SPECIFICITY AND POLYREACTIVITY OF THE ANTIBODY RESPONSE DURING NATURAL HIV-1 INFECTION

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ABSTRACT

The specificity and polyreactivity of the antibody response in natural HIV-1 infection were studied. First, to investigate the overall antibody response, overlapping linear peptides were used to screen sera taken from HIV-1-infected individuals. The polyclonal antibody response was relatively stable during long-term infection, compared with acute infection, and mostly directed against immunodominant regions. Low level, transient antibody responses were detected against membrane proximal external region of gp41. To test if these Abs are neutralizing, an affinity purification method was developed to isolate these serum antibodies.

Second, in terms of polyreactivity of antibody response, we found that two broadly-neutralizing monoclonal antibodies bound only weakly to self antigen, cardiolipin. Moreover, sera were screened against cardiolipin; no significant reactivity was observed.

We conclude that the antibody response in HIV-1 natural infection is relatively stable over time; the MPER is weakly immunogenic in vivo, and that broadly-neutralizing antibodies do not seem to be autoreactive.
DEDICATION

To my parents

and

my husband, Honggang,

for their unconditional love
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GLOSSARY

aa  Amino acid
Ab  Antibody
ABTS  2,2'-azino-bis(3-ethylbenzthiazoline-6) sulfonic acid
AIDS  Acquired immune deficiency syndrome
aMLV  amphotropic murine leukemia virus
AP  Alkaline phosphatase
Bio-  Biotinylated
bNt/Nt  Broadly neutralizing/neutralizing
BSA  Bovine serum albumin
CDR  Complementarity determining regions
CHR  C-terminal heptad repeat on gp41
CL  Cardiolipin
CTL  Cytotoxic T lymphocytes
ELISA  Enzyme-linked immunosorbent assay
FBS  Fetal bovine serum
H3  Hypervariable loops 3 (CDR3) of the heavy chain
HIV-1  Human immunodeficiency virus-type 1
HRP  Horse radish peroxidase
IC_{50}  Inhibitory concentration required to reduce ELISA signals to 50%
SC  Seroconverter
SP  Long-term, slow progressor
MAb  Monoclonal antibody
MHC  Major histocompatibility complex
MPER  Membrane proximal external region on gp41
NFDM/DM  Non fat dry milk
NHR  N-terminal heptad repeat on gp41
NP  Normal progressor
O.D.  Optical density
OVA  Ovalbumin
PBS  Phosphate-buffered saline
p-NPP  p-nitrophenyl phosphate substrate
RP  Rapid progressor
RT  Room temperature
SA  Streptavidin
TBS  Tris-buffered saline
TCLA  T-cell laboratory-adapted
CHAPTER 1: THESIS INTRODUCTION

1.1 The AIDS epidemic

The acquired immune deficiency Syndrome (AIDS) pandemic has been one of the most destructive epidemics in history and has killed more than 25 million people since the report of its first case. In spite of broader access to highly active anti-retroviral therapy (HAART) around the world, the AIDS epidemic claimed an estimated 2.8 million lives in 2005 of which more than a half-million were children. Globally, ~46 million people are currently living with HIV and Sub-Saharan Africa remains the worst affected region (UNAID, 2006, http://www.unaids.org/en/HIV%5Fdata/).

The first indication of AIDS epidemic was reported in 1981, with the outbreak of several rare pneumonia cases in homosexual men in Los Angeles, U.S.A. (CDC, 1981, http://www.cdc.gov/mmwr/preview/mmwrhtml/june_5.htm). In 1983, Montagnier et al. at the Pasteur Institute in France first discovered the pathogen causing AIDS, Human Immunodeficiency Virus (HIV) (Barre-Sinoussi et al. 1983). Based on sequence analysis and serology, HIV was divided into two types. Type 1 HIV (HIV-1) is more virulent, more easily transmitted and is the cause of the majority of HIV infections globally, whereas type 2 HIV (HIV-2) is less easily transmitted, has a different clinical picture, and is largely confined to West Africa (Reeves et al. 2002). HIV-1 has three subtypes / groups: M (majority), O (outliers), and N (non-M/non-O). Group M is further classified into 10 different sub-subtypes / clades, designated clades A through J, and a number of recombinant forms (RFs; e.g., the BC RF). Clade B is the most common viral isolate in
the United States and Western Europe; clades A, C, D, and E are most common in the developing world. The focus of work presented in this thesis is on HIV-1.

1.2 HIV-1 structure and life cycle

HIV-1 belongs to the *Lentivirus* genus, and *Retroviridae* family (Popovic *et al.* 1984). Lentiviruses have RNA genomes contained within a protein capsid surrounded by a lipid envelope. HIV-1 is ~120 nm in diameter (60 times smaller than a red blood cell). It is composed of two copies of a single-stranded RNA genome enclosed within a conical capsid, which is in turn surrounded by a lipid bilayer membrane (envelope) derived from the host cell. The p24 protein forms the capsid that encloses the two genomic RNA strands and viral enzymes, such as integrase, p32 and reverse transcriptase, p64. The matrix protein, p17, is anchored to the internal face of the envelope.

![Figure 1-1: Basic organization of HIV-1. See text for details.](Image)

HIV-1 targets T lymphocytes, macrophages, and dendritic cells, which all have important functions in the human immune system, via a primary receptor, the CD4
surface molecule, and a secondary chemokine receptor: either CXCR4 (X4) or CCR5
(R5) (Alkhatib et al. 1996; Deng et al. 1996; Doranz et al. 1996; Dragic et al. 1996; Feng
et al. 1996). The cellular preference (tropism) of an HIV-1 variant is largely determined
by its ability to use X4 and/or R5. The X4 coreceptor is mainly present on CD4\(^+\) T cells,
whereas the R5 coreceptor is presented on macrophage/dendritic cell lineages.

The life cycle of HIV-1 in CD4\(^+\) T cells begins with an effective infection, which
is mediated by binding of envelope protein, gp120, to the CD4 receptor and X4
coreceptor on the cell surface. This initiates the process of viral entry, which is described
in detail below. After successful entry into the target cell, the viral reverse-transcriptase
enzyme transcribes the single-stranded viral RNA into double-stranded DNA that is
integrated as a proviral genome into the genome of the host cell. In this state, the virus is
latent. When CD4\(^+\) T cell is activated by antigen, it is stimulated to translate the
integrated proviral DNA into proteins needed to build new viral particles. After the viral
components assemble inside the cell,

When the infected cell synthesizes new protein, integrated viral DNA is also
translated into the proteins needed to build new viral particles, such as Gag, Env and Pol
proteins. The Env protein (gp160) is cleaved into the mature gp120 and gp41 subunits by
cellular enzymes within the Golgi apparatus. After these proteins assemble at the
cytoplasmic side of the cell plasma membrane, the viral core buds through the membrane
and obtains an envelope composed of cell membrane components spiked with viral
envelope proteins (Freed et al. 1995).
1.3 **HIV-1 envelope proteins**

The HIV-1 envelope spike is a trimeric structure formed by three copies of the membrane-spanning protein, gp41, and three copies of the extracellular surface protein, gp120, non-covalently bound to gp41 (Lu et al. 1995). The envelope spike is encoded by the *env* gene, and is translated first as a single polypeptide precursor during viral replication. The *env* gene product subsequently enters the Golgi apparatus and undergoes oligomerization and extensive glycosylation to become gp160, which is then cleaved by cellular proteases into gp120 and gp41 during transfer via the Golgi network to the cell surface. The envelope proteins remain associated through weak, noncovalent (mainly hydrophobic) interactions after cleavage, and gp120-gp41 heterotrimers are incorporated into the envelope of the virion (Earl et al. 1991).

