

**AUTOLOGOUS AND HETEROLOGOUS
RECOGNITION OF MONOMERIC GP120
ANTIGENS DERIVED FROM HIV-1 INFECTED
PATIENTS**

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DECLARATION

I declare that this thesis and the work presented herein was done entirely by myself unless otherwise indicated. ✓

Camelia Gabriela Magureanu
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ABSTRACT

The research outlined in this thesis is mainly designed to develop a technology of producing a DNA prime-protein boost vaccine against HIV-1 infection. To reach this aim 1.7-kb fragment encoding gp120 antigen deriving from two groups of British HIV-1 infected persons (one consisting of homosexual individuals from Edinburgh, Newcastle and Belfast and the other consisting of haemophiliac patients from Edinburgh who became infected from a common batch of Factor VIII) were PCR amplified and subsequently subcloned into a cloning vector (pGEM T). A mammalian expression vector (pSRHS) was modified in order to include a polylinker to allow the transfer of 1.7-kb fragments from pGEM T to pSRHS. The recombinant clones were identified and the gp120 genes were expressed in mammalian cells (COS cells) by lipofectin protocol. The functional clones (i.e. those that contained intact open reading frames) were selected and their associated gp120 antigens were quantified by an "in house" ELISA method. Equivalent amounts of the gp120 antigens were used in an anti-gp120 ELISA to estimate the extent of recognition by the IgG antibodies from autologous and heterologous sera. The nucleic sequences of the functional clones were obtained and some properties such as their predicted NSI/SI phenotype, co-receptor usage and glycosylation sites were analysed. The phylogenetic relationship between the sequences derived from both cohorts was computed and the extent of gp120 antigen recognition by the IgG antibodies from the autologous and heterologous sera was analysed in conjunction with their degree of relatedness.

As a conclusion of this study, a high degree of cross-reactivity was noticed between the antigens and sera, the extent of the recognition of the antigens by the sera was given by the patients' immune status. No significant difference in recognition of the gp120 antigens by sera was observed. This result points towards the potential utilisation of a cocktail of such DNAs and their corresponding gp120 antigens as a DNA prime-protein boost vaccine.

ABBREVIATIONS

ELISA	Enzyme Linked Immunosorbent Assay
SI	Syncytium Inducing
NSI	Non-Syncytium Inducing
MCS	Multiple Cloning Site
TCLA	T Cell Line-Adapted Isolate
MAb	Monoclonal Antibody
IgG	Immunoglobulin Class G
EDTA	Ethylenediamine-Tetraacetic Acid
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
AMPAK	Amplification System
HRP	Horseradish Peroxidase
AP	Alkaline Phosphatase
p-NPP	para-Nitrophenyl Phosphate
OPD	ortho-Phenylene Diamine
CV	Coefficient of Variation
STDEV	Standard Deviation
MPMV	Mason-Pfizer Monkey Virus
DSS	Donor Splicing Site
SURE	Stop Undesired Recombinant Events
SV40	Simian virus 40
NADPH	Reduced Nicotinamide Adenine Dinucleotide
NAD ⁺	Oxidised Nicotinamide Adenine Dinucleotide
ARP	AIDS Reagent Programme
RRE	Rev Responsive Element
TAR	Trans-activation Response Element
CRS	cis-Acting Repressive Sequences
INS	Instability Sequences
ORF	Open Reading Frame

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1.1 The impact of HIV epidemic in the world

It is estimated that 30 million people are already infected with HIV-1 and more than 16,000 new infections occur annually. Although it was initially recognised in industrialised countries, the HIV epidemic is spreading nowadays in the developing world. More than 90% of new infections are occurring in less developed countries, with Sub Saharan Africa and South Asia experiencing the worst of the pandemic impact. The advent of highly active antiretroviral therapy (HAART) raised hopes of extending survival, but it is expensive (\$15-20,000 per person each year), may fail in the person treated and, although there is no clear evidence for it, it might lead to more virulent and pathogenic isolates. At the time being, a vaccine for HIV is desperately needed for all regions, but especially in developing countries where other interventions are limited.

1.2 Discovery of the HIV virus

In 1983, Barre-Sinoussi and his collaborators recovered, from the lymph node of a man with a polylymphadenopathy syndrome that was associated with AIDS, a virus containing reverse transcriptase. Because of many similarities this virus shared with the human T cell leukaemia viruses (HTLVs), Gallo and his co-workers (1983) suspected a member of the HTLV group. Other investigators (Montagnier *et al.*, 1984) found some distinctive characteristics of this virus and, therefore, they called it lymphadenopathy-associated virus (LAV). For instance, LAV agent was identified in haemophiliacs with AIDS in circumstances when HTLV can not be seen, as it does not exist as a free virion in blood and can not be transmitted by cell-free plasma products such as Factor VIII. Moreover, HTLV immortalises lymphocytes into continuous growth and could not be the cause for the CD4⁺ lymphocyte depletion in AIDS patients.

Subsequently, Levy *et al.*, (1984) also reported the identification from AIDS patients of a retrovirus, which was called AIDS-associated retrovirus (ARV), showing some cross-reactivity with the French LAV strain. In 1986, the International Committee on Taxonomy of viruses recognised all three viruses as members of the same group of

viruses that has been named Human Immunodeficiency Virus (HIV). Soon after HIV-1 was discovered, a distinct subtype, HIV-2, was identified in West Africa (Clavel *et al.*, 1986). These new viruses were included in the Lentivirinae subfamily of the Retroviridae family.

1.3 Genome and virion structure

HIV-1 is an enveloped, positive-strand RNA virus that is able to convert its RNA genome into a proviral DNA using a unique enzyme, reverse transcriptase (RT). The envelope is a lipid bilayer that is gained during virion budding from the cellular plasma membrane and, hence, contains, besides the viral Env glycoprotein, cellular proteins (i.e. major histocompatibility molecules, cyclophilin A, etc). The matrix is composed of p17 (myristylated protein) that encloses the capsid structure formed by p24 and contains the viral RNA and enzymes, encoded by *pol* (i.e. reverse transcriptase, integrase and protease).

The HIV-1 genome consists of a 10-kb RNA that has three coding regions common to all retroviruses: *gag*, *pol* and *env*. These regions encode for the capsid proteins (Gag), the viral enzymes necessary for replication (Pol) and the external glycoproteins (Env). Other regions encode other proteins that regulate directly viral gene expression (Tat, Rev), are part of the viral particle (Vif, Vpr) or interact with the cellular machinery to promote virus propagation (Vpu, Nef). Due to the presence of these regions, HIV was included in *the complex retrovirus subgroup*, together with the human T-cell leukaemia virus (Cullen *et al.*, 1991). HIV-1 RNA has two long terminal repeats (LTR), one at each end, that present a promoter at the 5'-end and a polyadenylation site at the 3'-end, and expresses one primary transcript. Many proteins are produced from this transcript by different mechanisms: generation and proteolytic processing of precursor proteins, ribosomal frameshifting or suppression of translational termination, alternative splicing of the primary transcript and bicistronic mRNA producing two proteins.

1.3.1 Elements involved in regulation of viral transcription

1.3.1.1 Promoters of transcription

There are several cis-acting regulatory sequences in the LTR, which regulate viral RNA transcription. The TATA box and three SP1 binding-sites are two important cis-acting regulatory elements in the U3 region of the HIV-1 LTR that control basal HIV-1 transcription.

TATA box, located between -24 and -27 relative to the start of transcription, facilitates the binding of the TATA-binding protein to the transcription (TFIID) complex during transcript initiation (Maldonado *et al.*, 1990, Peterson *et al.*, 1990).

SP-1-binding sites, consist of three regions located between -46 and -78 designated Site I, Site II and Site III that influence the basal HIV-1 transcription mechanism (Harrich *et al.*, 1989). All three sites involved in SP-1- binding function in concert. Site I alone does not support transcription of viral replication and is dispensable in the presence of Sites II and III (Parrott *et al.*, 1991, Ross *et al.*, 1991).

Leader-binding protein 1 (LBP-1) and upstream-binding protein (UBP-1) are also involved in basal HIV-1 transcription (Garcia *et al.*, 1987, Wu *et al.*, 1988, Harrich *et al.*, 1989). LBP-1 targets the transcription initiation site (-16 to +27) and also a binding site around TATA region (-38 to -16), which interferes with the interaction between TATA box and TFIID, inhibiting the transcription *in vitro* (Boris Lawrie *et al.*, 1992, Ross *et al.*, 1991). TATA box, SP1-binding sites and the regions involved in binding LBP-1 and UBP-1 form the basal promoter of transcription.

1.3.1.2 Enhancer and activation of transcription

The enhancer region is composed of two 10-base pair elements, located between -105 to -96 and -91 to -82, which are presented in tandem and are binding sites for NF- κ B (nuclear factor κ B) (Ross *et al.*, 1991). NF- κ B is a heterodimer of a 50-kd nucleic acid binding protein and a 65-kd transactivation protein. Its synthesis is strongly induced during T-lymphocyte activation (Bachelier *et al.*, 1991). As an inactive form, the NF- κ B heterodimer is complexed to an inhibitor protein (I κ B) that

blocks the nuclear localisation signal and DNA-binding capacity of NF- κ B (Baeuerle *et al.*, 1988). Phosphorylation of I κ B may result in the dissociation of the NF- κ B-I κ B complex (Shirakawa *et al.*, 1989), unbound NF- κ B being able to bind, after its translocation to the nucleus, to the NF- κ B enhancer element and thus activates HIV transcription.

1.3.1.3 Tat transactivation

A major control element of HIV transcription is TAR (trans-acting response element).

Trans-acting response element (TAR), located between +1 to +80, is a target of tat protein (transactivation of transcription). Tat is a 14-kd molecule that is translated from two adjacent coding exons in a multispliced mRNA. The function of the region encoded by the first exon (a 72 amino acids fragment), consists of transactivation and nuclear localisation. The function of the region encoded by the second exon is not yet defined. TAR RNA region has a predictive stem-loop secondary structure that is present at the 5'-terminus of all viral mRNAs. This structure contains a three-nucleotide bulge region in the stem that binds Tat protein *in vitro* and is necessary for transactivation *in vitro* (Gatignol *et al.*, 1989, 1991, Han *et al.*, 1992). In the absence of Tat protein, HIV transcription is non-processive. Tat functions by recruitment of specific cellular protein kinase complex called TAK (Tat-associated kinase). TAK complex comprises cyclin-dependent kinase 9 (Cdk9) and cyclin T (Cy T) and binds TAR RNA region via interactions between cyclin T and Tat. Binding of the TAK complex and Tat to the Tar region leads to the hyperphosphorylation of the CTD (C-terminal domain) of RNA polymerase II. As a consequence, the transcriptional elongation of the nascent viral RNA is upregulated and the virus production is maximised (Emerman *et al.*, 1998).

1.3.1.4 Posttranscriptional control of HIV RNA expression

HIV-1 proteins are produced by either monocistronic (e.g. gag-pol, tat) or bicistronic (e.g. rev/nef, vpu/env) mRNAs. Thus, single unspliced mRNAs produce Gag-Pol precursor by ribosomal frameshifting, whereas Env is produced at the second open

reading frame (ORF) by bicistronic vpu/env mRNAs (Schwartz *et al.*, 1990a). The HIV-1 primary transcript is alternatively spliced to produce more than 30 different species of viral proteins (Schwartz *et al.*, 1992a), all from a single promoter and sharing a common 5' end.

The cytoplasmic expression of intron-containing, unspliced and single-spliced viral mRNAs is regulated by Rev protein binding to a Rev responsive element (RRE). The RRE RNA is a 234-nucleotide region located in the *env* that for which a stable stem-loop secondary structure is predicted (Heaphy *et al.*, 1991). This region is contained in all viral mRNAs that encode the unspliced Gag-Pol and single spliced Vif, Vpr and Vpu-Env but not in multispliced mRNAs transcripts that encode Tat, Rev and Nef (Pomerantz *et al.*, 1992, Malim *et al.*, 1991a,b).

HIV-1 RNA contains, besides TAR and RRE, negatively acting elements that inhibit HIV expression (i.e. INS or CRS) by interfering with the stability, transport and translatability of the viral mRNAs. In addition to sites important for expression, the HIV mRNA also contains other sites necessary for other steps in the viral cycle: the genomic RNA dimerisation site, encapsidation site (ie. psi site) and priming sites for reverse transcriptase (primer binding site (PBS) for minus-strand priming and polypurine tract (PPT) for plus-strand priming).

1.3.2 Structural proteins

1.3.2.1 Gag proteins

Splicing of the primary transcript produces two gag containing precursor proteins: a gag precursor Pr55^{gag} and a Gag-Pol fusion protein (Pr 160^{gag-pol}).

The gag precursor Pr55^{gag} forms the core viral particle that interacts with other viral and cellular components (RNA, Pol and Env proteins, plasma membrane) and facilitates the incorporation of the viral proteins into viral particles during budding process. The Gag-Pol fusion protein (Pr 160^{gag-pol}) is produced by ribosome frameshifting to the first open reading frame (ORF). About 20 Pr55^{gag} molecules are produced for each Pr160^{gag-pol}.

During viral assembly the protease is activated and cleaves the Gag precursor into four proteins (matrix p17, capsid p24, nucleocapsid, p7 and p6) and two small peptides (p1 and p2).

The region between positions 178 and 300 in the capsid domain of the Gag precursor has been shown to bind cyclophilin A (Luban *et al.*, 1993). Mutation of the cyclophilin A-binding domain results in non-infectious viral particles (Franke *et al.*, 1994, Thali *et al.*, 1994). Cyclosporin and its analogues compete with the binding of cyclophilin A to Gag and inhibit HIV-1 replication.

1.3.2.2 Env proteins

The viral envelope contains, as spikes of trimers of two proteins, a surface external gp120 glycoprotein and a transmembrane gp41 glycoprotein.

Gp120 glycoprotein

Early studies have shown that gp120 has a complex three-dimensional structure that is held together by disulphide bonds whose number is conserved between divergent isolates (Myers *et al.*, 1991). Studies conducted by Leonard *et al.*, 1990 revealed the presence of five variable regions, designated V1 to V5, which are interspersed among five conserved regions. The V1/V2 domain has a double-loop disulphide-structure, whereas V3 and V4 regions exist as single loop structures that are formed by a single disulphide bond at the base of each. These regions were predicted by computer-assisted modelling to be exposed at the surface of the molecule and, hence, potentially antigenic (Modrow *et al.*, 1987). Also predicted to be exposed were some residues from conserved regions (ie. C2, C3 and C4) that are involved in binding to the CD4 molecule (Olshevsky *et al.*, 1990). Subsequently, studies employing immunochemical techniques have shown that the variable regions of the envelope are accessible to monoclonal antibodies and therefore exposed on the protein surface, whereas the conserved regions of gp120 are inaccessible to anti-gp120 monoclonal antibodies on the native protein (Moore *et al.*, 1993a, b, c, 1994a, b, c). In this respect it has been found that C1 and C5 conserved regions are exposed on the monomeric form of gp120 but inaccessible on the oligomeric form (Moore *et al.*, 1994b).

The structure of the gp120 core derived from HXBc2 strain of HIV-1 was ascertained by X-ray crystallography, when the core was complexed with two-domains: CD4 and neutralising antibody (Kwong *et al.*, 1998). The core contained deletions of the V1, V2 and V3 variable loops and the N- and C- termini, in comparison with the full-length gp120. Three domains were identified in this core structure: an inner, an outer and a bridging sheet. The inner domain is seen as a region that interacts with the gp41 glycoprotein. The outer domain that is variable and heavily glycosylated is exposed on the assembled envelope glycoprotein trimer (Wyatt *et al.*, 1998). Arbitrarily, a proximal and a distal side were delimited on the core structure. The “proximal” side of the gp120 core that includes the N- and C-termini is believed to reside near the viral membrane. The “distal” side is believed to face the target cell membrane after CD4 binding occurs. The density of glycosylation on the gp120 surface is seen to face outwards on the trimeric envelope glycoprotein spike. Three faces have been described on the oligomeric spikes: a neutralising face, a non-neutralising face and a silent face. The neutralising face includes the V2 and V3 loops and is adjacent to the surface. The non-neutralising face is poorly accessible on the assembled envelope glycoprotein trimer and therefore elicits only non-neutralising antibodies. The silent face corresponds to the highly glycosylated outer domain surface (Wyatt *et al.*, 1998).

Finally, residues located within V3 molecule are important for binding of gp120-CD4 complexes to the chemokine receptors (Rizzuto *et al.*, 1998).

Gp41 glycoprotein

Gp41 glycoprotein has three domains: a hydrophobic external domain at the N-terminal part of the molecule, a transmembrane domain and a cytoplasmic domain.

The external domain of gp41 interacts with gp120 through non-covalent bonds (Ivey-Hoyle *et al.*, 1991). It also contains a fusion domain that mediates the fusion of the viral envelope with the plasma membrane of the host cell (Freed *et al.*, 1990).

The cytoplasm domain of gp41 interacts with the matrix protein (MA) p17^{gag}. Deletions in this domain block incorporation of Env into virions (Freed *et al.*, 1996).

The core structure of gp41 glycoprotein has been recently elucidated (Chan *et al.*, 1998a,b). The external domain (also called ectodomain) of the molecule has a N-part that contains a hydrophobic glycine-rich “fusion” peptide, which has a role in the

membrane fusion, and also two other domains with a 4,3 hydrophobic (heptad) repeat motif, which is characteristic for a coiled-coil structure. These hydrophobic domains are designated N51 and C41 because they resulted from the N and C-terminal of the ectodomain by a limited proteolysis and consisted of a stretch of 51 and 41 amino acids, respectively (Lu *et al.*, 1997). By employing biophysical methods, Chan *et al.* (1997, 1998a) have shown that three of each N51 and C41 peptides associate to form a thermostable helical trimeric complex of N51/C41 heterotrimers, in which the N51 peptides from the inner helices form hydrophobic grooves in which fit three residues (Trp-628, Trp-631 and Ile-635) from the C41 peptides. Thus, the C41 peptides wrap obliquely the N51-peptide helices bundle, in an antiparallel manner (Lu *et al.*, 1997, Chan *et al.*, 1997).

The identification of the core structure of gp41 has brought light on the fusion mechanism. The interaction between gp120 and cellular receptors is followed by conformational changes in the envelope complex that lead to a pre-hairpin intermediate, in which the fusion domain of each N peptide region of the trimeric coiled-coil is inserted into the target membrane. The C41 and N51 peptide domains are not still associated, probably because there are still involved in binding to gp120 or to other regions of gp41. This pre-hairpin structure is long-lived and evolves into a fusion-active hairpin structure, when the C41 and N51 peptide regions associate and adopt a helical hexameric bundle structure. This event triggers the cellular and viral membranes in apposition followed by the membrane fusion, probably via fusion pores made by gp41 trimer aggregates (Chan *et al.*, 1998b).

The N-terminal region is the most highly conserved domain of Env and synthetic peptides that mimik the C41 region of gp41 protein, such as C34 and T20/DP179 peptides, have been used as effective inhibitors of HIV infection and syncytia formation. These C-peptides can associate with N-peptide domains of the pre-hairpin structure and inhibit this intermediate (Eckert *et al.*, 1999) in a dominant-negative manner. Thus, in a phase I clinical trial, Kilby *et al.* (1998) have shown that T20 has an antiviral effect when used as a therapeutic intervention in infected individuals.

Peptides such as T649 that do not target the pocket-binding residues can select for escape mutants, whereas those that act on the groove-binding residues are elusive to the emergence of the escape variants due to the fact that mRNA encoding those residues are part of the RRE and, hence, this region is highly conserved among different HIV-1 isolates. Also peptides containing D-amino acids such as IQN 17 have been identified as ligands for the hydrophobic pocket of gp41 and are able to inhibit gp41-mediated cell-cell fusion and HIV-1 infection (Eckert *et al.*, 1999). These peptides are insensitive to proteolytic degradation, mimic N51 peptide domain of gp41 and are fused to soluble trimeric-coiled coil to impair their aggregation.

1.3.3 Proteins with enzymatic activity

1.3.3.1 Protease

This protein contains a characteristic active centre (ie. Asp-Thr-Gly) that is active when the enzyme is in dimeric form, each subunit being of approximately 10kb (Debouck *et al.*, 1987, Copeland *et al.*, 1988, Kohl *et al.*, 1988). The enzyme becomes active only after Gag and Gag-Pol multimerisation occurs. Intracellular activation of HIV-1 protease, due to over expression of the HIV-1 gag-pol polyprotein inhibits the assembly and budding of virus-like particles (Karacostas *et al.*, 1993). Inhibitors of HIV protease such as saquinavir, indinavir, ritonavir, nelfinavir and amprenavir are now in widespread use as components of HAART.

1.3.3.2 Reverse transcriptase

This protein is a heterodimer of p66 and p51 subunits that is produced by proteolytic cleavage of Pr 160^{gag-pol} by the viral protease (Di Marzo Veronese *et al.*, 1986). Firstly, the p66 subunit is produced from the Gag-Pol precursor and dimerised. It is subsequently cleaved at the C-terminal part of one p66 subunit to produce a p66-p51 heterodimer. The protein has three enzymatic activities: RNA-dependent DNA polymerase, DNA-dependent DNA polymerase and ribonuclease H (RNase H). The N-terminal portion of p66 contains the polymerase activity, whereas the RNase H activity lies within the C-terminal portion of p66. The heterodimer is characterised by asymmetry (Kohlstaedt *et al.*, 1992, Jacobo-Molina *et al.*, 1993). Thus, p66 has a

large cleft that resembles a right hand with its subdomains designated fingers, palm and thumb. Reverse transcriptase does not have a 3' exonuclease proof reading activity and hence it has an error rate of one in 10^4 nucleotides. This means that each genomic RNA molecule of 10^4 nucleotides contains on average one misincorporation per each replication cycle.

Inhibitors of RT can be classified in nucleoside and non-nucleoside analogues (Debyser *et al.*, 1992a) The nucleoside analogues used are AZT (zidovudine), ddI (didanosine), ddC (zalcitabine), d4T (stavudine) and 3TC (lamivudine) (Debyser *et al.*, 1992a, Richman *et al.*, 1992, Kellam *et al.*, 1994). These analogues act after phosphorylation to nucleotide triphosphates to inhibit elongation and the active site of RT. Non-nucleotide analogues such as nevirapine, delaviridine and efaviranez inhibit directly reverse transcription activity (Debyser *et al.*, 1992b, De Clercq *et al.*, 1994). Non-nucleoside inhibitors target a region in RT-ase located between amino acid residues 181 to 188. Single point mutations at Tyr 181, 190 and 103 to Cys lead to drug-resistant escape mutants *in vitro* and *in vivo* (Balzarini *et al.*, 1993, 1994).

1.3.3.3 Integrase

This enzyme is a 31-kd protein that is produced after processing of Pr160^{gag-pol} from the C-terminal part of Pol. Integrase cleaves the ends of the linear viral RNA and also the cellular DNA randomly and covalently links the viral DNA to the host chromosomal DNA (Vink *et al.*, 1993a, Engelman *et al.*, 1993, Drelich *et al.*, 1993). It contains: an N-terminal zinc finger, a centrally located region responsible for catalytic activity and oligomerisation and a DNA-binding domain involved in the 3'-DNA processing activity and integration (Engelman *et al.*, 1993, Vink *et al.*, 1993b).

1.3.4 Regulatory proteins

HIV-1 has two regulatory proteins: Tat and Rev.

1.3.4.1 Tat protein

Tat is a 16-kd protein encoded from two separate exons of multiply spliced mRNAs (Dayton *et al.*, 1986, Fisher *et al.*, 1986a, Sodroski *et al.*, 1985). Another 14-kd form of Tat may be produced by single spliced mRNAs. Tat is located in the nucleus and acts as a transcriptional activator factor of HIV expression (Peterlin *et al.*, 1986,

Hauber *et al.*, 1989, Ruben *et al.*, 1989) through its binding to TAR. Tat contains distinct functional domains: an activation domain and an arginine-rich basic domain that are required for activation of transcription, specific binding to TAR RNA and nuclear localisation (Derse *et al.*, 1991). The activation domain lies in a region between amino acids 40 through 48 and contains a motif (RKGLGI) that is conserved between HIV-1, HIV-2 and SIV. The arginine-rich basic domain contains a RKKRRQRRR motif within the amino acids 49 through 72 that is responsible for RNA binding and for nuclear localisation of the protein, together with amino acid residues flanking the basic region (Churcher *et al.*, 1993, Luo *et al.*, 1993). Initiation of transcription is given by the interaction between the HIV-1 LTR and RNA polymerase II holoenzyme complex (RNAP II) via a bulged stem-loop structure called TAR. In the absence of Tat, transcription elongation is inefficient due to the hypophosphorylation of the C terminal domain (CTD) of RNAP II. Cdk9-CycT complex binds the activation domain of Tat via CycT, increasing the affinity of the interaction between Tat and TAR. After Tat binding to TAR, Cdk9 hyperphosphorylates the CTD of RNAP II, stimulating the transcription elongation of the nascent viral RNA (Emerman *et al.*, 1998).

1.3.4.2 Rev protein

Rev is a small, positively charged 18-kd protein that shuttles between the nucleus and the cytoplasm (Kalland *et al.*, 1994a,b, Meyer *et al.*, 1994, Richard *et al.*, 1994). Rev binds to RRE (Daly *et al.*, 1989, Holland *et al.*, 1990, 1992, Cook *et al.*, 1991) and stabilises the unspliced and partially spliced HIV mRNAs that are subsequently transported and translated into the viral structural proteins (Malim *et al.*, 1989, Felber *et al.*, 1989, Emerman *et al.*, 1989). In the absence of Rev, mRNAs are multiple spliced and the production of the structural proteins is very low. Rev antagonises with inhibitory/instability regions (ie. INS or CRS) that prevent RNA expression (Hadzopoulou-Cladaras *et al.*, 1989, Rosen *et al.*, 1988, Schwartz *et al.*, 1992b, c). Rev is located primarily in the nucleus (Cullen *et al.*, 1988, Cochrane *et al.*, 1989). Several functional domains have been identified in Rev by mutagenesis: an NH₂-terminal domain that mediates RRE-binding, Rev-Rev multimerisation as well as

nuclear localisation, a COOH-terminal leucine-rich domain that contains a nuclear export signal and an effector domain. Thus:

1. A region located between amino acid 35 through 50 (RQARRNRRRRWRERQR), rich in arginine residues, has been shown to be involved in nuclear-nucleolar localisation and Rev-Rev multimerisation (Zapp *et al.*, 1991, Olsen *et al.*, 1990, Malim *et al.*, 1991b).
2. The region between amino acids 34 through 50 binds specifically to RRE (Kjems *et al.*, 1992, 1991).
3. A region between amino acids 75 through 84 represents the effector domain. Four leucine residues (Leu78, Leu81, Leu83 and Leu75) and glutamine (Glu79) have been identified as being important for Rev function (Malim *et al.*, 1991b).

The effector domain is the signal for nuclear export after association with cellular factors. Rev multimerisation is essential and takes place even in the absence of RRE (Nalin *et al.*, 1990). Furthermore, formation of the RNA-binding site may depend on Rev multimerisation (Olsen *et al.*, 1990).

The nuclear export signal of Rev interacts co-operatively and in a leptomycin B sensitive reaction with exportin 1 (XPO)(a member of the importin β /kariopherin- β superfamily of shuttling nuclear transport receptors) and the Ran guanosine triphosphatase (Ran-GTPase). The Ran GDP-Ran GTP gradient that exists between the cytoplasm and nucleus triggers the RRE-Rev-XPO-Ran-GTP complex for nuclear export. In the cytoplasm, Ran GTPase-activating protein (RanGAP) stimulates GTP hydrolysis and triggers the dissociation of XPO, Ran-GDP from Rev-RRE-viral RNA. Subsequently, the viral RNA is released and is either translated or packaged into virions, whereas Rev shuttles back to the nucleus (Emerman *et al.*, 1998).

1.3.5 Accessory proteins

The four HIV-1 “accessory proteins” (ie. vif, vpr, vpu and nef) were so described because they were dispensable for virus replication *in vitro*.

1.3.5.1 Vif protein

This is a 23-kd protein that influences the infectivity but not the production of virus particles (Kan *et al.*, 1986, Lee *et al.*, 1986, Rabson *et al.*, 1985). It is a cytoplasmic protein that exists in both a soluble cytosolic form and a membrane-associated form (Michaels *et al.*, 1993, Goncalves *et al.*, 1994). Vif ensures a proper assembly of the virion and also an efficient Env-mediated infection of target cells. Thus, it has been shown that vif⁻ virus is approximately 25 to 100 times less infectious than wild-type virus produced in CEMx174 cells or PBMCs, respectively (Blanc *et al.*, 1993, Courcoul *et al.*, 1995).

The region of the C terminus of Vif is required for the stable association of Vif with membranes. Mutations of Cys114 and Cys133 confer a Vif⁻ infection phenotype (Ma *et al.*, 1994). The region between 103 to 115 and 142 to 150 is important for Vif function, although its function is not completely defined.

1.3.5.2 Vpr protein

This is a 14-kd protein that interacts with the p6^{gag} part of the Pr 55^{gag} precursor (Cohen *et al.*, 1990, Paxton *et al.*, 1993). Vpr is detected early after infection, being produced by multiply spliced (Rev-independent) and by singly spliced (Rev-dependent) mRNAs (Neumann *et al.*, 1994). The protein is involved in the nuclear import of the preintegration complex, in particularly non-dividing cells, cell growth arrest, transactivation of cellular genes and induction of cellular differentiation. The region between 17 and 34 on Vpr contains an amphipathic α -helical domain that is critical for the stability of Vpr and its efficient incorporation into the virion. HIV-1 is able to integrate its genome in non-dividing cells by transporting the preintegration complex into the nucleus via Vpr, integrase and p17^{MA} (Heinzinger *et al.*, 1994). Vpr can directly interact with nucleoporins, thus explaining the nuclear localisation of vpr during virion packaging via p6 protein, which is located at the C-terminus of the P55^{gag} precursor (Vodicka *et al.*, 1998, Paxton *et al.*, 1993). Vpr also binds Importin α (Imp α), increasing the affinity of the Imp α to the NLS present on HIV-1 PICs and, hence, enhancing PIC import to the nucleus (Popov *et al.*, 1998). Vpr alone arrests the cells at G2 in the cell cycle (Rogel *et al.*, 1995). The removal of phosphate residues on the p34^{cdc2}-cyclin B kinase by phosphatase cdc25C inhibits kinase

function that is critical for the transition from G2 to mitosis. In vpr-expressing cells, cdc25C is in an inactive form, which triggers the p34^{cdc2}–cyclin B kinase in an active form, event that leads to G2 arrest (Re *et al.*, 1995).

The Vpr homologue in HIV-1 and SIV is named Vpx and is important also in virus infectivity (Park *et al.*, 1995a, b).

Vpr is important for replication *in vivo*, as a Vpr⁻ molecular clone reverted to wild type in infected macaques monkeys (Lang *et al.*, 1993). This phenomenon explains the results obtained by Gibbs *et al.* (1995) who reported that animals infected with SIV isolates with deletion in Vpr or Vpx progressed to AIDS.

1.3.5.3 Vpu protein

This is a 16-kd protein that is produced by a bicistronic vpu/env mRNAs (Schwartz *et al.*, 1990). It is a membrane protein that has at least two functions: degradation of CD4 in the endoplasmic reticulum and enhancement of virion release from the plasmalema. Several regions on the cytoplasmic domain of CD4 molecules located between amino acid 402 and 420 and 418 to 425 are responsible for the susceptibility to Vpu-induced degradation (Willey *et al.*, 1994). The transmembrane domain of CD4 also provides sequences through which the Vpu protein could access CD4 for degradation in the endoplasmic reticulum (Buonocore *et al.*, 1994, Raja *et al.*, 1994). The C-terminal domain on Vpu is involved in interaction with CD4 molecule (Chen *et al.*, 1993). During viral infection, Env binds to CD4 in the ER and retards its transit to the plasma membrane. Vpu binds the CD4-Env complex in the ER and targets it for proteolysis by recruitment into the ubiquitin-proteasome mediated pathway (Schubert *et al.*, 1998) via interactions that are established between β -TrCP and Skp1p proteins (Margottin *et al.*, 1998), while vpu is apparently recycled. This leads to the release of Env from the ER and its incorporation into progeny virions.

1.3.5.4 Nef protein

This is a 27-kd myristylated protein that is found in the cytoplasm and is associated with the plasma membrane through the myristyl residue linked to the conserved

second amino acid (Gly). Nef seems to be important for viral replication *in vivo*. Its primary function consists of the CD4 down regulation and degradation in lysosomes (Garcia *et al.*, 1991, Aiken *et al.*, 1994). The dileucine motif on the cytoplasmic tail of CD4 initiates the interaction of Nef with CD4 and the μ subunit of the AP-2 adapter complex at the plasma membrane. The interaction between Nef and AP-2 increases the association of CD4 with clathrin-coated pits and promotes the formation of the pits themselves (Emerman *et al.*, 1998).

In addition to CD4, Nef induces downregulation of cell-surface MHC class I molecules (Le Gall *et al.*, 1998) and, thus, inhibits CTL-mediated lysis of HIV-1-infected cells (Collins *et al.*, 1998).

Nef also increases the infectivity of produced virions (Miller *et al.*, 1995, Chower *et al.*, 1994) through interactions between its proline-rich domain and cellular kinases. Thus, the PxxP motif in HIV-1 Nef binds to the SH3 domains of a subset of Src kinases and enhances the replication of Nef⁺ viruses but does not influence the CD4 downregulation. Nef PxxP motif binds specifically the biotinylated SH3 domains of Hck and Lyn proteins (Saksela *et al.*, 1995, Sawai *et al.*, 1994).

Other functions of Nef have been described such as: abnormal hematopoiesis through inhibition of bone marrow progenitor cells (Calenda *et al.*, 1994), B-cell activation and induction of IL-6 (Chirmule *et al.*, 1994), inhibition of the protein synthesis (Poulin *et al.*, 1994).

1.4 Viral replication cycle

HIV-1 virus makes primary contact to CD4 through its gp120 envelope glycoprotein spikes. This binding is followed by the interaction between V3 region of gp120 molecules with one of the chemokine receptors (Section 1.6.4). Conformational changes expose the N-terminus of gp41 (a stretch of hydrophobic amino acids) allowing the fusion of the cellular and viral membranes (Maddon *et al.*, 1988, Hunter *et al.*, 1990, Chan *et al.*, 1998) (Section 1.3.2.2). Afterwards, the viral core is uncoated and the HIV-1 nucleoprotein complex consisting of Gag and Pol proteins

and genomic RNA is released into the cytoplasm. The Pol protein comprises RT/RNase H enzyme, a 66- and 51-kd heterodimer that uses a single-stranded genomic viral RNA to synthesise a double-stranded linear proviral DNA. The reverse transcription process generates the LTR on the 5' and 3' ends of the proviral DNA, a requirement that is characteristic for integration of the proviral DNA into the cellular chromosomal DNA. The viral DNA integration into the chromosomal DNA requires a 32-kd integrase enzyme that is contained in the preintegration complex. The integrase removes two nucleotides at the 3'-end of both DNA strands and cuts the chromosomal DNA in such a way that produces a 5'-five nucleotide overhang on each end. Thus, the 3'-ends of the viral DNA are ligated with the 5'-ends of genomic DNA followed by the filling up the gaps in the genomic DNA. The resulting integrated form consists of a provirus minus two base pairs at the ends of both LTRs and an identical five-base pair sequence immediately upstream and downstream of the provirus. The integration of HIV-1 was until recently believed to occur randomly. However, in four persons infected with HIV-1 with non-B-cell lymphomas it was found that HIV-1 genome was integrated in *fur*, located upstream from the *fos-fps* protooncogene (Shiramizu *et al.*, 1994). Linear and circular double stranded DNA may be found unintegrated into the nucleus of a recently infected cell. When the infected cells are activated, the transcription of the proviral DNA occurs leading to the synthesis of the virus proteins that are included together with two copies of the RNA in the new virions during the virion assembling and budding.

1.5 HIV-1 subtypes

HIV-1 isolates that have significant public health importance were classified into several subtypes or clades designated A through K. Envelope sequences between subtype varied by more than 20%. Most of the HIV-1 subtypes have been found in sub-Saharan Africa, with subtypes A, C and D being more prevalent than the others. Subtype B showed distribution preponderance in the United States and Western Europe. Less heterogeneity was shown by the strains isolated in Asia. In Thailand, HIV-1 subtypes B and E spread into the population through different routes: subtype B was detected mainly among intravenous drug users, whereas subtype E was spread rapidly among heterosexuals. Similarly, in India subtype B spread among

intravenous drug users whereas subtype C was mainly found in heterosexuals. In general the more rapidly a strain spreads within a new population, the less viral diversity in that population was found (Weniger *et al.*, 1994).

A new strain designated HIV-0 was detected in Cameroon and Gabon (Peeters *et al.*, 1994, Nkengasong *et al.*, 1994). Analysis of its genome in comparison with those of HIV-1, HIV-2 and SIVs has shown that HIV-0 is more related to HIV-1 than HIV-2, being less related to HIV-1 subtype A through H than the other subtypes are related to each other (Gurtler *et al.*, 1994). For this reason HIV-1 subtypes A through H were designated M group (major group) and HIV-0 was designated the O group (outgroup) (Charneau *et al.*, 1994). A common human progenitor ancestor from whom they were derived and from which they diversified may explain the close relatedness of the subtypes from the M group. HIV-0 and HIV-2 probably entered into the human population through zoonotic transmission from chimpanzee and mangabey monkey, respectively (Hirsch *et al.*, 1989). A new branch of the HIV-1 cluster designated “N” was recently discovered (Simon *et al.*, 1998).

Subtype A, the most prevalent subtype in Africa, has recombined with many other subtypes giving recombinant viruses that were designated “Circulating Recombinant Forms” (CRFs). There are four CRFs of HIV-1 spread worldwide to a significant extent. AE virus from Southeast Asia (Carr *et al.*, 1996, Gao *et al.*, 1996), called AE (CM240), the AG recombinant from west and central Africa, called AG (IbNG), (Carr *et al.*, 1998), the AGI recombinant from Cyprus and Greece, called AGI (CY032) (Gao *et al.*, 1998, Kostrikis *et al.*, 1995, Nasioulas *et al.*, 1999) and the AB recombinant from Russia, called AB (Kal153) (Liitsola *et al.*, 1998). A variety of intersubtype recombinants combining segments such as A and C, A and D, B and F have also been documented.

1.6 Biological properties displayed by HIV-1 *in vitro*

HIV-1 displays three biological properties *in vitro*: replication rate, cytopathic effect and cellular host range.

1.6.1 Replication rate

The replication rate is the feature based on which the isolates were classified as “slow/low” and “rapid/high” (Asjo *et al.*, 1986). The classification was done by co-cultivation of HIV-positive PBMC with negative donor PBMC. The isolates able to replicate efficiently in PBMCs were designated “rapid/high”, whereas those able to yield low amounts of virus after a prolonged time in culture were defined as “slow/low” isolates. Generally, although not always, “rapid/high” but not “slow/low” isolates were found to induce syncytia and infect T cell lines (Fenyo *et al.*, 1988, 1989).

1.6.2 Cytopathic effect

The cytopathic effect distinguishes the isolates in syncytia inducing and non-syncytia inducing, based on their ability to induce cell fusion (or syncytia) in established T-cell lines (eg. MT-2 cells) (Lifson *et al.*, 1986). HIV infection of PBMC in culture results in the formation of the multinucleated cells within 2 to 3 days, accompanied by “ballooning” and degeneration of the cells. The cytopathic effect can be manifested by the single-cell death in the absence of syncytia as a consequence of pycnosis and degradation (Fenyo *et al.*, 1988).

1.6.3 Cellular host range

The cellular host range refers to the type of cells in which HIV-1 isolates can grow. Initially, several reports in which the impact of the envelope regions for the cellular host range was investigated, revealed different ability for different isolates to replicate in CD4-positive transformed cell lines and monocyte-derived macrophages (Gendelman *et al.*, 1990, Cheng-Mayer *et al.*, 1990a). In general, HIV-1 isolates able to grow in cultures of monocyte-derived macrophages do not grow well in transformed T cell line. These isolates are described as MT-tropic (for macrophage and primary T lymphocytes) or of broad host-range, or dual tropic (Moore *et al.*, 1995a). The isolates able to grow in CD4-positive cell lines but not monocyte-derived macrophage culture are referred to as T-cell-line tropic, T-tropic, or narrow-host range. This classification is not rigid. Thus, all isolates are able to infect activated peripheral blood CD4 cells and most if not all primary isolates are able to

infect macrophages with different replication kinetics (Cheng-Mayer *et al.*, 1988, Valentin *et al.*, 1994). Furthermore, dual-tropic viruses have been identified (ie. rapidly replicating, syncytium-inducing isolates capable of infecting macrophages) (Valentin *et al.*, 1994, Simmons *et al.*, 1996).

1.6.4 Receptors and co-receptor usage for HIV-1 entry

The main cellular receptor for HIV-1 was identified and showed to be CD4 molecule that is commonly found on a subset of T-lymphocytes (Th cells) and on cells of monocyte-macrophage lineage (Klatzmann *et al.*, 1984, Sattentau *et al.*, 1989, Maddon *et al.*, 1986, 1987). Some other molecules such as sphingolipid galactosyl ceramide are believed to mediate viral entry in the absence of CD4 molecule on CD4⁻ brain- and bowel-derived human cells with lower efficiency (Yahi *et al.*, 1994a, b, Harouse *et al.*, 1998). Early studies using murine cell lines that were transformed with a cDNA clone of the human CD4 gene have shown that the expression of the CD4 molecules alone on the murine cell lines did not render these cells permissive for HIV-1 infection (Maddon *et al.*, 1985). In 1995, it has been shown that CC chemokines RANTES, MIP-1 α , MIP-1 β are suppressive factors released by CD8⁺ T lymphocytes which are able to suppress infection by M-tropic HIV-1 strains but have no effect on a TCL-tropic strain (Cocchi *et al.*, 1995). The chemokine receptor with specificity for RANTES, MIP-1 α and MIP-1 β has been identified and called CCR5 (the fifth receptor for CC chemokines) (Samson *et al.*, 1996, Combadiere *et al.*, 1996, Raport *et al.*, 1996). It has been shown that CCR5 is the major coreceptor for macrophage-tropic HIV-1 strains (Deng *et al.*, 1996, Dragic *et al.*, 1996, Alkhatib *et al.*, 1996, Doranz *et al.*, 1996).

In 1996, a cDNA that rendered a CD4⁻ expressing murine cell permissive for fusion with cells expressing Env from a TCL-adapted strain was isolated (Feng *et al.*, 1996). The protein was identified and found to be a member of a superfamily of the seven transmembrane domain G-protein-coupled receptors. No ligand or functional activities were associated with this protein; therefore it has been called “orphan” receptor or “fusin”, based on its property to mediate Env-fusion (Feng *et al.*, 1996). Co-expression of the fusion with CD4 rendered non-human cells permissive for Env-

mediated cell fusion and anti-fusin antibodies were able to inhibit fusion and infection of primary CD4⁺ T lymphocytes. Thus, both results showed that fusion fit the criteria for the TCL-tropic HIV-1 receptor. Subsequently, stromal cell-derived factor-1 (SDF-1) has been characterised as a ligand for fusin (Bleul *et al.*, 1996, Oberlin *et al.*, 1996). Fusin was renamed CXCR4 (the fourth receptor for CXC chemokines).

In general, viruses from all clades are able to use both CCR5 and CXCR4 coreceptors (Bjorndal *et al.*, 1997, Bazan *et al.*, 1998). Thus, TCL-tropic strains use CXCR4, as T cell lines abundantly express this molecule. Conversely, macrophage-tropic strains use CCR5, a coreceptor that is expressed on macrophages. Finally, dual-tropic strains are able to use both coreceptors and can infect primary T cells that express both molecules.

Recently, the designation of HIV-1 phenotype was revised. Thus, X4 was conferred to isolates CXCR4-specific, TCL-tropic and syncytium inducing, R5 was given to isolates CCR5-specific, macrophage-tropic and non-syncytium-inducing, and finally R5X4 corresponds to isolates that use both coreceptors and are dual-tropic (Berger *et al.*, 1998).

Other human chemokine receptors have been demonstrated to have coreceptor activity: CCR2b (Doranz *et al.*, 1996), CCR3 (Choe *et al.*, 1996, Simmons *et al.*, 1998), CCR8 (Rucker *et al.*, 1997, Simmons *et al.*, 1998), GPR1 (Zhang *et al.*, 1998a), CX3CR1 (V28 or CMKBRL1) (Combadiere *et al.*, 1998a,b), STRL33/Bonzo (Deng *et al.*, 1997, Zhang *et al.*, 1998a, Simmons *et al.*, 1998), GPR15/BOB (Edinger *et al.*, 1998, Farzan *et al.*, 1997, Simmons *et al.*, 1998) and Apj (Choe *et al.*, 1998, Zhang *et al.*, 1998a) but CCR5 is the predominant receptor used in infected individuals. The identification of new coreceptors led to the elaboration of the sequential conformational changes of the Env protein to allow fusion/entry/infection of the CD4⁺ T lymphocytes (Wyatt *et al.*, 1998). In this model, CD4 binds to gp120, an event that induces conformational changes in gp120 to create or stabilise the determinants required for coreceptor binding. Subsequently,

gp120 interacts with the coreceptor, inducing further conformational changes in Env that result in exposing the fusion peptide of gp41 and in fusion of the viral bilipidic membrane with the plasma membrane of the target cell. The V3 loop plays an important role in the specificity of the gp120 receptor for coreceptor binding. Specifically, basic residues at particular positions on both sides of the V3 loop, in concert with other regions such as V1, V2 and C4 are important for gp120 binding to coreceptor. It has been shown by X-ray crystallographic studies (Kwong *et al.*, 1998) that coreceptors interact with the V3 loop and a conserved “bridging sheet” composed of the V1/V2 stem and an antiparallel, four-stranded structure that includes sequences in the C4 region.

The temporal evolution of HIV-1 tropism (ie. NSI-SI shift) during HIV-1 pathogenesis was supported by the discovery of the predominance of R5 viruses at early stages, with X4 and R5X4 isolates appearing at late stages. The early R5 restriction and the late R5-X4 evolution suggest an important role for CCR5 during initial viral transmission and for CXCR4 in disease progression at late stages.

Four coreceptor/chemokine genetic polymorphisms have been identified and shown to correlate with delayed rate of HIV-1 disease progression: CCR5 Δ 32 (Dean *et al.*, 1996), CCR5 59029 G/A (McDermott *et al.*, 1998), CCR2-64I (Kostrikis *et al.*, 1998) and SDF-1 3' UTR-801 G-A (abbreviated SDF-1 3'A) (Winkler *et al.*, 1998). Conversely, the presence of a CCR5 promoter allele, designated P1, has been reported to correlate with rapid progression to AIDS (Martin *et al.*, 1998).

Small molecules such as ALX-40 and T22 peptides or bicyclam compounds called AMD 3100 have been identified as specific ligands for CXCR4 (R4) receptors, being able to block the interaction between R4 and gp120 (Littman *et al.*, 1998).

1.7 Natural history of HIV infection

Usually, HIV-1 entry into its human host occurs through mucosal surfaces (Quinn *et al.*, 1996) and subsequently disseminates throughout the lymphatic tissue (LT). LT

becomes the reservoir where virus is produced and stored during the course of infection (Pantaleo *et al.*, 1991). The first few weeks of the infection is called the acute stage and is characterised by a high level of virus and viral antigen in the blood stream, accompanied in many individuals by an illness that resembles infectious mononucleosis (Daar *et al.*, 1991, Clark *et al.*, 1991, Ho *et al.*, 1985). The acute stage is followed by an asymptomatic, clinically latent stage, when the level of virus and viral antigen drops, due to the emergence of the cellular immune responses (Koup *et al.*, 1991, 1994a).

AIDS ensues after several years, when CD4⁺ T cell count in blood slowly declines to reach the level of 200cells/mm³, below which opportunistic tumours and infections can appear (Pantaleo, *et al.*, 1993, Fauci *et al.*, 1996).

HIV infection is defined by interconnected processes of viral replication, spread and infection of new cells (Wei *et al.*, 1995, Ho *et al.*, 1995). This means that the rate of progression to AIDS and death is correlated with the levels of viral RNA in the blood stream (Mellors *et al.*, 1995). That reflects virus production in LT (Haase *et al.*, 1999).

1.8 Reservoirs of the productively infected cells

HIV-1 is believed to pass through mucosal barriers by infecting dendritic cells (DC) or macrophages (MΦ) (Spira *et al.*, 1996). These cells convey the virus to LT where CD4⁺ T cells become infected. However, studies conducted by Haase (1999) revealed that more than 90% of the productively infected cells at the portal of entry were CD4⁺ T lymphocytes. In the second week of infection the productively infected cells in the LT are CD4⁺ T cells (Veazey *et al.*, 1998). From the clinically latent stage to AIDS, LT is the major reservoir of HIV virus. In AIDS, nearly every organ, including liver, kidney, adrenal, lung and central nervous system (CNS) harbour virus. In lung and CNS, MΦ and cells in the MΦ-lineage (eg, microglial cells, the resident MΦs in the CNS) become productively infected cells (Orenstein *et al.*, 1997). HIV-1 was also detected in CD8⁺ T cells in the late stages (Livingstone *et al.*, 1996). The number of productively infected cells during chronic HIV infection varies

between 10^7 to 10^8 cells. The number of copies of viral RNA per productively infected cell varies also from 20 copies to 200 copies per cell, with a mean of about 50-100 copies per cell (Haase *et al.*, 1999).

The advent of highly active antiretroviral therapy (HAART) has altered the outcome of HIV-1 infection by suppressing replication of HIV-1 to undetectable levels in the blood stream and LT reservoir (Cavert *et al.*, 1997). The level of viral RNA in FDC declines at rates that parallel the decline in numbers of productively infected cells. Immune complexes of virus are deposited on FDCs and degraded by complement-mediated virolysis and phagocytosis by MΦs (Spear *et al.*, 1990). Before HAART treatment, there is equilibrium between virus production, storage and clearance. Studies of 10 patients have shown that after HAART is initiated, the population of productively infected cells with more than 75 copies of HIV-1 RNA per cell can no longer be detected. This event is accompanied by a rapid decline of virus in circulation and in the FDC pool. This population consists of cells in the late stages of the viral life cycle in infections that have shortest life expectancy and succumb to the cytopathic effects or are eliminated by CTLs. These infected cells with highest intracellular concentration of HIV-1 turn over rapidly and are the source of the daily virus production. Secondly, cells with more than 20 but less than 75 copies of viral RNA per cell are eliminated at a slower rate and, thus, the decay rate of the virus is kept at a relatively constant level because of the continued production and deposition of virus by these cells. This population is believed to be MΦ, as they do not succumb as quickly, but may include $CD4^+$ T cells that are chronically infected, but produce small amount of virus and consequently have longer lifespans. After six months of HAART, a 2500-fold reduction in the FDC pool of virions has been documented (Cavert *et al.*, 1997). After 1 year, productively infected cells with more than 20 copies of viral RNA per cell have disappeared but those with a very few copies of viral RNA (less than 5) have not. Persistence of virus in these cells ensures a detectable pool of viral DNA even after 2.5 years of suppression of viral replication from which virus has been isolated (Wong *et al.*, 1997). The chronically and latently infected cells and residual virus in the FDC represent a potential source of wild type and drug resistant mutant viruses that are able to restart infection if treatment is

ineffective or is stopped. In the latter case, infection is quickly re-established in FDC with similar kinetics to acute infection (Wong *et al.*, 1997).

On HAART, the total CD4 count increases at a rate of 10^9 cells/day (Zhang *et al.*, 1998b) in both blood and LT, comparable to the CD4⁺ T cell turnover in PBMC (Ho *et al.*, 1995). The increase is in the RO⁺ subset until a plateau is reached, when a slow increase in RA⁺ subset in both blood and LT becomes significant (Autran *et al.*, 1997, Zhang *et al.*, 1998b). Thus, following treatment, mature CD4⁺ T cells from gut, lung and other compartments are redistributed to blood and peripheral lymph nodes (Mosier *et al.*, 1995, Rosenberg *et al.*, 1993). The restoration of the immune function has been reported even in patients with AIDS undergoing HAART therapy (Palella *et al.*, 1998).

1.9 Immune responses to HIV infection

1.9.1 Cellular mediated immunity (CMI)

After local replication in LT, virus is disseminated in a massive viremia, which usually is accompanied by symptoms that resolves without intervention. The onset of CTL response coincides with the fall in the virus level (Koup *et al.*, 1994b), suggesting an anti-viral effect of CD8⁺ cells. To support this finding, Schmitz *et al.* (1999) and Jin *et al.* (1999) have shown that virus replication cannot be controlled when CD8⁺ T cells are experimentally depleted during primary infection. Depletion of CD8⁺ cells in chronically infected monkeys resulted in high level of viremia. This level dropped coincidentally with the reappearance of CD8⁺ cells. An inverse correlation between the magnitude of CTL responses and viral load has also been observed by Ogg *et al.* (1998). These data support the idea that CD8⁺ T cells mediate the drop from the peak viremia in acute HIV infection. CTLs have been detected in peripheral blood, lymph nodes, skin and semen of infected persons (Bachelez *et al.*, 1998, Quayle *et al.*, 1998) and a general loss of CTLs occurs during disease progression (Klein *et al.*, 1995, Rinaldo *et al.*, 1995).

CTL responses have been reported in persistently uninfected people who have been exposed to HIV (Rowland Jones *et al.*, 1998) and also in HIV⁻ babies born to

seropositive mothers (Rowland Jones *et al.*, 1995). Moreover, long-term non-progressors who successfully manage to control viremia have showed a strong CTL responses and week to undetectable levels of neutralising antibodies (Harrer *et al.*, 1996). These individuals showed also strong virus-specific Th responses, Th1-like, that were accompanied by a high level of IL-2 and IFN- γ (Rosenberg *et al.*, 1997).

1.9.1.1 Effects of HAART on HIV-1 specific CMI

Institution of HAART has effects on CTL responses but data obtained to date is difficult to be interpreted. Thus, Gray *et al.* (1999) and Ogg *et al.* (1999) have shown that the onset of HAART is followed by a decline in CTL responses, suggesting that sustained viral replication is required to maintained high level of memory CTL. Two other factors are thought to lead to the loss of these cells. Firstly, the effect of the protease inhibitor on the proteasome mediated antigen processing that limits the antigen presentation and prevents effective stimulation of virus-specific CTLs (Andre *et al.*, 1998). Secondly, HAART therapy could have a direct effect on the proliferative capacity of CTLs (Ogg *et al.*, 1999) leading to the emergence of a short half-life CTL population.

1.9.1.2 Escape from CTL Responses

Disease progression has been reported to coincide with the emergence of CTL-escape variants *in vivo* (Borrow *et al.*, 1997, Goulder *et al.*, 1997a, b). Escape from CTL responses occurs both in the acute and late stages of the infection. Two studies investigating patients with acute infection showed that strong early CTL responses could rapidly select mutant virus. Thus, Borrow *et al.* (1997) investigated a patient early in infection, noticing a CTL clone specific for an epitope on gp160 (AENLWVTVYY), restricted by B44. This epitope mutated at the second position (E/K), seven weeks after infection. This mutation was rapidly fixed and a diversification of the CTL responses against other epitopes has followed.

Also, Goulder *et al.* (1997a) described two epitopes restricted by HLA B8. The first epitope was in gag p17 (GGKKKYKL) that rapidly mutated at the time of seroconversion at the third position (K/Q), abrogating peptide binding. The second

epitope was in Nef (FLKEKGGL) that was largely mutated (the third residue K/E/N/Q) or even completely deleted.

Late escape from CTL responses has also been documented. Thus, Goulder *et al.*, (1996b) studied a group of haemophiliac patients with HLA B27 over more than a decade. All individuals showed CTL responses to an epitope in gag p24 (KRWIILGLNK). In two patients a lysine replaced arginine at the second residue. This mutation was fixed 12 years after infection and represented 100% of activated provirus in one patient whose CD4 cell count rapidly dropped to 60 cells/mm³. Peptides with an Arg/Lys substitution bound weakly to HLA B27 impairing CTL recognition. Conversely, Brander *et al.* (1998), failed to find a strong immune pressure imposed by HLA-A*0201 restricted CTL response, concluding that CTL escape may occur in individual cases but is not a common phenomenon.

Several studies have shown that HLA type may be associated with rapid or slow disease progression. In this light, it has been shown that HLA-B27 and HLA-B57 were associated with slow progression of HIV infection (Kaslow *et al.*, 1996), whereas HLA A1-B8-DR3, HLA-B35 or HLA-Cw4 were associated with a more rapid progression (Steel *et al.*, 1988, Kaslow *et al.*, 1990, Carrington *et al.*, 1999).

Different mechanisms of CTL escape by HIV have been proposed. Firstly, mutation could occur during antigen presentation, especially in the amino acid residues flanking the epitope that could affect the CTL response (Koup *et al.*, 1994b). Secondly, mutations that change peptide orientation, distort the peptide-binding groove or alter residues exposed to the TCR give the virus the best chance to escape (Reid *et al.*, 1996). Finally, it has been shown *in vitro* that peptides with diminished antigenicity are able to drive proliferation of CTL with specificity for the wild-type peptides, that are not able to lyse cells presenting the altered peptides (Klenerman *et al.*, 1995). This mechanism, if manifested *in vivo*, could enhance immune escape. Escape mutants from CTL responses that appear during HIV infection support the model proposed by Nowak *et al.* (1995) according to which a diversity threshold

emerges beyond which the immune system can not control the infection, leading to the immune system failure.

1.9.1.3 Implication of CMI for vaccine development

It is widely accepted that vaccine-induced responses must broadly cross-neutralise multiple HIV-1 clades. Cellular immune responses that recognise conserved epitopes located in the structural and regulatory genes represent the immunologic arm that should be the response induced by an HIV vaccine. There is evidence showing that CTLs raised against viral antigens from different clades can cross-react extensively. Ferrari *et al.* (1997a, b) have investigated immune responses to canarypox-clade-B based ALVAC/HIV-1 vaccines containing full-length gp160MN, gp120 plus transmembrane portion of gp41, full-length gag and protease. Autologous CD4⁺ lymphoblasts that were infected with primary isolates from clade A to F were used as target cells. Both the gp160-based canarypox immunogen and that containing gag/pol genes could elicit CTL reactivities able to recognise target cells infected with genetically diverse HIV-1 primary isolates.

Other studies have investigated the ability of CTL responses elicited in HIV-1 infected individuals to recognise epitopes from clades different than those with which they were infected. Thus, Betts *et al.* (1997) have shown that individuals infected with HIV-1 clade C can elicit vigorous HIV-1 CTL responses reactive to HIV-1 clade B. Six of the eight Zambian individuals who were infected with clade C virus demonstrated high levels of HIV-1 specific CTL responses to HIV-1 clade B (strain IIIB) gag/pol/env constructs.

In another study, Cao *et al.* (1997) isolated CTL clones, specific for Gag, RT-ase or Env, with B- clade virus and tested their ability to recognise analogous A-, C-, D- and E- clade viral sequences. They have revealed that all CTL clones were able to cross-react with at least one non B-clade strain. They also investigated HIV-1 specific CTL responses in 14 individuals infected with A-, C- or G- clade viruses and pointed out that all CTL clones showed cross-reactivity with B- clade viral constructs expressing Gag, Env, Pol and Nef.

Also, Durali *et al.* (1998) investigated CTLs in eight patients with A- clade virus and seven infected with B- clade virus. Target cells were infected with recombinant vaccinia viruses expressing Env, Gag, Pol and Nef from A- or B- clades. The results showed that CTLs from all individuals cross-reacted with proteins from the heterologous clades.

Finally, McAdam *et al.* (1998) evaluated individuals infected with A-, B-, C- and D-clade HIV-1. They also observed extensive cross-reactivity in CTL responses to p55 in the majority of individuals.

The route by which the immunogen is administered is important for induction of a desired type of immune responses. Thus, Benson *et al.* (1998) induced a strong CTL response after intra-muscular vaccination of macaques with a vaccinia virus strain expressing SIV *gag*, *pol* and *env*. Co-administration of cytokines and the timing of cytokine addition may lead to increased CTL responses. Thus, co-administration of IL-12 and IL-2 increased the CTL responses when macaques were challenged intrarectally, but not intravenously with SIVmac 25strain (Benson *et al.*, 1998). A high level of CTL reactivity was obtained in mice when gp120 administration was followed by an IL-2 boost (Barouch *et al.*, 1998).

Studies concerning the effectiveness of vaccine-induced immune responses have also been performed. On this ground, two macaques were immunised with a mixture of lipopeptides and challenged with SIV. The vaccine induced strong CTL responses in two animals that selected escape variants (Mortara *et al.*, 1998). The ability of CTL responses to neutralise across the clades is a requirement for the HIV vaccine development, since most currently designed vaccines are based on B-clade immunogens and a vaccine for global use is highly desirable.

1.9.2 Humoral immunity

In contrast to CMI, HIV-1 specific antibodies are detected considerably later (Moore *et al.*, 1994d). Their appearance does not coincide with the reduction in viremia seen

during the acute infection. Particularly, neutralising anti-envelope antibodies develop very slowly at lower titres than non-neutralising antibodies to both continuous and discontinuous epitopes (Moore *et al.*, 1993c). The heterogeneity found within *env* gene during the infection is thought to be imposed by the antibody selective pressure. A high rate of replacement substitution in HIV envelope suggests a strong selective force of the humoral responses (Simmonds *et al.*, 1991).

Primary isolates and cell-line-adapted isolates have different sensitivities to neutralisation (Moore *et al.*, 1995a, b). These different neutralisation sensitivities are probably a result of different conformations of the Env of these viruses (Sattentau *et al.*, 1995a, b, Schutten *et al.*, 1993).

1.9.2.1 Neutralisation in the context of the relationship between the structure and function of *env* proteins

Very little gp120 protein is available to antibody attack, most of its surface being involved in gp41 binding, oligomerisation and chemokine binding or is occluded by glycosylation. Similarly, most of gp41 is occluded by gp120 binding or by the carbohydrate mass that surrounds the molecule.

Only two epitopes on gp120 have been identified as being accessible on different primary isolates. Monoclonal antibody (MAb) b12 and 2G12 target these epitopes. MAb b12 recognises an epitope overlapping the CD4 binding site and V2 and V3 domains (Roben *et al.*, 1994, Moore *et al.*, 1995c). MAb 2G12 binds a region composed of the V3 loop and the V4 region and is influenced by the presence of N-linked carbohydrates (Trkola *et al.*, 1995, 1996).

The gp41 protein has only one epitope that is exposed on the mature oligomer. This is located in the C-terminal part of the extracellular domain and is targeted by MAb 2F5 (Sattentau *et al.*, 1995a, Muster *et al.*, 1993, 1994). Specifically, its epitope is represented by the linear sequence ELDKWA that is conserved across many isolates of HIV-1, conferring a broad neutralisation capability.

1.9.2.2 Implications of neutralising antibody on vaccine development

Vaccine design must take into account the immunogenicity of the envelope protein. Subunit vaccine antigens based on soluble monomeric gp120 are poor inducers of antibodies able to neutralise primary isolate viruses. There are two explanations for this result. Firstly, monomeric gp120 used as immunogen could have been denatured and, hence, the antibodies elicited recognised this form of the protein but not the native Env (Moore *et al.*, 1993c, VanCott *et al.*, 1995). Secondly, the humoral immune responses induced by properly folded monomeric Env are of non-neutralising specificity. The reason for this is that the epitopes recognised by antibody elicited by monomeric Env are occluded on the trimeric form of primary isolates. This result suggested the idea of using oligomeric Env rather than monomeric Env as immunogen to elicit antibody able to neutralise primary isolates (VanCott *et al.*, 1997). In this regard, protection from challenge has been reported in macaques immunised with oligomeric, but not monomeric Env immunogens (Luke *et al.*, 1996), although other studies have reported protection of macaques achieved even by monomeric soluble gp120 proteins (Mooij *et al.*, 1998).

In natural infection, humoral immune response is directed to viral debris (ie. not to the mature envelope oligomer but to other conformations, especially to unprocessed gp160 precursor). This response is probably stimulated by the uncleaved gp160 that is released and acts by a mechanism known as original antigenic sin (Deutsch *et al.*, 1972). That means that immunisation with antigen 1 (in this case, unprocessed gp160 precursor) can establish a memory B cell population that is subsequently reactivated by a related antigen 2 (mature oligomer) to produce antibody with high affinity to antigen 1 and only moderate affinity to antigen 2.

An efficient vaccine approach should be based on that protocol that elicits neutralising antibody specific for primary isolates. Passive immunisation studies provide the evidence of the feasibility of such a vaccine. A series of studies have revealed that antibody to the V3 and CD4 binding domains on gp120 were able to protect chimpanzees and hu-PBL-SCID mice from challenge with TCLA viruses (Emini *et al.*, 1992, Safrit *et al.*, 1993, Parren *et al.*, 1995). Conversely, MAb 2F5

was able to delay disease progression but did not protect chimpanzees against challenge with a primary virus (Conley *et al.*, 1996). Disappointing results have been reported when passive transfer of pool Ig from HIV-1 seropositive donors was done to chronically infected humans. In this case HIVIG relatively weakly neutralised primary isolates (Burton *et al.*, 1994).

1.10 Antigenic diversity of HIV isolates

1.10.1 Quasispecies

HIV-1 replicates with a high mutation rate, leading to a genetically complex population designated quasispecies. Due to a high error-rate that is characteristic for RT-ase, a distribution of mutants in which a consensus or “master” sequence is generated, around which the mutants are distributed and reach an equilibrium (Domingo *et al.*, 1978). This mutant distribution rather than a single sequence is considered to be the target of selection.

The source of the genetic variation of HIV is the error prone polymerase enzyme RT-ase. This enzyme lacks a 3'→5'exonuclease ability and cannot repair nucleotide mismatches introduced into the sequence during the synthesis of the viral DNA (Preston *et al.*, 1988, Roberts *et al.*, 1988). Also, the provirus is transcribed by the cellular RNA polymerase II, another error-prone polymerase that provides another source of variation.

Hypermutation (a process where G to A transition occurs) was characterised as being another mutational process specific for HIV-1 (Goodenow *et al.*, 1989, Vartanian *et al.*, 1991). Other source of antigenic variability is given by recombination between non-identical viral genomes present in the same virion, due to template switching during reverse transcription (Clavel *et al.*, 1989).

More important, the emergence of quasispecies is seen as a consequence of a dynamic interaction between viral diversity and the humoral and cellular immune responses (Nowak *et al.*, 1990, 1991, 1996a, b, Wolinsky *et al.*, 1996). The

mathematical model of “the antigenic diversity threshold” elaborated by Nowak *et al.* (1991) may be of a conceptual importance. According to this concept, the selective pressure imposed by the immune system to select distinct antigenic variants finally destroys the immunity. The replication errors during reverse transcription produce antigenically distinct variants that, being under the control of the immune system may evolve in so-called “escape mutants”. These processes lead in time to the establishment of an antigenic diversity level that, if it is below a threshold, it is under the control of the immune system. If the antigenic diversity is above the threshold value, the immunity is overcome and the AIDS outbreaks. Conversely, Wolinsky *et al.* (1996) failed to see a correlation between the antigenic diversity and disease progression in 2 out of 6 patients followed up to 5 years of infection. In this study, two patients progressed rapidly and died within 36 and 46 months, respectively, after seroconversion, showing a relatively homogeneous population of variants. The other four individuals showed a more diverse heterogeneous viral population accompanied by a slow rate of CD4 T cell decline. The interpretation of Wolinsky’s results given by Nowak *et al.* (1996) would rather confirm the “antigenic diversity theory”. In this regard, two rapid progressors are seen to have a weak immunity, with a low diversity threshold, and may have developed disease rapidly in the presence of a low antigenic diversity. However, the “antigenic diversity threshold” theory still needs to be demonstrated experimentally (Nowak *et al.*, 1996).

1.10.2 Antigenic diversity and transmission

It has been documented that the usage of different coreceptors might be associated with infectivity and transmission. Tscherning *et al.* (1998) examined 81 primary isolates from nine different HIV-1 subtypes for their ability to use different coreceptors, suggesting subtype-specific differences in coreceptor usage. Soto-Ramirez *et al.* (1996) and Essex *et al.* (1997) have also shown that Langerhans cells in the genital tract were more easily infected with HIV-1 Env subtype E and C than subtype B. However, two other studies failed to show a difference between these HIV-1 subtypes and susceptibility to infection of Langerhans cells (Pope *et al.*, 1997, Dittmar *et al.*, 1997).

Conclusively, a correlation was rather found between transmissibility or pathogenesis and virological, host and sociobehavioral factors than genetic subtypes. Thus, Zhong *et al.* (1995) has shown that CCR5 is the major coreceptor for all primary macrophage tropic strains of HIV-1, irrespective of the genetic subtypes (ie. A, B and D).

1.10.3 Antigenic diversity and pathogenesis

The rate of disease progression varies hugely from one person to another one, being influenced by virological and host factors (Fauci *et al.*, 1996). Usually, infection with HIV-2 is characterised by a slower disease progression than HIV-1 infection or even HIV-1/HIV-2 dual infection (Marlink *et al.*, 1994). The existence of differences in pathogenesis and disease progression between different HIV-1 subtypes is still questioned. Thus, a study conducted by Kanki *et al.* (1999) who followed up seroconvertors infected with different type 1 subtypes, reported the AIDS onset by year 5 in women infected with subtype A in comparison to women infected with other subtypes who developed AIDS rapidly.

Conversely, two studies have shown no significant difference in the CD4 cell decline between subjects infected with different subtypes of HIV-1. Galai *et al.* (1997) found similar decline rates in CD4 cell count in two groups: a cohort of Israeli men infected with subtype B and another of Ethiopian immigrants into Israel infected with subtype C. Also, Mastro *et al.* (1999) investigated drug users infected with subtype B and E and found a higher viral load early post-infection in drug users infected with subtype E that was followed 1 year afterwards by similar levels of viral load, CD4 and CD8 cell count in persons infected with both subtypes.

Host immune responses to HIV do not correlate accurately with HIV-1 subtypes. Several studies have investigated the ability of both autologous and heterologous sera from HIV-1 infected persons to intra- and interclade neutralise different HIV-1 subtype isolates (Nyambi *et al.*, 1997). These studies have shown that whereas some primary isolates were widely neutralised by sera from persons infected with a broad range of genetic subtypes, others are weakly neutralised by the same sera (Moore *et*

al., 1996a, b, Kostrikis *et al.*, 1996). Similarly, many CTL-targeted epitopes appeared conserved between different subtypes, conferring the ability of CMI to broadly neutralise across the clades (Cao *et al.*, 1997).

1.10.4 Antigenic diversity and vaccine development

The high antigenic variability has hampered the vaccine development for more than a decade. It is still unknown how to best formulate a vaccine that is able to induce broadly effective humoral and cellular immune responses. A commonly held view is that a separate vaccine for each genetic subtype might be needed. However, the identification of three neutralising antibodies (ie. 2F5, 2G12 and IgG1b12), able to cross-neutralise different primary isolates and conserved CTL-epitopes across different clades brought great hopes that an effective prophylactic and therapeutic vaccine may be achieved. A combined regimen between HAART therapy and vaccine is currently viewed with optimism and expected to lead to the body clearance from HIV-1 infection.

