

CHAPTER 1: INTRODUCTION

1.1 HIV/AIDS pandemic

Acquired Immunodeficiency Syndrome (AIDS) is an infectious disease with a variable period between infection with human immunodeficiency virus type-1 (HIV-1) and the development of an AIDS-defining condition leading to death, ranging from months to more than 16 years (Hessol and Palacio, 1996). The first cases of HIV-1/AIDS were reported in the early 1980s (Barre-Sinoussi et al., 1983; Gallo et al., 1984; Gottlieb et al., 1981a; Gottlieb et al., 1981b; Montagnier et al., 1984). It was estimated that in 2004 there were 39.4 million people living with HIV/AIDS worldwide and 4.9 million newly infected people (<http://www.unaids.org>). During this time AIDS accounted for about 3.1 million deaths. The majority of these 25.4 (64% of the world's HIV-1-infected population) infections occurred in sub-Saharan Africa, with 3.1 million new infections and 2.3 million deaths from AIDS.

According to the United Nations, South Africa has the world's highest incidence of HIV-1 infections with 5.3 million people or an estimated one out of every five adults living with HIV/AIDS in 2004 (<http://www.unaids.org>). The highest prevalence rate of 30% was among young people between the ages of 21-29. In South Africa HIV-1 is spread predominantly through heterosexual contact, and subtype C is the predominant strain that circulates in the country (Bredell et al., 1998; Van Harmelen et al., 1999). The Actuarial Society of South Africa 2004 (ASSA2004) demographic modeling estimated that approximately 10% (500 000) of HIV-1 infected South Africans in 2004 were living with HIV-1/AIDS and required treatment.

This situation highlights the importance of undertaking scientifically proven interventions, preferably based on the circulating subtype in South Africa. There are 20 United States Food and Drug Administration (FDA) approved antiretroviral drugs, but neither diagnosis of nor treatment for HIV-1 infection has sufficiently penetrated the developing world to affect the pandemic (Graham and Mascola, 2005). For these reasons, many believe that the development of a vaccine will ultimately be necessary to combat the disease.

1.2 Natural history of HIV-1 infection

HIV-1 primarily infects human CD4⁺ T-cells and macrophages, although other cells can also be infected. The primary binding site for HIV-1 is the CD4 molecule on the target cell, and this interaction is mediated by the viral glycoprotein gp120 on the viral surface (Hill et al., 1997; Trkola et al., 1996a; Wu et al., 1996). Primary infection occurs between initial exposure (infection) and the appearance of HIV-1 antibodies at seroconversion (detection of HIV-1 antibodies in the blood stream of infected person). This acute phase of infection may be subclinical or accompanied by symptoms that last for a few days or up to a couple of weeks and is accompanied by a decrease of CD4⁺-T lymphocytes and rise in plasma viremia. In the 2-3 week incubation period following transmission (by sexual, oral or parental routes), the virus becomes established in a lymphatic tissue reservoir in CD4⁺ T-cells, macrophages, follicular dendritic cells (FDCs) and monocyte compartments where some of it is integrated as a silent provirus (Fig 1) (Blankson, Persaud, and Siliciano, 2002; Haase, 1999). From acute stage until the late stages of infection, fluctuating levels of virions are produced daily, mainly by activated CD4⁺ T cells (Douek et al., 2002). Following acute infection, cellular immune responses to HIV-1 develop and this serves to partially control viral replication

(Koup et al., 1994). Between 50-90% of HIV-1 infected individuals experience various opportunistic infections such as *Candida albicans* infection, *Pneumocystis carinii* pneumonia, lung disease caused by *Mycobacterium tuberculosis*, skin tumors *Kaposi sarcoma*, fatigue, headache and swollen lymph nodes with the onset of AIDS (Boshoff and Weiss, 2002; Vanhems et al., 1999).

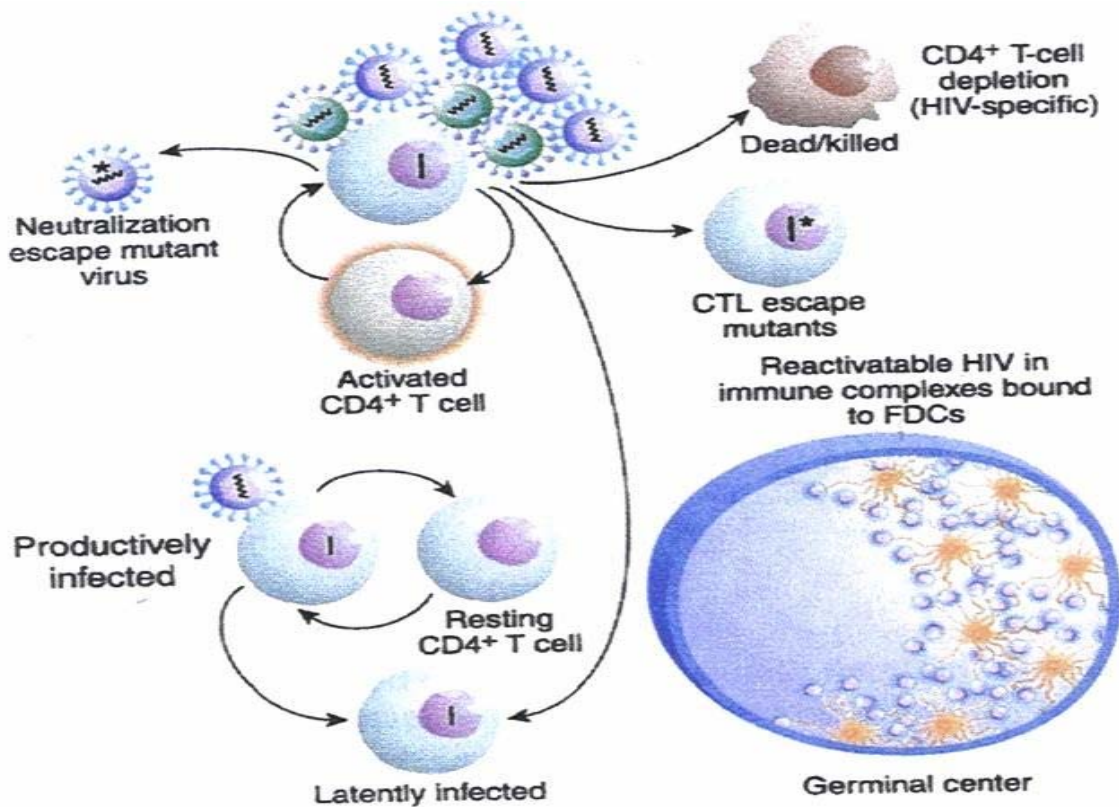


Figure 1. Lymphatic tissue reservoirs for HIV-1 and the potential obstacles the immune system faces in clearing the virus. HIV-1 is stored in immune complexes bound by FDCs where it persists in latently infected CD4⁺ T-cells. Replication of the virus generates antigenic escape mutants and depletes CD4⁺ T-cells. FDCs are also known to reactivate infectious virus from large FDC stores (Pope and Haase, 2003).

The onset of clinical symptoms differs among infected individuals with some individuals progressing rapidly towards AIDS and others remaining chronically infected over many

years. Some infected individuals, about 5%, remain asymptomatic for over 10 years, and are described as long-term non-progressors (LTNP), while others show signs of disease progression within a few years after seroconversion (fast progressors) (Buchbinder et al., 1994; Sheppard et al., 1993).

Depending on the viral load in the infected individual a viral set point is established that determines the rate of the disease progression (Mellors et al., 1996). The pace of disease progression can be measured through several immunological and virological predictive markers, namely: CD4 count and viral load (measured in RNA copies/ml). One strong predictive marker for disease progression to AIDS is the gradual decline in the total number of CD4⁺ T-cells (de Wolf et al., 1997), and this is accompanied by an increased RNA load (Koup et al., 1994; Mellors et al., 1997; Mellors et al., 1996). The appearance of X4 (syncitium-inducing in MT-2 cells) viral variants is also linked to a faster disease progression in subtype B (Connor et al., 1997; Karlsson et al., 1994). Ultimately, a progressive HIV-1 infection leads to immunosuppression, and AIDS in the majority of affected individuals.

Many researchers have focused their attention in elucidating the role of neutralizing antibodies (Nab) during the course of HIV-1 infection (Buchacher et al., 1994; Colman, 1988; Cornelissen et al., 1995; Ferrantelli and Ruprecht, 2002; Moore et al., 1996; Parren et al., 1998a). Higher titres in LTNP of Nab antibodies to HIV-1 can be detected (Pilgrim et al., 1997; Zhang et al., 1997). In these studies, it was observed that broadly Nabs develop over time and can be detected more frequently in asymptomatic individual than in patients in late stages of the disease (Albert et al., 1987). Nabs have been found in different individuals

regardless of the genetic subtypes of HIV-1 infecting the person (Burton and Montefiori, 1997; Weber et al., 1996).

1.3 Serology of HIV-1 infection

Infection with HIV-1 is accompanied by cellular and humoral immune response of different magnitude and specificities that participate in regulating plasma viremia during primary infection (Burton and Montefiori, 1997; Mackewicz et al., 1994). HIV-1 specific cytotoxic lymphocyte (CTL) responses are shown to be good correlate of the initial decline in plasma viremia and it is thought to be the dominant antiviral immune response during primary infection (Koup et al., 1994). Seroconversion occurs usually 1-3 months following infection with HIV-1. Between 1-2 weeks before seroconversion, HIV p24 antigens which are viral core proteins and one of the most abundant proteins produced by infected cells can be detected. This situation can be seen in acute infection and when viral load is high. As the antibody levels rise, the levels of p24 antigen declines as the antigen is bound by the antibodies. In the initial phase, IgM is the dominant immunoglobulin (Ig) and soon thereafter IgG takes over (Fig 2) (Van de Peere et al., 1992). However, there are questions as to the effectiveness of this early antibody response against the virus (Burton, 1997).

It was previously thought that most individuals do not develop antibodies capable of neutralizing their own virus (autologous neutralization) until some time after plasma viremia has declined (Burton and Montefiori, 1997; Koup et al., 1994). Antibodies that neutralize T-cell line adapted (TCLA) virus strains were detected sooner than autologous neutralizing antibodies, although these antibodies did not coincide with initial decline in plasma viremia

(Burton and Montefiori, 1997). Given these observations, it was believed that the slow development of neutralizing antibodies during primary HIV-1 infection might be one of the reasons why the virus is able to establish persistent infection. However, recent reports indicate that autologous Nab are found as early as 52 days after detection of HIV-1-specific antibodies (Richman et al., 2003; Wei et al., 2003). Here it is reported that viral inhibitory activity of Nab resulted in complete replacement of neutralization-sensitive virus by successive populations of resistant virus. Escape virus contained mutations in the *env* gene that did not map to known neutralizing epitopes, and involve primarily changes in N-linked glycosylation (Wei et al., 2003).

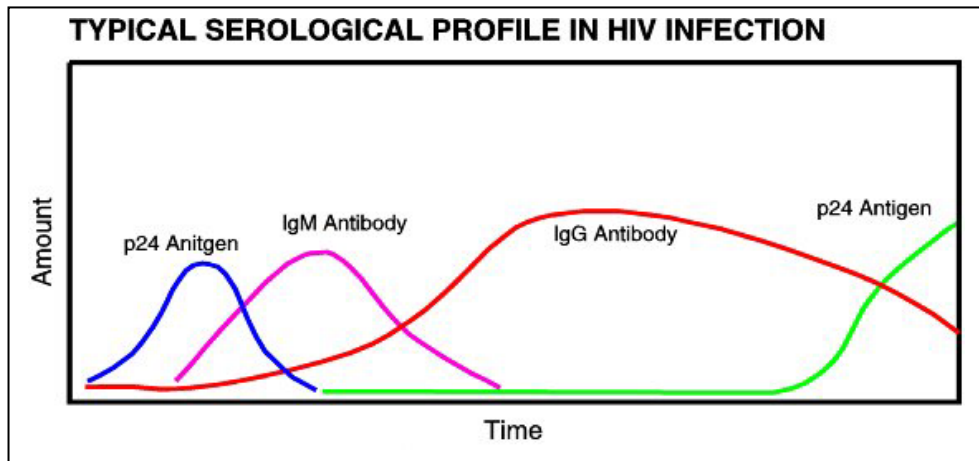


Fig 2. Serological profile of HIV-1 infection in human (<http://www.labcorp.com>).

1.4 Virus structure

HIV-1 is an enveloped virus with a target cell derived lipid membrane (Fig 3). In addition to many of the original, approximately 72 functional spikes, HIV-1 particles carry in their membranes numerous host cell-derived glycoproteins and serum proteins nonspecifically attached to the virion surface (Arthur et al., 1992; Cantin, Fortin, and Tremblay, 1996; Hoxie et al., 1987; Orentas and Hildreth, 1993). The envelope is made first as a monomeric precursor gp160 molecule which oligomerizes to a trimer for transport from the endoplasmic reticulum to plasma membrane (Wyatt and Sodroski, 1998). During transport, the gp160 is cleaved into gp120 and gp41 molecules. The gp41 becomes anchored to cellular or viral membranes and interacts non-covalently with extracellularly expressed gp120 to form the native oligomer. The native oligomer is said to undergo certain changes, to lose or shed gp120 molecules to generate free monomeric gp120 molecules and oligomeric gp41 anchored in the cell or viral membrane. Many of the original spikes have shed their gp120 subunits and may display a conformationally irrelevant postfusion gp41 (McMichael and Hanke, 2003; Wyatt et al., 1998; Wyatt and Sodroski, 1998). The remaining intact spikes are highly glycosylated, flexible on the viral surface and may differ by about 10% of amino acids between different HIV-1 virions within an individual at a particular time point.

