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Characterizing the immune response to HIV-1 using host derived epitope R7V

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On the side of the book:

Left side: **C. Bremnæs**

Right side: **2010**

In loving memory of my grandfather,

whose hunger for gaining knowledge never stopped. I know how much you would have loved to read my dissertation.

I miss you

Maj. Meidar Birger Martin Bremnæs

(1925 - 2008)

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ABBREVIATIONS

Units of measurement

kDa	KiloDalton
mg	Milligram
ml	Millilitre
ng	Nanogram
µl	Microlitre
µM	Micromolar
v/v	Volume per volume
w/v	Weight per volume

Amino acids

A	Alanine	M	Methionine
C	Cysteine	N	Asparagine
D	Aspartic acid	P	Proline
E	Glutamic acid	Q	Glutamine
F	Phenylalanine	R	Arginine
G	Glycine	S	Serine
H	Histidine	T	Tyrosine
I	Isoleucine	V	Valine
K	Lysine	W	Tryptophan
L	Leucine	Y	Tyrosine

Abs	Absorbance
ACK	Ammonium chloride potassium
β2m	Beta-2 microglobulin
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
APC	Antigen-presenting cell
ARV	Antiretroviral
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CA	Capsid
cc	Cell control
CDC	Center for Disease Control
cDNA	Complementary DNA
CIC	Circulating immune complexes
CFDA-SE	Carboxyfluorescein Diacetate, Succinimidyl Ester
CMV	Cytomegalovirus
CRF	Circulating recombinant forms
CTL	Cytotoxic T-lymphocyte
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Env	Envelope
ER	Endoplasmatic reticulum
ESI-MS	Electrospray ionization - mass spectroscopy
Fab	Fragment antigen-binding
FACS	Fluorescence-activated cell sorter
FBS	Fetal bovine serum
Fmoc	Fluorenylmethyloxycarbonyl
Gag	Group specific antigen

HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HRP	Horse-radish peroxidase
HTLV-III	Human T-lymphotropic virus type III
IL	Interleukin
IN	Integrase
IFN	Interferon
KLH	Keyhole limpet hemocyanin
LTNP	Long term non-progressor
LTR	Long terminal repeats
MA	Matrix
MAP	Multiple antigenic peptide
MHC	Major histocompatibility complex
MPER	Membrane-proximal external region
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide
MW	Molecular weight
NC	Nucleocapsid
NICD	National Institute for Communicable Diseases, Johannesburg, South Africa
NK	Natural killer
Nt	Nucleotide
OD	Optical density
OI	Opportunistic infection
ORF	Open reading frame
P	Probability
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCP	*Pneumocystis carinii* pneumonia
PCR	Polymerase chain reaction
PEG	Polyethylene glycol

PHA-P	Phytohaemagglutinin-protein
PI	Proliferation index
PMA	Phorbol 12-myristate 13-acetate
Pol	Polymerase
R^2	R squared
RLU	Relative light units
RNA	Ribonucleic acid
RP-HPLC	Reverse-phase high performance liquid chromatography
PR	Protease
RSD	Relative standard deviation
RT	Reverse transcriptase
scFv	Single-chain variable fragment
SD	Standard deviation
SIV	Simian immunodeficiency virus
SU	Surface unit
tBoc	Tert-Butoxycarbonyl
TCR	T-cell receptor
T_H	T-helper cell
T_H1	T-helper 1
T_H2	T-helper 2
TM	Transmembrane
USA	United States of America
vc	Virus control
VC	Virus codes
WIHS	The Women's Interagency HIV Study
(+)	Positive sense
(-)	Negative sense

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SUMMARY

Characterizing the immune response to HIV-1 using host derived epitope R7V

by

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Background: Host protein beta-2 microglobulin (β2m) is incorporated into the human immunodeficiency virus (HIV) -1 coat during budding. Antibodies directed to R7V, an epitope contained in β2m, increased with the duration of infection in long term non-progressor patients (LTNPs). Purified R7V antibodies neutralized HIV isolates and did not bind to human cells. These data suggested potential for R7V antibodies to be developed as therapeutic tools or prognostic markers and the R7V epitope as a vaccine candidate. However, the literature on R7V is still incomplete. For example, most published work on this epitope make no direct reference to HIV subtypes. The rationale for this study is the lack of information on whether all HIV-1 subtypes incorporate R7V and elicit immune responses to the same extent. In particular the response of HIV-1 subtype C infected individuals to R7V antigen is evaluated here.

Methodology and results: A synthetic peptide of the R7V epitope of HIV-1 was synthesized and an “in-house” enzyme-linked immunosorbent assay (ELISA)

developed. The peptide was able to detect antibodies generated during natural HIV-1 subtype C infection when used as antigen in the ELISA. This response was not as strong as that reported in the literature. A significantly lower ELISA response was observed for uninfected compared to infected sera (probability, *p,* value $\leq$ 0.000152), whereas no differences were noticed between antiretroviral (ARV) treated individuals compared to those who were treatment naïve or LTNPs compared to progressors. These data hold promise for the use of these antibodies as diagnostic rather than prognostic indicators. Polyclonal R7V antibodies produced in rabbits and recombinant R7V antibody fragments did not neutralize an HIV-1 subtype C isolate (Du151.2). However, the latter antibodies neutralized an HIV-1 subtype B strain (SF162), suggesting that the R7V epitope may be more exposed in this subtype. The recombinant R7V antibodies did not neutralize a vasicular stomatitis virus (VSV-G), indicating that no nonspecific neutralization occurred. Human immunodeficiency virus type 1 subtype C infected sera containing R7V antibodies (positive response in the R7V ELISA) neutralized Du151.2 while archive sera containing strong HIV-1 subtype C neutralizing antibodies did not recognize the R7V antigen ELISAs. The R7V peptide exogenously added to HIV-1 infected peripheral blood mononuclear cells (PBMCs) did not stimulate proliferation *in vitro* nor the production of interferon (IFN) gamma which if produced by $CD8^+$ T-cells would have been indicative of a cellular immune response. The parent protein β2m could not initiate these responses either.

Conclusion: Data collected here support a diagnostic rather than a prognostic application for R7V antibodies. R7V conjugated to keyhole limpet hemocyanin (KLH) induced non-neutralizing antibodies in rabbits, suggesting that other modifications (branching, lipid conjugation, etc.) may be needed before this epitope can be successfully utilized in vaccine studies.

CHAPTER 1
LITERATURE SURVEY

1. INTRODUCTION

Human immunodeficiency virus, the retrovirus known for disabling CD4 expressing T-cells and causing immune system disruption, incorporates host cell proteins into its envelope during budding. These virus-incorporated host proteins can be bystanders, assist in the viral life cycle or retain their functional ability to the detriment of the virus. One of these host proteins, β2m, is involved in the process of HIV-infection (Hoxie *et al*. 1987; Devaux *et al*. 1990; Corbeau *et al*. 1990; Arthur *et al*. 1992; Le Contel *et al*. 1996). The host immune response to β2m when it is presented as a virus-associated component, allows room for investigating the protein or antibodies directed at it as prognostic markers, therapeutic tools or potential vaccine candidates.

Current prognostic and diagnostic tools for HIV/ acquired immunodeficiency syndrome (AIDS) are not infallible. The most powerful marker for AIDS progression, CD4 cell count (Strathdee *et al*. 1996; Levy 1998), can be influenced by other diseases or conditions (Stein *et al*. 1992). Even though AIDS progression is associated with low levels of CD4 cells, some individuals have shown slow progression towards AIDS despite very low CD4 cell counts (Strathdee *et al*. 1996; Levy 1998). Diagnostic tools for HIV include detection of HIV antibodies, HIV antigens (p24) or nucleic acids/viral load (Hewer and Meyer 2007). Despite a selection of diagnostic tools available, there is a significant detection limit in the ability of these tools to diagnose exposure to HIV during early infection, commonly referred to as the window period. Even though drug therapies are effective in limiting HIV virulence after infection, there are still strains that manage to escape therapy. No curative or preventive treatment is currently available. Improved prognostic, diagnostic and therapeutic tools are therefore needed for improved management of HIV/AIDS and novel approaches to these may well lie in viral surface proteins including the incorporated host proteins (i. e. β2m) and/or antibodies produced in response to them.

Beta-2 microglobulin, is, as mentioned, one of the host proteins incorporated into HIV's envelope, and plays a role in major histocompatibility complex (MHC) class I

presentation of antigen to T-cells in the host. The levels of β2m in serum are viewed as a prognostic indicator for HIV/AIDS (Fahey *et al*. 1990). R7V is a peptide derived from β2m and following budding is situated on the exterior surface of HIV (Le Contel *et al*. 1996). This epitope has been suggested as a possible vaccine candidate for HIV because of its ability to elicit the production of HIV neutralizing antibodies (Le Contel *et al*. 1996; Galéa *et al*. 1999 a and b; Chermann 2001; Haslin and Chermann 2007 b). Natural production of R7V antibodies in individuals not progressing towards AIDS and an increase of these antibodies with the duration of infection in these individuals has been observed. The above mentioned observations have lead to the suggestion that the levels of antibodies to this peptide may serve as prognostic markers (Galéa *et al*. 1996; Chermann 2001; Ravanini *et al*. 2007; Kouassi *et al*. 2007; Sanchez *et al*. 2008) or therapeutic tools (Haslin and Chermann 2002, 2004 and 2007 b; Haslin *et al*. 2007 a). However, the numbers of reports on R7V are few (only 11 articles in peer reviewed journals and a number of patents) and lack essential details. In some of the reports, the sample numbers are limited and/or the percent positive responses compared to negative responses for R7V antibodies are few. Existing information on these antibodies is restricted to a few subtypes of HIV-1 and sometimes lack subtype information (reference to neither isolates infecting individuals from whom samples are collected nor isolates used in neutralizing assays provided) altogether. Published data on the R7V antibody prevalence is primarily based on samples from the United States of America (USA), selected countries in Europe, Cameroon and the Ivory Coast. Based on the geographical location of these patients, the subtypes involved are probably HIV-1 subtype A and B. No information exists on the presence of R7V antibodies in individuals infected with HIV-1 subtype C, which is dominant in South Africa. In addition, experiments performed are sometimes inadequately explained. Some reports even refer to data which are only discussed and not shown. Therefore more studies need to be done to clarify the potential roles for this epitope in HIV prognosis, therapy or vaccine development.

The purpose of this study was to investigate the potential role(s) of the R7V epitope of HIV-1 by characterizing the immune response in HIV-1 subtype C infected individuals using a synthetic peptide representing this epitope. The literature suggested R7V antibodies as prognostic markers and the epitope as a vaccine candidate. The work planned here will either confirm or contradict these suggestions. This study started out trying to answer whether R7V antibodies were produced in naturally HIV-infected

individuals living in South Africa (infected with HIV-1 subtype C) for which no published data presently exist. Secondly, the project investigated whether subtype C infected individuals not progressing towards AIDS had the same R7V antibody levels reported in patients infected with subtype A and B (referred to in existing literature). Thirdly, whether R7V antibodies (recombinant antibody fragments based on a synthetic R7V peptide and antibodies from R7V immunized rabbits) and serum from HIV-1 subtype C infections that recognize the synthetic R7V peptide could neutralize HIV-1 subtype C was analyzed. Lastly, the proliferation of cells and cytokine production in response to the synthetic R7V peptide was investigated. The cytokine of interest was IFN-γ because if this cytokine was produced in response to R7V and by $CD8^+$ T-cells in particular, it would allow for commentary on a possible influence on cellular immunity by this epitope. Given the origin of R7V (part of a MHC class I binding protein) these results may further clarify the potential roles of this epitope.

The R7V peptide (seven amino acids) was synthesised on solid phase by manual fluorenylmethyloxycarbonyl (Fmoc) chloride chemistry and tested in a variety of immunological assays. The data obtained allowed for commentary on the peptide's potential as possible vaccine target as well as antibodies to this peptide as prognostic markers or therapeutic tools.

This dissertation starts off by providing background information on HIV and the AIDS epidemic (in South Africa in particular). This is followed by introductory information on host proteins incorporated by HIV-1 and all the available information on the R7V epitope of HIV-1. The aims and research questions follows next. In subsequent chapters, the methodology and results are provided, ending off with a discussion of all observations and extra data provided in the appendix.

The title of this project is broad because *aspects* of *both humoral and cellular* immune responses to HIV-1 are investigated.

1.1 GENERAL INTRODUCTION TO HIV/AIDS

1.1.1 Discovery of HIV

In the early 1980's, unusual opportunistic infections (OIs) and Kaposi's Sarcoma, a rare cancer that tended to occur in elderly people, increased in otherwise healthy, young homosexual men in the USA. It was generally believed that the cause of these OIs and

cancer was due to infection with cytomegalovirus (CMV) or the use of anyl nitrate or butyl nitrate and immune overload (Center for Disease Control, CDC 1982). It soon became clear that signs of the same conditions were seen in injecting drug users and blood transfusion patients as well. In addition, cases of mother to child transmission were reported. Incidences of similar OIs and immune disorders were also reported in several other countries (Rozenbaum *et al*. 1982; Vilaseca *et al*. 1982; Kamradt *et al*. 1985). A report published by the Centre for Disease Control and Prevention (1981) was the first to take note of the syndrome and described the occurrence of *Pneumocystis (carinii)* pneumonia (PCP), now called *Pneumocystis jiroveci* pneumonia (Hoffmann *et al*. 2007), in a few homosexual men in the USA. Very little was known about the epidemiology and transmission of what seemed to be a new syndrome caused by a virus that could be transmitted between individuals. This was evident when the first cases of PCP were reported in injecting drug users. When it was reported that the virus could be transmitted heterosexually as well, the realization became all too inescapable that the number of people who could become infected was going to increase rapidly and investigation of the virus was necessary (Karim and Karim 2005). In 1982, the syndrome was named acquired immunodeficiency syndrome (AIDS). Luc Montagnier and his team (Institute Pasteur, France) reported in 1983 the isolation of a new virus believed to be the cause of AIDS. This virus was named lymphadenopathy-associated virus (LAV, Barre-Sinoussi *et al*. 1983). Soon thereafter, Robert C. Gallo (National Cancer Institute, USA) also reported the isolation of the virus believed to be the cause of AIDS and named it human T-lymphotropic virus type III (HTLV-III, Gallo *et al*. 1984). Detailed research on these two viruses in 1985 showed that the viruses were the same and in 1986 Dr. Gallo and Dr. Montagnier were both credited as co-discoverers of the virus which was re-named human immunodeficiency virus, HIV.

1.1.2 Taxonomy

Taxonomy and virus codes (VC) for all known viruses are described in the Sixth Report of the International Committee on the Taxonomy of Viruses (Murphy *et al*. 1995). Human immunodeficiency virus is a member of the genus Lentivirus that belongs to the Retroviridae (VC 61) family, a ribonucleic acid (RNA) virus so named because it encodes the enzyme reverse transcriptase (RT) that transcribes viral RNA into provirus deoxyribonucleic acid (DNA) which is subsequently integrated into the host genome (Sherman and Greene 2002). The Retroviridae family is divided into 7 genera, namely: alpha through epsilon retrovirus, lentivirus and spumavirus (Mahy 2001) and

characteristically contains three main coding domains, envelope (*env*), group specific antigen (*gag*) and polymerase (*pol)*, Lewis and Emerman 1994. Lentiviruses (VC 61.0.6) are non-oncogenic retroviruses that produce multi-organ diseases characterized by long incubation periods and persistent infection (Murphy *et al*. 1995). Lentiviruses are distinguished by the ability to infect non-dividing cells (Lewis and Emerman 1994) and in that they contain open reading frames (ORFs) between the *pol* and *env* genes and in the 3'env region (Trono 2002). Five serogroups (or complexes) of the lentivirus are recognized (ovine/caprine, bovine, equine, feline and primate), reflecting the mammalian hosts with which they are associated (Rwambo *et al*. 2001). Primate lentiviruses (VC 61.0.6.5) are further subdivided into several species, including HIV comprised of subspecies HIV-1 and HIV-2, VC 61.0.6.5.001, (Murphy *et al*. 1995).
The HIV lineage is therefore as follows:

VIRUSES
→ VERTEBRATE VIRUSES
→ RETROID VIRUS
→ FAMILY: Retroviridae
GENUS: Lentivirus
SEROGROUP: Primate lentivirus
SPECIES: Human Immunodeficiency Virus

1.1.3 HIV classification and diversity

All lentiviruses isolated from simian primates (e.g. chimpanzee) are named simian immunodeficiency viruses or SIV and those isolated from humans are named human immunodeficiency viruses or HIV. The human immunodeficiency virus has been divided into two types, HIV-1 (including the lineages named the M, main; O, outlier; N, non-M/non-O and P, proposed groups) and HIV-2 (including the lineages named the A-G groups, Simon *et al*. 2004), Gurtler *et al*. 1994; Simon *et al*. 1998; Plantier *et al*. 2009.
It is commonly believed that the HIV-1 epidemic originated when an ancestral virus was transmitted to humans from chimpanzees in equatorial West Africa during the 1930s (Karim and Karim 2005). The arisen genetic diversity of HIV-1 M has required subdivisions of the HIV-1 main group into 11 subtypes or clades (named A1, A2, B, C, D. F1, F2, G, H, J, and K) based on their phylogenetic relatedness (Robertson *et al*. 1999). However, with the increasing number of viral isolates available worldwide and

the improvement of sequencing methods, HIV-1 phylogenetic classifications are currently based either on nucleotide (nt) sequences derived from multiple subgenomic regions (*gag*, *pol* and *env*) of the same isolates or on full-length genome sequence analysis. This approach has revealed virus isolates in which phylogenetic relations with various subtypes switch along their genomes. These virus strains are believed to have originated in individuals multiply infected with viruses of two or more subtypes. An identical recombinant virus identified in at least three epidemiologically unlinked individuals and characterized by full-length genome sequencing is classified as circulating recombinant forms (CRFs). More than 20 CRFs are currently circulating (Buonaguro *et al*. 2007). These CRFs do not meet the criteria for designation as new subtypes (i.e. when partial sequences or less than three genomic sequences have been obtained) and remain unique unclassified recombinants (labelled as U).

Subtypes represent different lineages of HIV and have some geographical associations (Robertson *et al*. 1999). The HIV- M group accounts for the dominating global AIDS epidemic. HIV-2 is less pathogenic than HIV-1 and is mainly restricted to West Africa whereas HIV-1 subtype C viruses are dominant in South Africa (Karim and Karim 2005).

HIV-2 is distinct from HIV-1 and is closely related to SIV isolated from sooty mangabeys (Edinger *et al*. 1999) whereas the latter is most related to SIV from chimpanzees (Keele *et al*. 2006). SIV has been isolated from a number of non-human primates, all with natural hosts on the African continent, including the chimpanzees, sooty mangabey, *Macaca mulata*, African green monkey and Sykes monkey (Korber *et al*. 2000). The phylogenetic relationship between HIV-1, HIV-2 and SIV as well as the relationship of the subtypes within HIV-1 M is represented schematically in Figure 1.1.

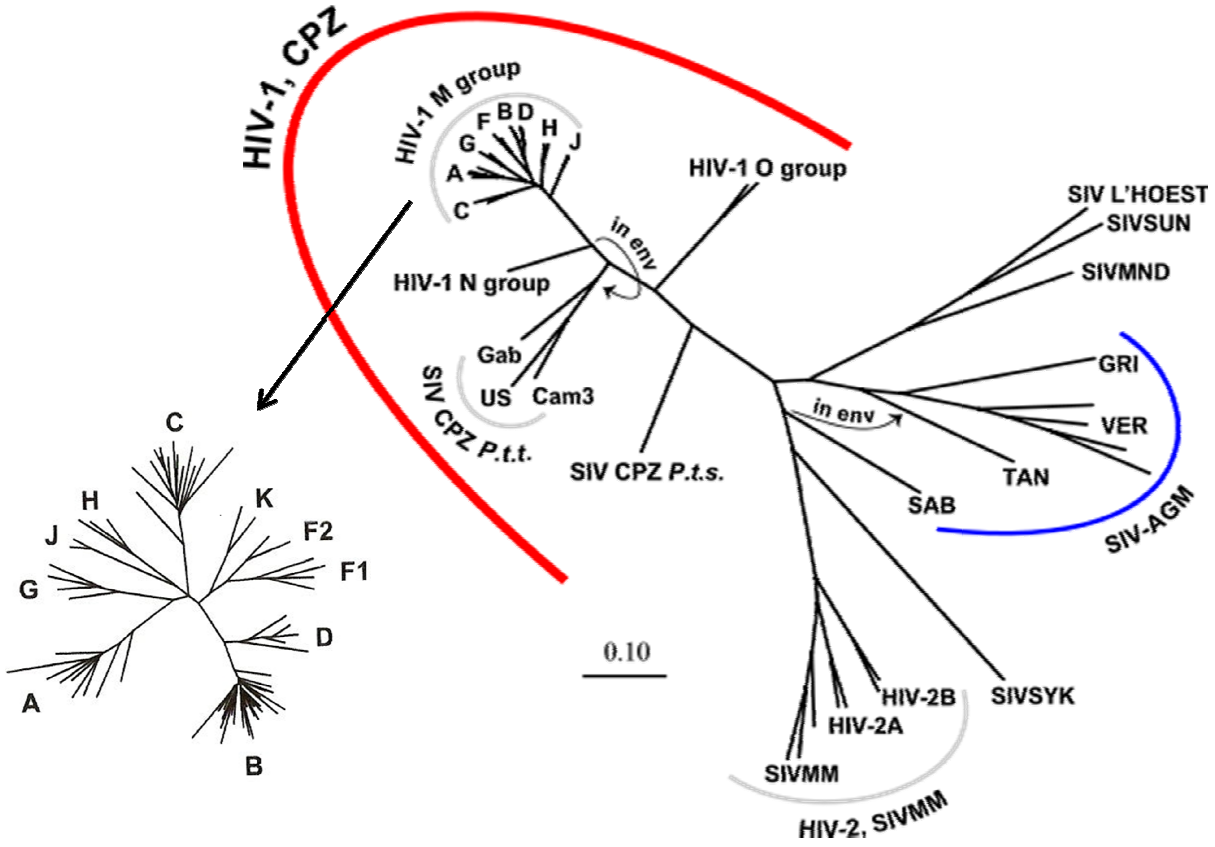


Figure 1.1. Phylogenetic relationships of the primate lentiviruses. This HIV-SIV-phylogenetic-tree illustrates the comparative relationship between HIV-1, HIV-2 and SIV as well as the relationship between subtypes of the HIV-1 main group. This figure was constructed utilizing *pol* gene sequences and small arrows indicate where sequences would branch in an *env* gene construction. The figure does not include the very new HIV-1 group designated P (Plantier *et al.* 2009). The figure was adapted from http://en.wikipedia.org/wiki/File:HIV-SIV-phylogenetic-tree.svg and Robertson *et al.* (1999).

1.1.4 Global geographic HIV-1 subtype distribution

As demonstrated in Figure 1.2, molecular epidemiological studies show that there has been an uneven spread of HIV strains out of Africa, leading to regional epidemics of very distinctive composition whereas sub-Saharan Africa contains almost all subtypes (McCutchan 2006). Founder effects, human genetic factors, social/behavioural factors (McCutchan 2006) as well as accidental trafficking (viral migration) or prevalent route of transmission which result in a strong advantage and local predominance of the subtype prevalently transmitted in the population (Buonaguro *et al.* 2007) have all been mentioned in this context. According to recent studies, the most prevalent HIV-1 genetic forms are the subtypes A, B and C, with subtype C accounting for almost 50% of all HIV-1 infections worldwide in 2004 (Hemelaar *et al.* 2006). Figure 1.2 graphically depicts the global distribution and prevalence of HIV-1 strains. Subtype A viruses find

the highest concentration in areas of Central/East Africa and Eastern Europe (countries formerly constituting the Soviet Union). Subtype B viruses are predominant in the Americas, Western and Central Europe and Australia, as well as in Southeast Asia. Subtype C is the overwhelming majority strain in Southern Africa, the Horn of Africa and in India. Subtype D dominates epidemics principally in East Africa and to lesser extent in West Africa. The subtypes F, G, H, J and K are restricted to West-Central Africa with a very low global prevalence (McCutchan 2006). The prevalence of CRFs is highest within Southeast Asia, West and West-Central Africa (McCutchan 2006) and all recombinant forms taken together are responsible for 18% of infections worldwide (Hemelaar *et al*. 2006). The principal concentrations of HIV-1 groups O and N are very rare in the pandemic and are restricted to Cameroon whereas HIV-2 is largely restricted to West-Africa (McCutchan 2006). HIV-1 group P is the variant that was recently discovered in a Cameroonian woman residing in France (Plantier *et al*. 2009).

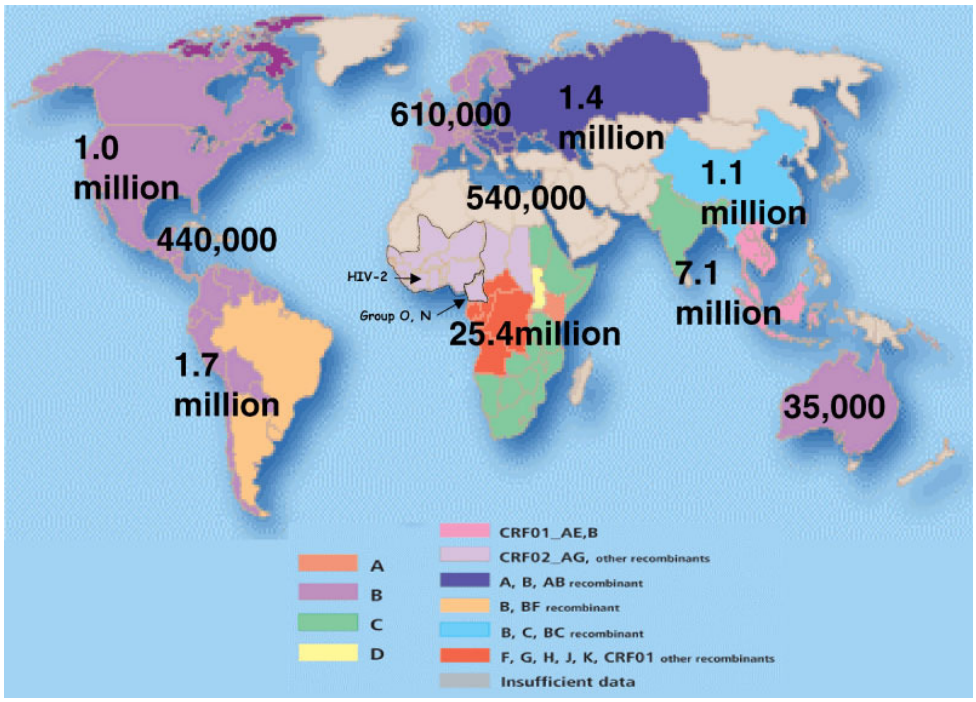


Figure 1.2. Global geographic HIV-1 subtype distribution. The colours depict regional patterns of HIV variation and areas were there is insufficient data. HIV-1 groups O and N in Cameroon and HIV-2 in West Africa are indicated by arrows. The estimated numbers of HIV-infected individuals are indicated with numbers on the respective continents. The figure was taken from McCutchan (2006).

1.1.5 Epidemiology of HIV in South Africa

Assessment of HIV prevalence and growth of the epidemic is made by national sentinel surveillance surveys of antenatal clinic attendees and calculations based on currently

approved methodologies are used to generate population prevalence values. HIV data from antenatal clinics in South Africa suggest that the country's epidemic might be stabilizing, but there is no evidence yet of major changes in HIV-related behaviour. An estimated 5.7 million (4.9 million – 6.6 million) South Africans were living with HIV in 2007, which makes this the largest HIV epidemic worldwide. Of these infections, 5 400 000 (4 700 000-6 200 000) are in adults (aged 15 and up) and 280 000 (230 000-320 000) in children (UNAIDS 2008). South Africa has an overwhelmingly dominant subtype C epidemic accounting for over 95% of the infections (Karim and Karim 2005). According to a report by UNAIDS (2008), the HIV prevalence data shows that the infection might be levelling off. Infection rates among young people (below age 20) have decreased significantly whilst the prevalence among pregnant women has shown signs of stabilization in the past three years to 2006. There is significant variation in the epidemic between provinces, ranging from 39.1% in KwaZulu-Natal to 15.1% in Western Cape (UNAIDS 2008).

The high HIV prevalence in South Africa is thought to be mostly driven by an overall low level of knowledge of HIV/AIDS and high-risk behaviour within the general population. Other possible reasons why the prevalence of AIDS is so high in the country are poverty, social instability and slow, visible government action. Misconceptions concerning HIV/AIDS (including modes of transmission and traditional beliefs around HIV) are commonly held. Controversial public stances held by the South African government during the last seven years, concerning the causative agent of AIDS, the therapeutic value of nevirapine and the dangers of ARV therapy, compounded the already held public misconceptions (Pembrey 2007). A radical shift in policy is evident in comments made by President Zuma in his World AIDS Day speech in 2009.

It was between 1993 and 2000 that the most rapid increase in South Africa's HIV prevalence took place. This was a time when the country was focused on major political changes, and while the impact of the epidemic was not acknowledged and HIV rapidly became more widespread, the attention of the South African people and the world's media was on the political and social changes occurring in the country. As the attention was focused on the country's transition from apartheid, HIV was rapidly becoming more widespread (Pembrey 2007).

The response to the AIDS epidemic in South Africa developed slowly at first followed by some initial momentum in the period just after the dawn of democracy in 1994.

However, the response to the AIDS epidemic has been gathering momentum once again with the announcement by the government in 2003 that it would make free ARV treatment available in the public health service (Karim and Karim 2005).

1.1.6 Structure of HIV

Morphology: The mature virion, as illustrated in Figure 1.3, is an enveloped, spherical particle with a diameter of approximately 100 nanometres. The outer lipid membrane is derived from the human host and consists of glycoprotein surface projections (formed from glycoproteins gp120 and gp41, collectively termed gp160) with a diameter of approximately 8 nanometres; dispersed evenly over the entire surface. The internal nucleocapsid (NC) core is isometric and the nucleod is concentric and rod-shaped, or shaped like a truncated cone (Murphy 1995).

Physicochemical properties: The HIV virion exhibits a buoyant density 1.16-1.18g cm^{-3} in sucrose gradients. Virions are sensitive to heat, detergents, and formaldehyde but infectivity is not affected by irradiation (Murphy 1995).

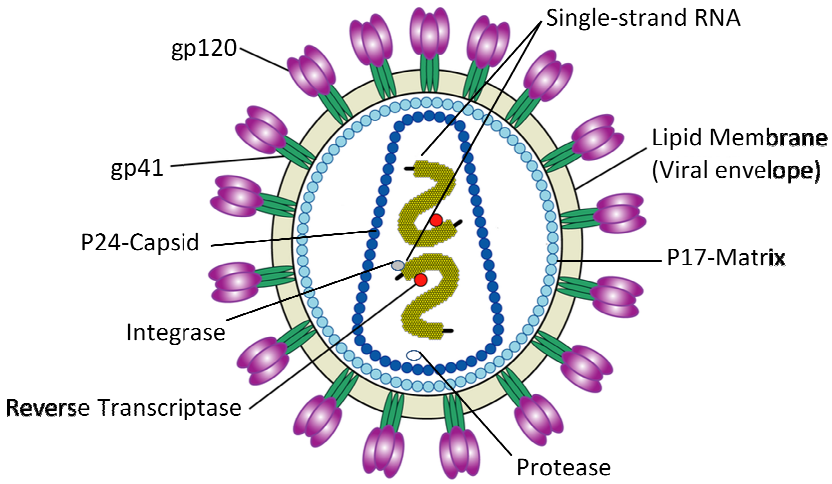


Figure 1.3. Schematic representation of the HIV-1 particle illustrating major viral components. The figure was adapted from http://en.wikipedia.org/wiki/File:HIV_Virion-en-2.png.

Genomic orientation: The viral genome, which constitutes 2% by weight, is composed of two identical linear positive-sense single-stranded RNA molecules attached together by hydrogen bonds. One monomer has a total genome length of 9200 nt with terminal repeated sequences named long terminal repeats (LTR) of about 600 nt. The 5' terminus of the genome has a cap (cap sequence of HIV-1 is m7G5ppp5' GmpNp) and

the 3' terminus of each monomer has poly (A) tract (Murphy, 1995). The genome is spliced to produce nine genes (Figure 1.4) which are categorized into structural (*gag*, *pol* and *env*), regulatory (*tat* and *rev*) and accessory (*vif*, *vpu*, *nef* and *vpr*/*vpx*) genes depending on the function of the encoded proteins (Table 1.1). Group specific antigen encode three non-glycosylated proteins; the 24 kiloDalton (kDa, p24) capsid (CA) protein that encloses the viral RNA, the 17kDa (p17) matrix (MA) protein that provides structure to the particle and the 7kDa (p7) NC protein (Coffin 1999). Polymerase encodes three enzymes remaining active and carried inside the viral particle; the 66kDa RT, the 32kDa integrase (IN) and the 14kDa protease (PR). Finally, *env* proteins encode two proteins that are linked together on the viral surface facilitating binding and entry to the host cell; the 120kDa gp120 glycosylated surface envelope (SU) protein and the 41kDa gp41 glycosylated transmembrane (TM) protein (Murphy 1995). The regulatory and accessory proteins are produced once the virus infects cells and are not present inside viral particles (Karim and Karim 2005). *Tat* (transactivation), *rev* (regulator of virion expression, Heaphy *et al*. 1991) and *nef* (necessary effector, Joseph *et al*. 2005) encode for 16 and 14kDa, 19kDa and 27-25kDa proteins respectively (HIV Sequence Compendium 2002). The *tat* and *rev* genes consist of two exons where exon 2 lies within the *env* open reading frame. The other genes have only one exon (Beer 1999). *Vif*, *vpr* and *vpu* encode proteins on 23kDa, 10-15kDa and 16kDa respectively. *Vpx* is a *vpr* homolog where *vpr* is unique to SIV_{CPZ} and HIV-1 and *vpx* is encoded by the SIV and HIV-2 genomes (HIV Sequence Compendium 2002).

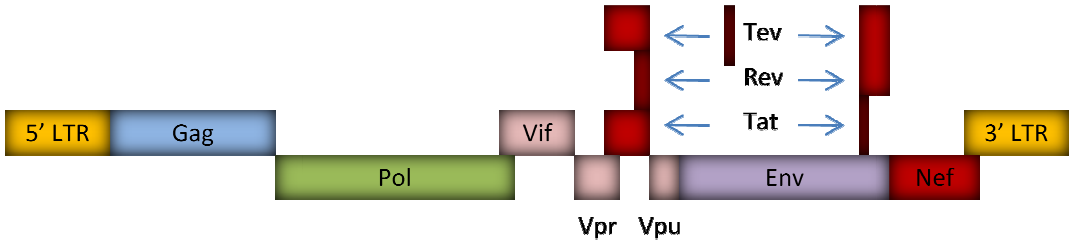


Figure 1.4. Diagram of the complete HIV-1 genome illustrating the nine genes including one additional gene, *tev*. The gene *tev* is only present in a few HIV-1 isolates and is a fusion of parts of the *tat*, *env* and *rev* genes. *Tev* codes for a protein with some of the properties of *tat* but little or none of the properties of *rev*. The figure was adapted from http://en.wikipedia.org/wiki/HIV_structure_and_genome.

Table 1.1. The table summarizes HIV/SIV genes and their corresponding sizes and functions of gene products. The table was adapted from the HIV Sequence Compendium (2002).

HIV/SIV genes

Gene	Gene products: Name	Size	Function
Structural			
Gag	Gag:		
	MA	p17	Membrane anchoring; Env interaction; nuclear transport of viral core
	CA	p24	Core capsid
	NC	p7	Nucleocapsid, binds RNA
		p6	Binds Vpr
Pol	Pol:		
	PR	p15	Gag/Pol cleavage and maturation
	RT	p66, p55	Reverse transcriptation, RNase H activity
	RNase H	p15	
	IN	p31	DNA provirus integration
Env	Env	gp120/ gp41	External viral glycoproteins, bind to CD4 and secondary receptors
Regulatory			
Tat	Tat	p16, p14	Viral transcriptional transactivator
Rev	Rev	p19	RNA transport, stability and utilization factor (phosphoprotein)
Accessory			
Vif	Vif	p23	Promotes virion maturation and infectivity
Vpu	Vpu	p16	Promotes extracellular release of viral particles, degrades CD4 in the ER (only in SIVCPZ and HIV-1)
Nef	Nef	p27- p25	Down regulates CD4 and class 1
Vpr	Vpr	p10-15	Promotes nuclear localization of preintegration complex, inhibits cell division, arrests infected cells at G2/M (in SIVCPZ and HIV-1)
Vpx	Vpx	p12-16	Vpr homolog (in SIV and HIV-2)

1.1.7 Life cycle of HIV

The life cycle of HIV can be described in six phases (adsorption, penetration, uncoating and provirus production, replication, assembly/maturation/release and re-infection). Figure 1.5 shows a schematic representation of the phases described below:

(i) **Adsorption phase**: Initial HIV-1 infection begins through the attachment of the envelope protein gp120 to the CD4 molecules predominately on the surface of helper T lymphocytes ($CD4^+$ T-cells) and macrophages (Karim and Karim 2005) and other antigen presenting cells such as monocytes (Weinberg *et al*. 1991) and dendritic cells (DC, Sleasman and Goodenow 2003). This interaction between CD4 and gp120 increases the affinity of virus for coreceptor molecules, which are seven TM, G-protein-coupled chemokine receptors (CCR5 receptors mainly on macrophages and dendritic cells, CXCR4 receptors mainly on $CD4^+$ T-cells and monocytes and CCR3 and CCR5 receptors on brain microglia cells, He *et al*. 1997; Sleasman and Goodenow 2003).

(ii) **Penetration phase:** Following the conformational changes, a previously hidden portion of the TM protein gp41 is exposed, allowing a fusion of the viral envelope and the target cell membrane. This fusion allows entry of the viral core, containing proteins, enzymes and diploid HIV-1 genome, into the cytoplasm of the cell (Sleasman and Goodenow 2003). Occasionally, the virus enters the cell by endocytosis, after binding the receptors, and then the envelope fuses through the endosome to release the genome-containing core into the cytoplasm of the cell (Miyauchi *et al*. 2009).

(iii) **Uncoating and provirus production phase:** During uncoating, the single-strand RNA genome within the core or capsid of the virus is released in the cytoplasm. Once within the cytoplasm, the RNA-dependent DNA polymerase (RT) transcribes positive sense (+) viral genomic RNA into negative sense (-) single stranded viral complementary DNA (cDNA). Ribonuclease activity associated with RT degrades the RNA genome and the (-) cDNA is used as a template to produce a (+) linear, double-stranded viral DNA intermediate (Sleasman and Goodenow 2003). The DNA intermediate is integrated into the host cell chromosomes to become a provirus through non-homologous recombination catalysed by the viral enzyme, IN. Once integrated, the proviral DNA can remain quiescent for extended periods of time or become transcriptionally active, particularly in cases where there is inflammation (Karim and Karim 2005).

(iv) **Replication phase:** Following activation of the provirus, the virus makes use of the host cell machinery to replicate itself. The integrated DNA provirus is transcribed into RNA by the host cell RNA polymerase II – dependent transcriptional machinery. Viral RNA is either single-spliced or unspliced to make structural proteins, or they are multiple-spliced to make regulatory (*nef*, *rev* and *tat*, which encourages new virus production) and accessory proteins. Full-length unspliced genomic RNA is transported to the plasma membrane to be included in new viral particles. As part of the assembly of new virions, which begins at the plasma membrane of the host cell, the precursor *gag-pol* polyprotein is spliced by viral PR and become individual proteins that aggregate beneath the plasma membrane surface for inclusion into the new virions. After passing through the endoplasmatic reticulum, the envelope glycoprotein is transported to the Golgi complex where it is cleaved by a PR and processed into the two envelope glycoproteins, gp41 and gp120 before they insert themselves into the cell membrane where gp41 anchors gp120 to the membrane of the infected cell (Earl *et al*. 1991).

(v) **Assembly, maturation and release phase:** The final step of the viral cycle, assembly of new HIV-1 virions, begins at the plasma membrane of the host cell. Mature viral particles are formed and the virus buds from the surface of the infected cell and is enveloped by the host cell membrane (Sleasman and Goodenow 2003). To become infectious, the forming bud or the newly released virion undergoes subsequent maturation. HIV PRs cleave the polyproteins into individual functional HIV proteins and enzymes. Further, the various structural components assemble to produce a mature virion which is then able to infect new susceptible host cells (Gelderblom 1997; Sleasman and Goodenow 2003).

(vi) **Re-infection: HIV can also infect through cell-to-cell contact.** This phenomenon occurs when an infected cell with gp120 on its cytoplasmic membrane attaches to CD4 molecules and chemokine receptors on the surface of an uninfected cell resulting in a fusion of the two cells.

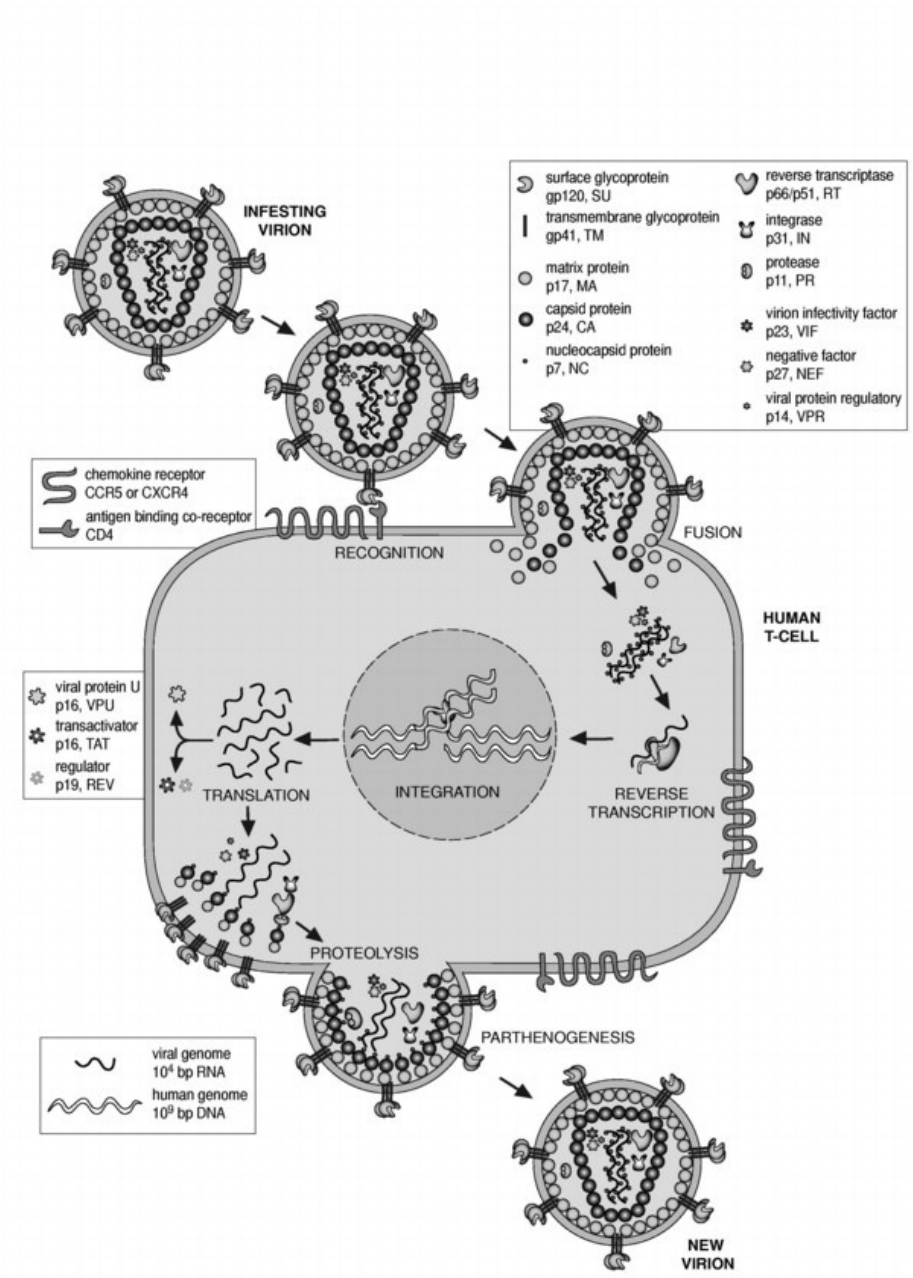


Figure 1.5. Schematic representation of the life cycle of HIV. The following six phases are illustrated; (i) adsorption, (ii) penetration, (iii) uncoating and provirus production, (iv) replication, (v) assembly/maturation/release and (vi) re-infection. The figure was taken from http://www.molmo.be/hiv_lifecycle.html.

1.2 AIDS PATHOGENESIS

1.2.1 Brief overview of the function and components of the immune system

The vertebrate immune system includes two essential defence systems that function both independently and cooperatively. The two immune systems are the non-specific (innate) immune system and the specific (adaptive) immune system. The innate response constitutes the *first line of defence*, the external physical barriers presented by intact skin and mucosal membranes. Here the pathogens will be eliminated by antimicrobial proteins and phagocytes. Pathogens able to penetrate these barriers will be met by the *second line of defence* (adaptive) consisting of immune cells which immediately target and recognize antigen structures on microorganisms and identify the pathogen as foreign. Here IFN, natural killer (NK) cells and macrophages become active (Karim and Karim 2005).

T-cells express CD3 on the surface along with the T-cell receptor (TCR). In addition, T-cells express either CD4 or CD8. $CD4^+$ T-cells coordinate and help the immune response to proceed by release of cytokines, hence named T-helper cells (T_H cells). Two systems of T_H responses exist. The T_H type 1 (T_H1) response consists of $CD4^+$ T-cells producing cytokines promoting cellular immunity and a cytolytic $CD8^+$ T-cell response and involve secretion of for example IFN-γ which has antiviral activity. A T_H type 2 (T_H2) response consists of $CD4^+$ T-cells releasing cytokines that direct humoral immunity and antibody responses and is involved in switching on B cell activity. Naïve T_H cells are able to secrete all the cytokines T_H1 and T_H2 cells secrete. It has been reported that T_H2 type responses predominate during HIV-1 infection and that explains the loss of cellular immunity and progression towards AIDS. $CD8^+$ T-cells, named cytotoxic T-lymphocytes (CTLs), protect the host from invading pathogens (Roitt *et al*. 2001).

It is the antigen-presenting cells (APC) which usually are macrophages or DCs and in some circumstances B-cells that engulf the foreign antigen and display fragments of the antigen on its surface. These fragments bind to one of two types of MHC cell surface proteins situated on the APC. The MHC (also known as the human leukocyte antigen, HLA) is a gene complex which functions in signalling between T-cells and APC and presents epitopes to the T-cells. The ability of an organism accepting grafts from a different strain is dependent on the donor and recipient sharing the same MHC haplotype. The molecules which determine graft rejection are termed class I and class II

molecules and are the molecules that present antigen. Although multiple class I and II genes exist within the MHC, class I and II gene products have similar overall structures. The remaining genes in the complex (which include genes encoding complement system molecules, some cytokines, enzymes, heat-shock proteins and some molecules involved in antigen processing) are very diverse and are collectively called class III (Roitt *et al*. 2001; Seder and Mascola 2003). It is important to highlight that MHC class I molecules consist of an MHC-encoded heavy chain bound to β2m (Roitt *et al*. 2001). Beta-2 microglobulin, the protein of importance in this dissertation, is essential for the expression of MHC class I (Roitt *et al*. 2001) and is also incorporated into HIV's envelope during budding and believed to be a functioning part of the virus (Hoxie *et al*. 1987; Devaux *et al*. 1990; Corbeau *et al*. 1990; Arthur *et al*. 1992; Le Contel *et al*. 1996). Antigen-presenting cells presenting class I MHC proteins are recognized by T-cell receptors which are present on the surface of immature cytotoxic T-cells. Antigen-presenting cells presenting class II MHC proteins are recognized by immature T_H cells (Roitt *et al*. 2001).

