 neutralized HIV-1_{Ba-L} with a 50% inhibition concentration (IC_{50}) of less than 1 nM, and at 100 nM more than 99.99% of the virus was neutralized. Colour-codes depict annotated degree of neutralization by each concentration of the aptamer used, compared to control aptamer.

Table 5.1: Alignment of HIV-1_{Ba-L} gp120 amino acid sequence of the break-through clones to the parental sequence (NIH 510) in relation to the database sequence (genbank accession M68893).

	10	20	30	40	50	60	70	80	
Ba-Lgp120 (M68893)	MRVTEIRKSYQHWWRWGIMLLGILMICNAEEKLWVTVYGVVPVWKEATTTLFCASDRKAYDTEVHNWATHACVPTDPNP								
Ba-Lgp120 (NIH510*)	-----					A			
Ba-Lgp120clone1	-----X		M			A			
Ba-Lgp120clone2	-----X		M			A			
Ba-Lgp120clone3	-----X					A			
Ba-Lgp120clone4	-----					A			
← → gp120 start									
	90	100	110	120	130	140	150	160	
Ba-Lgp120 (M68893)	QEVELKNVTENFNMWKNMVEQMHEDIISLWDQSLKPCVKLTPLCVTLNCTDLRNATNGNDTNTTSSSRGMVGGGEMKNC								
Ba-Lgp120 (NIH510*)									
Ba-Lgp120clone1	C					SR.V			
Ba-Lgp120clone2						S	V		
Ba-Lgp120clone3						S			
Ba-Lgp120clone4						S			
	170	180	190	200	210	220	230	240	
Ba-Lgp120 (M68893)	SFNITTNIRGKVQKEYALFYKLDIAPIDNNSNNRYRLISCNTSVITQACPKVSFEPIPIHYCAPAGFAILKCKDKKFNGK								
Ba-Lgp120 (NIH510*)	E..V								
Ba-Lgp120clone1	G.....E..V...KIDXX							E	
Ba-Lgp120clone2	E..V								
Ba-Lgp120clone3	G.....E..V...KIDXX								
Ba-Lgp120clone4	G.....E..V...KIDXX								
	250	260	270	280	290	300	310	320	
Ba-Lgp120 (M68893)	GPCTNVSTVQCTHGIRPVVSTQLLLNGSLAEEVVIRSANFADNAKVIIVQLNESVEINCTRPNNNTRKSIHIGPGRIFY								
Ba-Lgp120 (NIH510*)	...S.....E..TN...T								
Ba-Lgp120clone1	...S.....E..TN...T								
Ba-Lgp120clone2	...S.....E..TN...T						N		
Ba-Lgp120clone3	...S.....E..TN...T						N		
Ba-Lgp120clone4	...S.....E..TN...T						N		
	330	340	350	360	370	380	390	400	
Ba-Lgp120 (M68893)	TTGEIIGDIRQAHCNLSRAKWNDTLNKIVIKLREQFGNKTIVFKHSSGGDPEIVTHSFNCGGEFFYCNSTQLFNSTWNVT								
Ba-Lgp120 (NIH510*)									
Ba-Lgp120clone1	Y								
Ba-Lgp120clone2									
Ba-Lgp120clone3									
Ba-Lgp120clone4									
	410	420	430	440	450	460	470	480	
Ba-Lgp120 (M68893)	EESNNTVENNTITLPCRIKQIINMWQEVGRAMYAPPIRGQIRCSSNITGLLLTRDGGPEDNKTEVFRPGGDMRDNRSE								
Ba-Lgp120 (NIH510*)	K								
Ba-Lgp120clone1	K								
Ba-Lgp120clone2									
Ba-Lgp120clone3	K								
Ba-Lgp120clone4									

* Parental HIV-1_{Ba-L} (NIH request number 510) env gene region coding for gp120 was sequenced in advanced and compared to that of break-through clones. The alignment only shows that region of gp120 where there were amino acid differences. X denotes missing codon.

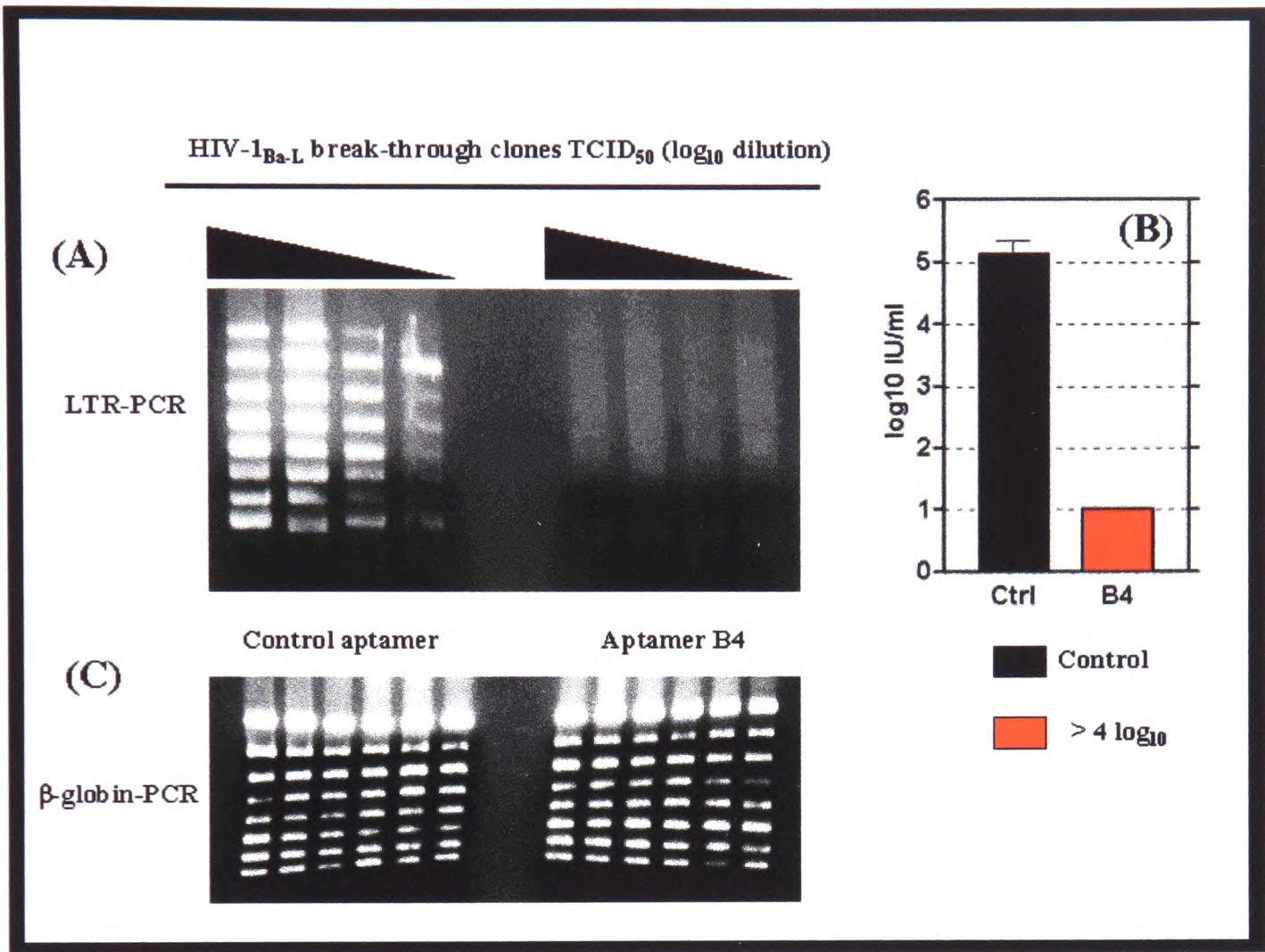


Figure 5.7: Failure of HIV-1_{Ba-L} to escape neutralization by aptamer B4. (A) Complete neutralization of clonal HIV-1_{Ba-L}-derived breakthrough stock with 100 nM of aptamer B4 in human PBMC as assayed by the TCID₅₀. (B) Graphical depiction of the TCID₅₀ data as statistically analysed with the ID50 software, showing neutralization of HIV-1_{Ba-L} breakthrough clone by more than 4 log₁₀ IU/ml, compared to control aptamer. The results are representative of all the clones in that all the four break-through clones were equally neutralized. (C) Corresponding β-globin PCR controls that monitored the uniformity of DNA preparation in the samples.

5.7. Neutralization of other HIV-1 strains with aptamers in PBMC

In addition to rapid development of drug resistance, another critical issue on HIV-1 neutralization centers on the ability of one molecule or a combination of selected molecules to neutralize a broad range of diverse primary isolates. To determine the breadth of neutralization by aptamers against the gp120 derived from HIV-1_{Ba-L} strain, I examined the sensitivity of another R5 strain, HIV-1_{ADA} against selected aptamers with a view to further test cross-neutralizing aptamers in a broad range of clinically relevant PIs. Despite the two strains being divergent representatives of clade B of HIV-1, based on sequence and phenotype, all HIV-1_{Ba-L} neutralizing aptamers I tested also neutralized HIV-1_{ADA} (Figure 5.8A). Although B4 neutralized HIV-1_{ADA} by 100-fold less than HIV-1_{Ba-L}, the strain from which the aptamer was isolated, HIV-1_{ADA} was equally sensitive to B19, B28, B36 and B44, each neutralized the virus by 3-4log₁₀ (Figure 5.8A). Reduced neutralization of HIV-1_{ADA} by B4 suggests that the aptamer primarily targets strain-specific neutralization epitope on the gp120, or perhaps the aptamer has lower affinity for HIV-1_{ADA}. While the uniform neutralization of HIV-1_{Ba-L} and HIV-1_{ADA} by the three aptamers suggest that the determinant(s) engaged by the aptamers may be conserved at least between the two R5 strains, and intimately tied to the basic functioning of the gp120 in binding and fusion, and consequently entry into PBMC.

Although X4 viruses fell out of the scope of this thesis, the fact that some of the aptamers tested also bound the gp120 derived from HIV-1_{IIIB} (section 4.5), tempted me to evaluate their neutralization ability in this strain. In this study I evaluated the ability of aptamers to preclude HIV-1_{IIIB} entry into PBMC using single cycle infections and LTR-PCR to detect early proviral DNA in the cytoplasm of infected cells. The detection of proviral DNA does not depend on the completion of a cycle of infection because it is an early event following viral entry. Single cycle infection was performed using a modification of the methods of (Kim et al., 1989) and (Collin, 1992) in PBMC. The assay required high multiplicity of infection so that as many cells as

possible are synchronously infected to increase LTR copy number and allow early detection of proviral DNA. At least 2 000 LTR copy number is required to be detected and visualized by 30 cycles of PCR on an ethidium bromide stained agarose gel (Montaner, 1995). High titer ($5\log_{10}$ IU/ml) HIV-1_{IIIB} that had been filtered and DNase-treated as described (2.8.3) was used to ensure that the DNA detected by LTR-PCR was newly formed. Day 7 PBMC seeded in a microtiter plate at 10^5 /well were infected with HIV-1_{IIIB} (m.o.i. of 0.1) that had been pre-incubated with 100 nM of test or control aptamer as described (2.8.4). 16 hours post-infection the cultures were lysed as described (2.8.5) and assayed for the presence of proviral DNA, which must have originated from *de novo* RT as a consequence of viral entry.

Although the data were not quantitative and hence preliminary, two aptamers, B5 and B36, like the soluble human CD4 control, inhibited HIV-1_{IIIB} entry into PBMC as evaluated by LTR-PCR of early proviral DNA (Figure 5.8B). B36 was shown to bind the gp120 derived from both HIV-1_{Ba-L} and HIV-1_{IIIB}, and to equally neutralize HIV-1_{Ba-L} and HIV-1_{ADA}. I did not test whether B5 bound the gp120 from HIV-1_{IIIB}, but it clearly did not neutralize HIV-1_{Ba-L}. Although there is no direct evidence, and inhibition of HIV-1_{IIIB} entry into PBMC by B5 is indeterminate, it could be deduced that B5 binds the region of the gp120 that is essential for X4 but not R5 virus entry, such as the gp120 epitope that interacts with CXCR4 coreceptor. On the other hand, the apparent ability of B36 to neutralize three phenotypically divergent representatives of subtype B of HIV-1, suggest that the aptamer probably overlaps functionally important site(s) on the gp120, conserved between at least the members of this subtype.

