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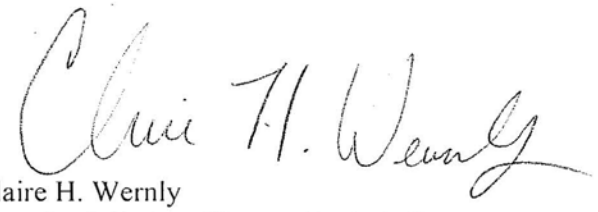

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Abstract

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Title of Dissertation:

"Evolution of the HIV-1 Envelope Glycoprotein Genes and Neutralizing Antibody Response in an Individual Whose Plasma Contains Broadly Neutralizing Antibodies."

The induction of broadly cross neutralizing (BCN) antibodies is likely to be a critical component of an effective immune response to an HIV-1 vaccine. Thus far, induction of such a response remains elusive. However, the identification of HIV-1 infected individuals who develop a BCN antibody response provides hope that an HIV-1 vaccine is possible. The characterizations of HIV-1 envelope genes from such individuals are of interest as they may contain epitopes responsible for the generation of the observed BCN response. For this project, we hypothesized that the study of HIV-1 envelope glycoprotein gene variation and evolution of the neutralizing antibody response from a patient (HNS2 donor) whose plasma contains BCN antibodies will reveal evidence of the mechanisms of induction and maintenance of a BCN response. DNA extracted from primary or co-cultured PBMC representing 21 years of sample availability from the patient was used as the source for envelope gene cloning throughout this study. Results indicated that the patient was infected early in his course with at least two strains of HIV-1, one of which differentiated into distinct neutralization sensitive (L1a) and neutralization resistant (L1b) lineages. L1a and variants derived from a second lineage (L2) remained neutralization sensitive for several years. Analysis of positive selection rates among the lineages did not indicate positive selection for L1a and L2, consistent with possible low levels of replication of these strains. The eventual displacement of neutralization sensitive L1a and L2 variants with neutralization resistant variants was accompanied by the loss of BCN antibodies and loss of circulating $CD4^+$ T cells. Findings in this patient that might be related to the BCN response include infection with more than one strain of HIV-1 and the apparent persistence of low levels of neutralization sensitive variants presenting cross-reactive neutralization epitopes.

Evolution of the HIV-1 Envelope Glycoprotein Genes and Neutralizing Antibody Response in an Individual whose Plasma Contains Broadly Neutralizing Antibodies.

Claire Heather Wernly

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Dedication

To my Parents

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Chapter 1. General Introduction

A new disease

The beginning of the AIDS epidemic in the United States was noted by a report in the Morbidity and Mortality Weekly Report (MMWR) on June 5, 1981, that described five cases of *Pneumocystis carinni* pneumonia (PCP) among previously healthy homosexual men in Los Angeles, CA. At the time of publication, two of the five men had died and the remaining three displayed "profoundly depressed numbers of thymus-dependent lymphocyte cells, and profoundly depressed responses to mitogens and antigens". The observations were remarkable, since in the United States and other industrialized countries, PCP had previously been observed almost exclusively in immunosuppressed individuals [1].

Within a month of the report, additional cases of PCP were reported among previously healthy homosexual men across the United States. Disturbingly, severe and aggressive cases of Kaposi's sarcoma, normally a slowly progressive cancer affecting elderly men of Mediterranean descent and observed in Africans without underlying conditions, began to be reported as well [2-4]. The unusual clustering of these diseases occurring within the homosexual male population led scientists to believe that acquisition of disease was related to behavior. The epidemic evolved further as similar cases of immunodeficiency coupled with opportunistic infections began to be reported in other populations as well, including intravenous drug users and hemophiliacs [5-7]. During the early years of the AIDS epidemic, individuals at greatest risk were men who had sex with men, intravenous drug users, and hemophiliacs. In fact, these early reports provided evidence that a blood borne agent was probably responsible for the disease. By 1982, it was clear that the new disease resulted in the impairment or elimination of T-lymphocyte

helper cells and as such the new disease was given the name AIDS (acquired immune deficiency syndrome) as reported in the MMWR on September, 3, 1982.

The etiological agent responsible for the new disease

Generalized lymphadenopathy, or swelling of the lymph nodes, was a recognized pre-AIDS manifestation. In 1983, Francoise Barre-Sinossi, working in the lab of Luc Montagnier at the Institute Pasteur, described the isolation of a novel retrovirus from the lymph nodes of a homosexual male patient presenting with lymphadenopathy [8]. As a result, the new virus was termed lymphadenopathy-associated virus (LAV), and was hypothesized to be the causative agent of AIDS. The following year, a group from the National Cancer Institute (NCI), published four papers detailing the isolation of a cytopathic, T-cell-tropic retrovirus they named HTLV-III, from a mixed culture of blood cells of patients suffering from AIDS like symptoms [9-12]. Subsequent, analysis indicated that the virus reported by the NCI laboratory was actually the LAV virus obtained from the Institute Pasteur [13]. Following years of controversy, the discovery of HIV has been attributed to Barre-Sinossi and Montagnier, earning them the 2008 Nobel Prize in Physiology or Medicine. In 1986, the International Committee on Taxonomy of Viruses recommended the currently accepted name of human immunodeficiency virus (HIV).

The global HIV/AIDS epidemic

By the time of the discovery of the virus in 1983, HIV infection was already a pandemic. Presently, according to the 2009 Joint United Nations Programme on HIV/AIDS (UNAIDS) report, approximately 33.4 million people are living with HIV. Additionally, there were an estimated 2.7 million new HIV infections in 2008 and an estimated 2.0 million AIDS-related deaths. Sub-Saharan Africa remains the region of the globe most heavily affected by HIV, and accounts for a staggering 67% of all people living with HIV and 72% of the AIDS related deaths. In 2003, the United States Government initiated the President's Emergency Plan for AIDS Relief (PEPFAR) to fight the global HIV/AIDS pandemic. The program aimed to provide antiretroviral treatment to HIV infected individuals in resource-limited settings with high HIV/AIDS prevalence rates, to prevent new infections and to care for individuals already infected with HIV. PEPFAR has been heralded as the largest health initiative ever initiated by one country to combat a disease. The response to PEPFAR has been largely positive and a 2009 study found that the program had successfully reduced the death rate due to AIDS in the countries involved by 10% [14].

HIV classification and genetic diversity

AIDS in humans is caused by either one of two lentiviruses, HIV-1 and HIV-2 [15-17]. The origins of HIV-1 and HIV-2 in humans were due to cross species transmission events from primates [18]. Indeed, phylogenetic analysis of Simian Immunodeficiency Viruses (SIV) compared to HIV indicate that SIV_{cpz}*Ptt*, found in

chimpanzees (*Pan troglodytes troglodytes* (*Ptt*)), is highly related to HIV-1 whereas SIV_{smm}, found in sooty mangabey monkeys (*Cercocebus torquatus atys*), is highly related to HIV-2 [16, 19, 20]. It is now well established that SIV_{cpz}*Ptt* gave rise to HIV-1 and that SIV_{smm}, gave rise to HIV-2. In addition to the different origins of HIV-1 and HIV-2, both viruses differ in their geographical distribution as well as their pathogenic potential. HIV-1 entered the human population in Central Africa and is responsible for most of the AIDS pandemic [19]. HIV-2 entered the human population in West Africa and remains largely confined to West Africa [16]. The pathogenic potential in humans of HIV-2 is less than that of HIV-1. In comparison to HIV-1, HIV-2 is associated with a lower plasma viral load set point, which may account for the observed slower progression of disease [21-23]. The more restricted replication in humans may also account for the more restricted spread of HIV-2, compared to HIV-1. Additionally, studies examining envelope sequences from asymptomatic HIV-2 infected patients demonstrated low sequence diversity; supporting the idea that HIV-2 replication in humans occurs at low levels [24, 25].

HIV-1 has been shown to have entered the human population through at least three different cross-species transmission events, spawning three commonly recognized HIV-1 groups known as: M (major), O (outlier) and N (nonmajor/nonoutlier). Group M accounts for the majority of HIV-1 infections (95%) and can be further divided into subtypes (or clades), denoted with letters and sub-subtypes, denoted by numbers. Currently recognized HIV-1 Group M subtypes include (A1, A2, A3, A4, B, C, D, F1, F2, G, H, J and K) [26, 27]. Additionally, infection by two or more HIV-1 strains may result in the formation of recombinant viruses [28-30]. Recombinant strains are reclassified as

Circulating Recombinant Forms (CRF) when they are identified in three or more people with no direct epidemiological linkage. HIV-1 Group O isolates share approximately 65% sequence homology with Group M isolates and are found primarily in individuals living in Cameroon, Gabon and Equatorial Guinea [31]. Similarly, HIV-1 Group N isolates are not pandemic, but rather have been isolated only from a few infected individuals living in Cameroon [32, 33]. In 2009, a putative new strain of HIV-1 was isolated from a Cameroonian woman [34]. This strain was designated HIV-1 Group P and was found to be related to SIVgor, a virus recently discovered in wild-living gorillas (*Gorilla gorilla gorilla*) [35]. Human to human transmission of this virus has not yet been described, but the observation indicates that gorillas are potential sources of immunodeficiency virus transmission to humans.

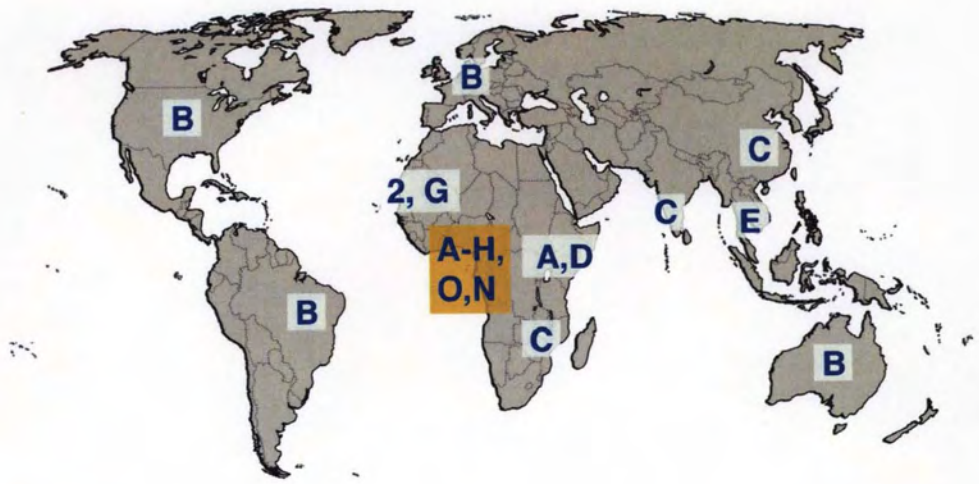
One of the many factors contributing to the formidable challenge of developing a globally effective HIV-1 vaccine is the enormous genetic diversity displayed by HIV-1. This diversity can be attributed in part to its highly error prone reverse transcriptase (RT), which lacks a 3'-5' exonuclease proofreading activity [36]. The process of reverse transcription results in approximately 3×10^{-5} mutations per nucleotide per replication cycle [37]. This high rate of mutation coupled with a short generation time results in sufficient variability that, on average, every possible mutation occurs at every position in the genome every day in an infected individual [28]. Additionally, insertions, deletions, duplications and recombination events contribute to the genetic heterogeneity of HIV-1 [30, 38]. Maintenance of the essential nucleotide sequence undoubtedly reflects that most possible mutations negatively affect viral fitness and cannot be retained. Some

mutations, such as those, which confer adaptive advantage in the context of an immune response or antiretroviral therapy, are subject to positive selection.

The various HIV-1 clades are not distributed equally around the globe as seen in Figure 1. All clades can be found in Africa, owing to the fact that diversification within the human population has been occurring, apparently in various isolated population groups, for a number of decades. The most predominant among HIV-1 infections are of subtype C. Subtype C accounts for 49.9% of the global prevalence and can be found primarily in India, as well as Eastern and Southern Africa. Conversely, Subtype B, which has been the most extensively studied, is found primarily in the Americas and Western Europe and only accounts for 10.2% of the global prevalence [39]. However the potential for global spread of all HIV subtypes indicates the need for a broadly effective vaccine.

Figure 1. Global distribution of HIV-1 and HIV-2

Pandemic HIV-1 can be divided into three groups M (major), O (outlier) and N (nonmajor/ nonoutlier). HIV-1 group M accounts for the majority of HIV infections and can be further subdivided into subtypes (A-K) and various circulating recombinant forms. In contrast, HIV-2 is largely confined to West Central Africa. The predominant subtypes associated with various geographical regions are indicated on the map. Image used with permission from [40].


B
B
2, G
A-H,
O,N
A,D
C
C
E
C
B
B

HIV-1 transmission and disease progression

There are three main transmission routes for HIV-1. Exposure to virus can occur as a result of sexual activity, exposure to contaminated blood and blood products and from an HIV-1 infected mother to her child [41]. HIV-1 transmission can be due to exposure to cell free virus and/or virally infected cells. It has been shown that in both blood and genital fluids HIV-1 infected cells are present in more substantial numbers than free virus, and the former may account for the majority of transmission events [42].

The rate of disease progression varies greatly among HIV-1 infected individuals. In most instances of untreated HIV-1 infection (80-90%), the median time period from primary infection to diagnosis of AIDS is approximately 10 years [43]. In a small proportion of persons infected with HIV-1 however, the progression to AIDS occurs very rapidly within months to a few years of primary infection [44]. Conversely, approximately 5% of HIV-1-infected individuals exhibit no signs of disease progression even after 12 years or more of infection [45-47]. The observed variability in disease course could be due to various host factors, such as age or genetics, virulence of the infecting HIV-1 strain, or dose of the virus inoculum.

There are three stages to HIV-1 infection. The first stage or acute phase begins the first few days after primary infection and lasts for approximately 7-8 weeks. During the acute phase, the virus establishes itself in the lymphatic tissues [48]. The lymphatic tissues then serve as the principle site of virus production and persistence [49-52]. Billions of virions are produced daily, mainly by activated $CD4^{+}$T cells [53]. The large production of virions results in viremia and a sudden and transitory drop in circulating

$CD4^+$T cells [54,55]. Within a few weeks the initial viremia is reduced, most likely due to the effects of the innate immune response and the actions of $CD8^+$ T cells [56-58].

The virus population during the acute phase is often relatively homogenous. Attempts to explain early HIV-1 transmission events following vaginal mucosal exposure, the main route of HIV-1 infections occurring worldwide, have been carried out using the rhesus macaque nonhuman primate (NHP) model along with high dose exposure to SIV [59, 60]. Results indicated that SIV rapidly crosses the mucosal barrier, within hours, and establishes a small founder population of infected cells [61,62]. The infected cells undergo local expansion, during the first week of infection, before seeding the secondary lymphatic tissues, which then serves as the main reservoir for continued viral replication [62, 63]. The small founder population and genetic bottleneck observed in HIV-1 and SIV transmission may be due to various factors such as dilution, trapping in cervical mucus, the presence of the mucosal epithelial barrier, the dose of the viral inoculum and the target cell availability.

The acute phase is followed by the chronic phase of infection that can last for a variable number of years and is characterized by relatively constant, low levels of viral genome in plasma along with a gradual decrease in numbers of circulating $CD4^+$T cells. This period is highly dynamic for both the host and the virus. During this time the virus is constantly evolving to escape the host immune response. In spite of the low levels of viral genome in the plasma, there is continuous and rapid HIV-1 replication and mutation [64, 65]. Accumulated mutations can be attributed partially to the highly error prone RT, ultimately resulting in the formation of diverse viral quasispecies circulating in the host

[66]. During the chronic phase there is a progressive increase in nucleotide diversity of the virus population [67, 68].

The end phase of infection is characterized by a precipitous decline in $CD4^+$ T cells to less than 200 cells/ml^3 and a substantial rise in plasma viral levels. Once $CD4^+$T cell numbers drop below 200 cells/ml^3, cell-mediated immunity is lost and infections with various opportunistic pathogens begin to occur. It is during this stage that the infected individual develops symptoms of AIDS. The first manifestations are often unexplained weight loss and recurring infections consisting of various opportunistic pathogens such as PCP. The appearance and recurrence of such infections as well as ulcerations and tumors are a direct result of the loss of cell-mediated immunity. In some cases, the virus population present during the end phase of infection shifts from being macrophage tropic to T cell tropic owing to mutations in the V3 loop of the gp120 subunit of the envelope glycoprotein [69, 70]. The tropism change results in altered co-receptor usage of the HIV-1 virus. HIV-1 co-receptors are members of the seven transmembrane spanning CC or CXC families of chemokine receptors. It has been determined that macrophage tropic HIV-1 utilizes CCR5 as a co-receptor, whereas T cell tropic HIV-1 utilizes CXCR4 as a co-receptor [71, 72]. This shift in tropism occurs in advanced infection and has been associated with a worsened disease prognosis and accelerated disease progression [73-75]. In addition it has been determined that the viral population once again becomes more homogenous during the end stage of disease. It is postulated that the drop in $CD4^+$ T cells results in a decrease in the primary target cell population for the virus, ultimately contributing to an evolutionary slowdown [68, 76].

Immune response to HIV-1 infection

During the acute phase of infection, HIV-1 replicates to high levels and elicits a robust immune response. The innate immune response provides the first line of defense against HIV-1 infection. HIV-1 RNA activates toll-like receptors present on dendritic cells (DC), resulting in the production of cytokines such as interferon alpha, interleukin-12, tumor necrosis factor alpha, and interleukin-6, which then serve to activate the immune system [77]. DCs provide an initial line of defense against HIV-1, however, DCs are also a target cell for HIV infection and their numbers are markedly reduced during the acute phase of infection [78, 79]. In addition, DCs can capture HIV-1 and transmit the virus to nearby $CD4^+$T cells thereby initiating productive HIV-1 infection in $CD4^+$T cells [80, 81].

The appearance of HIV-specific $CD8^+$T cells is an early adaptive immune response to HIV infection [56, 57, 82]. During the initial viremia, the HIV-specific $CD8^+$T cells expand to make up approximately 10% of all circulating T cells. It is this initial $CD8^+$T cell response that likely reduces the viral load during acute infection [82]. The humoral immune response to HIV infection is also significant and virtually all HIV-1 neutralizing antibodies are specific for the viral envelope glycoprotein spikes which are composed of a surface attachment glycoprotein (gp120) and a membrane spanning glycoprotein (gp41), both of which are discussed in greater detail in the following sections. The exact timing and potency of the antibody response varies greatly among HIV-1 infected individuals [83-85]. The majority of the first neutralizing antibodies to appear are directed against the variable loops present on the surface of gp120 subunit of

the envelope glycoprotein. However, due to the high amino acid sequence diversity exhibited in the variable loops, this initial neutralizing antibody response is generally virus strain specific and only effective against autologous virus [83-86]. Additionally, the nature of the HIV envelope glycoprotein also confounds the host's antibody response to the virus. The gp120 and gp41 subunits of the envelope glycoprotein are held together via weak noncovalent interactions, and as a result, various forms of nonfunctional envelope spikes can be found in the plasma, on the surface of the virion, and on the surface of infected cells. The presence of these nonfunctional spikes contributes to the generation of non-neutralizing antibodies [87-89].

As the course of infection progresses, HIV-1 rapidly and repeatedly escapes from the autologous neutralizing antibody response of the host [83, 84, 90]. This escape is due in part to the high mutation rate of HIV-1 and the rapid turnover of virus. In fact, it has been shown that the approximate half-life of HIV-1 in plasma is a mere six hours, and the half-life of productively HIV-1 infected cells is approximately 1.2 days [91]. Conversely the time required for generation of antibodies with new specificities is on the order of weeks. It is therefore not surprising that the immune response is forever lagging behind a rapidly evolving virus. Indeed, it has been demonstrated during the early phases of infection there is weak to no neutralization of autologous virus by contemporaneous host antibodies [83, 84, 90]. As a result, at any time during the course of infection, neutralizing antibody responses are more likely to recognize earlier versions of autologous virus rather than contemporaneous virus.

Over time, as the immune response evolves and the virus population diversifies,

neutralizing antibodies capable of recognizing heterologous isolates can also be detected [84, 92, 93]. It has been reported that in up to 10% of HIV infected individuals, a broadly cross-reactive neutralizing (BCN) antibody response is generated [94-99]. In some cases, these BCN antibodies are capable of neutralizing HIV-1 isolates representing multiple clades and provide hope that a cross-reactive neutralizing response against HIV-1 can be induced. Recently, a new subset of HIV-1 infected individuals termed "elite neutralizers" has been described [100]. In contrast to BCN patients, the elite neutralizers contain higher titers of cross-reactive neutralizing antibodies but only constitute a mere 1% of HIV-1 infected individuals. The so-called "elite neutralizers", were identified when researchers screened over 1000 serum and plasma samples from various geographical locations in a virus neutralization assay against a predetermined panel of heterologous primary isolates representing multiple clades.

The identification of broadly neutralizing and now "elite neutralizing" antibody responses provides evidence that effective broadly reactive HIV-1 virus neutralization can indeed occur. A better understanding of the mechanisms that lead some individuals to develop a broad heterologous neutralizing antibody response should aid in the development of a globally effective vaccine.

