Role of antibodies specific for the V3 region of gp120 in HIV-1 infection

by

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SUMMARY

On infection with HIV-1, the host exerts a highly specific immune response against the virus. Despite this, HIV-1 is able to persist in the host, resulting in the gradual deterioration of the immune system, leading to opportunistic infections characteristic of HIV-infection and ultimately death of the individual. To elucidate the mechanisms involved in the pathogenesis and persistence of the virus, it is important to understand what role if any, the immune response generated against the virus has in the control of HIV-1 infection or progression to disease.

This thesis describes the development of branched peptides for use in the detection of specific antibodies and describes two studies measuring the antibody responses of HIV-1 infected individuals against the V3 immunodominant region of HIV-1 gp120.

Branched peptides had previously been shown to be more immunogenic in rabbits and can detect antibodies at lower concentrations than can the equivalent monomeric peptides. The initial work described in this thesis, was designed to further characterise the observed increased sensitivity of branched peptides, to investigate the basis for it and to optimise branched peptides for use as serodiagnostic reagents. A series of experiments were performed to test the effect of varying the distance between the epitope and the core by introducing glycine spacers. The sensitivity of branched peptides in detection of specific antibodies was also compared with that of the native protein. Two systems were studied. The first, involved the HSV-1 UL42 protein and reactive monoclonal antibody Z1F11. The second, involved a peptide derived from the third hypervariable region (V3) of the HIV-1 gp120 protein. These experiments clearly demonstrate that the progressive addition of glycine spacers between the reactive epitope in UL42 and the polylysine core increased the reactivity of the branched peptides with Z1F11. In addition , optimal reactivity of the UL42 peptides with monoclonal antibody Z1F11, was observed with the addition of four or five glycine residues and further addition of glycine residues did not significantly increase the

sensitivity of these peptides. Comparison of the reactivity of the branched peptide with a five glycine spacer and the native UL42 protein demonstrated that both were equal in reactivity with monoclonal antibody Z1F11. The branched peptide derived from the V3 region of HIV-1 gp120 was eight-fold more sensitive than the native protein in detection of antibodies in the sera of HIV-1 infected individuals.

The second study presented in this thesis was undertaken to investigate whether the levels and affinities of antibodies present in infected mothers, directed against the V3 region of HIV-1 gp120 correlated with a lack of transmission of the virus from mother to child as had been previously reported. To do this, a retrospective study of the antibody responses in the sera taken from a cohort of seven mothers who transmitted HIV-1 to their children and 20 who did not, was performed. Maternal sera were titrated in a series of doubling dilutions and tested in an ELISA against both a V3 branched peptide encompassing the entire V3 loop region (amino acids 297-330) and a branched peptide containing an immunodominant region of gp41 (amino acids 596-614). Anti-gp41 titres were used to normalise the anti-V3 titres. Maternal sera were also screened for the presence of antibodies that bind to the V3 peptide with high affinity, using an antigen limited ELISA, in which the monomeric V3 peptide was titrated in a series of dilutions and screened for reactivity against the sera. A high affinity antibody would be detectable in ELISA at low peptide concentrations. No differences were observed in either titres or affinities of maternal antibodies to the V3 sequence from transmitters and non transmitters of HIV-1 to their children.

The third study was undertaken to test the hypothesis that the sequence variation that occurs in the principal neutralising determinant of HIV-1 gp120 (V3) represents antibody escape mutants. The reactivity of successive sera from patient 82, a member of the Edinburgh haemophiliac cohort with his previously identified variants found within the V3 region of HIV-1 gp120 during the natural course of infection was studied. Twelve branched peptides with a four glycine spacer, encompassing the immunodominant region of V3 were synthesised. These peptides correspond to the initial seroconversion sequence and to all subsequent variants identified in patient 82

over a seven year period. A further five branched peptides were synthesised, each containing just a single substituted amino acid of the multiple substitutions. Seven serum samples, collected over a seven year period from seroconversion, were tested in an ELISA to obtain their antibody titres against all of the peptides. The data indicates that patient 82 had high titres of antibodies against most of the V3-variants before they had been detected in the circulating virus population. All the sera showed very little reactivity against two of the peptides, nevertheless these sequences did not persist in the viral population. These data suggest that the newly-emerging replication-competent V3 variants do not represent V3-specific antibody escape mutants.

ABBREVIATIONS

AIDS	acquired immune deficiency syndrome
	acquired immunodeficiency syndrome
ADDC	antibody-dependent cell mediated cytotoxicity
agm	African green monkey
ARV	AIDS-related virus
AZT	zidovudine, 3'-azido-3' deoxythymidine
Boc	butyloxycarbonyl
BSA	bovine serum albumin
C-	carboxy-
^{0}C	degrees centigrade
CAEV	caprine encephalitis virus
CAT	chloramphenicol acetyltransferase
CDC	Centres for Disease Control
cDNA	complimentary deoxyribonucleic acid
cpz	chimpanzee
CRB	Cambridge Research Biochemicals
CTL	cytotoxic T lymphocytes
DCM	dichloromethane
ddC	zalcitabine, 2'3'-dideoxycytidine
ddI	didanosine, 2'3'-dideoxyinosine
DHBT	3,4-dihydro-3-hydroxy-4-oxobenzotriazin-3-yl
DMF	dimethyformamide
DNA	deoxyribonucleic acid
EDT	ethanedithiol
EDTA	ethlenediaminetetracetic acid
EIAV	equine infectious anaemia virus
ELISA	enzyme-linked immunosorbant assay

EMS	ethylmethylsulphide
env	envelope
FAB	fast atom bombardment
Fc	crystallisable fragment of immunoglobulin
FIV	feline immunodeficiency virus
FMDV	foot-and-mouth disease virus
Fmoc	9-fluorenyl-methoxycarbonyl
FPLC	fast performance liquid chromatography
Gal C	galactosyl ceramide
gp	glycoprotein
GM-CSF	granulocte/macrophage-colony stimulating factor
GTP	guanosine-5'-triphosphate
HAM	human T-cell leukemia virus associated myelopathy
HCMV	human cytomegalovirus
HEPES	N-2 hydroyethylpiperazine-N'-2-ethane sulphonic acid
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HOBt	1-hydroxybenotriazole
HPLC	high pressure liquid chromatography
HRP	horseradish-peroxidase
HSV-1	herpes simplex virus type-1
HTLV	human T-cell leukemia virus
Ig	immunoglobulin
ID	infectious dose
Kb	kilobase
KDa	kilodalton
LAV	lymphoadenopathy associated virus
LTR	long terminal repeat
mA	milliamps

MAb	monoclonal antibody
mac	macaque
MAP	multiple antigenic peptide
MHC	major histocompatibility complex
mM	millimolar
mmol	millimoles
MMTV	mouse mammary tumour virus
mnd	mandrill
m.o.i.	multiplicity of infection
mRNA	messenger ribonucleic acid
Mtr	methoxytrimethylbenzene sulphonyl
MuLV	murine leukemia virus
N-	amino-
ng	nanograms
nm	nanometres
NNRTI	non-nucleoside reverse transcriptase inhibitors
NSI	non syncytium inducing
OD	optical density
p	protein
PBLs	peripheral blood lymphocytes
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCP	pneumocystis carinii pneumonia
PCR	polymerase chain reaction
PFP	pentafluorophenyl
pfu	plaque forming units
PGL	persistent generalised lymphoadenopathy
%	percentage
Pmc	pentamethylchromane sulphonyl

PMSF	phenylmethylsulphonyl
PND	principal neutralising determinant
pol	polymerase
PyBOP	benzotriazolyloxy-tris[pyrrolidino]-phosphonium-hexafluorophoshate
RNA	ribonucleic acid
RNase H	ribonuclease H
rpm	revolutions per minute
RRE	REV response element
sCD4	soluble CD4
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sf	spodoptera frugiperda
SI	syncytium inducing
SIV	simian immunodeficiency virus
sm	sooty mangabey
SV5	simian virus five
TAR	transactivator response element
TBP	TATA binding proteins
TBS	tris buffered saline
TEMED	N,N,N'N-tetramethylethylenediamine
TFA	trifluoroacetic acid
TIBO	tetra hydro-imadazo[4,5,1-1-jk][1,4]-benzodiazepin-2H(1H)-one and thione
TIPS	triisopropylsilane
TMSB	trimethylsilylbromide
Tris	tris (hydroxymethyl)-amino-methane
tRNA	transfer ribonucleic acid
Trt	trityl
TSP	tropical spastic paraparesis

ug	micrograms
ul	microlitres
UL42	product of HSV-1 gene UL42
UV	ultraviolet
V	hypervariable
VSV	vesicular stomatitis virus

ONE AND THREE LETTER ABBREVIATIONS FOR AMINO ACIDS

Amino acid	Three letter code	One letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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Optimisation of branched peptides for use as serodiagnostic reagents

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Do antibodies play a role in selecting sequence variants within the V3 region of HIV-1 gp120

Chapter 1 INTRODUCTION

1.1 The Retroviruses

1.1.1 Description and origin

Viruses with RNA as genetic material are found in a wide variety of host species. They are designated retroviruses when they exhibit the ability to copy their RNA genome into double stranded DNA, that can be integrated into the DNA of an infected cell. This is catalysed by an RNA-dependent DNA polymerase or reverse transcriptase (RT) (Baltimore 1970, Temin and Mitzutani, 1970).

Although retroviruses have very different properties and pathogenicities they are structurally and genetically very similar. A retrovirus virion contains two identical copies of a plus strand RNA genome, 7,000-10,000 nucleotides long, enclosed in a nuclear core. This core is surrounded by a proteinaceous capsid layer which is in turn enclosed by a lipid bilayer in which are embedded the glycoproteins to form the 'knobs or spikes' of the membrane surface. The basic genes common to all retroviruses fall into three groups, shown in Figure 1.1: The *gag* gene encodes the structural proteins required by the virion, the *pol* gene encodes the reverse transcriptase, Rnase H, protease and integrase proteins and the *env* gene codes for the surface glycoproteins. When the virus is in the proviral state these genes are flanked by nucleotide sequences forming the long terminal repeats (LTR's) shown in Figure 1.1. The LTR sequences contain promoter and enhancer elements that govern transcriptional initiation and regulation and are also required for integration into the host cellular DNA. A number of other open reading frames (ORF's), specific for each retrovirus are present between the *gag, pol* and *env* genes. These play a role in regulation of viral gene expression, virion maturation and infectivity.

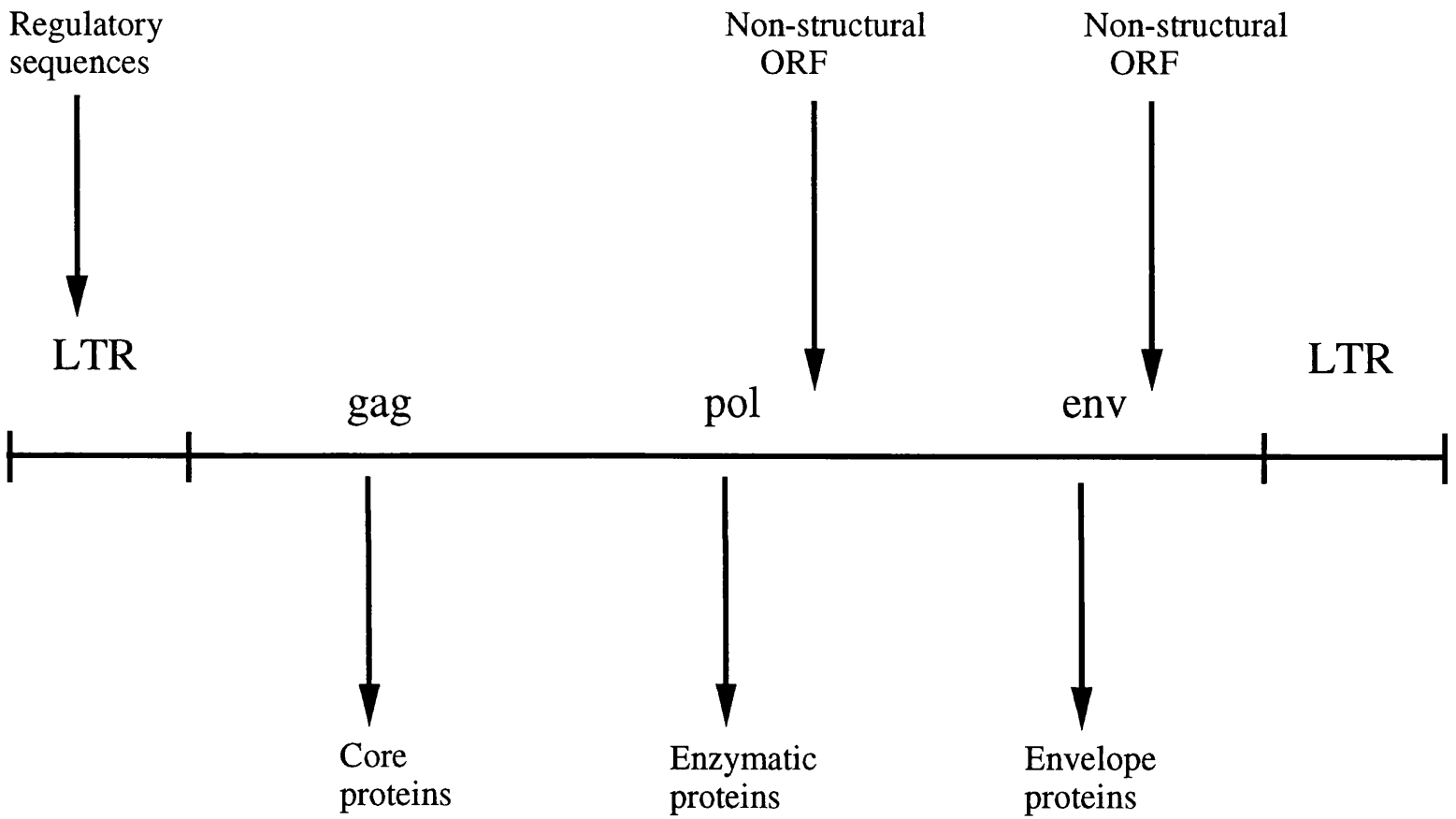


Figure 1.1 Basic proviral structure of the retroviral genome. The gag, pol and env genes are common to all retroviruses. The non-structural open reading frames are present only in complex retroviruses.

Two subtypes of retroviruses have been identified. Retroviruses that exists as viral sequences, usually carried in the germline and mainly defective for replication of infectious virus, are known as endogenous retroviruses. These retroviruses are passed in the germline from generation to generation in a Mendelian fashion. Exogenous retroviruses, in contrast, move freely from cell to cell by the production of infectious virus particles (reviewed by Doolittle *et al.,* 1989). Despite the differences in life cycle, endogenous and exogenous retroviruses share considerable sequence homology, which is perhaps surprising, as firstly, the present day strains of exogenous retroviruses, such as HIV appear to have been only recently introduced, whereas endogenous sequences are deeply rooted in primate evolution (Steele *et al.,* 1986; Shih *et al.,* 1991). Secondly, the mutation rate of exogenously replicating retroviruses is at least 10^5 times greater than that of endogenously replicating retroviruses.

To account for this observed sequence similarity it has been hypothesised that exogenous retroviruses exist as relatively short lived bursts (lasting upto decades or centuries) arising spontaneously from a backdrop of germline encoded endogenous retroviruses. Therefore the pool of endogenous retroviral sequences periodically contributes to the generation of exogenous virus (Doolittle *et al.*, 1989). The discovery of human immunodeficiency virus type 1, human T cell leukemia virus type 1 and type 2-like endogenous sequences, found in normal uninfected individuals provides supporting evidence for this model (Shih *et al.,* 1989; Horwitz *et al.,* 1992).

1.1.2 Classification of retroviruses

The family **retrovirinae** can be classified by a number of biological and morphological characteristics. Historically they have been divided into three taxonomic groups based on the *in-vivo* consequences of infection. (Teich, 1982, 1985)

Oncovirinae: These retroviruses can induce tumour formation upon infection. They can exist as exogenous viruses carrying *onc* genes derived from host DNA and directly

coding a protein involved in neoplastic transformation. Infection with this type of retrovirus usually results in rapid development of malignancy. Examples include Rous sarcoma virus (RSV) and Murine leukemia virus (MuLV).

Some exogenous oncoviruses can induce tumour formation without carrying a cellular oncogene, but rather express a viral protein that is directly involved in neoplastic transformation, therefore behaving more like a DNA tumour virus. Examples include the human T cell leukemia virus-1 (HTLV-1).

The oncoviruses can also exist as endogenous genetic material that is passed through the germline in a Mendelian fashion. An example of this type is the Mouse mammary tumour virus (MMTV), which can also exist as exogenous virus passed from mother to off-spring in breast milk.

Lentivirinae: These exogenous, non-oncogenic viruses are mainly found infecting ungulate mammals, in particular sheep (Visna virus), goats (Caprine Arthritis Encephalitis Virus, CAEV), cattle and horses (Equine Infectious Anaemia Virus, EIAV). Infection with a lentivirus is characterised by the slow appearance of the diseased state. In addition, infection with some types as mentioned above causes a slow progressive degenerative disease of the central nervous system. This group has been extended to include the immunodeficiency viruses found infecting cats (FIV), humans (HIV) and non-human primates (SIV) that cause a slow progressive degeneration of the immune system ultimately resulting in death.

Spumavirinae: These viruses, known as 'foamy' viruses induce persistent infection but are generally believed to be non-pathogenic. An example of this group is Simian foamy virus five (SFV5)

The retroviruses can be further classified on the basis of their morphology under electron microscopy into types A, B, C and D.

Type A: produce an immature form and are only found intracellularly, whereas all other types bud from the host cell membrane.

Type B: these have an eccentric core on budding, for example MMTV.

Type C: have a central spherical core on budding, for example FeLV, HTLV-1 and HTLV-11.

Type D: have a cylindrical central core such as the Mason Pfizer monkey virus. This group has been extended to include FIV, HIV and related primate lentiviruses that have a conical section shaped central core (Levy *et al.*, 1984).

Retroviruses may also be classified as being 'simple' or 'complex' depending on their mechanism of viral gene expression (reviewed by Cullen, 1991, 1993 in press). Simple retroviruses rely entirely on host DNA sequence specific transcription factors to regulate the level of proviral transcription. Complex retroviruses in contrast, encode potent transcriptional activators of their LTR promoter elements. All human retroviruses fall into the 'complex' retrovirus category.

1.2 Retroviral life cycle

This section describes in general the life cycle of the retroviruses, referring in particular to the human retrovirus HIV-1. A diagramatic representation of the retrovirus life cycle is shown in Figure 1.2

1.2.1 Attachment and entry

The first step in the retroviral replication cycle is the specific interaction of the outer membrane component of the virion glycoprotein with a specific cell-surface receptor. The receptor(s) therefore determine the target cell types and tissue tropism of the virus. After receptor binding occurs, the virion must enter the cell by a process of membrane fusion (reviewed by Marsh and Helenius, 1989) and it is believed that the hydrophobic amino terminus of the transmembrane component of the viral envelope (gp41 for HIV-1) mediates this process (Kowalski *et al.*, 1987; Freed *et al.*, 1990). A series of complex conformational changes in the virus receptor complex is required to

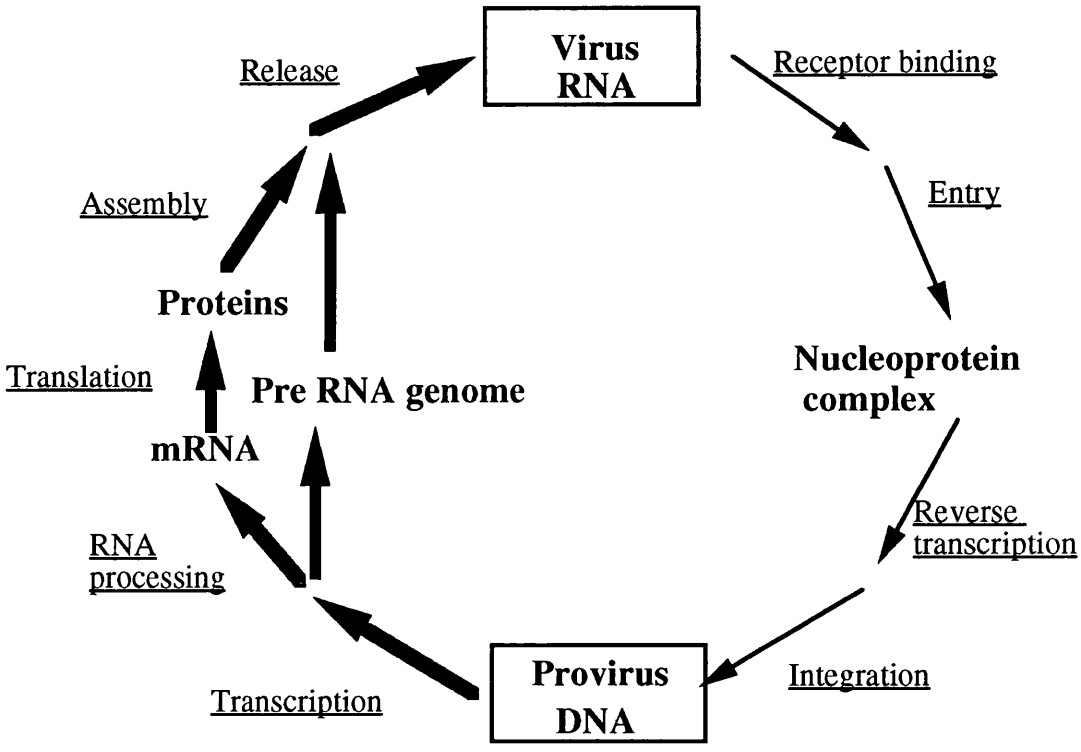


Figure 1.2 Adapted from B.R. Cullen, Human Retroviruses,in press. An overview of the retroviral replication cycle. Steps prior to proviral integration are indicated by light arrows: post integration events are denoted by thick arrows.

initiate this step. Retroviruses are known to enter cells via a pH-independent process (Maddon *et al.*, 1988; McClure *et al.*, 1988, 1990). Therefore the conformational shift required is not induced by exposure to low pH: one possible mechanism leading to membrane fusion could be a further interaction with a second cell-surface protein. A proteolytic cleavage site within the third hypervariable region (V3) of HIV-1 gp120 (discussed in detail later) also appears to be essential for viral fusion with the host cell membrane (Clements *et al.*, 1991).

1.2.2 Viral uncoating

After fusion with the host cell membrane, the retroviral virion undergoes a process of uncoating, resulting in the complete or partial loss of the envelope proteins and virion membrane. The 'nucleoprotein complex' is then competent to initiate the process of reverse transcription (Varmus and Brown, 1989). Essentially very little is known about retroviral uncoating *in-vivo* however, the recent identification of compounds that inhibit the process of HIV-1 uncoating in culture indicate that the process may be accessible to biochemical analysis (De Clercq *et al.*, 1992).

1.2.3 Reverse transcription

The next step in the replication cycle is the initiation of reverse transcription of the retroviral plus strand RNA genome, contained within the viral nucleoprotein complex. This process usually occurs in the cell cytoplasm. The primer for reverse transcription is a tRNA molecule that is specifically packaged into the virion. Different retroviruses use different tRNA molecules as their primers. The HIV-1 tRNA primer is $tRNA^{LYS}$ (Varmus and Brown, 1989).

The complex process of reverse transcription is carried out by the retroviral enzymes, reverse transcriptase and RNaseH, both encoded by the *pol* gene (Varmus and Brown, 1989; Varmus and Swanstrom, 1985). The final result is the conversion of

a single stranded RNA molecule into a double stranded DNA proviral intermediate. This process regenerates the long terminal repeats (LTR's), containing the promoter and enhancer elements required for viral gene expression. The mechanism involved in reverse transcription of the retroviral genome is highly conserved within the family retrovirinae.

An important aspect of the process of reverse transcription is that it provides a major source of rapid generation of genomic variability. This genomic variation is particularly apparent in the immunodeficiency viruses and is a real problem for the development of effective vaccines (Coffin, 1986a, b; Katz and Skalka, 1990; Hu and Temin, 1990).

During reverse transcription the reverse transcriptase enzyme jumps between the two strands of viral RNA carried in each retrovirus virion particle leading to the generation of a single proviral DNA intermediate (Katz and Skalka, 1990; Hu and Temin, 1990). However, random strand switches can also occur between the two copies of the viral RNA (Katz and Skalka, 1990; Hu and Temin, 1990). As a consequence of this random and non-random strand switching the RNA is highly resistant to agents designed to damage it, since any damage can potentially be avoided by transfer of the reverse transcriptase enzyme to the undamaged strand. This process also facilitates the high rate of recombination observed between similar but distinct retroviral genomes that may exist in dually-infected cells.

In the case of the lentiviruses HIV-1 and Visna virus, the persistence of virus in the face of the immune response mounted by the host and slow evolution of the disease can be partly explained by restriction in viral gene expression imposed at the transcriptional level. Most of the cells that harbor virus genomes have insufficient antigen to be detected and destroyed by immune surveillance, and limitation in the synthesis of virus gene products allows the virus to persist in the cells for extended periods, a feature characteristic of slow infections.

1.2.4 Integration into the host genome

After replication, the next step is the precise integration of the linear proviral DNA into the genome of the host cell (reviewed by Varmus and Brown, 1989). This process requires an interaction of the viral integrase protein, the third gene product of the *pol* gene and the two inverted DNA repeats (LTR's) one copy of which is present at each end of the proviral genome (Brown *et al.*, 1987, 1989; Katz and Skalka, 1990). The site at which the proviral genome is integrated into the host cell DNA appears to be essentially random. It has been suggested that in the case of Visna virus and SIV that proviral integration into the host genome may not be an essential step in the retroviral replication cycle (Harris *et al.*, 1984; Prakash *et al.*, 1992). Proviral unintegrated DNA from HIV-1 has been shown to be an efficient template for transcription of both core and envelope proteins but integration is necessary for productive infection of HIV-1 (Stevenson *et al.*, 1990a).

1.2.5 Control of viral gene expression

Expression of the integrated provirus is controlled by *cis*-acting promoter and enhancer elements that act as targets for cellular and viral regulatory proteins (Varmus and Brown, 1989; Cullen, 1991). In simple retroviruses, expression is controlled by cellular proteins alone whereas, complex retroviruses encode their own potent transcriptional activators. In the absence of these proteins each of the LTR's induce only a low or basal level of proviral transcription that is sufficient to maintain a productive retroviral replication cycle. Regardless of whether a complex or simple retrovirus, efficient gene expression is dependent on host cellular transcription factors.

Transcription gives rise to a single genome length initial transcript that also serves as the messenger RNA for the *gag* and *pol* gene products. All retroviruses encode a singly spliced transcript for the envelope proteins. In contrast to the 'simple' retroviruses, 'complex' retroviruses express several additional multiply spliced mRNA

species which encode the various regulatory and auxiliary proteins required by the virus. A summary diagram of HIV-1 and HTLV-1 gene regulation is shown in Figure 1.3.

Extensive post-transcriptional RNA processing occurs, controlled in complex viruses by virally encoded regulatory proteins. This protein, termed Rev in HIV-1 and Rex in T cell leukemia viruses, is required for the cytoplasmic expression of the incompletely spliced mRNA's that encode the retroviral structural proteins. The mRNA encoding the retroviral regulatory proteins is expressed in a Rev/Rex-independent manner (Greene and Cullen, 1990).

In simple retroviruses there is little or no temporal regulation, since proviral expression and the pattern of viral mRNA processing is governed by the availability of the cellular proteins. In contrast, gene expression in complex retroviruses is subject to marked temporal regulation since it is also controlled by viral regulatory proteins (Greene and Cullen, 1990).

At early times after infection, the provirus gives rise to low levels of viral messenger RNA mediated by cellular transcription factors. Subsequent expression of the transcriptional transactivator protein Tat (HIV) results in a positive feedback loop (Figure 1.3). At this time, a critical level of the Rev-like post transcriptional regulatory protein is achieved, thereby inducing expression of the viral structural proteins and subsequent release of progeny virus. This pattern of temporally regulated gene expression, featuring early regulatory and late structural phases is also characteristic of DNA tumour viruses such as the human papilloma viruses.

1.2.6 Assembly and release

The last step in the retrovirus replication cycle is the formation and release of mature virions. The precise mechanisms are not fully understood but the process occurs adjacent to the plasma membrane and involves the ordered assembly of the Gag and Gag-Pol capsid precurser polyproteins (Varmus and Brown, 1989) which in the case of

HIV-1/HTLV-1

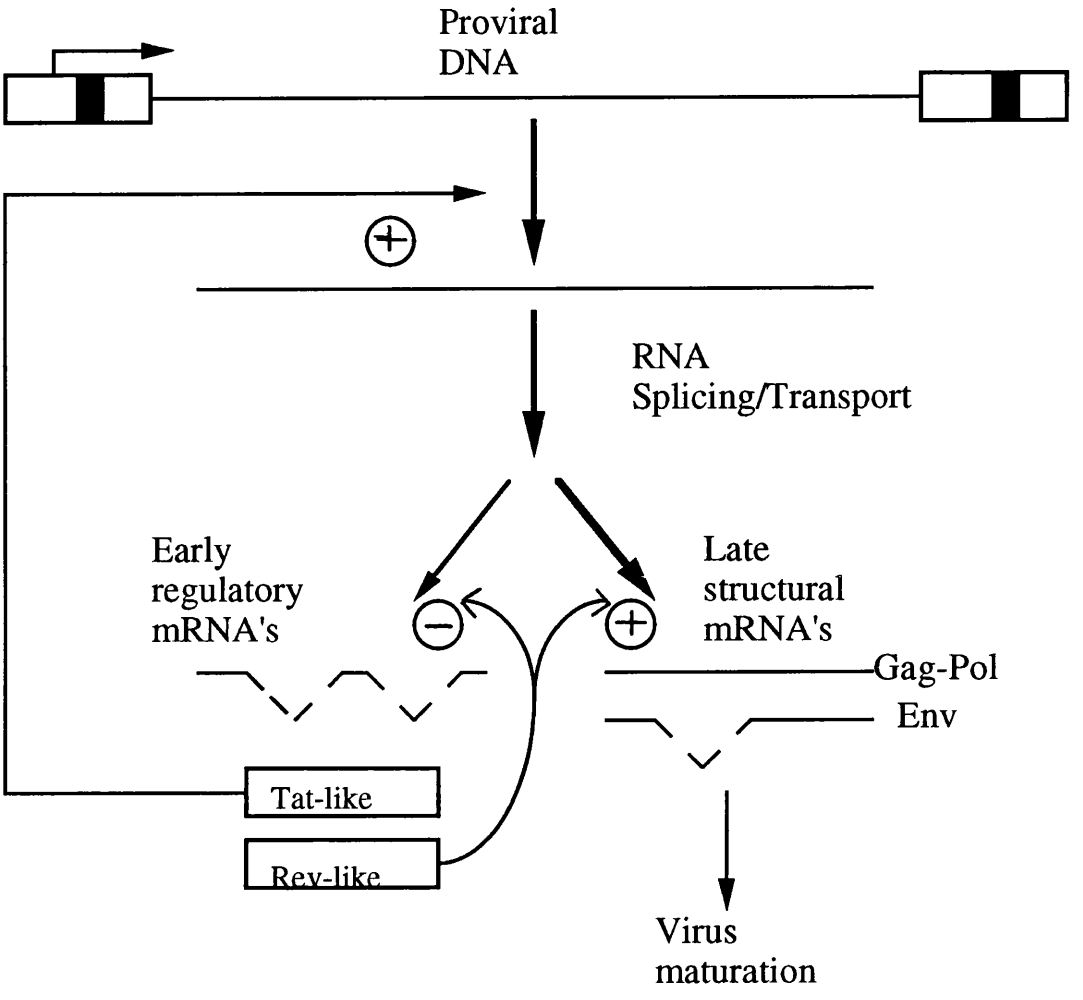


Figure1.3 Adapted from Green and Cullen (1990): An overview of the regulation of HIV-1 and HTLV-1 gene regulation. Infection first leads to a low level of viral RNA synthesis mediated by various host cell factors. The early viral mRNA's initially produced encode the viral regulatory proteins including Tat and Rev in the HIV-1 system and Tax and Rex in the HTLV-1 system. Tat and Tax serve as potent transcriptional activators of all genes and permit the 'late' structural gene expression important for virus maturation and assembly of infectious virions. This 'early' regulatory to 'late' structural transition is specifically controlled at the post-transcriptional level by the HIV-1 Rev and the HTLV-1 Rex proteins that regulate RNA transport and splicing. The sequence specific action of Rev and Rex induces cytoplasmic expression of the unspliced and singly spliced viral mRNA that encode the *gag*, *pol* and *env* gene products. Simultaneously, Rev and Rex down -regulate expression of the multiply spliced early regulatory mRNAs and thus inhibit their own production.

HIV-1 are targeted to the host cell membrane by myristylation of their amino-termini (Gottlinger *et al.*, 1989). During assembly two copies of the single stranded viral RNA genome are incorporated into the virion. This, in the case of HIV-1 involves the sequence-specific association of a zinc-finger nucleic acid-binding motif located in the nucleocapsid component of the viral gag protein (p7) with a packaging signal in the leader sequence of the genomic RNA (Aldovini and Young, 1990). Subsequently retroviral cores bud through regions of the plasma membrane bearing high levels of the envelope glycoproteins. During or shortly after budding the viral protease becomes activated, resulting in the highly ordered and specific cleavage of the Gag and Gag-Pol structural proteins and viral enzymes (Skalka, 1989). These newly released virus particles can then themselves infect available target cells.

1.3 Biology and epidemiology of human retroviruses

1.3.1 Human T cell leukemia virus type-1 (HTLV-1).

Human T cell leukemia virus was the first retrovirus to be found infecting humans and was isolated from established cell lines derived from an American patient with a cutaneous T cell lymphoma (Poiesz *et al* ., 1980). At approximately the same time a similar virus was isolated from a Japanese patient suffering from Adult T cell Leukemia (ATL) (Yoshida *et al* ., 1982). Both these viruses showed extensive sequence homology and collectively became known as human T cell leukemia virus type-1 (HTLV-1). Infection with HTLV-1 is known to cause adult T cell leukemia, a malignancy of mature $CD4^+$ T lymphocytes (Uchiyama *et al.*, 1977) and is also associated with a slowly progressive myelopathy, characterised by paraparesis and spasticity of the lower extremities, known as Tropical Spastic Paraparesis (TSP) (Gessain *et al.*, 1985) or HTLV-1 Associated Myelopathy (HAM) (Osame *et al.*, 1986). This disease can be referred to as TSP/HAM.

Identification of HTLV-II in intravenous blood donors in both San Francisco and Northern California has also recently been reported (Feigel *et al.*, 1991).

Infection with HTLV-1 is now endemic in South Western Japan, the Caribbean islands and parts of Central and Eastern Africa (Wong-Staal and Gallo, 1985). In addition HTLV-1 has been identified in populations within South Eastern and South Western United States, South America, the Philippines, Europe and the Middle East. (Manns and Blattner, 1991).

1.3.2 Human T cell leukemia virus type-II (HTLV-II)

Another virus sharing 65% nucleotide homology to HTLV-1 was isolated from an American patient suffering from a rare, relatively benign, T cell variant of hairy cell leukemia (Kalyanaraman *et al.*, 1982) and later from another American patient with both a T cell lymphoproliferative disease and B-cell type hairy cell leukemia (Rosenblatt *et al.*, 1988). This virus has been named HTLV-11.

The geographic distribution of HTLV-11 is still relatively unknown and as yet there is no consistent disease association with infection with HTLV-II. Serological evidence of HTLV-II has been found in intravenous drug users in the UK (Tedder *et al.*, 1984) and New Orleans (Lee *et al.*, 1989) and more recently in an isolated population of Guaymi Indians in Panama. (Lairmore *et al* ., 1990).

1.3.3 The human immunodeficiency viruses (HIV)

Infection with HIV results in a progressive disease with severe and irreversible immunosupression and acquired immune deficiency (AIDS) leading to eventual death. This chronic immunosupression results in a host defect that renders the body highly susceptible to opportunistic infection and malignant tumours associated with severe defects of cell-mediated immunity (Gottlieb *et al.*, 1981; Masur *et al.*, 1981; Nelson *et al.*, 1990). AIDS patients develop a range of infections such as persistent cytomegalovirus infection (CMV), persistent generalised lymphoadenopathy (PGL), Kaposi's sarcoma, a relatively rare cancer of the skin and pneumocystis carinii

pneumonia (PCP). Another feature of HIV-infection is that at least 50% of all AIDS patients develop encephalopathy resulting in memory loss, impaired speech and dementia (Price *et al.*, 1986; Navia *et al.*, 1986a, b).

The first cases of AIDS were described among homosexual men (Gottlieb *et al.*, 1981; Masur *et al.*, 1981; Siegal *et al.*, 1981). Following these reports AIDS cases were identified among haemophiliacs (CDC report, 1982a) , blood transfusion patients (CDC report, 1982b), heterosexual intravenous drug users IVDU's (CDC report, 1982c), infants born to infected mothers (CDC report, 1982d) and partners of infected individuals (CDC report, 1982e).

The causative agent of acquired immune deficiency syndrome (AIDS) was first identified by Montagnier and coworkers in 1983 (Barre-Sinoussi *et al.*, 1983). They isolated a retrovirus from a patient suffering from chronic lymphoadenopathy, thus the original name lymphoadenopathy associated virus, (LAV). At around the same time two other groups identified a human retrovirus in HIV-infected individuals named human T cell leukemia virus type three (HTLV-III) (Gallo *et al.*, 1984) and AIDS related virus (ARV) (Levy *et al.*, 1984). HIV-1 has been adopted as the accepted nomenclature by the International Committee of the Taxonomy of Retroviruses (Coffin *et al* ., 1986a, b) and is found infecting individuals in Southeast Asia, parts of Africa, America and Europe. Up to the end of 1991, it was estimated that approximately 9-11 million people have been infected with HIV worldwide with 1.5 million of these people already diagnosed as having AIDS (Chin, 1991). This number is continuing to rise with countries in Africa and Southeast Asia being the worst affected.

In 1986 a retrovirus was isolated in West Africa that was similar but distinct from HIV-1 (Clavel *et al.*, 1986). Antibodies directed against HIV-1 could immunoprecipitate the *Gag* and *Pol* products of these isolates but not the *Env*. This new virus was termed LAV-2 and is now referred to as HIV-2. It is mainly found in West Africa.

Sequence analysis of HIV-1 and HIV-2 has shown that these viruses are distinct from one another, sharing only 42% nucleotide sequence homology (Ratner *et al.*, 1985, Guyader *et al.*, 1987).

The human immunodeficiency viruses share many molecular characteristics with the human T cell leukemia viruses, including routes of transmission, formation of large multinucleated cells known as syncytia, a Mg^{2+} dependent reverse transcriptase, p24 core antigen and most strikingly the presence of viral encoded regulatory proteins that function at both transcriptional and post-transcriptional levels. Despite these similarities HIV is more appropriately placed in the lentivirinae sub-family of retroviruses on the basis of genetic and morphologic parameters (Gonda *et al.*, 1985).

HIV-1 and Visna virus which causes a neurodegenerative disease in sheep, share 66% nucleotide homology in the *pol* regions and 45% nucleotide homology in their transmembrane proteins (Gonda *et al.*, 1986). Both HIV and EIAV demonstrate rapid genetic shift, with the generation of microvariants in the course of a single infection and macrovariants in the course of an epidemic.

1.3.4 Transmission of the human retroviruses

The routes of transmission are shared between the human T cell leukemia viruses and the immunodeficiency viruses. Virus can be transmitted via cell-free virus and virus-infected cells through blood-blood contact or through mucosal routes in both homosexual and heterosexual intercourse (reviewed by Chin, 1991). HIV infection and the spread of AIDS was originally labelled as a homosexual problem, however, it is found in haemophiliacs infected with contaminated blood products, intravenous drug abusers, heterosexual partners of infected individuals and can be passed perinatally from mother to child (reviewed by Chin, 1991). The precise mechanisms involved in the stochastic nature of transmission of HIV are not yet understood.

1.4 Other primate lentiviruses

1.4.1 Simian immunodeficiency virus (SIV)

In the early 1980's a number of lymphomas and immunodeficiency related disorders were identified in a colony of captive macaques in a North American primate centre (Letvin *et al.,* 1983). The causative agent was identified as a retrovirus named SIV_{mac} (Daniel *et al.,* 1985, Kanki *et al.,* 1985) which is similar in nucleotide sequence to HIV-2 (Franchini *et al.,* 1987, Chakrabarti *et al.,* 1987, Hirsch *et al.,* 1987). However, SIV-infected macaques have not yet been identified in the wild (Lowestine *et al.,* 1986, Ohta *et al.*, 1988).

SIV has also been isolated from healthy sooty mangabeys (SIV_{sm}) both in captivity in North American primate centres (Murphey-Corb *et al.,* 1986, Fultz *et al.,* 1986) and in the wild in West Africa (Hirsch *et al.,* 1989) The fact that this virus is also closely related to HIV-2. (Hirsch *et al.,* 1989) provides a possible explanation as to the origin of infection of the captive macaques. Until 1968 these sooty mangabey monkeys were imported into the United States from west Africa and may have been housed with macaques where a possible cross-species transfer could have occurred (Hirsch *et al.,* 1989).

An HIV-1 like lentivirus has been identified in chimpanzees (Peeters *et al.,* 1989; Huet *et al.,* 1990) This virus is referred to as SIV_{cpz}.

SIV isolated from African green monkeys (SIV_{agm}) displays the highest genetic diversity (Johnson *et al.,* 1990, 1991, 1992a) compared to the human and other non-human immunodeficiency viruses, and appears to have been widely present in the population in the 1950's (Hendry *et al.,* 1986). Both findings suggest that this is the oldest primate lentivirus in existence (Johnson *et al.,* 1990; Fomsgaard *et al.,* 1991). It does not appear to cause an AIDS-like illness.

SIV has also been isolated from mandrills in Africa (Tsujimoto *et al.,* 1988). SIV_{mnd} appears to be another distinct non-human primate lentivirus. The primate

lentiviruses can be grouped as shown in Figure 1.4, based on sequence similarity in the *pol* gene (Desrosiers, 1990).

1.5 Molecular biology of HIV-1

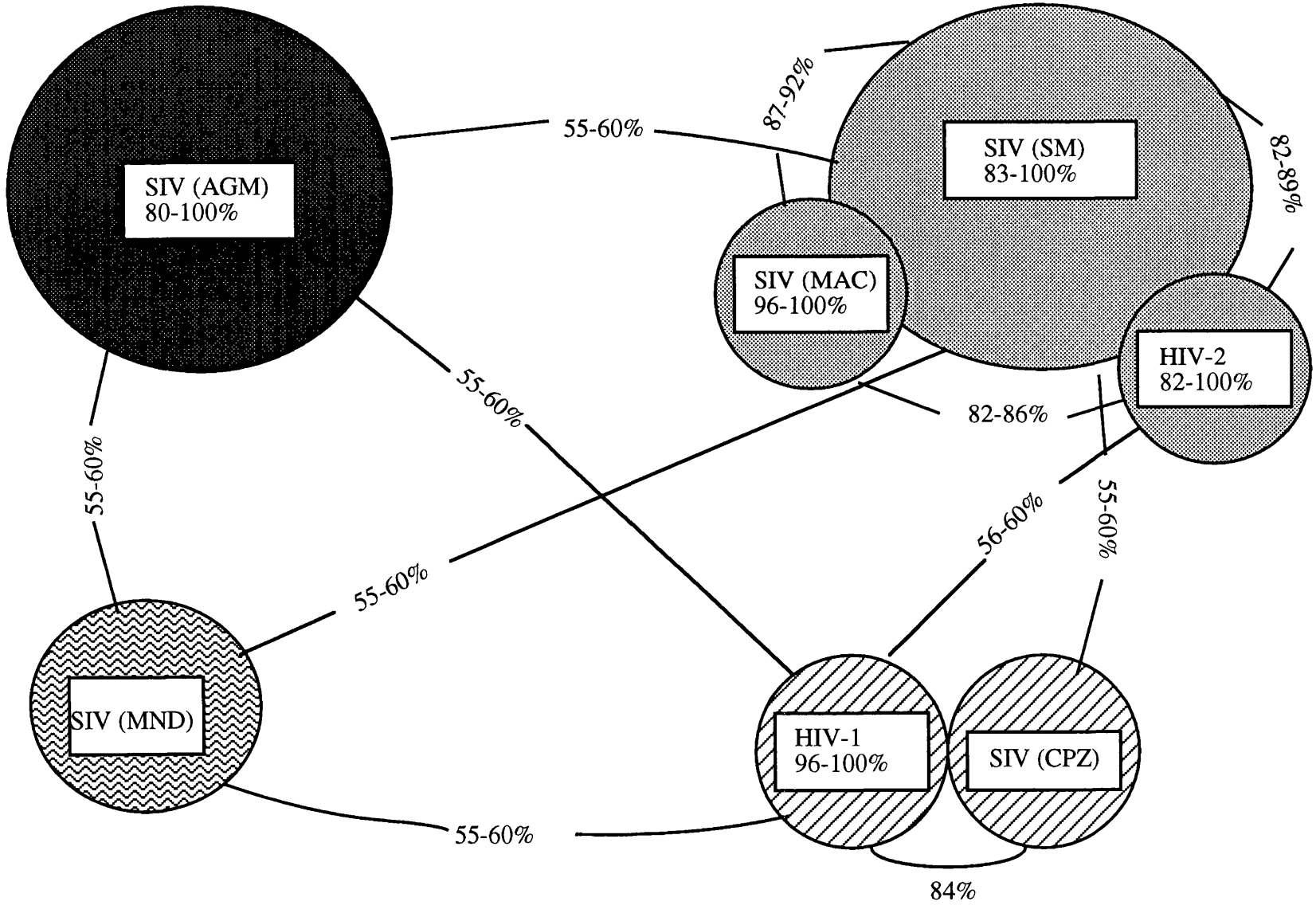
1.5.1 Structure of the HIV-1 virion

The HIV-1 virion is approximately 100nm in diameter with a double lipid bilayer derived from the host cell membrane from which the virus has budded. Forming the 'knobs or spikes' on the membrane surface are the glycoproteins made up from two components : gp41 which spans the membrane and gp120 the external glycoprotein anchored to the membrane via a non-covalent association with gp41 (Kowalski *et al.*, 1987; Helseth *et al.*, 1991). This membrane surrounds an icosahedral sphere shaped capsid made up of p17 (the myristylated protein) and contains the conical shaped nuclear core, made up of p24 (the major capsid protein). Each virion particle contains two identical copies of the viral RNA surrounded by p7 and p9 (the nucleocapsid proteins) as well as multiple copies of the enzymes reverse transcriptase, composed of two subunits (p66 and p51), protease (p22) and integrase (p32) (Gelderblom *et al.*, 1987). A schematic representation of the virus is shown in Figure 1.5.