Cellular immune responses play an important role in elimination of cell-associated virus (Figure 1.6). The immune response involves the activation of macrophages, NK cells, antigen-specific CTLs and the release of various cytokines in response to an antigen (e.g. virus). Cellular immunity protects the body in three ways. Firstly, by activating antigen-specific CTLs that are able to induce apoptosis in body cells displaying epitopes of foreign antigens on their surface. T-cells binding to these complexes multiply and a large number of mature cytotoxic T-cells or NK cells are generated. Secondly, cellular immunity can activate macrophages and NK cells thus enabling these cells to destroy the antigen. Lastly, cellular immunity can stimulate cells to secrete a variety of cytokines that influence the function of the antigen (Roitt *et al*. 2001; Seder and Mascola 2003). Cytokines are small signaling proteins which mediate interactions between cells. Interferon-γ has antiviral properties and is known as an immune IFN. Interferon-γ enhances the efficiency of the adaptive immune response by stimulating increased expression of MHC class I and II and serves to activate macrophages, cytotoxic T-lymphocytes, neutrophils, NK cells and T-cell and B-cell proliferation. In addition, IFN-γ activates APCs and promotes T_H1 differentiation. Interferon-γ displays a wide variety of antiviral, antiproliferative and immunomodulatory functions and is capable of inhibiting viral replication (Roitt *et al*. 2001).

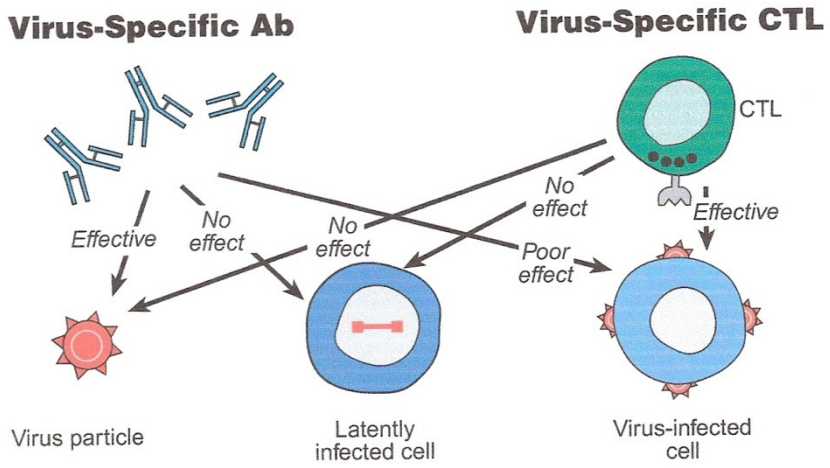


Figure 1.6. Schematic representation of the effectiveness of the components of the antiviral immune response. Effectiveness of neutralizing antibodies and CTLs against different variations of HIV-1 (free virus particles and virus-infected cells). The figure was taken from Pantaleo and Koup (2004).

The humoral immune response contributes in neutralizing cell free virus (Figure 1.6) and is mediated by secreted antibodies produced by the B-cells. Humoral immunity is called as such, because it involves substances found in the humours, or body fluids (cell-free body fluids or serum). B-cells display both immunoglobulins and class II MHC proteins on their surface. When a B-cell encounters an antigen that binds to its immunoglobulin, the antigen will be engulfed by the B-cell and fragments of the antigen will be displayed on the surface of the B-cell together with the MHC class II protein. Mature T_H cells with receptors specific for this complex attach to the complex and the release of interleukins will stimulate the B-cell to proliferate and differentiate. The B-cell progeny are mostly plasma cells that secrete the antigen-specific antibody. Secreted antibodies bind to antigens on the surfaces of invading microbes, which flag them for destruction either by phagocytosis or by activating the complement system involving proteins that lyse cells and trigger inflammatory reactions. The activation of B- cells and their differentiation into antibody-secreting plasma cells is triggered by antigen and usually requires T_H cells. Unless stimulated by their corresponding antigen, most T- and B-cells die through apoptosis. The remaining T- and B-cells become memory cells which in a faster and more efficient way respond to re-exposure to antigen (Roitt *et al*. 2001; Seder and Mascola 2003).

1.2.2 Disease progression

Human immunodeficiency virus-1 infection initiates a process that leads to progressive destruction of the target cell preference for HIV-1 infection, namely the $CD4^+$ T lymphocytes. The course of an HIV-1 infection varies widely from person to person but a typical pattern of HIV-1 infection *in vivo* is shown in Figure 1.7 and is characterized by three phases: the acute or primary infection, the asymptomatic or non-progressor phase and the symptomatic or progressor phase (Ferrantelli and Ruprecht 2002). The initial response to HIV-1 is generated by the adaptive immune response of the host. Virus-infected cells produce IFN-α and IFN-β which activate genes and enzymes that attempt to degrade viral RNA and inhibit synthesis of viral protein. Further, these IFNs mediate increased MHC class I expression and activate macrophages and NK cells (Roitt *et al.* 2001). The virus, however, continues to spread throughout the body, infecting various organs particularly the lymph nodes. This period is commonly referred to as the window period. During the acute stage, which generally last for 2 to 8 weeks, the viral load increases dramatically and the number of $CD4^+$ T-cells decline and clinical symptoms, including fever, cough, lymphadenopathy, pharyngitis, and macular skin rash can occur (Wiese and Guidry 2006).

However, within a few weeks an immune response to HIV-1 develops (attributed to specific cytotoxic T lymphocytes and to a lesser extent neutralizing antibodies) that curtails viral replication, resulting in a decline in the viral load and a return of $CD4^+$ T-cell numbers to near normal levels. As a result of these immune responses, some individuals remain clinically well for years (5-10 years or more). These individuals are called non-progressors (Borrow *et al*. 1994; Koup *et al*. 1994; Mackewicz *et al.* 1994). After about 6 months of infection, plasma HIV-RNA stabilizes around a so-called set point and it remains relatively constant during the asymptomatic phase (Ferrantelli and Ruprecht 2002). Kinetic studies have shown that during the asymptomatic phase a billion HIV particles and two billion of $CD4^+$ T-cells are destroyed and produced daily. Even though individuals may be clinical well, the virus continues to replicate, causing a gradual decline in $CD4^+$ T-cell numbers and the susceptibility for opportunistic infections, which signalling the onset of AIDS, increase. However, the rate of $CD4^+$ T-cell decline and the control over viral replication varies substantially between individuals. Some individuals never gain a temporary control over viral replication and progress to AIDS 2-3 years after infection (rapid progressors) while others have remained symptom-free for up to 20 years with often undetectable viral loads (long-term

nonprogressors, LTNPs). The latter patient group described here is of specific interest in this MSc project as antibodies produced in these individuals are investigated. It is worth mentioning that several definitions of patient groups not progressing to AIDS are found in the literature. For example, *LTNPs* are mostly defined based on their stable and high CD4 cell counts for up to 20 years in the absence of therapy whereas *elite controllers* are often defined based only on their ability to maintain undetectable viral loads (< 50 copies of virus/ml blood) in the absence of therapy regardless of their CD4 cell counts (Walker 2007). A selection of definitions of LTNPs is provided in Chapter 2 (Table 2.1). The level of $CD8^+$ T-cells increases during the acute phase and then returns to a level somewhat above normal until the final symptomatic phase where it drops dramatically. With this collapse of the immune system, the viral load increases and death ensues about 18 months to 2 years after an AIDS diagnosis. The median time to AIDS from the point of HIV-1 infection is 8-10 years in the absence of ARV therapy. This is for individuals living in Europe and the USA where there is generally good access to health care. However, there is evidence that the natural history of HIV-1 disease in Africa may be about 1-2 years shorter than in the developed countries. Whether this is related to viral factors such as differences in viral subtype, or to socio-economic factors such as poor access to health care, or to the generally higher burden of infectious diseases in Africa is not known (Karim and Karim 2005).

Nutrition may be one of the factors that influence disease progression. Insufficient dietary intake may lead to malabsorption, diarrhea, altered metabolism and nutrient storage. This may further lead to nutritional deficiencies causing increased oxidative stress and immune suppression, which, in turn, leads to increased HIV replication and hastened disease progression (Semba and Tang 1999).

Antiretroviral drugs and highly active ARV therapy (HAART, which is specific combinations of ARV drugs) are nucleoside RT inhibitors, nucleotide RT inhibitors, non-nucleoside RT inhibitors, PR inhibitors, entry inhibitors and fusion inhibitors that are aimed at delaying or preventing the progression to AIDS and death. However, successful treatment does not completely prevent clinical events, particularly when started in advance disease (CD4 cell counts < 50 cells/µl). The drugs are required for life since they cannot eradicate latent HIV which persists in the host, integrated within the genome of metabolically inactive but long-lived memory $CD4^+$ T-cells. Clinical benefits of treatment are hard to demonstrate in early HIV disease. Favourable responses to the drugs usually include a decline in plasma HIV-1 RNA and an increase

in CD4 cell counts accompanied by a continued viral suppression. Effective treatment also increases naïve and memory $CD4^+$ T-cells with partial restoration of immunity to some opportunistic infections. However, even though the drugs are effective in controlling viral replication, there are limitations including drug resistance because of the high mutation rate of the virus. Individual responses vary and the correlation between the number of CD4 cells and viral load is weak. In addition, CD4 cell counts can increase with incomplete viral suppression. Long-term nucleoside RT inhibitor use is associated with mitochondrial toxicities which can lead to life-threatening lactic acidosis, chronic myopathy or peripheral neuropathy. Protease inhibitors can cause metabolic toxicities leading to hyperlipidaemia, insulin resistance, diabetes and increased risk of cardiovascular diseases (Karim and Karim 2005).

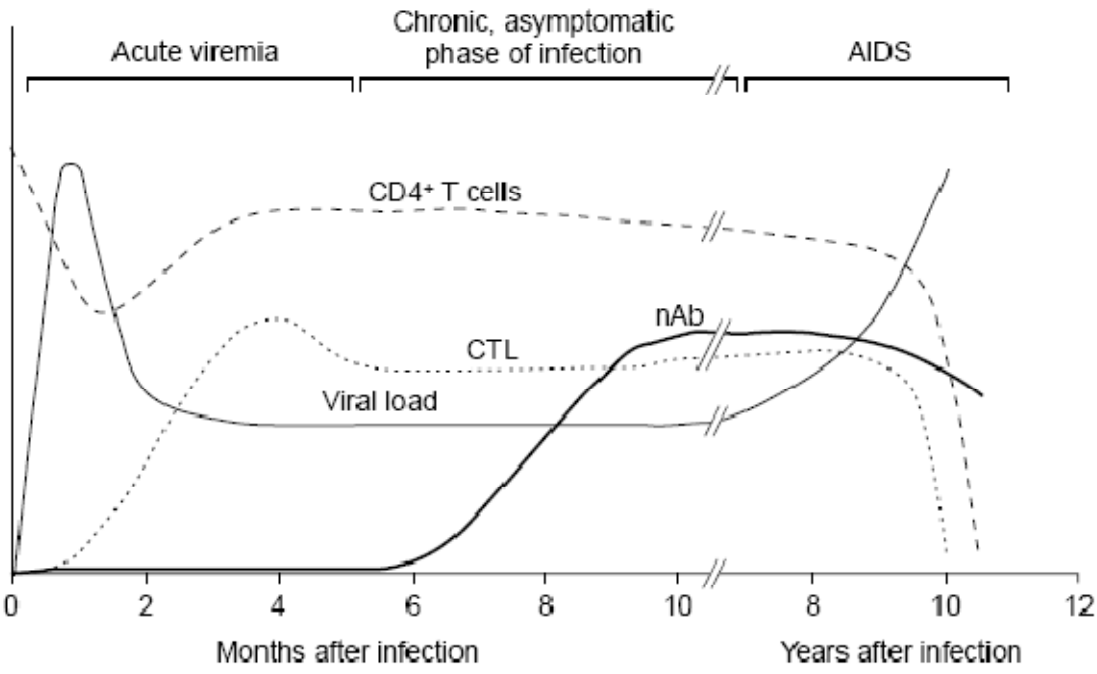


Figure 1.7. Schematic diagram showing the progression of natural HIV-1 infection. Also provided is the time course of adaptive immune responses in relation to viremia levels from initial infection to AIDS-defining conditions. The figure was taken from Ferrantelli and Ruprecht (2002).

1.3 LITERATURE REVIEW OF R7V

1.3.1 Cellular proteins in HIV

Cellular or host proteins can be incorporated by HIV-1 (see Figure 1.8) either on its surface or inside the viral lipid envelope (Ott 2008). Some host proteins present in the

virus retain their functional ability and can affect infectivity, tropism and pathogenesis. According to Ott (2008) there are three possible ways in which HIV-1 incorporates cellular proteins inside or on its surface. Most of the cellular proteins incorporated by the virus are taken up as simple bystanders because of their close proximity during the budding process. The presence of these non-specifically incorporated proteins provides important information about the local environment where HIV-1 assembles and the site of budding. The second possible mechanism of incorporation involves proteins that act as partners in the assembly and budding processes and these are incorporated by interacting with one of the viral proteins. Lastly, HIV-1 can hijack cellular proteins by incorporating them for a post-assembly step where they act as captives helping the virus replicate or evade the immune system (Ott 1997 and 2008). Selected cellular proteins detected in HIV-1 as well as their role in favour of the virus are detailed by Ott (1997 and 2008), see Table 1.2. The MHC class I is one of the proteins incorporated by HIV into its surface and since β2m, which is discussed in this report, is part of MHC class I, the assumption is that this protein is incorporated into HIV's envelope in the same manner as MHC class I. From information provided in the review by Ott (2008) it seems that HIV incorporates this protein as a bystander as there is no evidence that a specific HIV protein acts as a binding partner to bring the protein complex into the viral particle.

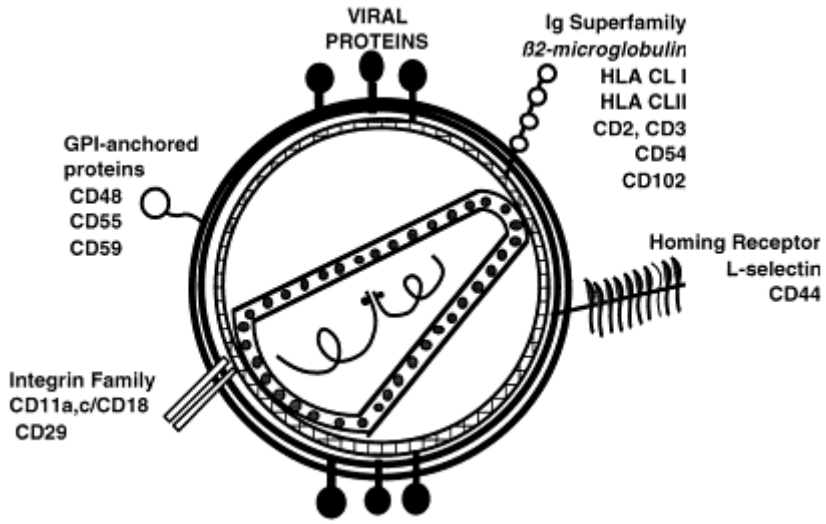


Figure 1.8. The figure illustrates the HIV structure with expression of various cellular proteins on the viral surface. The figure was taken from Galéa *et al.* (1999 b).

Table 1.2. Selected cellular proteins detected in HIV-1. The table was adapted from Ott (1997 and 2008).

Protein	Role for the virus or cellular function
HLA class II	MHC II restriction, cell signalling. Host cell binding, immune modulation.
HLA class I (α) and/or β2-microglobulin	MHC I restriction, cell signalling
CD2/LFA-2	Cell adhesion, T cell activation
CD3	T cell receptor subunit, T cell signalling
CD4	MHC II accessory, T cell signalling
CD5	Binds CD 72, T cell signalling
CD6	T cell activation
CD8	MHC I accessory, T cell signalling
CD9	Tetraspanin, cell signalling
CD11a/LFA-1	ICAM binding (host cell binding), cell adhesion
CD11b/CR3	ICAM binding, cell adhesion
CD11c/CR4	Fibrinogen binding, cell adhesion
CD14	LPS receptor
CD15	Cell adhesion
CD18	ICAM and fibrinogen binding, cell adhesion
CD19	B cell activation
CD21/CR2	Complement and EBV receptor, B cell signalling
CD25	IL-2 receptor
CD30	Lymphocyte marker
CD31	PECAM, cell adhesion
CD43	ICAM-1, cell signalling
CD44	Hyaluronic acid receptor, cell adhesion
CD46	Inhibition of virus lysis by complement
CD48	Lymphocyte regulation
CD53	Tetraspanin
CD54/ICAM-1	LFA-1 binding (host cell binding), cell adhesion/signalling
CD55	DAF, complement lysis inhibition
CD58/ICAM-1	Cell adhesion, T cell activation
CD59	Complement lysis inhibition
CD62/LAM-1	Cell adhesion
CD62L	Endothelial cell binding
CD63	Cell adhesion inhibitor, Tetraspanin
CD68	Unknown
CD71	Transferrin receptor, iron transport

Table 1.2 continues

CD80	Host cell binding/signalling
CD81	Tetraspanin
CD82	Tetraspanin, T-cell signalling
CD86	Host cell binding/signalling
CDw108	Unknown
ICAM-2	LFA-1 binding, cell adhesion/signalling
ICAM-3	LFA-1 binding, cell adhesion/signalling
Galectin-1	Host cell binding
INI/nSNF5	Chromatin organisation/viral assembly/reverse transcription
Lysyl-tRNA synthetase	tRNA primer packaging
Thioltransferase	Maintenance of PR activity
Assassin (APOBEC3G)	Proviral hypermutation
Actin	Cytoskeleton, protein transport
ALIX	Virus release
Cofilin	Actin filament regulation
eEF1A	Multiple functions-actin filament regulation
Ezrin	Membrane-actin linker
HS1	T-cell signalling
Moesin	Membrane-actin linker
Staufen	Genomic RNA packaging
Tal	Virus release, ubiquitination
Tsg101	Virus release, ESCRT pathway
Ubiquitin	Virus release ?, ESCRT pathway
Vps-4a	Virus release, ESCRT pathway
Vps-28	Virus release, ESCRT pathway
Annexin 2	Vasicle transport and fusion
Annexins (A5, A6, A11)	Various functions
CypA	Protein folding
Rap proteins	Vesicular trafficking, protein sorting
S100A10	Vesicle fusion
Tetraspanin-14	Tetraspanin
UNG2	Base excision repair

1.3.2 Beta-2 microglobulin

Beta-2 microglobulin (structure is shown in Figure 1.9) is a 12 kDa, 99 amino acid protein which is non-covalently bound to the 45 kDa heavy chain of the MHC class I molecule (Roitt *et al*. 2001) where it is essential for expression of this molecule. Major histocompatibility complex class I plays a central role in the immune system, is omnipresently expressed and binds peptide antigens for presentation to the $CD8^+$ T-cells (Rosano *et al*. 2005) during the initiation of a cellular immune response. Beta-2 microglobulin is also associated with CD1 (a protein related to MHC class I) for presentation of lipids, glycolipids and lipid antigens to T-cells (Roitt *et al*. 2001). Because β2m is expressed in almost all nucleated cells (Arthur *et al*. 1992) it can be found in all potential virus target cells. This protein along with neopterin and other serum and cellular markers that correlate with clinical progression of HIV disease (Fahey *et al*. 1990; Hofmann *et al*. 1990; Melmed *et al*. 1989) when found free in a variety of physiological fluids (Rosano *et al*. 2005), also serve as indicators of the degree of immune activation.

Beta-2 microglobulin is confirmed as one of the cellular proteins incorporated into the surface of HIV-1 (Arthur *et al*. 1992; Ott 1997) and possible other viruses like CMV (McKeating *et al*. 1987) and HTLV (Hoxie *et al*. 1897, more detail in Section 1.3.6). Because monoclonal antibodies to β2m have been shown able to neutralize HIV (Arthur *et al*. 1992), the protein is suggested to be involved in the process of HIV-infection (Hoxie *et al*. 1987; Devaux *et al*. 1990; Corbeau *et al*. 1990; Arthur *et al*. 1992; Le Contel *et al*. 1996) and will therefore be an important part of the HIV vaccine discussion later in this report.

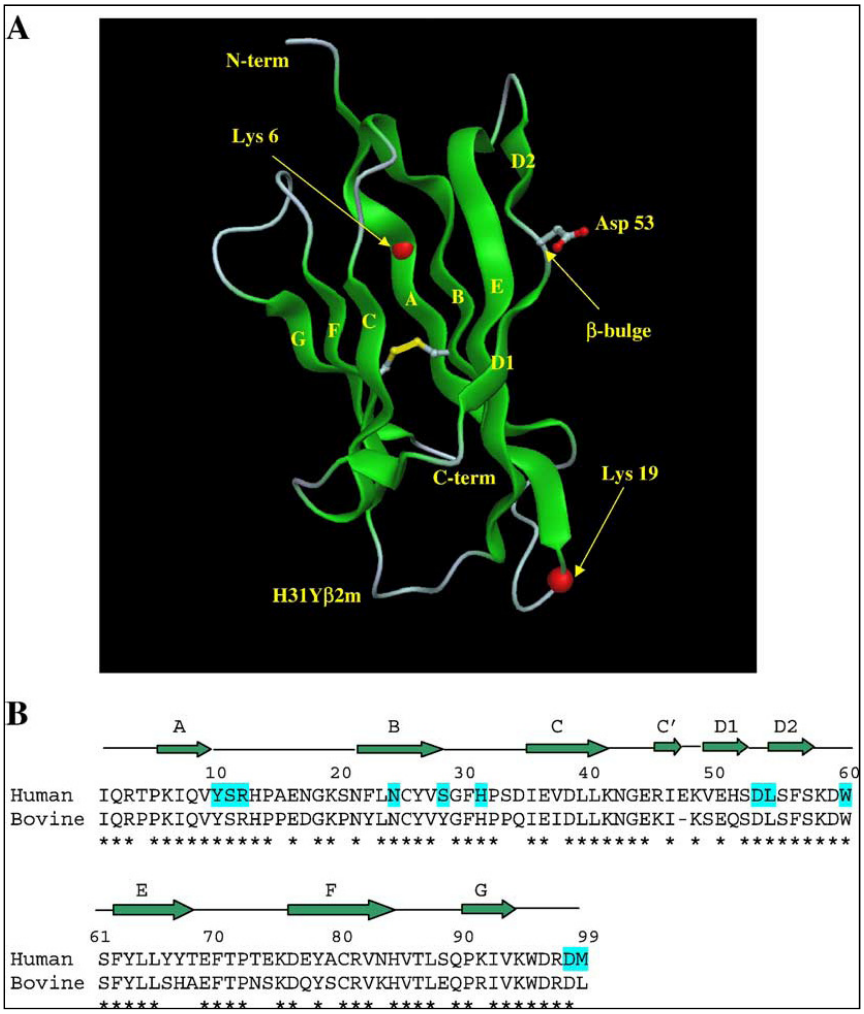


Figure 1.9. Stereo view of the β2m tertiary structure. The overall chain trace of human H31Y β2m is displayed here to highlight the overall β sandwich fold, the secondary structure elements (β strands labelled A, B, . . ., G), the loop regions (light grey), and the intra-chain disulphide bridge (yellow). The image also shows residues Lys 6 and Lys 19, where β2m can be proteolytically cut, and the location of the Asp53 h-bulge, in the D strand. (Drawn with DINO: Visualizing Structural Biology (2001). http://www.bioz.unibas.ch/~xray/dino).
B: Amino acid sequence alignments. This figure displays the primary structures of human and bovine β2m, highlighting the residue identities, the extension of secondary structure elements (on top), and the residues (marked in cyan) involved in hydrogen bonding to the MHC-1 heavy chain in the human complex. The figure was taken from Rosano *et al*. (2005).

1.3.3 Beta-2 microglobulin as vaccine target

Galéa *et al*. (1999 b) and Haslin and Chermann (2002) suggested that to prevent binding between HIV-1 and its host cell, it may be worth considering targeting β2m (a cellular protein) in vaccine design and possibly avoiding the problems associated with the variability of viral proteins. However, it is only possible to consider such a vaccine approach if epitopes in the cellular determinant used will only be exposed when it is carried away by the extracellular infectious agent, or the epitopes in question are

nonimmunogenic in their natural presentation by the cell and are modified when presented at the surface of the virion (Chermann *et al*. 2000). This appears to be the case with epitopes in β2m which do not bind to human cells (discussed in Section 1.3.9).

Monoclonal antibodies directed to β2m were able to immunoprecipitate intact viral particles and inhibit the life-cycle of HIV (Arthur *et al*. 1992). These data suggested β2m to be an integral and functioning part of the HIV-1 surface, involved in the process of HIV-infection and pathogenesis (Hoxie *et al*. 1987; Devaux *et al*. 1990; Corbeau *et al*. 1990; Arthur *et al*. 1992; Le Contel *et al*. 1996).

Most HIV protein-vaccine strategies to date focused on the variable viral envelope proteins, especially the V3 loop of gp120. Hewer and Meyer (2004 and 2005) hypothesized that exploiting the advantageous properties of V3 loop peptides and at the same time accounting for the variability of this region would aid in developing an effective vaccine component for inducing broadly cross-reactive neutralizing antibodies. Addressing variability by novel synthesis (synthesizing branched or circular peptides, synthesizing peptides with variable regions etc.) induced viral strain specific immune responses. The fact that synthetic approaches utilized up to now were unsuccessful underscores the need for circumventing viral variability by other means. Using non-variable virus incorporated host protein epitopes like those in β2m is being suggested as the “other means” in this report.

Studies demonstrating an inability of the potential vaccine antigen to induce autoimmune responses will of course have to be designed. Beta-2 microglobulin based vaccine formulas appear to be under investigation as is evident in the patent by Chermann *et al*. (2006) and in the references used in the patent by Chermann *et al*. (2000). These patents describe vaccine research using R7V formulations (or formulations of the other cryptic epitopes of β2m) conjugated to carrier proteins such as bovine serum albumin (BSA), KLH and multiple antigenic peptide (MAP).

1.3.4 The R7V epitope of beta-2 microglobulin

A seven amino acid epitope in β2m was shown to be present at the surface of divergent HIV isolates by Le Contel *et al*. in 1996. These authors studied several short overlapping peptides derived from β2m for their ability to reverse the neutralizing action of monoclonal antibodies directed to the protein. Among the tested peptides, the

heptamers R7V (Arg-Thr-Pro-Lys-Ile-Gln-Val), S7K (Ser-Gln-Pro-Lys-Ile-Val-Lys) and F7E (Phe-His-Pro-Ser-Asp-Ile-Glu) were efficient in reversing the action of these monoclonal antibodies, with R7V being the most efficient. The R7V peptide consists of fewer hydrophobic (PIV) than hydrophilic (RTKQ) amino acids. Although the tertiary structure of isolated human β2m (the protein containing R7V) is described as an antiparallel β-barrel fold (Figure 1.9, Rosano *et al*. 2005), the R7V section exhibits different secondary structure (Figure 1.10). Multiple Protein Sequence Analysis programs, including DeepView/Swiss-Pdb Viewer 3.7 (SP5), describe percentages of the 7 amino acids in the R7V peptide as either a random coil or an extended strand which are regions that could form part of a β-barrel (PDB entry 1LDS; Trinh *et al*. 2002).

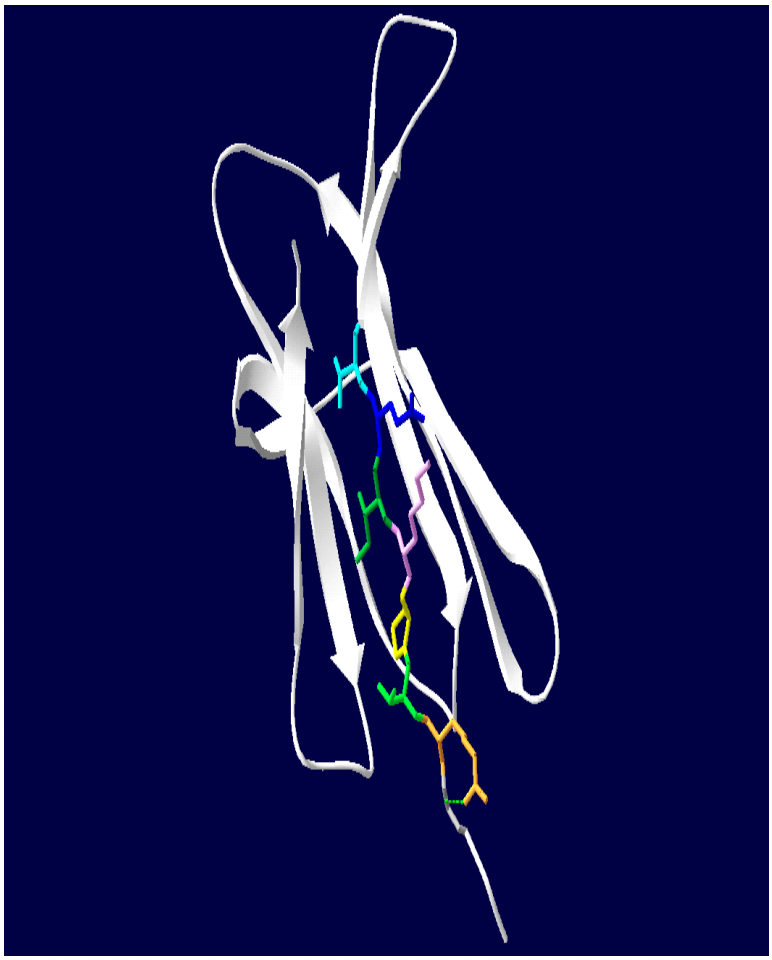


Figure 1.10. Tertiary structure of beta-2 microglobulin with the R7V peptide (RTPKIQV) indicated using different colours. The figure was drawn using the Protein Sequence Analysis program DeepView/Swiss-Pdb Viewer 3.7, SP5 with the PDB entry 1LDS which was taken from Trinh *et al*. (2002).

1.3.5 Polyclonal R7V antibodies detected in HIV positive individuals

Antibodies directed to R7V have been detected in HIV-infected individuals, primarily asymptomatic and long-term-infected patients naïve of treatment. In studies done by Galéa *et al.* (1996) and Sanchez *et al*. (2008), R7V antibodies were present in the majority of asymptomatic patients (HIV seropositive for 5-12 years, stage A1-A2 according to the 1993 CDC classification, Galéa *et al*. 1996; class A according to the 1993 CDC classification and naïve of HAART, Sanchez *et al*. 2008) and long-term-infected patients (infected for more than 10 years and not on HAART, Sanchez *et al.* 2008). The highest prevalence[1] of R7V antibodies were found in non-progressor patients compared to progressors who go on to develop AIDS with associated $CD4^+$ T-cell decreases and viral load increases (Galéa *et al*. 1996; Sanchez *et al*. 2008, Table 1.3). With regards to the correlation between R7V antibodies and the duration of HIV infection; asymptomatic or LTNP patients naïve of treatment and HIV-infected for 10 years or more had more R7V antibodies compared to patients HIV-infected for 5 years. Galéa and colleagues compared asymptomatic (HIV-infected 5-12 years) to long-term symptomatic (HIV-infected 7-13 years) patients or progressors (HIV-infected 2-6 years). This study reported an R7V antibody prevalence of 64.3% in asymptomatic and 72.7% in long-term symptomatic individuals with high titre sera (> 500 µg/ml) whereas for low titre sera (> 200 µg/ml) the respective percentages were 35.7% and 31.8%. The study done by Sanchez *et al*. (2008) demonstrated increased R7V antibody prevalences with the duration of HIV-infection (for 0-5 years: ~ 30%, 5-10 years: ~ 70% and 10-20 years: ~ 80%). This was in patients naïve of HAART. R7V antibodies were also found in individuals who were not infected with HIV, but at lower levels (Galéa *et al*. 1996; Sanchez *et al*. 2008, Table 1.3). This is interesting because R7V is believed to only be immunogenic when incorporated into the HIV envelope and the virus was absent in these patients. Sanchez *et al*. (2008) suggested that these antibodies could be cross-reactive or perhaps the HIV negative individuals were infected by other enveloped viruses containing host derived R7V-like epitopes (more detail in paragraphs to follow).

[1] The *prevalence of R7V antibodies* is often used in publications reporting on R7V antibodies. The *prevalence* has the meaning *occurrence* in this MSc dissertation.

Table 1.3. Summary of studies reporting on the presence of R7V antibodies in HIV-infected and uninfected individuals. HIV-1 subtypes listed were inferred from locations where samples were collected since this information was not always stated in the publications where data was obtained. The table was taken from Bremnæs and Meyer (2009).

Percent R7V antibody producing individuals				Total	HIV-1 subtype/country	References
HIV Positive vs. HIV negative						
%	**n**	**%**	**n**			
53,7	95	32,0	69	164	-	Galéa *et al.* 1996[3,4]
53,2	248	5,5	201	449	(B) USA	Sanchez *et al.* 2008[4,7]
33,5	507	3,0 [2]	201	708	(B) USA	Haslin and Chermann 2007 b[1,3,4]
9,1[7]	33	0,0	10	43	(B) Turkey	Ergünay *et al.* 2008[4,7]
Asymptomatic vs. Symptomatic						
%	**n**	**%**	**n**			
59,0	63	38,0	8	71	(B) Italy	Ravanini *et al.* 2007[1,4]
59,1	22	13,1	61	83	(A) Cameroon	Tagny *et al.* 2007[1,4]
42,0	36	9,0	53	89	(A) Ivory Coast	Kouassi *et al.* 2007[1,4]
64,45	177[5]	35,1	131	308	(B) USA	Sanchez *et al.* 2008[4]
35,76	29[6]	31,8[6]	22[6]	51	-	Galéa *et al.* 1996[2,3,4]
Treatment naïve vs. Treatment						
%	**n**	**%**	**n**			
67,0	45	35,0	17	62	(B) Italy	Ravanini *et al.* 2007[1,4]
56,8	88	21,3	159	247	(B) USA	Haslin and Chermann 2007 b[1,3,4]
38,0	50	2,0	50	100	(A) Ivory Coast	Kouassi *et al.* 2007[1,4]
71,6	88	43,1	160	248	(B) USA	Sanchez *et al.* 2008[4]
CD4 cell count:						
> 200 cells/µl vs. 0-200 cells/µl						
%	**n**	**%**	**n**			
59,1	22	13,1	61	83	(A) Cameroon	Tagny *et al.* 2007[1,4]
Years of HIV-infection (treatment naïve):						
		%	**n**			
< 5 years		14,3	-	-	(B) USA	Haslin and Chermann 2007 b[1,3,4]
5-10 years		50,0	-	-		
> 10 years		68,3	-	-		
0-5 years		~ 40,0	-	-	(B) USA	Sanchez *et al.* 2008[4]
5-10 years		~ 69,0	-	-		
10- 20 years		~ 80,0	-	-		
< 5 years		68,0	41	62[8]	(B) Italy	Ravanini *et al.* 2007[1,4]
5-10 years		31,0	13			
> 10 years		63,0	8			

Table 1.3 continues

Ethnic groups:	%	n			
African Americans	49,5	106	501[2]	(B) USA	Haslin and Chermann 2007 b[1,3,4]
Haitians	38,0	100			
Asian	31,6	95			
Caucasians	26,2	84			
Indians	25,4	67			
Hispanics	22,4	49			
Sex:	**%**	**n**			
Males	55,0	61	106	(B) USA, African American	Haslin and Chermann 2007 b[1,3,4]
Females	44,4	45			

[1]Conference proceedings. 3rd South African AIDS Congress, Durban, June 2007.
[2]Calculation errors: The authors stated that 10 of 201 subjects equal 3%. However, 10 of 201 subjects equal 5%. Adding all the ethnic groups equals 501, 507 is stated as the total number by the authors.
[3]Galéa *et al*. (1996) and Haslin and Chermann (2007 b) performed neutralizing assays with the R7V antibodies.
[4]The majority of studies were performed using the anti-R7V ELISA from Ivagen (Bernis, France) with the exception of Galéa *et al*. (1996) who used an "in-house" ELISA.
[5]Additional results in Sanchez *et al*. (2008): Long-term infected (more than 10 years) and naïve of treatment. Presence of R7V antibodies: ~ 80 %, n not mentioned.
[6] Error: n=29 stated in the text and n=28 stated in the summary. Additional results in Galéa *et al*. (1996): presence of R7V antibodies in progressors: ~ 9% (n=44).
[7]In Ergünay *et al*. (2008) all positive patients were on treatment. In Sanchez *et al*. (2008) 160 patients were on treatment and 88 patients were naïve of treatment.
[8]A sample number of 63 is stated in the poster. 18 of 63 patients were not on treatment.

Table 1.3 provides an indication of R7V antibody prevalence using different ELISAs. The study done by Galéa and colleagues (1996) used an "in-house" ELISA and the authors calculated concentrations of R7V antibodies from a standard curve while Sanchez *et al*. (2008) used the anti-R7V ELISA from Ivagen (Bernis, France) and determined the antibody ratio by normalizing the optical density (OD) value for the sample with the OD value for the internal calibrator. The Ivagen ELISA developed for detection of R7V in human serum and/or plasma of individuals confirmed as being seropositive for HIV-infection, is no longer commercially available. A letter from the Scientific Manager and CEO of Ivagen dated June 2008 was sent out to customers and investigators using the Ivagen ELISA. The letter stated that since early 2006, Ivagen launched several retrospective studies to determine the prevalence of these antibodies within several groups of HIV patients. However, these studies remained limited and the test could not be used as a prognostic tool (Da Costa Castro and Skorski 2008). The test was withdrawn from the market in 2008. Cross-reactivity between sera from both HIV-infected and uninfected individuals and the ELISA antigen, could be a possible explanation for the withdrawal.

The Sanchez *et al*. (2008) study contained several groups; A (201 HIV negative, 160 HIV positive on treatment and 88 HIV positive untreated patients from the USA), B (177 asymptomatic and 131 symptomatic HIV positive patients from the USA) and C (45 untreated Italian non-progressor patients infected with HIV-1). From the third study group Sanchez *et al.* (2008) noticed a direct positive correlation between R7V antibody ratio and viral load. On the other hand, no correlation between the R7V antibody ratios and the CD4 cell count was detected. Sanchez *et al*. (2008) also observed a higher prevalence of R7V Abs in untreated patients (71.6% n = 88) compared to patients on HAART (43.1% n = 160), Table 1.3. When treatment was considered successful, the authors reported a total disappearance in R7V antibodies for 77% (n = 21) of the patients**.** During antiviral treatment there is a decrease in newly formed virus particles budding from the host cell, it is therefore possible that fewer viruses containing the R7V epitope are produced in treated individuals. Sanchez *et al.* (2008) hypothesized that the R7V epitope may no longer be exposed on the virus and thus not visible to the host immune system after successful treatment and therefore suggested that the anti-R7V ELISA from Ivagen was better adapted to the detection of R7V antibodies in asymptomatic patients, still naïve of treatment.

Ergünay and colleagues (2008) following a smaller study (sera from 33 HIV positive individuals on treatment and 10 HIV negative compared to the 160 patients on HAART in the Sanchez *et al*. 2008 study) also reported the presence of R7V antibodies in HIV-infected individuals on HAART using the Ivagen ELISA (Table 1.3). Only 9.1% of 33 HIV positive individuals on treatment exhibited R7V antibodies and there was no correlation between the presence of the antibodies and disease progression. The Ergünay *et al*. (2008) report is in Turkish and the English abstract did not provide a description of the patients' disease status or duration of infection. It is difficult to directly compare the study done by Ergünay *et al*. (2008) and Sanchez *et al*. (2008) because the latter study used an HIV positive test group including patients both on HAART and not on treatment whereas the HIV positive test group in Ergünay's study (2008) were all on HAART. The overall agreement between the works done by these groups is a decrease in prevalence of R7V antibodies in the presence of treatment.

The other studies mentioned in Table 1.3 are conference proceedings that are discussed later (Section 1.3.8).

A study done by Lynne Webber from the Department of Medical Virology, University of Pretoria, South Africa, and presented at the HIV and AIDS Research symposium at the University of Pretoria in February 2009 (Webber 2009) reported on the prevalence of the R7V antibodies in HIV-1 infected individuals on HAART. The prognostic applicability of the anti-R7V ELISA from Ivagen (Bernis, France) was examined using a cohort of 25 HIV-infected patients on HAART. Nine participants were classified as LTNPs, defined as patients free of HIV-1-related disease and displaying stable $CD4^+$ T-lymphocyte counts (> 200 cells/µl for more than 10 years). The results indicated that 40% of all the HIV-infected patients and 56% of the LTNPs tested positive for R7V antibodies. Twenty eight percent of all the HIV-infected patients and 44% of the LTNPs were considered doubtful (data collected could not be classified as either positive or negative for R7V antibodies).

1.3.6 R7V antibodies and cross-reactivity

Data collected by Sanchez *et al*. (2008) demonstrated cross-reactivity of antibodies from individuals infected with other enveloped viruses when using the anti-R7V ELISA from Ivagen. The sample number and percent positive results in this study were limited. It is possible that individuals not infected with HIV but infected with other enveloped viruses which may also incorporate β2m in their membrane and also expose the R7V epitope, could produce antibodies to this epitope. Sanchez *et al*. (2008) observed that a few individuals uninfected with HIV or infected with the viruses causing mononucleosis or rubella gave positive R7V antibody results using the Ivagen ELISA. Three of 13 individuals (23.0%) infected with mononucleosis and 6/11 (54.5%) individuals infected with rubella responded positive for R7V antibodies (antibodies in the sera were able to bind the R7V antigen in an ELISA). Higher sample numbers are obviously needed to validate these data. Also, these antibodies were not isolated and purified before tested for an ability to neutralize or precipitate HIV-1 (which are properties of actual R7V antibodies), so it is possible that this response was due to some interference or cross-reactive antibodies. There is evidence that CMV (McKeating *et al*. 1987) and HTLV (Hoxie *et al*. 1987) incorporate β2m (see Section 1.3.2). Since R7V is part of β2m, this could mean that individuals infected with viruses other than HIV could test positive for R7V antibodies. However, the acquisition of β2m by HIV and CMV differs. HIV acquires β2m during budding while CMV acquires the protein after budding from the cell and monoclonal antibodies to β2m still bind to the latter virus (Grundy *et al*. 1987; Tysoe-Calnon *et al*. 1991; Le Contel *et al*. 1996). There is not enough evidence to form an

opinion on whether CMV (containing β2m) could not induce an R7V like antibody response during infection.

1.3.7 Data collected using assays other than ELISA

Most studies referred to here present conclusions based on ELISA data only (where an R7V peptide was used as antigen). Whether the polyclonal antibodies believed to be R7V antibodies could neutralize or precipitate HIV-1 is not always indicated. Neutralization or precipitation of HIV-1 with the isolated R7V antibodies serves as a means of verifying an actual R7V antibody response. However, a better means of validation would be to isolate and purify the antibodies before use in either assay. Neither Galéa *et al*. (1996), Sanchez *et al*. (2008) nor Ergünay *et al*. (2008) performed precipitation assays. Of these three studies only Galéa *et al*. (1996) demonstrated that the presence of R7V antibodies correlated with neutralization of various divergent HIV strains. A study done by Xu *et al*. (2002) used both ELISA and precipitation to investigate the prevalence of R7V antibodies in HIV-infected patients. However, that data is not referred to in this review because the article is in Chinese and the English abstract does not give information about sample numbers or percentages of R7V antibodies. R7V antibodies neutralized divergent HIV-1 strains and this neutralization was reversed by addition of R7V peptide (Le Contel *et al*. 1996). Galéa *et al*. (1996) hypothesized that R7V antibodies found in patients could have equivalent neutralizing activity as that observed *in vitro*. Extensive experiments with the two main target cells of HIV-1 infection, peripheral blood lymphocytes and blood derived macrophages, have shown that different T-lymphotropic as well as macrophage-tropic HIV-1 strains were neutralized by β2m monoclonal antibodies (Le Contel *et al*. 1996).

1.3.8 Evidence for R7V as potential vaccine target or therapeutic tool

Purified R7V antibodies from sera of rabbits injected with the peptide as well as in sera of non-progressor patients (HIV-1 subtype of infection virus not mentioned) precipitated and neutralized HIV-1 subtypes A, B, C, D and F (Galéa *et al*. 1999 a). The results obtained by Galéa *et al*. (1999 a) is supported by the studies done by Xu *et al*. (2002) who stated that R7V antibodies were found to inhibit the replication of HIV. Xu *et al*. (2002) also suggested that R7V antibodies prevented the virus from entering target cells by interfering with the binding of HIV to the co-receptors (CCR5 or CXCR4) of the target cell. In addition, human R7V IgG neutralized virus strains resistant to antiviral drugs and inhibited infection of cells by laboratory as well as primary viral isolates (Galéa *et al*.

1999 b). Furthermore, studies done by Haslin and Chermann (2007 b, poster presentation at a conference detailed below) showed R7V antibodies from HIV-1 subtype B-infected individuals to be able to neutralize HIV-1 subtype D. Collectively the presence of R7V antibodies in serum from HIV-infected individuals from different geographic areas suggests that R7V is naturally immunogenic and escapes variability and flexibility observed with the viral proteins in the HIV envelope (Galéa *et al*. 1999 a; Chermann 2001). These studies suggest R7V-like epitopes to have a potential role in an HIV/AIDS vaccine and the R7V antibodies in the treatment of patients in failure of HAART.

Production of R7V antibodies with antiviral properties has been demonstrated by Haslin and Chermann (2004) and Haslin *et al*. (2007 a). The antibodies were produced by infection of insect cells with a recombinant baculovirus in which the gene corresponding to the R7V antibody was introduced after isolation from EBV-immortalized B lymphocytes of non-progressor patients. These antibodies have been shown to be able to neutralize various clades of HIV-1 including drug-resistant viruses and should therefore be taken into consideration as therapeutic tools (Haslin and Chermann 2004; Haslin *et al*. 2007 a).

There are several studies reporting on the prevalence of the R7V antibodies in HIV-1 infected individuals which were presented at the 3rd South African AIDS Congress in Durban in June 2007 (Ravanini *et al*. 2007; Haslin and Chermann 2007 b; Tagny *et al*. 2007; Kouassi *et al*. 2007). Ravanini *et al*. (2007) studied a group of HIV- infected patients living in Italy including 63 asymptomatic (A class CDC, 18 ARV treated patients) and 8 symptomatic patients (B or C class CDC, 5 ARV treated patients). Haslin and Chermann (2007 b) studied 507 HIV-infected (on HAART or not) and 201 uninfected individuals from the USA. The study done by Tagny *et al*. (2007) was conducted with a group of HIV-infected individuals (ARV treatment naïve) living in Cameroon, including 22 asymptomatic (A1 and A2 class CDC 1993) and 61 symptomatic (A3, B or C class CDC 1993) patients. Lastly, 100 HIV-infected individuals (50 naïve of HAART and 50 on HAART) living in the Ivory Coast were used in the study done by Kouassi *et al*. (2007). Thirty six patients were at the clinical stage A according to CDC and classified asymptomatic and 53 patients classified symptomatic (CDC stage not stated). None of these four studies report on precipitation assays or any other validation study to confirm that the antibodies detected were actually R7V antibodies. These four studies are discussed in Table 1.3 with some additional observations

described below. Haslin and Chermann (2007 b) observed that R7V antibodies from HIV-1 subtype B-infected patients were able to neutralize a laboratory HIV-1 subtype D strain and that higher titres of R7V antibody plasma were more effective at neutralizing HIV-1, leading the authors to suggest that the neutralizing potential was most likely due to R7V antibodies. Why no subtype B neutralization data is provided, is not explained. A positive correlation between R7V antibody ratio and viral load for a group of asymptomatic ARV treatment naïve patients (n = 45) was observed by Ravanini *et al*. (2007) and supported by similar observations by Sanchez *et al*. (2008). Haslin and Chermann (2007 b) observed an increase in R7V antibodies with the duration of infection in untreated patients (< 5 years: 14.3%, 5-10 years: 50% and > 10 years: 68.3%).