1.3.1 **The structure of gp120**

The outer component of the trimeric spike, gp120, is a heavily glycosylated protein, with carbohydrate accounting for 40% to 50% of its molecular weight (Leonard et al. 1990). Glycosylation masks the protein surface of Env, and is important for viral evasion from host immune response (see section 1.6). Based on comparative sequence analyses, gp120 sequence has been divided into five alternating conserved and variable regions (C1–C5 and V1–V5) (Leonard et al. 1990; Starcich et al. 1986). Conserved regions of gp120 form a central core, whereas variable regions V1–V4, form loops that are anchored to the surface of the protein by disulfide bridged cysteine residues at their bases.

The V3 loop is a 35-residue-long, glycosylated, disulfide-bonded structure that has been extensively studied, since it is involved in viral infection and dominates the anti-
gp120 antibody (Ab) response (immunodominant region, see Chapter 2). The central segment of V3 has been represented by synthetic peptides (KRKRIHIGPGRAFYTT) (HIV-1 MN) (Hartley et al. 2005). In the literature, the “tip” or “crown” of the V3 loop has frequently been used to describe the beta-turn, GPGR/GPGQ, of the V3 loop and an unspecified number of residues flanking it. Although highly variable, V3 possesses some relatively conserved features, such as positively-charged patches and a predicted β-turn at the tip. The β-turn motifs, GPGR and GPGQ, are the most common motifs found among all HIV-1 strains, with the former being in subtype B viruses and the latter in viruses from other subtypes (Hartley et al. 2005).

It has long been recognized that the V3 loop has a major influence on HIV-1 tropism, which, in turn, substantially affects pathogenesis (Moore et al. 2004; Douek 2003). An early example of this influence dates from before the identification of the viral coreceptors. Huang et al. observed that some T-cell laboratory-adapted (TCLA) strains of HIV-1 replicated poorly in macrophages, but substitution of 20 residues from V3 of a “macrophage-tropic” viral strain conferred tropism to macrophages (Huang et al. 1991). Once different coreceptors (see 1.3.3) were identified for macrophage/lymphocyte tropic viruses, it was soon shown that if the V3 region of an X4 virus was genetically replaced by its counterpart from an R5 strain, the chimeric virus had the R5 phenotype, and vice versa (Moore et al. 2004; Choe et al. 1996; Cocchi et al. 1996).

The crystal structure of the gp120 core (comprising gp120 lacking the V1/V2 and V3 loops, and portion of the N and C termini) has been resolved in complex with the receptor CD4 domain and the Fab fragment of the chemokine receptor mimic, monoclonal (M) Ab, 17b (Kwong et al. 1998). Figure 1-2 shows that gp120 is
structurally organized into inner and outer domains that are linked by a bridging-sheet minidomain. The inner domain faces the axis of the trimeric complex, and contributes most of the contacts made with gp41. The outer domain of gp120 is heavily glycosylated, and is thought to face away from the trimer axis. The bridging sheet is formed by four antiparallel β-sheets. Among different clades of HIV-1, the inner domain is more conserved than the outer domain. The crystal structure also reveals that all three domains of the gp120 core contribute to receptor and coreceptor binding (Kwong et al. 1998). Packing of the envelope proteins into the trimeric spike structure, and the heavy glycosylation of gp120, lead to very little of the protein surface being exposed on infectious envelope spikes.

Figure 1-2   HIV-1 gp120 structure.
Crystal structure of gp120 core composed of inner domain, outer domain and bridging sheet.
1.3.2 The structure of gp41

The gp160 precursor is cleaved to form gp41. At the amino-acid sequence level, gp41 shares many features with the transmembrane proteins of other retroviruses. As shown in Figure 1-3, gp41 includes following regions: (a) a hydrophobic N-terminus forming the fusion peptide which comprises a characteristic stretch of about 15 hydrophobic amino acids (Gallaher 1987). The fusion peptide is believed to insert into and destabilize the target cell membrane during fusion process (see section 1.3.3). (b) C-terminal to the fusion peptide are two regions containing hydrophobic heptad repeats (HRs), the N-terminal (N) HR and C-terminal (C) HR, respectively (Gallaher et al. 1989). Both NMR and crystallographic studies have shown that three NHRs fold in parallel to form a trimeric coiled-coil structure surrounded by three antiparallel CHR helices. Each NHR-CHR pair is connected by an extended, disulfide-bridged loop, the cluster1 region (Chan et al. 1997; Caffrey et al. 1998; Skehel et al. 1998). Drawing from the extensive structural and functional studies done on the corresponding transmembrane proteins of human Influenza virus (Wilson et al. 1981; Carr et al. 1993; Bullough et al. 1994), it is currently believed that the six-helix bundle structure of gp41 represents post-fusion conformation (Melikyan et al. 2000; Chan et al. 1997; Tan et al. 1997; Weissenhorn et al. 1997). The “native” or “pre-fusion” conformation remains unknown. (c) The region C-terminal to C-NHR, named membrane-proximal external region (MPER), is highly conserved. (Zwick 2005). The MPER has been an important target for HIV-1 vaccine design because it binds to three broadly (b) neutralizing (Nt) MAbs and is one focus of this thesis. (d) Immediately C-terminal to the MPER is the transmembrane domain (TM) of gp41. (e) The C-terminus of gp41, cytoplasmic domain (CT), comprises a long, intercellular tail of about 150 amino acids.
1.3.3 Envelope proteins mediate HIV-1 entry

HIV-1 entry is a sequential multi-step process mediated by envelope protein gp120 and gp41. Surface protein gp120 is responsible for viral binding to receptors on target cells. The binding of gp120 to receptor CD4 molecule induces conformational changes in gp120, and leads to the exposure of the coreceptor binding site, also called the CD4-inducible (CD4i) site (Rizzuto et al. 1998). The binding of gp120 to the coreceptor (R5/X4) results in further conformational changes that lead to gp41 activation, fusion of the viral and cellular membrane, and entry of the nucleocapsid into the cell. These latter steps are mediated by gp41.