HIV-1 structure and function

HIV-1 structure

The Human Immunodeficiency viruses (HIV-1, HIV-2) are members of the family *Retroviridae* and belong to the genus *Lentivirinae*. Electron microscopy and morphological studies indicated they are enveloped viruses with a cone shaped nucleocapsid core with an approximate diameter of 80-130 nm [101-103]. As seen in Figure 2, the viral cone shaped core obtains its structure largely from the capsid protein (CA). Contained within the core are two copies of viral RNA, viral reverse transcriptase (RT), viral integrase (IN), and viral protease (PR). Also included within the core are three of the six viral accessory proteins (Vif, Vpr and Nef) and p6. Surrounding the core is the matrix protein (MA), lying just beneath the lipid bilayer of the virus membrane. The lipid bilayer is derived from the host cell membrane upon viral budding and therefore contains both viral and host cellular protein components. Protruding from the viral surface are the envelope glycoprotein spikes which are composed of the gp120 and gp41 subunits that associate to form a homotrimer of heterodimers.

HIV-1 genome

The HIV-1 genome consists of a single strand of positive sense RNA, approximately 9.8 kilobases (kb) in length, and encodes open reading frames for the structural genes (*gag*, *pol* and *env*) as well as six other accessory genes that encode the other viral accessory proteins making HIV a complex retrovirus.

The *gag* gene is translated from a full-length viral mRNA into a polyprotein precursor (p55Gag). The Gag proteins are structural components and include the MA, CA, NC, and p6 protein. It has been shown that production of the Gag precursor protein alone is sufficient for the assembly and release of virus like particles [104].

The *pol* gene (polymerase) is translated from a full length mRNA and encodes proteins essential for various viral enzymatic functions. The *pol* proteins include the viral PR, RT and IN. Due to an overlap between the *gag* and *pol* genes, a translational frame shift must occur to produce the Gag-Pol polyprotein precursor (p106). The frame shift is facilitated by a slippage sequence present in the gene overlap region [105]. The resulting Gag-Pol polyprotein precursor (p106) is cleaved by the viral protease into component subunits.

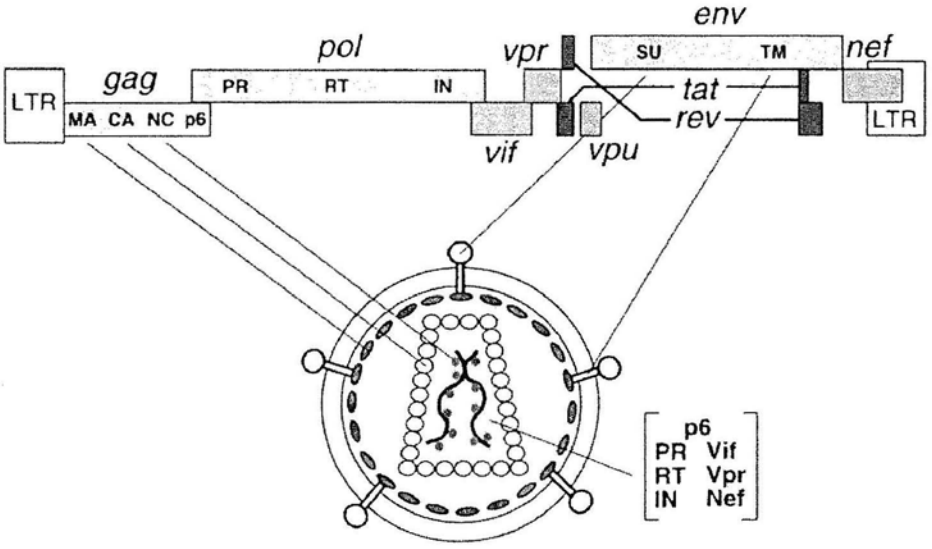
The *env* gene (envelope) is translated from a singly spliced mRNA and encodes for the two Env proteins, gp120 (attachment glycoprotein) and gp41 (transmembrane glycoprotein). The *env* gene is first translated into a gp160 glycoprotein precursor that is passed through the endoplasmic reticulum (ER) and the golgi, becoming heavily glycosylated in the process. The gp160 precursor is cleaved into the mature gp120 and gp41 subunits before transport to the host cell membrane where they remain associated noncovalently to form the HIV-1 envelope glycoprotein spikes on the surface of the virus. Details concerning the biosynthesis, maturation, structure and function of the Env glycoproteins will be discussed in more depth in the following sections.

The HIV-1 genome also encodes for six additional proteins known as accessory proteins. Three of the accessory proteins, Vif, Vpr and Nef, are packaged into the viral particle. The remaining three accessory proteins, Tat, Rev and Vpu, provide essential

regulatory functions for virus replication. There are differences between HIV-1 and HIV-2 in the accessory genes. The Vpu and Vpr genes are unique to HIV-1, compared to other retroviruses. The Vpr gene does share homology with the Vpx gene, unique to HIV-2, and they likely function in a similar fashion [106]. The accessory proteins and their functions are as follows: Vif promotes virion maturation and infectivity, Vpr promotes translocation of HIV nucleoprotein complexes into nucleus [107, 108], Nef down regulates CD4 expression [109, 110], Tat is a viral transcription activator, Rev is involved in viral RNA transport, and Vpu promotes degradation of CD4 contained in CD4/Env complexes present in the ER, thereby allowing transport of ENV molecules to the cell surface [111].

Figure 2. Organization of the HIV-1 genome and virion structure

HIV-1 is an enveloped, cone shaped virus with a diameter of 80-130nm. Contained within the cone shaped core are two copies of positive sense RNA genome. The HIV-1 genome encodes open reading frames for the structural genes (*gag*, *pol* and *env*) and for six accessory genes (*vif*, *vpr*, *nef*, *tat*, *rev* and *vpu*) making HIV-1 a complex retrovirus. The HIV-1 genome is flanked by long terminal repeats (LRT). On the surface of the HIV-1 virion are the envelope glycoprotein spikes, gp120 (attachment glycoprotein) and gp41 (transmembrane glycoprotein). Image used with permission from [111].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IN Nef

The HIV-1 envelope glycoprotein

The HIV-1 envelope glycoprotein present on the surface of the virus is essential for viral entry into target cells. In addition, the envelope glycoprotein is the main target for neutralizing antibodies generated during the course of HIV-1 infection. It is therefore not surprising that the envelope glycoprotein has evolved a variety of mechanisms to shield itself from the effects of neutralizing antibodies. Such mechanisms include; trimerization of the gp120/gp41 structure thereby occluding much of the conserved surface of gp120 from neutralizing antibodies [112], the addition of N-linked oligosaccharides resulting in a largely immunologically silent "glycan shield" [83], and the presence of five variable loop regions that are highly variable in sequence and continuously altered under selection pressure [113].

Envelope structure

The structure of the mature envelope glycoprotein consists of a homotrimer of gp120/gp41 heterodimers. Each heterodimer is composed of a cell surface receptor attachment glycoprotein (gp120), which binds to the CD4 receptor and to the co-receptor (CCR5 and/or CXCR4), and a membrane spanning protein (gp41), required for the fusion of the viral and cellular membranes [111, 114]. According to cryoelectron microscopy results performed by Roux et al, each HIV-1 virion may contain as few as 5-10 envelope glycoprotein spikes [115]. It has recently been estimated that only one functional envelope glycoprotein spike may be needed to mediate viral entry [116].

The envelope glycoprotein is generated as a polyprotein gp160 precursor [117]. As the gp160 precursor moves through the endoplasmic reticulum and the Golgi, the protein oligomerizes and becomes heavily glycosylated owing to the addition of N-linked high-mannose-type oligosaccharides [118, 119]. The resulting polyprotein is cleaved in the trans-Golgi network by furin and other endoproteases into the gp120 and gp41 subunits [117]. The resulting gp120/gp41 complexes are associated via noncovalent interactions and are transported to the cell surface where they are incorporated into the HIV virion in the course of viral budding from the infected cell.

HIV-1 surface glycoprotein (gp120)

The surface glycoprotein (gp120) is composed of five conserved regions (C1-C5) interspersed with five variable regions (V1-V5). Structural studies of gp120 suggested that the conserved regions form a central core and the variable regions, (V1-V2, V3, V4 and V5) extend from the surface of the protein [114, 120]. To date there are four reported crystal structures of gp120 for HIV-1 envelope glycoprotein and one crystal structure of the gp120 from SIV that have contributed to the understanding of the overall gp120 core structure as seen in Figure 3 [114, 121, 122]. These studies demonstrated that the core is composed of 25 beta-strands, 5 alpha-helices and 10 loop segments all folded into a heart-like shaped globular structure. The globular structure is composed of two major domains, an inner domain and an outer domain, linked to each other by a bridging sheet. The inner domain is composed primarily of the C1 and C5 regions and is largely devoid of glycosylation, whereas the outer domain is heavily glycosylated, thereby rendering itself a poor target for antibody neutralization [112, 114, 117, 123].

HIV-1 receptor binding sites

The receptor binding sites for the virus are present on the surface of gp120 and are attractive targets for therapeutic intervention, because an important aspect of the receptor binding sites is that they are conserved among the different clades of HIV-1. A second attractive quality is the necessary exposure of the receptor binding sites to the immune system in order for the virus to bind and infect a cell. However, in spite of these potential vulnerabilities, the virus has evolved mechanisms to protect these conserved regions from the reach of neutralizing antibodies.

The CD4 binding site is contained in a deep depression formed at the interface of the inner and outer domains along with the bridging sheet [114, 124]. The contact sites for CD4 on gp120 are formed by several discontinuous sequences distributed over six segments of the gp120 surface. Many of the CD4 contact sites on gp120 are, in fact, quite variable in sequence [125]. Furthermore, it has been reported that many primary isolates are inherently resistant to soluble CD4 binding, thereby limiting the potential efficacy of CD4 based therapies [126]. Additional contact sites include two large hydrophobic cavities; the localization of contact sites in these cavities likely reduces their immediate recognition by neutralizing antibodies. In addition, it has been shown that both CD4 and gp120 are quite flexible and this flexibility may have profound implications in the binding of gp120 to CD4 and the overall immunogenicity of the CD4 binding site (CD4bs) region [127, 128].

Crystal structures comparing the gp120 core liganded and unliganded to CD4 suggest that there may be significant conformational flexibility in the gp120 subunit prior

to CD4 binding. In particular there is flexibility in the inner domain, the bridging sheet and the V1/V2 stem of gp120 [114, 124]. In the unliganded state, the two beta-strand pairs that constitute the bridging sheet are separated from one another by 20 angstroms. One pair of beta strands, (β2-3) constitutes the V1/V2 stem, and models suggest that in the absence of CD4 the β2-3 strands oscillate such that they resemble both the liganded and unliganded structure, thereby in essence become a moving target. Conversely, the other beta strand pair (β20-21) largely remains in the same location near the outer domain for both the liganded and unliganded structures. Ultimately the conformational changes that occur in the inner domain of gp120 upon CD4 binding result in a 40 angstrom shift of the V1/V2 stem to form the bridging sheet [121].

The co-receptor-binding site is usually completely absent in the unliganded structure, and only appears after the conformational changes induced through the binding of gp120 to CD4 [121]. There are two regions important for the binding of gp120 to the co-receptor. They include portions of the intact bridging sheet as well as residues in the V3 loop sequence [69, 129]. Additionally, the V1/V2 loop region masks the co-receptor-binding site, providing an added shield of protection from neutralizing antibodies [130, 131].

HIV-1 entry and replication

The HIV-1 replication cycle begins with the attachment and adsorption of the virus particle to host cellular proteins [125]. HIV-1 first binds to CD4 [132]. The CD4 molecule is a member of the immunoglobulin superfamily and is expressed mainly on T-lymphocytes, macrophages and DCs. Binding of HIV-1 to CD4 is essential in most cases but not sufficient for infection to proceed. In addition to utilizing CD4 as a receptor,

HIV-1 requires the interaction of additional host proteins as co-receptors. The HIV-1 co-receptors are members of the seven transmembrane spanning CC or CXC families of chemokine receptors. Although multiple chemokine receptors have been shown to interact with one or more strains of HIV-1 the two principle co-receptors are CCR5 and CXCR4 [71, 72]. Chemokine specificity, or tropism, is determined mainly by the sequence of the third variable (V3) loop on the gp120 portion of HIV Env [69].

Entry of HIV-1 into target cells is a complex process. The first step involves binding of the gp120 subunit to the CD4 receptor on the host cell. This initial binding step involves substantial conformational changes in the Envelope spike. The process of gp120 binding CD4 results in movements within regions of the spike referred to as the bridging sheet, and the inner and outer domains of gp120 [114, 133-135]. These changes involve the movement of the V1-V2 variable loop stem, which acts to shield the underlying conserved receptor-binding site, as well as the movement of the V3 loop into position to aid in the binding of the co-receptor [112, 130]. Following co-receptor binding additional conformation changes ensue to allow insertion of a fusion peptide at the amino terminus of gp41 into the target cell membrane, ultimately leading to viral and cellular membrane fusion [136]. The gp41 ectodomain consists of alpha helical regions, which form a coiled-coil structure during the conformational changes leading to virus entry. The coiled-coil formation or 6-helix bundle formation acts as a spring-loaded mechanism to bring the cellular and viral membranes into close proximity leading to the formation of a fusion pore through which viral components enter the target cell. The membrane fusion event at merger is still poorly understood, and it is still unknown how many envelope glycoproteins are required for the formation of a fusion pore [137, 138].

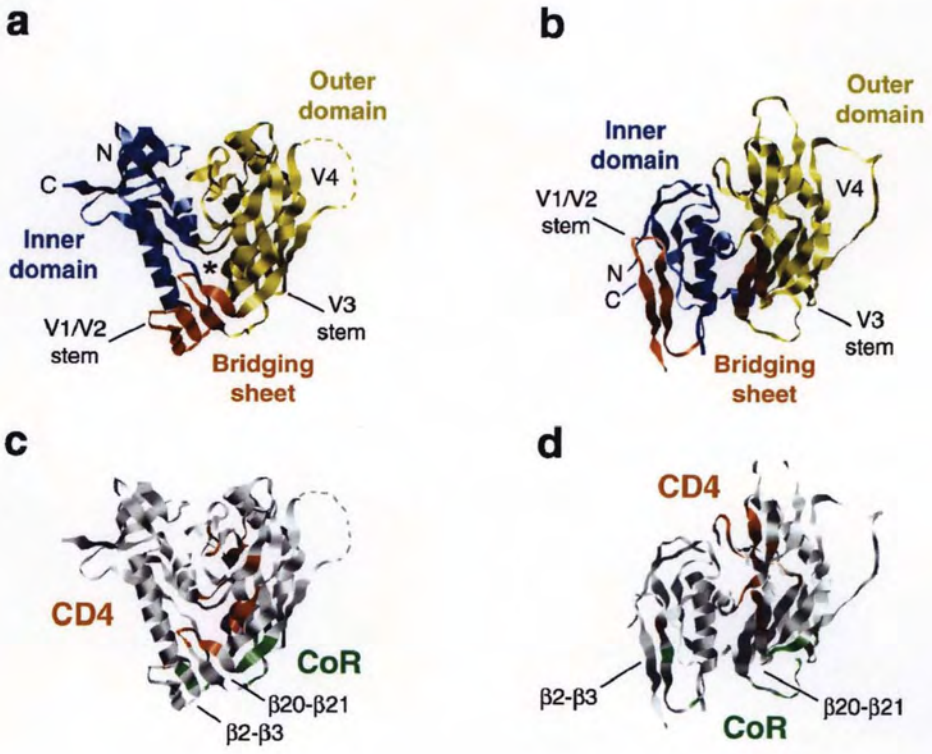
Following viral and cellular membrane fusion, the viral core is released into the cytoplasm and viral replication is initiated. Viral core uncoating and reverse transcription of the single stranded positive viral RNA genome into the double stranded DNA provirus begins simultaneously [139, 140]. The virus carries in with it both the RT as well as a $tRNA^{lys}$ to initiate reverse transcription. The process of reverse transcription generates a plethora of mutations due to a lack of a 3'-5' proofreading activity of the RT enzyme [36, 38]. Mutations include point mutations as well as insertions and deletions. Various mutations are maintained as a result of selective pressure brought on by the immune system, ultimately resulting in a large diverse quasispecies in the host [66].

The resulting double stranded HIV-1 provirus is translocated into the host cell nucleus with the aid of the viral MA and Vpr proteins [141-143]. The completed full-length double stranded DNA provirus is then integrated into the host cell genome by the viral IN protein [139, 140]. The 5' and 3' ends of the integrated provirus contain long terminal repeats (LTR). Transcription, enhanced by Tat, begins at the 5' end via promoter enhancer elements present in the LTR region and ends at the 3' end. The resulting transcripts are capped at the 5' end with 7-methylguanosine and polyadenylated at the 3' end with the aid of cellular enzymes [144, 145]. Full length genomic RNAs along with a set of spliced RNAs are transported, with the aid of Rev, from the nucleus to the cytoplasm where translation and packaging occur. Gag and Gag-Pol polyproteins are translated and processed in the cytoplasm before localizing to the cell membrane where they are involved in virus assembly. The Env (gp160) polyprotein is translated at the endoplasmic reticulum and passed through the Golgi where it becomes heavily glycosylated [118, 119]. The resulting Env polyprotein is cleaved in the trans-Golgi

network into the gp120 and gp41 subunits before localizing to the cell surface to be incorporated into the HIV virion upon virus budding. The final step involves the budding and maturation of the HIV virus. Maturation involves proteolytic processing of the Gag and Gag-Pol polyproteins by PR and Vif [111].

Figure 3. Structure of the HIV-1 gp120 core

Comparison of panels (a) and (c), which represent the HIV-1 gp120 core region in their CD4 bound conformation to panels (b) and (d), which represents the SIV gp120 core region in their unbound conformation, illustrates the extreme flexibility of the gp120 molecule. Comparison of panel (a) to panel (b) illustrates the locations and movements of the inner (blue) and outer (yellow) domains, the bridging sheet (orange) and the variable regions both before (d) and after (a) CD4 binding. Comparison of panel (c) to panel (d) illustrates the formation of the CD4 binding site (orange) and the co-receptor binding site (green) and the movement of the two beta pairs (β20-21) and (β2-3) which constitute the bridging sheet. Image used with permission from [146].


a
Outer domain
N
C
V4
Inner domain
*
V3 stem
V1/V2 stem
Bridging sheet
b
Outer domain
Inner domain
V1/V2 stem
V4
N
C
V3 stem
Bridging sheet
c
CD4
CoR
β20-β21
β2-β3
d
CD4
β2-β3
β20-β21
CoR

Antigenic determinants of the envelope glycoprotein

There are several key features to the structure of the HIV-1 envelope glycoprotein that result in the generation of both neutralizing and non-neutralizing antibodies throughout the course of infection. Neutralizing antibodies target the functional envelope glycoprotein, which is in the form of a homotrimer of heterodimers [115, 147]. However, other non-functional forms of the envelope glycoprotein may be present on the surface of HIV-1. These nonfunctional envelope glycoproteins may be in the form of monomers, dimers, tetramers, as well as gp41 stumps left behind from gp120 shedding. It is believed that such nonfunctional forms may arise from a break in the weak noncovalent interactions holding the trimer of heterodimers together, or they may be a result of inefficient trimerization in the Golgi [117, 148]. However they arise, the presence of these nonfunctional forms of the envelope glycoprotein in essence act as immunological decoys ultimately resulting in the generation of a non-neutralizing HIV-1 antibody response.

Studies suggest that gp120 contains three immunological "faces" known as the silent face, the non-neutralizing face, and the neutralizing face. The extensive glycosylation of the envelope glycoprotein renders a large proportion of the envelope glycoprotein to be either inaccessible to or invisible to antibodies [112]. It is believed that the carbohydrate chains act as poor immunogens, as they appear to the immune system as "self", rather than as antigenic determinants. Interestingly, the crystal structure of the gp120 core demonstrated that most carbohydrates were located on a single face of the outer domain of gp120. This location has been dubbed the "silent face" of gp120

owing to its poor immunogenicity brought upon by the heavy concentration of glycans [112]. Additional studies have documented that while the average number of glycans on the surface of gp120 remains about the same (n=25-30), their placement is constantly changing due to amino acid mutations in the underlying peptide. This has led to the theory of an ever evolving and movable glycan shield that prevents antibody binding via steric hindrance [83].

The second immunological face known as "the non-neutralizing face" of gp120 in fact generates a strong antibody response in infected individuals. Such antibodies however bind to epitopes that do not neutralize the virus. It has been demonstrated that the non-neutralizing face maps to the conserved inner domain of the gp120 core [114]. Models of gp120 trimers suggest that the non-neutralizing face is actually buried within the trimer and therefore not exposed on the functional oligomeric complex present on the viral particle. Therefore antibodies that bind to this surface cannot bind to functional envelope glycoprotein spikes or virions and exhibit no neutralizing activity [112, 123, 149]. It has been proposed that the non-neutralizing face is well exposed on gp120 monomers and that antibodies directed to this face are generated as a result of gp120 shedding from viral particles and/or infected cells [125].