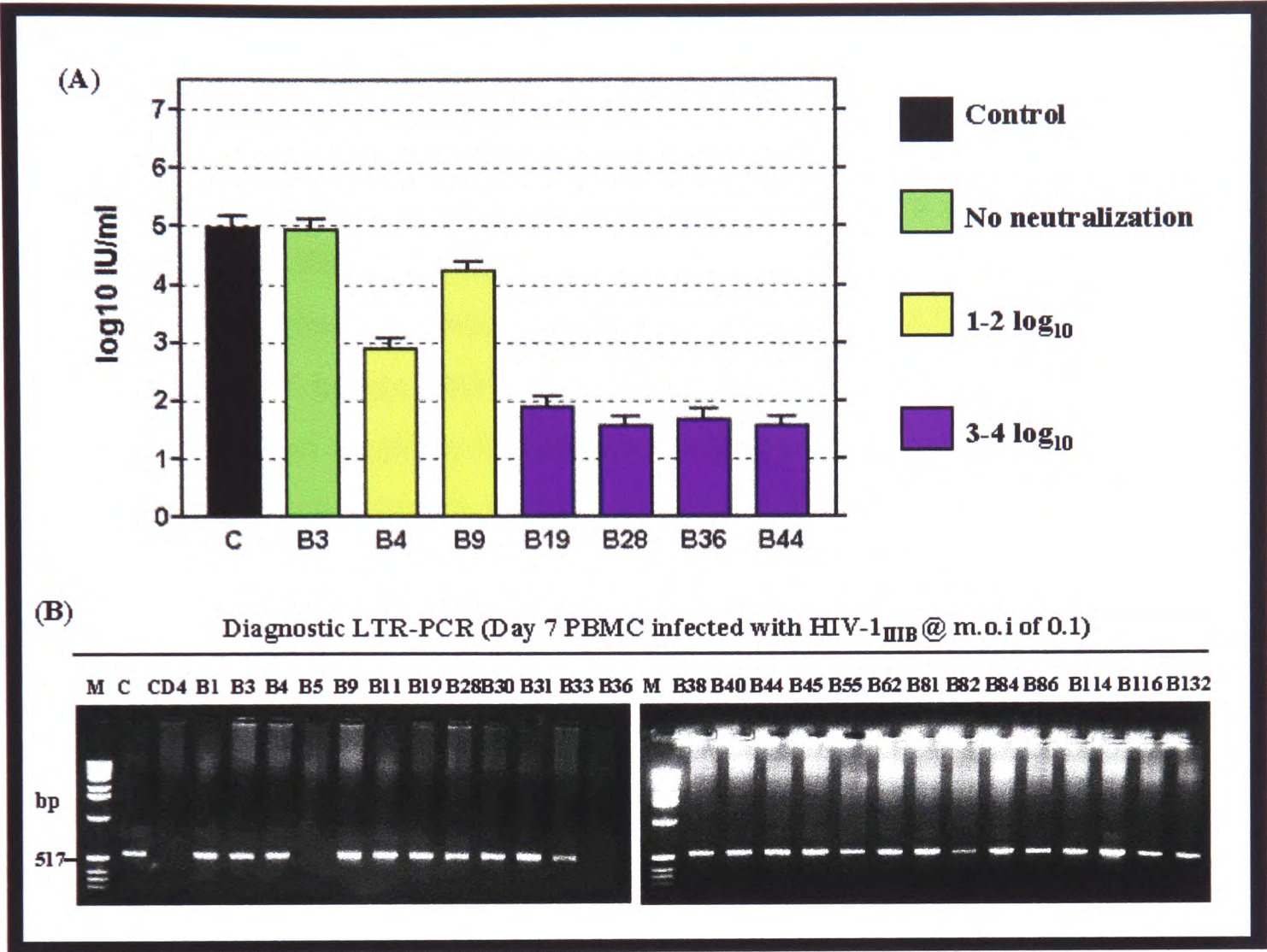


Figure 5.8: Neutralization of other stains of HIV-1 in human PBMC by aptamers generated against the gp120 derived from HIV-1_{Ba-L}. **(A)** Neutralization of HIV-1_{ADA} by aptamers that also neutralized HIV-1_{Ba-L}. B4 neutralized HIV-1_{ADA} by less than 100-fold compared to HIV-1_{Ba-L}, while the rest of the aptamers had similar neutralization profiles for the two strains, including B3 that did not neutralize either of the two strains. **(B)** *En masse*, rapid screening of aptamers in entry inhibition assay of HIV-1_{IIB} using single cycle infection of day 7 PBMC and LTR-PCR to detect early proviral DNA formation. B5 and B36, like the soluble human CD4 control, inhibited HIV-1_{IIB} entry below the limit of detection by 30 cycles of LTR-PCR as visualized by ethidium bromide staining on a 2% agarose gel. 1 μ M of soluble human CD4 compared to 100 nM of aptamer, was used to achieve complete inhibition because lower concentration (200 nM) was previously shown not to, or partially inhibit viral entry into PBMC and MDM (Collin, 1992).

5.8 Neutralization of HIV-1_{Ba-L} in MDM

There are variables at both the viral and cellular level that may profoundly alter neutralization ability of an antibody or a given aptamer for a given HIV-1 strain. Therefore, although PBMC mimic closely the conditions relevant to HIV-1 infectivity *in vivo*, for completeness, I evaluated the sensitivity of HIV-1_{Ba-L} to aptamers in purified cultures of human MDM. MDM were isolated from human PBMC as described (2.3.3) and single cycle infections and semi-quantitative LTR-PCR were used to rapidly screen aptamers for neutralization of HIV-1_{Ba-L}. Thus day 7 MDM were infected with high titer HIV-1_{Ba-L} (m.o.i. of 0.1) that has been incubated with either 1 μ M of soluble human CD4, 100 nM of test or control aptamer. 16 hours post-infection cell lysates were assayed for early proviral DNA using LTR-PCR as previously described, except that the lysate was serially diluted to estimate the degree of neutralization (Figure 5.9A). Following the rapid semi-quantitative LTR-PCR, some aptamers were selected from those that seemed to have inhibited HIV-1_{Ba-L} entry in MDM, and re-evaluated in a more quantitative manner using the TCID₅₀ assay as described (5.5). Although all the tested aptamers still neutralized HIV-1_{Ba-L} in MDM, they all lost at least 10 to 100-fold neutralization potency compared to when the virus sensitivity was studied in PBMC (Figure 5.9B). Most interesting, although aptamer B4 neutralized HIV-1_{Ba-L} by almost 5log₁₀ in PBMC, it neutralized the same virus by a modest log₁₀ in MDM. Taken together these data have several implications relating to the virus properties and the phenotype of macrophages grown and differentiated as a pure culture or a mixed population with T lymphocytes as PBMC.

First, these data may suggest that the phenotype of macrophages cultured as PBMC differ from that of those cultured and differentiated alone, thereby resulting in differential HIV-1 neutralization in these two cell types. Second there may exist distinct interactions between sites on gp120 and surface molecules that differ between forms of primary leukocyte, which have not been identified. Although I did not have the full history of the HIV-1_{Ba-L} used in both the PBMC and MDM studies, the virus was acquired in our laboratory over 10 years ago, and has been passaged in purified

MDM by my predecessors (Collin, 1992; Montaner, 1995), the tradition I maintained. Therefore several passages in MDM may have altered the properties of the HIV-1_{Ba-L} used in this study such that existed viral quasispecies that evolved to explore other surface molecules on MDM for entry, other than the typical ones. For instance it has become clear that HIV-1 incorporates cellular antigens into its envelope during the process of budding from the host cell membrane (discussed in section 1.5.1f). The incorporation of cellular adhesion molecules into the virion envelope has been implicated to increase virus-target cell binding avidity when the gp120-receptor interaction is relatively low or obstructed (Gomez and Hildreth, 1995), as could be the case when a neutralizing aptamer is used. MDM-derived cellular adhesion molecules may therefore contribute to HIV-1_{Ba-L} that has been passaged in MDM over 10 years, to be relatively resistance to aptamers neutralization when assayed in MDM, as compared to PBMC. Monocyte or macrophages constitutes only 10% in PBMC, therefore some of the above-mentioned theoretical possibilities are probably more manifested in an almost 100% purified population of MDM, including possible clearance of aptamers by macrophages. It is clear from this study, therefore, that neutralization of HIV-1 by a particular agent such as aptamers or antibodies must be defined in the context of the viral origin and passage history, as well as the cellular system being used to study it.

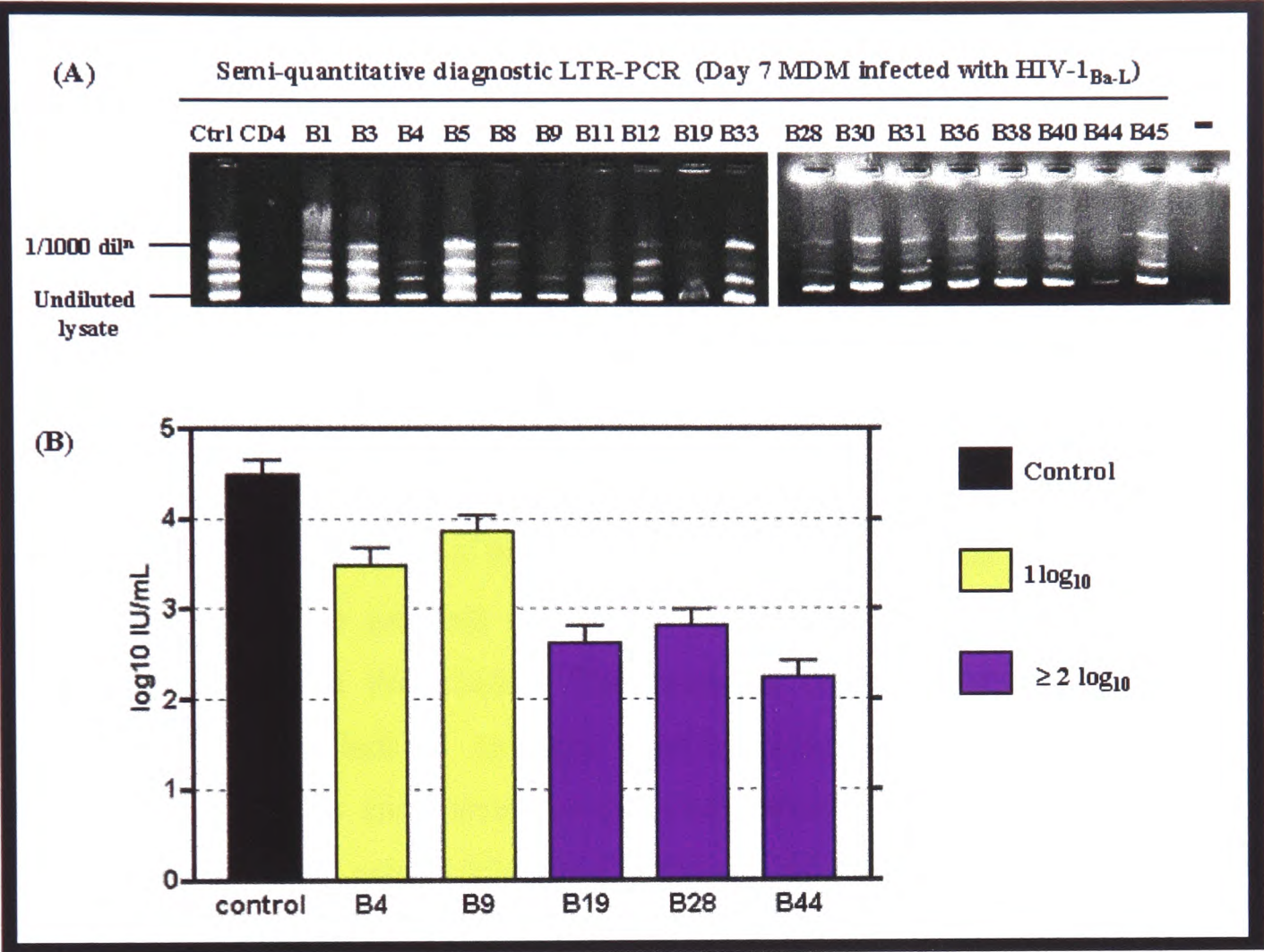


Figure 5.9: Neutralization profile of HIV-1_{Ba-L} by aptamers in day7 human MDM. (A) Semi-quantitative LTR-PCR screening of HIV-1_{Ba-L} neutralization in MDM. MDM were infected with high titer (1×10^5 IU/ml) HIV-1_{Ba-L} that has been incubated with 1 μ M of soluble human CD4, 100 nM of control or test aptamer. 24 hours post infection, neat and 10-fold serially diluted lysates were assayed for early proviral DNA using LTR-PCR. (B) TCID₅₀ assay to measure the extent of HIV-1_{Ba-L} neutralization in MDM by some of the aptamers that seemed to neutralize the virus as evaluated in (A). Degree of neutralization by each aptamer is indicated by annotated symbols.

5.9. Discussion

5.9.1. Monomeric gp120 as a target for HIV-1 neutralizing aptamers

The principal observation made in this chapter is that although the monomeric form of the gp120 does not function as an effective immunogen, as epitomized by the failure of vaccine candidates based on recombinant gp120 to elicit broad-neutralizing antibodies against HIV-1, some aspects of the critical epitopes important for viral entry are sufficiently represented on monomeric protein to allow generation of neutralizing aptamers. Thus I have shown that appropriately presented clade B, monomeric R5 gp120 can be used to generate potent neutralizing aptamers against at least three divergent members of this clade. This study is consistent with findings that a molecularly cloned clade B envelope protein presenting functional intermediate structures was able to elicit sera that cross-neutralized most primary isolates from multiple HIV-1 clades, using U87-CD4-CCR5 or -CXCR4 cells (LaCasse et al., 1999). It is however essential to determine whether neutralization by aptamers evaluated in this chapter can be extended to clinically relevant primary isolates, from diverse genetic clades and geographic locations.

5.9.2. Factors influencing neutralization *in vitro*

Another key observation made in this study is that HIV-1 neutralization *in vitro* depends on numerous factors. Both cellular and viral factors, and the assay system used to measure neutralization influence the outcome of the neutralization assay, a situation which may pose standardization problems between laboratories. An important factor in the measurement of neutralization is the passage history of the virus, the method and cell type used to passage the virus. Virus isolated directly from serum may have different properties from the same strain of virus passaged in MDM or PBMC, which in turn could be strikingly different from virus which has been passaged in immortalized cell lines (Moore and Ho, 1995; Sattentau, 1996). We know that the passage of virus in immortalized cell lines leads to the selection of variants

which have a phenotype that does not represent the majority of viral clones present in an infected individual. Although there is no direct experimental evidence, the virus that has been continuously passaged in one cell type, like MDM could have different properties and neutralization profile if assayed in a different cell type like PBMC or *vice-versa*, as it appeared to be the case in this study.

The method and cell type used in HIV-1 neutralization assay could also influence the outcome of the results. In this study human PBMC were used as the primary cell type to measure HIV-1 infectivity and evaluate sensitivity to aptamers because they emulate *in vivo* conditions better than other cell types, including MDM, which do not naturally exist in isolation. To mimic the *in vivo* conditions even more closely, unlike others (Trkola et al., 1998; Trkola et al., 2002), the PBMC used in this study were cultured as naturally as possible, without addition of a mitogen or IL-2. PCR-based TCID₅₀ assay as used in this study reliably allowed quantitative measurement of HIV-1 infectivity and evaluation of the magnitude of neutralization by aptamers. Endpoints measurement by PCR have been shown to be in good agreement with ELISA readings, demonstrating that LTR-PCR detects only productively infected cultures (Collin et al., 1993). Although the TCID₅₀ assay was the gold-standard method to quantitatively examine aptamers in neutralization assays, the method was labour intensive and costly, especially if all the 27 aptamers were individually tested. It was therefore necessary to perform single cycle infection and rapid screening of aptamers in HIV-1 entry inhibition assay, detected by diagnostic LTR-PCR (Figures 5.8 and 5.9), followed by TCID₅₀ evaluation of selected neutralizing aptamers.