1.10.5 Objectives of this thesis

The work presented in this thesis investigates the hypothesis regarding the high cross-reactivity between gp120 antigens and sera obtained from patients from which close related viruses were isolated. Two groups of subjects were selected in order to test this hypothesis. Namely, the first group consisted of nine homosexulas from Edinburgh, Newcastle and Belfast who were selected from a large group of subjects based on the similarity in gag region of the isolates with which they were infected and the second group was represented by seven haemophilic subjects from Edinburgh who had a common source of infection. Due to a high degree of relatedness of these isolates, the hypothesis regarding the presence of a high cross-reactivity between gp120 antigens and IgG antibody of sera from these subjects would be expected. To prove this, 1.7-kb fragments encoding gp120 antigen were amplified from patients PBMC's DNA, cloned and expressed in COS cells. The level of recognition of these primary isolate-derived monomeric glycoproteins by the IgG antibody from autologous and heterologous sera was assessed. The results obtained when taking this approach would reflect the situation when a group of persons would

be infected with similar isolates and elicit a cross-reacting IgG response. Furthermore, this high cross-reactivity would be desirable to be elicited in a group of vaccinees who would mount a level of IgG antibody response able to cross-react with isolates that are close related with the immunogen, following a DNA prime-protein boost immunisation. If the hypothesis questioned in this study is true than its applicability for an AIDS vaccine is of a considerable value. It may be worth mentioning that, by the time this work was performed, the assessment of recognition of gp120 antigens by IgG antibody from human sera was done using antigens from TCLA isolates or recombinant monomeric gp120 expressed in yeast or insect cells but not primary isolate-derived monomeric gp120s expressed in mammalian cells. This would be one aspect regarding the originality and the contribution of this work to HIV-1 biology. In spite of the fact that the methodology consists of assays and approaches that have been previously used in other studies, the main contribution of this work was to collect and present data in only one paper.

Specifically, Chapter 3 describes the steps taken to set up an ELISA assay which allows both the estimation of the amount of gp120 protein and the IgG antibody level from HIV-1 positive serum to gp120. Chapter 4 presents the construction of a mammalian expression cassette-vector into which gp120 genes can be subcloned. Chapter 5 gives all the clones obtained when cloning 1.7-kb fragments into a mammalian cassette-vector together with analysis of their sequence in conjunction with some of their biological properties. Chapter 6 examines the level of cross-reactivity between gp120 antigens and sera obtained from both groups of subjects investigated. Finally, Chapter 7 presents a discussion of the results obtained in the light of previous published data and their implication in the achievement of an AIDS vaccine.

CHAPTER 2

MATERIALS AND METHODS

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2.1 Collection and Processing of Blood Samples

Samples were obtained from HIV-1 seropositive homosexuals attending Genito-Urinary and infectious diseases' clinics in Edinburgh, Newcastle and Belfast between 1993 and 1995 (Leigh Brown *et al.*, 1997). The seroconversion dates were not available for these patients. However, they were asymptomatic and had not received any anti-viral therapy at the time of collection of the blood samples used in this study. Haemophilic subjects taken in this study were selected from the Edinburgh Haemophilic Cohort (Holmes *et al.*, 1995). Some details of the HIV-1 infected individuals are shown in Table 2.1.

2.1.1 Processing of blood samples

Blood samples were collected in tubes containing either Heparin or ethylenediaminetetra acetic acid (EDTA). Peripheral blood mononuclear cells (PBMC) were separated from plasma on the same day as collection. Blood was diluted two fold in phosphate buffer saline (PBS, OXOID or Gibco BRL). 10ml of diluted blood was gently layered over 10ml LymphoprepTM (NYCOMED) or NycoPrepTM 1.077 (NYCOMED) in a 20ml universal tube. Precautions were made to avoid mixing the two phases. The universals were centrifuged at 2,000rpm for 30 minutes (Beckman GP centrifuge, IEC Centra-4R). PBMCs and plasma layers were then carefully removed.

2.1.2 Sample storage

Plasma was stored in cryotubes (NUNC) at -70°C . PBMCs were washed twice with RPMI 1640 (HyClone^REurope LTD) and centrifuged at 2,000rpm for 5min. The pelleted cells were resuspended in freezing mix: 20% dimethyl sulphoxide (DMSO, Sigma) with 80% sterile heat inactivated fetal calf serum (FCS, Advanced Protein Products Ltd) in cryotubes (NUNC). The cryotubes were stored in freezer containers (NALGENE Cryo 1°C Freezing Containers), which allow tubes to cool down to -70°C at a rate of $1^{\circ}\text{C}/\text{minute}$. Next day the freezer boxes were moved to liquid nitrogen storage containers.

2.2 Extraction of the provirus DNA from PBMC

The cryopreserved PBMCs were thawed and washed once with 20ml RPMI 1640 and centrifuged at 15,000rpm for 10 minutes. The resulting cell pellet was resuspended in 400 μ l of lysis buffer (110mM NaCl, 55mM Tris pH8.0, 0.5% SDS, 1mg/ml Proteinase K (SIGMA Molecular Biology), 40 μ g/ml polyA) and incubated at 37⁰C for 2 hours. After cell lysis, DNA was extracted once with 450 μ l phenol, once with 450 μ l phenol: chloroform (1:1) and once with chloroform: isoamylalcohol (50:1). After each extraction the layers were vigorously mixed by vortexing and separated by centrifugation at 13,000rpm for 10 minutes (Heraeus SEPATECH BIOFUGE 15). Each time, the upper aqueous phase was removed and processed further. Finally, in order to precipitate the DNA, the aqueous phase was incubated with 800 μ l of 100% ethanol and 40 μ l of 3M Na-acetate pH5.2, overnight at -20⁰C. The next day, the DNA was pelleted by centrifugation at 15,000rpm for 20 minutes, the supernatant discarded and the pellet washed twice with 1ml ice-cold 80% ethanol. To prevent the occasional loss of DNA pellet, another centrifugation step at 15,000 rpm for 5 minutes was performed. The DNA pellet was air-dried and resuspended in 30 μ l of RNA-ase free water (dimethyl pyrocarbonate- (DEPC) treated water). The concentration of DNA was estimated spectrophotometrically and DNA was then stored in aliquots at -20⁰C or -70⁰C.

Table 2.1. Details of the HIV-1 infected individuals.

No	Sample	Age	Sex	Detection of infection	Antiviral Therapy*	No of CD4 Cell/mm3	Risk group	City
1	1363	31	M	1994	none	399	Homosexual	Edinburgh
2	1299	39	M	1994	none	580	Homosexual	Edinburgh
3	1260	32	M	1989	none	100	Homosexual	Edinburgh
4	1021	34	M	1992	none	226	Homosexual	Edinburgh
5	1020	34	M	1986	none	39	Homosexual	Edinburgh
6	1397	36	M	1992	none	210	Homosexual	Newcastle
7	1294	36	M	1992	none	640	Homosexual	Newcastle
8	1066	41	M	1993	none	ND	Homosexual	Belfast
9	1090	30	M	1993	none	290	Homosexual	Belfast
10	p80	ND	M	1984	none	ND	Haemophilia	Edinburgh
11	p82	ND	M	1984	none	ND	Haemophilia	Edinburgh
12	p74	ND	M	1984	none	ND	Haemophilia	Edinburgh
13	p84	ND	M	1984	none	ND	Haemophilia	Edinburgh
14	p86	ND	M	1984	none	ND	Haemophilia	Edinburgh
15	p89	ND	M	1984	none	ND	Haemophilia	Edinburgh
16	p79	ND	M	1984	none	ND	Haemophilia	Edinburgh

* at time of blood sample collection

2.3 Plasmid cloning vectors

2.3.1 pGEM-T

The pGEM-T vector (Mezei *et al.*, 1994) is a 3-kb plasmid-cloning vector that was purchased from Promega. It was derived from pGEM 5Zf(+) (Promega) by digestion with EcoRV at base 51 of its sequence and adding a 3-terminal thymidine to the 5' and 3'-ends. The vector contains T4 and SP6 Polymerase transcription initiation sites and promoters flanking a multiple cloning site (MCS) within the lacZ gene. Insertional inactivation by the PCR products allows recombinant clones to be directly identified from non-recombinant ones by blue-white colour screening of the plates. The pGEM T vector also contains the Amp^r gene that confers resistance to ampicillin allowing only those bacterial strains containing the region of replication of the filamentous phage f1 to grow, and thus preparation of single stranded copies of plasmid DNA. PGEM T vector can be used to clone PCR products generated by Taq polymerase, an enzyme that often adds a single deoxyadenosine to the 3'-ends of the amplicons in a template composition independent manner (Newton *et al.*, 1994a, b).

2.3.2 pSRHL

pSRHL is a mammalian expression vector that was modified from pSRHS (Dubay *et al.*, 1992) by replacing the wild-type gp160 encoded gene with a polylinker. In pSRHS, HIV gp120 gene expression is under the control of the SV40 late promoter and Mason-Pfizer Monkey Virus long terminal repeat (LTR) which polyadenilates and stabilises the message. In addition, a high level of gene expression is given by the SV40 origin of replication and an adjacent region encoding for large T antigen that acts as a DNA replication factor.

2.4 Bacterial strains: characterisation and storage

2.4.1 Characterisation of bacterial strains

The work involving cloning of the gp120 glycoprotein PCR products into pGEM T and subsequent subcloning into mammalian expression vector pEV6, was done using Epicurean Coli SURE (Stop Unwanted Rearrangement Events) Competent Cells (Stratagene). This strain was engineered to carry mutations able to inactivate certain

pathways catalysing rearrangements of cruciform and Z-DNA structures that occur frequently in eukaryotic DNA and are highly unstable. The SURE cells allow blue-white colour screening of recombinant plasmids due to the presence of *lacI^gZΔM15* gene on the F' episome. This strain genotype is:

e14⁻ (McrA⁻)Δ(mcrCB-hsdSMR-mrr)171 endA1 supE44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC::Tn5(Kan^r) uvrC(F' proAB lacI^g ZΔM15 Tn10(Tet^r)).

Modification of the mammalian expression vector, which was done in order to include a polylinker that subsequently allows exchanges of the gp120 genes from pGEM T into it, involved the manipulation of DH5α cells. DH5α Competent cells (Gibco) are capable of being transformed with high efficiency by different plasmids (Hanahan *et al.*, 1991). Their genotype is: F'φ80dlacZΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17(r_k⁻, m_k⁺) phoA supE44λ⁻ thi-1 gyrA96 relA1.*

2.4.2 Bacterial strain storage

For short-term storage (3-4 weeks) bacteria were maintained on solid Luria Broth medium (LB: 10g tryptone, 5g yeast extract, 5g NaCl, 1ml 1N NaOH, 15g agar or agarose per liter) plates at 4⁰C. For long-term storage (years), 2mls of a mid-log culture or 1ml of a freshly saturated culture were added to a cryovial (Nunc or Nalgene) containing 1ml-glycerol solution or 7% (v/v) dimethylsulfoxide (DMSO). Stored cells were revived by streaking a loop from frozen stock onto a fresh L-plate and incubated overnight at 37⁰C.

2.5 Polymerase Chain Reaction (PCR)

PCR is an *in vitro* technique which allows the exponential amplification of a DNA sequence template through a series of repetitive cycles involving template denaturation, primer annealing and primer extension by mean of a heat-stable DNA polymerase (Mullis *et al.*, 1987, 1990a, b, 1991).

2.5.1 Enzymes and primers

Two types of DNA polymerase were used in this study: Taq DNA Polymerase (Promega) and Pfu DNA Polymerase (Stratagene). Several features of these enzymes are presented in Table 2.2.

Table 2.2 Comparison of amplicons properties for Taq and Pfu Polymerase.

Characteristics	Taq Polymerase	Pfu Polymerase
resulting DNA ends	3'-A	blunt
5'-3' exonuclease activity	yes	no
3'-5' exonuclease activity (proof-reading capability)	no	yes
error rate	8.0×10^{-6}	1.3×10^{-6}
percentage of mutated PCR products	16	2.6

The primers design was based on the consensus of the HIV-1 HXB 2-D sequence, with the primer binding sites chosen to be as highly conserved as possible (Korber *et al.*, 1998). Oligonucleotides were synthesised by the Oswel DNA Service, Department of Chemistry, University of Edinburgh on an Applied BioSystems 394 and purified by high-performance liquid chromatography (HPLC). In the present study, two approaches to the polymerase chain reaction were taken: a limiting dilution nested PCR specific for V1/V2 region and a nested PCR specific for gp120 gene. Nested PCR involves two rounds of amplification. The first round employs outer primers, whereas the primers for the second round of amplification lay within the sequence delimited by the outer primers, therefore being called nested or inner primers. The primers for PCR both for amplification of V3 and env region are presented in Table 2.3. The layout of the primers for PCR amplification is shown in Figure 2.1.

2.5.2 Limiting dilution nested PCR

This method was carried out to quantify the target DNA (Simmonds *et al.*, 1990a, b) because its increased specificity and sensitivity that allows the amplification of a very limited concentration of DNA template. PBMC DNA was titrated into a series of dilutions (ten fold dilution first: 1/2, 1/3, 1/5, 1/10, 1/20, 1/30, 1/50, etc, and then two fold dilution was performed around the cut-off point). Multiple replicates (10-30) per each dilution were amplified in nested PCRs with primers that are specific for V1/2 region. The end point dilution for each sample was considered to be the dilution at which 20% of the reactions were consistently positive. The provirus copies per reaction were estimated based on the Poisson formula $-(\ln f_0) \times 1/d$, where f_0 is the frequency of negative reaction and d is the dilution of the DNA sample.

PCR reactions were performed in a 20 μ l reaction mixture containing 10mM Tris-HCl pH 8.8, 50mM KCl, 1.5mM MgCl₂, 0.1% Triton X-100, 33 μ M each dNTP, 100nM of each primer, 1U Taq Polymerase (Promega). A drop of paraffin overlaid the reaction mix. For the first round of amplification 1 μ l of DNA from each dilution was taken into the reaction and 1 μ l from the first PCR reaction was transferred into the second reaction. For both rounds, the amplification was done by using a programme involving 30 cycles of three segments: 94⁰C for 35sec, followed by 50⁰C for 35sec and 70⁰C for 2min 30sec and a final cycle in which the reaction tubes were held at 70⁰C for 10min.

2.5.3 Nested PCR Amplification with Pfu

Pfu polymerase was chosen for high-fidelity PCR amplification of the gp120 gene derived from patient PBMC DNA based on its lowest error rate (1.3×10^{-6} mutation frequency per base pair per duplication). The reactions were carried out in 100 μ l mix containing 250 μ M each dNTP, 250ng/reaction each primer, 5U Pfu Polymerase per reaction, 20mM Tris-HCl pH8.8, 2mM MgSO₄, 10mM KCl, 10mM (NH₄)₂ SO₄, 0.1% Triton X-100, 0.1 mg/ml nuclease-free BSA and 5 μ l of PBMC DNA. 5 μ l from the first round was transferred into the second round mix. The same programme was used for both rounds of amplification: 30 cycles including template denaturation at 94⁰C for 35sec, primer hybridisation at 50⁰C for 35 sec and primers extension at

72°C for 5min and one final cycle at 72°C for 10min. 10µl from each tube was run on the gel and the tubes, which gave positive bands, were selected for further cloning. For each amplification 5-10 negative samples were included in which the provirus DNA was substituted with distilled ultrapure water (TECHNE Thermal Cycler, GENEE Thermal Cycler).

2.5.4 Agarose gel electrophoresis

PCR reaction products were visualised on ethidium bromide stained 1-1.5% agarose gels. 300ml of 1xTAE buffer (40mM Tris-acetate and 2mM Na₂ EDTA x 2H₂O) and 3mg of agarose (Helena BioSciences) were heated until boiling point whilst continually stirring. The solution was then let to cool down to 60 °C at which point 0.5µg/ml ethidium bromide was added to each gel. The agarose was then poured onto the electrophoretic plate and left at room temperature to solidify. 20µl of secondary reaction were loaded onto each gel with 3µl of 6x FICOLL buffer together with pGEM marker (Promega) and the gel was fully immersed in 1x TAE buffer in the electrophoretic tank. The electrophoresis was run at 150 volts for 20-30min. The bands on the gel were visualised in a dark room on an ultra-violet transilluminator to visualise the bands. The gel was photographed using a Polaroid camera with Polaroid 667 film.

2.5.5 Procedure for purification of the PCR products

Purification of PCR products from amplification reactions was carried out using QIAquick PCR Purification Kit (Qiagen). DNA binds to the silica-membrane in the presence of 400µl of buffer PB (5 volumes) and 80µl (1 volume) of the PCR reaction, without removing the mineral oil. The QIAquick spin column was placed in a provided 2ml-collection tube. The sample was carefully applied to the centre of the column and centrifuged 30-60sec (Beckman Microfuge ETM). The flow through was then discharged. In the presence of a high concentration of chaotropic salts and a pH less than 7.5 DNA absorbs to the silica-membrane, whereas contaminants pass through the column. Pure DNA can then be eluted off with 10mM Tris-Cl buffer, pH 8.5

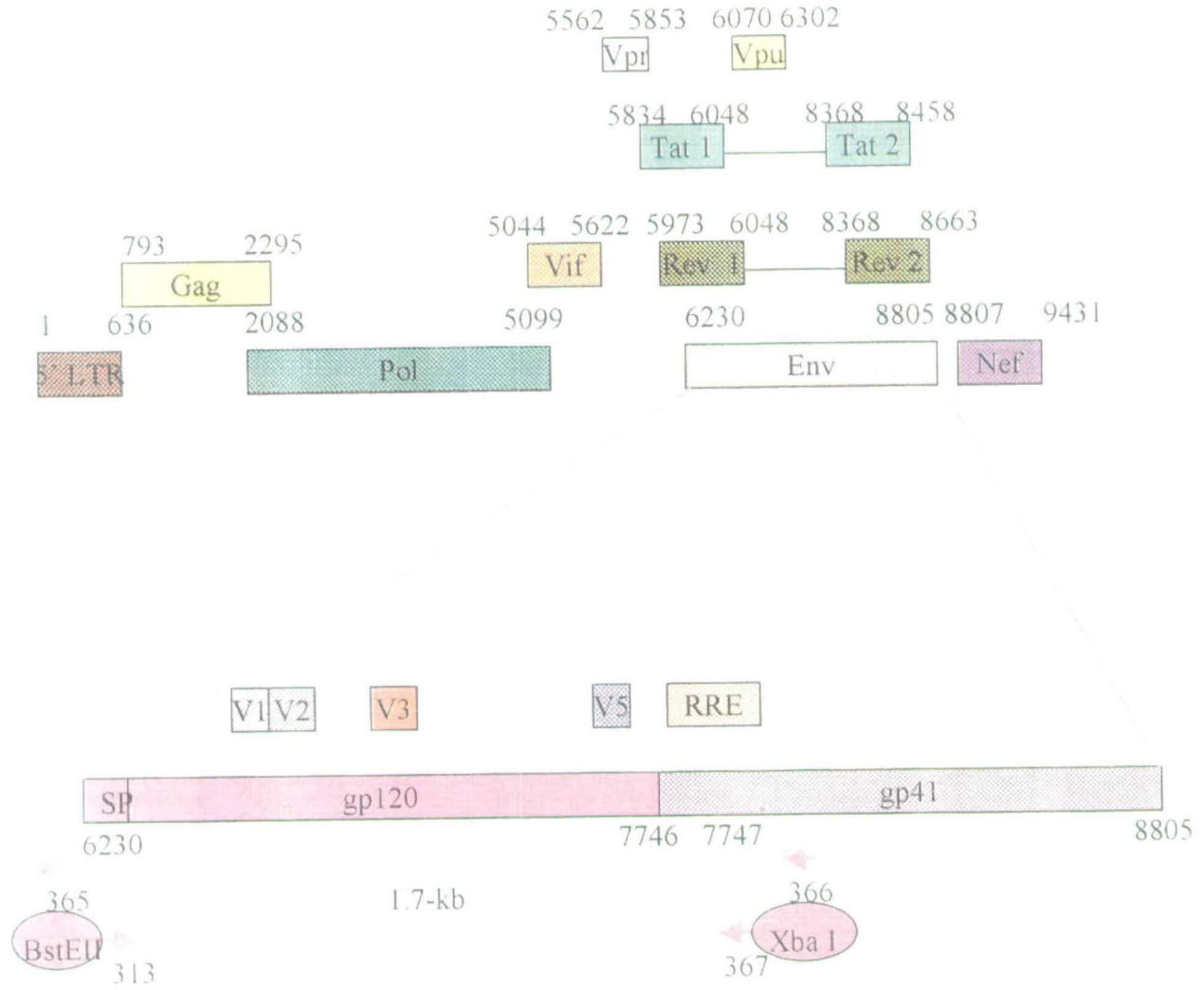
Table 2.3 Primers sequences and their co-ordinates

The table lists the primers used for PCR performed in V1/2 and V3 hypervariable regions and those used to amplify the gp120 gene. Figures showed in brackets refer to the primer co-ordinates in relation to the HIV-1 HXB2-D clone (Korber *et al.*, 1998). The restriction sites located within the primers are underlined.

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(1)	401	(+, 6540)	GAG GAT ATA ATC AG T TTA TGGG	SENSE (OUTER)	V1/V2
(2)	333	(-, 6945)	GTA CAT TGT ACT GTG CTG CTG ACA	ANTISENSE (OUTER)	V1/V2
(3)	402	(+, 6561)	GAT CAA AGC CTA AAG CCA TG	SENSE (INNER)	V1/V2
(4)	403	(-, 6855)	CAA TAA TGT ATG GGA ATT GG	ANTISENSE (INNER)	V1/V2
(5)	306	(+, 7009)	TGG CAG TCT AGC AGA AGA AG	SENSE	V3
(6)	308	(-, 7361)	AAT TTC TGG GTC CCC CTC CTG AGG	ANTISENSE	V3
(7)	365	(+, 6293)	GAT GTT GAT GAT CTG TAG	SENSE (OUTER)	gp120
(8)	366	(-, 8097)	ACT CCA TCC AGG TCG TGT	ANTISENSE (OUTER)	gp120
(9)	313	(+, 6327)	TGG <u>GTC ACC</u> GTC TAT TAT	SENSE (INNER)	gp120
(10)	367	(-, 8064)	CAT <u>CTA GAG</u> ATT TAT TAC TCC	ANTISENSE(INNER)	gp120





(Vogelstein *et al.*, 1979). Specifically, the QIA quick column was transferred into a clean 1.5ml-microfuge tube. DNA was eluted with 50µl buffer EB (10mM Tris-Cl, pH8.5) or for increased DNA concentration, 30µl elution buffer was added to the centre of the QIAquick column, it was allowed to stand for 1min and then centrifuged.

2.6 Cloning of the PCR products into pGEM T vector

2.6.1 Adding a 3'-A tail to the blunt end products

As mentioned previously, the thermostable proofreading Pfu polymerase generates blunt-end fragments during PCR amplification. PCR fragments obtained using this enzyme can be modified and ligated in pGEM T vector by adding 3'-A tail. 5µl of purified PCR fragment generated by polymerase was added to a 10µl mix containing 50mM Tris HCl pH 9.1, 16mM ammonium sulphate, 3.5mM MgCl₂ and 150µg/ml BSA, 0.2mM dATP, 5U of Taq Supreme (Helena BioSciences) and incubated at 70⁰C for 30min.

2.6.2 Cloning of A-tailed fragments into pGEM T vector

To achieve a high cloning efficiency, an insert-vector molar ratio of 3:1 was taken as optimal. The appropriate amount of PCR product (insert) taken into the ligation was estimated using the equation: ng of vector x size (kb) of insert/ size (kb) of vector x insert: vector molar ratio (i.e. 50ng of vector x 1.6kb:3kb x3:1=80ng insert). In order to accurately assess the performance of the ligation reactions 4ng of control insert DNA and 50ng of pGEM T vector were used in 10µl ligation reaction containing 10mM MgCl₂, 10mM DTT, 1mM ATP and 3U T4 DNA ligase.

To estimate the extent of background (i.e. blue colonies) that can arise due to the presence of non T-tailed or undigested pGEM vector, a background control was set up (50ng vector was taken in 10µl mix containing the same components as mentioned above). For standard ligation reactions, 80ng insert and 50ng vector were added into 10µl ligation reaction. All ligation tubes were mixed by pipetting and incubated overnight at 4⁰C.

2.6.3 Transformation of SURE cells

The ligation of fragments with a single-base overhang can be inefficient. For this reason, in order to obtain a reasonable number of colonies, cells, such as SURE cells, which have a high transformation efficiency of 1×10^8 cfu/ μ g DNA, were used. In addition, these cells carry lacI Δ M15, which is required in the blue/white colour screening process.

Falcon tubes containing 100 μ l of SURE competent cells were removed from -70 $^{\circ}$ C storage and placed on ice bath for 5min until they had just thawed. The cells were mixed by gently flicking the tubes. One positive control was set up for each experiment. SURE cells were transformed with 0.1ng uncut plasmid (pUC18) in order to calculate colony forming units (cfu)/ μ g DNA. 2 μ l of each ligation reaction was added to SURE cells. The cells were mixed by gently flicking the tubes and then placed on ice for 20 minutes. The cells were heat shocked for 45 seconds. After that, the tubes were returned to ice for 2 minutes. 950 μ l of room temperature LB medium were added to the tubes containing cells transformed with ligation reactions and 900 μ l of the same medium to the tube containing cells transformed with uncut plasmid. The tubes were incubated for 1.5 hours at 37 $^{\circ}$ C on a shaking platform (150rpm). 100 μ l of each transformation culture was plated onto LB plates with ampicillin/IPTG/X-Gal (LB supplemented with 0.5mM IPTG and 80 μ g/ml X-Gal was poured into the plates). For the transformation control, a 1:10 dilution with LB medium was made before 100 μ l of diluted ligation product were plated. The plates were incubated overnight (16-24 hours) at 37 $^{\circ}$ C. Next day, the white colonies were counted and transformation efficiency was calculated.

2.7 General manipulations during DNA cloning

2.7.1 Isolation of recombinant plasmid DNA

2.7.1.1 Small-scale plasmid DNA

Small amounts (e.g. less than 20 μ g) of DNA were obtained by alkaline lysis of bacteria cells (standard protocol) (Birnboim *et al.*, 1979). The pelleted bacterial cells were resuspended in 250 μ l of solution I (50mM Tris-Cl, pH 8.0, 10mM EDTA, 100 μ g/ml RN-ase). 250 μ l solution II were added followed by the inversion of the

tube several times. The lysis reaction was not allowed to exceed 5 min. 350µl of solution III (3M potassium acetate, pH 4.9) was used to precipitate the chromosomal DNA and proteins. The solution was mixed gently several times to avoid localised precipitation. The tubes were centrifuged 5 min at 13 000 rpm and the supernatant was transferred to a new tube. The precipitation of plasmid DNA was done with 2.5 volumes of absolute alcohol or 0.7 volumes of isopropanol. This was then centrifuged at 13000 rpm for 10min, and the resulting pellet was washed two times with 70% ethanol, dried and resuspended in 20-30µl of 10mM Tris-Cl, pH 8.0.

Plasmid DNA derived from recombinant clones were screened by three methods:

1. electrophoresis on 1-1.5% agarose gel using λ /HindIII as molecular marker and suitable negative and positive controls;
2. PCR reactions with primers designed based on the insert sequence;
3. digestions with appropriate restriction enzymes.

In experiments in which high quality plasmid DNA was required, a silica-gel membrane included in QIA prep Miniprep kits (Qiagen) was utilised.

The protocol for the first steps is identical to the one written beforehand (standard protocol). The supernatant obtained after the neutralisation step was applied to the QIA prep column by decanting or pipetting. The columns were centrifuged for 30-60 sec and then washed with 0.75ml of washing buffer (1M NaCl, 50mM MOPS, pH 7.0, 15% ethanol) and centrifuged again for 30-60 sec. An extra 1-min centrifugation step ensured that any residual wash buffer was not left in the column. The elution of plasmid DNA was done by adding 50µl of elution buffer (10mM Tris-Cl, pH 8.5) to the centre of the columns and spinning the tubes for 1min.

2.7.1.2 Large-scale plasmid DNA

Large amounts of plasmid DNA were obtained by using QUIAGEN-tip kits (QUIAGEN). A single colony was inoculated into 2ml of LB media containing ampicillin (100µg/ml) and grown for 8 hours. The miniculture was then diluted 1:100 to a final volume of 100ml for 12-16 hours (overnight). Next day, the culture was centrifuged at 4500 rpm for 10 min and the pellet resuspended in 4ml of buffer P1 (50mM Tris-HCl, pH 8.0, 10mM EDTA, 100µg/ml RN-ase A). When cell clumps

were no longer visible, 4 ml of buffer P2 (200mM NaOH, 1% SDS) were added and cells were left to lyse for 5min. The solution was neutralised by pipetting 4ml of chilled buffer P3 (3M potassium acetate, pH 5.5) and incubated on ice for 15 min to enhance the precipitation. The samples were centrifuged in non-glass tubes (Nalgene) at 20,000g for 30min at 4⁰C. The supernatant was subjected to another centrifugation step at 20,000g for 15min at 4⁰C. Meanwhile the QIAGEN-tip 100 columns were equilibrated by applying 4ml of buffer QBT (750mM NaCl; 50mM MOPS, pH 7.0, 5% ethanol, 0.15% Triton X-100). The buffer flowed through until the meniscus had reached the upper part of the column. The supernatant was applied to the QIAGEN-tip and allowed it to enter into the resin by gravity. The QIAGEN-tip were washed with 2x10 ml of buffer QC (1M NaCl, 50mM MOPS, pH 7.0, 15% ethanol) and plasmid DNA was eluted with 5ml of buffer QF (1.25M NaCl, 50mM Tris-HCl, pH 8.5, 15% ethanol) and precipitated with 0.7 volumes of isopropanol. After centrifugation at 15,000g for 30min at 4⁰C, the pellet was washed with 2 ml of 70% ethanol, air-dried and re-dissolved in 100µl of 10mM Tris-HCl pH 8.0 buffer.

2.7.2 Storage of plasmid DNA

Usually, plasmid DNA was stored in 10mM Tris-Cl, pH 8.0 at 4⁰C for several weeks. For long periods, samples were stored at -20⁰C or -70⁰C. However, the storage of plasmid DNA was preferred to the bacteria stock storage in order to avoid rearrangements that can occur during storage and revival of bacteria cells.

2. 7. 3 Digestion with restriction enzymes

Restriction digests of plasmid DNA employed enzymes such as: XbaI, XhoI, BstEII, NotI, Sall, SmaI, BHI (Promega, Stratagene or Boehringer). Digestions were carried out using 0.2µg DNA (when the purpose of the experiment was to check for new recombinants), or 5-10µg DNA (when the digestion product was used to set up a ligation reaction). Between 5-10U of enzyme (the amount of enzyme was calculated so that 1U could digest 1µg of DNA in 1 hour at the optimum concentration of that enzyme) and between 0.2µg and 5µg of DNA were added to 20µl final volume of reaction containing (1x) restriction enzyme buffer recommended by the manufacturer. The reactions were allowed to proceed for 0.5 to 2 hours at the

optimum temperature for each enzyme. A small volume from the digestion reaction (one tenth) was run on a gel to check for a complete digestion. When applicable, the enzyme was heat-inactivated (10 minutes at 65-70⁰C). Alternatively, the enzyme was removed by using QIA quick purification kit.

2.7.4 Filling in the recessed 3' tailed

To remove a restriction site, 5µg of completed digested plasmid were added into 20µl mix containing 15U of Klenow fragment of Escherihia coli DNA polymerase I (Boehringer), 1mM of each dNTP and 1x buffer (10mM Tris-Cl, 10mM MgCl₂, 1mM dithioerythritol). The reaction was incubated for 30 minutes at 37⁰C and the enzyme was inactivated with 2µl of 0.25M EDTA. The required DNA fragments were then purified using QIA quick purification kit.

2.7.5 Resolving the bands on agarose gel

After performing digestions, the reaction mix was loaded onto 1-% agarose gel and run at 25mA for 2 hours, to avoid damage to the ends. The required fragment was subsequently recovered from the gel by quickly cutting out the area around the band with a clean scalpel blade on a long-wave UV transiluminator (short-wave UV damage to the DNA must be minimised).

2.7.6 Purification of DNA from agarose slice

DNA extraction from agarose gel bands was done with GeneClean kit (Bio101). 3 volumes of NaI solution over the weight of the gel slice were added to keep the final concentration of NaI above 4M. The tubes were incubated in a water bath at 50⁰C until all the agarose had melted (about 5 minutes, mixing every two minutes).

The glassmilk suspension was resuspended by vortexing the vial for 1 minute. 5µl of glassmilk was added to the melted agarose solution (this is enough to extract 5µg DNA from 500µl of solution). For larger volumes of agarose containing solution, 10µl of silicagel was used. The mix was homogenised and incubated at room temperature for minimum 5 minutes, mixing the contents to ensure that the silicagel is always in suspension. This step allows an increased binding efficiency of the DNA

to silica matrix. The silicagel was pelleted by centrifugation for 5 seconds at full speed (approximately 14,000g). The pellet was then washed three times with 500µl New Wash Solution (provided by the kit) and pelleted by centrifugation as mentioned above. The tubes were left on the rack with the cap off for 5-10 minutes. Then the pellets were resuspended in a volume of Elution Solution (10mM Tris pH8.5) equal to that of the Glassmilk to ensure a good elution of the DNA. The silicagel was pelleted by centrifugation for about 30 seconds to 1 minute to make a solid pellet. The supernatant containing the eluted DNA was transferred in a new tube. Eventually, a second elution was done to recover additional DNA (less than 20% from the total amount of DNA). When necessarily, another centrifugation was carried out to ensure that any insoluble silica matrix remained in the eluate.

2.7.7 Oligonucleotides annealing and subsequent cloning of the polylinker

To discriminate between parental and new recombinants, polylinkers were subcloned into the vector. Table 2.4 describes the oligonucleotides used to assemble polylinkers.

Table 2.4. Polylinkers and the oligonucleotides from which they derive.

Polylinker	Oligonucleotides
BstEII/SmaI/XbaI	(+) 5' GTC ACC CCC GGG T3'
	(-) 5' CTA GAC CCG GGG3'
BstEII/SmaI/XhoI	(+) 5' GTC ACC CCC GGG C3'
	(-) 5' TCG AGC CCG GGG3'

The oligonucleotides synthesised by Oswell have neither a 3' nor 5' phosphate group. In order to improve the efficiency of ligation, they were phosphorylated at the 5'end. 15 pmoles of sense and antisense oligonucleotides were phosphorylated by setting up a 20µl reaction containing 2U of T4 polynucleotide kinase (Boehringer) in the presence of 1mM ATP, and 1x T4 polynucleotide kinase buffer (50mM TrisCl pH8.2, 10mM MgCl₂, 5mM EDTA, 5mM DTT and 0.1 mM spermidine) at 37⁰C for

30 minutes. Before annealing, the oligonucleotides were purified from enzymes, dATP and salt using QIA quick purification kit. The polylinkers were prepared as concentrates (10x) by heating the mix containing the phosphorylated sense and antisense oligonucleotides and 1x Taq polymerase buffer (Promega) at 90°C in a hot-block (Techne-DRI-BLOCK^R DB*2A). Once at 90°C, the metal block was removed and left on the bench to cool to room temperature, thus allowing the oligonucleotides to anneal. The subsequent ligation of the polylinker and the digested vector was set up in a 10µl mix in which the ratio of polylinker: vector was 1000:1.

2.7.8 Ligation

In all experiments that have been done to modify the ends of both the vector and insert, which were produced by restriction digestions, were compatible cohesive ends. To ligate them, a ratio vector: insert of 1:3 was generally applied. Ligation reactions were set up using 200ng vector in as small a volume as possible in two different ways. One way involved the usage of T4 DNA ligase (Promega) when 10µl mix containing vector:insert, 3U of T4 DNA ligase, 1x T4 DNA ligase buffer (30mM TrisCl, pH7.8, 10mM MgCl₂, 10mM DTT, 0.5mM ATP). The reaction tubes were incubated overnight (16 hours) at 4°C. The other way of doing ligation involved the utilisation of the Rapid ligation kit (Boehringer). 5µl of DNA (vector and insert) were added in 1x concentrated DNA dilution buffer (5x concentrated) together with 10µl T4 DNA ligation buffer (2x concentrated). The contents of the tubes were thoroughly mixed, then 1µl T4 DNA ligase was added and the solution was mixed again. The reaction was allowed to run for 5 minutes at room temperature. The ligation reaction mixture was used without heat inactivation of the T4 DNA ligase for the transformation of competent cells.

2.7.9 Transformation of competent cells. Transformation of DH5

Cells were removed from -70°C and left for 5 minutes on ice to thaw. 100µl of cells were added into each chilled 17x 100mm polypropylene tubes (Falcon 2054) on ice. For each transformation experiment, one positive control tube was set up in parallel with the experimental reaction to allow the efficiency of transformation to be certain.

For experimental ligation reaction, DNA was pre-diluted (1:5) in 10mM Tris-Cl, pH 7.5 and 1mM Na₂EDTA to increase the efficiency of transformation (Hanahan *et al.*, 1983, 1991). Usually, 1-2 µl of the dilution of the cells (1-10ng DNA) was added to the cells while dispensing. Tubes were gently tapped to mix; the cells were then incubated on ice for 30 minutes and heat-shocked for 45 seconds at 42⁰C, then placed back on ice for 2 minutes. 900µl of LB medium was added and the diluted cells were shaken at 225rpm at 37⁰C for 1/2-1 hour and 100-200µl spread on LB plates. The positive control was diluted 1:100 and 100µl of this dilution was spread onto LB plates. The plates were incubated overnight at 37⁰C. Next day, the colonies were counted on each plate. For a positive control, the transformation efficiency was expected to be minimum 10⁹ CFU/µg, while for experimental ligation reactions 10⁸ CFU/µg was normal.

Transformation of SURE cells - see Chapter 2.6.3.

2.7.10 Screening for recombinant clones

The colonies were picked in to 2ml LB (usually, 12 colonies per plate) and grown overnight in a shaking incubator at 37⁰C and 225rpm. Next day, a crude DNA miniprep was performed and a tenth of the resuspended DNA pellet was run on a 1-% agarose gel. The transformed colonies were discriminated from the previous one based on the difference of their mobility on the gel.

The second method employed was a restriction enzyme digestion of DNA clones. For the new recombinant clones, the band corresponding to the molecular weight of the insert should be present after digestion.

The third method used to ascertain the new recombinants was by performing one round of amplification with inner primers (131 and 167) using conditions mentioned in Section 2.5.3.

2.8 Automatic DNA Sequencing

Automated DNA sequencing was done by using the model 373 DNA sequencing system of Applied BioSystems Inc.

The method is based on the di-deoxy chain termination method of Sanger (Sanger *et al.*, 1988). In this study we employed dye-labelled dideoxynucleotides (dichloroRhodamine derived terminators), each of which emits a light at a different wavelength when excited by a laser that continually scans the gel while it is moving. The signal is transferred to the computer and is subsequently processed.

DNA sequencing involves the following steps:

- amplification of DNA by PCR,
- DNA sequencing,
- Data collection and analysis.

2.8.1 PCR amplification

Plasmid DNA used for DNA sequencing was obtained by QIA quick miniprep kit (Qiagen). 20µl of PCR reaction containing 8µl of Terminator Ready Reaction Mix (dye terminators, deoxynucleotides triphosphates, enzymes, MgCl₂ and buffer are premixed and ready to use), 200-500 ng double- stranded DNA, 3.2 pmoles of each primer and deionised water were spun briefly and 25 cycles of three segments: template denaturation at 96⁰C for 10 sec, primer annealing at 60⁰C for 4 minutes were performed. The PCR amplification was done using a Gene Amp PCR System 9600 or 2400. For each sequencing reaction, a 1.5ml microcentrifuge tube containing 2µl of 3M sodium acetate (NaOAc) pH 4.6, 50µl of 95% ethanol (EtOH) was prepared. The entire content of each extension reaction was pipetted into a tube of sodium acetate/ethanol solution and mixed thoroughly. The tubes were vortexed and placed on ice for ten minutes to precipitate the extension products. The tubes were spun in a microcentrifuge for 15-30 minutes at maximum speed. The supernatant was carefully aspirated with a pipette and discarded. The pellet was rinsed with 250µl of 70% ethanol, then spun for five minutes in a microcentrifuge at maximum speed. The supernatant was carefully aspirated and discarded and the pellet was dried in a

vacuum centrifuge for 1-3 minutes (until dry). Electrophoresis was done on the ABI PRISM 373 DNA Sequencer. First the pellet was resuspended in 4 μ l of 5:1 ratio of deionised formamide: 25mM EDTA pH8) with blue dextran (50mg/ml) and heated at 95^oC for two minutes (to denature the DNA) and then placed on ice until ready to load. 2 μ l of each sample was loaded into a separate lane of the gel.

2.8.2 Poly-acrylamide sequencing gel and DNA sequencing

Gel plates were cleaned thoroughly with detergent, rinsed with distilled water, dried and assembled. 3g urea, 0.5g Amber light resin, 20ml distilled water and 9ml of 40% Bis/Acrylamide stock solution (30g of 19:1 was dissolved in 48ml of H₂O, BIORAD), were dissolved and degassed by vacuum filtration through a 0.2 μ M cellulose acetate filter which was previously wetted with 6ml of TBE. 300 μ l of freshly prepared 10% Ammonium persulphate, and 33 μ l of TEMED (N,N,N',N'-tetramethylenediamine, EASTMAN KODAK COMPANY), were then added and the content was gently mixed, pouring into the gel plates and allowed to polymerase for 2 hours.

2.8.3 Data collection and analysis

An ABI data collection programme (ABI Inc) on a Macintosh computer was used to collect the data from the sequencing run. ABI sequence files were transferred to a SUN SPARC station computer via CAP (Columbia AppleTalk Package). Data was processed through a preliminary editor TED (which forms part of the Staden computer package, see below) using the Seqprocess script (written by Dr. Chris Wade, University of Edinburgh), then was introduced into the Xbap data base (Staden package) using the Seqedit script(Chris Wade, University of Edinburgh).

TED and Xbap are two programs that form part of the Staden Package (Roger Staden and Simon Dear, Cambridge). Sequences from both strands of DNA were aligned and contiguous sequences assembled within the Xpab program. The sequences were then edited by eye. The consensus sequence was output from Xpab and transferred to a VAX computer, where it was analysed using the GCG package. Sequences were collected and analysed by Macintosh Ici computer. Two programs were utilised: Data Collection and Analysis. Sample files created from the preliminary analysis of

data were transferred to a Sun SPARCstation computer and subsequently analyses using TED and Xbap.

2.9 Cell culturing

2.9.1 Continuous cell line

The cell line used in this study was COS-1. This is an adherent established kidney simian cell line transformed with the simian virus SV40 (Mellon *et al.*, 1981). These cells were a kind gift from Dr Natalia Gomez (ICAPB, University of Edinburgh). The cells have a single integrated copy of the complete early region of SV40 DNA and express large amounts of SV40 large T antigen.

2.9.2 Maintenance of the mammalian cell line

COS-1 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS, Advanced Protein Product Ltd, Brierley Hill, UK), 2mM L-glutamine (Hy Clone), 50U/ml penicillin (GIBCO BRL, Paisley, Scotland), 50µg/ml streptomycin (GIBCO).

One vial of frozen cells was rapidly incubated in a water bath at 37°C for several minutes to thaw the cells. The freezing medium was quickly removed by pipetting the content of the vial in a universal with 20µl of 1x PBS. The cells were spun down by centrifugation at 1,200rpm for 5 minutes. Another step of washing followed. Finally, the cells were resuspended in 5ml complete medium and seeded into a 25cm³ flask and let grow to become semiconfluent. Culture was split 1:3 using 0.02N EDTA pH7.00 and distributed in 15mls complete medium in 75cm³ flask.

2.10 Transfection of adherent cells

Transfection of COS cells requires preparation of small amount of DNA (less than 20µg of sufficient purity). This can be achieved by caesium chloride gradient centrifugation or using a conventional DNA purification column. Due to the fact that caesium chloride gradient centrifugation is a laborious method, QIA prep columns supplied by QUIAGEN were used. QIA prep columns use a silica-gel membrane for

selective adsorption of plasmid DNA in high salt buffer and elution in low salt buffer. RNA, cellular proteins and metabolites are not retained on the membrane but are found in the flow-through. The purified DNA is ready for immediate use in a range of applications such as transfection or automated DNA sequencing. The detailed protocol was given in Section 2.7.1.1.

2.10.1 Transfection with Lipofectin

In a six well plate, 1×10^5 cells were seeded in 2ml of the appropriate growth medium supplemented with serum. The cells were incubated at 37°C , in a CO_2 incubator until the cells were 40-60% confluent. For COS cells this usually takes 24 hours. Two types of solutions were prepared in Falcon 2054 tubes (Becton Dickinson):

- Solution A: 1-2 μg of DNA were diluted into 100 μl serum-free medium. OPTI-MEM^{RI} Reduced Serum Medium,
- Solution B: 5 μl of Lipofectin Reagent were diluted into 100 μl serum-free medium (OPTI-MEM^{RI} Reduces Serum Medium), and allowed to stand at room temperature for 1 hour in order for the liposomes to assemble.

The two solutions were combined, mixed gently and incubated at room temperature for 15 minutes. For each tube, 0.8 ml of serum-free medium were added and the tubes were tapped gently and added into the pre-washed cells (the cells were washed with 2ml of serum-free DMEM). The transfection was allowed to carry on for 5 hours at 37°C in a CO_2 incubator. Then the liposome-DNA complex containing medium was replaced with 2ml of completed medium and the cells were incubated at 37°C in a CO_2 incubator for 72 hours.

For 24 well plates, 1 μg DNA was added in each well seeded with $0.4\text{-}0.8 \times 10^5$ cells. The ratio DNA:Lipofectin used was also 1:5.

Lipofectin reagent (GIBCO, Life Technologies) is a 1:1 liposome formulation of the cationic lipid N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA) and dioleoyl phosphatidylethanolamine (DOPE). This positive charge and neutral lipids from liposomes can complex with nucleic acids. The lipid-nucleic acid complex, when applied to culture cells, facilitates the uptake of the nucleic acid into

the cells. Liposome mediated transfections are by 5 to 100-fold more efficient than calcium phosphate or DEAE-dextran transfected methods (Felgner *et al.*, 1987).

2.10.2 Transfection with SuperFect Transfection Reagent

SuperFect Transfection Reagent is an activated dendramer that possesses a defined spherical architecture, with branches radiating from a central core and terminating at charged amino-groups. SuperFect Reagent assembles DNA into compact structures, optimising the entry of DNA into the cells. The overall charge of SuperFect-DNA complexes is positive thereby allowing these complexes to bind to negatively charged receptors (e.g. sialylated glycoproteins) on the surface of eukaryotic cells. Inside the cells, the SuperFect Reagent buffer inhibits the lysosomal nucleases by stabilising the SuperFect-DNA complexes and enabling the transport of intact DNA to the nucleus.

The day before transfection, $2-8 \times 10^4$ cells were seeded into each well of 24 well plates in 1ml of completed DMEM and incubated at 37°C and 5% CO_2 in an incubator. $1\mu\text{g}$ of DNA dissolved in TE, pH 7.4 was diluted to a total volume of $150\mu\text{l}$ with cell growth medium containing no serum, proteins or antibiotics. The solution was mixed and centrifuged. $6\mu\text{l}$ of SuperFect Transfection Reagent was added and the mix was vortexed for 10 seconds. The samples were incubated for 5-10 minutes at room temperature (25°C) to allow complex formation. The growth medium from the 24 wells plate was gently aspirated and the cells were washed with 4ml PBS. The solution was mixed by pipetting up and down twice and then transferred immediately to the cells in the plate. The cells were incubated with complexes for 2-3 hours at 37°C and 5% CO_2 . Then, the medium containing the remaining complexes was removed from the cells by gentle aspiration. The cells were washed once with 4ml of PBS. Fresh medium containing serum and antibiotics was added and the transfected culture was maintained for 72 hours. Cells were then harvested and their supernatants collected and assayed for gene expression.

2.10.3 Transfection with Effectene Transfection Reagent

Effectene Transfection Reagent consists of non-liposomal lipids that together with a specific DNA-condensing Enhancer and buffer, produces high transfection efficiencies. Firstly, DNA is condensed by interaction with the Enhancer in a defined buffer system. Effectene Reagent is then added to the condensed DNA to produce condensed Effectene-DNA complexes. The Effectene-DNA complexes are mixed with medium and directly added to the cells.

One day before transfection, $2-8 \times 10^4$ cells were seeded per well. The cells were incubated at 37°C and 5% CO_2 in an incubator until they reached 40-60% confluence. $1\mu\text{g}$ of DNA dissolved in TE, pH 7.4 was diluted with DNA condensation buffer to a total volume of $150\mu\text{l}$. $8\mu\text{l}$ of Enhancer were added and the solution was mixed by vortexing for 1 second and incubated for 5 minutes at 25°C . $25\mu\text{l}$ of Effectene Transfection Reagent were added to the DNA-Enhancer mixture and the tubes were vortexed for 10 seconds. The sample was incubated for 10 minutes at room temperature in order to allow complex formation. The growth medium from the plate was gently aspirated and the cells were washed once with PBS. 1ml of complete growth medium was added to the reaction tube containing the transfection complexes. The contents were mixed by pipetting up and down twice and then transferred to the transfection complexes drop-wise into the wells of a 24 wells plate. The dish was gently swirled to homogenise the solution. The cells were incubated with the complexes at 37°C and 5% CO_2 for 72 hours when the cells were harvested and assayed for gene expression.

2.11 Assessment of gp120 gene expression

2.11.1 gp120 ELISA

2.11.1.1 Preparing ELISA plates

Immunolon II Microtitre plates (DYNATECH) were used for gp120 ELISA. Lyophilised D7324 (sheep polyclonal, Aalto BioReagent was resuspended in 2ml of distilled ultrapure water to give a concentration of 1mg/ml . This was diluted with 100mM NaHCO_3 pH 9.6 to a concentration of $2\mu\text{g/ml}$. $100\mu\text{l}$ of D7324 ($2\mu\text{g/ml}$)

were distributed in each well of a microtitre plate with a P200 multichannel pipett and incubated overnight at room temperature. Next day, the plates were washed four times with wash buffer (0.05% Tween 20 in TBS). Then the plates were blocked with 100µl per well of 2% BSA in TBS for 1 hour at 37⁰C. The blocking solution was removed by washing four times with wash buffer. Plates were then kept at 37⁰C until dry. The plates were wrapped in plastic bags containing silica gel sachets and stored at -70⁰C until required. Before using, plates were left on the bench to equilibrate to room temperature.

2.11.1.2 gp120 ELISA-main protocol

Three recombinant gp120s have been used in this study (IIIB, MN, SF2). Master solution (200µg/ml) was diluted with 0.1% Empigen in TBS to a concentration of 0.2µg/ml. A 2 fold serial dilution from this concentration was performed (100, 50, 25, 12.5, 6.25, 3.17, 1.65 ng/ml) and distributed in duplicates for each experiment. Also, a zero (in which all the reagents were missing but the substrate), a blank (in which recombinant gp120 was omitted) and the supernatants obtained after transfection were set up and incubated for 1 hour at 37⁰C. The plates were washed four times with wash buffer. Anti gp120 monoclonal antibody GP13 (ARP 3054) was diluted 1:25 with wash buffer supplemented with 1% BSA to an intermediate concentration of 40µg/ml. Aliquots of this concentration were kept at -70⁰C. Each aliquot was diluted to the working concentration of 200ng/100µl (2µg/ml). 100µl of this concentration were added in each well and incubated 30 minutes at 37⁰C. Then the plates were washed four times again with wash buffer. 0.4mg of lyophilised biotinylated F(ab')₂ Goat anti-human Ig (Zymed) were dissolved in 1ml of distilled water to a concentration of 0.4mg/ml, aliquoted and kept at -70⁰C. This stock was further diluted 1:1000 with wash buffer to give a working concentration of 0.4µg/ml. 100µl of this dilution were added per well and incubated 30 minutes at 37⁰C. The plates were washed four times with wash buffer. ExtrAvidin alkaline phosphatase (Sigma E-2636) was diluted 1:1000 in 1x TBS and 100µl were incubated in each well for 30 minutes at 37⁰C. Four time wash steps removed ExtrAvidin alkaline phosphatase. The substrate used was SIGMA FAST pNPP (N-1891 or N-2770). The

substrate was prepared by dissolving one tablet substrate N-1891 and one Tris buffer tablet in 5ml distilled water or 1 tablet substrate N-2770 and one Tris buffer tablet in 20ml distilled water. The tube that contained ready to use substrate was covered with tin foil and vortexed until dissolved and kept in darkness. The reaction was developed with the addition of 100 μ l pNPP for 20 minutes in a dark room and then stopped by addition of 100 μ l of 0.4N NaOH. The absorbance value (OD) of each sample was read with a Labsystems Multiskan Bicromatic Reader using the filter corresponding to the wavelength of 405 nm. Data processing was done with GENESIS computer program (GENESIS II Windows TM, Based Microplate Software, Labsystems).

2.11.2 Immunofluorescence assay

Monoclonal antibodies used to stain the cells for gp120 glycoprotein expression were: GP13 (ARP 3054, human MAb to HIV-1 gp120, which recognise an epitope within or topographically near to the CD4-binding site of gp120), GP68 (ARP 3055, human MAb to HIV-1 gp120, which recognise an epitope within or topographically near to the CD4-binding site of gp120), IgG1 b12 (ARP 3065, human MAb to HIV-1 IIIB gp120, is mapped to the CD4 binding site of gp120). After transfection the cells were removed by cell scraping (Greiner disposable cell scraper) and washed twice with PBS. The cells were pelleted by centrifugation at 1200rpm for 5 minutes and resuspended in 100 μ l of paraformaldehyde (this preserves the shape of the cells) and kept for 30 minutes at 4⁰C. 10 μ l of this suspension were dotted onto each well of the slide (Hendley-Essex Multispot microscope slides, PTFE and specialised coatings, PH-001) and air-dried for one hour in the laminar flow hood. The cells were then fixed by immersion in methanol:acetone solution (1:1) for 10 minutes at room temperature and then air-dried. The monoclonal antibody anti-gp120 was used in a dilution of 1:100 in normal sheep/goat serum (to decrease the non-specific backgrounds). 10 μ g of these solutions were dropped in each well and the slide was covered with tin foil and incubated for 1 hour, after which time the slides were washed four times with 1x PBS. Biotinylated goat anti-human IgG (Zymed) was diluted 1:50 and 10 μ l were added per well and incubated for 30 minutes. Then, the

slides were washed again three times with 1x PBS. Finally, 10 μ l - Streptavidin Fluorescein Isothiocyanate conjugate (StreptAvidin^{FITC}, Dako) diluted 1/150 were dotted into each well and incubated for 30 minutes. Slides were then washed four times with 1x PBS. Mounting medium (Dako) was added to each well along with a coverslip (22x64mm borosilicate cover glass, thickness no.1, BDH). The slides were examined by fluorescence microscopy (Nikot Optiphot microscope) with water lens immersion. The positive cells appeared green-fluorescent.

2.12 Detection and quantification of patients' sera IgG antibodies against patients-derived gp120s

Detection and semi-quantification of sera antibodies were done using the same ELISA assay presented in section 2.11.1, with some modifications: recombinant gp120s were substituted by clones expressing gp120 glycoproteins and Goat F(ab)₂ anti-human IgG with patient polyclonal antibodies (diluted 1:400).

ELISA first quantified gp120 glycoproteins secreted in the supernatants. Regardless of the clone from which they derived, the same amount of glycoprotein was used per well which were then recognised by the patients IgG antibodies which were present in sera (these were diluted 1/400). For each experiment eight replicates of a negative pool were worked in parallel. ODs for samples and all negative replicates were read at 405nm. A cut-off value was calculated based on the formula: the average OD (negatives) + 3 x standard deviations. The ratio between the sample OD and cut-off value gives the reactivity for that sample.

CHAPTER 3

A SENSITIVE ELISA FOR THE DETECTION AND QUANTIFICATION OF THE GP120 SURFACE GLYCOPROTEIN OF HIV-1

3.1 Introduction

3.1.1 The principle of the gp120 ELISA

3.1.2 Methodology

3.2 Results

3.2.1 ELISA assay employing F(ab)₂ anti-human IgG-biotin

3.2.1.1 AMPAK amplification system

3.2.1.2 Horseradish peroxidase (HRP)

3.2.1.3 Alkaline Phosphatase (AP)

3.2.1.3.1 Quantitative immunoassay

3.2.1.3.2 Statistical analysis and data interpretation

3.2.2 Optimisation of the "in house" gp120 ELISA

3.2.2.1 Titration of the capture antibody (D7324)

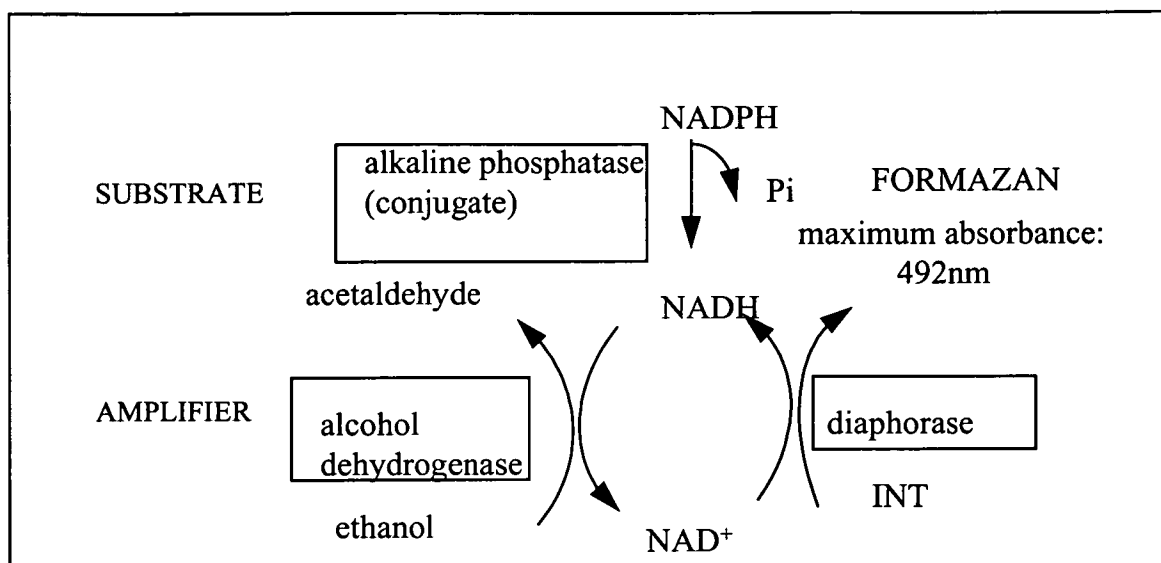
3.3 Summary of results

3.1 Introduction

The purpose of this study was to investigate the extent of recognition of gp120 protein obtained from HIV-1 infected individuals by the anti-gp120 IgG antibody from autologous and heterologous sera. In order to fulfil this aim, an “in house” ELISA method was set up to allow both the quantification of gp120 from the supernatants and the level of anti-gp120 IgG antibody from the patients derived sera. This method was based on that established by J. Moore (Moore *et al.*, 1988) that allows rapid detection of gp120 at a level of detection of 100-1000 pg per sample of transfection supernatant or estimation of gp120 in sera from HIV-1 infected individuals.

3.1.1 The principle of the gp120 ELISA

The method is very sensitive because involves a cascade amplification consisting of the sequential activation of enzymes ultimately leading to a response that is many orders of magnitude greater than the initial triggering event. A series of enzymes generates molecules such as cofactors that activate another reaction giving rise to a detectable product. One of these enzymes, alkaline phosphatase (AP), is suitable for use as a label in immunoassays, being readily available, quite stable, and having a relatively high specific activity. The enzyme is covalently bound to an antibody and acts directly on a substrate, which, in the AMPAK amplification system, is nicotinamide adenine dinucleotide phosphate (NADPH). NADPH is dephosphorylated by alkaline phosphatase (AP) to the reduced form nicotinamide adenine dinucleotide, (NADH). The interconversion of NADH and its oxidised form (NAD⁺) is catalysed by dehydrogenase and diaphorase, respectively. For each cycle, one molecule of formazan is produced, having a maximum of absorption around 492nm. Colour development increases in proportion to the length of the amplifier incubation time so that the initial signal that comes from the primary enzyme is multiplied many times and an amplification of 100-fold may easily be achieved. The cascade of enzyme amplification by the NAD-activated redox reaction is shown in the diagram below:



3.1.2 Methodology

The method uses the D7324 antiserum as a capture antibody. D7324 was produced by immunizing sheep with a synthetic peptide that has the following amino acid sequence: APTKAKRRVVQREKR. This sequence corresponds to amino acid number 497-511 in the envelope gene gp120 protein of the BH-10 strain of HIV-1 (Ratner *et al.*, 1985). D7324 antibodies were adsorbed onto Immunolon II microtitre wells (Dynatech Ltd) by incubation overnight at room temperature in 100µl per well of 100mM NaHCO₃, pH9.6. The wells were washed twice with TBS and blocked for 30 min with TBS solution containing 2% non-fat milk powder (Marvel, Cadbury Ltd) and then washed again with TBS. Recombinant gp120 IIIB was added to the wells in two fold dilutions in TBS containing 1% Empigen (alkyl dimethyl amine betaine) and incubated for 1h at room temperature. Unbound protein was removed by washing twice with TBS. Captured gp120 was detected by the addition of a second human biotinylated antibody anti-gp120 (GP13: ARP 3054), which was incubated for half an hour at room temperature. After another washing step with TBS + 0.05% Tween 20, 100 µl antihuman IgG -AP in TBS with 4% non-fat milk powder and 0.5% Tween 20 were added and incubated for 1 hour at room temperature. The unbound IgG-AP was removed by washing the wells six times with AMPAK wash buffer and the bound IgG-AP was detected with the AMPAK II ELISA amplification system using 50µl per well of substrate solution for 1 h followed by 50µl amplifier solution

for 5min. The reaction was stopped with 50 μ l of 0.5M HCl and the absorbance was read at 492nm.

3. 2 Results

3.2.1 ELISA assay employing biotinylated F(ab')₂ goat anti-human IgG (gamma chain specific)

3.2.1.1 AMPAK amplification system

The attempt to set up ELISA assay as described by Moore *et al.* (1986) failed because of high absorbance values recorded when D7324-gp120 complex was reacted with anti-human IgG-AP ($r = 1.92$ vs. $r_{\text{blank}} = 0.07$). These increased values suggested specific interactions between anti-human IgG-AP and capture antibody D7324. In order to reduce the interaction between anti-human IgG-AP and D7324, the anti-human IgG-AP was substituted with biotinylated F (ab) ₂ goat anti-human IgG (gamma chain specific) antibodies. The biotinylated F (ab') ₂ goat anti-human IgG (gamma chain specific) was obtained by conjugating the affinity purified F (ab') ₂ goat anti-human IgG (gamma chain specific) antibody with aminohexanoyl biotin N-hydroxysuccimide. The lyophilised powder was reconstituted with 1ml of distilled water to give a concentration of 0.4 mg/ml. ExtrAvidin-Alkaline Phosphatase used in conjunction with the biotinylated F(ab')₂ anti-human IgG, was prepared from egg white avidin, combining the high specific activity of avidin with the low background staining of StreptAvidin, a biotin binding protein produced by the bacteria *Streptomyces avidinii*. The protocol employed was similar to the previously mentioned protocol (see 3.1.2) with some modifications. After binding of gp120 and anti-gp120 antibody, biotinylated F(ab')₂-anti-human IgG (1:1000) was added and incubated for 30 min at 37⁰C. ExtrAvidin-AP (1:1000) was added for 30min at 37⁰C. The AMPAK amplification system was employed as mentioned before (substrate 20min, amplification 10min). The results are presented in the Table 3.1. For ELISA with the biotinylated F (ab') ₂ anti human IgG, ExtrAvidin-AP and AMPAK amplification system, a linear regression was obtained in the dose dependent curve

Table 3.1 Data obtained for ELISA assay when biotinylated F (ab') 2 anti-human IgG, ExtrAvidin-AP and AMPAK system were used.

gp120(ng/ml)	Status	OD492nm (detailed value)	OD492nm (calculated value)	CV (coefficient of variation)
0.5	median	1.1	0.12	1.08
1	median	1.24	0.26	2.64
2.5	median	1.38	0.4	3.5
5	median	1.56	0.57	12.2
10	median	1.71	0.73	2.85
20	median	2	0.9	2.32
40	median	2.15	1.1	4.5
80	median	2.15	1.17	2.99
160	median	2.15	1.17	4.1
Blank 1	median	0.27		3.6
Blank 2	median	0.26		5.2
Blank 3	median	0.28		4.3
Blank 4	median	0.98		8.6

Footnote: $OD_{405nm}(\text{calculated value}) = OD_{405nm}(\text{detailed value}) - OD_{405nm} \text{ Blank } 4$

The blanks used in table above are explained in the table below:

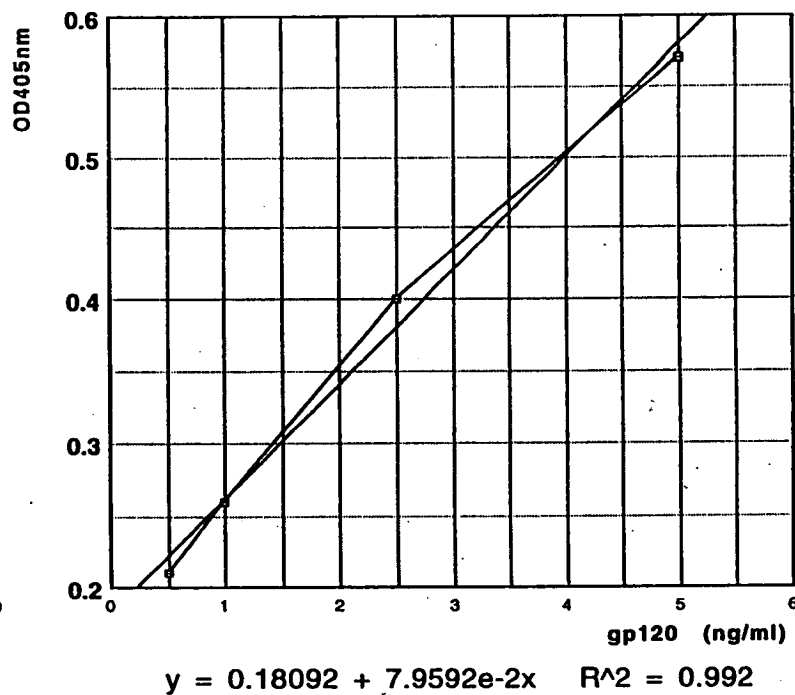
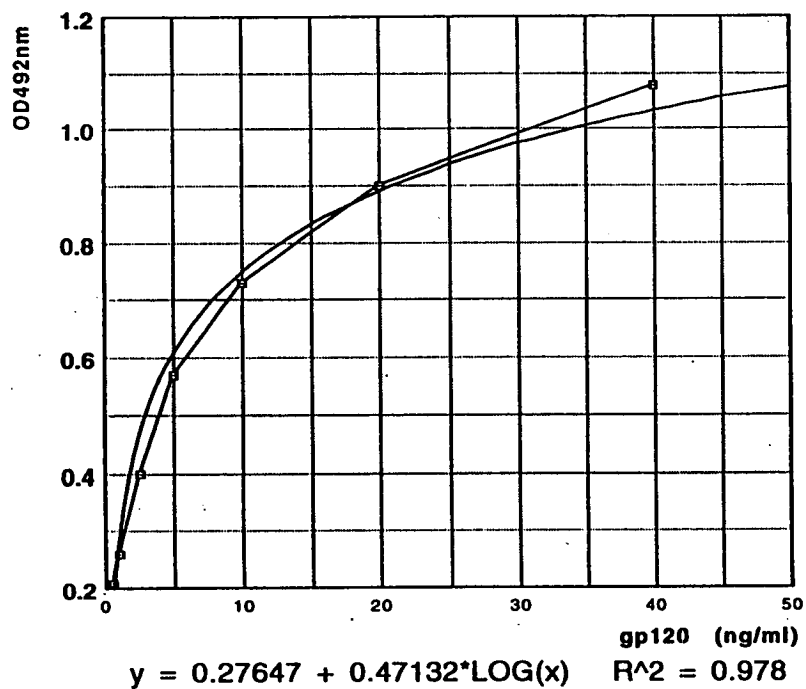
Blank	gp120	anti-gp120	F(ab)2 anti-human IgG-Biotin
Blank 1	-	-	-
Blank 2	+	-	-
Blank 3	-	+	-
Blank 4	-	-	+

for a range of protein concentration of between 1-5ng/ml (see Figure 3.1). As one may see in the Figure 3.1, the saturation point was reached for a concentration of the glycoprotein of 40ng/ml. This low saturation point may be inconvenient for quantifying the gp120 from transfection supernatants, because many dilutions of the supernatants would have to be done in order to record an absorbance that is in the linear part of this curve. Therefore, the amplification system was changed with a detection system. In this system, the cascade of amplification is replaced with the specific interaction between biotinylated F(ab)₂ anti-human IgG and Avidin labelled enzyme, with subsequent development of the colour. Two experiments were run to test if the nature of the enzyme influences the sensitivity of the assay and if so, to find what enzyme gives sensitivity suitable for the aim of this study.

3.2.1.2 Horseradish peroxidase (HRP)

HRP is a relatively cheap and widely available enzyme that has a high turnover rate. A large number of chromogens are commercially available that may be used with the HRP. Most commonly used are orthophenylene diamine (OPD), 2, 2-azino-di (3-ethylbenzothiazoline-6-sulphonate) (ABTS), 5-aminosalicylic acid (5-AS), and 3, 3', 5, 5'-tetramethylbenzidine hydrochloride (TMB). StreptAvidin-Peroxidase was chosen because it is easy manipulated and their carcinogen effect is minimised. The principle of the ELISA assay employing HRP as a substrate is similar to that presented in Section 3.1.2. After incubation with biotinylated F (ab')₂ anti-human IgG, the wells were washed and 100µl per well of StreptAvidin-HRP (1: 1000 diluted in PBS) was added for 30min at 37⁰C. The plates were washed four times and 100µl/well of OPD (1x 20mg tablet of OPD to 50ml substrate buffer and 100µl hydrogen peroxide) were added and the plate incubated at room temperature for 30min.