On native virus, gp41 exists in a metastable (pre-fusion) conformation and is largely buried by gp120 on its top. Following receptor and coreceptor binding, gp120 is shed, freeing the N-terminal fusion peptide of gp41 that is otherwise buried by gp120. The fusion peptide (Figure 1-3) then inserts into the target cell membrane. This extended intermediate form of gp41 is not stable, and is followed by the formation of a gp41 coiled-coil structure that acts as a spring-loaded mechanism to bring the viral and cell membranes into close proximity (Figure 1-4). The details involving membrane fusion, pore formation and entry of the viral capsid into the cell are poorly understood.
For example, it is not known how many gp41 trimers are required to form a fusion pore. After the pore has been formed, the viral capsid, including RNA genomes and various enzymes, is injected into the target cell through the pore.
Figure 1-4: A schematic model for HIV-1 entry. After gp120 binds to receptor CD4 and coreceptors on the target cell membrane, gp41 forms an extended conformation and the hydrophobic fusion peptide inserts into the target membrane (pre-hairpin intermediate). The next step is the formation of six-helix bundle conformation and the energy thereby released causes the membranes to bend towards each other. After two membranes fuse together, the viral capsid will then be injected into the target cell.
1.4 The structure of antibodies and their epitopes

Abs are globular proteins made by B lymphocytes. Recognition of an antigen by receptor on the B cell (surface antibody) triggers proliferation and differentiation into memory B cells and plasma cells that secret soluble Abs with identical specificity to the parent B cell surface receptor. Ab molecules have a common structure of four polypeptide chains (Figure 1-5), comprising two identical light (L) chains, and two identical heavy (H) chains. Each L chain comprises two homologous, ~110 amino acid (aa) domains, V_L and C_L, each of which folds into a two-layer sandwich of antiparallel β-strands arranged in two β-sheets, named immunoglobulin (Ig) domain. Depending on the isotype of the H chain, it comprises four to five Ig domains (V_H, C_H1, C_H2, C_H3 and sometimes C_H4); C_H1 and C_H2 are linked by a non-Ig domain, hinge region of varying length. Each light chain is bound to a heavy chain by a disulfide bond, and by noncovalent interactions between Ig domains that form H-L chain heterodimers. Similar noncovalent interactions and disulfide bridges link the two identical H-L chain combinations to each other to form the basic four-chain (H-L)_2 Ab structure (Edelman 1991; Janeway, 2005).
Sequence comparison revealed that there are variable regions and constant regions in each H and L chain (Wu et al. 1970). The amino-terminal variable (V) Ig domains of the H and L chains (VH and VL) together make up the V region of the Ab and confer the ability to bind specific antigen, whereas the constant (C) Ig domains of the H and L chains (CH and CL) make up the C-region (Janeway 2005). When an Ab binds to an antigen, only a very small part, representing the six hypervariable loops at the tips of the V domains is actually involved in binding the antigen. This region of antigen contact is called the paratope of the Ab; the region on the antigen it contacts is called the epitope. Since these loops determine what shapes on the antigen the Ab will bind to, they are referred to as complementarity determining regions (CDRs) (Janeway 2005). There are
three CDRs on both H and L chains, namely CDR1, CDR2 and CDR3. In this thesis, the CDR3 loop on the H chain will be referred to as “H3”.

An epitope is a site on an antigen recognized by an Ab (B cell epitope) or an antigen receptor (T cell epitope); epitopes are also called antigenic determinants (Laver et al. 1990). There are two types of protein epitopes, discontinuous and linear epitopes. A discontinuous epitope is composed of residues from different parts of the polypeptide chain that are brought together by the three-dimensional conformation of folded protein. Because denaturation of the protein normally changes such conformation, Abs that bind to discontinuous epitopes on a native protein typically do not bind to denatured protein. In contrast, a linear epitope is often composed of a single segment of polypeptide chain (also called linear epitope) and is present whether the protein is in its native or denatured state. However, linear epitopes may be conformational, requiring protein folding for Ab to bind. On the surface of a protein antigen (such as gp120 or gp41), a large number of potential epitopes are presented; the epitopes recognized by the immune system are called immunogenic. Furthermore, certain epitopes are said to be immunodominant, meaning they preferentially stimulate a stronger immune response than other epitopes within a particular antigen. The epitopes that are immunodominant for B cells tend to be on the surface because they are more available than internal epitopes on the antigen.

1.5 The antibody response during HIV-1 infection

In general, Abs provide protection from extracellular pathogens and cytotoxic T lymphocytes (CTLs) operate against intracellular pathogens. Both extracellular (before entry) and intracellular stages exist in the HIV-1 life cycle; therefore, an immune response involving both Abs and CTLs is produced in HIV-1 infection. The host
immune response can temporarily control but not eliminate the virus due to the large pool of latent infected cells (mainly CD4+ T lymphocytes) and the virus's ability to generate large numbers of mutants. There is a dynamic interplay between HIV-1 and the host immune system in which HIV-1 mutants arise and are suppressed by strain specific Ab and CTLs. However, eventually, the viral replication and evasion, and infected cell killing leads to decline of the CD4+ T cell population and to the clinical symptoms of AIDS.

HIV-1 CTLs have been shown to be well correlated with the initial decline of viremia (Pantaleo et al. 1994; Koup et al. 1994; Connick et al. 1996; D'Souza et al. 1996). In contrast, Nt Abs usually appear after viremia has begun to decline, suggesting that they are not responsible for the initial reduction in viremia (Ariyoshi et al. 1992; McKnight et al. 1992). However, studies have suggested that the NtAbs may be more pronounced in individuals with long-term, slowly-progressing infection (SP). NtAb responses in SP are improved in magnitude and breadth compared with other HIV-1 infected individuals (Montefiori et al. 1996). In addition, sera from SP have a greater probability of neutralizing heterologous primary isolates than sera from progressors (Cao et al. 1995; Montefiori et al. 1996). Note that whether the improved Ab response is a cause or effect of SP is still a question needs to be answered.

More direct evidence of the protective role of the Ab response comes from passive transfer studies. In chimpanzee models, it has been demonstrated that passive immunization with bNt MAbs followed by infection with a SIV-HIV chimeric virus (SHIV) can prevent chronic infections (Emini et al. 1992; Mascola 2003). In human, there is also direct evidence that passively transferred bNt MAbs have the capacity to
contain viremia in established HIV-1 infection (Trkola et al. 2005). Given such data, the general consensus is that an effective HIV-1 vaccine should produce bNtAbs in order to prevent new infections (i.e., that produces sterilizing immunity).

1.6 Mechanisms of HIV-1 evasion from Ab response

One reason that HIV-1 is not eliminated by the immune response during infection is that HIV-1 has evolved a capability to generate escape mutants. Many escape mechanisms from Ab-mediated neutralization have been described. First of all, the exceptionally high genetic variability of HIV-1 envelope proteins including the sites of glycosylations allows the virus to escape from Ab recognition by mutation of the epitopes on the envelope proteins (Moog et al. 1997; Wei et al. 2003; Richman et al. 2003). Second, both gp120 and gp41 are largely covered by carbohydrate glycans that are synthesized by the host and therefore considered "self" by the host's immune system at low density. As a result, the glycosylation renders the underlying “foreign” proteins gp120 and gp41 invisible to the immune surveillance (glycan-masking). The protective effect of the carbohydrate glycans has been demonstrated by Malenbaum et al., who showed that a gp120 mutant with a deletion of a single glycosylation site rendered much higher sensitivity to neutralization (Malenbaum et al. 2000). Third, the complex organization of gp120 and gp41 as heterotrimers, has important implications for immune evasion since functionally important sites for viral neutralization on envelope proteins are conformationally protected from binding to Abs; this has been called “conformational masking” (Kwong et al. 2002). In contrast, it has been suggested that in vivo, non-functional envelope falls apart due to non-covalent interactions that tether gp120 to gp41, thus exposing new immunogenic sites that are not present on functional envelope. These
non-infectious envelope proteins act as the predominant immunogen, and divert the immune system to produce Abs to surfaces that are occluded on the functional spike. For example, the gp41/gp120 interfaces and areas on gp120 buried along the trimer axis are now presented to the immune system. Both gp120 and gp41 may also change conformation upon dissociation. As a result, most Abs elicited in vivo are raised against these decoy epitopes and do not bind the functional envelope glycoprotein complex, therefore can not block viral infection (Parren et al. 1997a; Parren et al. 1997b; Burton 1997). In supportive of this idea, it has been shown that there is a clear correlation between antibodies that are capable of neutralization and those that bind to mature oligomeric Env protein (Fouts et al. 1997; Roben et al. 1994; Sattentau et al. 1995).