The third face, also known as "the neutralizing face" of gp120, can be thought of as the Achilles heel of the HIV-1 envelope glycoprotein. In order for HIV-1 to replicate, gp120 must associate with the appropriate cellular receptors thereby rendering certain aspects of gp120 to be both conserved and exposed. The two most conserved regions, the CD4 binding site and the co-receptor-binding site, are on the "neutralizing face". However, they are poorly accessible to neutralizing antibodies [112, 114]. Additionally,

studies performed by Kwong, et. al. have proposed that the CD4 binding site and the co-receptor binding site are further camouflaged from the immune system by a mechanism known as conformational masking [128]. The term of conformational masking reflects the observation that there is substantial energy required, or entropy involved when anti-CD4 and anti-co-receptor binding site antibodies bind to their respective epitopes. This entropy appears to reflect requirements for conformational change in gp120 for high affinity binding to occur.

Vulnerabilities in the envelope glycoprotein

In spite of the multiple immune evasion strategies employed by HIV-1, virus isolates representing different HIV-1 subtypes can be neutralized by the plasma of some patients who contain broadly cross-neutralizing antibodies [94, 96-99, 150]. In addition, monoclonal antibodies (mAbs) capable of neutralizing primary isolates representing multiple clades have also been identified. The epitope specificities of these mAbs are illustrated in Figure 4 [151-161,162]. This evidence of broadly cross-reactive neutralization provides hope that a cross-reactive neutralizing response against HIV-1 can be achieved and indicates that certain elements of the HIV-1 envelope glycoprotein structure are indeed well conserved.

Neutralization studies have defined conserved, cross neutralizing epitope regions present on the surface of the envelope glycoprotein including; the CD4 binding site, a unique glycan site on the silent face of gp120, and the membrane proximal (MPER) region of gp41. In addition, there are other important neutralization epitope regions on the surface of the envelope glycoprotein including the V3 region as well as the co-receptor-binding site.

CD4 binding site antibodies

Antibodies targeting the CD4 binding site recognize epitopes located at the interface of the inner and outer domains of gp120 and compete with CD4 for binding to gp120 [163]. A number of CD4bs antibodies have been described, however the majority

are only able to neutralize T cell line adapted (TCLA) isolates, which are known to be neutralization sensitive, rather than primary isolates, which tend to be neutralization resistant.

One particularly well characterized monoclonal antibody IgG1b12 (b12) is a highly potent BCN antibody that binds a discontinuous epitope region overlapping the CD4 binding site on gp120 and subsequently prevents CD4 attachment [156, 164]. Structural analyses have determined that b12 contains an extended CDR3 loop located on the variable region of the heavy chain. At the tip of the CDR3 loop, a protruding aromatic amino acid (tryptophan-100) has been implicated in binding to multiple amino acids in the CD4 binding site. Interestingly, it has also been determined that only the heavy chain of b12 makes contact with gp120 [165]. These unique structural and binding characteristics of the b12 may help to explain how it is able to bind to and neutralize the conformationally flexible gp120 envelope glycoprotein subunit.

Unique glycan epitope

The high level of glycosylation on the surface of the HIV-1 envelope glycoprotein, referred to as the glycan shield, confers protection from the attack of neutralizing antibodies. Nonetheless there exists a broadly neutralizing antibody, designated 2G12 that is capable of recognizing an epitope in the glycan shield. 2G12 binds to a cluster of high-mannose glycans located on the external surface of the "silent face" [152, 166, 167]. Mutagenesis and structural studies have demonstrated that amino acid residues N295, N332, N339, N386 and N392 in and around the C3/V4 region of gp120 are important for 2G12 binding [168]. While 2G12 represents a unique BCN mAb, its neutralizing activity

is mostly restricted to viruses from clade B. Indeed, 2G12 is unable to neutralize viruses from clade C, which are responsible for the majority of HIV infections worldwide. Additionally, 2G12 has been shown to contain minimal neutralizing activity against viruses from clades D as well as the circulating recombinant form, CRF AE [169]. The inability of 2G12 to neutralize as many viruses from other clades is likely due to interruptions in the glycan epitope region.

A gp120/gp41 complex epitope

A gp120/gp41 complex epitope region is recognized by mAb m43. The mAb m43 was selected along with four other mAbs m44, m45, m47 and m48 using a competitive antigen panning (CAP) technique from an immune phage library derived from long term nonprogressors who possessed high concentrations of broadly cross neutralizing antibodies [170]. Traditional phage panning techniques using soluble gp140 often result in the recovery of mAbs that preferentially recognize the gp120 subunit of the HIV-1 Envelope. The CAP technique was aimed at specifically identifying mAbs that preferentially recognize the gp41 subunit of the HIV-1 envelope glycoprotein. This is accomplished by panning with biotinylated, tethered gp140 subunits mixed together with 5-10 fold higher concentrations of gp120 subunits. Resulting mAbs selected with this technique have been found which neutralize primary HIV-1 Envelopes representing multiple clades [170]. In contrast to the linear 2F5 and 4E10 epitopes present in the MPER region of gp41, the newly identified mAbs recognize discontinuous, conformational epitopes. This specificity was determined using competition assays with

known linear gp41 peptides as well as observing the failure of the newly identified mAbs to bind to denatured gp140.

MPER region of gp41

The gp41 MPER region of the HIV-1 envelope glycoprotein is well conserved. However, due to its location on the functional spike, it is relatively inaccessible to neutralizing antibodies prior to the conformation changes induced following the binding of gp120 to CD4 and co-receptor. Nonetheless, highly potent mAbs against the gp41 MPER have been isolated. Of all the gp41 mAbs isolated to date, the two best characterized are 2F5 and 4E10. 2F5 and 4E10 recognize adjacent linear epitopes in the MPER region of gp41. MAb 2F5 recognizes the conserved linear sequence $_{662}$ELDKWA$_{667}$. The residues critical for binding are the triplet DKW motif [153, 154, 171, 172]. The 4E10 epitope lies directly adjacent to the 2F5 epitope and comprises the conserved linear sequence $_{671}$NWF(D/N) ITNWLW$_{680}$. The residues critical for 4E10 binding are dispersed throughout the epitope, and are as follows: W_{671}, F_{672} and W_{680} [151, 157, 172].

A recent comprehensive cross clade neutralization study utilizing a panel of primary HIV isolates and various mAbs was reported by Binley et al [169]. In that study it was shown that 2F5 neutralized 67% of all the isolates tested, but 2F5 did not neutralize any virus isolates in the panel representing HIV-1 clade C. This is not surprising considering that the epitope region sequence recognized by the 2F5 mAb is absent in a large proportion of viruses representing clade C. MAb 4E10, on the other hand, was able to neutralize more isolates than 2F5, albeit at a lower concentration [169].

While cross clade neutralization studies indicate that mAbs targeting the gp41 region are broadly cross neutralizing, attempts to develop immunogens that elicit such mAbs have so far been unsuccessful.

V3 binding site antibodies

To date there is one well-characterized BCN mAb designated 447-52D that targets the V3 region [155]. 447-52D recognizes the epitope sequence Gly-Pro-Gly-Arg (GPGR) present at the tip of the V3 loop [155, 173, 174]. MAb 447-52D exhibits somewhat broad cross neutralization making it a potential vaccine target [169]. However its specificity is somewhat restricted owing to the epitope sequence it recognizes. Indeed, the GPGR motif recognized by the 447-52D mAb is specific for clade B viruses only.

CD4 induced/co-receptor binding site antibodies

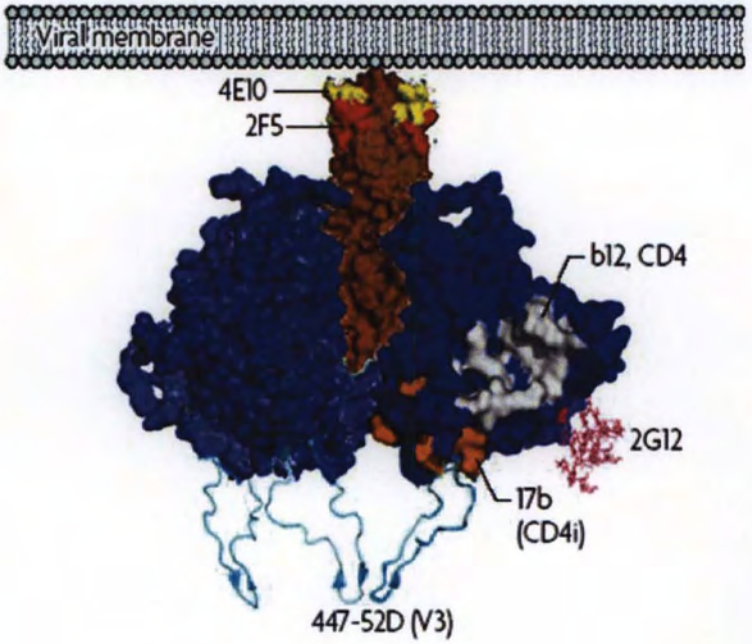
CD4 induced antibodies (CD4i), block the binding of HIV gp120 to the co-receptor. Antibodies that recognize the co-receptor-binding site are usually able to do so following the conformational change occurring after the binding of CD4 to gp120. As a result, CD4i mAbs generally neutralize primary viruses weakly if at all. The ability of CD4i mAbs to neutralize primary isolates can however be increased in the presence of sub-neutralizing amounts of sCD4 [175-177].

Epitopes targeted by CD4i antibodies are believed to be somewhat masked by the V2 loop. Indeed virus variants with V1/V2 loops deleted display increased sensitivity to neutralization using CD4i mAbs [130, 131, 178]. Nonetheless, mAbs targeting the CD4i

region have been identified. Two well characterized anti-CD4i mAbs are 17b and 48d. Both 17b and 48d exhibit neutralizing activity and recognize discontinuous epitopes in the bridging sheet [112, 124, 130, 163, 179]. More recently a novel anti-CD4 mAb denoted X5 was identified from a phage display library from a seropositive donor with a moderately high BCN titer [160]. It was shown that X5 inhibits HIV-1 entry and Env mediated cell fusion with a potency comparable to IgG1 b12 and binds to gp120-CD4 complexes better than to gp120 alone. In an attempt to identify mAbs with a higher potency than X5, a novel panning technique known as sequential antigen panning (SAP) was employed. SAP is a method by which the panning antigen is sequentially replaced during each subsequent round of panning with the hypothesis that this will lead to the identification of mAbs that preferentially bind to epitopes that are conserved among each of the respective panning antigens. This technique led to the identification of an additional CD4i mAb known as m9 [180]. MAb m9 targets the co-receptor binding region on gp120 and has been shown to be more potent than the previously described mAb X5 [180].

Figure 4. Epitope regions on the HIV-1 envelope glycoprotein targeted by cross reactive mAbs

Examples of epitope regions on the gp120 subunit of the HIV-1 envelope glycoprotein (blue) targeted by cross reactive mAbs include; the CD4bs, recognized by b12 and CD4 (grey), the co-receptor binding site/or CD4i, recognized by 17b (orange), the unique glycan specific epitope recognized by 2G12 (pink glycans), and the V3 region, recognized by 447-52D. Examples of epitope regions on the gp41 subunit of the HIV-1 envelope glycoprotein (brown) include the MPER region recognized by 4E10 (yellow) and 2F5 (red). Image used with permission from [162].


Viral membrane
4E10
2F5
b12, CD4
2G12
17b
(CD4i)
447-52D (V3)

Previous work relating to thesis project.

The patient (HNS2 donor) examined in this thesis research was an HIV-1 infected individual participating in a long-term cohort study at the Clinical Center of the National Institutes of Health (NIH). In 1995, an HIV-1 neutralizing serum denoted HNS2 was prepared from a plasma sample obtained from the individual in 1989 for its use as a reference reagent for laboratories conducting HIV-1 neutralizing antibody tests [94, 95]. The patient became of interest when it was recognized that neutralization studies using the HNS2 serum indicated that it contained cross-reactive neutralizing antibodies when tested against various primary HIV-1 strains representing different HIV clades [94, 95].

In addition to the preparation of the HNS2 serum, a functional envelope glycoprotein clone, designated R2, was amplified from proviral DNA extracted from lymphocytes collected from the patient in 1989. Sequence analysis of the R2 envelope glycoprotein clone demonstrated that it belonged to HIV-1 clade B and was similar to typical HIV-1 envelopes with respect to localization and numbers of potential N-linked glycosylation sites. Further analysis of the R2 envelope glycoprotein clone found that its V3 region was unique compared to other strains in the HIV database. Unusual features include a proline-methionine (PM) mutation at positions 313-314 and a glutamine (Q) at position 325. Subsequent analysis demonstrated that the PM mutation in the V3 loop caused virus to be capable of CD4-independent infection, albeit at lower efficiency, and was highly sensitive to neutralization using heterologous HIV-1 plasma and various monoclonal antibodies [181]. CD4 independence is a rarely recognized property of HIV-1, and is dependent on mutations of these residues in the V3 region [175]. Based on

these findings, further characterization of the R2 envelope glycoprotein for use as a potential immunogen were undertaken.

The generation of broadly cross neutralizing (BCN) antibodies is a major goal in the effort to develop a globally effective HIV-1 vaccine. Identifying and utilizing immunogens like the R2 envelope glycoprotein might provide crucial insights into the mechanisms needed to induce a BCN response. Various approaches utilizing the R2 envelope glycoprotein as an immunogen have been tried. The first approach led to the induction of cross-reactive HIV-1 neutralizing antibodies in mice and rabbits through the use of an alphavirus-derived expression system using Venezuelan equine encephalitis virus replicon particles (VEE-RP) [182]. Use of the VEE-RP system was employed, as this system is highly effective in inducing neutralizing antibodies against a number of glycoproteins including viral glycoproteins [183, 184]. Following inoculation into animals, VEE-RPs are taken up by dendritic cells and transported to lymphoid tissues where they express their transgenes with high efficiency [185]. VEE-RPs expressing the R2 envelope glycoprotein induced moderately cross-reactive responses in mice and rabbits [182]. Furthermore, immunization of Rhesus monkeys was carried out using the VEE-replicon particle system expressing the R2 envelope glycoprotein, followed by booster doses of the R2 envelope glycoprotein administered as a truncated oligomeric form of the envelope glycoprotein known as (gp140), which was produced in cell culture using a vaccinia virus expression system and purified to select for oligomeric envelope glycoproteins. The results indicated that antibodies induced by the VEE-RP/gp140 immunization regimen neutralized diverse primary strains of HIV-1 and offered protection of some of the monkeys after challenge with the partially pathogenic SHIV

strain DH12R $_{(Clone\ 7)}$ [186]. The soluble, oligomeric form of $gp140_{R2}$ possesses many of the functions of the mature Envelope spikes present on the surface of the HIV-1 virus [170]. Subsequently a study examining the immunization of rabbits with the soluble form of $gp140_{R2}$ in conjunction with a novel AS02A adjuvant, an oil and water emulsion containing 3D-monophosphoryl lipid A and saponin QS21 induced antibodies that achieved 50% neutralization of a large panel of primary strains of diverse HIV-1 subtypes tested, albeit with low neutralizing titers [170]. The extent of neutralizing cross reactivity induced in each of the studies was greater than has been reported in other HIV-1 vaccine studies.

Additionally, cross-reactive mAbs have been identified, characterized and added to the growing repertoire of HIV-1 neutralizing antibodies as a result of studies involving the R2 envelope glycoprotein or bone marrow from the patient. Phage panning with the R2 envelope glycoprotein against immune libraries from long-term nonprogressors resulted in the isolation of mAbs of various cross reactivity directed against conformational epitopes in both the gp120 region (m22 and m24) as well as the gp41 region (m46) [158]. Phage panning with the MN virus (HIV-1 clade B) against a 1996 bone marrow sample from the R2 donor resulted in the isolation of Z13, a gp41 specific mAb targeting a linear epitope in the MPER region and lying in close proximity to the 2F5 and 4E10 epitopes [172]. Phage panning using bone marrow from the same patient also resulted in the isolation of mAb 4KG5 [159]. Interestingly, 4KG5 is not a neutralizing mAb; rather it acts to enhance the neutralization capabilities of b12, a broadly cross-reactive CD4 binding site monoclonal antibody.

Considering the broad cross reactivity of neutralization in the HNS2 serum and the unusual nature and immunogenicity of the R2 envelope glycoprotein, we hypothesize that the neutralizing response in the patient may be the result of the phenotype of the infecting virus. Additionally, we hypothesize that the study of the evolution of the neutralizing antibody response and envelop glycoprotein gene variation in the patient over the course of infection will reveal evidence of the mechanisms of induction and maintenance of the BCN response. For this purpose we obtained sequential plasma and PBMC samples from the patient. The PBMC samples were available as primary cells collected on a yearly basis from 1986-1991 and as co-cultured cells prepared on a yearly basis from 1991-1993. All patient PBMC samples were stored in liquid nitrogen prior to use. Patient plasma samples were collected at the same time as the PBMC samples. All plasma samples were heat inactivated prior to use and stored at -80^0C. The cohort study ended in 1994 and it is believed that the patient initiated HAART therapy soon thereafter (personal communication with H. J. Alter). Due to the termination of the cohort study in 1994, there is a gap in the time sequence of this study. In 2007 when this thesis project was initiated, contact was reestablished with the patient in order to acquire an additional blood sample for the purpose of this study. The 2007 sample was obtained with IRB approval and informed consent. In spite of the gap in the time sequence of this study, important information regarding the evolution of the neutralizing antibody response and Envelope gene variation in the patient has been determined as described in the results chapters of this thesis.

In spite of our best efforts, there are some limitations to this research project. In most cases, the volumes of patient plasma available to us were not sufficient for use both

as a source of viral RNA for envelope gene cloning and for use in neutralization testing. For this reason, DNA extracted from primary or co-cultured PBMC was used as the source of proviral envelope gene cloning throughout this study. Traditionally, functional HIV-1 envelope genes are amplified from either proviral DNA or viral RNA through RT-PCR and the resulting envelope genes are cloned into expression vectors. The expression vectors are co-transfected along with a defective envelope clone, resulting in the production of pseudovirus, thereby allowing for various infectivity and neutralization studies of the amplified Envelopes [187]. There is a concern that such bulk PCR amplification methods using either proviral DNA or vRNA will result in various artifacts such as *Taq*-induced nucleotide substitutions, artificial recombination and resampling of viral genomes during the PCR process [188, 189]. Therefore, a technique involving single genome amplification (SGA) of HIV-1 Envelope genes was considered for use in this study [190]. The SGA method attempts to isolate a single molecule of viral RNA or copy DNA (cDNA) through serial dilution testing. However, the SGA method requires the use of larger plasma volumes that were not available to us for this study.

We reserved our limited amounts of patient plasma samples for use in our neutralization testing in order to determine the evolution of the neutralizing antibody response generated in the patient. We were able to test a limited number of the primary HIV-1 subtype B and C panel viruses, set up by the NIH and proposed for their use as standards for evaluating the potency of anti-HIV-1 neutralization activity [191, 192]. Similarly, we were only able to test a limited number of the sequentially cloned patient specific envelope glycoproteins for neutralization by the evolving autologous neutralization response in the patient. In spite of these potential limitations, we were able

to make significant observations regarding the evolution of gene variation in the patient that may be relevant to the induction and maintenance of the BCN response.

The results obtained in this study do not prove a relationship between phenotype of envelope glycoprotein of the infecting HIV-1 virus and breadth of neutralization response. They do however, indicate features of infection in this individual that may be related to the breadth of neutralization response and should be of interest for further evaluation in subsequent studies.

Specific Aims and Hypotheses

Building on previous work performed thus far, the specific aims of this thesis project are as follows:

Specific Aim 1

Characterize the evolution of the HIV-1 envelope glycoprotein gene and neutralizing antibody response in the HNS2 donor.

We hypothesize that the HIV-1 envelope glycoprotein gene variation and evolution of the neutralizing antibody response will reveal mechanisms of the induction and maintenance of the BCN response in the patient.

Specific Aim 2

Characterize the evolution of neutralization sensitivity of the HIV-1 envelope glycoproteins from the HNS2 donor to sCD4 and eight cross-reactive mAbs.

We hypothesize that the HIV-1 envelope glycoproteins will become progressively more resistant to neutralization as a result of mutations that contribute to global neutralization resistance. We anticipate that the nature of the development of neutralization resistance at various epitope regions will be related to the immunogenicity of the original infecting HIV-1 envelope glycoproteins.

Chapter 2. Materials and Methods

Patient samples

Peripheral blood mononuclear cells (PBMC) were collected from the patient on a yearly basis from 1986 to 1993 as part of a long-term cohort study conducted at the Clinical Center of the National Institutes of Health (NIH), division of Transfusion Medicine. PBMCs from the patient were available for study as primary PBMCs, representing years 1986-1991 as well as co-cultured PBMCs, representing years 1991-1993. A whole blood sample was collected from the patient in 2007. Primary PBMCs were collected from the 2007 sample using Ficoll-Isopaque density gradient centrifugation. All PBMCs were cryopreserved and stored in liquid nitrogen prior to use.