5.9.3. Neutralization of HIV-1 by antibodies compared to aptamers

Although high titer anti-gp120 antibodies are sustained in individuals throughout the course of HIV-1 infection, they have very poor neutralizing activity in these patients and are generated late in infection. However a large spectrum of anti-gp120 monoclonal antibodies readily neutralize T cell line adapted (TCLA) viruses that preferentially utilize CXCR4 as a coreceptor (reviewed in (Moore and Ho, 1995)). By contrast primary isolates, i.e., viruses obtained by passage in primary cultures such as PBMC and generally use the CCR5 (R5) or a combination of CCR5 and CXCR4 (R5X4) coreceptors generally display a high degree of resistance to neutralization by mAbs (Figure 5.10). The structural basis for the large difference in neutralization sensitivity between TCLA viruses and the majority of primary isolates can be understood as follows. It seems likely, based on the gp120 structure that the CD4BS and other neutralization epitopes on the primary isolate gp120 trimer, are more masked than in the TCLA viruses (Chapter 6).

The idea that gp120 oscillates between "closed" and "open" states is consistent with the dichotomy of primary and TCLA viruses. In this way, primary isolates would compromise some efficiency in receptor binding for increased resistance to antibody attack, whereas TCLA viruses would dispense with antibody resistance mechanisms and adapt for more efficient receptor interactions instead. Although primary isolates are generally resistant to antibody neutralization, R5 primary isolates assayed in this study, which are also refractory to antibody neutralization were highly sensitive to neutralization by aptamers (Figure 5.10). These data support the central hypothesis of this thesis that aptamers by virtue of their high affinities and compactness compared to antibodies would penetrate poorly accessible neutralization epitopes on the mature envelope of primary isolates.

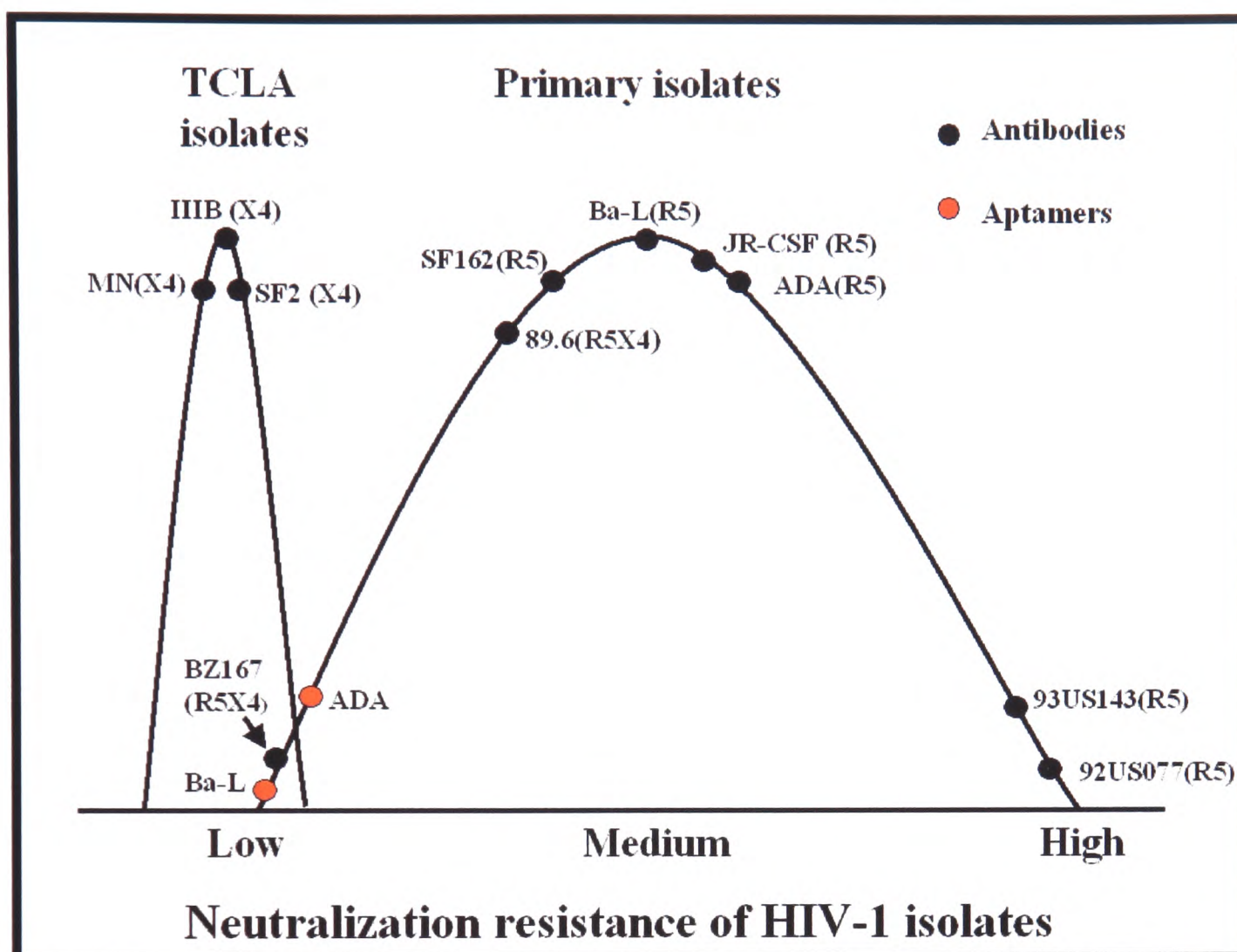


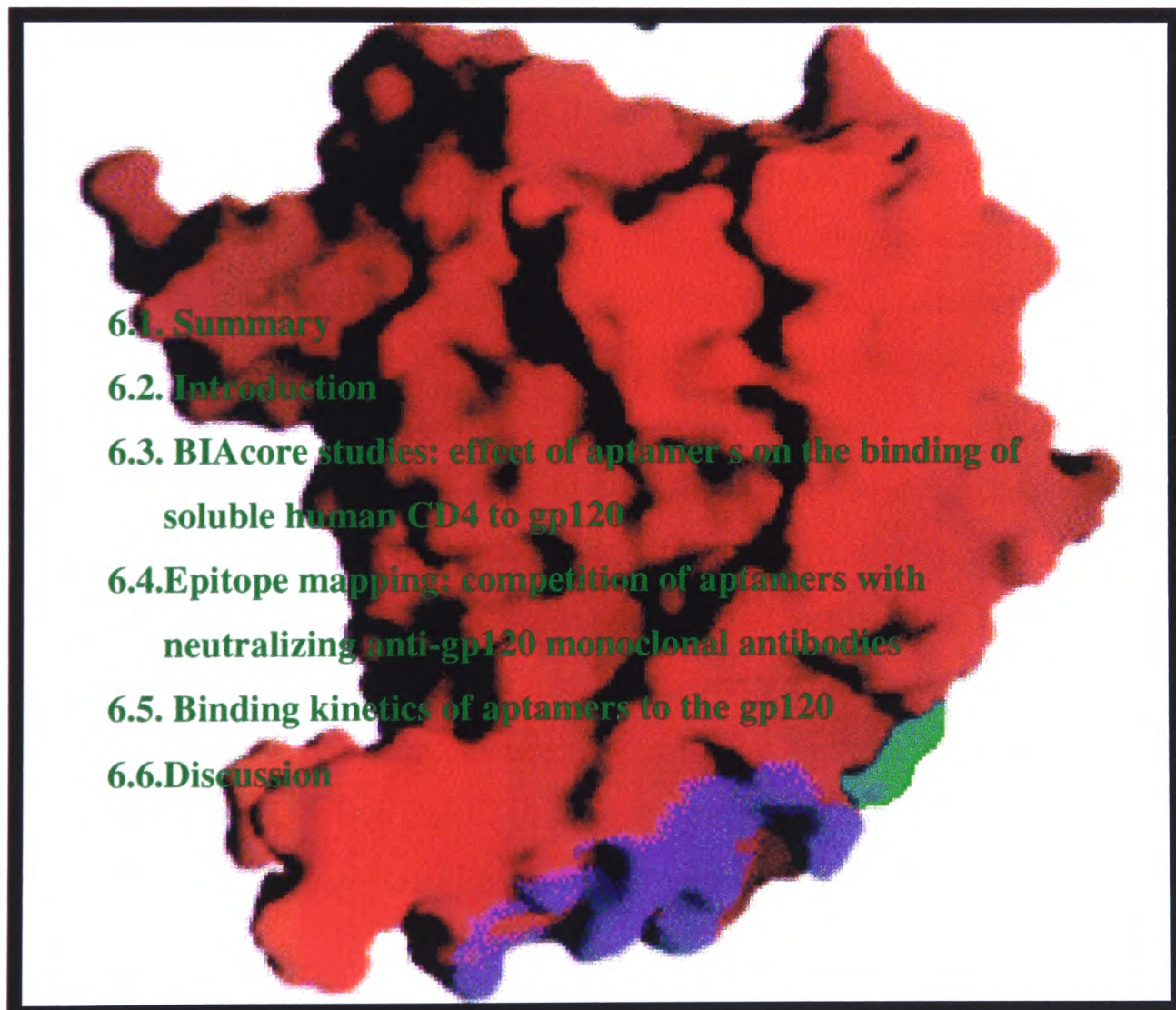
Figure 5.10: Depiction of neutralization resistance of HIV-1 isolates. TCLA isolates are highly sensitive to neutralization by antibodies in contrast to primary isolates, which are relatively neutralization resistant. Representatives of TCLA and primary HIV-1 isolates are indicated with their tropism as defined by the coreceptor use shown in parenthesis. A broad primary isolates curve depicts the fact that the degree of neutralization sensitivity can differ significantly between these isolates. Most of the primary isolates are resistant to antibody neutralization with the exception of some R5X4 isolates (e.g. BZ167) which are just as sensitive to antibody mediated neutralization as TCLA isolates (Moore and Montefiori, 1997). Other primary isolates like 93US143 are however extraordinarily resistant to antibody neutralization (Parren et al., 1998). R5 primary isolates, Ba-L and ADA, although antibody neutralization resistant, were highly sensitive to neutralization by anti-gp120 aptamers described in this thesis. Aptamer neutralization sensitivity of other strains was not done. (The figure was modified from Labrijn and Parren, 1999).

5.9.4. Implications

Generally, the findings described in this chapter show that aptamers could be used as effective HIV-1 entry inhibitors, and have a potential to be used as tools to understand further the interaction between the virus and host cell at entry. However much more research needs to be performed to understand the mechanism of aptamers' neutralization. Also, the neutralization ability of aptamers needs to be evaluated in a broad repertoire of clinically relevant primary isolates. Thus, the challenge is to map the epitope(s) on gp120 that interact with aptamers, and determine the exact mechanism of aptamer interaction with the aim of providing structural leads for the development of potent and even smaller molecules that can mimic neutralizing properties of these aptamers.

Chapter Six

Mechanism of HIV-1 neutralization by aptamers and binding kinetics to the gp120



Background: Neutralizing antibody 17b-gp120 interface. The surface of the gp120 is red, with any surface within 3.5Å of Fab 17b (surface-to-atom centre) colored blue and the surface of the V3 loop base colored green. (Source (Kwong et al., 1998))

6.0 Mechanism of HIV-1 neutralization by aptamers and binding kinetics to the gp120

6.1. Summary

The aptamers described in previous chapters, by virtue of their ability to neutralize HIV-1, possibly mimic the binding of gp120 to its natural receptor(s) or neutralizing antibodies. This chapter describes the probable mechanism of HIV-1 neutralization by selected aptamers, and binding kinetics to the gp120 as determined by epitope mapping in competition with soluble human CD4 and well-characterized neutralizing anti-gp120 monoclonal antibodies, and the biosensor binding assays, respectively. Aptamer B4 that was previously shown to completely neutralize HIV-1_{Ba-L} in PBMC did not affect binding of soluble or recombinant human CD4 to monomeric HIV-1_{Ba-L} gp120, but significantly reduced binding of 17b and 48d monoclonal antibodies to the gp120. Aptamer B4 bound the monomeric gp120 with affinity constants (K_d) of about 50nM. Competition with the 17b and 48d monoclonal antibodies suggests that B4 binding site (aptatope) overlaps the CD4-induced epitope recognized by these antibodies on the gp120. Aptamer B19, which was previously shown to neutralize equally both HIV-1_{Ba-L} and HIV-1_{ADA}, bound the gp120 with higher affinities (K_d value of 3nM). B19 mechanism of neutralization remains unclear because it did not affect binding of human CD4 or any tested monoclonal antibodies to gp120. Taken together the results suggest that B4 neutralizes HIV-1 by preventing interaction with the CCR5 coreceptor, following CD4 binding on target cells, while B19 could be interacting with a crucial yet undefined epitope on the gp120.

6.2. Introduction

Neutralizing antibodies are a major component of the host defense against HIV-1, and are particularly important in limiting the spread of cell-free virus (reviewed in (Poignard et al., 1996b)). The vast majorities of neutralizing antibodies are elicited by, and bind to the gp120 glycoprotein (reviewed in section 1.7.3). The production and characterization of monoclonal antibodies of human and rodent origin have allowed a precise determination of the neutralization epitopes on the envelope glycoprotein. Thus, the specificity of HIV-1 neutralization by anti-gp120 antibodies has been dissected by absorption studies using peptides and recombinant glycoproteins, and by the preparation and characterization of hundreds of mAbs from immunized animals and infected individuals (Bagley et al., 1994; Schutten et al., 1993).

To date, at least six distinct neutralization epitopes have been defined on gp120 (reviewed in (Poignard et al., 1996b; Sattentau, 1996)). These epitopes have been categorized as 'linear', based on the ability of the epitope to be presented in the form of a short peptide or on a denatured form of the glycoprotein, or 'conformational' for those epitopes that require conservation of the glycoprotein native structure. 'Discontinuous' epitopes require conservation of amino acid residues distant in the linear sequence. Epitope mapping for conformational and discontinuous groups of mAbs depended on analysis by site-directed mutagenesis to establish amino acids critical for the mAb-gp120 interaction (Kowalski et al., 1987; Lasky et al., 1987) (Olshevsky et al., 1990).