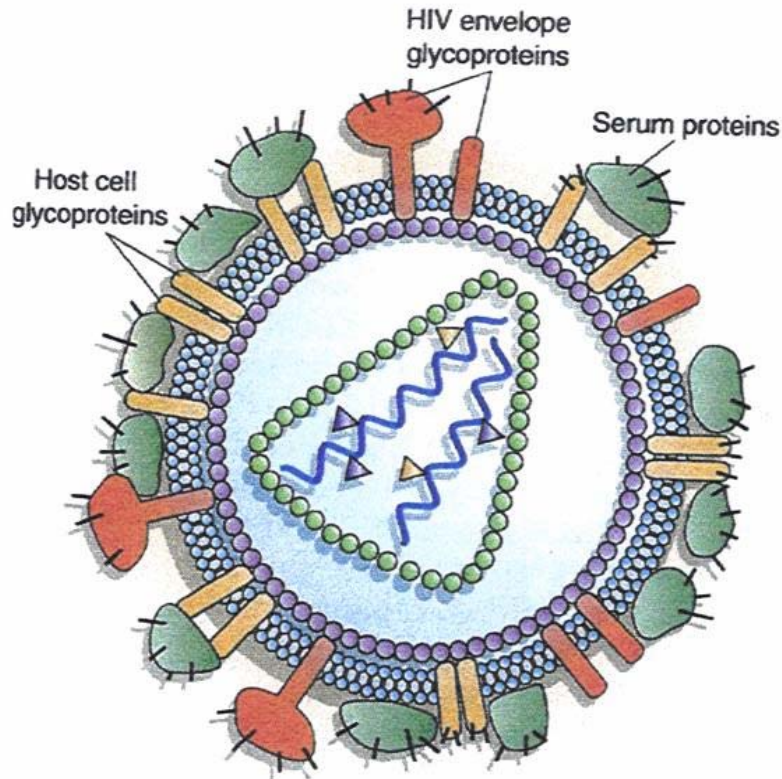


Figure 3. Diagram showing HIV-1 structure. The envelope spikes are indicated in red, numerous host cell-derived glycoproteins particles are shown in orange and an array of serum proteins non-specifically attached to the virion surface are coloured green. The envelope spikes are the major target for neutralizing antibodies (McMichael and Hanke, 2003).

1.5 Cellular tropism and interaction of HIV-1 with co-receptor molecules

In addition to its primary receptor molecule, CD4, the chemokine coreceptor molecules further regulate the type of cells HIV-1 can infect. These receptor molecules, mainly CCR5 and CXCR4 serve as obligate second receptors for viral entry (Berger, Murphy, and Farber, 1999). The chemokine receptors are large molecules spanning the cell membrane seven times, with the extracellular and intracellular loops connected by disulphide bonds forming a barrel-like structure (Bjorndal et al., 1997; Premack and Schall, 1996). CCR5 is a *beta* (β)

chemokine containing two cysteines linked (C-C) receptor, whereas *alpha* (α) or C-X-C chemokines have an intervening amino acid between the first two cysteines. CCR5 is an essential co-factor for fusion of HIV-1 strains of the non-syncytium inducing (NSI) phenotype with CD4⁺ T-cells, macrophages and primary T-cells (Dragic et al., 1996). In contrast, gp120 from TCLA syncytium inducing (SI) viral strains primarily use the CXCR4 coreceptor (Burton and Montefiori, 1997; Feng et al., 1996; Hung, Vander Heyden, and Ratner, 1999). In general, CCR5 is used by HIV-1 strains that dominate early in the infection (R5) while CXCR4 (X4) is used by viruses that emerge several years later or that are detectable only transiently (Feng et al., 1996). The presence of viruses able to use CXCR4 is associated with an accelerated disease course, due in part to the loss of naïve CD4⁺ T-cells that express CXCR4, but not CCR5. On the other hand, CCR5-using viruses target memory CD4⁺ CCR5⁺ T-cells and are lethal in their own right.

Implicit in these findings is that viral entry is an important stage of the virus life cycle that determines to a large extent the viral tropism and pathogenesis. This makes this stage a principal target for inhibition mainly because the participating molecules are exposed to the extracellular environment and therefore are relatively easier to reach than intracellular targets. Antibodies against virus proteins are produced in infected individuals as part of the natural immune response and therefore can serve as a paradigm for efficient entry inhibitors (Kuhmann et al., 2004). Other HIV-1 entry inhibitors have been developed, and these are discussed in detail later.

1.6 HIV-1 genomic organization

The HIV-1 has a genome size of approximately 9.8 Kb that contains open reading frames (ORF) for about a dozen virally encoded proteins that are flanked by long terminal repeats (LTR) regions (Los Alamos Database, 2002). The LTR contains binding sites for cellular transcriptional activators such as NF κ B. Furthermore, the HIV-1 genome encodes structural and enzymatic proteins (Gag, Pol, and Env) as well as non-structural RNA binding regulatory proteins (Tat, Rev and Nef) and accessory proteins (Vif, Vpr, Vpu). The *gag* (group associated antigen) gene codes for the Gag (p55) polyprotein precursor that is cleaved by the viral enzymes to the smaller p6 and p7 proteins, the capsid p24 and matrix p17 proteins. The *pol* gene codes for three viral enzymes reverse transcriptase (RT), integrase (IN), and protease (PR) that function in viral maturation. The *env* gene codes for the membrane associated proteins, the transmembrane protein gp41 and the surface glycoprotein gp120.

1.7 Classification of HIV-1

On the basis of phylogenetic analysis of numerous isolates obtained from diverse geographical origins, HIV-1 is subdivided into types, groups, subtypes, sub-subtypes, circulating recombinant forms (CRF) and unique recombinants (Louwagie et al., 1993; Peeters, Toure-Kane, and Nkengasong, 2003; Simon et al., 1998). Hence HIV-1 has been classified into 3 groups, namely: M for “major”, N for “new” and O for “outlier” (Robertson et al., 2000; Simon et al., 1998). Group M represents the vast majority of HIV-1 strains found worldwide and is responsible for the pandemic. This group is further subdivided into 9 subtypes, A-D, F-H, J, K and 15 CRFs each comprising strains isolated from different geographical sites worldwide. Phylogenetic variability is extended further by intragroup and

subtype (intraclade) variability and, within the same infected individual, by the emergence of viral quasi-species with time and interclade recombinants with superinfection. This means that effective prevention or treatment of HIV-1 infection should be broadly protective against highly divergent viral variants.

1.8 The HIV-1 envelope structure

The envelope protein of the HIV-1 is formed as a precursor gp160 that is proteolytically cleaved into two mature products, the exterior surface gp120 and transmembrane gp41 glycoproteins (Kwong et al., 1998; Wyatt et al., 1998). The gp41 protein anchors the gp120 molecule to the viral surface by means of noncovalent interaction and mediates oligomerization of gp120/41 (Chan et al., 1997; Wyatt and Sodroski, 1998). The amino-terminal portion of gp41 protein contains a stretch of hydrophobic amino acids with fusogenic properties essential for virus-cell and cell-cell fusion following gp120 binding to CD4 (Chan and Kim, 1998; Wyatt and Sodroski, 1998). Together, the viral envelope glycoproteins form a heteromeric structure that consists of triplets of the gp41, noncovalently linked to trimers of gp120 (Chan et al., 1997; Weissenhorn et al., 1996; Wyatt and Sodroski, 1998). The viral gp120 consists of five variable loop regions V1 to V5 forming structures that are interspersed with five conserved regions, C1 to C5 (Kwong et al., 1998; Lamers et al., 1993; Leonard et al., 1990; Starcich et al., 1986; Wyatt et al., 1998). In addition to other structures, the variable envelope loops are important for virus binding to the target cell receptors. The outer viral glycoprotein is highly glycosylated, a feature which masks viral proteins from recognition by the immune system (Bernstein et al., 1994; Leonard et al., 1990).

The HIV-1 envelope glycoprotein serves as the only viral target for NAbs (Wyatt and Sodroski, 1998). Structural studies of gp120-CD4 complexes reveal that gp120 core molecule consists of an inner domain, outer domain, and a bridging sheet (Fig 4) (Kwong et al., 1998; Wyatt and Sodroski, 1998). All three of the gp120 elements contact the most amino terminal of the four immunoglobulin domains of CD4 (Raja et al., 2003). In monomeric recombinant gp120, the V2, V3, C1, C5 and discontinuous CD4 binding domain (CD4bd) regions are immunodominant whereas the V1, V4, V5 and C2 regions are immunosilent (Basmaciogullari et al., 2002).

Binding of the HIV-1 envelope glycoproteins to CD4 triggers conformational changes that allow the binding of gp120 to chemokine coreceptor molecules, CCR5 and CXCR4, ultimately leading to membrane fusion and viral entry (Wyatt and Sodroski, 1998). These changes in the viral envelope result in the exposure of intermediate structures, i.e. epitopes that interact with coreceptor molecules. The significance of this is that some regions of the oligomeric envelope glycoprotein accessible on monomeric gp120 are not optimally exposed before CD4 binding (Sattentau and Moore, 1991; Sattentau et al., 1993; Thali et al., 1993). For instance, one of the important conformational changes induced by CD4 is the movement of the gp120 V1/V2 variable loops, which are thought to mask the chemokine receptor-binding site on gp120 (Wyatt and Sodroski, 1998). However, it has been shown that gp120 protein with deletions or alterations in the V1 and V2 loop often exhibit the ability to bind coreceptor in the absence of the receptor CD4 (Kolchinsky et al., 1999). To further strengthen these findings it has been observed that viruses with altered envelopes can infect CD4-negative cells that express the appropriate coreceptor. From these studies, it is

suggested that both full-length and core gp120 glycoprotein exhibit a high entropy in the free-state, thereby assuming multiple conformations. Upon CD4 binding, an unusually large reduction in entropy occurs, presumably locking the gp120 core into a specific conformation (Raja et al., 2003). These inaccessible regions of oligomeric envelope glycoprotein present an interesting area of research in mapping potential antibody neutralization-sensitive epitopes.

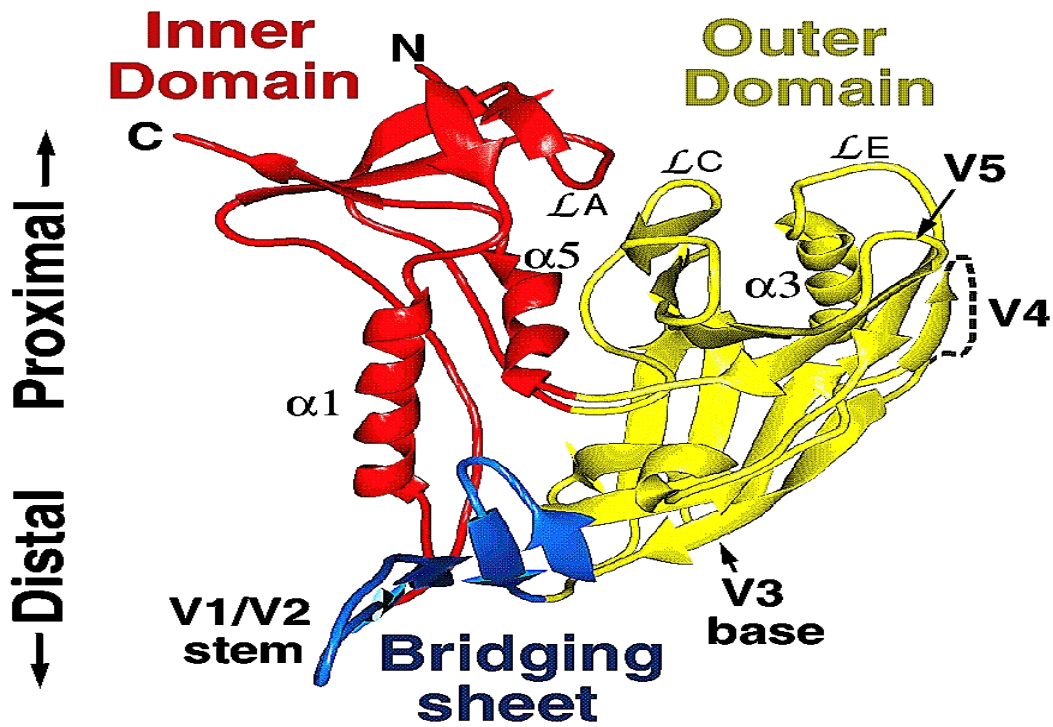


Figure 4. Structure of HIV-1 gp120 core. The inner domain (red), outer domain (yellow) and the bridging sheet (blue) are shown (hiv-web.lanl.gov).

1.9 Blocking viral entry

HIV-1 entry into a target cell is a multi-step process that involves the interaction of HIV-1 envelope glycoprotein with the CD4 receptor, and subsequent attachment to the co-receptor molecules, CCR5 and CXCR4 (Wyatt and Sodroski, 1998). Hence, the entry process occurs in three distinct phases, and thus allowing multiple opportunities for intervention: the attachment of gp120 to CD4, the interaction of the gp120-CD4 complex with either CCR5 or CXCR4 and the gp41 mediated membrane fusion process (fusion step). Agents that target these three steps have been developed. Neutralizing antibodies have also been shown to block viral entry (section 1.20.1).