A vaccine component should induce neutralizing antibodies and/or a cellular immune response depending on how it is presented to the immune system. Evidence summarized in this section demonstrates that R7V as antigen in animal studies induced neutralizing antibody production in rabbits (Galéa *et al*. 1999 a). Recombinant monoclonal antibodies to R7V neutralized viral isolates (Haslin and Chermann 2004; Haslin *et al*. 2007 a) and naturally produced R7V antibodies isolated from people did the same (Galéa *et al*. 1999 a and b; Haslin and Chermann 2007 a). In addition, monoclonal antibodies to β2m, the parent protein containing R7V, also neutralized multiple viral strains (Arthur *et al*. 1992). The above-mentioned evidence supports the initiation of large scale studies on either protein or peptide for induction of protective humoral immune responses. These types of studies should perhaps be preceded by studies demonstrating the extent to which β2m or R7V induce autoimmune responses.

However, as mentioned in the introduction, the listed studies explaining the role of R7V and antibodies directed to it in vaccine design or as prognostic or therapeutic tools are incomplete. Few reports published in peer reviewed journals, limited sample numbers in some of the studies, methods that are not explained and results that are not shown are some of the reasons why more research on this R7V epitope of HIV-1 is needed.

1.3.9 R7V antibodies and autoimmunity

A lot still remains to be done with regards to the peptide R7V and what it could mean for the prognosis of disease, therapeutics or HIV vaccine development. According to the patent of Chermann *et al*. (2006), vaccine research has been done with formulations of R7V conjugated to carrier proteins such as KLH, BSA and MAP. Because the epitope

is host-derived one may assume a concern of the possibility that R7V antibodies may initiate an autoimmune response. However, work by Galéa *et al*. (1996 and 1999 a and b), Haslin and Chermann (2002) and Haslin *et al*. (2007 a) suggest this to not be the case. Data are not shown in these papers, but according to the discussion there appears to be no self-recognition by R7V antibodies; no binding to the surface of human cells by purified R7V antibodies from either patients or immunized rabbits (Galéa *et al*. 1999 b). Nor did the recombinant R7V antibodies made by Haslin *et al*. (2007 a) bind to human cells. In addition, individuals with naturally high levels of R7V antibodies did not exhibit any autoimmune diseases (Galéa *et al*. 1996 and 1999 b). This suggests that R7V antibodies produced inside an individual were virus specific and therefore not a problem to the host. If there are no autoimmune antibodies it could be due to the fact that the R7V peptide is presented to MHC class II (for antibody production) in the context of viral molecules. R7V antibodies would therefore not recognize a corresponding epitope on the surface of the host cells.

1.3.10 R7V and cellular immunity

People exposed to HIV mount an immune response which in some individuals slows down disease progression. Strong neutralizing antibodies (Watkins *et al*. 1996; Pilgrim *et al*. 1997; Richman *et al*. 2003; Wei *et al*. 2003) and strong CTL responses (Wagner *et al*. 1999; Cao *et al*. 1995; Klein *et al*. 1995) have been detected in LTNPs. This indicates that both humoral and cellular immune responses slow down disease progression and must certainly be considered for establishing a good prognostic outcome through HIV vaccine development. Neutralizing antibodies as part of a humoral response should eliminate cell-free virus (Figure 1.11) while cellular immune responses (e.g. $CD8^+$ CTLs), on the other hand, should remove already infected cells that escape antibody-mediated neutralization (Lemckert *et al*. 2004), Figure 1.11. If R7V is to be considered in vaccine research, knowledge of possible cellular immune response epitopes within it is of importance. Cytotoxic T-lymphocyte responses are mostly related to viral core proteins (Buseyne *et al*. 1993; Cao *et al*. 1997; Nakamura *et al*. 1997; McAdam *et al*. 1998), but have been observed with some viral envelope proteins as well (Pinto *et al*. 1995). R7V (even though it is derived from a protein associated with and important for MHC class I presentation) is recognized as a foreign antigen by the host and induces a humoral immune response as is evident in all the reports on R7V antibodies. It would be interesting to know whether virus-associated R7V-like epitopes are capable of inducing a cellular immune response, especially given its β2m origins

and the role of this protein in MHC class I presentation of antigen. Major histocompatibility complex class I molecules are known to bind epitopes ranging from eight to eleven amino acids (Karim and Karim 2005) and therefore might bind the seven amino acid R7V peptide for presentation during natural infection. If R7V contains a CTL epitope visible during natural infection, exposing HIV-1 infected PBMCs to this epitope as a peptide *in vitro* should elicit the production of IFN-γ.

The frequency and specificity of IFN-γ secreting $CD8^+$ T-cells during primary and chronic HIV-1 infection can be determined by exposing PBMCs from ARV treatment naïve patients to peptides containing for example *Nef*, *Tat* or *Env* epitopes as stimulants (Cao *et al*. 2003) and enzyme-linked immunospot or flow cytometry as detection methods.

Usually peptide pools are used as antigens (when analyzing responses in out-bred populations) but on occasion single epitope peptides have successfully stimulated IFN-γ production *in vitro*. Peptides known to bind to MHC class I or II molecules are particularly successful (Caulfield *et al*. 2002) and given the origin of R7V it seemed worth the effort to determine whether this epitope could stimulate production of IFN-γ.
No HIV incorporated host proteins have yet been implicated in CTL responses nor has this been exhaustively investigated.

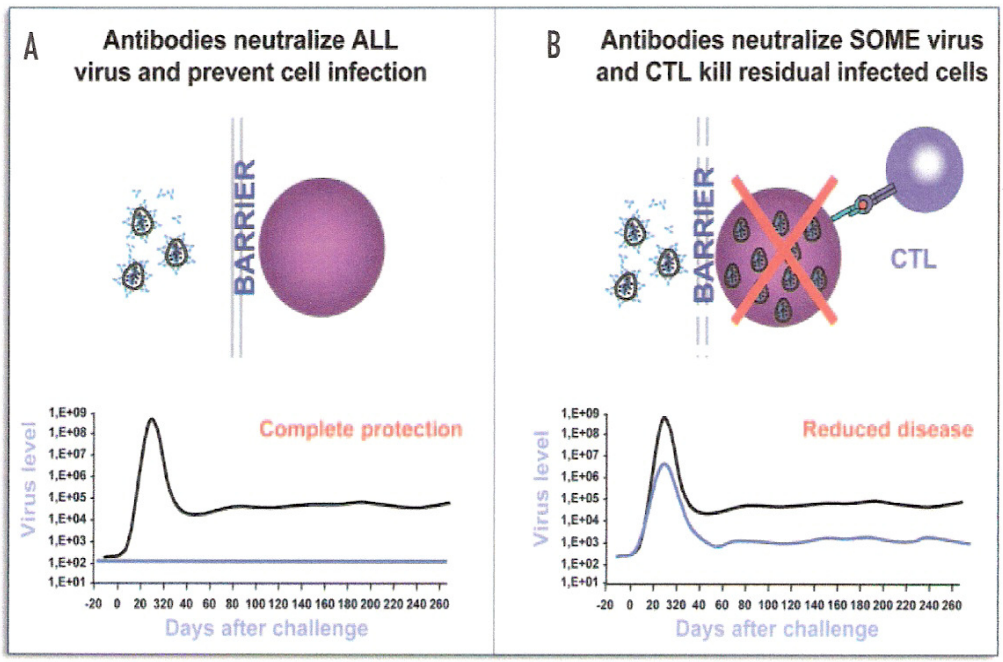


Figure 1.11. Illustration of humoral and cellular immunity – two potential outcomes for a preventive HIV vaccine. In the graphs, the black line shows the time course of viremia in the absence of vaccination. The blue line indicates the virologic outcome in the case of; A: complete protection and B: partial protection. The figure was taken from Srivastava *et al*. (2005).

1.3.11 Adding to the literature

To contribute to the existing literature, this study aims to comment on whether R7V antibodies are prevalent during HIV-1 subtype C infection. This will be done by using specimens from HIV-1-infected individuals living in South Africa where subtype C is prevalent. In these specimens the presence of R7V antibodies and the influence of HAART on the presence of these antibodies will be investigated. This project will also determine whether recombinant R7V single chain antibody fragments consisting of the binding domains of the heavy and light chain of the antigen-binding fragment (Fab) as well as polyclonal rabbit R7V antibodies could neutralize HIV-1 subtype C. In addition, the proliferation of HIV-1-infected cells and IFN-γ production in response to the synthetic R7V peptide will be analyzed.

1.4 STUDY HYPOTHESIS AND AIMS

Human immunodeficiency virus/AIDS has been defined and studied for more than two decades and although treatments and the means of detecting infection exists, prognostic markers like CD4 cell count and viral load are not always accurate. Further, better therapies against HIV are still under development because of side effects and drug resistance. Drug therapies have gained success in limiting viral virulence after infection, but no preventive or curative treatment is currently available. Vaccine research has primarily focused on HIV envelope proteins while more recent studies are shifting the focus to host proteins (i.e. β2m) incorporated by the virus as a radically different means of generating protective immunity (Le Contel *et al*. 1996; Galéa *et al*. 1999 a and b; Chermann 2001; Haslin and Chermann 2007 b). This is because it is believed that these host proteins are functional parts of the virion and involved in the process of HIV infection.

The R7V literature survey attempted to summarize findings on this epitope (contained in β2m) and antibodies directed at it. The production of these antibodies have primarily been observed in non-progressors and increased with duration of infection in these individuals. Many researchers also reported observing that R7V antibodies protect against HIV replication. Research that still has to be done on R7V, which may better explain the importance of this epitope, was also highlighted in the survey. Despite limited studies (reported on in the literature survey), the host immune response to this cellular peptide when it is presented as a virus-associated particle, allows room for investigating it as prognostic marker, therapeutic tool or vaccine candidate.

Because the studies done to date have not always specified the HIV-1 subtypes where R7V epitope/antibody activities were detected the work done here investigated whether the same level of R7V antibody prevalence would be detectable in a subtype C environment. An “in-house” R7V ELISA was developed to aid in this and other functions of the R7V epitope or antibodies was also explored by using a synthetic R7V peptide to characterize aspects of the humoral and cellular immune response in HIV-1 subtype C infection.

The aim of the work was therefore to provide data which may clarify the suggested roles of R7V (prognostic marker/vaccine candidate). In order to achieve this goal a R7V peptide was synthesized and used as antigen in an “in-house” ELISA. A positive control for the ELISA was developed by recombinant means and this recombinant antibody

fragment along with antibodies produced by immunized rabbits as well as sera that recognized the synthetic peptide in the ELISA were screened for viral neutralizing ability as indicators of the functionality or usefulness of the R7V humoral immunity. In addition, the synthetic peptide was used for characterizing aspects of cellular and humoral immune responses by means of cell proliferation and cytokine production in response to R7V.

The above aims were studied in support of **the following hypothesis;** Because R7V is incorporated by HIV and presented to the host immune system as foreign, a peptide representing this epitope should be able to detect humoral and perhaps cellular immune responses in infected individuals. In addition, R7V antibodies should neutralize HIV-1 and subtype C infected individuals (LTNPs) should have antibody levels comparable to that reported for HIV-1 subtype A and B.

Specific questions that were to be answered through this study in order to pursue the objective were as follows:

a) Were R7V antibodies produced during natural HIV-1 subtype C infection and to the same extent as in published literature?

b) Were the antibodies more prevalent in LTNPs compared to progressors?

c) Would HAART influence the prevalence of R7V antibodies?

The peptide was designed to mimic the HIV epitope, R7V. Numerous studies (Galéa *et al*. 1996; Xu *et al*. 2002; Ravanini *et al*. 2007; Haslin and Chermann 2007 b; Tagny *et al*. 2007; Kouassi *et al*. 2007; Ergünay *et al*. 2008; Sanchez *et al*. 2008; Webber 2009) demonstrated that R7V antibodies were produced in HIV-infected individuals and some of the studies demonstrated an elevated level of these antibodies in non-progressors compared to progressors (Galéa *et al*. 1996; Ravanini *et al*. 2007; Tagny *et al*. 2007; Kouassi *et al*. 2007; Sanchez *et al*. 2008). Existing studies did not always highlight subtype differences. Given the fact that the different subtypes of HIV-1 are very similar, it was expected that R7V antibodies should be produced in naturally HIV-1 subtype C infected individuals (to the same extent as in other subtypes) and the R7V peptide should be recognized by these antibodies found in human sera or plasma.

d) Were R7V antibodies *produced in uninfected individuals?*

In some of the studies reporting on R7V antibody prevalence it has been observed that individuals not infected by HIV tested positive for these antibodies but at lower

prevalence or at lower titre (Galéa *et al.* 1996; Haslin and Chermann 2007 b; Sanchez *et al*. 2008). Sanchez *et al*. (2008) suggest that these antibodies could be cross-reactive or that the production of R7V antibodies could be caused by other enveloped viruses. This research group tested a few samples containing antibodies against rubella and mononucleosis. Because only one study was done and with limited sample numbers and limited positive R7V antibody results, more research into the prevalence of R7V antibodies in uninfected individuals as well as during infection with other enveloped viruses is needed.

e) Were R7V antibodies *elicited in rabbits or R7V Fab as well as human polyclonal* antibodies *(detected by the R7V peptide) able to neutralize a primary HIV-1 isolate?*

f) Would R7V antibody fragments (and rabbit polyclonal antibodies) recognize the peptide as antigen in the "in-house" ELISA?

It has been demonstrated that R7V antibodies from purified sera of immunized rabbits and non-progressors (Galéa *et al.* 1999 a) as well as recombinant R7V antibodies produced in insects (Haslin and Chermann 2004; Haslin *et al*. 2007 a) were able to neutralize various clades of HIV-1. Therefore, sera from naturally HIV-1 subtype C infected individuals as well as purified R7V antibodies from immunized rabbits were evaluated for neutralizing ability in this study. In addition, recombinant single chain R7V antibody fragments based on R7V and consisting of the binding domains of the heavy and light domains of Fab were screened because these types of fragments have neutralized HIV-1 in other studies. Zhang and Dimitrov (2007) produced single-chain variable fragment (scFv) antibodies based on a region of gp120 and these had exceptional neutralizing ability against various isolates of HIV-1 including subtype C.

g) Could the R7V peptide stimulate in vitro proliferation of HIV-1 infected PBMCs?

Short HIV peptides have on numerous occasions been able to stimulate proliferation of PBMCs from HIV-1 infected patients, *in vitro* (Hewer and Meyer 2002 and 3003). Here we determine whether R7V would join the ranks of these envelope peptides. The difference, of course, being host-derived vs. virus-derived peptides. Because of how the immune system works, if there is antibody production to antigen, there should be proliferation and cytokine production.

h) Could the R7V epitope play a role in cellular immunity by stimulating the production of IFN-γ by $CD8^+$ T-cells?

Short HIV-1 envelope peptides when containing CTL epitopes will stimulate IFN-γ production if exogenously added to PBMCs in culture (Cao *et al*. 2003). Stimulation of IFN-γ by the peptide *in vitro* could suggest that R7V represented a CTL epitope visible *in vivo*.

1.5 OUTPUTS

1) The research presented in the dissertation has been presented at:

- The HIV and AIDS Research Symposium at the University of Pretoria, Pretoria, South Africa, hosted by the Centre for the Study of AIDS, 26-27 February 2009, oral presentation by Christiane Bremnæs.
- The 4th SA AIDS Conference in Durban, South Africa, 31 March – 3 April 2009, oral presentation by Christiane Bremnæs.

2) The literature review of R7V (Section 1.3 of Chapter 1 of this dissertation) has been published:

- Bremnæs C. and Meyer D. (2009). The HIV-based host derived R7V epitope; functionality of antibodies directed at it and the predicted implications for prognosis, therapy or vaccine development. *The Journal of Biotechnology and Molecular Biology Reviews* **3**, 071-080.

  A copy of the manuscript is included in the Appendix.

3) The experimental content of this dissertation has been compiled for publication and the manuscript entitled “Antibody responses of South African HIV-1 infected individuals determined using a synthetic peptide of host derived epitope R7V” is submitted for review along with this dissertation and will subsequently be submitted for publication to the *Journal of Vaccine*.

CHAPTER 2
MATERIALS AND METHODS

2.1 BACKGROUND TO METHODOLOGY USED

After more than two decades of the existence of HIV, the development of a vaccine remains a fundamental challenge. It is believed that some host proteins (e.g. β2m) and their epitopes (e.g. R7V) become functional parts of HIV and are involved in the process of infection. Therefore vaccine research today is giving consideration to host proteins incorporated by the virus as a radically different means of generating protective immunity (Le Contel *et al*. 1996; Galéa *et al*. 1999 a and b; Chermann 2001; Haslin and Chermann 2007 b). In addition, antibodies directed to these proteins offer possibilities for improved prognostic (Galéa *et al*. 1996; Chermann 2001; Ravanini *et al*. 2007; Kouassi *et al*. 2007; Sanchez *et al*. 2008) or therapeutic (Haslin and Chermann 2002, 2004 and 2007 b; Haslin *et al*. 2007 a) tools.

The first aim of this study was to investigate the presence of R7V antibodies in serum or plasma from naturally HIV-1 subtype C infected individuals. The ELISA is used both qualitatively and quantitatively to measure antigen-antibody binding. In this study an **"in-house" R7V ELISA** was developed and used for *in vitro* detection of R7V antibodies in serum or plasma of infected individuals. A synthetic R7V peptide was used as antigen in the ELISA.

Circulating immune complexes (CIC) have been detected in sera from patients with a wide variety of diseases, including autoimmune diseases, infectious diseases as well as neoplastic disorders (Diegon *et al*. 1972; Louzir *et al*. 1988). These immune complexes could interfere with the binding between the synthetic R7V peptide and the R7V antibodies in an ELISA. To develop the best possible R7V ELISA, CIC in sera was precipitated with polyethylene glycol (PEG, Chia *et al*. 1979) before sample analysis for R7V antibodies.

Another important step in the development of an "in-house" R7V ELISA was access to a positive control. Recombinant positive R7V antibody fragments were made using a recombinant antibody library together with DNA technology and M13 phage display.

These antibody fragments were single chains consisting of the binding domains of the light and heavy domains of the variable (scFvs) regions of Fab. Since scFv antibody fragments containing the Fab regions of whole antibodies are able to bind antigens, those types of antibody fragments were suited for this study. Phage display was used to produce scFvs, as it is known, to produce more stable antibody fragments compared to hybrinoma technology (Lorimer *et al.* 1996). Despite the lack of a Fragment crystallisable region scFv antibody fragments are still considered antibodies for this type of study because of their ability to bind antigens.

The second aim of this study was to evaluate the virus **neutralization ability of R7V antibodies**. Neutralization of HIV can be measured as a function of a reduction in luciferase reporter gene expression in infected cells. Molecularly cloned pseudoviruses are used in this assay because of reagent stability and greater reproducibility and precision in neutralizing assays. Molecularly cloned pseudoviruses were produced in cells by co-transfection with an *env* expression plasmid and a second plasmid expressing the entire HIV-1 genome except *env*. Only the latter plasmid replicates in the cells and is packaged by the pseudovirions for delivery of the *tat* gene to the cells to be infected. The infection is readly detectable in the cell lines that contain a *tat* -responsive reporter gene, such as luciferase. The cell line used in the study (TZM-bl) express CD4, CCR5 and CXCR4 and contains intergrated reporter genes for firefly luciferase. The cells are highly permissive to infection by most strains of HIV including primary HIV-1 isolates and molecularly cloned pseudoviruses (Montefiori 2004).

There are a number of techniques available to characterize aspects of the cellular and humoral immune responses by **measuring cell proliferation and cytokine production in response to R7V**. Cell division can be detected through cell counting using Typan Blue and a microscope, 3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) dye and spectrophotometry, Carboxyfluorescein Diacetate, Succinimidyl Ester (CFDA, SE) dye and flow cytometry or directly via radioactive or non-radioactive means. The use of cell counting in a microscope or MTT dye to detect cell proliferation are less sensitive methods than the use of CFDA, SE dye. The University of Pretoria has recently purchased a new flow cytometer (FACSAria, Becton Dickinson, San Jose, USA), and therefore this mechanism was preferred for this study to detect cell proliferation.

The CellTrace™ CFSE Cell Proliferation Kit (Molecular Probes™, Invitrogen Corporation, Carlsbad, USA) was used and provides an intracellular fluorescent label CFDA, SE to tag proliferating cells. The lipophilic dye, CFDA, SE, is a nonpolar molecule that diffuses into cells and spontaneously and irreversibly couples to cellular proteins (Urbani *et al*. 2006). It is colourless and non-fluorescent until the acetate groups are cleaved by intracellular esterases to yield highly fluorescent anionic CFSE (Figure 2.1). The succinimidyl ester groups reacts with intracellular amines, forming fluorescent conjugates that are well retained by the cells throughout development and meiosis. The label is inherited by daughter cells after cell division. The covalently bound CFSE is divided equally between daughter cells, allowing discrimination of successive rounds of cell division (Lyons and Doherty 2004). After each cell division the level of fluorescence halves for each daughter cell and measurement of the green fluorescence can be used to monitor the number of divisions that occur (Urbani *et al*. 2006). This is done by using flow cytometric analysis of the CFDA, SE labeling with a flow cytometer with 488 nm excitation and emission filters appropriate for fluorescein (Lyons and Doherty 2004).

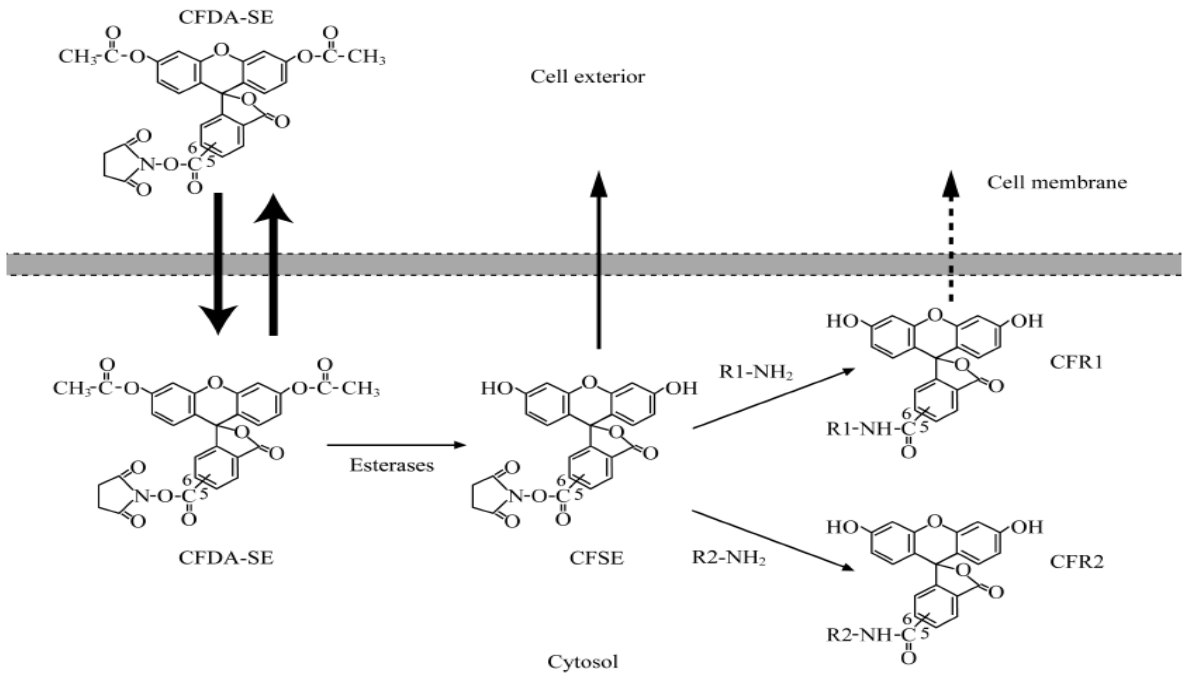


Figure 2.1 Schematic representation of the mechanism involved in fluorescent labelling of cells with CFDA-SE. CFDA, SE penetrates cell membranes and is converted to CFSE by intracellular esterases. Amine-reactive coupling of CFSE to proteins results in stable long-term intracellular retention. The figure was taken from Wang *et al*. (2005).

Flow cytometry is a technique used for counting, examining and sorting cells suspended in a stream. It allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of the cells flowing through an optical and/or electronic detection apparatus. A beam of light of a single wavelength is directed onto a stream with the cells to be analysed and a number of detectors are aimed at the point where the stream passes through the light beam. Each cell passing through the beam scatters the light and fluorescent chemicals attached on the cell may be excited into emitting light. The combination of scattered and fluorescent light is picked up by detectors and by analysing fluctuations in brightness at each detector it is possible to extrapolate the information about the physical and chemical structure of the cells (Figure 2.2, Roitt *et al.* 2001).

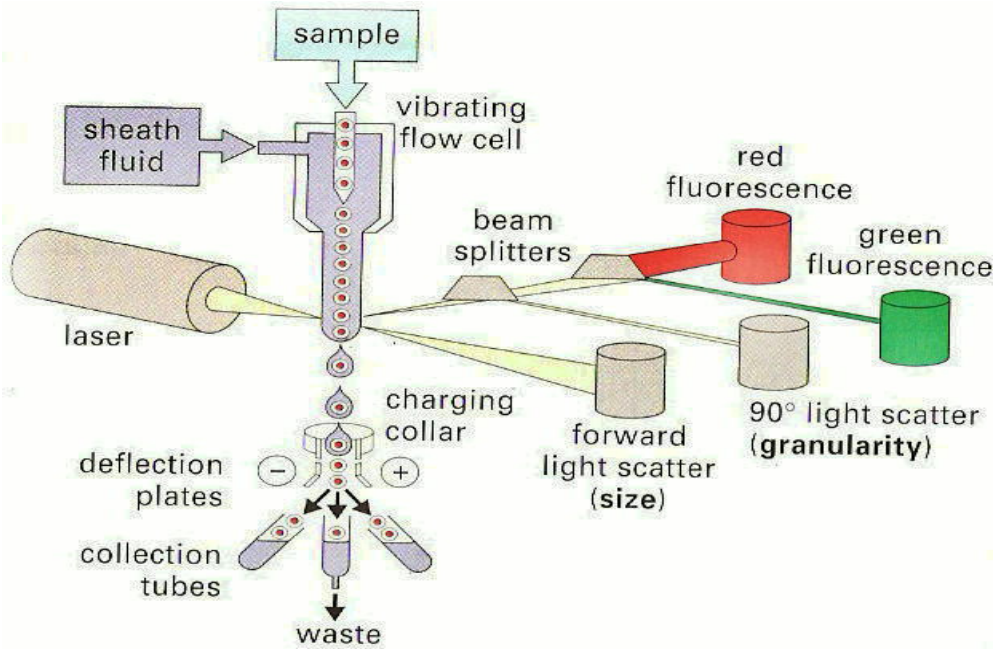


Figure 2.2. The figure illustrates the principle of a Fluorescence-activated cell sorter (FACS). Cells are stained with specific fluorescent reagents to detect surface molecules and are then introduced into the vibrating flow chamber. The cell stream passing out of the chamber is encased in a sheath fluid. The stream is illuminated by laser light and each cell is measured for size and granularity, as well as for green and red fluorescence, to detect two different surface markers. The vibration in the cell stream causes it to break into droplets which are charged and may then be steered by deflection plates under computer control to collect different cell populations according to the parameters measured. The figure was taken from Roitt *et al*. (2001).

The **production of IFN-γ** can be measured via ELISA, Western Blot, flow cytometry or polymerase chain reaction (PCR) alternatively. In this study we detected both intracellular and secreted IFN- γ by the use of flow cytometry and ELISA respectively.

There are many ways to define LTNPs. Elevated natural production of R7V antibodies have been associated with HIV-infected individuals not progressing to AIDS (commonly referred to as LTNPs) compared to progressors and uninfected individuals and will be an important sample group in this study. Long term non-progressors are HIV-infected individuals characterized by the absence of HIV-induced disease symptoms and no history of ARV therapy, with stable or normal CD4 cell count and low viral loads for prolonged periods of time. Long term non-progressors represent a minority (1-5%) of HIV-infected individuals (Easterbrook and Schrager 1998). Even though the phenomenon of long term non-progression in HIV infection is well studied and organized, there is an absence of a standardized case definition and a lack of consistency in the criteria used (Easterbrook 1999). The optimal definition for LTNPs should include the absence of evidence of disease progression after a sufficiently long duration of infection (Schrager *et al*. 1994; Easterbrook 1994; Cao *et al*. 1995) so that only the most stable non-progressors are included (Easterbrook 1999). A commonly used cut off is 10 years which represents the observed median time from HIV seroconversion to AIDS. The clinical criteria generally include a CD4 cell count above 500 cells/µl and/or the absence of a negative CD4-count slope (Easterbrook 1999). A selection of several definitions of a LTNP reported in the literature is summarized in Table 2.1. The majority of the definitions are based on individuals naïve of ARV treatment and with a stable or normal (≥ 500 cells/µl) CD4 cell count. The duration of HIV-infection for a LTNP varies from > 5 years to > 12 years and a few definitions have a CD4 cell count of > 200 or 200-500 cells/µl. While most of the definitions are based on CD4 cell counts, a few definitions are based on viral load as well. The Women's Interagency HIV Study (WIHS) managed by the University of San Francisco USA consists of a large number of scientists and institutions who've accumulated and uses research materials (sera, plasma, PBMCs, etc.) from one of the largest cohorts of LTNP samples in the USA. The LTNP definition used in this study agrees with what is generally accepted by the WIHS. That definition is:

"HIV-1 seropositive ≥ 5 years with a CD4 cell count ≥ 200 cells/µl

in the absence of ARV treatment*".*

This definition also agrees with what can be determined as a definition of LTNPs by most of the researchers who have published on R7V.

Table 2.1. A selection of the definitions of LTNPs found in the literature.

Definition	References
Plasma HIV viral load < 50 copies/ml in the absence of ARV	Tilton *et al.* (2007)
No symptoms of HIV infection, and CD4⁺ T-cell count > 500/µl over a 10-year period without any antiviral therapy (LTNPs I). *No symptoms of HIV infection, CD4⁺ T-cell count included between 200 and 500 cells/µl and remaining stable over a 10-year period without any antiviral therapy (LTNPs II).*	Lefrère *et al.* (1997)
No clinical manifestations of HIV infection for ≥ 7 years and CD4 cell count > 400/µl without therapy	Valdez *et al.* (2002); Paroli *et al.* (2001);
*HIV-1 seropositive **>** 7 years, undetectable plasma viral loads, > 500 CD4⁺ T-cells/µl in peripheral blood, ART[1]-naïve, and clinically healthy*	Sankaran *et al.* (2005)
Maintain CD4⁺ T-cell count ≥ 500/µl, healthy clinical parameters for 7-10 years, and control viral replication without treatment.	Rodes *et al.* (2004); Lopalco *et al.* (2004)
Certified seroconversion at ≥ 7 years, CD4⁺ T-cell count ≥ 500/µl, absence of ARV, asymptomatic HIV-1 infection, and good health conditions	Pastori *et al.* (2006)
*CD4⁺ T-cell count maintained > 500/µl after **>** 8 years of HIV-1 infection.*	Broström *et al.* (1998)
*No sign of HIV-1 disease progression for **>** 10 years and remain asymptomatic. CD4⁺ T-cell count > 500/µl over a long period of time without any antiviral therapy*	Buchbinder *et al.* (1994); Easterbrook (1994)
*No sign of HIV-1 disease progression for **>** 12 years and remain asymptomatic. CD4⁺ T-cell count > 500/µl over a long period of time without any antiviral therapy*	Cao *et al.* (1995)
*HIV-infected **>** 10 years and CD4 counts persistently > 500 cells/mm3 in the absence of ARV*	Vittinghoff *et al.* (1998)
*Patients free of HIV-1 related disease and displayed stable CD4+ T-lymphocyte counts > 200 cells/µl for **>** 10 years.*	Webber *et al.* (2009)
HIV-infected > 5 years and who had either a CD4 count > 500 cells/µl or a viral load < 10000 HIV-1 RNA copies/ml	National Institute for Communicable Diseases
A period of ≥ 5 years off-ART[1] with CD4 count ≥ 500 cells/µl regardless of duration of HIV-infection.	WIHS, University of San Francisco (USA)

[1] ART-antiretroviral treatment

2.2 SPECIMEN SOURCES AND SAMPLE PREPARATION

2.2.1 Human blood samples

Blood samples from HIV-1 infected (ARV treated or treatment naïve) and uninfected patients (negative control blood) living in South Africa were obtained from Prof. Anton Stoltz, Division of Infectious Diseases, Steve Biko Academic Hospital (Pretoria) and from the student clinic at the University of Pretoria as well as from patients attending the Fountain of Hope Clinic (Pretoria). In addition, HIV-1 positive blood was collected from patients attending the King's Hope Development Foundation (Olievenhoutbosch). To identify these samples they were numbered as follows; PAH for the Steve Biko Academic Hospital, SC for the student clinic, FOH for the Fountain of Hope Clinic, DS or KHC for the King's Hope Development Foundation. Negative control blood was also obtained from volunteers (staff members and students) at the Department of Biochemistry at the University of Pretoria. All samples used in this study are from South Africans if not stated otherwise. It is assumed that blood samples collected from individuals living in South Africa are HIV-1 subtype C, which was verified by sequencing a few samples. Archived plasma and serum samples from HIV-1 infected individuals were obtained from the National Institute for Communicable Diseases (NICD, Johannesburg) and Lancet Laboratories (Johannesburg). Archived samples were originally collected from South African patients for studies other than this one. Ethical approval for the collection of whole blood from patients was obtained from the Faculties of (1) Natural and Agricultural Sciences and (2) Health Sciences with the approval records E080-506-019 and 163/2008 respectively. Informed and consenting individuals were enrolled in this study and confidentiality and patient anonymity was maintained throughout. Blood was collected from both genders, over a wide age group (aged 21 to 56) and representing white and black individuals. The HIV-positive blood samples were primarily from black patients whereas the majority of the HIV-negative samples were donated from white individuals. 4 x 4 ml venous blood was collected from each patient in vacutainers with and without anti-coagulant (three vacutainers with anti-coagulant, Ethylenediaminetetraacetic acid, EDTA, and one vacutainer without anti-coagulant, tubes were purchased from the Scientific Group, South Africa).

2.2.2 Serum separation techniques

Blood in vacutainers without anti coagulant was allowed to clot at room temperature for 1 hour and then sera were recovered from the blood by centrifugation at 1610x g for 10

minutes. Sera were inactivated by heating at 56℃ for 30 minutes and stored at -70℃ until used. Sera and plasma used in the Anti-R7V ELISA test from the Ivagen company (Bernis, France) were not inactivated before use because of the company's specifications.

2.3 SYNTHESIS AND CHARACTERIZATION OF THE R7V PEPTIDE

Two peptides were made for this study and contained the seven amino acid (Arg-Thr-Pro-Lys-Ile-Gln-Val) region named R7V derived from the host protein β2m. One peptide was made by GenScript Corporation (Piscataway, USA) and was used to develop the "in-house" ELISA and the other by LifeTein LCC (Edison, USA) used to produce R7V antibodies in rabbits. The latter peptide is described in Section 2.5.

2.3.1 Synthesis of the R7V peptide

GenScript Corporation manufactured the peptide on solid phase by manual fluorenylmethyloxycarbonyl (Fmoc) chloride chemistry previously described by Goldenberg-Furmanov *et al*. (2004) using an automatic synthesizer. It was synthesized from its C-terminus by stepwise addition of amino acids. The first Fmoc-amino acid was attached to an insoluble support resin via an acid labile linker. After deprotection of Fmoc by treatment with piperidine, the second Fmoc-amino acid was coupled utilizing a pre-activated species or *in situ* activation. After the desired R7V peptide was synthesized, the resin-bound peptide was deprotected and detached from the resin via trifluoroacetic acid cleavage. The crude peptide was purified by reverse-phase C18 high-performance liquid chromatography (RP C18 HPLC).

2.3.2 Biochemical characterization of the R7V peptide

The R7V peptide was characterized by electrospray ionization - mass spectroscopy (ESI-MS) and RP-HPLC by the manufacturer.

2.4 EVALUATION OF THE ANTIGENICITY OF THE SYNTHETIC R7V PEPTIDE

R7V ELISAs were performed to characterize the humoral immune response to naturally HIV-1 infected and uninfected individuals by detection of R7V antibodies in serum or plasma. Two R7V ELISAs were used and are described below (the solid-phase "in-

house" R7V ELISA and the Anti-R7V ELISA test from Ivagen ref. IVR96000). The Ivagen ELISA is no longer commercially available and was kindly provided by Lancet Laboratories (Johannesburg). The latter ELISA is described in Section 2.4.6.

2.4.1. Detection of R7V antibodies using an "in-house" R7V ELISA

An indirect solid phase "in-house" R7V ELISA was developed to determine the presence of R7V antibodies in serum or plasma from naturally HIV-1 subtype C infected individuals. The standard protocol routinely used in the laboratory was performed as previously described (Hewer and Meyer 2002 and 2003; Sanchez *et al*. 2008). Briefly, 96-well Nunc-Immuno^{TM} Maxisorp^{TM} ELISA plates (Nunc, Roskilde, Denmark) were coated at 37°C for 1 hour with 100 µl/well of a 250 ng/ml R7V peptide (GenScript Corporation, dissolved in 10 mM Phosphate Buffered Saline, PBS pH 7.4). Thereafter, the plates were incubated overnight at 4°C with 300 µl blocking solution. The blocking solution was 1% (w/v) BSA Fraction V (Roche Diagnostics, Manheim, Germany), 5% (v/v) Fetal Bovine Serum (FBS, Highveld Biological, Lyndhurst, South Africa), 0.1% (v/v) Tween 20 (Highveld Biological, Lyndhurst, South Africa) in PBS. After washing 5x with 300 µl/well wash buffer (0.5%, v/v, Tween-20 in PBS) using a Wellwash 4 Labsystems (Thermo Labsystems, Helsinki, Finland) the plates were incubated for 1 hour at 37°C with 100 µl/well of a 100x diluted human sera or plasma samples (diluted in blocking solution). After washing as before, 100 µl/well of a 5000x diluted (diluted in blocking buffer) horse radish peroxidise (HRP)-labelled secondary antibodies (goat antihuman IgG H+L, Jackson ImmunoResearch laboratories, West Grove, USA) were added and the plates incubated for 1 hour at 37°C. A final washing step was followed by the addition of 100 µl/well of a O-Phenylenediamine substrate (Invitrogen Corporation, Carlsbad, USA) diluted in substrate buffer which was 0.05 M Citric Acid monohydrate (Saarchem, Wadeville, South Africa), 0.1 M di-Sodium hydrogen phosphate anhydrous (Merck, Darmstadt, Germany pH 5.0) containing 0.03% (w/v) hydrogen peroxide tablets (BDH Chemicals, Poole, England). One tablet substrate was dissolved in 12 ml substrate buffer. Enzymatic cleavage was determined on a Multiscan Ascent (Thermo Labsystems, Helsinki, Finland) plate reader with Multiscan Ascent software version 1.3.1, at a wavelength of 450 nm after 60 minutes. All samples were generally analyzed in triplicates and experiments repeated three times. Samples with OD values below or equal to 0.3 were considered negative and samples with OD values above 0.3 were considered positive for R7V antibodies. This decision was reached because almost all

(14/16) HIV-negative samples tested at OD values below 0.3. Information about the race, age and gender of the individuals tested is provided in Table A.1 in the appendix.

2.4.2 "In-house" ELISA protocol improvement

The precipitation of immune complexes and non-specific IgG was previously described (Yamashita *et al*. 1992). For serum precipitates, 8% (v/v) PEG (Sigma-Aldrich, Steinheim, Germany, diluted in 0.01 M PBS) was prepared to precipitate the immune complexes and IgG in human serum. Briefly, human serum (25 µl) was added to 25 µl 8% PEG and left overnight at 4°C. The following day the samples were centrifuged at 1600x g for 50 minutes at 4°C. The supernatant was removed from the described precipitation assay and saved in a separate tube to be analysed for the presence of R7V antibodies in the "in-house" R7V ELISA. The supernatant was diluted 10 times in blocking buffer to a final dilution of 1:100. One hundred µl of this dilution (containing serum without immune complexes) was analysed in the ELISA. Aliquots of the same samples not exposed to precipitation were also used for comparison.

2.4.3 Positive control

A recombinant R7V antibody fragment was produced as explained below (2.4.4) to serve as a positive control for the "in-house" R7V ELISA. To detect the recombinant R7V antibody fragment in the ELISA, the same procedure as described in Section 2.4.1 was performed with the following modifications:

The amount recombinant R7V antibody fragment and secondary antibodies per well was 50 µl (diluted in blocking solution). Two secondary antibodies were used to detect the R7V antibody fragment. Both secondary antibodies were diluted 1:1000. The first incubation was performed with a rabbit-anti-c myc antibody (AbD Serotec, Oxford, UK) and the second incubation was done with a mouse-anti rabbit-HRP-conjugated antibody (AbD Serotec, Oxford, UK).

2.4.4 Recombinant R7V antibody fragment - production

Recombinant single chain R7V antibody fragments that consisted of only the binding domains of the heavy and light domains of the Fab region based on a synthetic R7V sequence were made in the laboratory of Dr. Mervyn Beukes, Department of Biochemistry, University of Pretoria. Recombinant DNA technology and M13 phage display previously described by van Wyngaardt *et al*. (2004) were used with some

modifications. Briefly, a large library of recombinant filamentous bacteriophages displaying single chain antibody fragments derived from combinatorial pairings of chicken variable heavy and light chains was screened for binding to the synthetic R7V peptide (from GenScript Corporation, Section 2.3). Single chain antibody fragments that recognized the peptide were selected from this repertoire. In order to get variations in the fragments, sequences of the selected antibody fragments were cut out using PCR and the remaining sequences linked together. The fragments were then expressed in *Escherichia coli* after which, the antibody fragments were tested for binding to the selected antigen (R7V, GenScript Corporation) in an ELISA. The fragments were named recombinant R7V antibody fragments.

Quantitation of total protein in the recombinant R7V antibody fragment clones was done by the use of a Micro BCA^{TM} Protein Assay Kit (Thermo Scientific, Rockford, USA). Briefly, bicinchoninic acid (BCA) was utilized as detection reagent for Cu^{1+}, which was formed when Cu^{2+} was reduced by protein in an alkaline environment. A purple-coloured reaction product was then formed by the chelation of two molecules of BCA with one cuprous ion (Cu^{1+}). This water-souble complex exhibited a strong absorbance (Abs) at 562 nm that was linear with increasing protein concentrations.

2.4.5 Additional samples analyzed using the "in-house" ELISA

(i) Archived HIV-1 neutralizing antibodies

According to the literature, true R7V antibodies have HIV-1 neutralizing abilities. Therefore, archived plasma from NICD and sera (that respond to peptides from the gp41 membrane-proximal external region, MPER) previously collected by this laboratory (Philippeos, 2007) both with proven neutralizing antibodies, were analyzed for R7V content using the "in-house" ELISA.

(ii) Beta 2 –microglobulin

Beta-2 microglobulin is the parent protein from which R7V is derived, therefore this protein (purchased from ProSpec-Tany TechnoGene, Rehovot, Israel) was used as antigen in the "in-house" ELISA and screened against a purified mouse IgG2a monoclonal antibody to human β2m (purchased from BioLegend, San Diego, USA) as well as HIV positive sera. In addition, β2m antibodies were also screened for the ability to respond to the R7V peptide (GenScript Corporation) as antigen. The protein and antibodies were purchased from separate companies because these were the only

companies with these materials available. Most of the samples tested in this study were done with antibodies produced in humans, and an anti-human secondary antibody was used. For antibodies developed in mice, a peroxidise conjugated anti-mouse IgG (H&L) Goat antibody (Rockland, Gilbertsville, USA) diluted 1:1000 (in blocking buffer) was used.

2.4.6 R7V antibody prevalence detected by the Ivagen ELISA

A company in France developed an R7V ELISA originally planned for commenting on HIV/AIDS prognosis using the amount of R7V antibodies present in serum or plasma of infected individuals. That ELISA was evaluated here for comparison purposes. The Anti-R7V ELISA test (IVAGEN, Bernis, France) is a two-step immunoenzymatic assay allowing detection of R7V antibodies in human serum or plasma. The test was done according to the manufactures' protocol. Briefly, the native sera or plasma (HIV-negative and HIV-positive ARV treated or treatment naïve) were dispensed in a microtiter plate containing covalently fixed R7V peptide at the bottom of each well. An incubation time of 30 minutes is expected to capture R7V antibodies. A conjugate composed of goat anti-human IgG linked to HRP was added after washing. After a 30-minute conjugate incubation and another washing step, the colourimetric identification was made by addition of a tetramethylbenzidine substrate. The OD values were determined using a Multiscan Ascent (Thermo Labsystems, Helsinki, Finland) plate reader with Multiscan Ascent software version 1.3.1 at 450 nm with a reference wavelength at 620 nm after adding the "STOP" solution (diluted hydrochloric acid). After subtraction of the blank sample (sample diluent), the mean OD value observed for each sample (tested in duplicate) was normalized with the mean OD value of the internal calibrator (tested in triplicate). As a control of the functionality of the test, the test kit included a positive and a negative internal control (tested in duplicate). The material used for the calibrator and positive control was based on HIV-infected plasma from a selected asymptomatic donor with a high R7V antibody titre. The calibrator reagent was equilibrated and adjusted at a quality control criterion in a proprietary buffered solution. The result of the anti-R7V ELISA test (detection of the presence or absence of the R7V antibodies) was determined by ratios (mean OD value of the sample divided by the mean OD value of the calibrator). If the ratio was under 0.8, the sample was considered negative for R7V antibodies. Samples with ratios greater than 1.2 were defined as positive for R7V antibodies according to Ivagen. A ratio between 0.8 and 1.2 is

considered a grey zone and the samples responding in this region considered doubtful for the presence of R7V antibodies.

2.4.7 Comparing the "in-house" and Ivagen ELISAs

Four HIV-infected and heat-inactivated serum samples were used in the Anti-R7V ELISA test from Ivagen and the "in-house" R7V ELISA to compare the two ELISAs by analyzing the R squared (R^2) value. There are several definitions of R^2. In this case the R^2 value is the square of the sample correlation coefficient between the outcome and the values being used for prediction. The values vary from zero to one and give some information about the goodness of fit of a model. An R^2 value of 1.0 indicates that the regression line perfectly fits the data and 100% positive correlation between two variables is obtained. An R^2 of zero means there is no relationship between the two variables.

The functionality of the internal controls (Negative -, calibration- and positive control) from the Ivagen ELISA with the company's recommended dilution of 1:51 was also tested in the "in-house" R7V ELISA**.**

2.5 POLYCLONAL RABBIT R7V ANTIBODIES

Polyclonal rabbit R7V antibodies were produced to measure the ability of R7V antibodies in neutralizing HIV-1 subtype C. The antibodies' ability to recognize and bind to the antigen used for their production was confirmed with the "in-house" ELISA.