1.7 HIV-1 primary isolates and T-cell laboratory-adapted strains

There was an enigma misleading HIV-1 research for years, which is the difference in neutralization resistance between HIV-1 primary isolates and TCLA strains. Primary isolates represent viruses isolated directly from patients such as the molecular clones SF162, BaL, ADA, 89.6, JR-CSF, JR-FL, and YU2. In contrast, TCLA strains represent HIV-1 that has been cultured and passaged in immortalized T-cell lines in vitro for a period in the laboratory, such as the molecular clones HXBc2, IIIB, and MN. In general, primary isolates are more resistant to Ab neutralization compared to TCLA. Most of the early studies on NtAbs have used TCLA strains whereas it is believed now that primary isolates are more representative of the original in vivo HIV-1. The difference in neutralization resistance may be attributed to Ab-mediated selection pressure on primary isolate viruses in vivo. In other words, viruses grown in tissue culture without Ab pressure become more neutralization sensitive (Daar et al. 1990;

1.8 Broadly neutralizing antibodies and their epitopes on envelope proteins

During HIV-1 infection, strain-specific NtAbs are elicited early on. Once the Ab response reaches a certain level, a resistant virus emerges. Then, a neutralizing Ab response to this resistant virus develops and a new resistant virus emerges. In most cases, the virus always stays one step ahead of the evolving neutralizing Ab response and the autologous viruses are replaced quickly by populations of resistant viruses. To eliminate the established HIV-1 infection by natural immune response or to prevent a new infection by vaccines, the presence of Abs that can neutralize HIV-1 from a wide variety of clades and origins, the so-called bNt Abs, is important. Yet, up to now, only a small panel of bNt MAbs (b12, 2G12, 447-52D, 2F5, 4E10 and Z13) have been identified that can neutralize a broad range of HIV-1 primary isolates (Burton et al. 2005; Zolla-Pazner 2004). Therefore, the epitopes of these bNt MAbs have been attractive targets for vaccine design.

bNt MAb b12 binds to a conformational epitope that overlaps the CD4 receptor binding site on gp120. The CD4 binding site is a recessed pocket located at the interface of the outer and inner domains with the bridging sheet (Kwong et al. 1998). In the gp120-CD4 interaction, amino acid Phe43 on CD4 plays crucial role by protruding into the pocket on gp120 (Wyatt et al. 1998). The b12 crystal structure has been determined and at the same time, docked onto the gp120 structure with CD4-liganded (Saphire et al. 2001). In the crystal structure, a notable feature is that b12 has an unusually long
protruding H3 loop (18aa) compared with the average (13aa long) in anti-protein human Abs (Collis et al. 2003). It was suggested that this H3 loop inserts into the pocket of the CD4 binding site on gp120 and therefore blocks viral infection by preventing CD4 attachment.

bNt MAb 2G12 recognizes an epitope composed of oligomannose residues located on the outer domain of gp120 (Calarese et al. 2003). It has been hypothesized that the neutralizing activity of 2G12 is an indirect, steric effect manifested by a binding site that is physically close to the receptor-binding sites of gp120 (Scanlan et al. 2002). 2G12 is unique in terms of both specificity as well as structure. Crystallographic study has revealed that two Fabs of 2G12 assemble into an interlocked Vh domain-exchanged dimer, forming an extended antigen binding site that comprises two VH-VL interfaces and one VH-VH interface (Calarese et al. 2003). Thus, two conventional light chains are flanking this swapped heavy-heavy chain. The 2G12 structure indicates that the humoral arm of the immune system has the capacity to overcome the glycan-masking strategy (see section 1.6) that HIV uses to protect itself from Ab recognition.

The epitope of bNt MAb 2F5 was mapped to a linear epitope ELDKWA (aa662-667) on the MPER of gp41 (Muster et al. 1993). This epitope has been since confirmed and further expanded from the original 6-mer epitope to a longer 17-mer (655-671) linear epitope (Menendez et al. 2004; Conley et al. 1994; Parker et al. 2001; Tian et al. 2002; Barbato et al. 2003. Site-directed mutational study has demonstrated that the critical binding sites on the peptide are DKW (Zwick et al. 2005). Consistent with this, peptides bearing the DKW motif are repeatedly selected from phage-displayed peptide libraries (Menendez et al. 2004). In the crystal structure of 2F5 in complex with
the 17-mer peptide, a notable feature is that 2F5 has a 22 aa long CDR3 loop on heavy chain (Ofek et al. 2004). Curiously, only 10 out of 22 amino acids at the base of H3 show interactions with the peptide and the apex remains largely unbound. However, mutagenesis studies showed that mutation on a Trp at the tip of this CDR3 loop significantly decreases the binding affinity of 2F5 to both gp41 and its epitope peptide. This mutation also decreases neutralization activity, indicating that the long loop is not only required for the creation of the peptide binding site, but also that the tip of the loop may be involved in further interactions (Zwick et al. 2004).

Another bNt MAb 4E10 also binds to a linear epitope NWFDIT (aa 671-677) that is C-terminal to the 2F5 epitope within MPER (Zwick et al. 2001b). Mutagenesis has shown that W, F in the core epitope and another W at the C-terminal (7aa away) are important for 4E10 neutralization (Zwick et al. 2005). In the crystal structure of 4E10 in complex with a peptide (WNWFDITNW) (Cardoso et al. 2005), the peptide adopts an unusual helical conformation compared to the typical β-turn that is the predominant secondary structure of the Ab bound peptide (Stanfield et al. 1995). Furthermore, the binding site of 4E10 is remarkably hydrophobic. The hydrophobicity of the combining site is derived in part from the H3 loop as well as from an unusually hydrophobic H2 loop. This makes the 4E10 combining site considerably more hydrophobic than those of most antibodies. 4E10 H3 is also relatively long (18aa). Similar to 2F5, only two residues at its base contact the C-terminal region of the peptide epitope and the other part of the loop bends away. The tip of the H3 loop of 4E10 is composed of mainly nonpolar residues that form a hydrophobic, flat surface that has been suggested to interact with the adjacent viral membrane (Cardoso et al. 2005).
The existence of bNtAbs demonstrates the ability of the human immune response to recognize B cell epitopes on the HIV-1 envelope that are shared and that, when complexed with Abs, prevent virus infectivity. However, the fact that bNtAbs are produced in only few individuals, and all demonstrate quite extraordinary characteristics highlights the challenges that targeting bNtAbs poses to vaccine design.