1.9.1 Reagents preventing gp120:CD4 interactions

A number of agents that prevent gp120-CD4 interaction have been developed. PRO542, also known as CD4-IgG₂, is a tetravalent antibody coupled to the CD4 and binds to CD4 region on the gp120 thereby interfering with the interaction of CD4 and gp120 and potently neutralizes primary HIV-1 isolates (Allaway et al., 1995). BMS-378806 is a small compound found to have HIV-1 inhibitory activity by binding to or close to the CD4 binding site on gp120 (Guo et al., 2003). Its inhibitory activity is independent of the coreceptor usage. Cyanovirin (CV-N) is an 11 Kd protein derived from cyanobacterium *Nostoc ellisporum*, and also produced in recombinant form in *Escherichia coli*. It targets the HIV-1 virion attachment to the host cell and blocks the CD4-gp120 interaction (Boyd et al., 1997). Moreover, CV-N has been found to have similar binding site with 2G12 possibly by steric hindrance (Esser et al., 1999; Wyatt and Sodroski, 1998).

1.9.2 CCR5 and CXCR4 inhibitors

CCR5 has been shown to be inhibited by RANTES, TAK779 and PRO140 while CXCR4 is inhibited by T22, SDF-1 α and AMD3100. RANTES, an acronym derived from regulated upon activation, normal T-cell expressed and secreted is a β -chemokine is a natural ligand secreted by cytotoxic CD8⁺ T-cells and binds to CCR5 and other chemokine receptors (Cocchi et al., 1995). TAK779 binds into a pocket formed by trans-membrane helices 1-3, and 7 of the CCR5 and prevent HIV-1 infection (Baba et al., 1999; Dragic et al., 2000). PRO140 is a monoclonal antibody that has been shown to have high affinity for CCR5 coreceptor and effectively inhibit most R5 HIV-1 strains of different subtypes including subtype C isolates (Cilliers et al., 2003; Olson et al., 1999; Trkola et al., 2001). T22 is a precursor peptide of T-140 with specificity for X4 viruses while SDF-1 α is the natural ligand for CXCR4 (Bleul et al., 1996a; Bleul et al., 1996b; Tamamura et al., 1998). AMD3100 binds to the extracellular loops of CXCR4 to block infection (Labrosse et al., 1998).

1.9.3 Fusion inhibitor

The gp41 comprises a fusion peptide and two-terminal heptad repeat regions (HR1 and HR2) that are coiled and folded into each other (Chan et al., 1997). This structural arrangement enables the fusion process to take place. The heptad repeats contain conserved regions which make them attractive targets for the development of entry inhibitors. T-20 is a peptide derived from the HR-2 sequence of the HIV-1_{LAI} subtype B gp41 envelope glycoprotein (Wild et al., 1994a; Wild et al., 1994b). It prevents the fusion between the viral and host cell membranes by interfering with the formation of the six-helical region by binding to the HR-1 region of the gp41. It is the first entry inhibitor to receive FDA approval for use in humans.

1.9.4 Monoclonal antibodies to coreceptors

Several antibodies with different specificities have been described: anti-CCR5 and CXCR4 antibodies and antibodies targeting HIV-1 envelope glycoprotein. 2D7 was generated from a mouse and binds to the second extracellular loop of CCR5 thereby preventing binding of gp120 to CCR5 (Wu et al., 1996). 12G5 targets CXCR4 by binding to the second extracellular loop of CXCR4 and inhibits X4 viruses. MAbs that target neutralizing epitopes of HIV-1 envelope glycoprotein are described below (section 1.17).

1.10 Characteristic features of antibodies

1.10.1 Structure of antibody

The Igs have a basic four-chain monomeric structure consisting of two identical copies of each of heavy (H) and light (L) chains with covalent disulfide bonds (Fig 5) (<http://www-immuno.path.cam.ac.uk>). The L-chains are separated from the H-chains by disulphide (S-S) bonds. The intrachain S-S links divide H- and L-chains into domains which are separately folded. Hence, an IgG molecule contains 3-H chain domains CH1-3. Between CH1 and CH2 there are several cysteine and proline amino acid residues forming a hinge region that confers flexibility to the Fab arms of the Ig molecule, and the antibodies employ this feature when interacting with an antigen (Ditzel, Itoh, and Burton, 1996). There are five classes (isotypes) of Ig: IgG, IgM, IgD, IgE and IgA (Lewin, 1994; Roitt et al. 2001). The class and subclass of Ig molecule is determined by its heavy chain type. The four human IgG subclasses have heavy chains γ_1 , γ_2 , γ_3 and γ_4 that differ slightly, although all are recognizably γ heavy chains. These subclasses occur in the approximate proportion of 66%, 23%, 7% and 4%, respectively. The antibody classes differ in size, charge, amino acid and

carbohydrate content. In addition, there are two light chain isotypes, *kappa* (κ) and *lambda* (λ). Each antibody molecule has either *lambda* or *kappa* light chains, not both. The Ig light chain has a molecular weight of approximately 25 kilodaltons (Kd), and the heavy chain has a molecular weight (MW) of approximately 50 Kd.

Antibodies are proteins of MW 150-900 Kd, and the MW of IgG in particular is 150 Kd (Casali and Schettino, 1996). Antibody functions can be separated into two proteolytic fragments by cleavage with enzyme *papain* in the hinge region to produce the antibody binding fragment (Fab) and class-specific effector (Fc) fragment. In the regions concerned with antigen binding, the antibodies are extremely variable, whereas in other parts of the molecule the sequences are relatively constant. The hypervariable complementarity determining regions (CDRs) of the Fab fragment determines the specificity of the antibody and lies in the amino-terminal domains of the L- and H-chains. Thus each heavy and each light chain possesses a variable and constant region.

IgA represents 15-20% of the human serum Ig, and exists in monomeric or dimeric form. The IgA is the predominant Ig in seromucous secretions, and secretory IgA (s-IgA) may be of either subclass IgA1 or IgA2. This has sedimentation coefficient 11S and exists mainly as dimeric form MW of 385 Kd due to its association with another protein, known as secretory component. IgA molecules receive a secretory component from the epithelial cells into which they pass. The secretory protein is used to transport the IgA through the cell and remains attached to the IgA molecule within secretions at the mucosal surface. IgM accounts for approximately 10% of the Ig pool, and is found in pentameric form with molecular weight

of 970 Kd. IgG is the major Ig in normal human serum, accounting for 70-75% of the total Ig pool. There are four subtypes of IgG, IgG₁-IgG₄. IgG consists of a single four-chain molecule with a sedimentation coefficient of 7S. However, the γ_3 make IgG₃ protein slightly larger than the other subclasses. IgD glycoprotein accounts for less than 1% of the total plasma Ig but is a major component of the surface membrane of B cells. IgE is scarce in serum (except in hypersensitivity states), but found on the surface of membrane of basophils and mast cells in all individuals.

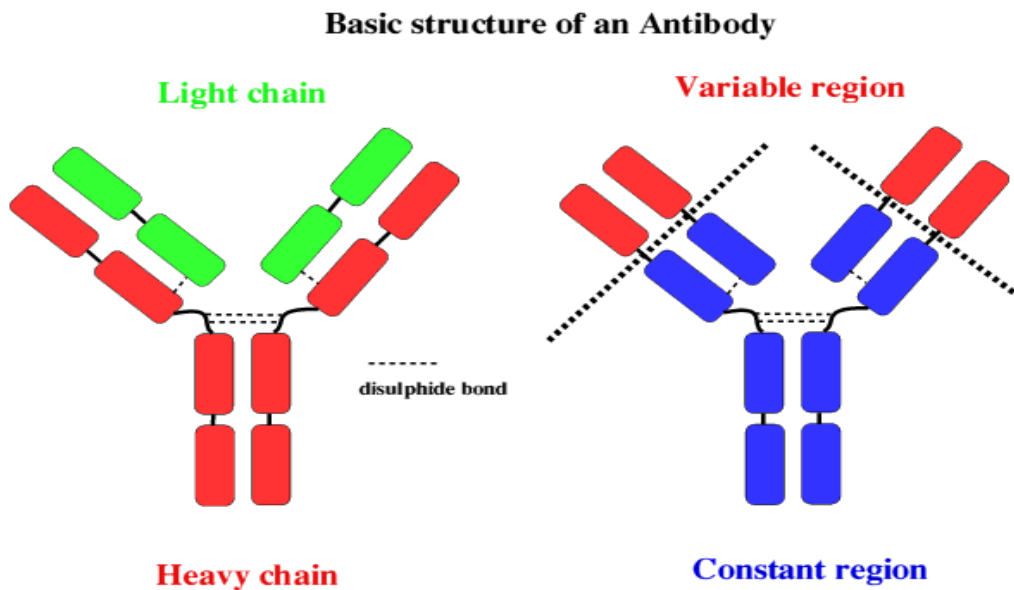


Figure 5. Diagram showing the basic structure of an IgG antibody. The diagram on the lefthand side shows the heavy (H) chains (indicated in red) and the light (L) chains in green; on the righthand side, the diagram shows the constant (C) regions indicated in blue while the red indicates the variable (V) regions. The chains are held together by disulphide bonds as indicated by dotted lines (<http://www.cartage.org.lb>).

1.10.2 Functions of antibodies

Functional Igs can either be secreted from B-cells or expressed on the surface of B-cells. The active complex on the cell surface is the B-cell receptor (BCR) and consists of an Ig associated with transmembrane protein called $Ig\alpha$ and $Ig\beta$ which provide connection to intracellular signalling component in response to antigen-antibody binding. The activation of BCR is also influenced by interaction with other receptors, for instance to mediate the interaction of antigen-activated B-cells with helper T-cells. The Fab fragment retains the antigen-binding activity, binding to a monovalent antigen with an affinity nearly as high as that of the entire antibody. Thus, each Ig molecule is bi-functional: Fab of the molecule is concerned with binding to antigen while the Fc region mediates class-specific effector function such as complement fixation exhibited by IgG and IgM antibodies. The effector functions include binding of the Ig to host tissue, to various cells of the immune system, to some phagocytic cells, and to first component (C1q) of the classical complement system. Antibodies exist in two forms: either membrane bound to B cells or secreted. Membrane bound antibodies interact with antigens. A B-cell makes antibodies of the same specificity able to interact with the same antigenic determinants. Mitotic division gives progeny B cells, i.e. clones that would continue making antibodies of the same specificity. Antigens have determinants called epitopes. Epitopes are molecular shapes recognized by antibodies, and an antibody will recognize one epitope rather than the whole antigen. An antigen may consist of many different epitopes and or may have many repeated epitopes. Free antibodies have the functions of: agglutination, opsonization, neutralization, complement activation, mucosal protection. Moreover, they can immobilize bacteria and hinder their movement and ability to escape phagocytosis or participate in expulsion as a consequence of mast cell degranulation.

They can also function by precipitating soluble antigens by forming immune complexes or in antibody dependent cellular cytotoxicity (ADCC) and confer immunity to the foetus (Roitt et al., 2001).

As a result of its ability to change its shape, IgM is particularly suitable to agglutinate particulate matter including bacteria and viruses. On the other hand, IgG specializes in coating of bacteria or antigen for which the antibody's Fab region has specificity (opsonization). This facilitates subsequent phagocytosis by cells possessing Fc receptors (polymorphonuclear leucocytes/neutrophils). Thus, for opsonization and phagocytosis both the Fab and Fc portions of an antibody are involved. Moreover, IgGs are involved in neutralization of antigen (e.g. toxins, viruses, bacteria) by binding to specific epitopes thus preventing the antigen from attaching to receptors on the host cells. The classical complement pathways is activated by the Fc region of both IgG and IgM and eventually lead to death of invading bacteria by terminal complement components which perforate the cell wall and lead to osmotic death. The complement system components also facilitate phagocytosis by cells possessing a receptor for C3b such as polymorphonuclear neutrophils. Mucosal protection is provided mainly by IgA, and to a lesser degree IgG. Through a Fab function, IgA inhibit pathogens from gaining attachment to mucosal surfaces. The mast cells release mediators when antigen bind to specific IgE that are attached (through Fc) to mast cells. Precipitation of soluble antigen by immune complex formation consists of antigen linked to antibody. Depending on the ratio of antigen to antibody, when the complex is fixed at one site, it can be removed by phagocytic cells. The circulation of the complex prior to localization and removal can also fix complement. Antibodies bind to invading organisms

via their Fab region and activate large granular lymphocytes (Natural Killer cells, NK) that attach through Fc receptors to release perforins to kill the organisms. IgG is the only class of Ig that can cross the placenta and enter the foetal circulation where it confers immune protection. The precise function of IgD is not known, however it serve as a maturation marker of B cells.

1.11 T-helper cells

T-cells secrete a number of cytokines that have powerful effect on B-cells. Differentiation into CD4⁺ T-helper cell subsets is an important step in selecting effector functions based on the profile of cytokine secretion (Roitt et al., 2001). Human T_{H1} cytokines include interferon gamma (IFN γ), tumor-necrosis factor *beta* (TNF β) and interleukin type-2 (IL-2) and these T_{H1} cells promote the production of IgG_{2a} opsonizing and complement-fixing antibodies, macrophage activation, ADCC and delayed-typed hypersensitivity. Hence, IL-2 is an inducer of proliferation for B cells. T_{H2} are mainly involved in the production of IL-4 and IL-5 as well as IL-6, IL-9, IL-10 and IL-13. IL-4 in particular acts on B-cells to induce activation and differentiation, hence providing optimal help for humoral immune responses, mucosal immunity, class-switching from IgM to other Ig classes, stimulation of mast cell and eosinophil growth and differentiation and IgA synthesis (Roitt et al., 2001). IL-4 also acts on T-cells as a growth factor and promotes differentiation of T_{H2} cells, and thus reinforcing the antibody response. Thus, T_{H1} cells are associated with cell-mediated inflammatory reactions and T_{H2} with strong antibody and allergic responses. In the absence of polarizing signal, CD4⁺ T_{H0} cells have a less differentiated cytokine profile and represent a heterogeneous

population with individual clones that can differentiate along the T_{H1} or T_{H2} pathways. Cytokines from T_{H1} cells inhibit the actions of the T_{H2} cells and vice versa.