(i) Peptide synthesis and characterization

LifeTein's PeptideSynTM platform was used for the synthesis of the R7V peptide (Arg-Thr-Pro-Lys-Ile-Gln-Val) that was used for the production of the polyclonal rabbit R7V antibodies. The peptide and polyclonal antibodies were commercially manufactured by LiteTein LCC. The synthesis of the peptide was carried out by Fmoc and tert-Butoxycarbonyl (tBoc) chemistry under the strictest quality control based on HPLC and MS.

(ii) Antibody production

LifeTein's AdjuBooster ^{TM} platform was used for the manufacture of the polyclonal rabbit R7V antibodies using two New Zealand Rabbits. LifeTein's AdjuBooster^{TM} adjuvants were used for the first injection with the R7V peptide and incomplete AdjuBooster ^{TM}

adjuvants for the subsequent injections. The peptide was conjugated to KLH and 50-100 µg immunogen was used for each immunization. The immunogen was diluted to 0.5 ml with sterile saline and combined with 0.5 ml of the AdjuBooster™. The mixed emulsion was injected beneath the skin of the rabbit in the area around the shoulders and intra-muscularly into the large muscle of the rear legs. About ¼ of the antigen was used in each area. Blood was collected from the central ear artery and allowed to clot and retract at 37℃ overnight. The clotted blood was further refrigerated for 24 hours before the serum was decanted and clarified by centrifugation at 2500 rpm for 20 minutes. An ELISA using the peptide as antigen was used to verify the binding between the polyclonal antibodies and the peptide before the antibodies were affinity purified. The R7V peptide was affinity bound to the resin in a column. The antibodies went through the column and the specific antibodies for R7V were bound to the column and other antibodies such as KLH antibodies could not bind to the antigen on the column. Further, the column was washed and the specific antibodies for R7V were eluted as purified antibodies and therefore the antibodies were R7V antibodies and not KLH antibodies.

(iii) Polyclonal R7V antibodies induced in rabbits - ELISA

The rabbit antibodies (LifeTein LCC) were tested in the "in-house" ELISA at various dilutions (1:8000, 1:32000 and 1:128000) using the R7V peptide that was used to make the antibodies (LifeTein LCC), as well as the other synthetic R7V peptide synthesized for this study (Genscript Corporation) as antigen. Other antigens used were β2m and BSA at 25 ng and 50 ng. In addition, negative rabbit serum (pre-bleed) at various dilutions (1:1000, 1:8000, 1:32000 and 1:128000) was tested against the R7V antigen. To detect the polyclonal rabbit R7V antibodies, the same procedure as described in Section 2.4.1 was performed except with the use of a different secondary antibody which was a peroxidise conjugated Goat IgG Fraction to Rabbit IgG (Organon Teknika Corporation, Durham, USA) antibody diluted 2500x.

2.6 EVALUATION OF THE VIRUS NEUTRALIZING ABILITY OF R7V ANTIBODIES

The neutralization assay was carried out at the AIDS Unit of NICD using a protocol described by Montefiori (2004). Briefly, three-fold serial dilutions of inactivated HIV-1 subtype C human serum (ARV treated or treatment naïve), recombinant single chain R7V antibodies or polyclonal rabbit R7V antibodies were made in 100 µl growth medium

(Dulbecco's modified Eagle Medium with L-glutamine, sodium pyruvate, glucose and pyridoxine, Gibco BRL Life Technologies, Grand Island, USA) supplemented with 10% (v/v) FBS, 50 µg/ml gentamycin and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid on 96-well Flat-bottom culture plates (Costar, AEC Amersham, Johannesburg, South Africa). To each well 50 µl virus was added. The viral samples used were 1:10 or 1:20 diluted Du151.2, which contains two HIV-1 subtype C isolates, 1:1500x diluted vesicular stomatitis virus (VSV-G) and 1:300x diluted subtype B virus entitled F162. The plates were then incubated at 37℃ and 5% CO_2 for 1 hour. VSV-G is a virus that contains the envelope of the vesicular stomatitis virus G which was used as a control for problems with toxicity and/or non-HIV-specific activity. SF162 is a very sensitive HIV-1 subtype B virus strain which is easily neutralized. Following incubation, 100 µl of adherent TZM-bl cells resuspended at $1x10^5$ cells/ml in growth media containing diethylaminoethyl dextran (8 µg/ml) was added to each well. Controls incorporated in this assay were cell controls (cc, which were cells and growth media), virus controls (vc, containing virus, cells and growth media) and positive control plasma (with known neutralizing antibodies referred to as Bbpool and/or IBU-21). Neutralizing antibody titres for the positive control plasma depends on the virus and it is therefore not a common practice to give a concentration to it and a 1:20 or 1:45 start dilution is normally used. Following incubation for 48 hours at 37℃ and 5% CO_2 150 µl of cell-culture supernatant was removed from each well. Bright Glo™ Reagent (100 µl, Promega, Madison, USA) was added to each well followed by incubation for two minutes at room temperature. From each well, 150 µl supernatant was transferred to the corresponding wells of a 96-well Flat-bottom black plate (Nunc, AEC Amersham, Johannesburg, South Africa) which was read immediately on a luminometer (PerkinElmer 1420 Multilabel Counter, $Victor^3{}_{TM}$ with software Wallac 1420 Manager version 3.00). Percent neutralization was determined by the ratio in average relative luminometry units (RLU) according to the following equation:

$$\% \text{ neutralization} = 1 - [(\text{test wells} - cc) / (vc - cc)] \times 100$$

Neutralizing antibody titres were expressed as the reciprocal of the serum dilution required to neutralize 50% of the virus.

2.7 CELL PROLIFERATION AND R7V

Proliferation of HIV-infected cells when exposed to dominant antigens in peptide form is well documented for envelope and core protein-based peptides. Therefore the R7V peptide was used in proliferation assays to determine whether R7V could be a dominant epitope in HIV infection.

2.7.1 Cell proliferation detected by flow cytometry

(i) Isolation of Peripheral Blood Mononuclear Cells

PBMCs were derived from human whole blood (HIV-positive and HIV-negative) as previously described (Burdon 1990) using gradient centrifugation and Histopaque-1077 (Sigma-Aldrich, Midrand, South Africa). Briefly, blood was diluted (1:1) in RPMI-1640 medium (Sigma-Aldrich, Midrand, South Africa) supplemented with 0.05% (v/v) Gentamycin (Sigma-Aldrich, Midrand, South Africa) and 0.05% (v/v) Antibiotic/Antimycotic (Sigma-Aldrich, Midrand, South Africa). Histopaque was added to the diluted blood in a 1:2 ratio followed by centrifugation and collection of PBMCs. After a washing step with media as described, the red blood cells were removed from the pellet by incubation with 5 ml ammonium chloride potassium (ACK, 150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) for 5 minutes at room temperature. Subsequently, the ACK was removed by centrifugation and the PBMCs resuspended in complete RPMI-1640 medium supplemented with 10% (v/v) FBS after a washing step. The cell concentration and viability were determined using Trypan blue (Sigma-Aldrich, Midrand, South Africa).

(ii) Staining of Peripheral Blood Mononuclear Cells with carboxylfluorescein diacetate succinimidyl ester

The flow cytometric analysis of cell division by dye dilution was performed as described by Lyons and Parish (1994) and Quah *et al*. (2007). The isolated PBMCs were washed 1x with staining buffer (0.1%, w/v, BSA in PBS). Thereafter, the pellet with PBMCs was labelled with 2 µl 5mM CFDA-SE (Invitrogen Corporation, Carlsbad, USA) stock solution diluted in 1 ml PBS pH 7.4 to a final working concentration of 10 µM CFSE for 10 minutes at 37°C and 5% CO_2. Following incubation, 5 volumes of ice-cold staining buffer were added followed by incubation on ice for 5 minutes. Unincorporated CFSE was removed by washing three times in staining buffer. Stained PBMCs were resuspended in complete media to a final concentration of 2 x 10^6 PBMCs/ml. The labelled PBMCs were incubated at 1 x 10^6 PBMCs/well with various amounts of antigen, 15, 30, 50 and 100 µg/ml R7V peptide (GenScript Corporation), 5 µg/ml mitogen

(Phytohaemagglutinin-protein, PHA-P, Sigma-Aldrich, South Africa) as positive control, or in the absence of stimulation as negative control for 3 and 6 days at 37℃ and 5% CO_2. In addition, labelled PBMCs were also incubated 1 x 10^6 PBMCs/well with various amounts of antigen (1, 5 and 10 µg/ml), human β2m (ProSpec-Tany TechnoGene), 5 µg/ml mitogen (PHA-P) or in the absence of stimulation for 3 and 6 days at 37℃ and 5% CO_2. Tables 2.2 and 2.3 show the samples used for the study with R7V and β2m respectively.

Table 2.2. Samples used in the cell proliferation analysis using R7V as antigen.

	Incubation time	3 days			6 days		
Sample	HIV status	R7V	PHA-P	No stimulation	R7V	PHA-P	No stimulation
13 PAH	+	N/A	N/A	N/A	√	√	√
14 PAH	-	N/A	N/A	N/A	√	√	√
20 PAH	-	√	√	√	√	√	√
21PAH	+	√	√	√	√	√	√

N/A means not applicable. These samples were only used for the 6 days incubation study.

Table 2.3. Samples used for the cell proliferation study with β2m as antigen.

	Incubation time	3 days			6 days		
Sample	HIV status	R7V	PHA-P	No stimulation	β2m	PHA-P	No stimulation
20 PAH	-	√	√	√	√	-	√
21PAH	+	√	√	√	√	-	√

(iii) Analysis of cell proliferation using flow cytometry

Following incubation, the PBMCs were harvested and then washed with 200 µl staining buffer and resuspended in 0.3 ml staining buffer. Cell proliferation of PBMCs was determined by analyzing ten thousand total events in the FITC detector (Blue lazer: 488 nm, BP 530/30, LP: 502, Ex.: 492, Em.: 517. Optical density filter: 1.0) of the FACSAria (Becton Dickinson, San Jose, USA), with FACSAria version 6.1.1 software. All samples were analyzed in triplicate.

2.8 THE R7V PEPTIDE AS STIMULANT OF INTERFERON-γ PRODUCTION

2.8.1 Intracellular cytokine staining (Interferon-γ)

The flow cytometric analysis of IFN-γ production stimulated by the R7V peptide and human β2m was investigated *in vitro* by means of the following fluorochromes from Becton Dickinson Biosciences Pharmagen™ (San Jose, USA): Pacific Blue™ Mouse Anti-Human CD3, PE Mouse Anti-Human CD4, CD8 PerCP (SK1) and FITC Mouse Anti-Human IFN-γ. PBMCs were isolated from human whole blood (HIV-infected and uninfected) as described in Section 2.7.1. After isolation, the cells were resuspended in complete media and incubated at 1 x 10^6 PBMCs/well with 50 µg/ml R7V peptide (GenScript Corporation), 5 µg/ml human β2m (ProSpec-Tany TechnoGene) or 50 µg/ml MPER 2b.b8 construct (GenScript Corporation) for 78 and 150 hours at 37°C and 5% CO_2. The MPER peptide successfully stimulated cell proliferation in other projects in this laboratory and was used as a control here. Stimulation in the absence of antigen served as negative control. Incubation with different concentrations of PHA-P (5 and 2 µg/ml) or (10 ng/ml) Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, Midrand, South Africa) plus (1mM/ml) Ionomycin calcium salt (Sigma-Aldrich, Midrand, South Africa) were used as positive controls. PHA-P was used as a positive control for the 78-hour incubation study. PMA/Ionomycin was used as a positive control for both the 78 and 150-hour incubation study but added to the PBMCs only for the last 6 hours of incubation and the last 5 hours of incubation with 1 µl BD GolgiPlug™ (Becton Dickinson Biosciences, San Jose, USA) which was added to all the samples including the R7V, MPER and PHA-P stimulated and unstimulated cells. GolgiPlug is a protein transport inhibitor that allows accumulation of cytokines in the Golgi complex enhancing the detectability of cytokine-producing cells with immunofluorescent staining and flow cytometric analysis. At the end of the incubation, the PBMCs were placed in 2-8°C over night. The next day the cells were gently mixed before heating at 37°C and 5% CO_2 for 10 minutes. The cells were then centrifuged for 10 minutes at room temperature (this centrifugation setting was used throughout the experiment) and the cell supernatant and cells were harvested and transferred into separate tubes. The cell supernatant (possibly containing secreted IFN-γ) was stored at -20°C until used. The PBMCs were pelleted by centrifugation and blocked with 180 µl ice-cold blocking buffer (10%, v/v, human serum in PBS) for 20 minutes at 2-8°C. Following washing with 180 µl blocking buffer, 50 µl of a staining solution (including the fluorochromes anti-Human CD3, 10 µg/ml, anti-Human CD4, 200 µl/ml, and anti-Human CD8, 1.25 µg/ml, diluted in staining buffer, 3%, v/v,

human serum in PBS) was added to the cell pellets and incubated for 15 minutes at RT in the dark. Subsequently, the cell pellets were washed with 200 µl staining buffer. To fix and permeabilizate the cells, Becton Dickinson Cytofix/Cytoperm™ Fixation/Permeabilization Kit (Becton Dickinson Biosciences Pharmagen, San Diego, USA) was used. Following incubation for 20 minutes at 2-8°C with 250 µl Becton Dickinson Cytofix/Cytoperm™ fixation and permeabilization solution, the cell pellet was washed with 200 µl 1x Becton Dickinson Perm/Wash™ Buffer. Further, the PBMCs were stained with 50 µl of a staining solution (consisting of the fluorochrome anti-Human IFN-γ diluted in Perm/Wash™ Buffer to a final concentration of 10 µg/ml) for 30 minutes at room temperature in the dark. Following washing with 200 µl 1 x BD Perm/Wash™ Buffer, the cells were resuspended in 250 µl fixing solution (3 %, v/v, Formaldehyde, formaldehyde 37% Solution Sigma-Aldrich, South Africa, in PBS). Intracellular IFN-γ staining was determined by analyzing 10,000 total events in the PerCP Cy5-5 (665 LP; 695/40), PE (556 LP; 585/42) and FITC (520 LP; 530/30) detector on the blue lazer and the DAPI (450/40) detector on the violet lazer for the fluorochromes CD8 PerCP (SK1), PE Mouse Anti-Human CD4, FITC Mouse Anti-Human IFN-γ and Pacific Blue™ Mouse Anti-Human CD3 respectively. (Optical density filter: 1.0) of the FACSAria (Becton Dickinson, San Jose, USA), with FACSAria version 6.1.1 software.

2.8.2 Secreted Interferon-γ ELISA

The amount of secreted IFN-γ produced by the PBMCs after stimulation with R7V or β2m (Section 2.8.1) was investigated *in vitro* by means of the Human IFNg (Interferon-gamma, IFN-g) ELISA Ready-SET-Go! (eBioscience, San Diego, USA). The cell supernatant collected after 78 and 150 hours of incubation (Section 2.8.1) was analyzed in the ELISA according to the manufacturer's protocol. Briefly, a Corning Costar 9018 ELISA plate was coated with 100 µl/well of capture antibody (pre-titrated, purified antibody) in Coating Buffer and incubated overnight at 4°C. Following 5x washing with 300 µl/well Wash Buffer (0.05%, v/v, Tween-20 in PBS), the wells were blocked with 200 µl/well of 1x Assay Diluent for 1 hour at room temperature and then washed again as described. Subsequently, 100 µl of cell culture supernatant from differently treated PBMCs were added and the plates incubated overnight at 4°C. A recombinant cytokine standard, diluted in assay diluent and added at 100 µl/well, was prepared by 2-fold serial dilutions and included at the same time. After incubation the samples were washed as described before. A detection antibody pre-titrated and biotin-conjugated

diluted in 1x Assay Diluent was added (100 µl/well) and the plate incubated for 1 hour at room temperature. Following another washing step, a 30-minute incubation at room temperature with 100 µl/well of Avidin-HRP diluted in 1x Assay Diluent was performed. After this step, the wells were washed 7 times and 100 µl/well of substrate solution (Tetramethylbenzidine) was added and incubateed for 15 minutes at room temperature. Finally, 50 µl of Stop Solution (2 Normal H_2SO_4) was added to each well and the plate was read at 450 nm. OD was determined on a Multiscan Ascent (Thermo Labsystems, Helsinki, Finland) plate reader with Multiscan Ascent software version 1.3.1. All samples were tested in triplicate.

2.9 STATISTICS

Calculations of average, standard deviation (SD) and percent relative standard deviation (% RSD) were made using Microsoft® Office Excel® 2007 (Microsoft Corporation, Redmond, USA). The SD based on a sample tested in triplicates and minimum three times measures how widely values are dispersed from the average value. Data is presented with bars showing the actual data point value for each sample including error bars indicating the plus and minus SD. A one-way Analysis of Variance (ANOVA) was performed using Statistical Package for the Social Sciences (SPSS) version 17 software. The ANOVA, invented by Sir R. A. Fisher (1935), is one of the most powerful statistical techniques and can be used to test the null hypothesis, whether the means among two or more independent groups are equal, under the assumption that the sampled populations are normally distributed. The statistically significant effect in the ANOVA was followed up by a Post hoc ergo propter hoc, post hoc, test (Benferroni multiple comparisons method) in order to assess which groups were different from which other groups. Benferroni's method is a simple method valid for equal and unequal sample sizes. The mean difference was declared as significant at the 0.05 level.

CHAPTER 3
RESULTS

3.1 SYNTHESIS AND CHARACTERIZATION OF THE R7V PEPTIDE

3.1.1 Synthesis of the R7V peptide

Two R7V peptides were synthesized for this project by GenScript Corporation and LifeTein LCC respectively. The results for the latter peptide are shown in Section 3.3 As described in the methods, GenScript Corporation manufactured the R7V peptide on solid phase by manual Fmoc chemistry and used RP C18 HPLC and ESI-MS for quality control of the product. HPLC and MS alone cannot predict the peptide structure. Multiple Protein Sequence Analysis programmes describe percentages of the 7 amino acids in the R7V peptide as either an extended strand or a random coil.

3.1.2 Biochemical characterization of the R7V peptide

In order to characterize the synthetic R7V peptide, ESI-MS and RP-HPLC were performed. Figures 3.1 and 3.2 depict the RP-HPLC chromatogram and the ESI-MS spectra of the synthesized R7V peptide respectively. The HPLC results indicate that a relatively pure majority product was obtained from the synthesis with a purity of 98.86% (Figure 3.1). Definite resolution was attained in the ESI-MS spectra. The [M+H] 1+ peak represents the R7V peptide's m/z of 841.55. The observed mass value (molecular weight, MW) for the R7V peptide was 841.55 - 1 = 840.55 (Figure 3.2) which correlated with the expected theoretical MW 841.01 calculated by using the software Peptide Property Calculator from GenScript Corporation. MW (and not relative molecular mass) is defined because the company described the peptide size in this manner.

The total amount of peptide received from Genscript Corporation was 29 mg which was stored at -20℃. According to the manufacturer's instructions, an aliquot was dissolved in 20x PBS and water was added to obtain a final concentration of 1mg/ml peptide in 10x PBS. The dissolved peptide was aliquoted and stored at -20℃ until use.

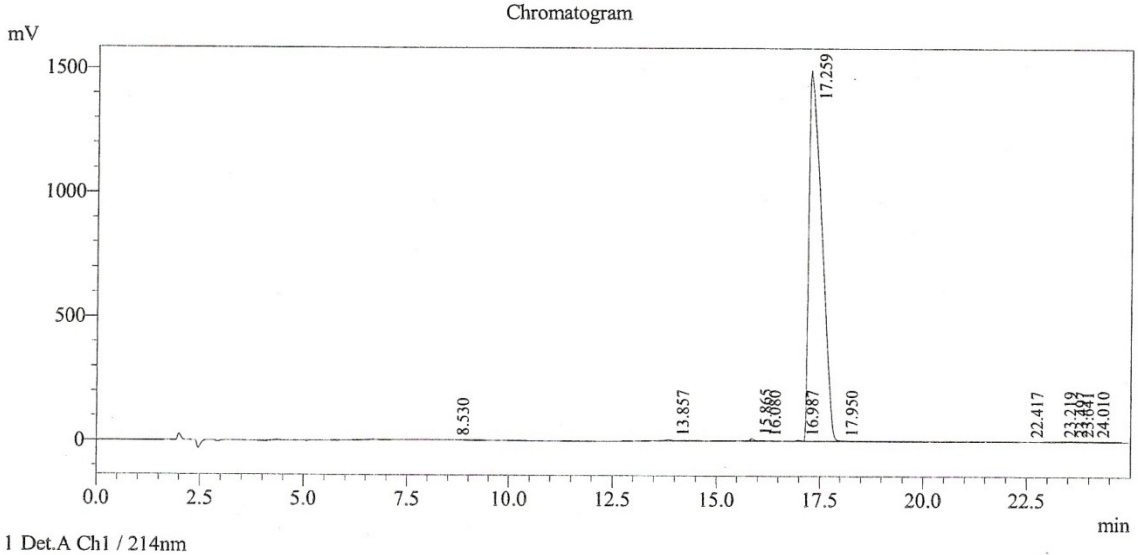
Sample Name : 60528-1
Sample ID : 60528-1

Pump A : 0.065% trifluoroacetic in 100% water
Pump B : 0.05% trifluoroacetic in 100% acetonitrile
Total Flow : 1 ml / min
Wavelength : 214 nm

Time	Unit	Command	Value	Comment
0.01	Pumps	Pump A B.Conc	5	
25.00	Pumps	Pump A B.Conc	65	
25.10	Pumps	Pump A B.Conc	95	
33.00	Pumps	Pump A B.Conc	95	
33.10	Pumps	Pump A B.Conc	5	
43.00	Pumps	Pump A B.Conc	5	
43.10	Controller	Stop		

<Detector A>
Column : SHIMADZU VP-ODS 4.6 x 150 mm

Chromatogram



1 Det.A Ch1 / 214nm

Peak Table

Detector A Ch1 214nm

Peak#	Ret. Time	Area	Height	Area %
1	8.530	12605	674	0.039
2	13.857	29344	2455	0.090
3	15.865	64820	8944	0.198
4	16.080	13438	1899	0.041
5	16.987	29281	3925	0.090
6	17.259	32290944	1494102	98.856
7	17.950	43951	2867	0.135
8	22.417	18346	654	0.056
9	23.219	46074	1713	0.141
10	23.497	45707	2482	0.140
11	23.641	27806	2051	0.085
12	24.010	42403	1370	0.130
Total		32664719	1523135	100.000

Figure 3.1. RP-HPLC chromatogram of the synthetic peptide. Substantial purity (98.86%) of the R7V peptide was achieved following the synthesis.

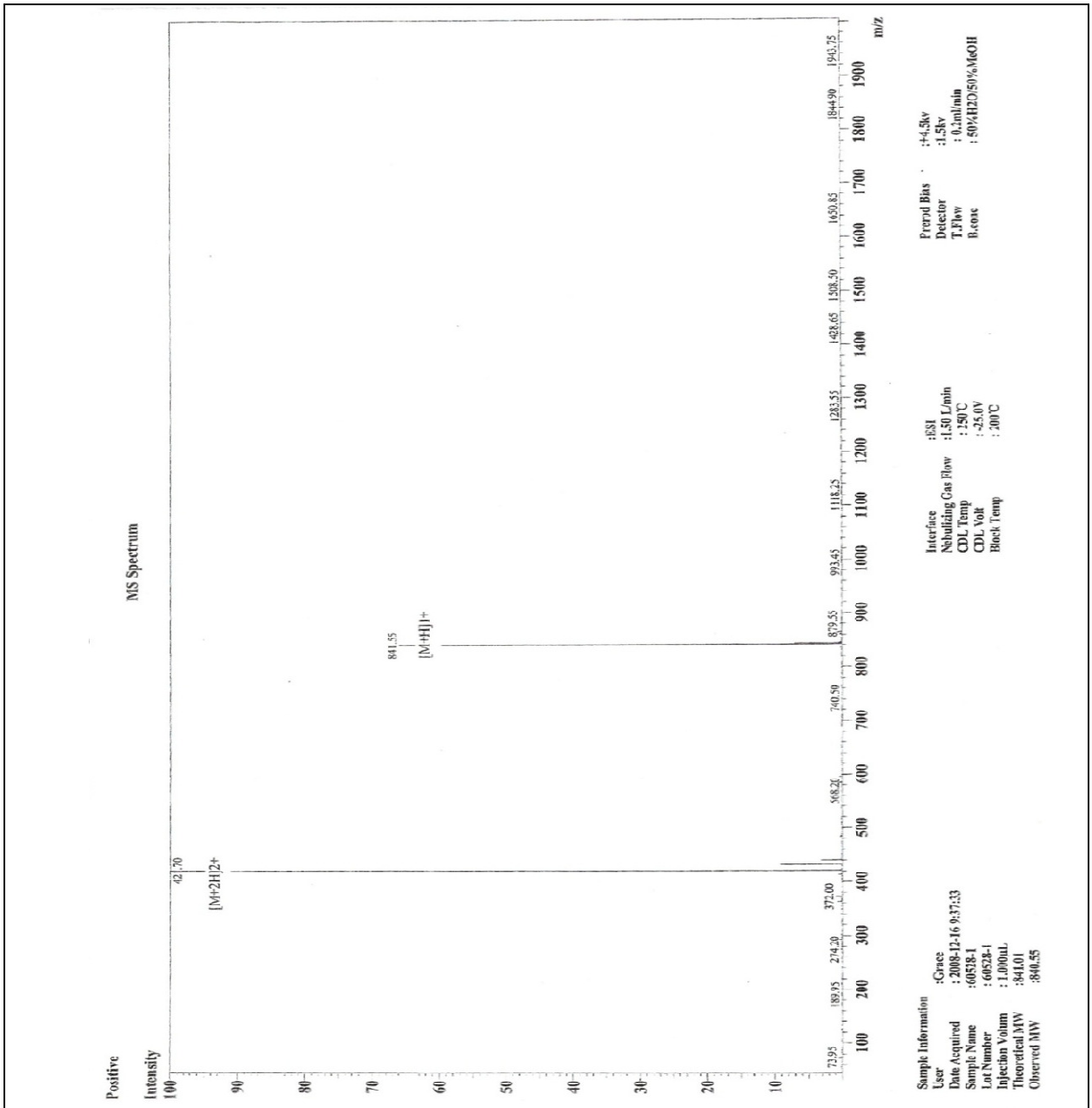


Figure 3.2. The ESI-MS spectrum of the R7V peptide. The [M+H] 1+ peak represents the R7V peptide's m/z of 841.55. The observed MW for the R7V peptide (840.55) correlated with the expected theoretical MW 841.01.

3.2 EVALUATION OF THE ANTIGENICITY OF THE SYNTHETIC R7V PEPTIDE

3.2.1 Detection of R7V antibodies using an "in-house" R7V ELISA

The R7V peptide was used as antigen in an "in-house" R7V ELISA to determine the amount of R7V antibodies in serum or plasma from naturally HIV-1-infected individuals. The amount of antigen used in the ELISA was 25 ng/well and all serum or plasma samples were diluted 100x. The selected antigen concentration and serum dilution was determined to be sufficient following the analysis of several dilutions and concentrations as shown in the appendix (Figures A.1, A2 and A.3). As shown in Figure 3.3, heat-inactivated sera from 78 individuals were tested in the R7V "in-house" ELISA. Sixteen HIV-negative, 62 HIV-1 positive (32 ARV treated and 30 treatment naïve with 11 considered LTNPs – CD4 cell count ≥ 200 cells/µl and infected ≥ 5 years) samples were analyzed. Six recombinant R7V antibody samples were used as positive controls. The positive controls were very responsive but some clones were more responsive than others (OD value above 2 at dilutions of 1:10). The recombinant antibodies do not maintain stability for very long and lose activity altogether when diluted higher than 1:10 (more detail in Section 3.2.4). Of the experimental samples, the HIV-negative sera consistently and repetitively responded at OD values of 0.3 or lower. The HIV-positive sera had OD values above 0.3 and were considered positive for R7V antibodies. Although the HIV-positive samples appear to have R7V antibodies present, few samples demonstrate OD values double that of the negative controls. No general trend was observed for the HIV-positive samples with or without ARV treatment. LTNPs had the same R7V antibody levels of AIDS patients (progressors). The same samples are also shown in Figure 3.4 together with only one of the positive controls for a better view of the differences between HIV-negative and HIV-positive samples. Figure 3.5 (A-F) shows that there was no correlation between (1) viral load and CD4 cell count, (2) CD4 cell count vs. Abs at 450 nm and (3) viral load vs. Abs at 450 nm for either infected samples on treatment or naïve of treatment. The figure also shows that there was no increase in R7V antibody prevalence with the duration of HIV infection (G).

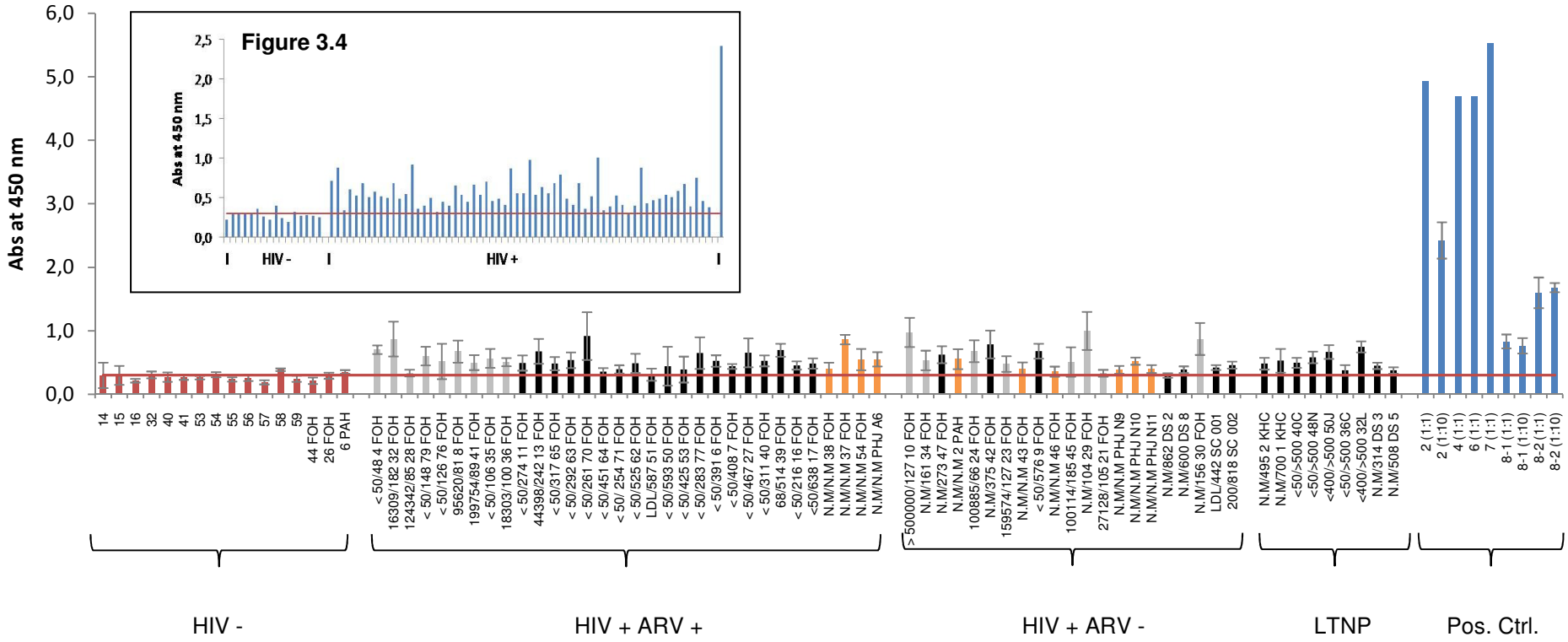


Figure 3.3. Seroprevalence, by using the R7V "in-house" ELISA, of R7V antibodies in heat-inactivated serum from naturally HIV-1 infected (HIV +) ARV treated (+) or treatment naïve (-) patients, LTNPs and uninfected (HIV -) individuals. The negative controls are numbered 14-59 (including 44 and 26 FOH and 6 PAH). For HIV positive samples, viral load (RNA copies/ml) and CD4 cell counts (cells/µl) are indicated in front of the sample number. Not mentioned (N.M) is indicated where information is not available. Colour codes indicate the following: pink, HIV -; grey, individuals with AIDS (CD4 cell count < 200 cells/µl); black, AIDS not yet developed (or recovered from AIDS after ARV treatment); orange, AIDS status unknown because of unknown CD4 cell counts. For the LTNP samples 40C, 48N, 50J, 36C and 32L the exact CD4 count is not known other than that they are above 500 cells/µl. Blue bars indicate positive controls: recombinant R7V antibody fragements. The red line indicates the cut-off value for determining positivity. Samples with OD values above 0.3 are considered positive for R7V antibodies. Samples with OD values below or equal to 0.3 are considered negative for R7V antibodies. An elevated but marginal reaction was observed for the HIV-positive samples. No general trend was observed for the ARV treated or treatment naïve HIV-positive samples. LTNPs demonstrated the same R7V antibody level of AIDS patients (progressors).

Figure 3.4. Seroprevalence of R7V antibodies. The figure shows all the HIV- and HIV+ samples from figure 3.3 with only one of the recombinant R7V antibody fragment clones (number 2) as positive control (at far right).

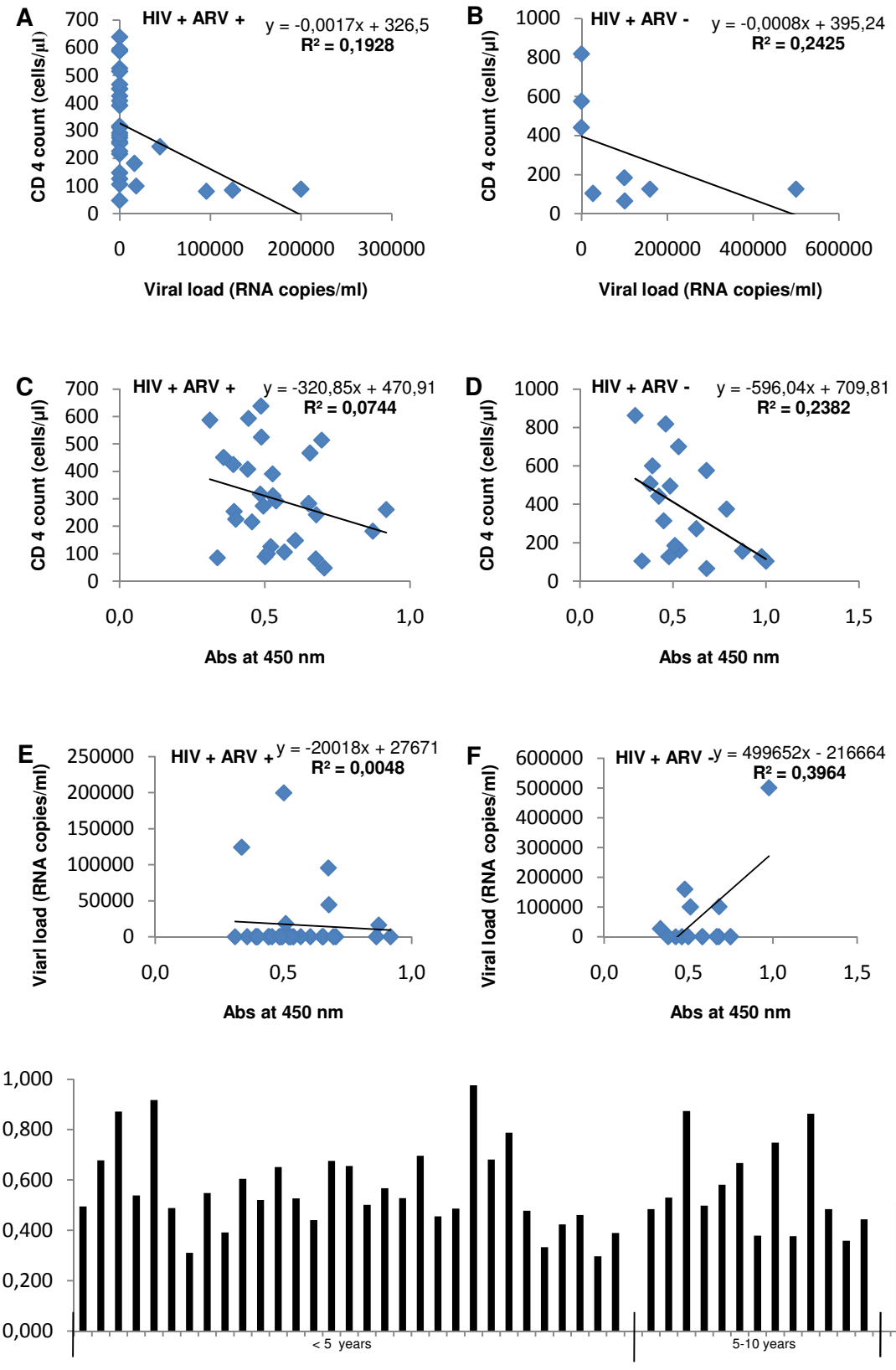


Figure 3.5. No correlation between CD4 cell count vs. viral load (A and B), CD4 cell count vs. Abs at 450 nm (C and D) and viral load vs. Abs at 450 nm (E and F) for HIV+ samples (with and without treatment) was obtained (samples were tested using the "in-house" ELISA). R^2 = 1 indicates a 100% correlation. No increase of the presence of R7V antibodies with the duration of HIV-infection (G).

Statistical analysis of the R7V antibodies detected by the "in-house" ELISA

One-way ANOVA was performed to analyze the statistical significant differences between sample groups analyzed in the "in-house" ELISA (data shown in Figure 3.3). The mean difference from the post hoc test (Figure 3.6 A) was declared as significant at the 0.05 level. Figures 3.6 A and B (demonstrating the mean values) show that there was a significant statistical difference between HIV-negative and HIV-positive samples. However, no significant statistical differences were observed between ARV treated samples, treatment naïve samples and LTNPs.

A

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.=P-value	95% Confidence Interval	
					Lower Bound	Upper Bound
HIVNeg	HIV+ARV	-.273281*	,046873	,000001	-,40036	-,14621
	HIV+No ARV	-.274535*	,052599	,000010	-,41713	-,13194
	LTNP	-.262729*	,058461	,000152	-,42122	-,10424
HIV+ARV	HIVNeg	.273281*	,046873	,000001	,14621	,40036
	HIV+No ARV	-,001253	,045104	1,000000	-,12353	,12102
	LTNP	,010552	,051820	1,000000	-,12993	,15104
HIV+No ARV	HIVNeg	.274535*	,052599	,000010	,13194	,41713
	HIV+ARV	,001253	,045104	1,000000	-,12102	,12353
	LTNP	,011806	,057052	1,000000	-,14286	,16648
LTNP	HIVNeg	.262729*	,058461	,000152	,10424	,42122
	HIV+ARV	-,010552	,051820	1,000000	-,15104	,12993
	HIV+No ARV	-,011806	,057052	1,000000	-,16648	,14286

B

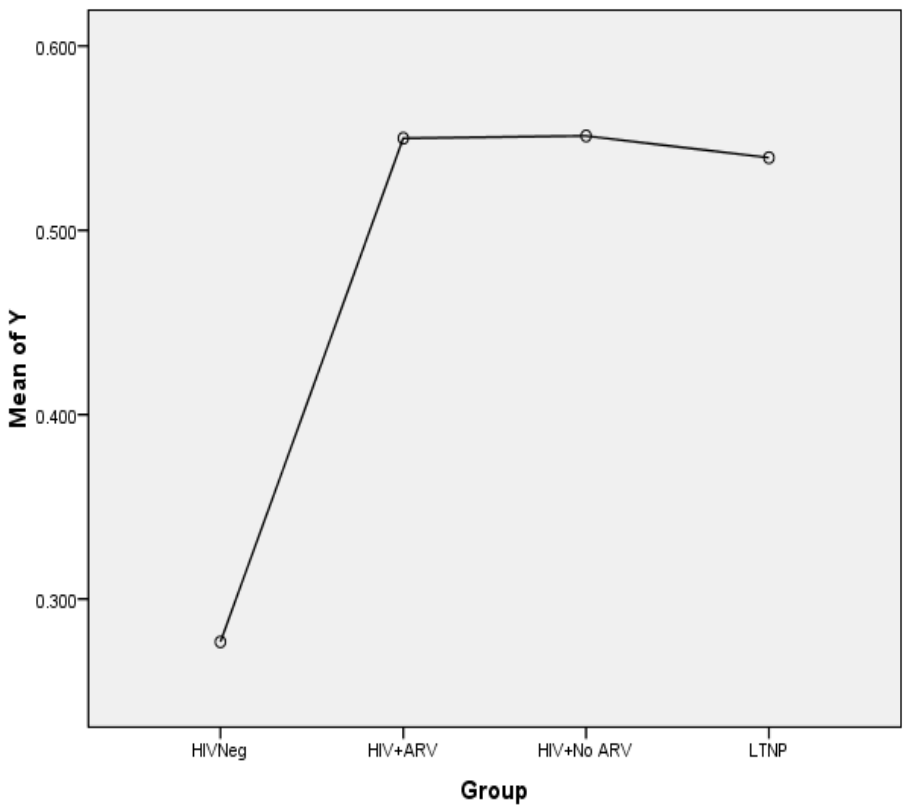


Figure 12. One-way ANOVA test analyzing the statistical significant differences between the sample groups: uninfected (HIV Neg), infected ARV treated (HIV + ARV), infected treatment naïve (HIV + No ARV) and LTNPs. The figure shows the results from the post hoc test demonstrating (A) the significant *p* values and (B) the mean values for each sample group. The mean difference was significant at the 0.05 level. Significant statistical difference was observed between HIV-negative and HIV-positive samples. No significant difference was observed between the infected ARV treated and treatment naïve samples or LTNPs.

3.2.2 "In-house" ELISA protocol improvement

In order to investigate whether immune complexes and other IgG interfered with the binding between the synthetic R7V peptide and R7V antibodies in the "in-house" ELISA, the immune complexes and IgG were precipitated from the human serum by using 8% PEG. The supernatant was then analysed for the presence of R7V antibodies in the "in-house" ELISA. Immune complexes were removed from two HIV-negative and 4 HIV-1-infected samples. Aliquots of the same samples not exposed to precipitation were used for comparison. In all cases, removal of the immune complexes from the serum did not cause an increase in OD of the R7V antibodies (Figure 3.7). The OD values for the HIV-negative samples used (22 FOH and negative control number 22) was much higher than that determined for 16 other HIV-negative samples (Figure 3.3) determined using the "in-house" ELISA. Longer incubation times, different assay conditions and sample storage are believed to be the reasons for this. As no other times did any negative control samples give such high absorbances again.

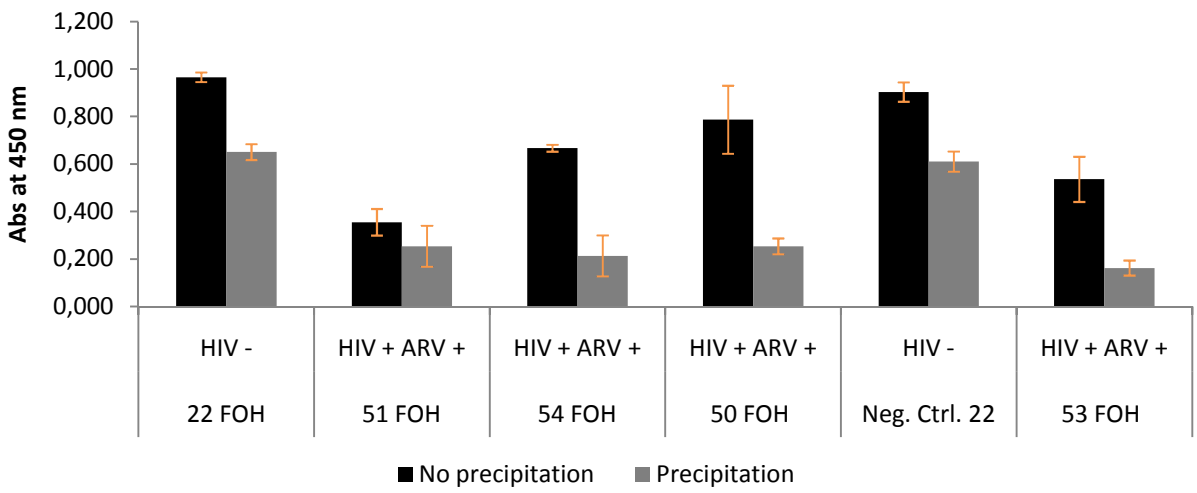


Figure 3.7. Precipitation assay with human serum. Samples with precipitation and without precipitation of immune complexes and IgG analyzed for the presence of R7V antibodies in the "in-house" ELISA. HIV and ARV status are indicated in the figure. Precipitation of immune complexes did not enhance an increase in OD of the R7V antibodies in all cases. The experiment was repeated twice giving the same pattern.

3.2.3 Production of the recombinant antibody fragments

Recombinant single chain R7V antibody fragments that consisted of only the binding domains of the heavy and light domains of Fab and based on a synthetic R7V sequence were made by means of recombinant DNA technology and M13 phage display (Section 2.4.4). Six recombinant R7V antibody fragment clones (recombinant R7V antibody

fragment clones 2, 4, 6, 7, 8-1 and 8-2) were produced by Dr. Mervyn Beukes, Department of Biochemistry, University of Pretoria.

A Micro BCA™ Protein Assay Kit was used for quantitation of total protein in the recombinant R7V antibody fragment clones (Table 3.1). The recombinant R7V antibodies were aliquoted (50 µl) and stored at -20°C.

These antibodies were designed to serve as positive controls in the "in-house" ELISA and were eventually also screened for neutralizing ability because they are R7V-specific antibodies.

Table 3.1. Total protein concentration in the recombinant R7V antibody fragment solutions.

Recombinant R7V antibody clone	Total protein concentration [µg/ml]
2	210.0
4	321.0
6	267.0
7	256.0
8-1	374.3
8-2	208.0

3.2.4 Stability of the positive control

The recombinant R7V antibody fragments served as positive controls for the "in-house" ELISA. Six clones (recombinant R7V antibody fragment clones 2, 4, 6, 7, 8-1 and 8-2) were tested in the ELISA (Figure 3.3). In addition, the antibody fragments were tested for stability in a time study as well as following storage in a fridge or freezer. Figure 3.8 shows a decrease in the stability of clones 2 and 8-1 at various dilutions over time. In addition, clone 7 was degraded when stored in the fridge rather than the freezer over a time period of 5 days.