1.9 Summary of my contribution to Chapter 2, 3 and 4

The work presented in this thesis was performed by a team of people that included myself. For Chapter 2, Chao Wang, an undergraduate student in our lab had finished a great amount of preliminary work before I started my project. However, I repeated most experiments to duplicate the data. I also analyzed the screening data. Sampson Wu, a technician in our lab, has helped me complete and replicate the serum screening experiments. For Chapter 3, I developed the initial affinity purification method. Amy Lubik, an undergraduate student under my supervision, optimized the method and compared two different purification systems. For Chapter 4, I was involved in experimental design and paper writing based on our data. Sampson Wu executed most experiments, and greatly helped Dr. Scott and me in writing this section as a manuscript for publication. I have revised it for this thesis.
CHAPTER 2: ANTIBODY RESPONSE AGAINST HIV-1 ENVELOPE PROTEINS IN NATURAL INFECTION

2.1 Introduction

A bNt Ab response is likely to be necessary for a preventive HIV-1 vaccine. The ideal Abs should have potent neutralizing activity against a broad range of HIV-1 primary isolates, and target conserved epitopes on the envelope protein that are not able to evolve in response to immune response. However, vaccine efforts have not been successful to elicit such bNtAbs in vivo (Belshe et al. 1998; Graham 1994; Hanson 1994; Letvin 1998; Mascola et al. 1996; Matthews 1994). The reason for this failure partly lies in our incomplete understanding of the Ab response in HIV-1 natural infection.

As with most pathogens, HIV-1 induces a polyclonal Ab response against various epitopes on different viral proteins, especially gp120 and gp41. Despite the ability of HIV-1 to evade the Ab response, as mentioned in the introduction, serology studies have shown that NtAbs are made in most HIV-1 infected individuals. Most patients during natural HIV-1 infection produce autologous NtAbs that neutralize the HIV-1 primary isolate the patients were infected with (Mascola et al. 1996; Cao et al. 1995; Ho et al. 1987; Moog et al. 1997; Richman et al. 2003; Weiss et al. 1985). In addition, such NtAbs appear during the acute phase of HIV-1 infection, within three months of seroconversion (Richman et al. 2003). Unfortunately, autologous NtAbs can not clear infection, and is followed by the appearance of escape variants. The continuous cycle of autologous neutralization followed by the emergence of escape variants, as shown in many studies
(Bradney et al. 1999; McKnight et al. 1992; Quinnan et al. 1998; Zhang et al. 1999; Richman et al. 2003; Wei et al. 2003), is particularly impressive in HIV-1 infection because of the virus’s extremely high mutation rate.

As the HIV-1 virus evolves, the Ab response some patients develop heterologous NtAbs that neutralize variants that are not present in the individual’s viral population. Heterologous neutralization always arises after autologous neutralization (Bradney et al. 1999; McKnight et al. 1992; Quinnan et al. 1998; Zhang et al. 1999; Wei et al. 2003; Moog et al. 1997; Richman et al. 2003). A few heterologous Nt sera have been found capable of Nt a very broad spectrum of primary HIV-1 isolates. Some examples of these bNt sera include FDA-2 (Vujcic et al. 1995), HNS2 (Polonis et al. 2001; Quinnan et al. 1999; Vujcic et al. 1995), and serum from a South African sex worker DU179 (Bures et al. 2002). Slow progressors of HIV-1 infection have been characterized, and some found to have high titre of NtAbs (Cao et al. 1995; Binley et al. 1998; Loomis-Price et al. 1998). A few potent bNt MAbs have been discovered, namely: b12, 2G12, 447-52D, 2F5, 4E10 and Z13 (Zolla-pazner 2004; Burton 2005). One important strategy for HIV-1 vaccine design therefore involves the design and optimization of immunogens that mimic these epitopes and thus possibly elicit bNtAbs in vivo.

Although it is known that neutralization activity becomes broader over time, it is still not known whether bNt activity is correlated with the breadth of the anti-HIV Ab response. Most previous studies that characterized the breadth of the Ab response against Env proteins were focused on peptide reactivity in natural infection. The immunodominant region has been shown to be the V3 region on gp120 (Garrity et al. 1997; Devash et al. 1990; Zwart et al. 1991; Zwart et al. 1992), and cluster1 on gp41
(Horal et al. 1991; Chiodi et al. 1987; Norrby et al. 1987; Smith et al. 1987; Wang et al. 1986). Other regions on Env proteins have also been reported to be immunogenic, such as MPER (Geffin et al. 1998; Calarota et al. 1996) and CT (Buratti et al. 1998) on gp41. In terms of the correlation of Ab response against certain peptides or proteins with different clinical outcomes (protective epitopes), the results are contradictory. Peptide reactivity, especially those regions NtAbs bind to (e.g., V3 and MPER), was related to better clinical outcomes in some studies (Loomis-price et al. 1998; Geffin et al. 1998; Srisurapanon et al. 2005; Ugen et al. 1997), but not others (Zwart et al. 1994; Moore et al. 1994).

The questions addressed in the study presented in this chapter were: First, what is the overall Ab response (in both strength and breadth) against Env proteins in different stages of HIV-1 infection? Second, is there reactivity against sites where bNt MAbs bind? And is there reactivity with peptide markers we have developed that are specific for the bNt MAbs? Third, is there a correlation between reactivity at sites bound by Nt MAbs, particularly the MPER, and stages of infection, clinical outcome, or neutralization activity in a serum?

We characterized the Ab response during natural HIV-1 infection by screening a large panel of serum samples (~150) with the envelope proteins, linear peptides derived from various regions on gp160 (MN strain), and peptides that are specific for the bNt MAbs. We used serum samples that were taken from 20 HIV-1 infected individuals. Some of these samples were grouped by clinical course as slow (SP), rapid (RP), and normal progressors (NP). Others were taken within the first 1-4 years of infection, starting at seroconversion (SC). Between four and eight samples were taken over time from each of these individuals, allowing the stability and breadth of each person’s Ab
response to be followed. We also used a set of single serum samples taken from 13 individuals; these sera had been characterized as bNt. The peptides used in our study include, (1) linear peptides representing immunogenic regions in literature and our previous screening of a panel of HIV+ IgGs to assess the breadth of reactivity. These peptides have been shown to be immunodominant or have moderate/low reactivity. (2) Peptides covering the regions where NtAbs possibly bind. (3) Peptides that are specific for the bNtMAbs (b12, 2G12, 2F5 and 4E10) developed in our lab.