Overall, the conclusion from the epitope mapping experiments is that all generally accepted, biologically relevant, neutralizing activity is directed against a limited number of epitope on the surface of the gp120, with the exception of one conserved linear epitope on the ectodomain of gp41 that is recognized by the mAb, 2F5 (Muster et al., 1993). In this chapter, I therefore exploited well-characterized neutralizing monoclonal antibodies that bind to defined epitopes of the gp120 to understand the mechanism of HIV-1 neutralization by aptamers. The selected neutralizing aptamers

(B4 and B19) were used in competition with these monoclonal antibodies for binding to the gp120 epitopes. Thus, in order to understand the mechanism of HIV-1 neutralization by these aptamers, I asked the following questions:

1. Does aptamer B4 or B19, like IgG1b12 mAb or CD4IgG for instance, inhibit virus attachment to the target cell, and if so in what way?
2. Does aptamer B4 or B19, like 17b mAb, interfere with coreceptor and post-CD4 binding events, including fusion of the virus and cell membrane?
3. Does aptamer B4 or B19 bind to neutralizing epitope(s) on the gp120 that are yet to be discovered?
4. Does HIV-1 neutralization potency correlates with binding affinity to the gp120?

6.3. BIAcore™ studies: effect of aptamers on the binding of soluble human CD4 to gp120

Human CD4 is a primary receptor for HIV-1 (Bedinger et al., 1988; Dalgleish et al., 1984; Klatzmann et al., 1984; Kowalski et al., 1987; McDougal et al., 1986). To distinguish among the possible mechanisms, by which aptamers could block gp120-mediated infection by HIV-1, I investigated the effect of two neutralizing aptamers on the binding of gp120 to soluble human CD4. Aptamer B4 and B19 were selected because the former had extreme neutralization potency of HIV-1_{Ba-L} in PBMC, and the latter was one of the aptamers that significantly neutralized HIV-1_{Ba-L} in both PBMC and macrophages, and also efficiently cross-neutralized HIV-1_{ADA}.

Optical biosensor interaction analysis was used to quantitate the binding of aptamer B4 and B19 to monomeric HIV-1_{Ba-L} gp120 and their effect on the subsequent interaction of gp120 with soluble human CD4. Soluble monomeric HIV-1_{Ba-L} gp120 at 100 nM final concentration was mixed with 15-fold molar excess of control or test aptamer at room temperature for 1 hour before injection. Excess aptamer was used to saturate the protein and maximize the formation of gp120–aptamer complex. Binding assays were then configured with soluble human CD4 immobilized on the CM5 sensor chip surface (Figure 6.1). Thus equal amounts, as measured by response units, of soluble human CD4 were simultaneously immobilized by amine coupling on the chip as previously described (2.7.2). Briefly, carboxymethyl groups on the chip were activated by injecting 50 µl of a solution containing 0.5 M NHS and 0.2 M EDC at a flow rate of 5 µl/min. Next, soluble human CD4 at a concentration of 100 µg/ml in 10 mM NaOAc, pH5 buffer was simultaneously passed over two flow cells of the chip surface at 25°C until the desired number of response units for immobilized ligand was reached (Figure 6.2A). A reference surface with BSA immobilized on the control flow cell was generated under the same conditions as a background to correct for instrument and buffer artifacts.

Binding experiments were performed at 37°C in HBS buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂ and 2.7 mM KCl). Associations were assessed by injecting the analyte (gp120-aptamer mixture) simultaneously over the test and control flow cells at a flow rate of 5 µl/min. The signal from the BSA reference flow cell was subtracted to correct for bulk effects and nonspecific binding to the surface. Crudely, the sensorgrams in panels B and C of Figure 6.2 show visually that, at the same concentration of gp120, there is about 30% increase in the level of gp120 binding to soluble human CD4 when B4 or B19 is present, compared to control aptamer. However SPR measures the mass of material binding to the sensor surface, and therefore the effect is not as dramatic when considering that fact that the mass of gp120-aptamer complex is four-thirds that of gp120 alone. Effectively the 30% increase in SPR was contributed by the aptamer that was bound to the gp120, and the results confirmed that the gp120-aptamer complex was the predominant form in the mixture. Taken together, the results show that both aptamer B4 and B19 had no effect on binding of HIV-1_{Ba-L} monomeric gp120 to soluble human CD4 (Figure 6.2 A and B). Thus, the data suggest that neither of the two aptamers neutralize HIV-1 by directly preventing CD4-dependent viral attachment to target cells.

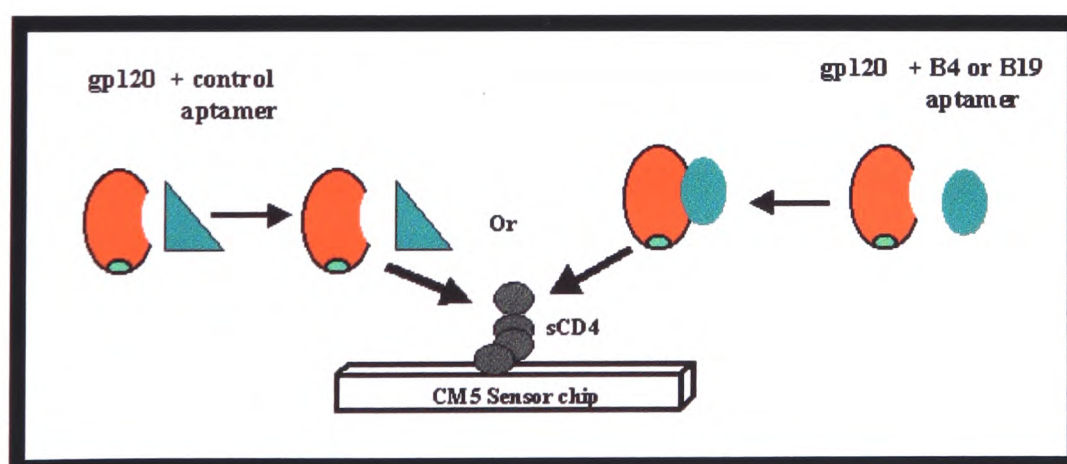


Figure 6.1: Schematic representation of the gp120-aptamer-sCD4 three component biosensor configuration, with soluble human CD4 immobilized on the sensor chip. Control aptamer has the same chemistry as both B4 and B19 but does not bind the gp120.

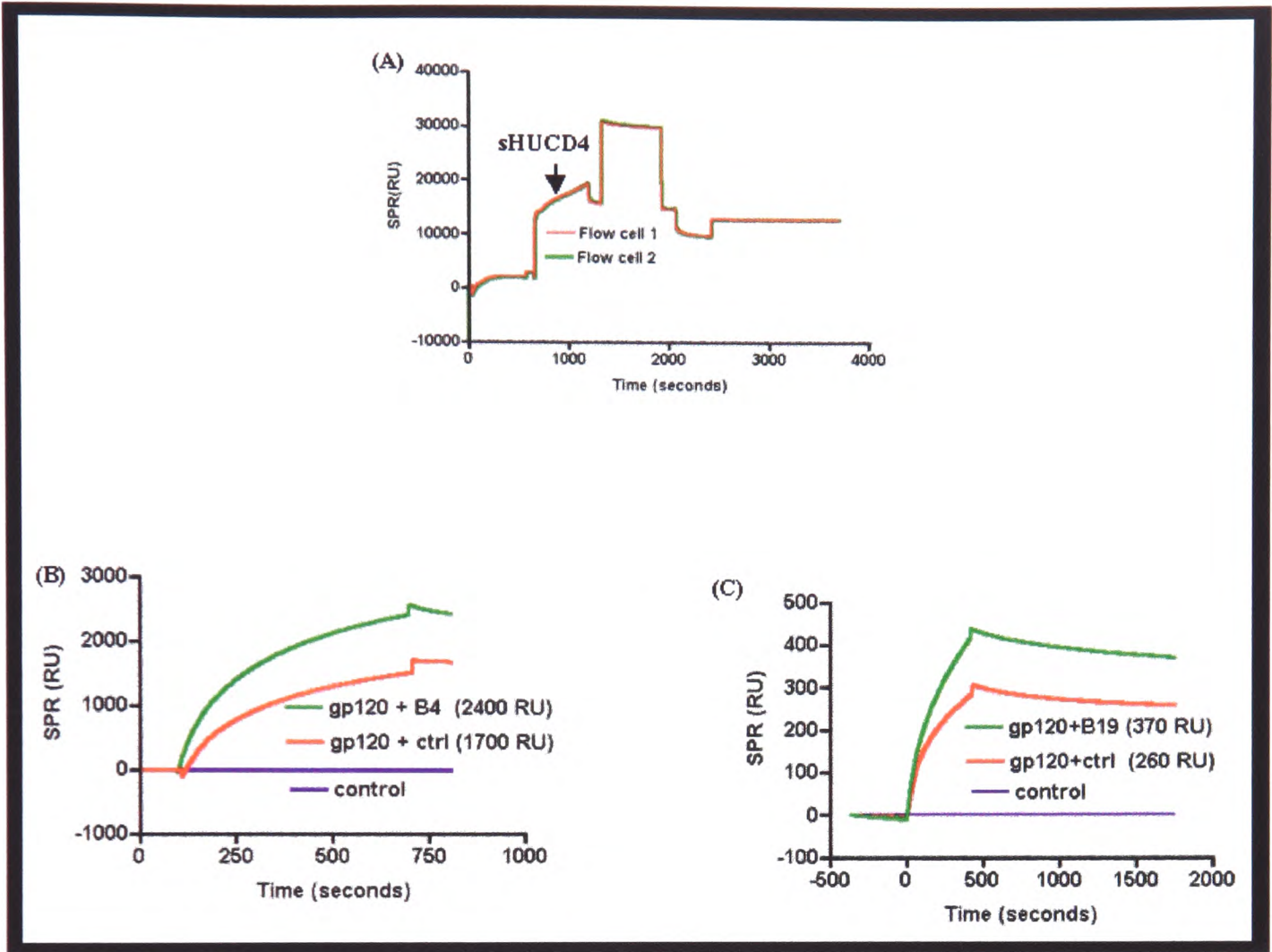


Figure 6.2: Effect of aptamers on the gp120-sCD4 interactions. (A) A BIAcore™ sensorgram depicting equal immobilization of soluble human CD4 on two flow cells of a biosensor chip. (B and C) Sensorgrams overlays for binding of monomeric HIV-1_{Ba-L} gp120 to soluble human CD4 in the presence of B4 and B19 respectively, compared to control aptamer. The molecular weight of each aptamer is 35 kDa, while that of the gp120 is 100 kDa, suggesting that there would be a proportional increase in response units if the aptamer did not interfere with binding of the gp120 to sCD4, and the gp120-B4/B19 complex predominate in the mixture. Inversely, if aptamer B4 or B19 interfered with binding of gp120 to soluble human CD4, there would be a significant decrease in the response units for that aptamer-gp120 complex, compared to the control aptamer.

6.4. Epitope mapping: competition of aptamers with neutralizing anti-gp120 monoclonal antibodies

6.4.1. Titration of antibodies against HIV-1_{Ba-L} gp120

To gain further insights into the mechanism of HIV-1 neutralization by aptamer B4 and B19, I used a large panel of anti-gp120 neutralizing monoclonal antibodies, described in sections 1.7.3 and 2.1.5, and whose epitopes had been defined with some precision (Figure 6.3). Monoclonal antibodies were titrated against the gp120 to determine the appropriate antibody concentration to be used. Antigen capture enzyme-linked immunosorbent assay was used for this study. Briefly, HIV-1_{Ba-L} gp120 was captured onto Immulon II ELISA plates, (Dynatech Ltd) via adsorbed sheep antibody D7324 to the carboxyl-terminal 15 amino acids of gp120 as described in chapter 2, section 2.10.1. The Env protein captured indirectly onto a solid phase via D7324 retains the ability to bind sCD4 and mAbs with high affinity (Moore, 1990; Moore et al., 1990; Moore and Sodroski, 1996; Thali et al., 1993). Serially diluted mAbs were reacted with the captured gp120 in triplicates, and bound antibody was detected as described (2.10.1). The monoclonal antibody concentration was selected on the basis of titration curves to give the mean optical density at 492 nm (OD₄₉₂) of approximately 0.8 units after correcting for background reading (Figure 6.4). Use of this antibody concentration resulted in signals of sufficient magnitude to be reliable while allowing sufficient sensitivity to small changes in the affinity of mAbs to the gp120 in the presence of aptamer that competed for the epitope.