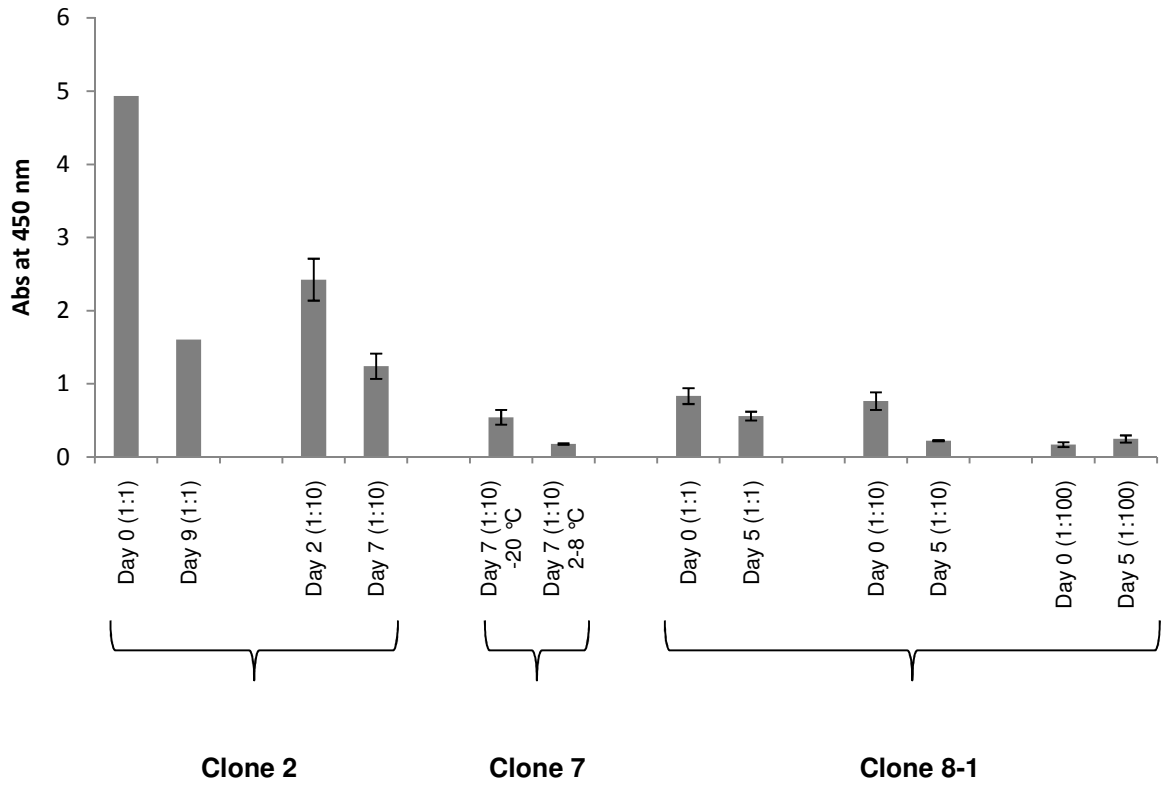


Figure 3.8. Stability analysis of various dilutions of recombinant R7V antibody fragment clone 2, 7 and 8-1 using the "in-house" ELISA. Clone 2 and 8-1 were degraded over time (when stored in the fridge, except the 1:1 dilution of clone 2 tested which was stored in the freezer). Clone 7 was degraded over time when stored in the fridge vs. freezer for a period of 5 days.

3.2.5 Additional samples analyzed using the "in-house" ELISA

(i) Archived HIV-1 neutralizing antibodies – R7V antibody levels

As mentioned in the literature review, true R7V antibodies have HIV-1 neutralizing abilities. For that reason, archived sera (Philippeos, 2007) and plasma (NICD) with both proven neutralizing antibodies were analyzed for R7V content using the "in-house" ELISA (Figure 3.9). Five of twelve samples had OD values of 0.3 or lower which as explained before are considered negative for R7V antibodies. Seven samples had OD values slightly above 0.3 and were still considered not responsive for R7V antibodies. Screening positive R7V antibodies for neutralizing ability is reported on in Section 3.4.

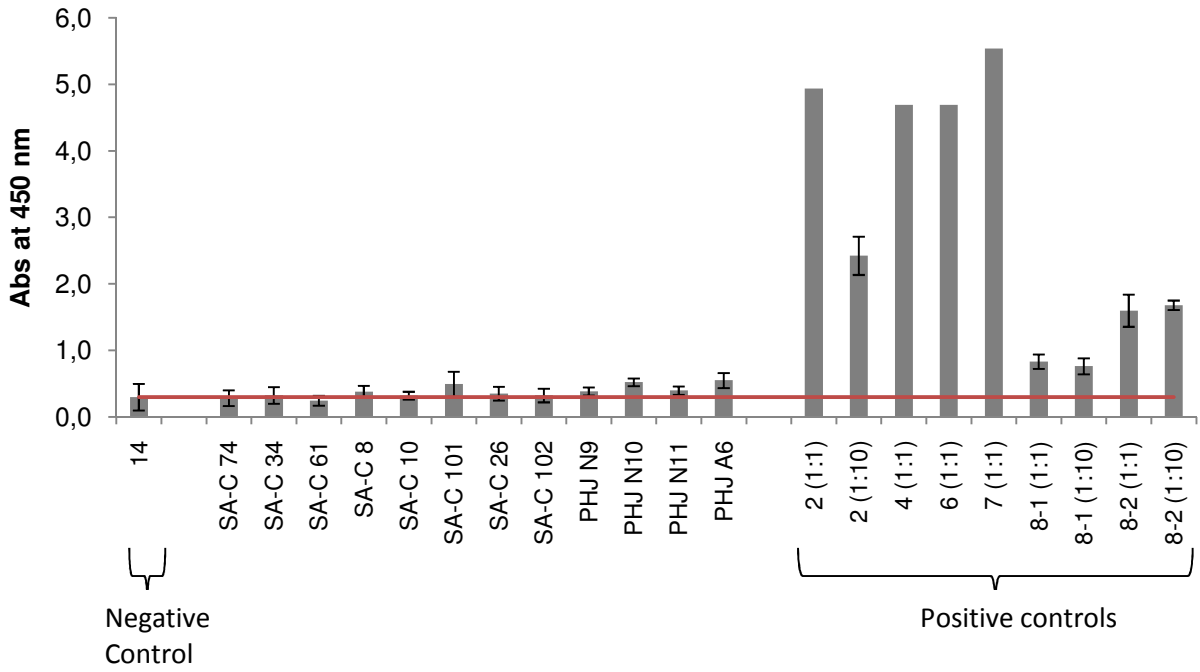


Figure 3.9. Archived sera and plasma samples were screened for R7V antibody prevalence. SA-C plasma (from NICD) and PHJ sera (Philippeos 2007) have strong HIV-1 subtype C (Du151) neutralizing capacity. The PHJ samples are samples that strongly respond to peptides from the gp41 MPER. All SA-C and PHJ samples were from ARV treatment naïve patients except for PHJ A6. The negative control is uninfected serum and the positive controls are recombinant R7V antibody fragments with different dilutions indicated. The red line indicates the cut-off value for determining positivity. Samples with OD value above 0.3 are considered positive for R7V antibodies. Samples with OD value below or equal to 0.3 are considered negative for R7V antibodies. All samples containing strong neutralizing antibodies did not respond with a strong R7V antibody response.

(ii) Beta 2 -microglobulin

Because β2m is the parent protein from which R7V is derived, this protein (purchased from ProSpec-Tany TechnoGene) was used as antigen in an ELISA and screened against β2m antibodies (purchased from BioLegend) and HIV-infected serum samples. In addition β2m antibodies were also screened for the ability to respond to R7V as antigen. These results are provided in Figure 3.10. The monoclonal β2m antibodies recognized the β2m antigen but not the R7V antigen (probably due to the R7V epitopes not being prominent/visible in the protein used to raise the β2m antibodies, more detail in chapter 4). The β2m antigen was also recognized by HIV-1-positive sera.

The ProSpec-Tany β2m protein was purified from the urine of patients with tubular proteinuria and although the company did not provide the amino acid sequence we assume it to be similar or identical to the published sequence of this protein. Two hundred µg was received and diluted in 400 µl 1x PBS pH 7.4 to a final concentration of 0.5 mg/ml which was stored at 2-8℃ until used.

The anti-human β2m antibodies were raised against purified human β2m. However, according to the company, the protein was purified in 1983 and it is no longer known from exactly what source (urine vs. serum) the protein was isolated. The antibodies were made against β2m with the following amino acid sequence: MSRSVALAVL ALLSLSGLEA IQRTPKIQVY SRHPAENGKS NFLNCYVSGF HPSDIEVDLL KNGERIEKVE HSDLSFSKDW SFYLLYYTEF TPTEKDEYAC RVNHVTLSQP KIVKWDRDM.

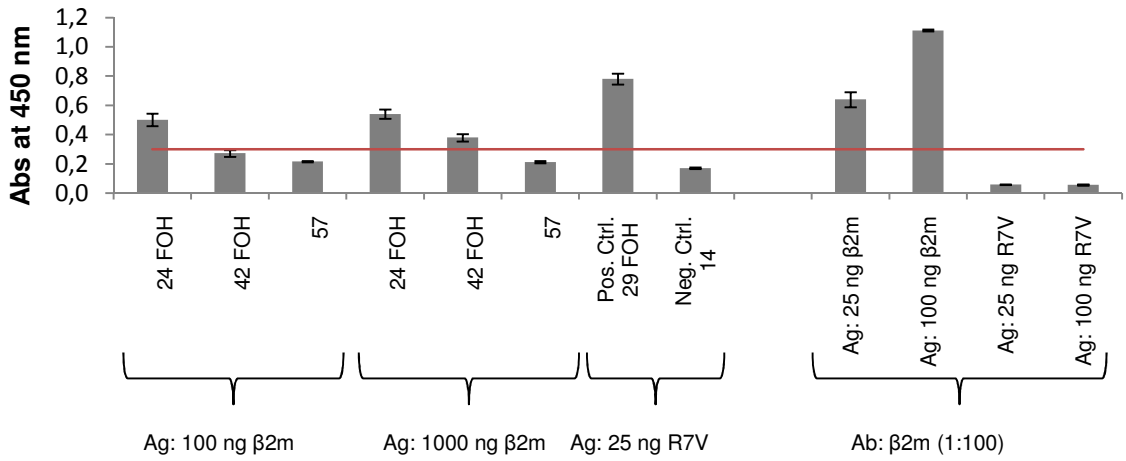


Figure 3.10. Test of interaction between β2m antigen and antibodies, human β2m and heat-inactivated HIV-infected serum (24 FOH ARV treated and 42 FOH treatment naïve) or uninfected serum (57). Also indicated is the response of β2m antibodies and R7V antigen at various concentrations using the "in-house" ELISA. Sample 29 FOH was used as a positive control for the experiment and sample number 14 as a negative control. The red line indicates the cut-off value for determining positivity. Samples with OD value above 0.3 are considered positive for R7V antibodies. Samples with OD value below or equal to 0.3 are considered negative for R7V antibodies. Sample numbers 24 FOH and 42 FOH recognized R7V (Figure 3.3) at OD values >0.3 The β2m antigen was recognized by human sera and β2m antibodies. The β2m antibodies did not respond to the R7V antigen. Even with higher concentrations of peptide tested, e.g. 100 ng, R7V gave an identical response to that indicated above.

3.2.6 R7V antibody prevalence detected by the Ivagen ELISA

Serum and plasma from naturally HIV-1-infected individuals living in South Africa were analyzed for the presence of R7V antibodies using the Anti-R7V ELISA test developed by Ivagen (described in section 2.4.6). Of the 26 samples tested (HIV-1-infected and uninfected samples) none tested positive for R7V antibodies (Figure 3.11). Using Ivagen's criteria, twenty four (92.3%) samples tested negative for R7V antibodies. Two HIV-infected samples (7.7%), one ARV treated and one treatment naïve, tested doubtful for R7V antibodies. Disregarding Ivagen's criteria (e.g. ratio > 1.2 is positive), an

elevated but marginal reaction was observed for the HIV-positive samples. No general trend between samples from individuals utilizing ARV and treatment naïve individuals was observed. Figure 3.11 also shows the seroprevalence of R7V antibodies in 5 HIV-1 infected samples where ARV treatment status was unknown. These were archived plasma samples provided by Lancet Laboratories (Johannesburg) and the response obtained with these samples was similar to that of the serum samples from HIV-1-infected individuals collected for this project. Lancet evaluated the ELISA on behalf of Ivagen and these samples were selected for this purpose.

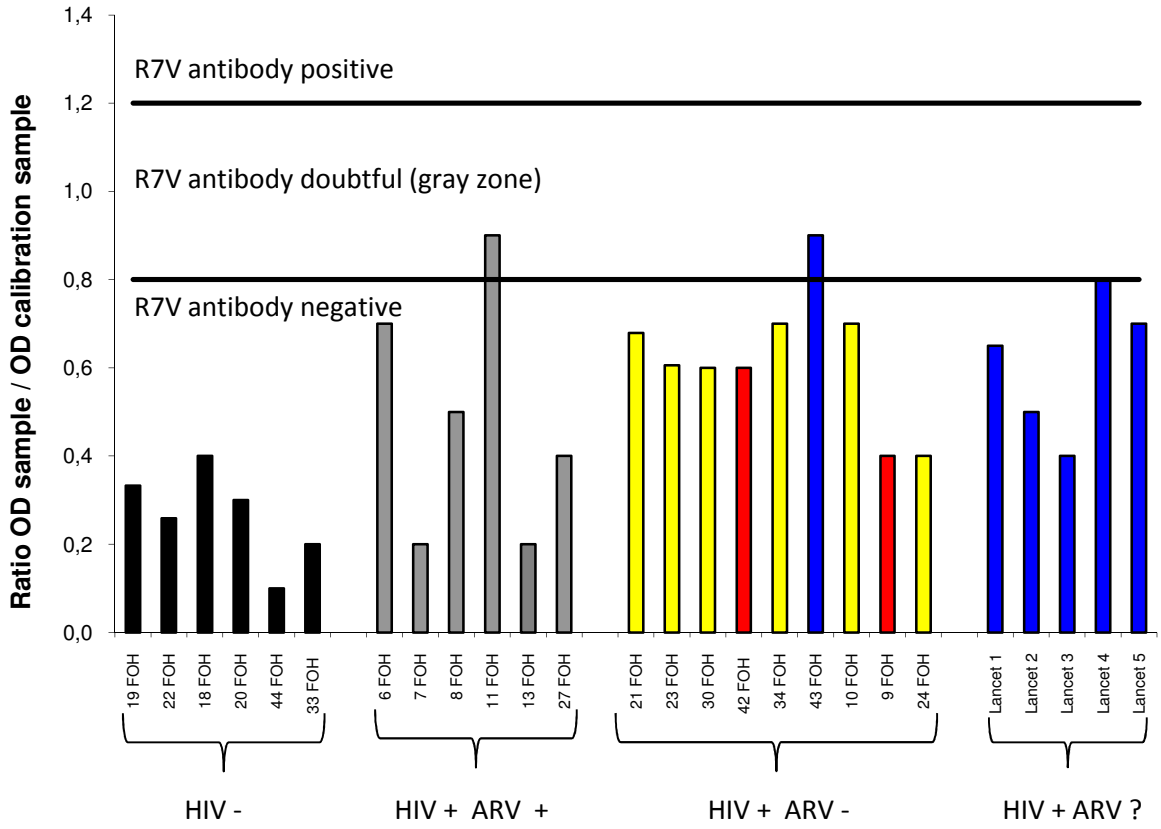


Figure 3.11. Seroprevalence of R7V antibodies as indicated by the Ivagen Anti-R7V ELISA test. Ratio OD sample/OD calibration sample is shown. Sample status is indicated on the x-axis. Colour codes for AIDS (CD4 cell count < 200 cells/µl) status of HIV + samples were as follows: red: no AIDS (CD4 cell count > 200 cells/µl), grey: recovered from AIDS after treatment, and blue: unknown AIDS status. According to Ivagen's criteria: ratio (OD sample / OD calibration sample) < 0.8; R7V antibody negative, ratio 0.8 - 1.2; R7V antibody doubtful and ratio > 1.2; R7V antibody positive. None of the samples tested positive for R7V antibodies.

3.2.7 Comparing the "in-house" and Ivagen ELISAs

Using 4 HIV-infected serum samples analyzed in the Anti-R7V ELISA from Ivagen (Section 2.4.6) and in the "in-house" R7V ELISA (Section 2.4.1) demonstrates a poor correlation between the two assays (Figure 3.12). The obtained R^2 was 0.2727. An R^2 =

0.429 was obtained when using 3 instead of 1 decimals after the point for the OD values (Figure A.4 in the appendix)

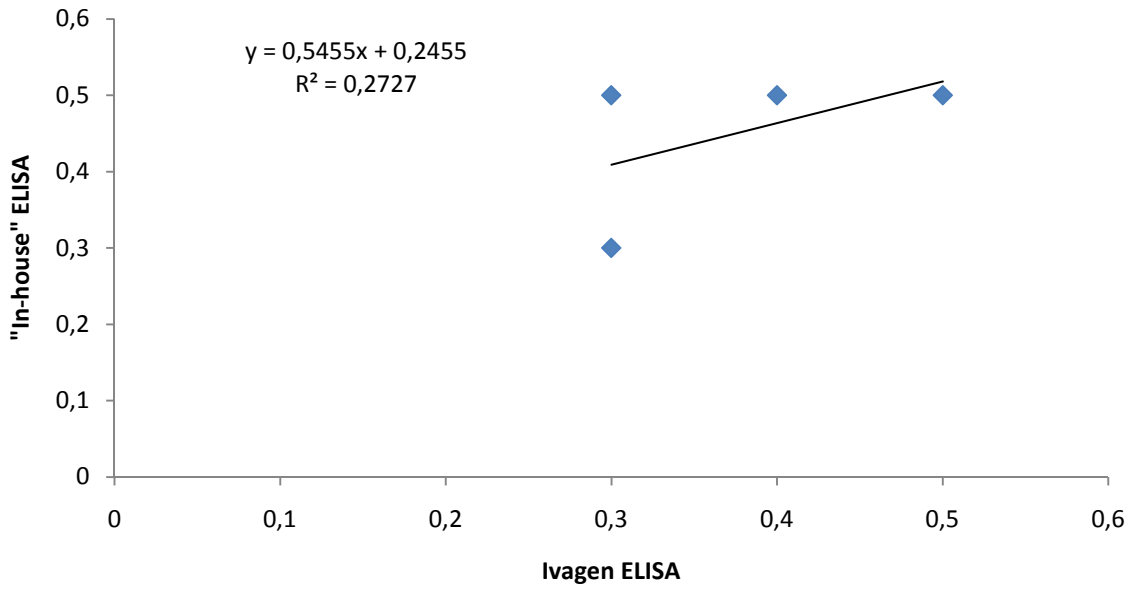


Figure 3.12. The diagram shows average OD values determined for 4 (HIV+) serum samples using the Anti-R7V ELISA test from IVAGEN (OD 450/260) and the "in-house" R7V ELISA (OD 450). Poor correlation (R^2 = 0.2727) between the two ELISA tests was observed. 100% correlation is obtained with an R^2 = 1.

The internal controls from the Ivagen ELISA was used with the company's recommended dilution (1:51) in the "in-house" R7V ELISA. The Ivagen internal controls did not work in the "in-house" ELISA (Figure 3.13). The negative control (an HIV-negative and R7V antibody negative diluted serum sample) should have responded with the lowest OD value but did not. The positive control was an HIV-positive and R7V antibody positive diluted serum sample and should have responded with the highest OD value. The calibrator which was also an HIV-positive and R7V antibody positive diluted serum should have responded with a lower OD value than the positive control and with an OD value > 2.5x OD value of the negative control. The Ivagen controls at 2 additional dilutions (1:20 and 1:100) were also tested in the "in-house" ELISA and the same trend was observed (Figures A.5 and A.6 in the appendix).

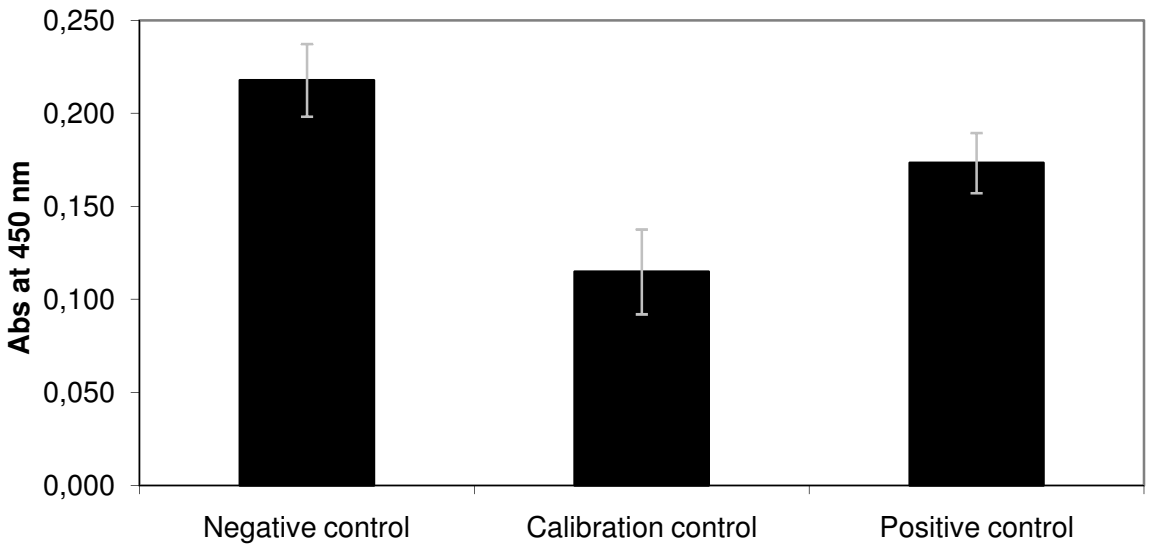


Figure 3.13. Results obtained using the Ivagen internal controls (negative -, calibration - and positive control) in the "in-house" R7V ELISA. The serum dilution used was 1:51. The Ivagen controls did not work in the "in-house" ELISA as the negative control failed to be lower than the calibration – and the positive control.

3.3 POLYCLONAL RABBIT R7V ANTIBODIES

(i) Characterization of the peptide

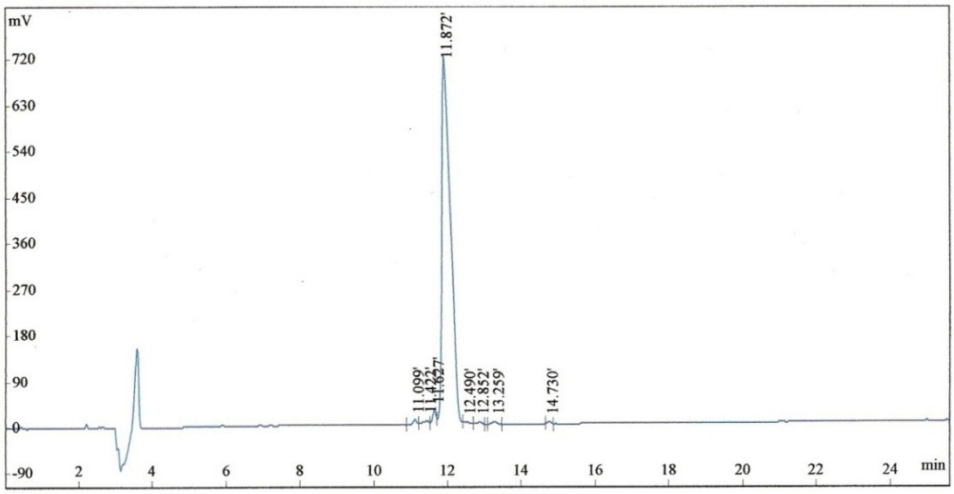
The R7V peptide used for the production of the polyclonal rabbit R7V antibodies was manufactured by Fmoc and tBoc chemistry (LifeTein LCC) and quality controlled by HPLC and by MS. The HPLC results indicated that a relatively pure majority product was obtained from the synthesis (Figure 3.14). Definite resolution was attained in the MS spectra. The observed MW (944.64) for the R7V peptide correlated with the expected theoretical MW 944.16 (Figure 3.15). The theoretical MW value was calculated by using a Peptide property calculator from Innovagen. To be able to conjugate the peptide to KLH, an additional Cys on the N terminus of the peptide was added. This explains why the MW for this peptide was higher than for the Genscript peptide (841.01), which was not used for antibody production and did not have to be conjugated to KLH (Section 2.3.1). A total of 2.5 mg unconjugated R7V peptide (purity 95.85%) was received and stored at -20℃. According to the manufacturer's instructions, 330 µg of the peptide was dissolved in 100 µl 25% acetic acid (Saarchem, Vadeville, South Africa) and 230 µl water was added to obtain a final concentration of 1mg/ml. The peptide solution was stored at 2-8℃.

Sample Description:
Product Name :R7V CV-8
Instrument No. :0200048
Analyst :LDD
Lot No. :P090804-WY163924
Column :4.6*250mn, VYDAC-C18
Solvent A :0.1%Trifluoroacetic in 100% Acetonitrile
Solvent B :0.1%Trifluoroacetic in 100% Water

Gradient :	A	B
0.01min	15%	85%
25min	40%	60%
25.01min	100%	0%
30min	Stop	

Flow rate :1.0ml/min
Wavelength :220nm
Volume :20ul

File opened: E:\WYL\CV-8-163924-F-20090807-021(00053,08;49;55).hw, where



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4	11.872		95.85	11553091	724954	12949
5	12.490		0.4207	50711	4934	34460
6	12.852		0.3727	44921	5158	50813
7	13.259		0.3977	47943	5782	59667
8	14.730		0.2131	25690	4642	165307
Total			100	12053547	790513	

Figure 3.14. The HPLC chromatogram of the synthesized peptide R7V used for production of the polyclonal rabbit R7V antibodies. Substantial purity of the R7V peptide was achieved following synthesis.

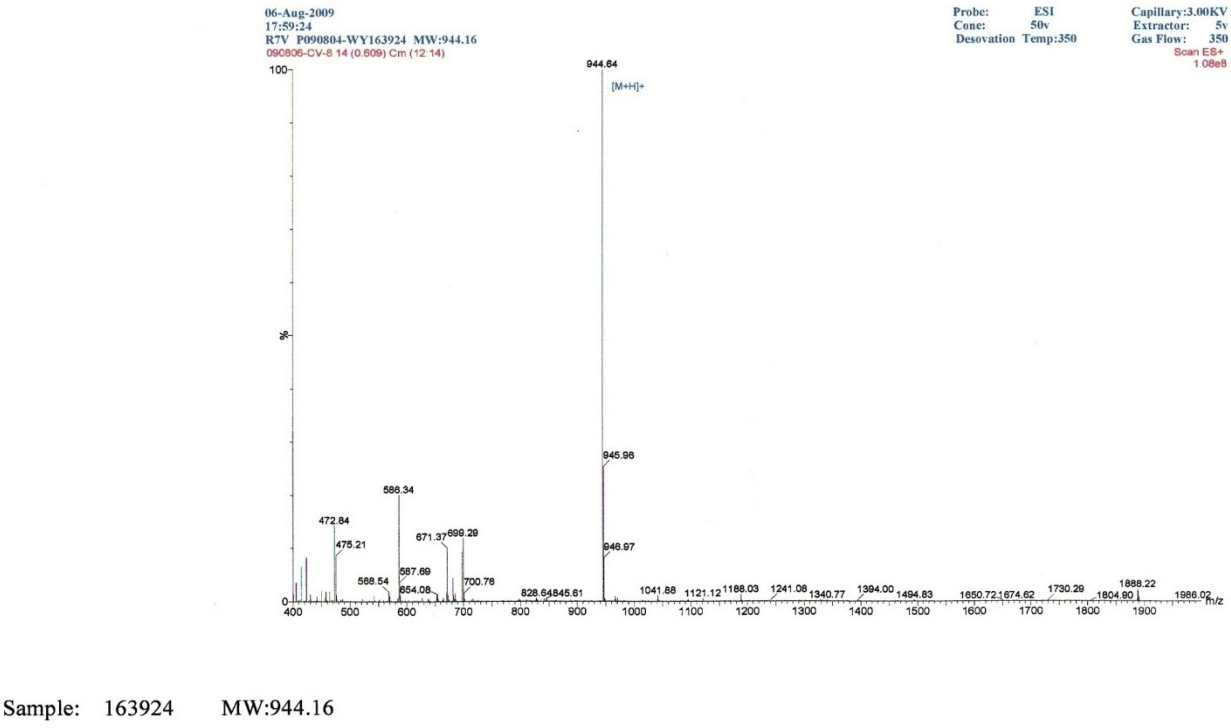


Figure 3.15. The MS spectrum of the synthetic peptide used for production of the polyclonal rabbit R7V antibodies. The [M+H] + peek represents the observed MW (944.64) for the R7V peptide which correlated with the theoretical MW 944.16.

(ii) Antibody production

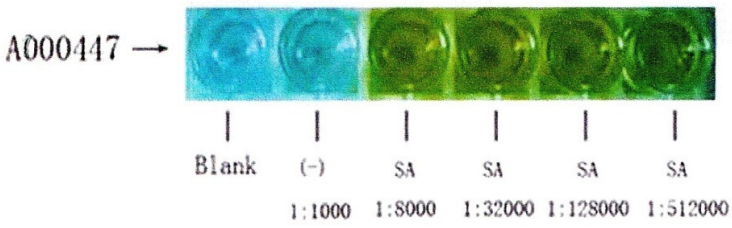
The company prepared the polyclonal rabbit R7V antibodies by using New Zealand White Rabbits. LifeTein's AdjuBooster^{TM} adjuvants were used for the first injection with the KLH conjugated R7V peptide followed by incomplete AdjuBooster TM adjuvants for the subsequent injections. ELISA against the peptide antigen was used to verify the binding between the polyclonal antibodies and the peptide before the antibodies were affinity purified. The LifeTein ELISA data demonstrated high OD value for various titres of the polyclonal rabbit R7V antibodies and showed that the antibodies responded well against the R7V peptide as antigen (Figure 3.16).

Four ml antibodies (0.72 mg/ml) and 0.75 ml pre-bleed from each of the two rabbits (RB269 and RB270) used for the production were received, aliquoted (50 µl) and stored at -20℃.

(iii) Polyclonal R7V antibodies induced in rabbits - ELISA

The polyclonal rabbit R7V antibodies were tested in the "in-house" R7V ELISA at various dilutions (1:8000, 1:32000 and 1:128000) using the R7V peptide that was used to make the antibodies (antigen and antibody made by LifeTein LCC), as well as the

other synthetic R7V peptide synthesized for this study (Genscript Corporation) as antigen. Other antigens used were β2m and BSA at 25 ng and 50 ng. In addition, negative rabbit serum (pre-bleed) at various dilutions (1:1000, 1:8000, 1:32000 and 1:128000) was tested against the respective antigens. Figure 3.17 shows that the polyclonal antibodies recognized the peptides produced by both sources. The rabbit antibodies showed no response to both β2m and BSA and with the same level of response as R7V negative rabbit serum. Figure 3.16 shows the ELISA data obtained by LifeTein and Figure 3.18 shows a comparison of the obtained ELISA results by LifeTein and the "in-house" ELISA for the rabbit antibodies using the R7V peptide.



Product ID	Blank	(-) 1:1,000	SA 1:8,000	SA 1:32,000	SA 1:128,000	SA 1:512,000
A000447	0.042	0.046	2.625	2.348	1.887	1.489

Figure 3.16. The figure shows the OD observed in an ELISA performed by LifeTein LCC with various titres of the polyclonal R7V antibodies. The blank was blocked with BSA and no peptide. SA stands for antigen (peptide) affinity purified antibody. The pre-bleed is indicated with (-). The polyclonal antibodies responded well against the R7V peptide in the ELISA.

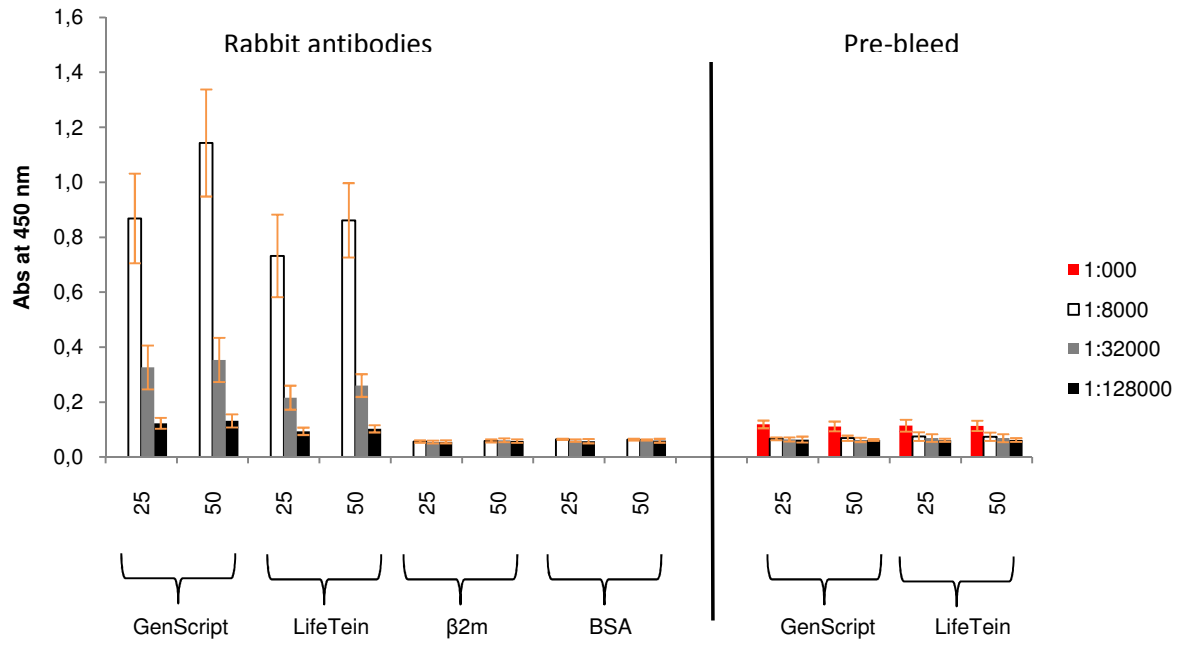


Figure 3.17. Response of polyclonal rabbit R7V antibodies and negative rabbit serum (pre-bleed) to the synthetic R7V peptide used for the production of these antibodies (LifeTein LCC), the R7V peptide from GenScript Corporation, human β2m and BSA at various concentrations of antigen (ng) and antibodies (indicated at the far right). Results obtained using the "in-house" ELISA. The polyclonal rabbit R7V antibodies recognized both R7V peptides. The rabbit antibodies showed no response to both β2m and BSA and with the same response as the pre-bleed.

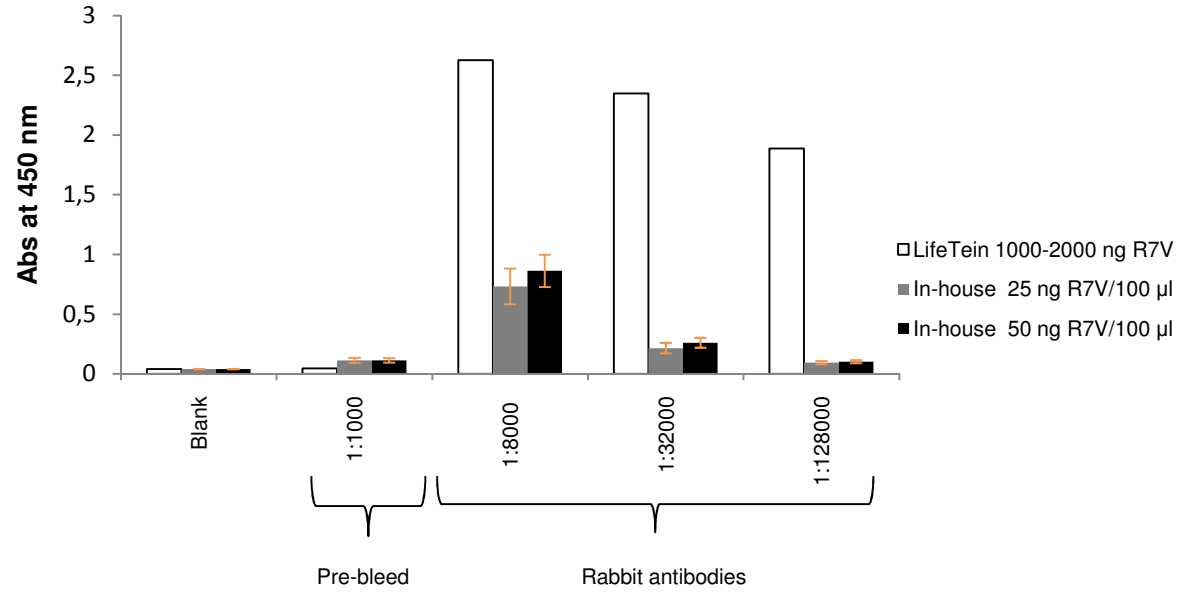


Figure 3.18. ELISA results obtained by LifeTein and the "in-house" ELISA by testing the rabbit R7V antibodies at various dilutions and pre-bleed using the LifeTein R7V peptide. The amount R7V antigen used in the tests is indicated in the figure. The blank was blocking buffer and no peptide. The antibodies responded well in both ELISAs.

3.4 EVALUATION OF VIRUS NEUTRALIZING ABILITY OF R7V ANTIBODIES

The following samples were analyzed for neutralizing antibodies against HIV Env-pseudovirus by measuring a reduction in luciferase gene expression after a single round of infection of TZM-bl cells (Montefiori 2004): serum from 3 naturally HIV-1-infected humans (ARV treatment naïve) that recognized the synthetic peptide R7V in ELISA (Figure 3.3) and three different clones (recombinant R7V antibody fragment clones 2, 8-1 and 8-2) of a recombinant single chain R7V antibody fragment. In addition, affinity purified polyclonal R7V antibodies from immunized rabbits were tested for neutralizing ability. Human sera were screened against a viral isolate consisting of two HIV-1 subtype C stains (referred to as Du151.2). The recombinant R7V antibody fragments were measured against three viruses: Du151.2, a sensitive HIV-1 subtype B strain (referred to as SF162) and a vesicular stomatitis virus (referred to as VSV-G). Polyclonal rabbit R7V antibodies were tested against Du151.2. Neutralizing antibody titres were calculated as the inhibitor concentrations (IC50) or reciprocal serum dilutions (ID50) causing a 50% reduction of RLU. Titres below IC50 indicate no neutralizing ability. A typical picture of a dose response curve obtained with positive controls (samples with known neutralizing ability against Du151) is shown in Figure 3.19. A summary of all data obtained is shown in Figures 3.20, 3.21 and 3.22. All HIV-positive human sera and the three positive control sera tested positive for the ability to neutralize the virus at various titres as the percent inhibition was above IC50 (Figure 3.20). No neutralizing ability against virus Du151.2 was detected for either dilution of the recombinant R7V antibody fragment clones, as titres were scattered with no dose response curve and the percent inhibition mostly below IC50 (Figure 3.21). The recombinant antibody fragments are indicated as 2, 8-1 and 8-2 which are the different clones from which the scFvs were derived. Recombinant R7V antibody fragment clone 2 was able to neutralize the sensitive HIV-1 subtype B virus (IC above 50), but a dose response curve was absent (Figure 3.21). The same clone did not respond to the vesicular stomatitis virus (VSV-G), Figure 3.21 (which would have been an indication of non specific neutralization). The lack of a dose response curve may be related to the fact that the recombinant antibodies are fragments and not whole antibodies. For all whole antibodies we were always able to see a dose response curve. The polyclonal rabbit R7V antibodies were not able to neutralize Du151.2 at any of the tested concentrations (Figure 3.22).

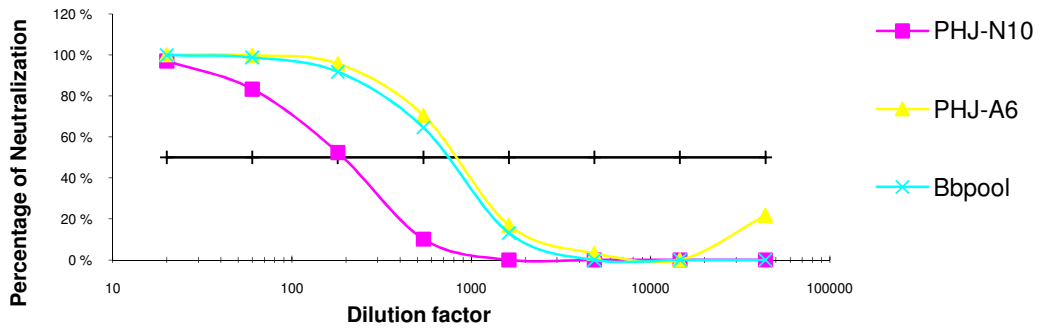


Figure 3.19. A typical picture of a dose response curve obtained for positive controls. The samples were tested for neutralizing ability against the viral isolate Du151.2 which consisted of two HIV-1 subtype C strains. Positive control sera used were the following: control sera from naturally HIV-1 infected individuals previously tested positive for neutralizing antibodies (PHJ-A6 on HAART treatment and PHJ-N10 treatment naïve) and an internal positive control (Bbpool) with known neutralizing ability. The start dilutions of the samples were 1:20 with further three-fold dilutions. The line of 50% represents the IC50. Titres below IC50 indicate no neutralization ability. All samples neutralized the virus at various titres.

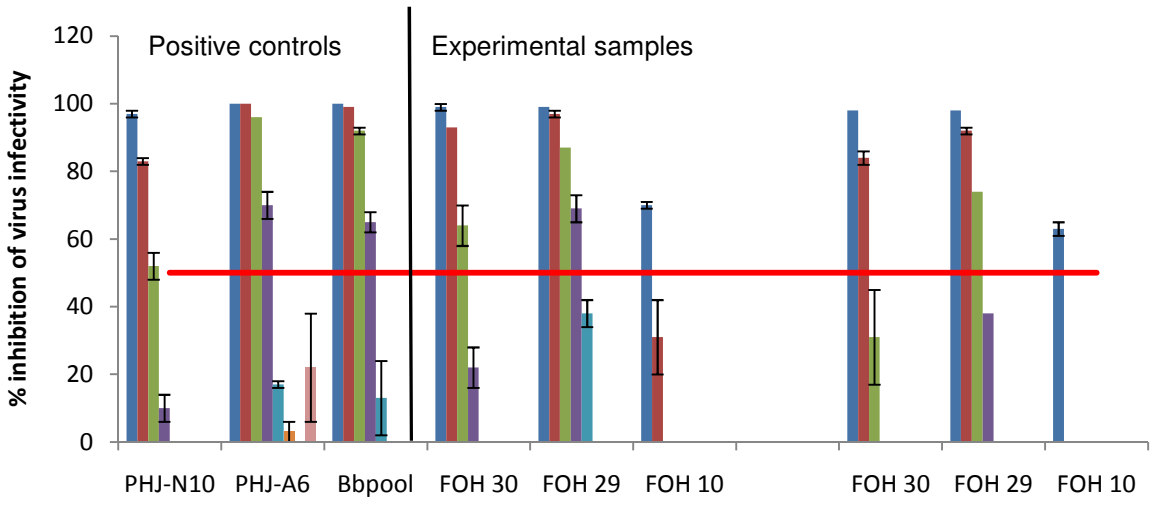


Figure 3.20. Neutralization ability of serum from naturally HIV-1 infected ARV treatment naïve patients (FOH 10, FOH, 29 and FOH 30). Positive control samples were PHJ-N10, PHJ-A6 and Bbpool with known neutralizing ability. The start dilutions of the samples were 1:20 with further three-fold dilutions. The samples were tested for neutralizing ability against the viral isolate Du151.2. The red line represents the IC50. No neutralization ability is obtained if the % inhibition is below IC50. The serum samples neutralized the virus at various titres.

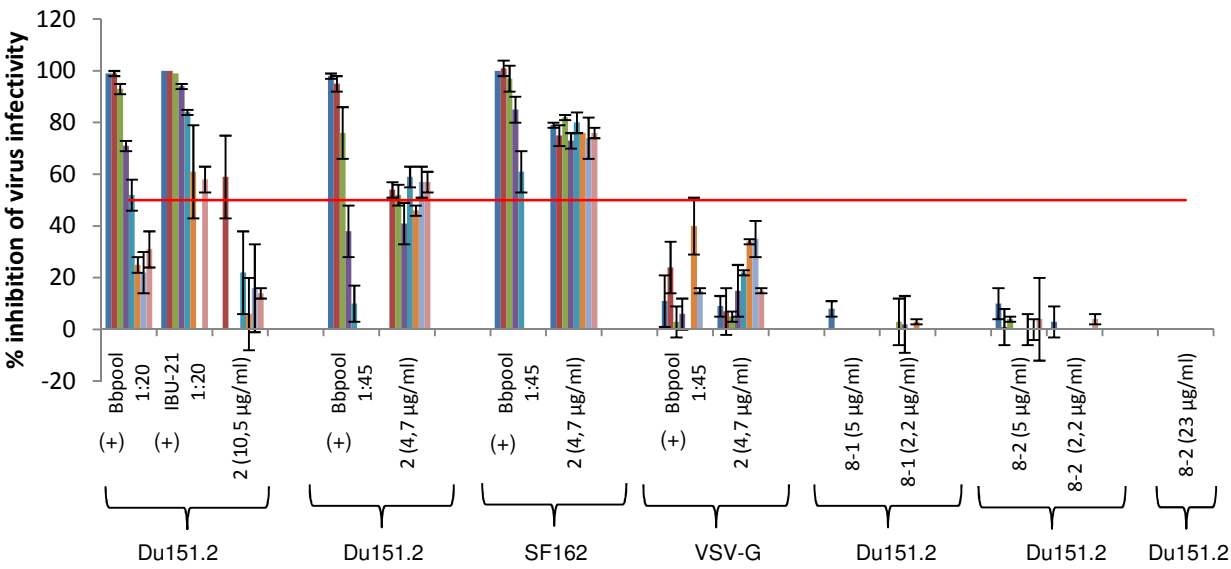


Figure 3.21. Neutralization ability of recombinant single chain R7V antibody fragments that consisted of only the binding domains of the heavy and light domains of Fab based on the R7V sequence (recombinant R7V antibody fragment clone 2, 8-1 and -8-2). The start dilution of each sample is indicated in the graph which was followed by three-fold dilutions. Internal positive control samples were Bbpool and IBU-21 with known neutralizing ability. The samples were tested for neutralizing ability against the viral isolates; Du151.2, SF162 (sensitive HIV-1 subtype B) and VSV-G (vesicular stomatitis virus). The figure shows results from 7 individual tests. The line of 50% represents the IC50. Titres below IC50 indicate no neutralization ability. No neutralizing ability of Du151.2 was detected for either dilution of the different recombinant R7V antibody fragment clones. Clone 2 did not respond to VSV-G and was able to neutralize SF162.

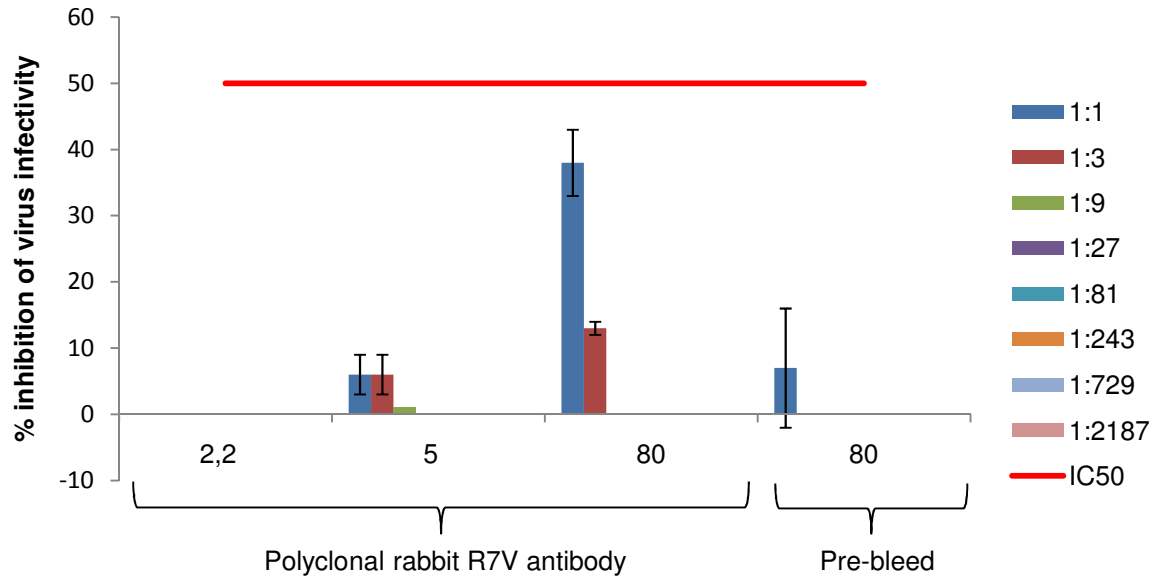


Figure 3.22. Neutralization ability of polyclonal R7V antibodies from immunized rabbits. Start dilution (µg/ml) of each sample is indicated in the graph with further three-fold dilutions. The antibodies were tested for neutralizing ability against the viral isolate Du151.2. Titres below IC50 indicate no neutralization ability. The rabbit R7V antibodies did not neutralize Du151.2 at any of the different concentrations.