In summary, we found that the Ab response broadens over time during the first 1-2 years after seroconversion, and for the most part, becomes stable. Surprisingly, the reactivity profiles of the bNt sera were less broad than RP sera, indicating that broad neutralization is likely due to high titer oligoclonal, or monoclonal, Nt Abs, in these sera. Strong Ab responses against sites where Nt MAbs are reported to bind (V3, cluster1 and NHR) were detected, but most of these Abs are probably not Nt, as they were produced in all or most HIV-1 infected people. The MPER was an exception to this; as expected, low and mainly transient reactivity was found against the MPER through all stages of infection, as well as in some of the bNt sera. No envelope specific reactivity was detected for any of the peptides that were specific for the bNt MAbs. Given that we observed reactivity in all of the sites of broad neutralization, including the MPER, this indicates that: (i) Reactivities against these sites are not bNt; or (ii) these epitopes are different from those of the bNt MAbs; (iii) these peptides binding to the bNt MAbs are too specific to cross-react with other bNtAbs against the same epitope.
2.2 Materials and methods

2.2.1 Serum samples

HIV-1 positive human sera from rapid progressor (RP), normal progressor (NP), and slow progressor (SP) groups were provided by Drs. L. Kingsley and C. Rinaldo (University of Pittsburgh) through the (Multicenter AIDS Cohort Study) MACS program. Samples from seroconverter group (SC) and the 11 bNt sera were provided by Drs. D. Richman and S. Little (UCSD). FDA-2 (#13 bNt serum), AD18 and AD28 sera were given by Drs. P. Parren and D. Burton (The Scripps Research Institute).

2.2.2 Reagents

High-binding microtiter plates (Corning Inc., Corning, NY) were used for all ELISA. Protein G (to determine serum IgG content), human IgG (as a standard), Bovine Serum Albumin (BSA) and 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma-Aldrich (St.Louis, MO). Horseradish peroxidase (HRP) conjugated protein A/G was purchased from Pierce (Rockford, IL). Tween-20 was from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ).

The reagents for the serum-screening ELISA are the same as above if not mentioned below. 15-mer synthetic peptides derived from HIV-1 envelope protein MN isolate (>80% HPLC purity) were provided by NIH AIDS Research and Reference Reagent Program; peptide sequences are listed in Table2-1. Ovalbumin (OVA) (Sigma-Aldrich, St. Louis, MO), and non-fat dried milk (DM) (Bio-Rad, Hercules, CA) were used as control antigens. Recombinant gp41 (MN strain) and gp120 (MN strain) envelope proteins were used as HIV-1 specific (ImmunoDiagnostics, Woburn, MA). Alkaline phosphatase (AP)-conjugated rabbit-anti-human IgG (Fc specific), (Pierce,
Rockford, IL), and the substrate, p-nitrophenyl phosphate (p-NPP) (Sigma-Aldrich, St.Louis, MO) were used to detect bound IgG.

2.2.3 Determination of serum IgG content

To IgG content was determined for each serum sample, using immobilized protein G (which binds to the Fc portion of IgG); this was used to capture IgG from the serum. One ug protein G was diluted in 35ul Tris-buffered saline (TBS) and used to coat microtiter wells. By overnight incubation at 4°C, wells were blocked with 200 ul TBS containing 2% BSA (w/v) (TBS/BSA) at 37 °C for 1 hour, then washed three times with TBS containing 0.01% (v/v) Tween-20 (TBST). Starting at a 1:300 dilution, 1:3 serial dilutions of serum samples were prepared in Ab diluent (TBST containing 1% (w/v) BSA). Second, 1:3 dilutions of 500 nM human IgG was used as standard with 35ul of each titration. The plates were incubated at room temperature for 2 hours. After six times wash with TBST, HRP conjugated proteinA/G, diluted 1 in 500 in Ab diluent was added with 35ul/well to the wells as secondary Ab. The plates were then incubated at room temperature for 45 minutes. All incubations were performed on a rocker.

After six washes with TBST, plates were developed with 0.03% (v / v) H₂O₂ and 400 ug/ml of ABTS in citrate/phosphate buffer. ELISA plate readings were done with a VersaMax Tunable Microplate Reader (Molecular Devices, Sunnyvale, CA). The optical densities (O.D.) at 405 nm and 490 nm were measured and reported as (OD₄₀₅₋₄₉₀) X 1000. Readings were typically taken every 10 minutes up to 50 minutes, and unless otherwise indicated, are presented at 30 minutes.
Calculation of serum IgG content was done by comparing the OD$_{405-490}$ of serum samples to a standard curve obtained from serial dilutions of human IgG with known concentrations. Three dilutions were used to obtain an average IgG content. Each serum sample was adjusted with TBST containing 1% (w/v) BSA to a final IgG concentration of 200 nM and used for all subsequent ELISA.

### 2.2.4 Direct enzyme-linked immunosorbent assay (ELISA) for epitope mapping

Microtiter wells were coated with 400 ng synthetic peptide in TBS, then incubated at 37°C for 6 hours to dry. Other antigens, 50 ng envelope proteins (gp120 and gp41), protein G (used as a quality control since its signal presents the amount of serum IgG), 2% OVA, 2% DM and 2% BSA in TBS, were then coated. Following the incubation at 4°C overnight, the plates were blocked with TBS/BSA at 37°C for 1 hour, then washed 3 times with TBST. Serum diluted to an IgG concentration of 200 nM was added, and rocked at room temperature for 2 hours; plates were then washed 6 times with TBST, and AP-conjugated rabbit anti-human IgG (Fc) (1:1000 dilution in Ab diluent) was added, and incubated for 1 hour at RT. Wells were developed by p-NPP, and was read in microplate reader at A$_{405}$nm.

### 2.2.5 Phage-displayed peptides ELISA

Phage ELISA were performed as described in Menendez et al. 2004. Briefly, wells were coated with phage clones ($10^{10}$ phage particles in TBS) overnight at 4°C, followed by blocking with TBS/BSA for one hour at 37°C. After washing 3 times with TBST, sera diluted to an IgG concentration of 200 nM were then added to the wells, and incubated for 2 hours at RT. After washing 3 times, HRP-conjugated goat-anti-human-
IgG was added (1:1000 dilution), and incubated for 1 hour at RT. The plates were developed with 0.03% (v/v) H₂O₂ and 400 µg/ml of ABTS in citrate/phosphate buffer. The data was reported at OD₄₀₅₋₄₉₀ as in 2.2.3.

2.3 Results

2.3.1 HIV-1 positive study population

Ab responses were studied over time among four groups of HIV-1 infected patients, namely the rapid progressor (RP), normal pregressor (NP), slow progressor (SP) and seroconverter (SC) groups. No patients from these groups have been treated with anti-retroviral therapy. The SP, NP, and RP groups have unknown seroconversion date, and were taken between 1984 to 1999. The RP samples span between one to five years, with three to five samples taken from each of six individuals. The NP samples span between nine years, with four to seven samples taken from each of five individuals. The last sample from both RP and NP groups was drawn shortly before patient death. The SP samples span fifteen years, with eight samples taken from each of four individuals. Patients from the SP group never developed symptoms of AIDS by the time the last samples were taken. Contrast to the SP, NP and RP groups, the serum samples in SC group were taken between 1998 to 2003 in a different cohort. Most samples in SC group were drawn within a year after seroconversion (six to eight samples for each individual) except two SC patients (SC1 and SC2, Figure 2-2), which have more samples taken up to four years (ten and eleven samples in these two individuals, separately).