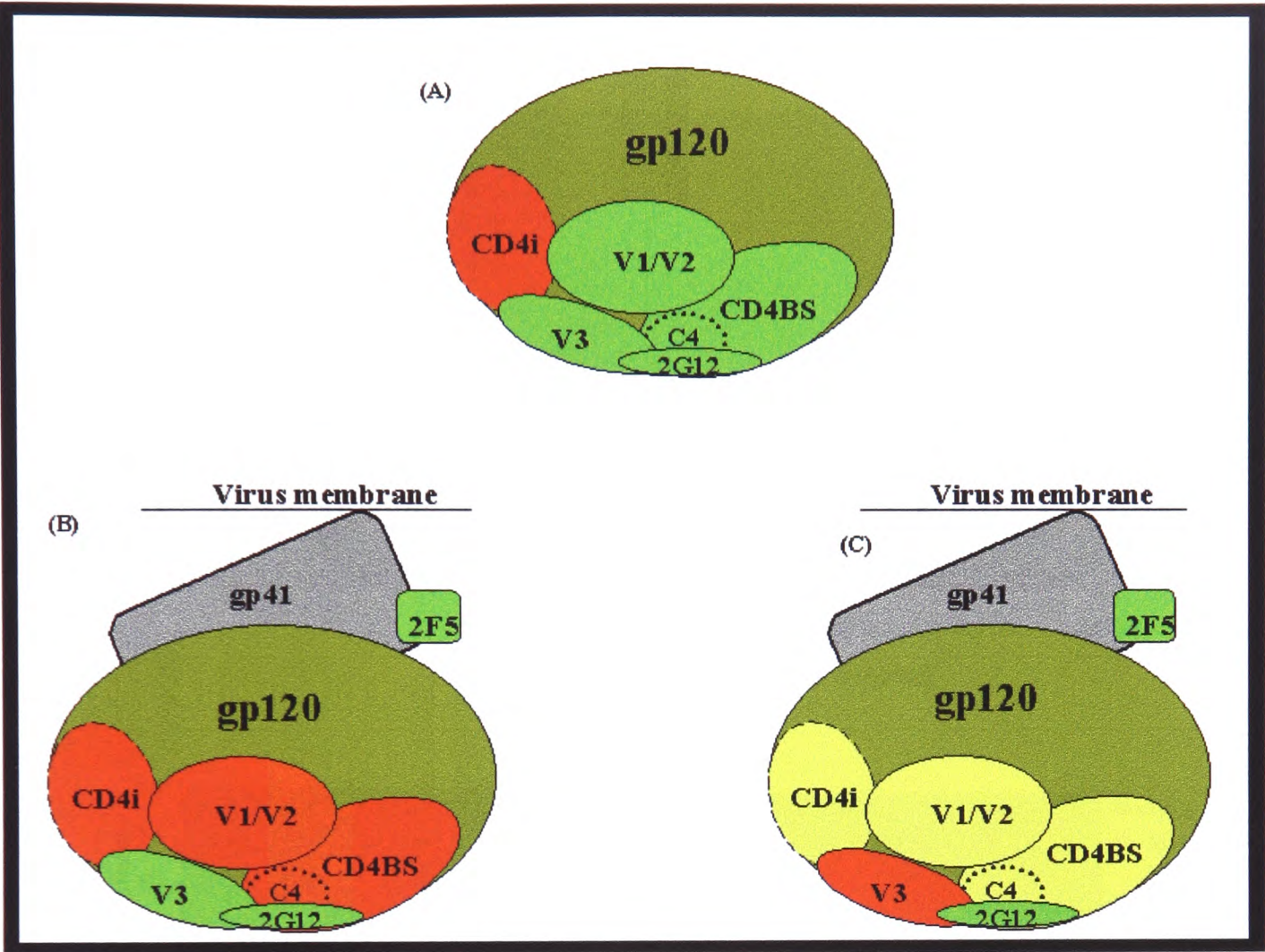


Figure 6.3: The topographic distribution of neutralization epitopes on the gp120 molecule. The scheme is based on epitope mapping and cross-blocking analysis of large number of mAbs. The schematic proximity of the regions reflects their estimated spacial proximity on the molecule. Epitope clusters shown are the CD4-binding site (CD4BS); the V1/V2 and V3 loops, the C4 and CD4-induced (CD4i) regions, the epitope for 2G12 mAb, and the 2F5 epitope on gp41. The V1/V2 and V3 loops, as well as the C4 region can be recognized as linear or conformational epitopes, while the 2F5 region can only be recognized as linear, and the rest as discontinuous epitopes. The colours represent relative exposure of mAb epitopes on the glycoprotein: green indicates strong exposure; red, partial exposure; yellow, poor exposure or completely masked. (A) Representation of the relative exposure of the different epitopes on soluble, monomeric gp120. All neutralization epitopes are well exposed with the exception of CD4i, which is partially masked. (B) Representation of the epitope exposure on oligomeric gp120 of the T cell-line adapted virus. The 2G12 region and V3 loop remain generally well exposed, but the V1/V2; CD4BS; C4 and CD4i epitopes are partially inaccessible. (C) Representation of the epitope exposure on oligomeric gp120 of the R5 primary isolate. The V1/V2 loop, CD4BS, C4 and CD4i regions are generally poorly exposed, and the V3 loop is partially masked. On these viruses the only well-exposed epitopes are likely to be 2G12 and rarely CD4BS. This model is modified from (Poignard et al., 1996b) and summarizes data from (Bou-Habib et al., 1994; Moore and Sodroski, 1996; Sattentau and Moore, 1995; Sattentau et al., 1995; Stamatatos and Cheng-Mayer, 1995).

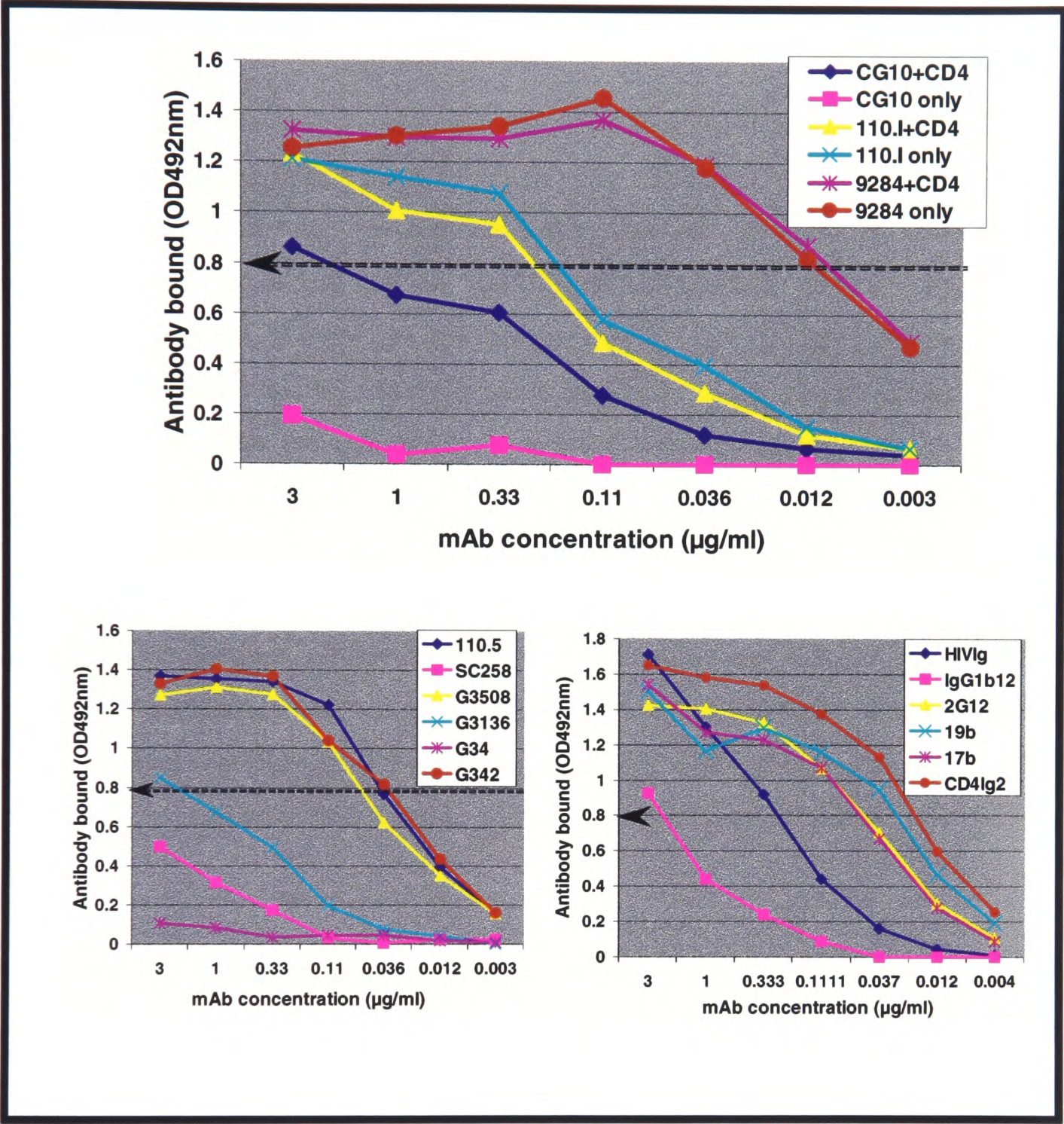


Figure 6.4: Titration of antibodies against HIV-1_{Ba-L} monomeric gp120. Note that CG10 rat mAb (upper panel) that maps to the CD4i epitope only bound the gp120 following CD4 binding. The rest except V2 loop mAbs, G34 and SC258, bound gp120 well, even in the absence of sCD4. In subsequent competition ELISA, all the antibodies were used at the concentration that corresponded to the optical density at 492 nm (OD₄₉₂) of about 0.8 unit (arrowhead). Each antibody titration was done in triplicate and mean OD₄₉₂ values were calculated after correcting for background reading.

6.4.2. Inhibition ELISA: competition of aptamers with antibodies for epitope binding

Inhibition ELISA was done to determine the binding site(s) of aptamer B4 and B19 on gp120, in competition with a panel of neutralizing antibodies that were selected from those titrated above (6.4.1). The protocol was carried out essentially the same way as in the titration assay except that the gp120 was pre-incubated with 1/3 dilutions of aptamer (i.e. 300 nM, 100 nM, 33 nM etc) made up in HBS binding buffer for 1 hour at room temperature before adding primary antibody. Thus all the preceding and subsequent steps after primary antibody addition were the same as in the titration assay, and the entire epitope mapping protocol is illustrated (Figure 6.5). Each test antibody was assayed in triplicates, and each test plate also contained background wells (no gp120 and no aptamer). The mean OD₄₉₂ values derived from the background wells were subtracted from all other mean test OD₄₉₂ values. After background correction, the mean OD₄₉₂ values, which expressed the amount of antibody bound was plotted as a function of aptamer concentration and compared to the control (no aptamer) to determine if there was inhibition of antibody binding to gp120.

The choice of mAbs for inclusion in the competition analysis presented a number of challenges. A large combination of different mAbs was needed for the assay to be as comprehensive as possible, but availability and costs limited this. Broad neutralizing antibodies that bind principal epitopes of native gp120 such as the CD4BS, the V3 loop and the CD4i regions were obvious choices. Candidates from the scores of rodents mAbs generated to various gp120 molecules, many in denatured states were excluded. Thus representative neutralizing antibodies that binds discontinuous and conformational epitopes of the gp120, generated in HIV-1 infected humans, were given a priority because they would have been elicited by native gp120 in either cell-associated or soluble forms.

For completeness, the influences of aptamer B4 and B19 on the CD4BS of the gp120 were probed with IgG1b12 and recombinant CD4IgG as competing ligands. Consistent with earlier findings using soluble human CD4, both B4 and B19 did not compete with the two antibodies for binding on the gp120 (Figure 6.6), further excluding the CD4BS as the possible epitope for the aptamers. Both aptamers also did not compete for binding with the 19b and 2G12 mAbs, which map to the V3 loop and the unique outer C3-V4 neutralizing epitope, respectively (Figure 6.6). Aptamer B4 did however inhibit binding of the 17b mAb to the gp120 in a concentration dependent manner (Figure 6.6), suggesting that the aptamer bind to, or overlaps the crucial CD4i neutralization epitope. The 17b mAb maps to the CD4i epitope, which is overlapped by the CCR5 binding site on the gp120. These results therefore suggest that aptamer B4 neutralize HIV-1 after attachment of the gp120 to the CD4, by blocking the CCR5 coreceptor-binding site on the gp120.

On the other hand, aptamer B19 that did not inhibit binding of monomeric gp120 to any of the tested antibodies must bind to an undefined gp120 neutralization epitope and possibly neutralize HIV-1 in a novel way, yet to be discovered. In this respect, although mAbs against the C4 region were not directly tested in competition assays, it was expected that 2G12, V3, or at least CD4BS mAbs would be inhibited if the aptamer bound the C4 epitope, because the C4 region overlaps the three epitopes, and contains elements of the CD4BS (Figure 6.3). It has been shown that several mAbs to the C4 sequences are able to inhibit gp120 binding to the CD4 or CD4-IgG (Sun et al., 1989). In addition C4-directed mAbs cross-block V3-directed mAbs, and amino acid changes in the V3 loop affect the binding of C4-directed mAbs and vice versa (Moore and Sodroski, 1996). Thus there is close association between segments of the C4 and V3 domains on the gp120, and V3 antibodies like 19b and G342 that were among mAbs not inhibited by aptamer B19 (Figure 6.6) actually recognize a discontinuous epitope spanning the C4 and V3 regions (Moore et al., 1993; Wyatt et al., 1992).

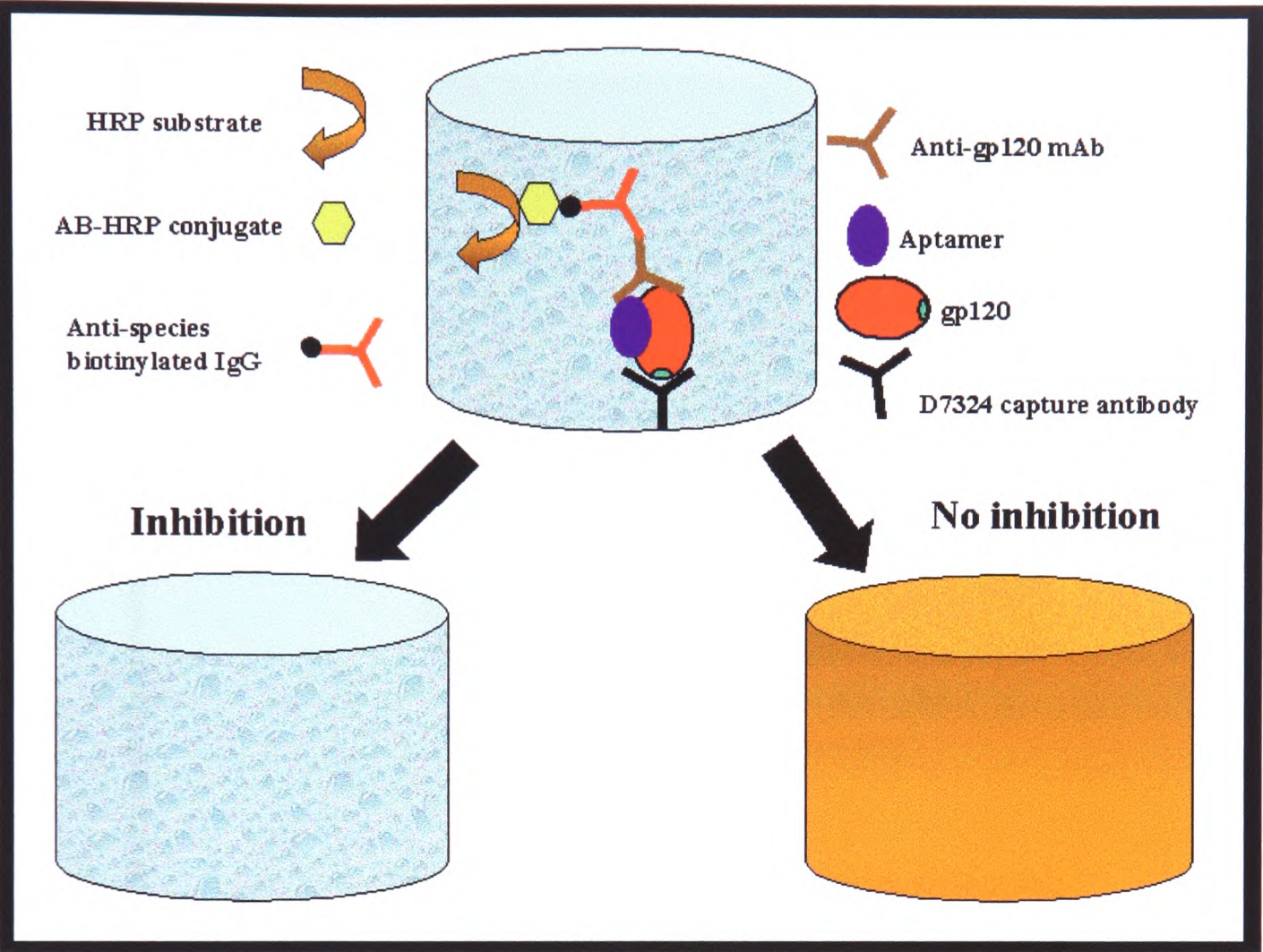


Figure 6.5: Simplified illustration of the gp120 epitope mapping ELISA, in which a panel of HIV-1 neutralizing antibodies individually competed with aptamer B4 or B19 for binding to the gp120. Inhibition of antibody binding to the gp120 epitope was indicated by no colour change of the reagents after addition of the substrate (Sigma fast HRP substrate kit), while colour change to orange indicated no inhibition. Quantitative data was obtained by measuring the optical density of the reagents at 492nm, after addition of the substrate.