Additionally, plasma samples were collected from the patient on a yearly basis from 1986 to 1993 as part of the above mentioned cohort study and from the 2007 time point. Due to sample limitation, plasma from 1987 was not tested. The R2 donor reference plasma HNS2, derived from a 1989 sample is available from the AIDS research ARRRP (catalog no. 1983) and was provided by L. Vujcic and G. Quinnan [94]. In this study we utilized additional HIV-1 positive human sera designated, MACS A, B, C and D derived from a longitudinal study examining the natural history of HIV-1 in homosexual men and used with permission from the Multicenter AIDS Cohort Study (MACS) at Johns Hopkins University (Baltimore, MD) [193]. Human plasma and sera were heat inactivated for 30 minutes at 56°C prior to use and subsequently stored at -20°C. All patient samples were collected with informed consent and IRB approval.

PCR amplification and cloning of gp160 envelope genes

Due to limited amounts of patient plasma, cryopreserved PBMCs were used as the source of HIV-1 proviral DNA. Genomic DNA was isolated using the Qiagen Blood and Tissue Kit (Qiagen Inc., Valencia, CA). All genomic DNA extractions and PCR reactions were performed in a separate PCR containment hood to prevent contamination. Control reactions without template were performed at each step to monitor for carryover contamination. Nested PCR was performed using the high fidelity rTth DNA polymerase (Applied Biosystems, Foster City, CA) using primers based on the consensus HIV-1 subtype B sequence. Primer sequences for first round PCR as well as the forward primer for second round PCR have been described previously [96]. The reverse primer for the second round PCR reaction is as follows:

5'-ATATCTCGAGCTCGAGATACTGCTCCCACCCCATCTGCTGCTGGC-3'.

Both first and second round PCR reactions were run as follows: Denaturation at 94°C for 1 min followed by 15 cycles of denaturation (94°C for 15 secs) with annealing and extension (68°C for 10 mins), followed by 11 cycles of denaturation (94°C for 15 secs) with annealing and extension (68°C for 10 mins) followed by a final extension (at 72°C for 10 min). Positive PCR products were gel purified using the Qiagen gel and electrophoresis kit (Qiagen Inc, Valencia, CA) and cloned into an expression vector as previously described [95]. Functional gp160 envelope glycoproteins capable of a single round of infection were selected as described below.

Sequencing and phylogenetic analysis

A total of 62 individual functional whole length HIV-1 gp160 envelope genes representing 21 years of sample availability were sequenced using a panel of forward and reverse primers based on consensus HIV-1 clade B sequences from the Los Alamos National Laboratory HIV sequence database (http://www.hiv.lanl.gov). Sequence products were purified using the Performa DTR gel filtration cartridge (Edge BioSystems, Gaithersburg, MD). Sequencing was performed using BigDye 3.1 reaction mix and an ABI-3100 automated DNA sequencer (Applied Biosystems, Foster City, CA). The resulting sequences were assembled and aligned using Clustal X as implemented in Vector NTI version 10 (Invitrogen). Pairwise evolutionary distances were estimated using Kimura's two-parameter method which corrects for multiple hits and takes into account transitional and transversional bias. Phylogenetic trees were constructed using the neighbor joining method as implemented in MEGA 4.0 and tested for reliability with 500 bootstrap replicates [194-196]. Neighbor joining trees incorporated consensus and ancestral clade B Envelope sequences which were obtained from the HIV database at (http://www.hiv-web.lanl.gov). Bootstrap values >70% were considered significant. Nonsynonymous substitution (dN) and Synonymous substitution (dS) rates were calculated using Synonymous Nonsynonymous Analysis Program (SNAP) at the HIV database website (http://www.hiv.lanl.gov/content/sequence/SNAP/SNAP.html) in accordance with the method of Nei and Gojobori [197].

Additional envelope clones used in this study

Five HIV-1 clade B envelopes (AC10.0.29, QH0692.42, CAAN5342.A2, RHPA4259.7, PVO.4) and three HIV-1 clade C (ZM2495M.PL1, ZM214M.PL15, Du422.1) envelopes representing neutralization resistant primary isolates designed for use in neutralization assay standardization have been described previously [191, 192]. Additional envelopes used in this study are as follows; GXC-44 (Clade C) was obtained from a Chinese donor [182], UG273 (Clade A) was obtained from a Ugandan donor [96], GXE14 (CRF01_AE) was obtained from a Chinese donor [182] and 14/00/4 (Clade F) was obtained from a Congolese donor [96].

Cell cultures

The Human Osteosarcoma (HOS)-$CD4^+$-$CCR5^+$ cell line stably expresses CD4 and the CCR5 co-receptor and was obtained from the NIH AIDS Research and Reference Reagent Program (NIH ARRRP) originally submitted by N. Landau. The human embryonic kidney cell line 293T, (HEK 293T) was obtained from the ATCC (Rockville, MD). 293T cells were maintained in Dulbecco's minimal essential medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS), L-glutamine, penicillin and streptomycin (Gibco). The (HOS)-$CD4^+$-$CCR5^+$ cells were maintained in Dulbecco's minimum essential medium (DMEM) (Sigma) supplemented with 10% FBS, L-glutamine, penicillin, streptomycin (Gibco), tylosin (Sigma), and puromycin. All cell cultures were maintained in a humidified atmosphere at 37°C and 5% CO_2.

Pseudovirus construction

Pseudotyped viruses were produced [95]using the calcium phosphate/HEPES buffer co-transfection method (Promega, Madison WI) in 40-80% confluent 293T cells grown in 25cm flasks (Costar, Corning NY) along with pNL4-3.luc.E-R (obtained from the ARRRP, contributed by N. Landau). Eighteen hours after co-transfection the medium was replaced with medium supplemented with 0.1mM sodium butyrate (Sigma) and cells were propagated for an additional 24 hours. Pseudovirus containing supernatant was clarified by centrifugation at 13,000 rpm for 10 minutes at 4°C, sterilized by passage through a 45-um pore size sterile filter (Millipore, Bedford, MA) and then used immediately in neutralization assays.

Infectivity assays

The ability of cloned gp160 HIV-1 envelope glycoproteins to mediate infection of HOS-$CD4^+$-$CCR5^+$ cells was measured using pseudotyped viruses as previously described [95]. Briefly, two fold serial dilutions of pseudotyped virus was added to 1-2 x 10^4 HOS-$CD4^+$-$CCR5^+$ cells and incubated for 3 days at 37C in 5% CO_2. After incubation the cells were washed with phosphate buffered saline (PBS) and lysed for 30 minutes with 15µl of Luciferase Assay System cell lysis buffer (Promega, Madison, WI). Luciferase activity was determined using a MicroLumat Plus luminometer (Wallac, Gaithersburg, MD) after the automatic addition of 50µl of Luciferase substrate per well (Promega, Madison, WI). Envelopes were considered functional and positive for infectivity if the luciferase activity was at least 10,000-fold greater than the negative control.

Monoclonal antibodies (mAbs)

A panel of neutralizing monoclonal antibodies (mAbs) was used in this study in order to assess the evolution of neutralization epitopes expressed on the surface of primary HIV-1 Envelopes isolated from the R2 patient over the course of sample collection. MAb m43 recognizes a complex epitope spanning gp120 and gp41 was kindly provided by D. Dimitrov [170]. MAbs 2F5 and 4E10 are directed against the MPER region of gp41 and were obtained from Polymun Scientific, Vienna, Austria [151, 153, 172]. MAbs directed against the CD4bs region m14 and b12 were kindly provided by D. Dimitrov and obtained from Polymun Scientific, Vienna, Austria, respectfully [156, 160]. Two-domain sCD4 was a gift from C. Broder (USUHS, Bethesda, MD) [198]. MAbs directed against the CD4 induced region (co-receptor binding site) m9, 4.8D, and 17b were kindly provided by D. Dimitrov and J. Robinson, respectfully [163, 180].

Neutralization assays

Neutralization was measured as a reduction of luciferase gene expression following a single round of infection in HOS-$CD4^+$-$CCR5^+$using Env-pseudotyped viruses. Neutralization assays were carried out in duplicate by preincubation of 25µl of two-fold serial dilutions of mAbs, sCD4, and heat inactivated human plasma and/or sera with 25µl pseudovirus suspensions for 1 hour at 4^0C in 96-well, white walled, flat-bottomed tissue culture plates (Costar, Corning NY). The virus-antibody mixtures were then combined with 1-2 x 10^4 HOS-$CD4^+$-$CCR5^+$ cells and incubated for 3 days at 37^0C in 5% CO_2. After incubation the cells were washed and lysed for 30 minutes with 15µl

of Luciferase Assay System cell lysis buffer (Promega, Madison, WI). Luciferase activity was determined using a MicroLumat Plus luminometer (Wallac, Gaithersburg, MD) after the automatic addition of 50µl of Luciferase substrate per well (Promega, Madison, WI). Neutralization titers were calculated as the inhibitor concentration (IC_{50}) or the reciprocal plasma dilution (ID_{50}) causing a 50% reduction of relative light units (RLU).

Statistical analysis

Evolution of neutralization sensitivity of envelope glycoproteins from the patient to various monoclonal antibodies was determined using linear regression as implemented in Graphpad prism version 4.0 (Graphpad Software Inc.). Evolution of neutralization sensitivities of envelope glycoproteins from the patient to autologous and heterologous plasma samples was determined using a 2-way ANOVA with cluster and plasma as factors to determine if the individual clusters were responsible for the main effect in observed sensitivity over time. The evolution of the neutralizing antibody response in the patient was determined using a 2-way ANOVA with heterologous panel viruses and plasma as factors. All 2-way ANOVA applications were performed in Graphpad prism version 4.0 (Graphpad Software Inc.). All statistics were additionally verified using SPSS (SPSS Inc.).

Chapter 3. Evolution of the HIV-1 Envelope Glycoprotein Genes and Neutralizing Antibody Response in the HNS2 Donor

Patient samples and lymphocyte profiles

Table 1 lists the dates of sample collection from the patient. The patient's lymphocytic cell counts were determined for each of the time points of sample collection for the cohort study. Figure 5 illustrates the thymocyte subset profiles over the course of long-term evaluation of the patient. Patient thymocyte cell counts were determined at the NIH as part of the cohort study (personal communication with H.J. Alter). At the start of the study in 1986, the patient was asymptomatic, naïve to antiretroviral therapy, and had a $CD4^+$ and $CD8^+$T cell count of 410 and 744 cells/mm^3, respectfully. Over the next consecutive seven years of study sample availability, the $CD4^+$ and $CD8^+$ T cell profiles fluctuated. $CD4^+$ and $CD8^+$ T cell profiles peaked in 1990 with values of 617 and 1301 cells/mm^3 respectfully. Following the peak in 1990, there was a gradual decrease in the numbers of circulating $CD4^+$ and $CD8^+$ T cells for the remainder of the cohort study, with values of 450 and 972 cells/mm^3 respectfully as seen with the 1993 time point. Upon acquisition of the 2007 time point sample, the patient appeared "healthy and asymptomatic" and presented with an increase in the number of $CD4^+$ and $CD8^+$T cell counts of 1047 and 1805 cells/mm^3 respectfully. This increase in the number of circulating $CD4^+$ and $CD8^+$ T cells seen in the patient was likely attributable to HAART treatment.

Patient infection history

The exact timing of the onset of HIV-1 infection in the patient is unknown. However, according to patient history collected at the time of enrollment into the study in

1986 the patient had two risk factors for HIV-1 infection. The first risk factor was intravenous drug use (IVDU) that reportedly occurred a "few years" prior to study entry. The second risk factor included multiple high-risk sexual activities occurring in 1984. Additionally, it was reported that his regular female partner had tested positive for infection with HIV-1. Based on this information, it is possible that the patient had multiple exposure events to HIV-1 through sex and/or shared needles during the few years prior to study entry in 1986, very possibly during the time period of 1983-1984.

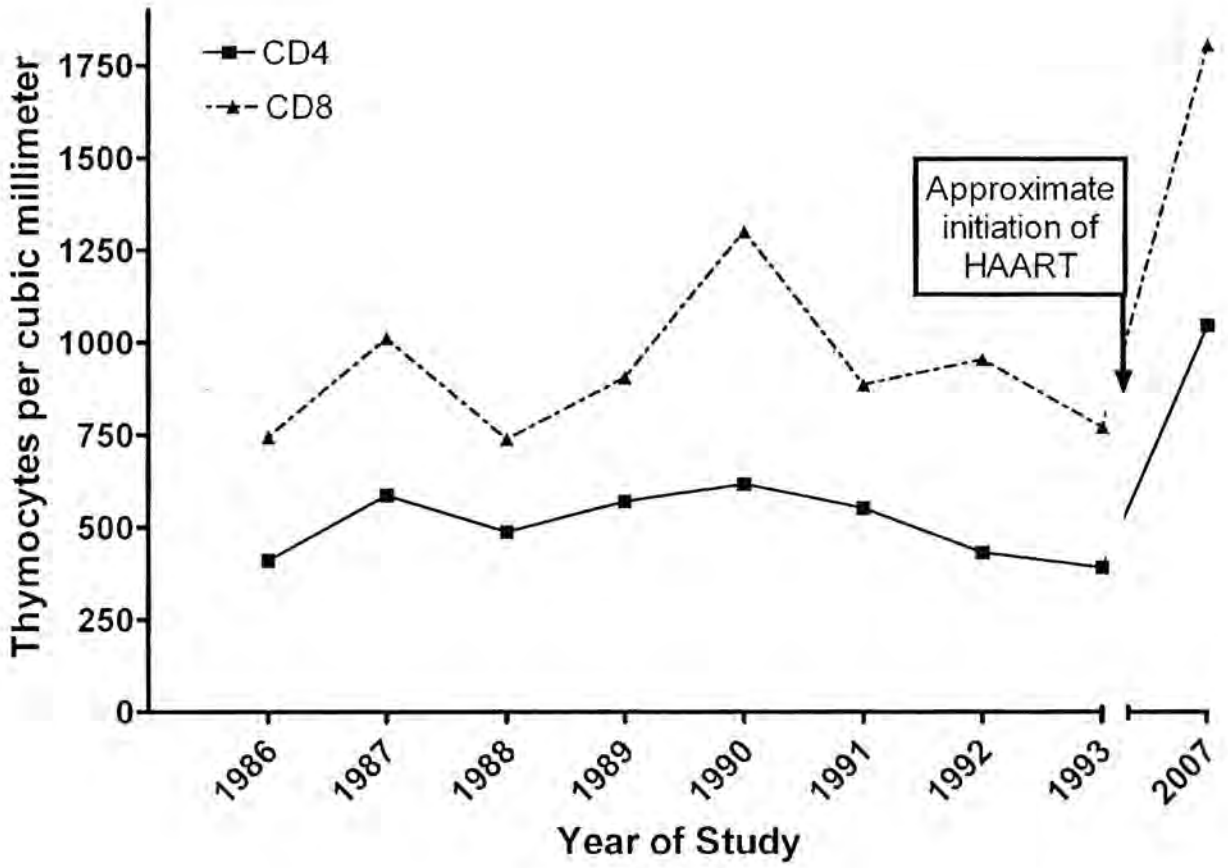
Table 1. Time points of sample collection from the HNS2 donor

Patient samples were available as PBMCs and plasma, and were collected and banked as part of a natural history cohort study at the Clinical Center, National Institutes of Health from 1986-1993. An additional sample was obtained in 2007, with IRB approval and informed consent. Patient PBMCs were available as both primary (1986-1991) and co-cultured (1991-1993) samples. In some cases, samples were collected more than once per year and are therefore designated with further differentiation by year and letter. Patient plasma samples were available in limited quantities for each time point of sample availability. The HNS2 sample was from a 1989 collection and was lyophilized. A recent 2007 whole blood sample from the patient yielded fresh primary PBMC and fresh plasma.

	PBMC		Plasma	
Date	**Type**	**Designation**	**Type**	**Volume (ml)**
12/23/86	Primary	1986	Frozen	0.3
7/14/87	Primary	1987	Frozen	0.2
8/9/88	Primary	1988	Frozen	1.2
3/14/89	Primary	1989a	Frozen	0.5
10/5/89	Primary	1989b	HNS-2	Lyophilized
6/12/90	Primary	1990	Frozen	1.1
1/22/91	Primary	1991a	Frozen	1.5
7/23/91	Co-Cultured	1991b	Frozen	1.5
8/25/92	Co-Cultured	1992	Frozen	0.5
4/6/93	Co-Cultured	1993a	Frozen	1.1
9/28/93	Co-Cultured	1993b	Frozen	1.5
6/4/07	Whole Blood	2007	Fresh	10

Figure 5. Thymocyte subset profiles over the course of long-term evaluation of the HNS2 donor

Patient thymocytes, $CD4^+$T cells (shown as black squares) and $CD8^+$ T cells (shown as black triangles) were collected and counted as part of the natural history cohort study at the Clinical Center, National Institutes of Health from 1986-1993 (personal communication H.J. Alter). $CD4^+$T cell counts fluctuated below physiologically normal levels (<1000 cells/mm^3) from 1986-1993. However, $CD4^+$T cell counts never fell below levels indicative of AIDS (<200 cells/mm^3) during the cohort study. The approximate time of onset of initiation of HAART is shown. The levels of circulating thymocytes increased following the initiation of HAART treatment, as seen with the additional 2007 timepoint sample.


CD4
CD8
Approximate initiation of HAART
Thymocytes per cubic millimeter
1750
1500
1250
1000
750
500
250
0
1986
1987
1988
1989
1990
1991
1992
1993
2007
Year of Study

Phylogenetic analysis of functional HIV-1 gp160 envelope genes

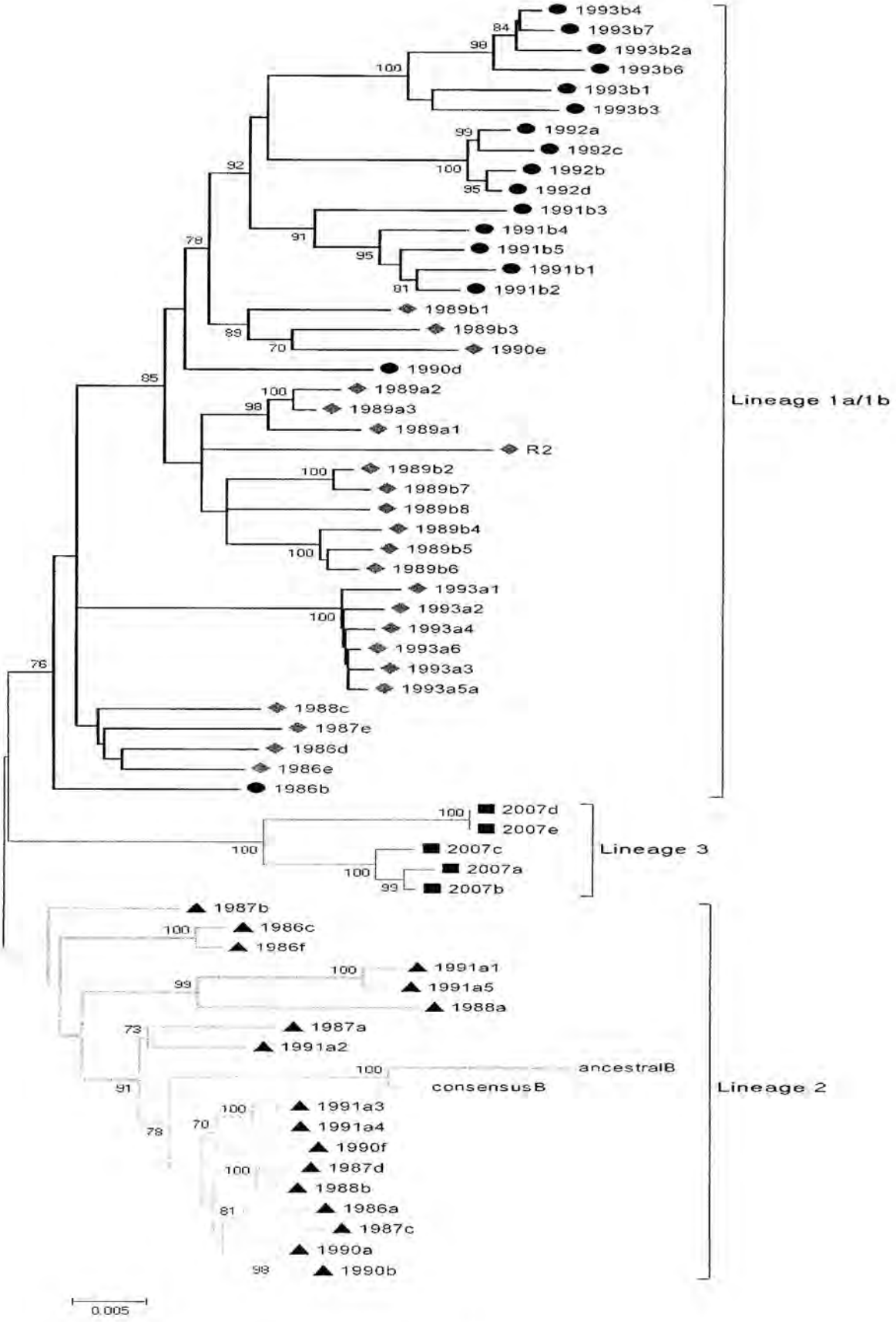
Functional HIV-1 gp160 envelope genes were cloned from amplified proviral DNA and tested for infectivity using HOS-CD4$^+$ CCR5$^+$ cells. Genes encoding functional envelope glycoproteins were used in subsequent studies. Figure 6 shows a midpoint rooted neighbor-joining (NJ) tree representing sequences of 62 functional HIV-1 gp160 Envelope genes forming three distinct lineages.