A set of 13 serum samples, taken after two to five years’ infection with known broadly neutralizing reactivity was also screened. Note that there is only one sample
available within each individual; thus, the Ab response over time within this group could not be studied. These 13 samples are named broadly neutralizing group (bNt). Serum #1~12 were tested to be bNt as described in Richman et al. 2003. Serum #13 (FDA-2) was also tested to be a bNt serum (Vujcic et al. 1995). The purpose of using these sera was to investigate the strength and breadth of Ab response in these samples, and to identify the difference between sera from bNt group and other groups.

2.3.2 General Ab response in natural HIV-1 infection – characterized by gp160 linear peptides in serum samples

2.3.2.1 Gp160 linear peptides

To investigate the breadth and stability of the overall Ab response against the HIV-1 envelope proteins, twenty nine 15-mer linear peptides representing distinct regions on gp120 and gp41 were chosen for study from the gp160 (MN strain). The sequences of these peptides are listed in Table 2-1. Some of the peptides were chosen by our previous screening of a panel of HIV+ IgGs to assess the breadth of reactivity (data not shown). These peptides have been shown to be immunodominant or have moderate/low reactivity. Other peptides were chosen since they had been reported to be immunogenic in HIV-1 natural infection or immunization studies (Klasse et al. 1988; Goudsmit et al. 1990; Neurath et al. 1990; Horal et al. 1991; Xu et al. 1991; Brolden et al. 1992; Ugen et al. 1992; Vanini et al. 1993; Calarota et al. 1996; Cleveland et al. 2003; Chanh et al. 1986; Loomis-Price et al. 1998). These linear peptides reflect specific Ab responses against linear (continuous) epitopes on envelope proteins. The non-specific control antigens, 2% non-fat dried milk (DM), 2% OVA were also used in the study, represented non-specific Ab response in HIV-1 natural infection.
Table 2-1: Table of HIV-1 MN Env peptides obtained from the NIH AIDS Research & Reference Reagent Program.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>HIV-1 MN Env 15-mer peptide #</th>
<th>Corresponding region on gp160</th>
<th>Peptide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>97-111</td>
<td>6233</td>
<td>C1</td>
<td>NNMVEQMHDIEISSLW</td>
</tr>
<tr>
<td>301-315</td>
<td>6284</td>
<td>V3</td>
<td>CTPNYNKRKRIHIG</td>
</tr>
<tr>
<td>305-319</td>
<td>6285</td>
<td>V3</td>
<td>NYKKKRIHIGPRA</td>
</tr>
<tr>
<td>357-371</td>
<td>6298</td>
<td>C3</td>
<td>FKNKITVFNQSSGGD</td>
</tr>
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<td>6313</td>
<td>C4</td>
<td>QCKIKQINMWQEVG</td>
</tr>
<tr>
<td>501-520</td>
<td>2015*</td>
<td>C5</td>
<td>TKAKRRVVQREKRAAIGALF</td>
</tr>
<tr>
<td>533-547</td>
<td>6342</td>
<td>Polar region</td>
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<td>NHR</td>
<td>EAQQHMLQLTVWGIK</td>
</tr>
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<td>LTVWGIKQLARVLA</td>
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<td>6352</td>
<td>NHR</td>
<td>GIKQLQARVL AVERY</td>
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<tr>
<td>577-591</td>
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<td>NHR</td>
<td>LQARVL AVERYLKDQ</td>
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<td>593-607</td>
<td>6357</td>
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<td>597-611</td>
<td>6358</td>
<td>Cluster 1</td>
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<tr>
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<td>6373</td>
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<td>MPER</td>
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<td>MPER</td>
<td>DKWASLWNWF DIT NW</td>
</tr>
<tr>
<td>669-683</td>
<td>6376</td>
<td>MPER (4E10)</td>
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<td>673-687</td>
<td>6377</td>
<td>MPER/TM</td>
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<tr>
<td>725-739</td>
<td>6390</td>
<td>CT</td>
<td>PRGPD RPEGIEE EGG</td>
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<td>729-743</td>
<td>6391</td>
<td>CT</td>
<td>DRPEGIEE GDR</td>
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<td>6392</td>
<td>CT</td>
<td>GIEEGGER DRT SG</td>
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<td>6401</td>
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<td>YHRDL LL IARIVE</td>
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<td>837-851</td>
<td>6418</td>
<td>CT</td>
<td>GRArifTRQGL</td>
</tr>
</tbody>
</table>

*All the peptides are 15-mer except C5 (20-mer) taken from another set.
Figure 2-1: Summary of peptides used in the serum screening.
Each red line represents one peptide. Different regions the peptides covered on gp120 and gp41 were shown in blue boxes. See Table 2-1 for the sequences of the peptides.
Figure 2-2: Summary of antibody response against envelope proteins in serum samples.

(1). The strength of serum Ab response in ELISA experiment is shown by different numbers with colours. "0" (white) means O.D.<0.1; "1" (grey) means 0.1<O.D.<0.5; "2" (green) means 0.5<O.D.<1; "3" (yellow) means 1<O.D.<1.5; "4" (orange) means 1.5<O.D.<2; "5" (brown) means 2<O.D.<2.5; "6" (red) means O.D. above 2.5 (2). The numbers in a column (#1, #2...etc) in SC, SP, NP, RP represents samples taken at different time points from a same patient. In bNt sera group, #1~13, represent 13 bNt serum samples taken from 13 different individuals.

2.3.2.2 The antibody response against gp120

The early timepoints within SC group represent acute HIV-1 infection; over time, Ab cross-reactivity with gp120 (MN strain) appeared at least 6 weeks after seroconversion and peaked at the end of the first year (Figure 2-2). The trend of increasing reactivity was obvious for every individual. Note that cross-reactivity is observed for all these studies since the TCLA MN strain sequence (may be different from primary viral sequence) was tested; this was thus testing “heterologous” reactivity. For the peptide response, Ab against one V3 region (6285) peptide appeared at the end of the first year and showed increasing trend afterwards. Note that the last three serum samples from number 1 and 2 individuals were taken at the 2nd, 3rd and 4th year after seroconversion. There was also some response against C4 region (6313 peptide); however this was low and not consistent over time. There was no reactivity against any other peptide on gp120, indicating a restricted Ab response against gp120 during early HIV-1 infection.

The RP, NP and SP groups were screened to represent the Ab response over the course of HIV-1 infection after diagnosis. Overall, the Ab response against both the gp120 protein and the peptides (if any) was stable over time regardless of clinical outcome. One exception was individual number 5 in the NP group, who had a strong Ab
response at the beginning, but which disappeared over time. Ab response against the V3 region (6284 and 6285 peptides) was the strongest and most cross-reactive (i.e., most individuals developed a V3-specific Ab response) in all three groups. The RP, but not the NP or SP groups, showed reactivity against the C1 (6233 peptide), C4 (6313 peptide) and C5 (2015 peptide) regions. Individual #1 in the RP group and #2 in the NP group developed a strong Ab response against gp120 protein, but showed no Ab response to any of the peptides. As discussed below, peptide mapping represents only a small fraction of the entire Ab response, as it detects only Abs that cross-react with linear peptide sequences. In comparison, the bNt serum group produced an overall strong Ab response against gp120 (except bNt serum #7, who did not show any reactivity against gp120 or its peptides). The Ab response against V3 region (6284 and 6285 peptides) was also the strongest among all the peptides. Note that only one sample available from each individual in this group.