Figure 3.1 Standard curve for ELISA employing biotinylated F(ab')₂ anti-human IgG, ExtrAvidin-AP and AMPAK



The reaction was stopped by adding 25µl per well of 3.4% H₂SO₄ solution and the absorbance values read at 492nm. The results obtained are presented in Table 3.2 and the plot of OD versus concentration of the glycoprotein is shown on Figure 3.2. In this particular ELISA assay, the linearity was obtained between 200-1000 ng/ml of gp120 glycoprotein, with the saturation point occurring around 1000ng/ml. This range of sensitivity may be suitable for quantifying the glycoprotein from supernatants and this ELISA was the first one that was taken into account as the best assay. Subsequently, HRP was substituted with AP to test if the sensitivity of the new method makes it a better candidate.

3.2.1.3 Alkaline Phosphatase (AP)

3.2.1.3.1 Quantitative immunoassay

Like HRP, AP is a good enzyme label for ELISA. Although it is expensive, it has the advantage of employing para-Nitrophenyl phosphate (p-NPP), which is a stable, non-mutagenic substrate. An experiment employing AP and p-NPP was performed to compare its sensitivity with that obtained for HRP and OPD. After incubation of biotinylated F(ab')₂ anti-human IgG (1:1000) and ExtrAvidin-AP (1:1000), the wells were washed and 100µl of pNPP (1 tablet N-1891 and 1 Tris buffer tablet dissolved in 5mls water) was added per each well and incubated for 60min at room temperature. The reaction was stopped by addition of 50µl 4N NaOH and the absorbance read at 405nm.

The method gives absolute quantitative results (weight per volume units of the tested sample) by means of a pre-calibrated standard curve. The response of the test sample is converted into a dose estimate by homologous interpolation. The homologous interpolation involves the use of a standard containing the homologous or identical reagent being measured in the test sample.

As a homologous standard, rgp120 IIIB glycoprotein was used in a serial dilution (i.e. two replicates per each dilution). The standard data points are fitted in a dose response curve (i.e. OD vs. gp120 concentration). The results obtained are presented in Table 3.3 and the plot OD vs. concentration of gp120 is shown in Figure 3.3.

Table 3.2. The data obtained for quantification of gp120 using biotinylated F(ab')₂ anti-human IgG and Avidin-HRP/OPD as a detection system.

gp120 (µg/ml)	Status	OD492nm (detailed values)	OD492nm (calculated values)	CV
12	mean	0.37	0.44	14
6.4	mean	0.36	0.431	3
3.2	mean	0.335	0.405	2.8
1.2	mean	0.434	0.364	8.3
0.6	mean	0.215	0.285	21
0.4	mean	0.306	0.236	29
0.2	mean	0.233	0.163	5.6
0.1	mean	0.207	0.137	24.2
Blank 1	mean	0.068		
Blank 2	mean	0.069		
Blank 3	mean	0.071		
Blank 4	mean	0.07		

Footnote: OD492nm (calculated value) = OD492nm (detailed value) - OD492nm Blank

The blank controls used in the experiment are presented in table below:

Blank	gp120	anti-gp120	anti-human IgG Biotin
Blank 1	-	-	-
Blank 2	+	-	-
Blank 3	-	+	-
Blank 4	-		

Figure 3.2. Standard curve that allows estimation of the concentration of gp120 protein when biotinylated F(ab')₂ anti-human IgG and Avidin-HRP was used as a detection system

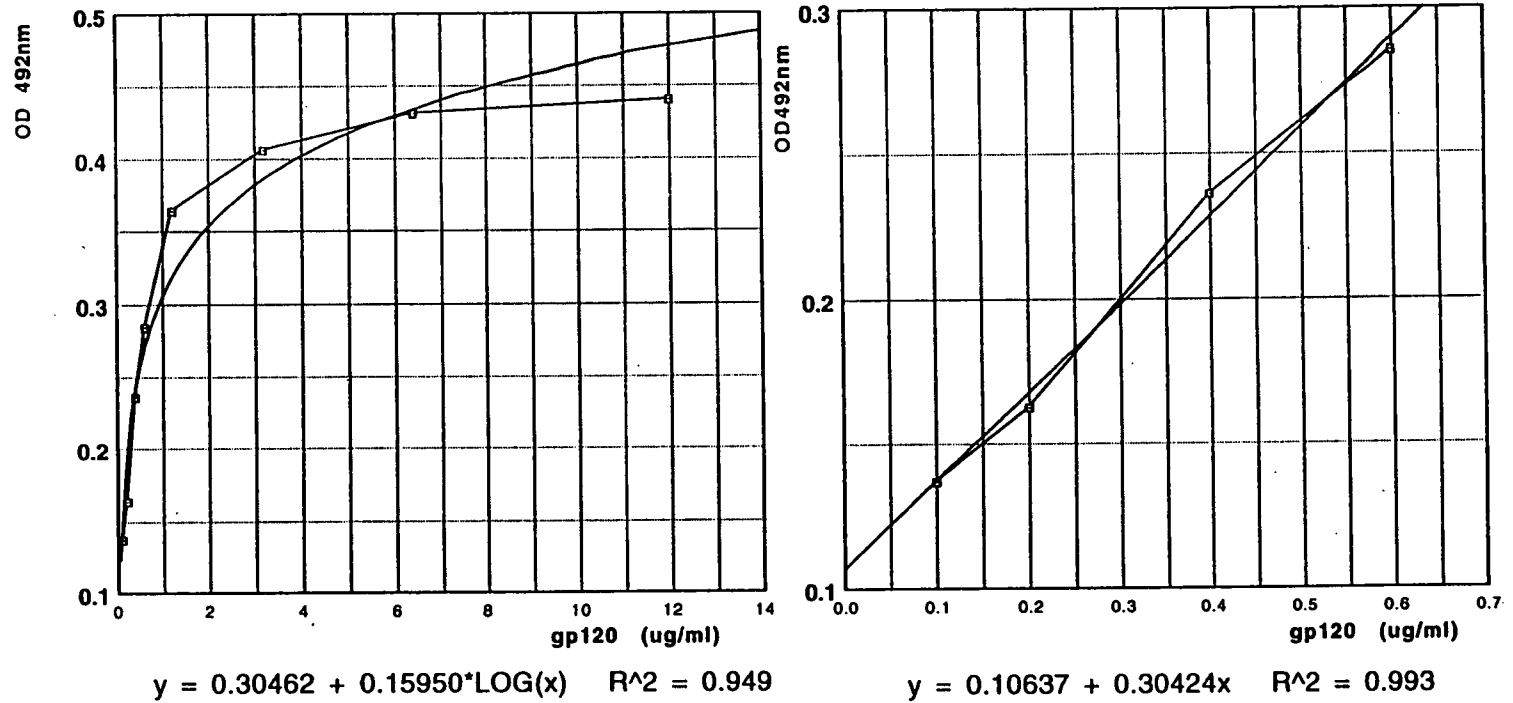


Table 3.3 The data obtained for the standard curve obtained when biotinylated F(ab')₂ anti-human IgG, Avidin-AP and pNPP were used as a detection system.

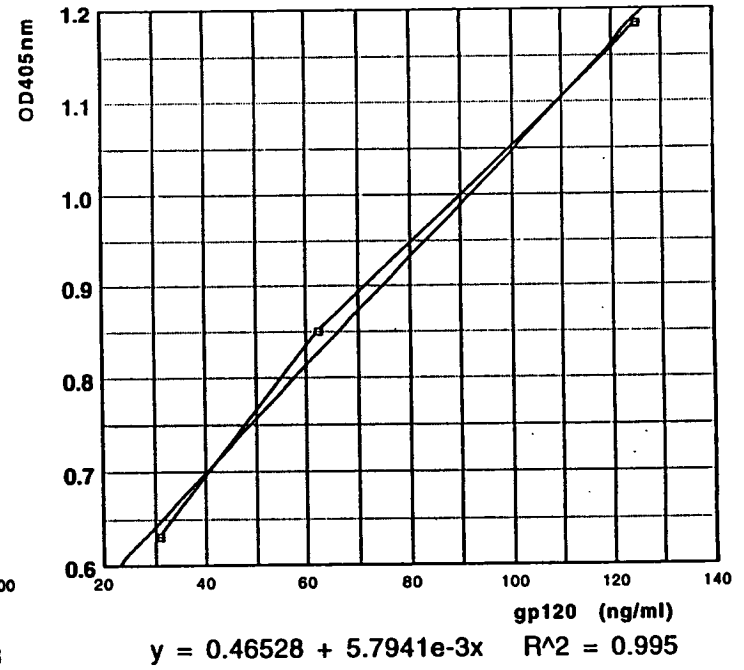
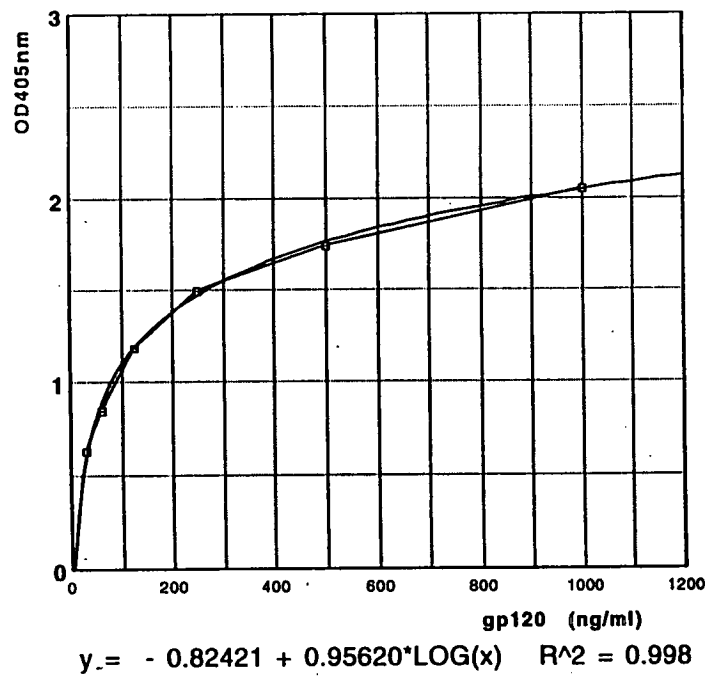
rgp120IIIB (ng/ml)	Status	Absorbance 405nm (detailed values)	Absorbance 405nm (calculated values)	% CV
31.2	mean	0.763	0.631	12%
62.5	mean	0.85	0.719	20%
125	mean	1.31	1.182	28%
250	mean	1.62	1.495	29%
500	mean	1.88	1.74	21%
1000	mean	2.19	2.05	20%
Zero		0.096	1.88	
Blank		0.145	2	

Footnote: CV refers to the coefficient of variation

The zero and blank controls which were used are shown in table below:

Blank	gp120	anti-gp120	F(ab') ₂ anti human IgG-Biotin	Avidin-AP	pNPP
Zero	-	-	-	-	+
Blank	-	+	+	+	+

Figure 3.3. Standard curve for quantification of the gp120 concentration when biotinylated F(ab')₂ anti-human IgG and Avidin-AP were used as a detection system



Linearity was obtained for this particular ELISA assay for a range of concentration of gp120 between 10 and 200ng/ml. The sensitivity of this method was between that which employed AMPAK amplification system and that that used OPD-HRP as a detection system and, therefore, more suitable for quantification of gp120 glycoprotein from transfection supernatants. The magnitude of the response (i.e. OD value) of the supernatant sample can be interpolated from the standard dose-response curve, producing a concentration of the gp120 glycoprotein expressed as a weight per volume units. When the supernatant samples were tested in several dilutions, the interpolated dose from each dilution, multiplied by their dilution, gave the same concentration within the reasonable statistical error (less than 10% inter-dilutional coefficient of variation). This result attests the accuracy of the “in house ELISA” developed in this study.

3.2.1.3.2 Statistical analysis and data interpretation

Variability of a method can be statistically assessed by testing at least two dilutions of the appropriate reference reagent to cover the upper, middle and bottom parts of the standard. The coefficient of variation of the inter- and intra - assay can be measured using the following equation:

Coefficient of variation (CV) = standard deviation (STDEV) / mean X 100

A good accuracy is given by a CV value less than 20. To test the intra-assay and inter-assay variability of the method, several concentrations of the recombinant glycoprotein gp120 IIIB were used (0.01, 0.05, 0.1, 0.5, 1, 5µg/ml) to obtain a standard curve. This curve was used to estimate the amount of gp120 glycoprotein from a series of diluted supernatants (non-diluted, 1/2, 1/4, 1/16, etc.), which were obtained after transfection of COS cells with HXB2-MCS. HXB2-MCS is an infectious molecular clone, which was obtained by Dr. Sarah Asherfold (Centre for HIV Research, University of Edinburgh). In order to test for reproducibility of the assay, a second experiment with the same supernatants was performed. Data from the first experiment are presented in Table 3.4 and Figure 3.4 and those from the second experiment are shown in Table 3.5 and Figure 3.5. A good reproducibility of the assay is proved by CV values presented in Table 3.6.

Table 3.4 Data obtained for titration of recombinant glycoprotein rgp120 IIIB in parallel with two fold dilution of a supernatant that was obtained by transfecting COS cells with HXB2-MCS (first experiment).

rgp120 (µg/ml)	IIIB	Status	OD405nm (detailed value)	OD405nm (calculated value)	CV	Dilution of the supernatant (HXB-2MCS)	Status	OD405nm (detailed value)	OD405nm (calculated value)	CV
0.01		mean	0.389	0.263	15	non-diluted	mean	0.89	0.75	22
0.05		mean	0.51	0.393	10	1/2	mean	0.82	0.68	10
0.1		mean	0.66	0.534	12	1/4	mean	0.63	0.49	17
0.5		mean	1.02	1.077	19	1/8	mean	0.5	0.36	5
1		mean	1.56	1.44	21	1/16	mean	0.44	0.3	9
5		mean	2.275	2.149	12	1/32	mean	0.34	0.2	11
Zero		mean	0.109							
Blank		mean	0.143							

OD calculate values = OD detailed values – OD blank

Footnote: The nature of the zero and blank controls is given in table below:

Blank	gp120	anti-gp120	anti-human IgG-Biotin	Avidin AP
Zero	-	-	-	-
Blank 1	-	+	+	+

Figure 3.4. Dose dependent curve for quantification of gp120 protein from a supernatant that derives from COS cells transfected with HXB2-MCS (first experiment)

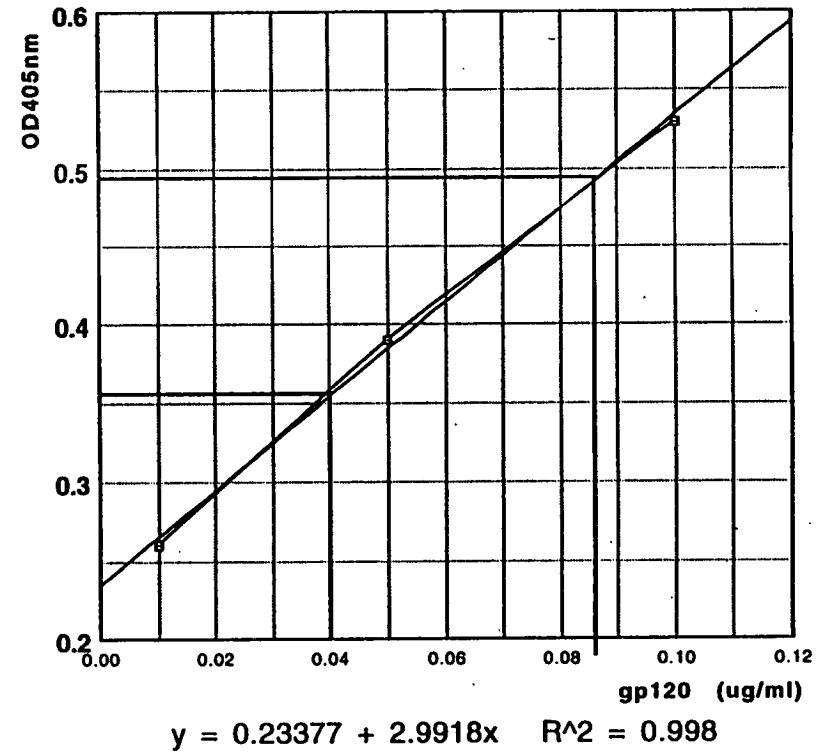
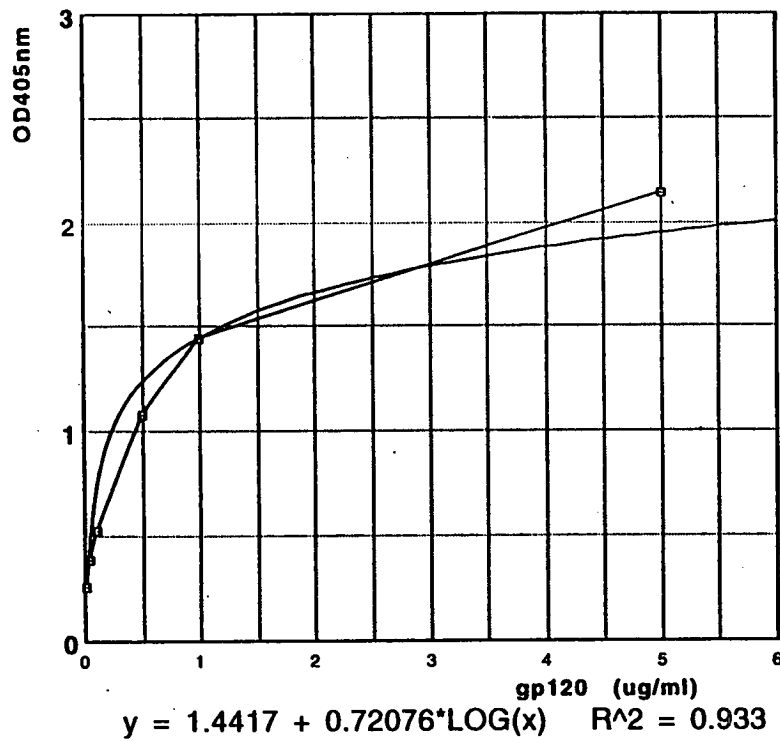


Table 3.5. Data obtained for titration of recombinant glycoprotein rgp120 IIIB in parallel with two fold dilution of a supernatant that was obtained by transfecting COS cells with HXB2-MCS (second experiment).

rgp120 (ug/ml)	IIIB	Status	OD405nm (detailed value)	OD405nm (calculated value)	CV	dilution of the supernatant (HXB-2MCS)	Status	OD405nm (detailed value)	OD405nm (detailed value)	CV
0.01		mean	0.297	0.113	14	non-diluted	mean	0.79	0.58	7
0.05		mean	0.411	0.227	15	1/2	mean	0.71	0.50	25
0.1		mean	0.532	0.348	21	1/4	mean	0.52	0.31	19
0.5		mean	0.801	0.617	23	1/8	mean	0.41	0.20	13
1		mean	0.914	0.73	8	1/16	mean	0.34	0.14	14
5		mean	1.279	1.09	14	1/32	mean	0.31	0.10	10
Zero			0.153							
Blank			0.215							

OD calculate value = OD detailed value – OD blank

Footnote: The nature of the controls (zero and blank) is given in table below:

Blank	gp120	anti-gp120	anti-human IgG-Biotin	Avidin AP
Zero	-	-	-	-
Blank	-	+	+	+

Figure 3.5. Dose dependent curve for quantification of gp120 glycoprotein from supernatant that derives from COS cells transfected with HXB2-MCS (second ELISA experiment)

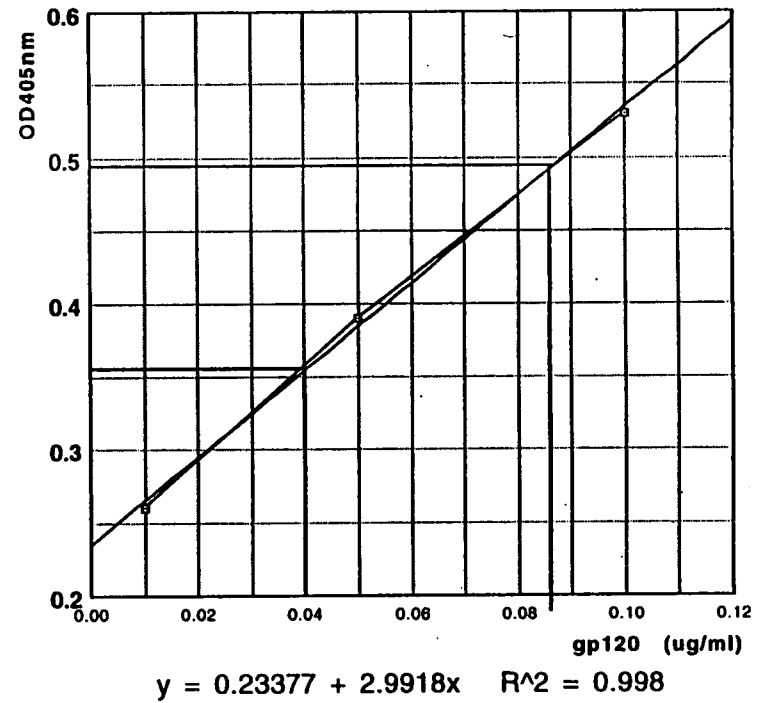
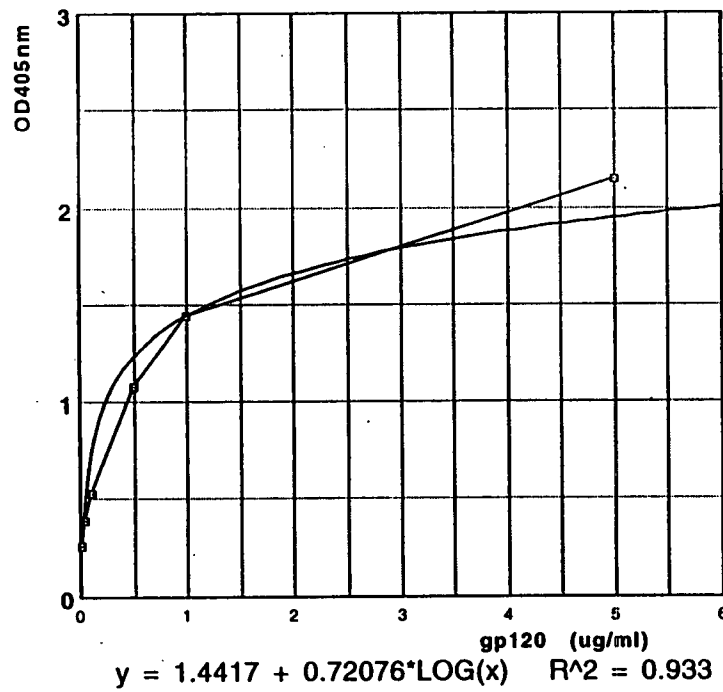


Table 3.6 Data processing for homologous interpolation ELISA method:

Dilution of the supernatant	OD405nm	interpolated gp120 concentration	final gp120 concentration
Experiment I/1: 1/4	0.49	83 ng / ml	83 x 4 = 332 ng / ml
Experiment I/2: 1/8	0.36	45 ng / ml	45 x 8 = 360 ng / ml
Experiment II/1: 1/4	0.31	78 ng / ml	78 x 4 = 312 ng / ml
Experiment II/2: 1/8	0.20	42 ng / ml	42 x 8 = 336 ng / ml

Analysis of the intra-assay variability:

Experiment	MEAN (ng/ml)	STDEV	CV
Experiment I	346	1.97	5.69
Experiment II	324	1.69	5.2

Analysis of the inter-assay variability:

Experiment	MEAN (ng/ml)	STDEV	CV
Exp I/1 + Exp II/1	322	1.41	4.3
Exp I/2 + Exp II/2	348	1.69	4.85
Exp I/1 + Exp II/2	334	0.28	0.8
Exp I/2 + Exp II/1	336	3.39	10

3.2.2 Optimisation of the "in house" gp120 ELISA

In this subchapter the experiments done in order to optimise the conditions of the ELISA assay, which employs biotinylated F (ab')₂ anti-human IgG, Avidin-AP and pNPP reagents, are described.

3.2.2.1 Titration of the capture antibody (D7324)

To optimise the concentration of D7324 antibody, three monoclonal antibodies were tested as a detection antibody to select that that gives the highest reactivity against gp120 glycoprotein. The following monoclonal antibodies (MAb) were used at the concentration of 2µg/ml:

- ARP 3054 (GP 13), a human monoclonal antibody to HIV-1 gp120 that recognises an epitope within or topographically near to the CD4-binding site of gp120 (amino acid: 256, 257, 262, 368, 370, 384) (Schutten *et al.*, 1993),
- ARP 3055 (GP68), a human monoclonal antibody to HIV-1 gp120, which recognises an epitope within or topographically near to the CD4 binding site of gp120 (amino acid: 117, 256, 262, 370, 384, 435) (Schutten *et al.*, 1993),
- ARP 3065 (IgG1 b12), a human monoclonal antibody to HIV-1 IIIB gp120, which was mapped to the CD4 binding site of gp120 (Burton *et al.*, 1991).

The results are presented in Table 3.7 and Figure 3.6. From Figure 3.6 it can be observed that, for concentrations of the capture antibody (D7324) lower than 6µg/ml, the reactivity of GP13 (ARP 3054) was slightly higher than GP 68 (ARP 3055). As the desirable concentration of the capture antibody should be as low as possible to allow a competition between high and low affinity anti-gp120 IgG antibody for binding to gp120 molecules, GP13 antibody seemed to be the best candidate as a detection antibody. The absorbance values obtained for antibodies GP68 and IgG1b12 when the concentration of D7324 was lower than 10µg/ml were below those recorded for GP13. The lowest reactivity values were recorded for IgG1b12 antibody, for all the concentrations of D7324 tested. As a consequence, GP13 (ARP 3054) was chosen as detection antibody in this ELISA assay.

Table 3.7 Parallel titration of capture antibody D7324 using three monoclonal antibodies: ARP 3054, ARP 3055, and ARP 3065:

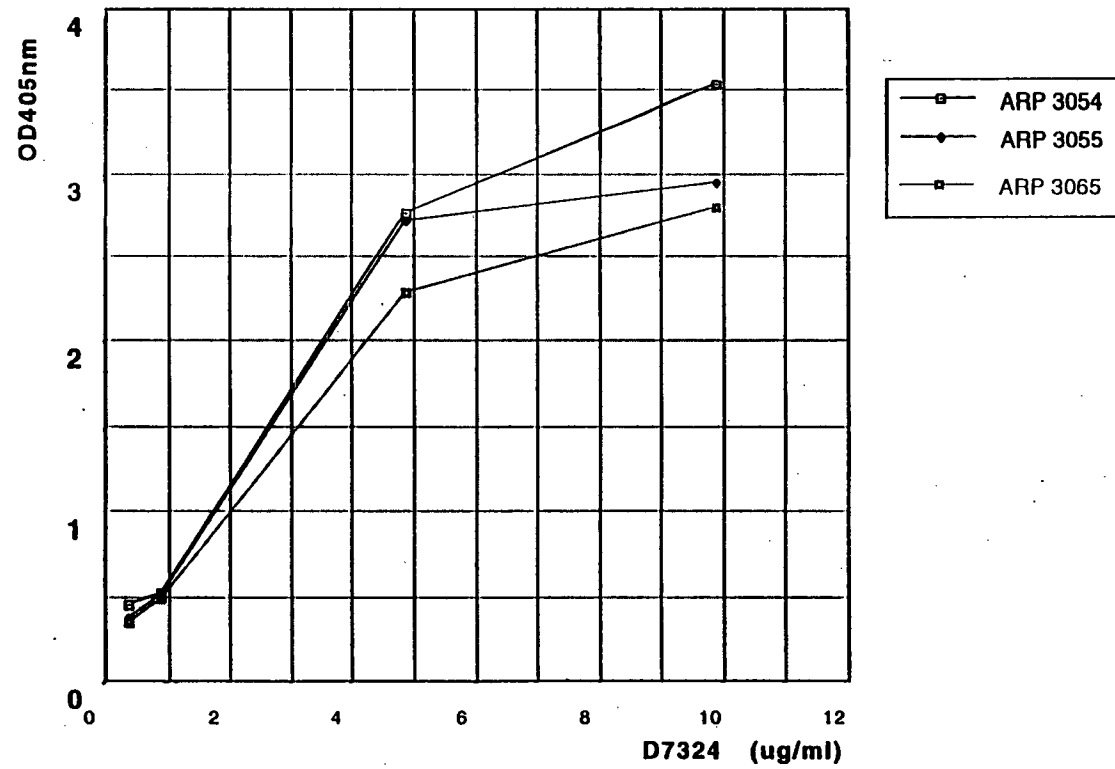
D7324 ($\mu\text{g/ml}$)	Status	OD405 ARP 3054		OD405 ARP 3055		OD 405 ARP 3065	
		calculated	detailed values	calculated	detailed values	calculated	detailed values
0.5	mean	0.5	0.81	0.41	0.6	0.393	0.673
1	mean	0.56	0.87	0.55	0.84	0.53	0.81
5	mean	2.8	3.11	2.76	3.05	2.33	2.61
10	mean	3.59	3.9	2.99	3.28	2.84	3.12
Zero	mean		0.12		0.16		0.13
Blank	mean		0.312		0.29		0.289

OD calculated value = OD detailed – OD blank

Footnote: The zero and blank controls were as presented in the following table:

Blank	gp120	anti-gp120	anti-human IgG-Biotin	Avidin AP
Zero	-	-	-	+
Blank	-	+	+	+

Figure 3.6. Titration of D7324 using three detection MAb: ARP 3054, ARP 3055, ARP 3065



A second experiment was performed to choose the optimum concentrations of the capture and detection antibodies.

The microtitre test format for this experiment is presented in the following diagram:



the values written in the columns represent the GP13 concentrations

0.1						0.1						
0.25						0.25						
0.5						0.5						
1						1						
2						2						
4						4						
8						8						
Blank						Blank						
8	4	2	1	0.5	0.25	8	4	2	1	0.5	0.25	



the D7324 concentrations are written on the bottom wells of the plate

The intersection between the diagonals which join the upper value of the concentration of the monoclonal antibody with lower value of the concentration of the capture antibody, and vice versa, indicated the well that have the optimum concentration of the reagents. For the ELISA assay that employs GP13 antibody as a detection antibody, the optimum concentration of the reagents was given by the well placed in the middle of the plate (i.e. $2\mu\text{g/ml}$ for D7324 and $2\mu\text{g/ml}$ for GP13). Data derived from this assay are presented in Table 3.8. Figure 3.7 depicts the curves when titration of D7324 was done simultaneously with a series of two-fold dilutions of the detection antibody (GP13). Taking $2\mu\text{g/ml}$ as being the optimum concentration for D7324, a titration of gp120 was done using three concentrations of GP13 ($0.5\mu\text{g/ml}$, $1\mu\text{g/ml}$ and $2\mu\text{g/ml}$). The results are presented in Table 3.9 and in Figure 3.8. The highest absorbance values were obtained for a concentration of GP13 of $2\mu\text{g/ml}$. Absorbance values less than 1 were obtained for a concentration of GP13 of $2\mu\text{g/ml}$ (the plates were incubated with the substrate for 20min in dark).

Table 3.8 Absorbance values when parallel titration of the capture antibody and detection antibody was performed.

D7324 ($\mu\text{g/ml}$)	Status	GP13 (0.25 $\mu\text{g/ml}$)	CV	GP13 (0.5 $\mu\text{g/ml}$)	CV	GP13 (1 $\mu\text{g/ml}$)	CV	GP13 (2 $\mu\text{g/ml}$)	CV	GP13 (4 $\mu\text{g/ml}$)	CV	GP13 (8 $\mu\text{g/ml}$)	CV
0.25	mean	0.139	2.5	0.17	3.3	0.21	8.4	0.37	4.2	0.6	3.11	0.95	6.7
0.5	mean	0.193	1.2	0.25	3.08	0.34	0	0.51	4.1	0.74	13	1.42	10.2
1	mean	0.25	2.3	0.36	5.5	0.53	2.4	0.76	2.5	1.03	5.6	1.90	2.5
2	mean	0.35	3.5	0.45	3.3	0.63	6.7	0.94	5.1	1.46	1	2.3	8
4	mean	0.45	8.3	0.59	11.6	0.81	18.25	1.28	19.1	1.65	3.4	2.54	3.9
8	mean	0.56	13.6	0.74	7.04	1.09	7.9	1.42	3.35	1.88	2.5	2.79	3.5
Zero	mean	0.091	5.2	0.102	5.6	0.130	7.4	0.150	3.6	0.172	1	0.196	2.5
Blank	mean	0.33	5.8	0.26	5.9	0.43	4.8	0.56	6.4	0.67	6.8	1.12	8.2

Footnote: Zero and blank controls are explained in table below:

Control	gp120	GP120	anti-human IgG-Biotin	Avidin AP
Zero	-	-	-	-
Blank	-	+	+	+

Figure 3.7. Titrations of the capture and detection antibody (D7324 and GP13)

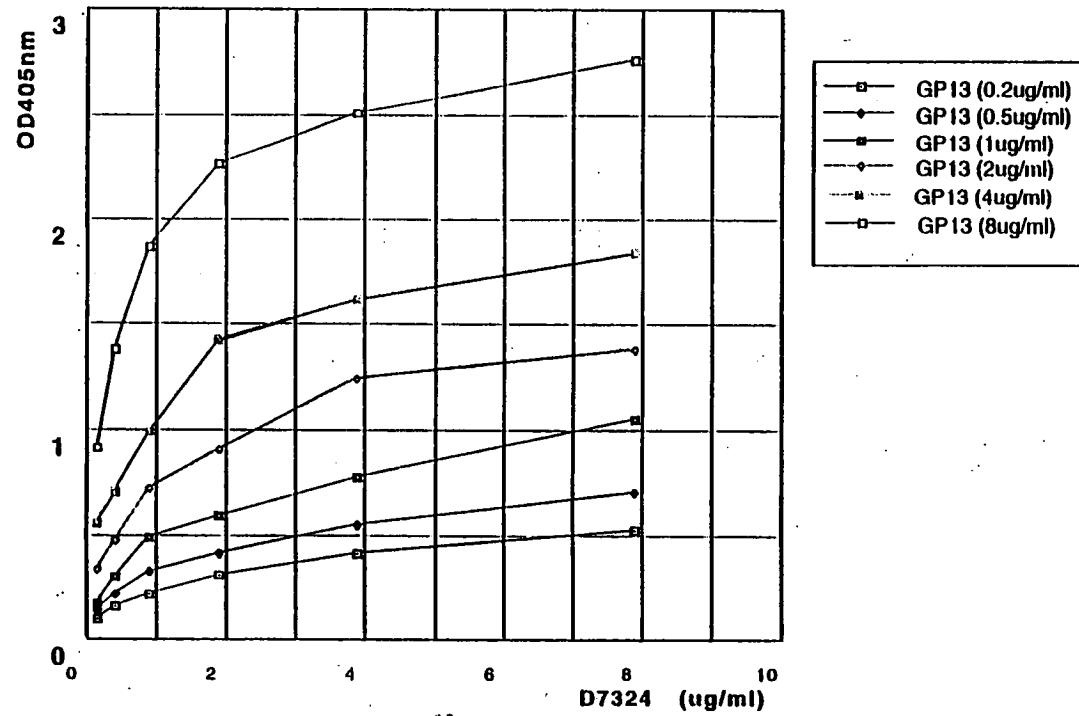


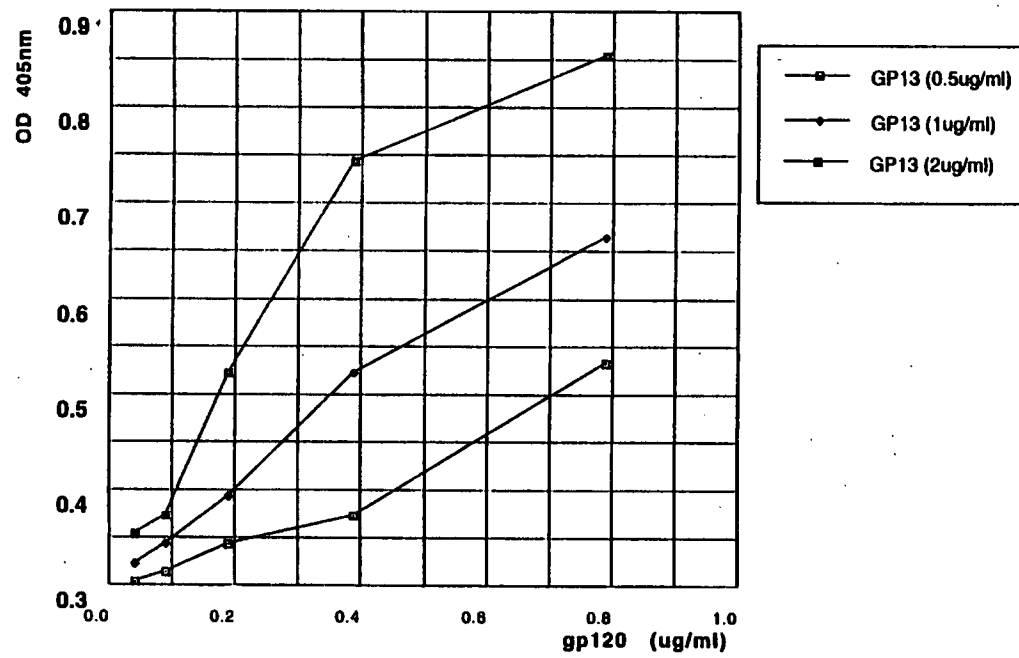
Table 3.9 Dose response curves for gp120 glycoprotein when using three concentrations of GP13:

gp120 ($\mu\text{g/ml}$)	Status	GP13 (0.5 $\mu\text{g/ml}$)	CV	GP13 (1 $\mu\text{g/ml}$)	CV	GP13 (2 $\mu\text{g/ml}$)	CV
0.8	mean	0.54	0.51	0.67	14.7	0.864	2
0.4	mean	0.38	0.72	0.53	3.65	0.759	5.6
0.2	mean	0.35	2.6	0.4	2.42	0.53	3.1
0.1	mean	0.31	4.5	0.35	7.1	0.38	4.6
0.05	mean	0.3	4.9	0.28	0	0.36	1.3
Zero	mean	0.094	8.9	0.093	5.3	0.094	2.6
Blank	mean	0.28	6	0.3	0	0.21	4.9

Footnote: The zero and blank controls are explained in table below:

Control	gp120	GP120	anti-human IgG-Biotin	Avidin AP
Zero	-	-	-	-
Blank	-	+	+	+

Figure 3.8. Dose dependent curve for quantification of gp120 protein when a titration of GP13 MAb was done



3.3 Summary of Results

The experiments done in order to reproduce the ELISA method described by Moore *et al.*, (1988) failed. This result given by the specific interactions between D7324 antiserum and anti-human IgG-AP made difficult to differentiate between the real signal and the background noise. To surmount this problem, anti-human IgG-AP was substituted with an biotinylated F(ab')₂ anti-human IgG.

Using biotinylated F(ab')₂ anti-human IgG, three ELISA methods were set up. Their sensitivities were compared and analysed, in order to choose that assay which allows the estimation of the gp120 concentration from the supernatants of COS cells transfected with an infectious clone (HXB2-MCS). We estimate that the level of transfection of COS cells with env encoding infectious clones will approximate that of transfection with a non-infectious clone.

ELISA assays using the AMPAK amplification system is probably more suitable as a serologic assay to estimate the amounts of gp120 glycoprotein in sera from HIV-1 infected individuals. The concentration of gp120 so far detected in these sera was reported not to exceed 20ng/ml, lower than the level of gp120 expression in COS cells (J. Moore *et al.*, 1988). The linear range of this assay (between 1-4 ng/ml) is not suitable to estimate the amount of gp120 protein from transfection supernatants unless many dilutions of these supernatants are done.

Consequently, the amplification system was replaced with a detection system. This was represented by the biotinylated F(ab')₂ anti-human IgG and Avidin-HRP or Avidin-AP, respectively.

When the sensitivity of both methods were compared, the ELISA assay that employed biotinylated F(ab')₂ anti-human IgG and Avidin-AP has shown a sensitivity that suits that needed to estimate the amount of gp120 glycoprotein in COS cell-derived supernatants. Statistical analysis of the results proved the accuracy and reproducibility of this method.

Chapter 4

Specification and construction of a suitable mammalian expression vector

4.1 Introduction

4.1.1 Control elements of gene expression

4.1.2 Transfer and expression of genes in COS cells

4.1.3 Alternative splicing pathway and cis-acting RNA splicing element of HIV-1

4.2 Results

4.2.1 Construction of pAA-MCS and pAAL from pSRHS

4.2.2 Transfection of COS cells with env constructs to check for gene expression

4.2.3 Identifying the reason for the lack of gene expression

4.2.4 Remodification required for reintroducing the 5' donor-splicing site into the mammalian expression vector

4.2.5 Testing the pEX2 and pEV5 for successful gene expression

4.2.6 Comments regarding the level of env gene expression

4.3 Summary of results

4.1 Introduction

Molecular clones of HIV may be generated either from viral DNA derived from virus amplified in cell culture or from PCR amplification *in vitro*. Virus amplification in cell culture may lead to a selection for (or against) a particular subset of HIV variants (Meyerhans et al, 1989). Virus propagation has the limitation of amplifying the virus in cell lines that may select for desired HIV variants (i.e. "rapid-high" (Asjo et al., 1986), tissue tropic, non-cytopathic, etc.). However, to obtain the most representative sampling of viral sequences present *in vivo*, cell culture should be kept to a minimum. An alternative method to the virus propagation in cell lines is DNA amplification *in vitro*, a method that is presented in detailed in Chapter 5. This alternative allows one to bypass potential cell culture selection against HIV variants.

PCR amplification gives the possibility to clone viral DNA derived from peripheral blood mononuclear cells in which the HIV DNA copy number is more than 1 in 10 down to 1 in 150.000 cells (Simmonds *et al.*, 1990a). Successful expression of these genes requires the choice of an appropriate vector and cells used for transfection.

4.1.1 Control elements of gene expression

Mammalian expression vectors have normally been used for investigating genes, which were already subcloned, for direct the synthesis of a desired protein, production of large amount of proteins and evaluation of the effect of specific mutations introduced into genes.

The mammalian expression vector (pSRHS) used in this study was modified in order to include a polylinker that has restriction sites compatible with the restriction enzymes used to clone the HIV-1 infected cellular DNA. The plasmid contains: the SV40 origin of replication (SV40ori), SV40 early and late promoters and the Mason-Pfizer Monkey Virus Long Terminal Repeat (LTR) (Dubay *et al.*, 1992). Mason-Pfizer Monkey Virus Long Terminal Repeat (LTR) contains the poly-A⁺ signal and, thus, stabilises the mRNA transcripts. The restriction map of the plasmid pSRHS is given in Figure 4.2.

4.1.2 Transfer and expression of genes in COS cells

The successful strategy for introducing foreign genes into mammalian cells relies upon COS cells for rapid, transient expression of protein from specific vectors. In transient system, the gene of interest is introduced into a population of cultured cells and its activity is assayed within a few hours to a few days. COS cells produce wild-type large T antigen but no viral particle. SV40ori containing plasmids are able to replicate in these cells to a high copy number (10,000 to 100,000 copies per cell) 48-72 hours post transfection. Thus, if the plasmid carries a genomic insert encoding a desired protein which is under the control of an appropriate promoter, COS cells will express the protein

at relatively high levels over a short period of time. Expressed eukaryotic proteins produced in COS cells are usually biologically active. Although COS cells are able to carry out some posttranslational modifications, they may not modify the expressed protein in exactly the same way as the cell that would normally produce it. Aruffo *et al.*, (1987a,b) showed that the lymphocyte cell-surface proteins tend to be underglycosylated in COS cells probably due to the lack of enzymes required to carry out the full posttranslational modifications. However, for gp120 glycoproteins expressed on COS cells, the glycosylation pattern is closer to gp120 glycoprotein which is found *in vivo* than the glycosylation pattern of the glycoproteins expressed in prokaryotic system (i.e. bacteria, Moore *et al.*, 1995b).

4.1.3 Alternative splicing pathway and cis-acting RNA splicing elements of HIV-1

Proviral DNA, being integrated into the host chromosome, is transcribed into a retroviral RNA that is further processed by cellular machinery (i.e. capped and polyadenylated). These mRNAs are alternately and incompletely spliced. A balance between unspliced and spliced transcripts is essential for viral replication.

The HIV-1 genome includes 20 exons bounded by 5 5' splice sites (called 1, 2, 3, 5 and 6d) and 11 3' splice sites (called 2, 3, 4, 4c, 4a, 4b, 5, 6d, 7a, 7b and 7) to yield more than 40 transcripts (Robert Guroff *et al.*, 1990). The open reading frames (A) and splice

sites (B) of HIV-1 BH10 are presented in Figure 4.1. The locations of the 5' splice sites are indicated above and of the 3' splice sites below the diagram. 5' splice site 5 is used by multiple exons, containing 3' splice sites 3-5.

Figure 4.1. HIV-1 BH10 genome organisation.

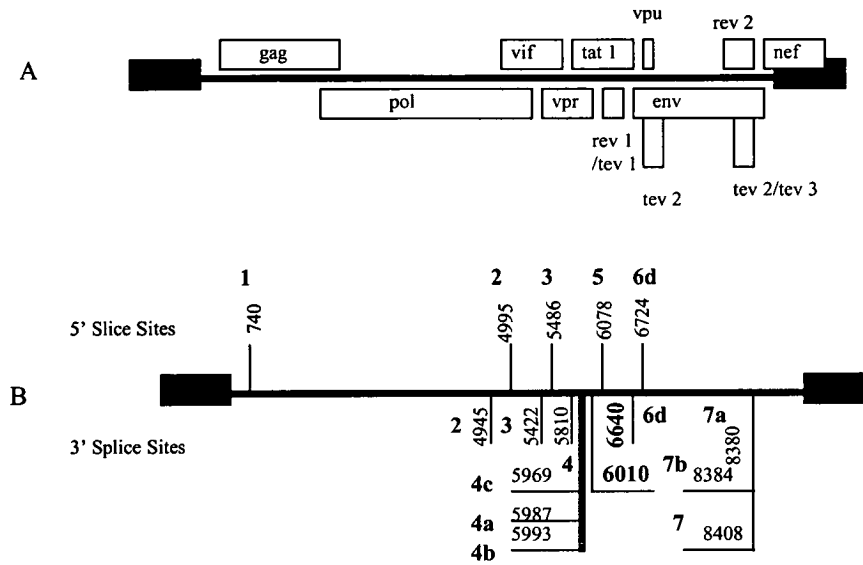
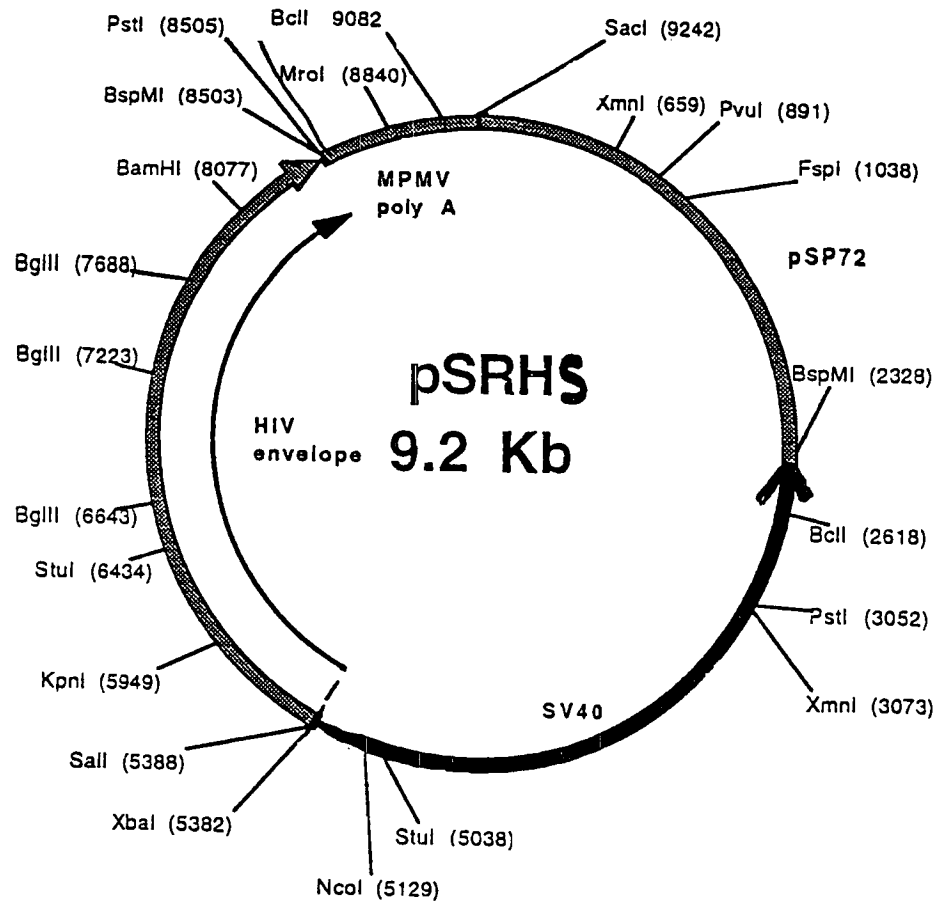


Figure 4.2 The restriction map of the mammalian expression vector pSRHS

The molecule is a double-stranded circle, 9200 base pairs in length. It contains SV40 ori, SV40 late promoter and enhancer, large T antigen, Mason Pfizer Monkey Virus polyadenylation signal, wild-type HIV envelope gene from clone HXB2 and the gene for resistance to ampicilline



4.2 Results:

4.2.1 Construction of pAA-MCS and pAAL from pSRHS

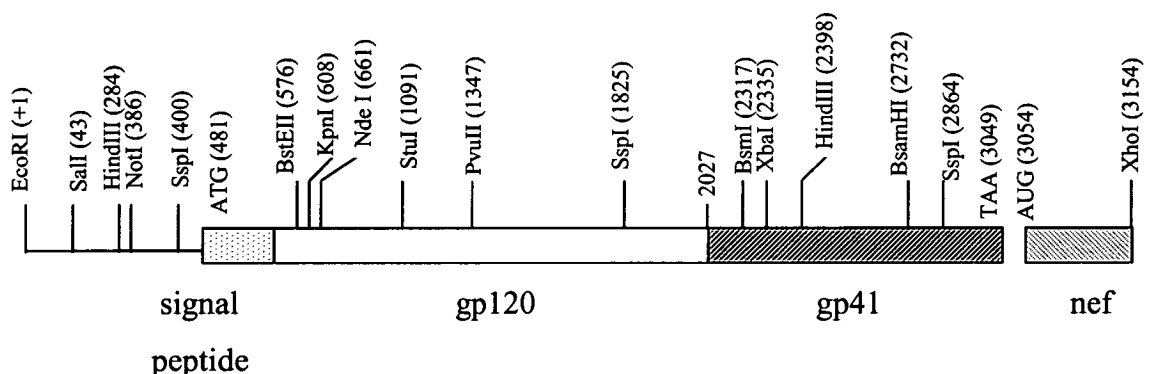
The original plasmid pSRHS was subject to a series of modifications in order to include a polylinker suitable for subsequent subcloning of the env gene amplification product. All the transformations, which have been done to pSRHS, are depicted in Annex 1.

Shortly, these steps involve:

1. deletion of 3.2 kbp fragment flanked by Sall and XhoI restriction sites that includes the env gene from pSRHS to generate pSRH,
2. cloning of the Sall/XhoI fragment containing the whole gp120 and gp41 gene and also the N-terminal of nef gene from the clone HXB2 into pSRH, giving pSRH-HXB2-MCS.
3. cloning of a polylinker (Sall/NotI/SmaI/XhoI) into pSRH, resulting pSRHL (L derives from linker),
4. insertion of the same Sall/XhoI fragment as mentioned before into pSRHL, generating pSRHL-MCS.
5. deletion of a XbaI site from pSRHL to produce pAA-MCS,
6. cloning of a polylinker (XbaI/SmaI/BstEII) into pAA-MCS that generate pAAL in which patient derived env PCR amplified fragments will be subcloned.

Plasmids pAA, pAA-MCS and pAAL were obtained by myself. All the other plasmids were obtained by Dr. A. Alonso and Mrs. P. Robertson.

The restriction map for pSRHS is given below:



This plasmid contains the second and third exon of *rev*, the 5' donor-splicing site, the whole *env* and the N-terminal part of *nef* and it expresses the *env* gene. PSRH-HXB2-MCSEnv has the same restriction sites as those showed in the restriction map given for pSRHS.

In pSRHL, the fragment between NotI(386) and XhoI(3154) (positions that correspond to the map given for pSRHS) was replaced by a polylinker (NotI/SmaI/XhoI/SalI).

PAA-MCS and pSRHL-MCS have the second and the third exon for *rev*, the whole *env* gene and the N-terminal part of *nef* but lacks a SalI(43)-NotI(386) fragment.

4.2.2 Transfection of COS cells with *env* constructs to check for gene expression

To check if the modifications done to pSRHS did not affect gene expression, transfection experiments were carried out on COS cells. The results are presented in Table 4.1.

Table 4.1. The level of gp 120 glycoprotein that was detected employing gp120 ELISA in transfection supernatants.

Transfectant DNA	Transfection 1 gp120 (µg/ml)	Transfection 2 gp120 (µg/ml)	Transfection 3 gp120 (µg/ml)	Mean+StDev gp120 (µg/ml)
pSRHL	0.01	0.02	0.05	0.026±0.02
pSRHS	0.46	0.23	0.20	0.29±0.14
pSRH-HXB2-MCS	0.33	0.16	0.11	0.2±0.11
pSRHL-MCS	0.03	0.05	0.06	0.04±0.01
pAA-MCS	0.01	0.03	0.07	0.03±0.03

Figures presented in Table 4.1 show a lack of expression of *env* recorded for pSRHL-MCS and pAA-MCS contrary to pSRHS and pSRH-HXB2-MCS.

4.2.3 Identifying the reason for the lack of gene expression

By analysing the restriction maps for pSRHL-MCS, pAA-MCS in comparison with those for pSRHS and pSRH-HXB2, it became obvious that the difference between these plasmids consisted of a small fragment that is flanked by Sall and NotI restriction sites with a size of 312 bp. It has been documented that the expression of HIV-1 env from SV40-based vector is dependent on the presence of this fragment containing a 5' donor-splicing site in the env mRNA (Lu *et al.*, 1990). Thus, the presence and integrity of this 5' donor-slicing site is essential for envelope gene expression.

4.2.4 Remodification required for reintroducing the 5' donor-splicing site into the mammalian expression vector

All the subsequent modifications were done in order to reintroduce the Sall-NotI fragment containing the 5' donor-splicing site responsible for the efficient env gene expression. These modification are shown in Annex 2 and the strategy used is summarised below:

1. deletion of BamHI (8750)-BamHI (2346) fragment containing the env gene, the SV40 ori and SV40 promoter elements from pSRHL-MCS having a size of roughly 2400 bp and comprising one Sall site was performed in order to ensure the presence of only one Sall site in the fragment left.
2. Sall site was deleted by digestion with Sall followed by the filling in of the 3' overhang ends with Klenow fragment.
3. BamHI-XhoI fragment from pSRHL that contains the SV40 ori, SV40 promoter and a polylinker Sall/NotI/SmaI/XhoI was subcloned into the previous plasmid.
4. NotI-XhoI fragment including the gp120 and gp41 genes and the amino-terminal of nef gene was introduced between NotI and XhoI sites carried by the polylinker.
5. Sall-NotI fragment that derived from the plasmid pRNBXX containing the donor-splicing site was included in the vector.
6. NotI-XhoI fragment from the vector was substituted with a short fragment of 190 bp that includes a linker NotI/BstEII/XhoI
7. Xba I site from the plasmid was deleted.

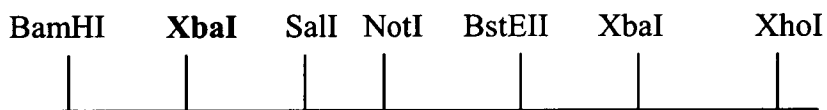
8. BstEII-XhoI fragment from pRNBXX containing the env and part of nef gene was subcloned in order to test if the XbaI deletion affected the gene expression.

9. BstEII-XhoI fragment was replaced with a BstEII/SmaI/XbaI polylinker to generate a suitable vector in which subcloning of the env amplified genes will become possible.

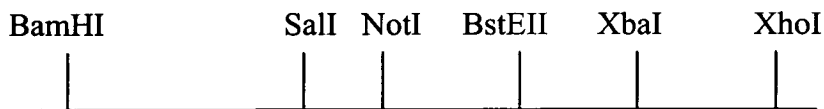
Plasmids pEV4, pEV5 and pEV6 were obtained by myself. The other modifications were done by Dr. A. Alonso and Mrs. P. Robertson.

The restriction sites for pEX, pEV5 and pEV6 are presented below:

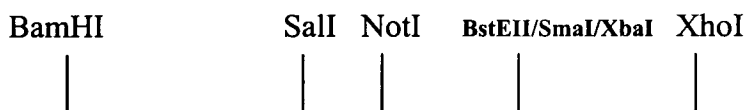
pEX2



pEV5



pEV6



pEV6 was used as an expression vector in which 1.7kb-fragments amplified from patients' PBMC DNA were subsequently subcloned. Therefore, it was a need to check if deletion of XbaI did not abolished gene expression and subcloning of the 5'donor-splicing site did have a consequence in re-establishing the ability of the plasmid to express the env gene.

4.2.5 Testing the pEX2 and pEV5 constructs for env gene expression

To check if the re-modifications described in Subchapter 4.2.4 re-establish the ability of the vector to express the env gene, a transfection experiment was done using pSRHL and pSRHL-MCS, as negative controls, and pSRH-HXB2-MCS, as a positive control. The results obtained are presented in Table 4.2.

Table 4.2. The level of gp120 glycoprotein detected in the transfection supernatant by ELISA.

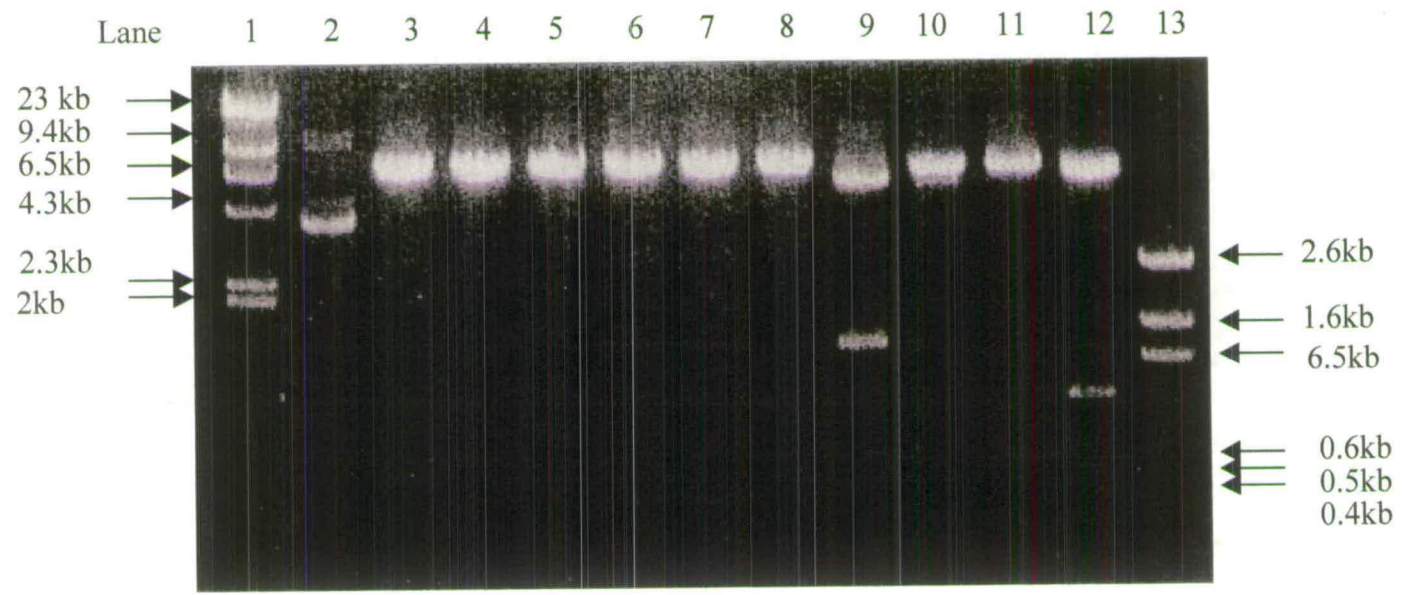
Plasmid	Transfection 1 gp120(μ g/ml)	Transfection 2 gp120(μ g/ml)	Mean+StDev gp120(μ g/ml)
pSRHL	0.048	0.127	0.087+0.055
PSRH-HXB2-MCS	0.292	0.431	0.361+0.098
pSRHL-MCS	0.126	0.139	0.132+0.009
pEX2	0.28	0.24	0.21+0.042
pEV5	0.2	0.24	0.22+0.042

Data presented in Table 4.2 show similar levels of gene expression for pEX2 and pEV5 and pSRH-HXB2-MCS, whereas pSRHL-MCS, plasmid that lacks the donor-splicing site, has a level of gene expression close to that recorded for the negative control pSRHL. Thus, experimental data obtained proved that the remodification done in order to include the donor-splicing site into the expression vector allowed env gene expression. Moreover, the removal of both Sall site at the position 2773 and XbaI site at the position 5382 did not have a negative impact on env expression. A restriction analysis of pEV6 is presented in Figure 4.2.

Figure 4.3 The restriction analysis of pEV6

Gel electrophoresis of the plasmid pEV6 (line 2), together with its single digests: Sall (line 3), Not I (line 4), BstEII (line 5), SmaI (line 6), XbaI (line 7), XhoI (line 8) and double digests: Sall/XhoI (line 9), SmaI/BstEII (line 10), SmaI/XbaI (line 11), BstEII/XhoI (line 12), along with the markers: λ /HindIII (line 1) and pGEM (line 13). The presence of only one site for BstEII., SmaI and XbaI and of only one band for the double digests: SmaI/BstEII and SmaI/XbaI shows the presence of the polylinker BstEII/SmaI/XbaI into the plasmid pEV6. Digestion of pEV6 with Sall/XhoI and BstEII/XhoI resulted in two band (details of the fragment sizes are presented in the table below):

Digestion	Fragment 1	Fragment 2
Sall/XhoI	5242bp	1358bp
BstEII/XhoI	5790bp	810bp



4.2.6 Comments regarding the level of env gene expression

All the clones described in Chapter 5 were obtained by subcloning 1.7-kb fragments amplified from patients' PBMC DNA into pEV6 and, hence, all of them contain 5'-donor splicing site, patient-derived gp120 encoding gene, HIV-HXB2-derived gp41 encoding gene, fragment encoding the N-terminal part of Nef, the second and third exons of Rev and RRE but not the acceptor-splicing site.

Levels of expression achieved by the env clones obtained in this study (see Table 5.3) were hugely variable, probably because of the polymorphism between patients. Low level of gene expression can be explained by the absence of functional Rev. Because the absence of the acceptor site, it is unlikely that Rev is produced by the activation of cryptic splices. Thus, in the absence of Rev, a significant part of env mRNA transcripts are intranuclear sequestered and degraded and only little will reach the cytoplasm. Therefore, a suggestion for increasing the gene expression could be co-transfection of the env constructs with Rev-expressing plasmids. Finally, another reason for low gene expression can be found in the presence of the Rev AUG 5' to the start of env (M.Kozak, 1995).

4.3 Summary of results

The first modifications that were done to pSRHS in order to replace the gp120 gene with a polylinker, failed to produce a plasmid able to express env gene. The explanation for the lack of env gene expression was found to reside in the absence of the 5' donor splicing site. Consequently, all the subsequent work was done in order to reintroduce this splicing site and to bring the vector in a form that is suitable for subcloning of patient-derived env genes.

CHAPTER 5

PCR AMPLIFICATION AND CLONING OF PATIENT DERIVED 1.7KB ENV SEQUENCES INTO THE pGEM T VECTOR AND THEIR SUBSEQUENT INCORPORATION INTO THE MAMMALIAN CASSETTE-VECTOR pEV6

5.1 Introduction

5.1.1 Abundance of HIV provirus molecules in patient material

5.1.2 Primer design

5.1.3 Cloning vector system

5.2 Results

5.2.1 Estimation of the provirus DNA copies number by end-point dilution nested PCR

5.2.2 PCR amplification of 1.7-kb env sequences and their incorporation into a cloning vector system (pGEM T)

5.2.3 Transfer of the 1.7-kb fragments from pGEM T into mammalian expression vector (pEV6)

5.2.4 Screening for a successful gp120 env gene expression by transfection of COS cells

5.2.4.1 Comparison between Effectine, SuperFect and Lipofectin transfection Protocol

5.2.4.2 gp120 cellular staining of COS cells

5.2.5 Sequence analysis of the env gene clones

5.2.5.1 NSI/SI phenotype

5.2.5.2 Prediction of the CCR5 co-receptor usage

5.2.5.3 Sequence analysis

5.3 Summary of Results

5.1 Introduction

The clones containing the uncultured patients PBMCs derived 1.7-kb sequences that were obtained by PCR amplification and their screening in order to select functional genes are presented in this chapter.

5.1.1 Abundance of HIV-1 provirus molecules in patient material

HIV-1 viral sequences are usually of low abundance *in vivo* (Harper *et al.*, 1986). The estimates of HIV DNA copy number in peripheral blood mononuclear cells and in tissues of infected individuals vary during the phase of the infection in the range between 1 per 10 PBMCs to as low as 1 per 150,000 PBMC (Psallidopoulos *et al.*, 1989, Simmonds *et al.*, 1990a). These levels are below the minimum required for cloning of the viral DNA without a method of amplification of the provirus copy number. A few reports documented the molecular cloning and characterisation of HIV-1 genomes directly from uncultured brain tissue from patients with AIDS dementia complex with an extremely high provirus load (Li *et al.*, 1991, 1992).

Because of the low abundance of proviral DNA in tissues and PBMCs of chronically infected individuals, most early studies were performed directly with small cultured material (i.e. viral cultures using infected PBMCs and uninfected PBMCs or CD4 immortalised cell lines). However, when infected PBMCs are stimulated to replicate, the viral copy number increases to a level that enables transfection-competent molecular clones to be obtained through conventional methods (Cheng Mayer *et al.*, 1988, 1989). Studies conducted by Overbaugh *et al.*, (1988), Meyerhans *et al.*, (1989) and Asjo *et al.* (1986) indicated that culture-derived isolates represent a selected subpopulation of viruses or a particular subset of HIV variants that have an *in vitro* growth advantage. Consequently, the subset with the highest replication rate will predominate in the cell culture, whereas the variants that hardly grow in the cell culture will be under-represented.

PCR technology enabled the genetic characterisation of HIV-1 directly from uncultured patient material (Balfe *et al.*, 1990, Epstein *et al.*, 1991, Meyerhans *et al.*, 1989, Vartanian *et al.*, 1991).

The method employs two oligonucleotide primers that flank the region of interest in the target DNA to initiate DNA synthesis followed by the strand elongation by means of a DNA polymerase (Mullis *et al.*, 1987). As it is complementary to the positive strand of denatured genomic DNA, one primer can anneal to that strand, an event that is followed by extension with the DNA polymerase and deoxynucleotide triphosphates, resulting in the synthesis of a negative strand fragment containing the target sequence. Simultaneously, a similar reaction occurs with the other primer, producing a new positive strand. These newly synthesised DNA strands will themselves be templates for the PCR primers. Consequently, a repetitive series of 20-30 cycles consisting of DNA denaturation, annealing of the primer to the DNA template and the extension of the annealed primers by DNA polymerase, results in an exponential amplification of the target sequence.

Hence, amplifying the PBMC HIV DNA via the polymerase chain reaction has the advantage of producing large quantities of a product suitable for direct cloning into plasmid vectors, bypassing potential cell culture selection against slower-replicating HIV-1 variants. In spite of bypassing the selection of a specific subset of isolates, the PCR amplification of provirus sequences has its own impediments. Firstly, the misincorporation of nucleotides during synthesis of the target sequences might produce errors associated with the PCR (Saiki *et al.*, 1988). Secondly, the presence of a heterogeneous population of the provirus DNA can lead to a recombination between non-identical templates during the PCR reaction (Meyerhans *et al.*, 1990).

5.1.4 Primer design

In order to amplify 1.7-kb env fragments from HIV-1 infected patients, the primers were designed to be complementary to the template DNA, with restriction endonuclease recognition sites being added at the 5'-ends of the oligonucleotides.

Thus, by restriction enzyme digestion, compatible overhangs for subcloning into the mammalian expression vectors are produced (BstEII at the 5' end of the sense inner primer and XbaI at the 5' end of the antisense inner primer). The presence of 3'-terminal mismatches was avoided since the consequence will be a dramatic reduction of PCR product yield. The primers used to amplify env genes are shown in Chapter 2 (Table 2.3). The presence of the restriction sites at the 5' ends of the primers are useful when considering the transfer of the DNA fragment that is between them from the cloning vectors into expression vectors.

5.1.5 Cloning vector system

The difficulty of cloning the amplified DNA directly into the expression vector, due to the inefficient cutting of restriction enzymes, impurities or blockages of the ends, is overcome by using a TA cloning vector such as pGEM T Easy Vector System (Mezei *et al.* 1994). TA cloning vectors overcome the problems associated with cloning of blunt-end ligation, a process that is less efficient and more time consuming. Blunt-ends fragments produced by Pfu polymerase are not suitable for ligation into pGEM T, unless an additional A' nucleotide at the 3' end of the PCR product is introduced by a round of amplification with Taq DNA Polymerase in the presence of only dATP.

5.2 Results

5.2.1 Estimation of the provirus DNA copies number by end-point dilution nested PCR

Proviral HIV-1 molecular copy number associated with each patient sample can be quantified by nested PCR, using primers for V1/V2 region in env gene (see Table 2.3). For each patient DNA extracted, test samples were titrated to an end-point (i.e. dilution when no signal was visible). The final dilution above the end point was then distributed into aliquots and the number of positive aliquots recorded (usually for this dilution 20% of the reactions produced a visible signal). Increasing the numbers of replicates at each dilution (Simmonds *et al.*, 1990a) can increase the accuracy of the titration. The frequency of positive reactions varies with the amount of target DNA amplified and can be converted into an estimated amount of target DNA (i.e. number of template molecules per reaction) by employing Poisson formula $-\ln(f_0) \times 1/d \times 100$, where f_0 is the frequency of negative reactions and $d \times 100$ is the dilution of the DNA \times the volume in which the DNA extracted from 10^6 PBMC was resuspended (i.e. 100 μ l). The results obtained for the patients used in this study are presented in Table 5.1.

In all V1/V2 PCR experiments, primers specific for HLA DQ α were used to amplify a 242bp fragment that is an indicative of a positive reaction. In negative reactions, patient DNA was substituted with distilled water.

In Figure 5.1 an agarose gel of a titration of DNA sample derived from patient 1090 is shown. When amplifying samples, using primers for V1/V2, a specific band corresponding to a fragment of 294 bp shows a positive reaction. The frequency of positive reactions less than 0.2 (defining the end point dilution) is reached in this case for sample diluted 1/35.