Sequences from lineage 1 represent infection with and evolution of a lineage 1 like progenitor that infected the patient sometime prior to 1986 and persisted through 1993. Further examination of the sequences indicates that lineage 1 is comprised of two related yet divergent lineages (L1a and L1b). Indeed, L1a and L1b form a monophyletic group supported by a bootstrap value of 85. Sequences belonging to L1a (grey diamonds) are related to the previously described R2 envelope (included in the NJ tree), they contain the rare PM mutation at position 313-314 in the V3 loop and represent genes amplified from 1989a, 1989b and 1990. Additionally, sequences from L1a contain similar polymorphisms in their V1/V2 regions (data not shown). Sequences belonging to L1b (black circles), form a monophyletic grouping supported by a bootstrap value of 92, contain the more commonly found HI residues at position 313-314 in the V3 loop, and represent genes amplified from 1991b, 1992 and 1993b. Additionally, sequences belonging to L1b all contain similar polymorphisms in their V1/V2 regions (data not shown). The L1a and L1b lineages likely derived from a common ancestor predating our earliest time point of sample availability collected in 1986 from the patient. Indeed, four L1 genes amplified from 1986-1988, also shown as grey diamonds, cluster together and contain similar characteristic polymorphisms to the L1a lineage. Additionally, a single

gene amplified from the 1986 time point (shown as a black circle), contains similar characteristic polymorphisms to the L1b lineage. Furthermore, six sequences amplified from a 1993 sample formed a monophyletic group with a bootstrap value of 100%, contain the rare PM mutation in the V3 loop and represent the continuation of the L1a lineage through the 1993a time point of sample availability. Sequences belonging to lineage 2 (shown as black triangles) represent infection with and evolution of a second heterologous HIV-1 clade B lineage and represent genes amplified from 1986, 1987, 1988, 1990 and 1991a. Sequences belonging to lineage 3 (shown as black squares) form a monophyletic cluster with a bootstrap value of 100%, represent genes amplified from 2007 and may represent a superinfecting HIV-1 clade B lineage. Due to the lack of patient samples between 1994 and 2007 we cannot precisely determine if or when the patient became superinfected with an additional strain of HIV-1.

Figure 6. Phylogenetic analysis and evolutionary relationships of functional HIV-1 gp160 envelope genes amplified from the HNS2 donor

The aforementioned HIV-1 envelope glycoprotein genes form three distinct clusters on a bootstrap consensus neighbor-joining tree indicating the presence and evolution of three distinct HIV-1 infections. Numbers (1986, 1987, 1988, etc...) indicate the sampling year from which a functional clone was amplified. Letters following the year of amplification (1986a, 1986b, 1986c, etc...) indicate the amplification of more than one functional clone from a particular sampling year. Sequences from lineage 1 (L1) represent infection with and evolution of an L1-like progenitor. Sequences from L1 were amplified from 1986-1993 and are comprised of two related yet divergent lineages (L1a and L1b). Sequences from L1a (grey diamonds) are related to the originally described R2 Env and contain the PM mutation in the V3 loop. Sequences belonging to L1b (circles) form a monophyletic grouping and contain the more commonly found HI residues in the V3 loop. Sequences from lineage 2 (L2) (shown as triangles) represent infection with and evolution of a second HIV-1 infection, and were amplified from 1986-1991. Sequences amplified from the 2007 time point sample form their own separate cluster and represent a possible superinfecting strain of HIV-1 strain, denoted as lineage 3 (L3) and are shown as squares. GenBank accession numbers: HQ110634-HQ110695.


1993b4
1993b7
1993b2a
1993b6
1993b1
1993b3
1992a
1992c
1992b
1992d
1991b3
1991b4
1991b5
1991b1
1991b2
1989b1
1989b3
1990e
1990d
1989a2
1989a3
1989a1
R2
1989b2
1989b7
1989b8
1989b4
1989b5
1989b6
1993a1
1993a2
1993a4
1993a6
1993a3
1993a5a
1988c
1987e
1986d
1986e
1986b
Lineage 1a/1b
2007d
2007e
2007c
2007a
2007b
Lineage 3
1987b
1986c
1986f
1991a1
1991a5
1988a
1987a
1991a2
ancestralB
consensusB
1991a3
1991a4
1990f
1987d
1988b
1986a
1987c
1990a
1990b
Lineage 2
0.005

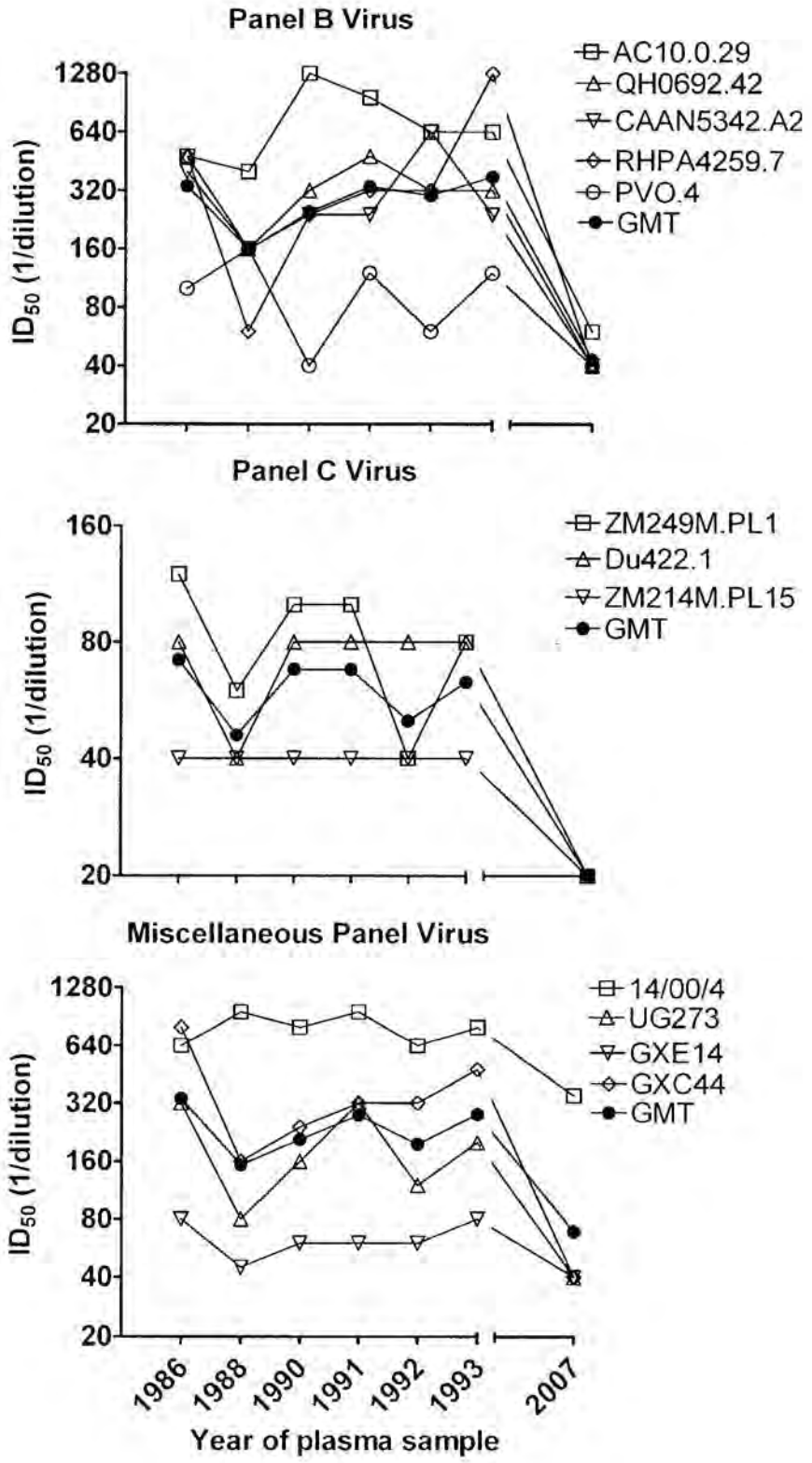
Evolution of the neutralizing antibody response using heterologous virus

The evolution of the neutralizing antibody response in the patient was examined using a panel of heterologous primary HIV-1 viruses pseudotyped with envelope glycoproteins representing viruses from various clades. Due to limited amounts of patient plasma, we were limited to testing twelve heterologous, primary envelope pseudotyped viruses including representatives from the established NIH panel of neutralization resistant viruses and other primary isolates of various clades [96, 182, 191, 192]. As seen in Figure 7, patient plasma collected from 1986-1993 contained cross-reactive neutralizing antibodies to envelope glycoproteins AC10.0.29, QH0692.42, CAAN5342.A2, RHPA4259.7, PV0.4 from the NIH clade B panel, envelope glycoproteins ZM249M.PL1, Du422.1, ZM214M.PL15 from the NIH clade C panel and to envelope glycoproteins comprising a miscellaneous panel of heterologous isolates, including one clade A, (UG273) from a Ugandan donor, one clade C, (GCX44), from a Chinese donor, one clade F (14/00/4) from a Congolese donor and one circulating recombinant, CRF01-AE, (GXE-14) from a Chinese donor. Geometric mean titers (GMTs), calculated for each time point of plasma sample availability from 1986-1993, representing the dilution of plasma at which 50% of virus infectivity was inhibited (ID_{50}), ranged from 1:158 to 1:376 against envelope glycoproteins from the NIH clade B panel, from 1:46 to 1:72 against envelope glycoproteins from the NIH clade C panel and from 1:153 to 1:338 against envelope glycoproteins from the miscellaneous panel. Comparison of the overall GMTs from 1986-1993, using a two-way ANOVA, indicates that the patient plasma contains significantly greater amounts of cross-reactive

neutralizing activity against envelope glycoproteins from the NIH clade B panel than to envelope glycoproteins from the NIH clade C panel ($p<0.0001$). Similarly, we observed that patient plasma contained greater amounts of cross reactivity against the miscellaneous panel of envelope glycoproteins than to envelope glycoproteins from the NIH clade C panel. Since members of the clade B and C panels are generally recognized as being neutralization resistant, the difference in GMT probably reflects greater neutralizing activity against subtype B than C. The miscellaneous panel is known to be more diverse, with respect to inherent neutralization sensitivity and statistical comparison of neutralization of that panel to the other panels is not meaningful regarding specificity of the neutralizing activity. The neutralizing activity in the 2007 time point sample was notably lower that in the earlier samples. This observed decrease is in agreement with another study which reported limited cross reactivity in a 2005 time point sample [199].

Figure 7. Evolution of the neutralizing antibody response using heterologous virus

Amounts of cross-reactive neutralizing activity present in plasma samples collected from the patient were calculated as the neutralization titer represented by the reciprocal plasma dilution (ID_{50}) causing a 50% reduction of relative light units (RLU) following a single round of infection against virus pseudotyped with twelve heterologous primary envelope glycoproteins from various clades. Due to limited sample availability, plasma from 1987 was not tested. Results are the average of two independent experiments. Geometric mean titers (GMT), represent levels of neutralizing activity calculated for each time point of plasma sample availability against each of the respective panels of viruses pseudotyped with HIV-1 envelope glycoproteins from clades B and C and a miscellaneous panel of clades.


Panel B Virus
AC10.0.29
QH0692.42
CAAN5342.A2
RHPA4259.7
PVO.4
GMT
ID50 (1/dilution)
1280
640
320
160
80
40
20
Panel C Virus
ZM249M.PL1
Du422.1
ZM214M.PL15
GMT
ID50 (1/dilution)
160
80
40
20
Miscellaneous Panel Virus
14/00/4
UG273
GXE14
GXC44
GMT
ID50 (1/dilution)
1280
640
320
160
80
40
20
1986
1988
1990
1991
1992
1993
2007
Year of plasma sample

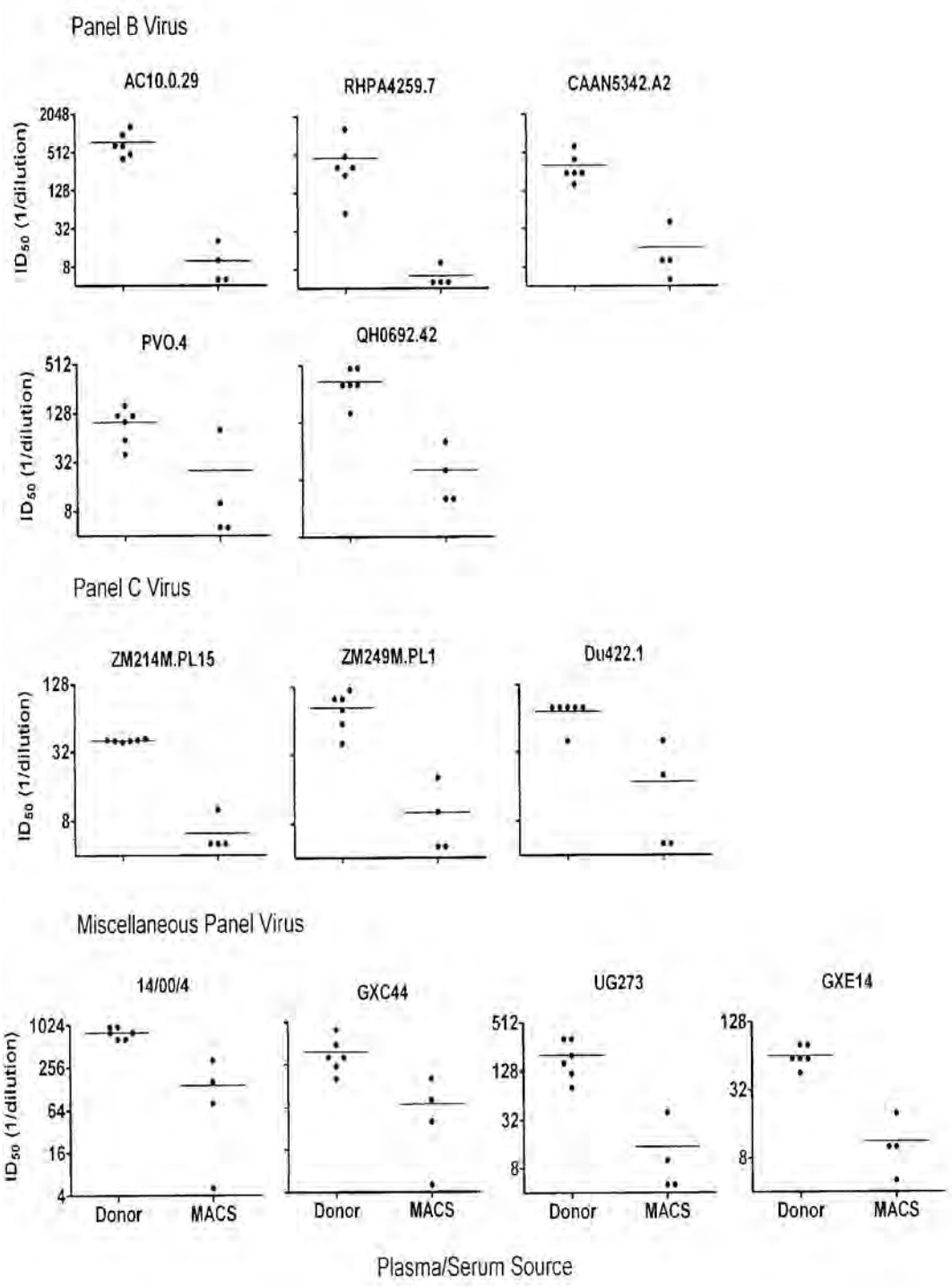
Comparison of neutralizing activity in sera or plasma of the HNS2 donor and other HIV-1 infected individuals

Figure 8 displays the comparison of neutralizing activity in plasma from the HNS2 donor to sera from other HIV-1 infected patients. For this comparison we utilized plasma samples from the HNS2 donor representing years 1986-1993 in comparison to sera from four patients enrolled in the Multicenter AIDS cohort study (MACS) at Johns Hopkins University (Baltimore, MD) [193]. The MACS patients (A-D) involved in this comparison were all male, HIV-1^+ at time of enrollment, selected from the cohort on the basis of having $CD4^+$T cell counts >400/mm^3 at time of enrollment, and remained clinically well with counts above >200/mm^3 for 5 years of study. All sera from the MACS patients represent the development of the neutralizing antibody response in those patients from at least 5 years of HIV-1 infection. The duration of HIV-1 infection and development of the neutralizing antibody response in the MACS patients is comparable to the duration and development of the neutralizing antibody response in the HNS2 donor. In all comparisons, the level of neutralizing activity in the plasma from the HNS2 donor was greater than the level of neutralizing activity in the sera from other HIV-1 infected patients. Indeed, viruses pseudotyped with envelope glycoproteins from the NIH clade B panel were neutralized by the HNS2 donor plasma at titers ranging from 1:40-1:1280 in comparison to neutralization titers ranging from 1:5-1:80 using sera from the MACS patients. The HNS2 donor plasma neutralized viruses pseudotyped with envelope glycoproteins from the NIH clade C panel at titers ranging from 1:40-1:120 while sera from the MACS patients neutralized at titers ranging from 1:5-1:40. The HNS2 donor plasma neutralized viruses pseudotyped with envelope glycoproteins from the

miscellaneous panel at titers ranging from 1:60-1:960 while sera from the MACS patients neutralized at titers ranging from 1:5-1:320.

Figure 8. Comparison of neutralizing activity in sera or plasma of the HNS2 donor and other HIV-1 infected individuals

Comparative amounts of neutralizing activity present in plasma samples from the HNS2 donor (1986-1993) versus sera from four other HIV-1 infected individuals (MACS A-D) were calculated as the reciprocal plasma dilution (ID_{50}) causing a 50% reduction of relative light units (RLU) following a single round of infection against virus pseudotyped with twelve heterologous primary envelope glycoproteins from various clades. Results are the average of two independent experiments. The horizontal lines indicate the geometric mean titers representing levels of neutralizing activity present in the HNS2 donor plasma (1986-1993) or the MACS (A-D) sera against virus pseudotyped with a particular HIV-1 envelope glycoprotein. In all cases, plasma samples from the HNS2 donor contained greater amounts of neutralizing activity than did sera samples from the HIV-1 infected MACS (A-D) patients.