1.5.2 HIV-1 genomic organisation

The genome of HIV-1 is approximately 10,000 nucleotides long and encodes at least 9 open reading frames (Ratner *et al.*, 1985; Wain-Hobson *et al.*, 1985; Green and Chen, 1990). A schematic representation of the genomic organisation of HIV-1 is shown in Figure 1.6. The genes encode the structural proteins Gag, Pol and Env, common to all replication competent retroviruses as well as genes involved in regulation of viral gene expression (*tat* and *rev*) and finally genes of uncertain function (*nef, vif, vpu* and *vpr*). Flanking these nine genes in the proviral state are the long

Figure1.4 Primate lentivirus taxonomy adapted from Desrosiers R.C.(1990). Percentages on the lines represent amino acid identities in the pol gene product between the different viruses. Numbers in the squares represent the degree of similarity between different isolates of each virus strain.


SIV (AGM)
80-100%
SIV (SM)
83-100%
SIV (MAC)
96-100%
HIV-2
82-100%
SIV (MND)
HIV-1
96-100%
SIV (CPZ)
55-60%
87-92%
82-89%
55-60%
55-60%
82-86%
55-60%
56-60%
55-60%
55-60%
84%

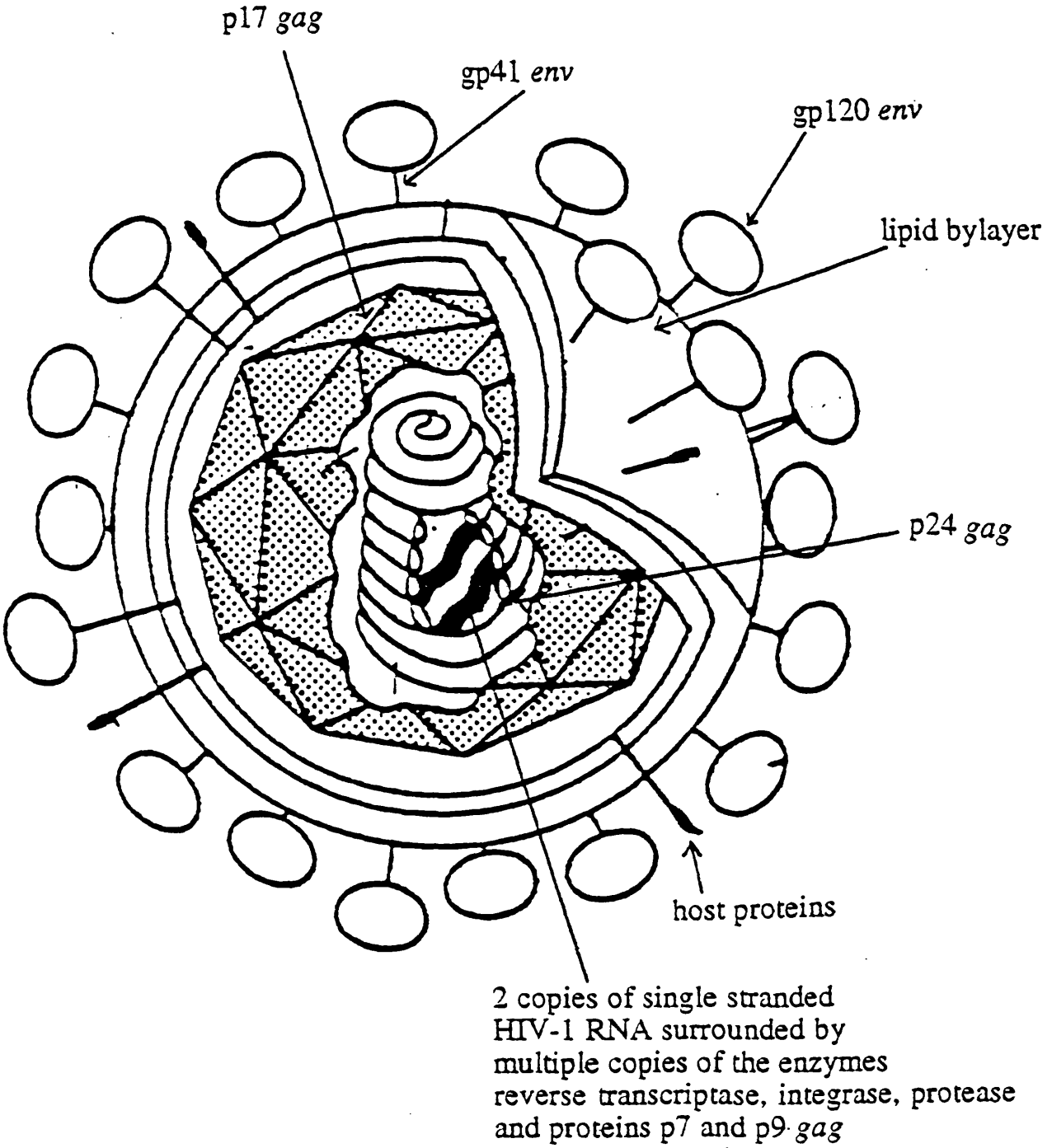
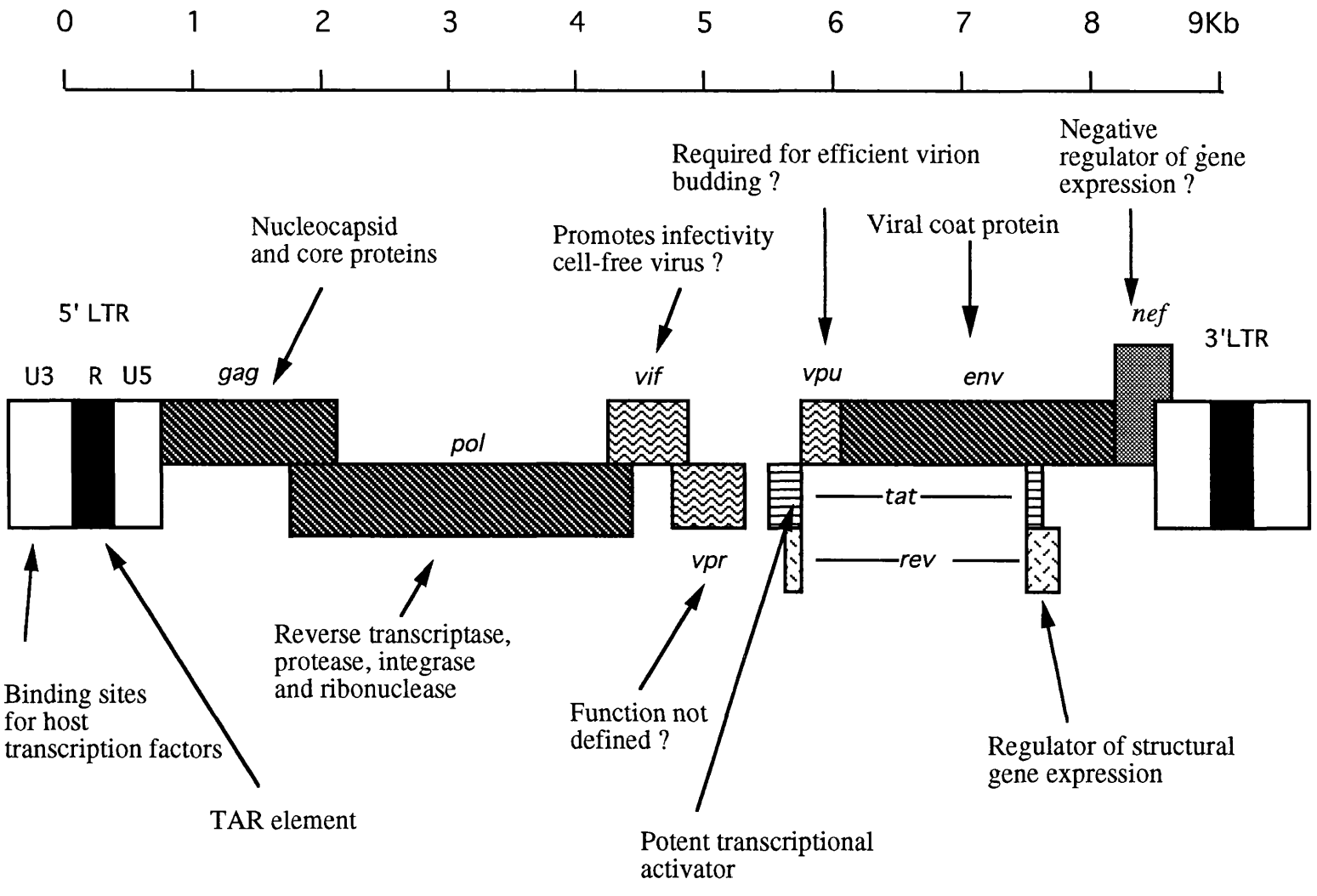


Figure 1.5 Schematic cross-sectional representation of a mature HIV-1 virion particle.

Adapted from Torrance, 1992.

Figure 1.6 Schematic representation of the proviral HIV-1 genomic organisation. The function of each gene marked with a ? indicates that the function of these genes is not fully understood.


0
1
2
3
4
5
6
7
8
9Kb
Required for efficient virion budding ?
Negative regulator of gene expression ?
Nucleocapsid and core proteins
Promotes infectivity cell-free virus ?
Viral coat protein
5' LTR
nef
3'LTR
U3
R
U5
gag
vif
vpu
env
pol
tat
rev
vpr
Reverse transcriptase, protease, integrase and ribonuclease
Binding sites for host transcription factors
Function not defined ?
Regulator of structural gene expression
TAR element
Potent transcriptional activator

terminal repeat sequences (LTR's) containing the promoter and enhancer elements involved in temporal regulation of viral gene expression (Sodroski *et al.*, 1984, 1985a, b; Arya *et al.*, 1985).

1.5.3 The structural proteins of HIV-1

Gag:

Expression of the HIV-1 *gag* gene products, p24 (the major capsid protein) and the core proteins p17, p7 and p9 results from translation from a gag-pol transcript, $p53_{gag}$ and subsequent cleavage of the translated polyprotein by viral proteases into the relevant virion proteins. Gag protein, p17 is myristylated at its N-terminus, therefore giving some indication of its function as a membrane bound protein (Veronese *et al.*, 1988). Phosphorylation of the p24 protein has also been described (Veronese *et al.*, 1988; Green and Chen, 1990). Both p17 and p24 are immunogenic in the natural host however, p24 is more immunogenic than p17 (Veronese *et al.*, 1988). The p7 protein contains a putative "zinc finger" domain that may be involved in direct interaction of the protein with the viral RNA (Aldovini and Young, 1990). Gag protein p9 in addition to p7 make up the nucleocapsid proteins.

Pol:

The *pol* gene products of HIV-1 are translated from an unspliced Gag-Pol transcript by a process of ribosomal frameshifting (Jacks *et al.*, 1987). Frameshifting moves the ribosome into the -1 reading frame in response to at least two sets of instructions in the Gag-Pol overlap region; a short sequence at the frameshift site and a secondary structure downstream of the site (Jacks *et al.*, 1987; reviewed by Varmus, 1988). The resultant Pol precurser protein is sequentially cleaved to yield the reverse transcriptase subunits p66 and p51, protease (p22) and integrase (p32). The reverse transcriptase enzyme contains an RNaseH activity which is required for the degradation of the RNA template during the synthesis of double stranded DNA (reviewed by

Varmus, 1988). The RNaseH activity is located in the C-terminal region of the p66 subunit (Hansen *et al.*, 1988).

Env:

The HIV-1 *env* gene is transcribed as a singly spliced mRNA that on translation yields a precurser protein of 160KDa. This polyprotein exists in the rough endoplasmic reticulum-golgi complex where it is cleaved into two components; the gp120 external glycoprotein and the gp41 transmembrane protein. These proteins then undergo extensive golgi-mediated carbohydrate modifications converting N-linked mannose-type glycans into complex oligosaccharides (Stein and Engelman, 1990). The heavily glycosylated gp120 molecules present on the surface of the virion particle as a multimeric unit are non-covalently linked to the transmembrane protein gp41 (Helseth *et al.*, 1991; Kowalski *et al.*, 1987). Gp41, as previously discussed, is involved in viral fusion with the host cell membrane via its hydrophobic N-terminal (Kowalski *et al.*, 1987; Freed *et al.*, 1990). The properties and functions of gp120 are discussed in detail later in this thesis.

1.5.4 The regulatory proteins of HIV-1

Tat:

In addition to the structural genes *gag*, *pol* and *env*, HIV-1 encodes two potent regulatory proteins Tat and Rev. The 14KDa Tat protein is translated from a double-spliced mRNA species and is comprised of 86 amino acids (reviewed by Cullen 1990, 1991; Sheridan *et al.*, 1993). This protein is primarily localised in the nuclei of infected cells (Hauber *et al.*, 1987) and is expressed early in the viral life cycle. The presence of the Tat protein dramatically increases the expression of all genes linked to the HIV-1 LTR's (Sodroski *et al.*, 1984, 1985a,b; Arya *et al.*, 1985). Tat exerts its effect by binding directly or indirectly to a 59 nucleotide RNA stem loop structure located between +1 and +60 in the viral RNA (Rosen *et al.*, 1985) both *in vitro* and *in vivo*

(Feng and Holland, 1988). This stem loop structure is transcribed from the TAR (transactivation response element) sequence downstream of the HIV-1 5' LTR. The TAR element is therefore located in the 5'-untranslated region of all HIV-1 mRNA's and functions in a position and orientation dependent manner (reviewed by Cullen, 1990, 1991; Sheridan *et al.*, 1993).

Nuclear run-on assays using plasmid constructs of the chloramphenicol acetyltransferase gene (CAT) under the control of the HIV-1 LTR were used to demonstrate that in the absence of Tat, transcripts that are initiated from the basal promoter are elongated inefficiently and are not terminated at discrete sites, but rather declined in a gradual manner as the distance from the promoter increased (Kao *et al.*, 1987; Laspia *et al.*, 1989). In contrast, highly processive transcription throughout the length of the region transcribed was found in the presence of Tat in both cultured cell lines (Kao *et al.*, 1987; Laspia *et al.*, 1989) as well as in infected primary T lymphocytes (Feinberg *et al.*, 1991). The function of Tat is therefore believed to enhance the ability of the RNA pol II initiation/elongation complex to efficiently traverse the length of the proviral genome. In the absence of Tat, a less processive form of the RNA pol II elongation complex is thought to be assembled at the HIV-1 promoter (Marciniak and Sharp, 1991; reviewed by Sheridan *et al.*, 1993). The precise mechanism by which Tat interacts with the TAR sequence is not fully understood but involves other cellular transcription factors not discussed in this summary.

Rev:

For efficient replication and production of infectious virion particles, three classes of mRNA must be produced corresponding to the unspliced genomic RNA also encoding the *gag* and *pol* gene products, the singly spliced mRNA encoding the envelope proteins and the multiply spliced mRNA encoding the various regulatory proteins including Rev (reviewed by Greene and Cullen 1990). Within the first 12-16 hours after experimental infection of human $CD4^+$ T lymphocytes the multiply spliced mRNA species encoding the regulatory proteins predominate in a one step growth curve

(Kim *et al.*, 1989). With longer culture periods, a progressive increase in the singly spliced *env* and unspliced genomic RNA's occurs, with peak levels obtained between 24 and 48 hours after infection (Cullen and Greene, 1989). This transition from 'early regulatory' to 'late structural' gene expression is controlled by the Rev protein (reviewed by Greene and Cullen 1990; Cullen, 1991).

As with Tat and Nef, Rev is translated from a double spliced 2kb transcript. Translation of this transcript yields the 19KDa Rev polypeptide composed of 116 amino acids (reviewed by Greene and Cullen 1990). Indirect immunofluorescence using polyclonal anti-Rev antiserum has shown that Rev is primarily localised in the nucleus (Cullen *et al.*, 1988) and predominantly in the nucleolus (Malim *et al.*, 1989a). The function of the Rev protein is to promote the nuclear export and cytoplasmic expression of the unspliced and singly spliced mRNAs that encode the various viral structural proteins. This is mediated through the direct binding to a specific response element, the RRE (Rev response element) (Zapp and Greene, 1989; Daly *et al.*, 1989; Malim *et al.*, 1989b, 1990) located within the env gene (Malim *et al.*, 1988). This element must be present in the mRNA species for Rev to act. The RRE forms an RNA stem loop structure (Malim *et al.*,1989b; Hanly *et al.*, 1989) as does the TAR sequence, however the position of this element is flexible but must be present in the sense orientation to function (Hanly *et al.*, 1989; Malim *et al.*, 1989b).

The Rev regulatory protein activates the cytoplasmic expression of the larger mRNA's, simultaneously down regulating the expression of the smaller RNA species, thereby inhibiting its own expression via an autoregulatory feed-back loop. The interaction of Tat and Rev have been previously summarised in Figure 1.3.

Host cellular factors also play a role in HIV-1 gene regulation:

In addition to the viral regulatory proteins, host cell factors also play a role in the regulation of HIV-1 viral gene expression. The HIV-1 LTR contains a typical, albeit compact, inducible RNA pol II promoter that can be activated by physiological agents including mitogenic stimulation and ultraviolet light (reviewed by Sheridan *et al.*, 1993)

as well as the HIV-1 trans-activator protein Tat. In addition, the HIV-1 promoter contains 3 tandem binding sites located between nucleotides -77 to -46, upstream of the TATA box, for the ubiquitous transcription factor Sp1. Mutation of these sites leads to a substantial reduction in HIV-1 LTR activity both *in vitro* and *in vivo* (Jones *et al.*, 1986). Immediately upstream of the Sp1 binding sites lie two tandem binding sites between nucleotides -104 and -81 (Rosen *et al.*, 1985) for the transcription factor NF_k-B (Nabel and Baltimore, 1987), a dimeric protein that becomes released from an inhibitory complex IkB as a result of T cell activation (Bauerle and Baltimore, 1988). Deletion or site-directed mutagenesis of this region impairs HIV-1 LTR activation induced by the NF_k-B protein (Greene and Chen, 1990). Other transcription factors such as the cytokine granulocyte-macrophage colony stimulating factor (GM-CSF) interleukin-2 and a potent T cell-specific activator, TCF-1 alpha, can also bind to sequences upstream of the NF_k-B sites, activating the HIV-1 LTR (Sheridan *et al.*, 1993). The HIV-1 TATA box is also recognised by TFIID, a general initiation factor that consists of a TATA binding protein (TBP) and a collection of diverse adaptor proteins termed TAF's, for TBP-associated factors, which mediate responsiveness to Sp1 (Dynlacht *et al.*, 1991).

1.5.5 HIV-1 proteins of poorly or undefined function

Nef:

Another protein encoded by HIV-1 is Nef, the product of the *nef* gene. This 27KDa protein is translated from a multiply spliced mRNA transcript resulting in a polypeptide constituted by 200-205 amino acids, depending on the virus isolate (Harris *et al.*, 1992). Nef is myristylated at the N-terminus (Allan *et al.*, 1985) and this acylation is required for its location on the cytoplasmic face of the plasma membrane of infected cells (Yu and Felsted, 1992). The precise role of myristylation and indeed of the Nef protein is not yet known and remains the subject of controversy. Suggested functions for Nef include: as a repressor of viral replication (Fisher *et al.*, 1986;

Terwilliger *et al.*, 1986), a component of the signal transduction pathway suggested by the protein's ability to bind and hydrolyse GTP (Guy *et al.*, 1987) and as a down-regulator of the surface expression of CD4 (Guy *et al.*, 1987; Garcia and Miller, 1991). Other workers have failed to confirm any of these attributed functions (Barchalerie *et al.*, 1990; Harris *et al.*, 1992; Cheng-Mayer *et al.*, 1989). It has been hypothesised that the apparent discrepancies observed in Nef function may be due to the significant degree of amino acid variability observed between different Nef isolates both *in vitro* and *in vivo* (Harris *et al.*, 1992).

Vif:

The precise function of this protein still remains unknown, however, homologues of the gene have been found in all lentiviruses with the exception of EIAV, suggesting an important functional role (Oberste and Gonda, 1992). The *vif* (viral infectivity factor) gene encodes a 23-27kDa polypeptide (Kan *et al.*, 1986; Lee *et al.*, 1986) and HIV-1 mutants with deletions of the *vif* gene showed delayed growth kinetics in $CD4^+$ T lymphocytes. In addition the virus produced from these cells was approximately 1000-fold less infectious than the wild type (Sodroski *et al.*, 1986). Vif is therefore thought to be important for viral infectivity (Strebel *et al.*, 1987).

Vpu:

The vpu gene encodes a 16KDa polypeptide (Strebel *et al.*, 1988) and in retroviruses examined to date, is only found in the HIV-1 and SIV_{cpz}. Several studies have shown that the absence of the *vpu* gene results in a decreased release of virion particles from infected cells and accumulation of the virion proteins in these cells (Strebel *et al.*, 1989; Terwilliger *et al.*, 1989; Klimkait *et al.*, 1990). Electron microscopic examination has revealed that mutations in the *vpu* gene lead to an accumulation of mutant virion particles at the membrane surface of infected cells indicating a role of Vpu in proper virion assembly, budding and release of infectious particles (Klimkait *et al.*, 1990). Recent reports have shown that the vpu protein

induces rapid degradation of CD4 in the endoplasmic reticulum (Willey *et al.*, 1992; Chen *et al.*, 1993). The mechanism(s) and biological significance of the observed CD4 degradation are not understood.

Vpr:

Essentially nothing is known about the function of this 11KDa protein, however, like *vif*, the *vpr* gene or a homologue of it is present in all known primate lentiviruses, suggesting an important function for this protein. Western blotting of subcellular fractions prepared from HIV-infected cells has indicated that Vpr is a membrane associated virion component, not present in high concentrations in the cytosol or nuclear fractions of the cell (Sato *et al.*, 1990). The Vpr protein is also not required for the formation and release of mature virions (Dedera *et al.*, 1989)

1.5.6 CD4 molecule as the principal receptor for HIV-1

Following the observation that infection with HIV-1 resulted in a numerical and functional depletion of T helper/inducer cells (Gottlieb *et al.*, 1981; Klatzmann *et al.*, 1984) it was hypothesised like the human T cell leukemia viruses, HIV-1 infected primarily $CD4^+$ bearing T lymphocytes, a vital subset of the host immune mechanism. HIV-1 infection however, does not cause neoplastic transformation of T cells as do HTLV-1 and HTLV-11 (Chen *et al.*, 1983) but has devastating cytotoxic effects (Popovic *et al.*, 1984). The major surface receptor for HIV-1 was subsequently defined as the CD4 molecule, present on the surface of T lymphocytes and cells of the monocyte/macrophage lineage, by a number of experiments described below.

Receptor-positive cells were identified by assessing induction of multinucleated cells, termed syncytia, and the susceptibility of various cell types to infection with psuedotypes of the vesicular stomatitis virus (VSV) bearing envelope antigens derived from HIV-1. This approach implicated the CD4 molecule as an essential component involved in HIV-1 attachment and tropism to target cells (Dalgleish *et al.*, 1984).

Involvement of CD4 in viral attachment was demonstrated directly through the use of monoclonal antibodies directed against CD4. These monoclonal antibodies inhibited HIV-1 activity in a variety of in-vitro assays (reviewed by Sattentau and Weiss, 1988). Firstly, anti-CD4 mab's prevented formation of syncytia, that result from fusion between HIV-infected and $CD4^+$ uninfected cells, and inhibited the infectivity of VSV psuedotypes carrying the HIV-1 envelope glycoprotein (Dalgleish *et al.*, 1984). Secondly, they reduced the infection of human peripheral blood mononuclear cells (PBL's), measured by decreased reverse transcriptase activity in cultures containing antibody (Klatzmann *et al* ., 1984). Thirdly, they inhibited binding of fluoresceinated HIV to $CD4^+$ cells (McDougal, 1985).

Further evidence of direct binding of HIV-1 to CD4 came from the observation that the external glycoprotein gp120 and the CD4 molecule could be immunoprecipitated as a complex from HIV-1-infected cells. Cross-linking prior to precipitation did not reveal any additional molecules in the complex, implying that only CD4 was necessary for infection with HIV-1 (McDougal *et al.*, 1986). Transfection experiments in which human $CD4^-$ cells, previously refractory to HIV infection, became permissive when a cloned CD4 cDNA was introduced by transfection, gave further evidence that CD4 acted as the receptor for HIV-1 (Maddon *et al.*, 1986). However, mouse cells manipulated in the same way to express human CD4, bound HIV-1 on their surface but were not infected by the virus. Therefore unlike human cells, the mere presence of CD4 on their surface does not render these cells permissive to HIV infection. The introduction of HIV-1 DNA into mouse fibroblast cells, allowed efficient replication and production of infectious virus, indicating that the block of infection of $CD4^+$ mouse cells occurs at an event prior to viral replication (Levy *et al.*, 1986). This observation confirms that the process of membrane fusion might well be mediated by the specific interaction of the retroviral envelope with a second cell surface receptor.

1.5.7 The CD4 molecule

The CD4 antigen is expressed as a 55KDa protein on the surface of mature thymus-derived lymphocytes and to a lesser extent on cells of the monocyte/macrophage lineage. T lymphocytes expressing CD4 act as helper cells, to promote antibody production or induce proliferation of CD8 bearing cytotoxic T cells. The best defined function of CD4 is that of an adhesion molecule binding with the class-11 major histocompatibility complex (MHC) (present on the surface of most cell types and in particular on antigen presenting cells) and the T lymphocyte. It has been proposed that the CD4 binds a conserved region of MHC class 11 molecules, and thereby stabilises the MHC class II T cell receptor complex. The function performed by CD4 on the surface of macrophages is not clear.

The molecule itself, a member of the immunoglobulin supergene family, consists of several structural domains: an N-terminal extracellular region containing a leader sequence and four immunoglobulin-like domains; a transmembrane region and a cytoplasmic region that is highly charged (Maddon *et al.*, 1987). The region of CD4 reactive with HIV gp120 has been mapped by monoclonal antibodies to the outermost V1 immunoglobulin-like region (Sattentau *et al* ., 1986).

1.5.8 Infection via Fc and complement receptors

Although the primary receptor for HIV-1 is the CD4 molecule, HIV-infection can be mediated *in vitro* by two additional mechanisms. The Fc and complement receptors present on the surface of macrophages and other cells of the immune system, can act to facilitate HIV-infection independently of one another in the presence of viral antibodies or complement factors (Takeda *et al.*, 1988, 1990; Homsy *et al.*, 1989; Boyer *et al.*, 1991; Robinson *et al.*, 1991; Haubrich *et al.*, 1992). This increased effciency of viral entry into cells in the presence of antibodies against the virus is termed "antibody-mediated enhancement". Two regions on the transmembrane protein gp41 have been

shown to elicit antibodies that enhance infection with HIV-1 (Robinson *et al.*, 1991). These findings imply that caution should be used when developing vaccine strategies that involve generating an antibody response against viral antigens (Takeda *et al.*, 1988, 1990; Homsy *et al.*, 1989; Boyer *et al.*, 1991; Robinson *et al.*, 1991; Haubrich *et al.*, 1992).

The expression of IgG Fc receptors can be induced in cells infected with some viruses. McKeating *et al.* (1990) demonstrated that the Fc receptors induced by cytomegalovirus infection allowed immune complexes of HIV-1 to infect fibroblasts not otherwise permissive to HIV-infection. Infection could be blocked by preincubation of the cells with human IgG but not soluble CD4 or anti-CD4 monoclonals. This finding indicates that the cellular tropism of HIV-1 might be altered by the presence of other viruses in HIV-infected individuals, however *in vivo* evidence for this speculation has yet to be obtained.

1.5.9 CD4 independent infection

A number of cell lines that do not detectably express the CD4 antigen on their surface are susceptible to HIV-infection, albeit less efficiently. These include a neuroblastoma cell line (Li *et al* ., 1990) and, as previously described, a fibroblast cell line (Tateno *et al.*, 1989: McKeating *et al.*, 1990). To identify other potential receptors involved in infection of neuronal cells with HIV-1, antibodies against neuronal cell-surface components were screened for their ability to inhibit viral internalisation (Harouse *et al.*, 1991). Antibodies against galactosyl ceramide (Gal C), a glycolipid common to oligodendrocytes and Schwann cells, blocked infection of neuronal cell lines. In addition, gp120 was shown to bind to Gal C but not to other glycolipids. Recently, antibodies against the third hypervariable region (V3) of HIV-1 gp120 have been shown to block the binding of gp120 to galactosyl ceramide (Cook *et al.*, 1993). The binding site for Gal C on gp120 has recently been mapped to amino acids 206-275, outside the V3 region , which together with the findings of Cook *et al.* (1993) suggest

an interaction of the this Gal C binding site with the V3 loop (Bhat *et al.*, 1993). It would appear that Gal C can bind HIV-1 as efficiently as can CD4 but facilitates less efficient viral entry. (Harouse *et al* ., 1991).

1.5.10 Persistence of HIV-1

Following infection with HIV-1 there is an initial viremia with or without clinical symptoms at the time of seroconversion. Then follows an asymptomatic period that can last many years during which the virus infection seems to be controlled by the host immune system. During the long asymptomatic phase and in some cases during the symptomatic phase of infection, HIV-1 is not readily isolated from the $CD4^+$ cells present in the peripheral blood. This apparent lack of virus in the cells that are drastically depleted in patients with AIDS, was the main argument of HIV sceptics, led by Dr Peter Duesberg against HIV being the etiological agent of AIDS.

It was first argued that HIV existed, during the asymptomatic period, in a non-replicating latent form, probably persisting in resting T cells that are deficient in the various cellular factors required either for efficient conversion of the viral RNA into double stranded DNA (Fritsch and Temin, 1977; Varmus *et al.*, 1977) or efficient integration of the double stranded DNA into the host genome (Green and Chen, 1990; Zack *et al.*, 1990, 1992; Stevenson *et al.*, 1990b). In addition, the virus may exist as a fully integrated proviral DNA in the small proportion of activated T cells that return to the G_0 phase to become memory T cells (Simmonds *et al.*, 1991). Because of the unlimited life span of these memory T cells, HIV may persist indefinitely in them, making the virus very inaccessible to potential antiviral agents and perhaps providing a viral reservoir during the long asymptomatic phase seen in HIV-infected individuals.

More recent data has shown, that although the virus can exist in the forms mentioned above, the virus population as a whole does not go completely latent, as viral mRNA can be isolated, reverse transcribed and amplified by PCR from the peripheral blood of individuals throughout the course of infection suggesting replication of the

virus in these tissues (Schnittman *et al.*, 1991). Virus has also been found actively replicating in the lymph nodes, not surprisingly the place where the $CD4^+$ cells usually reside (Pantaleo *et al.*, 1993; Embretson *et al.*, 1993). It is hypothesised that the observed depletion of $CD4^+$ cells can be partly explained by active replication of the virus in the lymph nodes, depleting the T cells in these organs and this partly explains the lack of infected T cells found in the peripheral blood (Pantaleo *et al.*, 1993; Embretson *et al.*, 1993; Tenner-Racz *et al.*, 1986).

Macrophages and monocytes are also believed to act as reservoirs for HIV-1 as these cells are relatively resistant to the cytolytic effects of HIV (Nicholson *et al.*, 1986; Harper *et al.*, 1986; McElrath *et al.*, 1989). In addition, infected macrophages and monocytes in the central nervous system (Ho *et al.*, 1985; Koenig *et al.*, 1986) may escape detection by certain immune effector cells because the MHC class 1 molecule is not expressed on their surface (Joly *et al.*, 1991). Some activated $CD4^+$ cells can also survive the cytolytic effects of HIV-1 infection further contributing to the pool of "latent" virus (Schnittman *et al.*, 1989).

The extensive sequence variation displayed by the virus may also play a role in the persistence of HIV-1 infection. During the asymptomatic phase, the highly specific humoral and T cell response mounted against the virus fails to clear it from the host. It is hypothesised that the host exerts an immune selection pressure against the virus that drives these sequence changes. This hypothesis has been supported by the *in vivo* isolation of viruses from individuals that were resistant to neutralisation by autologous sera (Albert *et al.*, 1990; Montefiori *et al.*, 1991). In addition, the cytotoxic T cell response against the virus has been shown to influence the sequence diversity in the *gag* gene products p17 and p24. (Phillips *et al.*, 1991). Wolfs *et al.* (1991), have shown that the sequence variants that occur within the V3 region of HIV-1 gp120 are accompanied by the emergence of a new population of V3-specific antibodies. Work presented in this thesis was designed to investigate the hypothesis that the sequence variants that arise in the V3 region during the course of infection represent antibody escape mutants.

1.5.11 Pathogenesis of HIV-1

It is known that during the asymptomatic phase of HIV-1 infection, slowly replicating, relatively non-cytopathic variants, that display tropism for macrophage derived cells predominantly exist, which, during the later stages of infection are either replaced by or evolve into, highly virulent viruses that display T cell tropism, the appearance of which, usually signals the rapid onset of disease. An important feature of infection with HIV-1 is the progressive depletion of the $CD4^+$ T lymphocytes (Gottlieb *et al.* ,1981; Barre-Sinoussi *et al.*, 1984; Popovic *et al.*, 1984), a subset of cells that in uninfected individuals constitute 50-70% of the circulating T cell population (Lifson and Engelman, 1989). The precise mechanisms by which these relatively non-cytopathic variants of HIV-1, present throughout the course of infection, cause this depletion of $CD4^+$ cells are as yet not fully understood, however, a number of mechanisms have been proposed.

As discussed in section 1.5.10 above, recent observations that HIV-1 is actively replicating in cells of the lymph nodes during the asymptomatic phase, may partly explain this observed depletion of $CD4^+$ cells in the apparent absence of high titres of virus in the peripheral blood of infected individuals (Embretson *et al.*, 1993; Pantaleo, *et al.*, 1993; Tenner-Racz *et al.*, 1986).

Direct cell killing by HIV-1 can occur by budding of infectious virus particles from the host cell membrane and it has been proposed that an interaction of gp120 and CD4 on the surface of HIV-1 infected cells, may result in both metabolic and osmotic disruption, ultimately leading to cell death (Haseltine, 1988). In addition, the presence of gp120 on the surface of infected cells can cause fusion with uninfected cells via interaction with the CD4 molecule, resulting in the formation of large multinucleated cells called syncytia (Lifson *et al.*, 1986a, b).

Immune responses resulting in the killing of uninfected $CD4^+$ cells, triggered by HIV-infection have also been suggested. Free gp120, that has been shed from the surface of virally infected cells or from the virus itself (Moore *et al.*, 1990) may be

selectively absorbed onto the surface of uninfected CD4-expressing cells through gp120-CD4 interactions. This is postulated to render $CD4^+$ cells expressing viral envelope determinants susceptible to lysis by gp120-specific cytotoxic T lymphocytes (Lanzavecchia *et al.*, 1988; Germain, 1988; Siliciano *et al.*, 1988), or killing via antibody-dependent cell mediated cytotoxic (ADDC) mechanisms (Lyerly *et al.*, 1987b).

Autoimmunity models of AIDS pathogenesis have also been suggested. These hypothesis are based on the fact that both gp120 and MHC class II bind to CD4 and that their binding sites must overlap in the outermost immunoglobulin like domain of CD4 (Sattentau *et al.*, 1986; Rosenstein *et al.*, 1990) as both recombinant gp120 and monoclonal antibodies that recognise the gp120 binding site on CD4 can block binding to MHC class II (Sattentau *et al.*, 1986; Rosenstein *et al.*, 1990). Therefore it has been hypothesised that antibodies against gp120 may cross-react with host MHC class II or elicit anti-idiotypic antibodies against CD4 (reviewed by Hoffman *et al.*, 1991). In support of this hypothesis, sequence homologies have been identified between MHC class II and a conserved region in gp120 (Young, 1988). Sequence homologies have also been identified between a region in the HIV-1 Nef protein and MHC class II which might contribute to pathogenesis in a similar fashion (Vega *et al.*, 1990). In addition, foreign T lymphocytes that are commonly passed during the transmission of HIV-1 may also induce antibodies or anti-idiotypic antibodies that may react with host MHC or other host cell surface components (reviewed by Hoffman *et al.*, 1991).

If these models of HIV-1 pathogenesis have any validity, then therapeutic interventions aimed at generating antibodies against gp120 may have adverse effects.

Selective depletion of $CD4^+$ T cells that bear specific V-beta sequences has been observed in HIV-1 infected individuals, suggesting that HIV-1 may encode a potential "superantigen" (Imbreti *et al.*, 1991). Most antigens, displayed on antigen presenting cells, are recognised through an interaction of the variable regions of the T cell receptor alpha and beta chains. Superantigens are however, recognised on the basis of the expressed B-beta chain only. This leads to a greater than 5-fold stimulation of T cell

proliferation, most of which are non-productive and may attack host cells resulting in a depletion of cells expressing that particular V-beta sequence, as has been observed in HIV-1 infected individuals (reviewed by Johnson *et al.*, 1992). $CD4^+$ T cells are a vital part of the immune repertoire and their function is intimately linked with most other cell types of the immune system, such that T cell dysfunction resulting from infection with HIV-1, will manifest a multitude of immunological deficits rendering the host susceptible to opportunistic infections, a feature characteristic of AIDS.

Polyclonal B-cell activation, as reflected by hypergammaglobulinaemia was documented in early descriptions of patients with AIDS (Gottlieb *et al.*, 1981). High levels of immunoglobulin (Pahwa *et al.*, 1985) and a poor response to novel antigens (Mizumi *et al.*, 1988) are characteristic in AIDS patients. This overproduction of non-specific gammaglobulin may also result in autoimmunity against cellular proteins (Lyerly *et al.*, 1987a, b; Endelman and Zolla Pazner, 1989).

Another mechanism has been proposed for the depletion of $CD4^+$ cells. Apoptosis or programmed cell-death is a process that occurs during the normal maturation of thymus-derived T lymphocytes and is required to eliminate autoreactive T cells, in order to establish self tolerance (reviewed by Gougeon and Montagnier, 1993). It has been proposed that binding of gp120 to CD4 primes the T cells to undergo apoptosis in response to further stimulation with antigen. Thus gp120 on the surface of infected cells may prime neighbouring uninfected T cells to undergo apoptosis. In addition, HIV-1 infected macrophages show altered cytokine production and can secrete high levels of tumour necrosis factor (TNF), which is known to induce apoptosis (reviewed by Levy, 1993). T cells taken from HIV-1 infected individuals can be induced *in vitro* to undergo apoptosis in response to antibodies against the T cell receptor, however this results in the depletion of both CD4 and CD8 bearing T cells, a phenomenon not reflected in the *in vivo* situation (reviewed by Gougeon and Montagnier, 1993). Therefore, the relevance of this hypothesis has yet to be fully understood.

HIV-1 not only infects $CD4^+$ T lymphocytes and cells of the monocyte-derived macrophage lineage in the peripheral blood and lymph nodes but is found in the macrophage cells present in several tissues of the perivascular compartment including brain, lung, gastrointestinal epithelium, skin, heart and kidney (Armstrong and Horne, 1984; Ho *et al.*, 1984, 1985; Levy *et al.*, 1985a, b; Koenig *et al.*, 1986; Popovic and Gartner, 1987; Koyanagi *et al.*, 1987; Nelson *et al.*, 1988; Cohen *et al.*, 1989). The clinical symptoms of AIDS, such as wasting, neurological and gastrointestinal dysfunction could be due, directly to primary infection of the corresponding tissues or to an indirect mechanism not yet determined. In either case, development of the disease appears to correlate with an increase in viral load and the emergence of HIV-1 variants that are more cytopathic and replicate in a wider variety of human cells (Cheng-Mayer *et al.*, 1988; Fenyo *et al.*, 1988; Asjo *et al.*, 1988; Tersmette *et al* ., 1988). This probably occurs when the $CD4^+$ T cells and the immune repertoire has been sufficiently depleted, allowing the unrestricted emergence of the rapidly replicating, cytopathic variants of the virus.

1.6 Genetic variability of HIV-1

1.6.1 Mechanisms of HIV variation

The high mutation rate of HIV-1 and other retroviruses reflects the infidelity of the RNA polymerase II, reverse transcriptase, and their lack of a proofreading activity (Moriyama *et al.*, 1991; Preston *et al* ., 1988; Roberts *et al.*, 1988; Coffin, 1992). In HIV-1 and other retroviruses, there is a high rate of mutation between adenine (A) and guanine (G) residues and in particular changes from G to A in both the *gag* and *env* genes (Moriyama *et al.*, 1991). This high rate of substitution between A and G appears to be specific for retroviruses and has been attributed to the poor recognition of pyrimidine residues by the reverse transcriptase enzyme during replication of the HIV-1 genome (Moriyama *et al.*, 1991). The rate of fixation of mutations (the

evolution rate) in the viral population is not only dependent on the mutation rate but also on environmental selection pressures such as the immune response and antiviral drugs and restrictions imposed by the functional constraints on the viral proteins. The observation that the rates of substitution are not constant across the HIV-1 genome but are high in the envelope gene (Li *et al.*, 1988) suggests that the probability of mutation fixation is not simply a reflection of the intrinsic mutation rate.

Recombination of retroviral genomes, requiring a heterozygous virion RNA (section 1.2.3) adds further to the complexity of genetic variation of HIV-1 (Hu and Temin, 1990). Based on experimental replication rates Hu and Temin (1990) estimated that at least one of three, two stranded 10-kb RNA viruses would experience at least one recombination event. In dually infected cells the possibility exists for recombination between similar but distinct viruses leading to a single proviral intermediate and adding further to the variation of the HIV-1 genome (Katz and Skalka, 1990; Hu and Temin, 1990).

1.6.2 Evolution of viral quasispecies

An hypothesis based on Darwin's theory of Natural Selection would imply that molecules that replicate with limited fidelity will generate enough diversity to code for some variants that may have a phenotypic selective advantage in the host at any particular time and that these variants would then replicate and become dominant in the virus population. Because of the error-prone replication machinery, not only these mutants would exist, but a swarm of other genotypes would also exist. The representation of each of these mutations in such a swarm is dependent on the rate at which that mutation occurs and the relative "fitness" of that mutation in the viral population. This distribution around a predominant sequence is termed the quasipecies distribution and was first described for the evolution of macromolecules in general by Eigen *et al.* (1971, 1988) and is applicable to the sequence distribution observed in most lentiviruses including HIV-1. This distribution allows the virus to adapt rapidly to host

selection pressures by allowing a switch in quasipecies distribution to favour the 'fittest' mutant. Because of the extensive sequence variation displayed by HIV-1, every genome has to be considered unique and consequently must be described in terms of populations of closely related genomes or quasispecies (Goodenow *et al.*, 1989).