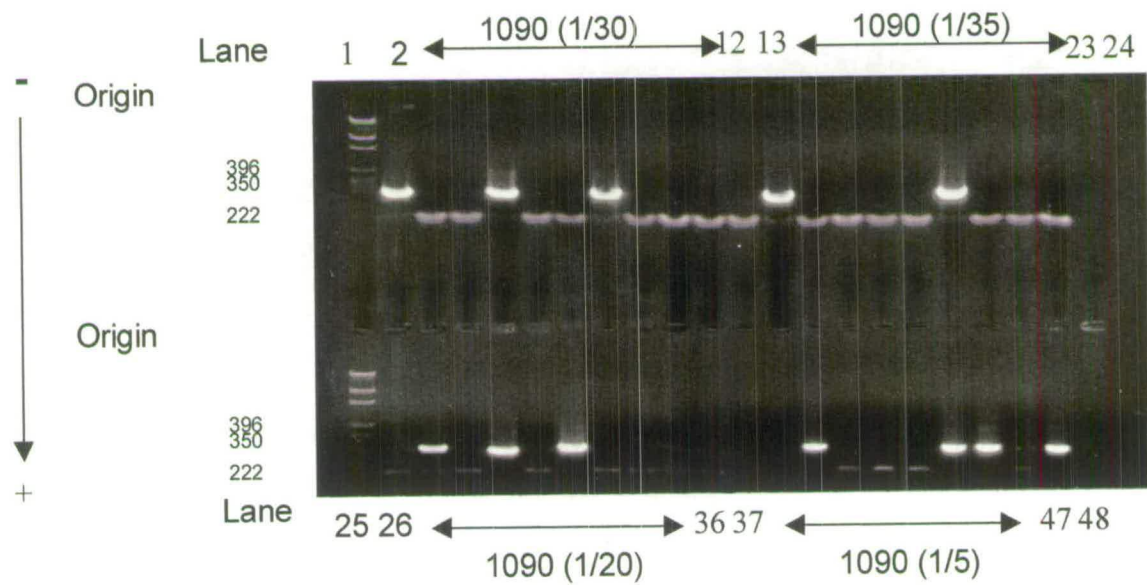
Table 5.1 Quantitation of target DNA per each patient sample

DNA no.	Patient PBMC DNA sample code	End-point dilution	Frequency of positive reactions	Estimated env copies/10 ⁶ PBMC
1	1363	1/30	30/50	1,532
2	1299	1/50	10/50	8,047
3	1260	1/50	8/50	9,162
4	1021	1/35	10/50	5,633
5	1020	1/10	5/50	2,302
6	1397	1/20	7/50	3,932
7	1294	1/15	11/50	5,868
8	1066	1/20	9/50	3,429
9	1090	1/35	30/50	510
10	p80 (115)	1/28	9/50	4,801
11	p80(158)	1/10	8/50	1,832
12	p80(191)	1/10	11/50	1,514
13	p80(270)	1/10	2/50	3,218
14	p80(301)	1/80	5/50	18,420

Figure 5.1 Gel electrophoresis of V1/V2 PCR amplification that was done when DNA from patient 1090 was used

The gel shows two runs, one above the other (from Origin 1 to Origin 2) of PCR reactions from material that derived from patient 1090. DNA sample extracted from PBMCs derived from patient 1090 was used in the following concentrations: 1/5, 1/20, 1/30, 1/35. Ten reactions were done per each dilution of DNA. The tube containing DNA diluted 1/35 gives the final dilution above end point. For this dilution another V1/V2 PCR was performed using 50 replicates with the same result (data not showed). The arrow showed at the left side of the gel gives the direction of migration. The sizes of the fragments provided by the marker are written. The number of wells is given in the lines written above or below the origins, with the distribution of each diluted sample or negative control specified in the legend presented on the right side of the gel. Positive bands resulted from V1/V2 amplification have a molecular weight of 294 bp.

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Lane 1, 25: pGEM marker
 Lanes 2-12: 1090 (1/30)
 Lanes 13-23: 1090(1/35)
 Lanes 26-36: 1090 (1/20)
 Lanes 37-47: 1090 (1/5)
 Lanes 24, 48: Negative controls

5.2.2 PCR amplification of 1.7kb env sequences and their incorporation into a TA cloning vector system

As presented in Section 5.1.1, recombination between the heterogeneous proviral DNA templates can occur. To avoid this putative event leading to hybrid molecules to be manifested during PCR, amplification of env gene from diluted DNA extracts was attempted. All the experiments failed to produce a detectable 1.7kb fragment on the agarose gel. However, despite a moderate viral load (as mentioned in the above section) which suggests a certain probability of DNA recombinants into PCR reaction that can be ascertained by screening and sequencing several env clones derived from the same PCR to establish the identity of each sequenced clone (i.e. if it is or not recombinant), the amplification of the gene from the bulk DNA extracts was undertaken. Many trials to amplify env gene from undiluted DNA sample failed (even when different annealing temperatures, concentrations of Mg^{2+} and addition of additives such as glycerol, DMSO were used), when using different batches of Pfu polymerase. The suspected cause for these failures was probably the low processivity of the Pfu enzyme from those particular batches. Finally, the amplification of the 1.7 kb fragment from several patients employing nested PCR with primers designed for env region (see Chapter 2) using hot start and a batch which provided a processive Pfu polymerase was successfully done. In hot start PCR, all the components of the reaction (i.e. the template, dNTPs, Pfu polymerase, and buffer) are added but not the primers. The reaction tubes were placed on GENEE Thermal Cycler hot block and heated to $92^{\circ}C$ for 1 min to denature the strands. The reaction is paused for 1min at $92^{\circ}C$, during which the primers were added, and then the annealing and extension steps performed normally. This first cycle is followed by other 30 cycles as described in Section 2.5.3. When the temperature dropped from $92^{\circ}C$ to $50^{\circ}C$, the primers recognise and specifically hybridise with the complementary strand leading to a very specific band after PCR reaction.

After PCR amplification, the reaction product was resolved in 1% agarose gel and the 1.7kb patient derived env genes were identified, cut and purified with GeneClean kit. A gel electrophoresis showing a 1.7kb amplicon is depicted in Figure 5.2. The purified bands and the mammalian expression vector pEV6 were digested with BstEII.

Figure 5.2 Agarose gel electrophoresis corresponding to PCR amplification when primers specific for env region were used

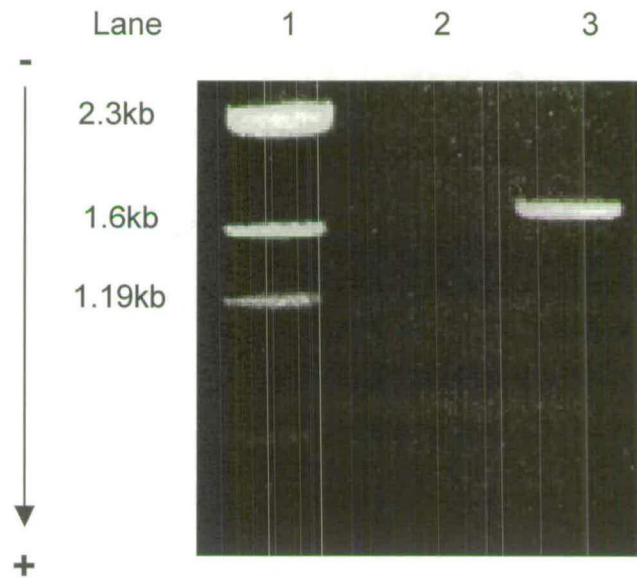
The arrow showed at the left side of the gel gives the direction of migration

Lane 1 shows the bands given by the pGEM Marker

Lane 2 represents the negative control

Lane 3 shows a 1.7kb fragment that represents env gene from patient 1090 amplified from bulk.

Opposite page number 120



Lane 1: pGEM marker
Lane 2: Negative control
Lane 3: 1.7-kb fragment

and XbaI and ligated with T4 DNA Ligation kit (standard protocols). All the attempts to ligate the amplified env genes directly into the mammalian expression vector (pEV6) were not successful. The failure of these experiments might be explained by the blockage of the ends, a process that can occur after electrophoresis, leading to a decrease in percentage of the functional ends. Another explanation might be given by the inefficient cutting of restriction enzymes that impaired the ligation to be carried out efficiently. In conclusion, a series of 1.7kb env fragments were obtained and lost because of the reasons mentioned above. A more efficient and quicker alternative to clone the 1.7kb env genes was provided by TA cloning vectors. Due to the lack of any A-overhang at the 3' ends of the amplicons, making it impossible to subclone the Pfu amplified env genes into TA vectors, 1.7kb fragments were subject to one more cycle of amplification with Taq polymerase in the presence of only dATP, followed by subcloning into the pGEM T vector system. Some of the PCR reactions were done employing Taq polymerase and the fragments produced were subcloned directly into pGEM T. The recombinant clones obtained were firstly differentiated from the parental pGEM T vector by agarose gel electrophoresis and the presence of the env genes was checked by both restriction digestion with BstEII and XbaI and PCR employing primers for env sequence. The clone nomenclature (recombinants deriving from pGEM T that contain env from patient sample) and the enzyme used to amplify the env gene are presented in Table 5.2.

5.2.3 Transfer of the 1.7kb fragments from pGEM T into the mammalian expression vector pEV6

The transfer of the pGEM T saved env genes from pGEM T into pEV6 was done according to standard protocols. pEV6 was linearised by double digestion with BstEII and XbaI. In order to minimise formation of the parental vector (pEV6) and thus to reduce the background after ligation of env genes with linearised pEV6, a third digestion with SmaI (which lies between BstEII and XbaI in polylinker) followed. Env fragments were obtained from env-containing pGEM T derived clones after double digestion with BstEII/XbaI, by separation of the digestion products on the 1.5-% agarose gel and gel purified.

Table 5.2. Recombinant clones obtained when 1.7kb genes were saved in pGEM T vector system

Patient PBMC	Enzyme used in amplification	Recombinant clone nomenclature
1363	Pfu	1363.1; 1363.2; 1363.4; 1363.5; 1363.6; 1363.7; 1363.8
1299	Pfu	1299.1; 1299.2 ; 1299.5; 1299.7 ; 1299.9; 1299.10
1260	Pfu	1260.1; 1260.3; 1260.4; 1260.5 ; 1260.7; 1260.9; 1260.10
1021	Pfu	1021.1; 1021.2; 1021.3; 1021.4; 1021.5; 1021.6 ; 1021.7; 1021.8; 1021.9
1397	Pfu	1397.1; 1397.2 ; 1397.3; 1397.4; 1397.6; 1397.7; 1397.8 ; 1397.9; 1397.10
1066	Pfu	1066.1
1090	Pfu	1090.4
1020	Pfu	1020.4; 1020.7; 1020.8
1299	Pfu	1299.10; 1299.5
158	Taq	158.1
115	Taq	115.2 ; 155.4; 155.6; 155.7 ; 155.8
191	Taq	191.3; 191.4; 191.5 ; 191.6; 191.9
270	Taq	270.1; 270.3; 270.4 ; 270.5; 270.6; 270.8
301	Taq	301.1; 301.2 301.4; 301.5; 301.6; 301.7 ; 301.8; 301.9 ; 301.10

The clones written in bold were selected for the env gene transfer from pGEM T into pEV6. The selection criteria were slightly biased on the quality and the yield of the DNA miniprep obtained but generally was a random process

After ligation, the recombinant clones containing env gene were screened and differentiated from the parental plasmid pEV6 by gel electrophoresis and the presence of env fragments was further confirmed by digestion with BstEII and XbaI and env PCR. The nomenclature of the clones obtained after swapping the genes from pGEM T to pEV6 is given in Table 5.3.

In order to identify the origin of each clone, the following nomenclature that reflects the order of the cloning steps was adopted. The first figure refers to the patient PBMC DNA samples. The suffix to this number refers to the number of the clone that derives from TA vector system in which the env gene was subcloned. The second suffix refers to the recombinant clone that derives from pEV6 in which the env gene was transferred from env containing pGEM T vector. For example, 1365.5.5 refers to the clone number 5, cloned from env containing cloning vector pGEM T number 5, amplified from PBMC DNA sample # 1365.

Figures in *Italics* in Table 5.3 refer to the level of the gp120 glycoprotein of the non-expressing clones or clones that have a very low level of expression that might be considered to be below the sensitivity of the gp120 ELISA employed, respectively. **Bold** figures are allocated to the recombinant clones which could express the gp120 gene and **underlined bold** figures correspond to those clones for which a high variation of the humidity was observed to have occurred (high values for gp120 level can be a result of an artefact given by the fact that supernatants evaporated and, thus, it does not reflect a high level of expression of the associated clones. Up to 500 µl (for example 100µl of each of 2-3 replicates, tested in parallel) out of 2ml of transfection supernatant were used in ELISA to estimate the concentration of gp120 expressed. The supernatant left was stored at -70⁰C and used later to quantify the antibody recognition from the patient sera against autologous and heterologous gp120 (see Chapter 6). As can be seen, all the clones obtained for patients 1021 (5/5) and 1397 (5/5) expressed the env gene, whereas for others such as patients 1299 (0/5), 1090 (0/1), 1020 (0/3) and all the samples deriving from patient p80 (158, 115, 191, 270 and 301) no functional clone could be selected. Interpretation of these results may consist of a relatively high level of defective proviruses into patients

Table 5.3 Recombinant clones able to express the env gene variants.
 This table shows the numbers of clones obtained when each selected pGEM T saved 1.7kb fragment presented in Table 5.2 was transferred to pEV6

Patient PBMC	Nomenclature of clones	gp120 (ng/ml) when 5µg transfectant DNA was used ^a	gp120 (ng/ml) when 1µg transfectant DNA was used ^a
1363	<i>1365.5.5</i>	0	0
	<i>1363.6.3</i>	0	5.8
	<i>1363.7.2</i>	4	5
	1363.8.2	32	56.35
<i>1299</i>	<i>1299.2.2</i>	0	6.9
	<i>1299.5.3</i>	0	0
	<i>1299.7.1</i>	8.6	15.4
	<i>1299.10.1</i>	0.9	2.5
	<i>1299.10.2</i>	1.6	2.8
1260	<i>1260.1.6</i>	5.3	11
	<i>1260.1.7</i>	2.1	4
	<i>1260.3.1</i>	0	0
	<i>1260.4.1</i>	0	2.4
	1260.5.1	49	19.5
1021	1021.6.1	110	45.9
	1021.6.2	26	42
	1021.6.3	34	ND
	1021.8.1	<u>1249</u>	<u>1685</u>
	1021.9.1	20	63
1397	1397.2.7	91	25.3
	1397.4.1	<u>395.3</u>	<u>337</u>
	1397.6.1	584	<u>458</u>
	1397.7.8	ND	180
	1397.8.1	601	<u>1216.4</u>
1066	1066.2.2	41	32
<i>1090</i>	<i>1090.4.2</i>	0	0
<i>1020</i>	<i>1020.4.1</i>	0	1.84
	<i>1020.7.1</i>	0	3
	<i>1020.8.1</i>	0	0
<i>158</i>	<i>158.1/2/3.1</i>	0	5.2
<i>115</i>	<i>115.1/2/3.1</i>	0	0
<i>191</i>	<i>191.1/2/3.1</i>	0	0
<i>270</i>	<i>270.1/2/3.1</i>	0	0
<i>301</i>	<i>301.1/2/3.1</i>	0	0

a. figures are the result of three paralleled transfections

PBMC DNA rather than a disruption of the open reading frame (ORF), as a consequence of a defective ligation or inactivating mutations introduced by PCR. 13 out of 44 clones amplified by Pfu polymerase were positive for env gene expression, corresponding an associated frequency of gene expression of 44.8%. Using Taq polymerase, 15 env clones originating from patient p80-derived samples were obtained, none of these expressed detectable levels of env gene, giving a frequency of gene expression of 0%.

The relationship between the number of expressing clones and the type of the enzyme employed during PCR amplification was analysed by chi square-test ($\chi^2 = 6.46$, $P < 0.01$) and Fisher test ($P < 0.01$). While clone selection was a fairly random process, the result suggests that there is a clear advantage of Pfu polymerase over Taq polymerase in terms of conferring a higher likelihood of gene expression for obtained env clones.

In light of the concepts presented in the previous chapter, the low number of clones able to express the env gene in a range that is detectable in ELISA assay may be explained by the lack of Rev. Therefore, both the number of expressing clones and their level of expression could be increased by co-transfection of these constructs with a Rev-expressing vector.

5.2.4 Screening for a successful gp120 env gene expression by transfection of COS cells

5.2.4.1 Comparison between Lipofectin, Effectine and SuperFect Transfection Protocols

The type of transfection vector and PCR product cloned within it, influences the transfection results. The configuration and size of the construct also determines the efficiency of transfection. Therefore, an experiment was performed to test different transfection protocols and compare their transfection efficiency. First, the Effectine protocol that uses a non-liposomal lipid that works together with a specific DNA-condensing Enhancer. Second, the SuperFect protocol that uses an activated

dendrimer. This is a compound that possessed a defined spherical architecture with branches radiating from a central core and terminating at charged amino groups able to bind transfectant DNA and to buffer the lysosomal nucleases after it has fused with the endosome. The third was Lipofectin that uses a liposome formulation of the cationic lipid N-(2,3-dioleoyloxy)propyl)-n,n,n-trimethylammonium chloride (DOTMA) and dioleoyl phosphatidylethanolamine (DOPE). pEV6 was used as a negative control and pEV5 as a positive control. Clone 1021.8.1 was chosen as a test sample because it gave a good expression level in one previous experiment, when Lipofectin was preliminary used. To test if Effectine and SuperFect give an advantage over the Lipofectin, regarding transfection and gene expression efficiency, clone 1021.8.1 derived plasmid was transfected in COS cells, employing all the methods in parallel. After transfection, COS cells were cultured for 72h and supernatants collected and tested for gp120 level using gp120 ELISA. The results obtained are given in Table 5.4.

Table 5.4 Transfection of COS cells using three transfection protocols

Sample	Effectine gp120(ng/ml) ^a	SuperFect gp120(ng/ml) ^a	Lipofectin gp120(ng/ml) ^a
pEV6	92	74	164
pEV5	13	7.4	14.5
1021.8.1	760	520	1231.5

a. values represent the results of one experiment using duplicates per sample.

Contrary to the expected results, the higher transfection efficiency was obtained for Lipofectin protocol. Therefore, all the transfection experiments to screen for gp120 env gene expression of the patient derived clones were done according to this protocol (see Table 5.3).

5.2.4.2 gp120 cellular staining of COS cells

An immunofluorescence assay to investigate gp120 expression was used as an alternative method for gp120 ELISA. After collecting the supernatants derived from COS cells transfected with patients derived env clones, transfected COS cells were detached from the transfection wells using 0.02M EDTA, washed two times in 1x PBS and treated with a hypotonic solution (PBS: H₂O = 40-60%). One drop of cell suspension was spotted per well on a multiwell microscope slide and allowed to dry thoroughly for 1h. The slides were fixed with a solution methanol: acetone (5:95) or acetone alone for 10min at room temperature, then washed four times with 1xPBS. After the slide had completely dried, one drop of mounting solution was distributed in each well and a cover slide put on top.

The staining procedure involved the sequential usage of the following reagents: antibody anti-gp120 and sheep anti-human IgG^{FITC} (γ chain specific) (Sigma). Three antibodies anti-gp120 were tested in parallel ARP 3054 (GP13), ARP 3055 (GP68) and ARP 3065 (IgG1b12). Supernatants obtained by transfection of COS cells with pEV6 were used as negative controls for each anti-gp120 antibodies being tested. Supernatants from pEV5 transfected COS cells were taken as positive controls. Different fixation protocols were tested to select one that gave a low background. Thus, treatment of the cells with either methanol: acetone = 5:95 or acetone alone was found to suit the purpose of the assay.

Titration of each reagent were done in order to establish the optimum concentration for each one. Dilutions of 1/100 in normal sheep serum for both anti-gp120 antibodies and sheep anti-human IgG^{FITC} were chosen as being optimum. From the intensity of the fluorescent staining of COS cells, ARP 3054 was selected as giving a better signal than ARP 3055 and ARP 3065 (see Figure 5.3).

When a transfection experiment was completed, COS cells were used in immunofluorescent assay to assess the gp120 staining and the supernatants were used to estimate the amount of the secreted glycoprotein. The results obtained by employing both methods were generally consistent, although variations in transfection and staining efficiency between experiments were noticed.

Figure 5.3. gp120 cellular staining of COS cells

The photographs show the cellular staining of COS cells transfected with a gp120 expressing plasmid (1021.8.1), with antibodies directed against HIV-1 gp120 as following:

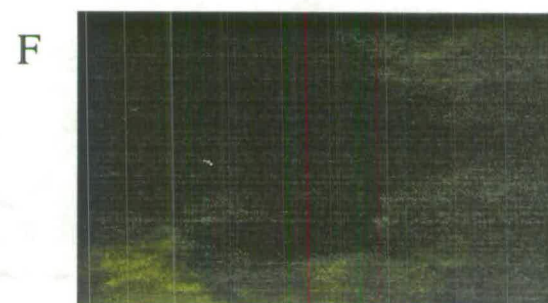
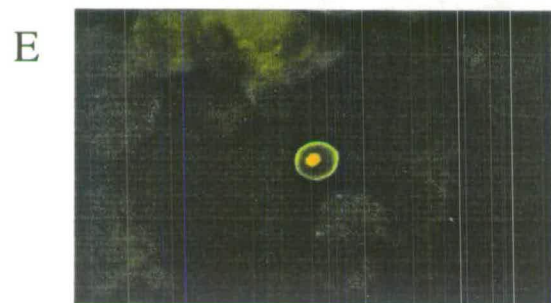
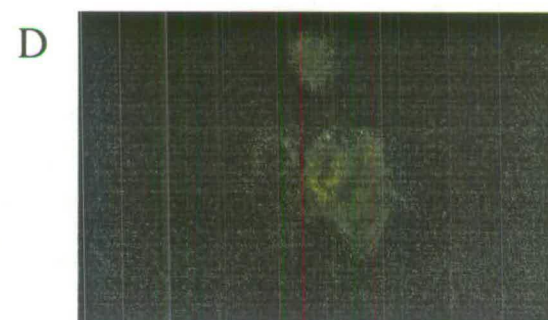
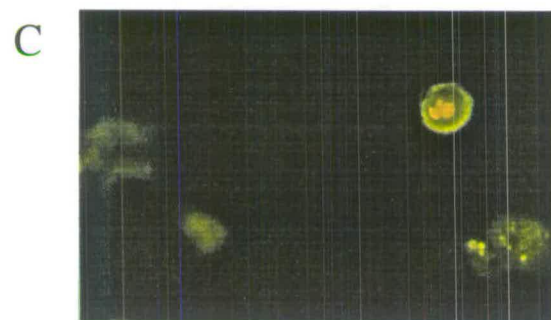
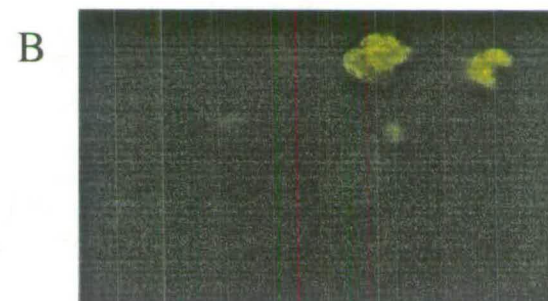
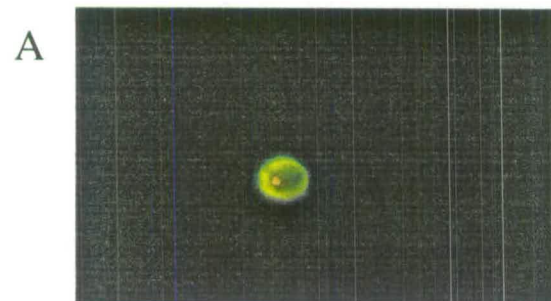
A. ARP 3054 (GP13) recognises V3 loop of gp120 MN, SF2 and other strains.

C. ARP 3025 (GP68) with specificity for V3 loop derived from HIV IIIB

E. ARP 3065 (IgG1 b12) that is mapped to the CD4 binding site of gp120

Mock transfected COS cells stain with antibodies GP13, GP68 and IgG1b12 are shown in photpgraphs B, D and F

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5.2.5 Sequence analysis of the env gene clones

5.2.5.1 NSI/SI Phenotype

The NSI/SI phenotype - associated sequence variation was assessed by employing two methods: Donaldson *et al.* (1994) and Fouchier *et al.* (1992).

In the first method, the phenotype of V3 sequences can be compared on the basis of predicted overall charge (x-axis) and number of amino acid changes from the subtype B consensus (y axis). In this model NSI isolates cluster in an area having a low charge on the V3 loop and a minimum difference from consensus, whereas SI isolates manifest a higher overall positive charge on the V3 loop and have more differences from subtype B consensus. A line (See Figure 5.4) can mark the delimitation between the two subgroups of isolates. Table 5.5 gives some features such as predicted overall positive charge and the number of differences from consensus for the env clones obtained from the homosexual patients and those derived from the patient 82. Figure 5.4 presents the phenotype of the same patients based on V3 loop sequences as predicted by Donaldson *et al.* (1994). The majority of sequences were predicted to be of NSI phenotype by both methods (see Figure 5.4 and Table 5.6). In order to simplify the nomenclature of env clones, the annotations p82a to p82m were written in the brackets following the old nomenclature (see Table 5.6). Clones 1066.2 and 1260.5 from homosexual patients and 82 and 82.3 from patient p82, were predicted to have derived from isolates with SI phenotype.

In Fouchier prediction, the SI isolates exhibit V3 sequences with a significantly higher positive charge than those of NSI isolates do with an obvious difference of the amino acids at positions 11 and 28. Thus, NSI variants usually present uncharged (S or G) residues at position 11 and either negatively charged (E or D) or uncharged residues (A or Q) at position 28. Conversely, in SI isolates, basic residues replace either one or both amino acid residues at positions 11 and 28. The NSI/SI phenotype of the env clones, deduced by Fouchier prediction, is presented in Table 5.6. Using this method, all the sequences but four (i.e. 1066.2, 1260.5, 82 and 82.3) have NSI phenotype.

Table 5.5 Overall charge and divergence from subtype B consensus of V3 loop presented by the patients derived clones and some well-known isolates

Overall charge (+)	Number of differences from consensus	Number of sequences with these properties	Clones from patient with these properties
2	4	2	139.6B 12.16
2	2	1	1363.8
3	0	2	r82r001r (p82a) 108.18
3	2	5	1021.89 12.08 108.10/108.11 108.17
3	4	1	139.05 JRCSF
4	1	1	108.19
4	2	2	108.15 108.42
4	3	1	1021.6.123/123.14 82
5	5	2	82.3 SF2
6	9	1	1260.5
7	12	1	1066.2
8	7	1	LAI
8	5	1	MN

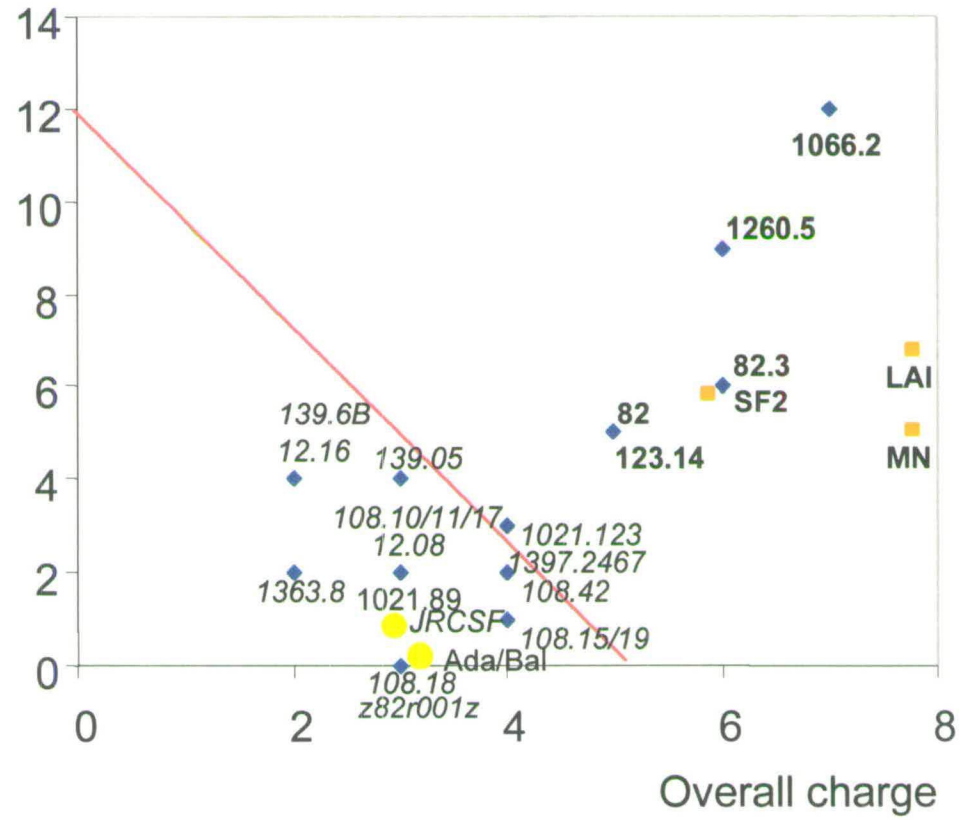
The clones written in bold are considered to derived from isolates with a predictive S1phenotype.

Figure 5.4 Predicted NSI/SI phenotype of the env isolates based on V3 loop sequences as compiled by Donaldson *et al.*, (1994)

Diagram shows the predictive NSI/SI phenotype of the env clones obtained from patients studied, based on the calculated overall positive charge and the degree of difference from the consensus B sequence (CTRPNNNTRKSI.HI..GPGRAFYTTGEIIGDIRQAHC) (Korber *et al.*, 1998). The V3 loop charge was calculated by assigning a unitary positive charge to Arg/Lys residues and a unitary negative charge to Glu/Asp residue. The potential charge contributed by histidine (His) residues was discounted. A line marks the split between NSI and SI isolates. Several well-defined isolates are also shown: Ada/Bal, JRCSF, MN, LAI, SF2, IIIB. When two samples gave the same values, they were written side by side, with the clones deriving from the same patient delimited by slash.

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No of changes from the type B consensus



5.2.5.2 Prediction of the CCR5 co-receptor usage

A correlation between amino acid substitutions at different positions in subtype B consensus was found between HIV-1 strains and CXCR4/CCR5 co-receptor usage. By analysing the influence of amino acid substitutions in the V3 loop on the effect of co-receptor usage, Speck *et al.*, (1997) observed a strong correlation between the presence of either His at position 13 or a Ile at position 33 or both of them simultaneously and the CCR5 co-receptor usage. The correlation between amino acid substitution and the ability to use CXCR4 co-receptor was not consistent, although the presence of either a lysine (K) at position 10 or one of the following residues: isoleucine (I) at position 31, aspartic acid (D) at position 32 and isoleucine (I) at position 33 was found to correlate well enough with viral entry via the CXCR4 co-receptor.

The CCR5/CXCR4 co-receptor usage prediction was applied to patient derived env sequences (see Table 5.6). All the isolates were predicted to use CCR5 as indicated by the score obtained when one unit was allocated for each His residue found at position 13 or Ile at position 29 for each isolate.

Generally the isolates had Lys (K) at position 10 (with only one exception, the isolate 1260.5 for which Lys is replaced by Arg (R) without altering the positive charge at this site), Ile (I) at position 33 and Asp (D) at position 31 (exception being made by clones 1021.6.123, 82, 82.3, 123.14), indicators of a predicted CXCR4 coreceptor usage.

5.2.5.3 Sequence analysis

5.2.5.3.1 Nucleic sequences from patients' PBMC DNA

A total of 12 sequences of the V3 domain and flanking regions (103bp fragment) were obtained directly from five homosexual patients PBMC DNA by nested PCR (i.e. 1021, 1066, 1260, 1363 and 1397). Sequencing was done by Dr. D.Yirrell, Centre for HIV Research, University of Edinburgh. All the clones derived from patient 1397 shown identical sequences (4 out of 4 sequences). The same result was noticed for all three clones that corresponded to patient 1021, with only one silent A→C transversion at position 7193, numbered according to HIV HXB2.

Table 5.6 The amino acid sequence alignment, predicted NSI/SI phenotype and co-receptor usage for patient derived env clones

HIV-1 env clones	1 10 20 28 32 38 CTRPNNNTRKSI.HI..GPGRAFYTGTGEIIGDIRQAHC	SI/NSI (Donaldson)	SI/NSI (Fouchier)	CXCR4	CCR5
1021.6.12	-----T-...-----D--N-----	NSI	NSI	+	+
1021.3	-----T-...-----D--N-----	NSI	NSI	+	+
1021.89	-----T-...-----D-----	NSI	NSI	+	+
1066.2	-----KIG-R.TM...-----V-----K-V---R-Y-	SI	SI	+	+
1260.5	-----RG-.V-...-----V-ARDK-----K---	SI	SI	+	+
1363.8	-E-----N-...-----	NSI	NSI	+	+
1397.2467	-----P-...-----S-----	NSI	NSI	+	+
Z82r001z (82a)	-----P-...-----S-----	NSI	NSI	+	+
108.10 (82b)	-----G-----Q-----	NSI	NSI	+	+
108.11 (82c)	-----T-...-----D-----	NSI	NSI	+	+
108.15 (82d)	-----P-...-----Q-----	NSI	NSI	+	+
108.17 (82e)	-----G-----Q-----	NSI	NSI	+	+
108.18 (82f)	-----P-...-----Q-----	NSI	NSI	+	+
108.19 (82g)	-----G-----Q-----	NSI	NSI	+	+
108.42 (82h)	-----P-...-----Q-----	NSI	NSI	+	+
12.08 (82i)	-----P-...-----D-----	NSI	NSI	+	+
12.16 (82j)	-----G-...-S--A-D-----	NSI	NSI	+	+
82	-----R-...-V--EQ--N-----	SI	SI	+	+
82.3 (82k)	-----R-.Y-...-V--ER--N-----	SI	SI	+	+
139.05 (82l)	-----G-...-S--A-G-----	NSI	NSI	+	+
139.6B (82m)	-----G-...-S--A-D-----	NSI	NSI	+	+

The sequences obtained from both homosexual cohort and haemophiliac patient p82 are presented in Figure 5.5, with alignments being preserved using dash to indicate the presence of gaps and dots to show the identity to the first sequence.

The 5'-end of sequences deriving from clones 108.10, 108.11, 108.17, 108.19, 12.08, 12.16, 82, 82.3 and 123.14 (between position 7051 and 7130) could not be read with accuracy and dashes were used to keep the alignment. Very few deletions and no insertions have been identified among the sequences. Conversely, a certain degree of variability has been noticed both within the nucleic acid sequence that encodes V3 loop and its flanking regions. This result may have been expected, since they were previously tested for their capability to express the env gene.

In flanking regions, the sequence variability was present at positions -9, -18, -19, -26, -38, -53, and -55 and +4, +14, +16, +23, + 25, +34, +35, +39, +44, +59 and +69 (the +/- signs reflect the direction from V3 region towards 5'/3' end of the sequences). The most frequent positions characterised by high variation within V3 region were: 37, 38, 58, 70, 80, 82, 91 (see Figure 5.5).

5.2.5.3.2 Nucleotide distances of the V3 and flanking regions within and between samples

Pairwise nucleotide distances were estimated for the sequences derived from both homosexuals and haemophiliac patient (p82) using the program DNADIST, as implemented by PHYLIP package (version 3.4), based on Kimura correlation. Average sequence diversity, both within and between sequences were estimated based on the pairwise nucleotide distances and is presented in Tables 5.7 and 5.8. Nucleotide distances of the V3 sequences within samples from the same haemophiliac patient (p82), at five points from seroconversion (year 3, year 4, year 6, year 7) were estimated by Dr. Lin Qi Zhang (1992) (PhD thesis, University of Edinburgh, 1992). The average intra-sample distances in year 3 was 3.2%, 3.6% in year 4, 2.5% in year 5, 5.7% in year 6 and 4.6% in year 7.

Figure 5.5 Alignment of the env clones sequences from homosexual patients and those from patient p82, encompassing V3 and flanking region.

The diagram shows the nucleic sequences for the V3 loop and flanking regions of homosexual patients derived env genes and those of the patient p82. Nucleotide positions are numbered according to HXB2. The sequences were aligned to the sequence of the clone 1021.6.12. Only nucleotides that differed from those that belong to the first sequence are shown. Gaps were used to maintain the alignment, when necessary, and were marked by dashes. The identity to the first sequence is shown by dots. All the sequences originating from patient p82 were written in the order in which they were chronologically found in the PBMC DNA (seroconversion: z82r001z, 1987: 108.10-108.42, 1988: 12.08, 12.16, 82, 82.3, 1989: 123.14, 1990: 139.05, 139.6b). 1021.6.12 represents the unique sequence obtained for both clones 1021.6.1 and 1021.6.2. The sequence TGT at positions -9 and 100 is given in bold to show the codons for Cys in the clones sequences.

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	7051											7149
		-55/53	-38	-26	-19/18	-9	1	10	20	30		
1021.6.12	AA	TTCTCGGACAATGCTAAA	ACTATAATAGTACATCTGAATAAA	TCTGTAGAAATTAATT	GTACAAGACCCCAACAACAATACAAGAAAA	AGTATAACT						
1021.6.3
1021.8	C.....	G.....
1021.9	C.....	G.....
1066.2	A.A.....	C.....	A.....	G.T.G.....	A.....
1260.5	A.....	A.....	C.....	G.....	C.....	T.....	C.....	G.G.....	GT.....
1363.8	A.....	C.....	G.....	C.....	A.C.....	C.....	GA.....	T.....
1397.2467	A.....	A.....	C.....	G.....	A.....	C.....
Z82r001z(82a)	A.....	C.....	G.....	GG.....	CA.....
108.10(82b)	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	CA.....
108.11(82c)	-----	-----	-----	-----	-----	-----	-----	G.-C.....	-----	-----	-----	CA.....
108.15(82d)	A.A.....	C.....	G.....	GG.....	T.....	C.....
108.17(82e)	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	CA.....
108.18(82f)	A.A.....	C.....	G.....	GG.....	A.....
108.19(82g)	-----	-----	C.....	G.....	G.....	T.....	CA.....
108.42(82h)	A.A.....	C.....	G.....	C.GG.....	C.....
12.08(82i)	-----	-----	C.....	G.....	GG.....	C.....	C.....
12.16(82j)	-----	-----	C.....	G.....	G.....	T.....	G.....
82	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	G.....
82.3(82k)	-----	-----	C.....	G.....	G.....	T.....	G.....
139.05(82l)	A.A.....	-C.....	G.....	G.....	T.....	G.....
139.6B(82m)	A.....	C.....	G.....	G.....	T.....	G.....

	7150	40	50	60	70	80	9	100	+4	+14/16	+24/25	7253
1021.6.12	ATCCAGAGAGGACCAGGGAGAGCATT	TTTATA	CAACAGGAGACATAATAGGAAATATAAGACAAGCACATT	TGTAACATTAGTAGAGAACAATGGAACAAACTT								
1021.6.3											C.....
1021.89						G.....					
1066.2	T.....			A.A.G.....	G.....	G.....	T.....		A.....		TGCC....
1260.5	G.....	G.....	G.ATA.A.....	G.....	A.....				C.G.....		TT.C....
1363.8				A.....	G.....				A.A.G.....		TG.C....
1397.2467	G.....	G.....	C.....	AGT.....	G.....				G.AC.C.....		C.T.-----
z82r001z(82a)	A.....			A.....	G.....				C.....		C.C.....CTG.C....
108.10(82b)				C.A.....	G.....				C.....		C.A.....TG.....
108.11(82c)				T.....	G.....				C.....		C.A.....TG.....
108.15(82d)				C.A.....	G.....				C.....		C.A.....TG.....
108.17(82e)				C.A.....	G.....				C.....		C.A.....TG.....
108.18(82f)				A.....	G.....				C.....		C.A.....TG.....
108.19(82g)				GA.....	G.....		G.....		C.A.....		TG.....
108.42(82h)				C.A.....	G.....				C.....		C.A.....TG.....
12.08(82i)					G.....				C.....		C.A.....TG.....
12.16(82j)	T.....		G.....		G.....				C.....		C.A.....TG.....
82.3		G.....		CGA.....					C.....		C.A.....TG.....
82.0		G.....		C.A.....					C.....		C.A.....TG.....
123.14(82k)		G.....		A.C.A.....					C.....		C.A.....TG.....
139.05(82l)	T.....		G.....	GA.....	G.....				C.....		C.A.....TG.....
139.6B(82m)	T.....		G.....		G.....		G.....		C.....		C.A.....TG.....

	7254	7293
	+34/35 +39 +44	+59 +69
1021.6.123	TAGAACAAATAGCTATAAAAATTAAGAAAACAATTCGGGAA	
1021.6.3	
1021.89	
1066.2	..AG..G....T.GA.....G.....--	
1260.5	..C...G.....GG.....T.C.....--	
1363.8	..C...G.....T.....T.C.....A.--	
1397.2467	-----	
Z82R001Z(82a)	..AG..G....T..G.....G.....G..	
108.10(82b)	..A...G....T.....G..G.....G..	
108.11(82c)	..AG..G....T.....G..G.....--	
108.15(82d)	..AG...-----	
108.17(82e)	..A...G....T.....G..G.....G--	
108.18(82f)	..AG..G....T.....G.....G..	
108.19(82g)	..A...G....T.....G.....G..	
108.42(82h)	..A...G....T.....-----	
12.16(82i)	..A...G....T.....G.....GC.	
12.08(82j)	..AG..G....T.....G..G.....G--	
82.3	..A...G-----	
82	..A...-----	
123.14(82k)	..A...G....T.....G.....G..	
139.05(82l)	..A...G....T.....G.....G..	
139.6B(82m)	..A...G....T..C.....G.....G..	

The same algorithms were applied for sequences that derived from the same patients (1021 and 1397) producing an intra-sample distance of 1.8% for patient 1021 and 0% for patient 1397 (see Table 5.7).

Figures presented in Table 5.7 suggest that patients 1021 and 1397 were investigated either early in infection (very close to the seroconversion time) or they are at a considerable time after seroconversion but they may not be progressors and hence their virions are not under the immune pressure to evolve into a heterogeneous population.

The mean inter-sample distances between sequences derived from patient p82 were estimated to be: 22.3%, between year 0 and year 3, 15.93%, between year 0 and year 4, 13.5%, between year 0 and year 5, 22.4%, between year 0 and year 6 and 20.38%, between year 0 and year 7. Thus, the mean intra-sample distance decreased after year 3 to reach the minimum value of 13.55% by year 5 and increased after year 6 to a value that was comparable to that between year 0 and year 3 (Dr. Lin Qi Zhang, 1992). For homosexual cohort, the inter-sample variation values, as computed by DNADIST program, are presented in Table 5.8. When analysing the distances between sequences taken from different patients, it became noticeable that all the values were less than that which was estimated for patient p82 between year 0 and year 7 (20.38%). This result suggests that the extent of V3 variation that appears within the individual over several years can reach the same order as that that is manifested between different persons. Holmes *et al.* (1995) reported the same result by investigating 132 V3 sequences obtained from HIV-1 infected persons who belong to different risk groups in Edinburgh (including sequences from patient p82).

The main conclusion of this analysis, regarding the homogeneous nature of the sequences obtained at or near after seroconversion with an enormous diversification of the sequences over time, is in agreement with that reported by Dr. Lin Qi Zhang (1992). The diversification of the sequences over time may be a consequence of a positive selection imposed by the host immune system or the need of the virus to use different co-receptors in order to gain the ability to infect different cell types, or a

Table 5.7 Mean pairwise nucleotide distances within the V3 sequences obtained for patient 1021.

Sample (a)	1021.6.1	1021.6.2	1021.6.3	1021.8.1	1021.9.1
1021.6.1	0 (b)				
1021.6.2	0	0			
1021.6.3	0.004	0.004	0		
1021.8.1	0.013	0.013	0.018	0	
1021.9.1	0.013	0.013	0.018	0	0

(a) env sequences obtained from patient 1021

(b) distances were estimated using DNADIST program, implemented in PHYLIP package. Mean distances from all pairwise inter-clone comparisons are presented below the diagonal.

Mean Intra Sample Variation **0.81%**
Range **0%-1.8%**

Table 5.8 Mean pairwise nucleotide distances for V3 sequences obtained for patient 1021, 1066, 1260, 1363 and 1397

Sample (a)	1021	1066	1260	1363	1397
1021	0.81 (b)				
1066	<i>0.1378</i>	0			
1260	<i>0.1570</i>	<i>0.1300</i>	0		
1363	<i>0.1206</i>	<i>0.2130</i>	<i>0.1590</i>	0	
1397	<i>0.1130</i>	<i>0.1680</i>	<i>0.165</i>	<i>0.1440</i>	0

(a) env sequences obtained from patients 1021, 1066, 1260, 1363 and 1397.

(b) distances were estimated using DNADIST program, implemented in PHYLIP package. Mean distances from all pairwise inter-sample comparisons are presented in italics below the diagonal.

Mean Inter Sample Variation **13.4%**
Range **0-19.4%**

neutral selection, as a consequence of the random activation of infected cells.

5.2.5.3.3 Phylogenetic relationship between V3 sequences

The phylogenetic relationship among sequences from homosexual patients, with reference to those obtained from patient p82 and three conventional subtype B isolates (HXB2, MN and SF2) was estimated by Neighbour-Joining method (NEIGHBOUR program, implemented in the PHYLIP package (version 3.4). A neighbour joining unrooted tree is presented in Figure 5.6, where a major division of the sequences into two main clusters is clearly seen.

Five sequences obtained from patient 1021 clustered together, whereas all the sequences originated from homosexual patients appeared on distinct branches, showing that no contamination of plasmid DNA occurred. All branch lengths of the tree are drawn to scale, allowing an assessment of the relative amount of evolutionary change between the isolates to be made.

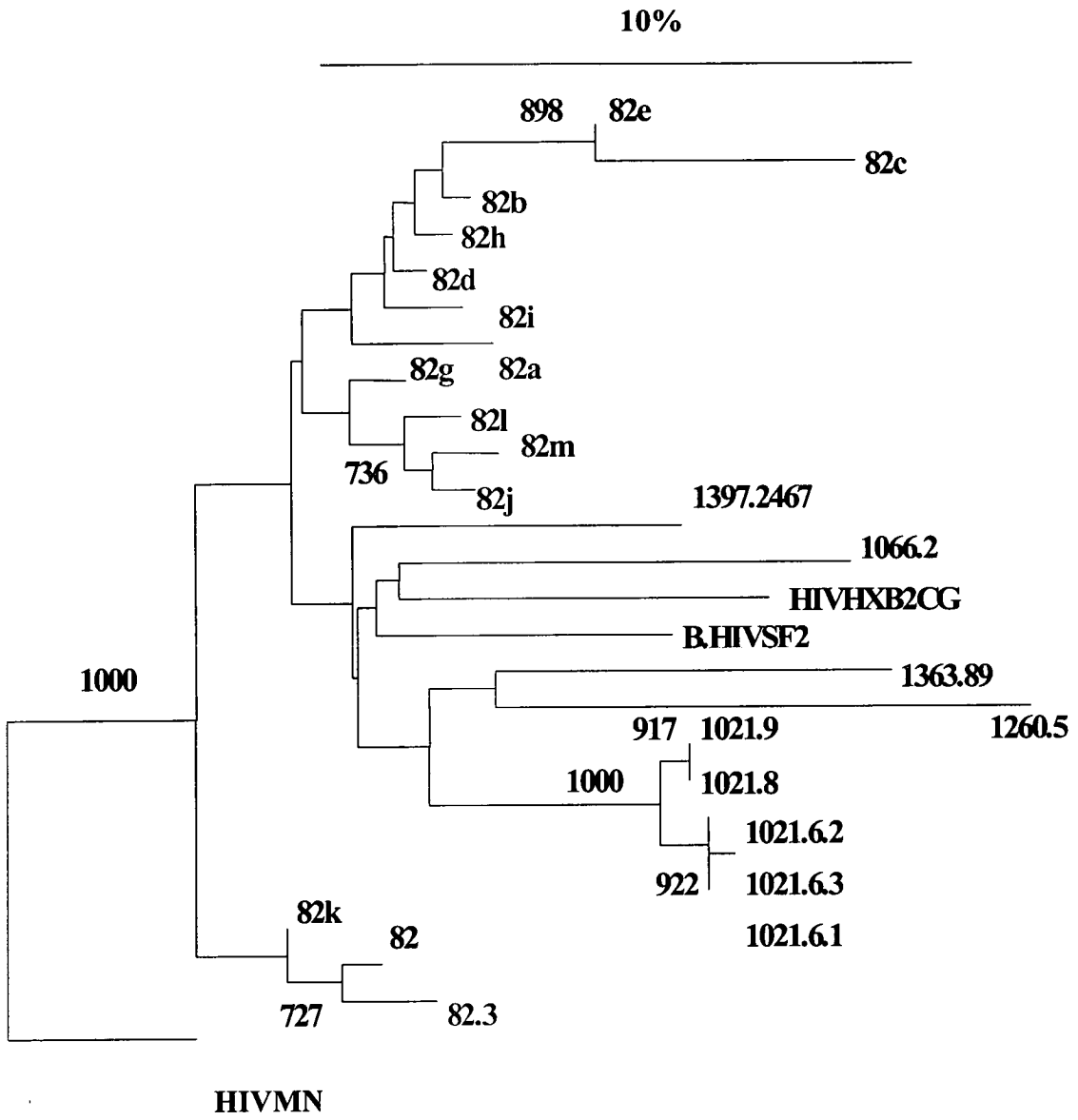
5.2.5.3.4 Amino acid sequence variation and glycosylation pattern in the V3 region of env sequences

The translated amino acid sequences of V3 domain and flanking regions of env sequences obtained from homosexual patients and patient p82 are presented in Figure 5.7. The V3 region consists of a 38 amino acid sequence whereas 5' and 3' regions comprise a stretch of 20 and 23 amino acid residues, respectively. The frequency with which the amino acid replacement was manifested (given by the total number of sites where a replacement is accounted) is very similar in all three regions investigated: 0.45 (9/20) in 5' region, 0.5 (19/38) in V3 domain and 0.52 (12/23) in 3' region. The amino acid replacements within V3 loop are largely concentrated at positions: 306, 308, 313, 315, 317, 319, 320, 324 and 327 (the numbering was done

Figure 5.6. The Neighbour-joining phylogenetic tree for env sequences

The diagram shows the unrooted phylogenetic tree of HIV-1 isolates that were obtained from homosexual individuals and from an HIV-1 positive haemophiliac patient infected from a contaminated batch of factor VIII (p82). The tree includes other three subtype B isolates (HIVHXB2, MN and SF2). The scale bar corresponds to 0.10% nucleotide sequence divergence. Horizontal branch lengths are drawn to scale. The method of bootstrap resampling (100 replications) was used on neighbour-joining trees (programs, SEQBOOT and CONSENSE). The bootstrap values higher than 50% are written in italics at the level of divergence, giving the approximate confidence limits on individual branches.

The sequences amplified and analysed by Dr. Sarah Ashelford (Ashelford (1996) PhD Thesis) and those deriving from homosexual patients clustered individually into two main lineages. To simplify the presentation the nomenclature p82a-p82m was adopted (see Figure 5.5 and Table 5.6). The sequences deriving from homosexual patients clustered together and apart from those originating from haemophiliac patient.



with reference to the HIV HXB2 isolate, Los Alamos, 1998). Some of these sites represent targets for neutralising antibodies (306, 308) and cytotoxic T cell, and are responsible for conversion from one phenotype to another (Milich *et al.*, 1993, Donaldson *et al.*, 1994, Fouchier *et al.*, 1992). The hypervariable nature of the V3 region was reported by many investigators (La Rosa *et al.*, 1990, Albert *et al.*, 1992), highlighting the high frequency of non-synonymous substitutions in this region, imposed by the host factors (immune system) or viral factors (diversification of viral tropism).

Comparison of amino acid sequences revealed common patterns of potential N-linked glycosylation in all sequences. Seven glycosylation sites were predicted to be present in the majority of sequences from both homosexual and haemophiliac patients, having the locations: 276, 289 (in 5' region), 295, 312 (in V3 domain) and 333, 339 (in 3' region).

Four glycosylation sites were perfectly conserved among the sequences (sites 276, 295, 312 and 331), with one exception being represented by the sequence derived from patient 1066.2, where Thr (T) was replaced by Ile (I) at position 312. By contrast, sites 289 and 339 (located at 5' and 3' regions) showed a greater variability. Thus, at position 275, Asn(N) was replaced by Thr (T), Lys (K) or Gln (Q) in sequences 1363.8, 108.18, 108.42 and z82r0001z. Position 339 is characterised by the Asn (N) replacement to His (H), Ile (I) or Thr (T), respectively, in sequences 1021.6.3, 1397, 1260.5 and z82r0001z. The loss of glycosylation sites may be relevant in connection with some biological properties such as antigenicity and infectivity (Montefiori *et al.*, 1988).

Some regions within V3 loop were characterised by the presence of highly conserved amino acid residues among the isolates, namely: CTTRPNNNTRK (positions 296 and 305), IGPG (positions 309 and 312) and IRQAHC (positions 324 and 329) suggesting the presence of constraints within V3 domain in order to preserve the glycoprotein quaternary structure (La Rosa *et al.*, 1990).

Figure 5.7 Alignment of the nucleic acid and amino acid sequences of the V3 loop and flanking regions

The diagram presents the alignment of deduced amino acid sequences for the V3 loop and flanking regions from the HIV-1 infected homosexual patients together with those deriving from a haemophiliac individual (designated p82). Nucleotide sequences from recombinant gp120 clones were translated, aligned and compared to the consensus env sequence 1021.6.12389. Dashes denote sequence identity with the first sequence, while dots represent gaps introduced to optimise alignments. Question marks above the first sequence indicate sites at which less than 50% of the viruses share the same amino acid residue. The arrows denote conserved cystein residues. V3 designates the third hypervariable HIV-1 envelope domain (Modrow *et al.*, 1987). Rectangles represent potential glycosylation sites.

V3 domain

	276	289?	295?	312*?	??	??	??	??	??	??	??	??	??	??	??	??	??	??
	□	□	□	□						□	□							
1021.6.123	NFSDNAKTIIV	HLNKSVEINC	TRPNNNTRKS	ITI..GPGRA	FYTTGDIIGN	IRQAHCNISR	EQWNKTLEQ	IAIKLRKQFG										
1021.89					D ₂													
1066.2	-T-----	Q-----	-----KIG-R	--M..---V	-----K-V-D	--R-Y-----	AE--A--R-	-VE---E---										
1260.5	-T-----	---E--V---	-----RG	-V-..---	V-ARDK---D	--K-----	-K-I---Q-	-AR--ST--D										
1363.8	-T-----	Q-TK--N-T-	E-----	-N-..---	-----E--D	-----K	KE--D--Q-	-----ST---										
1397.2467	-T-----	Q--E-I---	-----	-P-..---	-----S--D	-----G	T--H.....										
Z82r001z (82a)	-T-----	Q-KE--E---	-----	-H-..---	-----E--D	-----L--	AK-TD--R-	-VM---E---										
108.10 (82b)	-----	-H-..---	-----G-	-----Q--D	-----L--	AK--E--K-	-V---G---									
108.11 (82c)	-----	-----	-----L--	AK--E--R-	-V---G-...										
108.15 (82d)E--V---	-----	-P-..---	-----Q--D	-----L--	AK--E--R-	-V---G-...										
108.17 (82e)	-----	-H-..---	-----G-	-----Q--D	-----L--	AK--E--K-	-V---G---									
108.18 (82f)	-T-----	Q-KE-----	-----	-H-..---	-----E--D	-----	AK--E--R-	-V-----										
108.19 (82g)E--V---	-----	-H-..---	-----G--D	-----I--	AK--E--K-	-V-----E										
108.42 (82h)	-T-----	Q-QE-----	-----	-P-..---	-----Q--D	-----L--	AK--E--K-	-V-----										
12.08 (82i)	-----	-P-..---	-----D	-----L--	AK--E--R-	-V---G---										
12.16 (82j)	Q--E--V---	-----G	-H-..---	-----S-	--A-----D	-----L--	AK--E--K-	-V-----									
82	-----R	-----V--EQ	-----	-----AK--E--K-										
82.3 (82k)	-----R	-Y-..---	-----V--ER---	-----	-----AI--E--N-										
139.05 (82l)E--V---	-----G	-H-..---	-----S-	--A--G---D	-----AK--E--K-	-V-----E										
139.6B (82m)	-T-----	Q--E--V---	-----G	-H-..---	-----S-	--A-----D	-----AK--E--K-	-VT-----										

5.3 Summary of results

The simple nested PCR method using primers for V1/V2 region of env, applied to estimate the provirus copy number for each DNA sample, revealed a range of the provirus copy number of between 510 and 18,420 copies/10⁶ PBMC. This result suggested a certain probability of recombination between heterogeneous sequences being eventually present in DNA extracts. However, if present, it can be ascertained by sequencing the clones obtained from the same sample. Consequently, the amplification of env region was performed from bulk extracts using both Pfu and Taq polymerase, The strategy for efficient cloning was found to be that that employed pGEM T, as a cloning vector, from which the env sequences were subsequently exchange into the expression vector (pEV6). 13 out of 25 env clones, amplified with Pfu polymerase, which were obtained from five patients, were functional. For the other patients (1020, 1299, 1294 and 1090), no functional clone was produced. The same result was given by the patients p80-derived samples (158, 115, 191, 270 and 301). The number of defective env genes integrated in PBMC DNA is dependent on patient factors. Thus, for some patients, all the clones produced were functional, whereas for other only 20-25% or even 0% was functional. Biological characterisation was done using predictive methods for estimating SI/NSI phenotype and the ability to use CCR5/CXCR4 co-receptors. All the sequences obtained from homosexual patients were predicted to be NSI isolates with two exceptions: 1021.9 and 1066.2. The same NSI phenotype was estimated to be preponderant among the isolates obtained from patient p82 with three exceptions: 82, 82.3 and 123.14. All of the sequences investigated are predictably able to use both co-receptors (CCR4 and CXCR5).

Sequence analysis of env clones from the homosexual group was performed within and between patients in order to reveal the evolutionary distance between isolates derived from different HIV-1 positive individuals and those taken from the same patient. Nucleotide distances and the phylogenetic analysis of the sequences strongly suggested the relatedness of the sequences isolated from the homosexual patients in comparison to p82- derived sequences. This aspect is important because it will be correlated with the antibody recognition of the patient derived glycoprotein by the IgG antibodies that are present in autologous and heterologous sera (see Chapter 6).

CHAPTER 6

ANALYSIS OF THE REACTIVITY OF IgG ANTIBODIES FROM PATIENT'S PLASMA AGAINST AUTOLOGOUS AND HETEROLOGOUS GP120 ANTIGENS

6.1 Introduction

6.1.1 Overview of the ELISA method for quantifying the antibody response

6.2 Results

6.2.1 The extent of the IgG antibody response to autologous and heterologous patient-derived env glycoproteins

6.2.2 Analysis of the results obtained by ELISA in the presence of chaotropic compounds (8-M urea)

6.2.3 Correlation between mean antibody reactivities recorded for homosexual individuals and their CD4 cell count

6.2.4 Characterisation of the IgG antibody response of Edinburgh haemophiliac patients who received a common batch of factor VIII to autologous and heterologous gp120 glycoproteins

6.2.4.1. Longitudinal characterisation of reactivity of IgG antibody from different samples from a haemophiliac patient (p82) to autologous gp120 antigens

6.2.4.2 High degree of cross-reactivity between sera from other haemophiliac patients against gp120 antigens obtained from patient p82

6.2.5 Level of recognition of recombinant gp120 glycoproteins by IgG antibody from haemophiliac and homosexual-derived sera

6.3 Summary of results

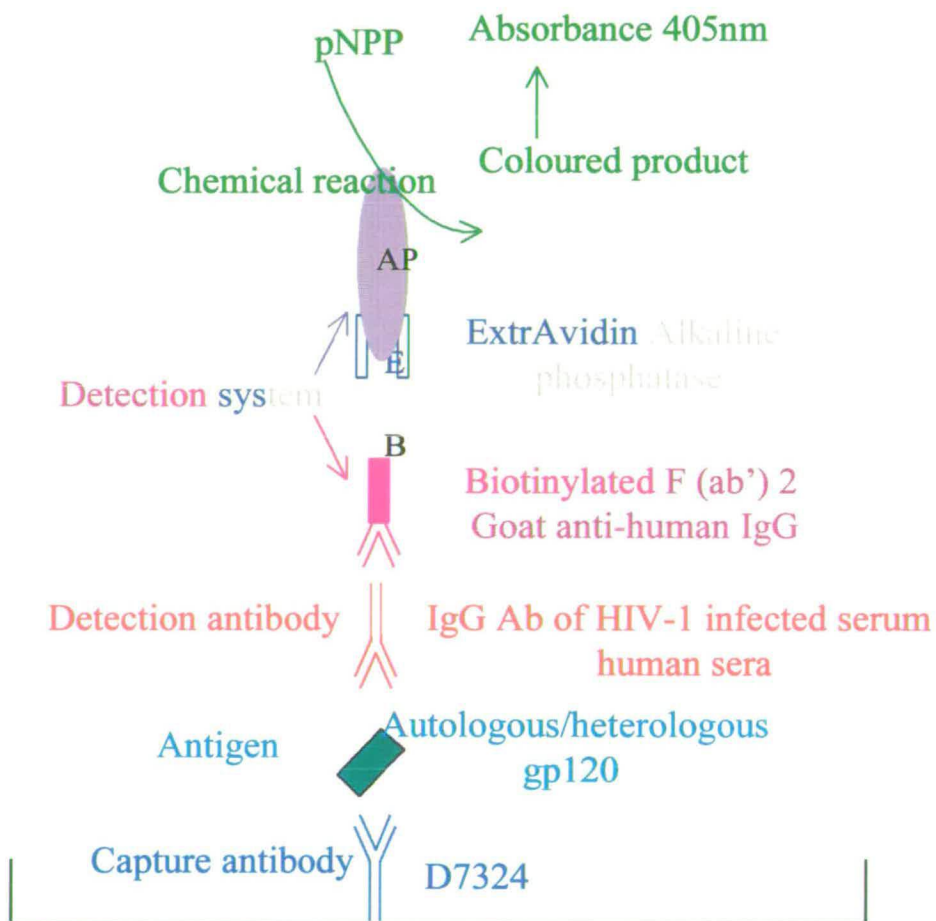
6.1 Introduction

This chapter describes results obtained using an ELISA assay in order to quantify the IgG antibody response of sera from homosexual and haemophiliac HIV-1 infected patients to autologous and heterologous gp120 antigens that were produced in mammalian cells (COS cells). The aims were: to measure the IgG antibody response from two groups of individuals (homosexual and haemophiliac) to monomeric, recombinant gp120 glycoproteins expressed in mammalian cells and derived from the same individuals, and to do this in autologous and heterologous combinations to allow comparison of these reactivities. Although these glycoproteins have a more exposed conformation than oligomeric molecules, their glycosylation pattern and hence their antigenic properties should be close to those produced by infected T lymphocytes, *in vivo*. Thus, the results obtained will approximate the *in vivo* degree of recognition of these antigens by patients' IgG antibody, being superior to all the results obtained by ELISA assay using V3 peptides, which quantify only the anti-V3 IgG antibody, and those produced when gp120 antigens were expressed in bacteria or insect cells, which have a glycosylation pattern different from those produced by the infected lymphocytes (see Chapter 7).

6.1.1 Overview of ELISA method for quantifying the antibody response

In order to estimate the reactivity of patients' sera against gp120 glycoproteins secreted in transfection supernatants, the ELISA method used to quantify gp120 glycoprotein described in Chapter 2.11.1 was employed with some modifications. The principle of this ELISA is given below. The capture antibody D7324 (a sheep polyclonal antiserum able to recognise and interact with C1 and C5 conserved regions of gp120 glycoprotein) was absorbed on each well of a microtitre plate. Between 100-500 ng/ml of recombinant gp120 glycoproteins, which had been expressed in transfection supernatants and had previously been quantified by the gp120 ELISA, was added in order to interact with capture antibodies. Subsequently, the patient's sera (diluted 1/400) were added in each well.

Figure 6.1. Diagram showing the principle of ELISA assay for estimation of IgG Ab response of patients sera to monomeric gp120 glycoprotein



This step was followed by the addition of biotinylated F(ab')₂ goat anti-human IgG that are able to interact with the IgG class from patient sera. Finally, ExtrAvidin alkaline phosphatase interacted with biotinylated F(ab')₂ anti-human IgG and pNPP was used as a substrate to produce a coloured product that has an absorbance read at 405nm. For each experiment, between 10-20 negative sera were used in order to establish a cut-off value calculated by employing the following formula: $CO = N^* + 3SD$, where N^* is the average of all the absorbance values recorded for the negative sera. The ratio between the absorbance of each test sample and the cut-off established for that experiment was used to define the reactivity of that sample so that results between different experiments could be compared. The antibody recognition expressed by the given reactivities referred to the extent of the IgG antibody response to gp120 antigens, without defining their functionality (i.e. if they are able to neutralise or not).

6.2 Results

6.2.1 The extent of the IgG antibody response to autologous and heterologous recombinant gp120 antigens

A panel of recombinant gp120s prepared from homosexual patients, as described in Section 5.2.4, was used to assess the magnitude of the IgG antibody reactivity from their sera to autologous and heterologous recombinant gp120 antigens. Sera used to conduct these experiments derived from nine homosexual patients: 1363, 1299, 1260, 1021, 1397, 1066, 1090, 1294 and 1020. Both positive and negative sera were diluted 1/400 in order to avoid non-specific interactions that appear when using concentrated samples. A panel of ten constructs that successfully expressed the *env* gene, deriving from five homosexual patients designated 1363.8.2, 1260.5.1, 1260.6.1, 1021.8.1, 1021.9.1, 1397.2.7, 1397.4.1, 1397.6.1, 1397.7.8 and 1066.2.2 was used. gp120 antigens were obtained directly from transfected COS supernatants, their amount having been estimated by employing the gp120 ELISA described in Chapter 2. A constant amount of gp120 antigen (50ng/ml) was added in each ELISA experiment in order to avoid the variation in the OD between different samples due to variation in the amount of antigen. The same experiment was repeated with 500ng/ml and

1000ng/ml of gp120 glycoprotein when a rise in both the magnitude of the reactivity and the degree of cross-reactivity between sera and gp120 antigens was noticed (data not shown). All the subsequent ELISA experiments were performed at low concentration of antigen (50ng/ml) to avoid the contribution of non-specific and low affinity interactions, which can be manifested at a high concentration of gp120 antigen (i.e. using a high antigen concentration both the high and low affinity antibody will bind equally to the antigen, whereas at limiting antigen concentration only high affinity antibodies bind to the antigen).

High level of variation in anti-HIV antibody

Table 6.1 gives the reactivities obtained for the individuals from the homosexual group when their sera were tested against autologous and heterologous gp120 antigens. These results were obtained by running three independent experiments, in which each sample was analysed in duplicate. The graph presented in Figure 6.2 shows the extent of cross-reactivity between all the patients' sera and the gp120 glycoproteins used, with error bars indicating the standard deviation between 6 replicates. In order to simplify the presentation of the results, the reactivities for all the antigens obtained from the same individual were averaged and data obtained in this way is given in Table 6.2. Plots of the mean reactivity of IgG antibody from each individual serum against gp120 antigens are drawn in Figure 6.3 A to I.

It was found that all the sera exhibited significant reactivity (r) against both autologous and heterologous recombinant antigens. The range of response was patient-specific (ie. some sera were more strongly reactive against all clones).

Several observations could be seen:

1. a high degree of cross-reactivity between all sera and both autologous and heterologous env clones,
2. the extent of the response to gp120 antigens was patient-specific (the recognition of the env clones depended on the immunocompetence of each individual). On the basis of the magnitude of the response to rgp120s, patients' sera could be classified into three groups of reactivity:
 - high, comprising the patient 1021 (mean r value 6.34),

Table 6.1 Data obtained from three ELISA experiments testing the overall recognition of gp120 glycoprotein by autologous and heterologous sera

Clone sera (a)	1363	1299	1260	1021	1397	1066	1090	1294	1020
1363.8.2	3.99±0.3(b)	1.98±0.35	4.31±0.0005	6.17±0.32	3±0.01	4.12±0.45	2.68±0.07	2.58±0.04	4.4±0.14
1260.5.1	2.12±0.42	1.12±0.05	2.78±0.49	5.92±0.06	2.31±0.31	2.54±0.62	2±0.14	1.62±0.1	2.29±0.45
1021.6.1	3.03±0.38	1.43±0.15	3.54±0.35	6.82±0.11	2.79±0.21	3.07±0.54	2.37±0.05	2.03±0.07	3.4
1021.8.1	2.92±0.19	1.1±0.24	3.25±0.11	6.18±0.14	2.68±0.02	3.23±0.15	2.41±0.01	2.03±0.09	3.53±0.18
1021.9.1	3.49±0.29	1.73±0.4	3.71±0.12	7±0.15	3.09±0.32	3.76±0.24	2.53±0.23	2.52±0.22	3.95±0.32
1397.2.7	3.7±0.32	1.11±0.1	3.85±0.03	6.44±0.2	3.52±0.2	4.22±0.5	2.6±0.14	2.75±0.29	4.16±0.48
1397.4.1	2.89±0.24	1.05±0.2	2.91±0.02	6.02±0.17	2.81±0.38	3.06±0.37	2.09±0.1	2.36±0.1	3.03±0.16
1397.6.1	3.89±0.02	1.22±0.28	3.8±0.16	6.4±0.18	3.12±0.18	4.09±0.06	2.56±0.14	2.36±0.18	3.03±0.03
1397.7.8	3.52±0.02	1.22±0.28	3.8±0.16	6.4±0.18	3.12±0.18	4.09±0.06	2.56±0.14	3.12±0.18	3.99±0.03
1066.2.2	2.56±0.16	1±0.01	3.03±0.03	6.11±0.21	2.73±0.09	3.18±0.04	2.35±0.01	1.94	3.29±0.11

(a) refers to the env clones obtained from homosexual patients

(b) figure indicates the reactivity for autologous combination of gp120 glycoprotein and serum

Figure 6.2. Relative cross-reactivity of sera to different recombinant gp120 antigens derived from homosexual patients.

The graph shows different extent of recognition of the gp120 antigens obtained from homosexual patients to autologous and heterologous sera

The reactivity value is calculated as a ratio between the optical density (OD) of a sample and the cut-off (CO) value that was calculated for each experiment based on the following formula:

$CO = \text{Mean } OD_{\text{negatives}} + 3 \times ST$, where ST stands for standard deviation. The OD values for the negative samples used in all experiments performed were less than 0.2.