Although B-cell can undergo activation, proliferation and differentiation in a T-cell independent fashion upon encountering an antigen, individual B-cell can receive help from T-cells. The interaction between T-cells and B-cells drive B-cell division and differentiation, with T-cells recognizing determinants on the antigen that are distinct from those recognized by the B-cells that subsequently differentiate and divide into antibody-forming cells.

1.12 Clonal selection and B-cell development

The antibody response is the culmination of a series of cellular and molecular interactions, and these events occur in an orderly sequence between a B-cell and a variety of other cells of the immune system. Hence, B-cell development, activation, proliferation and differentiation leading to the generation of plasma cells and memory cells involve immune cell cooperation (Lewin, 1994). B-cells are formed and develop in the bone marrow. The functions of these cells are: to interact with antigenic epitope using their Ig receptors, to develop into plasma cells and secrete large amounts of specific antibodies, to circulate as memory cells and to present antigenic peptides to T-cells. The B-cells evolve into plasma cells under the influence of cytokines released by T-cells (Fig 6). Plasma cells do not divide and exist in lymphoid tissues, not blood while other B cells circulate as memory cells.

Following exposure to an antigen, the immune system retains the ability to respond rapidly in the event of a re-infection. The pool of lymphocytes in an individual contains B-cells and T-cells carrying a large variety of Igs or T-cell receptors (Lewin, 1994). Any individual B

lymphocyte produces one Ig, which is capable of recognizing only a single antigen. On exposure to antigen, a B-cell whose antibody is able to bind the antigen is stimulated to divide and increase in size. The developmental pathway proceeds through a series of sequential stages: large pre-B cells express light surrogate chain and upon $V_H D_H J_H$ recombination heavy μ chain and light surrogate chains are produced. This is followed by the expression of heavy μ chains and light surrogate chain expression. Then small pre-B cells expressing only heavy μ chain are produced. Immature B-cells are formed as heavy μ and κ/λ light chains are expressed. Finally, large size mature B-cells are formed, and these express both μ and δ heavy chains in addition to the κ/λ light chains.

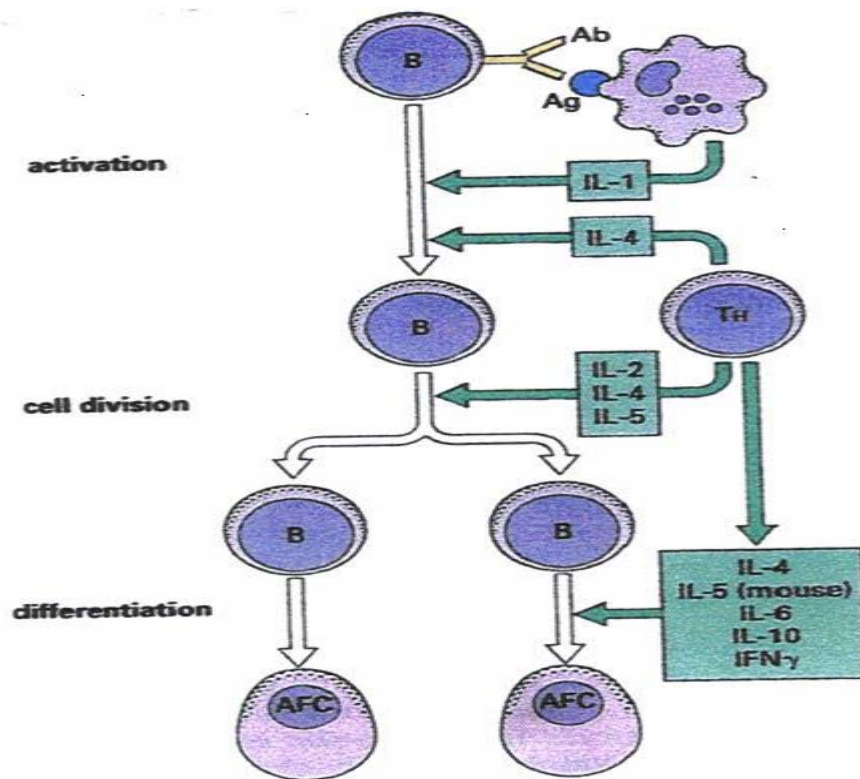


Figure 6. Diagrammatic representation of B-cell developmental pathways. B cells are activated by antigen-presenting cells in the presence of IL-4 and IL-1. This causes expression of receptors for several cytokines including that of IL-2. Many of these cytokines effect differentiation of antibody-forming cells (AFCs) (Roitt et al., 2001; 6th edit).

During B-cell development, genes encoding the variable (V) regions of the antibody molecules are assembled by somatic recombination. As a large number of gene segments are available for V(D)J recombination, and as additional diversity is generated at the joining sites, each B cell acquires a unique BCR representing a clonal marker (Kuppers, 2003a). During primary immune response, large numbers of B lymphocytes with specificity for the offending antigen are produced. This population represents a clone of the original responding B-cell. Antibody is secreted from the B-cells in large quantities. After a successful primary immune response, the B-cells are retained as memory cells representing an intermediate state between the immature cells and the mature cells and this allows a rapid secondary immune response during a second exposure to the same antigen. B-cells are derived from a self-renewing population of stem cells in the bone marrow. Maturation to give B-cells depends upon Ig gene rearrangement. If gene rearrangement is blocked mature B-cells are not produced. The antibodies carried by the B-cells have specificities determined by the particular combinations of V(D)J regions, and any additional nucleotides incorporated during the joining process. Exposure to antigen triggers two aspects of the immune response: primary and secondary immune responses. The primary response occurs by clonal expansion of B-cells responding to the antigen. This generates a large number of plasma cells that are specific for the antigen; isotype switching occurs to generate the appropriate type of effector response. In addition, somatic mutation generates B-cells that have increased affinity for the antigen. These cells remain inactive and do not trigger an immune response at this stage although pre-selected for the antigen, but can undergo isotype switching to select other forms of C_H region. Clonal selection allows rapid secondary immune response, but no further somatic mutation or isotype switching occurs during secondary response.

1.13 Organization of immunoglobulin gene and generation of diversity

During B-cell development in the bone marrow, gene segments encoding the variable (V) region of the antibody molecules are assembled by somatic recombination (Fig 7) (Lewin, 1994; Roitt et al., 2001). Each B-cell acquires a unique BCR because a large number of gene segments recombine from the diversification (D) mechanisms provided by the V(D)J recombination and additional diversity is further generated at the joining (J) sites. Recombination of D and J regions followed by V and DJ regions occurs in pre-B cells, and the recombination of C regions takes place in germinal centre (GC) B-cells. The B-cells expressing a functional BCR are released into the periphery as mature, antigen naïve B-cells. Subsequently, throughout their life, B-cells are constantly selected for expression of a functional BCR.

Mature B-cells are activated when they encounter cognate antigen. Activated B-cells undergo a process of somatic hypermutation, which generates mutations at a higher rate in the V genes. In this way, antibody variations are generated. The Ig genes of many germinal-centre B-cells are also remodelled by class switching, which replaces the originally expressed Ig heavy-chain constant region genes by those of another class. The selected germinal-centre B-cells differentiate to become memory B-cells or plasma cells that secrete functional Ig.

The immune system has to be capable of recognizing virtually any pathogen that the body has encountered, or might arise. The genetic solution to this problem of anticipation and unpredictable future environment involves the generation of a large number of different specific antigen receptors given the limited number of V, D, J and C genes in individual

humans coding for antibodies and T-cell receptors. The mechanisms involved in generating tremendous diversity from such limited gene pool have been examined: this includes intra-chain amplification of diversity, interchain amplification, and somatic hypermutation (Roitt et al., 2001).

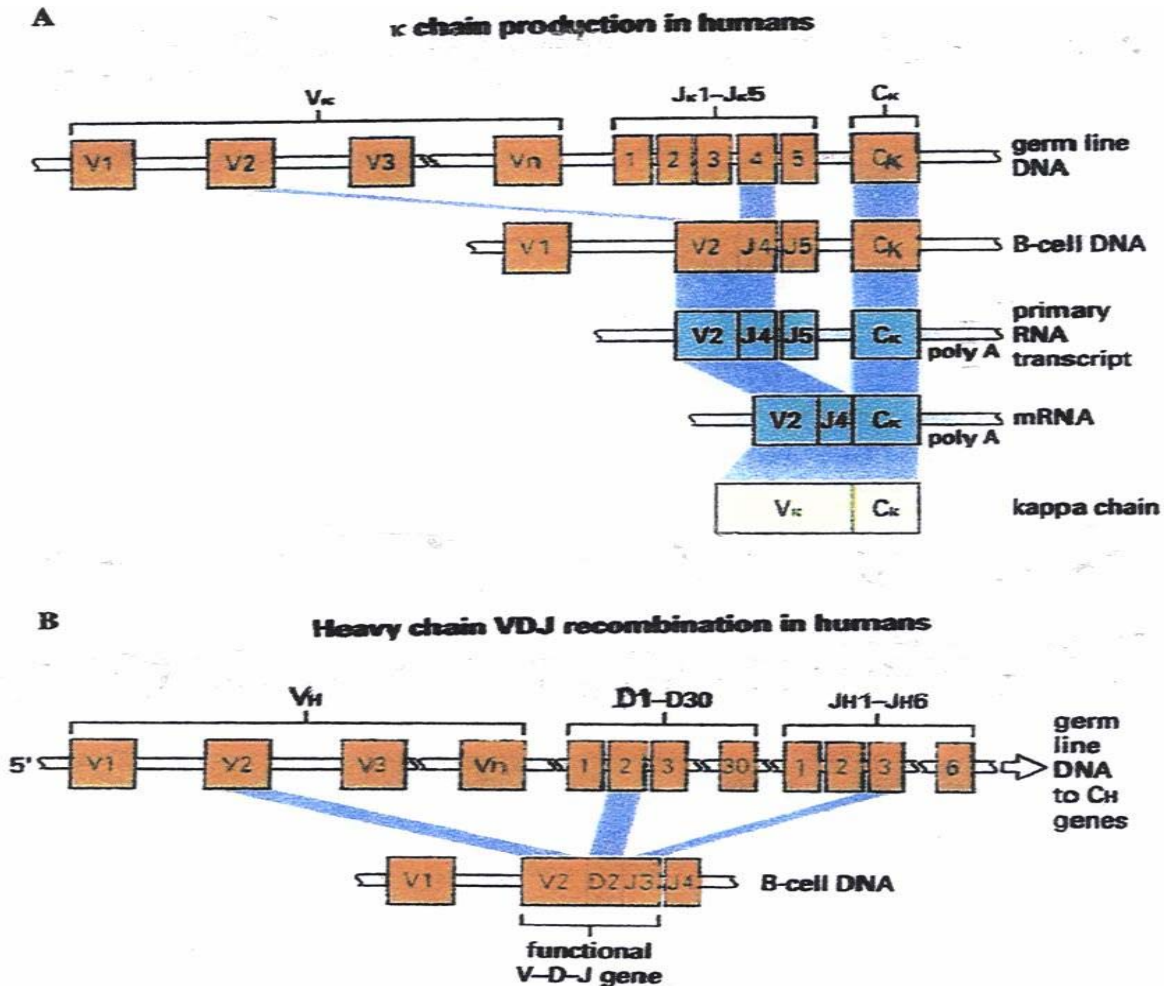


Figure 7. Organization of immunoglobulin genes. During differentiation of pre-B cell one of several V κ genes on the germ line DNA is recombined and apposed to J κ segment. The primary RNA transcript is processed into mRNA by splicing exons together and translated by ribosomes into kappa (κ) chains. (A) The heavy chain gene loci combine three segments to produce V-D-J gene exon coding for the V_H domain. (B) The V gene recombines with one of 30 D segments and one of six J segments to produce a functional V-D-J gene in the B cell (Roitt et al., 2001; 6th edit).

1.14 Intrachain amplification of diversity

The individual receptor gene segments serves as building blocks to fashion a multiplicity of antigen-specific receptors for both the B- and T-cells. The light chain variable regions (V_L) are created from the V and J segments, and the heavy chain variable regions (V_H) from the V, D and J segments (Roitt et al. 2001). The precise number of gene segments varies from one individual to another, but there are typically around 25 D, 6 J functional segments and approximately 50 V_H functional sequences (Lewin, 1994; Roitt et al., 2001). In addition to this geometric recombination, more variation out of germ-line repertoire involves variable boundary recombination of V, D and J to produce different sequences at junction. Further diversity results from the generation of palindromic sequences arising from the formation of hairpin structures during the recombination process and from the insertion of nucleotides at the N-region between the V, D and J segments, a process associated with the expression of terminal deoxynucleotidyl transferase. While these mechanisms add nucleotides to the sequences, yet more diversity can be created by nucleases chewing away at the exposed strand ends to remove nucleotides. Furthermore, the D segment can be read in three different reading frames and two D segments can join together. Such DD combinations produce a longer third complementarity determining region (CDR3) than is found in other TCR or antibody molecules. The CDR3 in the various receptor chains is essentially composed of the regions between the V(D)J segments, where junctional diversity mechanisms can introduce a very high degree of amino acid variability. Hence this hypervariable loop usually contributes the most to determining the fine antigen-binding specificity of these molecules.