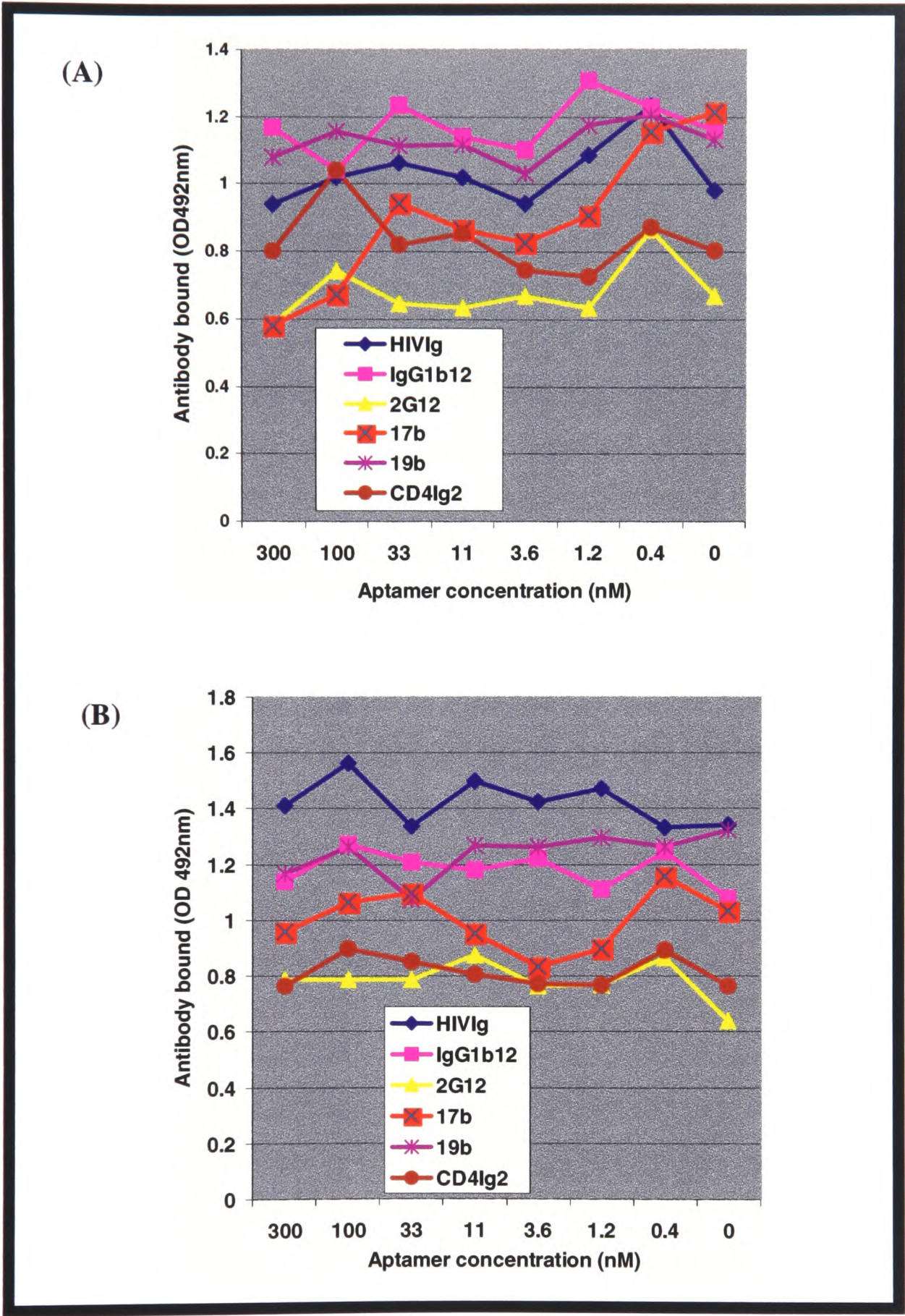


Figure 6.6: Competition of HIV-1 neutralizing antibodies with aptamers. **(A)** Effects of aptamer B4 on antibody binding to the gp120. **(B)** Effects of aptamer B19 on antibody binding to the gp120. Each antibody was tested in triplicates, and repeated at least twice, resulting in at least six ELISA wells for each competing antibody.

6.4.3. Inhibition ELISA: effects of sCD4 on competition of aptamer B4 with neutralizing mAbs that maps to the CD4i epitope of the gp120

To further determine the extent of competition between aptamer B4 and mAbs for the CD4i epitope on monomeric gp120, effect of soluble human CD4 on the assay was probed. The CD4i epitope is present on native gp120, but become more accessible upon CD4 binding, resulting in enhancement of 17b and competing 48d mAbs binding to the gp120 (Thali et al., 1993; Wyatt et al., 1995). In this study 10 μ M of soluble human CD4 were pre-incubated with D7324 captured monomeric gp120 for 1 hour at room temperature, followed by three washes as described (2.10.1), before adding aptamer B4. In addition to the 17b mAb, 48d and mouse CG10 mAb that also bind the CD4i epitope were tested in competition with aptamer B4 in the absence or presence of the human CD4.

Consistent with earlier findings, aptamer B4 inhibited 17b mAb binding to the gp120 by about 50% at the highest concentration used, however soluble CD4 did not enhance the inhibition or binding of 17b to monomeric gp120 in the presence or absence of B4, respectively (Figure 6.7). As expected B4 also inhibited binding of 48d to the gp120 by about 33% but soluble human CD4, again, did not increased inhibition of 48d by the aptamer (Figure 6.7). Interestingly, mouse CG10 mAb that only bound the gp120 in the presence of soluble human CD4 was not inhibited by aptamer B4 (Figure 6.7). The inability of soluble human CD4 to increase inhibition (in the presence of aptamer B4) or binding (in the absence of aptamer B4) of 17b and 48d mAbs to monomeric gp120 suggest that envelope glycoprotein oligomerization might be essential for the sCD4 induced enhancement of 17b and 48d binding. It has been shown that soluble human CD4 enhance binding of 17b and 48d to oligomeric HXBc2 envelope protein expressed on COS-1 cells by 200% and 150% respectively, (Thali et al., 1993). The CG10 mAb which strictly dependent on soluble human CD4 for binding to the gp120, implied that aptamer B4, which did not inhibit the antibody in the presence of sCD4 bind to a specific region of the CD4i-epitope. Thus the CG10 binding region is evidently more masked than the 17b and 48d regions of the CD4i epitope on monomeric gp120, and consequently not accessible to aptamer B4.

Differential effects of aptamer B4 on inhibition of 17b and 48d mAbs are consistent with others' findings, that the epitopes of these two antibodies overlaps, as 48d and 17b strongly cross-block each other but are not identical (Thali et al., 1993; Wyatt et al., 1995). Deletions within the V3 loop, for example, affect the integrity of 48d epitope more than that of the 17b epitope (Thali et al., 1993), and binding of mAb #49 that maps to the V3 loop, was enhanced by 17b but not 48d (Moore and Sodroski, 1996). Thus findings of this chapter taken together with others' data (Moore and Sodroski, 1996; Thali et al., 1993) argue that aptamer B4 binds a region of the CD4i epitope that is more distal from the 48d epitope and the V3 loop (Figure 6.3). By the same argument, the results of this study also suggest that although B4 inhibited 17b more than 48d binding to the monomeric gp120, B4 aptatope overlaps 17b binding region on the CD4i epitope, albeit much closer compared to the 48d region. Thus B4 probably binds a unique region within the CD4i epitope that overlaps the 17b and 48d binding sites.

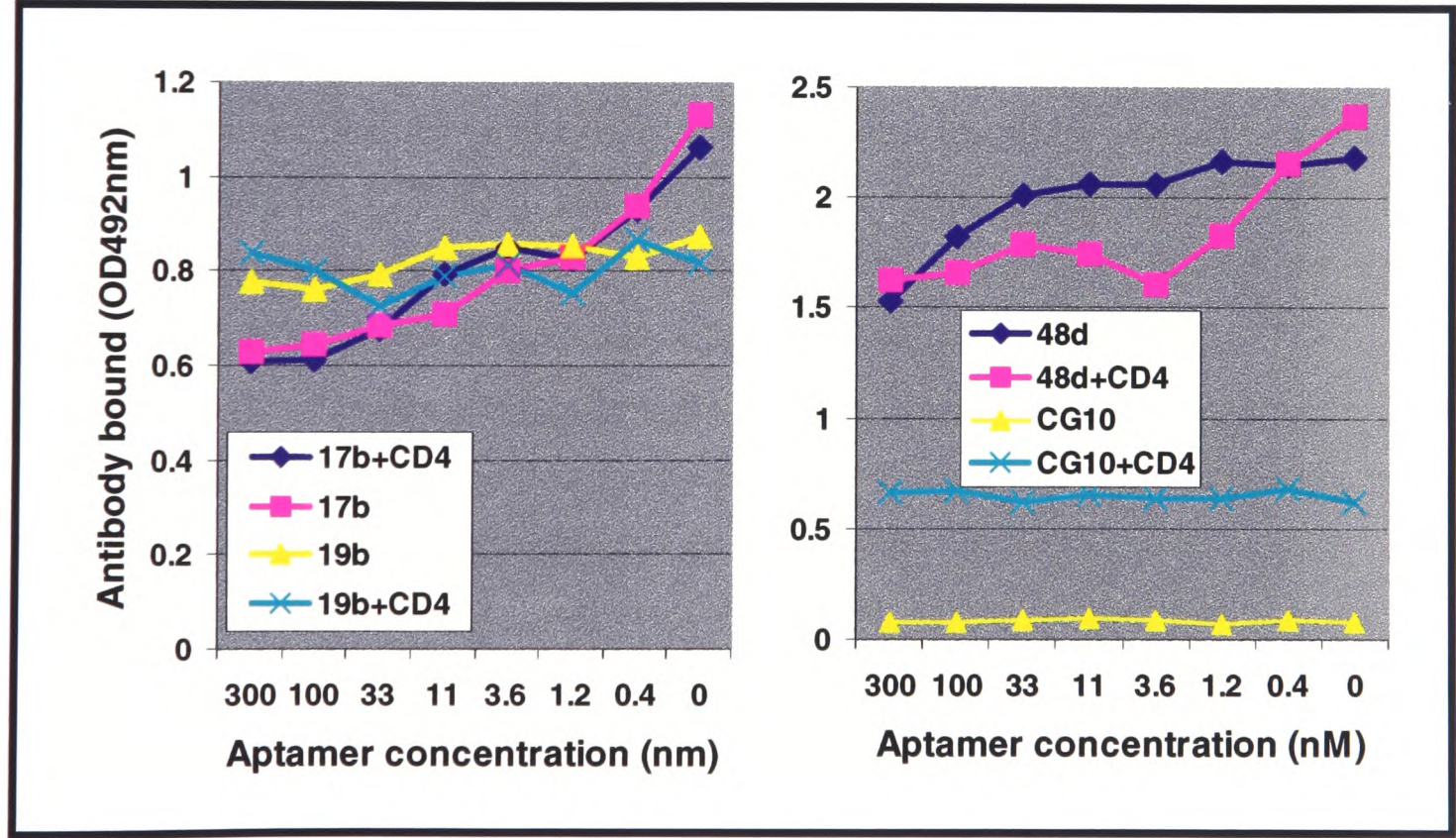


Figure 6.7: Effects of soluble human CD4 on competition of aptamer B4 with neutralizing mAbs that map to the CD4i epitope of the gp120. HIV-1_{Ba-L} monomeric gp120 was used in these assays, and the 19b mAb that maps to the V3 loop of the gp120 was used as a control.

6.5. Binding kinetics of aptamers to the gp120

Binding kinetics of the two selected aptamers, B4 and B19, to HIV-1_{Ba-L} monomeric gp120 were determined using the SPR analysis. The ligand, HIV-1_{Ba-L} gp120 was immobilized on the CM5 BIAcore™ chip using amine coupling as described (2.7.2). The concentration of the analyte (aptamer) was accurately measured, and the dilution series of the analyte (5nM – 3 200nM) were prepared in HBS binding buffer. Each concentration of the analyte (starting with the lowest) was injected at 5 µl/min and allowed to dissociate over 3 600 seconds. Analyte was also injected at different flow rates (10; 25 and 50 µl/min) on flow cells with varying surface densities of the ligand (2 500, 5 000 and 7 500 RU) to control for possible mass-transport effects. Data was analysed using the BIAevaluation 3.0 (BIAcore) and GraphPad Prism 3.00 (GraphPad software Inc., USA) software, and the affinity constant was calculated from the on-rates (k_{on}) and off-rates (k_{off}) as determined by non-linear regression curve fit to a simple bimolecular reaction model.