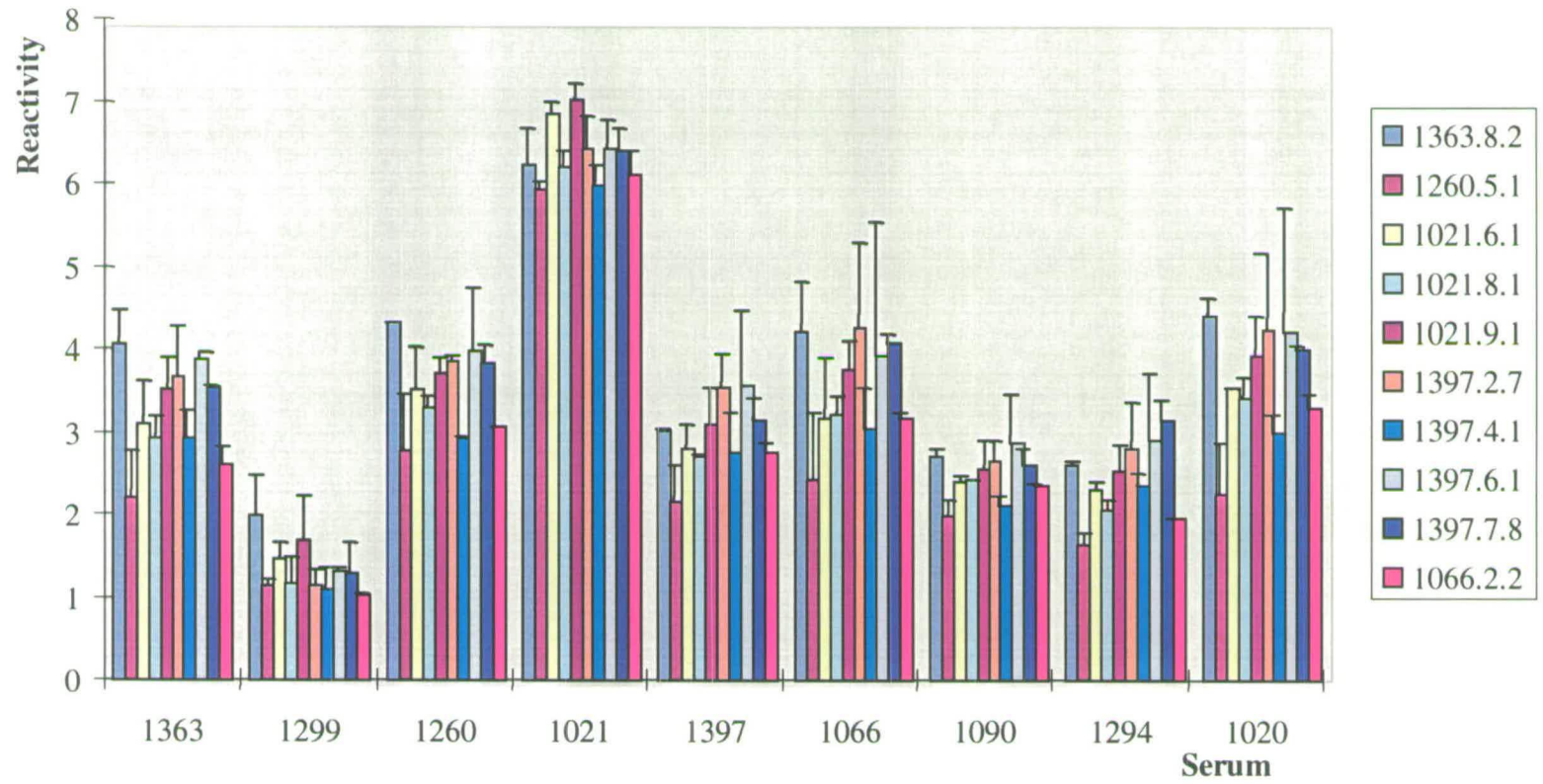
Data represent mean reactivity values measured at a single, selected serum dilution (1/400).

X-axis indicates the patients from which the sera derived

Y-axis indicates the magnitude of the IgG Ab reactivities against recombinant glycoproteins used

The legend given at the right side of the graph presents the gp120 antigens obtained from the homosexual men group. Each gp120 antigen is represented by a different colour.

Error bars represent one standard deviation



- moderate, containing four patients: 1363 (mean r value 3.21), 1260 (mean r value 3.49), 1066 (mean r value 3.53), 1020 (mean r value 3.5), 1397 (mean r value 2.91), 1090 (mean r value 2.41) and 1294 (mean r value 2.31).
 - low, represented by patient 1299 (mean r value 1.29), showing a low level of anti-HIV antibody, close to the threshold level, as detected with heterologous gp120 glycoprotein.
3. Some antigens obtained from one individual were slightly better recognised than others derived from the same individual.

6.2.1.2 Antibody responses to NSI vs. SI gp120s

As described in Section 5.2.5.1, the NSI/SI phenotype for each isolate from which gp120 antigens were obtained was predicted based on both Donaldson and Fouchier methods. Briefly, gp120 antigens designated 1260.5.1 and 1066.2.2 were derived from isolates with a predicted NSI phenotype, whereas those obtained from homosexual patients: 1363, 1021 and 1397 were derived from isolates with a predicted SI phenotype. Data from both Table 6.2 and Figure 6.3 suggest that the recognition of gp120 glycoproteins derived from isolates with predictive SI phenotypes was generally lower than those from isolates with a predicted NSI phenotype for both the autologous and heterologous systems. The reactivity values of all the sera derived from homosexual patients to the gp120 antigens from isolates with a predicted SI phenotype (e.g. 1066.2.2 and 1260.5.1) were lower than those with a NSI phenotype (e.g. 1363.8.2, 1021.689 and 1397.2467)(e.g. the reactivity values obtained for serum 1363 to env clones 1260.5.1 and 1066.2.2 were 2.12 and 2.56, respectively, whereas the reactivity values for env clones 1363.8.2, 1021.689 and 1397.2467 were 3.99, 3.14 and 3.5). The results regarding a higher recognition of the antigens deriving from isolates with predictive NSI phenotype than those from isolates with SI phenotype by autologous serum may be explained by a higher proportion of variants with NSI phenotype among HIV-1 isolates that were present in samples taken from infected individuals early in infection (see Subchapter 6.2.4.1). However, sera from patients in which SI variants appeared could strongly recognise the heterologous NSI derive isolates.

Table 6.2 The mean reactivity of sera from homosexual patients to autologous and heterologous gp120 antigens (data for the antigens originating from patients 1021 and 1397 were produced by calculating the mean of the reactivities presented in Table 6.1)

Clone ^{sera} (a)	136(c)	129	126	102	139	106	109	129	102
1363.8.2	3.99(b)	1.9	4.3	6.1	3.0	4.1	2.6	2.5	4.
1260.5.1	2.1	1.1	2.7	5.9	2.3	2.5	2.1	1.6	2.2
1021.689	3.1	1.4	3.	6.6	2.8	3.3	2.4	2.1	3.6
1397.2467	3.	1.1	3.5	6.3	3.1	3.8	2.4	2.6	3.5
1066.2.2	2.5	1.0	3.0	6.1	2.7	3.1	2.3	1.9	3.2

(a) refers to the env clones obtained from homosexual patients, with all suffixes written together for the clones originating from the same patient (e.g. all the reactivity values given for the env clone 1021.689 represent the average of all the individual reactivities of each sera with each clone deriving from patient 1021)

(b) bold italics figures indicate the reactivity for autologous combination of gp120 glycoprotein and serum

(c) figures refer to the mean of reactivity obtained for all the env clones classified in the same group

Figure 6.3. The extent of recognition of the sera from homosexual patients to autologous and heterologous gp120 glycoproteins

Graphs A to I present the mean reactivity of the IgG antibodies present in the homosexual patients' sera to autologous and heterologous glycoproteins. Each graph gives the reactivity values recorded for each individual serum, serum identity being written at the right side of the graph.

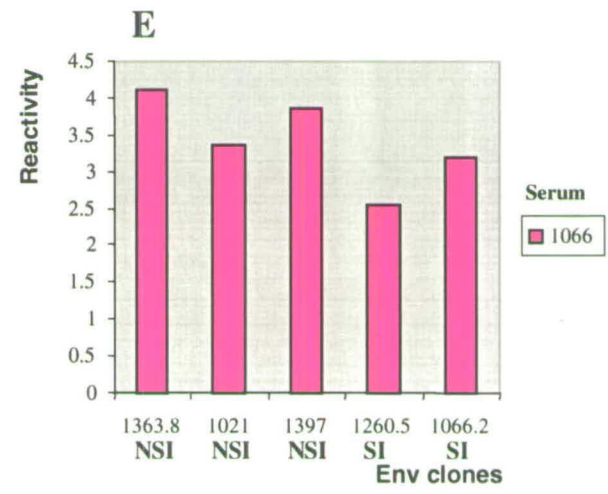
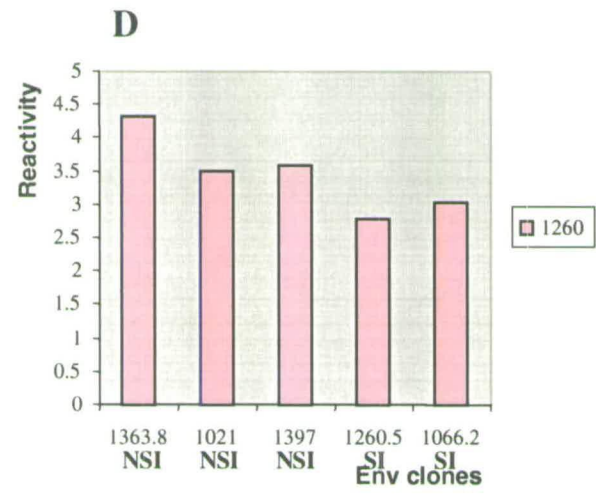
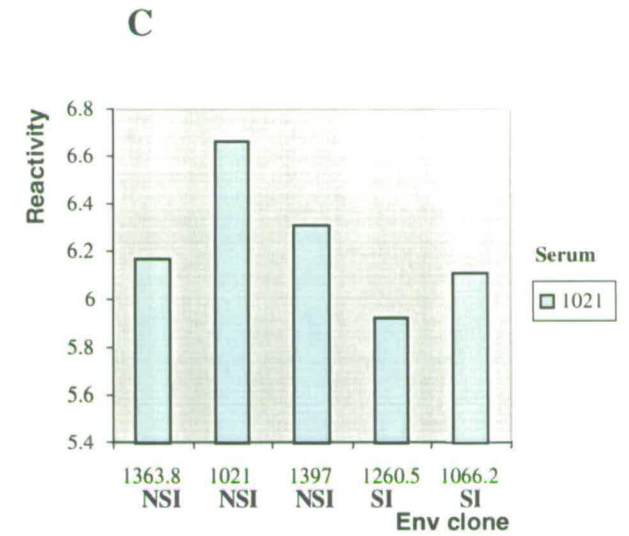
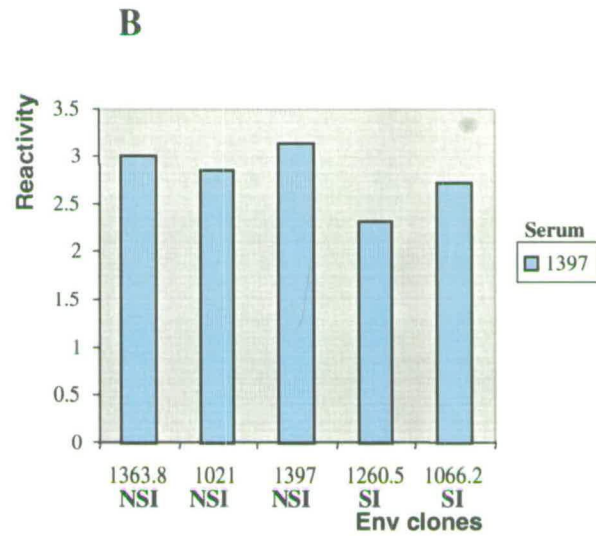
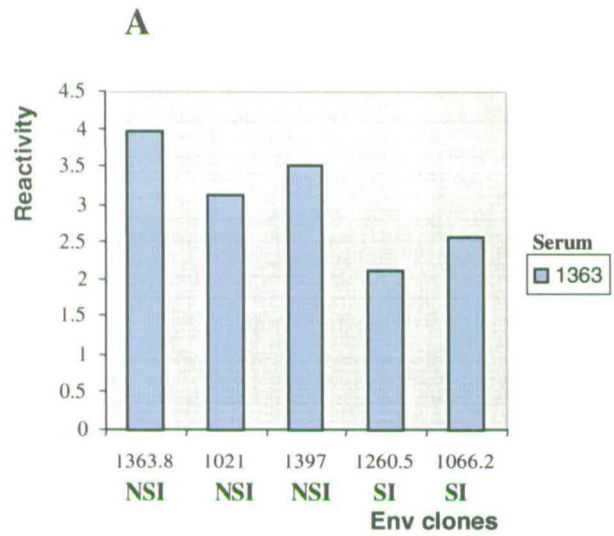
The predicted phenotype of the isolates from which the gp120 antigens derived is written in bold, below the X-axes.

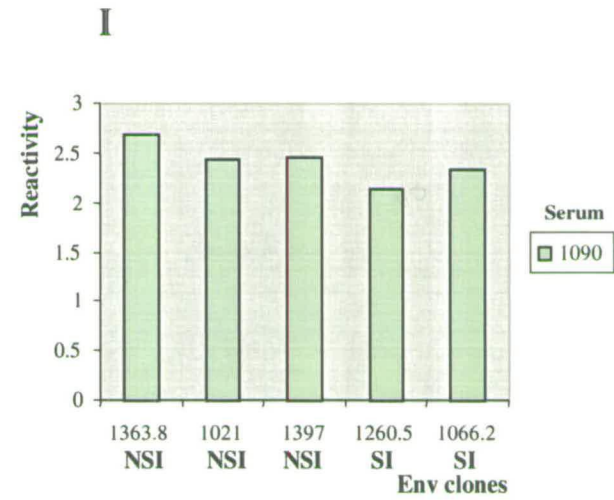
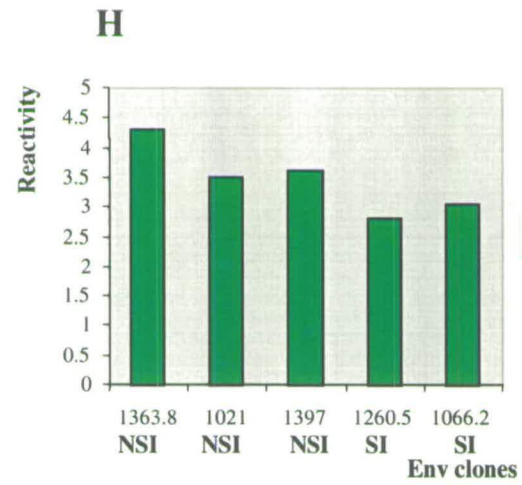
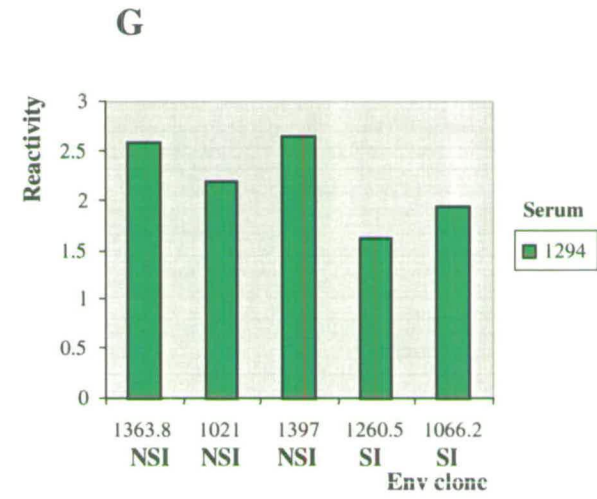
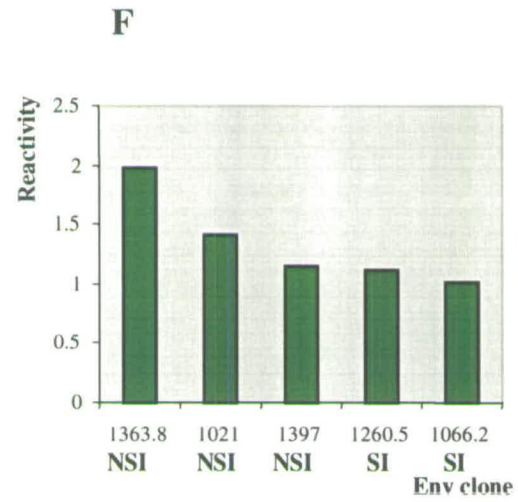
X-axes indicate the patients from which the sera derived.

Y-axes indicate the magnitude of the IgG Ab reactivities against recombinant glycoproteins used.

Data represent mean reactivity values measured at a single, selected serum dilution (1/400).

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6.2.1.3 Moderate differences between clones in level of recognition by different sera

Different levels of recognition of gp120 glycoproteins derived from the same patient were observed (e.g. 1021.6.1 and 1021.8.1 were moderately recognised, mean r value = 3.16 and 3.03, whereas 1021.9.1 was slightly better recognised, mean r value = 3.62; 1397.2.7, 1397.6.1 and 1397.7.8 were highly recognised, mean r value = 3.59, 3.37 and 3.53, while 1397.4.1 was slightly weakly recognised, mean r value = 2.91) by both autologous and heterologous samples might be explained by the fact that some of env clones (e.g. 1397.1) supported some mutations in certain positions imposed by the selective pressure of the immune response (see Chapter 1) or alternatively these results might be due to experimental variation.

Overall, the high level of cross-reactivity between different combinations of sera and gp120 antigens might suggest that conserved immunodominant epitopes are present on the immunising isolates, and that these are able to elicit broadly cross-reactive antibodies to gp120 glycoprotein in patients. This result is worth taking into account when considering a subunit vaccine design and the assessment of its efficacy. However, data presented here express only an estimation of IgG reactivity without investigating their function.

6.2.2 Analysis of the results obtained by ELISA in the presence of chaotropic compounds (urea)

It was documented by Kamoun *et al.* (1988) that the presence of urea, after antibody-antigen complexes are formed, reduces the strength of intramolecular hydrophobic-interactions and, hence, the consequence would be a catastrophic unfolding of the protein structure. Thus, urea is likely to have a major effect on the recognition of discontinuous or conformational epitopes.

Taking advantage of this property, it was possible to test the gp120 antigens, obtained from transfection supernatants, for their quaternary conformation. The experiment employed a modified gp120 ELISA. Briefly, gp120 secreted in transfection supernatants are bound via absorbed sheep antibody D7324 (Aalto Bio

Reagents, Dublin), as previously described (see Chapter 2.11). In each assay, after washing the plates with TBS containing 0.05% Tween 20, patient plasma diluted 1/400 was added to duplicate wells for 1h at room temperature. One of each duplicate was treated with 8M urea in TBS-0.05% Tween (urea elution) for 5 min at room temperature, followed by two washes with TBS. Bound antibody was detected using biotinylated F(ab')₂ anti-human IgG (gamma chain specific) and ExtrAvidin-AP as detection system. By urea elution, low affinity antibodies to linear epitopes and both low and high affinity antibodies directed to conformational epitopes were removed due to the high potential of urea to denature the proteins. Consequently, a drop in the OD values recorded when urea is employed might reflect the unfolding of the protein structure. The main disadvantage of this method would be the putative denaturation of the capture antibody (D7324), which may occur during urea treatment together with the denaturation of the antigens. It was difficult to find a control to show the effect of urea on the gp120-D7324 interaction and on the capture antibody alone, due to the fact that in the absence of gp120 molecules the value of the OD is the same as the negative control. In spite of the denaturing effect of urea on the capture antibody, which may be minimised by decreasing the time of urea elution, this method has been used previously to prove the presence of the antigens in a quaternary conformation (Moore *et al.*, 1996).

The results are presented in Table 6.3 and Figure 6.4, where the reactivity of plasma from homosexual patients against gp120 antigens (1363.8.2 and 1397.2.7), in the presence or absence of urea, is depicted. These two antigens were chosen at random from the group of homosexual patients whose sera reactivity was classified as being moderate. Mean reactivity values are shown for each plasma sample, in the presence or absence of urea treatment. It was observed that urea treatment causes a reduction in IgG anti-gp120 reactivity of between 1.5 to 4 folds. As the urea treatment has the consequence of unfolding the molecule and hence the conformational epitopes, by disrupting the hydrogen bonds, the OD values obtained in the presence of urea reflects the contribution of only the linear epitopes (the drop in OD is a consequence of the disturbance of the conformational epitopes in the presence of 8M-urea). Thus, ELISA in the presence of 8M-urea may be regarded as a test to demonstrate the

Table 6.3. Reactivity of the IgG antibody from plasma derived from homosexual subjects to two antigens (1363.8.2 and 1397.2.7) in the presence or absence of 8M urea.

Sera/Reactivity	1363.8.2	1363.8.2+urea	1397.2.7	1397.2.7+urea
1363	0,403051	0,579827561	0,622253967	0,367695526
1299	0,494975	0,127279221	0,219203102	0,325269119
1260	0,007071	0,445477272	0,070710678	0,45254834
1021	0,445477	1,046518036	0,395979797	0
1397	0,021213	0,084852814	0,424264069	0,657609307
1066	0,615183	0,091923882	1,025304833	1,13137085
1090	0,098995	0,084852814	0,240416306	0,473761543
1294	0,06364	0,127279221	0,579827561	0,502045815
1020	0,205061	1,011162697	0,961665222	0,219203102

Figure 6.4 Sera reactivity recorded for homosexual patients-derived sera with two gp120 antigens in the presence or absence of a denaturant agent (8M urea)

The graph presents the plot of the IgG antibody recognition of homosexuals' sera to mainly, heterologous gp120 glycoproteins in the presence or absence of 8M urea. Only two samples (1363 and 1397) were worked with autologousgp120 antigen.

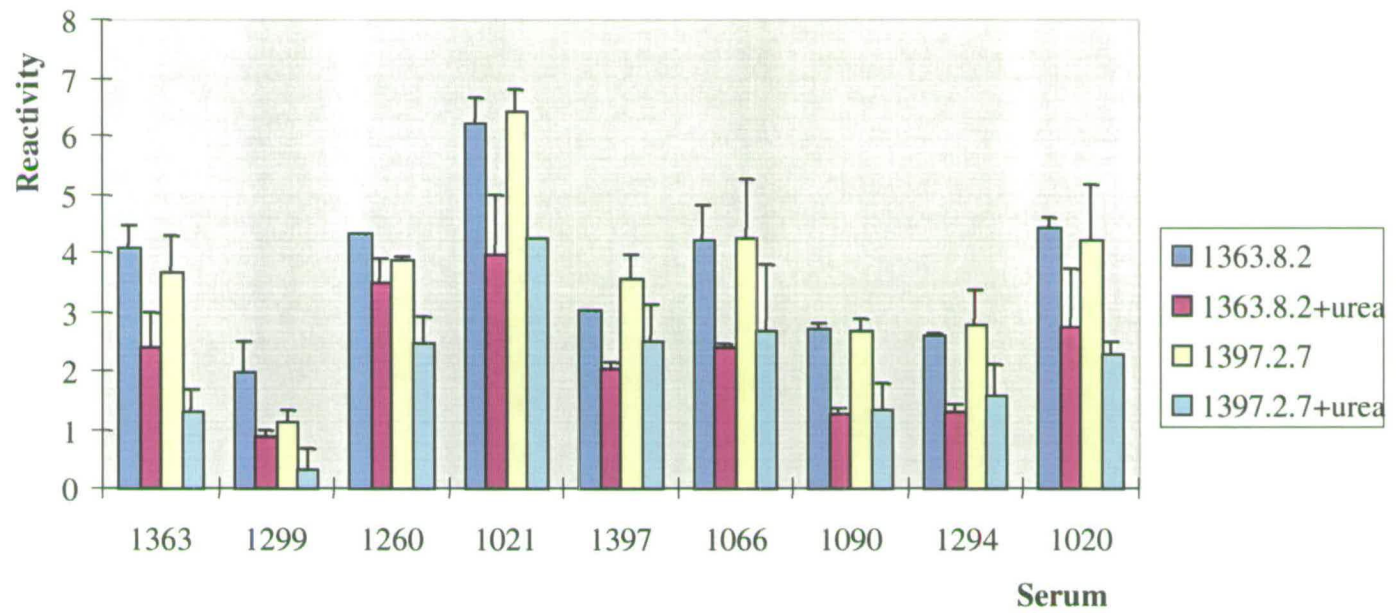
X-axis indicates the origin of the sera

Y-axis shows the calculated reactivity values (OD sample/OD cut-off, where OD cut-off is calculated using the following formula: OD cut-off = Average of OD Negatives +3xSD, where SD represents the standard deviation).

The env clones used are presented in the legend given at the right side of the graph. Error bars represent standard deviation obtained when the experiment was repeated twice, with two replicates per sample included in each experiment.

Figures written in **Italic** indicate the mean reactivity values obtained when an autologous combination between serum and gp120 antigens were used both with or without urea treatment

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quaternary conformation of the monomeric gp120 glycoproteins secreted in the transfection supernatants.

The OD sample/OD urea ratio values (rv) were calculated for all sera to gp120 antigens derived from patient 1363 and 1397 (corresponding to moderate reactive samples). A classification of sera containing high affinity antibodies (rv>2) and those with low affinity antibodies (rv<2) was adopted.

Antibody class (ie. if they are of high or low affinity) was analysed in connection with some parameters, which may reflect the stage of the disease. CD4 cell number, IgG level, viral load and provirus copy number/10⁶ PBMC for all the homosexual patients are presented in Table 6.4. Thus, the appearance of high affinity antibody in some patients but not others appears unrelated to clinical parameters including viral load, CD4 count and proviral copy number.

Table 6.4. The antibody affinity, CD4 cells count, viral load and provirus env copies/10⁶ PBMC of sera from homosexual cohort.

Sera	Affinity	CD4 cell count/mm ³ (d)	Viral load (b)	Env copies/10 ⁶ PBMC (c)
1294	High	640	>750,000	5,868
1299	Low	580	2,270	8,047
1363	High	339	315,000	1,532
1090	Low	290	77,900	510
1021	High	226	5,000	5,633
1397	High	210	105,000	3,932
1260	Low	100	253,000	9,162
1066	High	ND (a)	205,000	3,429
1020	High	39	336,000	2,302

(a) not done

(b) data kindly provided by Dr. Susan Nicoll, Lothian Regional Clinical virus laboratory, City Hospital, Edinburgh

(c) was determined by titration of the proviral DNA using primers for V1/V2

(d) data kindly provided by Dr A. Mc Millan, Royal Infirmary, Dr. K.N. Sankar, and Dr. M. Snow, Newcastle General Hospital, Belfast.

6.2.3 Correlation between mean antibody reactivities recorded for homosexual individuals and their CD4 cell count/mm³ blood and plasma viral load.

Results involving the IgG antibody level (IgG), CD4 cell count (CD4), plasma viral load (PVL) and provirus copy number (PCN) were analysed using Spearman rank correlation test. The patients were divided in two groups: one group containing patients 1021 and 1299 who have an atypical profile regarding the parameters analysed and the second group comprising the remaining patients who were more homogenous. Patient 1299 had a high plasma viral load (>750,000) but high number of CD4 cells. Patient 1021 had a very high IgG anti-gp120 antibody level, with a moderate viral load, proviral copy number and CD4 cell count. Therefore, the Spearman correlation test was done in parallel for three group-cases: a. for all the gay patients, b. for the situation when the patient 1299 was omitted from the comparison, c. for the case when both patients (1299 and 1021) were not considered. The correlation values obtained are presented in Table 6.5 and Figure 6.5.

Table 6.5 Spearman correlation coefficient for all the group-cases analysed.

Variables	All patients		All patients minus 1299		All patients minus 1299 and 1021	
	R	P	R	P	R	P
CD4 vs. IgG	-0.73	0.037	-0.52	0.18	-0.64	0.11
CD4 vs. VL	-0.08	0.831	-0.35	0.38	-0.46	0.29
CD4 vs. PVL	0.07	0.86	-0.14	0.76	-0.17	0.7
IgG vs. VL	0.06	0.86	0.52	0.18	0.6	0.14
IgG vs. PVL	-0.1	0.77	0.14	0.76	0	1
VL vs. PVL	-0.4	0.91	-0.42	0.33	-0.35	0.43

An inverse correlation between CD4 and IgG level was obtained when all patients were considered ($r=0.73$, $P=0.037$), but for the other two group-cases this correlation was not strongly supported, although still present.

Figure 6.5 Inverse correlation between mean IgG reactivity values from homosexual patients' sera to autologous and heterologous gp120 glycoproteins and their CD4 cell count

A. Table shows the calculated mean reactivity and CD4 cells/mm³ blood for each homosexual patient

B. The plot of Ab reactivity and CD4 cell count, for each patient sample, are presented as violet histograms and green filled circles.

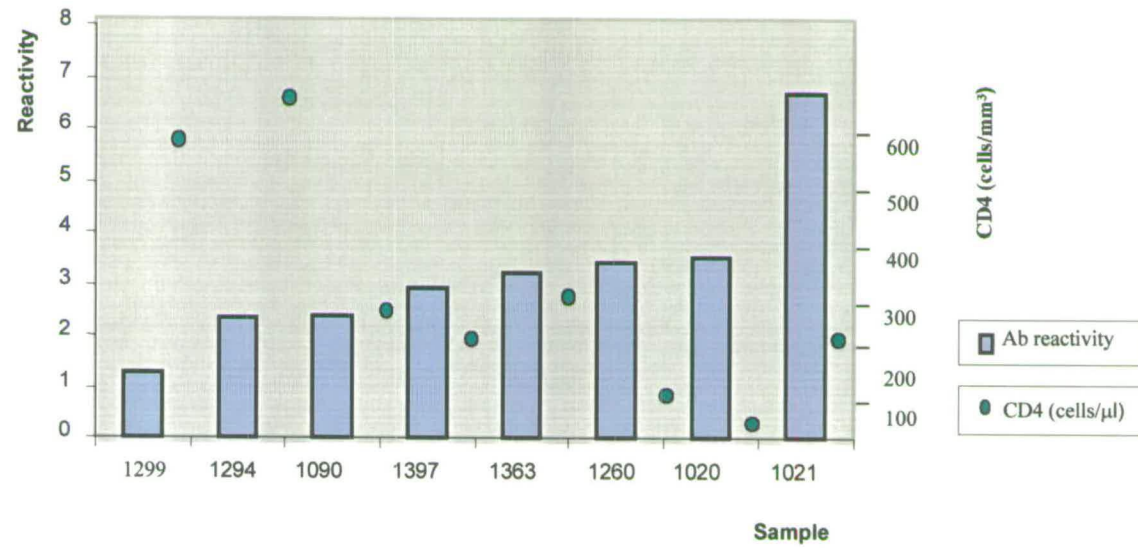
The Spearman correlation coefficient was computed using SPSS statistics package (-0.73, P=0.037) showing a negative correlation between Ab titres and CD4 cell number

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A

Serum	Antibody response	CD4cell/mm ³
1299	1.29	580
1294	2.33	640
1090	2.41	290
1397	2.91	210
1363	3.2	339
1260	3.4	100
1020	3.5	39
1021	6.68	226

B



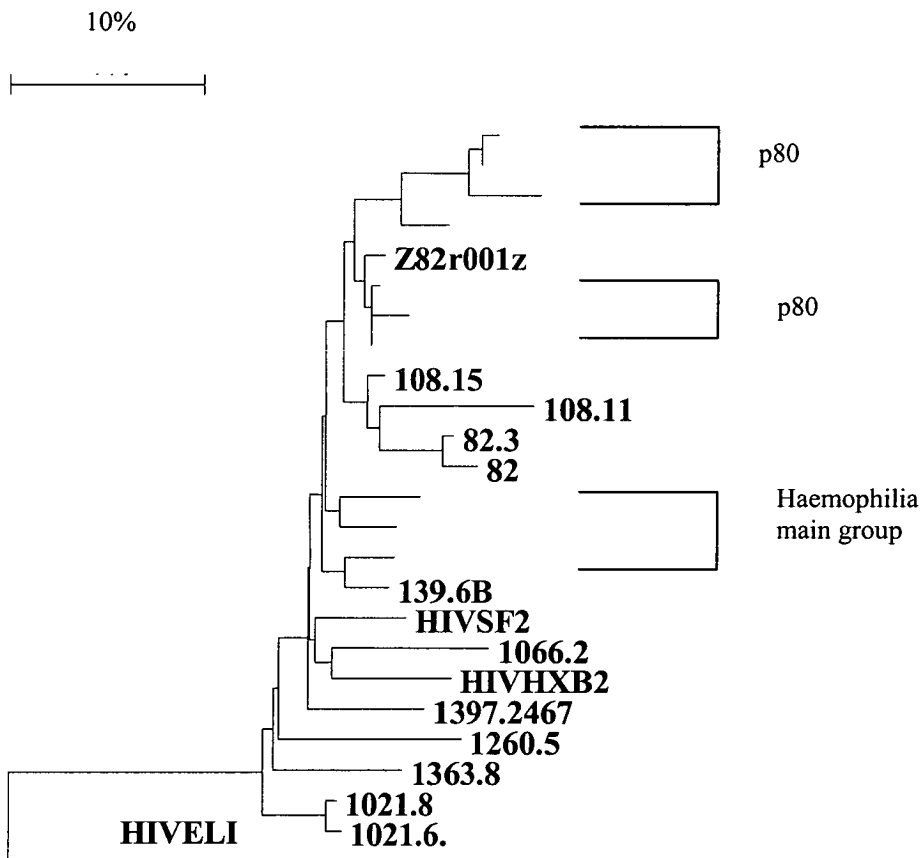
The correlation between a lower CD4 cell count/mm³ blood and an increase in antibody reactivity may be explained by the reactivation of the memory B cells and their differentiation into effector cells able to produce gp120-specific antibodies. When patients 1299 and 1021 were subtracted, the correlation was still present but not strongly supported. However, the interpretation of these results is difficult because of the scarcity of the samples available in this study.

6.2.4 Characterisation of the IgG antibody response from Edinburgh haemophiliac patients who received a common batch of factor VIII

Several reports revealed the existence of a small cohort of haemophilia A patients infected following exposure to non-commercial factor VIII, in 1984, in Edinburgh (Ludlam *et al.*, 1985, Balfe *et al.*, 1990, Simmonds *et al.*, 1990b). Among them, patient p82 was the most studied and characterised patient, because many samples at different time points during the infection were available and he never received antiretroviral treatment. Two patients (p82 and p79) had more divergent sequences, as estimated by phylogenetic analysis. One of them (p82) shared identical sequences in the V3 and V4 regions of gp120 with another HIV-1 infected haemophiliac (p80) who did not receive factor VIII from the main batch, but had at least one other batch in common with p82. The rooted neighbour-joining tree for 121 unique V3 sequences, taken from 25 Edinburgh patients, together with several world-wide isolates of HIV-1 subtype B and subtype D isolate HIV ELI (as outgroup), giving the relationship among the patients, is presented in Figure 6.6.

Thus, patients p28, p72, p77, p79, p82, p84, p86, p87, p89 and p91, clustered together to produce the main haemophiliac cohort, while patients p74, p79, p80 and p82 were separated from the main cohort. Although patients p80 and p82 grouped away from the main haemophiliac cohort, these patients are also believed to have been infected from the locally prepared factor VIII; p82 had received factor VIII from a small number of bottles from the common implicated batch, while p80 had not. Five gp120 antigens that originated from patient p82, obtained by Dr. Sarah Ashelford (PhD thesis, University of Edinburgh, 1996) were used in this study to investigate the extent of their recognition by the IgG class from autologous and heterologous sera using the “in house” ELISA assay. Their nomenclature, the years

Figure 6.5 Rooted neighbour-joining tree for 121 unique V3 sequences



when they were taken from patient p82, and the predicted phenotype of the isolates from which they derived are presented in Table 6.6. Their V3 amino acid and nucleotide sequence alignments are presented in Table 5.6 and Figure 5.5 (see Chapter 5).

Table 6.6. Designation of gp120 antigens from patient p82 together with the predicted phenotype of the variants from which they derived and years after seroconversion when they were isolated.

Env clones	Years after seroconversion	SI/NSI predictive phenotype
108.11	3	NSI
108.15	3	SI
82	5	SI
82.3	5	NSI
139.6B	6	NSI

Two independent ELISA experiments were conducted, in which p82-derived gp120 antigens were reacted with autologous and heterologous samples, as described in Section 6.1.2. Two replicates per sample were included in each experiment. The results are presented in Table 6.7, as mean reactivity \pm standard deviation. The extent of recognition of p82-derived gp120 glycoproteins, by the autologous and heterologous sera, are plotted as histograms, in Figure 6.7 and Figure 6.8 A to C. Figure 6.7 shows a significant cross-reactivity between sera from various haemophiliac patients to gp120 antigens from patient p82. Data obtained for each antigen with sera deriving from each patient were averaged. The following observations were made:

1. The mean reactivity value obtained for both gp120 antigens that derived from isolates with a predicted SI phenotype (i.e. 82.3 and 82) with sera from each patient were higher than those obtained for antigens that derived from isolates with NSI phenotype (108.11, 108.15 and 139.6b) (mean r values of 10.63 vs. 8.78 for p82 sera, 12.1 vs. 10 for p80 sera, 8.89 vs. 8.43 for the other haemophiliac sera).

2. One of the antigens that was derived from the SI isolate (82.3) appeared to be more reactive than the other (82), being recognised more strongly by the autologous, sequential sera as well as the heterologous p80-derived sera (mean r-value = 12.12 vs. 9.14 for p82 sera, 10.3 vs. 13.9 for p80 sera). These antigens are recognised to the same extent by the heterologous sera derived from the patients infected with unrelated virus from the main haemophiliac cohort, (mean r-value 9.38 vs. 8.4 for other haemophiliac sera). This result suggests the existence of specific epitopes on the gp120 antigens (probably localised at the level of the hypervariable regions, possibly the V3 loop), which were better recognised by related than unrelated sera. The extent of recognition by the unrelated sera gives the contribution of the group specific antibody able to recognise conserved epitopes on the glycoprotein.
3. Antigen 82.3, which was derived from an isolate taken at year 5 was recognised by the autologous samples from year 4 ($r = 11.5$ and 11.45), and showed higher levels of reactivity with the contemporaneous serum from year 5 ($r = 12.14$) and with samples from the following year (6) ($r = 13.87$). Both antigens derived from year 5 reacted strongly with sera from year 4. The significance of this result is analysed further in the following section.
4. One NSI derived isolate (139.6b) was recognised even more strongly than an SI-derived antigen (82) (mean r value = 13.41 vs. 9.21) and one NSI-derived antigen 108.15 had the poorest recognition among all the other NSI/SI-derived antigens (mean r value = 7.26 vs. 9.21, 9.21, 11.5 and 13.41).

Thus, in comparison to the homosexual group, samples were taken within 1 year from seroconversion, and probably therefore, the NSI-derived antigens were better recognised by the autologous sera. For haemophiliac patients p82 and p80, the antibody responses to NSI/SI variants suggest a dynamic evolution of the viral population, with the emergence of new SI variants that replaced the NSI isolates which consequently become less representative of the viral population. In order to simplify the interpretation of results, the data were analysed further as autologous pairs of sera and gp120 antigens, independently from the heterologous combination.

Figures 6.7 Cross-reactivity of sera from haemophiliac patients to a panel of env antigens obtained from patient p82

The patients from whom the sera derived are written on the x-axis.

Y-axis gives the magnitude of the IgG antibody reactivities against the gp120 antigens that were obtained from patient p82 (Sarah Asherfold, PhD thesis, 1996).

The gp120 antigens are presented in the legend on the right side of the graph, together with their predicted NSI/SI phenotype given between brackets. Each individual gp120 antigen is represented by a particular colour.

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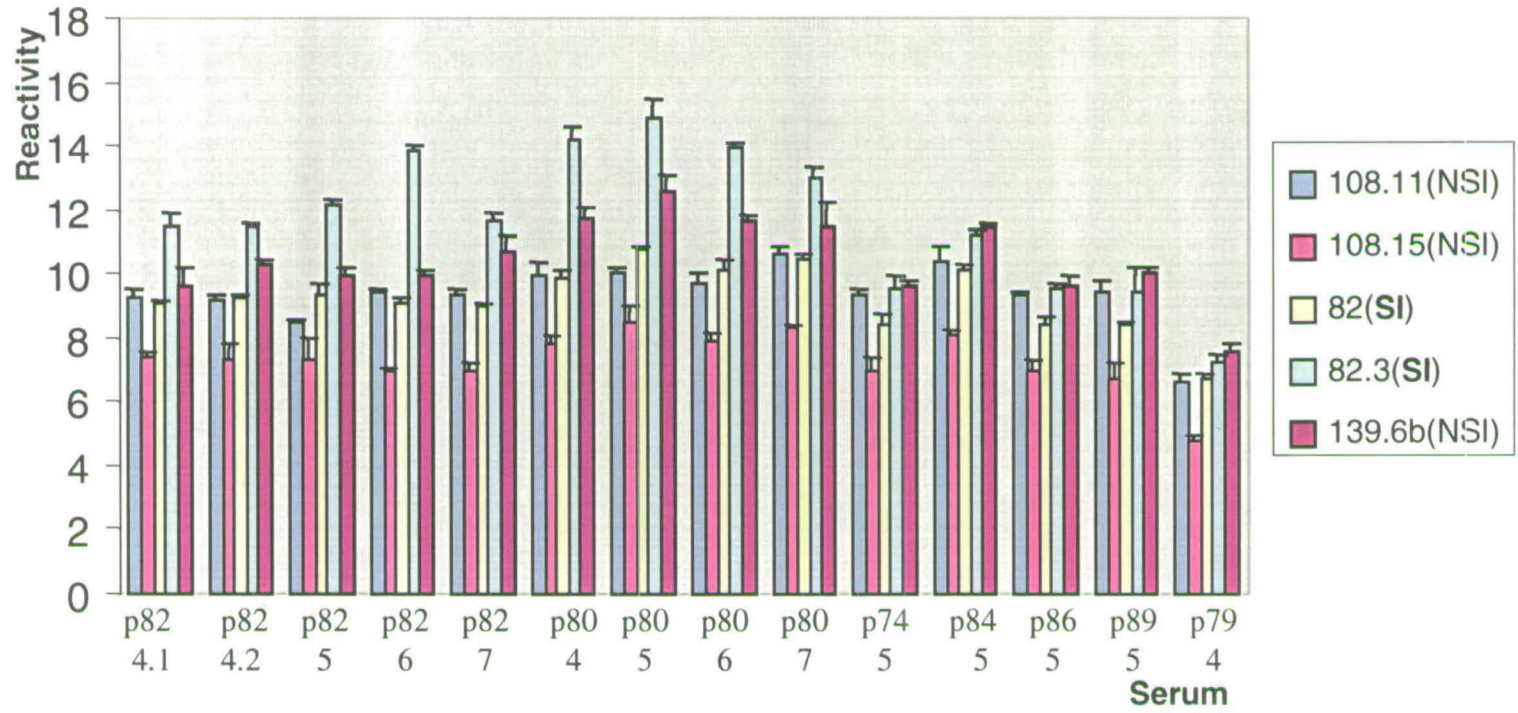


Figure 6.8 The reactivity of autologous and heterologous sera against gp120 antigens obtained from patient p82

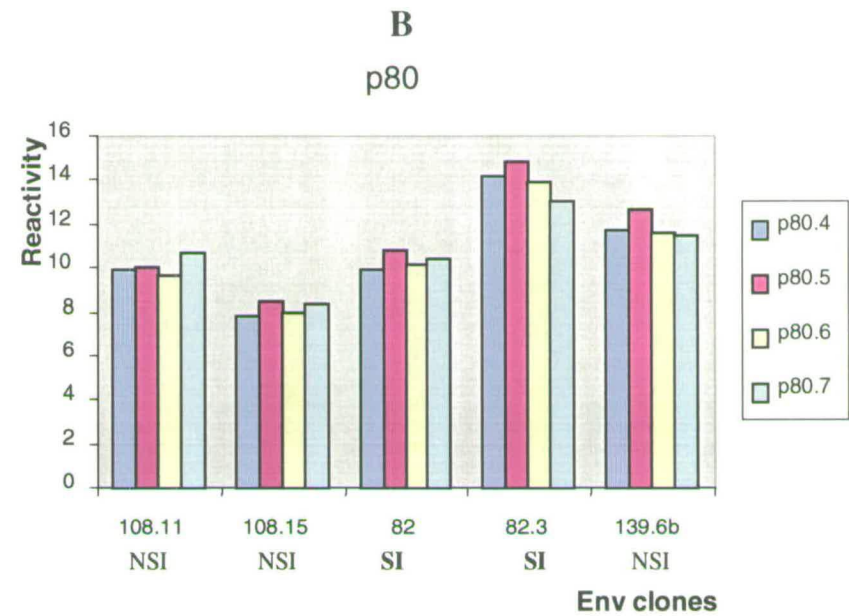
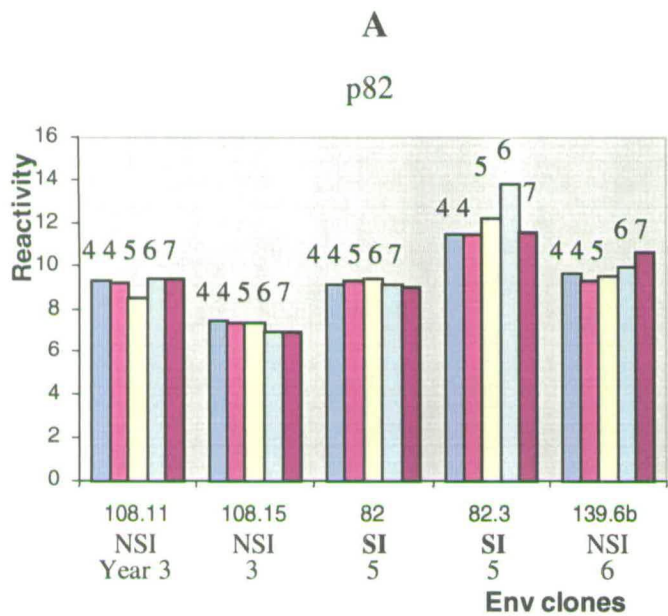
Graph A presents the plot of recognition of p82 –derived gp120 antigens by autologous sera.

Graph B presents the extent of recognition of the same gp120 antigens by heterologous sera obtained from a haemophiliac patient (p80) who was infected from the same batch as that shared by the patients from the main haemophiliac cohort but shared at least one other batch with patient p82.

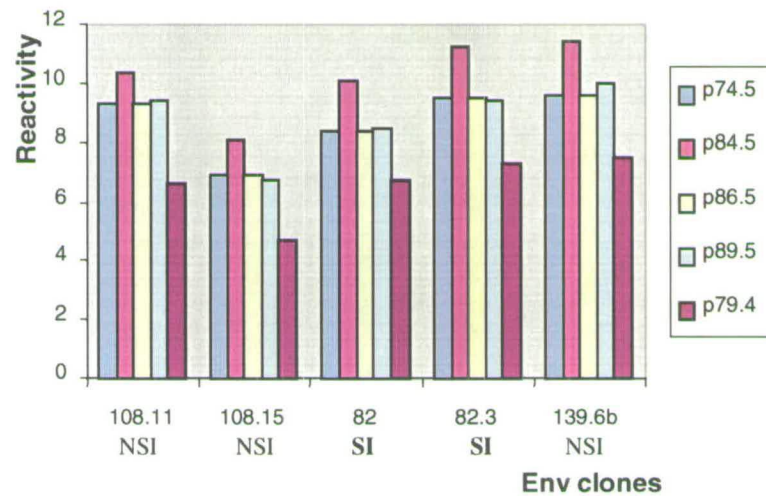
Graph C shows the reactivity recorded for patients belonging to the main haemophiliac cohort against the same p82-derived gp120 antigens
X-axes show the gp120 antigens used and their predicted phenotype.

Y-axes show the extent of the IgG antibody recognition of sera to the p82-derived gp120 antigens.

The sera used are given in the legend at the right side of each graph, each serum being represented by a particular colour.



C
p74, p84, p86, p89, p79



6.2.4.1 Longitudinal characterisation of the IgG antibody reactivity from different samples from a haemophiliac patient (p82) to autologous gp120 antigens

It was found by Simmonds *et al.* (1991) that V3 sequences amplified from patient p82 were characterised by a high percentage of non-synonymous substitutions, reflecting a high positive selection that is often assumed to be imposed by the immune system to select escape mutants. To estimate the degree of amino acid replacement mutations, which is manifested within the V3 loop, mean number of synonymous and non-synonymous substitutions for the env sequences from patient p82 were calculated using the MEGA program (Kumar *et al.*, 1993). The results are presented in Table 6.8.

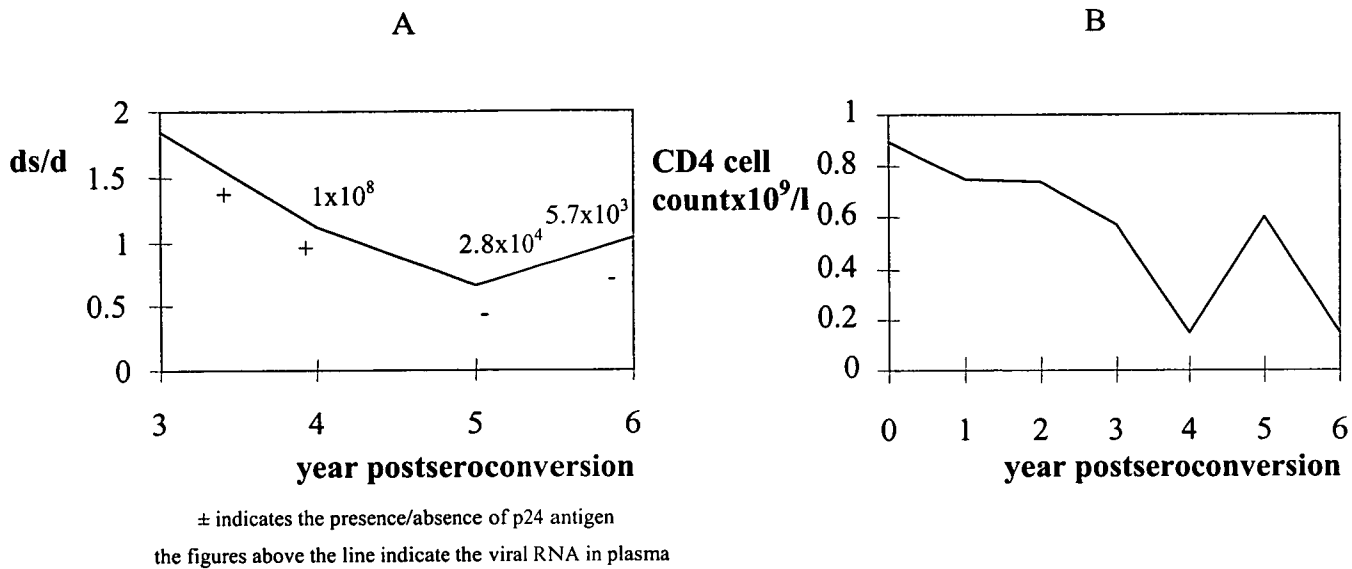
Table 6.8. Mean number of synonymous and non-synonymous substitutions for sequences derived from patient p82 at year 3, 4, 5 and 6.

Year postseroconversion	Synonymous (ds) $\times 10^{-3}/\text{site}/\text{year}$	Non-synonymous (dn) $\times 10^{-3}/\text{site}/\text{year}$	ds/dn
3 years	47.2	25.6	1.84
4 years	46.9	41.75	1.12
5 years	63	94.05	0.66
6 years	71.9	69.3	1.03

The plot of ds/dn that corresponded for years 3, 4, 5 and 6 is given in Figure 6.9 A and the CD4 cell count for each year post seroconversion, as shown by Bonhoeffer *et al* (1995a and b) for sequences isolated from patient p82 is plotted in Figure 6.9 B (also Lin Qi Zang, PhD thesis, University of Edinburgh, 1992). The results presented in Figure 6.8A and B were analysed together with the IgG antibody reactivity of sera obtained from patient p82 against autologous gp120 antigens plotted in Figure 6.8A.

Figure 6.9A. The ratio of ds/dn in p82 for each year postseroconversion.

B. Changes in CD4 cell count after seroconversion (Bonhoeffer *et al.*, 1995, Lin Qi Zang, PhD thesis, 1992).



As it can be seen, by year 5, there was a significant drop in ds/dn ratio, pointing towards a sustained positive selection for amino acid replacement, at a time when p24 antigen was not detected. Furthermore, with the antigen which was isolated in year 5 (82.3), a high reactivity was given by the sera taken at years 5 and 6, with a subsequent drop of reactivity at year 7, to the same level given by sera taken from year 4.

Regarding CD4 cell count, an increase in CD4 cells was reported by Bonhoeffer *et al.* (1995) to occur in year 5, when analysing sequential samples from patient p82 (Figure 6.9B), although these data were obtained over 10 years ago and may represent an experimental artefact associated with occasional variability in reagents over 10 years ago, they may also represent a real spike associated with an acute infection. Nevertheless, taking the findings mentioned above at face value, they suggest a scenario according to which a more virulent isolate with SI phenotype (represented by env clone 82.3) was able to elicit a strong and rapid humoral immune response which neutralised the initial variant and reduced the viremia to below the sensitivity of the p24 detection assay. Consequently, a sustained Th function may be

manifested over 7 years of the infection in the absence of the antiretroviral therapy. The immune responses may select for new mutants that might be more virulent and have a destructive effect on Th cells and impairs the immune system function. This continuous dynamism of the viral population may also be reflected by the fact that the antigen derived from year 5 was recognised by the IgG antibody from serum taken in year 4, a result that may be explained by the fact that the isolates circulating in plasma in year 4 that were able to mount an immune response were integrated into the chromosomal DNA in the subsequent year. In this light, Simmonds *et al.*, (1991) initially reported that the integrated provirus might reflect the viral variant that were found in previous years.

A detailed analysis of Figure 6.8A highlights also, the existence of a high cross-reactivity between gp120 antigens and heterologous sera, since 82.3 derived from year 5 could be recognised by sera taken one year before (the reactivity values were in range between 6 and 10, similar to those recorded for heterologous sera, see Chapter 6.2.4.2 and Figure 6.8C). Another interpretation of this result could be the presence of the isolates with SI and NSI phenotypes (designated 82 and 82.3 and 139.6b, respectively) as plasma free virions that were present as a minority virus population among the HIV-1 isolates at year 3 or 4. Being under pressure of the humoral immune responses, these isolates became integrated into chromosomal DNA of long lived cells as provirus, therefore being detected in year 5 and 6, respectively, among PBMC DNA derived provirus isolates (Holmes *et al.*, 1992, Sarah Ashelford, PhD thesis, University of Edinburgh, 1996). As described by these two investigators, V3 sequences that corresponded to antigens 82 and 82.3 were indeed detected among V3 RNA isolates in plasma in years 1988/1989 (four/five years after seroconversion) and those corresponding to the antigen 139.06b arose in plasma in 1987/1988 (three/four years after seroconversion), being preponderant in year 1989/1990 (four/five years after seroconversion).

As mentioned before, another parameter that reflects the viral dynamism consists of the replacement in time of the NSI-phenotype isolates with more virulent SI variants. On this basis, a variety of responses of p82-derived sera to gp120s derived from

isolates with SI and NSI phenotype were observed. Thus, both antigens from SI isolates (82 and 82.3) were recognised better than one deriving from NSI isolates: 108.15 (mean reactivity 9.1 and 12.1 vs. 7.18). The other NSI isolate-derived gp120 antigen (108.11) reacted with the p82 sera to same extent as 82 (9.11 vs. 9.1) but lower with 82.3 (mean reactivity 9.11 vs. 12.1). Finally, one NSI isolate-derived gp120 antigen, 139.6b, reacted better than SI isolate-deriving antigen 82 (mean reactivity 10.7 vs. 9.1) but poorer than the other antigen 82.3 (mean reactivity 10.07 vs. 12.1). A striking difference in responses was also noticed between the SI isolate deriving-gp120 antigens (82 and 82.3). Specifically, the antigen 82 gave a lower level of recognition with all the p82 sera, similar to those that derived from isolates with a NSI phenotype, whereas for the antigen 82.3, a broader variation with sequential sera was observed. To find an explanation for differences observed between them, I looked at the synonymous and non-synonymous substitutions between 82 and 82.3 and the sequence found at the seroconversion (82a) and also I analysed the differences regarding the amino acid sequences of the V3 region from antigens 82 and 82.3.

Thus, whereas a slight difference between gp120 antigens 82 and 82.3 regarding the rate of replacement mutations ($111 \times 10^3/\text{site}/\text{year}$ vs. $97 \times 10^3/\text{site}/\text{year}$) was noticed, a two-fold difference for the rate of synonymous substitutions ($46 \times 10^3/\text{site}/\text{year}$ vs. $96 \times 10^3/\text{site}/\text{year}$) was evident, although the error on this is very large.

Shioda *et al.* (1994) investigated the impact of basic amino acid substitutions in the V3 region of the HIV-1 Env protein on the antigenic properties of the virus, revealing that the basic substitution at position 323, numbered according to HXB2 sequence (or 28, numbered from the first Cys(C) residue at the 5' terminal end of the V3 loop), correlated with a shift in the antigenicity of that protein. Therefore I have compared the V3 amino acid sequences from antigens 82 and 82.3. Thus, the presence of a basic amino acid (Arg, R) at position 28, on the V3 sequence from the antigen 82.3 correlated with a higher recognition of this glycoprotein by the IgG antibody from both autologous and heterologous samples, in comparison with the

other SI isolate-derived antigen 82, that has a Gln (Q) at position 28, which is uncharged at physiological pH. This result, as those reported by other investigators, is consistent with the proposal that the presence of a basic amino acid at position 323 could change the antigenicity of that isolate. However, the amino acid replacement that could modify the antigenicity of the glycoproteins might have occurred outside the V3 region.

In conclusion, the poor antibody responses to the NSI-derived antigens and the enhanced recognition of some SI-derived antigens by the IgG antibody from sera taken over the several years of the infection may show the switch from the less virulent, macrophage tropic, NSI phenotype-isolates to more virulent, T cell tropic, SI phenotype-isolates that was reported to occur during HIV-1 infection.

6.2.4.2 High degree of cross-reactivity between sera from haemophiliac patients against gp120 antigens obtained from patient p82

As mentioned before, p82 shared at least one batch of factor VIII with patient p80, who himself was infected from the main batch of factor VIII as was the main haemophiliac cohort. From Figure 6.6, it can be seen that p80 sequences were clustered into two groups, one close to, and interspersed among, sequences derived from patient p82, although these positions are not strongly supported in this tree. p74 and p79 sequences were placed between p82 sequences and the sequences from the main haemophiliac cohort. Therefore, the extent of IgG antibody recognition of sera from patient p80 and main haemophiliac patients against p82-derived gp120 antigens was plotted for p82-derived antigens versus p80 sera, separately from p82-derived antigens versus sera taken from the main haemophiliac group (see Figure 6.8B and C). The extent and the pattern of responses for p80 were in the same range (or even slightly higher) as those given by autologous sera. The highest value was obtained for the SI isolate-derived antigen 82.3, a result which was similar to that recorded for patient p82. This may reflect the contribution of type-specific antibody. Conversely, for patient p74, p84, p86, p89 and p79, the extent of recognition decreased, result that could have been predicted based on the basis of position these patients occupied on the phylogenetic tree. There is a common background level of recognition of p82-

derived gp120 antigens by each individual serum that may reflect cross-reactivity between these antigens and less related sera, as observed in the homosexual group (Figure 6.8C). The lowest level of recognition of p82-derived gp120 antigens was obtained for the p79 serum (mean $r = 6.5$). The explanation for the low level of recognition of p82-derived gp120 antigens by p79 serum may consist of the immunocompromised status of this subject. However, the recognition of the p82-derived antigens by the less-related sera might reflect the contribution of group-specific antibody. High reactivities recorded for the most related patient p80 and p82 against p82-derived gp120 antigens may be a consequence of recognition of less conserved epitopes, that act as an additive factor to the value given by the recognition of more conserved epitopes.

6.2.5 IgG antibody recognition of three recombinant gp120 antigens (rgp120) from prototypic HIV strains by haemophiliac and homosexual derived sera

In order to compare the total IgG antibody response of sera from both groups (haemophiliac and homosexual) to HIV-1 monomeric gp120 glycoprotein, two independent ELISA experiments, with samples in duplicate, were performed, using three rgp120s at the concentration of 1µg/ml. The recombinant gp120 glycoproteins were: HIV-1 IIIB and MN-derived gp120s, produced in Baculovirus expression system and HIV-1 SF2-derived gp120, expressed in Chinese hamster ovary (CHO) cells (Levy *et al.*, 1984). The protocol employed was presented in Chapter 2.11 and Chapter 6.1.2. The results obtained are presented in Table 6.9 and Figure 6.10A and B and Figure 6.11 A, B and C.

The reactivity values recorded for all three recombinant gp120 glycoproteins were averaged for each serum obtained from homosexual individuals and, based on the mean reactivity values, sera were classified into the following groups:

- highly reactive group, containing serum 1021 (mean $r = 8.92$),
- moderately reactive group, comprising sera deriving from patients: 1363 (mean $r = 6.14$), 1397 (mean $r = 5.81$), 1066 (mean $r = 6.54$), 1090 (mean $r = 4.42$), 1020 (mean $r = 6.64$), 1294 (mean $r = 5.68$).
- poorly reactive group, represented by serum obtained from patient 1299 (mean r

Figure 6.10 Reactivity of homosexual and haemophilic sera against three recombinant glycoproteins: gp120 IIIB, gp120 MN and gp120 SF2

Graph A shows the extent of recognition of recombinant gp120 glycoproteins IIIB, MN and SF2 by the IgG Ab of haemophilic patients (p80, p82, p86, p74, p89, p79 and p84). Graph B shows the extent of recognition of recombinant gp120 glycoproteins IIIB, MN and SF2 by the IgG Ab of homosexual patients (1363, 1299, 1260, 1021, 1020, 1397, 1297, 1066, 1090).

X-axes show the patients' sera used in ELISA assay

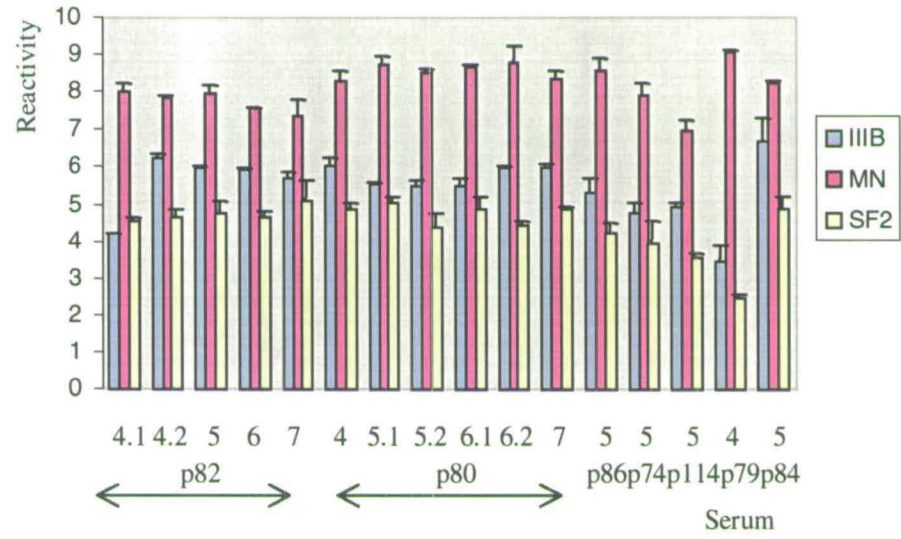
Y-axes give the magnitude of IgG Ab recognition of these gp120 glycoproteins by patients' sera.

Both positive and negative sera used in this experiment were used at a dilution of 1/400. Reactivity values for negative samples were averaged and a cut-off (CO) was established by applying the formula: $CO = \text{average } OD_{\text{Negatives}} + 3SD$.

Ratio between OD_{Sample} and CO value gave the reactivity of that sample.

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A.



B.

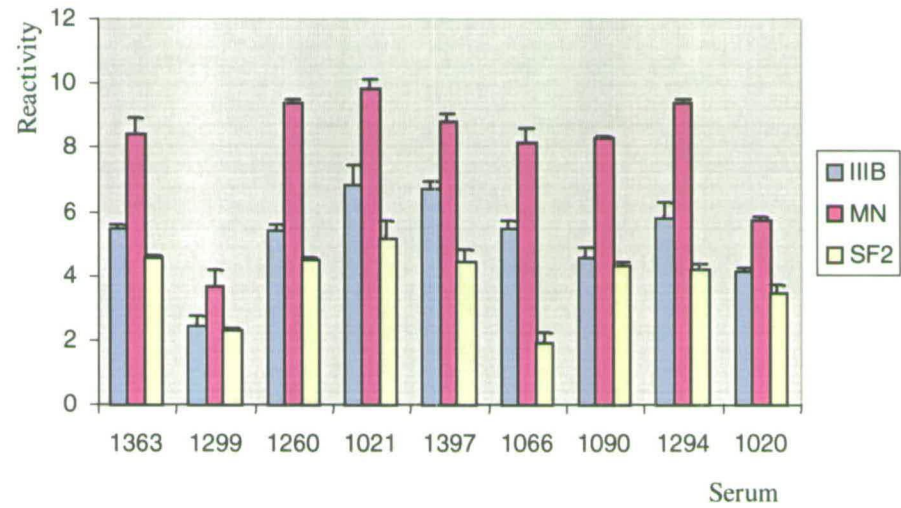


Figure 6.11 Reactivity of homosexual and haemophilic sera against three recombinant glycoproteins: gp120 IIIB, gp120 MN and gp120 SF2

Graphs A, B and C show the extent of recognition of three recombinant gp120 glycoproteins: IIIB, MN and SF2 by the IgG antibody from the haemophilic patients (p80, p82, p86, p74, p89, p79 and p84) and homosexual patients (1363, 1299, 1260, 1021, 1020, 1397, 1297, 1066, 1090).

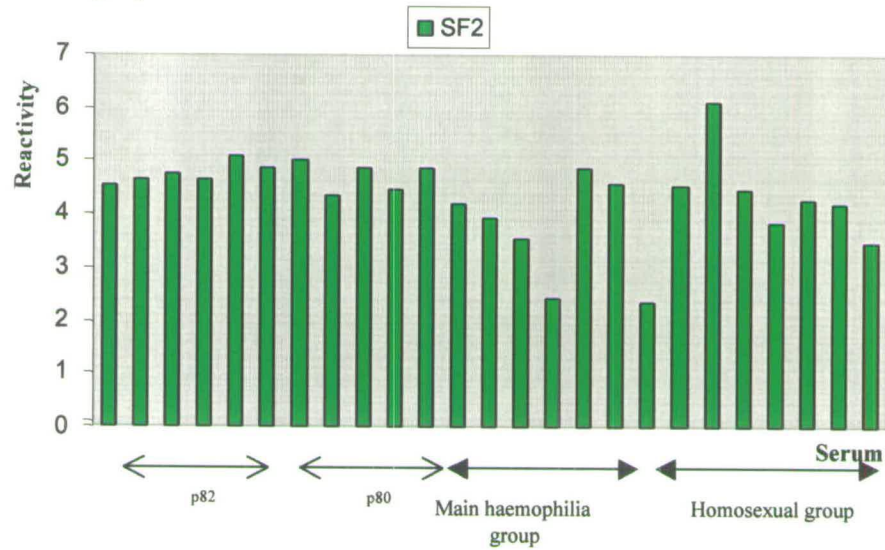
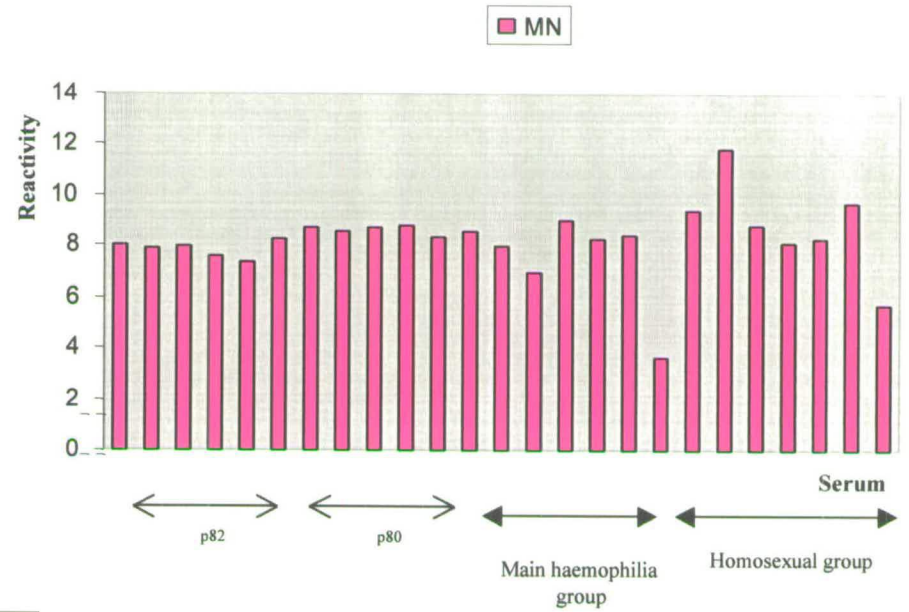
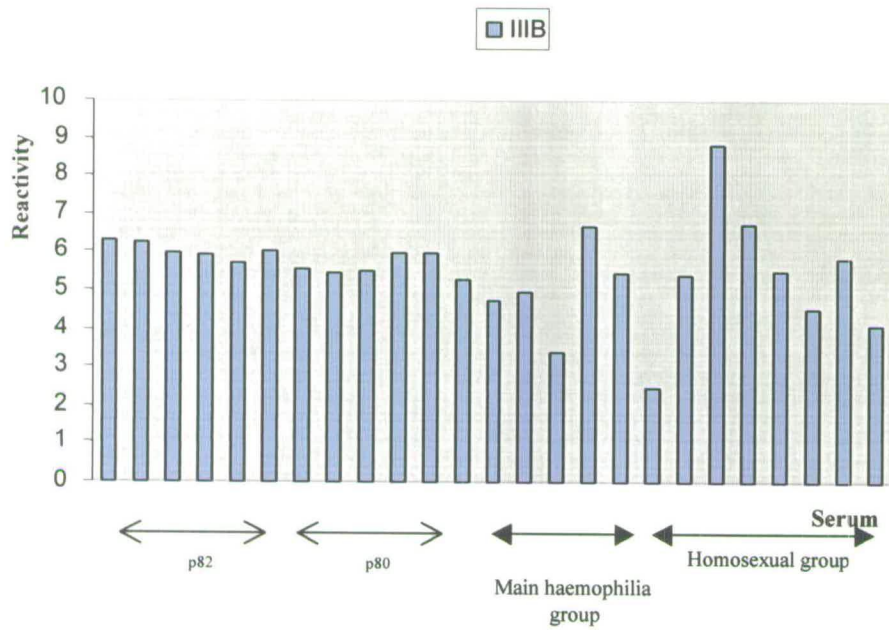
X-axes show the patients' sera used in ELISA assay

Y-axes give the magnitude of IgG antibody recognition of these gp120 glycoproteins by patients' sera.