3.5 CELL PROLIFERATION AND R7V

HIV-infected cells routinely proliferate when exposed to dominant viral antigens in peptide form. This has been demonstrated for envelope and core protein peptides. To determine whether R7V is a dominant epitope in HIV-infection, the peptide was used in proliferation assays as described previously.

Cellular division induced by the synthetic R7V peptide or its host protein, human β2m, was investigated by labelling isolated PBMCs from naturally HIV-1 infected and uninfected individuals with the fluorescent cell proliferation dye CFDA-SE (Section 2.7.1). The decreased CFSE fluorescent signal following cell division was measured using flow cytometry. Labelled cells were incubated with various concentrations of the synthetic R7V peptide (Figures 3.23 and 3.24) or the human β2m protein for 3 and 6 days (Figures 3.25 and 3.26). Cells stimulated with the mitogen PHA-P served as positive controls and cells naïve of stimulation served as negative controls. Proliferation of cells (infected and uninfected) incubated with their respective antigen (R7V or β2m) exhibited similar levels of stimulation when compared to the negative control, and no antigen-specific proliferation was observed for any of the antigens at the various antigen concentrations and incubation days. The Proliferation Index (PI), indicating the average number of divisions a cell from the original population (parent) has undergone, for each sample stimulated with R7V or β2m was 0 or 1. Some background or proliferation of unstimulated cells and some unstained cells were observed for some of the samples. Figures 3.23 and 3.25 are representative graphs while Figures 3.24 and 3.26 show the average responses of all samples analyzed.

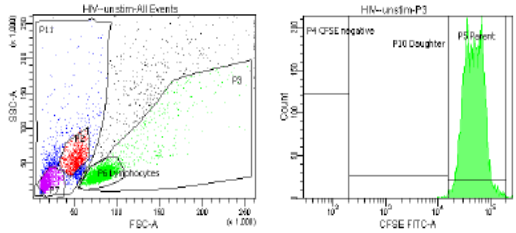
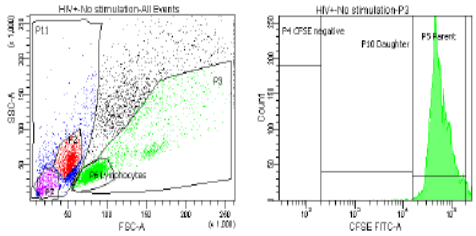
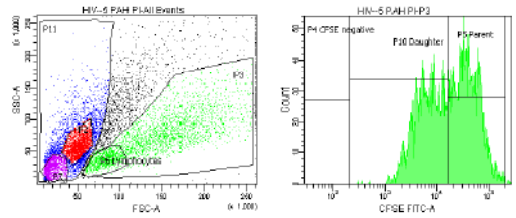
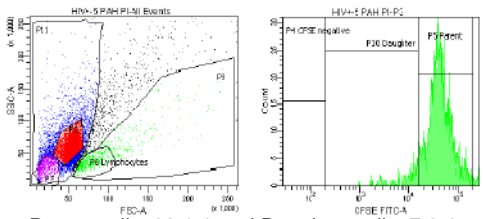
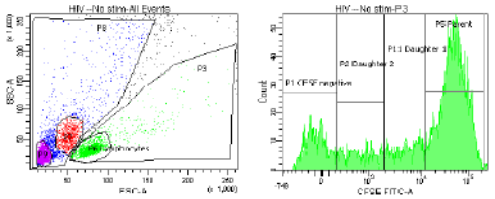
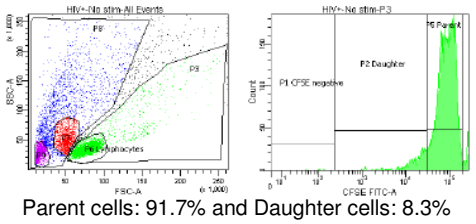
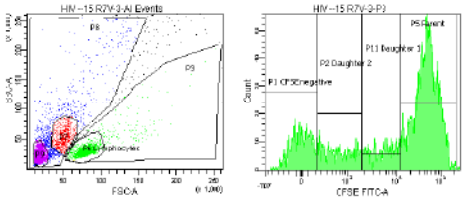
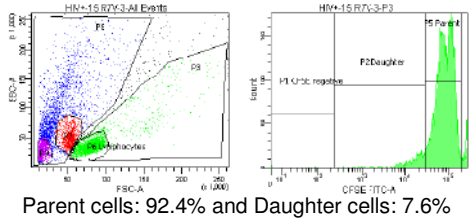
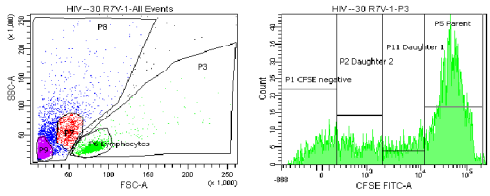
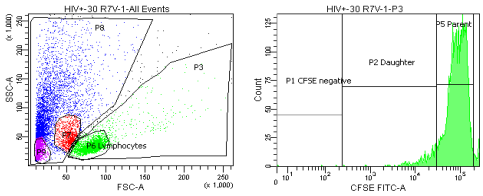
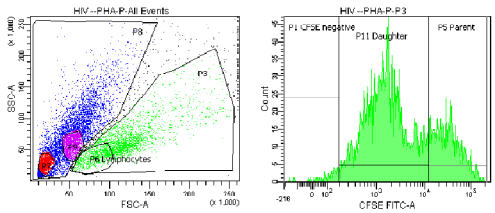
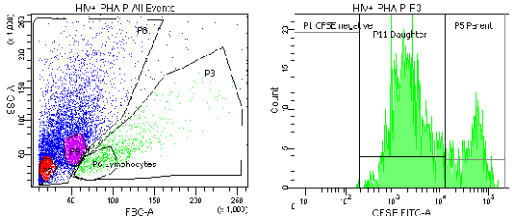
	(i) HIV -	(ii) HIV +
(A) No stimulation	 Parent cells: 99.7% and Daughter cells: 0.3% Sample: 14 PAH (HIV -)	 Parent cells: 99.7% and Daughter cells: 0.3% Sample: 13 PAH (HIV + ARV -)
(B) 5 µg/ml PHA-P 3 days	 Parent cells: 54.6% and Daughter cells: 45.4% Sample: 14 PAH (HIV -)	 Parent cells: 92.1% and Daughter cells: 7.9% Sample: 13 PAH (HIV + ARV -)
(C-1) *No stimulation 3 days	 Parent cells: 72.4% and Daughter cells: 27.6% Sample: 20 PAH (HIV -)	 Parent cells: 91.7% and Daughter cells: 8.3% Sample: 21 PAH (HIV + ARV -)
(C-2) *15 µg/ml R7V 3 days	 Parent cells: 70.7% and Daughter cells: 29.3% Sample: 20 PAH (HIV -)	 Parent cells: 92.4% and Daughter cells: 7.6% Sample: 21 PAH (HIV + ARV -)
(C-3) *30 µg/ml R7V 3 days	 Parent cells: 67.8% and Daughter cells: 32.2% Sample: 20 PAH (HIV -)	 Parent cells: 92.6% and Daughter cells: 7.4% Sample: 21 PAH (HIV + ARV -)
(D) 5 µg/ml PHA-P 6 days	 Parent cells: 25.7% and Daughter cells: 74.3% Sample: 20 PAH (HIV -)	 Parent cells: 25.0% and Daughter cells: 75.0% Sample: 21 PAH (HIV + ARV -)

Figure 3.23 continues.

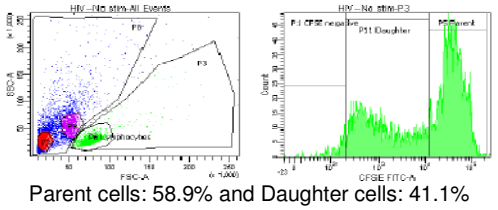
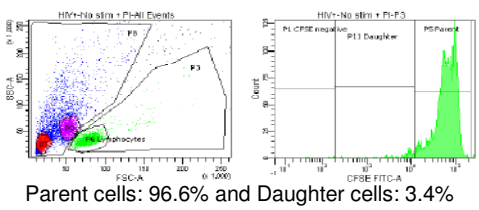
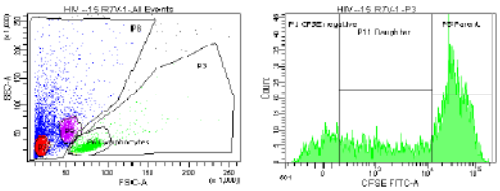
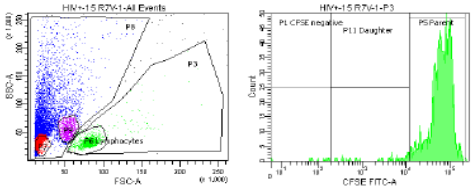
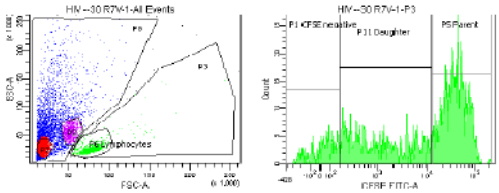
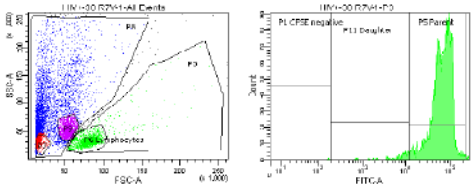
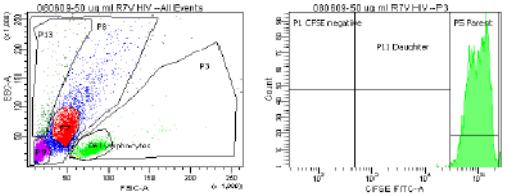
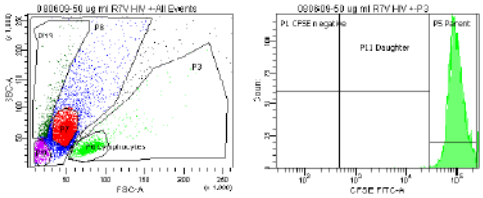
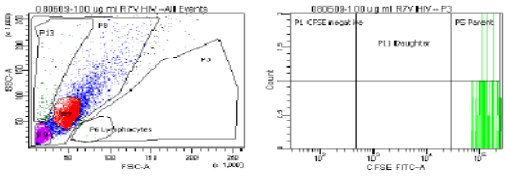
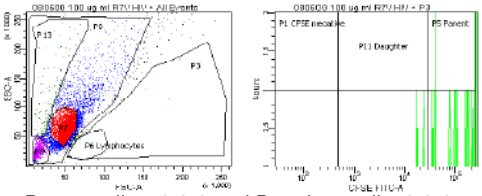
	HIV -	HIV +
(E-1) ****No stimulation** **6 days**	 Parent cells: 58.9% and Daughter cells: 41.1% Sample: 20 PAH (HIV -)	 Parent cells: 96.6% and Daughter cells: 3.4% Sample: 21 PAH (HIV + ARV -)
(E-2) ****15 µg/ml R7V** **6 days**	 Parent cells: 64.9% and Daughter cells: 35.1% Sample: 20 PAH (HIV -)	 Parent cells: 96.7% and Daughter cells: 3.3% Sample: 21 PAH (HIV + ARV -)
(E-3) ****30 µg/ml R7V** **6 days**	 Parent cells: 60.0% and Daughter cells: 40.0% Sample: 20 PAH (HIV -)	 Parent cells: 97.0% and Daughter cells: 3.0% Sample: 21 PAH (HIV + ARV -)
(F) **50 µg/ml R7V** **6 days**	 Parent cells: 99.8% and Daughter cells: 0.2% Sample: 14 PAH (HIV -)	 Parent cells: 99.7% and Daughter cells: 0.3% Sample: 13 PAH (HIV + ARV -)
(G) **100 µg/ml R7V** **6 days**	 Parent cells: ~0.0% and Daughter cells: 0.0% Sample: 14 PAH (HIV -)	 Parent cells: ~0.0% and Daughter cells: 0.0% Sample: 13 PAH (HIV + ARV -)

Figure 3.23. Flow cytometric analysis of human PBMCs stimulated with the synthetic peptide R7V from (i) naturally HIV-1 infected and (ii) uninfected individuals. The forward/side scatter (FSC/SSC) dot plots show the lymphocyte population and the FITC/count histograms show the gated lymphocyte population from the FSC/SSC dot plots for each sample (CFSE detected in FITC detector). Each image shows a representation of each R7V concentration and incubation time. Ten thousand total events were recorded per sample. A) No stimulation. B) 5 µg/ml PHA-P stimulation (3 days). C-1, 2, 3) No stimulation, 15 and 30 µg/ml R7V stimulation (3 days) respectively. D) 5 µg/ml PHA-P stimulation (6 days). E-1, 2, 3) No stimulation and 15 and 30 µg/ml R7V stimulation (6 days) respectively. F) 50 µg/ml R7V stimulation (6 days). G) 100 µg/ml R7V stimulation (6 days). Fluorescent counts in the gated parent generation represent the number of naïve (undivided) cells. Fluorescent counts in the gated daughter generation represent the number of divided cells. The gate representing CFSE negative cells are also indicated. Cells stimulated with the mitogen proliferated as expected. Cells stimulated with the antigen, R7V, did not proliferate. *These samples are from the same experiment. Because some background was observed, the unstimulated cells are also shown to compare these cells with the R7V-treated cells. ** These samples are also from the same experiment and the unstimulated cells are shown because of the background observed so one can better compare with the R7V-stimulated cells.

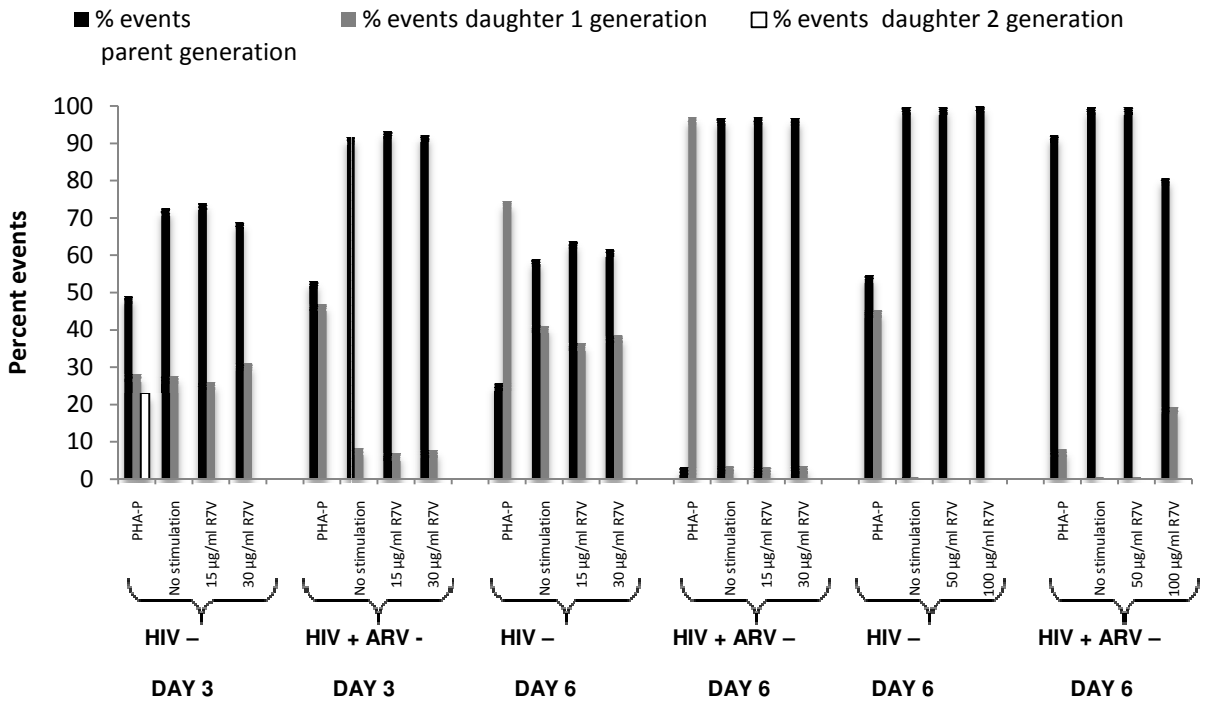


Figure 3.24. Flow cytometric analysis of human PBMCs from uninfected (HIV -) and HIV-1 infected ARV treatment naïve (HIV + ARV -) individuals stimulated with 15, 30, 50 or 100 µg/ml R7V (for 3 and/or 6 days). The figure shows percent events of undivided cells (parent) and divided (daughter) cells. Daughter 1 generation indicates cells divided once. Daughter 2 generation indicates cells divided twice. The data presented represents the average of triplicates. The mitogen, PHA-P, stimulated cell proliferation. Cells naïve of stimulation or stimulated with R7V showed the same low amount or no cell proliferation. PI, indicating the average number of division a cell from the original population (parent) has undergone, for each sample stimulated with R7V was 0 or 1.

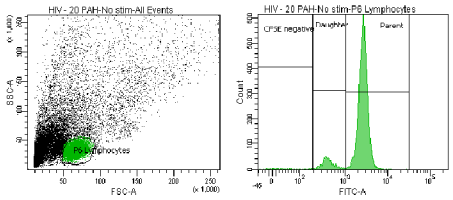
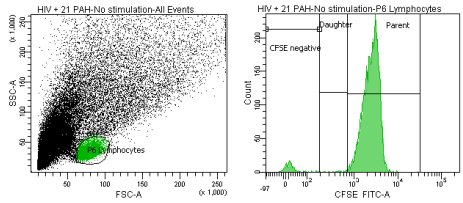
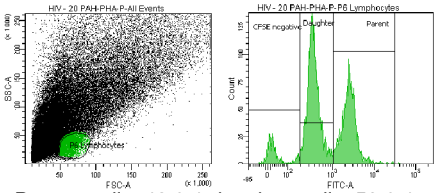
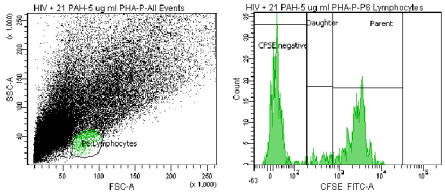
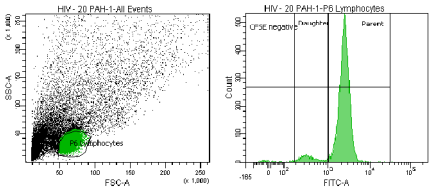
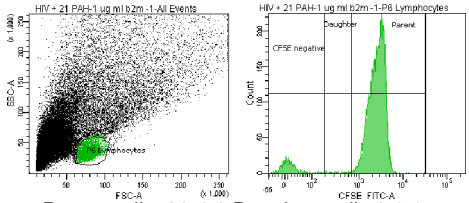
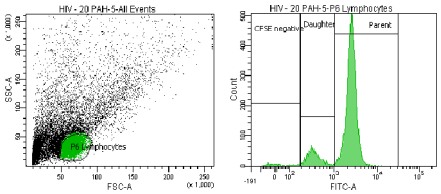
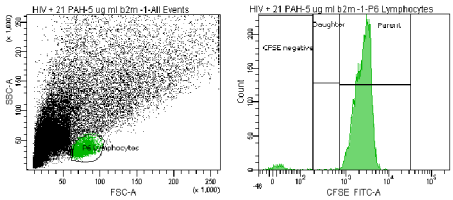
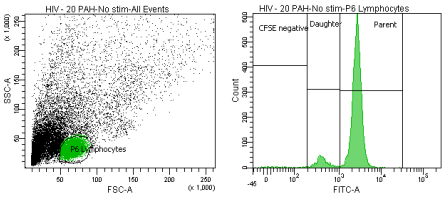
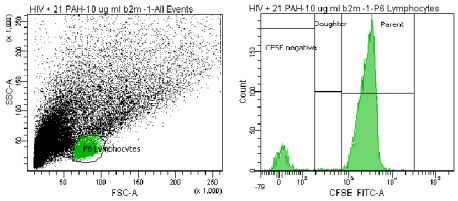
1	(i) HIV -	(ii) HIV +
A) No stimulation **3 days**	 Parent cells: 91.3% Daughter cells: 8.7% Sample: 20 PAH (HIV -)	 Parent cells: 99.4% Daughter cells: 0.6% Sample: 21 PAH (HIV + ARV -)
B) 5 µg/ml PHA-P **3 days**	 Parent cells: 42.0% daughter cells: 58.0% Sample: 20 PAH (HIV -)	 Parent cells: 88.9% Daughter cells: 11.1% Sample: 21 PAH (HIV + ARV -)
C) 1 µg/ml β2m **3 days**	 Parent cells: 93.6% Daughter cells: 6.4% Sample: 20 PAH (HIV -)	 Parent cells: 99.4% Daughter cells 0.6% Sample: 21 PAH (HIV + ARV -)
D) 5 µg/ml β2m **3 days**	 Parent cells: 86.7% Daughter cells: 13.3% Sample: 20 PAH (HIV -)	 Parent cells: 99.5% Daughter cells 0.5% Sample: 21 PAH (HIV + ARV -)
E) 10 µg/ml β2m **3 days**	 Parent cells: 91.3% Daughter cells: 8.7% Sample: 20 PAH (HIV -)	 Parent cells: 99.3% Daughter cells: 0,7% Sample: 21 PAH (HIV + ARV -)

Figure 3.25 continues.

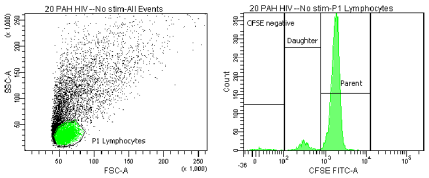
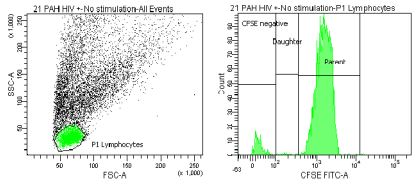
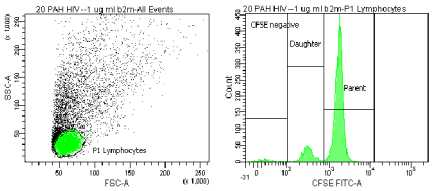
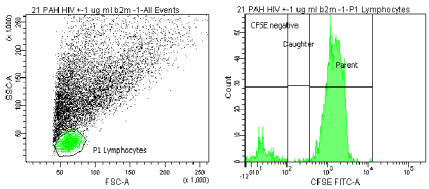
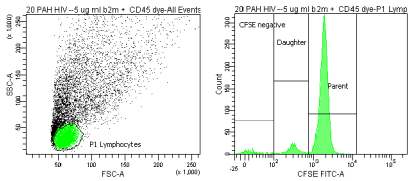
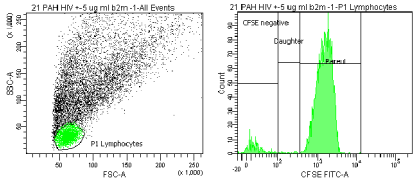
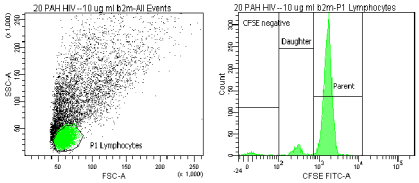
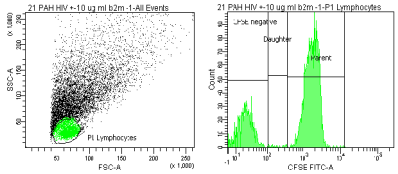
2	(i) HIV -	(ii) HIV +
A) No stimulation **6 days**	 Parent cells:91.4% Daughter cells: 8.6% Sample: 20 PAH (HIV -)	 Parent cells: 99.4% Daughter cells: 0.6% Sample: 21 PAH (HIV + ARV -)
B) 1 µg/ml β2m **6 days**	 Parent cells: 84.5% Daughter cells: 13.5% Sample: 20 PAH (HIV -)	 Parent cells: 99.4% Daughter cells: 0.6% Sample: 21 PAH (HIV + ARV -)
C) 5 µg/ml β2m **6 days**	 Parent cells: 90.5% daughter cells 9.5% Sample: 20 PAH (HIV -)	 Parent cells: 99.3% Daughter cells: 0.7% Sample: 21 PAH (HIV + ARV -)
D) 10 µg/ml β2m **6 days**	 Parent cells: 91.1% Daughter cells: 8.9% Sample: 20 PAH (HIV -)	 Parent cells: 99.1% Daughter cells: 0.9% Sample: 21 PAH (HIV + ARV -)

Figure 3.25. Flow cytometric analysis of human PBMCs stimulated with β2m from (i) naturally HIV-1 infected and (ii) uninfected individuals. FSC/SSC scatter dot plots show the lymphocyte population and the FITC/count histograms show the gated lymphocyte population from the FSC/SSC dot plots for each sample (CFSE detected in FITC detector). Each image shows a representative of each β2m concentration and incubation time. Ten thousand live events were recorded for the 3 days experiment. 1) 3 days incubation with a) no stimulation; b) mitogen PHA-P; c) 1 µg/ml β2m; d) 5 µg/ml β2m and e) 10 µg/ml β2m. 2) 6 days incubation with a) no stimulation; b) 1 µg/ml β2m; c) 5 µg/ml β2m and d) 10 µg/ml β2m. Events fluorescent counts in the gated parent generation represent the number of naïve (undivided) cells. Fluorescent counts in the gated daughter generation represent the number of divided cells. The gate representing CFSE negative cells are also indicated. Cells stimulated with the mitogen proliferated whereas cells naïve of stimulation did not proliferate much. Cells stimulated with the antigen, β2m, did not show proliferation.

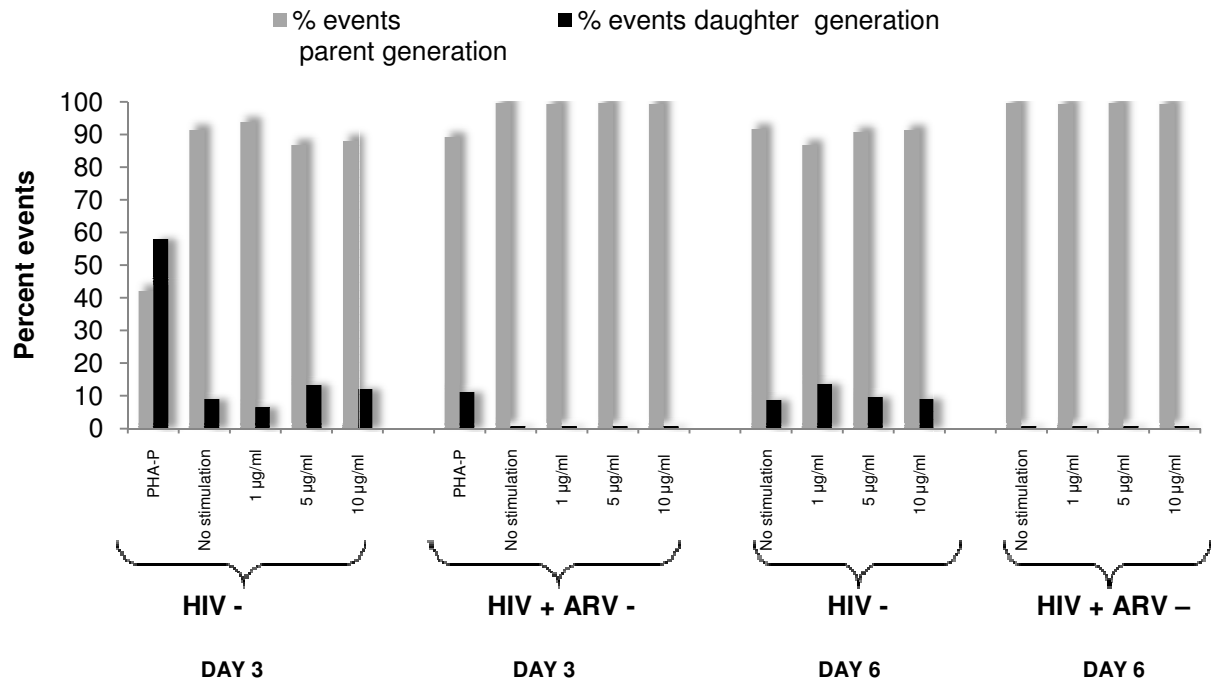


Figure 3.26. Flow cytometric analysis of human PBMCs from naturally HIV-1 infected ARV treatment naïve (HIV + ARV -) and uninfected (HIV -) individuals stimulated with β2m (for 3 and 6 days) The figure shows percent events of undivided cells (parent) and divided (daughter) cells. The presented data represents the average of triplicates. The PI, indicating the average number of division a cell from the original population (parent) has undergone, for each sample tested was 1.

3.6 THE R7V PEPTIDE AS STIMULANT OF INTERFERON-γ PRODUCTION

3.6.1 Intracellular cytokine staining (Interferon-γ)

Interferon-γ production induced by the synthetic R7V peptide or its host protein, human β2m, was investigated by the use of intracellular staining and flow cytometry (Section 2.8.1). Isolated PBMCs from naturally HIV-1 infected individuals were incubated with 50 µg/ml R7V peptide or 5 µg/ml β2m, for 3 (Figures 3.28 and 3.29) and 6 (Figures 3.30 and 3.31) days at 37℃ and 5% CO_2. Figure 3.27 shows the hierarchical gating strategy used for both studies (3 and 6 days). For both infected and uninfected cells, stimulation in the absence of antigen served as negative controls and stimulation with PHA-P (5 and 2 µg/ml) or (10 ng/ml) PMA plus (1 mM/ml) Ionomycin calcium salt were used as positive controls. BD GolgiPlug™ was used to accumulate the cytokine in the Golgi complex and cells were fixed using BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit. Cells were stained with CD3 Pacific Blue, CD4 PE, CD8 PerCP and IFN-γ FITC and analyzed for intracellular cytokine staining on a flow cytometer. Production of IFN- γ in cells (infected and uninfected) incubated with the respective antigen (R7V or β2m)

exhibited similar levels of production as the negative control. No cytokine production was observed for any of the two antigens at the different incubation days. Adding PMA/Ionomycin for the last 6 hours to HIV-positive cells stimulated with R7V for 78 hours did not enhance IFN-γ production. Lenarczyk *et al*. (2000) did further stimulation with PMA/Ionomycin without achieving enhanced cytokine production by antigen stimulated cells, which did not work in this case either. A peptide from the gp41MPER of HIV-1 which previously stimulated IFN-γ secretion in mouse splenocytes (Philippeos, 2007) did not stimulate cytokine production (Figures 3.30 and 3.31) in this case after 150 hours. Figures 3.28 and 3.30 show representative graphs and Figures 3.29 and 3.31 show the summaries.

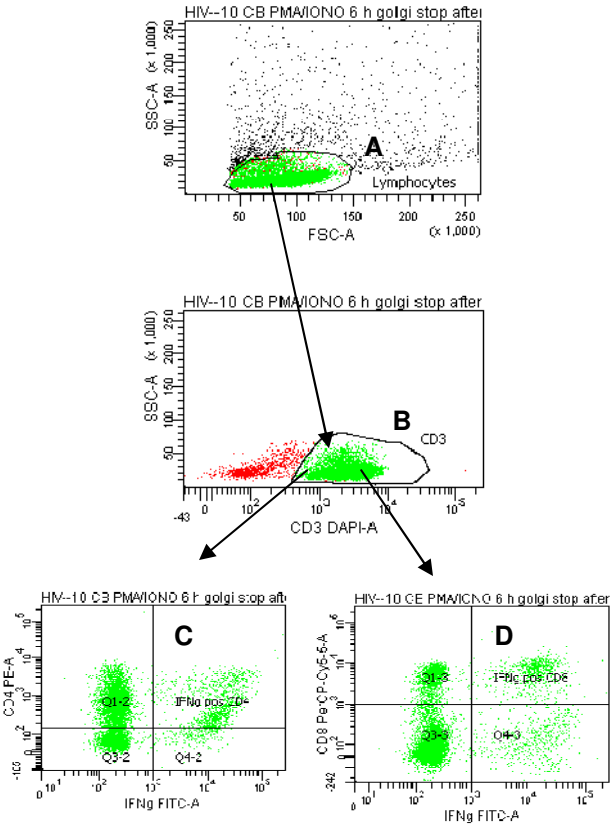


Figure 3.27. Hierarchical gating strategy for intracellular cytokine (IFN-γ) staining. SSC/FSC dot plot indicated all live cells including gate A indicating the lymphocytes. After applying an acquisition threshold on $CD3^+$ lymphocytes, gate B was constructed to include $CD3^+$ cells. $CD3^+$ cells were identified either as $CD4^+$ INF- γ^+ cells (gate C) or $CD8^+$ IFN- γ^+ cells (gate D). The example shown is from PMA/Ionomycin stimulated HIV negative PBMCs for 6 hours. The same gating strategy was used for both intracellular staining studies (Figures 3.28 and 3.30).

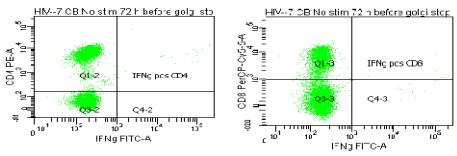
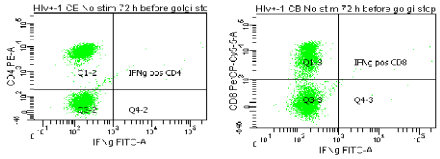
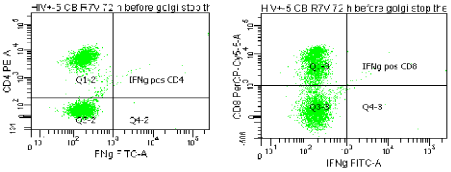
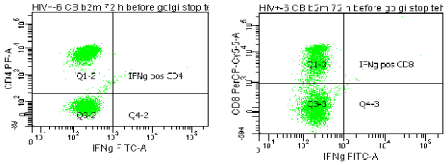
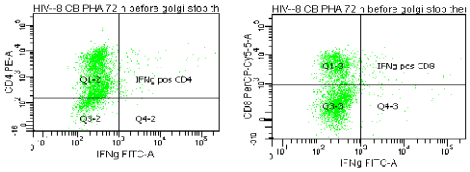
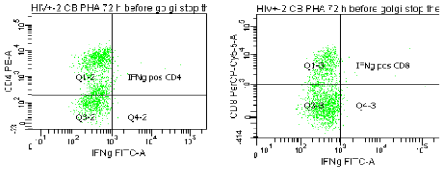
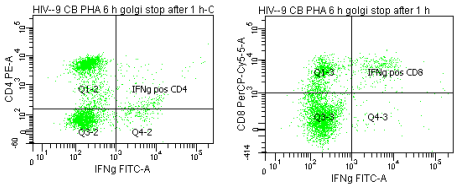
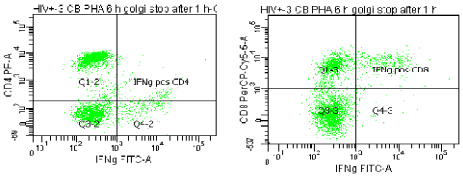
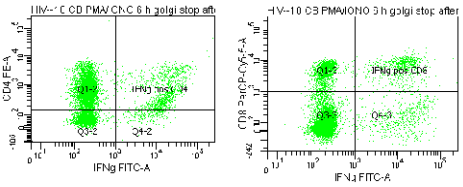
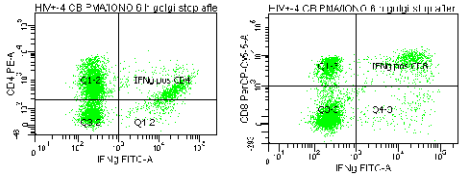
	HIV -	HIV +
(A) **No stimulation** **78 hours**		
(B) **50 µg/ml R7V** **78 hours**	N/A	
(C) **5 µg/ml β2m** **78 hours**	N/A	
(D) **5 µg/ml PHA-P** **78 hours**		
(E) **2 µg/ml PHA-P** **6 hours**		
(F) **PMA/Ionomycin** **6 hours**		

Figure 3.28. Flow cytometric analysis of IFN-γ production of PBMCs from uninfected (HIV-) and naturally HIV-1 infected (HIV+) individuals stimulated with the synthetic peptide R7V or its parent protein β2m for 78 hours. CD4/IFNg and CD8/IFNg dot plots show the positive and negative CD4 and CD8 population either positive or negative for IFN-γ. CD4, CD8 and IFN-γ were detected in the PE, PerCP-Cy5-5 and FITC detector respectively. Each image shows a representative of R7V, β2m, PHA-P, PMA/Ionomycin stimulated or unstimulated cells after 78 hours. Unstimulated cells were negative controls. Cells stimulated with PHA-P or PMA/Ionomycin were positive controls. A) No stimulation for 78 hours; B) stimulation with 50 µg/ml R7V for 78 hours; C) stimulation with 5 µg/ml β2m for 78 hours; D) stimulation with 5 µg/ml PHA-P for 78 hours and E) stimulation with 2 µg/ml PHA-P for 6 hours. F) stimulation with PMA/Ionomycin for 6 hours. Fluorescent counts in the IFNg pos. CD4 gate represent the number of CD4 cells positive for IFN-γ. Fluorescent counts in the IFNg pos. CD8 gate represent the number of CD8 cells positive for IFN-γ. Cells stimulated with PHA-P for 6 hours or PMA/Ionomycing for 6 hours induced cytokine production whereas cells naïve of stimulation did not stimulate much IFN-γ production. Cells stimulated with R7V or β2m did not produce much IFN-γ compared to the unstimulated cells.

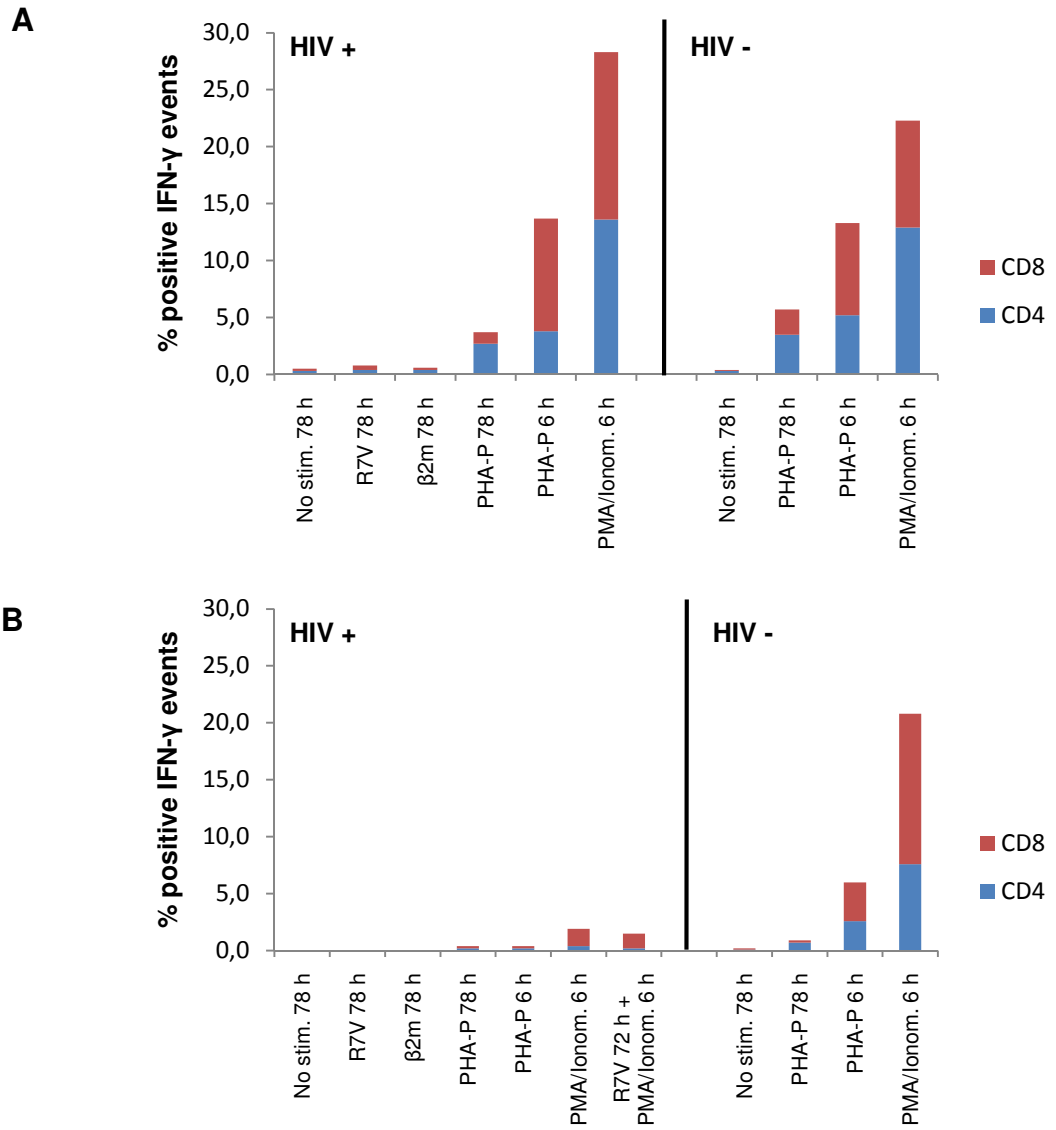


Figure 3.29. Flow cytometric analysis of IFN-γ production of PBMCs from uninfected (HIV-) and naturally HIV-1 infected (HIV+) individuals stimulated with the synthetic peptide R7V or its parent protein β2m for 78 hours. A: Specimen 1 (HIV+ ARV+) and 2. B: Specimen 3 (HIV+ ARV-) and 4. The figure illustrates percent events CD4 and CD8 cells positive for IFN-γ. Unstimulated cells and cells stimulated with PHA-P or PMA/Ionomycin calcium salt were used as negative and positive controls respectively. The data presented represents the averages of the results obtained. R7V and β2m did not stimulate cytokine production after 78 hours. Further stimulation with PMA/Ionomycin by antigen stimulated cells did not enhance production of IFN-γ.

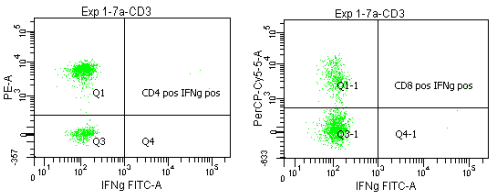
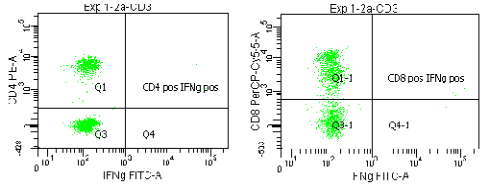
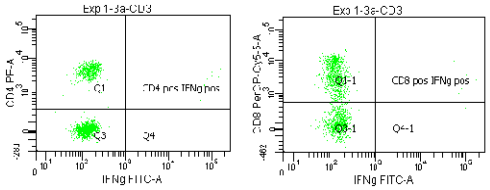
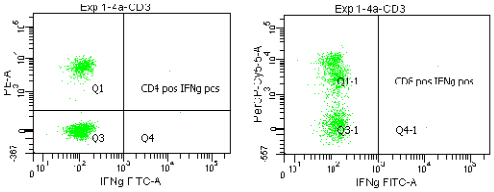
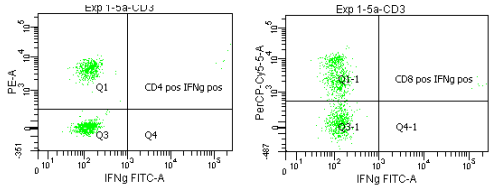
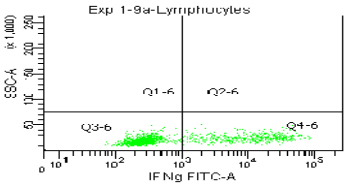
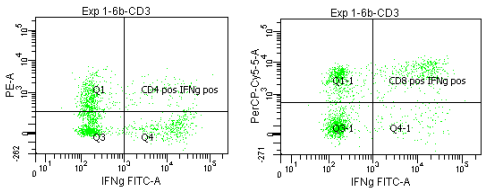
	HIV -	HIV +
(A) **No stimulation** **150 hours**		
(B) **50 µg/ml R7V** **150 hours**	N/A	
(C) **5 µg/ml β2m** **150 hours**	N/A	
(D) **50 µg/ml MPER** **150 hours**	N/A	
(E) **PMA/Ionomycin** **6 hours**		

Figure 3.30. Flow cytometric analysis of IFN-γ production of PBMCs from uninfected (HIV-) and naturally HIV-1 infected (HIV+) individuals stimulated with the peptide R7V, its parent protein β2m or the MPER construct for 150 hours. CD4/IFNg and CD8/IFNg dot plots indicate the positive and negative CD4 and CD8 population either positive or negative for IFN-γ. CD4, CD8 and IFN-γ are detected in the PE, PerCP-Cy5-5 and FITC detector respectively. Each image shows a representative of R7V, β2m, MPER, PMA/Ionomycin stimulated or unstimulated cells after 150 hours. Only 2500 total events were recorded because duplicates were selected out of the well. A) No stimulation for 150 hours; B) stimulation with 50 µg/ml R7V for 150 hours; C) stimulation with 5 µg/ml β2m for 150 hours; D) stimulation with 50 µg/ml MPER for 150 hours and E) stimulation with PMA/Ionomycin for 6 hours. Fluorescent counts in the IFNg pos. CD4 gate represent the number of CD4 cells positive for IFN-γ. Fluorescent counts in the IFNg pos. CD8 gated generation represent the number of CD8 cells positive for IFN-γ. Cells stimulated with PMA/Ionomycin calcium salt for 6 hours induced cytokine production whereas cells naïve of stimulation did not stimulate much IFN-γ production. Cells stimulated with R7V, β2m or the MPER construct did not produce much IFN-γ compared to the unstimulated cells.