1.6.3 Variation between HIV-1 isolates

The genomic diversity of HIV-1 was first shown using restriction enzyme mapping and analysis of thermal denaturation of heteroduplexes to compare various geographically distinct viral isolates, including the MN and RF strains to the original HTLV-IIIB cloned isolate (Shaw *et al.*, 1984, 1985; Hahn *et al* ., 1984, 1985). These studies demonstrated that virus isolated from patients with AIDS or AIDS related complex (ARC) displayed considerable genetic variation and that this variation was predominantly found in the envelope coding region rather than the *gag* and *pol* coding regions. Later studies confirmed that among 18 isolates from individuals with AIDS, each showed a different restriction enzyme pattern (Wong-Staal *et al.*, 1985). The complete genomic sequence for the original isolates of HIV-1 (Barre-Sinoussi *et al.*, 1983; Gallo *et al.*, 1984) was later identified (Ratner *et al.*, 1985; Wain-Hobson *et al.*, 1985). Further diversity than previously observed between the North American and European isolates was seen in the nucleotide sequences obtained from two Zairian patients infected with HIV (Alizon *et al.*, 1986) indicating a divergent evolution of the virus in Africa. The sequence diversity of HIV-1 is now well documented (Simmonds *et al.*, 1991; Leigh Brown, 1991; Coffin, 1992). These findings along with analogous data for the Equine Infectious Anaemia Virus (EAIV) and Visna virus, two lentiviruses in which it has been suggested that variation in the envelope region correlates with demonstrable changes in antigenicity and pathogenicity, and the finding that variants arise progressively during infection (Scott *et al.*, 1979; Montelaro *et al.*, 1984; Salinovich *et al.*, 1986; Clements *et al.*, 1988) suggested perhaps that the genetic

variation in HIV-1 could alter viral pathogenesis via the emergence of immunological escape mutants and mutants with altered tissue tropism and virulence.

1.6.4 Variation within the envelope protein

The comparison of independent HIV-1 isolates has shown that the region of the envelope gene encoding gp120 is characterised by considerable sequence variation (Hahn *et al.*, 1986; Simmonds *et al.*, 1990; Leigh Brown, 1991; Coffin, 1992). This variation is not uniformly spread across the whole protein but is clustered into five hypervariable regions (V1-V5) displaying less than 30% sequence homology, interspersed between four highly conserved sequences (C1-C4) and regions of intermediate variability (Starcich *et al*, 1986; Willey *et al.*, 1986; Modrow *et al.*, 1987). These regions have been mapped and are shown schematically in Figure 1.7. The precise numbering of the individual amino acids varies depending on the virus strain.

Despite its high divergence, the 21 cysteine residues present in the uncleaved gp160 molecule are completely conserved in all reported isolates including SIV and HIV-2, which share only 30-40% identity with HIV-1 (Tschachler *et al.*, 1990). Substitution of these cysteine residues by other amino acids resulted in non-infectious virus that were blocked in the viral life cycle (Tschachler *et al.*, 1990) indicating that they have an indispensable role in viral function, probably at least in part through the contribution of disulphide bridges to the tertiary structure of the envelope protein.

1.6.5 Variation within an individual

An important feature of HIV-1 infection is that genetic variation exists not only between viral isolates from different individuals but between isolates from within individuals and that this heterogeneity becomes more apparent during the course of infection (Saag *et al.*, 1988; Fisher *et al.*, 1988; Simmonds *et al.*, 1990, 1991; Balfe *et al* ., 1990; Wolfs *et al.*, 1992). Hahn *et al.* (1986) examined sequential isolates from

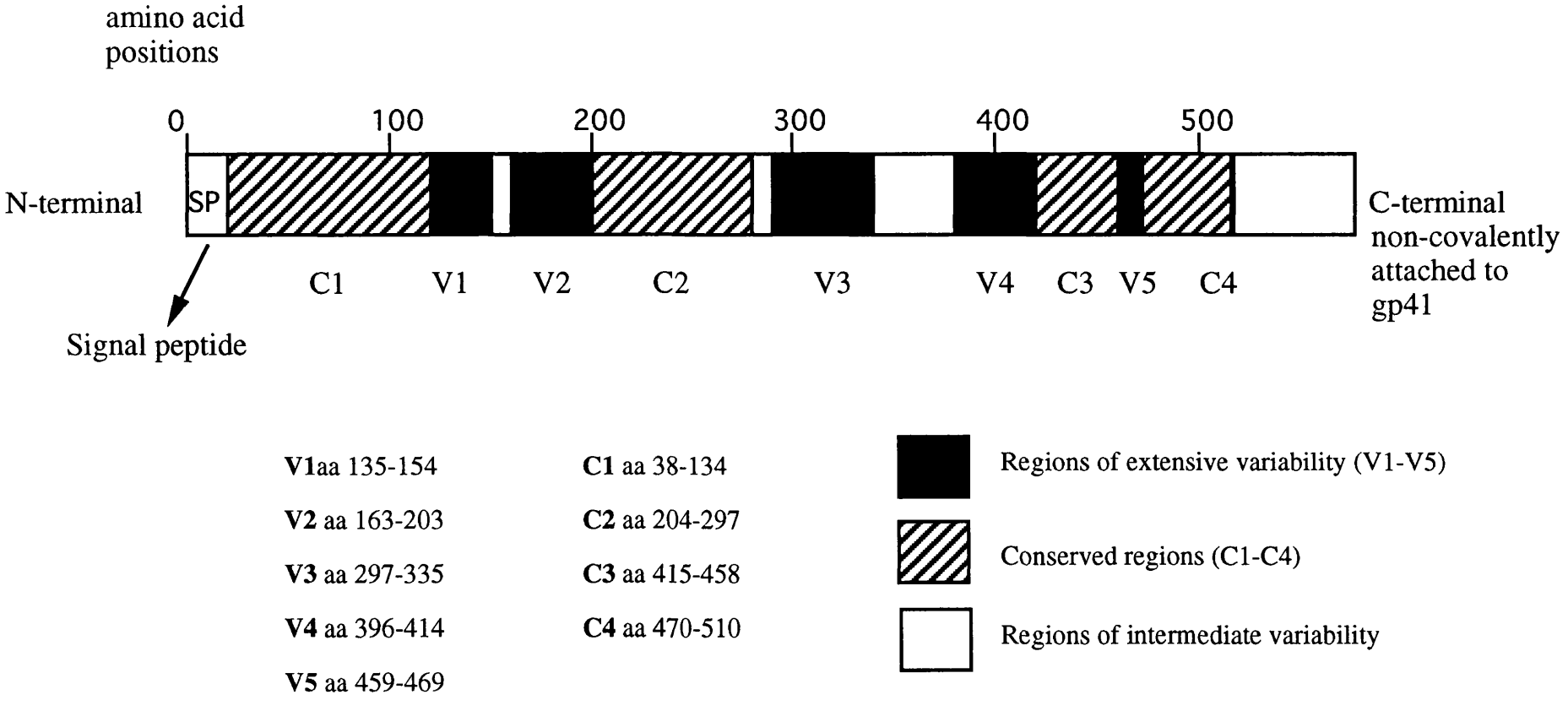


Figure 1.7 Schematic representation of the conserved and variable regions of gp120 based on the numbering of the HXB-2 strain according to Modrow *et al.* (1987).

three individuals infected via different routes of transmission and identified single amino acid changes in the hypervariable regions of gp120 and found that the level of heterogeneity appeared to increase over time. This is consistent with the more recent findings that on initial infection with HIV-1 the virus population appears to be relatively homogenous in the envelope region (Wolfs *et al.*, 1991, 1992; Zhang *et al.*, 1993). Indeed, virus isolated from pre-seroconversion samples from nine infected patients showed no heterogeneity in either the V3 or V4 regions of gp120, in contrast to major variability observed in the *gag* gene or in the regions flanking V3 and V4 (Zhang *et al.*, 1993). Similarly, the observed reduction of sequence variability of the *env* gene found on mother-child transmission (Wolinsky *et al.*, 1992) suggests, a selection for specific *env* sequences either upon transmission or in the interval between exposure and seroconversion. This apparent requirement for a specific env determinant on primary infection may play a part in the stochastic nature of virus transmission.

During the long asymptomatic phase the viral population is highly heterogenous in the envelope region (Hahn *et al.*, 1986; Simmonds *et al.*, 1990, 1991; Balfe *et al.*, 1990; Wolfs *et al.*, 1992). Sequence variation in HIV-1 isolates between individuals infected from a common source has also been reported (Simmonds *et al.*, 1990; Balfe *et al.*, 1990; Burger *et al.*, 1990). Such variation was less extensive than the variation observed between epidemiologically unlinked individuals. Within these patients infected from a common source, heterogeneity in the envelope region again increased over time. It was also found that although sequence variants were detected within both the viral RNA isolated from blood plasma and the proviral DNA isolated from PBMCs, the novel sequence variants appeared first in the plasma RNA populations and then subsequently in the proviral population and the proviral sequences persisted in the population for much longer periods of time (Simmonds *et al.*, 1991). Furthermore, the evolution of HIV-1 sequences does not appear to accumulate steadily with time. In a patient of the Edinburgh haemophiliac cohort (p82), also studied in the work presented in this thesis, sequence variation over a five year follow-up period consisted of a series of replacements in three hypervariable regions of gp120 of one particular sequence type

with another, not directly derived by a single substitution from the previously observed sequence. This suggests either the existence of a hidden mechanism of evolution such as a requirement for covariance between specific amino acids for the sequence changes in HIV-1 gp120 (Korber *et al.*, 1993) or that the interval between sampling sequential isolates is such that intermediate sequences have been missed (Simmonds *et al.*, 1991).

Nowak *et al.* (1991) hypothesise that there is a threshold level of diversity that can be supported by the host immune system. Beyond this diversity threshold, the individual is unable to mount an effective immune response against new variants, resulting in the outgrowth of the most virulent viruses and the subsequent progression to AIDS.

1.6.6 Effect of variation on biological phenotype

The sequence variation that occurs in gp120, in particular within the V3 region, has an effect on the pathological phenotype of the virus in terms of tropism, ability to induce syncytia and ability to replicate *in vitro* (Levy *et al.*, 1984; Anand *et al.*, 1987; Asjo *et al.*, 1986; Evans *et al.*, 1987; Briesen *et al.*, 1987; Tersmette *et al.*, 1988; Fenyo *et al.*, 1988). Viruses isolated from early stages of infection in general, grow more slowly, do not induce syncytia between infected cells (non syncytium-inducing or NSI) and replicate in primary macrophages as well as in activated $CD4^+$ cells but do not grow well in immortalised T cell lines. Isolates recovered from patients with AIDS or ARC however, show higher replication rates than isolates recovered from asymptomatic individuals , can induce syncytia (syncytium-inducing or SI) and are also capable of replicating in immortalised T cell lines but not in primary macrophage cell lines (Asjo *et al.*, 1986; Evans *et al.*, 1987; Briesen *et al.*, 1987; Tersmette *et al.*, 1988, 1989; Fenyo *et al.*, 1988). Although these three properties of HIV-1 are distinct, it is likely that rapid-high, T cell tropic and SI, all define the same state, which is distinct from the less virulent state characterised as slow-low, macrophage tropic and NSI. It is also unlikely that all HIV-1 isolates can be unambiguously assigned to one or other of these two

distinct groups, and more probable that some isolates will display a spectrum of these properties (Simmonds-personal communication). In longitudinal studies, it has been shown that disease progression is linked with a transition from NSI isolates such as the primary macrophage tropic isolates to SI T cell tropic isolates (Cheng-Mayer *et al.*, 1988; Tersmette *et al.*, 1989a, b; Schuitemaker *et al.*, 1991, 1992). In one study, 3 out of 19 virus isolates collected before seroconversion were SI and non-macrophage tropic. The detection of this more virulent phenotype correlated with a more rapid progression to AIDS than observed in those patients infected with NSI variants (Roos *et al.*, 1992). It has also been argued that macrophage tropic, NSI isolates are responsible for the persistence of HIV-infection (Meltzer *et al.*, 1990).

1.7 Properties and functions associated with the V3 region of gp120

1.7.1 Structure of the V3 loop

The V3 loop region consists of 33-35 amino acids, depending on the HIV-1 strain, bound to form a loop structure by a disulphide bridge between two invariant cysteines (Rusche *et al.*, 1988; Goudsmit *et al.*, 1988a; Palker *et al.*, 1988; Leonard *et al.*, 1990). Although this region is highly variable, it is better described as a mosaic of variable and conserved regions with a highly conserved amino acid triplet, Gly-Pro-Gly, common to all HIV-1 strains at the tip of the loop (La Rosa *et al.*, 1990). It has been predicted from a consensus of V3 sequences that the loop structure is: cysteine-beta strand-type II beta turn-beta strand-alpha helix-cysteine (La Rosa *et al.*, 1990).

1.7.2 The V3 loop as the principal neutralising determinant of HIV-1

Patients infected with HIV elicit potent neutralising antibodies against the external glycoprotein gp120 (reviewed by McKeating, 1992) and in particular the V3 loop (Palker *et al.*, 1988; Goudsmit *et al.*, 1988a; Rusche *et al.*, 1988; Matsushita *et al*, 1988; Javaherian *et al.*, 1989). Initial findings identified antibodies that could cross neutralise a number of laboratory isolates of HIV-1 (Weiss *et al.*, 1985; Robert-Guroff *et al.*, 1985), however, as more isolates became available both isolate restricted and non-isolate restricted neutralising antibodies were observed (Weiss *et al.*, 1986). Antibodies that can cross neutralise divergent strains of HIV-1, the so-called "group-specific" antibodies are in general directed against conserved conformational epitopes on gp120 (Haigwood *et al.*, 1990a; Profy *et al.*, 1990; Steimer *et al.*, 1991; Kang *et al.*, 1991; Hariharan *et al.*, 1993) and in particular against the CD4 binding site that has been mapped to between amino acids 397-439 in the C-terminal portion of gp120 (Lasky *et al.*, 1987). These antibodies show the capacity to inhibit the binding of gp120 to the cellular receptor molecule CD4 (Steimer *et al.*, 1991; Kang *et al.*, 1991). Antibodies that neutralise autologous virus, the so-called "type-specific" neutralising antibodies in general map to the V3 loop (Palker *et al.*, 1988; Goudsmit *et al.*, 1988a; Rusche *et al.*, 1988; Matsushita *et al.*, 1988; Javaherian *et al.*, 1989) and are produced early in infection. More recently however, a number of monoclonal antibodies that recognise conserved features of the V3 domain can neutralise a broader range of isolates (Javaherian *et al.*, 1989; Ohno *et al.*, 1991; Gorny *et al.*, 1992). The neutralisation ability of HIV-1 infected sera appears to broaden with time (Nara *et al.*, 1987; Zagury *et al.*, 1988; Goudsmit *et al.*, 1988b), probably in response to the exposure of the individual to extensive sequence variation in gp120 during the course of infection.

Antibodies that are reactive against the V3 region can account for up to 50% of the total native gp120-specific antibodies in infected sera and can account for nearly all of the antibodies that bind to denatured gp120, suggesting that V3 acts as a linear

determinant. (Moore and Ho, 1993). In addition, peptides mimicking the V3 region of the MN strain of HIV-1, elicit high titres of virus neutralising antibodies and can, when attached to a solid phase support absorb out most of the neutralising activity of HIV-1_{MN} infected sera (Profy *et al.*, 1990). For these above reasons the V3 region of HIV-1 is defined as the principal neutralising determinant or PND (La Rosa *et al.*, 1990).

The reactive epitope in V3 has been mapped to a stretch of 8-14 amino acids, incorporating the highly conserved Gly-Pro-Gly-Arg domain and amino acids that directly flank this region (Goudsmit *et al.*, 1988a; Javaherian *et al.*, 1989; Meloen *et al.*, 1989). The *in vivo* relevance of neutralising antibodies against the V3 loop has yet to be fully established. Monoclonal antibodies directed against the PND prevent HIV-1 infection of MT4 cells in vitro , while antibodies in polyclonal IgG when pre-incubated with the IIIB strain of the virus were shown to inhibit infection of chimpanzees in vivo (Emini *et al.*, 1990). Immunisation of chimpanzees with recombinant forms of gp120 were protected from challenge with HIV-1 and were shown to contain high titres of neutralising antibodies directed against the V3 region (Berman *et al.*, 1990; Emini *et al.*, 1990; Girard *et al.*, 1991). The role V3 antibodies have in the observed protection of chimpanzees from infection was not directly measured in these experiments, however, a mouse monoclonal antibody specific for the V3 loop domain could protect chimpanzees against infection with HIV-1 when administered before or after challenge (Emini *et al.*, 1992). Vaccine trials in rhesus macaques has however, shown no correlation between the level of neutralising antibodies and protection from infection with challenge SIV (Desrosiers *et al.*, 1989).

Viruses that can escape neutralisation by V3 monoclonal antibodies have been identified *in vivo* in HIV infected chimpanzees (Emini *et al.*, 1990; Nara *et al.*, 1990) and *in vitro* with V3-specific monoclonal antibodies (McKeating *et al.*, 1989). In most cases, sequence changes in the V3 region can account for the differences in neutralisation however, sequencing of the V3 region of resistant viruses that emerged in chimpanzees, experimentally infected with HIV-1_{IIIB}, revealed that some of these

mutants had no amino acid changes in the V3 region or that amino acid changes that emerged in the V3 region, occurred at positions outwith the monoclonal antibody binding site (Nara *et al.*, 1990). This finding suggests, that other regions of gp120 may interact with the V3 region in some way altering its conformation and neutralisation ability.

1.7.3 Other neutralisation epitopes in gp120

In addition to the well defined neutralisation epitopes located in both the V3 region and the CD4 binding site, a linear neutralisation epitope in the V2 region and a discontinuous epitope, dependent on the structure of the V1/V2 region, have been identified using monoclonal antibodies (Fung *et al.*, 1992; McKeating *et al.*, 1993). Of the monoclonal antibodies that were dependent on the structure of the V1/V2 region, additional mutations in the C4 region of gp120 could be shown to have an effect on the neutralisation properties of these monoclonal antibodies (McKeating *et al.*, 1993). It was therefore suggested, that amino acid changes in the C4 region may alter the structure of the V1/V2 domain recognised by conformation-dependent antibodies (McKeating *et al.*, 1993). The conformation of gp120 is therefore important for the efficient neutralisation of HIV-1. Recent studies have identified discontinuous epitopes in gp120, proximal to or overlapping the CD4 binding site that only become properly exposed to neutralising antibodies upon binding to CD4 (Thali *et al.*, 1991, 1992, 1993). Linear neutralisation epitopes have also been identified in the V4 and V5 regions of HIV-1 gp120 (Haigwood *et al.*, 1990b).

1.7.4 The role of the V3 region in viral entry into target cells

Monoclonal antibodies against the V3 region (Matsushita *et al.*, 1988; Scott *et al.*, 1990) do not act to neutralise the virus by blocking binding to the CD4 molecule but are believed to work via a post binding event (Javaherian *et al.*, 1990). Deletion of most of

the V3 region does not appear to affect the binding of monomeric gp120 to CD4 (Pollard *et al.*, 1992). These findings implicate V3, acting directly or indirectly, as having an important role in viral fusion (Freed *et al.*, 1991a, b). It is hypothesised that V3 interacts with cell surface proteases on target cells which may act to facilitate infection. This hypothesis is based on experiments showing that both HIV-1 and HIV-2 have proteolytic cleavage sites at the tip of the V3 loop (Stephens *et al.*, 1990; Clements *et al.*, 1991) and that neutralising antibodies raised against this region can block the proteolytic cleavage of the V3 loop by thrombin (Clements *et al.*, 1991) In addition, a serine protease inhibitor, trypstatin, has been shown to block HIV infection *in vitro* (Kioto *et al.*, 1989; Hattori *et al.*, 1989; reviewed by McKeating, 1992). This suggests that neutralising antibodies against V3 interfere with the cleavage event required for viral entry. Sattentau and Moore (1991) have also shown that the V3 loop on the surface of virions is not accessible to proteolytic cleavage (thrombin) until the virus has bound sCD4, confirming the hypothesis that a conformational change is required in gp120 to allow viral entry. If this model is correct then viral tropism could be influenced by the presence of specific-cellular proteases on the surface of target cells.

1.7.5 Involvement of the V3 region in viral tropism and biological phenotype of HIV-1

As discussed previously in 1.6.5 and 1.6.6, virus isolates obtained from infected individuals during the course of infection, display not only considerable genetic diversity (Hahn *et al.*, 1986; Saag *et al.*, 1988; Fisher *et al.*, 1988; Simmonds *et al.*, 1990, 1991; Balfe *et al* ., 1990; Wolfs *et al.*, 1992) but also differences in their biological phenotype (Levy *et al.*, 1984; Anand *et al.*, 1987; Asjo *et al.*, 1986; Evans *et al.*, 1987; Briesen *et al.*, 1987; Tersmette *et al.*, 1988, 1989a, b; Fenyo *et al.*, 1988; Cheng-Mayer *et al.*, 1988; Schuitemaker *et al.*, 1991, 1992). The existence of macrophage tropic isolates early in infection may suggest that viruses of this type are transmitted more frequently from individual to individual perhaps due to their inability

to induce syncytia and slow replication in both macrophages and PBMCs and therefore decreased recognition by the host immune system as compared to the more virulent T cell tropic isolates. Evidence supporting this hypothesis has not yet been obtained and the importance of these macrophage tropic clones is not fully understood.

A number of studies aimed to determine the minimum region of the HIV-1 genome that conferred this macrophage tropism (Chesebro *et al.*, 1991; Hwang *et al.*, 1991; O'Brien *et al.*, 1990; Shioda *et al.*, 1991; Westervelt *et al.*, 1991, 1992). These studies followed essentially the same protocol, involving chimeric gp120 molecules with different regions of the protein derived from macrophage tropic clones, cloned into T cell tropic clones by the use of shared restriction enzyme sites. These chimeric viruses were then tested for their ability to grow in primary macrophages, PBMCs and transformed T cell lines. The studies showed that amino acids between 202-358 incorporating the V3 loop were sufficient to confer macrophage tropism. Hwang *et al.* (1991) further determined the minimal region capable of controlling macrophage tropism to be a 20 amino acid sequence contained within the V3 loop. In addition, similar studies have shown that amino acid changes both in the highly conserved Gly-Pro-Gly-Arg motif and flanking amino acids in the V3 region were sufficient to change virus tropism to a T cell tropic cell line and to confer syncytium inducing properties (Chesebro *et al.*, 1992; De Jong *et al.*, 1992a). Evident from all these studies was that no consensus V3 sequence can determine macrophage tropism, as T cell tropism could be eliminated by site directed mutagenesis with concomitant replication in macrophages with as few a two or three amino acid substitutions in the V3 region, none of which appeared in any of the macrophage tropic isolates (Chesebro *et al.*, 1992). More recent studies have identified the V1 and V2 regions in addition to the V3 region of gp120 of to be involved in tropism of HIV-1 (Kioto *et al.*, 1993; Andeweg *et al.*, 1993; Sullivan *et al.*, 1993 ; Boyd *et al.*, 1993).

Studies have also been performed to assess the role of the V3 region in syncytium formation. De Jong *et al.* (1992a) provide evidence that SI viruses have a higher net charge associated with their V3 sequences than do NSI viruses. This has been further

extended by comparison of the V3 sequences from sets of biological clones isolated sequentially from 12 patients. These sequences showed a correlation between a basic charge (usually R or H) at amino acid position 11 (gp120 amino acid 306, based on the MN strain of HIV-1) and R or K at amino acid position 28 (gp120 amino acid 320) in the V3 region and the transition form an NSI to an SI phenotype. In NSI variants, the amino acid residue at position 11 was uncharged (in general S or G) and the residue at position 28 was either negatively charged (E or D) or uncharged (A or Q) (Fouchier *et al.*, 1992). In addition, these mutations were shown to potentially alter the structure of the V3 region, suggesting that SI and NSI variants may have distinct V3 structures. Mutational analysis of these two amino acids abolished syncytium-inducing ability of these clones (De Jong *et al.*, 1992b). In a recent study it was shown that changes outwith V3 are also capable of altering the biological phenotype, and that an interaction between several regions of gp120 may be required (Cheng-Mayer *et al.*, 1991; Stomatatos and Cheng-Mayer, 1993). This suggests that the overall conformation of gp120 is important for its biological functions. Amino acid changes in the V1 and V2 region in addition to V3 have been shown to alter the replicative capacity of viral isolates (Groenink *et al.*, 1993).

1.8 Factors affecting maternal-foetal transmission of HIV-1

The rate of mother to child transmission of HIV appears to vary depending on the study cohort. Transmission rates ranging from 7-65% have been reported, with the highest transmission rates being reported from African countries (Rubinstein and Bernstein, 1986; Piot *et al.*, 1988; Mok *et al.*, 1989; Blanche *et al.*, 1989; Ades *et al.*, 1991). The factors that affect this transmission rate are not yet understood, however, the European collaborative study (1992), set up to analyse transmission data from nineteen European countries between December 1984 and August 1991, aimed to focus on the risk factors associated with maternal-foetal transmission. This study

established that low CD4 counts, p24 antigenemia and high virus load in the mothers were predictive markers of vertical transmission in this study group. In addition, this and one other study (Goedert *et al.*, 1991) established that infants born before 38 weeks gestation were more likely to have acquired HIV-1-infection from their mothers. This finding could be compatible with these infants having been infected *in-utero* and HIV-infection having affected foetal development, causing premature birth. Goedert *et al.* (1991) also reported that these HIV-1 infected infants born prematurely had low concentrations of maternal anti-gp120 antibodies, raising the possibility that antibodies against gp120 may have a protective role in vertical transmission of HIV-1. A number of studies discussed in detail in results section 2, suggested that high titre or high affinity antibodies against the V3 region of HIV-1 gp120 correlated with a lack of transmission of the virus to the child (Broliden *et al.*, 1989; Rossi *et al.*, 1989; Devash *et al.*, 1990). Work presented in this thesis and by others (Parekh *et al.*, 1991; Geffin *et al.*, 1992; Halsey *et al.*, 1992) however, contradicts these findings.

The precise time at which vertical-transmission occurs is not yet known, however, transmission data collected from twin-studies (Goedert *et al.*, 1991) has suggested that transmission occurs in as many as 50% of the infants in their studies at the time of birth. These studies involved 66 sets of twins from 9 countries. HIV-1 was diagnosed in 50% of first born twins delivered vaginally and in 38% delivered by caesarian section, compared with only 19% for both modes of delivery in the second born twins. This increased risk for the first born twin may be due to increased exposure of that infant to maternal blood and fluids in both the birth canal and via caesarian section. The data from this study and others (Chiodo *et al.*, 1986; Lindgren *et al.*, 1991) shows no significant difference in the relative risk of transmission between the two modes of delivery, however, a more recent study, which analysed all the published data, suggests that birth by caesarian section may reduce the risk of vertical HIV-1 transmission (Villari *et al.*, 1993). If this is the case, then by modification of obstetric techniques used on HIV-infected women , advising a planned caesarian section may help reduce the risk of vertical HIV-1 transmission.

Vertical transmission of HIV-1 has also been reported to occur through breast feeding, following primary infection of the mother after delivery (Van de Perre *et al.*, 1992). In an extension to this study, a correlation was found between increased vertical transmission and higher numbers of HIV-1 infected cells in early breast milk (Van de Perre *et al.*, 1993). It was also found that none of the mothers in this study had any detectable HIV-1 specific IgA (IgA being the main component of humoral immunity in breast milk (Welsh and May, 1979)). However HIV-1 specific IgM appeared to compensate for the absence of IgA, in that mothers with higher levels of IgM had a decreased risk of vertical transmission of the virus. The results of these finding are however difficult to interpret , as it is not known if infection of the child occurred either before or after delivery. The authors recognise this limitation and conclude only that the presence of HIV-1 in breast milk is a risk factor for vertical transmission of the virus.

1.9 Vaccination against AIDS

1.9.1 Approaches to AIDS vaccines

The minimal elements of an effective HIV-1 prophylactic or therapeutic vaccine are, safety, long term protective immunity and the ability to protect against globally diverse isolates (reviewed by Barrett and Dorner, 1993). A number of strategies have generally been used for the development of viral vaccines including, the use of whole inactivated viruses, live attenuated viruses, live recombinant viruses, subunits purified from whole virus or subunits derived by recombinant DNA technology and synthetic peptides. Prophylactic vaccination has proved highly successful in the control of a range of diseases caused by viruses such as smallpox, yellow fever and poliomyelytis. The nature of HIV-1 infection makeseffective vaccination against this virus both scientifically and ethically more difficult. HIV-1 has mechanisms that enable it to establish a persistent infection in man which are more elaborate than those of viruses

against which successful vaccines have been produced. Non cytopathic deletion mutants of HIV-1 could be developed that could grow and induce immune responses against HIV-1 without causing disease. However, as HIV-1 can integrate into the host genome, these viruses could be retained for life and may result in malignant transformation. In addition, inactivation of HIV-1 could present problems as the viral genome could remain functionally intact following inactivation and integrate into the host genome resulting in latency or virus production. Due to the ethical restrictions of vaccinating seronegative individuals with live attenuated or non cytopathic deletion mutants most of the vaccine development studies involve viral protein subunits derived by recombinant DNA technology.

It is important to note that infection with HIV-1 can result from transmission of both cell-free virus and through virus-infected cells that display little or none of the viral antigens on their surface. Therefore a successful vaccine against HIV-1 must induce both humoral and cellular immunity. This again is no simple task as immunogenic regions of the virus have been shown to induce both antibody-mediated enhancement of viral infection (Takeda *et al.*, 1988, 1990; Homsy *et al.*, 1989; Robinson *et al.*, 1991.; Haubrich *et al.*, 1992) and anti-idiotypic antibodies against cellular components (Hoffman *et al.*, 1991).

At present both prophylactic and therapeutic vaccines are being tested with the aim of either protecting against or controlling HIV-1 infection and the progression to AIDS.

1.9.2 Prophylactic vaccine trials

The majority of prophylactic vaccine trials that have been initiated in America, Europe and Africa involve immunisation of seronegative individuals with preparations of either recombinant HIV-1 gp120 or the uncleaved gp160 molecule. These trials so far demonstrate the production of high titres of neutralising antibodies and memory T cells against HIV-1 (Barrett *et al.*, 1989; Dolin *et al.*, 1991; reviewed by Barrett and

Dorner, 1993) with the most recent vaccine trial reporting induction of both neutralising and syncytium-inhibiting antibodies (Schwartz *et al.*, 1993). However, without actually challenging these individuals with live HIV-1 it is impossible to assess the preventative capacity of these preparations. Animal models are therefore important in the development of potential vaccines against HIV-1. Monkeys infected with HIV-1 show none of the clinical signs of disease associated with infection in humans and for this reason SIV-infection in both chimpanzees and macaques which do show AIDS-like symptoms are widely used animal models. However, given the dissimilarity of the principal neutralising epitopes for HIV-1 and HIV-2 /SIV and differences in cell tropism for these viruses operating at the level of entry (Clapham *et al.*, 1991) experiments on SIV may not be predictive of humoral protection against HIV-1 (Moore and Weiss, 1991).

As discussed previously in section 1.7.2, monoclonal antibodies against the principal neutralising determinant of gp120 protect chimpanzees from infection when administered either before or 10 minutes after virus challenge (Emini *et al.*, 1992). However, immunisation of chimpanzees with attenuated SIV showed no correlation between the *in vitro* level of neutralising antibodies and protection against challenge virus (Desrosiers, *et al.*, 1989).

Several groups have independently demonstrated that macaques could be protected from infection with SIV by immunisation with inactivated vaccines, based on either whole inactivated SIV or fixed SIV-infected cells (Desrosiers *et al.*, 1989; Murphey-Corb *et al.*, 1989; Stott *et al.*, 1990) raising expectations that the development of a vaccine against HIV-infection in man could be possible. However, it has now been shown that inactivated SIV-based vaccines were unable to protect against challenge with SIV grown in monkey cells but could protect against SIV grown in human cells. In addition, vaccination of monkeys with uninfected human cells protected against challenge against SIV derived from human cells, despite the fact that all the monkeys had specific SIV neutralising antibodies (Stott, 1991; Langlois *et al.*, 1992; Le Grande *et al.*, 1992; Osterhaus *et al.*, 1992; Cranage *et al.*, 1992). The protection conferred

against virus grown in human cells is now known to be due to the recognition of human HLA type 1 on the surface of these virions as foreign molecules by the monkey immune system. These studies demonstrate the absolute necessity of designing vaccine experiments where viruses used for challenge are grown in PBLs from the target host species and bring into question the importance of neutralising antibodies in protection against HIV-infection.

The current experimental vaccine studies using recombinant gp120 or gp160 have overlooked a number of important features of infection with HIV-1 (reviewed by Sabin, 1992). Firstly, the two most important vehicles of infection in humans are semen and blood. These contain large numbers of virus-infected cells that display little or none of the viral antigens on their surface. Such virally-infected cells would not be detected by neutralising antibodies against gp120 or gp160. Indeed, Johnson *et al.* (1992b) reported that an inactivated whole SIV vaccine that protected macaques against an intravenous challenge of 50 ID_{50} of cell-free SIV did not protect against challenge with 50 ID_{50} of SIV-infected PBMCs. Secondly, one of the major routes of transmission of HIV-1 is via the introduction of semen into the colorectal area of men and women through receptive anal intercourse. A successful vaccine must be able to prevent infection via this route.

In addition to the prophylactic vaccine studies, studies to develop a vaccine that will have a therapeutic effect in those individuals already infected with HIV is of vital importance and the subject of much investigation. Vaccine therapy could either clear the virus from the host, prevent the progression to AIDS or improve the quality of life for those patients with AIDS.

1.10 Antiviral agents against HIV-1

1.10.1 Inhibition of HIV-1 infection with soluble recombinant CD4 (sCD4)

The observation that the CD4 molecule is the principal receptor for HIV-1, HIV-2 and SIV in both T lymphocytes and monocytic macrophages led to the hypothesis that a soluble form of the CD4 receptor might inhibit infection. To test this hypothesis a number of groups made recombinant soluble CD4 and demonstrated its ability to block HIV-1 infection *in vitro* (Smith *et al.*, 1987; Deen *et al.*, 1988; Hussey *et al.*, 1988; Traunecker *et al.*, 1988; Clapham *et al.*, 1989; Byrne *et al.*, 1989). The effect of recombinant CD4 was tested in Rhesus macaques infected with SIV_{mac}. Virus could be readily isolated from PBLs and bone marrow cells of these animals prior to treatment but became difficult to isolate after treatment with sCD4. In addition, diminished growth of granulocyte/macrophage and a rise to normal levels of erythrocyte progenitor colonies in the bone marrow during treatment was observed (Watanabe *et al.*, 1989). This evidence and the finding that sCD4 could also protect chimpanzees against experimental infection with HIV-IIIB (Ward *et al.*, 1991) implicated sCD4 as a potential therapeutic agent in infected individuals (Kahn *et al.*, 1990; Schooley *et al.*, 1990). Soluble CD4 may act to neutralise HIV-1 in a number of ways. At low concentrations, sCD4 binds competitively for the gp120 on the virion surface to weakly neutralise the virus (Lasky *et al.*, 1987). However, at higher concentrations of sCD4, the virus is effectively neutralised by a process of shedding of the gp120-sCD4 complex from the surface of the virion, rendering it non-infectious (Moore *et al.*, 1990, 1991). In addition, the binding of soluble CD4 could alter the conformation of gp120 such that viral fusion with the host cell membrane cannot occur. Inaccessibility of the V3 loop to proteolytic cleavage may be one of the mechanisms involved (O'Brien *et al.*, 1992).

The initial enthusiasm for the use of sCD4 in HIV-infected individuals was perhaps dampened by the observations that some viral isolates even from the same individual are more susceptible to neutralisation by sCD4 than others (Daar *et al*, 1990; Gomatos *et al.*, 1990; Byrne *et al.*, 1989). Primary isolates of HIV-1 which are generally believed to be macrophage tropic (Gartner and Popovic, 1990; Schuitemaker *et al.*, 1991, 1992) were found to be less susceptible to neutralisation by sCD4 *in vitro* than laboratory isolates that have been selected for efficient replication in transformed T cell lines (Daar *et al.*, 1990). The concentrations of sCD4 required to neutralise primary isolates was 100-1000-fold higher than that required to neutralise laboratory strains (Daar *et al.*, 1990; Moore *et al.*, 1991) This is probably due to the lower binding affinity of the primary isolates to sCD4 (Moore *et al.*, 1991). Recent studies have shown that adaptation of two HIV-1 primary isolates to grow in transformed T cell lines also results in the viruses becoming more sensitive to neutralisation by sCD4 (Moore *et al.*, 1993). It is important to note that progression to disease is believed to be associated with a gradual switch from a virus population that is in predominantly macrophage tropic to one which is T cell tropic (Schuitemaker *et al.*, 1992). The observed phenotypic differences between macrophage and T cell topic viruses and their susceptibility to neutralisation by sCD4, has been attributed to sequence variation found in the V3 loop and does not include regions of gp120 that are directly involved in binding to the CD4 binding site (Hwang *et al.*, 1992; O'Brien *et al.*, 1992).

1.10.2 Chemotherapeutic inhibitors of HIV reverse transcription

The current therapeutic compounds being tested for their inhibitory properties of HIV replication fall into two categories. Firstly, the deoxynucleoside analogues, with AZT (zidovudine, 3'-azido. 3'deoxythymidine), ddC (zalcitabine, 2'3'-dideoxycytidine) and ddI (didanosine,2'3'-dideoxyinosine) being the most well known, which inhibit the retroviral enzyme reverse transcriptase, and secondly, non-nucleoside inhibitors of reverse transcriptase (NNRTI) such as neviraprine and TIBO (tetra hydro-

imadazo[4,5,1-1-jk][1,4]-bezodiazepin-2(1H)-one and thione) (reviewed by Richman, 1993). Initial studies on the efficacy of AZT, the only currently licensed drug against HIV, concluded that treatment of HIV-infected individuals with this drug, prolonged the disease-free interval in asymptomatic patients, improved the quality of life and delayed death. However, these studies based their conclusions on data obtained from a trial that was cut short in the first year (Volberding *et al.*, 1990). This was due to the initial findings that the patients who were receiving AZT showed a lower mortality rate and a slower decline in CD4 counts, believed to be an accurate marker for disease progression. The results of the recently published Concorde trial (Aboulker and Swart, 1993), that involved over 1700 asymptomatic patients, in which some were given AZT and the rest were given a placebo until the onset of disease, showed no significant difference in disease progression and eventual death in the patients from the two groups, even though patients who were receiving AZT maintained higher levels of CD4 cells than those taking the placebo drug. These findings have proved very disheartening for both AIDS researchers and ultimately individuals infected with HIV and suggest caution in using the CD4 count as a reliable marker for the progression of AIDS in infected people (Maddox, 1993).

The failure of these drugs to effectively inhibit viral replication for extended periods of time is probably due to the emergence of mutants that show resistance to these compounds (Larder *et al.*, 1989a, b, c; Nunberg *et al.*, 1991; Richman *et al.*, 1991). Mutations in the RT of HIV-1 that account for the reduced susceptibility to drugs have been identified by sequencing isolates of resistant virus, and the contribution of identified mutations confirmed by using site-directed mutagenesis to place these mutations in an infectious plasmid containing a sensitive HIV-1 genome (reviewed by Richman, 1993). Mhori *et al.* (1993), have recently identified isolates that show resistance to AZT from patients that were never treated with the drug. These variants will be selected for rapidly on treatment with AZT. Chow *et al.* (1993a), have proposed the concept of combination therapy, which implies that simultaneous multiple-inhibitor therapy against the HIV-1 reverse transcriptase would delay the emergence of multiply

resistant virus strains. They also reported the emergence of a variant in the presence of multiple-inhibitors that was non-viable in human cells, concluding that the mutations that arose in that variant, in response to combination therapy, were not compatible with one another. Based on this finding they put forward a strategy to turn this evolutionary survival mechanism against the virus. They proposed that by selecting certain combinations of mutations for drug resistance, that it may be possible to force the virus into inviability. However, other studies have failed to confirm these results, demonstrating that the variant described by Chow *et al.* (1993a) was in fact resistant to combination therapy and exhibited growth kinetics similar to the wild type, indicating that drug resistant variants are likely to be selected eventually during combination therapy. (Emini *et al.*, 1993; Larder *et al.*, 1993). Chow *et al.* (1993b) have now revealed that other unintended mutations in reverse transcriptase could explain why viruses derived from these clones were not viable.

1.10.3 Other potential antiviral targets

In addition to the reverse transcriptase inhibitors, molecules or chemicals that could inhibit either the retroviral protease enzyme, essential for the generation of mature infectious virions or the retroviral regulatory protein Tat are being investigated (Reviewed by Crumpacker, 1992). Peptide analogue-based inhibitors that mimic the sites at which the protease enzyme acts could inhibit HIV-1 replication in infected cells by blocking proteolytic processing and virion maturation. A favourable feature of protease inhibitors is that they could lead to a decreased frequency of drug resistant mutants arising as the protease molecule recognises different stretches of amino acids during its normal function and therefore single amino acid substitutions may not have an effect on its ability to bind to peptide analogues. Current peptide based protease inhibitors have low solubility and are readily degraded in human serum (reviewed by Crumpacker, 1992).

Inhibition of the Tat protein would effectively render proviral DNA unable to produce infectious virus or efficient quantities of viral proteins. A recent study, based on the knowledge that Tat exerts its effects through the TAR sequence present downstream of the HIV-1 LTR (Feng and Holland, 1988) has shown that the introduction of plasmids expressing copies of TAR in transient transfection assays in a human T cell line, when challenged with HIV-1 or SIV, inhibited viral replication by as much as 99% (Lisziewicz *et al.*, 1993).

The demonstration of the efficacy of a candidate vaccine, whether for therapeutic or prophylactic purposes as discussed in section 1.9 and 1.10, must await the results of phase 1 studies designed to determine the safety, phase II studies which determine immunogenicity, and phase three trials which determine the efficacy in protecting against infection or the onset of disease. This requires the monitoring of a large volunteer group over an extended period of time. Therefore despite the extensive studies aimed at developing an effective vaccine, licensing for general use is probably still many years away.

1.11 Structure of branched or multiply antigenic peptides (MAPs)

Branched or multiply antigenic peptides (MAPs) were originally described by Tam (1988) and their antigenic properties together with the initial observations which suggested their potential for serodiagnosis are discussed in detail in results section 1. The octameric structure of these peptides is achieved by the sequential addition of three bivalent lysine residues onto an alanine core attached to the resin on which the peptide is to be synthesised. This branched configuration, shown in Figure 1.8, allows the sequential addition of amino acids by solid-phase peptide synthesis onto eight free amino groups rather than one as with the monomeric peptides.

1.12 Aims of the work presented in this thesis

The aims of the work presented in this thesis are three fold. The initial studies aimed to optimise branched peptides for use as serodiagnostic reagents based on the observation in our laboratory that peptides made with this configuration could detect antibodies at lower concentrations than could monomeric peptides (Owsianka and Marsden-personal communication). The work performed, aimed to examine the nature of this increased sensitivity and to optimise the form of branched peptide that would give maximal reactivity with specific antibodies. In addition to this I sought to compare the reactivity of these branched peptides corresponding to a known epitope with that of the native protein from which the epitope is derived. The information obtained from this work was then applied in two separate studies that involved the synthesis of branched peptides derived from the V3 region of HIV-1 gp120. The first of these studies aimed to determine whether antibodies against the entire V3 region of HIV-1 gp120 correlated with protection against maternal-foetal transmission of HIV-1, as had been previously suggested on the basis of limited data (Broliden *et al.*, 1989; Rossi *et al.*, 1989; Devash *et al.*, 1990). The second study aimed to test the hypothesis that the sequence variants that arose in the V3 region of HIV-1 gp120 during the course of infection in a patient of the Edinburgh haemophiliac cohort represented antibody escape mutants. This work involved the synthesis of all the peptides corresponding to sequence variants that emerged in patient 82 over a seven year period. These peptides were tested for their reactivity against sequential sera taken from patient 82. The finding of these studies are of considerable interest in terms of the use of peptide based assays for serodiagnosis and in the design of vaccines against HIV-infection.