Both positive and negative sera used in this experiment were used at a dilution of 1/400. Reactivity values for negative samples were averaged and a cut-off (CO) was established by applying the formula: $CO = \text{average } OD_{\text{Negatives}} + 3SD$.

Ratio between OD_{Sample} and CO value gave the reactivity of that sample.

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- = 2.82).

The same classification of sera was obtained when sera were reacted with autologous and heterologous glycoproteins (see Section 6.2.1).

To simplify the interpretation of the results, the reactivity values obtained for all sera from patients p80 and p82, those from the main haemophiliac cohort (p86, p74, p89, p79 and p84) and those from the high, moderate and low reactive groups, in which the homosexual subjects, were clustered were averaged. The values are presented in Table 6.9.

Table 6.9 The mean reactivity values obtained for groups of sera to three recombinant gp120 antigens (IIIB, MN and SF2).

Group of sera	Mean r (MN)	Mean r (IIIB)	Mean r (SF2)
p82	7.82	6.02	4.74
p80	8.6	5.68	4.71
Other haemophiliac	8.1	5	3.79
High reactive homosexuals	11.84	8.83	6.11
Moderate reactive homosexuals	8.33	5.34	4.19
Low reactive homosexuals	3.67	2.47	2.34

It can clearly be seen that SF2 was less well recognised by all groups of sera, in comparison with the other two recombinant glycoproteins: IIIB and MN (overall mean $r=4.31$ vs. 5.55 and 8.06, respectively). One possible explanation of a low recognition of SF2 gp120 glycoprotein by all sera may be differences in the antigenic properties of gp120 SF2, due to its expression in mammalian cells (CHO cells) as opposite to the insect cells in which gp120 IIIB and MN were expressed. Insect cells lack the machinery involved in the metabolic pathways of fucose and galactosamine,

which are added during complex oligosaccharide synthesis (see Chapter 7). The presence of complex oligosaccharides on the SF2 gp120 may therefore occlude some epitopes from the antibody recognition, in comparison to the IIIB and MN-derived gp120 antigens, which have only high mannose-type oligosaccharide).

Of the glycoproteins expressed in the Baculovirus system, gp120 MN was better recognised than IIIB by all sera (overall mean $r=8.06$ vs. 5.55). Previous studies have also described SF2 strain as having a weak antigenicity in comparison with other strains such as: MN and IIIB (Hariharan *et al.*, 1993, Boudet *et al.*, 1996). This result is also in agreement with data reported by other investigators (Zwart *et al.*, 1994).

From Figure 6.10 A and B and Figure 6.11 A to C it can be seen that p80 and p82-derived sera reacted consistently with all recombinant gp120 glycoproteins (IIIB, MN and SF2). For p82, the variation was in a range between 5.69 to 6.3 for IIIB, 7.35 to 8.28 for MN and 4.52 to 5.09 for SF2, whereas for patient p80 the range was: between 5.29 to 5.98 for IIIB, 8.32 to 8.81 for MN and 4.36 to 5.03 for SF2.

Among the patients clustered in the main haemophilic group, p79 reacted poorly with IIIB and SF2 ($r=3.41$ for IIIB, 2.43 for SF2) but strongly recognised gp120 MN ($r=9.04$). Sera from this patient also reacted poorly with the heterologous gp120 glycoproteins derived from patient p82. A low IgG anti-env antibody level in serum taken from p79, which was still able to strongly cross-react with MN gp120 antigen, might explain this result.

6.3 Summary of results

Ten gp120 antigens obtained from homosexual individuals and five similar antigens from a haemophiliac patient (p82) were used in ELISA experiments in order to measure the extent of recognition by autologous and heterologous sera.

For the homosexual cohort, the antigens derived from isolates with predictive NSI phenotype were better recognised by autologous and heterologous sera than those which derived from isolates with SI phenotype, suggesting that the patients may have been at an early stage in infection, when NSI isolates are preponderant. However, a high level of cross-reactivity between gp120 antigens and different sera was evident, showing the presence of highly conserved epitopes on the immunising isolates. Envelope antigens derived from the same patient were recognised to different extents by autologous samples, suggesting the presence of escape mutants or mutants with lower antigenicity, during HIV-1 infection.

By employing an ELISA in the presence of denaturing agent (8M urea), it has been shown that the gp120 antigens expressed after transfection in supernatants have a proper quaternary structure. No correlation between the affinity of antibody as expressed by the reactivity recorded in the presence of urea, and CD4 cell count was found. Conversely, an inverse correlation between the extent of antibody reactivity given by the homosexual patients' sera against gp120 antigens and their CD4 cell count was observed (Spearman $r=0.73$, $P= 0.037$). This finding supports the idea that a high IgG level in patients with impaired Th function may be a consequence of the activation of the memory B cells by the antigen.

A dynamic relationship between viremia and humoral immune responses was also found in a longitudinal study of a haemophiliac patient (p82). From this patient, two SI isolates-derived gp120 antigens were recognised to different extents by antibody from autologous and heterologous sera (i.e. sera that was derived from a closely related individual, p80).

Two closely related patients (p80 and p82) gave similar patterns and extent of reactivity to the p82-derived gp120 antigens, higher than those recorded for non-related patients sera (as shown by phylogenetic analysis), indicating the contribution of type-specific antibody. The recognition of p82-derived antigens by sera from less related patients might indicate the presence of group-specific antibody.

The level of the IgG antibody response of sera from both haemophiliac and homosexual patients was assessed against three control antigens: IIIB, MN and SF2 recombinant gp120 glycoproteins. All sera reacted poorly with gp120 SF2, a glycoprotein expressed in CHO cells, in comparison with baculovirus-expressed IIIB and MN gp120s, probably showing a lower antigenicity of the molecule due to the presence of complex oligosaccharide chains on this antigen that might occlude epitopes from antibody recognition. Homosexual patients' sera fell into the same groups (high reactive, medium reactive and low reactive) when employing both autologous and heterologous gp120 glycoproteins or recombinant gp120 glycoproteins (IIIB, MN and SF2). Consistent results were obtained for all the haemophiliac-derived sera, when reacting with both p82-derived antigens and control antigens.

A sustained Th function might be present during the infection in the absence of the antiretroviral therapy.

CHAPTER 7

GENERAL DISCUSSION

- 7.1 The use of gp120 ELISA to estimate the amount of gp120 glycoprotein from transfected cell culture or body fluids**
- 7.2 ELISA-based methods for assaying anti-HIV antibodies**
- 7.3 Expression of the HIV-1 gp120 glycoprotein**
- 7.4 Intracellular processing and transport of env proteins**
- 7.5 *in vivo* variation of the gp120 genes in the homosexual group**
- 7.6 Strategies for AIDS vaccines**

As stated in Chapter 3, an “in house” gp120 ELISA was set up to fulfil two aims. The first one was to estimate the amount of gp120 glycoprotein in transfection supernatants. The second aim was to estimate the extent of the gp 120 recognition by the autologous and heterologous sera. Chapter 4 described the modifications performed to pSRHS to make it suitable for the subsequent subcloning of the 1.7-kb fragments encoding gp120 glycoprotein. In Chapter 5, the env-expressing clones were presented together with their nucleic and amino acid sequences and some biological features (NSI/SI-phenotype, co-receptor usage, glycosylation sites). In Chapter 6, the reactivity values obtained when homosexual and haemophiliac-derived gp120 antigens were reacted with autologous and heterologous sera and their significance were shown.

Consequently, in this chapter the results obtained using ELISA assay in comparison to the other methods used to quantify the gp120 proteins are discussed. Also, the discussion covers the level of gene expression achieved by env-containing clones in comparison to other systems used to express the HIV-1 gp120 antigens, together with their limitation, which consists of an inefficient intracellular processing and transport of env proteins. The discussion further reveals the significance of the *in vivo* variation of the gp120 genes in the homosexual group. Finally, comments on strategies for AIDS vaccines that are currently ongoing, with the achievements obtained to date regarding DNA/prime-protein/boost vaccine, are presented.

7.1 gp120 ELISA

An HIV specific ELISA assay was needed to allow the estimation of the degree of recognition of the gp120 antigens produced in this study by the autologous and heterologous sera. It was first necessary to quantify gp120 antigens obtained from the transfection supernatants to ensure that the ELISA microtitre wells are loaded with

similar amounts of antigen. The ELISA assay set up fulfils both requirements. Previous studies have adopted different approaches for quantifying gp120. Two different ways of quantifying gp120 glycoprotein were used by Weiler *et al.*, (1991). One consisted of an HIV protein ELISA where gp120 glycoprotein and other viral proteins were directly adsorbed to the solid phase through the interactions between the negatively charged residues on vinyl and styrene molecules of the microtitre plates and positive charge residues on the surface of the protein. Subsequently, the protein was detected using enzyme coupled-*Narcissus pseudonarcissus* lectin (NPL), with an associated sensitivity of between 3 to 600ng/ml. The main disadvantage of this method resides in a possible alteration of the conformation of gp120 as a consequence of direct absorption of the molecules onto the solid phase that might obscure or distort the epitopes. The second method consisted of a mannose binding lectin-based ELISA, when NPL lectin was bound to the solid phase to capture gp120 glycoprotein and *Galanthus nivalis* agglutinin-coupled peroxidase were used as a detector system. The linear relationship between the absorbance and the concentration of the glycoprotein was between 0.6-20,000ng/ml. Despite a relatively unaltered conformation of the glycoprotein due to its binding to the lectin, the orientation cannot be rigorously controlled because lectin binding may occur through mannose binding at any N-linked carbohydrate residue.

Quantitative studies of gp120 were also performed by Moore *et al.*, (1988) who used, as a capture antibody, a sheep antiserum (D7324) able to specifically interact with the C1 and C5 region of the gp120 molecule. Gilbert *et al.* (1991) also developed an ELISA assay to quantify the amount of the gp120 glycoprotein employing recombinant soluble CD4 as a substitute for D7324.

In the study described in this thesis, an “in house” sandwich enzyme-linked immunosorbent assay (ELISA) was developed. This was based on Moore’s ELISA assay with the only one modification consisting of the replacement of the AMPAK amplification system with a specific detection system, biotin-F (ab’) 2 anti-human IgG

and avidin-alkaline phosphate. When using AMPAK system a high background was recorded which was subsequently reduced by replacing the whole biotin-anti-human IgG with more specific biotin-F (ab')₂ anti-human IgG. The results suggested non-specific interactions that occurred between Fc fragments of the anti-human IgG and the capture antibody D7324, which have been removed by using F (ab)₂ fragments. A high background due to the interactions between biotin-anti-human IgG and the capture antibody D7324 was also observed by Moore *et al.* (1993a) who removed from the sheep antiserum D7324 the fraction that was able to react with the human IgG. Thus, biotin-anti-human IgG was used as an alternative to reduce the background. Optimisation with F (ab)₂ anti-human IgG was performed, finally resulting a linear relationship between optical density and gp120 concentration between 50 and 400ng/ml. Precision analysis of the "in house"gp120 ELISA provided a coefficient of variation between 5 and 20, indicating that the method had a good reproducibility.

When transiently transfecting COS cells with a construct encoding gp120 and gp160 glycoprotein from a T cell line adapted isolate (pHXB2-RC), Moore *et al.*, (1988) estimated an equivalent of 2.5×10^6 molecules of gp120 to be produced per cell, 40-50% of which can be recovered from the culture medium after 24 hours, leading to a total of between 500-700ng gp120/ 10^6 cells/72 hrs being produced. A similar level of expression was obtained in this study, when using both homosexual and haemophilic-derived env-encoding constructs to transfect COS cells (average value of 500ng/ 10^6 cells/72hrs). Consequently, the linear relationship between the absorbance value and the gp120 concentration of the ELISA method should be in the range of 50-500 ng/ml, a requirement that was fulfilled by the "in house" ELISA assay developed in the present study.

Adaptations of gp120 ELISA have been used to assess the magnitude of reactivity between gp120 glycoprotein and IgG antibody from HIV-1 infected persons. Early ELISA assays for testing human sera for antibodies to HIV-1 used purified HIV

particles passively absorbed to plastic surfaces in order to replace the radioimmunoprecipitation (RIP) assay, which is complicated and more time consuming to perform. The main disadvantage of these methods resides in the retention of only a proportion of gp120 on the virus surface, due to the fact that the gp120-gp41 interaction is non-covalent in HIV-1 and, hence, a significant proportion of gp120 is shed during virus purification. In addition, a lower specificity that might be a consequence of the sera containing antibodies reacting to cellular antigens that appeared as contaminants of the viral preparation due to their incorporation to the virion envelope during budding was reported.

7.2 Development of an ELISA-based method for assaying anti-HIV antibodies

As already mentioned, the development of an ELISA assay to estimate the extent of the recognition of the patients-derived gp120 antigens by the autologous and heterologous sera was a need to test the degree of cross-reactivity between antigens and sera.

ELISAs utilising synthetic peptides representing segments of gp120 glycoprotein have been used (Thorn *et al.*, 1987, Jansson *et al.*, 1994, Warren *et al.*, 1992) to test their degree of recognition by the HIV-1- infected sera. This method was developed in response to the need to find efficient tools to identify HIV-1 subtypes rapidly for epidemiological studies (see Chapter 1). While sequencing regions of the genome represents the most reliable method for classification, simpler and quicker assays were required to investigate subtype distribution. The V3 domain was chosen as an antigen for serologic subtyping for several reasons. Firstly, in spite of the great genetic variation within the env gene, the V3 domain seems to be sufficiently conserved within subtypes allowing the identification of a V3 consensus sequence for each HIV-1 subtype (Korber *et al.*, 1998). Secondly, the epitopes that reside within this region are highly antigenic *in vivo* and a humoral immune response to this region can be detected in most of the HIV-1 infected persons. Therefore, short linear V3 peptides of subtype A to I were extensively

used as antigens in peptide binding enzyme immunoassays. In general, it was found that V3 peptide serology could predict HIV-1 genotypes, a conclusion that was supported by higher recognition of the peptides corresponding to the genotypes A, B, C and E by the sera that had the same genotype (Cheingsong-Popov *et al.*, 1994). The use of V3 serotyping for epidemiological studies has been questioned by other studies (Nkengasong *et al.*, 1998, Plantier *et al.*, 1999) due to the great extent of serological cross-reactivity to peptides representing heterologous genotypes, leading to incorrect classification of subtypes. Nowadays, V3 serology is considered to be limited to epidemiological monitoring of HIV-1 epidemics in large populations.

Use of V3 peptides for serological purpose had another shortcoming that derived from differences in conformation of the V3 peptide absorbed on the solid-phase, in comparison to the same peptide in solution and the conformation of that V3 domain on the intact gp120 glycoprotein. Moore *et al.*, (1993d, 1994e) showed that reactivity of positive sera with solid-phase V3 peptides could, therefore, be a poor predictor of reactivity with the same domain on native gp120 molecules. The level of anti-V3 antibody, as measured by a V3 peptide ELISA, did not correlate with the stage of disease (Zwart *et al.*, 1994). Conversely, Fenouillet *et al.*, (1995) reported a correlation between the antibody reactivity to the North American/European consensus sequences: V3 (MN) or V3 (Cs) and clinical stage of the disease, using a V3 specific antigen limited ELISA. In their study, the level of anti-V3 antibody declined in relation to progression to AIDS. This result might be explained by the emergence of escape mutants with V3 sequences very different from those used as antigens (a situation which, if it is true, shows a limitation of the V3 peptide based ELISA assay) or by a general decline in the numbers of memory B cells.

In spite of these limitations associated with V3 serology, it is worth mentioning a study of Zwart *et al.* (1992) where the V3 region from viral RNA and proviral DNA from 13 asymptomatic individuals (samples were taken less than one year after seroconversion)

and 14 symptomatic individuals were cloned and sequenced. Firstly, they observed that sera taken early after seroconversion reacted specifically with the peptides that reproduced the viral population early in infection. Sera taken at the later points during the infection reacted to the same extent or even more strongly with the peptides that were prevalent early in the infection than with those specified to the sample of contemporary virus. That means that the specificity of anti-V3 antibodies in most sera taken late during the infection does not reflect the isolates circulating at that time point. Only 7% of individuals developed serum specificity for new variants that appeared over the five years, whereas 50% of individuals had the same specificity (both early and late sera) to the isolate found early in infection. This result was explained by the phenomenon named “original antigenic sin”, in which the humoral immune response over the course of an infection is generated predominantly against the first immunogenic isolate, with a new, related, antigen that is slightly different from the original “activator” causing the reactivation of the same B cells (Nara *et al.*, 1991, Kohler *et al.*, 1992).

To surmount the problems involved with quantifying the total serum HIV-specific IgG elicited against the V3 domain, V3 serology was replaced with an ELISA that employed recombinant gp120. Different systems to capture gp120 on the solid-phase were used. The first set of ELISA methods employed lectins such as Concanavalin A (ConA) (Robinson *et al.*, 1990), *Galanthus nivalis* agglutinin (GNA) (Hinkula *et al.*, 1994) or *Narcissus pseudonarcissus* (NPL) lectin (Weiler *et al.*, 1991). The second set of ELISAs was based on the same principle as the ELISA for quantification of gp120 glycoprotein. Thus, sheep antiserum raised to gp120 glycoprotein (D7324) was used as a source of capture antibodies able to specifically interact with the C1 and C5 regions of monomeric gp120. Similar to the lectin ELISA, an ELISA employing a capture antibody offers the chance of detecting antibodies able to react with conformational epitopes (i.e. epitopes within the C2, C3 and C5 regions), since the gp120 glycoprotein is immobilised unaltered and with a precise orientation, via the D7324 antibodies (Moore *et al.*, 1993a, 1994b, Binley *et al.*, 1998). This was demonstrated by a 100-fold reduction in the titres

of sera seen when monomeric IIB gp120 was denatured before performing the assay (Moore *et al.*, 1993c).

A variation of the ELISA method used to quantify gp120 antigens, consisting of a replacement of the anti-gp120 MAb with IgG antibodies from HIV-1 infected sera, was employed in order to estimate the degree of recognition of autologous and heterologous gp120 antigens by sera taken either cross-sectional or longitudinal from both groups of subjects studied. In comparison to V3 peptide ELISA, this assay allows detection of IgG antibodies that are raised against regions other than the V3 loop (region that was referred to as the Principal Neutralisation Determinant) such as V1/V2 loop. A limitation of this assay would come from the conformation of the monomeric gp120 molecules that is more exposed than that presented by the oligomeric forms and, hence, would have different antigenic properties. In spite of this shortage, the purpose of this study can be satisfactorily fulfilled with the “in house” anti-gp120 ELISA, taking account the fact that monomeric gp120 antigens obtained from transfection supernatants derived from primary isolates that have a conformation closer to those presented by the oligomeric gp120 molecules than monomeric or oligomeric gp120 antigens derived from T cell line adapted isolates (TCLA).

Due to its rapidity and simplicity this ELISA assay was also used by other investigators to map epitopes on the monomeric and oligomeric gp120 molecules. The C1, C2, C3 and C5 domains appeared poorly accessible on the native monomeric gp120 glycoprotein (Moore *et al.*, 1994b), whereas the V1, V2 and V3 domains were found to be exposed on its surface and accessible to antibody (Moore *et al.*, 1993a). In oligomeric gp120, the epitope exposure mapped somewhat differently (Stamatos *et al.*, 1998). The C1 and C5 regions that were accessible on monomeric gp120 molecules became occluded on the oligomers, whereas the C4 region was partially exposed (Moore *et al.*, 1994b) while the V1/2 and V3 loop have a good exposure. A study conducted by Binley *et al* (1998) revealed that gp120 V1/2 and V3 variable loops contribute to the occlusion

of some of the conserved gp120 epitopes. These results were obtained by reacting two forms of the gp120 glycoproteins (i.e. a gp120 protein derived from the LAI strain of HIV-1, HXBc2 clone and a deleted Δ V1/2/3 glycoprotein, both expressed in COS cells) with four CD4bs MAb (15e, F91 and IgG1b12) and CD4-IgG2. Thus a 6- to 15-fold higher affinity was recorded when the CD4bs MAbs and CD4-IgG2 bound to the Δ V1/2/3 glycoprotein than to the HXBc2 glycoprotein.

Subsequent studies conducted on crystals of gp120 glycoproteins performed by Kwong *et al.* (1998) and Wyatt *et al.* (1998) revealed the structure of a truncated gp120 protein derived from HXBc2 strain of HIV-1, with 52 and 19 residues deleted from the N- and C-termini and Gly-Ala-Gly tripeptide substitutions for 67 V1/V2 loop residues and 32 V3 loop residues. About 80% of the gp120 core was deglycosylated. The analysis revealed that the polypeptide chain of gp120 is folded in two major domains: an inner domain and an outer domain. The proximal end of the outer domain includes variable loops V4 and V5 and a protrusion represented by the β -hairpin consisted of β 20– β 21 that is hydrogen bonded with the V1/V2 stem emanating from the inner domain. This structure represented by an antiparallel, four-stranded region called the “bridging sheet” is involved in the interaction of gp120 with both CD4 and the 17e antibody. Interatomic contacts between gp120 and CD4 are mediated by Phe 43 and Arg 59 protruding from CD4 and residues Asp 368, Glu 370 and Trp 427 on gp120 which form a water-filled, hydrophobic cavity. The inaccessibility of these amino acids explains the failure to find almost any neutralising antibody that prevents CD4-gp120 binding directly. In the light of their accessibility to the neutralising antibody, three regions could be described: a neutralising face that includes the V2 and V3 loops that are found adjacent to the surface, a non-neutralising face, represented by the regions that are poorly accessible on the assembled envelope glycoprotein trimer and a silent face that roughly correspond to the highly glycosylated outer domain surface.

Conclusions of this section

The ELISA developed in this study employing a set of reagents represented by the capture antibody D7324, anti-gp120 IgG, Biotinylated F (ab')₂ goat anti-human IgG and ExtrAvidin Alkaline phosphatase fulfilled two purposes:

1. quantification of the gp120 glycoproteins from the supernatants of transfected COS cells
2. estimation of the sera IgG level able to react to the gp120 antigens.

The reproducibility and sensitivity of the method was in the range that was suitable for quantification of both gp120 molecules from transfection supernatants and IgG level from HIV-1 infected sera (i.e. 50-200ng/ml). This assay overcomes the shortage of the V3 peptide based ELISA residing in their recognition by a narrow group of antibody and their inability to reproduce the conformation of the V3 domain on native gp120 glycoproteins. The glycoproteins are absorbed on the microtitre plates in a precise orientation, via D7324 capture antibody, thus presenting the epitopes in an unaltered conformation.

7.3 Systems used to express the HIV-1 gp120 glycoprotein

Different expression systems have been used to express env gene in different cell types. Thus, HIV-1 envelope antigens have been produced in bacterial cells (*Escherichia coli*) (Crowl *et al.*, 1985a,b, Chang *et al.*, 1985), yeast (Barr *et al.*, 1987), insect cells (Weiss *et al.*, 1993) and also in mammalian cells (Rekosh *et al.*, 1988).

1. A number of plasmids have been used to express HIV-1 env genes in *E.coli* (Crowl *et al.*, 1985a, b). These plasmids derived from pBR322 and contained the phage lambda P_L promoter, a synthetically derived ribosome-binding site, convenient cloning sites, downstream to the initiation codon, and the ampicillin resistance gene. The main advantage of this system is the large amount of the protein produced in bacteria,

although the bacterial products are not modified after translation and are therefore of much lower molecular weight in comparison to naturally occurring *env* gene product. In spite of a completely different glycosylation pattern that gp120 molecule produced in bacteria have in comparison to homologous mammalian cells produced molecules, Crowl *et al.*, (1985b) showed that the *env* glycoproteins synthesised in *E.coli* cells are recognised by antibody present in the sera of AIDS patients. These results may suggest that some antibodies to gp120 glycoprotein are directed against the protein backbone of the molecule rather than against the carbohydrate moieties. However, the significance of this reactivity for viral neutralisation has not been established.

2. Expression of envelope glycoprotein was achieved in insect cells such as *Drosophila melanogaster* Schneider 2 cells (Ivey-Hoyle *et al.*, 1990) and *Spodoptera frugiperda*-8 lepidopteran insect cell line (Rusche *et al.*, 1987, Wells *et al.*, 1990, Hu *et al.*, 1987). The DNA construct used to produced HIV-1 BH10 gp160 protein in *Drosophila* cells contains an expression cassette that has the inducible *Drosophila* metallothionein promoter, the beginning of the human tissue plasminogen activator (tPA) gene fused in frame with gp160 coding sequences and SV40 early polyadenylation signal (Ivey-Hoyle *et al.*, 1990) on a pBR322-based plasmid. *Drosophila* cells stably transfected with this construct expressed 1 to 2 mg of gp120 per litre of culture medium, upon metal induction of the metallothionein promoter. In spite of the high level of *env* expression in insect cells, in comparison to the other systems employed in vertebrate cells, there is evidence to suggest that the processing pathway is different between insect and vertebrates cells, since N-linked oligosaccharides from insect cells are deficient in sialic acid, galactose and fucose (Butters *et al.*, 1981a, b, Hsieh *et al.*, 1984). Studies of oligosaccharide processing in insect cells reveals that the core structures represented by three mannose residues, one glucose residue and two N-acetylglucosamine residues (Man₃ Glu NAc₂), which arise from high mannose oligosaccharides, represent the most extensively processed oligosaccharides in these cells, whereas those from virus grown in vertebrate cells are characterised by the large sialic acid-containing complex-type

oligosaccharides. It is not clearly understood the extent to which difference in glycosylation pattern affects the antigenic property of the insect cell product.

The *env* gene of HIV-1 was also inserted into the genome of the baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) to produce a recombinant baculovirus (Hu *et al.*, 1987). The principle of constructing a recombinant baculovirus consists of the insertion of a foreign DNA into a plasmid vector (pAc610) downstream from the promoter for the baculovirus polyhedrin gene. The chimeric gene in the recombinant plasmid is flanked by additional AcNPV sequences around the polyhedrin gene. This plasmid is then transfected with AcNPV DNA into insect tissue culture cells, an event that is followed by the homologous recombination between the plasmid and the viral DNA, which occurs in the region flanking the chimeric gene and allows its insertion into the genome of AcNPV. The recombinant viruses lack the ability to synthesise polyhedrin and, therefore, exhibit a polyhedrin negative phenotype. Thus, Hu *et al.*, (1987) inserted an *env* gene of HIV-1 into the genome of an insect virus vector (*Autographa californica* nuclear polyhedrosis virus) and expressed the *env* gene by infecting *Spodoptera frugiperda* cells with the recombinant virus, the presence of HIV-specific proteins in the infected cell lysates being detected by Western Blots. Recombinant baculovirus-produced *env* proteins were immunoreactive, being successfully used as antigens in diagnostic tests for AIDS and also to immunise animals that subsequently developed neutralising antibodies (Rusche *et al.*, 1987).

In addition to transient expression of *env* genes, stable expression of the envelope glycoprotein has been described in human CD4-positive lymphocyte cell lines such as Jurkat and CEM (Stevenson *et al.*, 1988), HELA cells (Terwilliger *et al.*, 1988) and human B cell line (Ahmad *et al.*, 1993).

3. Both transient and stable expressions were performed in mammalian cells. Some vectors used in other systems such as the amphotropic retrovirus vector, Semliki Forest

virus and recombinant adenovirus vectors are less commonly used in comparison to the SV40 based and recombinant vaccinia virus vectors.

a. Amphotropic retrovirus vectors were used to expressed the HIV envelope glycoproteins in a human CD4⁺ cell line (CEM cells). The *env* gene was inserted into the retrovirus expression vector, being under the control of the highly active cytomegalovirus (CMV) immediate early promoter (Stevenson *et al.*, 1988).

Using a novel expression system based on Semliki Forest virus (SFV) expression of HIV-1 *env* glycoprotein was performed. Different genes have been cloned into the expression system, based on the cDNA of SFV. These vectors can be used either to transfect the heterologous gene directly or to produce a recombinant virus by co-expression of a helper plasmid (Paul *et al.*, 1993). An SFV expression system is composed of the pSFV1 expression vector and SFV-helper1. Both constructs have the SP6 promoter, SP6 origin of replication and the ampicillin resistant gene. The pSFV1 encodes SFV non structural genes (nsP1-nsP4), HIV-1 *env* gene and packaging site for incorporation into SFV particles, whereas pSFV-helper1 encodes a deleted form of nonstructural gene, the capsid (C) and envelope (p62, 6k and E1) genes. The SFV system has several advantages for HIV-1 envelope glycoprotein expression. The recombinant proteins are produced efficiently, the recombinant viruses have a broad host range of wild-type SFV, being able to infect many eukaryotic cells, including human T cell lines, the system allows the expression of a high amount of glycoprotein without the requirement of Rev protein, since the replication of SFV occurs in the cytoplasm, the cells may express the recombinant glycoprotein for longer period of time (more than 75 hrs), in comparison to vaccinia expression, case in which the cytopathic effect is manifested early after infection. Thus, Paul *et al.*, (1993) induced high level of expression of HIV-1 envelope glycoproteins in Baby Hamster Kidney (BHK) cells, HeLa and MOLT-4 cells, using pSFV1 vectors, glycoprotein being properly folded,

processed and transported and able to interact with CD4 molecules and mediate cellular fusion.

Another way to induce HIV-1 env glycoprotein involved the manipulation of recombinant adenovirus expressing env/rev genes of the HIV-1. They were constructed by inserting the gene into an expression cassette containing the adenovirus type 7 major late promoter, following by leaders 1 of the adenovirus tripartite leader and a portion of intron between leaders 1 and 2, leaders 2 and 3, and a hexon polyadenylation signal. The cassette was then inserted in the terminal region between the E 4 and ITR domains of the adenovirus 7 genome with a concomitant E3 region deletion. A 549 cells were co-infected with a recombinant Ad7 viruses containing *env* and *rev* genes or with a recombinant virus that had both the *rev* and *env* genes. HIV-1 envelope gene expression was greatly enhanced (20 to 50-fold) when *env* gene was expressed in the presence of Rev protein, as measured by ELISA and Western blotting (Chanda *et al.*, 1990), showing the role of Rev protein in preventing the nuclear retention of incompletely spliced mRNA (see Introduction).

b. Env glycoprotein synthesis from SV40-based systems

Major systems that were extensively used to express the env glycoprotein in mammalian cells are represented by the SV40 based and vaccinia-based vectors. The vector used in this study belong to the group of vectors that contain the entire SV40 early/late promoter, the SV40 origin of replication, Mason-Pfizer Monkey virus polyadenylation signal and ampicillin resistance gene, driven by the SV40 promoter, and are able to replicate to high copy numbers in cells permissive for SV40, such as COS cells (Dubay *et al.*, 1992). This lead to high levels of expression of the cloned gene. Since the Env proteins are expressed from a vector that might be easily modified by site-directed mutagenesis, this system also provides a convenient source of mutated proteins for purification and further characterisation.

A similar env-encoding SV40 based eukaryotic expression vector, able to express Env proteins both in *E. coli* and eukaryotic cells permissive for SV40 replication was used by Rekosh *et al.*, (1988). The level of gp120 expression obtained by Rekosh *et al.* (1988) was similar to those found in this study, transfected COS cells producing approximately 200 times more protein than HIV-1 infected cells (Robey *et al.*, 1986). The vector used contained Sall-XhoI fragment from the BH10 clone of HIV-1, under the control of SV40 late promoter, large T antigen, under the control of the SV40 early promoter, the SV40 enhancer, the rabbit β -globin gene containing the splice donor and acceptor surrounding the second intron as well as the β -globin polyadenylation signal. The initial vector, called pBABE, contained the entire envelope protein-coding region (Env) from SstI site at position 6035 to the XhoI site at position 8920 (GENBANK numbering system). It contained about 200 nucleotides of sequence upstream from the start of ORF encoding the envelope protein but did not contained the donor-splicing site (6043, 6044) (GENBANK numbering system). Their attempts to demonstrate synthesis of the envelope proteins with this vector were unsuccessful. Consequently, a fragment from the Sall site (5820) to the SstI (6035) was subcloned in the previous plasmid to produce a plasmid called pSVSX1. This construct was able to express the env gene, in the presence of Tat and Rev proteins, at the level of 500ng/10⁶ cells/72hrs, with the frequency of positive cells of 20% to 30%, as detected by immunofluorescence assay. This study revealed the importance of the donor-splicing site in the synthesis of the envelope proteins. Similar results were obtained in the present study when a fragment containing the donor-splicing site (DSS) was removed by digestion with Sall. The ability of the plasmid to express the *env* gene was restored only when donor-splicing site was subcloned into the plasmid (see Chapter 4).

Similar vectors were used to produce the influenza hemagglutinin, in CV1 cells, with a level of expression comparable with those obtained with env constructs (Gething *et al.*, 1981, 1982). Also, a derivative of the vector used by Rekosh *et al.*, (1988) lacking the β -globin splice and polyadenylation signal was used to express EBNA (Hammarskjold *et*

al., 1986) and the human myc protooncogene products (Classon *et al.*, 1987) even in several human lymphoid cell lines.

Another *env* gene construct (pSVT *env* (tat⁺rev⁺), containing SV40 ori, SV40 early promoter, Amp^R, SV40 polyA, was used by Bird *et al.*, (1992) to induce *env* expression in murine L cell lines along with many other expression vectors. Thus, Weiss *et al.*, (1993) used *env* sequences inserted into an expression vector which has a glutamine synthetase minigene for selection and amplification and CMV promoter to obtain a stable CHO cell line, able to express both the wild-type, secreted form of gp120 or a glycosylphosphatidyl inositol-anchored enveloped glycoprotein of HIV-1. Ahmad *et al.*, (1993) transfected Raji cells (human lymphoid B cell clone) with a vector (pSL 3Env⁺Vpu⁻Neo) which contained the Epstein-Barr virus origin of replication, the neomycin resistance gene, driven by the SV40 promoter, PolyA signal, provided by the SV40 early region intron (SV40 polyA) and *env* fragment of HXBc-2 ligated to the 3' LTR of SL 3-3 murine leukemia virus. Another vector (pSV2gpt) that had mycophenolic acid resistance gene and *env* gene driven by the HIV LTR was used to express *env* glycoprotein in HeLa cell lines (Terwilliger *et al.*, 1988).

c. Production of gp120 from infectious molecular clones

Infectious molecular clones, such as pHXB2D, have been used to produce wild-type virus, after transfection into COS-1 cells (Fisher *et al.*, 1986a, Guo *et al.*, 1990). pHXB2D plasmid contains an SV40 origin of replication, HIV-1 LTR directing expression of full-length and spliced viral transcripts. The same construct was used to express viral proteins in CD4⁺HeLa cells (Guo *et al.*, 1990). A modified form of gp120 (Δ V1/2/3), containing a deletion of the V1/V2 stem-loop and one of the V3 loop and a deglycosylated Δ V1/V2/V3 that resulted by treatment of the Δ V1/V2/V3 glycoprotein with a combination of endoglycosidase D and H, were obtained by transfecting insect cells (*Drosophila* Schneider 2 cells) with a derivative of pHXB2D in order to investigate the interaction of antibodies with a conserved, deglycosylated core of the HIV-1 Env

glycoprotein. This study revealed that deletion of V1, V2 or V3 regions only minimally altered the binding of the most antibodies examined, when compare with the full-length, correctly folded gp120 monomer, a result which was exploited in crystallography studies of deglycosylated, truncated gp120 glycoprotein (Wyatt *et al.*, 1998).

The other major class of the expression systems is represented by the vaccinia virus vectors, which have been intensively used to induce the *env* gene expression in mammalian cells. Schwartz *et al.*, (1993) expressed *env* glycoprotein in CEM cells using this vector together with a retroviral vector containing murine leukemia virus LTR and SV40 early promoter-neo gene cassette able to express Nef protein. The study revealed a downregulation of the CD4 cell surface localisation in the presence of Nef protein together with a significant decrease of cell surface levels of gp120. Down regulation of CD4 by Nef and Vpu confers protection of the virus-producing cells from superinfection and prevents inappropriate gp120/CD4 interactions prior to viral budding. Vaccinia virus vectors with *env* gene under the control of vaccinia virus promoter and with a thymidine kinase- negative phenotype were used to express gp120 gene in Green monkey kidney cells (BSC-40), H9 and HeLa cells (Chakrabarti *et al.*, 1990, Hu *et al.*, 1986). The synthesis, glycosylation, processing and membrane transport of these *env* glycoproteins were identical with those recorded for infected T cell lymphocytes, *env* proteins being recognised by heterologous HIV positive sera and were able to induce antibodies to gp120 in mice.

Conclusion of this section

The main advantage of expressing the *env* genes in eukaryotic cell lines consists of the similarity of the posttranslational processes between the expressing cells and infected T lymphocytes. The glycoproteins expressed in this type of cells have a high mannose and complex oligosaccharide type N-linked carbohydrates moiety, similar to those found *in vivo*, being thus a good reagent for eliciting an antibody response or for epitope

mapping. The level of *env* gene expression was 500-1000 ng/10⁶cells/72hrs comparable to those reported by other investigators.

7.4 Inefficient intracellular processing and transport of env proteins

The results obtained by immunofluorescence assay showed the presence of the env protein intracellularly suggesting gp160 precursor degradation in lysosomes, accounting for a moderate level of expression achieved by transfecting COS cells with gp120 encoding vector. A similar result was reported by Bird *et al.*, (1990) who evaluated the kinetic and efficiency of intracellular processing and transport of the envelope glycoprotein in COS cells by pulse chase metabolic labelling of the cells with pSVT env (tat⁻ rev⁺). It has been proved that in transfected COS-1 cells, the conversion of gp160 precursor to gp120 and gp41 begins very soon after synthesis, but quantitatively is highly inefficient. If the processing rate was initially rapid, it slowed considerably after 3hrs of chase, suggesting that gp160 is no longer subject to further conversion. Only 40% of the newly synthesised gp160 precursor appeared to be converted to gp120 and gp41 at the completion of the chase period (20hrs) and only 30% of the total gp120 protein was found in culture supernatant. Thus, approximately 35% of the total gp160 precursor synthesised cannot be accounted for after a 20hrs chase, being intracellularly degraded. The inefficient processing and transport of env proteins is not a feature limited to COS cells. Similar results have been reported by Willey *et al.*, (1988) in infected T lymphocytes, where less than 20% of gp160 was cleaved to gp120 and gp41. Also, another study conducted by Bird *et al.*, (1992) revealed that most of the env proteins had a perinuclear Golgi or endoplasmic reticulum distribution, as indicated by indirect immunofluorescent staining of fixed and permeabilised pSVT env (tat⁻ rev⁺) gpt transfected murine L cells. It has been suggested that inefficient env processing might be a consequence of subsequent destruction of complexes of CD4 and gp160, which occurs in the absence of Rev protein (Emerman *et al.*, 1998). However, since transfected COS

cells do not constitutively express CD4, the inefficient processing of gp160 must occur independently of CD4-gp160 complex formation.

Significance of the *in vivo* variation of the gp120 genes in the gay group

Discussion of the variation of the sequences obtained for individuals from the male homosexual group may be done both within and between samples.

a. Intra-samples distances

As given in Chapter 5, the nucleotide distance of the gp120 env sequences derived from the same patients were computed to produced an intra-sample distance of 1.8% for the patient 1021 and 0% for 1397. These results were analysed in conjunction with data reported by Dr. Lin Qi Zhang (1992) (University of Edinburgh, PhD thesis, 1992), regarding the nucleotide distances of the V3 sequences amplified and sequenced directly from samples from the same haemophiliac patient (p82), at five sequential time points (year 3, year 4, year 6 and year 7). The average intra-sample distance in year 3 was 3.2%, 3.6% in year 4, 2.5% in year 5, 5.7% in year 6 and 4.6% in year 7. Analysing the patients 1021 and 1397-derived sequences relative to those from a single patient, taken longitudinally the data are consistent with the fact that these patients were investigated early in infection (last negative 03.92, first positive and the date of sample collection 03/94, suspected date of seroconversion 1993). However, the procedure employed in this study sampled a limited number of proviruses. Nevertheless, the results suggest that the viable complete coding sequences are not less diverse than all sequences taken at similar time points.

b. Inter-sample distances

The value of 13.4% obtained for inter-samples distances associated with the sequences obtained for gay individuals was analysed relative to the mean inter-samples distances computed for sequences obtained sequentially from patient p82, in a longitudinal study (Holmes *et al.*, 1992). In this study, the mean inter-sample distances between sequences derived from patient p82 were estimated to be: 22.3%, between year 0 and year 3, 15.93,

between year 0 and year 4, 13.5% between year 0 and year 5, 22.4%, between year 0 and year 6 and 20.38%, between year 0 and year 7. Thus, the mean intra-sample distance decreased after year 3 to reach the minimum value of 13.55% by year 5 and increased after year 6 to a value that was comparable to that between year 0 and year 3. It was found that the mean inter-samples distances for sequences from homosexual subjects were lower than the value obtained for the patient p82 between year 0 and year 7. This result may suggest that the extent of the sequence variation that appears within the individual over several years can reach the same extent as that that is manifested between different persons. A similar result was reported by Holmes *et al.* (1995), who investigated 132 V3 sequences obtained from HIV-1 infected persons from different risk groups in Edinburgh (including sequences from patient p82). The main conclusion of this analysis, regarding the homogeneous nature of the sequences obtained at or near after seroconversion with the diversification of the sequences over time, is in agreement with that reported by Holmes *et al.* (1992). The diversification of the sequences over time may be a consequence of a positive selection imposed by the host immune system or the need of the virus to use different co-receptors in order to gain the ability to infect different cell types, or a neutral evolution, as a consequence of the random activation of infected cells. Some of these sites (e.g. V3 hypervariable region) represent targets for neutralising antibodies (Moore *et al.*, 1996a, b, Javaherian *et al.*, 1989) and cytotoxic T cell (Doe *et al.*, 1994, Kameoka *et al.*, 1994), and are responsible for conversion from one phenotype to another (Milich *et al.*, 1993, Donaldson *et al.*, 1994, Fouchier *et al.*, 1992). The hypervariable nature of the V3 region was also described by many investigators (La Rosa *et al.*, 1990, Albert *et al.*, 1992). They reported a high frequency of non-synonymous substitutions in this region, probably imposed by the host factors (immune system) or viral factors (diversification of viral tropism).

Mean nucleotide distances reported for more conserved regions (i.e. gag and pol) were lower than those found for env gene ($7.2 \pm 0.12\%$ for a group of gays from Edinburgh, Newcastle and Belfast and $3.3 \pm 0.29\%$ for haemophiliac individuals from Edinburgh (Leigh Brown *et al.*, 1997).

7.5 Strategies for AIDS vaccines focusing on the achievements obtained to date regarding prime-boost vaccines

Inactivated virus vaccines

Desrosiers *et al.* (1989) and Murphy-Corb *et al.* (1989) tested the first vaccines against SIV infection in macaques. These vaccines consisted of formalin inactivated whole SIV administered with the appropriate adjuvant, giving substantial protection 24 weeks after vaccination, with no evidence of infection detected even by PCR.

Subsequently, Stott *et al.* (1991) revealed that in vaccinated monkeys the antibody level correlated with protection, but the protective immunity was not virus-specific as protection of macaques was achieved by immunisation with uninfected cells alone. Later, Cranage *et al.* (1992) and Le Grand *et al.* (1992) have reported that monkeys vaccinated with inactivated SIV grown in human T cells were protected against challenge with SIV grown in human T cells but not against SIV grown in simian cells. Antibodies specific for the cells in which the virus used as a vaccine was cultivated gave protection seen in these macaques. They were elicited by the cell-surface molecules that co-purified with the virus or by those that became incorporated into the lipid bilayer of the virions, after budding from the membrane of the infected cells. Thus the protection was due to the response to xenoantigens (Chan *et al.*, 1995). Also the antibody response to xenoantigens in sera from vaccinated monkeys may induce complement-mediated virolysis of HIV and SIV (Spear *et al.*, 1993). As a result of these limitations, the study of efficacy of inactivated virus vaccines against AIDS was abandoned. However, the clearance of the SIV in the vaccinated monkeys might be due to the alloreactivity given by the normal activation of Th cells by the ALLO MHC molecules taken by the HIV virion after budding from the donor infected cells (Shearer, *et al.*, 1999). This was the basis of a suggestion for an allovaccine against HIV. Thus, approximately 40% of asymptomatic, HIV-1 infected individuals do not generate *in vitro* Th-cell responses to recall antigens such as influenza A virus and HIV envelope peptides but do respond to irradiated allogeneic peripheral blood mononuclear cells. The intact Th-cell function to

alloantigens could be used to drive a CD8⁺ T-effector-cell response specific for HIV-1 antigens by alloimmunisation.

Live attenuated virus vaccines

In 1991, Desrosiers *et al.* (1992) reported that an irreversible genetic disruption of the *nef* gene of the pathogenic clone SIV_{mac239} attenuated the course of infection of six rhesus macaques that remained clinically healthy for more than 3 years, despite the persistence of the virus. In the following year, Daniel *et al.* (1992) observed that macaques chronically infected with attenuated viruses were protected against challenge with wild-type SIV. Subsequent reports confirmed the protection from superinfection conferred by the immunised macaques. Thus, using an attenuated clone of SIV_{mac32H}, called C8, Almond *et al.* (1995) and Rud *et al.* (1994) induced protection of the macaques against superinfection with wild-type SIV presented as either cell-free virus or virus infected cells. Almond *et al.* (1995) also observed a protective immunity in monkeys infected with attenuated viruses, which was effective against a broad range of viruses, including SHIV chimeras carrying *env*, *tat* and *rev* genes of HIV-1. More precisely, eight cynomolgus macaques infected with attenuated SIV were challenged with cell-free and cell-associated SIV and they were protected against infection in comparison with the eight controls that were infected after challenge.

Thus, live attenuated virus vaccines fulfilled many of the criteria expected of an effective AIDS vaccine, although debates regarding safety issues have tempered the enthusiasm for using this approach as a prophylactic vaccine in humans. In this light, a study done by Deacon *et al.* (1995) on a cohort of Australian patients called Sydney Bloodbank cohort, infected from the same donor with a *nef*-defective virus, remained free of the HIV-1 related disease, with stable, normal CD4 lymphocyte counts for between 10 to 14 years postinfection. The cohort consisted of seven HIV-1 infected recipients of HIV-1 infected blood from the same donor (a homosexual male who became infected between December 1980 and April 1981) who have a stable CD4 cell count of more than 500/ μ l of blood for more than 10 years. In this time no member

developed any AIDS-defining condition or HIV-related symptoms or received any antiretroviral chemotherapy. The amount of HIV-1 DNA in the cohort members was very low, which is a characteristic feature for long-term non-progressors (LTNP) and ranged from <10 to 400 copies of HIV-1 DNA per 10^5 CD4 cells in the absence of therapy. Sequencing amplified fragments from the nef gene-LTR region indicated deletions in the nef-LTR region. However, recent evidence showed that LTNP infected with attenuated viruses manifested the disease after 14-16 years postinfection, which raises awareness of the safety issue of attenuated-virus vaccine. Firstly, this vaccine could initiate cancer through insertional mutagenesis of the provirus in the host cell genome with the activation of the oncogenes, as MMTV does in mice (Hardiman *et al.*, 1996, van Leeuwen *et al.*, 1995). Secondly, attenuated SIV_{mac} in adult macaques was capable of causing AIDS when transmitted orally to neonates (Baba *et al.* 1995). Thirdly, Whatmore *et al.* (1995) observed the regeneration of a complete nef gene from a 12-nucleotide nef gene deletion during the course of infection in macaques. However, if the revertant viruses were injected into a macaque infected with an attenuated virus, the animal remained resistant (Sharpe *et al.*, 1997). Clearly there is a limitation on this type of vaccine coming from the inability of the immunity elicited by live attenuated virus to eliminate or prevent the emergence and pathogenesis of revertant virus arising endogenously, despite the good protection against virulent strains introduced exogenously.

The mechanism by which live attenuated SIV induces such protection is still unknown, although there is evidence that the immunity elicited by infection with nef-deleted SIV might be CTL-based (Johnson *et al.*, 1997).

Subunit vaccine

Considerable efforts have been made to evaluate proteins as immunogens because of the greater safety of non-replicating molecules. Early studies performed by Berman *et al.* (1988) showed protection of chimpanzees vaccinated with recombinant gp120 against a

cell-free TCLA inoculum. The result was not convincing due to the fact that HIV isolates do not replicate to high levels in chimpanzees. Subsequent studies conducted by Mills *et al.* (1993) and Giavedoni *et al.* (1993) failed to show protection of the macaques induced by subunit vaccines comprising env and gag proteins. Also Mascola *et al.* (1996) showed that this vaccine from a cell-line-adapted strain does not elicit viral-specific CTL and does not generate antibody responses that can neutralize primary patient isolates of HIV-1.

So far, several different gp120 and gp160 vaccines have been tested in phase I and II clinical trials. More than 1000 individuals have been tested in phase I trials of gp120 and gp160 vaccines in USA. However, the shift of the project from phase I to phase II (efficacy) trial was postponed due to the finding that the neutralising antibody could inactivate TCLA-adapted viruses but not primary isolates (Mascola *et al.*, 1996). Thus, Connor *et al.*, (1998) reported a lack of protection of such vaccines in immunogenicity trials in a high-risk human population in USA, the immunity elicited by the vaccines did not exert selective pressure on the infecting virus. Moreover, several individuals who received these vaccines have subsequently been infected with HIV-1 (Kahn *et al.*, 1995). In spite of these results, vaccine trials with bivalent immunogens are currently proceeding in Thailand, the immunogens including env sequences typical for clade E viruses that have a high prevalence in this region.

In conclusion, the efficacy of subunit vaccines in stimulating an efficient neutralising response is impaired by the more occluded conformation of the oligomers on the surface of the primary isolates that consequently shields the neutralising epitopes (Bou Habib *et al.*, 1994). Nevertheless such immunogens may be useful in eliciting effective immune responses in combination with other vaccine strategies such as live vectors-based vaccines or DNA vaccines.

DNA vaccines

DNA vectors were successfully used to elicit immune responses able to confer protective immunity against influenza challenge in mice and ferrets. After intramuscular or intradermal injection by means of a gene gun, DNA vaccines are taken up by local cells that become antigen-presenting cells able to express viral protein-derived antigens. As a consequence, strong and persistent humoral and cellular immune responses are generated. The advantage of this vaccine consists of eliciting humoral and CTL activity without the pathogenic risk, which characterise the immunisation with live vectors.

DNA vaccines have been used both in primates and human trials. Thus, Wang *et al.* (1995a, b) obtained good protection of the cynomolgous macaques against a challenge with SIV_{mac}/HIV-MN env chimera after immunisation with DNA vaccines comprising *env* and *rev* from HIV-1MN. Three out of four monkeys cleared the virus in 60 days, whereas the fourth animal developed a viral load at the same level as the control animals. DNA vaccines were also used in chimpanzee by Boyer *et al.* (1996). Immunising chimpanzees with DNA containing MN env and gag/pol both humoral and cellular immune responses were elicited. In a subsequent study, Boyer *et al.* (1997) challenged two DNA vaccinated and one negative control chimpanzees with HIV-1 SF2. Both vaccinated chimpanzees were protected against HIV-1 SF2 inoculum: one of them developed a high antibody response, whereas the other had a higher CTL response. The type of the response that is elicited by DNA vaccines clearly varies from one chimp to the other.

The prophylactic effect of DNA vaccines was tested in HIV-1 infected chimpanzees by Boyer *et al.* (1997) who reported the clearance of the virus from the PBMC by DNA-PCR and viral culture assays and from plasma by RT-PCR after the third intramuscular vaccination with DNA encoding *env*, *rev* and *gag/pol*. The immunised animals developed both humoral and cellular immune responses. Subsequently, animals were challenged with a heterologous stock of HIV-1 SF2 virus and followed for 48 weeks

after challenge. Animals vaccinated with DNA constructs were protected from the establishment of infection, whereas RT-PCR results indicated infection in the control animals. Nowadays, DNA vaccines are currently in trials for both prophylactic and therapeutic purpose.

Live vector-based vaccines

These vaccines consist of recombinant organisms that are produced when genes encoding viral proteins are inserted into the genomes of other viruses or bacteria. By infecting experimental animals or humans with such vectors an immune response against the parental organism and the products of the inserted viral genes is elicited. First attempts to produce a live-vector-based vaccine were based on poxviruses as an HIV gene vector. Thus, Hu *et al.* (1991) reported the establishment of an AIDS virus-specific cellular and humoral immunity in macaques when immunising with vaccinia virus in which HIV genes were inserted. Moreover, subsequent studies recorded a good protection against infection by some SIV isolates conferred by immunising macaques with such constructs followed by boosting with recombinant proteins (Zolla-Pazner *et al.*, 1998a, b). However, there is a reluctance to use this vector system in large-scale human trials because of the life-threatening dissemination of the vaccinia infections in immunosuppressed humans. Therefore, later studies were focused on poxviruses with limited *in vivo* replicative capacity and limited pathogenic potential in humans. Thus, modified vaccinia ankara (MVA), a vaccinia strain attenuated by extensive passaging *in vitro* with deletions in certain genes associated with its pathogenicity has been used as a vector virus for SIV genes to immunise macaques (Hirsch *et al.*, 1996). Immunisation of 12 rhesus macaques with MVA carrying SIV-env and gag-pol genes showed a sustained suppression of the virus which promoted a long-term, asymptomatic survival of two immunised SIV-infected macaques, but did not confer sterilising immunity to any of the immunised macaques (Hirsch *et al.*, 1996).

Contrary to the MVA-based vaccines that were used as an HIV-1 vaccine in non-human primates, avian pox viruses (APV) have undergone extensive preclinical and early-phase human trial because avian pox viruses do not complete an entire replication cycle in human cells but they initiate protein synthesis and therefore, can elicit an immune response. Among APV, canarypox vectors encoding HIV env and gag have been used in different dosages and immunisation protocol. Ferrari *et al.* (1997a, b) reported a low titre env-and gag-specific antibodies elicited in 70% of vaccinees, with 30% of individuals developing an env or gag-specific CTL response, after repeated vaccination with both envelope and gag-expressing canarypox constructs. However, the responses elicited by this vaccine were not durable, some vaccinees showing only a sporadic CTL responses. Therefore, the poor consistency of HIV-1 specific CTL elicitation impairs testing of this vector strategy in large-scale human efficacy trial.

Other viruses have been used as vaccine vectors such as adenoviruses that are able to elicit mucosal immune response. Wang *et al.* (1997) revealed a low and non-effective immune response elicited by recombinant serotype 5 and 7 adenoviruses. Also, poliovirus and the alpha viruses (such as Semliki Forest Virus and Venezuelan Equine Encephalitis virus) are being evaluated as a potential HIV- vaccine vectors (Moldoveanu *et al.* 1995).

Recombinant bacteria have also been evaluated as HIV vaccine vectors. BCG (baccille Calmette-Guerin), a bacterium that is used to vaccinate against Mycobacterium tuberculosis infection was found to be an effective vaccine vector candidate because it establishes a chronic persistent infection and is safe in worldwide use. Winter *et al.* (1995) obtained rBCG strains expressing both regulatory (*nef*) and structural (*gag p26* and *env*) genes that were used to immunised Balb/c mice. Immune responses induced in the experimental animals involved strong cellular immune responses, both proliferative and cytotoxic T-cell responses, accompanied by a strong production of IFN- γ , indicator of a Th1 immune response. In addition, the elicitation of a strong humoral immune

response against Gag p26 and Env gp120 was reported by Lim *et al.* (1997). In their study, mice or guinea pigs were inoculated with rBCG strain expressing the N-terminal half of the SIV mac251 *env* gene, having the consequence the induction of serum IgGs against *env*, able to neutralise *in vitro* virulent SIVmac 251 strain. Enteric bacteria such as Salmonella and Shigella, which are able to elicit a strong mucosal immune responses were also investigated as HIV-vaccine vectors (Wu *et al.*, 1997). A summary of the viral vectors used for HIV vaccine trials are presented in Table 7.1. The main shortcoming of the live vector-based vaccines for therapeutic purpose resides in the direct correlation between the immunogenicity of that vector and the extent with which that vectors replicate *in vivo*. Thus, the most immunogenic of the live vector is usually the most pathogenic, especially for immunocompromised individuals. Also, pre-existing immunity or a vigorous anamnestic response make the use of these vectors problematic where significant proportion of the population have been exposed to the virus or bacterium, because the vector could be cleared by the host memory immunity before immune response to recombinant immunogens is induced.

Table 7.1 Viral vectors used for HIV vaccine trials

Vector	Immune response	Safety issue	Trials
Vaccinia virus	NA+CTL	Non safe in IC	humans
Modify vaccinia Ankara	NA+CTL	safe	monkeys
Avian pox viruses (canarypox vectors)	NA+CTL	safe	humans
BCG	NA+CTL+MI	-	mice
Salmonella	CMI	-	mice
AdV, AAV, HSV, VZV, SV40, IV, polio, SIN, VEE	Not tested	Not tested	conceptual

IC stands for immunocompromised individuals

Prime-boost regimens

Since 1994, interest in HIV vaccine research has been shifted towards mixed-modality vaccines called prime-boost regimens. Early mixed-modality prime-boost strategies consisted of immunisation with a live-recombinant virus vector to induce CTL activity and to prime for humoral responses, followed by a vaccine boost or boosts with recombinant subunit proteins (Pialoux *et al.* 1995, Fleury *et al.* 1996, Lubeck *et al.* 1997). Newer combinations that have been evaluated are naked DNA prime-recombinant protein boost, naked DNA prime-vaccinia boost and more recently DNA prime/MVA boost. First studies in this respect were conducted in mice and guinea pigs to evaluate a vaccine approach consisting of naked plasmid DNA encoding gp120 from HIV-1 SF2 and recombinant gp120 SF2 protein (rgp120 SF2) with MF 59 adjuvant (oil in water emulsion) (Barnett *et al.*, 1998). Results showed that the mixed modality DNA prime/protein boost regimen performed better than DNA/DNA or protein/protein alone. Importantly, gp120 DNA immunisation induced potent and persistent CTL responses in all animals tested, regardless of the fact that DNA was administered alone or in combination with a protein boost. Relatively low neutralising titres were seen following env DNA alone, which were substantially enhanced by subsequent immunisation with protein.

The function of the prime is to induce strong and persistent cell-mediated responses to CD4 and CD8 T cell epitopes and also a broad neutralisation response. Different DNA vectors comprising env, gag, pol, tat, nef and rev genes are currently under evaluation for their ability to prime the immune responses (Lubeck *et al.*, (1997), Barnett *et al.*, (1997), Fuller *et al.*, (1997a, b), Hanke *et al.*, (1998), Hinkula *et al.*, (1997). The main advantage conferred by priming with a DNA vaccine resides in their ability to elicit CTL responses to recognise similar peptides that have conserved amino acid residues at certain position. For instance, studies done by Ferrari *et al.*, (1997a), Betts *et al.*, (1997) and Cao *et al.*, (1997) to evaluate the CTL responses in volunteers receiving env-based

DNA vaccines and patients infected with clade B strains of HIV has demonstrated cross-clad recognition. This points towards the hope of achieving an international vaccine including env, gag and pol genes from more prevalent HIV-1 strains. Furthermore, results reported by Hu *et al.*, (1996) revealed that priming the animals with DNA encoding gp160 gene is superior to gp120 for protection of vaccinia primed/protein boosted animals.

The function of the boost is to enhance the immune responses to the primary immunisation through activation of specific T and B memory responses. Early studies done in mice showed that antibody responses were augmented when the animals were primed with recombinant vaccinia encoding env protein followed by a recombinant gp160 protein boost but were not increased in priming with protein first followed by vaccinia (Hu *et al.*, 1991). Recombinant protein boosts with nucleic acid as a prime was also a potent regimen for inducing neutralising antibody responses (Barnett *et al.*, 1997). DNA priming followed by MVA boosting has been shown to be effective for the induction of anti-malaria CTL responses in a mouse model, whereas other regimens such as: DNA alone, MVA alone or MVA as prime and DNA as boost were less effective (Schneider *et al.*, 1998). Finally, recombinant protein or vaccinia vaccines were effective in augmenting antibody responses when used to boost in macaques after gene-gun DNA immunisation (Fuller *et al.*, 1997a).

The most common boost regimen both in animals and human models used adjuvant-supplemented env gp120 and gp160 proteins from either HIV-1 or SIV strains expressed in baculovirus expression system, CHO cells or other mammalian cells (Barnett *et al.*, 1998). Significant results showed that boosting with either form of env is effective for the induction of neutralising antibody against TCLA viruses (Hu *et al.*, (1991), Graham *et al.*, (1993), Barnett *et al.*, (1997), Lubeck *et al.*, 1997, Hu *et al.*, (1996)). However, only few reports revealed the neutralising potential of such vaccine-induced antibodies to primary HIV-1 strains, especially, those with NSI phenotype which are

preponderantly transmitted in HIV-1 infection (Zolla-Pazner *et al.*, 1997, 1998a, b). This result may be explained by a different conformation adopted by the gp120/gp160 molecules on the TCLA isolates in comparison to primary isolates. However, the gp120 protein from TCLA strains SF2 was capable of eliciting neutralising antibody able to bind to the primary isolates (Mascola *et al.*, 1996). Currently, both monomeric gp120 and its oligomeric form (gp140 or gp160) derived from primary HIV-1 strains are evaluated as boost candidates for DNA prime recombinant glycoprotein vaccines (VanCott *et al.*, 1997, Mascola *et al.*, 1997).

Conclusion of this section:

The prime boost regimen elicits strong CTL and neutralising antibody response and it has been shown to protect macaques from challenge with SIV strains. Immune responses are elicited in circumstances where the risk of pathogenicity associated with the live-attenuated viruses and live-vector vaccines is eliminated. Furthermore, this regimen is virus-specific and long lasting due to the enhancement of the antibody responses by boosts with recombinant proteins.

Reference List

- Ahmad, A., A. Ladha, E.A. Cohen, and J. Menezes.** 1993. Stable expression of the transfected HIV-1 env gene in a human B cell line: characterization of gp120-expressing clones and immunobiological studies. *Virology* **192**:447-457.
- Aiken, C., J. Konner, N.R. Landau, M.E. Lenburg, and D. Trono.** 1994. Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain. *Cell* **76**:853-864.
- Albert, J., L. Franzen, M. Jansson, G. Scarlatti, P.K. Kataaha, E. Katabira, F. Mubiro, M. Rydaker, P. Rossi, and U. Pettersson.** 1992. Ugandan HIV-1 V3 loop sequences closely related to the U.S./European consensus. *Virology* **190**:674-681.
- Alkhatib, G., C. Combadiere, C.C. Broder, Y. Feng, P.E. Kennedy, P.M. Murphy, and E.A. Berger.** 1996. CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* **272**:1955-1958.
- Almond, N., K. Kent, M. Cranage, E. Rud, B. Clarke, and E.J. Stott.** 1995. Protection by attenuated simian immunodeficiency virus in macaques against challenge with virus-infected cells [see comments]. *Lancet* **345**:1342-1344.
- Andre, P., M. Groettrup, P. Klenerman, R. de Giuli, B.L. Booth, JR, V. Cerundolo, M. Bonneville, F. Jotereau, R.M. Zinkernagel, and V. Lotteau.** 1998. An inhibitor of HIV-1 protease modulates proteasome activity, antigen presentation, and T cell responses. *Proceedings of the National Academy of Sciences of the United States of America* **95**:13120-13124.
- Aruffo, A. and B. Seed.** 1987a. Molecular cloning of two CD7 (T-cell leukemia antigen) cDNAs by a COS cell expression system. *EMBO Journal* **6**:3313-3316.
- Aruffo, A. and B. Seed.** 1987b. Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system. *Proceedings of the National Academy of Sciences of the United States of America* **84**:8573-8577.
- Arya, S.K., C. Guo, S.F. Josephs, and F. Wong-Staal.** 1985. Trans-activator gene of human T-lymphotropic virus type III (HTLV-III). *Science* **229**:69-73.
- Asjo, B., L. Morfeldt-Manson, J. Albert, G. Biberfeld, A. Karlsson, K. Lidman, and E.M. Fenyo.** 1986. Replicative capacity of human immunodeficiency virus from patients with varying severity of HIV infection. *Lancet* **2**:660-662.
- Autran, B., G. Carcelain, T.S. Li, C. Blanc, D. Mathez, R. Tubiana, C. Katlama, P. Debre, and J. Leibowitch.** 1997. Positive effects of combined antiretroviral therapy on CD4+ T cell homeostasis and function in advanced HIV disease [see comments]. *Science* **277**:112-116.
- Baba, T.W., Y.S. Jeong, D. Pennick, R. Bronson, M.F. Greene, and R.M. Ruprecht.** 1995. Pathogenicity of live, attenuated SIV after mucosal infection of neonatal macaques [see comments]. *Science* **267**:1820-1825.

- Bachelierie, F., J. Alcamí, F. Arenzana-Seisdedos, Virelizier, and JL.** 1991. HIV enhancer activity perpetuated by NF-kappa B induction on infection of monocytes [see comments]. *Nature* **350**:709-712.
- Bachelez, H., F. Hadida, C. Parizot, B. Flageul, M. Kemula, L. Dubertret, P. Debree, and G. Gorochov.** 1998. Oligoclonal expansion of HIV-specific cytotoxic CD8 T lymphocytes in the skin of HIV-1-infected patients with cutaneous pseudolymphoma. *Journal of Clinical Investigation* **101**:2506-2516.
- Baeuerle, P.A. and D. Baltimore.** 1988. I kappa B: a specific inhibitor of the NF-kappa B transcription factor. *Science* **242**:540-546.
- Balfe, P., P. Simmonds, C.A. Ludlam, J.O. Bishop, and A.J. Brown .** 1990. Concurrent evolution of human immunodeficiency virus type 1 in patients infected from the same source: rate of sequence change and low frequency of inactivating mutations. *J. Virol.* **64**:6221-6233.
- Balzarini, J., A. Karlsson, A.M. Vandamme, M.J. Perez-Perez, H. Zhang, L. Vrang, B. Oberg, K. Backbro, T. Unge, and A. San-Felix.** 1993. Human immunodeficiency virus type 1 (HIV-1) strains selected for resistance against the HIV-1-specific [2',5'-bis-O-(tert-butylidimethylsilyl)-3'-spiro-5''-(4''-amino-1'',2''-oxathiole-2'',2''-dioxide)]-beta-D-pentofurano syl (TSAO) nucleoside analogues retain sensitivity to HIV-1-specific nonnucleoside inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **90**:6952-6956.
- Balzarini, J., J.P. Kleim, G. Riess, M.J. Camarasa, C. De, and A. Karlsson.** 1994. Sensitivity of (138 Glu-->Lys) mutated human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) to HIV-1-specific RT inhibitors. *Biochemical & Biophysical Research Communications* **201**:1305-1312.
- Barnett, S.W., S. Rajasekar, H. Legg, B. Doe, D.H. Fuller, J.R. Haynes, C.M. Walker, and K.S. Steimer.** 1997. Vaccination with HIV-1 gp120 DNA induces immune responses that are boosted by a recombinant gp120 protein subunit. *Vaccine* **15**:869-873.
- Barnett, S.W., J.M. Klinger, B. Doe, C.M. Walker, L. Hansen, A.M. Duliege, and F.M. Sinangil.** 1998. Prime-boost immunization strategies against HIV. [Review] [106 refs]. *AIDS Research & Human Retroviruses* **14 Suppl 3**:S299-S309
- Barouch, D.H., S. Santra, T.D. Steenbeke, X.X. Zheng, H.C. Perry, M. Davies, D.C. Freed, A. Craiu, T.B. Strom, J.W. Shiver, and N.L. Letvin.** 1998. Augmentation and suppression of immune responses to an HIV-1 DNA vaccine by plasmid cytokine/Ig administration. *Journal of Immunology* **161**:1875-1882.
- Barr, P.J., K.S. Steimer, E.A. Sabin, D. Parkes, C. George-Nascimento, J.C. Stephans, M.A. Powers, A. Gyenes, G.A. Van Nest, and E.T. Miller.** 1987. Antigenicity and immunogenicity of domains of the human immunodeficiency virus (HIV) envelope polypeptide expressed in the yeast *Saccharomyces cerevisiae*. *Vaccine* **5**:90-101.
- Barre-Sinoussi, F., J.C. Chermann, F. Rey, M.T. Nugeyre, S. Chamaret, J. Gruest, C. Dauguet, C. Axler-Blin, B. Vezinet, C. Rouzioux, W. Rozenbaum, and L.**

- Montagnier.** 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* **220**:868-871.
- Bazan, H.A., G. Alkhatib, C.C. Broder, and E.A. Berger.** 1998. Patterns of CCR5, CXCR4, and CCR3 usage by envelope glycoproteins from human immunodeficiency virus type 1 primary isolates. *J.Virol.* **72**:4485-4491.
- Benson, J., C. Chougnet, M. Robert-Guroff, D. Montefiori, P. Markham, G. Shearer, R.C. Gallo, M. Cranage, E. Paoletti, K. Limbach, D. Venzon, J. Tartaglia, and G. Franchini.** 1998. Recombinant vaccine-induced protection against the highly pathogenic Simian immunodeficiency virus SIV(mac251): Dependence on route of challenge exposure. *J.Virol.* **72**:4170-4182.
- Berger, E.A., R.W. Doms, E.M. Fenyo, B.T. Korber, D.R. Littman, J.P. Moore, Q.J. Sattentau, H. Schuitemaker, J. Sodroski, and R.A. Weiss.** 1998. A new classification for HIV-1 [letter]. *Nature* **391**:240
- Berman, P.W., J.E. Groopman, T. Gregory, P.R. Clapham, R.A. Weiss, R. Ferriani, L. Riddle, C. Shimasaki, C. Lucas, and L.A. Lasky.** 1988. Human immunodeficiency virus type 1 challenge of chimpanzees immunized with recombinant envelope glycoprotein gp120. *Proceedings of the National Academy of Sciences of the United States of America* **85**:5200-5204.
- Betts, M.R., J. Krowka, C. Santamaria, K. Balsamo, F. Gao, G. Mulundu, C. Luo, N. N'Gandu, H. Sheppard, B.H. Hahn, S. Allen, Frelinger, and JA.** 1997. Cross-clade human immunodeficiency virus (HIV)-specific cytotoxic T-lymphocyte responses in HIV-infected Zambians. *J.Virol.* **71**:8908-8911.
- Binley, J.M., H. Arshad, T.R. Fouts, and J.P. Moore.** 1997. An investigation of the high-avidity antibody response to glycoprotein 120 of human immunodeficiency virus type 1. *AIDS Research & Human Retroviruses* **13**:1007-1015.
- Binley, J.M., R. Wyatt, E. Desjardins, P.D. Kwong, Hendrickson, J.P. Moore, and J. Sodroski.** 1998. Analysis of the interaction of antibodies with a conserved enzymatically deglycosylated core of the HIV type 1 envelope glycoprotein 120. *AIDS Research & Human Retroviruses* **14**:191-198.
- Bird, C., J. Burke, P.A. Gleeson, and J. McCluskey.** 1990. Expression of human immunodeficiency virus 1 (HIV-1) envelope gene products transcribed from a heterologous promoter. Kinetics of HIV-1 envelope processing in transfected cells. *Journal of Biological Chemistry* **265**:19151-19157.
- Bird, C., P.A. Gleeson, A. Ramsay, P. Li, and J. McCluskey.** 1992. Stable expression of the human immunodeficiency virus type 1 envelope glycoprotein in transfected L cells. *AIDS Research & Human Retroviruses* **8**:1999-2009.
- Birnboim, H.C. and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7**:1513-1523.
- Bjorndal, A., H. Deng, M. Jansson, J.R. Fiore, C. Colognesi, A. Karlsson, J. Albert, G. Scarlatti, D.R. Littman, and E.M. Fenyo.** 1997. Coreceptor usage of primary human immunodeficiency virus type 1 isolates varies according to biological phenotype. *J.Virol.* **71**:7478-7487.