Panel B Virus
AC10.0.29
RHPA4259.7
CAAN5342.A2
PVO.4
QH0692.42
Panel C Virus
ZM214M.PL15
ZM249M.PL1
Du422.1
Miscellaneous Panel Virus
14/00/4
GXC44
UG273
GXE14
ID50 (1/dilution)
2048
512
128
32
8
1024
256
64
16
4
Donor
MACS
Plasma/Serum Source

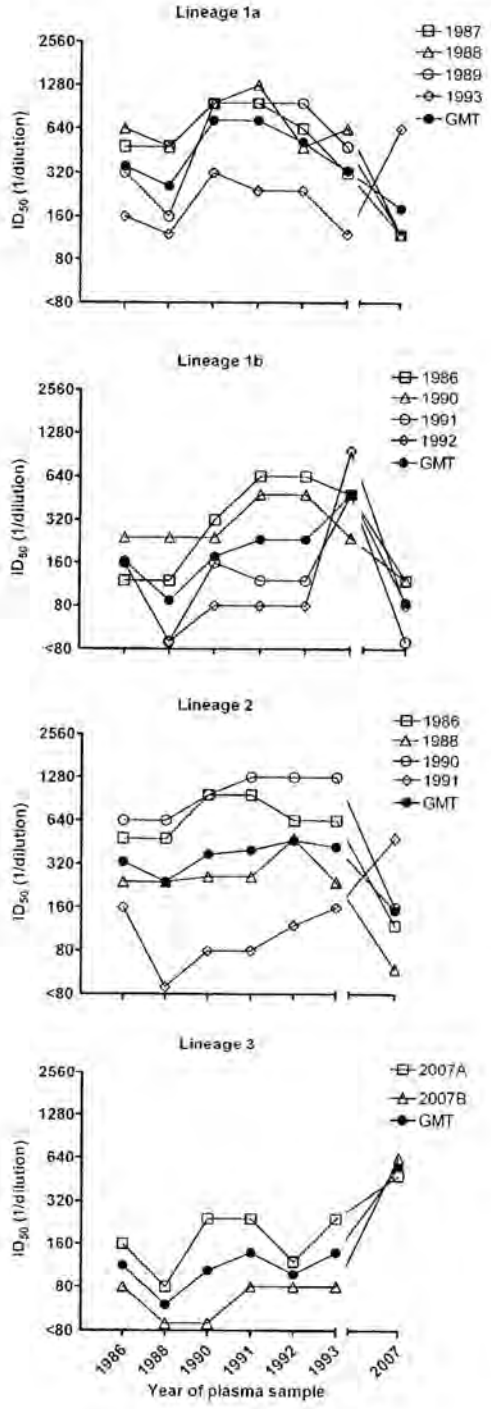
Evolution of the autologous neutralizing antibody response

We determined the evolution of the autologous neutralizing antibody response by testing the neutralization sensitivities of viruses pseudotyped with envelope glycoproteins representing each of the HIV-1 lineages identified in the patient. Due to limited amounts of plasma sample volumes, only four envelope glycoproteins for lineages L1a, L1b, L2 and two envelope glycoproteins for lineage L3 were chosen to represent the evolution of each of the respective lineages. Additionally, due to limited amounts of plasma volume, plasma from 1987 was not tested. The results shown in Figure 9 illustrate that all lineages are sensitive to autologous plasma. Furthermore, the data illustrates an increase in the neutralizing antibody (nAb) response in the patient from 1986-1991 for lineages L1a, L1b and L2. The increasing nAb response is most evident against the earlier time point clones, including envelope glycoproteins from 1987, 1988 and 1989 representing L1a, envelope glycoproteins 1986 and 1990 representing L1b and envelope glycoproteins 1986, 1988 and 1990 representing L2. In contrast, later time point clones including a 1993 clone from L1a, a 1991 and 1992 clone from L1b and a 1991 clone from L2 were approximately two to four fold less sensitive to the autologous nAb response from 1986-1993. Furthermore, the nAb response in the patient began to decrease in 1992 and was low to absent in 2007, with the exception of a 1993 time point envelope glycoprotein clone representing L1a, a 1991 envelope glycoprotein clone representing L2 and two, 2007 envelope glycoprotein clones representing L3. Comparison of the overall GMTs from 1986-1993, using a 2-way ANOVA, indicates that envelope glycoproteins representing lineage L1a, all of which contain the rare PM mutation in the V3 loop are significantly more sensitive to autologous neutralizing antibodies than are envelope

glycoproteins representing lineage L1b, all of which contain the more commonly found HI residues in the V3 loop ($p<0.0001$). Additionally, envelope glycoproteins derived from the second lineage, L2 were significantly more sensitive to autologous neutralizing antibodies than envelope glycoproteins representing lineage L1b ($p<0.0001$). No significant difference in sensitivity to autologous neutralizing antibodies was observed between lineages L1a and L2 ($p=0.6$). Lineage L3, which represents a possible superinfecting strain of HIV-1 was significantly more resistant to autologous neutralizing antibodies from the 1986-1993 time frame when compared to lineages L1a, L1b and L2 ($p<0.0001$). The observed increase in neutralization sensitivity of the two 2007 time point envelope glycoproteins clones to the 2007 plasma sample may indicate the development of a strain specific response against the L3 lineage.

Figure 9. Evolution of the autologous neutralizing antibody response

Viruses pseudotyped with envelope glycoproteins representing each of the lineages (L1a, L1b, L2 and L3) present in the HNS2 donor were neutralized by autologous plasma. Neutralization titers were calculated as the reciprocal plasma dilution (ID_{50}) causing a 50% reduction of relative light units (RLU) following a single round of infection. Results shown are the average of two independent experiments. Geometric mean titers (GMTs) were calculated for each time point of plasma sample availability against virus pseudotyped with envelope glycoproteins representing each of the respective lineages. Comparison of the GMTs among the lineages represents the evolution of the neutralizing antibody response generated in the patient.


Lineage 1a
1987
1988
1989
1993
GMT
2560
1280
640
320
160
80
<80
ID50 (1/dilution)
Lineage 1b
1986
1990
1991
1992
GMT
Lineage 2
1986
1988
1990
1991
GMT
Lineage 3
2007A
2007B
GMT
1986
1988
1990
1991
1992
1993
2007
Year of plasma sample

Evolution of neutralization sensitivities of the HIV-1 envelope glycoproteins to heterologous HIV-1$^+$ sera

The R2 envelope was previously reported to be sensitive to neutralization using heterologous HIV-1$^+$ sera [95, 181]. In Table 2 we show neutralization of the same envelope glycoprotein clones shown in Figure 9 by sera from four patients from the MACS cohort as previously described. Briefly, the MACS cohort patients were enrolled in the study after diagnosis of infection without known dates for seroconversion. The sera were obtained during the final three years of follow up. None of the MACS patients were receiving antiretroviral therapy and none had manifestation of AIDS. Neutralization by the reference plasma HNS2 is shown in comparison. In all cases, envelope glycoprotein clones from L1a, Llb, L2 and L3 were more sensitive to autologous HNS2 plasma than to the MACS patient sera. Envelope glycoproteins from 1987, 1988, 1989 (R2) and 1993 representing L1a were neutralized by the MACS sera with GMTs ranging from 1:12 to 1:95. Envelope glycoproteins from 1986, 1990, 1991 and 1992 representing L1b were neutralized by the MACS sera with GMTs ranging from 1:7 to 1:24. Envelope glycoproteins from 1986, 1988, 1990 and 1991 representing L2 were neutralized by the MACS sera with GMTs ranging from 1:20 to 1:80. Two envelope glycoproteins representing L3 were resistant to neutralization using the MACS sera with a GMT of 1:10. We did not notice a significant decrease in neutralization sensitivity of the envelope glycoproteins from the patient representing lineages L1a, L1b and L2 to the heterologous sera over the course of infection. Rather we did notice that the comparison of the overall GMTs, using a 2-way ANOVA, indicates that envelope glycoproteins representing lineage L1a, are significantly more sensitive to the MACS

sera than are the envelope glycoproteins representing lineage L1b ($p=0.01$). Additionally, envelope glycoproteins representing lineage L2 were also found to be significantly more sensitive to the MACS sera than envelope glycoproteins representing lineage L1b ($p=0.005$). No significant difference in sensitivity to the MACS sera was observed between envelope glycoproteins representing lineages L1a and L2 ($p=0.3$). Additionally, envelope glycoproteins representing lineage L3, are significantly more resistant to the MACS sera when compared to envelope glycoproteins representing lineages L1a ($p=0.006$), L1b ($p=0.001$) and L2 ($p=0.002$).

Table 2. Evolution of neutralization sensitivities to heterologous HIV-1$^+$ sera

Viruses pseudotyped with envelope glycoproteins from each of the time points of sample availability (indicated as clones) representing each of the lineages present in the patient (L1a, L1b, L2 and L3) were tested for their neutralization sensitivities using heterologous HIV-1$^+$ sera (MACS A-D). Neutralization results using HNS2 and a negative control human serum are shown in comparison. Neutralization titers were calculated as the reciprocal plasma dilution (ID_{50}) causing a 50% reduction of relative light units (RLU) following a single round of infection. All results are the average of two independent experiments.

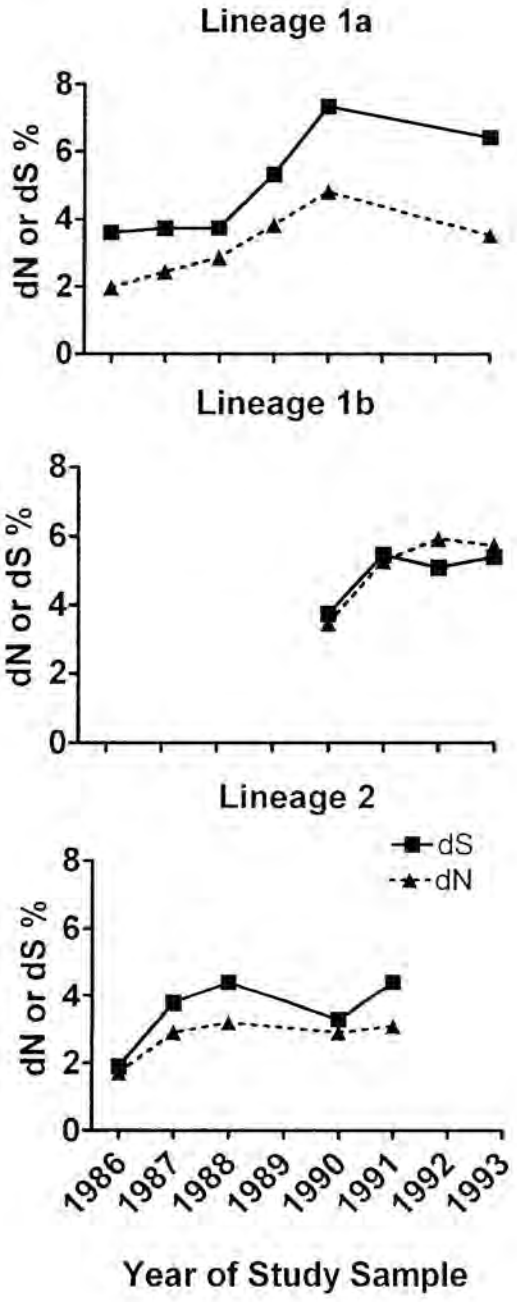
	Clone	HNS-2	MACSA	MACSB	MACSC	MACSD	Negative
L1A	1987	640	40	160	40	80	<10
	1988	640	10	160	160	80	<10
	1989	160	20	80	40	40	<10
	1993	80	10	40	20	40	<10
L1B	1986	160	10	40	10	20	<10
	1990	160	20	80	80	80	<10
	1991	40	10	10	10	10	<10
	1992	40	10	10	10	10	<10
L2	1986	640	10	40	20	20	<10
	1988	320	20	160	320	40	<10
	1990	640	320	160	320	160	<10
	1991	40	10	10	20	10	<10
L3	2007A	40	<10	10	<10	10	<10
	2007B	40	<10	<10	<10	<10	<10

Analysis of synonymous and nonsynonymous mutations

The rates of nonsynonymous (dN) to synonymous (dS) mutations were examined in each of the lineages in order to determine whether the changes resulted from positive section. A ratio of dN/dS exceeding 1 was considered to be evidence of positive selective pressure. As seen in Figure 10, synonymous nucleotide substitutions accumulated more rapidly than nonsynonymous nucleotide substitution for lineages L1a and L2 resulting in dN/dS ratios <1, indicating low levels of positive selection. In contrast, beginning in 1992 and continuing in 1993, the accumulation of nonsynonymous nucleotide substitutions occurred more rapidly than synonymous nucleotide substitutions for cluster C1b, resulting in a dN/dS ratio >1, indicating high levels of positive selection. While increasing resistance to neutralization over time was most likely due to immunological selection, the number of mutations resulting from the selective pressure did not exceed synonymous mutations in L1a and L2.

Figure 10. Analysis of synonymous and nonsynonymous mutations

The proportions of nucleotide substitutions indicating levels of selective pressure were calculated as synonymous (dS) mutations, as indicated on the graph as squares and nonsynonymous (dN) mutations, as indicated on the graph as triangles, for lineages L1a, L1b and L2 over the course of infection and evolution of each of the respective lineages from 1986-1993.


Lineage 1a
dN or dS %
8
6
4
2
0
Lineage 1b
dN or dS %
8
6
4
2
0
Lineage 2
dS
dN
dN or dS %
8
6
4
2
0
1986
1987
1988
1989
1990
1991
1992
1993
Year of Study Sample

Chapter 4. Evolution of Neutralization Sensitivities of the HIV-1 Envelope Glycoproteins to sCD4 and Eight Cross-reactive Monoclonal Antibodies

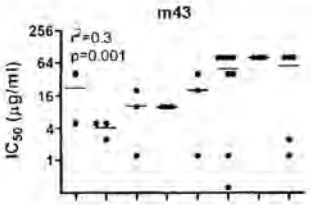
Evolution of sensitivity to neutralization by sCD4 and cross-reactive mAbs

The results in Figure 11 show the neutralization of envelope glycoproteins from all lineages combined obtained from 1986-1993 to sCD4 and eight cross-reactive mAbs targeting distinct neutralization epitope regions. We chose representative envelope glycoprotein clones from each lineage per time point with the highest infectivity titers to be tested. Statistically significant evidence for progressive neutralization resistance development was observed with mAb m43, targeting the complex gp120/gp41 epitope ($p=0.001$), mAbs b12 ($p=0.02$) and m14 ($p=0.002$) targeting the CD4bs regions, and mAbs m9 ($p=0.0001$), 48d ($p<0.0001$) and 17b ($p=0.01$) targeting the co-receptor binding site region. In contrast, progressive neutralization resistance development was not observed with mAbs 2F5 and 4E10 targeting the MPER region of gp41, or to sCD4. Neutralization resistant viruses began to emerge in 1991 against mAbs m43, m14, m9, 48d and 17b. Indeed, 5/7, 5/7, 4/7, 7/7 and 5/7 pseudoviruses expressing HIV-1 envelope glycoproteins from 1991 were resistant to neutralization by these antibodies with IC_{50} titers $\geq 40\mu g/ml$. Subsequent envelope glycoproteins remained resistant to neutralization with these mAbs with the exception of two envelope glycoproteins from the 1993 time point and mAbs m43, m14 and 17b. Sequence analysis of these two neutralization sensitive clones indicated an apparent recombination event in the V1/V2 region of gp120 between clones from L1a and clones from L1b. It is likely that this recombination event led to the observed neutralization sensitivity of these clones. Envelope glycoproteins remained sensitive to neutralization throughout the study period to mAbs 2F5 and 4E10 and to sCD4, at $\leq 10\mu g/ml$.

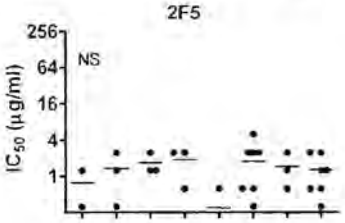
Figure 11. Evolution of neutralization sensitivity of the HIV-1 envelope glycoproteins to sCD4 and eight cross-reactive mAbs.

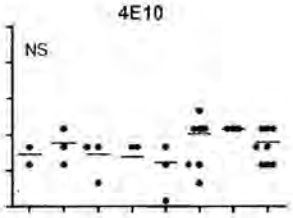
Viruses pseudotyped with envelope glycoproteins (circles) from the patient were tested for their sensitivity to neutralization by sCD4 and cross-reactive mAbs targeting various regions on the HIV-1 envelope glycoprotein including; the complex gp120/gp41 epitope (m43), the MPER region (2F5 and 4E10), the CD4 binding site (sCD4, b12, m14), and the co-receptor binding site (m9, 48d, 17b). Neutralization titers were calculated as the inhibitor concentration causing a 50% reduction of luciferase activity (IC_{50}) following a single round of infection. All experiments were performed in duplicate. Longitudinal changes in neutralization sensitivity over the course of infection (1986-1993) indicate the development of progressive neutralization resistance (p value ≤ 0.05) as assessed using linear regression analysis (r^2) as implemented in Graphpad and verified using Excel (Microsoft).

gp120/gp41 complex epitope

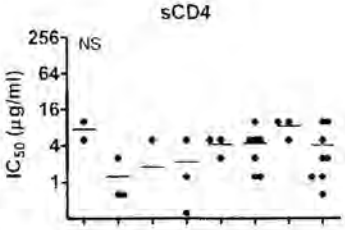


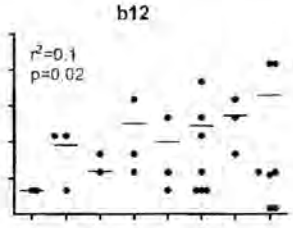
Membrane proximal epitope

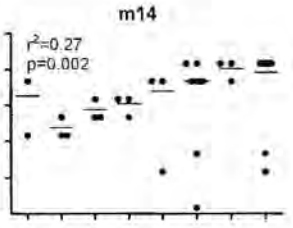




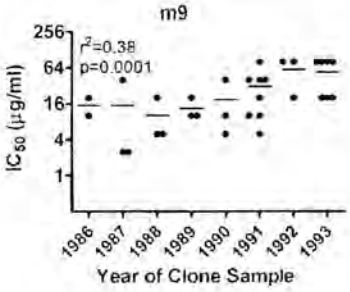
CD4 binding site

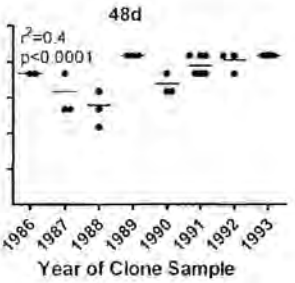


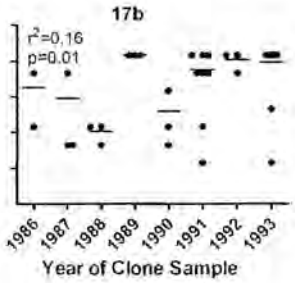




Coreceptor binding site





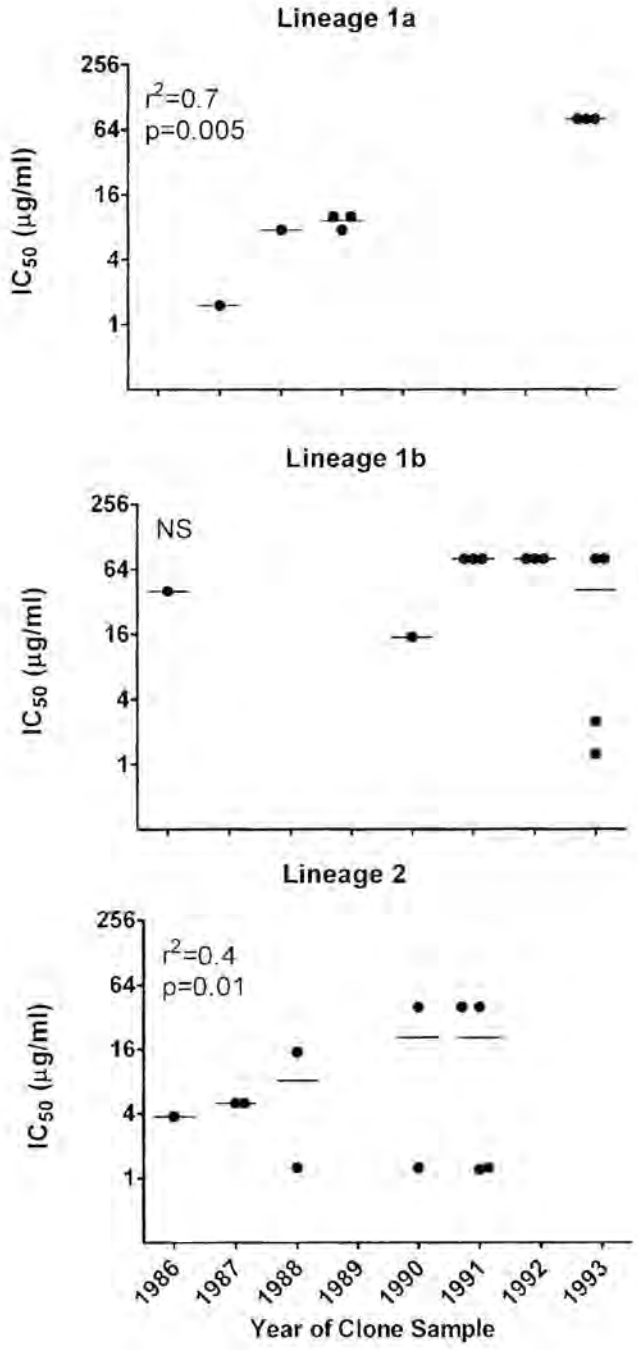


Evolution of neutralization sensitivities according to each respective lineage

We next sought to determine if the evolutionary patterns shown in Figure 11 were reflected in the individual HIV-1 lineages amplified and identified in the patient. The results shown in Figures 12-15 are the same as those shown in Figure 11, but are shown separately for lineages L1a, L1b and L2. Each of these figures illustrates results for a particular epitope region. Figure 12 illustrates that the progressive resistance to the complex epitope mAb m43, as demonstrated in Figure 11, was attributable to resistance development in lineages L1a ($p=0.005$) and L2 ($p=0.01$) only, while lineage L1b remained resistant to m43 throughout the course of infection, with the exception of the two 1993 recombinant clones discussed above.

Figure 12. Evolution of neutralization sensitivities to mAb m43.

The data from Figure 11 was reanalyzed to account for the relative contributions of the development of progressive neutralization resistance to the complex gp120/gp41 epitope (m43) according to each respective lineage (L1a, L1b and L2). Longitudinal changes in neutralization sensitivity over the course of infection (1986-1993) indicate the development of progressive neutralization for lineages L1a ($p=0.005$) and L2 ($p=0.01$) to m43.