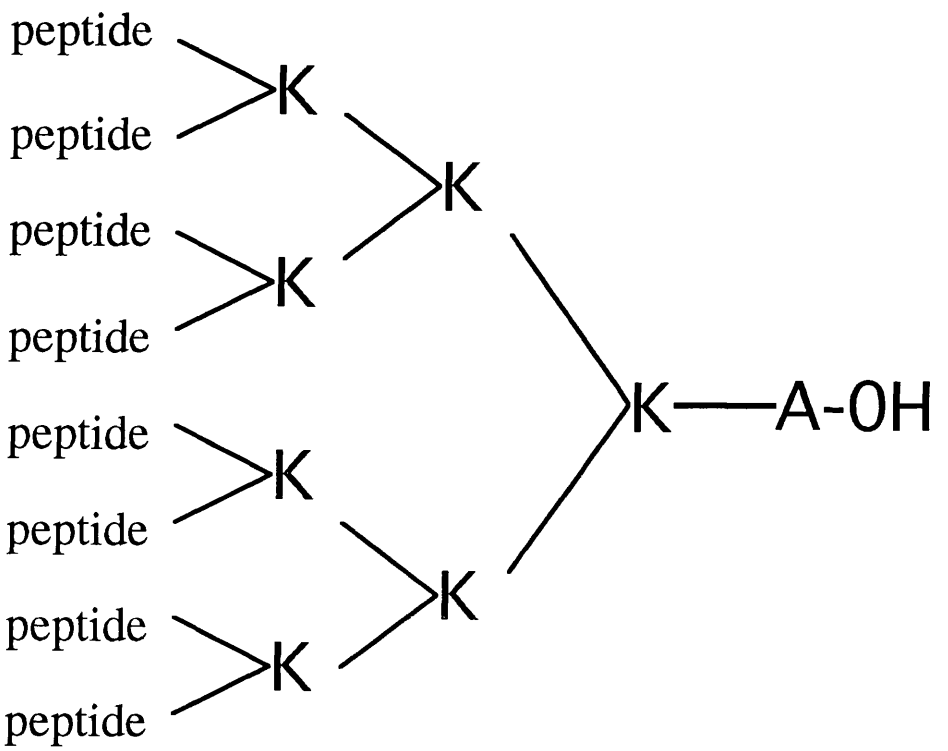


Figure 1.8 Schematic representation of a branched or multiple antigenic peptide (MAP) (Tam, 1988)

Chapter 2 MATERIALS

2.1 0ligopeptide synthesis

Dimethylformamide, t-amyl alchohol, piperidine, triflouroacetic acid, acetic acid diethyl ether.	Rathburn Chemicals Ltd, Walkerburn, Scotland, UK.
Anisole, thioanisole, dichloromethane, EDTA, phenol, triisopropysilane.	Aldrich Chemical Company, Gillingham, Dorset, UK.
Fmoc amino acids 1-hydroxybenzotriazole (HOBt), Fmoc Ultrosyn C resin	Novabiochem (UK) Ltd, Nottingham, UK.
Fmoc8 K7A resin	Peptide and Protein Research, Exeter, Devon, UK.

2.2 High pressure liquid chromatography (HPLC)

HPLC grade methanol and acetonitrile	Rathburn Chemicals Ltd, Walkerburn, Scotland, UK.
HPLC reverse phase columns: Dynamax 300A C8 analytical column (4.6mm internal diameter x 250mm) catalogue number 83-303-C.	Anachem, Luton, Bedfordshire UK.

Vydak-FB1520 preparative column (20mm internal diameter x 50cm) catalogue number C-8-83-323-C	HiChrom Ltd, Reading Berkshire, UK.

2.3 Fast performance liquid chromatography (FPLC)

Double stranded deoxyribonucleic acid (DNA) cellulose	Sigma Chemical Company, Poole, Dorset, UK.

2.4 Enzyme-linked immunosorbant assay (ELISA)

Immunlon-2 flat bottom UV irradiated plates	Dynatech Laboratories, Billingshurst, West Sussex, UK.
HRP conjugated-protein A, 2,2'Azino-bis (3-ethylbenz-thiazoline-6-sulphonic acid), (ABTS), HRP conjugated goat-antimouse IgG, 30% hydrogen peroxide solution	Sigma Chemical Company, Poole, Dorset, UK.
HRP conjugated goat-antihuman IgG	Scottish Antibody Production Unit, (SAPU) Carluke, Lanarkshire Scotland

2.5 Tissue culture

TC-100 medium, penicillin/ streptomycin, neomycin, foetal calf serum	Gibco- Biocult, Paisley, Scotland, UK.

2.6 Electrophoresis

Acrylamide, N,N'-methylene bisacrylamide	BDH Laboratory Supplies, Poole, England,UK.
Ammonium persulphate, N,N,N'N- tetramethylethylenediamine TEMED	BioRad Laboratories Ltd, Richmond, California, USA.
Rainbow markers (Mwt range 2,350-46,000)	Amersham International PLC, Amersham, UK.

2.7 Micellaneous materials

Gp120 protein from insect cells infected with recombinant baculovirus	Kindly provided by the MRC AIDS Reagent Project.
Peptide 284: (GCSGKLICTTAVPWNAS $G_5)_8K_7A$	Made in house by Dr Susan Graham.
Peptide 216: VSHGDPEDLDGAARAGE	Made in house by Dr Ania Owsianka.
Monoclonal antibody Z1F11	Made in house by Dr Anne Cross from hybridoma cells provided by H. Ludwig (Schenk *et al.*, 1988)
HIV-1 Western blotting diagnostic strips	DuPont (USA)

2.8 Human sera

Control HIV-1 negative sera were provided by Dr E. Follet, Ruchill Hospital, Glasgow and Dr Karin Froebel, HIV Immunology Unit, Edinburgh. These sera were defined negative by Western blot analysis.

Maternal Sera described in section 2 of the results were provided by Dr Jackie Mok and Dr Shiela Burns of the City Hospital, Edinburgh. These sera were defined positive by Western blot analysis.

Patient 82 sera described in section 3 of the results were provided by Dr Andrew Leigh Brown, Institute of Cell and Population Biology, Edinburgh. These sera were defined as positive firstly, by Western blot analysis and subsequently, by detection of viral RNA using PCR.

2.9 Commonly used solutions

Phosphate buffered saline:

170mM NaCl, 3.4mM KCl, 10mM Na_2HPO_4, pH7.2 supplemented with 6.8mM $CaCl_2$ and 4.9mM $MgCl_2$.

Tris buffered saline:

20mM Tris, pH 7.5, 500mM NaCl.

Electrophoresis buffer:

52mM Tris, 53mM glycine, 0.1% SDS.

Denaturation buffer:

50mM Tris , pH6.7, 2% SDS, 700mM 2-mercaptoethanol, 10% glycerol, bromophenol blue to colour.

Protein Stain:

0.2% coomassie brilliant blue R250 in methanol:water:acetic acid (50:50:7).

Destain:

5% methanol ,7% acetic acid in water.

PBS Tween 20 wash buffer x 10

1.45MNaCl, 0.075M Na_2HPO_4, 0.028M NaH_2PO_4 +0.5% glycerol, pH adjusted to 7.2 with NaOH or HCl.

Blocking buffer:

1xPBS + 2% bovine serum albumin.

Antibody dilution buffer:

1xPBS + 0.5% bovine serum albumin.

Citrate phosphate buffer:

0.1M Na_2HPO_4, 0.08M citric acid, pH adjusted to 4.0 using citric acid or NaOH.

Extraction buffer:

20mM hepes,pH 7.9, 0.5mM DTT, 0.5mM $MgCl_2$, 10mM $NaHSO_3$ + protease inhibitors 0.5mM PMSF, 1.0uM pepstatin

High salt extraction buffer:

As above + 3.4M NaCl.

Dialysis buffer:

20mM hepes, pH7.5, 0.5mM DTT, 0.5mM EDTA, 10% glycerol, 50mM NaCl +protease inhibitors, 0.5mM PMSF, 1.0uM pepstatin.

Buffer A:

20mM hepes, pH 7.6, 0.5mM DTT, 0.5mM EDTA, 10 % glycerol, 50mM NaCL + protease inhibitors, 0.5mM PMSF, 1.0uM pepstatin

Buffer B:

As A + 0.7 mM NaCl.

Buffer C:

As A + 1.0 M NaCl.

Chapter 3 METHODS

3.1 Oligopeptides

3.1.1 Oligopeptide synthesis

All the peptides used in the following studies were made by continuous flow Fmoc chemistry on Kieselguhr polyamide resin (Atherton and Sheppard 1989), using a Novabiochem peptide synthesiser. This involves sequential addition of Fmoc protected amino acids. The Fmoc group was removed at the end of every cycle using 20% piperidine in DMF for 15 to 20 minutes. DHBT or PFP esters in conjunction with HOBt were used for coupling most amino acids except where stated in the text. For the latter stages of the work described in this thesis PyBOP amino acids became commercially available. This form of amino acids is highly reactive and permits a reduction of standard coupling times from 4 hours to 1.5 hours and was therefore used in preference to the DHBT and PFP esters. Side chain protecting groups were as follows: trityl (trt), side chains of asparagine, cysteine, glutamine and histidine; butlyoxycarbonyl (Boc), side chains of tryptophan and lysine; tertiary-butyl (t-Bu), side chains of aspartic acid, glutamic acid, serine and tyrosine. Two forms of arginine were used that differed in side chain protecting groups. Arg(mtr) (methoxytrimethylbenzenesulphonyl) was used for synthesis performed using DHBT and PFP esters and Arg(pmc) (pentamethylchromanesulphonyl) was used in synthesis performed using PyBOP chemistry.

3.1.1.1 Synthesis of octameric peptides: These peptides were synthesised on a branched polylysine core allowing the formation of 8 chains of homologous peptide (Tam, 1988).

3.1.1.2 Synthesis of monomeric peptides: These peptides were synthesised on Ultrosyn C which is specifically designed to make peptide carboxy amides.

In all cases, the scale of synthesis was approximately 0.1 mmol of free Fmoc groups at a concentration of 0.1 mmol per gram of functionalised resin for monomeric peptides and 0.02 mmol per gram of functionalised resin for branched peptides. Sometimes up to three peptides were synthesised at the same time using three columns of the synthesiser in series. In these cases the amount of resin was adjusted to keep the total scale at 0.1mmol. Prepacked vials containing 0.5 mmol of Fmoc amino acid esters were used giving a 5-fold excess of amino acid during coupling reactions.

3.1.2 Cleavage and deprotection

After synthesis, the peptide resin was removed from the column and transferred into a sintered glass funnel and washed with the following solvents: DMF, t-amyl alcohol, acetic acid, t-amyl alcohol and finally diethyl ether. The resin was then transferred to a 100ml round bottomed flask and dried in an evacuated desiccator. At this point half the resin was stored at -20 ^{o}C in case of an unsuccessful cleavage.

For peptides that did not contain Arg(Mtr), Cys(Trt), Met or Trp a 95% aqueous solution of TFA was used, while for peptides containing any of the above amino acids, appropriate scavengers were used as summarised in Table 3.1. Peptides were cleaved from the resin and side chain protecting groups removed using 25 ml of the cleavage solution. After the reaction time, the mixture was poured into a sintered glass funnel and allowed to drip through into a 100 ml round bottomed flask. Any residual peptide on the resin was washed through with approximately 5 ml of TFA. The filtrate was reduced on a rotary evaporator to a volume of 1-2 ml and the peptide precipitated out of solution by the addition of 25ml of diethyl ether. The precipitate was centrifuged, the ether removed and washed thoroughly a further twice with 25ml of ether. The peptide was then dissolved in 25ml of milli-Q water and after removal of any residual ether by rotary evaporation, shell frozen and lyophilised overnight.

Table 3.1 **PEPTIDE CLEAVAGE CONDITIONS**

Arg	Cys	Met	Trp	TFA (%)	Scavengers (%)	Time (hrs)
-	-	-	-	95	Water (5)	1-1.5
+	-	-	-	95	Phenol (5)	>6
-	+	-	-	95	EDT (5)	1-1.5
-	-	+	-	95	EMS (5)	1-1.5
-	-	-	+			1-1.5
-	+	-	+	94	Phenol (5) EDT (1)	1-1.5
+	+	-	-			>6
+	+	+	-	93	Phenol (3)	>6
+	-	+	-		EDT (1) EMS (3)	
+	-	+	+		TMSB (12.5)	
+	+	-	+	71	EDT (4.5)	0.5
+	+	+	+		*m*-creosol (1)	
					Thioanisole	

Scavenger abbreviations

EDT	Ethanedithiol
EMS	Ethylmethylsulphide
TMSB	Trimethylsilylbromide

Arg(pmc) was used in place of Arg(mtr) in PyBOP amino acids as this side chain protecting group can be removed in less than two hours rather than overnight as with Arg(mtr). As a simplification to peptide cleavage Novabiochem recommended a single cleavage mixture for all peptides made with the PyBOP amino acids. This mixture is termed reagent K and contains 81.5% TFA, 5% phenol, 5% thioanisole, 5% water, 2.5% EDT and 1% TIPS. Peptides were cleaved in this reagent for 1.5 hours and are then treated as above.

3.1.3 Analysis of peptides

3.1.3.1 Mass spectrometry

The molecular weight of the monomeric peptides was determined by M-Scan Ltd, Ascot, England, using the fast atom bombardment (FAB-mass) ionisation technique (Barber *et al,* 1981).

3.1.3.2 Amino acid analysis

Amino acid analysis was carried out on the branched peptides by Cambridge Research Biochemicals Ltd, Cheshire England, UK. This provides the molar ratio of each amino acid contained in the peptide.

3.1.3.3 Reverse phase high pressure liquid chromatography (HPLC).

The purity of each peptide was determined by reverse phase HPLC monitored at 225nm on a Beckman System Gold HPLC using a dynamax 300 A C8 analytical column (4.6mm internal diameter x 250mm, catalogue number 83-303-C). A gradient of 0-95% acetonitrile (plus 0.05% TFA) in water (plus 0.1% TFA) run over 20 minutes at a flow rate of 0.5ml/min.

3.1.4 Purification of peptides

Peptides were purified by reverse phase HPLC on a Beckman System Gold system using a Vydak-FB1520 preparative column (20mm internal diameter x 50cm, catalogue number C-8-83-323-C) and a gradient of 0-95 % acetonitrile (plus 0.05% TFA) in water (plus 0.1% TFA), run over 20 minutes at a flow rate of 10 ml/min. Fractions were analysed by reverse phase HPLC as above, peak fractions were pooled, lyophilised and reanalysed by reverse phase HPLC. The purified peptide, depending on whether it was in the monomeric or branched form was sent for either FAB-mass spectrometry (M-Scan Ltd, Ascot) or amino acid analysis (CRB, Cheshire) respectively.

3.1.5 Storage of peptides

Peptides were stored at -20 ^{o}C in a dry sealed universal . When required, the peptides were warmed to room temperature prior to opening to prevent any moisture damaging the peptide.

3.1.6 Dissolution of peptides

Most peptides were dissolved in Milli-Q water, for analysis and purification and most dissolved in PBS for use in assays. Those peptides insoluble in water were treated as follows: acidic peptides were suspended in water and bubbled with ammonia vapour, basic peptides were dissolved in a small amount of either 10% formic acid or 33% acetic acid and made up to the required volume with water or PBS.

3.2 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Vertical polyacrylamide gels with a gradient either 5-12% or 10-20% acrylamide were used to analyse purified peptides and the UL42 protein Stocks of 30% and 60% acrylamide were prepared in water with a 20:1 ratio of acrylamide to the crosslinking agent, N,N'-methylene bisacrylamide. After filtering through Watman N°1 filter paper these solutions were used to prepare the appropriate gel concentration in a buffer of final concentration 375mM Tris.HCl, pH8.9, 0.1% SDS. Glycerol (final concentration 15 %) was added to the 60% acrylamide solution to stabilise the gradient. The gel solutions were polymerised with a final concentration of 3.65mM ammonium persulphate and 2.75mM TEMED. Following polymerisation a stacking gel consisting of 5% acrylamide in 122mM Tris.HCl, pH 6.7, 0.1% SDS was added and a teflon comb inserted to form the sample wells.

The peptide or protein samples to be run were boiled for 3 minutes in denaturing buffer, final concentration 50mM Tris.HCl, pH 6.7, 2% SDS, 700mM 2-mercaptoethanol and 10% glycerol with sufficient bromophenol blue to see the dye front. Electrophoresis was carried out in a buffer containing 52mM Tris, 53mM glycine and 0.1% SDS at 12 mA overnight or 60 mA for 3-4 hours.

3.2.1 Coomassie brilliant blue staining

Following electrophoresis gels were stained for 30 minutes in methanol:acetic acid:water (50:7:50) containing 2% Coomassie brilliant blue R250. The gels were then destained for at least an hour in 5% methanol and 7% acetic acid.

3.3 Human sera

3.3.1 Heat inactivation of human sera

To comply with Institute safety regulations, all HIV-1 positive sera were heat treated at 56^{o}C for one hour. This was performed before sera were brought into the Institute. HIV-1 negative sera were heated in the same way.

3.3.2 Storage of human sera

All human sera were stored at -20^{o}C in small sealed aliquots. When required, the sera were allowed to warm to room temperature before being made up to the required dilution in 0.5% BSA in PBS. A fresh aliquot of serum was used in each experiment.

3.3.3 Confirmation of HIV-1 negative control serum by Western blot

All human HIV-1 negative control sera used in the studies of the human sera analysis described in this thesis were confirmed to be negative for antibodies to HIV-1 by Western blot analysis using commercially available strips of nitrocellulose membrane with the HIV-1 proteins already attached. Free protein binding sites on the membrane were blocked with PBS containing 2% for an hour at 37 ^{o}C. The sera to be tested were diluted 1:50 in PBS containing 0.5% BSA. They were then incubated with individual strips at room temperature for 1 hour. The strips were then washed 5 times (5 minutes each wash) in PBS-Tween 20 wash buffer and reacted with an 1:1000 dilution of horseradish peroxidase conjugated-protein A in 0.5% BSA in PBS for 1 hour at room temperature and washed a further 5 times in PBS-Tween 20. 60mg of colour development reagent (DuPont) were dissolved in 20ml of ice cold methanol and added to 100ml PBS + 60ul of 30% hydrogen peroxide solution immediately before use and

the resulting solution incubated with the strips for no longer than 40 minutes. None of the negative control sera described in this thesis reacted to any of the HIV-1 proteins present on the strips. An HIV-1 known positive sera was tested as a control and showed reactivity against the proteins bound to the strips.

3.4 Enzyme linked immunosorbant assay (ELISA)

Peptides were dissolved as described above (section 3.1.6) at the desired concentration (see results) and allowed to adsorb to microtitre ELISA plates overnight at 37^{o}C. The plates were then washed five times in PBS-Tween 20 solution and blocked with 2% BSA in PBS for 1 hour at 37^{o}C. Antibodies or human sera were diluted in PBS containing 0.5% BSA and 50ul of the appropriate dilution added to the wells. After incubation at room temperature for 1 hour, the plates were then washed five times in PBS-Tween 20 solution. Horseradish peroxidase conjugated-goat antimouse or antihuman IgG or horseradish peroxidase conjugated-protein A (see results) was diluted 1:1000 in PBS containing 0.5% BSA and 50ul added to each well. After incubation for 1 hour at room temperature the plates were then washed a further ten times in PBS-Tween 20 solution and reacted with 100ul of a 50mg/ml solution of enzyme substrate 2,2,'Azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) in citrate phosphate buffer (0.1M Na_2HPO_4, 0.08M citric acid, pH4), containing 0.01% hydrogen peroxide. After 15-30 minutes of colour development the absorbance at 405nm was read on a Multiskan plate reader (Titertek; ICN Biomedicals, High Wycombe, UK). In most cases the results are means of duplicate determinations. The background absorbance for each serum dilution was determined by incubating the sera on a well with no peptide Where stated this background value was subtracted from the appropriate readings.

3.5 Statistical analysis

Statistical analysis was performed on the data produced in the maternal antibody study (results section 2) by Dr Karin Froebel (HIV Immunology unit, Edinburgh). Where the data from two groups does not show normal distribution, a non-parametric test must be used. The test employed was the Mann Whitney U-test, described by Goldstein (1965). The anti-V3 and anti-V3/gp41 titres collected from the two groups are placed in rank order and the sum of the rank order values calculated for each group. From this, the median value from each group is obtained. The null hypothesis states, "the difference in the median values for each of the two groups is no greater than can be reasonably explained by random sampling fluctuations". In other words this test calculates the probability of two sets of data points coming from the same or different populations.

3.6 Purification of HSV-1 UL42

3.6.1 Growth of insect cells

Spodoptera frugiperda (sf) insect cells were grown in 800 ml flasks in TC-100 medium containing 5% foetal calf serum, 1% penicillin/streptomycin and 0.5% neomycin at 28^{o}C until confluent. The cells were split 1:4 by gently knocking them off the flask bottom and pipetting them into the desired number of flasks. 50mls of fresh medium were then added and the cells placed at 28^{o}C until confluent again. This was repeated until a minimum of 10 flasks of confluent cells were obtained.

3.6.2 Infection of cells with recombinant baculovirus

The recombinant baculovirus expressing the HSV-1 UL42 gene was supplied by Dr Nigel D Stow. (Stow 1992). The medium was poured off the cells and 1ml of virus

suspension added to each of the 10 flasks to give a m.o.i of approximately 5 p.f.u./cell. Virus was allowed to adsorb to the cells for 1 hour at room temperature. The cells were then overlaid with 20ml of fresh TC-100 medium containing 5% foetal calf serum 1% penicillin/streptomycin and 0.5% neomycin and incubated at 28 ^{o}C for 50 hours (a time found by Mrs Mary Murphy to be optimal for the yield of UL42 protein). Sterility checks on blood agar plates were carried out on cells after each passage and on the virus stock used for infection.

3.6.3 Harvesting the UL42 protein

Infected cells were agitated off the flasks and transferred to 50ml conical tubes. The flasks were then rinsed with cold TBS and the remaining cells collected with a pipette. * The cells were pelleted by centrifugation at 1,000rpm for 5 minutes and the supernatant was removed under low vacuum. Fresh TBS was added to the tubes and the cells were resuspended by pipetting up and down.* The process *, * was repeated a further three times. If a virus stock was to be made, the supernatant from the first centrifugation step was aliquoted and stored at -70^{o}C. After the final centrifugation the cells were resuspended in 10ml of extraction buffer containing 20mM HEPES pH7.9, 0.5mM $MgCl_2$, 10mM $NaHSO_3$. 0.5mM PMSF, 1uM pepstatin and 5uM bestatin and allowed to swell on ice for 10 minutes. The cells were then lysed on ice using a dounce homogeniser and the nuclei pelleted by low speed centrifugation (5 minutes at 4,000rpm). The supernatant was discarded and the nuclei were lysed by the addition of 5ml of high salt extraction buffer (as above containing 3.4mM NaCL) and incubation on ice for 30 minutes. The nuclear extract was obtained by centrifugation at 40,000rpm for 1 hour. The supernatant was collected. and either stored at -70^{o}C or further processed as following.

The nuclear extract was then dialysed extensively overnight in a buffer containing 20mM HEPES, 0.5 mM DTT, 0.5mM EDTA, 10% glycerol, 50mM NaCl plus the protease inhibitors, 0.05mM PMSF, 1uM pepstatin and 5uM bestatin. The extract was

aliquoted, stored at -70^{0}C and one aliquot was thawed then analysed on a 5-12% SDS polyacrylamide gradient gel.

3.6.4 Preparation of the DNA cellulose column

The column was prepared from 4g of double stranded DNA cellulose (Sigma Chemicals Ltd) which was first washed three times in PBS and twice in buffer A in a sintered glass funnel. The DNA cellulose slurry (15ml) was poured into a 1.5cm diameter column.

Chapter 4 RESULTS: Section 1

Optimisation of branched peptides for use as serodiagnostic reagents.

Branched or multiply antigenic peptides (MAP's) were originally developed by Tam (1988). Following immunisation, animals were found to have higher titres of antipeptide and antiprotein antibodies than sera obtained using equivalent monomeric peptides covalently linked to a carrier protein (Tam, 1988; Posnett *et al.*, 1988; McLean *et al.*, 1991; Wang *et al.*, 1991). Tam and Zavala (1989) observed that antibodies could be detected by lower amounts of branched peptides than monomeric peptides. Whilst titrating sera from animals immunised with branched or monomeric peptides it was observed that branched peptides could detect lower amounts of antibodies than could monomeric peptides (Owsianka and Marsden-personal communication). Work described in this chapter was designed to further characterise the observed increased sensitivity of branched peptides, to investigate the basis for it and to optimise branched peptides for use as serodiagnostic reagents. It was considered that the increased sensitivity might be due in part to bivalent binding between the two arms of the antibody and epitopes on two arms of the branched peptide. Such bivalent binding would give a considerable increase in stability compared to the monovalent binding of the antibody to a monomeric peptide. Such a model would predict that if the epitope contained within the peptide lies too close to the polylysine core then the paratopes on the two arms of the reactive antibody may not be able to contact two epitopes simultaneously. A series of experiments were therefore performed to test the effect of varying the distance between the epitope and the core by introducing glycine spacers. The sensitivity of branched peptides was also compared with that of the of native protein for detection of antibodies. Two systems were studied. The first, the HSV-1 UL42 protein and reactive monoclonal antibody Z1F11, was chosen as the reagents were either readily available or could be readily generated

Gene UL42 of HSV-1 is one of seven (UL5, UL8, UL9, UL29, UL30, UL42 and UL52) essential for origin dependent viral DNA synthesis (Wu *et al.*, 1988) The UL42 gene product is necessary for viral growth and DNA synthesis (Marchetti *et al.*, 1988; Johnson *et al.*, 1991). It physically associates with Pol to form a heterodimer (Gallo *et al.*, 1988; Crute and Lehman, 1989; Gottlieb *et al.*, 1990) and functions as an accessory protein to increase the rate of incorporation of deoxyribonucleoside triphosphates into activated DNA (Gallo *et al.*, 1989) and to increase Pol processivity on a defined template primer (Gottlieb *et al.*, 1990); Hernandez *et al.*, 1990).

The second involved a branched peptide derived from the third hypervariable region (V3) of the HIV-1 gp120 protein. The peptide sequence shown in Table 4.1 (peptide 306) is similar to the consensus sequence of the MN strain of the virus, which is representative of the population of HIV-1 found infecting individuals in both North America and Europe and was the subject of detailed studies described later in this thesis.

4.1 HSV-1 UL42 protein

The effect of varying the distance between the epitope and the core on the reactivity of branched peptide with detecting antibody was tested using as a model system monoclonal antibody Z1F11 (Schenk *et al.*, 1988) previously shown to recognise the sequential epitope GDPEDLD on HSV-1 UL42 protein (Murphy *et al.*, 1989). A series of peptides were synthesised in which different numbers of glycine residues were added to the lysine core followed by the sequence GDPEDLD on each of the branches. These peptides can be represented by the general formula $(GDPEDLDGn)_8\ K_7\ A$ and are shown in Table 4.1 where the number of glycine spacers n = 0,1,2,4,5,7,10,15 and 20.

Table 4.1 Summary of peptides utilised in chapter 4

Protein	amino acid position in protein	peptide sequence	Peptide number
HSV-1 UL42	360-366	$(GDPEDLDG_n)_8K_7A$ n= 0,1,2,4,5,7,10,15 and 20	G0-G20
	357-373	VSHGDPEDLDGAARAGE	216
HIV-1 gp120	297-330	$(TRPNNNTRKRIHIGPGRAFYTTGQIIGDIRQAH)_8K_7A$	306
	297-330	TRPNNNTRKRIHIGPGRAFYTTGQIIGDIRQAH	347
HCMV UL45	514-525	DLGQESAKEKEY	171A
		$(DLGQESAFEKEY)_8K_7A$	171C

4.1.1 Amount of peptide required to detect MAb Z1F11

In the first experiment shown in Figure 4.1, the peptides were tested in ELISA assays at concentrations ranging from 100ug down to 100fg for reactivity with Z1F11 (diluted 1:2000). The controls used included: monoclonal antibody HCMV-3, peptide 171 (Table 4.1) in the monomeric (A) and branched (C) forms and monomeric peptide 216 (VSHGDPEDLDGAARAGE) which corresponds to amino acids 357-373 of the UL42 protein (McGeoch *et al*, 1988a, b) and contains the Z1F11-reactive epitope. The figure shows that all branched peptides containing the sequence GDPEDLD reacted more strongly with Z1F11 than did the monomeric peptide 216. However, there was a marked difference between the branched peptide with no glycine spacer (G_0), which was only about ten fold more reactive than the monomeric peptide, and the branched peptide with one glycine spacer (G_1): at 100ug peptide per well G_1 gave twice the absorbance produced by G_0, while 10^4-fold more G_0 than G_1 was needed to produce an absorbance of 0.5 units. Additional glycine residues progressively increased the absorbance for amounts of peptide above 10^{-5}ug per well although peptides G_5 and G_7 were most sensitive at low concentrations. The controls behaved as expected: firstly, the non-related antibody HCMV-3 did not react with any peptide at any concentration (only the data from G_{10} is plotted); secondly, Z1F11 did not react with unrelated 171A (monomeric) and 171C (branched) peptides; and thirdly, in the absence of peptide, Z1F11 was not reactive.

4.1.2 Sensitivity of detection of MAb Z1F11

In the experiments shown in Figures 4.2A, B and C, the effect of glycine spacer length on the sensitivity with which the branched G_0, G_5 and G_{10} peptides could detect monoclonal antibody Z1F11 was examined. Three different amounts of peptide were tested (100ug, 1ug and 0.01 ug) against doubling dilutions of Z1F11 ranging from 1:250 to 1:64,000. Because the controls included in Figure 4.1 behaved as expected

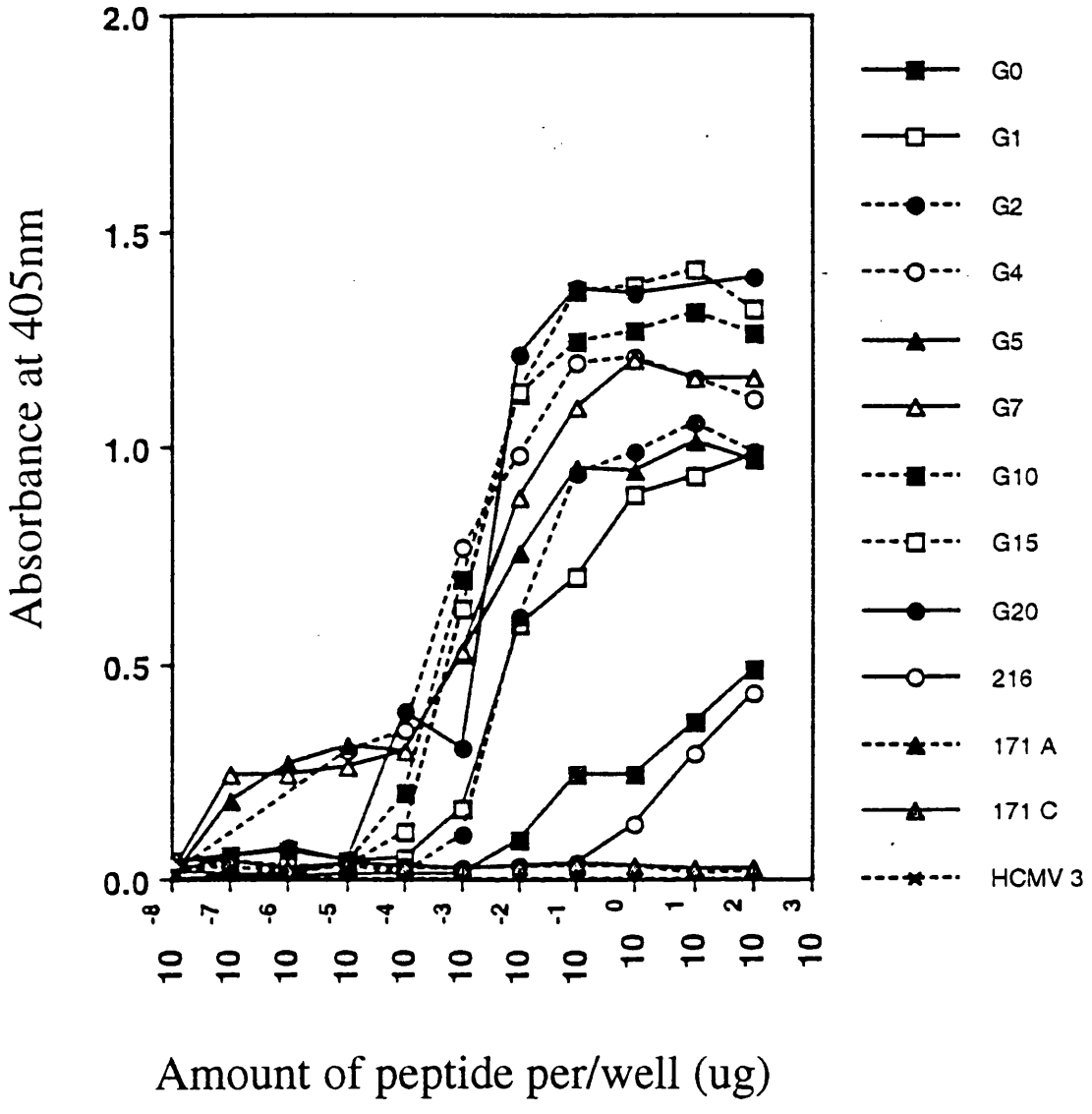
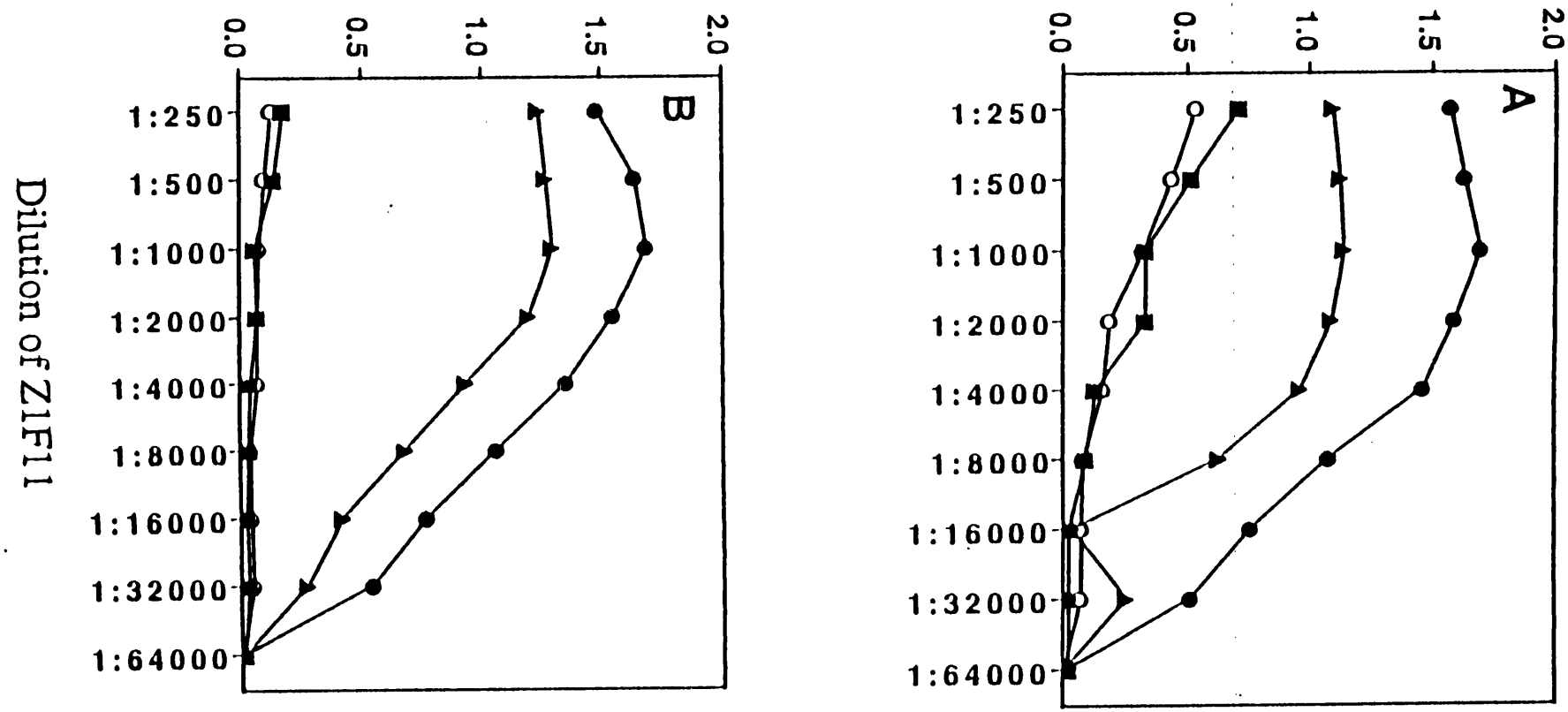


Figure 4.1 Effect of spacer glycines on recognition of monoclonal antibody Z1F11 at a dilution of 1:2000, by peptides containing the epitope GDPEDLD in an ELISA. Peptides G_0-G_{20} which are labelled on the right hand side of the figure, represent branched peptides containing 0-20 spacer glycines preceding the reactive epitope. Peptide 216, VSHGDPEDLDGAARAGE, is monomeric and represents the natural sequence of HSV-1 protein UL42 and contains the Z1F11-reactive epitope. Control peptides 171A (monomeric) and 171C (branched) (Table 4.1) do not react with Z1F11. HCMV3 is an unrelated antibody and was unreactive with all of the peptides. Only the data for G10 is plotted.

they were not retested in this experiment. At all concentrations tested the G5 and G10 forms of the branched peptide were significantly more sensitive than the monomeric peptide 216 (Fig 4.2). In addition, the G10 peptide was more sensitive in detecting Z1F11 than the G5 peptide, which in turn was more sensitive than the G0 peptide as would be expected according to the data shown in Figure 4.1. 100ug of peptide/well is however an amount considerably above that normally used for coating microtitre plates with proteinaceous material (Fig 4.2A). Therefore some the observed binding of Z1F11 to the peptides at low antibody concentrations could be due to a non specific interaction of the antibody with such high amounts of peptide. At the lower concentrations of peptide (Figures 4.2B and C) the conditions are more stringent and the observed reactivity of the peptides with Z1F11 should not represent any non-specific interaction . This experiment was extended to measure the sensitivity of all the branched peptides, G_0-G_{20} at a concentration of 1ug peptide per well with doubling dilutions of Z1F11 from 1:250 to 1:64,000. The results are shown in Figure 4.3. All the branched peptides showed greater sensitivity than the monomeric peptide. The effect was particularly marked with four or more glycine spacer residues. When diluted 64,000-fold, Z1F11 yielded an absorbance of 0.5 with G4 peptide whereas to produce the same absorbance with peptide G_0 the antibody could only be diluted 250-fold. Additional glycine residues did not significantly increase the reactivity of the branched peptide. It was concluded from these results that a spacer of 4 or more glycine residues optimally increases the sensitivity for detection of antibody Z1F11 by 256-fold. The observed reactivity of the G0 peptide is considerably higher than that obtained in the previous experiment at the same peptide concentration, shown in Figure 4.2B. It might be that around 1ug/well is close to some critical concentration of the G0 peptide required for detection of Z1F11, which was sufficient for some reactivity shown if Figure 4.3 but not in the experiment shown in Figure 4.2B.


Absorbance at 405nm
0.0
0.5
1.0
1.5
2.0
A
B
1:250
1:500
1:1000
1:2000
1:4000
1:8000
1:16000
1:32000
1:64000
Dilution of Z1F11

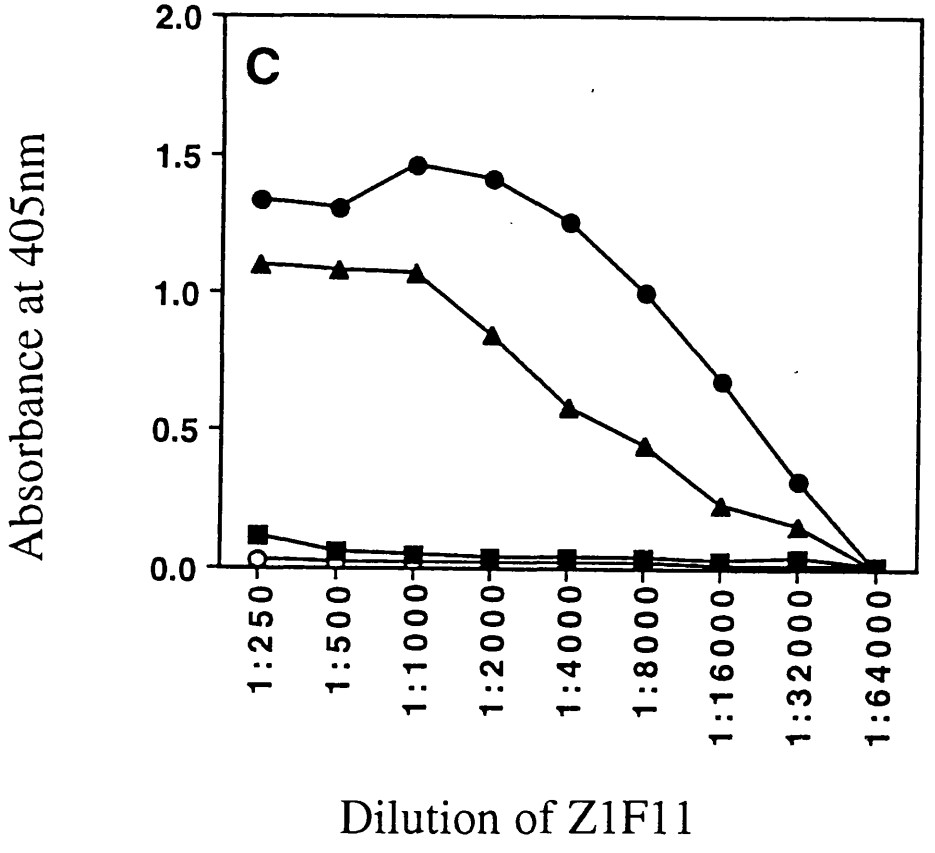


Figure 4.2 Effect of spacer glycines on the sensitivity of detection of monoclonal antibody Z1F11 by G_0 (■), G_5 (▲) and G_{10} (●) branched peptides containing the epitope GDPEDLD at 100ug (**A**), 1ug (**B**) and 0.01ug (**C**) per well, tested by ELISA. Z1F11 is titrated in a series of doubling dilutions (1:250-1:64,000). Peptide 216 (○), VSHGDPEDLDGAARAGE is monomeric and represents part of the natural sequence of HSV-1 protein UL42 and contains the Z1F11-reactive epitope.