- Blanc, D., C. Patience, T.F. Schulz, R. Weiss, and B. Spire.** 1993. Transcomplementation of VIF- HIV-1 mutants in CEM cells suggests that VIF affects late steps of the viral life cycle. *Virology* **193**:186-192.
- Bleul, C.C., R.C. Fuhlbrigge, J.M. Casasnovas, A. Aiuti, and T.A. Springer.** 1996. A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1) [see comments]. *Journal of Experimental Medicine* **184**:1101-1109.
- Bonhoeffer, S., E.C. Holmes, and M.A. Nowak.** 1995. Causes of HIV diversity [letter]. *Nature* **376**:125
- Boris-Lawrie, K.A., J.N. Brady, and A. Kumar.** 1992. Sequences within the R region of the long terminal repeat activate basal transcription from the HIV-1 promoter. *Gene Expression* **2**:215-230.
- Borrow, P., H. Lewicki, X. Wei, M.S. Horwitz, N. Pfeffer, H. Meyers, J.A. Nelson, J.E. Gairin, B.H. Hahn, M.B. Oldstone, and G.M. Shaw.** 1997. Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus [see comments]. *Nature Medicine* **3**:205-211.
- Bou-Habib, D.C., G. Roderiquez, T. Oravec, P.W. Berman, P. Lusso, and M.A. Norcross.** 1994. Cryptic nature of envelope V3 region epitopes protects primary monocytotropic human immunodeficiency virus type 1 from antibody neutralization. *J.Virol.* **68**:6006-6013.
- Boudet, F., J.J. Lasarte, P. Sarobe, F. Borrás-Cuesta, and Theze .** 1996. Fine analysis of immunoreactivity of V3 peptides: antibodies specific for V3 domain of laboratory HIV type 1 strains recognize multiple V3 sequences synthesized from field HIV type 1 isolates. *AIDS Research & Human Retroviruses* **12**:1671-1679.
- Boyer, J.D., B. Wang, K.E. Ugen, M. Agadjanyan, A. Javadian, P. Frost, K. Dang, R.A. Carrano, R. Ciccarelli, L. Coney, W.V. Williams, Weiner, and DB.** 1996. In vivo protective anti-HIV immune responses in non-human primates through DNA immunization. *Journal of Medical Primatology* **25**:242-250.
- Boyer, J.D., K.E. Ugen, B. Wang, M. Agadjanyan, L. Gilbert, M.L. Bagarazzi, M. Chattergoon, P. Frost, A. Javadian, W.V. Williams, Refaeli, R.B. Ciccarelli, D. McCallus, L. Coney, and D.B. Weiner.** 1997. Protection of chimpanzees from high-dose heterologous HIV-1 challenge by DNA vaccination [see comments]. *Nature Medicine* **3**:526-532.
- Brander, C., K.E. Hartman, A.K. Trocha, N.G. Jones, R.P. Johnson, B. Korber, P. Wentworth, S.P. Buchbinder, S. Wolinsky, B.D. Walker, and S.A. Kalams.** 1998. Lack of strong immune selection pressure by the immunodominant, HLA-A*0201-restricted cytotoxic T lymphocyte response in chronic human immunodeficiency virus-1 infection. *Journal of Clinical Investigation* **101**:2559-2566.
- Brown, A.J., D. Lobidel, C.M. Wade, S. Rebus, A.N. Phillips, R.P. Brettler, A.J. France, C.S. Leen, J. McMenamin, A. McMillan, R.D. Maw, F. Mulcahy, J.R. Robertson, K.N. Sankar, G. Scott, R. Wyld, and J.F. Peutherer.** 1997.

- The molecular epidemiology of human immunodeficiency virus type 1 in six cities in Britain and Ireland. *Virology* **235**:166-177.
- Buonocore, L., T.G. Turi, B. Crise, and J.K. Rose.** 1994. Stimulation of heterologous protein degradation by the Vpu protein of HIV-1 requires the transmembrane and cytoplasmic domains of CD4. *Virology* **204**:482-486.
- Burton, D.R., C.F. Barbas, M.A. Persson, S. Koenig, Chanock, RM, and R.A. Lerner.** 1991. A large array of human monoclonal antibodies to type 1 human immunodeficiency virus from combinatorial libraries of asymptomatic seropositive individuals. *Proceedings of the National Academy of Sciences of the United States of America* **88**:10134-10137.
- Burton, D.R., J. Pyati, R. Koduri, S.J. Sharp, G.B. Thornton, P.W. Parren, L.S. Sawyer, R.M. Hendry, N. Dunlop, and P.L. Nara.** 1994. Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. *Science* **266**:1024-1027.
- Butters, T.D. and R.C. Hughes.** 1981a. Isolation and characterization of mosquito cell membrane glycoproteins. *Biochimica et Biophysica Acta* **640**:655-671.
- Butters, T.D., R.C. Hughes, and P. Vischer.** 1981b. Steps in the biosynthesis of mosquito cell membrane glycoproteins and the effects of tunicamycin. *Biochimica et Biophysica Acta* **640**:672-686.
- Calenda, V., P. Graber, J.F. Delamarter, and J.C. Chermann.** 1994. Involvement of HIV nef protein in abnormal hematopoiesis in AIDS: in vitro study on bone marrow progenitor cells. *European Journal of Haematology* **52**:103-107.
- Cao, H., P. Kanki, J.L. Sankale, A. Dieng-Sarr, G.P. Mazzara, S.A. Kalams, B. Korber, S. Mboup, and B.D. Walker.** 1997. Cytotoxic T-lymphocyte cross-reactivity among different human immunodeficiency virus type 1 clades: implications for vaccine development. *J.Virol.* **71**:8615-8623.
- Carr, J.K., M.O. Salminen, C. Koch, D. Gotte, A.W. Artenstein, P.A. Hegerich, D. St Louis, D.S. Burke, and F.E. McCutchan.** 1996. Full-length sequence and mosaic structure of a human immunodeficiency virus type 1 isolate from Thailand. *J.Virol.* **70**:5935-5943.
- Carr, J.K., M.O. Salminen, J. Albert, E. Sanders-Buell, Gotte, D.L. Birx, and F.E. McCutchan.** 1998. Full genome sequences of human immunodeficiency virus type 1 subtypes G and A/G intersubtype recombinants. *Virology* **247**:22-31.
- Carrington, M., G.W. Nelson, M.P. Martin, T. Kissner, D. Vlahov, J.J. Goedert, R. Kaslow, S. Buchbinder, K. Hoots, and S.J. O'Brien.** 1999. HLA and HIV-1: Heterozygote advantage and B*35-Cw*04 disadvantage. *Science* **283**:1748-1752.
- Cavert, W., D.W. Notermans, K. Staskus, S.W. Wietgreffe, M. Zupancic, K. Gebhard, K. Henry, Z.Q. Zhang, R. Mills, H. McDade, Schuwirth, CM, J. Goudsmit, S.A. Danner, and A.T. Haase.** 1997. Kinetics of response in lymphoid tissues to antiretroviral therapy of HIV-1 infection [see comments] [published erratum appears in *Science* 1997 May 30;276(5317):1321]. *Science* **276**:960-964.

- Chakrabarti, S., T. Mizukami, G. Franchini, and B. Moss.** 1990. Synthesis, oligomerization, and biological activity of the human immunodeficiency virus type 2 envelope glycoprotein expressed by a recombinant vaccinia virus. *Virology* **178**:134-142.
- Chan, D.C., D. Fass, J.M. Berger, and P.S. Kim.** 1997. Core structure of gp41 from the HIV envelope glycoprotein. *Cell* **89**:263-273.
- Chan, D.C., C.T. Chutkowski, and P.S. Kim.** 1998a. Evidence that a prominent cavity in the coiled coil of HIV type 1 gp41 is an attractive drug target. *Proceedings of the National Academy of Sciences of the United States of America* **95**:15613-15617.
- Chan, D.C. and P.S. Kim.** 1998b. HIV entry and its inhibition. *Cell* **93**:681-684.
- Chan, W.L., A. Rodgers, R.D. Hancock, F. Taffs, P. Kitchin, G. Farrar, and F.Y. Liew.** 1992. Protection in simian immunodeficiency virus-vaccinated monkeys correlates with anti-HLA class I antibody response. *J.Exp.Med.* **176**:1203-1207.
- Chan, W.L., A. Rodgers, C. Grief, N. Almond, S. Ellis, B. Flanagan, P. Silvera, J. Bootman, J. Stott, and K. Kent.** 1995. Immunization with class I human histocompatibility leukocyte antigen can protect macaques against challenge infection with SIVmac-32H. *AIDS* **9**:223-228.
- Chanda, P.K., R.J. Natuk, B.B. Mason, B.M. Bhat, L. Greenberg, S.K. Dheer, K.L. Molnar-Kimber, S. Mizutani, M.D. Lubeck, and A.R. Davis.** 1990. High level expression of the envelope glycoproteins of the human immunodeficiency virus type I in presence of rev gene using helper-independent adenovirus type 7 recombinants. *Virology* **175**:535-547.
- Chang, N.T., P.K. Chanda, A.D. Barone, S. McKinney, D.P. Rhodes, S.H. Tam, C.W. Shearman, J. Huang, T.W. Chang, R.C. Gallo, et, and al.** 1985. Expression in *Escherichia coli* of open reading frame gene segments of HTLV-III. *Science* **228**:93-96.
- Charneau, P., A.M. Borman, C. Quillent, D. Guetard, Chamaret, J. Cohen, G. Remy, L. Montagnier, and F. Clavel.** 1994. Isolation and envelope sequence of a highly divergent HIV-1 isolate: definition of a new HIV-1 group. *Virology* **205**:247-253.
- Cheingsong-Popov, R., S. Lister, D. Callow, P. Kaleebu, S. Beddows, and J. Weber.** 1994. Serotyping HIV type 1 by antibody binding to the V3 loop: relationship to viral genotype. WHO Network for HIV Isolation and Characterization. *AIDS Research & Human Retroviruses* **10**:1379-1386.
- Chen, M.Y., F. Maldarelli, M.K. Karczewski, R.L. Willey, and Strebel.** 1993. Human immunodeficiency virus type 1 Vpu protein induces degradation of CD4 in vitro: the cytoplasmic domain of CD4 contributes to Vpu sensitivity. *J.Virol.* **67**:3877-3884.
- Cheng-Mayer, C., D. Seto, M. Tateno, Levy, and JA.** 1988. Biologic features of HIV-1 that correlate with virulence in the host. *Science* **240**:80-82.
- Cheng-Mayer, C., C. Weiss, D. Seto, and J.A. Levy.** 1989. Isolates of human immunodeficiency virus type 1 from the brain may constitute a special group of

- the AIDS virus. Proceedings of the National Academy of Sciences of the United States of America **86**:8575-8579.
- Cheng-Mayer, C., M. Quiroga, J.W. Tung, Dina, and J.A. Levy.** 1990. Viral determinants of human immunodeficiency virus type 1 T-cell or macrophage tropism, cytopathogenicity, and CD4 antigen modulation. *J.Virol.* **64**:4390-4398.
- Chirmule, N., N. Oyaizu, C. Saxinger, and S. Pahwa.** 1994. Nef protein of HIV-1 has B-cell stimulatory activity. *AIDS* **8**:733-734.
- Choe, H., M. Farzan, Y. Sun, N. Sullivan, B. Rollins, Ponath, PD, L. Wu, C.R. Mackay, G. LaRosa, W. Newman, N. Gerard, C. Gerard, and Sodroski.** 1996. The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* **85**:1135-1148.
- Choe, H., M. Farzan, M. Konkel, K. Martin, Y. Sun, L. Marcon, M. Cayabyab, M. Berman, M.E. Dorf, N. Gerard, C. Gerard, and J. Sodroski.** 1998. The orphan seven-transmembrane receptor apj supports the entry of primary T-cell-line-tropic and dualtropic human immunodeficiency virus type 1. *J.Virol.* **72**:6113-6118.
- Chowers, M.Y., C.A. Spina, T.J. Kwah, N.J. Fitch, D.D. Richman, and J.C. Guatelli.** 1994. Optimal infectivity in vitro of human immunodeficiency virus type 1 requires an intact nef gene. *J.Virol.* **68**:2906-2914.
- Churcher, M.J., C. Lamont, F. Hamy, C. Dingwall, S.M. Green, A.D. Lowe, J.G. Butler, M.J. Gait, and J. Karn.** 1993. High affinity binding of TAR RNA by the human immunodeficiency virus type-1 tat protein requires base-pairs in the RNA stem and amino acid residues flanking the basic region. *Journal of Molecular Biology* **230**:90-110.
- Clark, S.J., M.S. Saag, W.D. Decker, S. Campbell-Hill, Roberson, JL, P.J. Veldkamp, J.C. Kappes, B.H. Hahn, and G.M. Shaw.** 1991. High titers of cytopathic virus in plasma of patients with symptomatic primary HIV-1 infection [see comments]. *New England Journal of Medicine* **324**:954-960.
- Classon, M., M. Henriksson, J. Sumegi, G. Klein, Hammaskjold, and ML.** 1987. Elevated c-myc expression facilitates the replication of SV40 DNA in human lymphoma cells. *Nature* **330**:272-274.
- Clavel, F., M. Guyader, D. Guetard, M. Salle, L. Montagnier, and M. Alizon.** 1986. Molecular cloning and polymorphism of the human immune deficiency virus type 2. *Nature* **324**:691-695.
- Clavel, F., M.D. Hoggan, R.L. Willey, K. Strebel, M.A. Martin, and R. Repaske.** 1989. Genetic recombination of human immunodeficiency virus. *J.Virol.* **63**:1455-1459.
- Cocchi, F., A.L. DeVico, A. Garzino-Demo, S.K. Arya, R.C. Gallo, and P. Lusso.** 1995. Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells [see comments]. *Science* **270**:1811-1815.

- Cochrane, A., R. Kramer, S. Ruben, J. Levine, and C.A. Rosen.** 1989. The human immunodeficiency virus rev protein is a nuclear phosphoprotein. *Virology* **171**:264-266.
- Cohen, E.A., E.F. Terwilliger, Y. Jalinoos, J. Proulx, Sodroski, JG, and W.A. Haseltine.** 1990. Identification of HIV-1 vpr product and function. *Journal of Acquired Immune Deficiency Syndromes* **3**:11-18.
- Collins, K.L., B.K. Chen, S.A. Kalams, B.D. Walker, and D. Baltimore.** 1998. HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. *Nature* **391**:397-401.
- Combadiere, C., S.K. Ahuja, H.L. Tiffany, and P.M. Murphy.** 1996. Cloning and functional expression of CC CKR5, a human monocyte CC chemokine receptor selective for MIP-1(alpha), MIP-1(beta), and RANTES. *Journal of Leukocyte Biology* **60**:147-152.
- Combadiere, C., J. Gao, H.L. Tiffany, and P.M. Murphy.** 1998a. Gene cloning, RNA distribution, and functional expression of mCX3CR1, a mouse chemotactic receptor for the CX3C chemokine fractalkine. *Biochemical & Biophysical Research Communications* **253**:728-732.
- Combadiere, C., K. Salzwedel, E.D. Smith, H.L. Tiffany, Berger, EA, and P.M. Murphy.** 1998b. Identification of CX3CR1. A chemotactic receptor for the human CX3C chemokine fractalkine and a fusion coreceptor for HIV-1. *Journal of Biological Chemistry* **273**:23799-23804.
- Conley, A.J., J.A. Kessler, II, L.J. Boots, P.M. McKenna, Schleif, WA, E.A. Emini, G.E. Mark, III, H. Katinger, E.K. Cobb, S.M. Luncford, S.R. Rouse, and K.K. Murthy.** 1996. The consequence of passive administration of an anti-human immunodeficiency virus type 1 neutralizing monoclonal antibody before challenge of chimpanzees with a primary virus isolate. *J.Virol.* **70**:6751-6758.
- Connor, R.I., B.T. Korber, B.S. Graham, B.H. Hahn, D.D. Ho, Walker, BD, A.U. Neumann, S.H. Vermund, J. Mestecky, S. Jackson, E. Fenamore, Y. Cao, F. Gao, S. Kalams, K.J. Kunstman, D. McDonald, N. McWilliams, A. Trkola, Moore, JP, and S.M. Wolinsky.** 1998. Immunological and virological analyses of persons infected by human immunodeficiency virus type 1 while participating in trials of recombinant gp120 subunit vaccines. *J.Virol.* **72**:1552-1576.
- Cook, K.S., G.J. Fisk, J. Hauber, N. Usman, T.J. Daly, Rusche, and JR.** 1991. Characterization of HIV-1 REV protein: binding stoichiometry and minimal RNA substrate. *Nucleic Acids Research* **19**:1577-1583.
- Copeland, T.D. and S. Oroszlan.** 1988. Genetic locus, primary structure, and chemical synthesis of human immunodeficiency virus protease. *Gene Analysis Techniques* **5**:109-115.
- Courcoul, M., C. Patience, F. Rey, D. Blanc, A. Harmache, J. Sire, R. Vigne, and B. Spire.** 1995. Peripheral blood mononuclear cells produce normal amounts of defective Vif- human immunodeficiency virus type 1 particles which are restricted for the preroetrotranscription steps. *J.Virol.* **69**:2068-2074.
- Cranage, M., J. Stott, K. Mills, T. Ashworth, F. Taffs, G. Farrar, L. Chan, M. Dennis, P. Putkonen, and G. Biberfeld.** 1992. Vaccine studies with the 32H

- reisolate of SIVmac251: an overview. [Review] [5 refs]. *AIDS Research & Human Retroviruses* **8**:1479-1481.
- Crowl, R., C. Seamans, P. Lomedico, and S. McAndrew.** 1985a. Versatile expression vectors for high-level synthesis of cloned gene products in *Escherichia coli* [published erratum appears in *Gene* 1986;45(1):117]. *Gene* **38**:31-38.
- Crowl, R., K. Ganguly, M. Gordon, R. Conroy, M. Schaber, R. Kramer, G. Shaw, F. Wong-Staal, and E.P. Reddy.** 1985b. HTLV-III env gene products synthesized in *E. coli* are recognized by antibodies present in the sera of AIDS patients. *Cell* **41**:979-986.
- Cullen, B.R., J. Hauber, K. Campbell, J.G. Sodroski, Haseltine, WA, and C.A. Rosen.** 1988. Subcellular localization of the human immunodeficiency virus trans-acting art gene product. *J.Virol.* **62**:2498-2501.
- Cullen, B.R.** 1991. Human immunodeficiency virus as a prototypic complex retrovirus. [Review] [20 refs]. *J.Virol.* **65**:1053-1056.
- Daar, E.S., T. Moudgil, R.D. Meyer, and D.D. Ho.** 1991. Transient high levels of viremia in patients with primary human immunodeficiency virus type 1 infection [see comments]. *New England Journal of Medicine* **324**:961-964.
- Daly, T.J., K.S. Cook, G.S. Gray, T.E. Maione, and J.R. Rusche.** 1989. Specific binding of HIV-1 recombinant Rev protein to the Rev-responsive element in vitro. *Nature* **342**:816-819.
- Daniel, M.D., F. Kirchhoff, S.C. Czajak, P.K. Sehgal, Desrosiers, and RC.** 1992. Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene [see comments]. *Science* **258**:1938-1941.
- Dayton, A.I., J.G. Sodroski, C.A. Rosen, W.C. Goh, and W.A. Haseltine.** 1986. The trans-activator gene of the human T cell lymphotropic virus type III is required for replication. *Cell* **44**:941-947.
- De Clercq, E.** 1994. New developments in the chemotherapy of lentivirus (human immunodeficiency virus) infections: sensitivity/resistance of HIV-1 to non-nucleoside HIV-1-specific inhibitors. [Review] [65 refs]. *Annals of the New York Academy of Sciences* **724**:438-456.
- Deacon, N.J., A. Tsykin, A. Solomon, K. Smith, M. Ludford, D.J. Hooker, D.A. McPhee, A.L. Greenway, A. Ellett, and C. Chatfield.** 1995. Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients [see comments]. *Science* **270**:988-991.
- Dean, M., M. Carrington, C. Winkler, G.A. Huttley, M.W. Smith, R. Allikmets, J.J. Goedert, S.P. Buchbinder, E. Vittinghoff, E. Gomperts, S. Donfield, D. Vlahov, R. Kaslow, A. Saah, C. Rinaldo, R. Detels, O'B, and SJ.** 1996. Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study [see comments] [published erratum appears in *Science* 1996 Nov 15;274(5290):1069]. *Science* **273**:1856-1862.
- Debouck, C., J.G. Gorniak, J.E. Strickler, T.D. Meek, Metcalf, BW, and M. Rosenberg.** 1987. Human immunodeficiency virus protease expressed in

- Escherichia coli* exhibits autoprocessing and specific maturation of the gag precursor. Proceedings of the National Academy of Sciences of the United States of America **84**:8903-8906.
- Debysers, Z., R. Pauwels, K. Andries, and E. De Clercq.** 1992a. Specific HIV-1 reverse transcriptase inhibitors. [Review] [17 refs]. *Journal of Enzyme Inhibition* **6**:47-53.
- Debysers, Z., K. De Vreese, R. Pauwels, N. Yamamoto, J. Anne, E. De Clercq, and J. Desmyter.** 1992b. Differential inhibitory effects of TIBO derivatives on different strains of simian immunodeficiency virus. *Journal of General Virology* **73**:1799-1804.
- Deng, H., R. Liu, W. Ellmeier, S. Choe, D. Unutmaz, Burkhardt, P. Di Marzio, S. Marmon, R.E. Sutton, C.M. Hill, C.B. Davis, S.C. Peiper, T.J. Schall, D.R. Littman, and N.R. Landau.** 1996. Identification of a major co-receptor for primary isolates of HIV-1 [see comments]. *Nature* **381**:661-666.
- Deng, H.K., D. Unutmaz, V.N. KewalRamani, and D.R. Littman.** 1997. Expression cloning of new receptors used by simian and human immunodeficiency viruses [see comments]. *Nature* **388**:296-300.
- Derse, D., M. Carvalho, R. Carroll, and B.M. Peterlin.** 1991. A minimal lentivirus Tat. *J.Virol.* **65**:7012-7015.
- Desrosiers, R.C., M.S. Wyand, T. Kodama, D.J. Ringler, Arthur, LO, P.K. Sehgal, N.L. Letvin, N.W. King, and M.D. Daniel.** 1989. Vaccine protection against simian immunodeficiency virus infection. Proceedings of the National Academy of Sciences of the United States of America **86**:6353-6357.
- Desrosiers, R.C.** 1992. HIV with multiple gene deletions as a live attenuated vaccine for AIDS. [Review] [69 refs]. *AIDS Research & Human Retroviruses* **8**:411-421.
- Deutsch, S. and A.E. Bussard.** 1972. Original antigenic sin at the cellular level. I. Antibodies produced by individual cells against cross-reacting haptens. *European Journal of Immunology* **2**:374-378.
- di, M., V. T.D. Copeland, DeVico, al, R. Rahman, S. Oroszlan, R.C. Gallo, and M.G. Sarngadharan.** 1986. Characterization of highly immunogenic p66/p51 as the reverse transcriptase of HTLV-III/LAV. *Science* **231**:1289-1291.
- Dittmar, M.T., G. Simmons, S. Hibbitts, M. O'Hare, S. Louisirirothanakul, S. Beddows, J. Weber, P.R. Clapham, and R.A. Weiss.** 1997. Langerhans cell tropism of human immunodeficiency virus type 1 subtype A through F isolates derived from different transmission groups. *J.Virol.* **71**:8008-8013.
- Doe, B., K.S. Steimer, and C.M. Walker.** 1994. Induction of HIV-1 envelope (gp120)-specific cytotoxic T lymphocyte responses in mice by recombinant CHO cell-derived gp120 is enhanced by enzymatic removal of N-linked glycans. *European Journal of Immunology* **24**:2369-2376.
- Domingo, E., D. Sabo, T. Taniguchi, and C. Weissmann.** 1978. Nucleotide sequence heterogeneity of an RNA phage population. *Cell* **13**:735-744.
- Donaldson, Y.K., J.E. Bell, E.C. Holmes, E.S. Hughes, H.K. Brown, and P. Simmonds.** 1994. In vivo distribution and cytopathology of variants of human

- immunodeficiency virus type 1 showing restricted sequence variability in the V3 loop. *J. Virol.* **68**:5991-6005.
- Doranz, B.J., J. Rucker, Y. Yi, R.J. Smyth, M. Samson, Peiper, SC, M. Parmentier, R.G. Collman, and R.W. Doms.** 1996. A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell* **85**:1149-1158.
- Dragic, T., V. Litwin, G.P. Allaway, S.R. Martin, Y. Huang, K.A. Nagashima, C. Cayanan, P.J. Maddon, R.A. Koup, J.P. Moore, and W.A. Paxton.** 1996. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5 [see comments]. *Nature* **381**:667-673.
- Drelich, M., M. Haenggi, and J. Mous.** 1993. Conserved residues Pro-109 and Asp-116 are required for interaction of the human immunodeficiency virus type 1 integrase protein with its viral DNA substrate. *J. Virol.* **67**:5041-5044.
- Dubay, J.W., S.J. Roberts, B. Brody, and E. Hunter.** 1992. Mutations in the leucine zipper of the human immunodeficiency virus type 1 transmembrane glycoprotein affect fusion and infectivity. *J. Virol.* **66**:4748-4756.
- Durali, D., J. Morvan, F. Letourneur, D. Schmitt, N. Guegan, M. Dalod, S. Saragosti, D. Sicard, J.-P. Levy, and E. Gomard.** 1998. Cross-reactions between the cytotoxic T-lymphocyte responses of human immunodeficiency virus-infected African and European patients. *J. Virol.* **72**:3547-3553.
- Eckert, D.M., V.N. Malashkevich, L.H. Hong, P.A. Carr, and P.S. Kim.** 1999. Inhibiting HIV-1 entry: Discovery of D-peptide inhibitors that target the gp41 coiled-coil pocket. *Cell* **99**:103-115.
- Eckert, K.A. and T.A. Kunkel.** 1990. High fidelity DNA synthesis by the *Thermus aquaticus* DNA polymerase. *Nucleic Acids Research* **18**:3739-3744.
- Edinger, A.L., T.L. Hoffman, M. Sharron, B. Lee, B. O'Dowd, and R.W. Doms.** 1998. Use of GPR1, GPR15, and STRL33 as coreceptors by diverse human immunodeficiency virus type 1 and simian immunodeficiency virus envelope proteins. *Virology* **249**:367-378.
- Emerman, M., R. Vazeux, and K. Peden.** 1989. The rev gene product of the human immunodeficiency virus affects envelope-specific RNA localization. *Cell* **57**:1155-1165.
- Emerman, M. and M.H. Malim.** 1998. HIV-1 regulatory/accessory genes: keys to unraveling viral and host cell biology. [Review] [41 refs]. *Science* **280**:1880-1884.
- Emini, E.A., W.A. Schleif, J.H. Nunberg, A.J. Conley, and Y. Eda .** 1992. Prevention of HIV-1 infection in chimpanzees by gp120 V3 domain-specific monoclonal antibody. *Nature* **355**:728-730.
- Engelman, A., F.D. Bushman, and R. Craigie.** 1993. Identification of discrete functional domains of HIV-1 integrase and their organization within an active multimeric complex. *EMBO Journal* **12**:3269-3275.
- Epstein, L.G., C. Kuiken, B.M. Blumberg, S. Hartman, L.R. Sharer, M. Clement, and J. Goudsmit.** 1991. HIV-1 V3 domain variation in brain and spleen of

- children with AIDS: tissue-specific evolution within host-determined quasispecies. *Virology* **180**:583-590.
- Essex, M., L.E. Soto-Ramirez, E. Renjifo, W.K. Wang, and T.H. Lee.** 1997. Genetic variation within human immunodeficiency viruses generates rapid changes in tropism, virulence, and transmission. *Leukemia* **11 Suppl 3**:93-94.
- Fang, G., B. Weiser, A. Visosky, T. Moran, and H. Burger.** 1999. PCR-mediated recombination: a general method applied to construct chimeric infectious molecular clones of plasma-derived HIV-1 RNA. *Nature Medicine* **5**:239-242.
- Farzan, M., H. Choe, K. Martin, L. Marcon, W. Hofmann, G. Karlsson, Y. Sun, P. Barrett, N. Marchand, N. Sullivan, N. Gerard, Gerard, and J. Sodroski.** 1997. Two orphan seven-transmembrane segment receptors which are expressed in CD4-positive cells support simian immunodeficiency virus infection. *Journal of Experimental Medicine* **186**:405-411.
- Fauci, A.S.** 1996. Host factors and the pathogenesis of HIV-induced disease. *Nature* **384**:529-534.
- Feinberg, M.B., R.F. Jarrett, A. Aldovini, R.C. Gallo, and F. Wong-Staal.** 1986. HTLV-III expression and production involve complex regulation at the levels of splicing and translation of viral RNA. *Cell* **46**:807-817.
- Felber, B.K., M. Hadzopoulou-Cladaras, C. Cladaras, Copeland, and G.N. Pavlakis.** 1989. rev protein of human immunodeficiency virus type 1 affects the stability and transport of the viral mRNA. *Proceedings of the National Academy of Sciences of the United States of America* **86**:1495-1499.
- Felgner, P.L., T.R. Gadek, M. Holm, R. Roman, H.W. Chan, M. Wenz, J.P. Northrop, G.M. Ringold, and M. Danielsen.** 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proceedings of the National Academy of Sciences of the United States of America* **84**:7413-7417.
- Feng, Y., C.C. Broder, P.E. Kennedy, and E.A. Berger.** 1996. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor [see comments]. *Science* **272**:872-877.
- Fenouillet, E., N. Blanes, A. Benjouad, and J.C. Gluckman.** 1995. Anti-V3 antibody reactivity correlates with clinical stage of HIV-1 infection and with serum neutralizing activity. *Clinical & Experimental Immunology* **99**:419-424.
- Fenyo, E.M., L. Morfeldt-Manson, F. Chiodi, B. Lind, von, A. Gegerfelt, J. Albert, E. Olausson, and B. Asjo.** 1988. Distinct replicative and cytopathic characteristics of human immunodeficiency virus isolates. *J.Virol.* **62**:4414-4419.
- Fenyo, E.M., J. Albert, and B. Asjo.** 1989. Replicative capacity, cytopathic effect and cell tropism of HIV. [Review] [85 refs]. *AIDS* **3 Suppl 1**:S5-12.
- Ferrari, G., W. Humphrey, M.J. McElrath, J.L. Excler, Duliege, AM, M.L. Clements, L.C. Corey, D.P. Bolognesi, and K.J. Weinhold.** 1997a. Clade B-based HIV-1 vaccines elicit cross-clade cytotoxic T lymphocyte reactivities in uninfected volunteers. *Proceedings of the National Academy of Sciences of the United States of America* **94**:1396-1401.

- Ferrari, G., C. Berend, J. Ottinger, R. Dodge, J. Bartlett, J. Toso, D. Moody, J. Tartaglia, W.I. Cox, E. Paoletti, and K.J. Weinhold.** 1997b. Replication-defective canarypox (ALVAC) vectors effectively activate anti-human immunodeficiency virus-1 cytotoxic T lymphocytes present in infected patients: implications for antigen-specific immunotherapy. *Blood* **90**:2406-2416.
- Fisher, A.G., M.B. Feinberg, S.F. Josephs, M.E. Harper, Marselle, LM, G. Reyes, M.A. Gonda, A. Aldovini, C. Debouk, and R.C. Gallo.** 1986a. The trans-activator gene of HTLV-III is essential for virus replication. *Nature* **320**:367-371.
- Fisher, A.G., L. Ratner, H. Mitsuya, L.M. Marselle, M.E. Harper, S. Broder, R.C. Gallo, and F. Wong-Staal.** 1986b. Infectious mutants of HTLV-III with changes in the 3' region and markedly reduced cytopathic effects. *Science* **233**:655-659.
- Fleury, B., G. Janvier, G. Pialoux, F. Buseyne, M.N. Robertson, J. Tartaglia, E. Paoletti, M.P. Kieny, J.L. Excler, and Y. Riviere.** 1996. Memory cytotoxic T lymphocyte responses in human immunodeficiency virus type 1 (HIV-1)-negative volunteers immunized with a recombinant canarypox expressing gp 160 of HIV-1 and boosted with a recombinant gp160. *J.Infect.Dis.* **174**:734-738.
- Fouchier, R.A., M. Groenink, N.A. Kootstra, M. Tersmette, H.G. Huisman, F. Miedema, and H. Schuitemaker.** 1992. Phenotype-associated sequence variation in the third variable domain of the human immunodeficiency virus type 1 gp120 molecule. *J.Virol.* **66**:3183-3187.
- Franke, E.K., H.E. Yuan, and J. Luban.** 1994. Specific incorporation of cyclophilin A into HIV-1 virions [see comments]. *Nature* **372**:359-362.
- Freed, E.O., D.J. Myers, and R. Risser.** 1990. Characterization of the fusion domain of the human immunodeficiency virus type 1 envelope glycoprotein gp41. *Proceedings of the National Academy of Sciences of the United States of America* **87**:4650-4654.
- Freed, E.O. and M.A. Martin.** 1996. Domains of the human immunodeficiency virus type 1 matrix and gp41 cytoplasmic tail required for envelope incorporation into virions. *J.Virol.* **70**:341-351.
- Fuller, D.H., L. Simpson, K.S. Cole, J.E. Clements, D.L. Panicali, R.C. Montelaro, M. Murphey-Corb, and J.R. Haynes.** 1997a. Gene gun-based nucleic acid immunization alone or in combination with recombinant vaccinia vectors suppresses virus burden in rhesus macaques challenged with a heterologous SIV. *Immunology & Cell Biology* **75**:389-396.
- Fuller, D.H., M.M. Corb, S. Barnett, K. Steimer, and J.R. Haynes .** 1997b. Enhancement of immunodeficiency virus-specific immune responses in DNA-immunized rhesus macaques. *Vaccine* **15**:924-926.
- Galai, N., A. Kalinkovich, R. Burstein, D. Vlahov, and Bentwich.** 1997. African HIV-1 subtype C and rate of progression among Ethiopian immigrants in Israel [8]. *Lancet* **349**:180-181.
- Gallo, R.C., P.S. Sarin, E.P. Gelmann, M. Robert-Guroff, E. Richardson, V.S. Kalyanaraman, D. Mann, G.D. Sidhu, R.E. Stahl, P. Zolla, J. Leibowitch,**

- and M. Popovic.** 1983. Isolation of human T-cell leukemia virus in acquired immune deficiency syndrome (AIDS). *Science* **220**:865-867.
- Gao, F., D.L. Robertson, S.G. Morrison, H. Hui, S. Craig, J. Decker, P.N. Fultz, M. Girard, G.M. Shaw, B.H. Hahn, and P.M. Sharp.** 1996. The heterosexual human immunodeficiency virus type 1 epidemic in Thailand is caused by an intersubtype (A/E) recombinant of African origin. *J. Virol.* **70**:7013-7029.
- Gao, F., D.L. Robertson, C.D. Carruthers, Y. Li, E. Bailes, L.G. Kostrikis, M.O. Salminen, F. Bibollet-Ruche, M. Peeters, D.D. Ho, G.M. Shaw, P.M. Sharp, and B.H. Hahn.** 1998. An isolate of human immunodeficiency virus type 1 originally classified as subtype I represents a complex mosaic comprising three different group M subtypes (A, G, and I). *J. Virol.* **72**:10234-10241.
- Garcia, J.A., F.K. Wu, R. Mitsuyasu, and R.B. Gaynor.** 1987. Interactions of cellular proteins involved in the transcriptional regulation of the human immunodeficiency virus. *EMBO Journal* **6**:3761-3770.
- Garcia, J.V. and A.D. Miller.** 1991. Serine phosphorylation-independent downregulation of cell-surface CD4 by nef. *Nature* **350**:508-511.
- Gatignol, A., A. Kumar, A. Rabson, and K.T. Jeang.** 1989. Identification of cellular proteins that bind to the human immunodeficiency virus type 1 trans-activation-responsive TAR element RNA. *Proceedings of the National Academy of Sciences of the United States of America* **86**:7828-7832.
- Gatignol, A., A. Buckler-White, B. Berkhout, and K.T. Jeang.** 1991. Characterization of a human TAR RNA-binding protein that activates the HIV-1 LTR. *Science* **251**:1597-1600.
- Gendelman, H.E., L.M. Baca, H. Husayni, J.A. Turpin, D. Skillman, D.C. Kalter, J.M. Orenstein, D.L. Hoover, and M.S. Meltzer.** 1990. Macrophage-HIV interaction: viral isolation and target cell tropism. *AIDS* **4**:221-228.
- Gething, M.J. and J. Sambrook.** 1981. Cell-surface expression of influenza haemagglutinin from a cloned DNA copy of the RNA gene. *Nature* **293**:620-625.
- Gething, M.J. and J. Sambrook.** 1982. Construction of influenza haemagglutinin genes that code for intracellular and secreted forms of the protein. *Nature* **300**:598-603.
- Ghosh, P.K., P. Lebowitz, R.J. Frisque, and Y. Gluzman.** 1981. Identification of a promoter component involved in positioning the 5' termini of simian virus 40 early mRNAs. *Proceedings of the National Academy of Sciences of the United States of America* **78**:100-104.
- Giavedoni, L.D., V. Planelles, N.L. Haigwood, S. Ahmad, Kluge, JD, M.L. Marthas, M.B. Gardner, P.A. Luciw, and T.D. Yilma.** 1993. Immune response of rhesus macaques to recombinant simian immunodeficiency virus gp130 does not protect from challenge infection. *J. Virol.* **67**:577-583.
- Gibbs, J.S., A.A. Lackner, S.M. Lang, M.A. Simon, P.K. Sehgal, M.D. Daniel, and R.C. Desrosiers.** 1995. Progression to AIDS in the absence of a gene for vpr or vpx. *J. Virol.* **69**:2378-2383.

- Gilbert, M., J. Kiriwara, and J. Mills.** 1991. Enzyme-linked immunoassay for human immunodeficiency virus type 1 envelope glycoprotein 120. *Journal of Clinical Microbiology* **29**:142-147.
- Goncalves, J., P. Jallepalli, and D.H. Gabuzda.** 1994. Subcellular localization of the Vif protein of human immunodeficiency virus type 1. *J.Virol.* **68**:704-712.
- Goodenow, M., T. Huet, W. Saurin, S. Kwok, J. Sninsky, and S. Wain-Hobson.** 1989. HIV-1 isolates are rapidly evolving quasispecies: evidence for viral mixtures and preferred nucleotide substitutions. *Journal of Acquired Immune Deficiency Syndromes* **2**:344-352.
- Goulder, P., C. Conlon, K. McIntyre, and A. McMichael.** 1996. Identification of a novel human leukocyte antigen A26-restricted epitope in a conserved region of Gag [letter]. *AIDS* **10**:1441-1443.
- Goulder, P., D. Price, M. Nowak, S. Rowland-Jones, Phillips, and A. McMichael.** 1997a. Co-evolution of human immunodeficiency virus and cytotoxic T-lymphocyte responses. *Immunological Reviews* **159**:
- Goulder, P.J., R.E. Phillips, R.A. Colbert, S. McAdam, G. Ogg, M.A. Nowak, P. Giangrande, G. Luzzi, B. Morgan, A. Edwards, A.J. McMichael, and S. Rowland-Jones.** 1997b. Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. *Nature Medicine* **3**:212-217.
- Graham, B.S., T.J. Matthews, R.B. Belshe, M.L. Clements, R. Dolin, P.F. Wright, G.J. Gorse, D.H. Schwartz, M.C. Keefer, and D.P. Bolognesi.** 1993. Augmentation of human immunodeficiency virus type 1 neutralizing antibody by priming with gp160 recombinant vaccinia and boosting with rgp160 in vaccinia-naive adults. The NIAID AIDS Vaccine Clinical Trials Network. *J.Infect.Dis.* **167**:533-537.
- Gray, C.M., J. Lawrence, J.M. Schapiro, J.D. Altman, M.A. Winters, M. Crompton, M. Loi, S.K. Kundu, M.M. Davis, and T.C. Merigan.** 1999. Frequency of class I HLA-restricted anti-HIV CD8+ T cells in individuals receiving highly active antiretroviral therapy (HAART). *Journal of Immunology* **162**:1780-1788.
- Guo, H.G., F.M. Veronese, E. Tschachler, R. Pal, V.S. Kalyanaraman, R.C. Gallo, and M.S. Reitz, Jr.** 1990. Characterization of an HIV-1 point mutant blocked in envelope glycoprotein cleavage. *Virology* **174**:217-224.
- Gurtler, L.G., P.H. Hauser, J. Eberle, A. von Brunn, S. Knapp, L. Zekeng, J.M. Tsague, and L. Kaptue.** 1994. A new subtype of human immunodeficiency virus type 1 (MVP-5180) from Cameroon. *J.Virol.* **68**:1581-1585.
- Haase, A.T.** 1999. Population biology of HIV-1 infection: viral and CD4+ T cell demographics and dynamics in lymphatic tissues. [Review] [158 refs]. *Annual Review of Immunology* **17**:625-656.
- Hadzopoulou-Cladaras, M., B.K. Felber, C. Cladaras, A. Athanassopoulos, A. Tse, and G.N. Pavlakis.** 1989. The rev (trs/art) protein of human immunodeficiency virus type 1 affects viral mRNA and protein expression via a cis-acting sequence in the env region. *J.Virol.* **63**:1265-1274.

- Hammarskjold, M.L., S.C. Wang, and G. Klein.** 1986. High-level expression of the Epstein-Barr virus EBNA1 protein in CV1 cells and human lymphoid cells using a SV40 late replacement vector. *Gene* **43**:41-50.
- Han, X.M., A. Laras, M.P. Rounseville, A. Kumar, and P.R. Shank.** 1992. Human immunodeficiency virus type 1 Tat-mediated trans activation correlates with the phosphorylation state of a cellular TAR RNA stem-binding factor. *J.Virol.* **66**:4065-4072.
- Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. *Journal of Molecular Biology* **166**:557-580.
- Hanahan, D., J. Jessee, and F.R. Bloom.** 1991. Plasmid transformation of *Escherichia coli* and other bacteria. [Review] [83 refs]. *Methods in Enzymology* **204**:63-113.
- Hanke, T., T.J. Blanchard, J. Schneider, C.M. Hannan, M. Becker, S.C. Gilbert, A.V. Hill, G.L. Smith, and A. McMichael.** 1998. Enhancement of MHC class I-restricted peptide-specific T cell induction by a DNA prime/MVA boost vaccination regime. *Vaccine* **16**:439-445.
- Hardiman, G., S. Albright, J. Tsunoda, T. McClanahan, and F. Lee .** 1996. The mouse Wnt-10B gene isolated from helper T cells is widely expressed and a possible oncogene in BR6 mouse mammary tumorigenesis. *Gene* **172**:199-205.
- Hariharan, K., P.L. Nara, V.M. Caralli, F.L. Norton, N. Haigwood, and C.Y. Kang.** 1993. Analysis of the cross-reactive anti-gp120 antibody population in human immunodeficiency virus-infected asymptomatic individuals. *J.Virol.* **67**:953-960.
- Harouse, J.M. and F. Gonzalez-Scarano.** 1996. Infection of SK-N-MC cells, a CD4-negative neuroblastoma cell line, with primary human immunodeficiency virus type 1 isolates. *J.Virol.* **70**:7290-7294.
- Harper, M.E., L.M. Marselle, R.C. Gallo, and F. Wong-Staal.** 1986. Detection of lymphocytes expressing human T-lymphotropic virus type III in lymph nodes and peripheral blood from infected individuals by in situ hybridization. *Proceedings of the National Academy of Sciences of the United States of America* **83**:772-776.
- Harrer, T., E. Harrer, S.A. Kalams, T. Elbeik, S.I. Staprans, M.B. Feinberg, Y. Cao, D.D. Ho, T. Yilma, A.M. Caliendo, Johnson, RP, S.P. Buchbinder, and B.D. Walker.** 1996. Strong cytotoxic T cell and weak neutralizing antibody responses in a subset of persons with stable nonprogressing HIV type 1 infection. *AIDS Research & Human Retroviruses* **12**:585-592.
- Harrich, D., J. Garcia, F. Wu, R. Mitsuyasu, J. Gonazalez, and R. Gaynor.** 1989. Role of SP1-binding domains in in vivo transcriptional regulation of the human immunodeficiency virus type 1 long terminal repeat. *J.Virol.* **63**:2585-2591.
- Hauber, J., M.H. Malim, and B.R. Cullen.** 1989. Mutational analysis of the conserved basic domain of human immunodeficiency virus tat protein. *J.Virol.* **63**:1181-1187.
- Heaphy, S., J.T. Finch, M.J. Gait, J. Karn, and M. Singh.** 1991. Human immunodeficiency virus type 1 regulator of virion expression, rev, forms nucleoprotein filaments after binding to a purine-rich "bubble" located within the

- rev-responsive region of viral mRNAs. *Proceedings of the National Academy of Sciences of the United States of America* **88**:7366-7370.
- Heinzinger, N.K., M.I. Bukinsky, S.A. Haggerty, A.M. Ragland, V. Kewalramani, M.A. Lee, H.E. Gendelman, L. Ratner, M. Stevenson, and M. Emerman.** 1994. The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. *Proceedings of the National Academy of Sciences of the United States of America* **91**:7311-7315.
- Hinkula, J., M. Gidlund, C. Persson, A. Osterhaus, and B. Wahren .** 1994. Enzyme immunoassay (ELISA) for the evaluation of antibodies directed to the CD4 receptor-binding site of the HIV gp120 molecule. *Journal of Immunological Methods* **175**:37-46.
- Hinkula, J., P. Lundholm, and B. Wahren.** 1997. Nucleic acid vaccination with HIV regulatory genes: a combination of HIV-1 genes in separate plasmids induces strong immune responses. *Vaccine* **15**:874-878.
- Hirsch, V.M., R.A. Olmsted, M. Murphey-Corb, R.H. Purcell, and P.R. Johnson.** 1989. An African primate lentivirus (SIVsm) closely related to HIV-2. *Nature* **339**:389-392.
- Hirsch, V.M., T.R. Fuerst, G. Sutter, M.W. Carroll, L.C. Yang, S. Goldstein, M.J. Piatak, W.R. Elkins, W.G. Alvord, D.C. Montefiori, B. Moss, and J.D. Lifson.** 1996. Patterns of viral replication correlate with outcome in simian immunodeficiency virus (SIV)-infected macaques: effect of prior immunization with a trivalent SIV vaccine in modified vaccinia virus Ankara. *J.Virol.* **70**:3741-3752.
- Ho, D.D., M.G. Sarngadharan, L. Resnick, F. Dimarzo-veronese, T.R. Rota, Hirsch, and MS.** 1985. Primary human T-lymphotropic virus type III infection. *Annals of Internal Medicine* **103**:880-883.
- Ho, D.D., A.U. Neumann, A.S. Perelson, W. Chen, J.M. Leonard, and M. Markowitz.** 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection [see comments]. *Nature* **373**:123-126.
- Holland, S.M., N. Ahmad, R.K. Maitra, P. Wingfield, and Venkatesan.** 1990. Human immunodeficiency virus rev protein recognizes a target sequence in rev-responsive element RNA within the context of RNA secondary structure [published erratum appears in *J Virol* 1992 Feb;66(2):1288]. *J.Virol.* **64**:5966-5975.
- Holland, S.M., M. Chavez, S. Gerstberger, and S. Venkatesan.** 1992. A specific sequence with a bulged guanosine residue(s) in a stem-bulge-stem structure of Rev-responsive element RNA is required for trans activation by human immunodeficiency virus type 1 Rev. *J.Virol.* **66**:3699-3706.
- Holmes, E.C., L.Q. Zhang, P. Simmonds, C.A. Ludlam, and A.J. Brown.** 1992. Convergent and divergent sequence evolution in the surface envelope glycoprotein of human immunodeficiency virus type 1 within a single infected patient. *Proceedings of the National Academy of Sciences of the United States of America* **89**:4835-4839.

- Holmes, E.C., L.Q. Zhang, P. Robertson, A. Cleland, E. Harvey, P. Simmonds, and A.J. Leigh Brown.** 1995. The molecular epidemiology of HIV-1 in Edinburgh, Scotland. *J.Infect.Dis.* **171**:45-53.
- Hsieh, P. and P.W. Robbins.** 1984. Regulation of asparagine-linked oligosaccharide processing. Oligosaccharide processing in *Aedes albopictus* mosquito cells. *Journal of Biological Chemistry* **259**:2375-2382.
- Hu, S.L., S.G. Kosowski, and J.M. Dalrymple.** 1986. Expression of AIDS virus envelope gene in recombinant vaccinia viruses. *Nature* **320**:537-540.
- Hu, S.I., S.G. Kosowski, and K.F. Schaaf.** 1987. Expression of envelope glycoproteins of human immunodeficiency virus by an insect virus vector. *J.Virol.* **61**:3617-3620.
- Hu, S.L., J. Klaniecki, T. Dykers, P. Sridhar, and B.M. Travis.** 1991. Neutralizing antibodies against HIV-1 BRU and SF2 isolates generated in mice immunized with recombinant vaccinia virus expressing HIV-1 (BRU) envelope glycoproteins and boosted with homologous gp160. *AIDS Research & Human Retroviruses* **7**:615-620.
- Hu, S.L., P. Polacino, V. Stallard, J. Klaniecki, S. Pennathur, B.M. Travis, L. Misher, H. Kornas, A.J. Langlois, W.R. Morton, and R.E. Benveniste.** 1996. Recombinant subunit vaccines as an approach to study correlates of protection against primate lentivirus infection. *Immunology Letters* **51**:115-119.
- Hunter, E. and R. Swanstrom.** 1990. Retrovirus envelope glycoproteins. [Review] [478 refs]. *Current Topics in Microbiology & Immunology* **157**:187-253.
- Ivey-Hoyle, M. and M. Rosenberg.** 1990. Rev-dependent expression of human immunodeficiency virus type 1 gp160 in *Drosophila melanogaster* cells. *Molecular & Cellular Biology* **10**:6152-6159.
- Ivey-Hoyle, M., R.K. Clark, and M. Rosenberg.** 1991. The N-terminal 31 amino acids of human immunodeficiency virus type 1 envelope protein gp120 contain a potential gp41 contact site. *J.Virol.* **65**:2682-2685.
- Jacobo-Molina, A., J. Ding, R.G. Nanni, Clark, A.D. Jr, X. Lu, C. Tantillo, R.L. Williams, G. Kamer, A.L. Ferris, P. Clark, et, and al.** 1993. Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 A resolution shows bent DNA. *Proceedings of the National Academy of Sciences of the United States of America* **90**:6320-6324.
- Jansson, M., B. Wahren, J. Albert, G. Scarlatti, L. Franzen, P.K. Kataaha, E. Katabira, F. Mubiro, H. Wigzell, and P. Rossi.** 1994. Peptide serology for analysis of the inter- and intra-individual variation in the HIV-1 V3 domain. *AIDS* **8**:413-421.
- Javaherian, K., A.J. Langlois, C. McDanal, K.L. Ross, Eckler, LI, C.L. Jellis, A.T. Profy, J.R. Rusche, D.P. Bolognesi, and S.D. Putney.** 1989. Principal neutralizing domain of the human immunodeficiency virus type 1 envelope protein. *Proceedings of the National Academy of Sciences of the United States of America* **86**:6768-6772.
- Jin, X., D.E. Bauer, S.E. Tuttleton, S. Lewin, A. Gettie, J. Blanchard, C.E. Irwin, J.T. Safrit, J. Mittler, L. Weinberger, L.G. Kostrikis, L. Zhang, A.S.**

- Perelson, and D.D. Ho.** 1999. Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. *Journal of Experimental Medicine* **189**:991-998.
- Johnson, R.P., R.L. Glickman, J.Q. Yang, A. Kaur, J.T. Dion, M.J. Mulligan, and R.C. Desrosiers.** 1997. Induction of vigorous cytotoxic T-lymphocyte responses by live attenuated simian immunodeficiency virus. *J.Virol.* **71**:7711-7718.
- Kahn, J.O., K.S. Steimer, J. Baenziger, A.M. Duliege, Feinberg, T. Elbeik, M. Chesney, N. Murcar, D. Chernoff, and F. Sinangil.** 1995. Clinical, immunologic, and virologic observations related to human immunodeficiency virus (HIV) type 1 infection in a volunteer in an HIV-1 vaccine clinical trial [see comments]. *J.Infect.Dis.* **171**:1343-1347.
- Kalland, K.H., A.M. Szilvay, K.A. Brokstad, W. Saetrevik, and G. Haukenes.** 1994a. The human immunodeficiency virus type 1 Rev protein shuttles between the cytoplasm and nuclear compartments. *Molecular & Cellular Biology* **14**:7436-7444.
- Kalland, K.H., A.M. Szilvay, E. Langhoff, and G. Haukenes.** 1994b. Subcellular distribution of human immunodeficiency virus type 1 Rev and colocalization of Rev with RNA splicing factors in a speckled pattern in the nucleoplasm. *J.Virol.* **68**:1475-1485.
- Kameoka, M., Y. Nishino, K. Matsuo, N. Ohara, T. Kimura, A. Yamazaki, Yamada, and K. Ikuta.** 1994. Cytotoxic T lymphocyte response in mice induced by a recombinant BCG vaccination which produces an extracellular alpha antigen that fused with the human immunodeficiency virus type 1 envelope immunodominant domain in the V3 loop. *Vaccine* **12**:153-158.
- Kamoun, P.P.** 1988. Denaturation of globular proteins by urea: breakdown of hydrogen or hydrophobic bonds? *Trends in Biochemical Sciences* **13**:424-425.
- Kan, N.C., G. Franchini, F. Wong-Staal, G.C. DuBois, W.G. Robey, J.A. Lautenberger, and T.S. Papas.** 1986. Identification of HTLV-III/LAV sor gene product and detection of antibodies in human sera. *Science* **231**:1553-1555.
- Kanki, P.J., D.J. Hamel, J.-L. Sankale, C.-C. Hsieh, I. Thior, F. Barin, S.A. Woodcock, A. Gueye-Ndiaye, E. Zhang, M. Montano, T. Siby, R. Marlink, I. Ndoye, M.E. Essex, and S. Mboup.** 1999. Human immunodeficiency virus type 1 subtypes differ in disease progression. *J.Infect.Dis.* **179**:68-73.
- Karacostas, V., E.J. Wolffe, K. Nagashima, M.A. Gonda, and B. Moss.** 1993. Overexpression of the HIV-1 gag-pol polyprotein results in intracellular activation of HIV-1 protease and inhibition of assembly and budding of virus-like particles. *Virology* **193**:661-671.
- Kaslow, R.A., R. Duquesnoy, M. VanRaden, L. Kingsley, Marrari, H. Friedman, S. Su, A.J. Saah, R. Detels, J. Phair, and C. Rinaldo.** 1990. A1, Cw7, B8, DR3 HLA antigen combination associated with rapid decline of T-helper lymphocytes in HIV-1 infection. *Lancet* **335**:927-930.
- Kaslow, R.A., M. Carrington, R. Apple, L. Park, A. Munoz, Saah, AJ, J.J. Goedert, C. Winkler, S.J. O'Brien, C. Rinaldo, R. Detels, W. Blattner, J. Phair, H. Erlich, and D.L. Mann.** 1996. Influence of combinations of human major

- histocompatibility complex genes on the course of HIV-1 infection. *Natural Medicines* **2**:405-411.
- Kellam, P., C.A. Boucher, J.M. Tijnagel, and B.A. Larder.** 1994. Zidovudine treatment results in the selection of human immunodeficiency virus type 1 variants whose genotypes confer increasing levels of drug resistance. *Journal of General Virology* **75**:341-351.
- Khoury, G. and P. Gruss.** 1983. Enhancer elements. [Review] [0 refs]. *Cell* **33**:313-314.
- Kilby, J.M., S. Hopkins, T.M. Venetta, B. DiMassimo, G.A. Cloud, J.Y. Lee, L. Allredge, E. Hunter, D. Lambert, D. Bolognesi, T. Matthews, M.R. Johnson, M.A. Nowak, G.M. Shaw, and M.S. Saag.** 1998. Potent suppression of HIV-1 replication in humans by T-20, a peptide inhibitor of gp41-mediated virus entry [see comments]. *Nature Medicine* **4**:1302-1307.
- Kjems, J., M. Brown, D.D. Chang, and P.A. Sharp.** 1991. Structural analysis of the interaction between the human immunodeficiency virus Rev protein and the Rev response element. *Proceedings of the National Academy of Sciences of the United States of America* **88**:683-687.
- Kjems, J., B.J. Calnan, A.D. Frankel, and P.A. Sharp.** 1992. Specific binding of a basic peptide from HIV-1 Rev. *EMBO Journal* **11**:1119-1129.
- Klatzmann, D., E. Champagne, S. Chamaret, J. Gruest, Guetard, T. Hercend, J.C. Gluckman, and L. Montagnier.** 1984. T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. *Nature* **312**:767-768.
- Klein, M.R., C.A. van Baalen, A.M. Holwerda, G.SR. Kerkhof, R.J. Bende, I.P. Keet, J.K. Eeftinck-Schattenkerk, A.D. Osterhaus, Schuitemaker, and F. Miedema.** 1995. Kinetics of Gag-specific cytotoxic T lymphocyte responses during the clinical course of HIV-1 infection: a longitudinal analysis of rapid progressors and long-term asymptomatics. *Journal of Experimental Medicine* **181**:1365-1372.
- Klenerman, P., U.-C. Meier, R.E. Phillips, and A.J. McMichael.** 1995. The effects of natural altered peptide ligands on the whole blood cytotoxic T lymphocyte response to human immunodeficiency virus. *European Journal of Immunology* **25**:1927-1931.
- Kobs, G. Promega Notes. (62, 15). 1997. (GENERIC)
Ref Type: Catalog
- Kohl, N.E., E.A. Emini, W.A. Schleif, L.J. Davis, J.C. Heimbach, R.A. Dixon, E.M. Scolnick, and I.S. Sigal.** 1988. Active human immunodeficiency virus protease is required for viral infectivity. *Proceedings of the National Academy of Sciences of the United States of America* **85**:4686-4690.
- Kohler, H., J. Goudsmit, and P. Nara.** 1992. Clonal dominance: cause for a limited and failing immune response to HIV-1 infection and vaccination. [Review] [80 refs]. *Journal of Acquired Immune Deficiency Syndromes* **5**:1158-1168.
- Kohlstaedt, L.A., J. Wang, J.M. Friedman, P.A. Rice, and T.A. Steitz.** 1992. Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science* **256**:1783-1790.

- Korber, B., C. Kuiken, B. Foley, B.H. Hahn, F.E. McCutchan, J.W. Mellors, and J. Sodroski.** 1998. Human Retroviruses and AIDS. Theoretical Biology and Biophysics, Group T-10, Mail Stop K710,
- Kostrikis, L.G., E. Bagdades, Y. Cao, L. Zhang, D. Dimitriou, and D.D. Ho.** 1995. Genetic analysis of human immunodeficiency virus type 1 strains from patients in Cyprus: identification of a new subtype designated subtype I. *J.Virol.* **69**:6122-6130.
- Kostrikis, L.G., Z.-H. Michalopoulou, Y. Cao, J.P. Moore, and D.D. Ho.** 1996. Determining neutralization serotypes of HIV type 1 by neural networks. *AIDS Research & Human Retroviruses* **12**:1667-1669.
- Kostrikis, L.G., Y. Huang, J.P. Moore, S.M. Wolinsky, L. Zhang, Y. Guo, L. Deutsch, J. Phair, A.U. Neumann, and D.D. Ho.** 1998. A chemokine receptor CCR2 allele delays HIV-1 disease progression and is associated with a CCR5 promoter mutation [see comments]. *Nature Medicine* **4**:350-353.
- Koup, R.A., C.A. Pikora, G. Mazzara, D. Panicali, and J.L. Sullivan.** 1991. Broadly reactive antibody-dependent cellular cytotoxic response to HIV-1 envelope glycoproteins precedes broad neutralizing response in human infection. *Viral Immunology* **4**:215-223.
- Koup, R.A., J.T. Safrit, Y. Cao, C.A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, and D.D. Ho.** 1994a. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J.Virol.* **68**:4650-4655.
- Koup, R.A.** 1994b. Virus escape from CTL recognition. *Journal of Experimental Medicine* **180**:779-782.
- Kozak, M.** 1995. Adherence to the first-AUG rule when a second AUG codon follow closely upon the first. *Proceedings of the National Academy of Sciences of the United States of America* **92**:2662-2666.
- Kozarsky, K., M. Penman, L. Basiripour, W. Haseltine, J. Sodroski, and M. Krieger.** 1989. Glycosylation and processing of the human immunodeficiency virus type 1 envelope protein. *Journal of Acquired Immune Deficiency Syndromes* **2**:163-169.
- Kumar, S., Tamura, K., Nei, M.** 1993. *Molecular Evolutionary Genetics Analysis*, version 1.01, University Park, Philadelphia:Pennsylvania State University.
- Kwong, P.D., R. Wyatt, J. Robinson, R.W. Sweet, J. Sodroski, and W.A. Hendrickson.** 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody [see comments]. *Nature* **393**:648-659.
- Lang, S.M., M. Weeger, C. Stahl-Hennig, C. Coulibaly, Hunsmann, J. Muller, H. Muller-Hermelink, D. Fuchs, H. Wachter, and M.M. Daniel.** 1993. Importance of vpr for infection of rhesus monkeys with simian immunodeficiency virus. *J.Virol.* **67**:902-912.
- LaRosa, G.J., J.P. Davide, K. Weinhold, J.A. Waterbury, Profy, AT, J.A. Lewis, A.J. Langlois, G.R. Dreesman, R.N. Boswell, and P. Shadduck.** 1990. Conserved sequence and structural elements in the HIV-1 principal neutralizing

- determinant [published erratum appears in *Science* 1991 Feb 15;251(4995):811]. *Science* **249**:932-935.
- Le Gall, S., L. Erdtmann, S. Benichou, C. Berlioz-Torrent, L. Liu, R. Benarous, J.M. Heard, and O. Schwartz.** 1998. Nef interacts with the mu subunit of clathrin adaptor complexes and reveals a cryptic sorting signal in MHC I molecules. *Immunity* **8**:483-495.
- Le Grand, R., G. Vogt, B. Vaslin, P. Roques, F. Theodoro, A.M. Aubertin, and D. Dormont.** 1992. Specific and non-specific immunity and protection of macaques against SIV infection. *Vaccine* **10**:873-879.
- Lee, T.H., J.E. Coligan, J.S. Allan, M.F. McLane, J.E. Groopman, and M. Essex.** 1986. A new HTLV-III/LAV protein encoded by a gene found in cytopathic retroviruses. *Science* **231**:1546-1549.
- Leigh Brown, A.J., D. Lobidel, C.M. Wade, S. Rebus, A.N. Phillips, R.P. Brettle, A.J. France, C.S. Leen, J. McMenamain, A. McMillan, R.D. Maw, F. Mulcahy, J.R. Robertson, K.N. Sankar, G. Scott, R. Wyld, and J.F. Peutherer.** 1997. The molecular epidemiology of human immunodeficiency virus type 1 in six cities in Britain and Ireland. *Virology* **235**:166-177.
- Leonard, C.K., M.W. Spellman, L. Riddle, R.J. Harris, J.N. Thomas, and T.J. Gregory.** 1990. Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. *J.Biol.Chem.* **265**:10373-10382.
- Levy, J.A., A.D. Hoffman, S.M. Kramer, J.A. Landis, Shimabukuro, JM, and L.S. Oshiro.** 1984. Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. *Science* **225**:840-842.
- Li, Y., J.C. Kappes, J.A. Conway, R.W. Price, G.M. Shaw, and B.H. Hahn.** 1991. Molecular characterization of human immunodeficiency virus type 1 cloned directly from uncultured human brain tissue: identification of replication-competent and -defective viral genomes. *J.Virol.* **65**:3973-3985.
- Li, Y., H. Hui, C.J. Burgess, R.W. Price, P.M. Sharp, B.H. Hahn, and G.M. Shaw.** 1992. Complete nucleotide sequence, genome organization, and biological properties of human immunodeficiency virus type 1 in vivo: evidence for limited defectiveness and complementation. *J.Virol.* **66**:6587-6600.
- Lifson, J.D., G.R. Reyes, M.S. McGrath, B.S. Stein, and E.G. Engleman.** 1986. AIDS retrovirus induced cytopathology: giant cell formation and involvement of CD4 antigen. *Science* **232**:1123-1127.
- Liitsola, K., I. Tashkinova, T. Laukkanen, G. Korovina, T. Smolskaja, O. Momot, N. Mashkilleysen, S. Chaplinskis, H. Brummer-Korvenkontio, J. Vanhatalo, P. Leinikki, and M.O. Salminen.** 1998. HIV-1 genetic subtype A/B recombinant strain causing an explosive epidemic in injecting drug users in Kaliningrad. *AIDS* **12**:1907-1919.
- Lim, E.M., M. Lagranderie, R. Le Grand, J. Rauzier, Gheorghiu, B. Gicquel, and N. Winter.** 1997. Recombinant *Mycobacterium bovis* BCG producing the N-terminal half of SIVmac251 Env antigen induces neutralizing antibodies and

- cytotoxic T lymphocyte responses in mice and guinea pigs. *AIDS Research & Human Retroviruses* **13**:1573-1581.
- Littman, D.R.** 1998. Chemokine receptors: keys to AIDS pathogenesis?. [Review] [20 refs]. *Cell* **93**:677-680.
- Livingstone, W.J., M. Moore, D. Innes, J.E. Bell, and P. Simmonds.** 1996. Frequent infection of peripheral blood CD8-positive T-lymphocytes with HIV-1. Edinburgh Heterosexual Transmission Study Group [see comments]. *Lancet* **348**:649-654.
- Lu, M. and P.S. Kim.** 1997. A trimeric structural subdomain of the HIV-1 transmembrane glycoprotein. *Journal of Biomolecular Structure & Dynamics* **15**:465-471.
- Lu, X.B., J. Heimer, D. Rekosh, and M.L. Hammarskjold.** 1990. U1 small nuclear RNA plays a direct role in the formation of a rev-regulated human immunodeficiency virus env mRNA that remains unspliced. *Proceedings of the National Academy of Sciences of the United States of America* **87**:7598-7602.
- Luban, J., K.L. Bossolt, E.K. Franke, G.V. Kalpana, and S.P. Goff.** 1993. Human immunodeficiency virus type 1 Gag protein binds to cyclophilins A and B. *Cell* **73**:1067-1078.
- Lubeck, M.D., R. Natuk, M. Myagkikh, N. Kalyan, K. Aldrich, F. Sinangil, S. Alipanah, S.C. Murthy, P.K. Chanda, S.M.J. Nigida, P.D. Markham, S. Zolla-Pazner, K. Steimer, M. Wade, M.S.J. Reitz, L.O. Arthur, S. Mizutani, A. Davis, P.P. Hung, R.C. Gallo, J. Eichberg, and M. Robert-Guroff.** 1997. Long-term protection of chimpanzees against high-dose HIV-1 challenge induced by immunization. *Nature Medicine* **3**:651-658.
- Ludlam, C.A., J. Tucker, C.M. Steel, R.S. Tedder, R. Cheingsong-Popov, R.A. Weiss, D.B. McClelland, I. Philp, and R.J. Prescott.** 1985. Human T-lymphotropic virus type III (HTLV-III) infection in seronegative haemophiliacs after transfusion of factor VIII. *Lancet* **2**:233-236.
- Luke, W., C. Coulibaly, U. Dittmer, G. Voss, R. Oesterle, B. Makoschey, U. Sauermann, E. Jurkiewicz, C. Stahl-Henning, H. Petry, and G. Hunsmann .** 1996. Simian immunodeficiency virus (SIV) gp130 oligomers protect rhesus macaques (*Macaca mulatta*) against the infection with SIVmac32H grown on T-cells or derived ex vivo. *Virology* **216**:444-450.
- Lundberg, K.S., D.D. Shoemaker, M.W. Adams, J.M. Short, J.A. Sorge, and E.J. Mathur.** 1991. High-fidelity amplification using a thermostable DNA polymerase isolated from *Pyrococcus furiosus*. *Gene* **108**:1-6.
- Luo, Y., S.J. Madore, T.G. Parslow, B.R. Cullen, and B.M. Peterlin.** 1993. Functional analysis of interactions between Tat and the trans-activation response element of human immunodeficiency virus type 1 in cells. *J.Virol.* **67**:5617-5622.
- Ma, X.Y., P. Sova, W. Chao, and D.J. Volsky.** 1994. Cysteine residues in the Vif protein of human immunodeficiency virus type 1 are essential for viral infectivity. *J.Virol.* **68**:1714-1720.