Kinetic constants obtained from data analysis are summarized in Table 6.1. Aptamer B4 had 20-fold faster on-rates, and 120-fold faster off-rates than aptamer B19, and overall bound monomeric HIV-1_{Ba-L} gp120 with about 10-fold lower affinity than B19 (Figure 6.8). Comparison of these data with soluble human CD4 kinetics to HIV-1_{Ba-L} gp120 showed that aptamer B4 had higher K_d value (affinity constant) than the CD4, while aptamer B19 had more than 2-fold K_d value to the gp120 than soluble human CD4 (Table 6.1). These data support the notion that, while the three analytes are obviously different from each other, they also bind to different regions of the gp120. Further comparison of B4 and B19 data to published kinetics of 17b mAb binding to HIV-1_{JRFL} monomeric gp120 (Zhang et al., 2001), showed that B4 kinetic constants fell within the range of 17b affinity constants to the gp120, while B19 had lower kinetic constant values (Table 6.1). Taken together these data advance the argument that aptamers can have as good affinities to the gp120 as mAbs and HIV-1 natural ligands such as the human CD4.

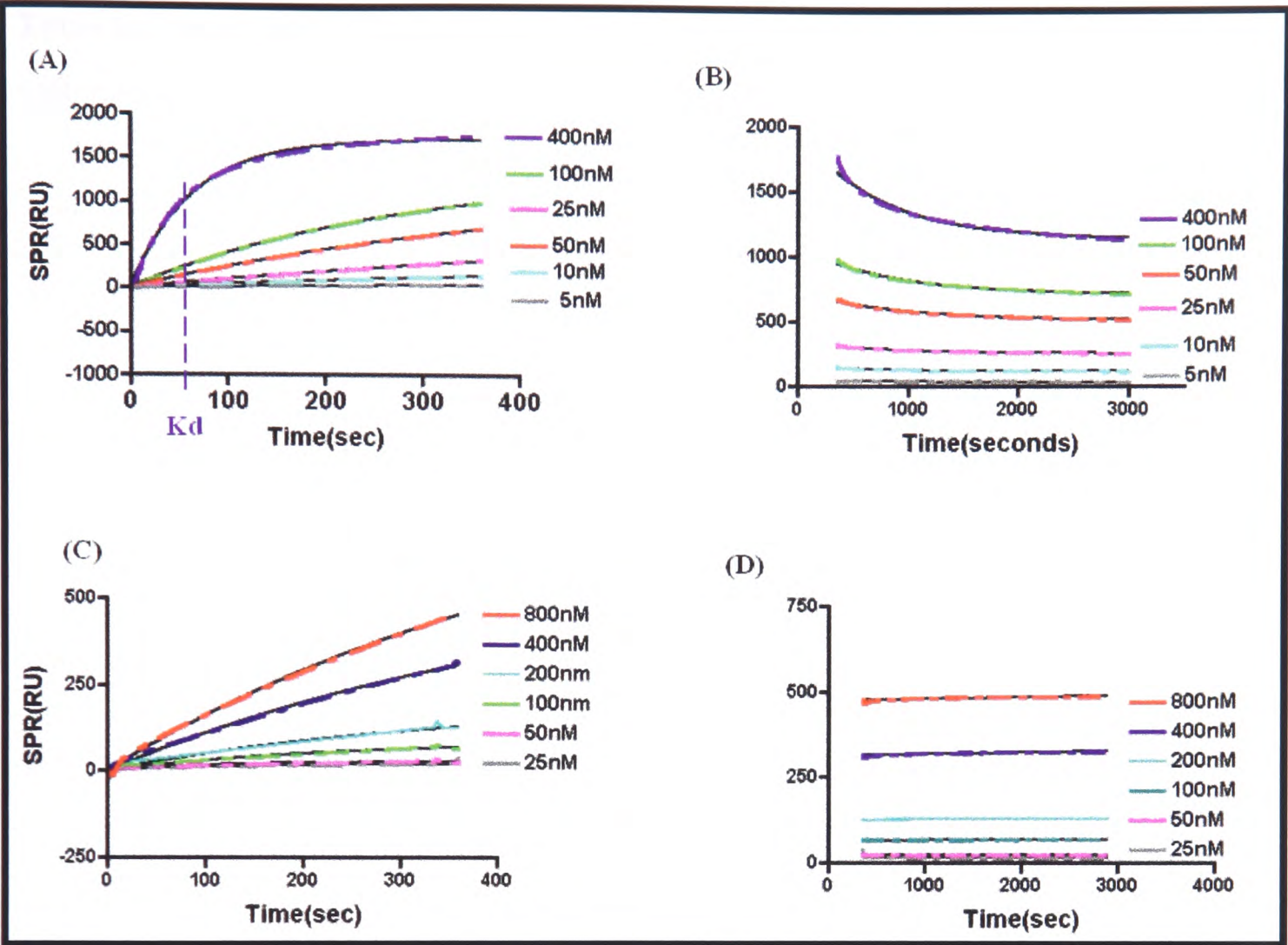
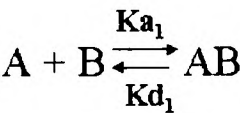


Figure 6.8: Global curve-fitting analysis of aptamers kinetics to immobilized R5 monomeric gp120 (2 500 RU). Broken colour-coded lines represent experimental data for shown concentration of analyte used, and superimposed solid black lines represent the fitting results. (A and B) Sensorgram overlays of aptamer B4 observed association rates (k_{ob}) and off-rates, respectively. (C and D) Aptamer B19 observed on-rates and off-rates, respectively. Although the K_d in these experiments was determined from nonlinear global curve-fitting analysis using GraphPad Prism software, it could also be determined by linear regression of Scatchard transformed data. Thus, for instance, K_d of B4 at 400nM (blue line in A) could be estimated as the negative reciprocal of the slope of transformed linearized data (slope = $-1/K_d$). However, the Scatchard transformation distorts the experimental error, and thus violates several assumptions of linear regression.

Table 6.1: Summary of biosensor-derived kinetic constants of aptamers interaction with monomeric gp120, compared with shuCD4 and 17b kinetics^a.

Interaction	K _{on} (x10 ⁶ M ⁻¹ s ⁻¹)	K _{off} (x10 ⁻³ s ⁻¹)	K _d (x10 ⁻⁹ M)
B4-gp120(Ba-L)	0.027 ± 0.0062	1.298 ± 0.118	48.8 ± 7.21
B19-gp120(Ba-L)	0.0015 ± 0.0006	0.01 ± 0.0014	6.66 ± 1.46
shuCD4-gp120(Ba-L)	0.34 ± 0.06	5.28 ± 0.084	15.52 ± 2.9
17b-gp120(JRFL) ^b	0.087 ± 0.002	2.43 ± 0.24	28.1 ± 3.5

^aThese data are representative of experiments done with different densities of ligand immobilized to control for mass transport effects which anyway did not seem to be limiting in the system used. Data is expressed as average ± S.D. Kinetic constants were obtained from data fitting analyses following a 1:1 binding model:



Where $k_{on} = k_{a_1}$ and $k_{off} = k_{d_1}$. Affinity constant of ligand binding (K_d) was calculated as the ratio of k_{off} to k_{on} , in line with the law of mass action.

^bBecause of limiting amounts of mAbs, 17b-gp120 kinetic constants published elsewhere (Zhang et al., 2001) were used for comparison. I perceived the 17b-gp120 kinetic data as reliable for comparison because it was also determined using the SPR and global-curve fitting analysis. In addition the gp120 used was monomeric and was derived from a clade B R5 virus, though a different strain.

6.6. Discussion

6.6.1. Neutralizing epitopes

The availability of mAbs that are directed against neutralization epitopes of the gp120 allowed me to investigate important aspects of the mechanism of HIV-1 neutralization by some of the aptamers. Key findings of this study are that aptamer B4 binds or overlaps a highly conserved but poorly immunogenic CD4i epitope as defined by competition with 17b and 48d mAbs, while B19 probably binds to an undefined, novel neutralization epitope. All the six distinct clusters of neutralization epitopes identified so far on the gp120 are defined in terms of neutralizing mAbs. It can therefore not be excluded that more potent neutralization epitopes do exist, which could be dissected by ligands possessing higher resolution such as neutralizing aptamers, like B19.

Although all the currently identified epitopes are neutralizing in TCLA, far fewer are accessible by antibodies on the mature envelope of primary isolates. Neutralization of a range of primary isolates has only been observed with two mAbs specific for two epitopes on the gp120. The V3 and V1/V2 loops, which have been considered as the principal and secondary neutralizing domain of HIV-1, respectively, due to their dominant role in neutralization of TCLA strains, have insignificant role in neutralization of primary isolates (Spence et al., 1998; Vancott et al., 1995). In addition, they also display a highly strain-specific neutralization in TCLA isolates because of hypervariability and polymorphism of the loops (Wang et al., 1995). Moreover, linear epitopes, like the V1/V2 and V3 loops, and the C4 epitope, have a restricted neutralization range compared with discontinuous or conformational epitopes, like the 2G12 and CD4BS epitopes, which depend on native structure of gp120 (Steimer et al., 1991). For example, IgG1b12 that recognizes the conformational dependent epitope on the CD4BS (Burton et al., 1994; Roben et al., 1994) and mAb 2G12 (Trkola et al., 1995; Trkola et al., 1996b) that recognizes a discontinuous epitope located at the base of the V3 and V4 loop, are the only gp120 epitopes confirmed to stimulate a neutralizing response in primary isolates, in addition to TCLA viruses.

The CD4i epitope, although it is discontinuous like the CD4BS and the 2G12 epitopes, has been reported to only neutralize TCLA strains of HIV-1 (reviewed by (Labrijn and Parren, 1999; Poignard et al., 1996b; Sattentau, 1996)). Neutralization of primary isolates by mAbs against the CD4i epitope has not been observed. Thus the study carried out in this chapter, is the first to describe neutralization of HIV-1 primary isolates in a manner that is dependent on the CD4i epitope. This novel finding suggests that aptamers could provide a useful alternative, where antibodies have been insufficient thus far.

6.6.2 Binding kinetics of aptamers to the gp120 and neutralization

Fundamentally, neutralization of HIV-1 by at least aptamer B4 and B19, could be explained with an incremental model (Schonning et al., 1999), in which neutralization occurs incrementally as aptamer binds each envelope molecule. Thus neutralization of HIV-1 primary isolates by aptamers probably occurs as the result of the reduction of the number of functional envelope protein below a critical threshold. Relatively slow dissociation rates and high affinity of aptamers to the gp120 derived from a primary isolate (Table 6.1) partly explain the neutralization potency by these molecules. It has previously been shown that HIV-1 neutralization correlates broadly, although not absolutely with affinity of the gp120 to antibodies (Sattentau and Moore, 1995), and dissociation but not association rates of antibody-gp120 binding is predictive of neutralization (VanCott et al., 1994). Although the studies above were performed with mAbs, there is no reason that the general conclusion from these studies could not apply to aptamers.

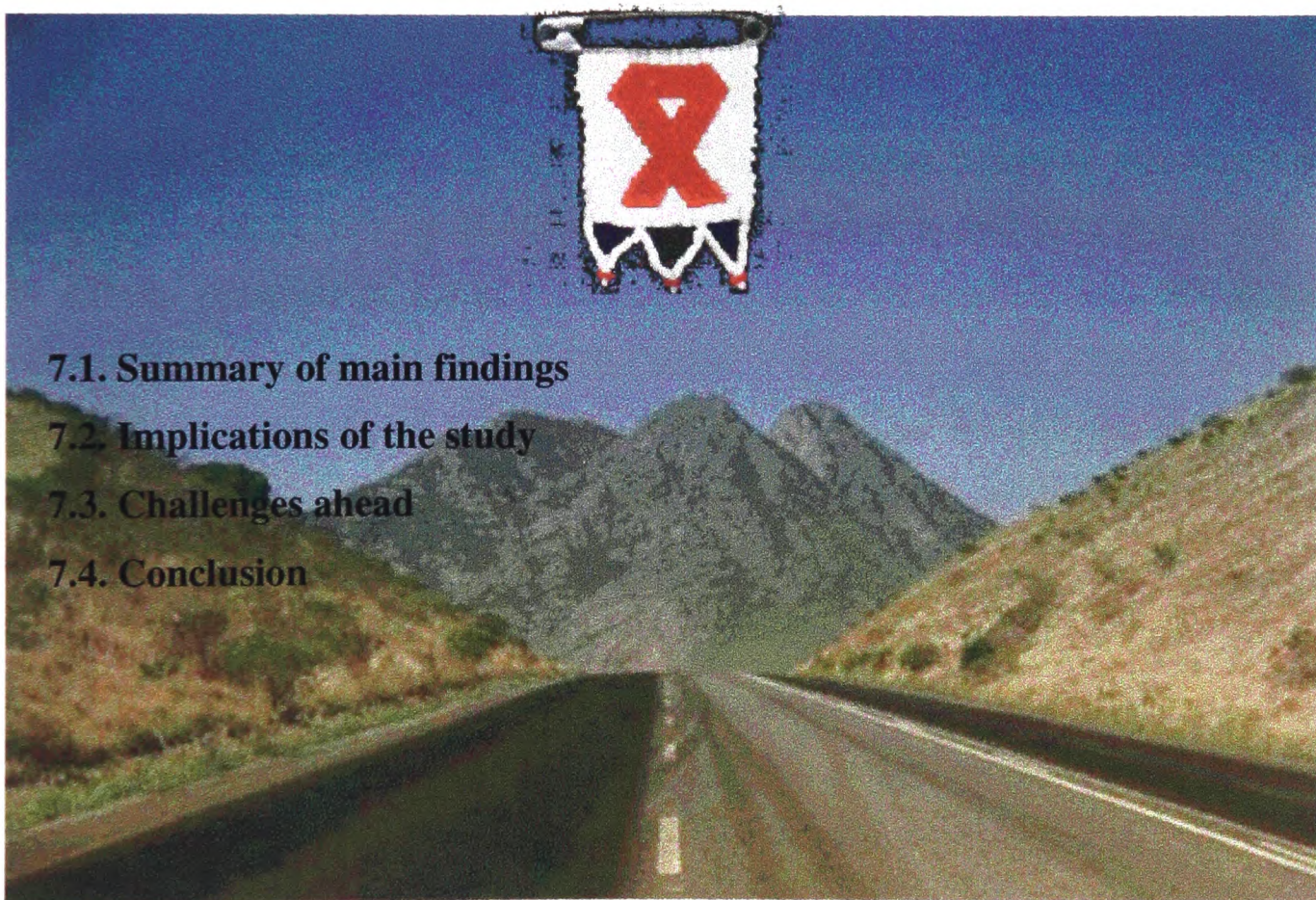
6.6.3. Probable mechanism of HIV-1 neutralization by aptamers

Although it is practically difficult to directly determine the mechanism of HIV-1 neutralization during infection of target cells, the mechanism could be reliably inferred from the gp120 epitope mapping of neutralizing aptamers. From epitope mapping ELISA and SPR experiments, both aptamer B4 and B19 did not inhibit binding of monomeric gp120 to soluble human CD4, suggesting that both aptamers interfere after viral attachment to the CD4 of target cells. Although the mechanism of neutralization by B19 remains unclear, competition of aptamer B4 with 17b and 48d mAbs suggests that the aptamer block binding of the CCR5 coreceptor to the gp120, because the two antibodies inhibit interaction of the gp120 with CCR5 (Trkola et al., 1996a; Wu et al., 1996).