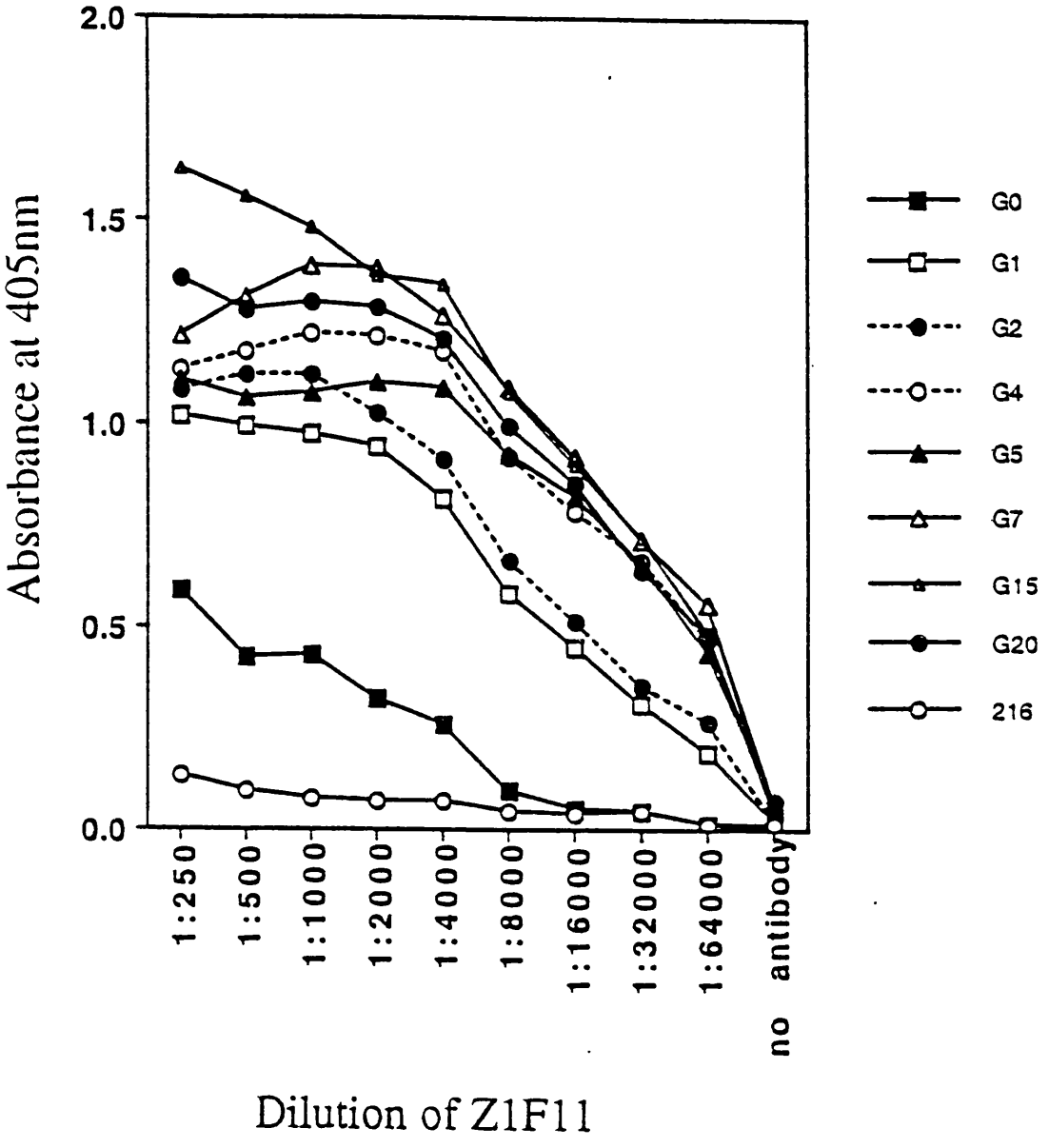


Figure 4.3 Effect of spacer glycines on the sensitivity of detection of monoclonal antibody Z1F11 branched peptides containing the epitope GDPEDLD used at 1ug per well in an ELISA. Peptides G_0-G_{20} which are labelled on the right hand side of the figure, represent branched peptides containing 0-20 spacer glycines preceding the reactive epitope. Peptide 216, VSHGDPEDLDGAARAGE, is monomeric and represents part of the natural sequence of the HSV-1 protein UL42 and contains the Z1F11-reactive epitope. Z1F11 is titrated in a series of doubling dilutions from 1:250-1:6400. The final point on each curve is a measure of the reactivity of the peptide incubated with 0.5% BSA in PBS alone to give a "no antibody" control. This showed no reactivity as expected.

4.1.3 Purification of the UL42

To compare the reactivity of the branched peptides with that of the native UL42 protein, the protein was purified from a nuclear extract of 10 flasks of confluent insect cells infected with recombinant baculovirus expressing UL42 (Stow *et al.*, 1992) according to the protocol described in section 6 of the methods. Figure 4.4 shows the partially purified extract (track B) and a previously purified UL42 preparation used as a marker for the protein. This partially purified extract was run through a double stranded DNA cellulose column and the resulting fractions collected and run on a 10-15% gradient SDS polyacrylamide gel and stained with Coomassie brilliant blue. Unfortunately however, the protein had degraded and was not apparent on the gel. Due to time limitations a previously quantified preparation of the UL42 protein purified by Mrs Mary Murphy was used in the following experiments.

4.1.4 Relative sensitivity of the branched G5 peptide and native UL42 protein

In the experiment shown in Figure 4.5, the reactivity of the branched peptide with 5 glycine spacers and the native UL42 protein with monoclonal antibody Z1F11 was compared in ELISA. The peptide and protein were coated to microtitre plates at a concentration of 25ng per well and the reactivity of both the peptide and protein tested against doubling dilutions of Z1F11 ranging from 1:500 to 1:512,000. A negative control peptide, 306 (Table 4.1), derived from the V3 region of HIV-1 gp120 was also tested in the ELISA assay with Z1F11. The data show that at antibody dilutions greater than 1:16,000 the reactivity of the peptide and protein are indistinguishable (Figure 4.5). At the higher concentrations of Z1F11 the native protein shows slightly greater sensitivity to Z1F11 than the peptide, giving an absorbance reading at 405nm approximately 0.05 units higher than the peptide. These finding suggest the use of branched peptides as serodiagnostic reagents as the relative cost and time to produce the

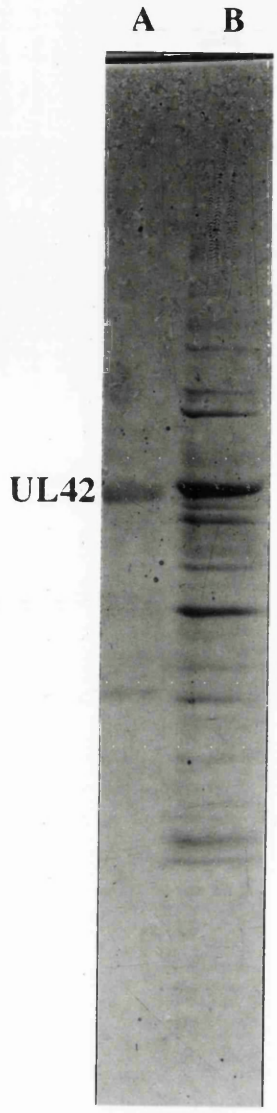


Figure 4.4 Coomassie blue stained 10-15% SDS polyacrylamide gradient gel of baculovirus expressed, HSV-1 gene UL42 protein: Track A shows a previously purified and quantified extract provided by Mrs Murphy. Track B shows the partially purified extract prior to purification on a double stranded DNA cellulose column.

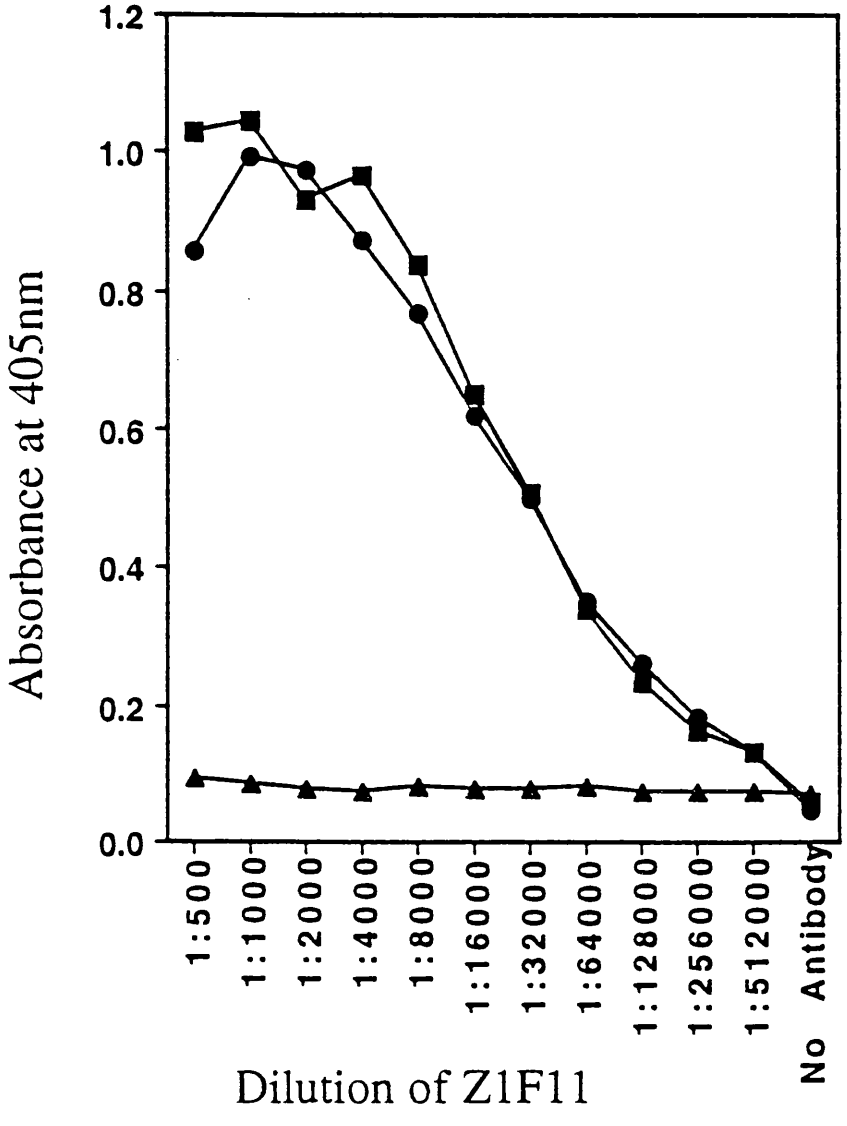


Figure 4.5 Comparison of the sensitivity of the branched G5 (●) peptide containing the epitope GDPEDLD and the baculovirus expressed and purified UL42 protein (■) used at 25ng per well, tested in an ELISA. Monoclonal antibody Z1F11 is titrated in a series of doubling dilutions (1:500-1:512,000). Control V3 peptide (306, Table 4.1) (▲) shows no reactivity with monoclonal antibody Z1F11. The final point on each curve is a measure of the reactivity of the peptide incubated with 0.5% BSA in PBS alone to give a "no antibody" control. This showed no reactivity as expected.

peptide is far less than that involved in making preparations of recombinant protein. In addition the branched peptides should have greater specificity (fewer false-positives) than native protein.

4.2 HIV-1 gp120 peptide

4.2.1 Oligopeptides

A peptide spanning the entire third hypervariable region (V3) of the HIV-1_{MN} strain between amino acids 297-330 was synthesised in branched (Table 4.1, peptide 306) and monomeric (peptide 347) forms. Both peptides were used in the study presented in section 2 of the results. In this case a four glycine spacer was not inserted between the peptide and the lysine core, as the region reactive with HIV-1 infected sera is located in the middle third of the peptide (results, section 3) and is therefore pushed out from the core by the natural amino acid sequence. Figure 4.6, shows an analysis of the branched peptide by reverse-phase HPLC. The trace indicates that this peptide is 99.7% pure. The yield of peptide was 345mg and its sequence was confirmed by amino acid analysis performed by Cambridge Research Biochemicals.

The monomeric form of the peptide (347) was also analysed by reverse-phase HPLC (Figure 4.7). Multiple peaks were observed suggesting a heterogenous mixture of peptides. These peaks were assigned numbers 1-12, shown if Figure 4.7, with retention times in minutes of 12.8, 13.8, 14.6, 14.8, 15.5, 16.0, 16.5, 17.1, 17.8, 18.4, 18.9 and 19.8 respectively. Preparative HPLC was used to isolate the individual peaks and fractions from the preparative run were collected and rerun on an analytical column. Those fractions corresponding to each individual peak were pooled, dried and peaks 6-12 were sent to M-Scan Ltd for fast atom bombardment mass spectrometry. Peaks 1-5 were not sent for analysis as the quantities and retention times suggested that they were unlikely to represent the desired peptide. The molecular weights obtained for each pooled fraction suggested that one of the peaks (peak 7) represented the desired

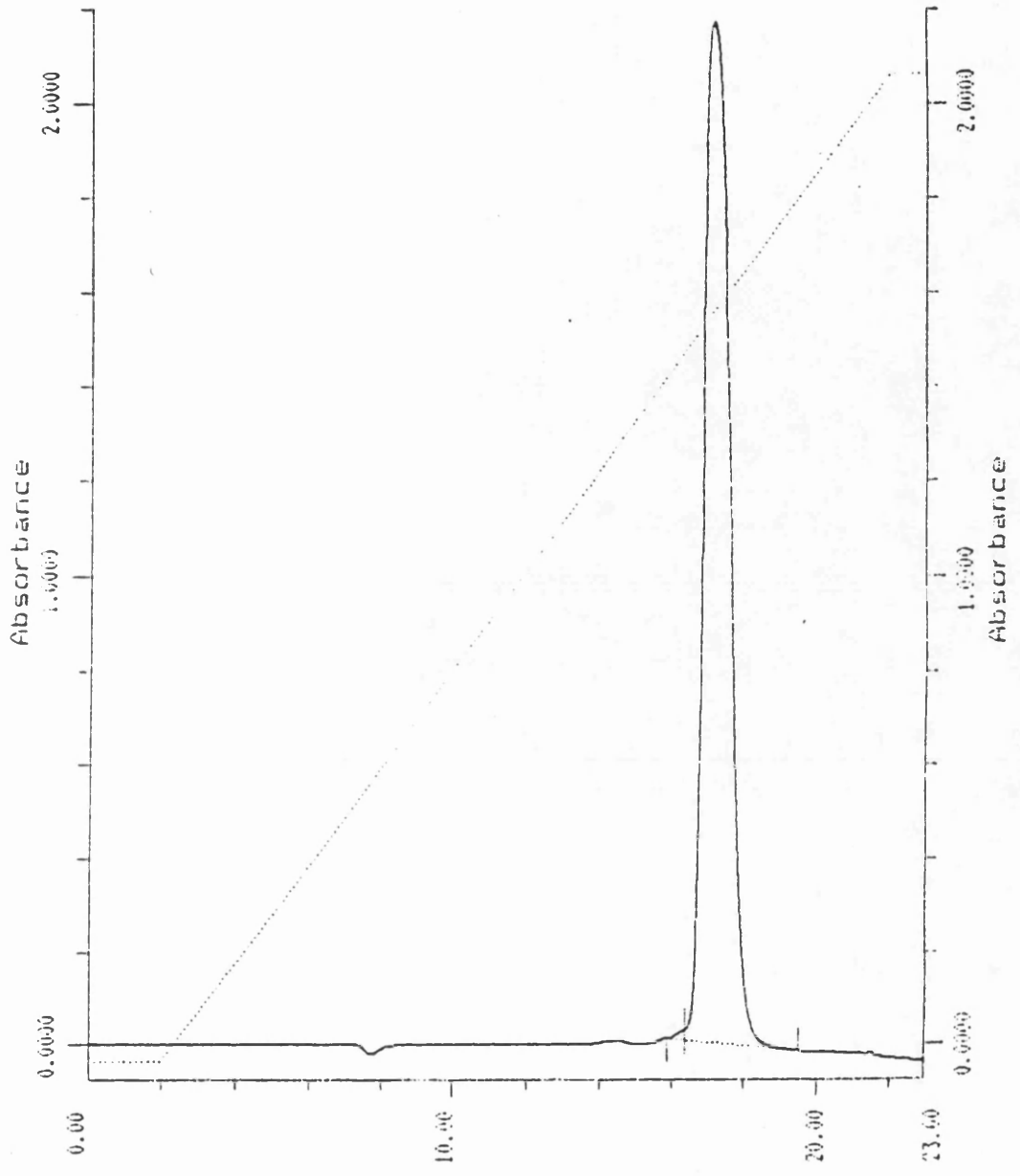


Figure 4.6 Chromatogram of the analysis of branched V3 peptide 306 (Table 4.1) by reverse-phase HPLC . The eluate was monitored at 225nm and the peak represents 99.7% of the total material.

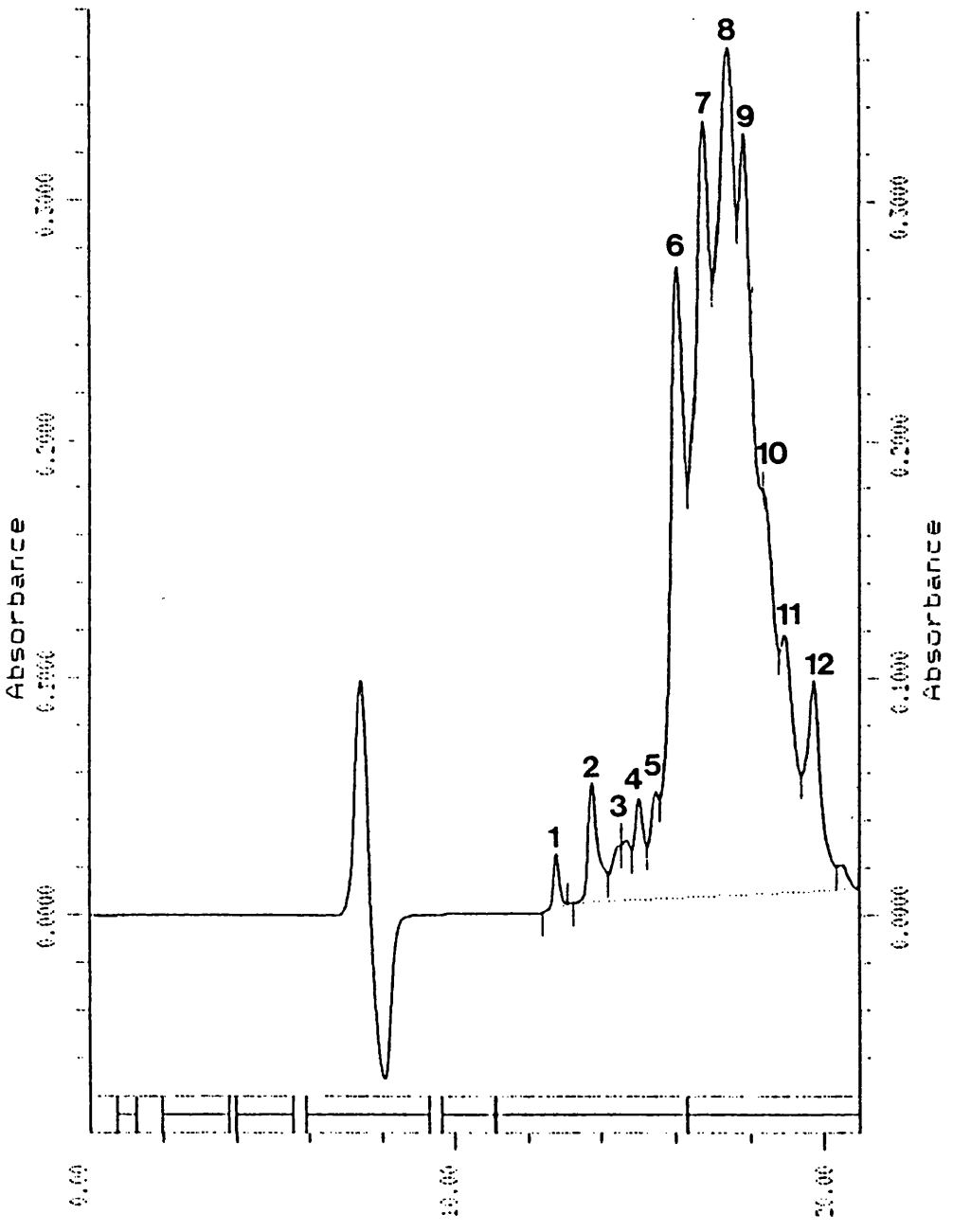


Figure 4.7 Chromatogram of the analysis of the monomeric V3 peptide 347 (Table 4.1) by reverse-phase HPLC. The eluate was monitored at 225nm and the trace indicates a heterogenous mixture of peptide. The peaks labelled 1-12 with retention times in minutes of 12.8, 13.8, 14.6, 14.8, 15.5, 16.0, 16.51, 17.13, 17.8, 18.4, 19.0 and 19.8 respectively were further separated by preparative HPLC. Fractions corresponding to peaks 6-12 were sent for FAB-mass spectrometry analysis.

peptide with a molecular weight of 3,757. The chromatographic analysis for this peptide is shown in Figure 4.8 and indicates a purity of 92%. Sufficient peptide was obtained to perform the experiments discussed in section 2 of the results.

The molecular weights of the remaining six peaks were larger by multiples of the molecular weight of the Mtr side chain protecting group, present on arginine residues, indicating that each peak represented the desired peptide with one or more of the uncleaved Mtr groups. To remove the remaining Mtr groups, the peptide was recleaved overnight in a mixture containing 95% TFA plus 5% phenol and the resulting peptide rerun on an analytical reverse-phase HPLC column. Figure 4.9 shows the HPLC trace for this peptide and indicates that the retention time (16.15 minutes) for the predominant peak is identical to the retention time for peak 7 (16.15 minutes). To further confirm that the peptides are the same, a mixture containing the peptide obtained from peak 7 and the recleaved peptide were analysed by reverse-phase HPLC (Figure 4.10). The trace indicated one predominant species. The experiments using the monomeric peptide described in this chapter all involved the recleaved peptide.

4.2.2 Analysis of the branched and monomeric peptides by SDS-PAGE

To further analyse the branched and monomeric peptides, both were run on a 10-20% SDS-polyacrylamide gradient gel. Figure 4.11, shows the Coomassie blue stained gel run with low molecular weight rainbow markers, 2,350-46,000 molecular weight (Amersham) (track M). Track A, contains the branched peptide, calculated molecular weight 30,898 and track B, contains the monomeric peptide, molecular weight 3,757. Both peptides migrate to positions on the gel compatible with their calculated molecular weight. The branched V3 peptide shows a slight laddering effect, possibly arising from amino acid deletions in some of the chains. This slight heterogeneity was not detected by analysis on reverse-phase HPLC, perhaps suggesting that the analytical column was not sufficiently resolving enough to detect these minority species. A possible not mutually exclusive cause for the apparent heterogeneity may be incomplete

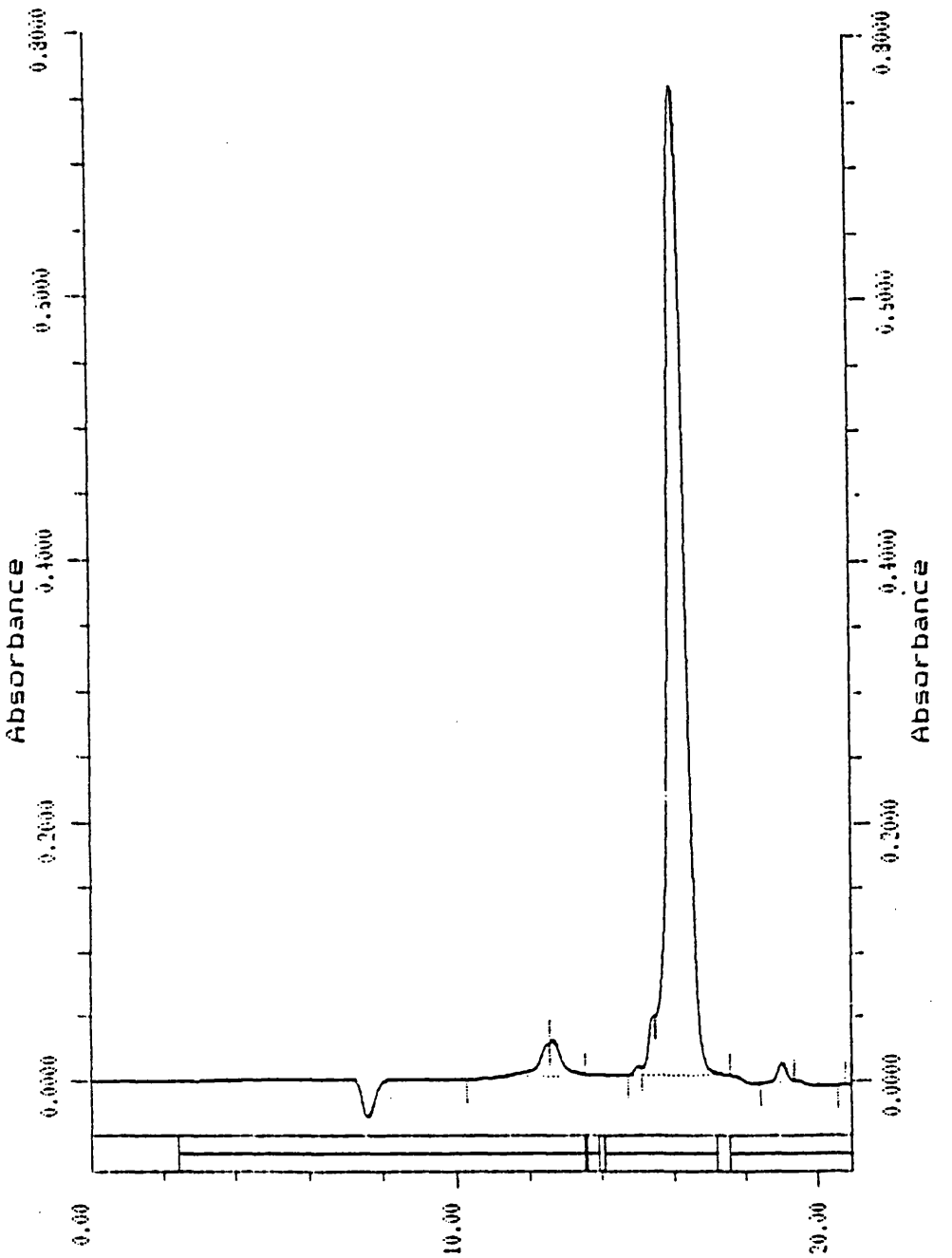


Figure 4.8 Chromatogram of the analysis of peak 7 of the monomeric V3 peptide after purification by reverse-phase HPLC. The eluate was monitored at 225nm and the peak with a retention time of 16.15 minutes represents 92.4% of the total mixture.

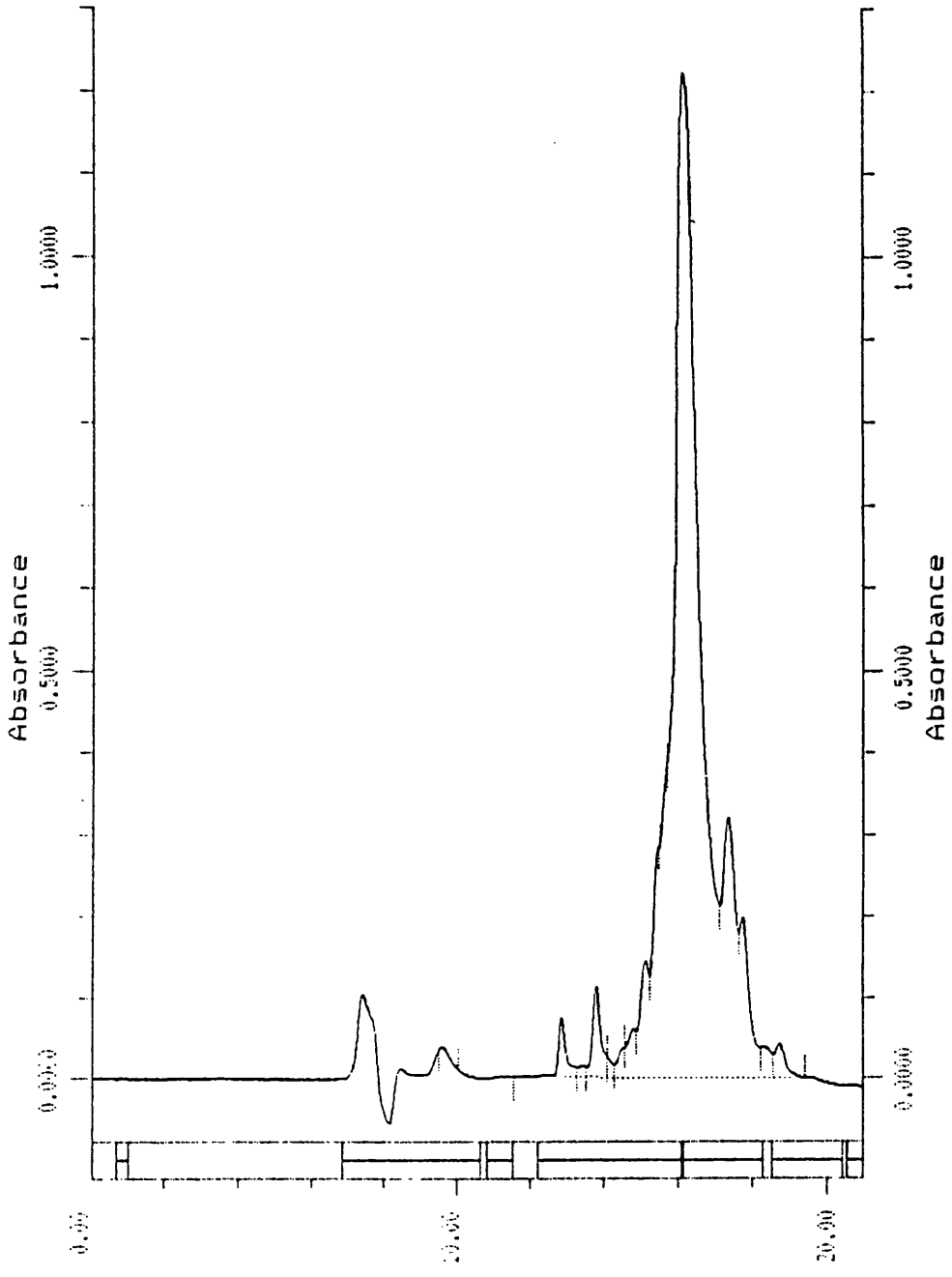


Figure 4.9 Chromatogram of the unpurified monomeric V3 peptide recleaved overnight in 95% TFA, 5% phenol by reverse-phase HPLC. The eluate was monitored at 225nm and the retention time of the predominant peak is identical to that of the purified monomeric peptide (Fig 4.8) eluting from the column at 16.15 minutes.

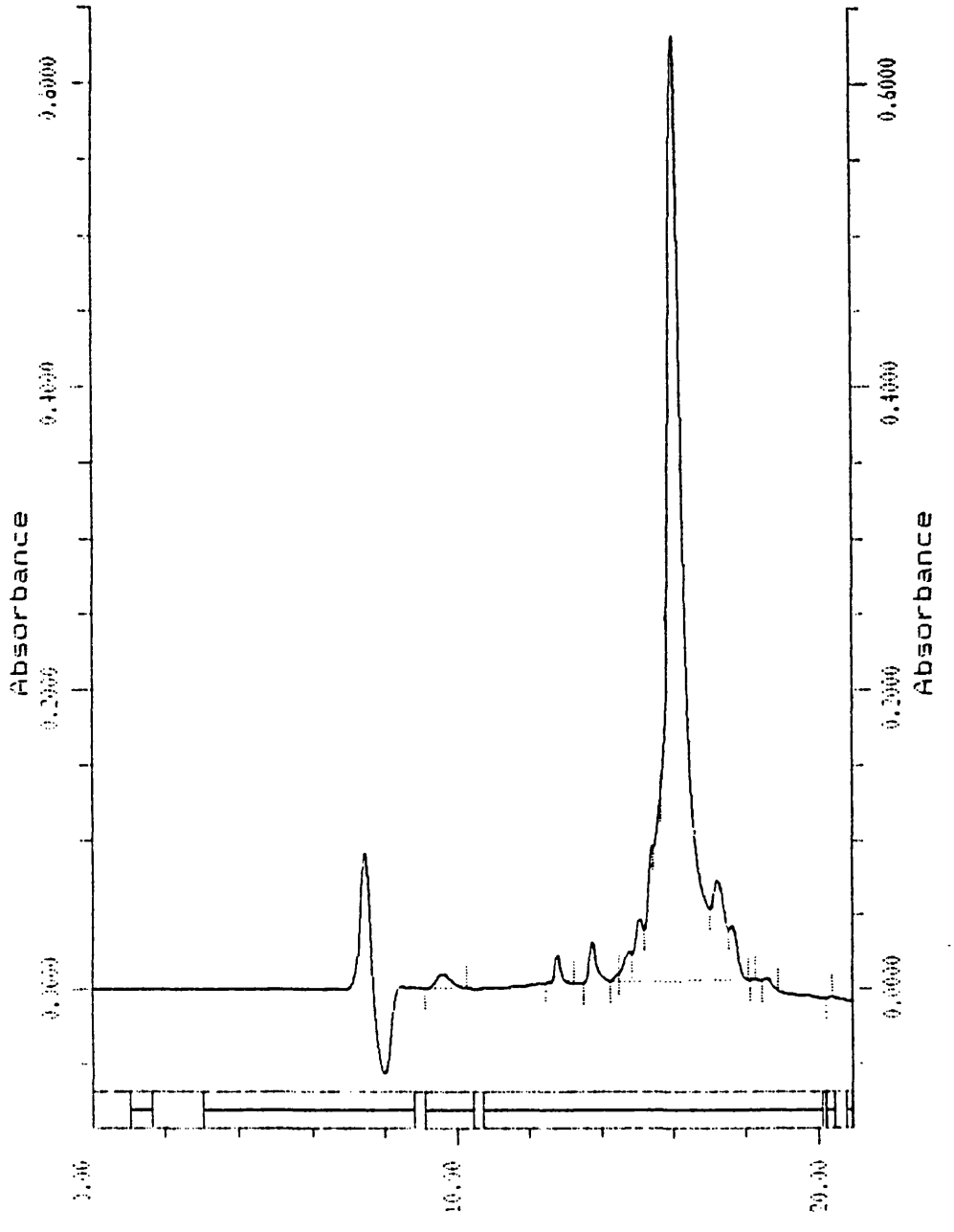


Figure 4.10 Chromatogram of a mixture of the recleaved monomeric V3 peptide and the purified peak 7 of the monomeric peptide analysed by reverse-phase HPLC. The eluate was monitored at 225nm. The chromatogram indicates one predominant peptide species with a retention time of 16.05 minutes, almost identical to the retention times for the purified monomeric peptide (peak 7) and the recleaved monomeric peptide individually shown if Figures 4.8 and 4.9 respectively.

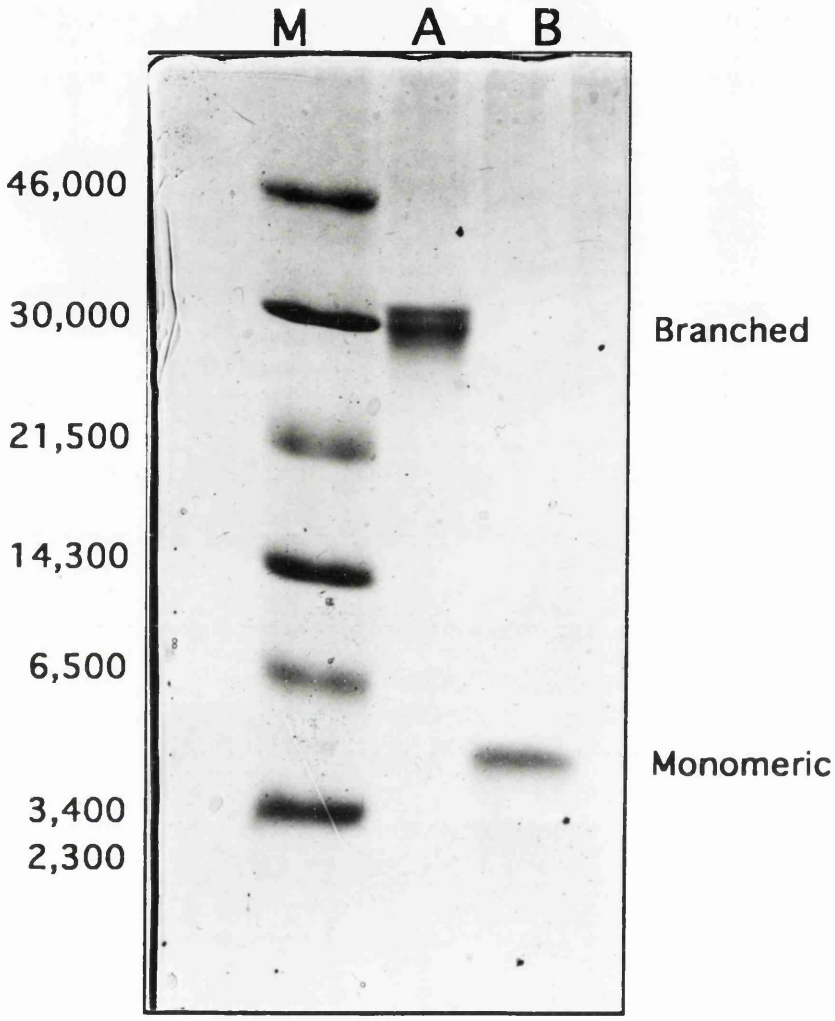


Figure 4.11 Coomassie blue stained 10-20% SDS-polyacrylamide gradient gel run with low molecular weight markers (2,350-46,000KDa) (track M). Track A is the branched V3 peptide (306) molecular weight 30,898KDa. Track B is the monomeric V3 peptide (347) molecular weight 3,757KDa. Both peptides migrate to the expected positions on the gel.

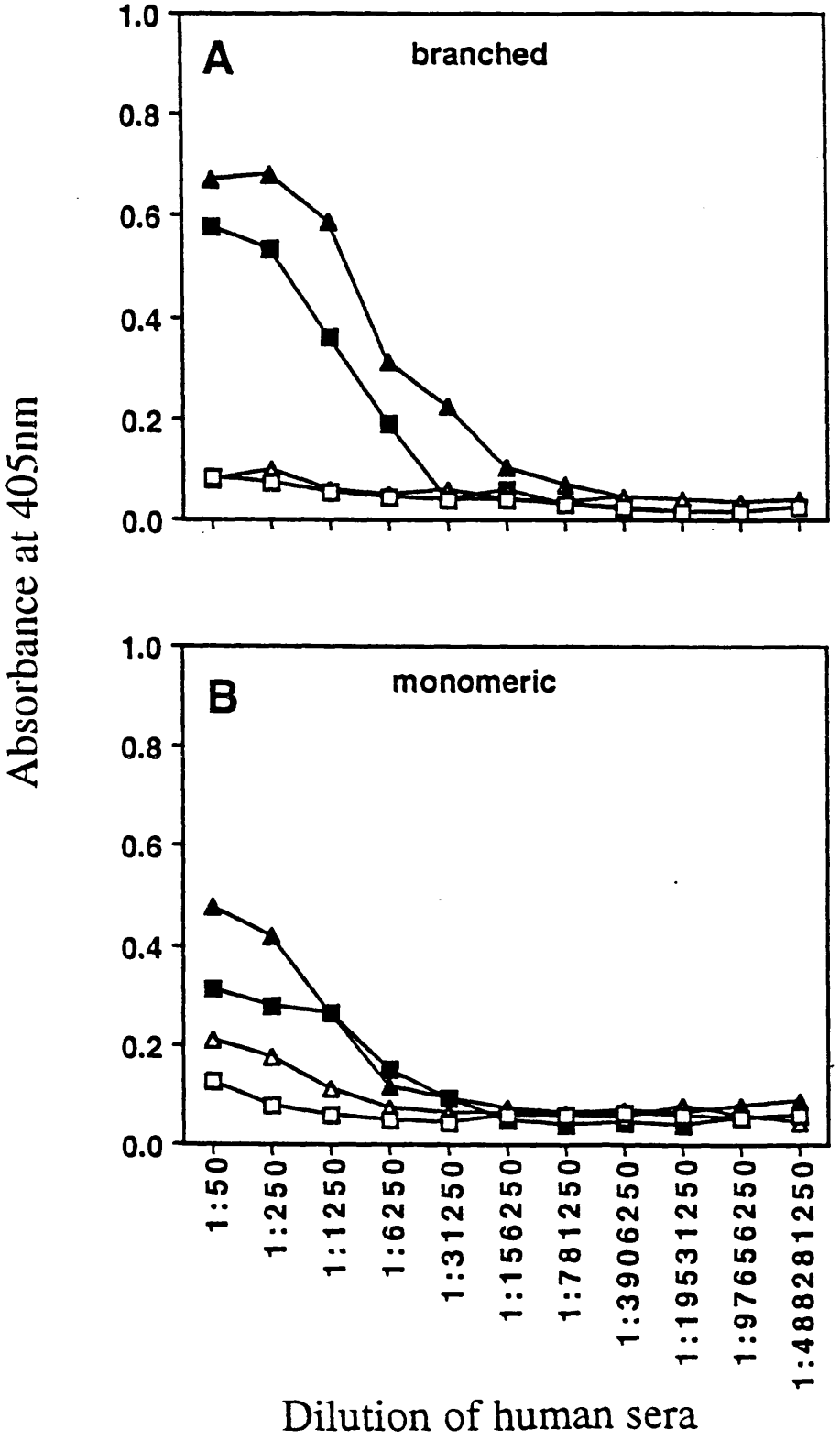


Figure 4.12 ELISA showing comparison of the sensitivity of the branched V3 peptide (panel **A**) and monomeric V3 peptide (panel **B**) at 100ng per well. HIV-1 positive sera (▲,■) and HIV-1 negative sera (△,□), were titrated in a series of twelve doubling dilutions from 1:50 to 1:488,281,250.

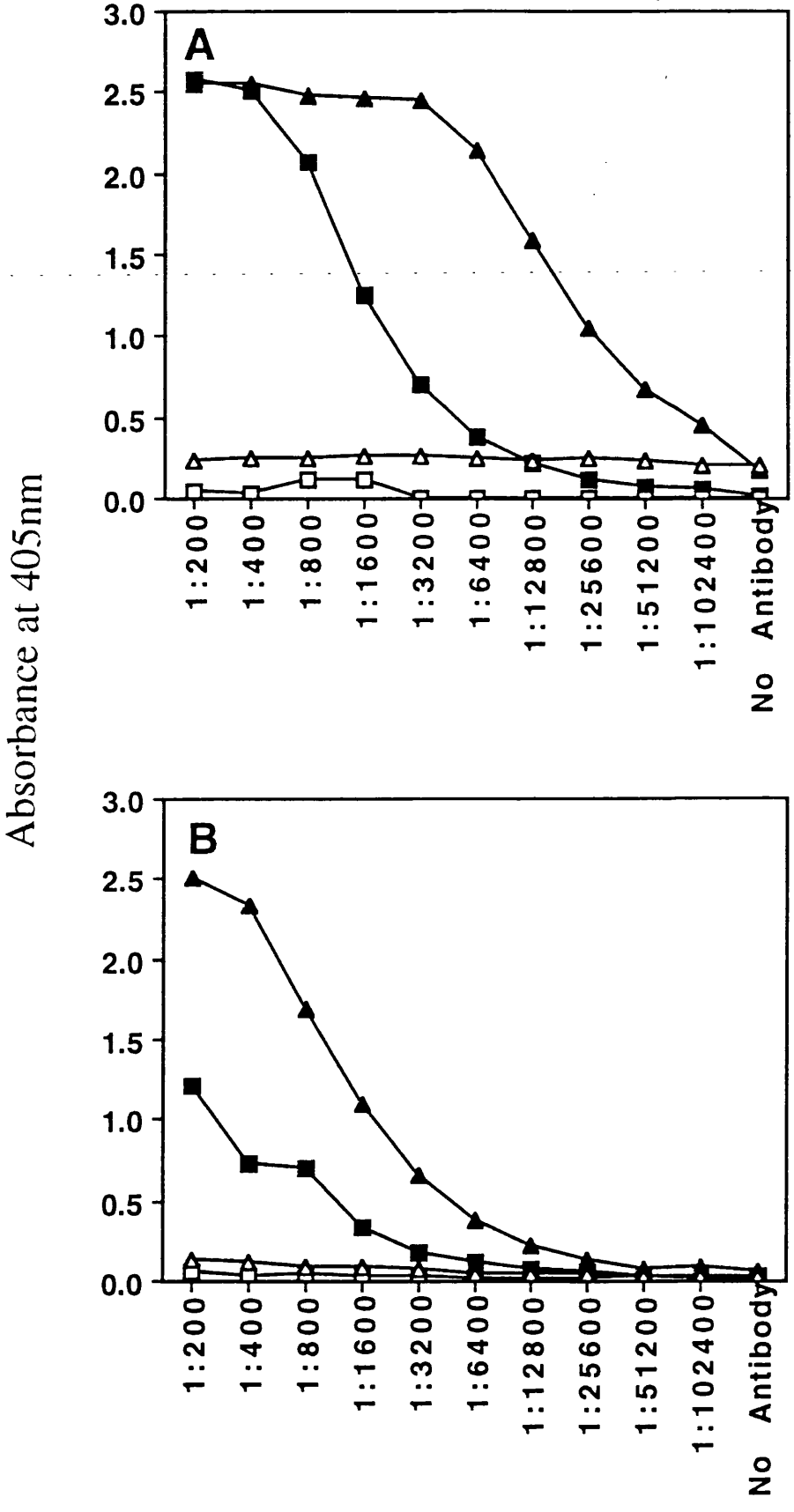
denaturation of different conformational forms of the branched peptide. The monomeric V3 peptide runs as a single band confirming its homogeneity.