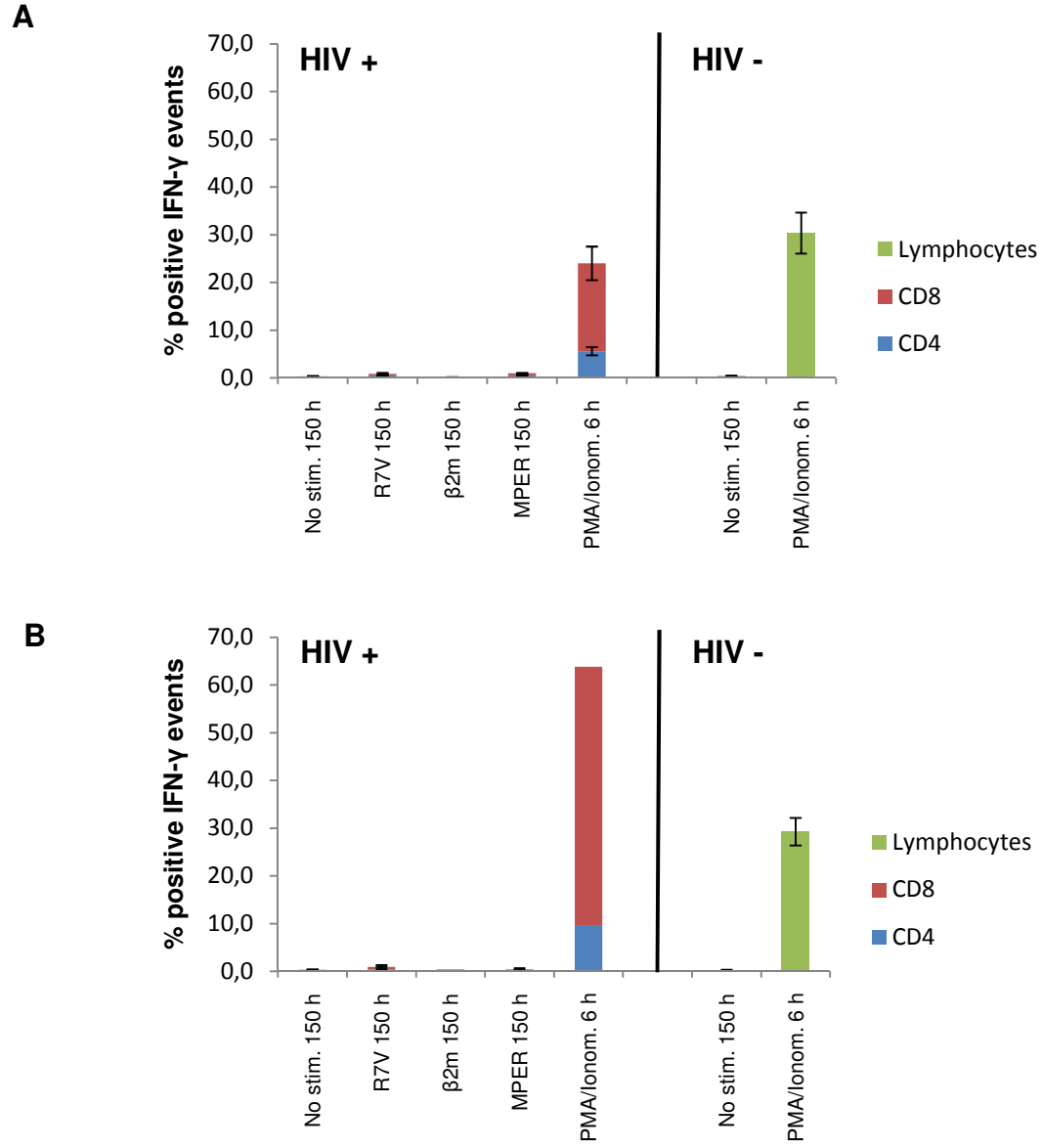


Figure 3.31. Flow cytometric analysis of IFN-γ production of PBMCs from uninfected (HIV-) and naturally HIV-1 infected (HIV +) individuals stimulated with the R7V peptide, its parent protein β2m or a MPER peptide for 150 hours. A: Specimen 5 (HIV+ ARV+) and 6. B: Specimen 7 (HIV+ ARV-) and 8.The figure shows percent events CD4 and CD8 cells positive for IFN-γ. Negative controls were unstimulated cells and cells stimulated with PMA/Ionomycin were positive controls. The data presented represents the averages of the results obtained. Uninfected cells stimulated with PMA/Ionomycin calcium salt were only stained with IFN-γ and total IFN-γ is shown only for the lymphocytes. R7V, β2m or the MPER construct did not stimulate cytokine production after 150 hours.

3.6.2 Secreted Interferon-γ

HIV-positive and uninfected PBMCs were analyzed for IFN-γ secretion following incubation with 50 µg/ml R7V peptide, 5 µg/ml β2m or 50 µg/ml MPER-construct for 78 and 150 hours (Section 2.8). Cytokine secretion was measured by the use of a Human IFNg (Interferon-gamma, IFN-g) ELISA Ready-SET-Go! kit. Secretion of IFN-γ was not

detected in both unstimulated cells and cells treated with R7V peptide and β2m for 78 and 150 hours or MPER-treated infected cells for 150 hours. Incubating infected PBMCs stimulated with R7V for 78 hours with PMA/Ionomycing for the last 6 hours did not enhance the cytokine secretion. IFN-γ was not secreted from cells stimulated with PHA-P for 6 hours. Cells stimulated for 6 hours with PMA/Ionomycin calcium salt did secrete some amount of the cytokine in some of the cases. Following an incubation of 78 hours with PHA-P, a large amount of cytokine secretion was observed in both infected and uninfected cells (Figure 3.32). The incubation times used correlated with what was used in the literature for similar studies (Artavanis-Tsakonas and Riley 2002; Till *et al*. 1997).

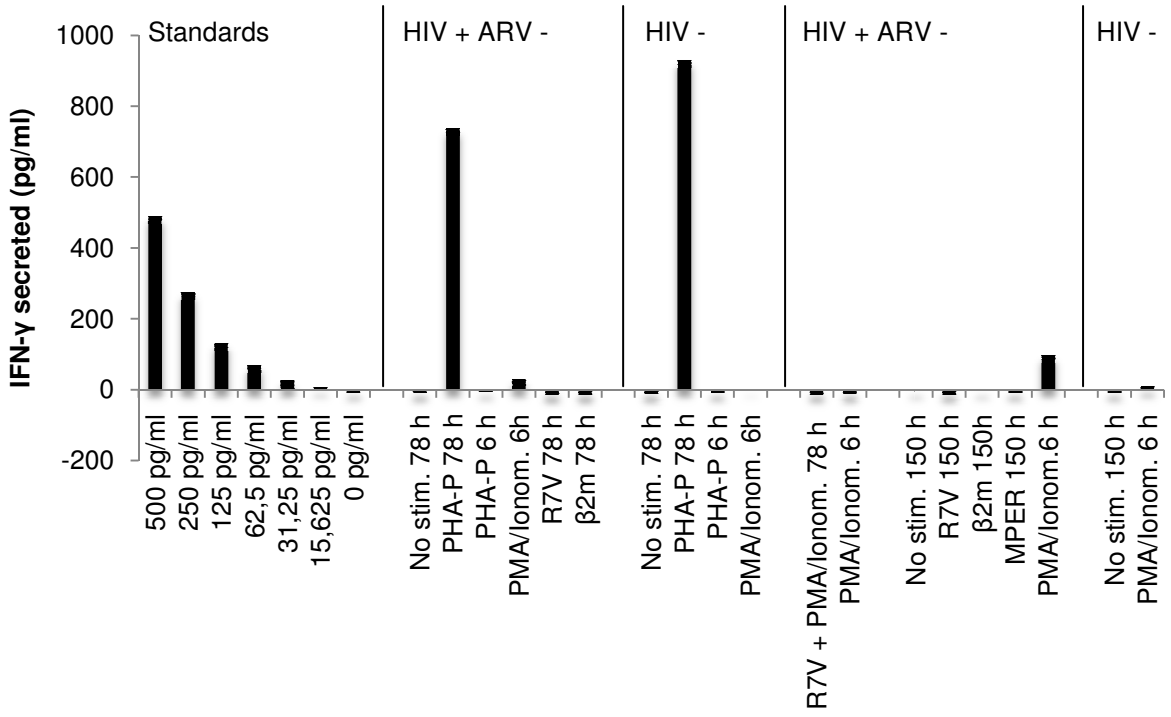


Figure 3.32. Cytokine secretion of PBMCs from uninfected and naturally HIV-1 infected individuals using Human IFNg (Interferon-gamma, IFN-g) ELISA Ready-SET-Go! kit treated with 50 µg/ml R7V peptide, 5 µg/ml β2m or 50 µg/ml MPER-construct for 78 and 150 hours. The standard from the kit with two-fold dilutions is shown. Antigen had no effect on IFN-γ secretion. Cells naïve of stimulation for 78 and 150 hours and cells stimulated with PHA-P for 6 h did not secrete the cytokine. Cells stimulated with and PMA/Ionomycin for 6 h secreted a little amount of the cytokine in some of the cases and cells stimulated with PHA-P for 78 h secreted a large amount of IFN-γ.

CHAPTER 4
DISCUSSION

Since the early years of the HIV epidemic, attempts have been made to determine factors which predict progression to AIDS and overall survival in those infected with the virus. The rate of progression and survival varies greatly, with some patients rapidly progressing to AIDS and death, while others remain asymptomatic for years and are designated LTNPs. Because current preventative and therapeutic regimens have still not been able to eradicate the disease, an HIV vaccine is generally considered as the most likely, and perhaps the only way by which the AIDS pandemic can be halted. One obstacle to the development of an effective HIV vaccine has been the difficulty in inducing potent broadly cross-reactive neutralizing antibodies with protective functions against the virus. According to the literature the host derived R7V epitope induced antibodies prevalent in LTNPs, suggesting a possible role in disease prognosis. Being host derived the epitope is constant (not variable) and induced neutralizing antibodies in rabbits suggesting a role in HIV-1 vaccine development.

Here we report on the fact that the host immune response recognizes a “self” epitope (R7V) as foreign once it is incorporated by the HIV-1 envelope. Unlike already published reports, data collected here does not support a prognostic but rather diagnostic role for R7V antibodies. In its entirety, this study described the design rationale, synthesis, immunological ability, vaccine competency and prognostic/therapeutic value of the R7V peptide (and antibodies directed to it) that mimic the R7V epitope of HIV-1.

4.1 DESIGN RATIONAL OF THE R7V PEPTIDE

Divergent HIV-1 isolates contain a seven amino acid epitope, R7V, at the surface according to Le Contel *et al*. (1996). R7V was able to induce HIV neutralizing antibodies (Galéa *et al*. 1999 a and b; Haslin and Chermann 2007 b) and, in addition, natural production of antibodies directed at this epitope has been observed in individuals not progressing towards AIDS (Galéa *et al*. 1996; Tagny *et al*., 2007; Ravanini *et al*. 2007; Kouassi *et al*. 2007; Sanchez *et al*. 2008). An increased production of these antibodies

with the duration of infection has also been reported (Ravanini *et al*. 2007; Sanchez *et al*. 2008; Haslin and Chermann 2007 b). Based on these reports this study was designed to determine the prevalence of R7V antibodies in HIV-1 infected individuals in South Africa.

Two R7V peptides (one made for use as an ELISA antigen and one made for production of polyclonal rabbit R7V antibodies) were synthesized and an "in-house" ELISA to detect the prevalence of R7V antibodies in HIV-1 subtype C infected serum or plasma was developed. Polyclonal R7V antibodies produced in rabbits were screened in the "in-house" ELISA and tested for the ability to neutralize HIV-1 subtype C isolates. The R7V peptide was used to produce a positive control for the "in-house" ELISA by recombinant means and this antibody fragment was also tested for neutralizing ability of the virus. Lastly, the synthetic peptide was used for characterizing aspects of cellular and humoral immune responses by means of cell proliferation and cytokine production in response to R7V.

4.2 The "IN-HOUSE" ELISA

An "in-house" ELISA to detect R7V antibodies in naturally HIV-1 subtype C infected individuals, was successfully developed as were positive controls (recombinant R7V antibody fragments). This ELISA repeatedly showed negligible levels of R7V antibodies in negative control sera and marginal to high levels in HIV-1 infected people (Figure 3.3). PEG precipitation, employed to make sure that immune complexes and any other proteins in sera/plasma did not interfere with R7V antibody binding its antigen, was deemed unnecessary. This step made no difference to the ELISA data obtained (Figure 3.7) and was therefore omitted. Other modifications (e.g. higher antigen or antibody concentrations) may have improved the ELISA, but was not necessary because of the distinct difference obtained in the responses between uninfected and infected samples. Quantification of the antibodies could have been done by preparing calibration curves using the positive control antibodies but in absence of precipitation or isolation of R7V antibodies this would only indicate the concentration of total antibodies detected. Also because of the c-myc tagged recombinant R7V antibody fragments, a different secondary antibody was used compared to what was used to detect the polyclonal antibodies which make quantification difficult. The R7V antibodies would have to first be

isolated from the polyclonal antibodies and then a c-myc tag added. This is time consuming and would have to be for future work.

Contrary to what is shown in the literature (Galéa *et al*. 1996; Tagny *et al*., 2007; Ravanini *et al*. 2007; Kouassi *et al*. 2007; Sanchez *et al*. 2008), the R7V antibodies could not be viewed as prognostic markers as no difference in R7V antibody levels was observed in normal progressors compared to LTNPs.

An ANOVA test showed a significant difference between HIV-negative and HIV-positive samples but not between the samples on treatment vs. those not on treatment and LTNPs (Figure 3.6). The significant statistical difference seen between uninfected and infected samples support the use of the prevalence of R7V antibodies as a diagnostic rather than a prognostic tool. Whether HIV-positive males and females produce R7V antibodies differently (based on R7V ELISA results) has been documented with contradictory results. Haslin and Chermann (2007) documented that between black African Americans there was no difference in the R7V antibody prevalence between the genders. Ravanini *et al.* (2007) stated that more men than women produced R7V antibodies (using Italian patients) while Kouassi *et al.* (2007) observed that more women than men tested positive for these antibodies using patients from the Ivory Coast. In this study 69% of the HIV-positive samples were from women and 27% from men and no difference between the genders was observed (Figure A.7 in the appendix). Whether race among HIV-positive patients matters in regards to positivity with the R7V ELISA, no studies have reported on this between black and white individuals. Only one study could separate 3 significantly different groups of ethnicities (with HIV-positive samples) where black African Americans had the highest percentage of R7V antibody producing patients compared to a group of Haitians and Asians and a group containing Hispanics, Caucasians and Indians (Haslin and Chermann 2007). In this study the majority (95%) of HIV-positive samples were from black individuals (3% with unknown race). No studies reporting on differences in capacities according to age of the patient has been documented. In this study the age of the HIV-positive individuals ranged from 21 to 56 (majority of patients were below 40) years and no increase in the “in-house” ELISA response was observed with the increase of the age of the patient (Figure A.8 in the appendix).

An increase in R7V antibodies with the duration of infection has been reported (Ravanini *et al*. 2007; Sanchez *et al*. 2008; Haslin and Chermann 2007 b). This was not

observed in this study (Figure 3.5 G). However, it is important to mention that the sample number or percent positive results for R7V antibodies were limited in some of the published studies. Also, none of these studies were done with specimens from HIV-1 infected individuals living in South Africa (primarily infected with HIV-1 subtype C) as instead samples were collected from individuals living in the USA, Europe, Cameroon and the Ivory Coast, (individuals most likely infected with HIV-1 subtype A or B, McCutchan 2006). HIV-1 subtype C do not have the same characteristics as subtype B (Ping *et al.* 1999) and may not have the same β2m epitopes incorporated. Data provided here suggest the R7V epitope to be present in subtype C but it may be less prominent than in other subtypes. Also the exact point of incorporation may differ between subtypes and the conformation of the region of the protein where incorporation occured may influence epitope exposure. Two other hydrophilic peptides (Le Contel *et al*. 1996), both 7 amino acid (S7K; Ser-Gln-Pro-Lys-Ile-Val-Lys and F7E; Phe-His-Pro-Ser-Asp-Ile-Glu) derived from β2m have also been found in the envelope of HIV-1 and were capable of reversing the neutralizing action of the monoclonal antibodies directed to β2m (Le Contel *et al*. 1996). Perhaps one of these two epitopes are better exposed in HIV-1 subtype C. Future work should investigate whether HIV-1 subtype C infected individuals have antibodies directed at these two epitopes and if these antibodies are present to a larger extent than R7V antibodies.

All the aims stated for detecting R7V antibodies in HIV-1 infected individuals were met; however the antibodies detected appear to be prevalent at much lower levels than that reported in the literature.

4.2.1 Comparisons with the Ivagen ELISA

The data in this study used an “in-house” ELISA that differs from that used in previous studies. The Ivagen ELISA was used by Xu *et al*. (2002), Tagny *et al*. (2007), Ravanini *et al*. (2007), Kouassi *et al*. (2007), Haslin and Chermann (2007 b), Sanchez *et al*. (2008), Ergünay *et al*. (2008) and Webber (2009) and Galéa *et al*. (1996) used their own “in-house” ELISA. The strong recombinant R7V antibody fragment response in this report’s “in-house” ELISA indicates that enough antigen was present in the wells to quickly detect concentrated R7V antibodies. The percent RSD between triplicates (8.3%, 6.5% and 5.8% for uninfected, infected on treatment and infected naïve of treatment respectively) and between tests (19.6%. 25.7% and 20.5 % for uninfected,

infected on treatment and infected naïve of treatment respectively) in the "in-house" R7V ELISA is considered acceptable.

E-mail communication with researchers working with the Anti-R7V ELISA from Ivagen (Ravanini *et al.* 2007; Haslin and Chermann 2007 b; Kouassi *et al*. 2007; Tagny *et al*. 2007; Ergünay *et al*. 2008; Sanchez *et al*. 2008; Webber 2009) was unsuccessful. The Ivagen ELISA was also removed from the market in 2008 with little explanation. The data collected using that ELISA indicates that as it was, the product was not market ready.

Ivagen S.A (Ref. IVR 96000, Bernis, France) developed an ELISA to detect non-progression towards the disease in HIV-infected patients. Since early 2006, IVAGEN launched several retrospective studies to determine the prevalence of these antibodies within several groups of HIV patients. The test was recalled from the market and is now dedicated to research use only (Personal communication with Da Costa Castro and Skorski 2008). A letter written by the scientific manager and CEO of Ivagen (Da Costa Castro and Skorski 2008, see Section 1.3.5) to customers and investigators explained why the kit was recalled from the market. In the letter Ivagen stated that the presence of R7V antibodies detected by this ELISA could not be considered at all as predictive of a future slower progression nor non-progression of HIV disease for a patient newly diagnosed HIV positive. Our data using the Ivagen ELISA and our own "in-house" ELISA supported these observations; R7V antibody levels are not more prominent in non-progressors and are therefore not a prognostic marker detectable by ELISA.

4.2.2 The recombinant R7V antibody fragments

The recombinant antibody fragments, single chain R7V antibody fragments that consisted of only the binding regions of the heavy and light chain domains of Fab, were made by means of recombinant DNA technology and M13 phage display using a library based on chicken immunoglobulin genes. Large recombinant antibody libraries allow specific monoclonal antibody fragments to be obtained without immunizing animals or employing hybridoma technology. Even though most existing antibody repertoires are derived from human immunoglobulin genes, genes from other species can also be used. Because of the way in which gene conversion introduces diversity, the naïve antibody repertoire of the chicken can easily be accessed using only two sets of primers (van Wyngaardt *et al*. 2004). A Micro BCA™ Protein Assay Kit indicated that the total protein in the recombinant R7V antibody fragment clones was always ~ 270 µg/ml

(Table 3.1). The recombinant antibody fragments responded very well to the R7V antigen in the ELISA

The recombinant antibody fragment was unstable and lost the ability to bind the antigen within 5 days. Antigen binding antibody fragments/scFvs made by phage display are supposed to be more stable than scFvs made by hybridoma technology (Lorimer *et al.* (1996) because in phage display, peptides are expressed on the surface of phage as fusion proteins and allows selection and amplification of phage clones with specific binding activities (O'Neil and Hoess 1995). Stability of scFvs can be improved by inactivation of the unstable variants by denaturing treatment. By combining denaturation with reduction of the intra-domain disulfide bridges between the heavy and light variable domains, a favourable condition for selection of clones with improved stability can be created (Brockmann *et al.* 2004). This was not done here, but is planned for the future of the recombinant R7V antibody fragments. Dilution in an incorrect storage buffer may also cause the instability of the antibody fragment and some scFv antibodies have been shown to be unstable during long-term storage in aqueous solutions (Moss *et al*. 2003). Although the recombinant antibody fragments prepared for this study were not stable, we dealt with this by making new antibodies when needed and used them within 5 days.

4.2.3 Responses to β2m antigen and antibodies

Antibodies raised against the parent protein of R7V, recognized β2m but not the R7V peptide as ELISA antigens (Figure 3.10). Prospec purified the protein from human urine. The purified protein has a different conformation than when it is incorporated by the envelope of HIV-1. R7V may not be exposed in the purified protein, explaining why antibodies against the protein do not recognize the epitope in ELISA. Beta-2 microglobulin as antigen was recognized by polyclonal antibodies from HIV-1 infected individuals (3 out of 4 samples). These 3 samples bind both R7V and β2m as antigen. However, when these samples bind to β2m they do so to epitopes other than R7V (because R7V is hidden in the purified protein).

4.3. THE RABBIT R7V ANTIBODIES

LifeTein produced R7V antibodies in rabbits which were responsive in both the company's and the "in-house" ELISA at very high serum dilutions (Figures 3.16, 3.17 and 3.18). These antibodies did not bind to β2m as antigen (Figure 3.17) suggesting

that the R7V epitope was not visible in purified β2m. These data agrees with the fact that β2m antibodies could also not recognize R7V as antigen (Figure 3.10). This means the R7V epitope is not visible in the parent protein β2m when this protein is in its natural form as host protein but does become visible once the parent protein is incorporated by the envelope of HIV-1. This fact is also why Galéa *et al*. (1999 a and b) suggest this epitope for consideration as vaccine candidate because it should not be able to elicit autoimmune responses as in its virus-associated form it will differ from what it looks like when part of the host's β2m.

Beside the above suggestions the following consideration is also plausible; R7V antibodies in rabbits were made against a linear epitope which cannot recognize the conformational epitope on β2m antigen. Also the β2m antibodies may be against conformational epitopes which does not recognize the linear R7V antigen for binding. These are plausible but less likely explanations.

Polyclonal R7V antibodies from immunized rabbits recognized R7V antigens from 2 different sources which makes sense because a 7 amino acid peptide is not difficult to synthesize and purify. Both sources demonstrated successful synthesis and comparable levels of peptide purity.

4.4 EVALUATION OF VIRUS NEUTRALIZING ABILITY OF R7V ANTIBODIES

The published literature (Section 1.3.8) argues for the consideration of R7V as vaccine candidate. An HIV-1 vaccine protein or peptide should elicit neutralizing antibodies following antigen immunization. In this study neither recombinant antibody fragments nor polyclonal rabbit R7V antibodies had viral neutralizing abilities against HIV-1 subtype C. The recombinant R7V antibody fragments were not able to neutralize a subtype C isolate but were capable of neutralizing a subtype B isolate (Figure 3.21). This also supports the possibility that the R7V epitope is more exposed in HIV-1 subtype B.

It has previously been shown that purified R7V antibodies from rabbits immunized with the peptide R7V as well as in sera of HIV-1 infected non-progressors, neutralized various subtypes of HIV-1 including subtype C (Galéa *et al*. 1999 a and b; Haslin and Chermann 2007 b). In addition, recombinant R7V antibodies produced in insects were

able to neutralize various subtypes of HIV-1 (Haslin and Chermann 2004; Haslin *et al*. 2007 a).

Possible reasons for why data collected here, differs from that in the literature is explained below. The rabbit polyclonal antibodies produced by Galéa *et al*. (1999 a) used a different carrier protein, a different adjuvant and different immunization and blood collection strategies. In addition, different virus strains were used in the neutralization assay.

The recombinant antibody fragments used here were prepared by different means than those employed by Haslin and Chermann (2004) and Haslin *et al*. (2007 a). Recombinant antibodies produced in insects are whole antibodies and the recombinant antibody fragments produced here only consist of the variable fragments of the binding domains of the heavy and light chains of the Fab. Zhang and Dimitrov (2007) produced HIV-1 envelope gp120 human monoclonal antibody fragments with exceptional neutralizing activity and breadth of neutralization against different subtypes including subtype C and these were scFvs. Because the scFvs produced here were only stable for 5 days modifications to improve the stability of the scFv may also improve its neutralizing abilities. Finally, these scFvs inhibited the HIV-1 subtype B strain (Figure 3.21) which could suggest that the R7V epitope is more exposed in subtype B compared to subtype C.

Why some antibodies neutralized the subtype B isolate and not the primary subtype C virus could also be because the subtype C virus (consisting of a mixture of 2 subtype C isolates) used in this study may be unusual in its own right, and that ideally more representative subtype C isolates (a virus consisting of only one isolate) should be tested in the future. Another point is (with regards to neutralizing antibody capacity) that viruses that are passaged in cell lines are more easily neutralized than primary isolates. The subtype B isolate used in this study is a lab-adapted virus that has been passaged in cell lines and known to be very neutralization-sensitive. This may be why the subtype B virus neutralized as easily as it was in this study. A last question that arises is if there might be differences based on co-receptor usage preference (CCR5-utilizing vs. CXCR4-utilizing strains of the same subtype). The subtype C isolate Env clone used here is from PBMC-grown virus that uses the CCR5 co-receptor for entry.

Archived samples with known neutralizing ability were found to have very little R7V antibodies and sera (collected in this study) with high R7V antibody levels did neutralize

the HIV-1 subtype C isolate. The samples chosen to measure neutralizing were not LTNPs but had the highest presence of antibodies recognizing R7V according to the "in-house" ELISA data. These antibodies would have to be purified to determine whether the neutralizing antibodies present were indeed R7V antibodies.

4.5 CELL PROLIFERATION AND R7V

Since R7V is an epitope in HIV's envelope (Le Contel *et al*. in 1996) and R7V antibodies have been detected in HIV-infected individuals (Galéa *et al.* 1996; Haslin and Chermann 2007 b; Sanchez *et al*. 2008; Ergünay *et al*. 2008), one could assumed that the R7V peptide should be recognized/remembered by antigen-specific memory T-cells. This possibility was evaluated by measuring proliferation of HIV-1 infected cells in the presence of R7V, using flow cytometry. The fact that PBMCs did not proliferate to R7V *in vitro* could be because whole virus infected cells did not recognize the epitope if it is presented in the absence of other viral components. Another possible explanation could be that the R7V peptide's conformation (the 7 amino acids are described as either a random coil or extended strand) is very different from what it looks like in the whole virus. The peptide may not contain enough secondary structure for immune system recognition *in vitro*. That the R7V peptide is too small to be presented to immune cells could not be a reason as MHC molecules are known to bind epitopes ranging from eight to eleven amino acids (Karim and Karim 2005) and therefore should bind the seven amino acid R7V peptide for presentation. As a general rule one can estimate that detectable $CD4^+$ T-cell responses to HIV-1 peptides/antigen may only be detected in about 25% of HIV-1 infected individuals (if using large pools of peptides or whole viral antigens); but more among individuals who have good control of the virus. $CD8^+$ T-cell responses, on the other hand, would generally be more measurable. However, when one considers responses to individual HIV-1 peptides there is variability in peptides recognized by different individuals. In this study one particular epitope and HIV-infected patients naïve of treatment with a CD4 cell count of less than 200 cells/µl and in addition few patients were used and might explain the absence on an immune response. The parent protein of R7V, β2m, is much larger (99 amino acid) and described as an antiparallel β-barrel fold (Rosano *et al*. 2005) and should have a bigger chance of inducing cell proliferation. The absence of antigen-specific proliferation by this

protein can again be due to conformational issues and may indicate the absence of autoimmune consequences.

Carboxyfluorescein Diacetate Succinimidyl Ester and flow cytometry are not the most sensitive methods to detect cell proliferation. It has been shown that CFSE in concentration of 37 nM to 10 µM decreases the proliferative capacity due to the decreased viability of proliferating cells in a concentration dependent manner. Thus, CFDA-SE can kill dividing cells and could give a high proportion of false positive results. In addition, the dye modulates expression of activation markers (Laštòvička *et al*. 2009). In this study a CFDA-SE concentration of 10 µM was used and could explain the number of dead cells and the negative CFSE peak observed with some of the samples. A more sensitive method, radioactive standard assays with ^{3}H-thymidine incorporation (Laštòvička *et al*. 2009) may have detected something different. However, PBMCs naïve of stimulation are also able to proliferate (Geiger *et al*. 2001; Morgera *et al*. 2003) which explains the background proliferation observed in some of the unstimulated samples. As discussed in Chapter 1, HIV-1 might not be the only virus incorporating the R7V epitope in its envelope. We do not know the clinical status of the uninfected samples other than that the donors were not infected by HIV-1. However, the aim was to detect cell proliferation in response to R7V or β2m compared to unstimulated cells and we did not observe more cell proliferation with the antigen-treated cells vs. unstimulated cells. The mitogen. PHA-P, induced proliferation of positive samples was less than that of HIV negative cells. Decreased proliferation responses of human PBMCs to PHA-P have previously been shown for HIV-infected cells (Resino *et al.* 2003). The literature supports incubation for 3 (Kardinal *et al*. 2000; Troger *et al*. 2003; Trantor *et al*. 1995) and 6 (Trantor *et al*. 1995; Li *et al*. 2006; Yu et *al*. 2001) days with peptides for cell proliferation assays. In this study, increased incubation days (5 µg/ml R7V for 7 and 10 days, 30 µg/ml R7V for 7 days, 50 µg/ml R7V for 7 and 10 days and 100 µg/ml R7V for 8 and 10 days) did not change the observations.

4.6 THE R7V PEPTIDE AS STIMULANT OF IFN-γ PRODUCTION

Cytokine production of HIV-infected cells exposed to R7V was measured by means of intracellular cytokine staining and flow cytometry and the cytokine secretion by means of a human IFN-γ ELISA. No IFN-γ production was observed either intracelluarly (Figures 3.28, 3.29, 3.30 and 3.31) or secreted (Figure 3.32). The production of IFN-γ

by $CD8^+$ T-cells would have suggested that R7V is recognized as a cellular immune response epitope. Many peptides based on HIV-1 envelope proteins contain CTL epitopes, however, R7V does not appear to be one of these. This is however not necessarily a negative quality of the epitope. Since the assumption is that R7V is only exposed when β2m is incorporated by the envelope of HIV-1 during budding (Galéa *et al.* 1996; 1999 a and b), this epitope is hidden during normal host activities (binding of β2m to MHC class I) and therefore does not bind to MHC class I and has no CTL epitope. However, like discussed in section 4.5, there is variability in peptides recognized by different individuals when using individual peptides and therefore a larger number of samples should be tested to conclude that the immune response was in fact absent. In this study both ARV-treated and treatment naïve HIV-infected individuals (using both patients with CD4 cell count below and over 200 cells/µl) were used and it may be that the immune response (IFN-γ production) was absent because of the influence of treatment or viral load.

4.7 CONCLUDING DISCUSSION

In this study it was hypothesized that a peptide representing the R7V epitope of HIV, which is presented to the host immune system as foreign, should be able to detect humoral and perhaps cellular immune responses in infected individuals. In addition, it was hypothesized that R7V antibodies should neutralize HIV-1 isolates and that subtype C infected individuals (LTNPs) should have antibody levels comparable to other HIV-1 subtypes.

Although not able to stimulate *in vitro* cell proliferation or IFN-γ production, the synthetic peptide R7V evaluated here induced humoral immune responses (in rabbits) and mimicked the *in vivo* R7V epitope of HIV-1 by detecting antibodies produced during natural infection in an ELISA. The peptide was not able to induce neutralizing antibodies in rabbits. The lack of sufficient secondary structure of the peptide or that the peptide is only experienced as something foreign when part of the virus could be the reason for it not inducing antigen specific proliferation of cells or IFN-γ production. The inability of the polyclonal rabbit antibodies to ihibit an HIV-1 subtype C isolate could be because this subtype does not efficiently express the R7V epitope. This is supported by the recombinant R7V antibodies that were not able to neutralize the same virus but an HIV-1 subtype B strain. If it is true that the R7V epitope is exposed on subtype C, it is

possible that modification of the antigen binding domain of the recombinant antibodies may improve their neutralizing ability. The R7V peptide showed immunogenicity as it was able to characterize R7V antibody responses in naturally HIV-infected humans in an ELISA. However, LTNP subtype C sera responded to R7V at lower levels than what the literature describes for other subtypes. It may be that subtype C does not have the R7V epitope with the same conformation as that exposed on the surface of subtypes A and B.

The following conclusions can be made with regards to the questions raised by the objectives of the study:

4.7.1 Were R7V antibodies produced during natural HIV-1 subtype C infection and to the same extent as in published literature?

The R7V "in-house" ELISA detected antibodies generated in natural HIV-1 subtype C infection however the response was lower than that reported in the literature. The use of different R7V ELISAs or that the R7V epitope is not that exposed in subtype C are possible reasons for these observations. Validation assays like isolation and purification of the antibodies must be done to confirm the antibody responses. The anti-R7V response in subtype C may simply be lower because of a weaker presence of the R7V epitope in this subtype or it may be possible to ameliorate the data by improving binding conditions in the assay itself.

4.7.2 Were the antibodies more prevalent in LTNPs compared to progressors?

LTNPs had the same R7V antibody levels compared to that of progressors (AIDS patients). No significant statistical difference between the sample groups was observed. Increasing the number of LTNPs and obtaining sera from subtype A/B LTNPs to evaluate in the "in-house" ELISA may further clarify the differences observed in this study compared to other published work.

4.7.3 Would HAART influence the prevalence of R7V antibodies?

No significant difference in the R7V antibody response was observed between individuals on treatment compared to individuals naïve of treatment. These results suggest that other factors e.g. duration of infection may influence the antibody response.

4.7.4 Were R7V antibodies produced in uninfected individuals?

The R7V antibody response of uninfected individuals was repetitively and consistently lower than the infected individuals and was considered R7V antibody negative. A significant statistical difference was observed between uninfected and infected samples suggesting that R7V antibodies might constitute a diagnostic rather than a prognostic tool. However, the extent to which R7V is incorporated by other viruses must be clarified. ELISA is not the most sensitive assay especially when one has low affinity antibodies. In this study, biosensor analysis was attempted but the R7V peptide did not attach to the biosensor chip even though the company (GenScript Corporation) who synthesized the peptide confirmed that the N-terminal was not blocked. Several studies were done to trouble shoot the binding problem with no success. This method is suggested for future consideration.

4.7.5 Were R7V antibodies elicited in rabbits or R7V Fab as well as human polyclonal antibodies (detected by the R7V peptide) able to neutralize a primary HIV-1 isolate?

Polyclonal R7V rabbit antibodies and recombinant R7V antibody fragments were not able to neutralize an HIV-1 subtype C isolate. As mentioned earlier this could be because the R7V epitope is not very well exposed in subtype C. This could be supported by the fact that the latter antibodies did neutralize a subtype B strain. Why the recombinant antibody fragments did not neutralize the subtype C isolate could also be because the antigen binding region of the antibody is only suited for the synthetic R7V peptide and not the natural R7V epitope. The human polyclonal antibodies neutralized the HIV-1 subtype C isolate.

4.7.6 Would R7V antibody fragments (and rabbit polyclonal antibodies) recognize the peptide as antigen in the “in-house” ELISA?

The R7V antibody fragments and the rabbit polyclonal antibodies responded very well to the R7V antigen in the “in-house” ELISA.

4.7.7 Could the R7V peptide stimulate in vitro proliferation of HIV-1 infected PBMCs and could the R7V epitope play a role in cellular immunity?

The R7V peptide or its parent protein, β2m, were not able to stimulate cell proliferation or IFN-γ production suggesting that the antigens are only immunogenic when associated with the virus. However, the number of samples tested here may preclude

any likelihood of detecting such response particularly if such responses were only in a small number of individuals; or these immune responses have not developed as T-cells cannot "see" the epitope in the context of specific HLA class I (CD8) and class II (CD4). Arguably T-cells would need to have the epitope presented on infected cells (processing of viral peptides through the endogenous pathway) in the context of class I to $CD8^+$ T-cells. Virus or virus components would need to be exogenously presented by HLA class II antigen presenting cells to $CD4^+$ T-cells. The lack of an immune response to the parent β2m protein could indicate that there are no autoimmune consequences.

4.8 FUTURE PERSPECTIVES

R7V antibodies have been suggested as a potential therapeutic tool (Haslin and Chermann 2002, 2004 and 2007 b; Haslin *et al*. 2007 a) since 2002. To date no studies report on passive infusion of animal models (or human volunteers) with these R7V antibodies. Nor have recombinant R7V antibodies (Haslin and Chermann 2004; Haslin *et al*. 2007 a) been used in *in vivo* therapeutic studies. Passive infusion of animals (Binley *et al*. 2000; Mascola *et al*. 2000 and 2003; Mascola 2002) or humans (Armbruster *et al*. 2004) with HIV antibodies is not uncommon but data presented here suggest that more empirical research is necessary before application research can be done. In this study the polyclonal rabbit R7V antibodies and the recombinant R7V antibodies did not neutralize an HIV-1 subtype C isolate. More research into verifying whether subtype C really has the R7V epitope exposed needs to be done. The antigen binding domain of the recombinant antibodies needs to be modified for it to recognize and bind the natural R7V epitope of HIV for neutralization purposes.

Suggestions of a synthetic R7V peptide being considered as a vaccine has been made since 1996 (Le Contel *et al*. 1996; Galéa *et al*. 1999 a and b; Chermann 2001; Haslin and Chermann 2007 b). No studies evaluating the *in vivo* value of R7V antibodies have yet been reported. By this we mean eliciting *in vivo* neutralizing antibodies in animal models (using synthetic R7V as antigen) and challenging this response with live virus. The literature also does not report on any R7V based (phase I/II) vaccine trials. It appears that vaccine development studies have been done and some of it has been published as patents (Chermann *et al*. 2000 and 2006). These studies are done with R7V conjugated to carrier proteins such as KLH and BSA. This project, however, demonstrated that R7V alone did not induce a cellular immune response in terms of

IFN-γ production which is essential for a successful vaccination. Modification of the R7V peptide by for example lipid conjugation may improve its antigenicity and presentation via MHC class I. Meyer and Torres (1999) demonstrated that lipid conjugation of envelope peptides induced a CTL response in mice.

Because the prevalence of the R7V antibodies was shown to correlate with non-progression to AIDS (Galéa *et al*. 1996; Ravanini *et al*. 2007; Kouassi *et al*. 2007; Sanchez *et al*. 2008), it has been postulated that patients who have elevated levels of R7V antibodies have a lower likelihood of progression to AIDS. Data provided here did not show increased levels of R7V antibodies in LTNPs. Further studies are therefore needed to clarify the use of R7V antibodies as possible prognostic markers.

Finally, whether the R7V epitope or the entire β2m protein is incorporated by other or all enveloped viruses has implications for the use of the epitope or antibodies to it in prognosis or therapy. Limited sample number studies commenting on possible cross-reactivity between R7V antigen and antibodies from other enveloped viruses exist (Sanchez *et al*. 2008), but needs to be expanded. There are similarities between HIV and HTLV and there are reports of non-random incorporation of host proteins by the latter which further supports clarifying the extent to which β2m or R7V is incorporated by other viruses. In addition, the HIV-1 subtype issue needs to be clarified as most of the published studies were done with HIV-1 infected individuals most likely infected with subtype A or B. Lastly, validation studies such as isolation and purification of the antibodies believed to be R7V antibodies must be done to confirm that the observed antibody response is actually by R7V antibodies.

R7V and antibodies to the epitope holds promise but more extensive and clarifying autoimmunity and cross-reactivity studies are needed to maintain optimism on its use. That a host epitope when incorporated in a viral envelope is immunogenic has been sufficiently demonstrated. What remains to be rigorously shown is the usefulness of this immunogenicity (challenging the neutralization response with live virus) after autoimmunity due to these host antigens have been shown to not be a concern.

CHAPTER 5
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FULL INTERNET SOURCES

The following websites were all accessed and active 30 January 2010.

CHAPTER 1

UNAIDS (2008):
http://www.unaids.org/en/KnowledgeCentre/HIVData/GlobalReport/2008/2008_Global_report.asp

Figure 1.1: http://en.wikipedia.org/wiki/File:HIV-SIV-phylogenetic-tree.svg

Figure 1.3: http://en.wikipedia.org/wiki/File:HIV_Virion-en-2.png

Figure 1.4: http://en.wikipedia.org/wiki/HIV_structure_and_genome

Figure 1.5: http://www.molmo.be/hiv_lifecycle.html

CHAPTER 2

WIHS:
http://statepiaps.jhsph.edu/wihs/

CHAPTER 3

Peptide Property Calculator (Genscript): https://www.genscript.com/ssl-bin/site2/peptide_calculation.cgi

Peptide property calculator (Innovagen): http://www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp

APPENDIX

THE APPENDIX CONTAINS THE FOLLOWING:

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A copy of the manuscript of the published review article:

Bremnæs C., Meyer D. (2009). The HIV-based host derived R7V epitope; functionality of antibodies directed at it and the predicted implications for prognosis, therapy or vaccine development. *The Journal of Biotechnology and Molecular Biology Reviews*. **3**, 071-080.

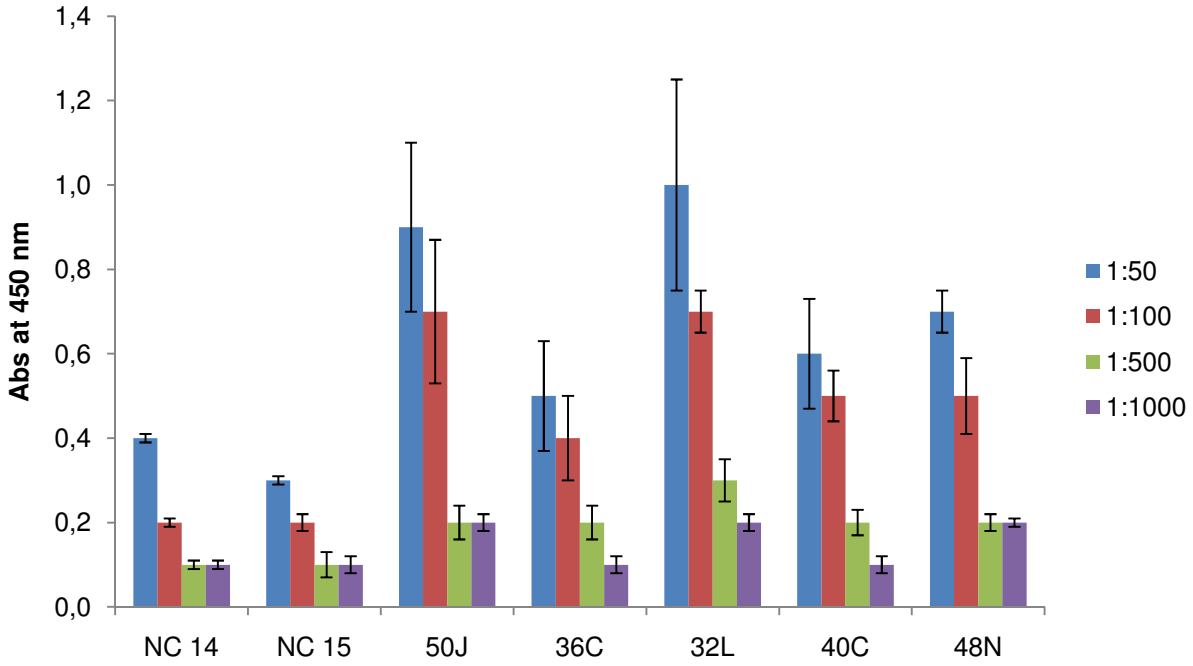

Figure A.1. Serum titration in the "in-house" ELISA. Two HIV negative samples and 5 HIV positive (LTNPs) samples were analyzed with the following titrations: 1:50, 1:00, 1:500 and 1:1000. 25 ng antigen was plated. The results indicated that 1:500 was too diluted and that a 1:100 dilution was acceptable to use for this ELISA.

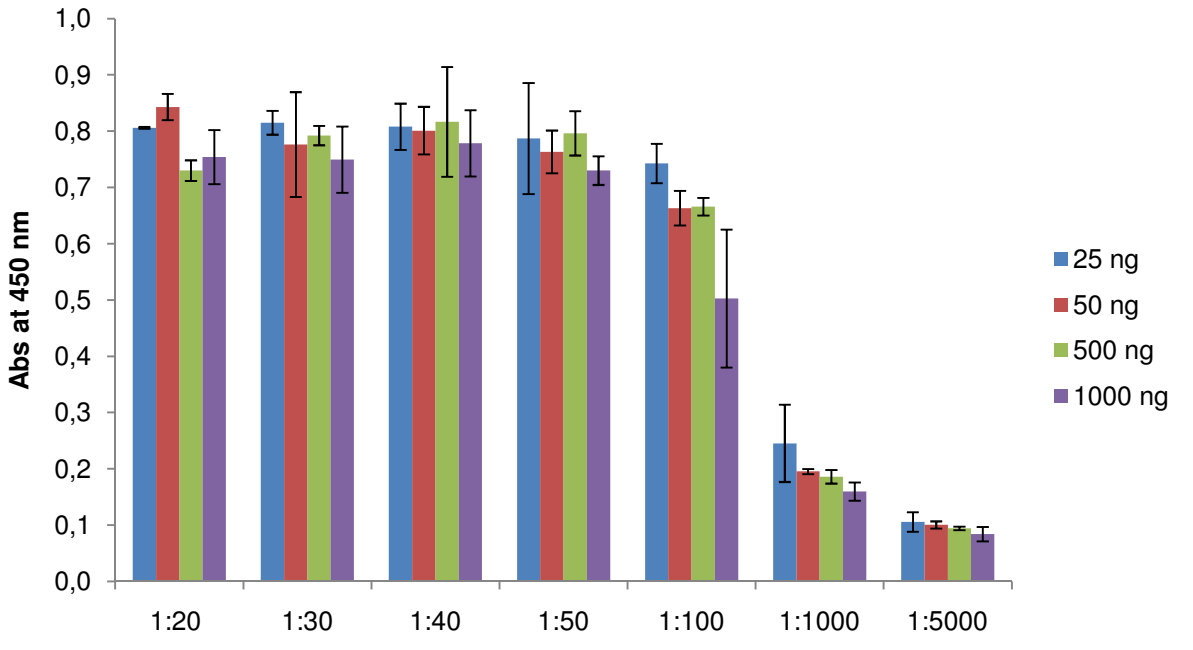

Figure A.2. Serum antigen and serum titration in the "in-house" R7V ELISA. One HIV positive sample (ARV treatment naïve) was used (FOH 10). The antigen titrations plated were the following: 25, 50, 500 and 1000 ng. The serum titrations are indicated on the x-axis. The use of 25 ng antigen and serum dilution of 1:100 was determined to be sufficient.