- Maddon, P.J., D.R. Littman, M. Godfrey, D.E. Maddon, L. Chess, and R. Axel.** 1985. The isolation and nucleotide sequence of a cDNA encoding the T cell surface protein T4: a new member of the immunoglobulin gene family. *Cell* **42**:93-104.
- Maddon, P.J., A.G. Dalgleish, and J.S. McDougal.** 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. *Cell* **47**:
- Maddon, P.J., J.S. McDougal, P.R. Clapham, A.G. Dalgleish, Jamal, R.A. Weiss, and R. Axel.** 1988. HIV infection does not require endocytosis of its receptor, CD4. *Cell* **54**:865-874.
- Maldonado, E., I. Ha, P. Cortes, L. Weis, and D. Reinberg.** 1990. Factors involved in specific transcription by mammalian RNA polymerase II: role of transcription factors IIA, IID, and IIB during formation of a transcription-competent complex. *Molecular & Cellular Biology* **10**:6335-6347.
- Malim, M.H., J. Hauber, S.Y. Le, J.V. Maizel, and B.R. Cullen.** 1989. The HIV-1 rev trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. *Nature* **338**:254-257.
- Malim, M.H. and B.R. Cullen.** 1991a. HIV-1 structural gene expression requires the binding of multiple Rev monomers to the viral RRE: implications for HIV-1 latency. *Cell* **65**:241-248.
- Malim, M.H., D.F. McCarn, L.S. Tiley, and B.R. Cullen.** 1991b. Mutational definition of the human immunodeficiency virus type 1 Rev activation domain. *J.Virol.* **65**:4248-4254.
- Margottin, F., S.P. Bour, H. Durand, L. Selig, S. Benichou, V. Richard, D. Thomas, K. Strebel, and R. Benarous.** 1998. A novel human WD protein, h-beta TrCp, that interacts with HIV-1 Vpu connects CD4 to the ER degradation pathway through an F-box motif. *Molecular Cell* **1**:565-574.
- Marlink, R., R. Kanki, I. Thior, K. Travers, G. Eisen, Siby, I. Traore, C.-C. Hsieh, M.C. Dia, E.-H. Gueye, J. Hellinger, A. Gueye-Ndiaye, J.-L. Sankale, I. Ndoye, S. Mboup, and M. Essex.** 1994. Reduced rate of disease development after HIV-2 infection as compared to HIV-1. *Science* **265** :1587-1590.
- Martin, M.P., M. Dean, M.W. Smith, C. Winkler, B. Gerrard, N.L. Michael, B. Lee, R.W. Doms, J. Margolick, S. Buchbinder, J.J. Goedert, T.R. O'Brien, M.W. Hilgartner, D. Vlahov, S.J. O'Brien, and M. Carrington.** 1998. Genetic acceleration of AIDS progression by a promoter variant of CCR5. *Science* **282**:1907-1911.
- Mascola, J.R., S.W. Snyder, O.S. Weislow, S.M. Belay, R.B. Belshe, D.H. Schwartz, M.L. Clements, R. Dolin, B.S. Graham, G.J. Gorse, M.C. Keefer, M.J. McElrath, M.C. Walker, K.F. Wagner, J.G. McNeil, F.E. McCutchan, and D.S. Burke.** 1996. Immunization with envelope subunit vaccine products elicits neutralizing antibodies against laboratory-adapted but not primary isolates of human immunodeficiency virus type 1. The National Institute of Allergy and Infectious Diseases AIDS Vaccine Evaluation Group. *J.Infect.Dis.* **173**:340-348.

- Mascola, J.R., M.K. Louder, T.C. VanCott, C.V. Sapan, J.S. Lambert, L.R. Muenz, B. Bunow, D.L. Birx, and M.L. Robb.** 1997. Potent and synergistic neutralization of human immunodeficiency virus (HIV) type 1 primary isolates by hyperimmune anti-HIV immunoglobulin combined with monoclonal antibodies 2F5 and 2G12. *J.Virol.* **71**:7198-7206.
- Mastro, T.D., S. Vanichseni, and D. Kitayaporn.** 1999. Viral load and CD4/CD8 cell count in injecting drug users (IDUs) newly infected with HIV-1 subtypes B and E, Bangkok. 6th Conference on Retroviruses and Opportunistic Infections. Chicago, January (Abstract)
- McAdam, S., P. Kaleebu, P. Krausa, P. Goulder, N. French, B. Collin, T. Blanchard, J. Whitworth, A. McMichael, and F. Gotch.** 1998. Cross-clade recognition of p55 by cytotoxic T lymphocytes in HIV-1 infection. *AIDS* **12**:571-579.
- McDermott, D.H., P.A. Zimmerman, F. Guignard, C.A. Kleeberger, S.F. Leitman, and P.M. Murphy.** 1998. CCR5 promoter polymorphism and HIV-1 disease progression. Multicenter AIDS Cohort Study (MACS). *Lancet* **352**:866-870.
- Mellon, P., V. Parker, Y. Gluzman, and T. Maniatis.** 1981. Identification of DNA sequences required for transcription of the human alpha 1-globin gene in a new SV40 host-vector system. *Cell* **27**:279-288.
- Mellors, J.W., L.A. Kingsley, C.R.J. Rinaldo, J.A. Todd, B.S. Hoo, R.P. Kokka, and P. Gupta.** 1995. Quantitation of HIV-1 RNA in plasma predicts outcome after seroconversion. *Annals of Internal Medicine* **122** :573-579.
- Meyer, B.E. and M.H. Malim.** 1994. The HIV-1 Rev trans-activator shuttles between the nucleus and the cytoplasm. *Genes & Development* **8**:1538-1547.
- Meyerhans, A., R. Cheynier, J. Albert, M. Seth, S. Kwok, J. Sninsky, L. Morfeldt-Manson, B. Asjo, and S. Wain-Hobson.** 1989. Temporal fluctuations in HIV quasispecies in vivo are not reflected by sequential HIV isolations. *Cell* **58**:901-910.
- Meyerhans, A., J.P. Vartanian, and S. Wain-Hobson.** 1990. DNA recombination during PCR. *Nucleic Acids Research* **18**:1687-1691.
- Mezei, L.M. and D.R. Starts.** 1994. PCR Technology: Current Innovations.
- Michaels, F.H., N. Hattori, R.C. Gallo, and G. Franchini.** 1993. The human immunodeficiency virus type 1 (HIV-1) vif protein is located in the cytoplasm of infected cells and its effect on viral replication is equivalent in HIV-2. *AIDS Research & Human Retroviruses* **9**:1025-1030.
- Milich, L., B. Margolin, and R. Swanstrom.** 1993. V3 loop of the Human Immunodeficiency Virus Type 1 env protein: interpreting sequence variability. *J.Virol.* **67**:5623-5634.
- Miller, M.D., M.T. Warmerdam, K.A. Page, M.B. Feinberg, Greene, and WC.** 1995. Expression of the human immunodeficiency virus type 1 (HIV-1) nef gene during HIV-1 production increases progeny particle infectivity independently of gp160 or viral entry. *J.Virol.* **69**:579-584.
- Mills, K.H., M. Page, P. Kitchin, L. Chan, W. Jones, Silvera, T. Corcoran, B. Flanagan, C. Ling, and C. Thiriart.** 1993. Immunisation of macaques with SIV

- env recombinants: specificity of T cell and antibody responses and evaluation of protective efficacy. *Journal of Medical Primatology* **22**:104-109.
- Modrow, S., B.H. Hahn, and G.M. Shaw.** 1987. Computer-assisted analysis of envelope protein sequences of seven human immunodeficiency virus isolates: Prediction of antigenic epitopes in conserved and variable regions. *J.Virol.* **61**:
- Moldoveanu, Z., D.C. Porter, A. Lu, S. McPherson, and C.D. Morrow.** 1995. Immune responses induced by administration of encapsidated poliovirus replicons which express HIV-1 gag and envelope proteins. *Vaccine* **13**:1013-1022.
- Montagnier, L., J.C. Chermann, F. Barre-Sinoussi, D. Klatzmann, S. Wain-Hobson, M. Alizon, F. Clavel, F. Brun-Vezinet, E. Vilmer, and C. Rouzioux.** 1984. Lymphadenopathy associated virus and its etiological role in AIDS. [Review] [34 refs]. *Princess Takamatsu Symposia* **15**:319-331.
- Montefiori, D.C., W.E.J. Robinson, and W.M. Mitchell.** 1988. Role of protein N-glycosylation in pathogenesis of human immunodeficiency virus type 1. *Proceedings of the National Academy of Sciences of the United States of America* **85**:9248-9252.
- Mooij, P., M. van der Kolk, W.M. Bogers, P.J. ten Haaf, Van, M.P. Der, N. Almond, J. Stott, M. Deschamps, D. Labbe, P. Momin, G. Voss, P. Von Hoegen, C. Bruck, and J.L. Heeney.** 1998. A clinically relevant HIV-1 subunit vaccine protects rhesus macaques from in vivo passaged simian-human immunodeficiency virus infection. *AIDS* **12**:F15-F22
- Moore, J.P. and R.F. Jarrett.** 1988. Sensitive ELISA for the gp120 and gp160 surface glycoproteins of HIV-1. *AIDS Research & Human Retroviruses* **4**:369-379.
- Moore, J.P., Q.J. Sattentau, H. Yoshiyama, M. Thali, M. Charles, N. Sullivan, S.W. Poon, M.S. Fung, F. Traincard, and M. Pinkus.** 1993a. Probing the structure of the V2 domain of human immunodeficiency virus type 1 surface glycoprotein gp120 with a panel of eight monoclonal antibodies: human immune response to the V1 and V2 domains. *J.Virol.* **67**:6136-6151.
- Moore, J.P., M. Thali, B.A. Jameson, F. Vignaux, G.K. Lewis, S.W. Poon, M. Charles, M.S. Fung, B. Sun, and P.J. Durda.** 1993b. Immunochemical analysis of the gp120 surface glycoprotein of human immunodeficiency virus type 1: probing the structure of the C4 and V4 domains and the interaction of the C4 domain with the V3 loop. *J.Virol.* **67**:4785-4796.
- Moore, J.P. and D.D. Ho.** 1993c. Antibodies to discontinuous or conformationally sensitive epitopes on the gp120 glycoprotein of human immunodeficiency virus type 1 are highly prevalent in sera of infected humans. *J.Virol.* **67**:863-875.
- Moore, J.P.** 1993d. The reactivities of HIV-1+ human sera with solid-phase V3 loop peptides can be poor predictors of their reactivities with V3 loops on native gp120 molecules. *AIDS Research & Human Retroviruses* **9**:209-219.
- Moore, J.P., Q.J. Sattentau, R. Wyatt, and J. Sodroski.** 1994a. Probing the structure of the human immunodeficiency virus surface glycoprotein gp120 with a panel of monoclonal antibodies. *J.Virol.* **68**:469-484.

- Moore, J.P., R.L. Willey, G.K. Lewis, J. Robinson, and J. Sodroski.** 1994b. Immunological evidence for interactions between the first, second, and fifth conserved domains of the gp120 surface glycoprotein of human immunodeficiency virus type 1. *J.Virol.* **68**:6836-6847.
- Moore, J.P., F.E. McCutchan, S.W. Poon, J. Mascola, J. Liu, Cao, and D.D. Ho.** 1994c. Exploration of antigenic variation in gp120 from clades A through F of human immunodeficiency virus type 1 by using monoclonal antibodies. *J.Virol.* **68**:8350-8364.
- Moore, J.P., Y. Cao, D.D. Ho, and R.A. Koup.** 1994d. Development of the anti-gp120 antibody response during seroconversion to human immunodeficiency virus type 1. *J.Virol.* **68**:5142-5155.
- Moore, J.P., Y. Cao, A.J. Conley, R. Wyatt, J. Robinson, Gorny, MK, S. Zolla-Pazner, D.D. Ho, and R.A. Koup.** 1994e. Studies with monoclonal antibodies to the V3 region of HIV-1 gp120 reveal limitations to the utility of solid-phase peptide binding assays. *Journal of Acquired Immune Deficiency Syndromes* **7**:332-339.
- Moore, J.P., Y. Cao, L. Qing, Q.J. Sattentau, J. Pyati, R. Koduri, J. Robinson, C.F. Barbas, D.R. Burton, and D.D. Ho.** 1995a. Primary isolates of human immunodeficiency virus type 1 are relatively resistant to neutralization by monoclonal antibodies to gp120, and their neutralization is not predicted by studies with monomeric gp120. *J.Virol.* **69**:101-109.
- Moore, J.P. and D.D. Ho.** 1995b. HIV-1 neutralization: the consequences of viral adaptation to growth on transformed T cells. [Review] [271 refs]. *AIDS* **9 Suppl A**:S117-S136
- Moore, J.P., A. Trkola, B. Korber, L.J. Boots, J.A. Kessler, F.E. McCutchan, J. Mascola, D.D. Ho, J. Robinson, and A.J. Conley.** 1995c. A human monoclonal antibody to a complex epitope in the V3 region of gp120 of human immunodeficiency virus type 1 has broad reactivity within and outside clade B. *J.Virol.* **69**:122-130.
- Moore, J.P., Y. Cao, J. Leu, L. Qin, B. Korber, and D.D. Ho.** 1996. Inter- and intraclade neutralization of human immunodeficiency virus type 1: genetic clades do not correspond to neutralization serotypes but partially correspond to gp120 antigenic serotypes. *J.Virol.* **70**:427-444.
- Mortara, L., F. Letourneur, H. Gras-Masse, A. Venet, Guillet, JG, and I. Bourgault-Villada.** 1998. Selection of virus variants and emergence of virus escape mutants after immunization with an epitope vaccine. *J.Virol.* **72**:1403-1410.
- Mosier, D.E.** 1995. HIV results in the frame. CD4+ cell turnover [letter; comment]. *Nature* **375**:193-194.
- Mukund, S. and M.W. Adams.** 1990. Characterization of a tungsten-iron-sulfur protein exhibiting novel spectroscopic and redox properties from the hyperthermophilic archaeobacterium *Pyrococcus furiosus*. *Journal of Biological Chemistry* **265**:11508-11516.

- Mullis, K.B. and F.A. Faloona.** 1987. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods in Enzymology* **155**:335-350.
- Mullis, K.B.** 1990a. Target amplification for DNA analysis by the polymerase chain reaction. *Annales de Biologie Clinique* **48**:579-582.
- Mullis, K.B.** 1990b. The unusual origin of the polymerase chain reaction. *Scientific American* **262**:56-61.
- Mullis, K.B.** 1991. The polymerase chain reaction in an anemic mode: how to avoid cold oligodeoxyribonuclear fusion. [Review] [0 refs]. *Genome Research* **1**:1-4.
- Murphey-Corb, M., L.N. Martin, B. Davison-Fairburn, Montelaro, RC, M. Miller, M. West, S. Ohkawa, G.B. Baskin, J.Y. Zhang, and S.D. Putney.** 1989. A formalin-inactivated whole SIV vaccine confers protection in macaques. *Science* **246**:1293-1297.
- Muster, T., F. Steindl, M. Purtscher, A. Trkola, A. Klima, G. Himmler, F. Ruker, and H. Katinger.** 1993. A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. *J.Virol.* **67**:6642-6647.
- Muster, T., R. Guinea, A. Trkola, M. Purtscher, A. Klima, F. Steindl, P. Palese, and H. Katinger.** 1994. Cross-neutralizing activity against divergent human immunodeficiency virus type 1 isolates induced by the gp41 sequence ELDKWAS. *J.Virol.* **68**:4031-4034.
- Myers, G., B. Korber, j. Berzofsky, T.F. Smith, and G.N. Pavlakis.** 1991. *Human Retroviruses and AIDS.* Los Alamos Laboratory, Los Alamos, New Mexico, USA.
- Nalin, C.M., R.D. Purcell, D. Antelman, D. Mueller, L. Tomchak, B. Wegrzynski, E. McCarney, V. Toome, R. Kramer, and M.C. Hsu.** 1990. Purification and characterization of recombinant Rev protein of human immunodeficiency virus type 1. *Proceedings of the National Academy of Sciences of the United States of America* **87**:7593-7597.
- Nara, P.L., R.R. Garrity, and J. Goudsmit.** 1991. Neutralization of HIV-1: a paradox of humoral proportions. [Review] [111 refs]. *FASEB Journal* **5**:2437-2455.
- Nasioulas, G., D. Paraskevis, E. Magiorkinis, M. Theodoridou, and A. Hatzakis.** 1999. Molecular analysis of the full-length genome of HIV type 1 subtype I: evidence of A/G/I recombination. *AIDS Research & Human Retroviruses* **15**:745-758.
- Neumann, M., J. Harrison, M. Saltarelli, E. Hadziyannis, V. Erfle, B.K. Felber, and G.N. Pavlakis.** 1994. Splicing variability in HIV type 1 revealed by quantitative RNA polymerase chain reaction. *AIDS Research & Human Retroviruses* **10**:1531-1542.
- Newton, C.R. and A. Graham.** 1994. *PCR.* Bios Scientific Publishers, Ltd.,
- Newton, C.R. and A. Graham.** 1994. *PCR:Instrumentation, Reagents and Consumables,* Anonymous Bios Scientific Publisher,
- Nkengasong, J.N., W. Janssens, L. Heyndrickx, K. Fransen, P.M. Ndumbe, J. Motte, A. Leonaers, M. Ngolle, J. Ayuk, and P. Piot.** 1994. Genotypic subtypes of HIV-1 in Cameroon. *AIDS* **8**:1405-1412.

- Nkengasong, J.N., B. Willems, W. Janssens, R. Cheingsong-Popov, L. Heyndrickx, F. Barin, P. Ondoa, K. Fransen, J. Goudsmit, and G. van der Groen . 1998.** Lack of correlation between V3-loop peptide enzyme immunoassay serologic subtyping and genetic sequencing. *AIDS* **12**:1405-1412.
- Nowak, M.A., R.M. May, and R.M. Anderson. 1990.** The evolutionary dynamics of HIV-1 quasispecies and the development of immunodeficiency disease. *AIDS* **4**:1095-1103.
- Nowak, M.A., R.M. Anderson, A.R. McLean, T.F. Wolfs, J. Goudsmit, and R.M. May. 1991.** Antigenic diversity thresholds and the development of AIDS [see comments]. *Science* **254**:963-969.
- Nowak, M.A., R.M. May, R.E. Phillips, S. Rowland-Jones, D.G. Lalloo, S. McAdam, P. Klenerman, B. Koppe, K. Sigmund, C.R.M. Bangham, McMichael, and A.J. 1995.** Antigenic oscillations and shifting immunodominance in HIV-1 infections. *Nature* **375**:606-611.
- Nowak, M.A., R.M. Anderson, M.C. Boerlijst, S. Bonhoeffer, May, RM, and A.J. McMichael. 1996a.** HIV-1 evolution and disease progression [letter; comment]. *Science* **274**:1008-1011.
- Nowak, M.A. and C.R. Bangham. 1996b.** Population dynamics of immune responses to persistent viruses. *Science* **272**:74-79.
- Nyambi, P.N., B. Willems, W. Janssens, K. Fransen, Nkengasong, M. Peeters, K. Vereecken, L. Heyndrickx, P. Piot, and G. van der Groen. 1997.** The neutralization relationship of HIV type 1, HIV type 2, and SIVcpz is reflected in the genetic diversity that distinguishes them. *AIDS Research & Human Retroviruses* **13**:7-17.
- Oberlin, E., A. Amara, F. Bachelerie, C. Bessia, Virelizier, JL, F. Arenzana-Seisdedos, O. Schwartz, J.M. Heard, I. Clark-Lewis, Legler, DF, M. Loetscher, M. Baggiolini, and B. Moser. 1996.** The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1 [published erratum appears in *Nature* 1996 Nov 21;384(6606):288]. *Nature* **382**:833-835.
- Ogg, G.S., T. Dong, P. Hansasuta, L. Dorrell, J. Clarke, Coker, G. Luzzi, C. Conlon, A.P. McMichael, and S. Rowland-Jones. 1998.** Four novel cytotoxic T-lymphocyte epitopes in the highly conserved major homology region of HIV-1 Gag, restricted through B*4402, B*1801, A*2601, B*70 (B*1509) [letter]. *AIDS* **12**:1561-1563.
- Ogg, G.S., X. Jin, S. Bonhoeffer, P. Moss, M.A. Nowak, Monard, J.P. Segal, Y. Cao, S.L. Rowland-Jones, A. Hurley, M. Markowitz, D.D. Ho, A.J. McMichael, and D.F. Nixon. 1999.** Decay kinetics of human immunodeficiency virus-specific effector cytotoxic T lymphocytes after combination antiretroviral therapy. *J.Virol.* **73**:797-800.
- Olsen, H.S., A.W. Cochrane, P.J. Dillon, C.M. Nalin, and C.A. Rosen. 1990.** Interaction of the human immunodeficiency virus type 1 Rev protein with a structured region in env mRNA is dependent on multimer formation mediated through a basic stretch of amino acids. *Genes & Development* **4**:1357-1364.

- Olshevsky, U., E. Helseth, C. Furman, J. Li, W. Haseltine, and J. Sodroski.** 1990. Identification of individual human immunodeficiency virus type 1 gp120 amino acids important for CD4 receptor binding. *J. Virol.* **64**:5701-5707.
- Orenstein, J.M., C. Fox, and S.M. Wahl.** 1997. Macrophages as a source of HIV during opportunistic infections. *Science* **276**:1857-1861.
- Overbaugh, J., N. Riedel, E.A. Hoover, and J.I. Mullins.** 1988. Transduction of endogenous envelope genes by feline leukaemia virus in vitro. *Nature* **332**:731-734.
- Palella, F.J.J., K.M. Delaney, A.C. Moorman, M.O. Loveless, J. Fuhrer, G.A. Satten, D.J. Aschman, S.D. Holmberg, J.C. Von Bargen, C. Gardner, K. McKettrick, B.G. Yangco, K.D. Halkias, C. Lapierre, D.J. Ward, R. Deighton, L. Aronson-Ryan, J.B. Marzouk, R.T. Phelps, P. Joseph, M. Rachel, R.E. McCabe, W.A. Alexander, S. Lingam, K.A. Lichtenstein, K.S. Greenberg, P. Zellner, Widick, C. Stewart, K.M. Delancy, and K.M. Ragland.** 1998. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. *New England Journal of Medicine* **338**:853-860.
- Pantaleo, G., C. Graziosi, L. Butini, P.A. Pizzo, Schnittman, SM, D.P. Kotler, and A.S. Fauci.** 1991. Lymphoid organs function as major reservoirs for human immunodeficiency virus. *Proceedings of the National Academy of Sciences of the United States of America* **88**:9838-9842.
- Pantaleo, G., C. Graziosi, and A.S. Fauci.** 1993. The role of lymphoid organs in the pathogenesis of HIV infection. [Review] [42 refs]. *Seminars in Immunology* **5**:157-163.
- Park, I.W. and J. Sodroski.** 1995a. Amino acid sequence requirements for the incorporation of the Vpx protein of simian immunodeficiency virus into virion particles. *JAIDS: Journal of Acquired Immune Deficiency Syndromes* **10**:506-510.
- Park, I.W. and J. Sodroski.** 1995b. Functional analysis of the vpx, vpr, and nef genes of simian immunodeficiency virus. *JAIDS: Journal of Acquired Immune Deficiency Syndromes* **8**:335-344.
- Parren, P.W., H.J. Ditzel, R.J. Gulizia, J.M. Binley, C.F. Barbas, RD, D.R. Burton, and D.E. Mosier.** 1995. Protection against HIV-1 infection in hu-PBL-SCID mice by passive immunization with a neutralizing human monoclonal antibody against the gp120 CD4-binding site. *AIDS* **9**:F1-F6
- Parrott, C., T. Seidner, E. Duh, J. Leonard, T.S. Theodore, A. Buckler-White, M.A. Martin, and A.B. Rabson.** 1991. Variable role of the long terminal repeat Sp1-binding sites in human immunodeficiency virus replication in T lymphocytes. *J. Virol.* **65**:1414-1419.
- Paul, N.L., M. Marsh, J.A. McKeating, T.F. Schulz, P. Liljestrom, H. Garoff, and R.A. Weiss.** 1993. Expression of HIV-1 envelope glycoproteins by Semliki Forest virus vectors. *AIDS Research & Human Retroviruses* **9**:963-970.

- Paxton, W., R.I. Connor, and N.R. Landau.** 1993. Incorporation of Vpr into human immunodeficiency virus type 1 virions: requirement for the p6 region of gag and mutational analysis. *J.Virol.* **67**:7229-7237.
- Peeters, M., J. Nkengasong, B. Willems, E. Karita, Delaporte, M. Van den Haesevelde, P. Piot, and G. van der Groen.** 1994. Antibodies to V3 loop peptides derived from chimpanzee lentiviruses and the divergent HIV-1ANT-70 isolate in human sera from different geographic regions. *AIDS* **8**:1657-1661.
- Peterlin, B.M., P.A. Luciw, P.J. Barr, and M.D. Walker.** 1986. Elevated levels of mRNA can account for the trans-activation of human immunodeficiency virus. *Proceedings of the National Academy of Sciences of the United States of America* **83**:9734-9738.
- Peterson, M.G., N. Tanese, B.F. Pugh, and R. Tjian.** 1990. Functional domains and upstream activation properties of cloned human TATA binding protein [published erratum appears in *Science* 1990 Aug 24;249(4971):844]. *Science* **248**:1625-1630.
- Pialoux, G., J.L. Excler, Y. Riviere, G. Gonzalez-Canali, V. Feuillie, P. Coulaud, J.C. Gluckman, T.J. Matthews, B. Meignier, and M.P. Kiény.** 1995. A prime-boost approach to HIV preventive vaccine using a recombinant canarypox virus expressing glycoprotein 160 (MN) followed by a recombinant glycoprotein 160 (MN/LAI). The AGIS Group, and l'Agence Nationale de Recherche sur le SIDA [published erratum appears in *AIDS Res Hum Retroviruses* 1995 Jul;11(7):875]. *AIDS Research & Human Retroviruses* **11**:373-381.
- Plantier, J.C., F. Damond, M. Lasky, J.L. Sankale, C. Apetrei, M. Peeters, L. Buzelay, S. M'Boup, P. Kanki, E. Delaporte, F. Simon, and F. Barin .** 1999. V3 serotyping of HIV-1 infection: correlation with genotyping and limitations. *JAIDS: Journal of Acquired Immune Deficiency Syndromes* **20**:432-441.
- Pomerantz, R.J., T. Seshamma, and D. Trono.** 1992. Efficient replication of human immunodeficiency virus type 1 requires a threshold level of Rev: potential implications for latency. *J.Virol.* **66**:1809-1813.
- Pope, M., S.S. Frankel, J.R. Mascola, A. Trkola, F. Isdell, D.L. Birx, D.S. Burke, D.D. Ho, and J.P. Moore.** 1997. Human immunodeficiency virus type 1 strains of subtypes B and E replicate in cutaneous dendritic cell-T-cell mixtures without displaying subtype-specific tropism. *J.Virol.* **71**:8001-8007.
- Popov, S., M. Rexach, G. Zybarth, N. Reiling, M.A. Lee, Ratner, C.M. Lane, M.S. Moore, G. Blobel, and M. Bukrinsky.** 1998. Viral protein R regulates nuclear import of the HIV-1 pre-integration complex. *EMBO Journal* **17**:909-917.
- Poulin, L., M. Fauchon, A. Darveau, and J.A. Levy.** 1994. Inhibition of protein synthesis by the human immunodeficiency virus type 1 nef gene product. *Journal of General Virology* **75**:2977-2984.
- Prakash, K., R.L. Hodinka, D.M. Hullihen, and S.A. Plotkin.** 1991. Isolation and characterization of an infectious molecular clone of the MN strain of HIV-1. *Biochemical & Biophysical Research Communications* **179**:1377-1383.
- Preston, B.D., B.J. Poiesz, and L.A. Loeb.** 1988. Fidelity of HIV-1 reverse transcriptase. *Science* **242**:1168-1171.

- Psallidopoulos, M.C., S.M. Schnittman, L.M. Thompson, Baseler, A.S. Fauci, H.C. Lane, and N.P. Salzman.** 1989. Integrated proviral human immunodeficiency virus type 1 is present in CD4+ peripheral blood lymphocytes in healthy seropositive individuals. *J.Virol.* **63**:4626-4631.
- Purcell, D.F. and M.A. Martin.** 1993. Alternative splicing of human immunodeficiency virus type 1 mRNA modulates viral protein expression, replication, and infectivity. *J.Virol.* **67**:6365-6378.
- Quayle, A.J., W.M. Coston, A.K. Trocha, S.A. Kalams, K.H. Mayer, and D.J. Anderson.** 1998. Detection of HIV-1-specific CTLs in the semen of HIV-infected individuals. *Journal of Immunology* **161**:4406-4410.
- Quinn, T.C.** 1996. Global burden of the HIV pandemic [see comments] [published erratum appears in *Lancet* 1996 Jul 27;348(9022):276]. [Review] [50 refs]. *Lancet* **348**:99-106.
- Rabson, A.B., D.F. Daugherty, S. Venkatesan, K.E. Boulukos, Benn, SI, T.M. Folks, P. Feorino, and M.A. Martin.** 1985. Transcription of novel open reading frames of AIDS retrovirus during infection of lymphocytes. *Science* **229**:1388-1390.
- Raja, N.U., M.J. Vincent, and J.M. Abdul.** 1994. Vpu-mediated proteolysis of gp160/CD4 chimeric envelope glycoproteins in the endoplasmic reticulum: requirement of both the anchor and cytoplasmic domains of CD4. *Virology* **204**:357-366.
- Raport, C.J., J. Gosling, V.L. Schweickart, P.W. Gray, and I.F. Charo.** 1996. Molecular cloning and functional characterization of a novel human CC chemokine receptor (CCR5) for RANTES, MIP-1beta, and MIP-1alpha. *Journal of Biological Chemistry* **271**:17161-17166.
- Ratner, L., R.C. Gallo, F. Wong-Staal.** 1985. HTLV-III. LAV, ARV are variants of same AIDA virus. *Nature.* **313**: 636-7.
- Re, F., D. Braaten, E.K. Franke, and J. Luban.** 1995. Human immunodeficiency virus type 1 Vpr arrests the cell cycle in G2 by inhibiting the activation of p34cdc2-cyclin B. *J.Virol.* **69**:6859-6864.
- Reid, S.W., S. McAdam, K.J. Smith, P. Klenerman, O'C, CA, K. Harlos, B.K. Jakobsen, A.J. McMichael, J.I. Bell, D.I. Stuart, and E.Y. Jones.** 1996. Antagonist HIV-1 gag peptides induce structural changes in HLA B8. *Journal of Experimental Medicine* **184**:2279-2286.
- Rekosh, D., A. Nygren, P. Flodby, M.L. Hammar skjold, and Wigzell .** 1988. Coexpression of human immunodeficiency virus envelope proteins and tat from a single simian virus 40 late replacement vector. *Proceedings of the National Academy of Sciences of the United States of America* **85**:334-338.
- Richard, N., S. Iacampo, and A. Cochrane.** 1994. HIV-1 Rev is capable of shuttling between the nucleus and cytoplasm. *Virology* **204**:123-131.
- Richman, D.D.** 1992. Emergence of mutant HIV reverse transcriptase conferring resistance to AZT. [Review] [24 refs]. *Journal of Enzyme Inhibition* **6**:55-64.
- Rinaldo, C., X.L. Huang, Z.F. Fan, M. Ding, L. Beltz, A. Logar, D. Panicali, G. Mazzara, J. Liebmman, and M. Cottrill.** 1995. High levels of anti-human immunodeficiency virus type 1 (HIV-1) memory cytotoxic T-lymphocyte

activity and low viral load are associated with lack of disease in HIV-1-infected long-term nonprogressors. *J. Virol.* **69**:5838-5842.

- Rizzuto, C.D., R. Wyatt, N. Hernandez-Ramos, Y. Sun, P.D. Kwong, W.A. Hendrickson, and J. Sodroski.** 1998. A conserved HIV gp120 glycoprotein structure involved in chemokine receptor binding. *Science* **280**:1949-1953.
- Roben, P., J.P. Moore, M. Thali, J. Sodroski, C.F. Barbas, and D.R. Burton.** 1994. Recognition properties of a panel of human recombinant Fab fragments to the CD4 binding site of gp120 that show differing abilities to neutralize human immunodeficiency virus type 1. *J. Virol.* **68**:4821-4828.
- Robert-Guroff, M., M. Popovic, S. Gartner, P. Markham, R.C. Gallo, and M.S. Reitz.** 1990. Structure and expression of tat-, rev-, and nef-specific transcripts of human immunodeficiency virus type 1 in infected lymphocytes and macrophages. *J. Virol.* **64**:3391-3398.
- Roberts, J.D., K. Bebenek, and T.A. Kunkel.** 1988. The accuracy of reverse transcriptase from HIV-1. *Science* **242**:1171-1173.
- Robey, F.A., T. Harris-Kelson, M. Robert-Guroff, D. Batinic, B. Ivanov, M.S. Lewis, and P.P. Roller.** 1996. A synthetic conformational epitope from the C4 domain of HIV Gp120 that binds CD4. *Journal of Biological Chemistry* **271**:17990-17995.
- Robey, W.G., L.O. Arthur, T.J. Matthews, A. Langlois, Copeland, TD, N.W. Lerche, S. Oroszlan, D.P. Bolognesi, R.V. Gilden, and P.J. Fischinger.** 1986. Prospect for prevention of human immunodeficiency virus infection: purified 120-kDa envelope glycoprotein induces neutralizing antibody. *Proceedings of the National Academy of Sciences of the United States of America* **83**:7023-7027.
- Robinson, J.E., D. Holton, J. Liu, H. McMurdo, A. Murciano, and R. Gohd.** 1990. A novel enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to HIV-1 envelope glycoproteins based on immobilization of viral glycoproteins in microtiter wells coated with concanavalin A. *Journal of Immunological Methods* **132**:63-71.
- Rogel, M.E., L.I. Wu, and M. Emerman.** 1995. The human immunodeficiency virus type 1 vpr gene prevents cell proliferation during chronic infection. *J. Virol.* **69**:882-888.
- Rosen, C.A., E. Terwilliger, A. Dayton, J.G. Sodroski, and W.A. Haseltine.** 1988. Intragenic cis-acting art gene-responsive sequences of the human immunodeficiency virus. *Proceedings of the National Academy of Sciences of the United States of America* **85**:2071-2075.
- Rosenberg, Y.J., P.M. Zack, B.D. White, S.F. Papermaster, Elkins, WR, G.A. Eddy, and M.G. Lewis.** 1993. Decline in the CD4+ lymphocyte population in the blood of SIV-infected macaques is not reflected in lymph nodes. *AIDS Research & Human Retroviruses* **9**:639-646.
- Rosenberg, E.S., J.M. Billingsley, A.M. Caliendo, S.L. Boswell, P.E. Sax, S.A. Kalams, and B.D. Walker.** 1997. Vigorous HIV-1-specific CD4+ T cell

responses associated with control of viremia [see comments]. *Science* **278**:1447-1450.

- Ross, E.K., A.J. Buckler-White, A.B. Rabson, G. Englund, Martin, and MA.** 1991. Contribution of NF-kappa B and Sp1 binding motifs to the replicative capacity of human immunodeficiency virus type 1: distinct patterns of viral growth are determined by T-cell types. *J.Virol.* **65**:4350-4358.
- Rowland-Jones, S.L. and A. McMichael.** 1995. Immune responses in HIV-exposed seronegatives: have they repelled the virus?. [Review] [63 refs]. *Current Opinion in Immunology* **7**:448-455.
- Rowland-Jones, S.L., T. Dong, K.R. Fowke, J. Kimani, P. Krausa, H. Newell, T. Blanchard, K. Ariyoshi, J. Oyugi, E. Ngugi, J. Bwayo, MacDonald, KS, A.J. McMichael, and F.A. Plummer.** 1998. Cytotoxic T cell responses to multiple conserved HIV epitopes in HIV-resistant prostitutes in Nairobi [see comments]. *Journal of Clinical Investigation* **102**:1758-1765.
- Ruben, S., A. Perkins, R. Purcell, K. Joung, R. Sia, Burghoff, W.A. Haseltine, and C.A. Rosen.** 1989. Structural and functional characterization of human immunodeficiency virus tat protein. *J.Virol.* **63**:1-8.
- Rucker, J., A.L. Edinger, M. Sharron, M. Samson, B. Lee, J.F. Berson, Y. Yi, B. Margulies, R.G. Collman, B.J. Doranz, M. Parmentier, Doms, and RW.** 1997. Utilization of chemokine receptors, orphan receptors, and herpesvirus-encoded receptors by diverse human and simian immunodeficiency viruses. *J.Virol.* **71**:8999-9007.
- Rud, E.W., M. Cranage, J. Yon, J. Quirk, L. Ogilvie, N. Cook, S. Webster, M. Dennis, and B.E. Clarke.** 1994. Molecular and biological characterization of simian immunodeficiency virus macaque strain 32H proviral clones containing nef size variants. *Journal of General Virology* **75**:529-543.
- Rusche, J.R., D.L. Lynn, M. Robert-Guroff, A.J. Langlois, Lyerly, HK, H. Carson, K. Krohn, A. Ranki, R.C. Gallo, and D.P. Bolognesi.** 1987. Humoral immune response to the entire human immunodeficiency virus envelope glycoprotein made in insect cells. *Proceedings of the National Academy of Sciences of the United States of America* **84**:6924-6928.
- Safrit, J.T., M.S. Fung, C.A. Andrews, D.G. Braun, W.N. Sun, Chang, TW, and R.A. Koup.** 1993. hu-PBL-SCID mice can be protected from HIV-1 infection by passive transfer of monoclonal antibody to the principal neutralizing determinant of envelope gp120. *AIDS* **7**:15-21.
- Saiki, R.K., S. Scharf, F. Faloona, K.B. Mullis, G.T. Horn, H.A. Erlich, and N. Arnheim.** 1985. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230**:1350-1354.
- Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis, and H.A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239** :487-491.
- Saksela, K., G. Cheng, and D. Baltimore.** 1995. Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the

- enhanced growth of Nef+ viruses but not for down-regulation of CD4. *EMBO Journal* **14**:484-491.
- Samson, M., O. Labbe, C. Mollereau, G. Vassart, and M. Parmentier.** 1996. Molecular cloning and functional expression of a new human CC-chemokine receptor gene. *Biochemistry* **35**:3362-3367.
- Sanger, F.** 1988. Sequences, sequences, and sequences. *Annual Review of Biochemistry* **57**:1-28.
- Saragosti, S., S. Cereghini, and M. Yaniv.** 1982. Fine structure of the regulatory region of simian virus 40 minichromosomes revealed by DNAase I digestion. *Journal of Molecular Biology* **160**:133-146.
- Sattentau, Q.J., J. Arthos, K. Deen, N. Hanna, D. Healey, P.C. Beverley, R. Sweet, and A. Truneh.** 1989. Structural analysis of the human immunodeficiency virus-binding domain of CD4. Epitope mapping with site-directed mutants and anti-idiotypes. *Journal of Experimental Medicine* **170**:1319-1334.
- Sattentau, Q.J., S. Zolla-Pazner, and P. Poignard.** 1995a. Epitope exposure on functional, oligomeric HIV-1 gp41 molecules. *Virology* **206**:713-717.
- Sattentau, Q.J. and J.P. Moore.** 1995b. Human immunodeficiency virus type 1 neutralization is determined by epitope exposure on the gp120 oligomer. *Journal of Experimental Medicine* **182**:185-196.
- Sawai, E.T., A. Baur, H. Struble, B.M. Peterlin, J.A. Levy, and C. Cheng-Mayer.** 1994. Human immunodeficiency virus type 1 Nef associates with a cellular serine kinase in T lymphocytes. *Proceedings of the National Academy of Sciences of the United States of America* **91**:1539-1543.
- Schmitz, J.E., M.J. Kuroda, S. Santra, V.G. Sasseville, Simon, MA, M.A. Lifton, P. Racz, K. Tenner-Racz, M. Dalesandro, B.J. Scallon, Ghayeb, M.A. Forman, D.C. Montefiori, E.P. Rieber, N.L. Letvin, and K.A. Reimann.** 1999. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science* **283**:857-860.
- Schneider, J., S.C. Gilbert, T.J. Blanchard, T. Hanke, Robson, KJ, C.M. Hannan, M. Becker, R. Sinden, G.L. Smith, and A.V. Hill.** 1998. Enhanced immunogenicity for CD8+ T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia virus Ankara. *Nature Medicine* **4**:397-402.
- Schubert, U., L.C. Anton, I. Bacik, J.H. Cox, S. Bour, Bennink, JR, M. Orłowski, K. Strebel, and J.W. Yewdell.** 1998. CD4 glycoprotein degradation induced by human immunodeficiency virus type 1 Vpu protein requires the function of proteasomes and the ubiquitin-conjugating pathway. *J.Virol.* **72**:2280-2288.
- Schutten, M., A. McKnight, R.C. Huisman, M. Thali, McKeating, JA, J. Sodroski, J. Goudsmit, and A.D. Osterhaus.** 1993. Further characterization of an antigenic site of HIV-1 gp120 recognized by virus neutralizing human monoclonal antibodies. *AIDS* **7**:919-923.
- Schwartz, O., Y. Riviere, J.M. Heard, and O. Danos.** 1993. Reduced cell surface expression of processed human immunodeficiency virus type 1 envelope glycoprotein in the presence of Nef. *J.Virol.* **67**:3274-3280.

- Schwartz, S., B.K. Felber, E.M. Fenyo, and G.N. Pavlakis.** 1990. Env and Vpu proteins of human immunodeficiency virus type 1 are produced from multiple bicistronic mRNAs. *J.Virol.* **64**:5448-5456.
- Schwartz, S., B.K. Felber, and G.N. Pavlakis.** 1992a. Mechanism of translation of monocistronic and multicistronic human immunodeficiency virus type 1 mRNAs. *Molecular & Cellular Biology* **12**:207-219.
- Schwartz, S., B.K. Felber, and G.N. Pavlakis.** 1992b. Distinct RNA sequences in the gag region of human immunodeficiency virus type 1 decrease RNA stability and inhibit expression in the absence of Rev protein. *J.Virol.* **66**:150-159.
- Schwartz, S., M. Campbell, G. Nasioulas, J. Harrison, Felber, BK, and G.N. Pavlakis.** 1992c. Mutational inactivation of an inhibitory sequence in human immunodeficiency virus type 1 results in Rev-independent gag expression. *J.Virol.* **66**:7176-7182.
- Sharpe, S.A., A.M. Whatmore, G.A. Hall, and M.P. Cranage.** 1997. Macaques infected with attenuated simian immunodeficiency virus resist superinfection with virulence-revertant virus. *Journal of General Virology* **78**:1923-1927.
- Shearer, G.M., L.A. Pinto, and M. Clerici.** 1999. Alloimmunization for immune-based therapy and vaccine design against HIV/AIDS. [Review] [49 refs]. *Immunology Today* **20**:66-71.
- Shioda, T., S. Oka, S. Ida, K. Nohikara, Toriyoshi, S. Mori, Y. Takebe, S. Kimura, K. Shimada, Y. Nagai *et al.*** 1994. A naturally occurring single basic amino acid substitution in the V3 region of the human immunodeficiency virus type 1 env protein alters the cellular host range and antigenic structure of the virus. *J. Virol.* **68**: 7689-7696.
- Shirakawa, F. and S.B. Mizel.** 1989. In vitro activation and nuclear translocation of NF-kappa B catalyzed by cyclic AMP-dependent protein kinase and protein kinase C. *Molecular & Cellular Biology* **9**:2424-2430.
- Shiramizu, B., B.G. Herndier, and M.S. McGrath.** 1994. Identification of a common clonal human immunodeficiency virus integration site in human immunodeficiency virus-associated lymphomas. *Cancer Research* **54**:2069-2072.
- Simmonds, P., P. Balfe, J.F. Peutherer, C.A. Ludlam, J.O. Bishop, and A.J. Brown.** 1990a. Human immunodeficiency virus-infected individuals contain provirus in small numbers of peripheral mononuclear cells and at low copy numbers. *J.Virol.* **64**:864-872.
- Simmonds, P., P. Balfe, C.A. Ludlam, J.O. Bishop, and A.J. Brown .** 1990b. Analysis of sequence diversity in hypervariable regions of the external glycoprotein of human immunodeficiency virus type 1. *J.Virol.* **64**:5840-5850.
- Simmonds, P., L.Q. Zhang, F. McOmish, P. Balfe, C.A. Ludlam, and A.J. Brown.** 1991. Discontinuous sequence change of human immunodeficiency virus (HIV) type 1 env sequences in plasma viral and lymphocyte-associated proviral populations in vivo: implications for models of HIV pathogenesis. *J.Virol.* **65**:6266-6276.

- Simmons, G., D. Wilkinson, J.D. Reeves, M.T. Dittmar, Beddows, J. Weber, G. Carnegie, U. Desselberger, P.W. Gray, R.A. Weiss, and P.R. Clapham.** 1996. Primary, syncytium-inducing human immunodeficiency virus type 1 isolates are dual-tropic and most can use either Lestr or CCR5 as coreceptors for virus entry. *J.Virol.* **70**:8355-8360.
- Simmons, G., J.D. Reeves, A. McKnight, N. DeJucq, S. Hibbitts, C.A. Power, E. Aarons, D. Schols, E. De Clercq, A.E. Proudfoot, and P.R. Clapham.** 1998. CXCR4 as a functional coreceptor for human immunodeficiency virus type 1 infection of primary macrophages. *J.Virol.* **72**:8453-8457.
- Simon, F., P. Mauclore, P. Roques, I. Loussert-Ajaka, M.C. Muller-Trutwin, S. Saragosti, M.C. Georges-Courbot, F. Barre-Sinoussi, and F. Brun-Vezinet.** 1998. Identification of a new human immunodeficiency virus type 1 distinct from group M and group O. *Nature Medicine* **4**:1032-1037.
- Sodroski, J., C. Rosen, F. Wong-Staal, S.Z. Salahuddin, M. Popovic, S. Arya, R.C. Gallo, and W.A. Haseltine.** 1985. Trans-acting transcriptional regulation of human T-cell leukemia virus type III long terminal repeat. *Science* **227**:171-173.
- Soto-Ramirez, L.E., B. Renjifo, M.F. McLane, R. Marlink, C. O'Hara, R. Sutthent, C. Wasi, P. Vithayasai, V. Vithayasai, C. Apichartpiyakul, P. Auewarakul, C. Pena, V. D.S. Chui, R. Osathanondh, K. Mayer, T.H. Lee, and M. Essex.** 1996. HIV-1 Langerhans' cell tropism associated with heterosexual transmission of HIV [see comments]. *Science* **271**:1291-1293.
- Spear, G.T., B.L. Sullivan, A.L. Landay, and T.F. Lint.** 1990. Neutralization of human immunodeficiency virus type 1 by complement occurs by viral lysis. *J.Virol.* **64**:5869-5873.
- Spear, G.T., D.M. Takefman, B.L. Sullivan, A.L. Landay, Jennings, MB, and J.R. Carlson.** 1993. Anti-cellular antibodies in sera from vaccinated macaques can induce complement-mediated virolysis of human immunodeficiency virus and simian immunodeficiency virus. *Virology* **195**:475-480.
- Speck, R.F., K. Wehrly, E.J. Platt, R.E. Atchison, I.F. Charo, D. Kabat, Chesebro, and M.A. Goldsmith.** 1997. Selective employment of chemokine receptors as human immunodeficiency virus type 1 coreceptors determined by individual amino acids within the envelope V3 loop. *J.Virol.* **71**:7136-7139.
- Spira, A.I., P.A. Marx, B.K. Patterson, J. Mahoney, R.A. Koup, S.M. Wolinsky, and D.D. Ho.** 1996. Cellular targets of infection and route of viral dissemination after an intravaginal inoculation of simian immunodeficiency virus into rhesus macaques. *Journal of Experimental Medicine* **183** :215-225.
- Stamatos, N.M., J.R. Mascola, V.S. Kalyanaraman, M.K. Louder, L.M. Frampton, D.L. Birx, and T.C. VanCott.** 1998. Neutralizing antibodies from the sera of human immunodeficiency virus type 1-infected individuals bind to monomeric gp120 and oligomeric gp140. *J.Virol.* **72**:9656-9667.
- Steel, C.M., C.A. Ludlam, D. Beatson, J.F. Peutherer, Cuthbert, RJG, P. Simmonds, H. Morrison, and M. Jones.** 1988. HLA haplotype A1 B8 DR3 as a risk factor for HIV-related disease. *Lancet* **1**:1185-1188.

- Stevenson, M., C. Meier, A.M. Mann, N. Chapman, and A. Wasiaik.** 1988. Envelope glycoprotein of HIV induces interference and cytolysis resistance in CD4+ cells: mechanism for persistence in AIDS. *Cell* **53**:483-496.
- Stott, E.J.** 1991. Anti-cell antibody in macaques [letter] [see comments]. *Nature* **353**:393
- Terwilliger, E., J. Proulx, J. Sodroski, and W.A. Haseltine.** 1988. Cell lines that express stably env gene products from three strains of HIV-1. *Journal of Acquired Immune Deficiency Syndromes* **1**:317-323.
- Thali, M., A. Bukovsky, E. Kondo, B. Rosenwirth, C.T. Walsh, J. Sodroski, and H.G. Gottlinger.** 1994. Functional association of cyclophilin A with HIV-1 virions [see comments]. *Nature* **372**:363-365.
- Thorn, R.M., G.A. Beltz, C.H. Hung, B.F. Fallis, S. Winkle, Cheng, KL, and D.J. Marciani.** 1987. Enzyme immunoassay using a novel recombinant polypeptide to detect human immunodeficiency virus env antibody. *Journal of Clinical Microbiology* **25**:1207-1212.
- Trkola, A., M. Purtscher, T. Muster, C. Ballaun, A. Buchacher, N. Sullivan, K. Srinivasan, J. Sodroski, J.P. Moore, and H. Katinger.** 1996. Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. *J.Virol.* **70**:1100-1108.
- Tscherning, C., A. Alaeus, R. Fredriksson, A. Bjorndal, Deng, D.R. Littman, E.M. Fenyo, and J. Albert.** 1998. Differences in chemokine coreceptor usage between genetic subtypes of HIV-1. *Virology* **241**:181-188.
- Valentin, A., J. Albert, E.M. Fenyo, and B. Asjo.** 1994. Dual tropism for macrophages and lymphocytes is a common feature of primary human immunodeficiency virus type 1 and 2 isolates. *J.Virol.* **68**:6684-6689.
- van Leeuwen, F. and R. Nusse.** 1995. Oncogene activation and oncogene cooperation in MMTV-induced mouse mammary cancer. [Review] [91 refs]. *Seminars in Cancer Biology* **6**:127-133.
- VanCott, T.C., V.R. Polonis, L.D. Loomis, N.L. Michael, P.L. Nara, and D.L. Birx.** 1995. Differential role of V3-specific antibodies in neutralization assays involving primary and laboratory-adapted isolates of HIV type 1. *AIDS Research & Human Retroviruses* **11**:1379-1391.
- VanCott, T.C., J.R. Mascola, R.W. Kaminski, V. Kalyanaraman, P.L. Hallberg, P.R. Burnett, J.T. Ulrich, D.J. Rechtman, and D.L. Birx.** 1997. Antibodies with specificity to native gp120 and neutralization activity against primary human immunodeficiency virus type 1 isolates elicited by immunization with oligomeric gp160. *J.Virol.* **71**:4319-4330.
- Vartanian, J.P., A. Meyerhans, B. Asjo, and S. Wain-Hobson.** 1991. Selection, recombination, and G----A hypermutation of human immunodeficiency virus type 1 genomes. *J.Virol.* **65**:1779-1788.
- Veazey, R.S., M. DeMaria, L.V. Chalifoux, D.E. Shvetz, Pauley, DR, H.L. Knight, M. Rosenzweig, R.P. Johnson, R.C. Desrosiers, and A.A. Lackner.** 1998.

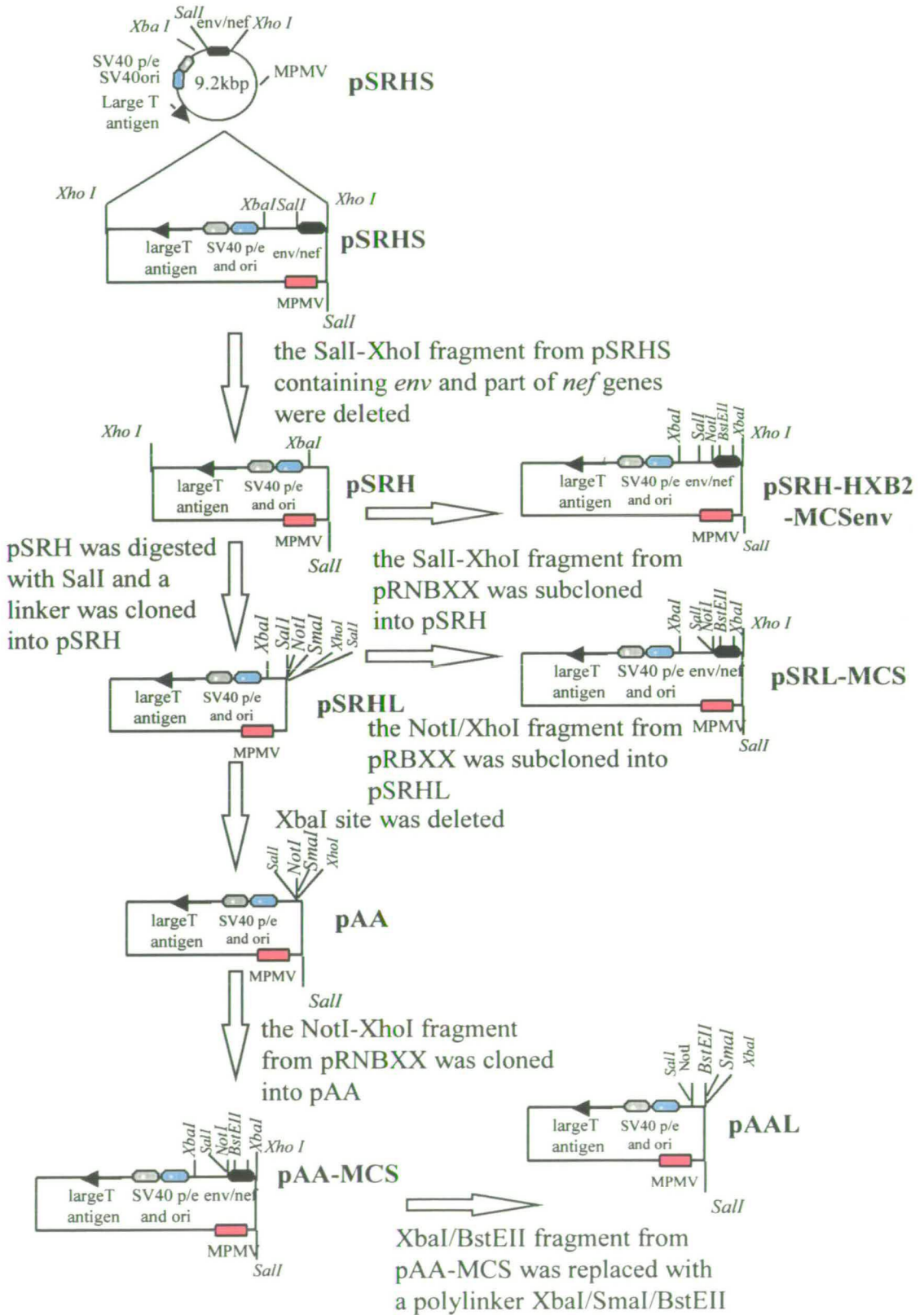
- Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. *Science* **280**:427-431.
- Vink, C. and R.H. Plasterk.** 1993a. The human immunodeficiency virus integrase protein. [Review] [47 refs]. *Trends in Genetics* **9**:433-438.
- Vink, C., G.A. Oude, and R.H. Plasterk.** 1993b. Identification of the catalytic and DNA-binding region of the human immunodeficiency virus type I integrase protein. *Nucleic Acids Research* **21**:1419-1425.
- Vodicka, M.A., D.M. Koepf, P.A. Silver, and M. Emerman.** 1998. HIV-1 Vpr interacts with the nuclear transport pathway to promote macrophage infection. *Genes & Development* **12**:175-185.
- Vogelstein, B. and D. Gillespie.** 1979. Preparative and analytical purification of DNA from agarose. *Proceedings of the National Academy of Sciences of the United States of America* **76**:615-619.
- Wang, B., J. Boyer, V. Srikantan, K. Ugen, L. Gilbert, Phan, K. Dang, M. Merva, M.G. Agadjanyan, and M. Newman.** 1995a. Induction of humoral and cellular immune responses to the human immunodeficiency type 1 virus in nonhuman primates by in vivo DNA inoculation. *Virology* **211**:102-112.
- Wang, B., J.D. Boyer, K.E. Ugen, V. Srikantan, V. Ayyaroo, M.G. Agadjanyan, W.V. Williams, M. Newman, L. Coney, and R. Carrano.** 1995b. Nucleic acid-based immunization against HIV-1: induction of protective in vivo immune responses. [Review] [66 refs]. *AIDS* **9 Suppl A**:S159-S170
- Wang, Y., Z. Xiang, S. Pasquini, and H.C. Ertl.** 1997. The use of an E1-deleted, replication-defective adenovirus recombinant expressing the rabies virus glycoprotein for early vaccination of mice against rabies virus. *J.Virol.* **71**:3677-3683.
- Warren, R.Q., S.A. Anderson, W.M. Nkya, J.F. Shao, C.W. Hendrix, G.P. Melcher, R.R. Redfield, and R.C. Kennedy.** 1992. Examination of sera from human immunodeficiency virus type 1 (HIV-1)-infected individuals for antibodies reactive with peptides corresponding to the principal neutralizing determinant of HIV-1 gp120 and for in vitro neutralizing activity. *J.Virol.* **66**:5210-5215.
- Wei, X., S.K. Ghosh, M.E. Taylor, V.A. Johnson, E.A. Emini, P. Deutsch, J.D. Lifson, S. Bonhoeffer, M.A. Nowak, and B.H. Hahn.** 1995. Viral dynamics in human immunodeficiency virus type 1 infection [see comments]. *Nature* **373**:117-122.
- Weiler, B.E., H. Schacke, M. Bachmann, L. Brigido, M. Gilbert, J. Mills, E. Matthes, J.M. Forrest, and W.E. Muller.** 1991. Human immunodeficiency virus: novel enzyme-linked immunoassays for quantitation of envelope glycoprotein 120. *Journal of Virological Methods* **32**:287-301.
- Weiss, C.D. and J.M. White.** 1993. Characterization of stable Chinese hamster ovary cells expressing wild-type, secreted, and glycosylphosphatidylinositol-anchored human immunodeficiency virus type 1 envelope glycoprotein. *J.Virol.* **67**:7060-7066.

- Wells, D.E. and R.W. Compans.** 1990. Expression and characterization of a functional human immunodeficiency virus envelope glycoprotein in insect cells. *Virology* **176**:575-586.
- Wen, W., J.L. Meinkoth, R.Y. Tsien, and S.S. Taylor.** 1995. Identification of a signal for rapid export of proteins from the nucleus. *Cell* **82**:463-473.
- Weniger, B.G., Y. Takebe, C.Y. Ou, and S. Yamazaki.** 1994. The molecular epidemiology of HIV in Asia. [Review] [156 refs]. *AIDS* **8 Suppl 2**:S13-S28
- Whatmore, A.M., N. Cook, G.A. Hall, S. Sharpe, E.W. Rud, Cranage, and MP.** 1995. Repair and evolution of nef in vivo modulates simian immunodeficiency virus virulence. *J.Virol.* **69**:5117-5123.
- Willey, R.L., J.S. Bonifacino, B.J. Potts, M.A. Martin, Klausner, and RD.** 1988. Biosynthesis, cleavage, and degradation of the human immunodeficiency virus 1 envelope glycoprotein gp160. *Proceedings of the National Academy of Sciences of the United States of America* **85**:9580-9584.
- Willey, R.L., A. Buckler-White, and K. Strebel.** 1994. Sequences present in the cytoplasmic domain of CD4 are necessary and sufficient to confer sensitivity to the human immunodeficiency virus type 1 Vpu protein. *J.Virol.* **68**:1207-1212.
- Winkler, C., W. Modi, M.W. Smith, G.W. Nelson, X. Wu, M. Carrington, M. Dean, T. Honjo, K. Tashiro, D. Yabe, S. Buchbinder, E. Vittinghoff, J.J. Goedert, T.R. O'Brien, L.P. Jacobson, R. Detels, Donfield, A. Willoughby, E. Gomperts, D. Vlahov, J. Phair, and S.J. O'Brien.** 1998. Genetic restriction of AIDS pathogenesis by an SDF-1 chemokine gene variant. ALIVE Study, Hemophilia Growth and Development Study (HGDS), Multicenter AIDS Cohort Study (MACS), Multicenter Hemophilia Cohort Study (MHCS), San Francisco City Cohort (SFCC) [see comments]. *Science* **279**:389-393.
- Winslow, D.L., E.D. Anton, R.A. Horlick, R.J. Zagursky, R.J. Tritch, H. Scarnati, K. Ackerman, and L.T. Bachelier.** 1994. Construction of infectious molecular clones of HIV-1 containing defined mutations in the protease gene. *Biochemical & Biophysical Research Communications* **205**:1651-1657.
- Winter, N., M. Lagranderie, S. Gangloff, C. Leclerc, M. Gheorghiu, and B. Gicquel.** 1995. Recombinant BCG strains expressing the SIVmac251nef gene induce proliferative and CTL responses against nef synthetic peptides in mice. *Vaccine* **13**:471-478.
- Wolinsky, S.M., B.T. Korber, A.U. Neumann, M. Daniels, Kunstman, KJ, A.J. Whetsell, M.R. Furtado, Y. Cao, D.D. Ho, and J.T. Safrit.** 1996. Adaptive evolution of human immunodeficiency virus-type 1 during the natural course of infection [see comments]. *Science* **272**:537-542.
- Wong, J.K., H.F. Gunthard, D.V. Havlir, Z.Q. Zhang, A.T. Haase, C.C. Ignacio, S. Kwok, E. Emini, and D.D. Richman.** 1997. Reduction of HIV-1 in blood and lymph nodes following potent antiretroviral therapy and the virologic correlates of treatment failure. *Proceedings of the National Academy of Sciences of the United States of America* **94**:12574-12579.

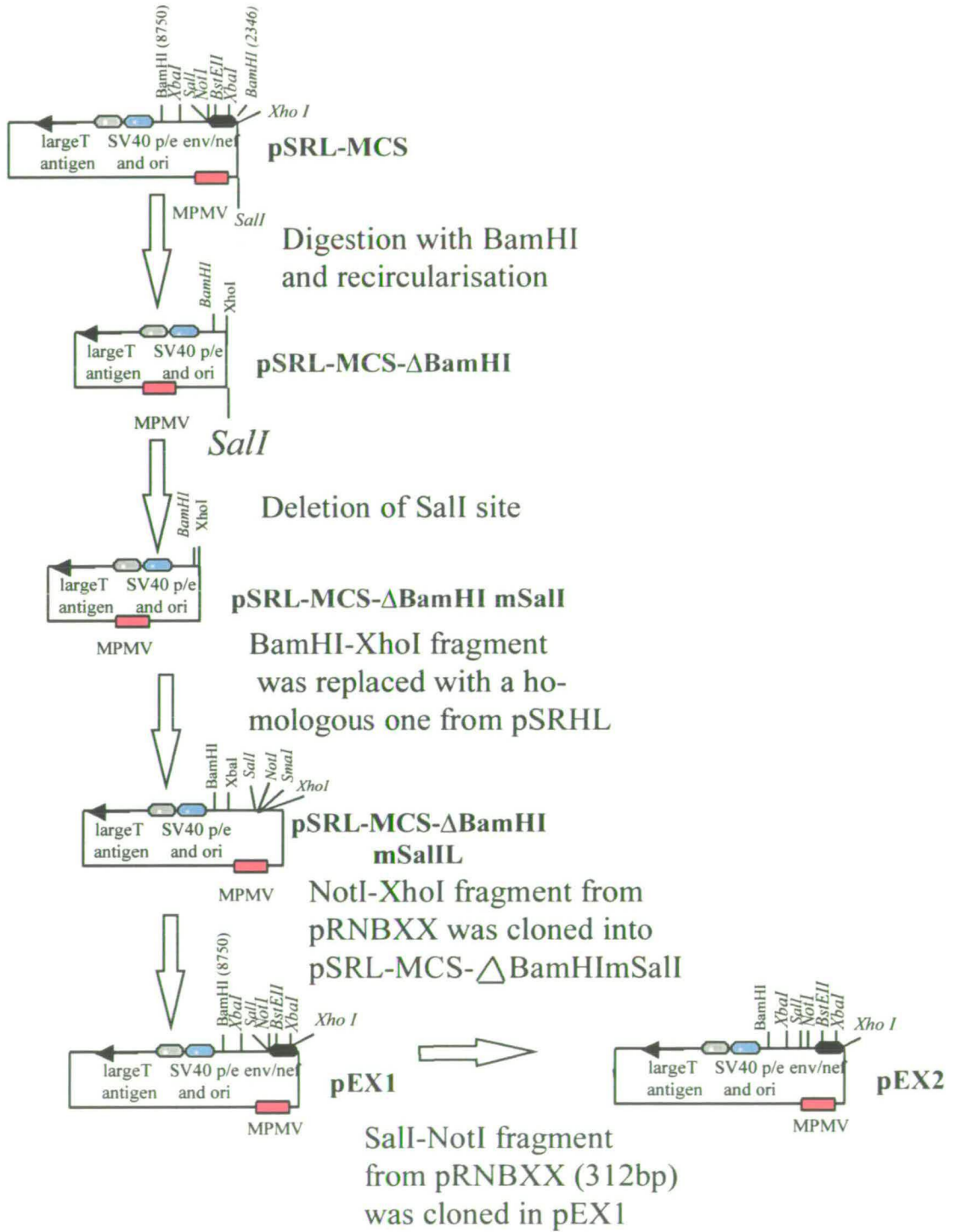
- Wu, F.K., J.A. Garcia, D. Harrich, and R.B. Gaynor.** 1988. Purification of the human immunodeficiency virus type 1 enhancer and TAR binding proteins EBP-1 and UBP-1. *EMBO Journal* 7:2117-2130.
- Wu, S., D.W. Pascual, G.K. Lewis, and D.M. Hone.** 1997. Induction of mucosal and systemic responses against human immunodeficiency virus type 1 glycoprotein 120 in mice after oral immunization with a single dose of a Salmonella-HIV vector. *AIDS Research & Human Retroviruses* 13:1187-1194.
- Wyatt, R., P.D. Kwong, E. Desjardins, R.W. Sweet, J. Robinson, W.A. Hendrickson, and J.G. Sodroski.** 1998. The antigenic structure of the HIV gp120 envelope glycoprotein [see comments]. *Nature* 393:705-711.
- Yahi, N., J.M. Sabatier, P. Nickel, K. Mabrouk, F. Gonzalez-Scarano, and J. Fantini.** 1994a. Suramin inhibits binding of the V3 region of HIV-1 envelope glycoprotein gp120 to galactosylceramide, the receptor for HIV-1 gp120 on human colon epithelial cells. *Journal of Biological Chemistry* 269:24349-24353.
- Yahi, N., S.L. Spitalnik, K.A. Stefano, P. De Micco, F. Gonzalez-Scarano, and J. Fantini.** 1994b. Interferon-gamma decreases cell surface expression of galactosyl ceramide, the receptor for HIV-1 GP120 on human colonic epithelial cells [published erratum appears in *Virology* 1995 Feb 20;207(1):343]. *Virology* 204:550-557.
- Zapp, M.L., T.J. Hope, T.G. Parslow, and M.R. Green.** 1991. Oligomerization and RNA binding domains of the type 1 human immunodeficiency virus Rev protein: a dual function for an arginine-rich binding motif. *Proceedings of the National Academy of Sciences of the United States of America* 88:7734-7738.
- Zhang, Y.J., T. Dragic, Y. Cao, L. Kostrikis, D.S. Kwon, D.R. Littman, V.N. KewalRamani, and J.P. Moore.** 1998a. Use of coreceptors other than CCR5 by non-syncytium-inducing adult and pediatric isolates of human immunodeficiency virus type 1 is rare in vitro. *J. Virol.* 72:9337-9344.
- Zhang, Z.Q., D.W. Notermans, G. Sedgewick, W. Cavert, Wietgreffe, M. Zupancic, K. Gebhard, K. Henry, L. Boies, Z. Chen, M. Jenkins, Mills, H. McDade, C. Goodwin, C.M. Schuwirth, S.A. Danner, and A.T. Haase.** 1998b. Kinetics of CD4+ T cell repopulation of lymphoid tissues after treatment of HIV-1 infection. *Proceedings of the National Academy of Sciences of the United States of America* 95:1154-1159.
- Zhong, P., M. Peeters, W. Janssens, K. Fransen, L. Heyndrickx, G. Vanham, B. Willems, P. Piot, and G. van der Groen.** 1995. Correlation between genetic and biological properties of biologically cloned HIV type 1 viruses representing subtypes A, B, and D. *AIDS Research & Human Retroviruses* 11:239-248.
- Zolla-Pazner, S., C. Alving, R. Belshe, P. Berman, S. Burda, P. Chigurupati, M.L. Clements, A.M. Duliege, J.L. Excler, C. Hioe, J. Kahn, M.J. McElrath, S. Sharpe, F. Sinangil, K. Steimer, Walker, MC, N. Wassef, and S. Xu.** 1997. Neutralization of a clade B primary isolate by sera from human immunodeficiency virus-uninfected recipients of candidate AIDS vaccines [see comments]. *J. Infect. Dis.* 175:764-774.

- Zolla-Pazner, S., S. Xu, S. Burda, Duliege, AM, J.L. Excler, and M.L. Clements-Mann.** 1998a. Neutralization of syncytium-inducing primary isolates by sera from human immunodeficiency virus (HIV)-uninfected recipients of candidate HIV vaccines. *J.Infect.Dis.* **178**:1502-1506.
- Zolla-Pazner, S., M. Lubeck, S. Xu, S. Burda, R.J. Natuk, F. Sinangil, K. Steimer, R.C. Gallo, J.W. Eichberg, T. Matthews, and M. Robert-Guroff.** 1998b. Induction of neutralizing antibodies to T-cell line-adapted and primary human immunodeficiency virus type 1 isolates with a prime-boost vaccine regimen in chimpanzees. *J.Virol.* **72**:1052-1059.
- Zwart, G., T.F. Wolfs, M. Valk, L. Van der Hoek, C.L. Kuiken, and J. Goudsmit.** 1992. Characterization of the specificity of the human antibody response to the V3 neutralization domain of HIV-1. *AIDS Research & Human Retroviruses* **8**:1897-1908.
- Zwart, G., N.K. Back, C. Ramautarsing, M. Valk, H. van der, and J. Goudsmit.** 1994. Frequent and early HIV-1MN neutralizing capacity in sera from Dutch HIV-1 seroconverters is related to antibody reactivity to peptides from the gp120 V3 domain. *AIDS Research & Human Retroviruses* **10**:245-251.

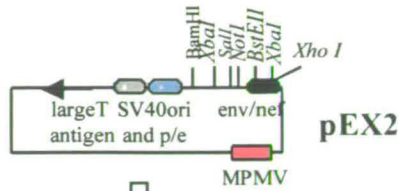
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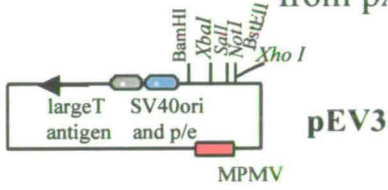
Annex 2 (page 1)



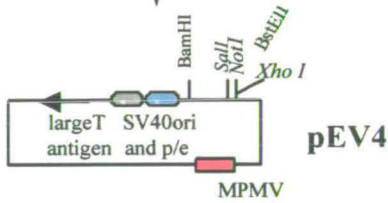
Annex 2 (page 2)



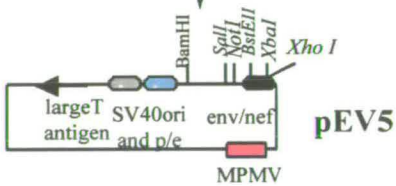
NotI-XhoI from pEX2 was replaced with a fragment containing a linker NotI/BstEII/XhoI from pAD5



Xba I site was deleted



BstEII-XhoI fragment from pRNBXX containing env was subcloned into pEV4



BstEII-XbaI fragment from pEV5 was replaced by a polylinker (BstEII/SmaI/XbaI)