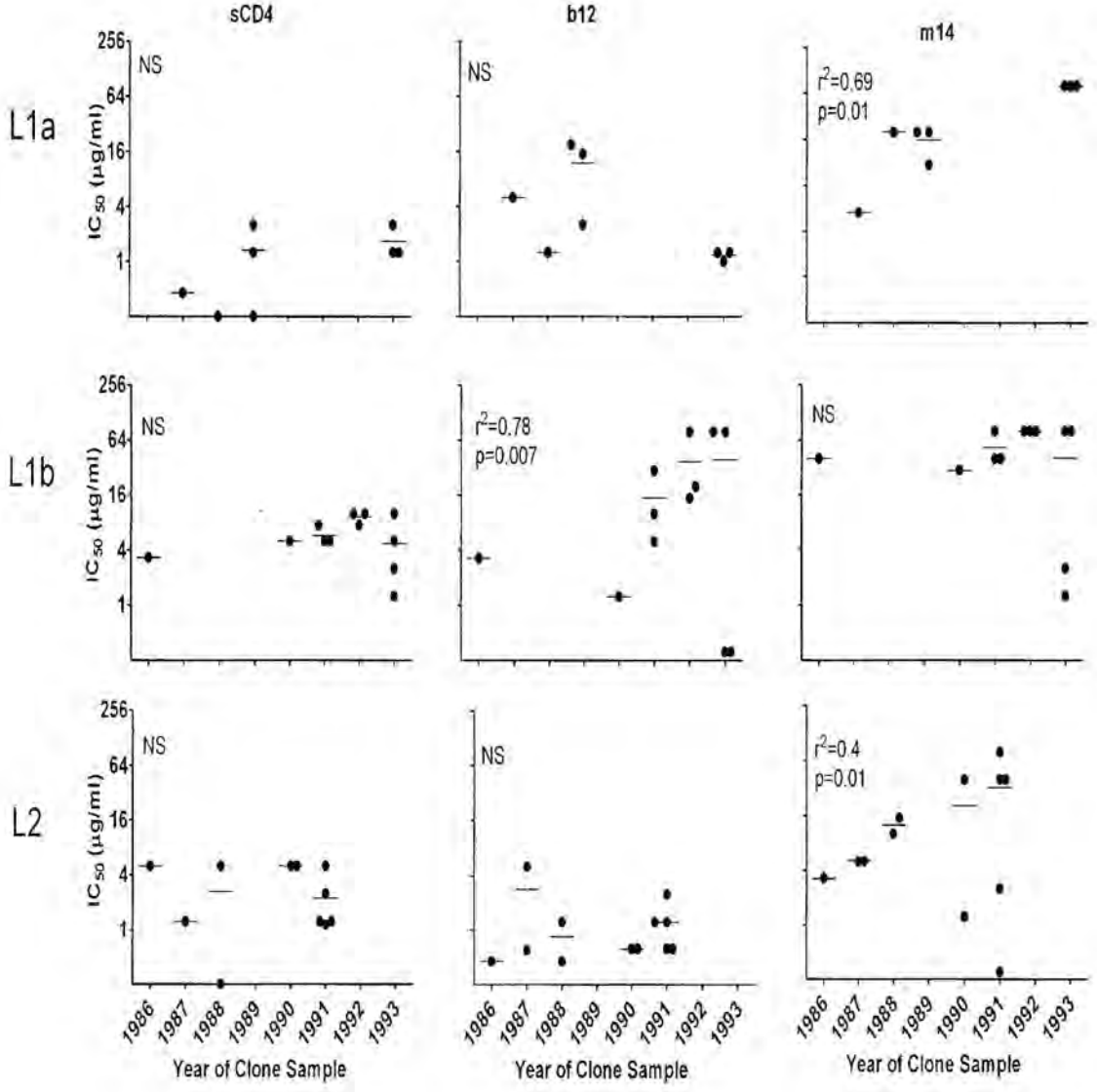

Lineage 1a
256
64
16
4
1
r^2=0.7
p=0.005
IC_{50} (µg/ml)
Lineage 1b
256
64
16
4
1
NS
IC_{50} (µg/ml)
Lineage 2
256
64
16
4
1
r^2=0.4
p=0.01
IC_{50} (µg/ml)
1986
1987
1988
1989
1990
1991
1992
1993
Year of Clone Sample

Evolution of neutralization sensitivities of HIV-1 envelope glycoproteins to sCD4 and CD4bs mAbs according to each HIV-1 lineage

The results shown in Figure 13 are those obtained using sCD4 and the CD4bs mAbs b12 and m14. Resistance to sCD4 did not develop over the course of the study period for any of the HIV-1 lineages. Interestingly, the two L1b, 1993 envelope glycoproteins representing the possible V1/V2 recombination, were two to four fold more sensitive to sCD4 than the non recombinant L1b envelope glycoproteins. Envelope glycoproteins of L1a and L2 did not develop progressive resistance to b12 over the course of sample availability and remained sensitive throughout the study period. In contrast, envelope glycoproteins of L1b became progressively more resistant to neutralization beginning in 1991 ($p=0.007$). Interestingly, the V1/V2 recombinant L1b envelope glycoproteins from the 1993 time point were sensitive to neutralization. With respect to mAb m14, L1a ($p=0.01$) and L2 ($p=0.01$) became progressively more resistant to neutralization. In contrast, with the exception of the two V1/V2 recombinant L1b envelope glycoproteins from 1993 pseudoviruses expressing L1b envelope glycoproteins remained resistant to neutralization over the entire study period. In summary, all lineages remained sensitive to sCD4, without progressive resistance developing. Only L1b envelope glycoproteins became resistant to IgG1b12 and L1b envelope glycoproteins were also resistant to m14 throughout, while L1a and L2 lineages became progressively resistant to m14.

Figure 13. Evolution of neutralization sensitivities to sCD4 and CD4bs mAbs

The data from Figure 11 was reanalyzed to account for the relative contributions of the development of progressive neutralization resistance to the CD4 binding site as seen using (sCD4, b12, m14) according to each respective lineage (L1a, L1b and L2). Longitudinal changes in neutralization sensitivity over the course of infection (1986-1993) indicate the development of progressive neutralization resistance to b12 for lineage L1b ($p=0.007$) and for the development of progressive neutralization resistance to m14 for lineages L1a ($p=0.01$) and L2 ($p=0.01$).

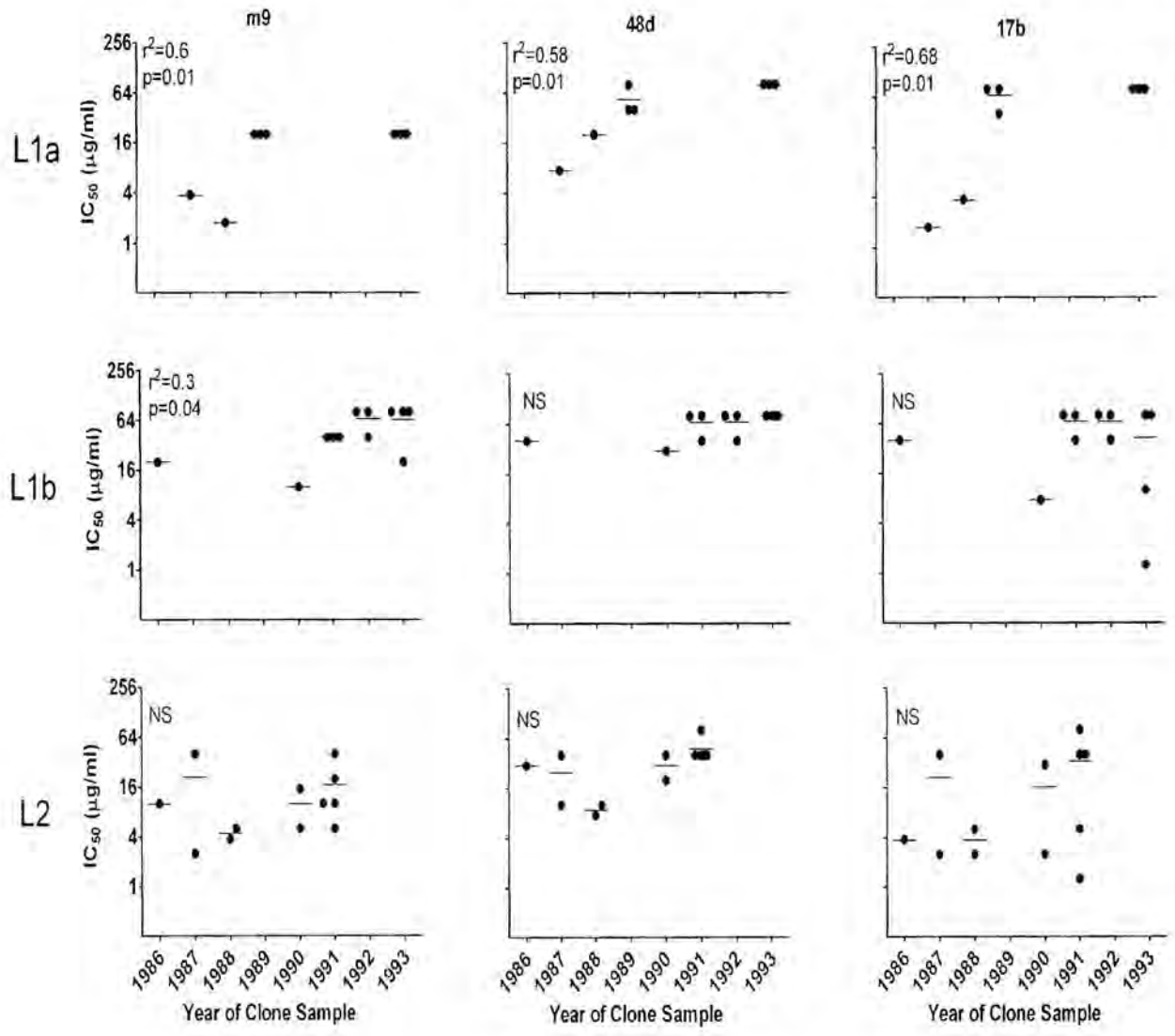

sCD4
b12
m14
L1a
L1b
L2
NS
NS
r²=0.69
p=0.01
NS
r²=0.78
p=0.007
NS
NS
r²=0.4
p=0.01
IC50 (µg/ml)
256
64
16
4
1
1986
1987
1988
1989
1990
1991
1992
1993
Year of Clone Sample

Evolution of neutralization sensitivities to co-receptor binding site mAbs according to each HIV-1 lineage

The results shown in Figure 14 are those obtained using co-receptor binding site mAbs m9, 48d and 17b. Envelope glycoproteins representing L1a and L1b developed progressive neutralization resistance to m9 over the study period with p values of p=0.01 and p=0.04 respectfully. In contrast, envelope glycoproteins representing L2 remained sensitive to m9 throughout the period of study. Envelope glycoproteins from L1a became progressively resistant to 48d over the course of study (p=0.01) while envelope glycoproteins from L1b remained resistant. In contrast, envelope glycoproteins from L2 remained sensitive to 48d throughout the study period. With respect to 17b, envelope glycoproteins from L1a became progressively resistant (p=0.01) while envelope glycoproteins from L1b remained resistant over the study period with the exception of the two 1993 recombinant clones. In contrast, envelope glycoproteins from L2 remained sensitive to neutralization with 17b throughout the study period. In summary, envelope glycoproteins from lineage L1a became progressively resistant to neutralization with regards to the co-receptor binding site mAbs tested. Envelope glycoproteins from L1b became progressively resistant to m9 only and remained resistant to mAbs 48d and 17b over the course of the study period with the exception of the two 1993 recombinant envelope glycoproteins. In contrast, envelope glycoproteins from L2 remained sensitive to all the co-receptor binding site mAbs tested.

Figure 14. Evolution of neutralization sensitivities to co-receptor binding site mAbs

The data from Figure 11 was reanalyzed to account for the relative contributions of the development of progressive neutralization resistance to the co-receptor binding site as seen using (m9, 48d, 17b) according to each respective lineage (L1a, L1b and L2). Longitudinal changes in neutralization sensitivity over the course of infection (1986-1993) indicate the development of progressive neutralization resistance to m9 for lineage L1b ($p=0.04$) to 48d for lineage L1a ($p=0.01$) and to 17b for lineage L1a ($p=0.01$).

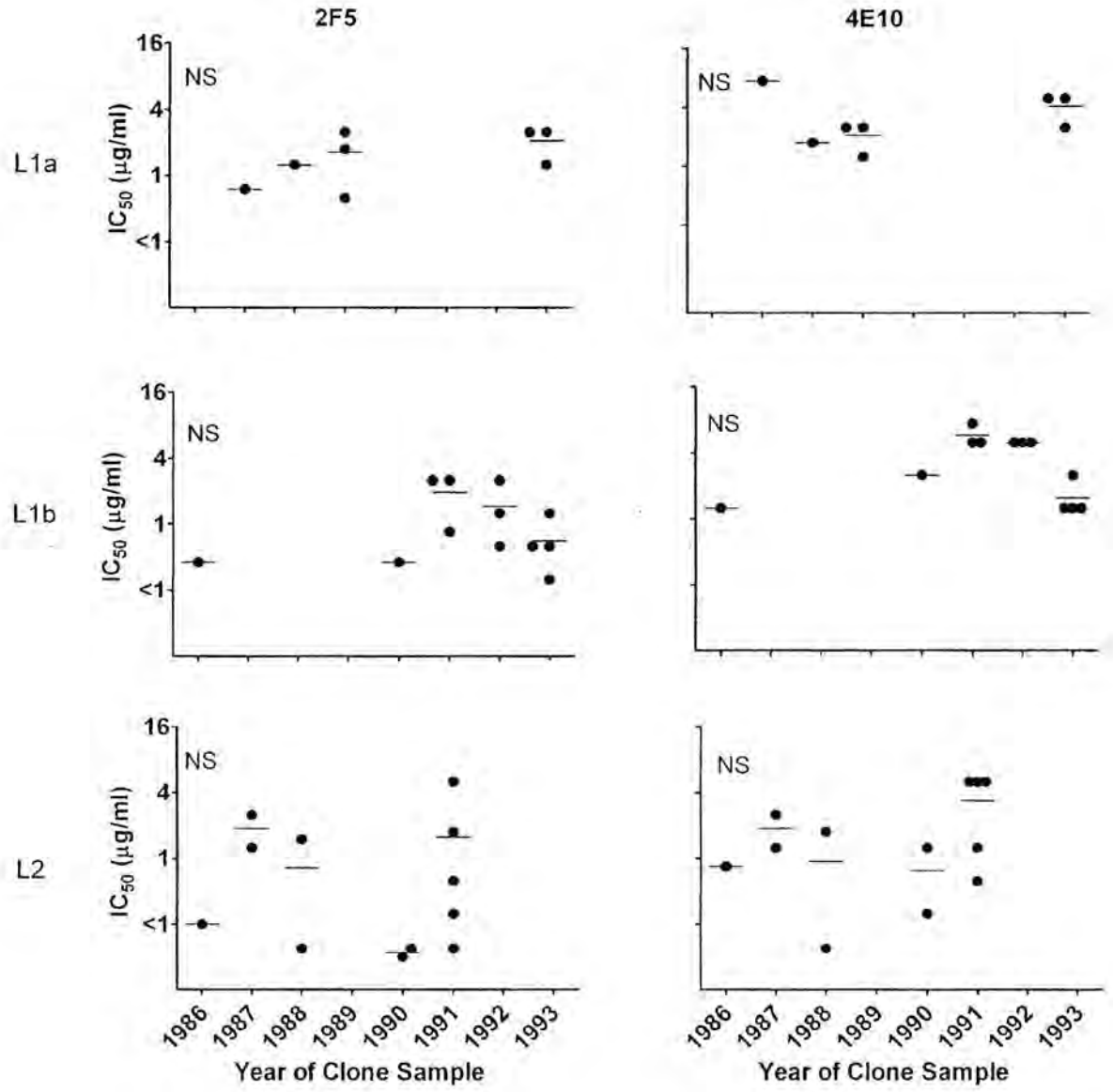

m9
48d
17b
L1a
L1b
L2
IC50 (µg/ml)
256
64
16
4
1
r2=0.6
p=0.01
r2=0.58
p=0.01
r2=0.68
p=0.01
r2=0.3
p=0.04
NS
1986
1987
1988
1989
1990
1991
1992
1993
Year of Clone Sample

Evolution of neutralization sensitivities to MPER mAbs according to each HIV-1 lineage

The results shown in Figure 15 are those obtained using the MPER mAbs 2F5 and 4E10. Resistance to 2F5 did not develop over the course of study period in any of the HIV-1 lineages. Indeed, envelope glycoproteins representing L1a, L1b and L2 were neutralized at $IC_{50} \leq 5.0\mu g/ml$. Sequence analysis of the envelope glycoprotein genes representing lineages L1a, L1b and L2 indicated there were no mutations to the critical (DKW) core sequence of the 2F5 epitope. With respect to 4E10, progressive resistance did not develop in any of the HIV-1 lineages. Envelope glycoproteins representing L1a, L1b and L2 were neutralized at $IC_{50} \leq 10.0\mu g/ml$. Sequence analysis of the envelope glycoprotein genes indicated there were no persistent mutations to the critical residues (W_{671}, F_{672} and W_{680}) of the 4E10 epitope sequence, with the exception of the 1987 envelope glycoprotein gene representing L1a. Sequence analysis revealed that in 1987, the phenylalanine normally found at position 672 was replaced by a leucine; however this particular mutation was not maintained as part of the viral sequence over the course of sample availability. In summary, envelope glycoproteins from each of the lineages amplified and identified in the patient did not develop progressive resistance to either of the MPER mAbs.

Figure 15. Evolution of neutralization sensitivities to MPER region mAbs

The data from Figure 11 was reanalyzed to account for the relative contributions of the development of progressive neutralization resistance to the MPER region as seen using (2F5 and 4E10) according to each respective lineage (L1a, L1b and L2). Longitudinal changes in neutralization sensitivity over the course of infection (1986-1993) indicate the absence of development of progressive neutralization resistance to 2F5 and 4E10 for viruses pseudotyped with envelope glycoproteins representing lineages L1a, L1b and L2.

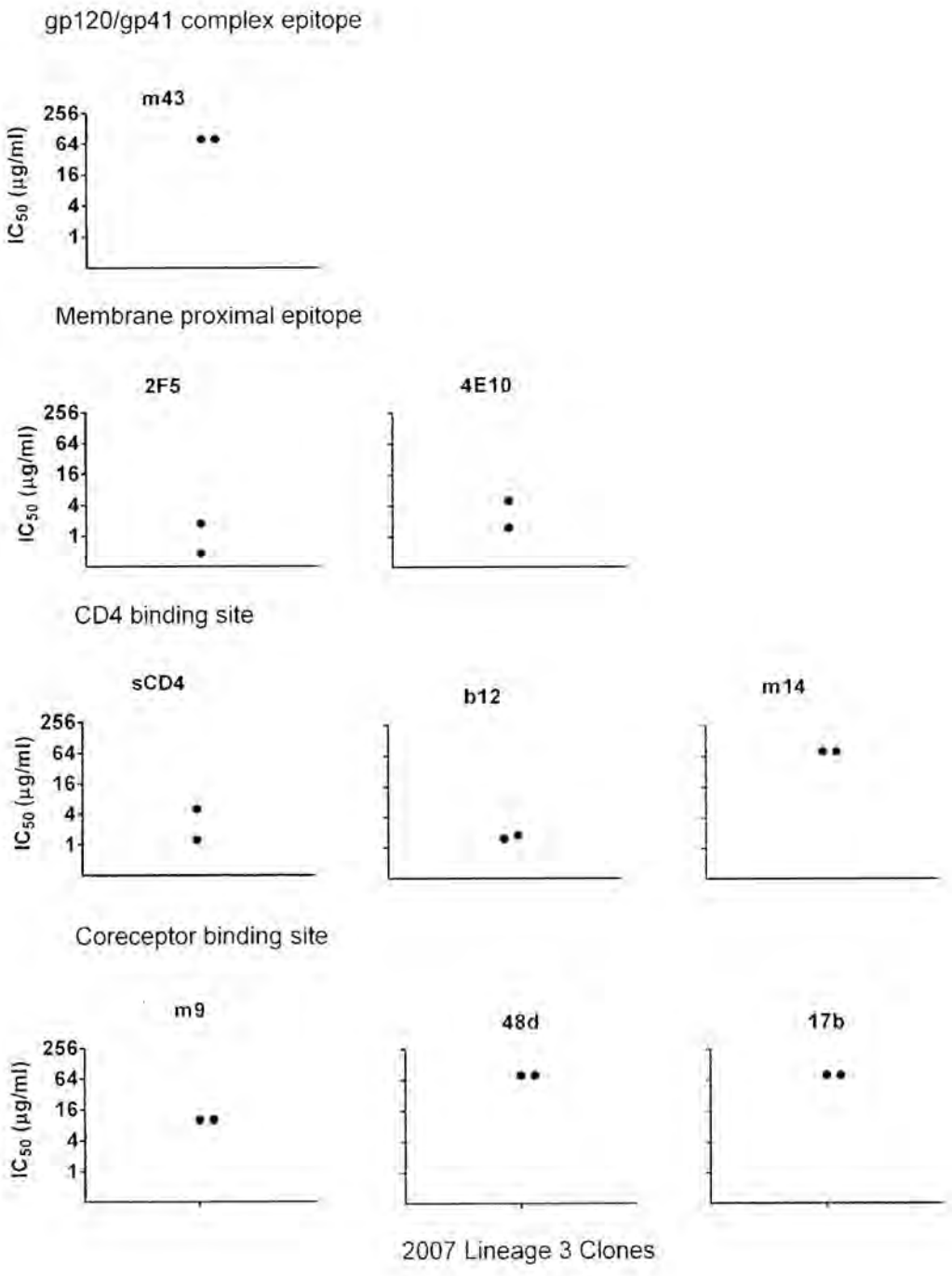

2F5
4E10
L1a
L1b
L2
NS
IC_{50} (µg/ml)
16
4
1
<1
1986
1987
1988
1989
1990
1991
1992
1993
Year of Clone Sample

Neutralization sensitivities of 2007 clones representing a possible superinfecting strain of HIV-1

As the HIV-1 envelope glycoproteins amplified from 2007 may represent a superinfecting or possibly antiretroviral resistant strain, we chose to analyze the neutralization sensitivities of clones from this time point separately. For our analysis we chose two envelope glycoproteins from the 2007 time point for analysis of their neutralization patterns. As seen in Figure 16, the two independent envelope glycoproteins were resistant to m43, m14, 48d, and 17b all at $IC_{50} \geq 40 \mu g/ml$. In contrast, both clones were found to be sensitive to 2F5, 4E10, b12, m9 and sCD4 all at $IC_{50} \leq 10.0 \mu g/ml$.