It has also been established that lymphocytes are not necessarily fixed with the antigen receptor they initially make. They may adapt to conditions and change their initial receptor (Lewin, 1994). The replacement of an undesired receptor with the one which has more acceptable characteristics is referred to as receptor editing. This process allows the replacement of either non-functional rearrangements or autoreactive specificities. In the periphery, receptor editing is thought to be able to rescue low affinity B-cells from apoptotic cell death by replacing a low affinity receptor with a selectable one of higher affinity.

1.15 Interchain amplification

Two different types of chains are utilized for the recognition molecules, and this produces not only a larger combining site with potentially greater affinity, but also new variability. Heavy-light chain pairing amongst Igs appears to be largely random, suggesting that two B-cells can employ the same heavy chain but different light chains either to fine tune or to alter the specificity of the final antibody (Lewin, 1994). The major contribution to diversity and specificity is said to come from the heavy chain largely as the result of heavy chain CDR3 participation.

1.16 Somatic hypermutation

The Ig V-region genes undergo significant somatic hypermutation (Lewin, 1994). A number of features of this somatic mutation phenomenon add to the diversification of the Ig population. The mutations are the result of single nucleotide substitutions, they are restricted to the variable as distinct from the constant regions and are said to occur in both framework and hypervariable regions. The mechanism of the mutation is thought to be in some way

connected to the class-switch system since hypermutation is more frequently in IgG and IgA than in IgM antibodies, affecting both the heavy and light chains. In general, V_H genes are found to be more mutated than V_L genes. Somatic hypermutation does not appear to add significantly to the repertoire available in the early phases of the primary response, but occur during generation of memory and probably thought to be responsible for tuning the response towards higher affinity.

The mechanism behind the greatly enhanced mutation frequency in Ig genes is unknown. However, it is generally thought that somatic hypermutation is associated with an error-prone DNA polymerase which is coupled to RNA transcription (Lewin, 1994; French, Laskov, and Scharff, 1989; Tonegawa, 1983). It is also suggested that another mechanism for creating further diversity involves the insertion and deletion of short stretches of nucleotides within the Ig V gene sequence of both heavy and light chains. The latter mechanism is perceived to have an intermediate effect on antigen recognition, being more dramatic than single point mutation, but considerably more subtle than receptor editing.

1.17 HIV-1 envelope glycoprotein as a target for the immune response

1.17.1 Glycosylation as a means to evade immune surveillance

The HIV-1 gp120 is heavily glycosylated with about 50% of its molecular weight being contributed by carbohydrate (Parren et al., 1999). Several types of carbohydrates are found: mannose, complex carbohydrates, or a mixture of both. Glycosylation is found to be an important strategy used by HIV-1 to evade neutralizing antibody responses (Wei et al., 2003). The number of glycosylation sites varies between isolates and up to 8 O-linked and 24 N-

linked glycans are known to be essential for correct folding and processing of gp120 during synthesis (Bernstein et al., 1994; Leonard et al., 1990). HXB2 and SF2 are common HIV-1 subtype B reference strains for which the N-linked carbohydrates additions to Env gp120 have been biochemically defined (Leonard et al., 1990). HIV-1 replication in the presence of inhibitors of glycosylation is found to be associated with decreased affinity of gp120 for CD4 as well as the reduction of viral infectivity (Lee et al., 1992; Pal, Hoke, and Sarngadharan, 1989). However, it has been demonstrated that removal of carbohydrates from the mature oligomeric envelope does not affect binding to soluble CD4 (Li et al., 1993). Most of the individual glycosylation sites may be eliminated without affecting the infectivity of HIV-1 virus. It is against this background that the presence or absence of carbohydrate may have a dramatic influence on the antigenicity of viral glycoprotein, suggesting that carbohydrates play a critical role in the masking of neutralizing epitopes on the protein backbone and diversion of the humoral immunity. Viruses that have been passaged in PBMC cultures have gp120 of a higher molecular weight as a result of modified glycosylation, and they are even less susceptible to neutralization than TCLA viruses (Gorny et al., 2002). Other reports have shown that removal of N-glycan in the V3 loop of HIV-1 gp120 increases the sensitivity of mutant virus to neutralization by V3-loop and CD4BS mAbs (LaCasse et al., 1998; Parren et al., 1998a; Trkola et al., 1998). Such mutant viruses cultured in the presence of neutralizing V3-loop antibody have been shown to rapidly escape the antibody by re-acquiring N-linked carbohydrate (Kwong et al., 1998).

On the other hand, Wei and coworker report on escape virus that contained mutations in *env* gene that were unexpectedly sparse and did not map to known neutralizing epitopes (Wei et

al., 2003). These changes primarily involved N-linked glycosylation sites. Given these observations, a “glycan shield” mechanism of neutralization escape whereby selected changes in glycan packing prevent Nab binding but not receptor binding was postulated. It was shown that Nab-selected alterations in glycosylation conferred escape from both autologous antibody and epitope-specific monoclonal antibodies. The evolving glycan shield is presented as a new mechanism contributing to HIV-1 persistence in the face of an evolving antibody repertoire.

1.17.2 Structural elements of gp120

From the immunological diagram, three structural elements are identified: the outer domain, the inner domain and the bridging sheet (Fig 8) (Kwong et al., 1998; Parren et al., 1999; Wyatt et al., 1998). The conserved chemokine receptor binding site is comprised of the bridging sheet with additional contributions from the base of the V2 loop. Although coordinates for the V1/V2, V3 and V4 loops are said to be missing from the structure (Fig 8B), the approximate positions are placed on the basis of experimental data in combination with the position of the bases of the loops. The inner domain is said to be much more conserved between isolates than the outer domain. The region proximal to the V4 and V5 loops in the outer domain is highly variable and potential N-linked glycosylation site. Figure 8C represents the antigenic surface of gp120. The inner domain is involved in the trimerization of gp120 and binding to the trimeric transmembrane glycoprotein gp41. The “neutralizing face” represents the relatively restricted area to which neutralizing antibodies such as 2G12 have been mapped (Moore and Sodroski, 1996; Sanders et al., 2002; Scanlan et al., 2002; Wyatt and Sodroski, 1998). Part of the heavily glycosylated outer domain

represents an immunologically silent face that seem to be protected against antibody responses.

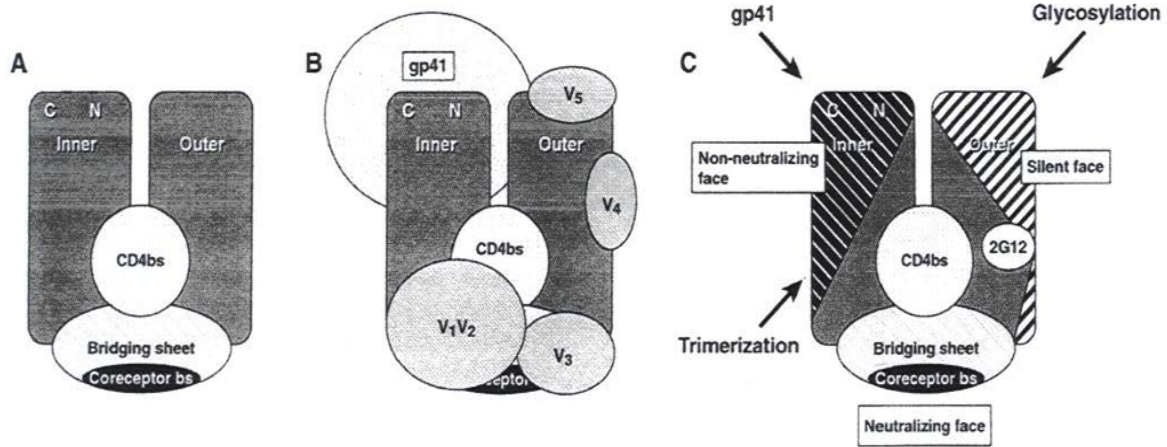


Figure 8. Model of mimeric gp120 core structure; based on X-ray crystal of HIV-1 HXBc2 complexed with CD4 and 1.7B. (A) Three structural elements are described: the inner-, outer-domains and bridging sheet. The CD4BS is located at the interface of the two domains and the bridging sheet. The conserved chemokine receptor binding site is made of the bridging sheet and base of the V2 loop. (B) The positions of V1/V2, V3, V4 and V5 are indicated. (C) Shows representation of the antigenic surfaces of the gp120 with the inner domain involved in the trimerization of gp120 and binding to the trimeric transmembrane glycoprotein gp41 (Parren et al., 1999).

1.17.3 HIV-1 Nab antibody response

The HIV-1 envelope glycoproteins elicit an antibody response during natural infection although elicitation of broadly reactive neutralizing antibodies is inefficient (Burton and Montefiori, 1997). This is revealed in various studies in which the degree of glycosylation, exposed variable loops and the lability of the trimeric envelope glycoprotein complex have been studied (Parren et al., 2000; VanCott et al., 1997; Wyatt and Sodroski, 1998). It has

also been observed that many of the naturally elicited HIV-1 specific antibodies do not recognize the functional oligomeric envelope protein and fail to neutralize the virus (Sattentau and Moore, 1995). Neutralizing antibodies are raised against both variable and conserved regions of the envelope glycoproteins (Moore and Sodroski, 1996).

Characterization of gp120 conformational epitopes is considerably more difficult, but has been done successfully for a number of antibodies. Several epitopes spanning this region have been described, namely: the CD4 binding site (CD4BS) (Ditzel et al., 1995; Thali et al., 1992; Thali et al., 1991), regions induced by CD4 binding (CD4-induced, CD4i) epitope (Thali et al., 1993), the carbohydrate-dependent 2G12 epitope (Trkola et al., 1996b), and the conformationally-dependent IgG1b12 (Barbas et al., 1992a; Barbas et al., 1992b; Burton et al., 1991; Burton et al., 1994; Roben et al., 1994). Along with 2F5 (Muster et al., 1995; Muster et al., 1994; Muster et al., 1993; Purtscher et al., 1994) and 4E10 (Stiegler et al., 2001) these four mAbs are reportedly capable of broad neutralisation of primary isolates.

1.17.3.1 CD4BS mAbs

It is thought that within an envelope oligomer, gp120 probably fluctuates between CD4BS “open” and “closed” conformations as the result of movement of the V1/V2 loops. Multivalent binding between an “open” gp120 oligomer and a cluster of CD4 molecules further displaces the V1/V2 and V3 loops, inducing the chemokine receptor binding site and “loosening” the gp120 association with gp41 (Wyatt et al., 1998). The CD4BS is located within a depression, and is at the interface of the three elements that comprise the gp120 structure: the outer domain, the inner domain and the bridging sheet (Fig 9) (Parren et al.,

1999). This would seem to form an excellent target for neutralizing antibodies. It constitutes an epitope that is said to be well exposed on gp120 and unprocessed gp160. Its exposure is decreased in native oligomer of TCLA viruses and probably on primary isolates as well, thus resulting in poor neutralisation efficacy for anti-CD4bd antibodies. The CD4BS-specific mAb epitopes overlap the CD4-gp120 binding surface, but unlike the CD4 itself, they also make contacts with a number of more variable residues, many of which line the hydrophilic cavity or surround the hydrophobic cavity (Kwong et al., 1998; Parren et al., 2000). Taken together, these studies serve to illustrate that free gp120 molecules are capable of assuming many different conformations.

1.17.3.2 CD4i mAbs

CD4i antibodies recognize conserved bridging sheet structures on gp120 that are induced by CD4 binding and are near a conserved gp120 region that has been shown to be involved in coreceptor binding (Moore and Sodroski, 1996; Sattentau and Moore, 1995; Thali et al., 1992). The CD4i-specific mAbs such as 1.7B bind epitopes that overlap the chemokine receptor-binding surface to varying degree and may also contact residues in the V3 loop (Fig 9) (Rizzuto et al., 1998).

1.17.3.3 2G12 mAb

2G12 recognizes a unique epitope on the neutralizing face within the outer domain of gp120 (Fig 9) (Trkola et al., 1996b). The ability of 2G12 to neutralize a range of primary isolates is remarkable considering the variability in this region. Several mannose carbohydrate moieties are implicated in the binding site for 2G12. The 2G12 epitope is mapped to the N-

glycosylation sites, and it is known to be exposed on monomeric gp120. Based on positions on HXB2 strain, the sites that comprise the epitope require the mannose at positions of N295, N332, N339, N386 and N392 (Moulard et al., 2002; Sanders et al., 2002; Scanlan et al., 2002). These sites are well conserved in most subtypes, and tend to show comparable levels of glycosylation. Recognition of carbohydrate structure that is relatively well conserved in comparison with the underlying primary amino acid sequences in this region help explain the conservation of the 2G12 epitope between a wide range viral isolates, even from different subtypes (Rizzuto et al., 1998; Wyatt et al., 1998). The exception is subtype C, which only rarely has a glycosylation site immediately next to a cysteine at the base of the V3 loop (N295) (Binley et al., 2004; Bures et al., 2002). It is thought that the 2G12 epitope may be conserved because its structure enhances gp120-mannose interaction with the human protein DC-SIGN, an interaction that facilitates efficient HIV-1 infection (Sanders et al., 2002).

1.17.3.4 IgG1b12 mAb

The immunoglobulin (Ig)G1b12 (b12) is a recombinant anti-CD4bd antibody that is capable of potent broad neutralisation activity of primary isolates (Burton, 1997; Burton and Montefiori, 1997; Burton et al., 1994; Parren et al., 1995; Roben et al., 1994). This mAb was identified using a phage display system. The b12 antibody has roughly equivalent affinities for gp120 and unprocessed gp160 as well as for native oligomer on TCLA viruses, and hence is highly efficient at neutralisation. Its neutralisation potency against primary viruses is considerably reduced relative to the TCLA viruses. The epitope is also thought to contain residues from the base of the V3 loop region (C2 and C3) and the V4 region. It has a conformational epitope overlapping the CD4 binding site and thus prevents CD4-gp120

interaction (Burton and Montefiori, 1997; Roben et al., 1994). Its neutralization activity has been found to be effective against most viral strains but not all HIV-1 subtype C isolates are sensitive to this mAb (Binley et al., 2004; Bures et al., 2002).