4.2.3 Relative sensitivity of the V3 branched and monomeric peptides for detection of antibodies

To compare the sensitivity of each of the two peptides, both were tested in ELISA with HIV-1 positive and negative human sera. The peptides were dissolved in PBS and coated to microtitre plates at a concentration of 100ng per well and tested with the control sera titrated in a series of 5-fold dilutions from 1:50 to 1:488,281,250. Figure 4.12 shows the readings at 405nm for the branched (A) and monomeric (B) peptides tested with HIV-1 positive and negative sera. The results indicate that the branched peptide shows greater sensitivity than the monomeric peptide, detecting HIV-1 specific antibody at a maximum dilution of 1:31,250 compared to 1:1,250 for the monomeric peptide. It is also clear that the difference between the signal produced by positive and negative sera is greater with the branched peptide.

4.2.4 Relative sensitivity of the V3 branched peptide and native gp120 for detection of antibodies

The reactivity of purified gp120 and branched V3 peptide with HIV-1 positive and negative sera were examined by ELISA. Both peptide and protein were compared at three concentrations (1ug, 100ng and 10ng of peptide or protein per well). The results are the readings at 405nm of HIV-1 positive and negative sera in a series of 2-fold dilutions (Figure 4.13A, B and C). Both peptide and protein were incubated with 0.5% BSA in PBS alone to give "no antibody" controls., shown as the last point on each of the curves . At all three concentrations, the branched V3 peptide shows greater sensitivity for detection of antibodies in HIV-1 positive sera than does gp120 At a concentration of 1ug per well (Figure 4.13A) the maximum dilution of HIV positive


A
B
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1:200
1:400
1:800
1:1600
1:3200
1:6400
1:12800
1:25600
1:51200
1:102400
No Antibody
Absorbance at 405nm
Dilution of human sera

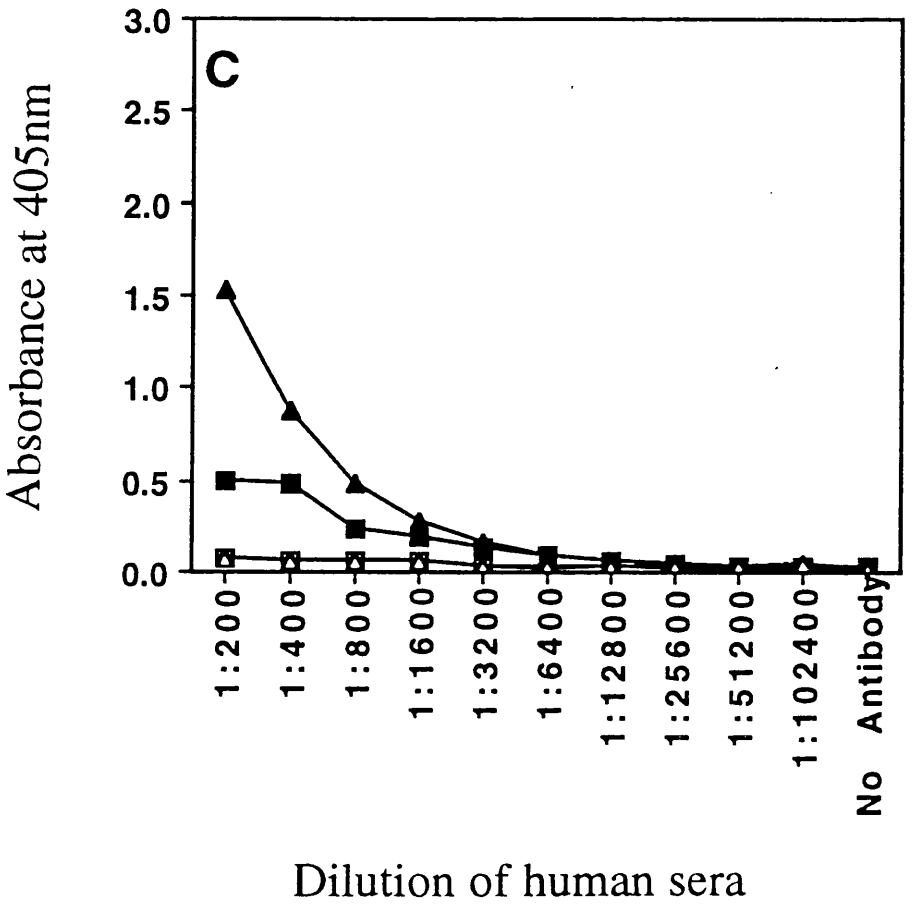


Figure 4.13 Comparison of the sensitivity of the branched V3 peptide (triangle symbols) and recombinant HIV-1 gp120 (square symbols) with an HIV-1 positive (filled symbols) and an HIV-1 negative (empty symbols) serum at three different peptide and protein concentrations. 1ug per well **(A)**, 100ng per well **(B)**, 10ng per well **(C)**. The HIV-1 positive and negative sera were titrated in a series doubling dilutions from 1:200-1:102,400. The final point on each curve is a measure of the reactivity of the peptide incubated with 0.5% BSA in PBS alone to give a "no antibody" control.

sera which gives an absorbance at 405nm of 2.0 OD units is 1:800 for the protein compared to 1:6,400 for the branched V3 peptide, indicating weight for weight that the peptide is 8 times more sensitive than gp120. A similar conclusion can be drawn for peptide and protein coated at a concentration of 100ng per well (Figure 4.13B). Therefore the maximum dilution of HIV positive sera which gives an absorbance at 405nm of between 1.0 and 1.5 OD units with the protein is, 1:200 compared to 1:1,600 with the branched V3 peptide. In Figure 4.13C, where the peptide and protein are at a concentration of 10ng per well the reactivity of both is greatly reduced giving a maximum absorbance of 1.5 and 0.5 OD units respectively. Again however , the branched V3 peptide shows greater sensitivity. The higher optical densities obtained for the branched V3 peptide in this experiment compared with those shown in Figure 4.12A is due to the use of HRP conjugated-protein A rather than goat anti-human IgG as the second antibody.

4.3 DISCUSSION

4.3.1 Effect of glycine spacers on the reactivity of branched peptides with specific antibodies

The work presented in this chapter was undertaken to further understand and increase the sensitivity of branched peptides for the detection of specific antibodies. To do this branched peptides were synthesised with varying numbers of glycine spacers to test the effect of increasing the distance of the reactive epitope from the polylysine core. The initial experiments involved the epitope GDPEDLD, derived from the HSV-1 UL42 protein and its monoclonal antibody Z1F11. The results shown in Figures 4.1 and 4.3, clearly demonstrate that a spacer of four or five glycine residues markedly increased the reactivity with the monoclonal antibody Z1F11.

This increased sensitivity might also be achieved with the use of alternative spacers, such as other small uncharged amino acids or non-natural amino acids. The natural sequence of the peptide could also act as a spacer depending on where the epitope lies in the peptide. For the branched V3 peptides used in this study, which encompasses 33 amino acids spanning the entire V3 region of HIV-1 gp120, the addition of a glycine spacer would probably have no effect on its reactivity with HIV-1 infected sera as the reactive epitope lies in the central eight to fifteen amino acids of the peptide (Goudsmit *et al.*, 1988a; Javaherian *et al.*, 1989; Meloen *et al.*, 1989 and this thesis). This expectation was not tested. Both peptides derived from the V3 region of HIV-1 gp120 and the HSV-1 UL42 when synthesised in the branched form were considerably more reactive with their respective antibodies than the monomeric peptides, confirming the initial observations of Marsden, Graham and Owsianka (personal communication) that branched peptides can detect lower concentrations of antibody than can their monomeric equivalent. This increased sensitivity and overall superiority of branched peptides might be due to a number of factors. Firstly, if one imagines the steric interaction of both branched and monomeric peptides with the

surface of a microtitre plate, it is easy to hypothesise that the octameric structure of branched peptides, when immobilised to the plate might allow multimeric binding sites to remain freely exposed to antibodies. In contrast, the binding of the antibodies to monomeric peptides may be impaired by the physical constraints imposed on the peptide by binding to the surface of the plate and prevent it from freely interacting with the antibody. Secondly, multivalent binding between antibody and branched peptides could result in an increase in stability of the antibody-peptide interaction, with both arms of the antibody binding to separate chains of the peptide, compared to the simple monovalent binding of the antibodies to monomeric peptides. Thirdly, branched peptides may bind more readily to the plate than monomeric peptides (Tam and Zavala, 1989) and therefore the observed increased sensitivity in ELISA may be due to the presence of more peptide actually bound to the plate. To test this hypothesis, branched and monomeric peptides would have to be labelled in some way so as to provide a quantitative measure of the relative binding capabilities of each type. This will be an important experiment to do however, it will not explain the observed increase in immunogenicity of branched peptides in animals.

The increased sensitivity of the GDPEDLD, UL42 branched peptides, observed with the addition of varying numbers of glycine spacers preceding the reactive epitope (Figures 4.1 and 4.3) could be due to the epitope being extended away from any physical constraints imposed on antibody binding imposed at the polylysine core, thereby making the epitope more accessible to binding by Z1F11.

4.3.2 Comparison of the reactivity of branched peptides with that of the native protein.

The reactivity of the branched peptides were compared in ELISA with the recombinant native protein from which the epitope is derived. The reactivity of the (GDPEDLDG5)8K7A peptide and the baculovirus expressed UL42 recombinant protein demonstrated that on a weight for weight basis both showed equal reactivity

with the monoclonal antibody Z1F11 (Figure 4.5). In a similar experiment, the comparison of the branched V3 peptide and the native HIV-1 IIIB gp120 protein at three different concentrations indicated that the peptide is a least 8-fold more sensitive than the native protein (Figure 4.13). Caution must be applied when interpreting this result, as this increase in sensitivity may be in part due to the fact that the MN strain of HIV-1, from which the peptide is derived is the predominant strain found infecting individuals from both North America and Europe. In contrast, the recombinant gp120, is derived from the IIIB strain of the virus, which is not commonly found and contains two additional amino acids (QR), conserved in all IIIB isolates, close to the highly conserved GPGR in the immunodominant region of V3. It is unlikely that the Edinburgh donors of the HIV-1 positive sera used in this experiment will have been infected with the IIIB strain of HIV-1.

The relative sensitivities of branched and monomeric peptides and the native protein can also be considered on a molarity basis. With equal weights of a monomeric peptide and a branched peptide with 8 arms, the monomeric peptide contains approximately 8 times more molecules. However, because each branched peptide contains 8 epitopes, the molarity of epitopes in equal weights is approximately the same.

The experiments described in this chapter indicate that branched peptides have considerable potential advantages over the use of both monomeric peptides and native proteins as serodiagnostic reagents. Peptides can be synthesised relatively inexpensively and purified to yield large quantities that can be easily stored at -20^{o}C or possibly even room temperature for extended periods of time without losing reactivity. This contrasts with the labour intensive and relatively costly protein production and purification protocols and the more demanding conditions, lyophilisation or storage at -70^{o}C, required for stability of the protein. Because peptides contain only few amino acids they are less likely than proteins to elicit false-positive reactions with spurious cross-reactive antibodies. Peptides will also be free of other contaminating proteins from the overexpression system which might also give false positives. All of these

aspects suggest that branched peptides should become the preferred reagent of choice for serodiagnosis and they therefore were used in the following studies described in this thesis.

4.3.3 Future studies

A better understanding of the properties of branched peptides, should make it possible to further improve them for use as serodiagnostic reagents. It may well be that peptides with fewer branches would be equally, or even more sensitive, in serodiagnosis. Fewer branches might have additional potential advantages as it might be expected that some branched peptides will present difficulties in synthesis and/or epitope detection due to steric hindrance caused by close proximity of the peptide chains. Reducing the number of peptide chains to four, six or even two, could relieve any steric constraints on the peptide and open up the structure, making it more accessible for recognition by specific antibodies. For long peptides (greater than fifteen amino acids), reducing the number of peptide chains may have detrimental effects on the peptides reactivity with specific antibodies and will depend on how the reactive epitope is recognised in that particular peptide. For long peptides the interaction of eight chains may stabilise the linear structure of the peptide. Four branches however could reduce any steric constraints imposed on the peptide and may result in the individual peptide chains folding in on themselves. Depending on the epitope contained in the peptide, this could increase or decrease the recognition of that particular epitope with specific antibodies. Although these possibilities are highly speculative, it will be important to test how sensitivity is affected by the number of branches using a number of different epitopes and their cognate monoclonal antibodies or polyclonal sera.

The future for branched peptides as serodiagnostic reagents so far looks very promising. In the last year of this project the peptide chemistry available for the automated synthesis of oligopeptides improved so much as to have allowed the reduction in time taken to synthesise and cleave the peptide from the resin by at least

one third. In addition, the purity of the peptides obtained is now improved (Susan Graham, personal communication) At present, many of the serodiagnostic kits available for screening HIV-1 infected blood rely on the reactivity of sera against HIV-1 proteins by Western blot. From the data presented in this chapter one could imagine that a panel of HIV-1 peptides derived from immunodominant regions of the virus, precoated to microtitre plates could provide a more sensitive test for the detection of HIV-1 specific antibodies in human sera. An experiment not described in this thesis, where the branched V3 peptide was used to screen blindly a panel of 30 HIV-1 infected and uninfected human serum samples, demonstrated that this peptide could successfully distinguish between infected and uninfected sera (collaboration with Dr E. Follet, Ruchill Hospital, Glasgow.). The high reactivity of the HIV-1 infected sera and total unreactivity of HIV-1 negative sera against the V3 peptide, suggest that this peptide itself may prove very useful in the development of serdiagnostic kits.

Chapter 5 RESULTS:Section 2

Maternal antibody levels to the V3 region of HIV-1 gp120 and transmission of HIV-1 to the foetus

This study was undertaken to investigate whether high levels of maternal antibodies present in the mother, directed against the V3 region of HIV-1 gp120 correlated with lack of vertical transmission of HIV-1.

A significant proportion of children born to HIV-infected mothers are infected in utero and transmission rates vary between 7% and 65% depending on the study cohort (Rubinstein *et al.*, 1986; Mok *et al.*, 1989; Ades *et al.*, 1991; Piot *et al.*, 1988; Blanche *et al.*, 1989). It has been reported that mothers who have high-affinity antibodies to the V3 domain of HIV-1 gp120 are less likely to transmit HIV-1 to their children (Broliden *et al.*, 1989; Rossi *et al.*, 1989; Devash *et al.*, 1990). In contrast, Parekh *et al.* (1991) found no correlation between levels of maternal antibodies against the V3 loop and maternal-foetal transmission. The resolution of these apparently contrasting results is of importance for prognostic serodiagnosis and vaccine development. Such a correlation would be helpful in predicting the outcome of at-risk pregnancies and would lend support to vaccine strategies aimed at increasing antibody levels to the V3 region.

The work presented in this chapter aimed to further examine the relationship between maternal antibodies to the V3 sequence and the risk of vertical transmission by examining the titres of antibodies against a branched V3 peptide in sera from two groups of mothers, identified as transmitters or nontransmitters. The study differs from earlier studies in three aspects. First, antibody titres were determined as opposed to classifying sera as positive or negative on the basis of whether a single dilution of maternal serum gave an ELISA titre above a cut-off value. Second, a long peptide representing the whole of the V3 sequence between the conserved cysteines was used, as it might have been expected to contain conformational as well as sequential epitopes.

In the event, this potential advantage was not realised as the V3 region is now believed to behave as a sequential epitope as denaturation of the native gp120 does not reduce binding of antibodies to this region (Moore *et al.*, 1993). Third, this study was designed to investigate whether differences in antibody levels to the V3 sequence reflected differences in the overall antibody levels or were peculiar for those against V3. To do this the antibody levels to a peptide corresponding to an immunodominant and conserved domain of gp41 were also measured. In this way the maternal antibody levels against the variable V3 loop could be normalised to the antibody levels against the constant gp41 region.

5.1 Oligopeptides

Three peptides were used in this study. The branched and monomeric V3 peptides, 306 sequence (TRPNNNTRKRIHIGPGRAFYTTGQIIGDIRQAH)$_8$K$_7$A and 347 with sequence TRPNNNTRKRIHIGPGRAF YTT GQIIGDIRQAH respectively are described in the previous chapter and shown in Table 4.1. This V3 sequence is essentially that described by LaRosa *et al.* (1990) and corresponds closely to amino acids 297-330 of the HIV-1$_{MN}$ strain gp120. This sequence was chosen because it corresponds most closely to that of isolates from the Edinburgh area and it is likely that the women enrolled in this study would have been exposed to virus containing such sequences. The monomeric V3 peptide was used for the antibody-affinity assays. Another peptide, 284 with sequence (GCSGKLICTTAVPWNASG$_5$)$_8$K$_7$A, was synthesised by Dr Susan Graham. The sequence GCSGKLICTTAVPWNAS in this peptide corresponds to residues 596-614 , the immunodominant region of gp41 (Gnann *et al*, 1987; Narvanen *et al*, 1988; Yamada *et al*, 1991). Both forms of the V3 peptide were purified to greater than 92% homogeneity and their sequences were confirmed as described in the previous chapter. The gp41 peptide, synthesised by Dr Susan Graham was found to be 91% pure and its sequence was confirmed by amino acid analysis performed by CRB. This peptide is insoluble in water and was dissolved in a 100ul of

33% acetic acid and made up to volume with PBS for use in the experiments discussed in this chapter.

Before beginning the study on the maternal sera it was important to optimise the conditions for the experimental assay. As sera is in such short supply it is of the utmost importance to maximise its use. The following experiments were designed to optimise the amount of peptides to be used for screening the sera.

5.2 Determining the optimal concentration of branched V3 peptide

Branched V3 peptide was titrated in a series of eight 10-fold dilutions against two HIV-1 positive control sera (EBH6 and EBH4), kindly provided by Ruchill Hospital and four HIV-1 negative sera (1,2,3 and 4) diluted 1:100 (Figure 5.1). Serum, EBH6, showed high reactivity with the V3 peptide, giving an absorbance at 405nm of 1.0 OD unit with as little as 10ng of peptide. Serum, EBH4 showed lower reactivity with the peptide compared to EBH6 but also reached an optimal absorbance at a peptide concentration of 10ng. All the HIV-1 negative sera behaved as expected, giving lower reactivity than with the HIV-1 positive sera. At peptide concentrations greater than 10ng per well the reactivity of the HIV-1 negative control sera increased in all cases to give a background absorbance of around 0.2 OD units. At peptide concentrations of 10ng per well there is good discrimination between the HIV-1 positive and negative sera giving background absorbance of < 0.1 OD unit with each of the negative controls. Therefore a concentration of V3 peptide of 10ng per well was used in subsequent experiments.

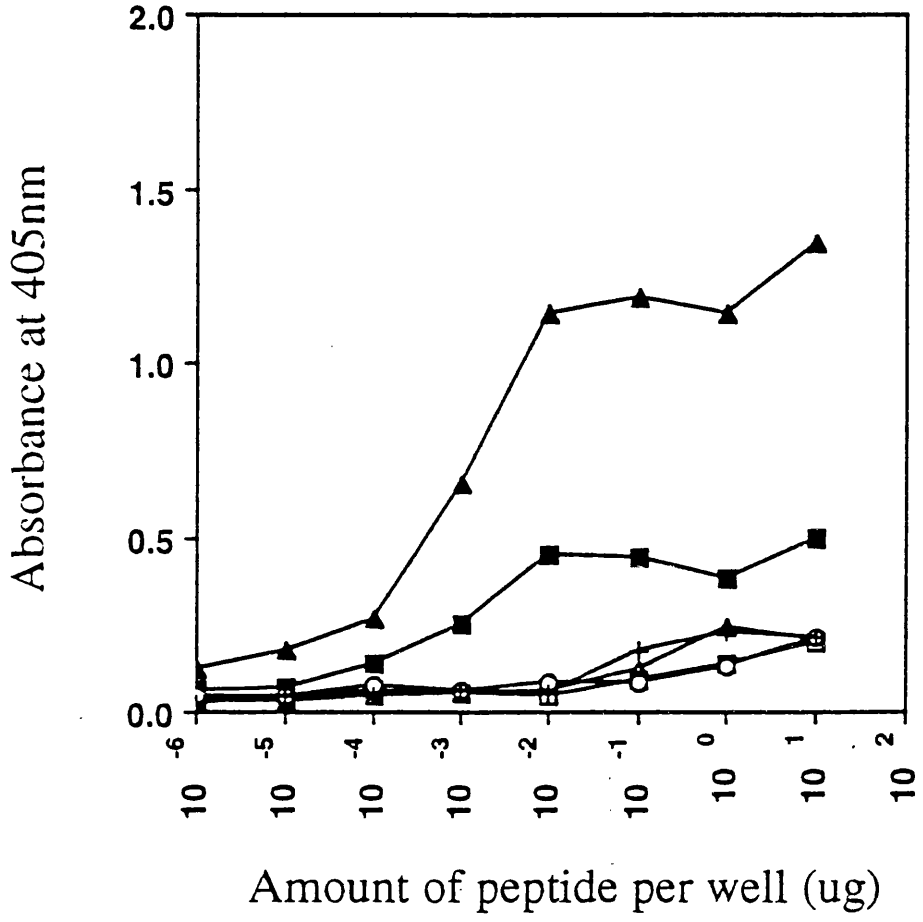


Figure 5.1 ELISA reactivity of HIV-1 positive control sera EBH4 (■) and EBH6 (▲) and HIV-1 negative control sera, 1 (△), 2, (□), 3 (○), and 4 (+).The sera were used at a dilution of 1:100 against the branched V3 peptide (TRPNNNTRKRIHIGPGRAFYTTGQGDIRQAH)8K7A, titrated in a series of eight 10-fold dilutions.

5.3 Determining the optimal concentration of the gp41 branched peptide

To optimise the amount of gp41 peptide, it was titrated in a series of eight, 5-fold dilutions from an initial concentration of 1ug per well to $1.28x10^{-5}$ug per well against a single dilution (1:100) of HIV-1 positive and negative sera . The results shown in Figure 5.2 indicate that like the V3 peptide, the concentration of gp41 peptide required to give optimal absorbance without giving high background readings with the HIV-1 negative control sera was 10ng per well.

5.4 Screening a larger panel of HIV-1 human sera against the V3 branched peptide

Before the V3 peptide was used to screen the HIV-1 infected maternal sera it was considered important to examine its reactivity with a larger number of HIV-1 negative sera and so establish mean and standard deviation values for the background. The peptide was screened in ELISA at the concentration of 10ng/well established by the previous experiment, against a panel of 14 HIV-1 negative sera and 2 HIV-1 positive sera (EBH4 and EBH6) all diluted in 0.5% BSA in PBS at dilutions of 1:10 and 1:100. The results for the V3 peptide are shown in Figure 5.3. At both serum dilutions the two positive sera EBH4 and EBH6 show high reactivity with the peptide. The fourteen negative control sera again behaved as expected giving low reactivity with the peptide. At the lower dilution of sera (1:10), the background absorbance increased to approximately 0.2 OD units. In both cases however, the discrimination between positive and negative sera is clear. At a serum dilution of 1:100 and a peptide concentration of 10ng/well the ODs at 405nm for both the V3 and gp41 peptides against the fourteen seronegative controls were OD= 0.040+/- 0.026 (from Figure 5.3) and 0.066+/- 0.030 (data not shown) respectively.

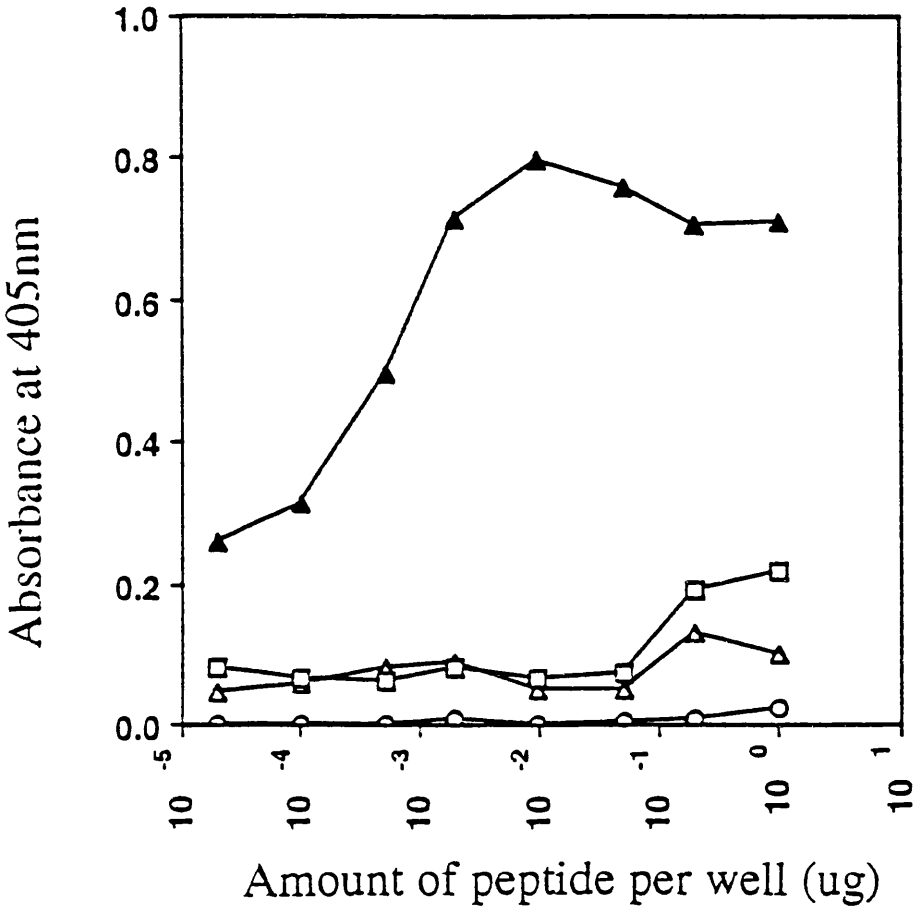


Figure 5.2 ELISA reactivity of an HIV-1 positive control sera, EBH6 (▲) and two HIV-1 negative sera (□, △) at a dilution of 1:100 against the gp41 peptide with sequence (GCSGKLICTTAVPWNAS)8K7A. The peptide was titrated in a series of eight 5-fold dilutions. Peptide alone without human sera (○) was included to give a "no antibody" control.

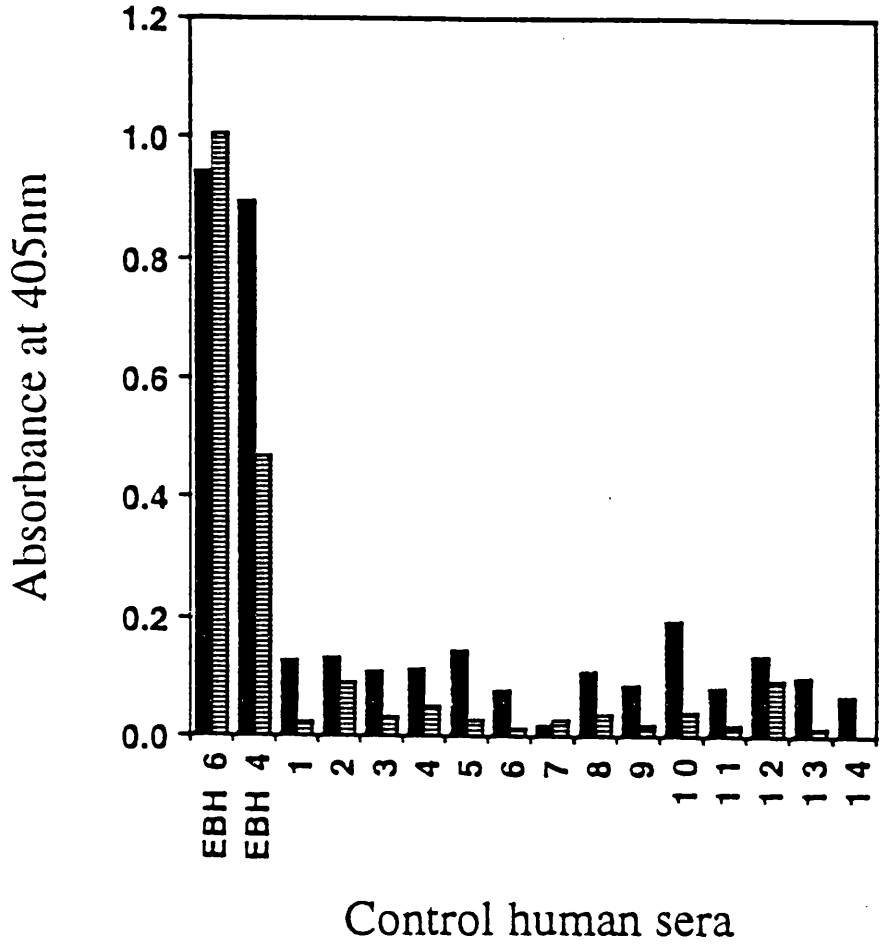


Figure 5.3 ELISA reactivity of two HIV-1 positive control and fourteen HIV-1 negative control sera at dilutions 1:10 (■) and 1:100 (▤) against the V3 branched peptide with sequence (TRPNNNTRKRIHIGPGRAFYTTGQIIGDIRQAH)$_8$K$_7$A at a concentration of 10ng per well.

5.5 Test titration of control human sera against the V3 branched peptide

This preliminary experiment was done to ensure that the assay system proposed for the maternal antibody study, using this V3 branched peptide would effectively discriminate between positive and negative sera and enable titres to be determined. Figure 5.4 shows an ELISA titration of HIV-1 positive and negative sera against the V3 peptide at a concentration of 10ng per well using a series of twelve 2-fold dilutions of the sera from 1:25 to 1:51,200. All HIV-1 negative control sera showed low reactivity with the peptide even at the lowest dilution of 1:25. A cut-off value (0.118) was calculated from the optical densities obtained with the negative sera as the mean plus 3 standard deviations. Both HIV-1 positive sera react strongly with the peptide. In the case of sample EBH6, which is consistently more reactive in all the experiments described so far, detection of specific antibody can be demonstrated at dilutions of sera as high as 1:25,600. Antibodies present in EBH4 can be detected at a 1:1,600 dilution of sera. These data suggests that the assay should provide meaningful data when used to screen the maternal sera.

5.6 The study cohort

The maternal sera used in this collaborative study were provided by Dr Jacqueline Mok and Dr Shiela Burns of the City Hospital in Edinburgh, who also collected the clinical data.

All infants born to HIV-1-infected women in the Lothian region of Scotland were enrolled in a prospective study to examine the risk of mother-child transmission of HIV-1, as well as to evaluate the natural history of vertically acquired HIV-1 disease (Mok *et al.,* 1989). All women were clinically well during pregnancy, with the exception of two, who had the opportunistic infection common to HIV-1 infected individuals, *pneumocystis carinii* pneumonia at the time of delivery. After a median

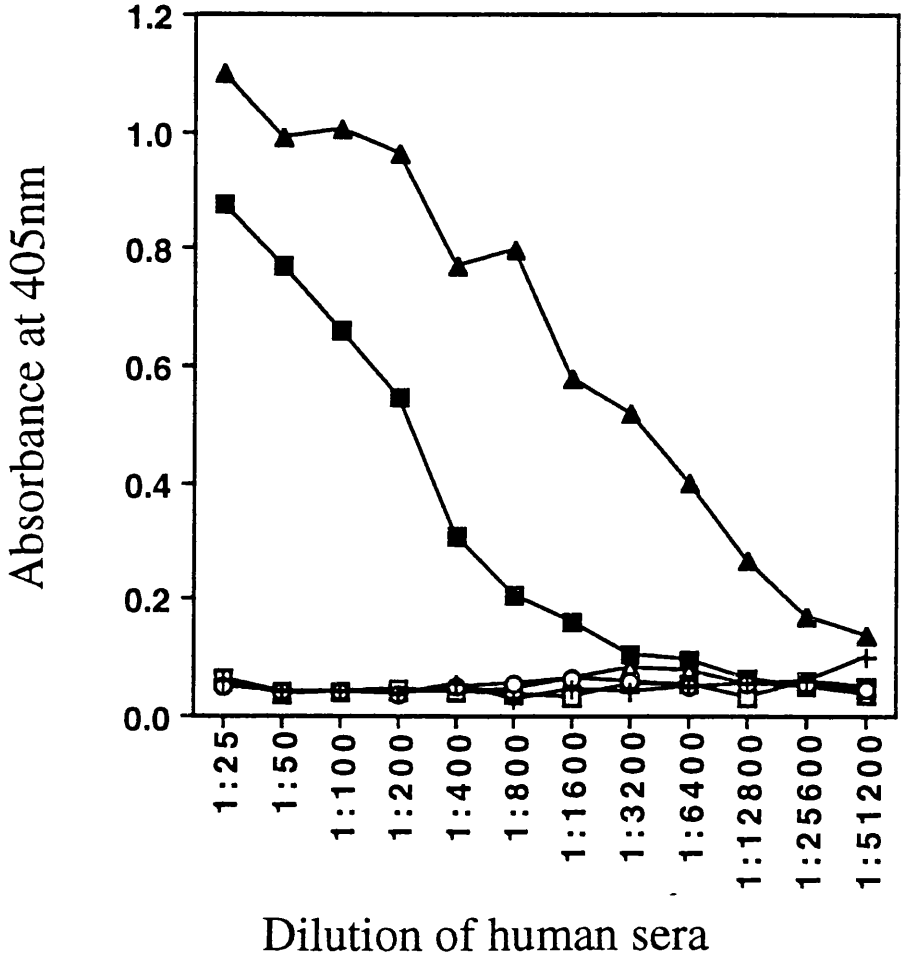


Figure 5.4 Test titration of HIV-1 positive control sera, EBH4 (■) and EBH6 (▲) and negative control sera , 1 (△), 2 (□), 3 (○), and 4 (+) in a series of doubling dilutions (1:25-1:51,200) against the V3 branched peptide at a concentration of 10ng per well in an ELISA.

follow-up of 48 months (range 4-73), infants were diagnosed as HIV-1-infected on the basis of two of the following criteria : persistence of HIV-1 antibody beyond 18 months of age, positive HIV culture or HIV antigen tests on more than two occasions, and clinical evidence of HIV infection. Children were presumed uninfected when the were > 2 years old, remained clinically healthy, had normal immune function tests and tested negative for HIV antibody, antigen and culture.

Mothers were divided into 2 groups: Group 1 (n=7) were those who had given birth to infected children (HIV-1 transmitters); group 2 (n=20) were those who delivered uninfected children (HIV-1 nontransmitters). Maternal sera were collected during pregnancy or soon after delivery. Also present in the study cohort were two women who had each given birth to both an infected and uninfected child from separate pregnancies. Sequential serum samples were available from these women during the time that each child was born.

In the following study the sera were coded by Dr Mok and screened blind against the branched V3 and gp41 peptides. Only after all the data had been collected was the code broken.

5.7 Measuring maternal antibody titres against the V3 and gp41 peptides

Both peptides were coated to microtitre ELISA plates at the optimal concentration of 10ng per well. Each of the 27 maternal sera were tested for reactivity against both peptides on the same day in a series of twelve 2-fold dilutions from 1:25 to 1:51,200. Controls for the experiment included adding each serum dilution to wells with no peptide bound. These readings (typically 0.1+/-0.03) were subtracted as background from each of the corresponding test readings at 405nm. The subtraction of background from each reading should compensate for any difference in time between addition of enzyme substrate solution and reading at 405nm on a Multiskan plate reader.

After screening all the sera against the V3 and gp41 peptides, the code was broken and each of the samples placed into the categories, transmitter or nontransmitter. Representative data from 6 transmitters and 6 nontransmitters are shown in Figure 5.5. High titres of antibody to the V3 peptide were seen in both groups of mothers regardless of whether their children were infected (panel A and C). Sera from both transmitters (panel B) and nontransmitters (panel D) also reacted with the gp41 peptide although the maximum optical densities were lower than with the V3 peptide. One point clearly demonstrated in panel B, is the importance of titrating the sera rather than defining the sera as reactive or non-reactive based on a single dilution. Sample 2995 at a dilution of 1:25 would be defined as non-reactive with the gp41 peptide yet is strongly reactive at a 1:400 dilution.

To allow a better interpretation of the data, the maximum dilution of each of the 27 sera which would to give an absorbance of 0.4 OD units with both peptides were measured from each of the graphs. Table 5.1 gives the titre defined as the reciprocal of this maximum dilution and the ratio of the titres against the V3 and the gp41 peptides for each sample.

5.8 Statistical analysis of the median values

To determine whether there was any statistical difference in antibody titres to the V3 peptide between the two groups of mothers, the non parametric Mann-Whitney statistical test was performed on the data by Dr Karin Froebel according to the method described by Goldstein (1965). The test showed no significant difference between the median values for anti-V3 titres ($p= 0.82$) or the anti-V3/anti-gp41 titres ($p=0.21$) at 0.4 OD units. Similar analysis was done for the anti-V3 titres at 1.0 OD units. but not for the gp41 titres as the reactivities of some of the sera did not reach an absorbance of 1.0 OD units. Again no significant difference was seen between the median values for the anti-V3 titres.

Figure 5.5 Titration of maternal sera against the V3 and gp41 branched peptides. Maternal sera, representative of 6 HIV-1 transmitters (**A**, **B**) and 6 HIV-1 nontransmitters (**C**, **D**) were titrated in an ELISA against the V3 peptide (**A**, **C**) and gp41 peptide (**B**, **D**). Individual sera for **A** and **B**: 1384 (⊡), 2924 (●), 2995 (◘), 2017 (○), 737 (■) and 2961 (□); for C and D: 432 (⊡), 847 (●), 2084 (◘), 3625 (○), 1896 (■) and 2751 (□). Reactivity of 1 representative seronegative subject is also shown (▲). The sera were titrated in a series of doubling dilutions from 1:25-1:51,200.

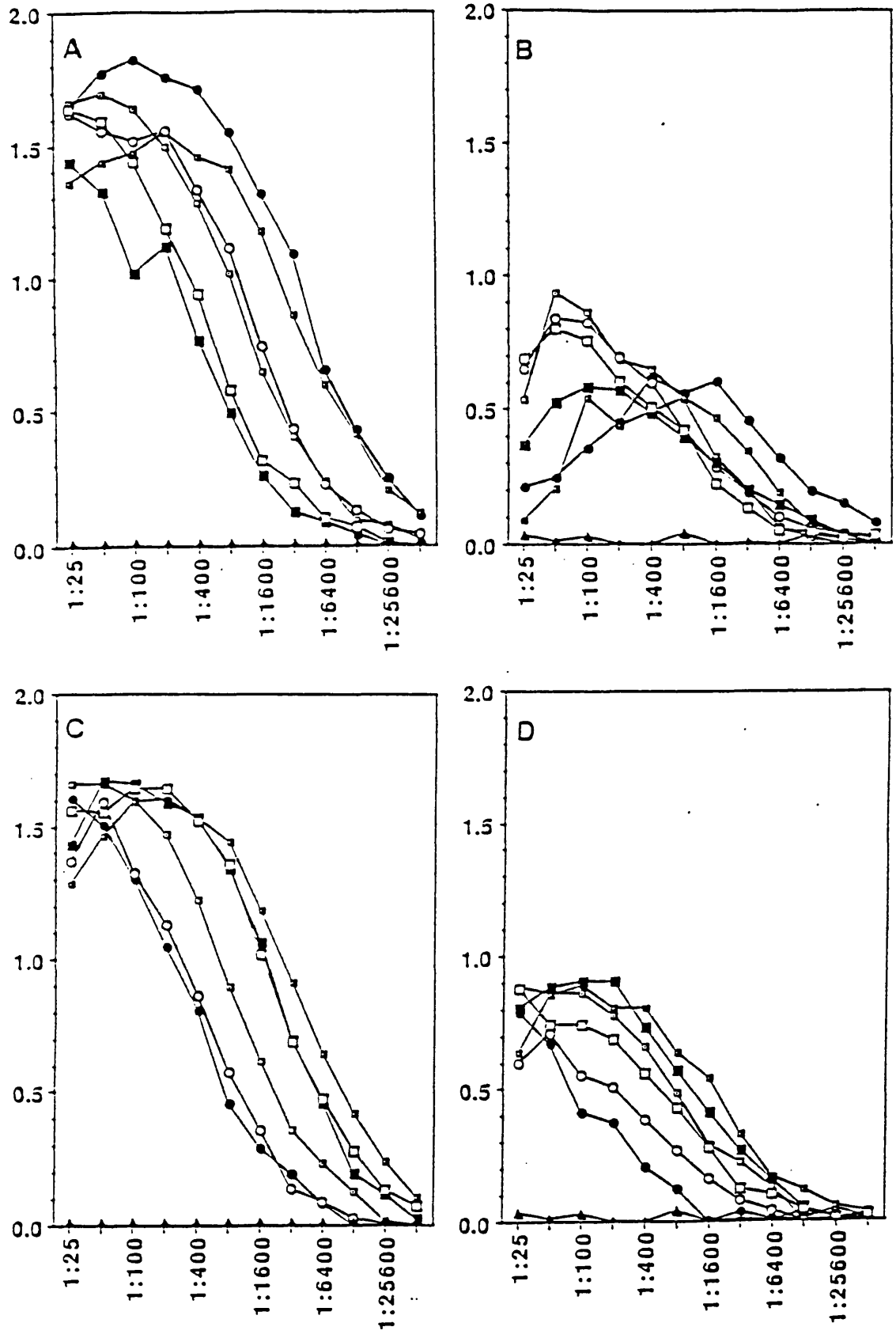

A
B
C
D
2.0
1.5
1.0
0.5
0.0
1:25
1:100
1:400
1:1600
1:6400
1:25600
Absorbance at 405nm
Dilution of maternal sera

Table 5.1 Maternal antibody titres against the branched V3 and gp41 peptides

	Titre giving absorbance of 0.40 OD @ 405 nm		
Mother (Patient No.)	gp120-V3 peptide	gp41 peptide	ratio[a]
Transmitters[b]			
737	1:1200	1:800	1.50
1384	1:3200	1:1200	2.66
2017	1:3200	1:800	4.00
2924	1:12800	1:4800	2.66
2961	1:1200	1:800	1.50
2995	1:12800	1:2400	6.33
3164	0	0	NC
Nontransmitters[d]			
343	1:6400	1:1200	5.33
393	1:3200	1:800	4.00
432	1:3200	1:200	16.00
613	1:3200	1:1600	2.00
847	1:800	1:100	8.00
888	1:12800	1:6400	2.00
1373	1:3200	1:50	64.00
1633	1:3200	1:800	4.00
1896	1:6400	1;1600	4.00
2017	1:3200	1:800	4.00
2084	1:12800	1:3200	4.00
2601	1:800	1:100	8.00
2632	1:3200	1:800	4.00
2652	1:6400	1:3200	2.00
2751	1:6400	1:800	8.00
2898	1:25600	1:12800	2.00
3100	1:600	1:800	0.75
3309	1:200	1:200	1.00
3625	1:1600	1:400	4.00
3938	0	0	NC

a **gp120 V3 loop peptide titre divided by gp41 peptide titre**

b **HIV-1 infected mothers with an infected child**

NC **not calculatable**

d **HIV-1 infected mothers with an uninfected child**

5.9 Measurement of V3 titres in sequential maternal serum samples

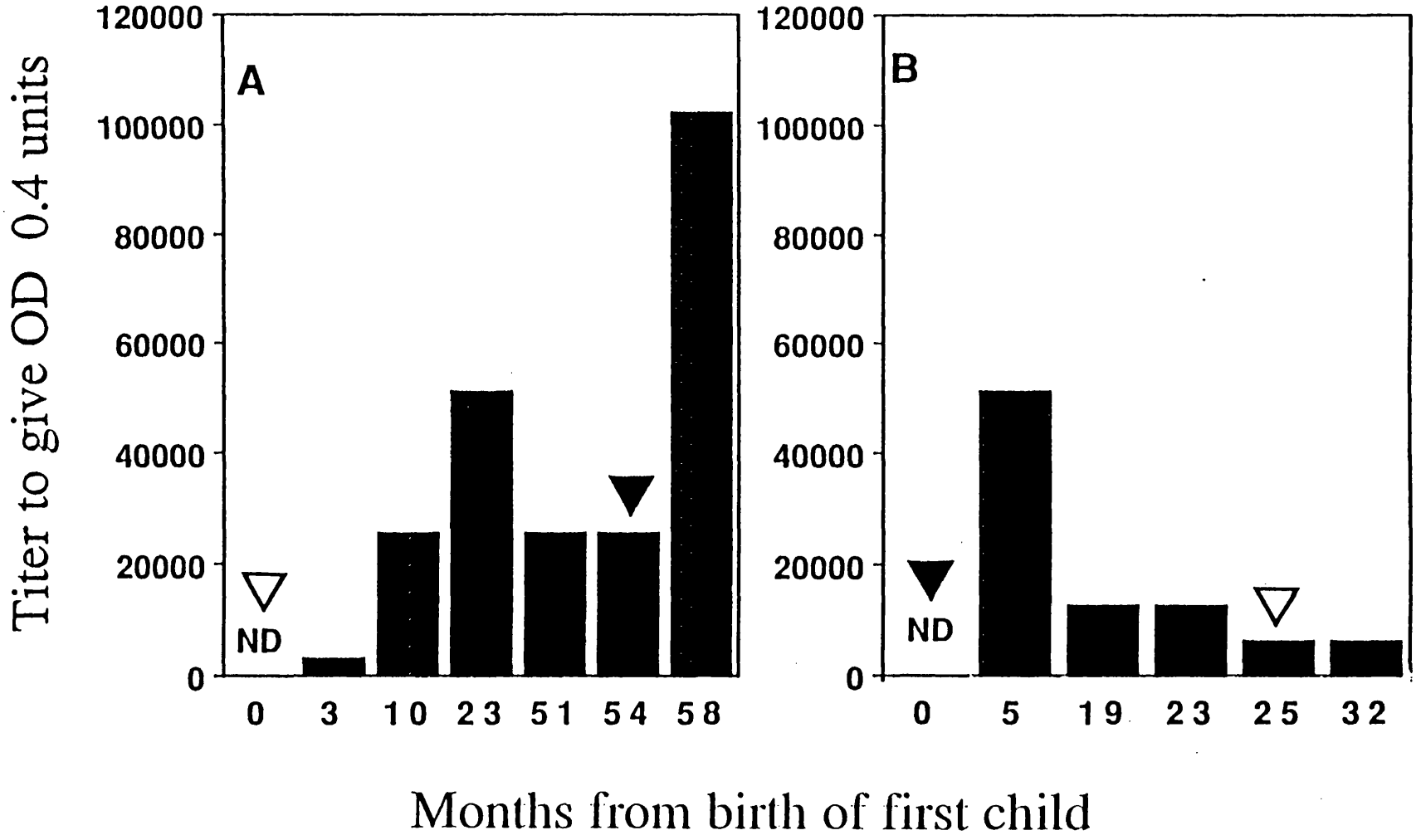
Anti-V3 and anti-gp41 titres were measured as described above for sera from two women who each gave birth to 1 infected and 1 uninfected child (Figure 5.6). The x-axis represents months from the birth of the first child. Panel A shows the anti-V3 titres from a mother who 3 months after delivery of an uninfected child had low titres against the V3 peptide and during a period of increasing anti-V3 titres, gave birth to an infected child. Panel B demonstrates that 5 months after giving birth to an infected child this woman had high titres of antibodies against the V3 region and during a period of decreasing anti-V3 titre gave birth to an uninfected child. The data presented here shows no positive correlation between anti-V3 titre and transmission of HIV-1 to the child.