Figure 16. Neutralization sensitivities of the 2007 superinfecting HIV-1 strain

Viruses pseudotyped with two independent envelope glycoproteins (circles) from the 2007 sample were tested for their sensitivity to neutralization by sCD4 and cross-reactive mAbs targeting various regions on the HIV-1 envelope glycoprotein including; the complex gp120/gp41 epitope (m43), the MPER region (2F5 and 4E10), the CD4 binding site (sCD4, b12, m14), and the co-receptor binding site (m9, 48d, 17b). Neutralization titers were calculated as the inhibitor concentration causing a 50% reduction of luciferase activity (IC_{50}) following a single round of infection. All experiments were performed in duplicate.


gp120/gp41 complex epitope
m43
256
64
16
4
1
IC50 (µg/ml)
Membrane proximal epitope
2F5
4E10
CD4 binding site
sCD4
b12
m14
Coreceptor binding site
m9
48d
17b
2007 Lineage 3 Clones

Chapter 5. General Discussion

The goal of this project was to characterize the evolution of the HIV-1 envelope glycoprotein genes and neutralizing antibody response in an individual with broadly cross neutralizing (BCN) antibodies [95]. We hypothesized that the HIV-1 envelope glycoprotein gene variation and evolution of the neutralizing antibody response would reveal mechanisms of the induction and maintenance of the BCN response. In addition to an evaluation of gene sequence evolution, we sought to characterize the evolution of the neutralization sensitivity of HIV-1 envelope glycoproteins from the individual to sCD4 and eight cross-reactive monoclonal antibodies (mAbs). We hypothesized that the HIV-1 envelope glycoproteins would become progressively more resistant to neutralization as a result of mutations that contribute to global neutralization resistance. We anticipated that the nature of the development of neutralization resistance at various epitope regions would be related to the immunogenicity of the original infecting HIV-1 envelope glycoproteins. Despite over approximately 30 years of HIV-1 research, it is still not understood why some HIV-1 infected individuals develop a BCN response while others do not.

In order to better understand why or how the individual (HNS2 donor) in this study developed a BCN response we amplified functional gp160 HIV-1 envelope genes from proviral DNA present in PBMC samples collected on a yearly basis from 1986-1993 as part of a long-term cohort study at the NIH and from the PBMC of a whole blood sample collected in 2007. Plasma samples collected at the same time as the PBMC were used to characterize the evolution of the neutralizing antibody response in the patient. We discovered that the patient was infected early in his course of infection with at least two strains of HIV-1, one of which differentiated into distinct neutralization sensitive

(L1a) and neutralization resistant (L1b) lineages. L1a and variants derived from another lineage, L2, remained neutralization sensitive for several years. Positive selection analysis indicated low levels of positive selection for L1a and L2, consistent with possible low levels of replication for these strains. The progressive emergence of L1a and L2 envelope genes that encode envelope glycoproteins shown to be resistant to neutralization by plasma and multiple mAbs was followed by the decline of BCN antibodies and a decrease in circulating $CD4^+$T cells. This study does not prove a relationship between the phenotype of the HIV-1 envelope glycoprotein and the breadth of the neutralizing antibody response. However, the results from this study could indicate a mechanism whereby the presence of one or more virus lineages that effectively present cross-reactive neutralization epitopes may persist despite the development of BCN antibodies and may account for induction and maintenance of the BCN response

It is not known exactly when the patient became infected with HIV-1, therefore we cannot directly classify the patient as a long term non-progressor (LNTP). Nonetheless, the $CD4^+$T cell counts for the patient never fell below 200 cells/mm^3 for the seven-year study period and it is reported that the patient remained asymptomatic throughout. The relevance of the rate of disease progression to breadth of the neutralizing antibody response is unknown.

The identification of patients harboring multiple HIV-1 infections has profound implications in the understanding of HIV-1 transmission and vaccine development [200]. Multiple infections can occur either before or after initial seroconversion [201-207]. In the case of the HNS2 donor, it is unclear whether the patient was simultaneously or sequentially infected with multiple HIV-1 strains. However, the patient acknowledged

IVDU and having multiple sexual partners, thereby establishing the existence of multiple risk factors for infection. Phylogenetic analysis indicated the presence of multiple HIV-1 infections from as early as 1986, including the divergence of one variant into two distinct lineages. Therefore it is likely that the patient was multiply infected sometime prior to 1986, and perhaps as early as 1983 as based on patient interviews conducted at the time of sample acquisition as part of the cohort study at NIH (personal communication with H.J. Alter). The lack of patient samples before 1986 precludes us from determining exactly when the two lineage 1 strains (L1a) and (L1b) diverged, or when multiple infection occurred. The distinctions between L1a and L1b strains are based on polymorphisms in the V1/V2 and V3 regions. The sequence analysis of envelope genes amplified from the 2007 sample indicates a possible superinfecting strain of HIV-1. It must be considered that the superinfecting strain may have been present and circulating at low levels prior to detection. Furthermore, the envelope genes from 2007 may represent an antiretroviral drug-resistant lineage. We did not examine any of the patient samples for antiretroviral resistance sequence motifs.

Previous studies indicated that the HNS2 serum, collected in 1989, contained moderate to high levels of neutralizing antibodies against a panel of heterologous HIV-1 envelope glycoproteins [94, 95]. In this study we observed cross-reactive neutralizing activity against a panel of heterologous HIV-1 envelope glycoproteins known to be neutralization resistant using patient plasma samples collected from 1986-1993. In contrast to a recent study examining the neutralizing antibody specificities of broadly neutralizing sera from HIV-1 infected individuals against a panel of 40 isolates representing multiple HIV-1 clades [199], our limited plasma volumes constrained us to

testing a panel of only 12 heterologous HIV-1 isolates. The Clade B and Clade C panels were recently established for their use in determining the cross reactivity of neutralizing antibody responses induced by candidate vaccines and are known to be relatively neutralization resistant [191, 192]. Therefore the difference in GMT titers probably reflects greater neutralizing activity of the patient plasma against the clade B panel than the clade C panel. Envelopes from the miscellaneous panel are more heterogeneous in their neutralization sensitivity and therefore may not be compared directly to the clade B and C panels. Overall, these results demonstrate substantial cross reactivity of the initial response in this patient. The potency of the BCN response began to wane in 1992 and was markedly reduced in 2007. A report by Dhillon et al, demonstrating only modest neutralizing serum activity in a 2005 sample, may also reflect the waning of the BCN response in this patient [199].

Over time, as the immune response matures and the virus population diversifies, HIV-1 infected individuals develop a more cross-reactive neutralizing antibody response [83, 85, 92]. Approximately 10% of HIV-1 infected individuals develop a BCN response. [96-98, 199]. Progressive development of limited cross reactivity has been described previously in the MACS patients [94, 193]. The results of this study demonstrate that the BCN response of the HNS2 donor distinguishes this response from the that of the MACS patients, and that the BCN response of the HNS2 donor developed early, probably within the first three years of infection.

It is possible that the development of the neutralizing antibody response in the patient was influenced by the presence of the multiple HIV-1 lineages circulating in the patient from 1986-1993. Indeed, all lineages were sensitive to neutralization using

autologous plasma. Furthermore, we noticed an increase in the neutralizing antibody response from 1986-1991 for all lineages L1a, L1b and L2. It is possible that the presence of distinct neutralization sensitive envelope glycoproteins as seen from lineages L1a (1987, 1988 and 1989) L1b (1986 and 1990) and L2 (1986, 1988 and 1990) were responsible for inducing and driving the evolution of the autologous neutralizing antibody response in the patient. As was previously described [95] [181], the PM mutation found in the V3 loop region of the R2 envelope results in an unusually neutralization sensitive phenotype. It is therefore also possible that the presence and persistence of the PM mutation, which was maintained in envelope glycoprotein genes representing lineage L1a, may have significantly contributed to the development of the BCN response in the patient. The replacement of the neutralization sensitive L1a, L1b and L2 clones with the emergence of neutralization resistant envelope glycoproteins, suggests that the neutralizing antibody response developed by the patient may have selected for the emergence of the neutralization resistant envelope clones, as seen with a 1993 clone (L1a), 1991 and 1992 clones (L1b) and a 1991 clone (L2). Ultimately the decrease in the neutralizing antibody response seen in 1992-1993 and the loss of the neutralizing antibody response seen in 2007 may reflect the emergence of neutralization resistant envelope glycoproteins and a decrease in the viral load due to HAART treatment respectfully. The difference in GMT representing the sensitivity of envelope glycoproteins from a particular lineage to the autologous plasma indicate that envelope glycoproteins representing lineage L1b may in fact represent a significantly more neutralization resistant lineage.

Sensitivity of HIV-1 envelope glycoproteins to heterologous sera provides further evidence of the exposure of various neutralization sensitive epitopes on the surface of the HIV-1 envelope glycoprotein. The heterologous MACS sera used in this study was collected from males who were HIV-1^{+} and had seroconverted at the time of enrollment and remained clinically well for five years with CD4^{+}T cell counts over 200 cells/mm^{3} [193]. While the envelope glycoproteins from the HNS2 donor were more sensitive to the autologous than heterologous plasma, comparison of the GMTs indicated that the lineage L1b stain was also more resistant to neutralization using heterologous sera than were the other lineages. The inherent neutralization resistant phenotype of the L1b envelope glycoproteins to the heterologous plasma correlates with their inherent resistance to the autologous plasma and may indicate that envelope glycoproteins from the L1b strain already contain a "globally neutralization resistant" phenotype [208].

The emergence of neutralization resistant envelope glycoproteins was likely due selective pressures brought on by various aspects of the immune system including the neutralizing antibody response. Due to the limited amounts of patient plasma we were unable to examine the antibody specificities present in each sample. We were able to examine the number of synonymous and nonsynonymous mutations occurring in each lineage over the course of infection. It is possible that the levels of nonsynonymous mutations did not exceed the levels of synonymous mutations in the neutralization sensitive lineages (L1a and L2) due to low levels of replication of these strains. Furthermore, the sensitivity of the L1a and L2 strains to neutralizing antibodies may have also contributed to their low levels of replication in comparison to the neutralization resistant L1b lineage. The actions of CD8^{+}T cells may have also shaped the evolution of

the L1b strain. Indeed, the possible higher levels of replication of the L1b variants may have subjected them to greater selective pressures by $CD8^+$T cells. However, we cannot confirm this possibility, as we have not examined the sequences of the L1b strain for CTL escape mutations. Overall, findings in the HNS2 donor that may have contributed to the induction and maintenance of the BCN response include infection with and persistence of multiple HIV-1 strains presenting neutralization sensitive epitopes to the host immune system.

The second aim of this research project was to characterize the evolution of the neutralization sensitivity of HIV-1 envelope glycoproteins from the HNS2 donor to sCD4 and eight cross-reactive monoclonal antibodies (mAbs). We hypothesized that the HIV-1 envelope glycoproteins would become progressively more resistant to neutralization as a result of mutations that contribute to global neutralization resistance. Additionally, we anticipated that the nature of the development of neutralization resistance at various epitope regions would be related to the immunogenicity of the original infecting HIV-1 envelope glycoproteins.

The development of progressive neutralization resistance of the HIV-1 envelope glycoproteins from the HNS2 donor to mAbs targeting the CD4bs region, the co-receptor binding site and the unique gp120/gp41 complex epitope region, suggest that the HIV-1 envelope glycoproteins in this patient are evolving toward a global neutralization resistant phenotype. Indeed, the concept of global neutralization resistance has been previously observed for other HIV-1 envelope glycoproteins [208-210]. Global neutralization resistance is hypothesized to occur as non-epitope mutations occurring at

multiple sites in the HIV-1 envelope lead to a generalized neutralization resistant phenotype.

The lack of progressive neutralization resistance development to the MPER region mAbs 2F5 and 4E10 is not surprising as antibodies with such specificities are generally not found in HIV-1 infected individuals. The hypothesis is that these mAbs contain homology to self proteins and therefore mechanisms of self tolerance may select against the development of neutralizing antibodies with such specificities in HIV-1 infected individuals [211]. Due to our limited plasma volumes we were unable to test the samples for antibodies of such specificities and therefore we cannot be certain as to whether or not the patient developed any neutralizing antibodies targeting the MPER region. However, based on the lack of progressive evolution against the two MPER antibodies, it is likely that the patient never developed neutralizing antibodies of such specificities.

Lineage specific neutralization resistance was determined to be involved in the development of progressive neutralization resistance over the course of infection. Indeed, the emergence of neutralization resistant variants in the inherent neutralization sensitive lineages, especially in lineage L1a, was largely responsible for the observed development of progressive neutralization resistance of the HIV-1 envelope glycoproteins in the HNS2 donor. In contrast the inherent neutralization resistant L1b lineage remained largely resistant to neutralization using mAbs over the course of infection with the exception of the development of neutralization resistance to b12, a CD4bs mAb. In most cases, envelope glycoproteins from lineage L1b were resistant to neutralization by various mAbs with the exception of the two V1/V2 recombinant envelope glycoprotein genes

from the 1993 time point. As has been shown in a previous study, the V1/V2 region is important in regulating the overall neutralization sensitivity of primary isolates [212]. It is hypothesized that epitopes targeted by co-receptor antibodies are somewhat masked by the V2 loop. Indeed virus variants with their V1/V2 loops deleted display increased sensitivity to neutralization using CD4i mAbs [130, 131, 178]. Additionally, it was demonstrated that the neutralization phenotypes of two primary HIV-1 clade B envelope glycoproteins, SF162 (neutralization sensitive clone) and JR-FL (neutralization resistant clone) were reversed when chimeric envelope genes containing the V1/V2 domains from each respective envelope gene were exchanged [212]. In this study, we observed a similar reversal in neutralization sensitivity when the V1/V2 region from two independent envelope genes from the 1993 time point of L1b recombined with the V1/V2 region of envelope genes from the 1993 time point of L1a for mAbs m43, b12, m14 and 17b. We must take into consideration that the recombinants are in fact artifacts of amplification from using proviral DNA and were generated during the bulk PCR reaction.

Finally we examined the neutralization characteristics of pseudoviruses expressing two functional envelope glycoproteins isolated from the 2007 time point of sample availability (L3), which may represent a possible superinfecting and/or antiretroviral strain of HIV-1. Due to the lack of samples from 1994-2007 we cannot directly infer any evolutionary trends leading to the observed neutralization resistance of the two envelope glycoproteins to m43, m14, 48d or 17b.

One limitation of this study was that the volume of patient plasma available to us was not sufficient for use in both envelope gene cloning and for neutralization studies. As a result, proviral DNA from patient PMBCs collected at the same time as the plasma

was used for functional envelope gene cloning. There is a concern that the use of bulk PCR will result in various artifacts such as Taq-induced nucleotide substitutions, artificial recombination and resampling of viral genomes [188, 189]. One potential result of this limitation can be seen with the fact that envelope genes representing lineages L1a, L1b and L2 were not recovered from all time points spanning the cohort study period from 1986-1993. This pattern could be the result of a sampling artifact and may be a consequence of using proviral DNA as a source of envelope gene cloning rather than vRNA. It is possible that in some cases the HIV-1 envelope genes amplified from proviral DNA were from a few latently infected cells. Alternatively, the failure to amplify each cluster from each time point could be an artifact of the *in vitro* co-culture conditions as seen with the 1991b-1993b PBMC samples. Indeed, a study examining the diversity of HIV-1 envelope genes *in vivo* and after co-cultivation *in vitro*, found that a predominant sequence was found following co-culture of PBMC [213]. Another factor regarding the recovery of each lineage from each time point may be differing levels of viral replication. Our limited amounts of patient plasma precluded us from examining vRNA levels to determine the relative presence of each lineage from each time point of sample availability. The absence of L1a, L1b and L2 envelope genes from the 2007 time point of sample availability may be due to the initiation of antiretroviral therapy by the patient following the termination of the cohort study in 1994. It is likely that envelope genes representing lineage L3 represent an antiviral resistant strain and the disappearance of the L1a, L1b and L2 clusters may be due to sensitivity of these clusters to the antiretroviral therapy. Nonetheless, we observed progressive evolution of gene sequence in each of the amplified HIV-1 lineages, and evolution of neutralization resistance in L1a

and L2 over time. This pattern also coincided with the maintenance and loss of the BCN response in the patient over the course of infection, further demonstrating that the Envelope genes amplified from proviral DNA were representative of progressive evolution over the course of infection.

Another limitation of this study concerns the lack of patient samples prior to 1986. Ideally, the study of evolution of viral sequences would have been conducted from the onset of viral infection. In this study, we did not have access to the sequences representing the initial infecting strain/s of HIV-1 and cannot definitively determine which strains infected before the observed BCN response. Additionally, we cannot define the precise time at which the BCN response was developed.

In conclusion, we have shown that the donor of the HNS2 serum was infected early in his course with at least two strains of HIV-1, one of which differentiated into distinct neutralization sensitive (L1a) and neutralization resistant (L1b) lineages. L1a and variants from a second strain (L2) remained neutralization sensitive for several years. Analysis of positive selection rates among the lineages did not indicate positive selection for L1a and L2. The eventual emergence of neutralization resistant variants was accompanied by the loss of cross-reactive neutralizing antibodies and a loss of circulating $CD4^{+}$T cell numbers. Additionally, HIV-1 envelope glycoproteins from the patient became progressively more resistant to neutralization by mAbs targeting the complex gp120/gp41 epitope (m43), the CD4bs region (b12 and m14) and to the co-receptor binding site region (m9, 48d and 17b), but not to sCD4 or to mAbs targeting the MPER region of gp41 and that the development of progressive neutralization was unique with

respect to each of the clusters of HIV-1 sequences identified in the patient. It is likely that the development of progressive resistance was a result of mutations that contributed to global neutralization resistance and that the emergence of neutralization resistant viruses at various epitope regions was related to the immunogenicity of the original infecting HIV-1 strains.

Our overall conclusions indicate some significant findings in regards to the evolution of the HIV-1 envelope glycoprotein genes and neutralizing antibody response in the HNS2 donor. Indeed, we demonstrated that the autologous nAb response increased from 1986-1991. During this time we also demonstrated that the neutralization sensitive strains (L1a and L2) remained sensitive to autologous plasma, while the neutralization resistant L1b strain remained relatively resistant to the early plasma. Following the peak nAb response in 1991; we demonstrated that the emergence of neutralization resistant variants in lineages (L1a and L2) coincided with the development of progressive neutralization resistance to various mAbs targeting the gp120 subunit of the HIV-1 envelope glycoprotein, but not to sCD4 or to b12, a CD4bs mAb. In contrast, we demonstrated that the neutralization resistant strain (L1b) did develop progressive neutralization resistance to b12. The development of progressive neutralization resistance to CD4bs mAbs may indicate the development of anti-CD4bs antibodies in the patient. Furthermore, the persistent sensitivity of all three lineages (L1a, L1b and L2) to sCD4 and the persistent sensitivity of L1a and L2 to b12 could explain their corresponding sensitivities to the autologous plasma. It is therefore likely that the BCN activity in the patient may be due to the presence of b12-like or other CD4bs-like antibodies. Recently, two new highly potent and cross-reactive CD4bs mAbs were

identified from HIV-1 infected individuals [214, 215]. The identification of these new CD4bs antibodies as well as the possibility that the BCN activity in the HNS2 donor may be due to CD4bs-like antibodies indicates that the CD4bs region may be of great interest and could be an important region for inclusion as part of an immunogen that elicits a BCN antibody response.

Findings in this patient that may be related to the induction and maintenance of the BCN response may include infection with more than one lineage of HIV-1 and the low-level persistence of neutralization sensitive variants from lineages L1a and L2 that may be presenting cross-reactive neutralization sensitive epitopes. Features of infection from this patient that should be of interest for future evaluation include: the examination of phenotypes of HIV-1 envelope glycoproteins from patients who develop a BCN response in comparison to the phenotypes of HIV-1 envelope glycoproteins from patients who do not develop a BCN response in an effort to better understand which epitope regions may be effectively presented to B cells, the examination of whether or not the presence of multiple HIV-1 infections in an individual may induce a BCN response, and the examination of the whether or not the presence and persistence of neutralization sensitive HIV-1 strains in a patient are able to induce a BCN response due to the continued presentation of cross-reactive neutralization epitopes to the host immune system.

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