1.17.3.5 Soluble CD4-1.7B

A novel approach to inhibit HIV-1 was to link sCD4 with 1.7B by using a polypeptide linker so that the complex can prevent coreceptor binding (Dey, Del Castillo, and Berger, 2003). 1.7B is an antibody able to bind well to its epitope after gp120 is bound to sCD4. The sCD4-1.7B was found to be neutralizing against a broad range of HIV-1 subtype including both R5 and X4 strains, and more potent than 2G12, 2F5 and b12.

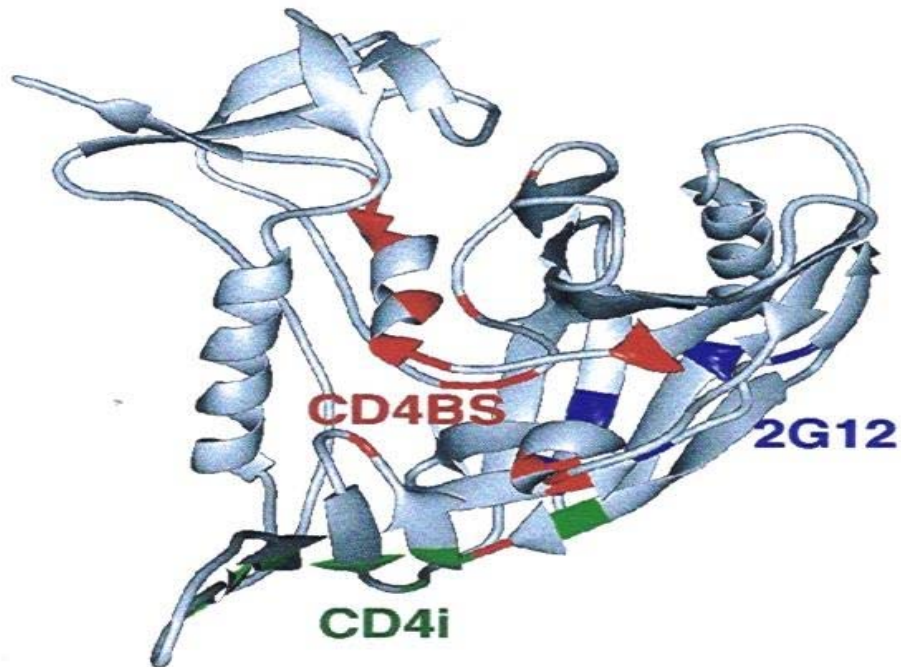


Fig 9. The core structure of gp120 indicating the binding sites for selected neutralizing antibodies (hiv-web.lanl.gov).

1.17.3.6 Other selected mAbs

A32 is a non-neutralizing and yet broadly reactive mAb as it reacts with virtually every gp120 monomer epitope of every subtype (Moore et al., 1994; Wu et al., 1996). The epitope for this mAb is better exposed upon CD4 binding to gp120, and binding of A32 enhances binding of 1.7B (Wyatt et al., 1995). A32 enhances the binding of CD4i mAbs 1.7B (and 48d) (Sullivan et al., 1998a; Sullivan et al., 1998b). The antibody conformationally dependent mAb that recognizes discontinuous epitopes involving C2 but C1 and C4 also contribute (Boots et al., 1997).

The mAb 1.7B has a similar epitope to 48d which is better exposed upon soluble CD4 (sCD4) binding to gp120, and competes with 1.5E, 2.1H and other anti-CD4 binding site mAbs (Thali et al., 1993). Deletion of V1/V2 enhances the binding of the mAb in the presence of CD4, similar effect observed for 48d and A32 (Wyatt et al., 1995). The mAb has been found to bind with higher affinity to both monomers and oligomers (Sattentau and Moore, 1995). 1.7B bound oligomeric Env and neutralizes JR-FL in the presence of sCD4, but in the absence of sCD4, 1.7B only bound monomers and does not neutralize and can also neutralize TCLA strains and not primary isolates (Fouts et al., 1997; Parren et al., 1997b). It has been demonstrated that 1.7B binds to soluble gp120 efficiently, but not soluble gp120+gp41, which suggested that its CD4i gp120 epitope is blocked by gp41 binding.

C11 is a non-neutralizing mAb and it binds efficiently to sgp120 but not soluble gp120+gp41. This suggests that it is a gp120 discontinuous epitope, in C1-C5, that is blocked by gp41 binding (Parren et al., 1997a; Trkola et al., 1996a; Wyatt et al., 1997). MAb 7B2 binds to the

gp41 immunodominant region (James Robinson, unpublished data).

1.17.3.7 Anti-V3 antibodies

The V3 of the HIV-1 gp120 envelope glycoprotein is one of the critical elements for the formation of syncytia and for the virus entry into target cells (Wyatt et al., 1993). These functions are mediated by the interaction of the V3 loop with the chemokine receptors and are maintained despite the sequence variation that characterizes this region of the virus envelope (Hill et al., 1997; Trkola et al., 1996a). The V3 loop is characterized by a constant size of 30-35 amino acids, a conserved type II β -turn at its tip, a disulfide bond at its base, and a net positive charge (Kwong et al., 2000; LaRosa et al., 1990). Nuclear magnetic resonance studies also suggest conserved features of the structure of the V3 loop (Sharon et al., 2003) and conserved elements in the V3 crown and stem are mandatory features for coreceptor interaction (Cormier and Dragic, 2002; Suphaphiphat et al., 2003). All of these structural constraints appear to be imposed by the required interaction of the V3 loop with the coreceptors for the HIV-1, CCR5 or CXCR4, and suggest that this region of the virus envelope should induce antibodies that are cross-reactive among isolates and that are inhibitory to virus infectivity.

In addition to being hypervariable in amino acid sequence, earlier studies have implicated the V3 loop to contain principal neutralising determinants of the virus (Cavacini et al., 1993; Forthal et al., 1995; Hogervorst et al., 1995; Tilley et al., 1992). Virus infectivity and syncytial formation were reported to be effectively blocked by antibodies that recognise the V3 loop determinants, although in an isolate specific manner (Ivanoff et al., 1992). While

these mAbs could potentially neutralize TCLA strains, most of them displayed weak and sporadic neutralization against most primary isolates (Gorny et al., 1992; Hioe et al., 1997; Moore and Ho, 1995). Within the V3 loop of different HIV-1 gp120 envelope proteins, the tetrapeptide sequence motif GPGQ in the case of HIV-1 subtype C and GPGR for subtype B, has been found to be highly conserved. The V3 loop is also known to be well-exposed on monomeric gp120 glycoprotein, unprocessed gp120 and TCLA viruses (Nabatov et al., 2004; Roderiquez et al., 1995; Yang et al., 2004). This epitope has been identified as a major determinant of cellular tropism and coreceptor specificity (Hung, Vander Heyden, and Ratner, 1999). However, it is not clear which region or residues within this stretch of 30-35 amino acids loop are responsible for the observed phenotype. A substantial proportion of the antibody in sera from infected individuals that is capable of binding to TCLA HIV-infected cells or viruses is said to be V3-specific (Burton and Montefiori, 1997). However, neutralisation activity against primary isolates has also been found to be poor. Several studies suggested that this could be due to limited exposure of the V3 loop on the surfaces of primary isolates (Bou-Habib et al., 1994; Chen et al., 2001; Stamatatos and Cheng-Mayer, 1995). However, studies that examined the ability of the anti-V3 mAbs to binding to intact virus particles showed that V3 exposure is the rule rather than the exception (Nyambi et al., 1998; Nyambi et al., 2000a; Nyambi et al., 2000b). Other studies reveal a highly significant correlation between the affinity of binding of anti-V3 mAbs to primary isolates and neutralization potency (Gorny et al., 2002). However, significant variations suggest that there are additional factors that contribute to the ability of a given antibody to neutralize a particular virus. Clearly, from these contradictory findings, it is still not clear how the presence and exposure of the V3 loop affect neutralization sensitivity and how the specificity

of anti-V3 antibodies contributes to this phenomenon. Like other epitopes, the accessibility of V3 in native envelope on primary isolates is decreased. Moreover, the overall charges of the V3 loop have been suggested to influence both cellular and coreceptor tropism of HIV-1.

1.17.4 Epitopes of gp41

Vigorous human antibody response to gp41 protein during HIV-1 infection has been reported (Binley et al., 1996; Burton and Montefiori, 1997; Burton et al., 1994). Three epitope clusters are found within the gp41 protein: a disulfide-bridge loop defining an immunodominant cluster I epitope, cluster II formed by a putative helical region, and cluster III that shows overlapping reactivity pattern with the other two clusters (Fig 10) (Kent and Robinson, 1996). However, none of these clusters has significant neutralisation activity as most of these epitopes on gp41 are thought to be masked in the native oligomer. The apparent vigorous antibody response to gp41 protein during infection is believed to be elicited not by intact virions but by unprocessed gp160 or spikes where the gp120 has been stripped away. It is these molecular forms which appear to expose the gp41 epitopes that are recognised by the clusters I-III antibodies. Three broadly neutralizing antibodies that have been described within the gp41 regions are 2F5, 4E10 and Z13.

1.17.4.1 2F5 mAb

An important epitope of the gp41 protein is found to be located towards the carboxy-terminal part of the extracellular domain. This epitope is defined by 2F5 human antibody which has been shown to be potent and broadly neutralising. The epitope recognised by 2F5 is a linear amino acid sequence, ELDKWA, which has been found to be conserved in many HIV-1

isolates (Muster et al., 1995; Pinter, Honnen, and Tilley, 1993; Purtscher et al., 1994; Stiegler et al., 2001). It has also been demonstrated that only changes at the first and last amino acid position of ELDKWA, that is E and A, did not significantly reduce the binding capacity of the 2F5 mAb to the sequence (Purtscher et al., 1996). However, the binding of the 2F5 to infected cells is relatively weak with a binding apparently below that predicted from neutralisation titres. However, most subtype C viruses do not have the ELDKWA epitope and thus 2F5 is ineffective against this subtype (Binley et al., 2004; Bures et al., 2002).

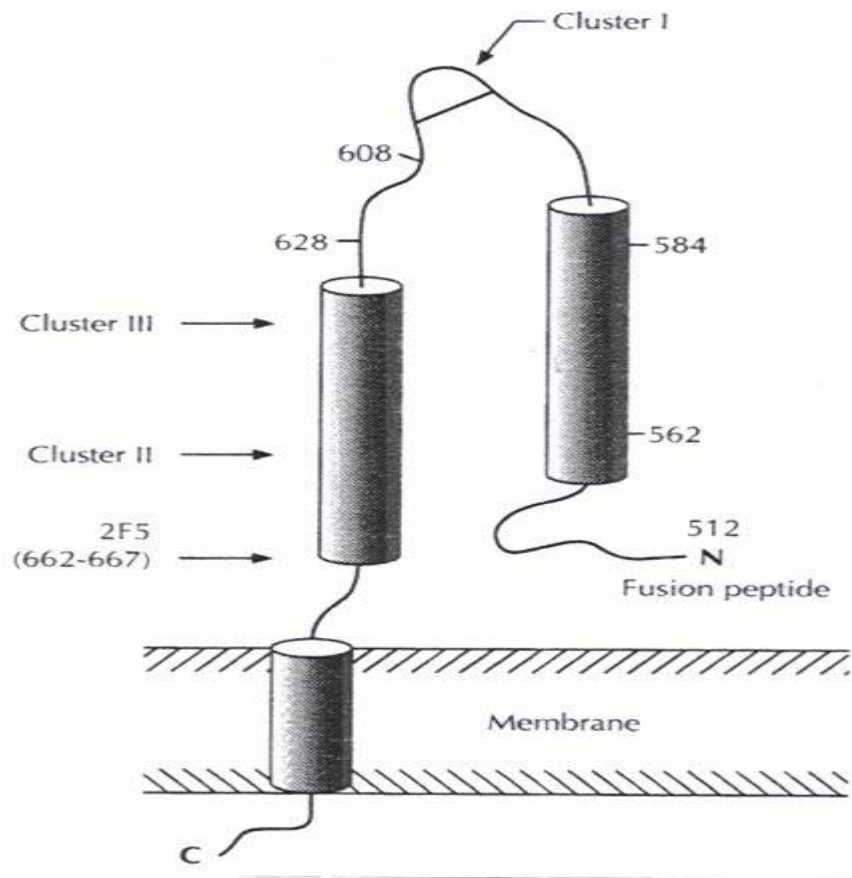


Figure 10. Schematic representation of gp41 structure. Cluster I antibodies recognize an immunodominant epitope including a disulfide-bridged loop while Cluster II antibodies recognize regions at the distal helix. Cluster III antibodies recognise a region overlapping Cluster I & II epitopes (Burton and Montefiori, 1997).

1.17.4.2 4E10 and Z13 mAbs

Another interesting mAb in terms of virus neutralization activity that has been recently described is 4E10 (D'Souza et al., 1994; Stiegler et al., 2001). 4E10 was originally identified as an IgG₃ antibody isotype, and was shown to neutralize some laboratory strains (Rajewsky, 1996). The 4E10 was subsequently cloned as IgG₁ containing the identical constant regions of the heavy chains as the recombinants 2F5 and 2G12. After class-switch from IgG₃ to IgG₁, 4E10 showed increased neutralizing potency and thus its significance in neutralization gained attention. 4E10 and Z13 recognize a novel epitope on gp41 carboxy-terminal to the 2F5, an epitope defined by NWF(D/N)IT (Buchacher et al., 1994; Stiegler et al., 2001; Xu et al., 2001; Zwick et al., 2001a). The antibodies were found to be broadly neutralizing including subtype C strains (Binley et al., 2004; Stiegler et al., 2001).