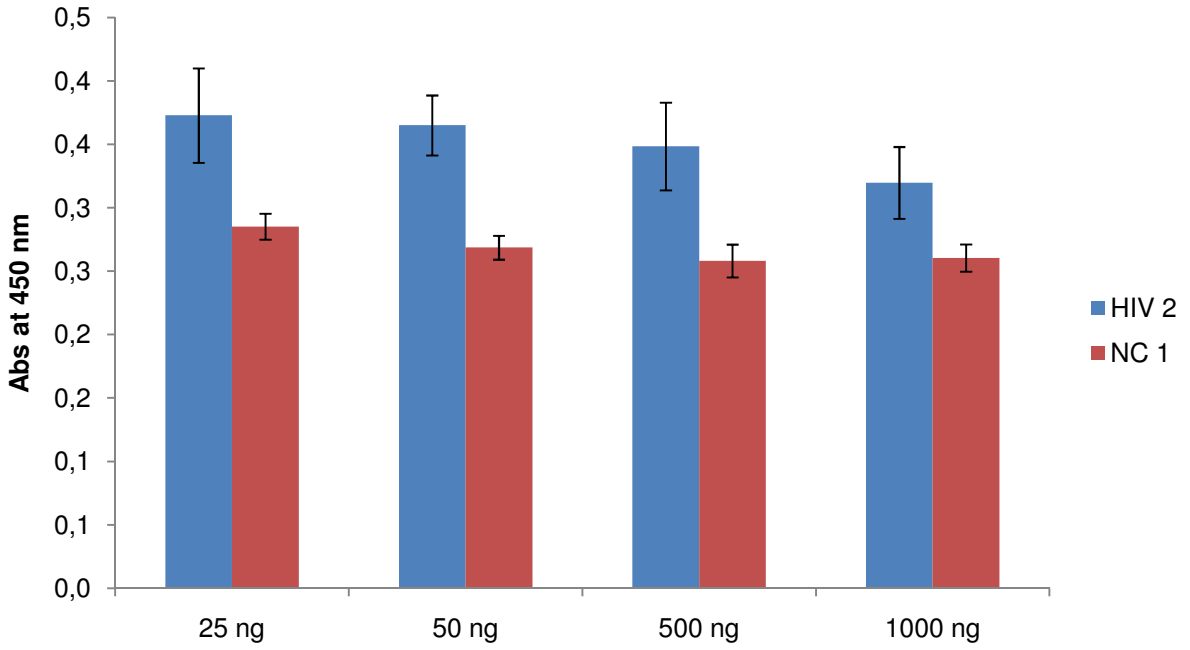

Figure A.3. Serum antigen titration in the "in-house" R7V ELISA. One uninfected (NC 1) and 1 HIV positive sample were used (HIV 2). The antigen titrations 25, 50, 500 and 1000 ng were used. Serum was diluted 1:100. The use of 25 ng antigen was determined to be sufficient.

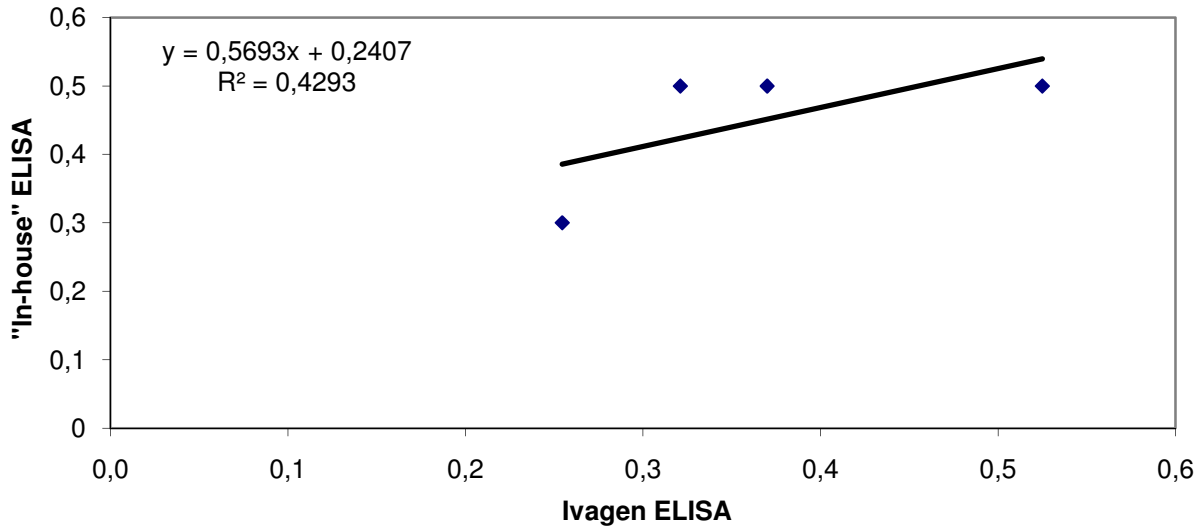

Figure A.4. The diagram shows average OD values determined for 4 naïve (HIV+) serum samples using the Anti-R7V ELISA test from Ivagen (OD450 nm/OD260 nm) and the "in-house" ELISA (OD 450 nm). Three decimals after comma are used for the OD values. Poor correlation (R^2=0.4293) between the two ELISAs was observed. 100% correlation is obtained with a R^2 =1.

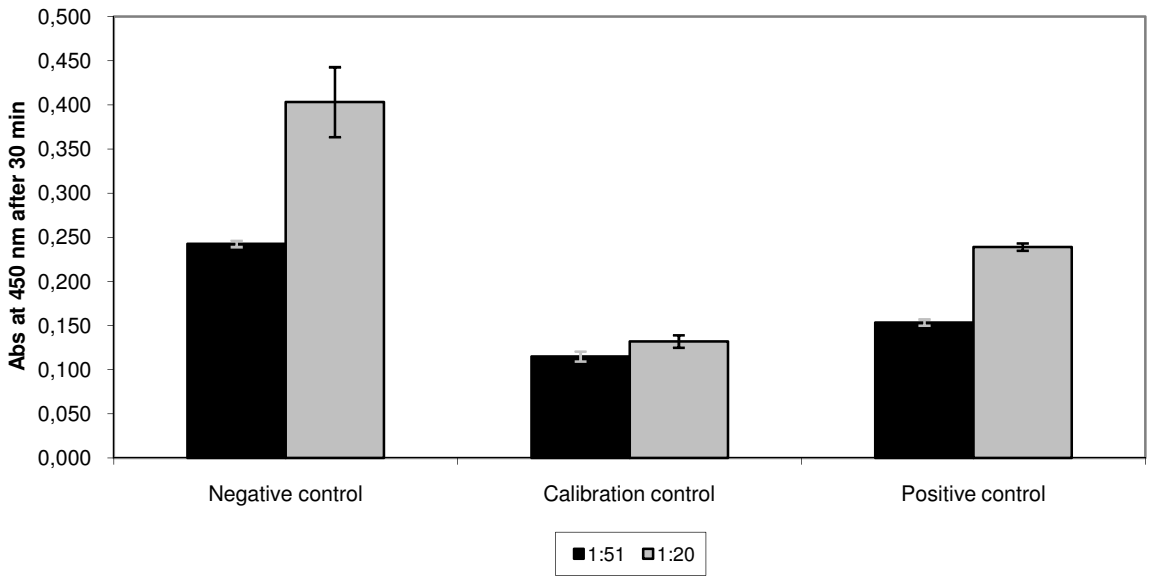

Figure A.5. Results obtained using the Ivagen internal controls (negative -, calibration - and positive control) from the Anti-R7V ELISA test in the "in-house" R7V ELISA. OD value determined after 30 minutes. The serum dilutions were 1:51 and 1:20. The controls did not work in the "in-house" R7V ELISA as the negative control failed to be lower than the calibration – and the positive control.

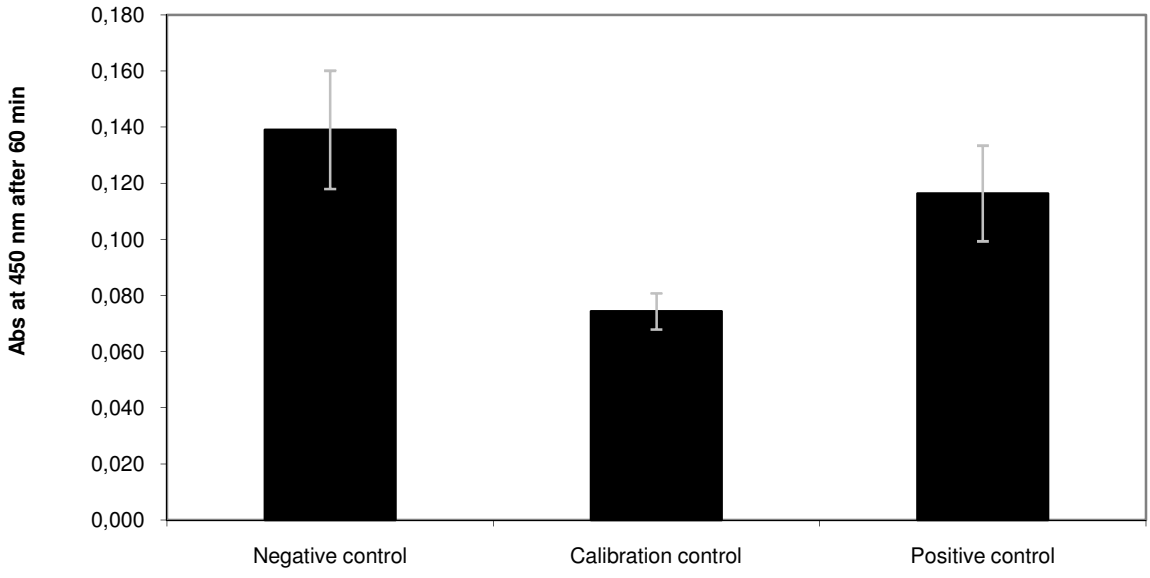

Figure A.6. Results obtained using the Ivagen internal controls (negative -, calibration - and positive control) from the Anti-R7V ELISA in the "in-house" ELISA. OD value determined after 60 minutes. The serum dilution was 1:100. The controls did not work in the "in-house" R7V ELISA as the negative control failed to be lower than the calibration – and the positive control.

Table A.1. Information about the race, age and gender of the samples in figure 3.3.

Sample	Race	Age	Gender
HIV -			
Control 14	White	32	F
Control 15	White	36	F
Control 16	*	*	*
Control 32	*	*	*
Control 40	*	21	F
Control 41	Black	21	F
Control 53	White	24	F
Control 54	Black	*	*
Control 55	White	*	*
Control 56	White	22	F
Control 57	White	*	*
Control 58	White	24	M
Control 59	White	*	*
44 FOH	*	*	*
26 FOH	Black	39	F
6 PAH	Black	22	F
HIV + ARV +			
4 FOH	Black	34	F
32 FOH	Black	50	F
28 FOH	Black	40	M
79 FOH	Black	37	M
76 FOH	Black	50	F
8 FOH	Black	25	M
41 FOH	Black	40	F
35 FOH	Black	39	F
36 FOH	Black	46	M
11 FOH	Black	35	F
13 FOH	Black	26	F
65 FOH	Black	30	F
63 FOH	Black	52	F
70 FOH	Black	40	F
64 FOH	Black	26	F
71 FOH	Black	50	F
62 FOH	Black	40	F
51 FOH	Black	26	F
50 FOH	Black	49	F
53 FOH	Black	30	M
77 FOH	Black	26	F
6 FOH	Black	43	F
7 FOH	Black	40	M

Table A.1 continues.

Sample	Race	Age	Gender
HIV + ARV +			
27 FOH	Black	33	F
40 FOH	Black	40	F
39 FOH	Black	38	F
16 FOH	Black	36	F
17 FOH	Black	30	F
38 FOH	Black	43	M
37 FOH	Black	43	F
54 FOH	Black	53	M
PHJ A6	Black	42	F
HIV + ARV -			
10 FOH	Black	34	M
34 FOH	Black	34	F
47 FOH	Black	43	F
2 PAH	*	*	*
24 FOH	Black	32	M
42 FOH	Black	31	M
23 FOH	Black	29	M
43 FOH	Black	42	M
9 FOH	Black	44	F
46 FOH	*	*	*
45 FOH	Black	36	M
29 FOH	Black	33	F
21 FOH	Black	33	M
PHJ N9	Black	41	M
PHJ N10	Black	48	F
PHJ N11	Black	38	F
DS 2	Black	25	F
DS 8	Black	56	F
30 FOH	Black	25	F
SC 001	Black	21	F
SC 002	Black	21	F
LTNP			
2 KHC	Black	31	F
1 KHC	Black	37	M
40 C	Black	55	F
48 N	Black	55	F
50 J	Black	37	F
36 C	Black	38	F
32 L	White	47	F
DS 3	Black	45	F
DS 5	Black	39	F

*Race, age or gender unknown.

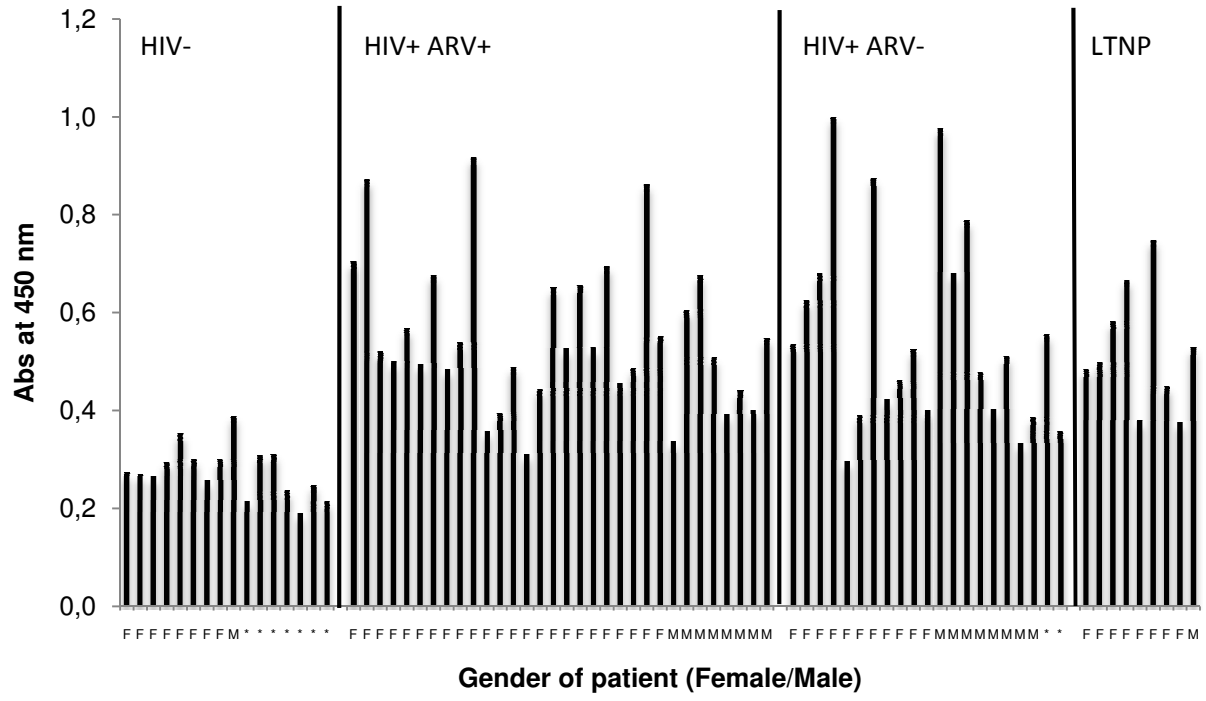

Figure A.7. Comparison of R7V antibody prevalence between genders. The samples are taken from figure 3.3. No difference in the "in-house" ELISA response was observed between females and males. For the samples labelled with *, the gender is unknown.

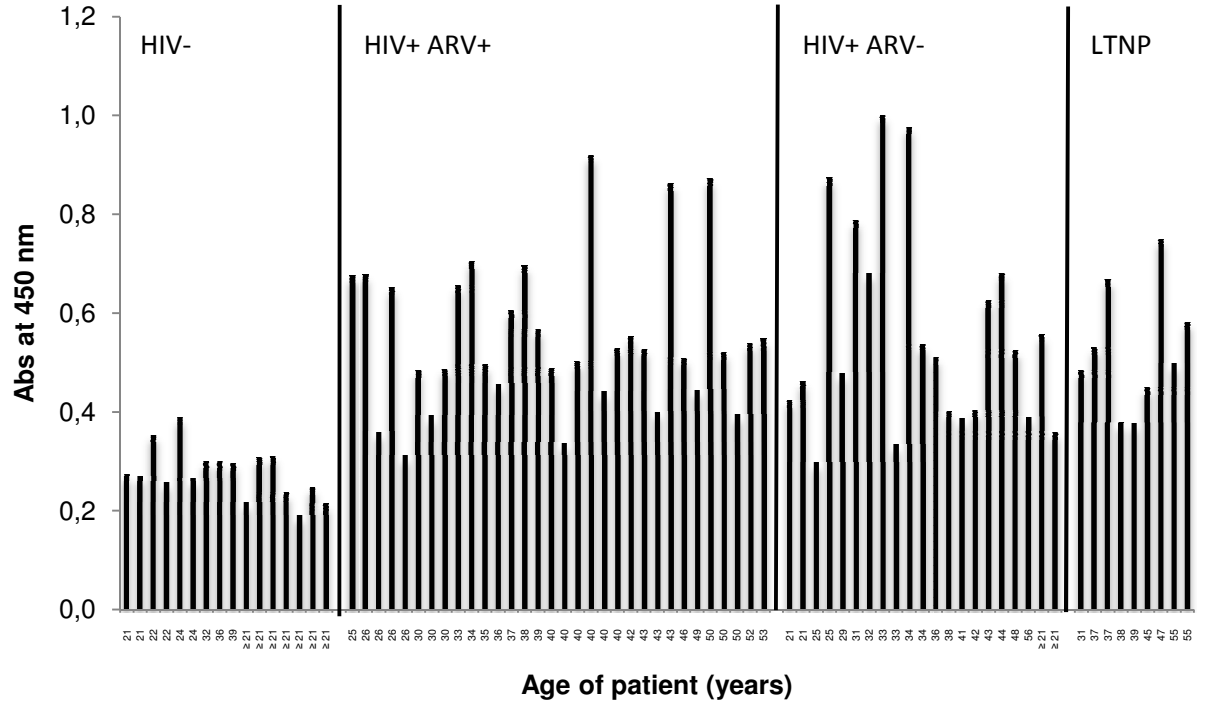

Figure A.8. Comparison of R7V antibody prevalence and age of patient. The samples are taken from figure 3.3. No increase in the "in-house" ELISA response was observed with the increase of the age of the patient. For some of the samples the exact age is unknown other than that the individual was 21 years old or older.

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Standard Review

The HIV-based host derived R7V epitope; functionality of antibodies directed at it and the predicted implications for prognosis, therapy or vaccine development

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Host protein beta-2 microglobulin (β2m) is incorporated into the HIV-1 coat during budding. Individuals not progressing to AIDS produce antibodies directed to an epitope contained in β2m which is designated R7V. These antibodies increased with duration of HIV-infection in non-progressor patients and protected against HIV replication. Purified R7V antibodies neutralized different HIV-1 isolates and did not bind to human cells. In individuals progressing to AIDS or using antiretroviral treatment, a lower prevalence of R7V antibodies was observed. This review summarizes findings on the R7V epitope and antibodies directed at it. Suggestions are also made as to necessary research on R7V which may clarify the importance of this epitope in HIV therapy, prognosis or vaccine development.

Key words: R7V, epitope, antibodies, β2m, HIV, ELISA.

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INTRODUCTION

HIV, the retrovirus known for disabling CD4 expressing T cells and causing immune system dysfunction, incorporates

host cell proteins into its envelope during budding. These virus incorporated host proteins can be bystanders, assist in the life cycle and viral ability to avoid immune system detection, while some of these proteins retain their functional ability or engage in responses that are detrimental to the pathogen. The host immune response to these cellular proteins when they are presented as virus-associated particles allows room for investigating them as prognostic markers, therapeutic tools or potential vaccine candidates. Existing AIDS prognostic markers like CD4 cell count and viral load are not infallible and better therapies against HIV is still under development because of shortcomings in existing treatment regimes. In addition, the uneven record of success of HIV vaccine strategies leaves room for considering host proteins incorporated by the virus as a radically different means of generating protective immunity.

Beta-2 microglobulin (β2m) is one of the host proteins incorporated into the envelope of HIV-1 and under discussion in this review are the possible uses of antibodies to R7V, an epitope within this protein. R7V is incorporated into the envelope of HIV-1 and located on the exterior surface (Le Contel et al., 1996). This epitope has been suggested as possible vaccine target (Le Contel et al., 1996; Galéa et al., 1999 a and b; Chermann, 2001; Haslin and Chermann, 2007b) and antibodies induced by it as prognostic (Galéa et al., 1996; Chermann, 2001; Ravanini et al., 2007; Kouassi et al., 2007; Sanchez et al., 2008) or therapeutic markers (Haslin and Chermann, 2002, 2004 and 2007b; Haslin et al., 2007a). Data that lead to these suggestions are reviewed here. Because reports on R7V are limited (only 11 articles in peer reviewed journals and a number of patents), data contained in posters (4 posters reviewed by a scientific committee and presented at international conferences) as well as personal communications with researchers (Webber, 2009) are also referenced to provide a complete picture of work done on this epitope thus far.

Cellular proteins in HIV

Cellular or host proteins can be incorporated by HIV-1 either on its surface or inside the viral lipid envelope (Ott, 2008; Figure in Galéa et al., 1999b). Some host proteins present in the virus retain their functional ability and can affect infectivity, tropism and pathogenesis. According to Ott (2008) there are three possible ways in which HIV-1 incorporates cellular proteins inside or on its surface. Most of the cellular proteins incorporated by the virus are taken up as simple bystanders because of their close proximity during the budding process. The presence of these non-specifically incorporated proteins provides important information about the local environment where HIV-1 assembles and the site of budding. The second possible mechanism of incorporation involves host proteins that act as partners in the assembly and budding process and these are incorporated by interacting with one of the viral proteins. Lastly, HIV-1 can hijack cellular proteins by incorporating them for a post-assembly step where they act as captives helping the virus replicate or evade the immune system (Ott, 1997 and 2008). Selected cellular proteins detected in HIV-1 as well as their role in favour of the virus are detailed by Ott, 1997 and 2008. Since β2m is part of the major histocompatibility complex (MHC) class I, the assumption is that this protein is incorporated into HIV's envelope in the same manner as MHC class I. From information provided in the review by Ott, 2008 it seems that HIV incorporates this protein as a bystander as there is no evidence that a specific HIV protein acts as a binding partner to bring the protein complex into the viral particle.

Beta-2 microglobulin

β2m is a 12 kDa, 99 amino acid protein which is non-covalently bound to the 45 kDa heavy chain of the MHC class I molecule (Roitt et al., 2001) where it is essential for expression of this molecule. MHC class I plays a central role in the immune system, is omnipresently expressed and binds peptide antigens for presentation to the $CD8^+$ T-cells (Rosano et al., 2005) during the initiation of a cellular immune response. β2m is also associated with CD 1 (a protein related to MHC class I) for presentation of lipids, glycolipids and lipid antigens to T-cells (Roitt et al., 2001). Because β2m is expressed in almost all nucleated cells (Arthur et al., 1992), it can be found in all potential virus target cells. This protein along with neopterin and other serum and cellular markers that correlate with clinical progression of HIV disease (Fahey et al., 1990; Hofmann et al., 1990; Melmed et al., 1989) when found free in a variety of physiological fluids (Rosano et al., 2005), also serve as indicators of the degree of immune activation.

Beta-2 microglobulin as vaccine target

Arthur et al. (1992); Ott (1997) and Haslin et al. (2002) confirmed β2m as one of the cellular proteins incorporated

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Abbreviations: AIDS, acquired immunodeficiency syndrome; **ARV,** antiretroviral; **β2m,** beta-2 microglobulin; **CMV,** cyto-megalovirus; **CTL,** cytotoxic T-lymphocyte; **ELISA,** enzyme-linked immunosorbent assay; **HAART,** highly active anti-retroviral therapy; **HIV,** human immunodeficiency virus; **HLA,** human leukocyte antigen; **LTNP,** long term non-progressor; **MAbs,** monoclonal antibodies; **MHC,** major histocompatibility complex; **OD,** optical density.

into the surface of HIV-1. Monoclonal antibodies (MAbs) directed to β2m were able to immunoprecipitate intact viral particles and inhibit the life-cycle of HIV (Arthur et al., 1992). These data suggests β2m to be an integral and functioning part of the HIV-1 surface, involved in the process of HIV-infection and pathogenesis (Hoxie et al., 1987; Devaux et al., 1990; Corbeau et al., 1990; Arthur et al., 1992; Le Contel et al., 1996). β2m is immunogenic only when exposed on the viral surface and not when it is part of the human leukocyte antigen (HLA)/MHC class I at the host cell surface or when it is part of circulating β2m. MAbs against β2m reacts with free urinary β2m (Liabeuf et al., 1981) but not with the protein when it is associated with cell surface proteins.

To prevent binding between HIV-1 and a host cell, it may be worth considering targeting β2m in vaccine design and possibly avoiding the problems associated with the variability of viral proteins (Galéa et al., 1999b; Haslin et al., 2002). However it is only possible to consider such a vaccine approach if epitopes in the cellular determinant used will only be exposed when it is carried away by the extracellular infectious agent, or the epitopes are nonimmunogenic in their natural presenta-tion by the cell and is modified when it is presented at the surface of the virion (Chermann et al., 2000). This appears to be the case with epitopes in β2m. Studies demonstrating an inability of the potential vaccine antigen to induce autoimmune responses need to be designed. β2m based vaccine formulas appear to be under investigation as is evident in the references used in the patent by Chermann et al. (2000).

Most HIV protein-vaccine strategies to date focused on the variable viral envelope proteins, especially the V3 loop of gp120. Hewer and Meyer (2004 and 2005) hypothesized that exploiting the advantageous properties of V3 loop peptides and at the same time acounting for the variability of this region would aid in developing an effective vaccine component for inducing broadly cross reactive neutralizing antibodies. Addressing variability by novel synthesis induced viral strain specific immune responses with some amount of cross-reactivity which underscores the need for circumventing viral variability by other means.

The R7V epitope of beta-2 microglobulin

A seven amino acid epitope in β2m was shown to be present at the surface of divergent HIV isolates by Le Contel et al. in 1996. These authors studied several short overlapping peptides derived from β2m for their ability to reverse the neutralizing action of MAbs directed to the protein. Among the tested peptides, the heptamers R7V (Arg-Thr-Pro-Lys-Ile-Gln-Val), S7K (Ser-Gln-Pro-Lys-Ile-Val-Lys) and F7E (Phe-His-Pro-Ser-Asp-Ile-Glu) were efficient in reversing the action of these MAbs, with R7V being the most efficient. The R7V peptide consists of fewer hydrophobic (PIV) than hydrophilic (RTKQ) amino acids. The tertiary structure of isolated human β2m is described as an antiparallel β-barrel fold (Rosano et al., 2005). Multiple Protein Sequence Analysis programs, including DeepView/Swiss-Pdb Viewer 3.7 (SP5), describe percentages of the 7 amino acids in the R7V peptide as either a random coil or an extended strand which are regions that could form part of a β-barrel (PDB entry 1LDS; Trinh et al., 2002).

Polyclonal antibodies detected using an R7V peptide as antigen

Antibodies directed to R7V were detected in HIV-infected individuals, primarily asymptomatic and long term infected patients naïve of treatment. In studies done by Galéa et al. (1996) and Sanchez et al. (2008), R7V antibodies were present in the majority of asymptomatic patients (HIV seropositive for 5 - 12 years, stage A1-A2 according to the 1993 CDC classification, Galéa et al., 1996; class A according to the 1993 CDC classification and naïve of highly active antiretroviral therapy, HAART, Sanchez et al., 2008) and long-term infected patients (infected for more than 10 years without HAART, Sanchez et al., 2008). The highest prevalence of R7V antibodies were found in non-progressor patients compared to progressors who go on to develop AIDS with associated CD4 cell decreases and viral load increases (Galéa et al., 1996; Sanchez et al., 2008, Table 1). R7V antibodies were also found in individuals who were not infected with HIV but at lower levels (Galéa et al., 1996; Sanchez et al., 2008, Table 1). This is interesting because R7V is believed to only be immunogenic when incorporated into the HIV envelope and the virus was absent in these patients. These antibodies could be cross-reactive or perhaps the HIV negative individuals were infected by other enveloped viruses containing host derived R7V-like epitopes (more detail in paragraphs to follow).

The study done by Galéa and colleagues (1996) used an “in-house” enzyme-linked immunosorbent assay (ELISA) and the authors calculated concentrations of R7V antibodies from a standard curve while Sanchez et al. (2008) used the anti-R7V ELISA from Ivagen (Bernis, France) and determined the antibody ratio by normalizing the optical density (OD) value for the sample with the OD value for the internal calibrator. The Ivagen ELISA developed for detection of R7V in human serum and/or plasma of individuals confirmed as being seropositive for HIV-infection is not commercially available.

The Sanchez et al. (2008) study contained several groups; A (201 HIV negative, 160 HIV positive on treatment and 88 HIV positive untreated patients from USA), B (177 asymptomatic and 131 symptomatic HIV positive patients from USA) and C (45 untreated Italian non-progressor patients infected with HIV-1). From the

Table 1. Summary of studies reporting on the presence of R7V antibodies in HIV-infected and uninfected individuals. HIV-1 subtypes listed were inferred from locations where samples were collected since this information was not always stated.

Percent R7V antibody producing individuals				Total	HIV-1 subtype/country	References
HIV Positive vs. HIV negative						
%	n	%	n			
53.7	95	32.0	69	164	-	Galéa et al., 1996[3,4]
53.2	248	5.5	201	449	(B) USA	Sanchez et al., 2008[4,7]
33.5	507	3.0 [2]	201	708	(B) USA	Haslin and Chermann, 2007b[1,3,4]
9.1[7]	33	0.0	10	43	(B) Turkey	Ergünay et al., 2008[4,7]
Asymptomatic vs. Symptomatic						
%	n	%	n			
59.0	63	38.0	8	71	(B) Italy	Ravanini et al., 2007[1,4]
59.1	22	13.1	61	83	(A) Cameroon	Tagny et al., 2007[1,4]
42.0	36	9.0	53	89	(A) Ivory Coast	Kouassi et al., 2007[1,4]
64.4[5]	177[5]	35.1	131	308	(B) USA	Sanchez et al., 2008[4]
35.7[6]	29[6]	31.8[6]	22[6]	51	-	Galéa et al., 1996[2,3,4]
Treatment naïve vs. Treatment						
%	n	%	n			
67.0	45	35.0	17	62	(B) Italy	Ravanini et al., 2007[1,4]
56.8	88	21.3	159	247	(B) USA	Haslin and Chermann, 2007b[1,3,4]
38.0	50	2.0	50	100	(A) Ivory Coast	Kouassi et al., 2007[1,4]
71.6	88	43.1	160	248	(B) USA	Sanchez et al., 2008[4]
CD4 cell count:						
> 200 cells/µl vs. 0-200 cells/µl						
%	n	%	n			
59.1	22	13.1	61	83	(A) Cameroon	Tagny et al., 2007[1,4]
Years of HIV-infection (treatment naïve):						
		%	n			
< 5 years		14.3	-	-	(B) USA	Haslin and Chermann, 2007b[1,3,4]
5 - 10 years		50.0	-	-		
> 10 years		68.3	-	-		
0 - 5 years		~ 40.0	-	-	(B) USA	Sanchez et al., 2008[4]
5 - 10 years		~ 69.0	-	-		
10 - 20 years		~ 80.0	-	-		
< 5 years		68.0	41	62[8]	(B) Italy	Ravanini et al., 2007[1,4]
5 - 10 years		31.0	13			
≥ 10 years		63.0	8			

third study group Sanchez et al. (2008) noticed a direct correlation between R7V antibody ratio and viral load. On the other hand, no correlation between the R7V antibody ratios and the CD4 T-cell count was detected. Sanchez et al. (2008) also observed a higher prevalence of R7V anti-bodies in untreated patients (71, 6% n = 88) compared to patients on HAART (43.1% n = 160), Table 1. When treatment was considered successful, these authors re-

Table 1. Continues.

Ethnic groups:	%	n			
African Americans	49.5	106	501[2]	(B) USA	Haslin and Chermann, 2007b[1,3,4]
Haitians	38.0	100			
Asians	31.6	95			
Caucasians	26.2	84			
Indians	25.4	67			
Hispanics	22.4	49			
Sex:	%	n			
Males	55.0	61	106	(B) USA, African American	Haslin and Chermann, 2007b[1,3,4]
Females	44.4	45			

[1]Conference proceedings. 3rd South African AIDS Congress, Durban, June 2007.
[2]Calculation errors: The authors stated that 10 of 201 subjects equal 3%. However, 10 of 201 subjects equal 5%. Adding all the ethnic groups equals 501, 507 is stated as the total number by the authors.
[3]Galéa et al. (1996) and Haslin and Chermann (2007b) performed neutralizing assays with the R7V antibodies.
[4]The majority of studies were performed using the anti-R7V ELISA from Ivagen (Bernis, France) with the exception of Galéa et al. (1996) who used an "in-house" ELISA.
[5]Additional results in Sanchez et al. (2008): Long-term infected (more than 10 years) and naïve of treatment. Presence of R7V antibodies: ~ 80%, n not mentioned.
[6] Error: n = 29 stated in the text and n = 28 stated in the summary. Additional results in Galéa et al. (1996): presence of R7V antibodies in progressors: ~ 9% (n = 44).
[7]In Ergünay et al. (2008) all positive patients were on treatment. In Sanchez et al. (2008) 160 patients were on treatment and 88 patients were naïve of treatment.
[8]A sample number of 63 are stated in the poster. 18 of 63 patients were not on treatment.

ported a total disappearance in R7V antibodies for 77% (n = 21) of the patients. During antiviral treatment there is a decrease in newly formed virus particles budding from the host cell, it is therefore possible that fewer viruses containing the R7V epitope are produced in treated individuals. Sanchez et al. (2008) hypothesized that the R7V epitope may no longer be exposed on the virus and thus not visible to the host immune system after successful treatment and therefore suggested that the anti-R7V ELISA from Ivagen was better adapted to the detection of R7V antibodies in asymptomatic patients, still naïve of treatment.

Ergünay and colleagues (2008) following a smaller study (33 HIV positive and 10 HIV negative compared to the 160 patients on HAART in the Sanchez et al., 2008 study) also reported the presence of R7V antibodies in HIV-infected individuals on HAART (Table 1). Only 9.1% of 33 HIV positive individuals on treatment exhibited R7V antibodies and there was no correlation between the presence of the antibodies and disease progression. The Ergünay et al. (2008) report is in Turkish and the English abstract did not provide a description of the patients' disease status or duration of infection. It is difficult to directly compare the study done by Ergünay et al. (2008) and Sanchez et al. (2008) because the latter study used an HIV positive test group including patients both on HAART and not on treatment whereas the HIV positive test group in Ergünay`s study (2008) were all on HAART. The overall agreement between the works done by these groups is a decrease in prevalence of R7V antibodies in the presence of treatment.

A study reporting on the prevalence of the R7V antibodies in HIV-1 infected individuals on HAART was done by Professor Lynne Webber from the Department of Medical Virology, University of Pretoria, South Africa and presented at the HIV and AIDS Research symposium at the University of Pretoria in February 2009 (Webber, 2009). The prognostic applicability of the anti-R7V ELISA from Ivagen (Bernis, France) was examined using a cohort of 25 HIV-infected patients on HAART. Nine participants were classified as long term non-progressors (LTNPs), defined as patients free of HIV-1 related disease and displaying stable CD4+ T-lymphocyte counts (> 200 cells/µl for more than 10 years). The results indicated that 40% of all the HIV-infected patients and 56% of the LTNPs tested positive for R7V antibodies. Twenty eight percent of all the HIV-infected patients and 44% of the LTNPs were considered doubtful (data collected could not be classified as either positive or negative for R7V antibodies).

R7V Antibody and cross-reactivity

Data collected by Sanchez et al. (2008) demonstrated cross-reactivity of antibodies from individuals infected with other enveloped viruses when using the anti-R7V percent positive results in this study were limited. It is

possible that individuals not infected with HIV but infected with other enveloped viruses which may also incorporate β2m in their membrane and also expose the R7V epitope, could produce antibodies to this epitope. Sanchez et al. (2008) observed that a few individuals uninfected with HIV or infected with the enveloped viruses causing mono-nucleosis or rubella gave positive R7V antibody results using the Ivagen ELISA. Three of thirteen individuals (23.0%) infected with mononucleosis and six of eleven (54.5%) individuals infected with rubella responded positive for R7V antibodies (antibodies in the sera were able to bind the R7V antigen in an ELISA). Higher sample numbers are obviously needed to validate these data. Also these antibodies were not tested for an ability to neutralize or precipitate HIV-1 (which are properties of actual R7V antibodies) so it is possible that this response was due to some interference or cross-reactive antibo-dies. There is evidence that cytomegalovirus (CMV, McKeating et al., 1987) and HTLV (Hoxie et al., 1987) incorporate β2m. Since R7V is part of β2m this could mean that individuals infected with viruses other than HIV could test positive for R7V antibodies. However the acquisition of β2m by HIV and CMV differs. CMV acquires β2m after budding from the cell (in a non-HLA-like manner) but still binds MAbs to β2m (Grundy et al., 1987; Tysoe-Calnon et al., 1991; Le Contel et al., 1996). There is not enough evidence to form an opinion on whether CMV (containing β2m) could not induce an R7V like antibody response during infection.

Data collected using assays other than ELISA

Most studies referred to here present conclusions based on ELISA data only (where an R7V peptide was used as antigen). Whether the polyclonal antibodies believed to be R7V antibodies could neutralize or precipitate HIV-1 is not always indicated. Neutralization or precipitation of HIV-1 serves as a means of verifying an actual R7V antibody response. Better validation would be to purify the antibodies before use in either assay. Neither Galéa et al. (1996), Sanchez et al. (2008) nor Ergünay et al. (2008) performed precipitation assays. Of these three studies only Galéa et al. (1996) demonstrated that the presence of R7V antibodies correlated with neutralization of various divergent HIV strains. A study done by Xu et al. (2002) used both ELISA and precipitation to investigate the prevalence of R7V antibodies in HIV-infected patients. However, that data is not referred to in this review because the article is in Chinese and the English abstract does not give information about sample numbers or percentages of R7V antibodies. Since R7V antibodies neutralized divergent HIV-1 strains and this neutralization was reversed by addition of R7V peptide (Le Contel et al., 1996). Galéa et al. (1996) hypothesized that R7V antibodies found in patients could have equivalent neutralizing activity as that observed *in vitro*. Extensive experiments with the two main target cells of HIV-1 infection, peripheral blood lymphocytes and blood derived macrophages, have shown that different T-lymphotropic as well as macrophage-tropic HIV-1 strains was neutralized by β2m MAbs (Le Contel et al., 1996).

Evidence for R7V as potential vaccine target or therapeutic tool

Purified R7V antibodies from sera of rabbits injected with the peptide as well as in sera of non-progressor patients (HIV-1 subtype of infection virus not mentioned) precipitated and neutralized HIV-1 subtypes A, B, C, D and F (Galéa et al., 1999a). The results obtained by Galéa et al. (1999a) is supported by the studies done by Xu et al. (2002) who stated that R7V antibodies were found to inhibit the replication of HIV. Xu et al. (2002) also suggested that R7V antibodies prevent the virus from entering target cells by interfering with the binding of HIV to the co-receptors (CCR5 or CXCR4) of the target cell. In addition, human R7V IgG neutralized virus strains resistant to antiviral drugs and inhibited infection of cells by laboratory as well as primary viral isolates (Galéa et al., 1999b). Furthermore, studies done by Haslin and Chermann (2007b, poster presentation) showed R7V antibodies from HIV-1 subtype B infected individuals to be able to neutralize HIV-1 subtype D. Collectively the presence of R7V antibodies in serum from HIV-infected individuals from different geographic areas suggests that R7V is naturally immunogenic and escapes variability and flexibility observed with the viral proteins in the HIV envelope (Galéa et al., 1999a; Chermann, 2001). These studies suggest R7V-like epitopes to have a potential role in an HIV/AIDS vaccine and the R7V antibodies in the treatment of patients in failure of HAART.

Production of R7V antibodies with antiviral properties has been demonstrated by Haslin and Chermann (2004) and Haslin et al. (2007a). The antibodies were produced by infection of insect cells with a recombinant baculovirus in which the gene corresponding to the R7V antibody was introduced after isolation from EBV-immortalized B lymphocytes of non-progressor patients. These antibodies have been shown to be able to neutralize various clades of HIV-1 including drug-resistant viruses and should therefore be taken into consideration as therapeutic tools (Haslin and Chermann, 2004; Haslin et al., 2007a). There are several studies reporting on the prevalence of the R7V antibodies in HIV-1 infected individuals which were presented at the 3rd South African AIDS Congress in Durban in June 2007 (Ravanini et al., 2007; Haslin and Chermann, 2007b; Tagny et al., 2007; Kouassi et al., 2007). Ravanini et al. (2007) studied a group of HIV- infected patients living in Italy including 63 symptomatic (A class CDC, eighteen patients on antiretroviral, ARV, therapy) and 8 symptomatic patients (B or

C class CDC, 5 patients on ARV therapy). Haslin and Chermann (2007b) studied 507 HIV-infected (on HAART or not and 201 uninfected individuals from USA. The study done by Tagny et al. (2007) was conducted with a group of HIV-infected individuals naïve of ARV therapy and living in Cameroon including 22 asymptomatic (A1 and A2 class CDC 1993) and 61 symptomatic (A3, B or C class CDC 1993) patients. Lastly, 100 HIV-infected individuals (50 naïve of HAART and 50 on HAART) living in the Ivory Coast were used in the study done by Kouassi et al. (2007). Thirty six patients were at the clinical stage A according to CDC and classified asymptomatic and 53 patients classified symptomatic (CDC stage not stated). None of these four studies report on precipitation assays or any other validation study to confirm that the antibodies detected were R7V antibodies. These four studies are discussed in Table 1 with some additional observations described below. Haslin and Chermann (2007b) observed that R7V antibodies from HIV-1 subtype B infected patients were able to neutralize HIV-1 subtype D and that higher titres of R7V antibody plasma were more effective at neutralizing HIV-1, suggesting that the neutralizing potential most likely was due to R7V antibodies. A positive correlation between R7V antibody ratio and viral load for a group of asymptomatic patients naïve of ARV therapy (n = 45) was observed by Ravanini et al. (2007) and supported by similar observations by Sanchez et al. (2008).

A vaccine component should induce neutralizing antibodies and/or a cellular immune response depending on how it is presented to the immune system. Evidence summarized in this review demonstrates that R7V as antigen in animal studies induced neutralizing antibodies in rabbits (Galéa et al., 1999a). Recombinant MAbs to R7V neutralized viral isolates (Haslin and Chermann, 2004; Haslin et al., 2007a) and naturally produced R7V antibodies isolated from humans did the same (Galéa et al., 1999a and 1999b; Haslin and Chermann, 2007a). In addition, MAbs to β2m, the parent protein of R7V, also neutralized multiple viral strains (Arthur et al., 1992). This evidence supports large scale studies on either protein or peptide for induction of protective humoral immune responses. These studies should perhaps be preceded by studies demonstrating the extent to which β2m or R7V induce autoimmune responses.

R7V antibodies and autoimmunity

A lot still remains to be done with regards to the peptide R7V and what it could mean for prognosis of disease, therapeutics or HIV vaccine development. According to the patent of Chermann et al. (2006) vaccine research has been done with formulations of R7V conjugated to carrier proteins such as KLH and BSA. Because the epitope is host derived one may assume a concern of the possibility that R7V antibodies may initiate an autoimmune response. However, work by Galéa et al. (1996 and 1999a and b), Haslin and Chermann (2002) and Haslin et al. (2007a) suggest this not to be the case. Data are not shown in these papers but according to the discussion there appears to be no self recognition by R7V anti-bodies; no binding to the surface of human cells by purified R7V antibodies from either patients or immunized rabbits (Galéa et al., 1999b). Nor did the recombinant monoclonal R7V antibody made by Haslin et al. (2007a) bind to human cells. In addition, individuals with naturally high levels of R7V antibodies did not exhibit any autoimmune diseases (Galéa et al., 1996 and 1999b). This suggests that R7V antibodies produced inside an individual were virus specific and therefore not a problem to the host. If there are no autoimmune antibodies it could be due to the fact that the R7V peptide is presented to MHC class II (for antibody production) in the context of viral molecules. R7V anti-bodies would therefore not recognize a corresponding epitope on the surface of the host cells. Also, as men-tioned before, the R7V epitope is only visible to the immune system when this epitope is contained in HIV and not in the natural protein.

R7V and cellular immunity

People exposed to HIV mount an immune response which in some individuals slows down disease progression. Strong neutralizing antibodies (Watkins et al., 1996; Pilgrim et al., 1997; Richman et al., 2003; Wei et al., 2003) and strong cytotoxic T-lymphocyte (CTL) responses (Wagner et al., 1999; Cao et al., 1995; Klein et al., 1995) have been detected in LTNPs. This means that immune responses slow down disease progression and is certainly of value for prognosis of disease and vaccine development. An HIV vaccine should presumably induce humoral and cellular responses. Neutralizing antibodies as part of a humoral response should eliminate cell-free virus. Cellular immune responses (e.g. CD8+ CTLs) on the other hand should remove already infected cells that escape antibody-mediated neutralization (Lemckert et al., 2004). If R7V is to be considered in vaccine research, knowledge of possible cellular immune response epitopes within it is of importance. CTL responses are mostly related to viral core proteins (Buseyne et al., 1993; Cao et al., 1997; Nakamura et al., 1997; McAdam et al., 1998), but it has been observed with some viral envelope proteins as well (Pinto et al., 1995). R7V (even though it is derived from a protein associated with and important for MHC class I presentation) is recognized as a foreign antigen by the host and induces a humoral immune response as is evident in all the reports on R7V antibodies. It would be interesting to know whether virus associated R7V-like epitopes are capable of inducing a cellular immune response especially given its β2m origins and the role of this protein in MHC class I presentation of antigen. MHC class I molecules are known to bind epitopes ranging from eight to eleven amino acids (Karim and Karim, 2005) and therefore might

bind the seven amino acid R7V peptide for presentation during natural infection. No HIV incorporated host proteins have yet been implicated in CTL responses nor has this been exhaustively investi-gated.

CONCLUSION

R7V antibodies have been suggested as a potential therapeutic tool (Haslin and Chermann, 2002, 2004 and 2007b; Haslin et al., 2007a) since 2002. To date no studies report on passive infusion of animal models (or human volunteers) with these R7V antibodies. Nor have recombinant R7V antibodies (Haslin and Chermann, 2004; Haslin et al., 2007a) been used in *in vivo* therapeutic studies. Passive infusion of animals (Binley et al., 2000; Mascola et al., 2000 and 2003; Mascola, 2002) or humans (Armbruster et al., 2004) with HIV antibodies is not uncommon. Suggestions of a synthetic R7V peptide being considered as a vaccine has been made since 1996 (Le Contel et al., 1996; Galéa et al., 1999a and b; Chermann, 2001; Haslin and Chermann, 2007b). No studies evaluating the *in vivo* value of R7V antibodies have yet been reported. By this we mean eliciting *in vivo* neutralizing antibodies in animal models (using synthetic R7V as antigen) and challenging this response with live virus. Subsequently the literature also does not report on any R7V based (phase I/II) vaccine trials. It appears that vaccine development studies have been done and some of it has been published as patents (Chermann et al., 2000 and 2006). Because the prevalence of the R7V antibodies was shown to correlate with non-progression to AIDS it has been postulated that patients who have elevated levels of R7V antibodies have a lower likelihood of progression to AIDS. Further studies are needed to clarify the use of R7V antibodies as possible prognostic markers. The presence of these antibodies in uninfected individuals needs to be clarified as well since it has implications for the former statement. Finally, whether the R7V epitope or the entire β2m protein is incorporated by other or all enveloped viruses has implications for the use of the epitope or antibodies to it in prognosis or therapy. Limited sample number studies commenting on possible cross-reactivity between R7V antigen and antibodies from other enveloped viruses exist but needs to be expanded. There are similarities between HIV and HTLV and there are reports of non-random incorporation of host proteins by the latter which further supports clarifying the extent to which β2m or R7V is incorporated by other viruses.

R7V and antibodies to the epitope holds promise but more extensive and clarifying autoimmunity and cross-reactivity studies are needed to maintain optimism on its use. That a host epitope when incorporated in a viral envelope is immunogenic has been sufficiently demonstrated. What remains to be rigorously shown is the usefulness of the immunogenicity (challenging the neutralisation response with live virus) after autoimmunity due to these host antigens have been shown to not be a concern.

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