The mechanism of HIV-1 neutralization by aptamer B4 at the level of viral-cell interactions would therefore be analogous to that proposed for antibodies directed at the CD4i epitope (reviewed by (Labrijn and Parren, 1999; Sattentau, 1996)), but probably much efficiently. Thus, subsequent to viral binding to the target cell initiated by gp120-CD4 interaction, probable events leading to HIV-1 neutralization by B4 would include inhibition of virus binding to the CCR5 coreceptor. Inhibition of CCR5 binding by the aptamer, would then induce the dissociation of gp120 from gp41, in a manner similar to that of antibodies that act after CD4 binding (Poignard et al., 1996a; Poignard et al., 1996b). B4 induced dissociation of gp120 from gp41 would lead to inappropriate exposure of the gp41 fusion domain and virus inactivation. Thus whatever the exact molecular mechanism, one may speculate that B4, as proposed for mAbs against the CD4i epitope, inhibit fusion of the virus and cell membrane by mimicking CCR5-triggering of fusion activation, which when elicited prematurely, renders the virus ineffective. In conclusion, while the use of aptamers in understanding the molecular interaction of HIV-1 and target cells at entry is still in its infancy and neutralization epitopes on gp120 are defined by mAbs, aptamers are promising tools possessing higher resolution to refine the precise boundaries and positions of these epitopes.

Chapter Seven

General discussion and conclusions:
implications of the study and challenges ahead



Uncertain as the road seems, there are challenges ahead in a fight against HIV-1 and AIDS, but in the face of uncertainty hangs a cloud of hope. Surely like the devastating 1347 bubonic plague a.k.a. "the Black Death" and many more defunct communicable diseases, AIDS will be conquered.

7.0. General discussion and conclusions: implications of the study and challenges ahead

7.1. Summary of main findings

The primary aim of this thesis was to develop synthetic nucleic acid ligands, aptamers, against gp120 envelope glycoprotein of an R5 strain of HIV-1, with a view of using aptamers as novel neutralization molecules and analytical tools to study HIV-1 entry. Thus the aim included use of aptamers to chart the early steps in the HIV-1 life cycle and understand better the molecular mechanisms and pathways by which R5 isolates enter natural target cells.

Using the SELEX protocol and SPR technology the primary aim of this thesis was fulfilled by isolating at least 26 distinct aptamer sequences that bind gp120 with as high affinity constants as mAbs, and had very slow dissociation rates which were predictive of HIV-1 neutralization. This study showed that many of these aptamers neutralized the infectivity of R5 primary isolates on average by approximately 1,000-fold, and two of which neutralized HIV-1_{Ba-L} by more than 100,000-fold, truly unprecedented when compared with ligands such as antibodies. Thus HIV-1 primary isolates, defined as viruses obtained by passage in primary cultures such as PBMC, are relatively refractory to antibodies neutralization, yet this study has shown that the two R5 primary isolates tested, HIV-1_{Ba-L} and HIV-1_{ADA}, were as sensitive to aptamers neutralization as TCLA isolates are to antibodies. One aptamer called B4 has defined a conserved site of the gp120 that could not mutate to escape neutralization following stringent selection for break-through virus. This was corroborated by a finding that B4 binds to a highly conserved CD4i epitope as determined by competition with 17b and 48d mAbs that map to this neutralization epitope on the gp120. Although the CD4i neutralization epitope has been demonstrated to neutralize in TCLA viruses, neutralization of primary isolates by mAbs against this epitope has not been observed, hitherto. This study is the first of its kind to describe neutralization of primary isolates by a ligand against the CD4i epitope. Another key finding was that although aptamer

B4 potently neutralized HIV-1_{Ba-L} in PBMC, which is a mixed T cell and macrophage population, it modestly neutralized infection of the same virus in a purified culture of macrophages. These findings are intriguing in that they suggest that aptamers could be used to dissect unique sites on the virus that interact with target cell surface in ways that have not been revealed heretofore, and would help understand better HIV-1 entry pathways, especially in macrophages.

7.2. Implications of the study in relation to HIV-1 therapy

The use of combinatorial chemistry as epitomized by the SELEX process has moved serendipity-based research towards a rational, target-based approach for drug discovery. In this spirit, this thesis presented a novel way of potently neutralizing HIV-1 primary isolates in human PBMC using anti-gp120 aptamers. While intensive research is focused on vaccine strategies, novel therapeutics are urgently needed to supplement current anti-retroviral drugs and ameliorate disease course in infected individuals as pointed out by others (LaBranche et al., 2001; Michael and Moore, 1999). In this regard, the small size and biophysical properties of aptamers enabled them, among others, to bind to a highly conserved CD4i epitope on the gp120 and produce highly efficient neutralization of infectivity by blocking interaction of the virus with the β chemokine CCR5 receptor on target cells. Inhibition via CCR5 coreceptor is a particularly attractive option because, like other molecules that target gp120, it precludes viral entry. Most importantly, as discussed in the introduction (1.6.2), viruses that establish a new infection are generally R5 tropic. Therefore a successful inhibition of viral interaction with the CCR5 coreceptor like in this study might significantly decrease the probability of HIV-1 transmission in real life situations. In addition direct blocking of the gp120 epitope that interacts with CCR5, as opposed to blocking the CCR5 itself has a number of advantages. Although the CCR5 receptor has been shown to have a dispensable physiological function, as evidenced by individuals lacking the functional receptor ($\Delta 32$ homozygous) and yet apparently immunologically normal, ligands that target wild-type CCR5 might induce aberrant signaling cascades, thereby leading to increase viral infection.

For an example, while CCR5 ligands like β chemokines have been reported to inhibit HIV-1 infection of macrophages *in vitro* by competing with the gp120 for binding to the coreceptor (Alkhatib et al., 1996; Capobianchi et al., 1998; Coffey et al., 1997), others have found them to act as stimulators, enhancing viral infection instead (Gordon et al., 1999; Trkola et al., 1999). Stimulatory effects of CCR5 ligands are dependent on cell signaling events that induce a number of intracellular mechanisms in macrophages and CD4⁺ T cells, which lead to increased HIV-1 replication (Kelly et al., 1998; Taub et al., 1996). Another observed problem, common to CCR5-targeted antagonists is the rapid emergence of drug resistance.

Escape mutants of HIV-1_{JR-CSF} with more than a 100-fold resistance to the anti-CCR5 mAb 2D7 have been generated (Aarons et al., 2001). Recently another R5 primary isolate, HIV-1_{CC1/85}, escape mutant with more than 20,000-fold resistance to AD101, a CCR5-specific entry inhibitor was reported (Trkola et al., 2002). The same virus was cross-resistant to a related inhibitor, SCH-C and partially resistant to RANTES. Common to all these escape mutants is that they continued to use the CCR5 in an inhibitor-insensitive manner (Aarons et al., 2001; Trkola et al., 2002). The authors argued that although it can take only a few amino acid substitutions to convert an R5 to an X4 virus (Fouchier et al., 1992), and CXCR4 was available on PBMC, the HIV-1 did not follow seemingly the simple pathway of switching to CXCR4 use in the presence of CCR5-targeted inhibitor (Trkola et al., 2002). Instead the virus accumulated multiple mutations on the gp120 over time, creating a mutant that continued to use CCR5 but was almost completely insensitive to the selecting CCR5 specific inhibitor.

Although the problem of drug resistance is not unique to CCR5-targeted inhibitors, and has been reported with anti-retroviral drugs currently in clinical use (discussed in 1.5.4), HIV-1_{Ba-L} mutants remained completely sensitive to aptamer B4, which binds the gp120 epitope that interacts with CCR5 receptor (section 5.6). This finding supports the suggestion that the CD4i epitope could be the virus' Achilles' heel. For this reason the virus has evolved an efficient strategy to protect it from antibody attack

and transiently expose it only during infection. In retrospect the findings of this study imply that aptamer B4 is able to readily gain access into the CD4i epitope, even without being induced by CD4, supporting a central hypothesis of this thesis that aptamers can reach parts within gp120 that antibodies cannot reach. Another implication of this study, as noted elsewhere (Trkola et al., 2002), is that suppression of HIV-1 with a potent agent like aptamer B4 that prevents CCR5 utilization does not readily drive viral evolution towards CXCR4 use. These data argue against the notion that coreceptor switching to CXCR4 use must necessarily be a rapid consequence of the inability to use the CCR5 as seen in about 50% of HIV-1 infected individuals. Other unknown viral or host cell factors probably dictate coreceptor switching.

The viral tropism as well as entry mechanisms and pathways are much complex, and clinical experience has taught us that the plasticity of HIV-1 will enable it to almost always mutate to escape from the selection pressure of any one drug (Perrin and Telenti, 1998). Although HIV-1 might eventually escape neutralization by aptamers, from *prima facie* evidence aptamers seem to have properties of an effective antiviral agent which include delayed drug resistance and slow dissociation rates. Arguably an entry inhibitor with even less than 50% efficacy could have a significant impact in reducing HIV-1 transmission.

Entry inhibitors such as aptamers, whether coreceptor targeted or otherwise, would intervene at a hitherto clinically undisturbed part of the viral life cycle. It is assumed that in therapeutic circumstances any HIV-1 entry inhibitor would be combined with existing therapy to achieve maximum suppression of the HIV-1 replication (LaBranche et al., 2001; Michael and Moore, 1999; Trkola et al., 2002). Thus, the most prudent antiviral strategy will involve drugs that target multiple steps in the viral life cycle. Conceptually, this strategy should slow the emergence of escape mutants because the speed with which mutations occur is directly dependent on the rate of HIV-1 replication.

7.3. Challenges ahead

Although aptamers potently neutralized R5 HIV-1 primary isolates and seemed attractive candidates for therapy, the high cost of their synthesis and their poor bioavailability make it unlikely that aptamers in the form described in this thesis could be readily used as anti-retroviral therapy. The challenge is to exploit aptamers to provide structural leads for the development of potent or even smaller molecules that can mimic their HIV-1 neutralizing properties.

First, it would be important to determine how many functionally distinct aptamer binding sites are present on the gp120 by using all the different sequences of HIV-1_{BaL} neutralizing aptamers to generate a competition matrix. Representatives of each competition group could then be screened to determine if their neutralization ability of HIV-1_{BaL} could be extended to include a diverse group of clinical isolates, including those that are extraordinarily resistant to antibody neutralization, mentioned in chapter 5 (5.9.3). Arguably, although others feel that the efficacy of HIV-1 neutralization when evaluating a candidate vaccine or agent like aptamers should be assessed in primary isolate of intermediate neutralization sensitivity (Labrijn and Parren, 1999), I believe inclusion of those strains that are extraordinarily resistant is important, because they predominate in patients.

Another immediate challenge would be to determine the structural determinants of aptamer functionality. Thus detailed structural analysis of selected aptamers with a broad-neutralizing activity against HIV-1 primary isolates would provide further molecular insight into the mechanism of action and the possibility for subsequent structure-based drug design. In addition, definition of the minimum sequence element required for aptamer function would allow possible solid phase synthesis of aptamers with reasonable efficiency and cost-effectiveness, and the solution of neutralizing aptamer–gp120 complex structure might enable one to develop more conventional drugs through structure-based drug design.

Although availability of a good HIV-1 animal model is a limiting factor, and human PBMC to an extent resemble the *in vivo* conditions as these cells are natural targets of HIV-1, it would be prudent to determine the HIV-1 neutralization activity of aptamers in a more dynamic *in vivo* system. Thus, it would be important to find out whether aptamers *in vitro* neutralization data would support the growing body of evidence that inactivation of HIV-1 in animal models such as hu-SCID mice and macaques generally correlates well with *in vitro* results (Gauduin et al., 1997; Mascola et al., 1999; Parren et al., 1995; Shibata et al., 1999).

7.4. Concluding remarks

HIV-1 infection remains a significant issue in global health, and while it would not drive us to extinction, it would systematically continue to destabilize society until a safe and effective vaccine and/or therapy is found. Therefore echoing sentiments of others (LaBranche et al., 2001; Michael and Moore, 1999), given recent demonstrations of both viral resistance and persistence in the face of current anti-retroviral drug regimens, ongoing assessment of HIV-1 entry inhibitors such as aptamers should gain momentum. These novel ligands or their structural mimetic would provide hope for salvage therapy for individuals failing HAART, as well as alternatives for initial therapy for newly infected individuals. In conclusion this study has pointed to the value of aptamers been as good as antibodies, only much neater, and could provide a useful alternative where antibodies failed.

"The tragedy in life does not lie in not reaching a goal. The tragedy lies in having no goal to reach.

It is not a calamity to die with dreams unfulfilled, but it is a calamity not to dream.

It is not a disaster to be unable to capture your ideal, but it is a disaster to have no ideal to capture.

It is not a disgrace not to reach the stars, but it is a disgrace to have no stars to reach for.

Not failure, but low aim is sin".

Benjamin Elijah Mays (1894–1984)

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