1.18 Virus neutralization

1.18.1 TCLA versus primary virus neutralization

Based on the gp120 structure, the CD4bs on primary viral isolates gp120 trimer appears to be more completely masked by the V1/V2 loops than of TCLA viruses. That gp120 oscillates between 'closed' and 'open' states is consistent with the dichotomy of primary and TCLA viruses: thus, gp120 of primary isolates would have the equilibrium biased in favour of a 'closed' conformation, whereas TCLA gp120 would be biased towards 'open' (Parren et al., 1999). In this way, the virus *in vivo* would sacrifice some efficiency in receptor binding for increased resistance to antibody attack, whereas cell line-passaged virus would dispense with some now unnecessary antibody resistance mechanisms and adapt for more efficient receptor interaction instead.

Several research studies have demonstrated that the resistance of primary isolates compared with TCLA viruses does not seem to be dependent on coreceptor usage since X4, R5 and R5X4 primary viruses overall exhibit similar neutralization sensitivity (LaCasse et al., 1998; Trkola et al., 1998). Analysis of syncytium-inducing and non-syncytium-inducing viruses of different subtypes showed that there was little or no phenotype-dependent distribution of neutralization sensitivity to potent neutralization antibodies and sera (Losman et al., 2001; Parren et al., 1998a).

1.18.2 Mechanisms of neutralization

There are a number of mechanisms that have been proposed for the neutralization of HIV-1. The simplest mechanism involves inhibition of virus-receptor binding, manifested as inhibition of virus attachment to its target cells (Parren et al., 1999). Although some research groups initially thought that the mechanism of neutralization fulfils this, this mode of neutralization is now known not to apply to HIV-1 (Ugolini et al., 1997). There is a direct correlation between antibody occupancy of its binding site on the virus, irrespective of the particular epitope involved, and infectivity neutralization (Parren et al., 1998a). On the basis of inhibiting soluble gp120-CD4 binding, V3 loop-specific mAbs were once thought to interfere with the late stages of the HIV-1-cell fusion process (Linsley et al., 1988; Moore and Nara, 1991; Skinner et al., 1988), other studies have suggested that they mediate post-attachment neutralization (Armstrong et al., 1996; Lu, Putney, and Robinson, 1992; Pelchen-Matthews, Clapham, and Marsh, 1995). Antibodies to the V3 loop have been shown to effectively inhibit HIV-1 attachment (Valenzuela et al., 1997).

Parren and coworker assert that the principal neutralization mechanism for HIV-1 is that coating of the virus by antibody obstructs the close approach of virion and target cell, thereby effectively inhibiting virus-cell attachment (Parren et al., 1998a). While inhibition of HIV-1 attachment by CD4BS-specific mAbs can be explained by competition for CD4 binding, but how the binding and neutralization by mAbs that bind to epitopes spatially distinct from the CD4BS interfere with HIV-1 attachment to cells needs to be elucidated. Steric hindrance model has been suggested as the major mechanism of HIV-1 neutralization since the face of gp120 exposed to antibody attack is very limited in size (Kwong et al., 1998; Parren et al., 2000). Consistent with this model, it has been demonstrated that antibody affinity for the oligomer is the single most important determinant of neutralization (Fouts et al., 1997), since epitope occupancy by a minimum number of antibody molecules would correspond with blocking of a critical number of receptor binding sites on the virus (Parren et al., 1999). The critical number of receptors binding sites to be blocked may be dependent on the number of envelope spikes per virion (Klasse and Moore, 1996). The possibility that an antibody may have more than one activity, and that an immunoglobulin molecule bound to a virion at a concentration insufficient to prevent attachment might exert other antiviral effects or even enhance infectivity for certain viruses (Sullivan et al., 1998a).

The 2F5 mAb is an exception in that it does not inhibit HIV-1-cell binding (Ugolini et al., 1997). This mAb is thought to neutralize at the stage subsequent to virus attachment by interfering with the assembly of the leucine zipper that occurs during gp41 activation, or with the insertion of the fusion peptide in the membrane (Binley and Moore, 1997). The 2F5 epitope overlaps with the N-terminal end of a conserved tryptophan-rich region implicated in

envelope-mediated fusion and HIV-1 infectivity (Salzwedel, West, and Hunter, 1999). While it is thought unlikely that 2F5 epitope is directly involved in a molecular interaction during the fusion event, some evidence point to the existence of a pre-hairpin intermediate in the conformational transition of gp41 from its native non-fusogenic state to its post-fusogenic state that 2F5 may act by interfering with the completion of this process.

The induction of conformational changes in the gp120 that can lead to gp120 dissociation from gp41 is believed to be another factor that influences HIV-1 neutralization (Hart et al., 1991; Moore et al., 1991; Moore et al., 1990; Sattentau and Moore, 1991). The induction of conformational changes in gp120, potentially without causing shedding, would be consistent with the notion of synergistic neutralization induced by pairs of ligands to different gp120 epitopes. The CD4BS and V3 loop mAbs added together have been shown to neutralize to a greater extent than the sum of their neutralization activities separately (Laal et al., 1994; McKeating et al., 1992; Potts et al., 1993). The same has been shown for a variety of combinations of several others mAbs including V3 loop mAb, 2F5 and 2G12 (Li et al., 1998). While the extent of synergy with antibodies tested is generally weak, the *in vivo* relevance of this synergy is unknown. Nonetheless, synergy in the context of a polyclonal response would be highly significant to HIV-1 vaccine design, and further research in this regard would be appropriate.

Overall, occupation by antibody of a minimum number of binding sites on the envelope may be the principal factor determining neutralization, implying that antibody affinity and concentration are more important criteria than epitope specificity (Parren et al., 1999).

1.18.3 Neutralization escape

HIV-1 has shown an ability to escape from the selective pressure exerted by any single replication inhibitor based on blocking its entry into target cells. Hence, it is prudent to study escape pathways *in vitro* in order to understand what might happen in clinical use. The role of antibodies to influence ongoing viral replication has been suggested from the emergence on neutralization escape mutants during the course of HIV-1 and SIV infection (D'Costa et al., 2001; Ohgimoto et al., 1998; Parren et al., 1999; Parren et al., 1998b). It is however, unclear whether the slow emergence of neutralization escape variants in natural infection is causally related to immune pressure from neutralizing antibodies, or whether is merely a consequence of viral variation, or both. There are at least two mechanisms through which escape mutants are thought to occur. First, local changes through point mutations can occur that reduce the affinity of the neutralizing antibody for the virion. The second mechanism is more global conformational changes that occur through mutations in distal sites, which create viruses more refractory to neutralization by globally altering the envelope antigenic makeup (Parren et al., 1999). Nonetheless, details of this proposed global mechanism remain obscure. However, it is thought that certain “difficult to neutralize viruses” may acquire resistance to neutralizing antibodies directed at multiple antigenic sites by operating through such mechanisms. Parren et al. (1999) identified some primary isolates resistant to multiple potent neutralizing antibody preparations, in which neutralization escape could not generally be explained by loss of the relevant epitope on the envelope subunits. Such viruses are deemed as a major threat for antibody-based vaccine strategies against HIV-1.

1.19 Passive immunization and treatment

1.19.1 Treatment

Several studies suggest that neutralizing antibodies exert some pressure to control HIV-1 replication in individuals with established infections (Dacheux et al., 2004; Richman et al., 2003; Trkola et al., 2005; Wei et al., 2003). Immunodeficient mice reconstituted with human lymphoid tissue have been infected with HIV-1 and then evaluated after infusion with neutralizing monoclonal antibodies derived from HIV-1-infected individuals. In this model, the antibody treatment had little effect on viral replication (Poignard et al., 1999). Similarly, it is reported that in HIV-1 infected individuals, intravenous infusion of hyperimmune globulin with high titres of HIV-1 specific antibodies had little effect on viral load or disease progression (Jacobson and French, 1998). In contrast, pre-existing circulating neutralizing antibody has been shown to alter the clinical development of SIV and HIV-1 hybrid (SHIV) infections in macaques. In these studies, infusion of either serum IgG or combinations of monoclonal antibodies that neutralize these viruses attenuates the pathogenicity or even blocks the establishment of infection by these lentiviruses (Baba et al., 2000; Mascola et al., 2000). To define the activity of neutralizing antibodies in established infection a proof-of-principle study was recently conducted (Trkola et al., 2005). Three Nab 2G12, 2F5 and 4E10 were passively administered over 11 weeks to HIV-1-infected patients who carried viral isolates sensitive to the three antibodies. Although viral escape were reported, the study provided direct evidence that antibodies have the capacity to contain viremia in established human HIV-1 infection. Overall, these findings suggested that such antibodies will be very important in any strategy to prevent HIV-1 infection.

The rationale for immune-based therapy in HIV-1 infection arise from the observation that prolonged highly active antiretroviral therapy (HAART) leads to increases in naïve cells (Autran et al., 1997), as well as from the improvement in observed functional defects in CD4⁺ and CD8⁺ T cells normally found in this infection (Letvin and Walker, 2003). The restoration of immune responsiveness to other pathogens with administration of HAART indicates that immune suppression is reversible after prolonged HIV-1 infection. Numerous other approaches to address immune augmentation in HIV-1 infection are being investigated. These include: adoptive therapy, cytokine therapy, therapeutic immunization and combination of HAART and treatment interruption to boost immune responses to autologous virus (Letvin and Walker, 2003). Adoptive therapy has been done using both antibodies and cells. Infusion of cocktails of neutralizing mAbs has led to marked protection from infection in nonhuman primates (Mascola et al., 1997). Infused antigen-specific CTLs can home to sites of virus replication (Brodie et al., 1999). Infusion of interleukin-2 by a number of dosing schedules and routes has resulted in increases in CD4⁺ T cell counts, possibly affecting disease progression (Kovacs et al., 1996). However, immunogenicity of therapeutic antibodies is a significant problem and severely limits their widespread and repeated application to treat many diseases.

1.19.2 Passive immunization

Broadly neutralizing antibodies have been demonstrated to be protective against intravenous and mucosal challenges with immunodeficiency viruses in animal models (Berman et al., 1990; Fultz, 1992; Lubeck et al., 1997; Mascola et al., 2002). These efforts further reinforce the view that vaccine development against HIV-1 should be capable of eliciting such

antibodies (Mascola, Frankel, and Broderick, 2000; Parren et al., 2001). These animal models have provided a number of guidelines regarding the types of antibodies that should be elicited. That protection is generally provided by those antibodies which are capable of effectively neutralizing the HIV-1 virus *in vitro*. Also, that serum-neutralizing antibody levels at the time of virus challenge need to be relatively high (1:100) to achieve sterile protection. Thirdly, that protection by broadly-neutralizing human monoclonal antibodies (mAbs) against a number of viruses suggests that protection against the different strains of HIV-1 may be achievable.

1.20 Vaccine strategies

1.20.1 Role of neutralizing antibodies

One of the main reasons for understanding the humoral immune response to HIV-1 infection is to guide the design and evaluation of vaccines based, at least in part, on the induction of relevant humoral immunity. A vaccine that stimulates a sterilizing neutralizing antibody response would clearly be desirable: if a vaccine were able to induce antibodies with the combined specificities of neutralizing monoclonal antibodies such as b12, 2G12, 2F5 and 4E10 would most likely have a major impact on reducing HIV-1 transmission, including viruses from multiple genetic subtypes (Parren et al., 1999). It is also believed that if sterilizing immunity turns out to be unachievable, neutralizing antibody responses of lesser, but still significant, potency could still make an important contribution to a multi-modal vaccine in which induction of broadly active cellular immune response is sought. Other studies have shown that non-sterilizing antibody response can reduce the infectivity of a viral inoculum enough to allow the cellular immune response sufficient time to develop (Dittmer,

Brooks, and Hasenkrug, 1998). This would mean that for HIV-1, with its capacity to replicate in the helper T-cells that coordinate humoral and cellular immune responses, the retarding effect of pre-existing antibodies might be especially beneficial. However, creating such a vaccine remains a challenging aspect of HIV-1 research. The crucial aspect is to design immunogens capable of inducing sufficiently potent antibody as well as cellular immune responses.

A vexing question is to what strength and breadth neutralizing antibody response will be sufficient to contribute to a combined modality vaccine? There is a body of evidence emanating from passive antibody immunization experiments that strongly suggests that probably at least a 100-fold or greater reduction in viral infectivity that is greater than 99% neutralization, *in vitro* will be necessary for significant protective effects to be observed *in vivo* (Parren et al., 1999). In HIV-1 vaccine development, it is sometimes suggested that any measurable neutralizing antibody response, such as a two-fold reduction in HIV-1 infectivity *in vitro*, that is 50% neutralization, by a low dilution of serum may indicate a significant contribution of antibody to protection. For HIV-1, 90% or greater neutralization is rarely achievable and the reasons for this are not clearly understood. On the other hand, 50% *in vitro* neutralization titres may not be appropriate for estimating likely *in vivo* antibody efficacy. It has been estimated that a vaccine of at least 95% efficacy would be required to abolish productive infection of this magnitude in the acute stage (Little et al., 1999).