5.10 Antigen-limited V3 ELISA

To test for the presence of high affinity antibodies, the antigen-limited ELISA described by Devash *et al* (1990) was tested on eight of the maternal sera (four transmitters and four nontransmitters). This test measures the reactivity of a fixed dilution of human sera against different amounts of peptide. Antibodies are classified as being of high-affinity or avidity if they reacted with low amounts of peptide. Typically, Devash *et al.* (1990) designated a serum as having high affinity if it retained 50% of the maximum observed reactivity at a peptide concentration of $5x10^{-4}$ ug/well. For this assay the monomeric V3 peptide was used (peptide 347, Table 4.1) to follow the conditions of the published assay and because branched peptides probably detect low-affinity antibodies more readily (Marsden *et al.*, 1992). The mean reactivity of the monomeric V3 peptide with fourteen seronegative controls was 0.082+/-0.037.

Four transmitter and four nontransmitter maternal samples were chosen at random from the 7 transmitters and 20 nontransmitters at and tested in the antigen-limited ELISA at a dilution of 1:100. The peptide was titrated in a series of seven 10-

Figure 5.6 Longitudinal study of serum antibody titres to the V3 peptide in two HIV-1 infected mothers . Serum samples were taken at different times after the birth of the first child. ▽ and ▼ denote births of uninfected and infected children respectively. ND, titres not determined.


Titer to give OD 0.4 units
120000
100000
80000
60000
40000
20000
0
A
ND
0 3 10 23 51 54 58
B
ND
0 5 19 23 25 32
Months from birth of first child

fold dilutions from an initial concentration of 1ug per well to 10^{-7}ug per well. No differences were observed in the affinities of antibodies in the maternal sera from the four transmitters and four nontransmitters in the study group (representative data are shown in Figure 5.7).

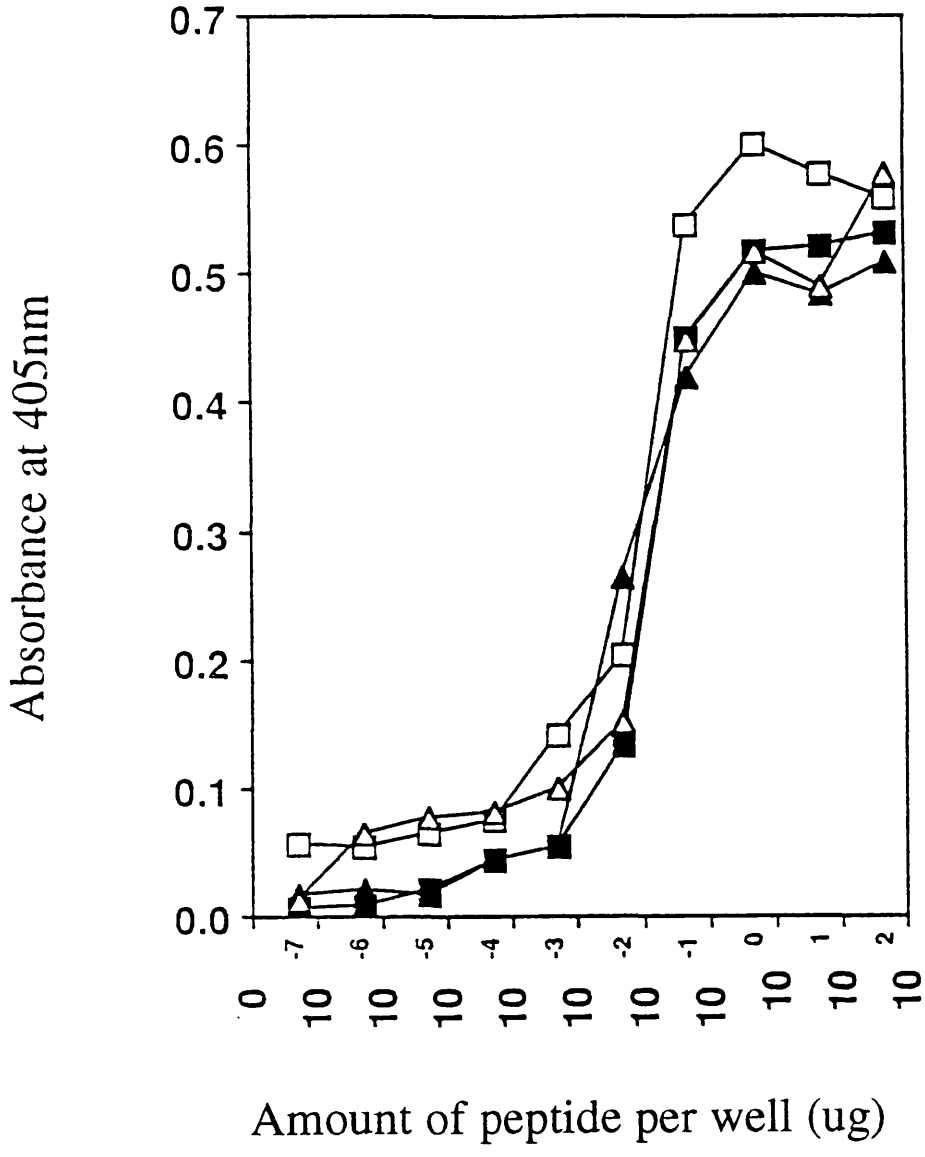


Figure 5.7 Reactivity of maternal sera with varying amounts of the monomeric V3 peptide with sequence TRPNNNTRKRIHIGPGRAFYTTGQIIGDIRQAH. Sera from HIV-1 transmitters (▲, ■) and HIV-1 nontransmitters (△,□) were diluted 1:100 for use in the ELISA. The monomeric peptide was titrated in a series of ten, 10-fold dilutions.

5.11 DISCUSSION

5.11.1 Maternal antibody levels against the V3 region of HIV-1 gp120

Prior to this investigation several reports had been published suggesting that the presence of maternal antibodies against specific epitopes in the V3 region of HIV-1 gp120 correlated with protection of the foetus against HIV-1 infection in utero (Broliden *et al.*, 1989; Rossi *et al.*, 1989; Devash *et al.*, 1990). The peptides used to screen the mothers or infants sera are listed in Table 5.2. Broliden *et al.* (1989) examined the reactivity of childrens sera taken prior to six months of age, as these sera will still contain maternal antibodies', with a series of eight peptides spanning the consensus sequence peptide from the HIV-IIIB strain of the virus. The sera from the infants born to the HIV-1 infected mothers were screened at a single dilution for reactivity against each of these peptides. Peptide C57, which encompasses the far right hand side and extends beyond the V3 loop region was found to react with sera from 6 out of 19 uninfected infants, but showed no reactivity with sera from any of fourteen infected children. Similarly, among the four peptides used by Rossi *et al.* (1989) to screen sera of newborn children or HIV-1 positive mothers, peptide C57 showed significantly greater reactivity with sera from 5 out of 19 uninfected children and no reactivity with any of the sera from fourteen infected children. When sera from HIV-1 positive mothers were screened against the four peptides, peptides C51 and C57, but not C53 or C58 showed significantly greater reactivity with the sera from mothers who gave birth to uninfected children than that from mothers of infected children. The reactivity seen against peptide C57 and not other peptides spanning the conserved GPGR motif, common to all HIV-1 isolates is perhaps surprising as the immunodominant epitope of gp120 encompasses this tetrameric domain (LaRosa *et al.*, 1990) and it would be expected that infected sera should recognise this region.

In another study (Devash *et al.*, 1990), the reactivities of both maternal and neonatal sera (age less than four weeks) were measured in an antigen-limited ELISA.

Using this technique they demonstrated a correlation between high-affinity or high-avidity antibodies in the sera of both mothers and infants with a lack of transmission of HIV-1 from mother to child. All the groups were able to establish that low immunoreactivity to the principal neutralising determinant was not due to an overall loss of humoral response by demonstrating reactivity to other HIV-1 peptides derived from other immunogenic proteins of the virus.

During the course of the work described in this chapter, Parekh *et al.* (1991), reported a study of maternal antibody levels, using peptides similar to that of Rossi *et al.* (1989), but derived from the MN rather than the IIIB strain. They found that only the peptide KRIHIGPGRAFYT, the same as used by Devash *et al.* (1990) was recognised by the maternal sera. Using the same antigen-limited ELISA, these authors found no correlation between transmission and V3 antibody affinity or avidity. In addition, Parekh sent coded sera to the laboratory of Devash *et al.*, who was unable to correctly classify them as from transmitting or nontransmitting mothers.

The experiments described in this chapter were designed to examine quantitatively whether there is a correlation between maternal antibodies to the V3 peptide and lack of transmission of the virus to the child. Antibody titres to the V3 peptide were determined against doubling dilutions of the maternal sera. No correlation was observed with reactivity against this peptide, nor was any observed when the anti-V3 peptide titres were normalised by anti-gp41 peptide titres. When the relative affinities of the antibodies from four representative samples taken from each of the two groups were assayed using the antigen-limited ELISA as described by Devash *et al.* (1990), no differences in antibody affinity were found. These results support the findings of Parekh *et al.* (1991) and not those of Devash *et al.*, (1990). Two other studies also fail to show a correlation between maternal antibodies against V3 and lack of transmission to the foetus (Geffin *et al.*, 1992; Halsey *et al.*, 1992).

The V3 peptide described in this study contains all the amino acids between the two conserved cysteines of the V3 loop and encompasses the peptides used by both Devash *et al.* (1990) and Parekh *et al.* (1991). It only part overlaps the C57 peptide

Table 5.2 Comparison of V3 peptide sequences used to screen maternal or children's sera

Study	Peptide	Peptide sequence	Correlation between serum reactivity and uninfected status of the child: Child*	Maternal
Broliden *et al.*, 1989	C51	INCTRPNNNTRKSIR	-	NT
	C52	PNNNTRKSIRIQRGP	-	NT
	C53	RKSIRIQRGPGRAFV	-	NT
	C54	IQRGPGRAFVTIGKI	-	NT
	C55	GRAFVTIGKI.GNMRQ	-	NT
	C56	TIGKI.GNMRQAHCNI	-	NT
	C57	GNMRQAHCNISRAKW	+	NT
	C58	AHCNISRAKWNNTLK	-	NT
Rossi *et al.*, 1989	C51	INCTRPNNNTRKSIR	-	+
	C53	RKSIRIQRGPGRAFV	-	-
	C57	GNMRQAHCNISRAKW	+	+
	C58	AHCNISRAKWNNTLK	-	NR
Devash *et al.*, 1990		KRIHI..GPGRAFYT	+	+
Parekh *et al.*, 1991		INCTRPNNYNKRRI	NT	NR
		KRIHI..GPGRAFYT	NT	-
		GTIRQAHCNISRAKW	NT	NR
		AHCNISRAKWNDTL	NT	NR
THIS STUDY		TRPNNNTRKRIHI..GPGRAFYTTGQIIGDIRQAH	NT	-

NT not tested
NR not reactive with maternal sera and therefore not tested
no amino acid at that position
* sera taken in the first six months of age

It should be noted that Broliden *et al.*, 1989 and Rossi *et al.*, 1989 are essentially the same group.

with which Broliden *et al.* (1989) and Rossi *et al.* (1989) detected a correlation, as the carboxy-terminal 7 amino acids of C57 lie outside the V3 loop. The use of different peptides from the V3 sequence could perhaps explain the apparent conflict between these results and those of Broliden *et al.* (1989) and Rossi *et al.* (1989) but cannot explain the disagreement with the findings of Devash *et al.* (1990). If there is a correlation between reactivity of peptide C57 (spanning gp120 amino acids 323 to 337) and vertical transmission then the data presented in this study imply that the important epitopes on peptide C57 is specific for the IIIB strain, GNMRQ, versus GTIRQ in the MN strain or includes residues that lie outside V3.

5.11.2 The involvement of other factors in vertical transmission of HIV-1 from mother to child

The data presented in this chapter do not support the view that high levels or high affinity antibodies to sequences in the V3 region of HIV-1 gp120 correlate with protection against vertical transmission. The cases of twins in which one was infected and one was not (Goedert *et al.*, 1991 and this study) argues strongly for the involvement of other factors in the protection against vertical transmission of HIV-1. It is perhaps naive to suggest that only one variable will determine the outcome of at risk pregnancies, but more likely that an interplay of factors will be involved. These factors have previously been discussed (Introduction, section 1.8). In addition, antibodies against other regions of the gp120 and the specificity of the maternal cytotoxic T cell response may also play a role in the transmission of HIV-1 to the foetus.

To determine the factors involved in maternal-foetal transmission it may be important to define the time point at which transmission occurs. In this study, the blood samples were taken from the mothers immediately before, during or after the birth of the child. It is possible that at transmission, the levels of antibodies against the V3 region between the two groups may have been different. This possibility is unlikely as transmission is believed to occur in the later stages of pregnancy and it is difficult to

imagine that the antibody levels at this time will be significantly different from those at birth. It would, however, be interesting to measure maternal antibody levels throughout the entire pregnancy to determine whether they remain relatively constant and if not, whether they correlate with the outcome of the child.

5.11.3 Future Studies

The findings of Wolinsky *et al.*, (1992), that only certain variants observed in the mother were detected in the infected child and the findings that on initial infection of haemophiliacs with HIV-1, the envelope sequences in the V3 region are completely homogenous (Zhang *et al.*, 1993), suggest that there is a selection for specific env sequences either upon transmission or in the interval between infection and seroconversion. Perhaps the presence of a specific *env* determinant is required to allow transmission of the virus. In addition to this initial genotypic homogeneity, the viruses that are found in infected individuals early in infection appear to be predominantly of the same biological phenotype; slow low, NSI and macrophage tropic. Specific regions of gp120, including V3, have been shown to be involved in these phenotypic characteristics. It would therefore be of interest to isolate the viruses from infected infants early after infection and if possible prior to seroconversion, to determine their phenotypic properties. The findings from such experiments should indicate whether the virus(es) involved in vertical transmission of HIV-1 have properties similar to those transmitted by other modes.

The results presented in this chapter do not lend support to the notion that immunotherapy aimed at increasing anti-V3 antibody titres in pregnant HIV-1 infected mothers would have an effect on the clinical status of the child. Moreover, these results demonstrate that the level of anti-V3 antibodies in infected mothers could not be used to serve as a prognostic marker in predicting the outcome of at-risk pregnancies. It is however important to note that in this study, the levels of neutralising antibody against the principal neutralising determinant were not measured, and indeed it has not yet been

determined what role neutralising antibodies play in HIV-1 infection. Although no correlation has been shown between the levels of antibodies against gp120 and an clinical status of the patient (Holmback *et al.*, 1993), two groups have shown that the titres of neutralising antibodies against the V3 region in infected children did not correlate with p24 antigen levels, CD4 count, age and sex but showed a significant correlation with an improved clinical status (Robert-Guroff, *et al.*, 1987, 1993; Ljunggren *et al.*, 1990). In addition, the presence of antibodies capable of inhibiting syncytium formation may also improve the clinical status of the HIV-1 infected child (Brenner *et al.*, 1991). Their study involved 34 children born to HIV-1 infected mothers: infants who had syncytium-inhibiting antibodies had an overall better clinical outcome than infants with lower titres of these antibodies. Because the V3 region is involved in the SI phenotype, induction of V3-specific antibodies in infected infants may improve their clinical status. This hypothesis remains to be tested, but raises the possibility that hyperimmunoglobulin therapy or subunit vaccines derived from the V3 region may have a therapeutic effect in infected infants. Further, larger studies will be necessary to confirm these findings.

The work described in this chapter was done to determine whether support could be found for the view that antibodies against the V3 region of HIV-1 gp120 might protect against maternal-foetal transmission and/or whether such antibodies might serve as a prognostic marker for transmission. The results obtained have clinical relevance in that they provide no support for a protection strategy aimed at raising anti-V3 antibody titres and indicate that measuring anti-V3 titres is not useful in predicting the outcome of an HIV-1 infected mother giving birth to an infected child.

Chapter 6 Results: Section 3

Do antibodies play a role in selecting sequence variants within the V3 region of HIV-1 gp120.

The work presented in this chapter was undertaken to investigate whether the sequence variants that arise in the third hypervariable region (V3) of HIV-1 gp120 during infection, represent antibody escape mutants. This work was conducted as part of a collaboration with Dr Peter Simmonds and Dr Andrew Leigh Brown from the University of Edinburgh.

On infection with HIV-1 the virus population appears to be relatively homogenous in the V3 region of the envelope protein (Wolfs *et al.,* 1991,1992; Zhang *et al.,* 1993), with only certain genotypes being passed from individual to individual (Wolinsky *et al.,* 1992). This homogeneity is believed to be due to the existence of strong selection for specific *env* sequences either upon transmission or in the interval between exposure and seroconversion in the infected individual. The homogeneity is maintained prior to seroconversion for reasons that are not yet clear but could be due to little or no immunoselective pressure exerted by the host on the virus, resulting in the clonal outgrowth of the most viable isolate. Following seroconversion and during the long asymptomatic phase, the virus population displays considerable genetic diversity, particularly in the external glycoprotein gp120 (Hahn *et al.,* 1986; Simmonds *et al.,* 1990, 1991; Balfe *et al.,* 1990), not only in terms of genetic composition but also in terms of cell and tissue tropism, syncytium-inducing properties and replication ability in-vitro (Levy *et al.,* 1985; Anand *et al.,* 1987; Asjo *et al.,* 1986; Evans *et al.,* 1987; Briesen *et al.,* 1987; Tersmette *et al.,* 1988; Fenyo *et al.,* 1988). The viral-specific antibody and T-cell responses which can be readily demonstrated during the asymptomatic period are consistent with the possibility that continuous replication of the virus is accompanied by immune selection.

To test the hypothesis that sequence variation in the V3 region of HIV-1 gp120 is driven by humoral immune selection, peptides corresponding to the variants found during the natural course of infection in one patient were synthesised. The peptides were tested in ELISA for reactivity against sequential serum samples taken from that individual.

6.1 Study patient

The HIV-1 V3 sequence variants which emerged during the natural course of infection of patient 82, a member of the Edinburgh haemophiliac cohort had previously been identified (Simmonds *et al*, 1991) and were made available to me before their publication. These sequences were obtained by extraction of viral RNA from the plasma of serum samples taken at intervals from the time of seroconversion in 1984 through to 1991. The viral RNA was reverse transcribed to give cDNA copies of the replicating virus genomes found in patient 82. The cDNA was diluted to give single copies for amplification by nested PCR. (Simmonds *et al* , 1990). Nested PCR involves two rounds of amplification using primers having the sense and antisense orientations. The first round utilises primers spanning the V3 region and the second involves internal primers. This procedure yielded sufficient DNA from single isolated molecules for direct sequencing. The DNA products obtained from the amplification were purified and then sequenced to deduce the amino acids at residues 297-330, which are listed in Table 6.1 (adapted from Holmes *et al.*, 1992). The predominant V3 sequence found at the time of seroconversion is termed the *seroconversion sequence* and the variants are defined as having amino acid substitutions deviant from this sequence. Table 6.1 shows the 24 V3 sequencies detected in patient 82 over the sampling period. Only the amino acid residues, deviant from the seroconversion sequence are represented by their appropriate single letter amino acid code and identical residues are denoted with a dot. The frequency in each year, that an individual sequence was detected is shown, with a value of 1.000 representing 100% of the sequenced population. The total number of

Table 6.1 Amino acid sequences found in patient 82 are listed according to their theoretical evolutionary lineage (A-F) assigned by Holmes *et al.*(1992). Residues that differ from the seroconversion sequence (A) are shown, with a dot denoting identical residues. The frequencies of each sequence, in each year, from which a sample was available are presented, with a value of 1.000 representing 100% of the sequenced viral population. The total number of sequences obtained for each time point are shown along the bottom. A blank space indicates that the sequence was not detected in that particular year.

Table 6.1 The 24 V3 loop amino acid sequences and their sample frequencies in the plasma of patient 82

Lineage	Sequence	Frequency in year 0	3	4	5	6	7
	296 330						
A	CTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAH	1.000					
B	.....D.....		0.067				
C1	.....P.....D.....		0.067				
C2	.....P.....Q.....		0.267				
C3	.....G.....Q.....		0.267				
C4	.....P.....D.T.....		0.067				
C5	.....D.T.....		0.067				
D1	.....V....Q.....			0.091			
D2	.....R.....V...EQ...N.....			0.455			
D3	.....G.....V...EQ...N.....			0.091			
D4	.....R.Y.....V...EQ...N.....				0.087		
D5	.....R.Y....S.V...EQ...N.....				0.043		
D6	.....R.Y.....V...DQ.....					0.067	
D7	.....R.Y.....V...DQ...N.....					0.200	0.077
D8	.....Y...R.G.....SV..AEQ...N.....					0.200	0.077
E1	.....G.....S...A..D.....		0.067		0.043	0.333	0.769
E2	.....G.....S...A..G.....			0.182	0.696		
E3	.....G.....S...A..R.....			0.091			
E4	.....G.....S.V.A..G.....				0.043		
E5	.....G.....S...A..G...N.....				0.087		
E6	.....G.....S...A..D.....					0.067	
E7	.....G.....A..D.....					0.067	
E8	.....G.....V....D.....					0.067	
F	.....G.....		0.133	0.091			0.077
	TOTAL	12	15	11	23	15	11

sequenced V3 regions for each time point is shown along the bottom of the table. These sequencies have been placed in a theoretical evolutionary tree (Figure 6.1), with proposed relationships between the different sequences represented by arrows (Holmes *et al.*, 1992). Each of the 24 sequences have thus been assigned to specific lineages (listed in Table 6.1) termed A (the seroconversion sequence) through to F, making the assumption that all of the sequencies have evolved from the seroconversion sequence (Holmes *et al.*, 1992). This assumption is based on the observation that at the time of seroconversion the V3 region is highly homogenous (Wolfs *et al.*, 1991, 1992; Zhang *et al.*, 1993).

Seven sequential serum samples were taken from patient 82 over the period 1984-1991. These samples were coded and are listed in Table 6.2. During the period of sampling patient 82 remained asymptomatic for the clinical symptoms of HIV-1 infection, but showed a continual decline in CD4 counts to below 200 at the latter time points.

Before beginning the study, preliminary experiments were done to firstly, define the region of V3 which is reactive with sera from patient 82, so that peptides shorter than 33 amino acids could be synthesised and secondly, to determine the optimal form of peptide to best detect V3 specific antibody in the sera of patient 82.

6.2 Defining the region of V3 reactive with sera from patient 82

To define the region of V3 reactive with HIV-1 infected sera, four overlapping peptides spanning the region between amino acids 297 and 330 were synthesised in the monomeric form. The sequence of these peptides is shown in Table 6.3. Peptide 344A, synthesised by Dr Susan Graham, and peptide 416 were soluble in PBS, however, peptides 353A, 353B and 353C with basic properties had to be dissolved in 100ul of 10% formic acid and made up to the required volume with PBS. Figure 6.2 shows each of the five peptides titrated in a series of eight, 10-fold dilutions from a concentration of 50ug to $5x10^{-6}$ug peptide per well. All four peptides were tested in ELISA for

Figure 6.1 Predicted evolutionary framework relating the 24 different sequencies found in the V3 loop (adapted from Holmes et al, 1992). Distinct evolutionary lineages are designated by numbers. Proposed relationships are indicated by arrows. Lineages that persist are indicated by dashed arrows. Time scale is given along the top of the diagram.

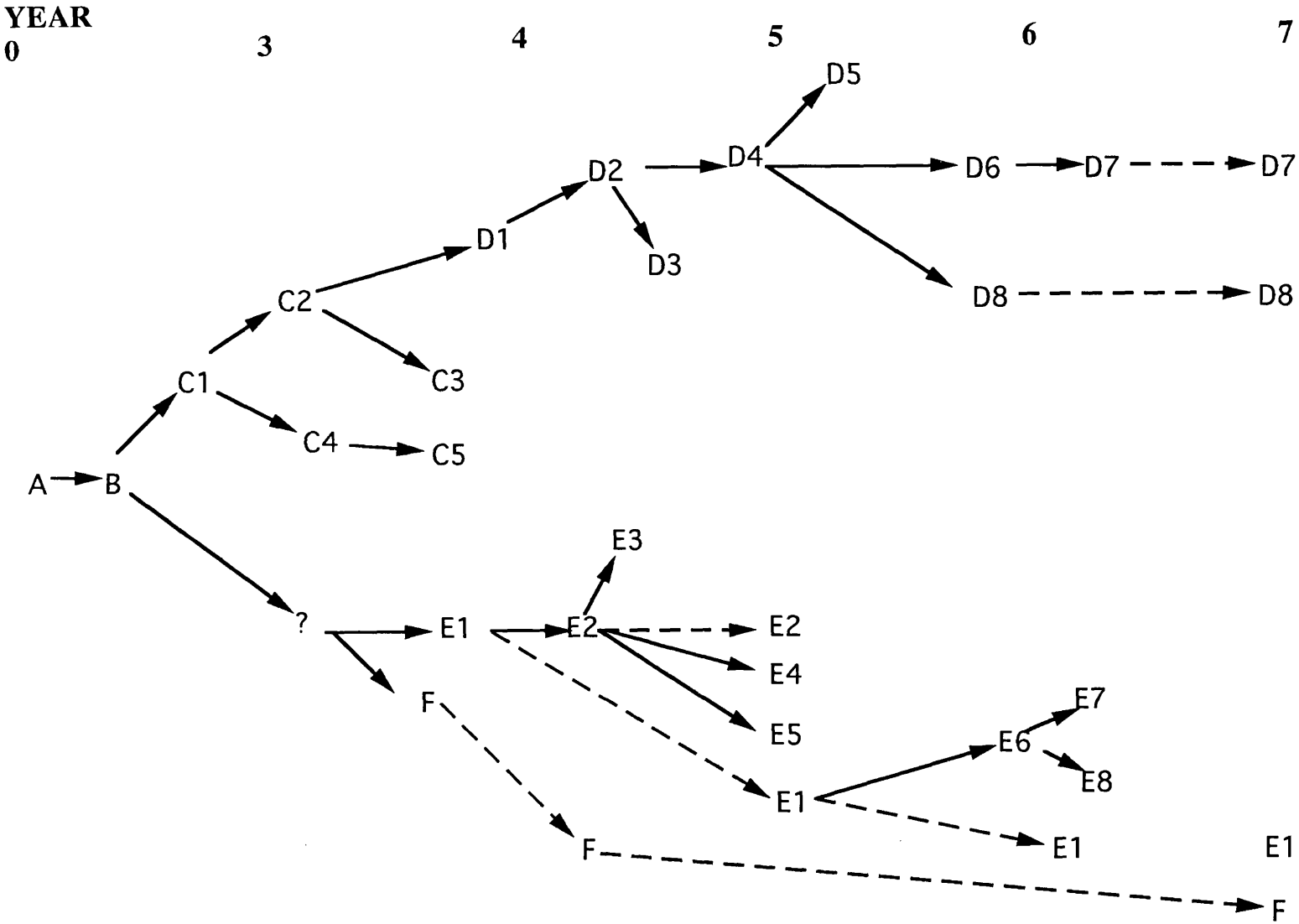

YEAR
0
3
4
5
6
7
A
B
C1
C2
C3
C4
C5
D1
D2
D3
D4
D5
D6
D7
D7
D8
D8
?
E1
E2
E3
E2
E4
E5
F
E1
E6
E7
E8
E1
E1
F
F

Table 6.2

Serum samples taken from patient 82 (1984-1991)

Sample number	Sample code	Date sample taken
1	JA53190	4-12-84
2	H5640/87	30-7-87
3	H530/88	26-1-88
4	H5326/88	27-7-88
5	H1094/89	15-2-89
6	H6092/89	18-8-89
7	H1164/91	8-2-91

Table 6.3 Peptides corresponding to regions of patient 82 seroconversion sequence

Seroconversion sequence

gp120 aa	297	330

TRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAH

Peptide No

416	TRPNNNTRKSI
344A	RKSIHIGPGRAFY
353A	GPGRAFYTTGEIIGDIRQAH
353B	RAFYTTGEIIGDIRQAH
353C	YTTGEIIGDIRQAH

All peptides were synthesised as monomers

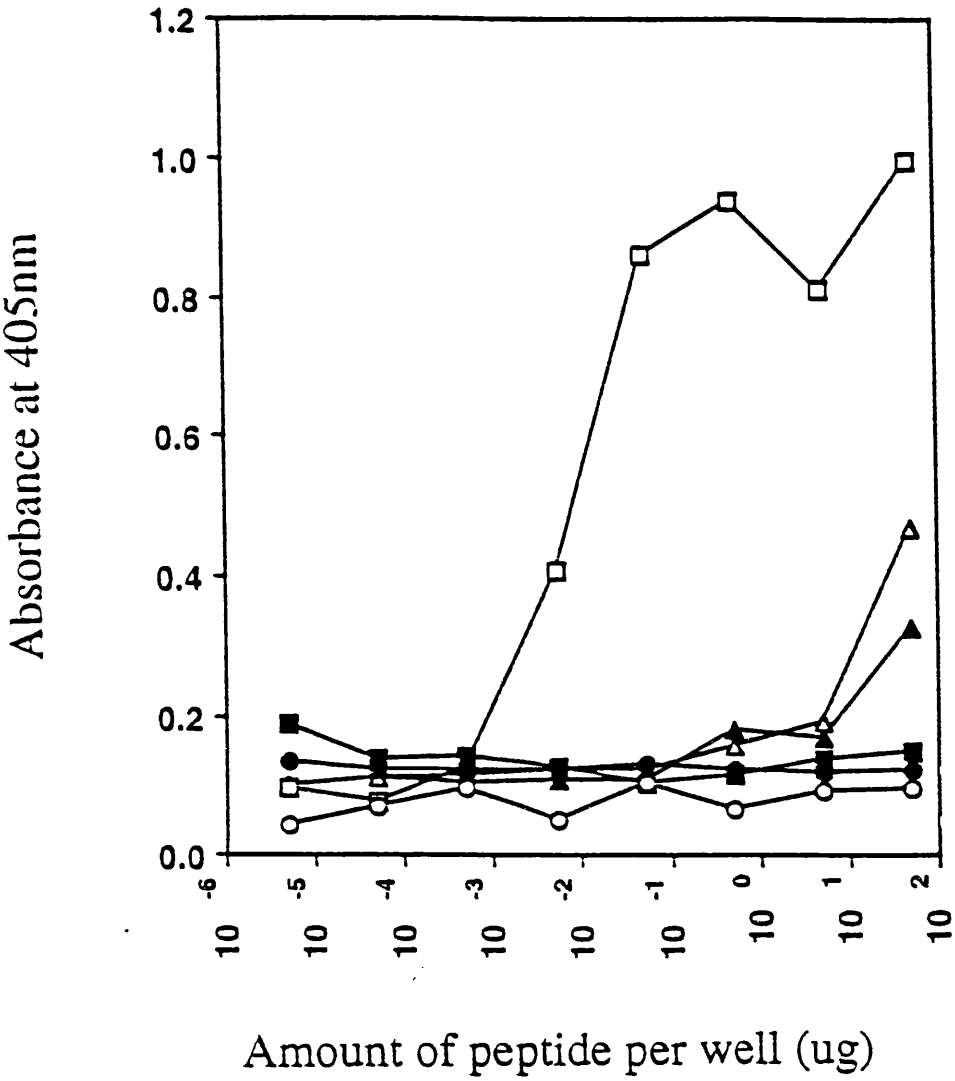


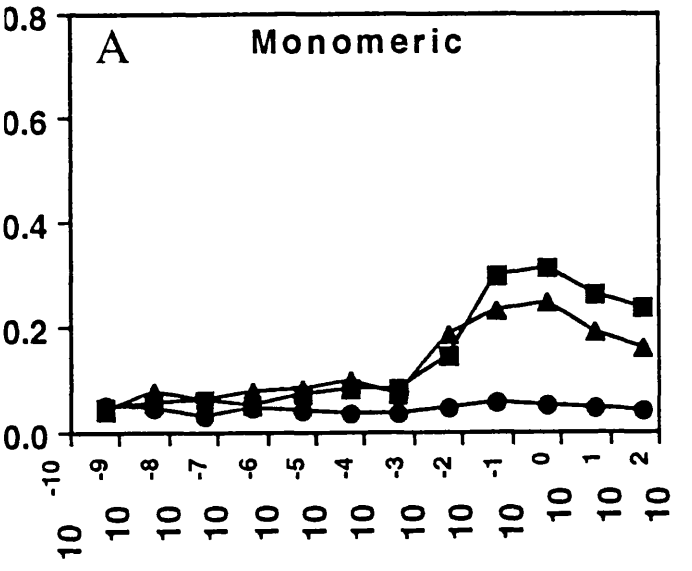
Figure 6.2 Reactivity of the monomeric peptides (shown in Table 6.3) spanning the seroconversion sequence of patient 82 in an ELISA. Peptide 416 with sequence TRPNNNTRKSI (●), 344A with sequence RKSIHIGPGRAFY (□), 353A with sequence GPGRAFYTTGEIIGDIRQAH (▲), 353B with sequence RAFYTTGEIIGDIRQAH (△) and 353C with sequence YTTGEIIGDIRQAH (■) were titrated in a series of eight 10-fold dilutions from a concentration of 50ug to 5x10-6ug peptide per well and screened for reactivity with the serum sample (diluted 1:100) taken at seroconversion (JA53190, Table 6.2). The reactivity of peptide 344A with an HIV-1 negative serum is shown (○).

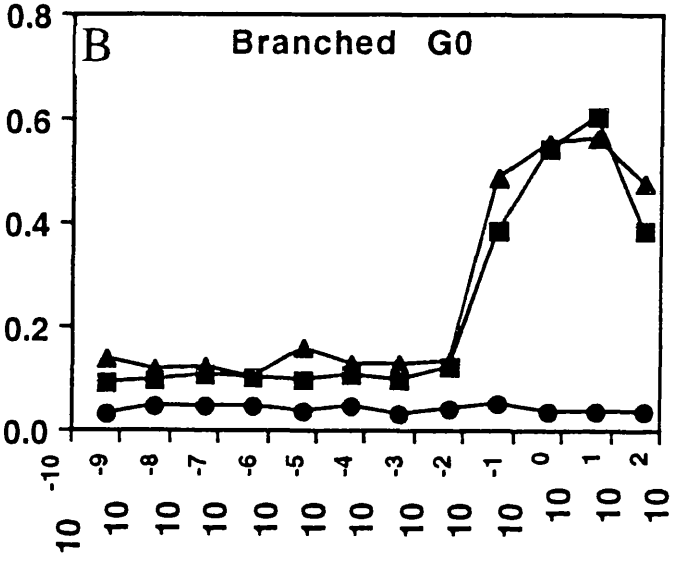
reactivity with the 1984 serum sample JA53190 and an HIV-1 negative control serum at a 1:100 dilution. The HIV-1 positive serum was highly reactive against the 344A peptide, still detecting antibody at 10^{-2}ug of peptide, giving an absorbance at 405nm of 0.4 OD units. Peptides 416, 353A, B and C showed very little reactivity with the serum however, peptides 353A and B showed reactivity at the highest peptide concentrations. The HIV-1 negative control serum showed no reactivity with any of the peptides though only the data from peptide 344A is shown. These findings confirm previously published work showing that the immunodominant region of V3 maps to the highly conserved GPGR motif at the tip of the loop and the surrounding amino acids (Goudsmit *et al.*, 1988a; Javaherian *et al.*, 1989; Meloen *et al.*, 1989). Peptides spanning this immunodominant region of V3 (gp120 aa 304-316) containing all variant sequences found in patient 82 were subsequently made.

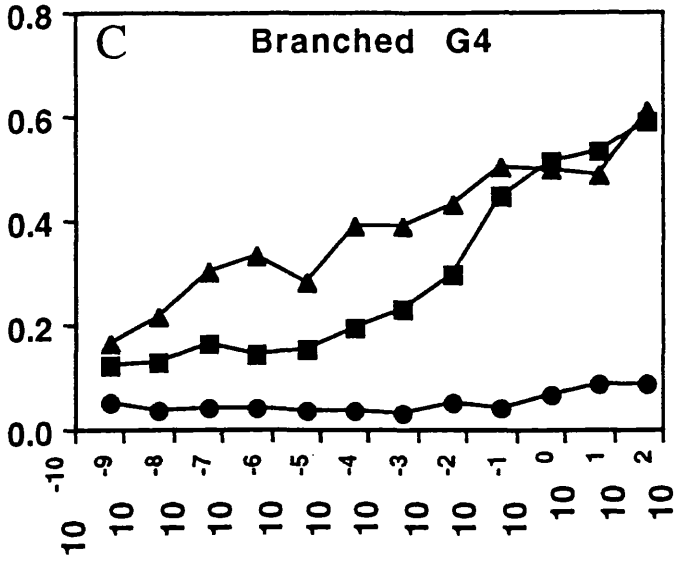
6.3 Reactivity of the short 13-mer seroconversion peptide in the monomeric, branched and branched form with a four glycine spacer

The short V3 monomeric seroconversion peptide (344A), sequence RKSIHIGPGRAFY, highly reactive with the initial sample from patient 82, was synthesised in branched form with and without four glycine spacers. Both peptides like 344A, were soluble in PBS. Figures 6.3A, B and C show each form of peptide titrated in a series of twelve, 10-fold dilutions from $5x10^{-10}$ug/well to 50ug/well and tested in ELISA with HIV-1 positive and negative control sera at a dilution of 1:100. Both forms of branched peptide showed greater reactivity with the HIV-1 positive control sera than did the monomeric peptide. Using a cut-off absorbance value of 2.2 (= 5x the mean of the absorbance values of the negative serum), the monomeric and branched peptide without glycine spacers could detect specific antibody at peptide concentrations as low as $5x10^{-2}$ug per well whereas, the branched peptide with the four glycine spacer could detected antibodies in one serum with $5x10^{-9}$ug peptide per well and in the other serum with $5x10^{-4}$ug peptide per well. From the graphs , the concentration of all three

Figure 6.3 Comparison of the reactivity in an ELISA, of the short V3, seroconversion peptides, synthesised in the following forms, monomeric (A), branched (B), and branched with a four glycine spacer (C) with control HIV-1 positive (■,▲) and negative sera (●) at a dilution of 1:100. Peptides were titrated in a series of twelve, 10-fold dilutions from a concentration of 50 ug to 5x10-10ug per well.







Absorbance at 405nm

Amount of peptide per well (ug)

peptides required to give the optimal reactivity with minimal background against HIV-1 negative control sera was considered to be 10^{-1}ug per well.

6.4 Sensitivity of the V3 monomeric and branched G4 peptides

The branched G4 and monomeric seroconversion peptides, were compared in ELISA for sensitivity in detecting antibody in HIV-1-infected sera. The peptides were dissolved in PBS and coated to microtitre plates at the optimum concentration of 100ng per well and the sera titrated in a series of doubling dilutions from 1:25 to 1:51,200. The serum titre was defined as the reciprocal of the maximum dilution which would give an absorbance of 0.3. The data shown in Figure 6.4A and B and summarised in Table 6.4 demonstrate that the branched G4 peptide has an 8-fold greater sensitivity than the monomeric peptide for detection of antibodies in one of the sera and a 32-fold greater sensitivity for the other. Based on these data, the branched form of the peptide with four glycine spacers was considered the best form of peptide for detection of antibodies against the V3 region.

6.5 V3 variant peptides

Peptides corresponding to all the variants in the V3 region between gp120 aa 304-316 that had been identified in patient 82, during the period 1984-1991, were synthesised in the branched form with four glycine spacers. The sequence, relative frequency and the years in which the variants were found in the circulating virus population are shown in Table 6.5. The frequency at which each individual sequence was present has been deduced from the values shown in Table 6.1, taking into account only amino acids between residues 304-316. The peptides were designated V3(1)-V3(17), V3(1) being the seroconversion sequence peptide. Peptides V3(2)-V3(12) correspond to all the variants found in patient 82 over the seven year period. Some of

Figure 6.4 Comparison of the sensitivity of the short branched V3 peptide with a four glycine spacer (B) and the monomeric V3 peptide (A) coated to microtitre plates at a concentration of 100ng of peptide per well. The peptides were screened against doubling dilutions of control HIV-1 positive (■ , ▲) and negative sera (●) from 1:25 to 1:51,200.