The role of antibodies in preventing virus infection and re-infection has been established (Pantophlet et al., 2003). However, their contribution to the resolution of the viral disease

has been at the centre of much controversy, despite the findings that they exert immune selection pressure (Richman et al., 2003; Wei et al., 2003). It is thought that natural antibodies contribute to innate response to both bacteria and viruses (Barbas et al., 1991). These antibodies are typically IgM, but can also be IgG and are usually characterized by moderate affinity to antigen and poly-reactive behaviour (Casali and Schettino, 1996; Ditzel, Itoh, and Burton, 1996). While each arm of the immune system may be sufficient in certain situations, it has become clear that humoral responses act in concert with cellular immunity in the control of viral disease.

1.20.2 Creating vaccine immunogens

Efforts to create an effective HIV-1 vaccine have been undermined by the lack of an appropriate immunogen capable of eliciting reasonable levels of available broadly neutralizing antibodies. The inability of the humoral immune system to mount a good primary isolate neutralizing response against HIV-1 in natural infection is a paradox in view of the very strong and sustained antibody response against gp120 monomer present in most infected individuals (Binley et al., 1997; Moore and Ho, 1995). The basis of these problems is believed to be that most antibodies against the envelope are elicited by viral debris rather than virions (Parren, Burton, and Sattentau, 1997; Parren et al., 1997a). Viral debris are defined as antigenic forms of HIV-1 envelope distinct from the mature, oligomeric envelope glycoprotein complex on the surface of infected cells or virion, such as unprocessed gp160 envelope precursor, 'shed' monomeric gp120 dissociated from the virion/infected cell surface, and gp41 exposed on the virion/infected cell surface after shedding of gp120 (Cormier and Dragic, 2002). Unmodified monomeric gp120 subunits alone have been found

to be inadequate to elicit reasonable levels of broadly neutralizing antibodies (Connor et al., 1998; Graham et al., 1998).

It has also been suggested that the limitation of individual monomeric gp120 proteins can be overcome by a mixture of proteins from different virus isolates drawing analogies to other vaccines that contain multiple antigens, each designed to counter a particular viral or bacterial serotype (Zwick et al., 2001b). However, this view is strongly challenged on the basis that for HIV-1 there is currently no rational basis for choosing which individual components to add to a multivalent cocktail as neutralizing serotypes do not exist for primary HIV-1 isolates (Parren et al., 1999). Conceptually, these antibodies should be targeted to relatively conserved and exposed regions of the HIV-1 envelope. Several studies seem to indicate that in natural infection the virus may present these regions to the immune system in such a way as to minimize an effective antibody response. Against this background, a molecular understanding of the regions of the HIV-1 envelope that are exposed and conserved and how these regions can be recognized by antibodies would greatly assist in the design of an immunogen that can elicit broadly neutralizing antibodies.

Overall, for envelope-based vaccines to be effective and efficient, it is believed that they will need to elicit antibodies capable of neutralizing virus infectivity. The conformation-dependent CD4 and chemokine receptor-binding domains of gp120 represent important targets for neutralization. However, the 3-dimensional conformation of gp120 shows that these regions are located in the inner core of gp120 and thus rendering them poorly accessible to antibody (Amit et al., 1986). The presence of carbohydrate glycans on the outer domain of

gp120 and the possibility of subunit-subunit interactions within the native trimeric complex further occlude these regions (Yang, Wyatt, and Sodroski, 2001).

1.20.3 Existing vaccines

Several HIV-1 vaccine formats have entered phase I/II clinical trials in various parts of the world (McMichael and Hanke, 2003). These vaccines include HIV-1-derived immunogens as adjuvant-associated peptides and proteins (AIDSVAX B/E: gp120, AIDSVAX B/B: gp120, NefTat fusion/gp120: Nef-Tat, gp120); as DNA in plasmid form (VCR-HIVDNA009-99-VP: Env) (Boyer et al., 2000); and as insert in recombinant canarypox (ALVAC vCP205: Env-Gag-Pol, 1452: Env-Gag-Pol-CTL) (Cao et al., 2003), MVA (MVA.HIVA: Gag-CTL) (Wee et al., 2002), and adenovirus (Poly-env1 vaccinia: Env) (<http://63.126.3.84/2002>). Lower doses of these vaccine preparations have been administered and as a result the immune responses in human trial participants have been small as compared with responses in macaques (McMichael and Hanke, 2003). These constructs have been determined to be immunogenic, and it is thought that it is possible to improve the immune responses by increasing the dose and number of immunizations or by testing different routes of the challenge. It is also believed that combinations of these vaccines in prime-boost approaches may show additive effects. Except for Poly-env1 vaccinia and VCR-HIVDNA009-99-VP which have subtype C virus Env component the rest of these formulations are mainly based from subtype B.

1.21 Approaches to generate mAbs

Since early reports by Olson and Kaplan and others in the 1980s on the generation of human-human hybridomas, different techniques have been described for the generation of human monoclonal antibodies. This includes Epstein-Barr virus (EBV) transformation, somatic cell hybridization (fusion), and phage-display systems (Burton et al., 1991). For EBV-transformation and fusion, the yield of specific clones after the immortalization methods depends on the number of antibody-producing cells in the lymphocyte source, the fusion efficiency, and the efficiency of the fusion line. Regarding the fusion method, rates of optimized protocols are between 10^{-4} and 10^{-5} and specific antigen-binding B-cells in blood are between 10^{-4} and 10^{-5} . For this reason the yield of fused antibody-producing B-cells is expected to be in the range of 10^{-8} to 10^{-10} , using PBMCs as source. An additional problem arises from the genomic instability within the first 6-8 weeks after immortalization. The loss of antibody-producing cells often caused by overgrowing nonproducers can be avoided by early subcloning steps.

1.21.1 EBV immortalization of human B-cells

EBV consists of a linear double-stranded DNA genome with a size of 172kb (Kuppers, 2003a; Kuppers, 2003b). After infection of B cells, the virus DNA circularises and persists as an episome in the nuclei of infected cells, thereby establishing a latent infection. The virus can switch to a lytic cycle, and make progeny viruses that ultimately lyse to infect other cells. EBV is a ubiquitous human B-lymphotropic herpesvirus capable of efficiently immortalizing primary B cells into continuously growing lymphoblastoid cells (Nakayama et al., 2002; Yoshie and Ono, 1980) although in some cases it can infect T-cells and squamous epithelial

cells via the complement receptor, CR2 (CD21) (Guigou et al., 1991). The CD21 receptor for C3d/EBV is present on all mature B-cells and late pre-B-cells. The resulting cell lines retain several markers of B cell such as surface Igs and complement receptor and continue to produce secretory Ig. B-cell specific transformation with EBV could be established to generate human cell lines producing antibodies with known specificities.

Although EBV is generally considered a harmless passenger, its transforming capacity promotes the development of B-cell lymphomas (Zimber-Strobl and Strobl, 2001). In latently infected B cells, up to nine EBV-encoded proteins are expressed, viz: six EBV nuclear antigens (EBNA) EBNA1, EBNA-2, EBNA-3A-C, and EBNA-LP as well as 3 latent membrane proteins (LMP) LMP1, LMP-2A and LMP-2B. In addition, two types of EBV-encoded non-translated RNA are transcribed in latently infected B-cells, the EBER1 and EBER-2 (EBV-encoded RNAs). LMP1 is said to resemble a constitutively active CD40 receptor while LMP2A mimics a BCR (Kuppers, 2003b). The EBNA1 binds to the origin of replication of the EBV episome (Yates, Warren, and Sugden, 1985). This interaction is required for the replication of the virus DNA and its distribution to the daughter cells in proliferating cells. It is a trans-activating protein that regulates several virus genes such as LMP1 and LMP2A and many cellular genes. LMP1 inhibits apoptosis by up-regulating expression of the anti-apoptotic proteins BCL2 and A20, and induces an activated phenotype. The LMP1 is an oncogene whose effects are caused by the activation of the nuclear factor- κ B (NF- κ B) pathway. This signalling pathway is said to be similar to that of CD40 (Brown, Hostager, and Bishop, 2001), which plays a major role in the activation and differentiation of B cells. LMP2A prevents unwanted antigen-triggered activation of EBV-positive B cells that

would cause entry into the lytic cycle (Miller et al., 1995). Lastly, EBERs are thought to participate in stimulating growth of infected B cells and suppress cytotoxic T cells. Against this background, the immortalization which involves the implementation of the virus encoded programme that results in unlimited survival and proliferation of infected B-cells occur as a consequence of establishment of latency (Kuppers, 2003b).

Immediately after EBV transformation, specific antibodies can be detected but the isolation of antibody-producing cells raises more difficulties. The loss of antibody-producing cell lines is often caused by transient activation of B lymphocytes by EBV binding without subsequent transformation. Contamination and gene shut-off (class-switching) could be among some of the reasons for loss of antibody-producing cell lines. The problem could be further compounded by lack of quality feeder layer in the cultures. Early and repeated subcloning steps are necessary for successful establishment of human monoclonal antibody-producing cell lines. In addition, antibody production of EBV-transformed lymphocytes can be rescued by subsequent fusion with an efficient myeloma or myeloma-like parent line. Transformation rates after EBV transformation have been found to vary considerably and always not reproducible. (Gorny et al., 1998) reported that the frequency of EBV transformation declines with increasing patient disease progression. Anti-HIV-1 producing cell lines could be generated only from lymphocytes of patients in the early stage. MAbs such as A32, 1.7B, C11 and 7B2 were produced by EBV-transformation.

1.21.2 Cell fusion

Cell fusion involves direct hybridization of unstimulated B-cells from PBMC with a fusion partner. After the isolation of PBMC by centrifugation on Ficoll-Hypaque gradients the cells can be mixed with an appropriate cell line such as human B cell immortalizing heteromyeloma, CB-F7, in a ratio of 5:1 and fused by combined polyethylene glycol (PEG) electrofusion method (Buchacher et al., 1994). The cell mixtures are pulsed with 2 kV before being seeded and cultivation for 2-3 weeks after which actively growing hybridomas are evaluated for IgG and further identified for reaction with HIV-1 proteins. Clones such as 2G12, 2F5 and 4E10 were produced using this technique.

Electrofusion immortalization method is reported to be always accomplished successfully and hybrids formed independent of the clinical state of the blood donors. In general, establishment of anti-HIV-1 antibody-producing cell lines is faster and simpler by electrofusion and as such it could be a method of choice for the generation of monoclonal antibodies.

1.21.3 Phage display technologies

The phage display system, originally developed by Parmley and coworkers (Parmley and Smith, 1988) can be used to mimic the immune response *in vitro* (Geoffroy, Sodoyer, and Aujame, 1994). The technology is used for the construction of large phage-displayed antibody repertoires (Fab fragments) through the recombination of two separate immunoglobulin V_H and V_L chain gene libraries amplified from hybridomas or B-cells using polymerase chain reaction and cloned into expression vectors (Barbas et al., 1991;

McCafferty et al., 1990). The process makes use of λ phage *att* recombination sites, leading to irreversible physical association between plasmid and phagemid vector carrying, respectively V_L and V_H sequences. Theoretically, all possible associations between the diverse repertoires of V_L and V_H gene sequences from lymphocytes should be obtained, and it should be possible to generate multicombinatorial libraries of close to 10^{12} clones, to represent natural antibody repertoire. Thus, the use of filamentous bacteriophage represents a powerful alternative to the hybridoma technology. The b12 is one mAb that has been produced using this method.

1.22 Study background

HIV-1 subtype C is one of the most prevalent genetic subtypes of HIV-1 in the world today. Most information on HIV-1 neutralization is derived from subtype B. The identification of immunogenic sites within HIV-1 subtype C envelope that are able to elicit neutralising antibodies against primary isolates was of interest in this study. Several other studies indicate that there are significant differences between HIV-1 subtype B and C viruses (Binley et al., 2004; Burton and Montefiori, 1997). In the case of subtype B, the conserved tetrapeptide sequence amino acid sequence at the tip of V3 loop is GPGR while GPGQ predominate with subtype C viruses. Since the V3 loop and its overall charge character have been identified as a major determinant of cellular tropism and coreceptor specificity the differences in subtype B and C V3 loop impact on the functionality of the virus strain.

Neutralising mAbs such as 2G12 and 2F5 that have been found to be broadly neutralizing for subtype B and vaccines developed based on information derived from the studies are not

effective on subtype C. It is therefore imperative to undertake complimentary studies based on HIV-1 subtype C viral strains. Identifying such epitopes with broad neutralising activity has proven difficult mainly because many of the epitopes are discontinuous and buried within the conformational structure of the envelope glycoprotein which is further shielded from immune surveillance by a large amount of glycans. Although not unique to subtype C viruses, it is estimated that the exposure of these sensitive epitopes during viral entry is in the order of minutes, giving the immune system very limited opportunity to mount an immune response to these regions.

1.23 Study objectives

The purpose of this research project is to identify immunogenic sites within HIV-1 subtype C, and possibly define epitopes that are able to elicit potent neutralising antibodies against primary viral isolates. We proposed to generate monoclonal antibodies against HIV-1 subtype C epitopes by immortalisation of B cells from HIV-1 subtype C seropositive patients with relatively strong neutralising antibody titres using EBV transformation, and then screen for anti-HIV-1 envelope antibodies. By using cell lysates derived from HIV-1 subtype C infected cell lines as the antigen source in our screening system, we aimed to capture viral particles in their near native state. These viral particles may expose novel epitopes that mimic an *in vivo* situation in HIV-1 infection. The resulting mAbs are characterized in terms of their binding specificity to the gp120 or anti-gp41, as well as assessing their functional ability.

Generation of monoclonal antibodies that are able to block HIV infection in vitro will be useful reagents for studying virus neutralization and would also provide clues to the antigenic sites within HIV-1 that elicit antibodies possessing anti-viral activity. Moreover, the identification of the neutralization sensitive epitopes will provide important information for designing HIV vaccines that aim to induce neutralizing antibodies.