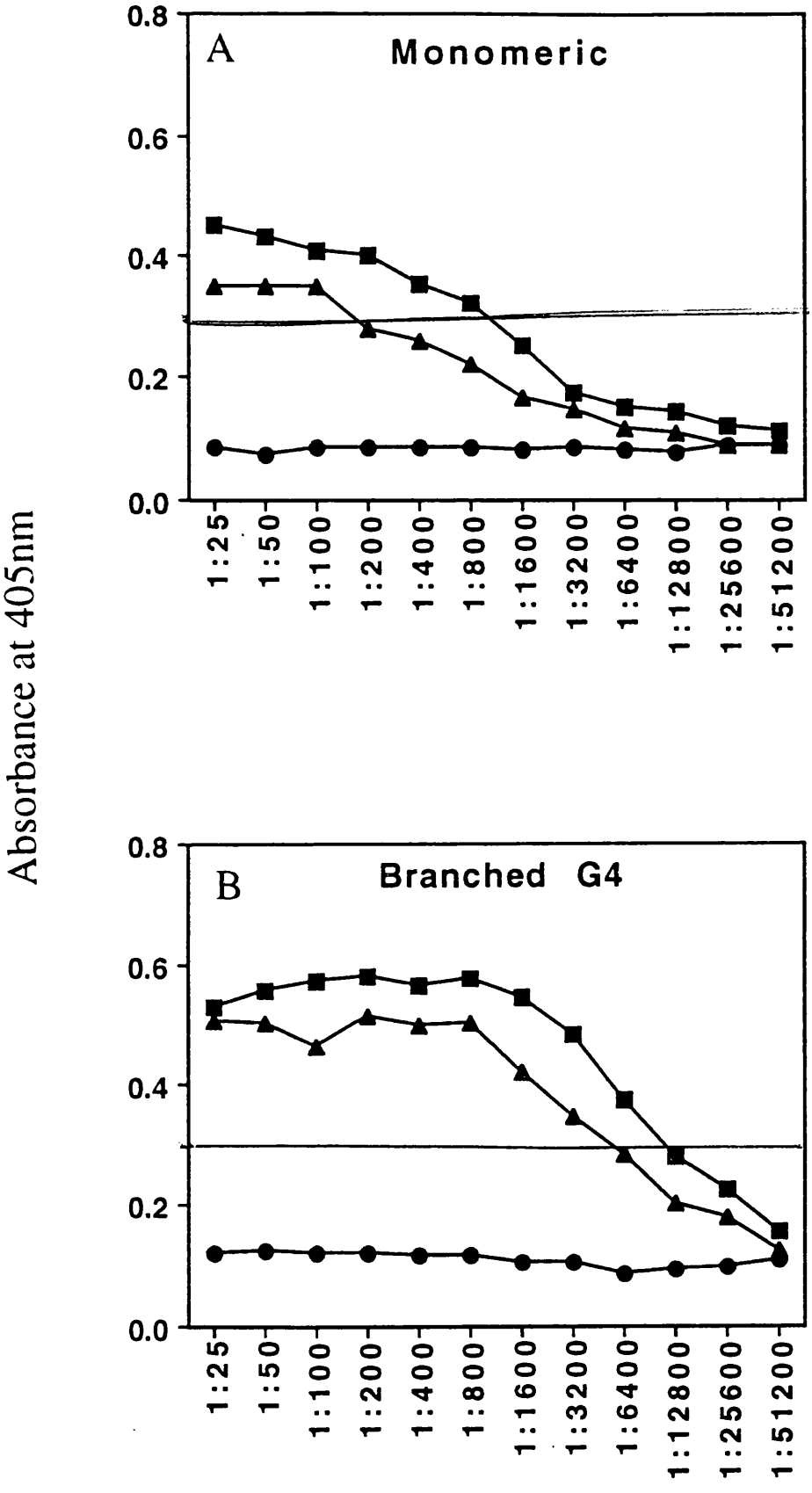

A
Monomeric
B
Branched G4
0.8
0.6
0.4
0.2
0.0
1:25
1:50
1:100
1:200
1:400
1:800
1:1600
1:3200
1:6400
1:12800
1:25600
1:51200
Absorbance at 405nm
Dilution of human sera

Table 6.4

Titration of HIV-1 positive sera against a branched peptide with a 4 glycine spacer and a monomeric peptide , both corresponding to residues 304-316 of HIV-1 gp120.

Serum	Titre against [a]	
	monomeric peptide	branched peptide
EBH4	100	3200
EBH6	800	6400

a The titre is defined as the reciprocal of the maximum dilution giving an absorbance of 0.3 OD units at 405nm

Table 6.5

a All peptides were made with the general structure (Sequence GGGG)$_8$K$_7$A. Peptide V3(1) corresponds to the seroconversion sequence. Peptides V3(2)-V3(12) correspond to variants found in patient 82, while peptides V3(13)-V3(17) represent the individual amino acid changes found in variants V3(6)-V3(11) containing multiple amino acid substitutions.

b Purity was determined by reverse phase HPLC.

c Frequency with which the V3 sequence variants were present in patient 82 (adapted from Holmes *et al.*, 1992). The number of genomes sampled in each year was: 1984, 12; 1987, 15; 1988, 11; 1989, 23; 1990, 15; 1991, 11. A blank space indicates that the genotype was not present in that year.

d The peptide was insoluble in water and was not included in the study.

e Not found in the circulating virus population of patient 82.

ND Not determined.

Table 6.5

V3 peptide	Sequence [a]	Purity %[b]	frequency found in patient 82[c]					
			1984	1987	1988	1989	1990	1991
V3(1)	RKSIHIGPGRAFY	98	1.000	0.267	0.091			0.077
V3(2)	RKSI**P**IGPGRAFY	96		0.401				
V3(3)	RKSIHIGPG**G**AFY	97		0.267				
V3(4)	RKSIHIGPGRA**V**Y	98			0.091			
V3(5)	RK**G**IHIGPGRA**V**Y	95			0.091		0.077	
V3(6)	RK**R**IHIGPGRA**V**Y	98			0.455			
V3(7)	RK**G**IHIGPG**S**AFY	98		0.067	0.273	0.826	0.333	0.769
V3(8)	RK**R**I**Y**IGPGRA**V**Y	93				0.087	0.267	0.077
V3(9)[d]	RK**R**I**Y**IGPG**S**A**V**Y	ND				0.043		
V3(10)	RK**R**I**G**IGPGR**SV**Y	97					0.200	0.077
V3(11)	RK**G**IHIGPG**S**A**V**Y	100				0.043	0.067	
V3(12)	RK**G**IHIGPGRAFY	99					0.067	
V3(13)[e]	RK**R**IHIGPGRAFY	99						
V3(14)[e]	RKSIHIGPG**S**AFY	97						
V3(15)[e]	RKSI**Y**IGPGRAFY	94						
V3(16)[e]	RKSI**G**IGPGRAFY	96						
V3(17)[e]	RKSIHIGPGR**S**FY	86						

these variants, V3(5)-V3(11), contained multiple changes from the founder amino acid sequence. For this reason a further 5 peptides, V3(13)-V3(17), were synthesised, with sequences not detected in patient 82, but which contain single changed amino acids found in the variants with multiple amino acid substitutions. All the peptides were purified by reverse phase HPLC to greater than 93% homogeneity with the exception of the V3(17) peptide (85.8%). The composition of the peptides sequences was confirmed by amino acid analysis (Cambridge Research Biochemicals). All peptides with the exception of V3(9) were soluble in PBS. Thus to avoid possible complications arising from the need to use a different solvent to dissolve this peptide it was not included in the study.

6.6 Antibody titres in patient 82 against the V3 peptides

All sixteen V3 peptides were then tested in ELISA against the seven serum samples taken from patient 82. The peptides were screened two at a time for reactivity against a series of eleven doubling dilutions (from 1:100 to 1:102,400) of the seven sera plus an HIV-1 negative control serum. The twelfth well on each of the plates was incubated with PBS alone to provide a "no antibody" control. To allow for any non-specific binding of sera to the microtitre plates, each serum dilution was incubated against wells with no peptide prebound and the optical density at 405nm subtracted from each of the appropriate test readings . A typical value was 0.166 +/- 0.129, obtained on the day that the seroconversion peptide was titrated. As a control for day-to-day variation the long branched V3 peptide (peptide 306) was screened daily against additional HIV-1 positive and negative control sera. This control allowed any increase or decrease in reactivity of the sera to be attributed to real change in their titres and discount experimental factors. In practise, over the 3 weeks taken to screen the sera, the titre against this peptide varied within a two-fold range and the maximum absorbance varied by less than 10%.

Representative data from two peptides V3(1) (RKSIHIGPGRAFYG4)8K7A and V3(6), (RK**R**IHIGPGRA**V**YG4)8K7A, with all the patient 82 sera are shown in Figure 6.5. All of the peptides with the exception of V3(4), (RKSIHIGPGRA**V**YG4)8K7A and V3(11), (RK**G**IHIGPG**S**A**V**YG4)8K7A, show reactivity with the sera similar to that seen with the V3(1) and V3(6) peptides. The reactivity of peptides V3(4) and V3(11) with patient 82 sera are shown in Figure 6.6. The titre of each of the seven sera, defined as the reciprocal of the maximum dilution giving an absorbance of 1.0 OD units at 405nm was determined by interpolation of the data from the graphs obtained for each of the peptides (Table 6.6) and is plotted in Figure 6.7A and B. Figure 6.7A shows the titres obtained for the peptides V3(1)-V3(12), corresponding to all the variants that were detected in patient 82 and Figure 6.7B shows the reactivity of the peptides V3(13)-V3(17), the sequences of which were not detected in patient 82.

All seven sera were reactive to varying degrees with the eleven peptides V3(1)-V3(12). In each case, with the exception of peptide V3(12), the initial serum sample showed low reactivity against the peptides. This observation is consistent with the existence of a limited immune response to the virus around the time of seroconversion. The reactivity of the seroconversion sample with peptide V3(12) may represent some fortuitous cross-reaction with pre-seroconversion antibodies as has been suggested by Davis *et al.* (1990) but this speculation could not be tested. Similar reactivity, again with the exception of the seroconversion sample with peptides V3(14)-V3(17) was observed for the patient 82 sera against the five peptides corresponding to the single changed amino acids in the variants with multiple substitutions (Figure 6.7B).

The data summarised in Figure 6.7A can be used to test the hypothesis that newly emerged variants represent antibody escape mutants. The hypothesis would suggest that peptides corresponding to such variant sequences should show lower reactivity with sera taken around the time that variant arose. This is clearly not so for patient 82. High titres of antibodies are present against most of the variants prior to their first detection in the circulating virus population. This effect is particularly marked for peptides V3(5) and V3(6), the sequences of which first became detectable in 1988, yet

Figure 6.5 Representative data from two of the V3 peptides which show high reactivity with patient 82 sera: peptide V3(1) (panel **A**), the seroconversion peptide (RKSIHIGPGRAFYG$_4$)$_8$K$_7$A and peptide V3(6), (panel **B**), (RK**R**IHIGPGRA**V**YG$_4$)$_8$K$_7$A. Peptides were tested in ELISA at a concentration of 100ng per well against doubling dilutions of the seven sequential sera from patient 82: 4/12/84 (■), 30/7/87 (□), 26/1/88 (●), 27/7/88 (○), 15/2/89 (▲), 18/8/89 (△), 8/2/91 (×) and an HIV-1 negative serum (▫).

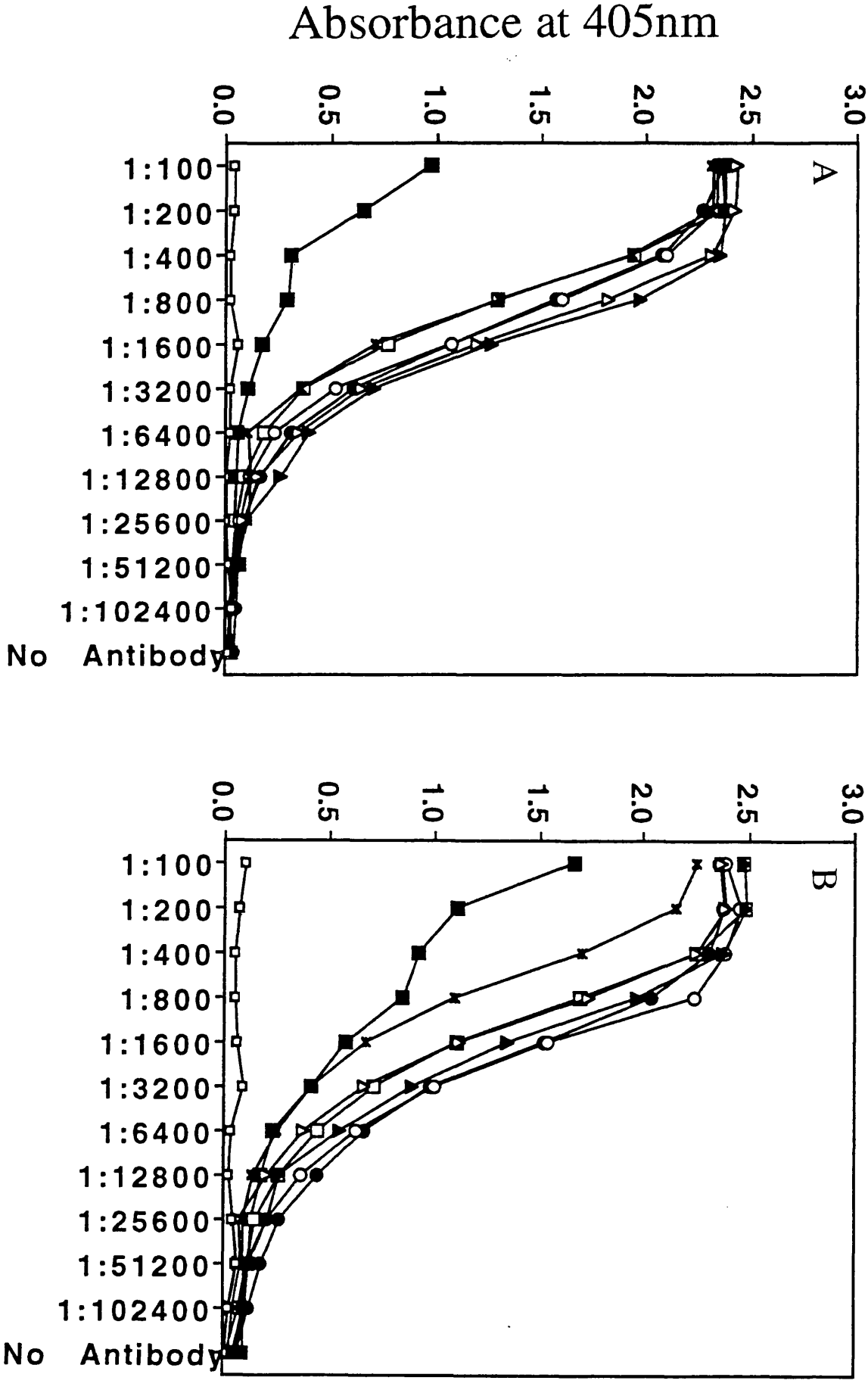

Absorbance at 405nm
0.0
0.5
1.0
1.5
2.0
2.5
3.0
1:100
1:200
1:400
1:800
1:1600
1:3200
1:6400
1:12800
1:25600
1:51200
1:102400
No Antibody
A
B
Dilution of patient 82 sera

Figure 6.6 ELISA reactivity of peptides, V3(4), (panel **A**), (RKSIHIGPGRA<u>V</u>YG4)8K7A and V3(11), (panel **B**), (RK<u>G</u>IHIGPG<u>S</u>A<u>V</u>YG4)8K7A with the seven sequential sera frompatient 82: 4/12/84 (■), 30/7/87(□), 26/1/88 (●), 27/7/88 (○), 15/2/89 (▲), 18/8/89 (△), 8/2/91 (×) and an HIV-1 negative sera (▫). Peptides were tested at a concentration of 100ng per well against doubling dilutions of human sera. Patient 82 showed low reactivity against both these peptides.

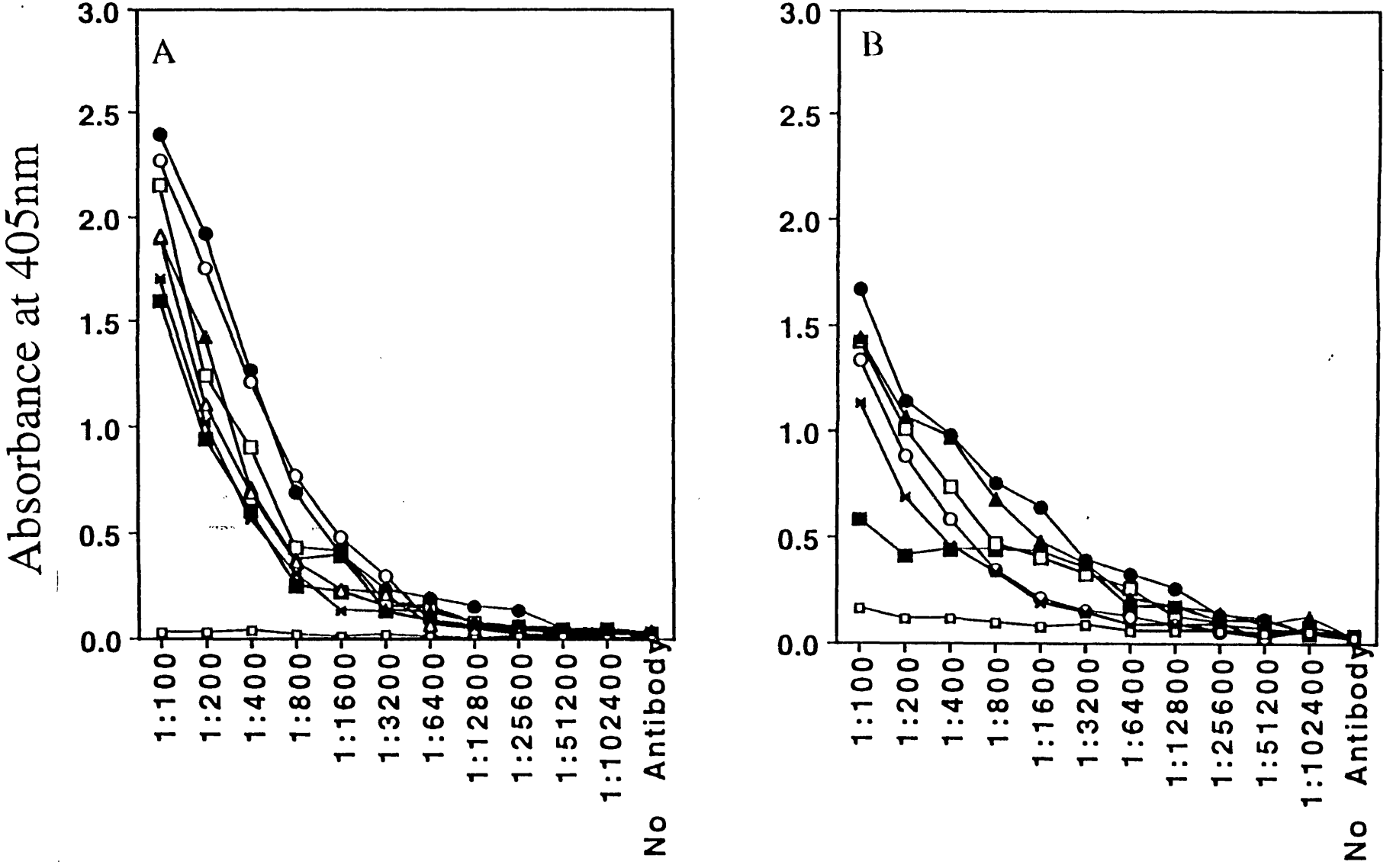

A
B
Absorbance at 405nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1:100
1:200
1:400
1:800
1:1600
1:3200
1:6400
1:12800
1:25600
1:51200
1:102400
No Antibody
Dilution of patient 82 sera

Table 6.6

Titres of sequential sera from patient 82 [a]

Peptide	Sample date						
	4/12/84	30/7/87	26/1/88	27/7/88	15/2/89	18/8/89	8/2/91
V3(1)	<100	1100	1700	1700	2100	1900	1100
V3(2)	<100	1000	1500	1250	1250	1250	380
V3(3)	540	1000	1150	1600	470	580	580
V3(4)	190	320	540	300	250	200	200
V3(5)	290	1700	2900	2900	2400	1700	1500
V3(6)	310	1900	3100	3100	2700	1900	950
V3(7)	<100	1100	1200	900	1000	900	750
V3(8)	<100	800	900	900	750	750	560
V3(10)	<100	700	1500	600	1700	1600	1600
V3(11)	<100	200	360	180	320	410	120
V3(12)	3200	2800	4200	1900	4200	3400	2800
V3(13)	2200	2300	3200	2900	2900	2800	2900
V3(14)	<100	780	1400	1400	1050	950	950
V3(15)	<100	950	2300	1600	1050	950	950
V3(16)	<100	1020	1700	900	1100	1200	950
V3(17)	200	1800	2400	1800	1800	1500	1100

a The titre is defined as the reciprocal of the maximum dilution giving an absorbance of 1.0 OD units at 405nm

high titres of antibody (1:1,700 and 1:1,900 respectively) were present against both sequences already in 1987. V3(12), the sequence of which was only ever detected in 1990, already had antibody titres of 1:3200 in 1984 and this high reactivity persisted throughout the sample period. The antibody escape hypothesis would predict that variants with this sequence would not emerge in the population even if the antibodies reactive with V3(12) are only fortuitously cross-reactive. The low reactivity observed against the V3(4) and V3(11) peptides, according to the antibody escape hypothesis, would predict that these variants should persist in the population, yet they did not. Their failure to persist could be explained if these sequences were obtained from replication defective virus, but argues strongly against the variants being antibody escape mutants.

Figure 6.7A Titres of antibodies in sequential sera from patient 82 against peptides V3 (1)-V3(12), corresponding to the V3 variants observed in replication-competent virus over a seven year period. The titres were obtained from the data shown in Figures 6.5 and 6.6 and similar graphs for the other sequences and correspond to the reciprocal of the dilution giving an absorbance at 405nm of 1.0. The titres for each of the peptides is shown in Table 6.6. Each panel displays the titres of all seven sera, A (4/12/84), B (30/7/87), C (26/1/88), D (27/7/88), E (15/2/89), F (18/8/89), G (8/2/91) against a single peptide, the peptide number V3(1)-V3(12), the sequence of that peptide and the year(s) that sequence was detected in the circulating virus population. The absorbance produced by the seroconversion serum with some of the peptides was less than 1.0 OD units and for these samples the titre is plotted as 100.

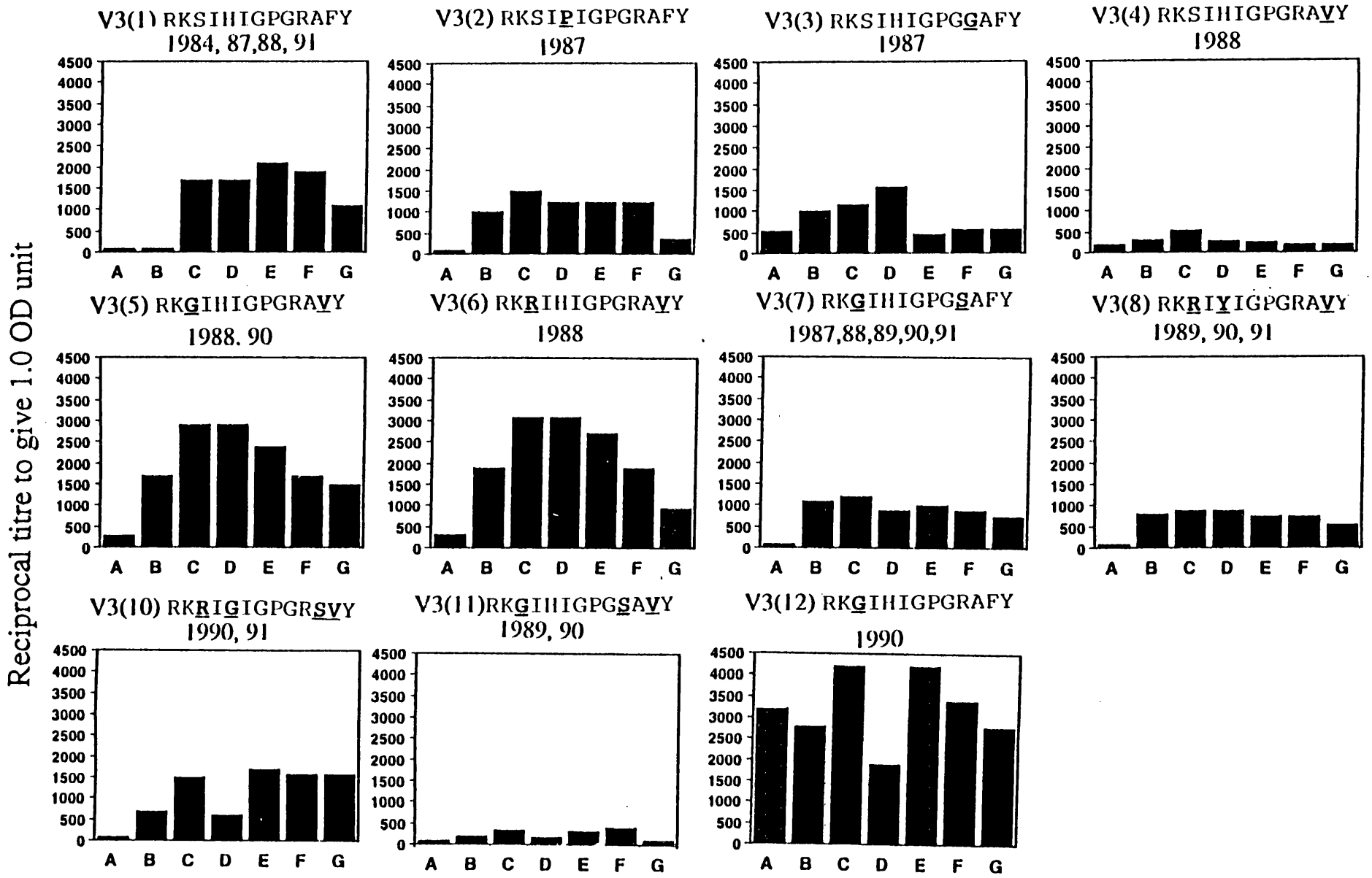
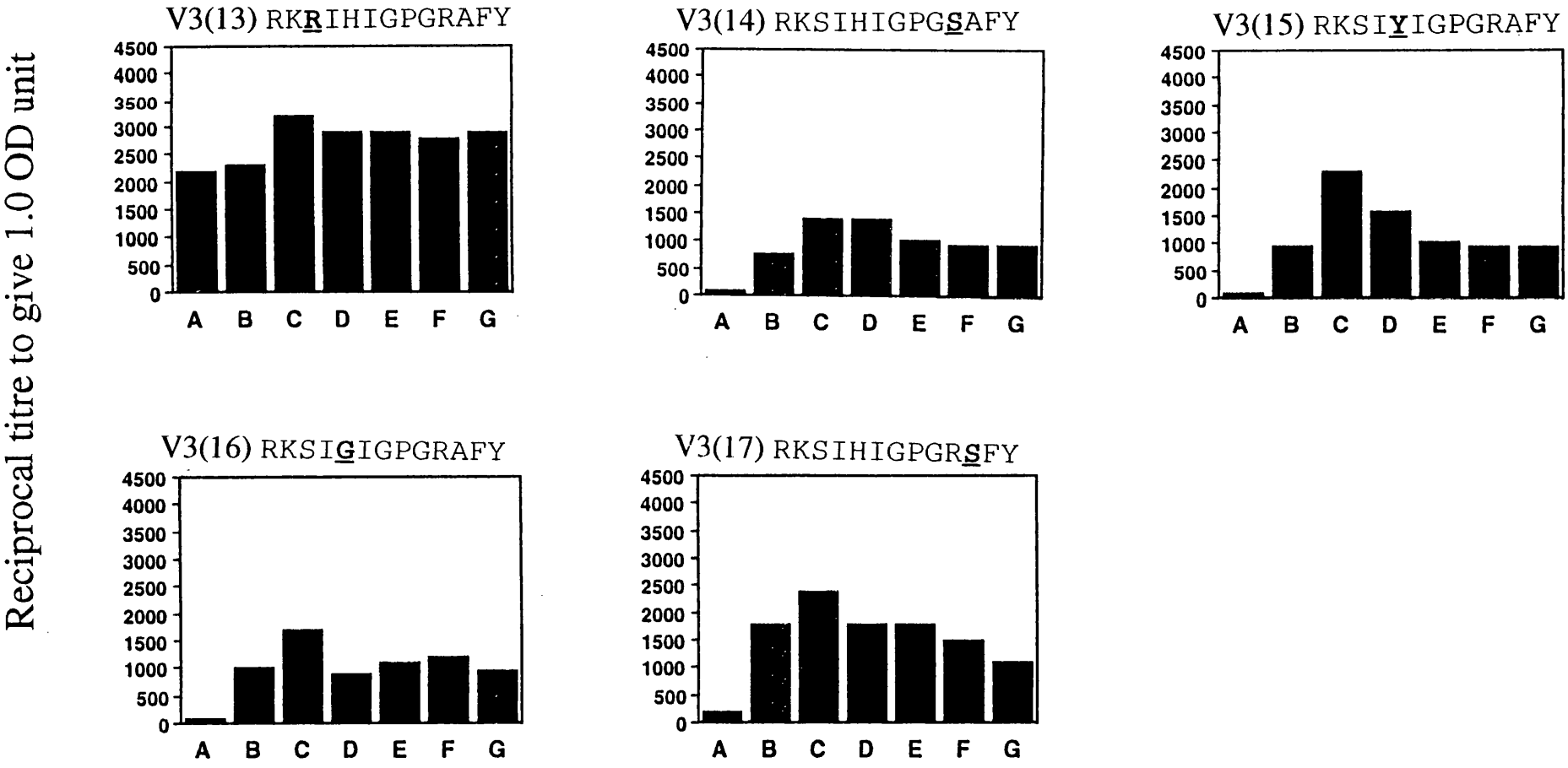

V3(1) RKSIHIGPGRAFY
1984, 87,88, 91
V3(2) RKSIPIGPGRAFY
1987
V3(3) RKSIHIGPGGAFY
1987
V3(4) RKSIHIGPGRAVY
1988
V3(5) RKGIHIGPGRAVY
1988. 90
V3(6) RKRIHIGPGRAVY
1988
V3(7) RKGIHIGPGSAFY
1987,88,89,90,91
V3(8) RKRIYIGPGRAVY
1989, 90, 91
V3(10) RKRIGIGPGRSVY
1990, 91
V3(11)RKGIHIGPGSAVY
1989, 90
V3(12) RKGIHIGPGRAFY
1990
Reciprocal titre to give 1.0 OD unit
0
500
1000
1500
2000
2500
3000
3500
4000
4500
A B C D E F G
Patient 82 sera

Figure 6.7B Titres of antibodies in sequential sera from patient 82 against peptides V3(13)-V3(17), corresponding to sequences not detected in patient 82 but contain the single changed amino acid found in the variants with mutiple amino acid substitutions. The graphs are plotted from the titres shown in Table 6.6 and correspond to the reciprocal of the dilution giving an absorbance at 405nm of 1.0. Each panel displays the titres of all seven sera, A (4/12/84), B (30/7/87), C (26/1/88), D (27/7/88), E (15/2/89), F (18/8/89), G (8/2/91) against a single peptide, the peptide number V3(13)-V3(17), the sequence of that peptide and the year(s) that sequence was detected in the circulating virus population. The absorbance produced by the seroconversion serum with some of the peptides was less than 1.0 OD units and for these samples the titre is plotted as 100.


Reciprocal titre to give 1.0 OD unit
V3(13) RKRIHIGPGRAFY
V3(14) RKSIHIGPGSAFY
V3(15) RKSIYIGPGRAFY
V3(16) RKSIGIGPGRAFY
V3(17) RKSIHIGPGRSFY
4500
4000
3500
3000
2500
2000
1500
1000
500
0
A B C D E F G
Patient 82 sera

6.7 DISCUSSION

6.7.1 Antibody responses against the V3 sequence variants detected in patient 82

The work presented in this chapter provides a comprehensive study of the antibody responses against the V3 region of HIV-1 gp120 in one patient and aimed to determine whether the sequence variants that arose in this region during the course of infection represented antibody escape mutants. The experimental protocol used to detect the V3-specific antibodies utilised an optimised form of branched peptide developed and discussed in chapter four of this thesis. Peptides corresponding to all the V3 variants that were detectable in patient 82 during the course of infection were synthesised onto a branched lysine core with a four glycine spacer preceding the reactive epitope, defined in this chapter and elsewhere (Palker *et al.*, 1988;Rusche *et al.*, 1988; Goudsmit *et al*, 1988a; Matsushita *et al.*, 1988) as lying between amino acids 304-316 of HIV-1 gp120. These peptides were then screened against doubling dilutions of sera taken from patient 82 over a seven year period. Using branched peptides in this way, V3-specific antibodies can be detected in very low concentrations of human sera. This is of great importance to any retrospective study of the antibody responses in HIV-infected individuals as early serum samples are usually in very short supply. In this study, only 34ul of each serum sample were needed to obtain all the data presented.

The hypothesis that sequence variants that arise during infection represent antibody escape mutants, would suggest that peptides corresponding to such variant sequences should show lower reactivity with sera taken around the time that variant arose. The data obtained for patient 82, summarised in Figure 6.7A and B, clearly indicates that this is not the case for this individual. High titres of antibodies were present against most of the variants that arose during infection.

An antibody escape mutant would be expected to be a variant with a novel sequence which emerges in the viral population and is then maintained in the absence of antibodies which recognise that particular sequence. The data obtained provide clear examples of variants which do not behave in this way. For example, the seroconversion sequence represented by peptide V3 (1) was found in 1987, 1988, and 1991 in spite of good titres of antibodies which recognised the sequence. Another example is the variant represented by peptide V3(7) which emerged in 1987 and persisted through to 1991 in the presence of a constant titre of antibodies which recognised the sequence.

Relevant to these findings is an earlier study by Wolfs *et al.* (1991), who obtained V3 sequence data on two patients and demonstrated that an amino acid substitution at position 308 (H to P in one patient and P to H in another) led to a changed antibody population within the individual. The data obtained in this chapter are not in conflict with this earlier study, as the experiments were not designed to examine whether different antibody populations exist within patient 82, nor whether newly emerged variants resulted in a new antibody population.

The ability of each of the peptides to compete for the V3-specific antibodies in an ELISA would determine whether the antibodies present in each serum sample were cross-reactive for the V3 peptides or were specific to individual variant sequences that arose during infection. Regardless of the outcome of such experiments, the high titres of antibodies observed in patient 82 that react with all of the variants that were detected, provide a strong argument that these sequence variants do not represent antibody escape mutants.

It would also appear that the amino acids within the V3 peptide do not always act independently in determining its reactivity with serum antibodies. This is evidenced by the low reactivity of peptides V3(4) and V3(11). For V3(4) the low reactivity is brought about by the single amino acid substitution F^{316}-V. This same substitution occurs in peptides V3(5), V3(6), V3(8), V3(10) and V3(11) and reactivity against the serum antibodies can be regained by changes elsewhere in all of them except V3(11), even though all of the changes seen in V3(11), can be found in part in those other

peptides. Moreover, the high titres observed against peptides V3(12),V3(14) and V3(16), which each contain one of the amino acid substitutions found in V3(11), indicate that no single amino acid change can account for the reduced reactivity seen against peptide V3(11).

6.7.2 Variation in the absence of humoral immune selection

The conclusion from this study, that the sequence variants that emerge in patient 82 do not represent antibody escape mutants, is perhaps surprising as most of the antibodies in HIV-1 infected sera that recognise linear epitopes are directed against the V3 region (Moore and Ho, 1993) and this region is the principal neutralising determinant of the virus (La Rosa *et al.*, 1990). For these reasons, one would expect that extensive sequence variation in a highly immunogenic region of the virus would be driven by immune selection and would subsequently have an effect on its recognition by antibodies. This however, does not appear to be the case.

Antigenic variation and the emergence of immunological escape mutants has been proposed for a number of viruses, including Visna virus and Equine infectious anaemia virus, both members of the lentivirus family (reviewed by Clements *et al*, 1988). Perhaps the best documented example of a virus demonstrating immunological escape is the influenza virus which has the ability to undergo both antigenic drift and antigenic shift resulting in the emergence of distinct viruses that are unrecognised in previously infected hosts. Sequence variation in the antigenic sites of the haemagglutinin (H) and neuraminidase (N) surface proteins can account for this antigenic diversity and has resulted in several distinct outbreaks of influenza epidemics in Britain, for example the Spanish flu epidemic in 1918 (H1N1), the Asian flu epidemic in 1957 (H2N2) and the Hong Kong epidemic in 1968 (H2N3) (reviewed by Wiley and Skehel, 1987).

Mutations leading to immunological escape do not necessarily occur as a result of immune selection in all RNA viruses. Indeed, extensive antigenic variation in the

absence of any immune selection has been reported for the Foot-and-Mouth Disease Virus (FMDV), a member of the picornaviridae family (Diez *et al.*, 1989, 1990; reviewed by Domingo *et al.*, 1993) that causes an economically important disease in cattle. In one study in which plaque purified FMDV was passaged in cell culture, viruses emerged with single amino acid substitutions in antigenically important sites of the viral proteins. These variant viruses when characterised by an *in situ* plaque immunotest were not recognised by specific monoclonal antibodies directed against regions of antigenic importance (Diez *et al.*, 1989). In addition, repeated passage of the virus resulted in an outgrowth of the viruses displaying this antigenic diversity, despite the absence of any immune selection. It has been proposed that this antigenic variation arose randomly by the quasispecies evolution of viral sequences and the variants displaying the observed antigenic variation were selected for because of properties unrelated to their antigenicity. A recent extension to this study , in which the same FMDV isolates were passaged in the presence of a limited amount of neutralising polyclonal antisera showed that substitution of an antigenically critical amino acid occurred only in the clones passaged with antibody (Borrego *et al.*, 1993). These results suggests that antigenic variation in this virus occurs in both the presence and absence of immune selection.

6.7.3 Basis for emergence of V3 variants

How do the observations described above relate to the sequence variation that arises in the V3 region of HIV-1 gp120? Apparently, the sequence changes that emerge in patient 82 are not driven by the humoral immune response and are unlikely to represent random fluctuations in the viral population. The variants may play an important role in other properties that are selectively advantageous for the virus or could represent CTL escape mutants. As discussed previously, the V3 region is involved in a number of important biological features of the virus. Sequence variation in the V3 region of this patient occurs at amino acid positions known to be important

for the ability of the virus to induce syncytia and display altered cell tropisms. As these amino acid changes have no apparent effect on the recognition of these variants by specific antibodies, it would be interesting to examine whether the substitutions have any effect on the biological properties of the virus. To test this, molecular clones of these variant V3 sequences in a common background virus would have to be made and screened for their ability to induce syncytia and to replicate in different cell types.

6.7.4 Future studies

To provide a more general basis for the conclusion that sequence variants that arise in the V3 region do not represent antibody escape mutants, it is important that this study be extended from a single individual to a number of patients.

This work does not exclude the possibility that the newly emerged variants represent mutants escaping from a subset of antibodies, such as neutralising antibodies. Indeed, mutations arising in vitro have been identified which are resistant to neutralisation by anti-V3 loop antibodies (McKeating *et al.*, 1989; Masuda *et al.*, 1990). However it is perhaps unlikely that the variants detected in patient 82 are neutralisation escape mutants, for two reasons. Firstly, of the sixteen monoclonal antibodies which have been mapped to the V3 loop, all are neutralising (Scott *et al.*, 1990; Gorny *et al.*, 1991; Tilley *et al.*, 1991; Laal and Zolla Pazner, 1993). Second antibodies from other species, directed against the V3 loop are also invariably neutralising (Rusche *et al.*, 1988; Palker *et al.*, 1988; Goudsmit *et al.*, 1988a, b; Zagury *et al.*, 1988). However, the neutralising ability of antibodies detected in patient 82 has not been directly measured. Construction of HIV-1 clones in which the variant sequences are inserted into a constant HIV-1 background are required to test this hypothesis.

The neutralising activities of serial samples with sequential virus isolates from HIV-1 infected individuals have been examined by Adrenup *et al.* (1993). In contrast to the conclusions from this study, these authors suggest a participation of the neutralising antibody response against the V3 loop in the immunoselection of escape

virus. However, in that study, viruses were isolated from the PBMCs, where proviral genome would be inaccessible to antibody selection, whereas virus sequences in the study of patient 82 were obtained from virus in the plasma. Second, the V3 sequence of only one virus isolate was obtained, which does not permit the frequency with which the sequence was present in the individual to be estimated. Third, the observed differential neutralisation by an anti-V3 monoclonal antibody of sequential isolates confirms the existence of antigenic variation but does not constitute evidence for lack of recognition of the V3 variant sequences by the patients own sera.

It is also possible that sequence variation in the V3 region is accompanied by variation in other regions of the gp120 protein and that an interaction of the V3 region with other such regions is important for escape from immune recognition. If this is the case, then the study described here, which examined only small regions of the protein will not be able to determine what effect these individual changes have on the antigenicity of the virus. One way to investigate this possibility would be to clone the entire gp120 from each individual virus isolate into a general background virus and test its ability to be neutralised by autologous sera. Although this would undoubtedly be a very difficult and laborious task, I feel that it would provide a better understanding of the mechanisms involved in viral pathogenesis. However, such considerations do not affect the finding that V3 variants are not antibody-escape mutants.

The amino acid changes observed in the V3 variants in patient 82 could also represent mutants escaping from cytotoxic T cells. Indeed, the V3 loop contains epitopes that elicit cytotoxic T cell responses, where the amino acid at residue 325 (three amino acids C-terminal of the conserved GPGR motif) can define peptide epitope specificity (Clerici *et al.*, 1991; Takahashi *et al.*, 1988, 1989, 1990). CTL escape mutants in V3 have yet to be demonstrated *in vivo.*

6.8 GENERAL DISCUSSION

The question that arises from the studies described in the previous two chapters is, what role do V3-specific antibodies play in the control of HIV-1 infection ? The answer to this question and indeed what role, if any, do anti-HIV-1 antibodies in general play in the control of HIV-1 infection, is one of increasing controversy. Some researchers believe that determining the regions of the virus that are important in eliciting a protective antibody response will be useful in developing vaccines. The findings of this thesis lead me to believe that the development of potential vaccines that involve immunisation with V3 peptides which elicit only V3-specific antibodies will be of little use in protecting against transmission of HIV-1. The study described in chapter 5 showed no correlation between the levels or affinities of V3-specific antibodies and protection of the child from vertical transmission of the virus. Additionally (chapter 6), there appears to be no effect on antibody recognition of the immunodominant V3 region of extensive sequence variation, suggesting either that the virus does not need to escape from the humoral immune response to persist in the host or that the mechanisms by which the virus escapes humoral immune recognition are much more complex than those investigated . It is however, important to note that this patient had high titres of V3-specific antibodies throughout the sampling period and although remaining asymptomatic for the clinical symptoms of AIDS, had shown a continual decline in $CD4^+$ cells to below 200 at the latter time points. Holmback *et al.* (1993) report no correlation between the stage of disease progression and the titres of V3-specific antibodies.

It has been argued that a vaccine which induces only an antibody response will not be sufficient to protect against the transmission of virus in virally infected cells that bear none of the viral antigens on their surface (reviewed by Sabin, 1992). For a vaccine to be effective against HIV infection it should probably stimulate both arms of the immune response. The cytotoxic T cell response generated against the virus has not been investigated in this thesis but will perhaps play an important role in the

development of potential vaccines against HIV-1. It has become apparent that there are some individuals, in high risk behavioural groups, that have been exposed to the virus yet appear not to be infected. Studies performed on individuals of this kind have shown that some of them (two out of five sexual partners of HIV-infected individuals and 8 out of 24 uninfected children born to HIV-infected mothers) had CTL activity in response to HIV peptides, despite having no detectable virus by PCR or antibody reactivity against HIV proteins (Levy, 1993). In other studies (reviewed by Salk *et al*, 1993), as many as 49% of a total of 97 presumed exposed individuals , compared with 2% of 163 individuals not known to be exposed to HIV, displayed CTL activity against HIV-derived peptides in the absence of detectable virus or antibodies against HIV-1 proteins. The question then arises as to whether these individuals are immune to HIV-infection or whether they were perhaps exposed to defective virus or HIV proteins and therefore, infection was not established ? What can be derived from such a study is that the antibody response and the cytotoxic T cell response appear to be induced separately.

It is known that two subtypes of $CD4^+$ T-helper cells are induced in response to antigen (reviewed by Levy, 1993; Salk *et al.*, 1993). These different cell types secrete specific cytokines which stimulate different arms of the immune response. T-helper 1-type cells (TH-1) secrete cytokines, such as interferon-gamma and interleukin-2, which stimulate the production of cytotoxic T cells. T-helper 2-type cells (TH-2) secrete cytokines such as interleukin-4 and 10, which stimulate a humoral response. These different responses are believed to be in some way competitive, in that stimulation of one type will eventually lead to a down-regulation of the other. If cytotoxic T cell activity against HIV provides a protective response against the virus then strategies aimed at increasing the levels of neutralising antibody response against HIV envelope proteins, could lead to a susceptibility to infection, rather than having any protective effect. It is interesting to note that during the asymptomatic stage of HIV-infection the levels of $CD8^+$ cells, remains high and falls off on progression to AIDS, suggesting that the CTL response may play a role in the control of HIV-infection during the asymptomatic phase. It is the opinion of some researchers that a prophylactic vaccine

designed to induce a strong CTL response will provide better protection against infection (Salk *et al.*, 1993).

It is also argued that the symptoms and features, characteristic of HIV-1 infection, are similar to those observed in autoimmune diseases and that progression to AIDS may not be caused directly by viral infection, but by the immune response against the virus destroying its own uninfected cells (Hoffman *et al.*, 1991; reviewed by Weiss, 1993). Such a process could account for the massive depletion of $CD4^+$ cells in the apparent absence of high numbers of infected cells. If this is the case then stimulating the immune response against the virus will have detrimental effects and indeed speed the progression to AIDS. Perhaps then, selective immunosupression of certain subsets of immune effector cells will slow the onset of disease. This theory though highly speculative is one to be considered when developing therapeutic vaccines against HIV.

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