Compared to the SC group, other groups showed a more stable Ab response. This is mainly indicated by reactivities against whole gp120 and the V3 region (6284 and 6285 peptides). In all five groups, the Ab response against V3 region was immunodominant for gp120, consistent with many previous reports (see 2.4, discussion). While the stability of the Ab response seemed to be independent of clinical course after initial seroconversion, the breadth of the Ab response varied among RP, NP, SP and bNt groups. The RP group had the broadest Ab response, which is shown by a higher frequency of reactivity against the C1 (6233 peptide), C4 (6313 peptide) and C5 (2015 peptide) regions, but not the SP or NP groups. Interestingly, the breadth of the Ab response in bNt serum group did not seem to be greater than other groups, though there
was only one sample available from each individual. The breadth of peptide reactivity in bNt sera is more comparable to NP and SP groups than the RP group, indicating the breadth of neutralization activity in the sera does not correlate with the breadth of Ab response or more specifically, Ab response that cross-reacts with overlapping peptides studied here.

2.3.2.3 Antibody response against gp41

Reactivity against gp41 (MN strain) in all serum samples in the SC group appeared earlier than that against gp120. While gp41 reactivity in the SC group was strong throughout time, it appeared weaker in the NP and SP groups (especially SP). In all five groups, the Ab response against the cluster I region (6357 and 6358 peptides) was immunodominant; however in the SC group, this reactivity appeared to increase over time. In addition, reactivity against the region N-terminal to the NHR (6342 peptide), and within the NHR (6349 peptide) was very crossreactive, with this reactivity being the weakest in the SC group, and strongest in the RP group. Interestingly, there was also a difference in the extent of cross-reactivity against some MPER peptides (6373-6376) and cytoplasmic regions (6401 and 6402 peptides), with the RP group showing the strongest and broadest reactivity. Thus, compared to other groups, the RP group showed stronger and broader Ab responses against peptides in the gp41 region.

As mentioned in the introduction, the MPER is of particular interest because this region includes the epitopes for three bNt MAbs, 2F5, 4E10 and Z13. It is also the most conserved region on gp41; therefore, if other NtAbs are discovered against this region, it is possible they will neutralize a broad range of HIV-1 isolates. For these reasons, we affinity purified specific Abs using the 2F5 and 4E10 epitope peptides (6373 and 6376,
respectively). The purpose was to see if the purified Abs that bind to 2F5 and 4E10 linear epitopes will have 2F5 or 4E10-like neutralization activity or, will neutralize HIV-1 at all (see Chapter 3).

2.3.2.4 Non-specific reactivity against OVA, DM and BSA

The reactivity against dried milk (DM) and ovalbumin (OVA) in this study indicates the presence of non-specific or polyspecific Abs in serum samples. In acute infection, represented by SC group, a relatively clean response was shown by minimal non-specific reactivity with these antigens. Over the longer-term, the SP, NP and RP groups showed stronger non-specific reactivity, with the RP group showing the strongest. The bNt serum group also showed minimal non-specific response, similar to the SCs. Given the time period over which sera were collected for the bNt serum group (after 2~3 years of infection), its serum samples are most comparable to the NP group, yet the DM and OVA reactivity of the bNt serum group was most comparable to the SC group. This is consistent with the results for the gp120 and gp41 reactivity profiles, with the SC and bNt serum groups having the most restricted Ab response, and the RP group the broadest and strongest ones.

2.3.3 Serum reactivity with peptides specific for broadly neutralizing monoclonal antibodies

Our group has selected and optimized phage-displayed peptides that specifically bind to bNt MAbs b12, 2G12, 2F5 and 4E10 (Zwick et al. 2001a; Menendez et al. 2004; Menendez 2005; Bahr 2004). These phage-displayed peptides bind to the bNt MAbs at affinities similar to their cognate epitopes on the envelope proteins. In this thesis, these phage-displayed peptides were used to determine if those having similar reactivities to
the bNt MAbs were present in our panel of sera. These bNt MAb-specific peptides included one specific for the b12 MAb (B2.1); one specific for the 2G12 MAb (D10, ACPPSHYLDMKSGTCR); five for 2F5 MAb (AL4.22, ELDKWSFWPGDDSTLLT; AA 4.22, AADKWSFWPGDDSTLLT; EL1.1, ELDKWSHLSLTEFSPSHQ; EL10.5, ELDKWSDWPNIAQYTTTL; and AA10.5, AADKWSDWPNIAQYTTTL), and two for the 4E10 MAb (E6.8, RCRTIDVFRNCI; and 10A.3, AEPAETSWFYLTFTFL).

In addition, as positive controls, three phage clones were used, with each displaying a peptide that commonly react with a significant fraction of sera from HIV-1 infected people. Briefly, the IgG from two sera, AD18 and AD28, were used to screen phage library, clones with high binding were chosen to screen sera. Three clones were selected and sequenced due to their common reactivity. These phage clones are, A4.3 (AQKQIKCGDACNFYF), A5.4 (KCMWTSPFQTCV) and A7.4 (TCSGKILCSLPIRSWEQ).

A serum panel, comprising sera from the SC, RP, NP and SP patient groups, was screened to determine whether they contain Abs that recognize peptides specific for the bNt MAbs. Phage displaying-peptides that were known to react with HIV positive sera, were used as a positive control. While the entire serum panel was screened, the results in Figure 2-3 are a summary, and represent the highest ELISA signal produced from the serum samples of a given individual; thus, they are biased toward depicting the strongest reactivities in the serum panel. In all groups, the Ab response against bNt MAb-specific peptides was minimal (green and yellow colors) compared to the positive controls, which included gp120, gp41 and the A4.3, A5.4 and A7.4 phage; reactivity with control phage bearing no recombinant peptide was also minimal (data not shown). While some serum
samples showed reactivity with clones AA4.22 and 10A.3, it was not gp41 specific since the ELISA signal for these phage were unchanged in the presence of soluble gp41 (data not shown). In contrast, there was significant reactivity with phage clones A4.3, A5.4, and especially A7.4. Previous experiments had shown that serum binding to these clones is blocked by gp120 (A4.3 and A5.4) and gp41 (A7.4). Thus, while the sera reacted well with phage displaying peptide markers for reactivities commonly produced in HIV-1 positive sera, no envelope-specific reactivity was observed for any of the phage bearing peptides specific for the bNt MAbs. This is especially striking for the peptides specific for MPER-binding Nt MAbs, 2F5 and 4E10, since reactivity with these epitope peptides (6373 and 6376, respectively) was present in a number of sera (see Figure 2-2).
### Figure 2-3: Summary of serum screening with bNt MAb peptide markers.

X-axis represents bNt MAb peptide markers and controls (see the text for details). Y-axis represents serum samples from SC, RP, NP and SP groups. As a summary of each box represents the strongest reactivity in multiple serum samples taken from one person. ** NC: no competition, peptides do not compete against gp120 or gp41 for binding to serum Abs.

### 2.4 Discussion

#### 2.4.1 The stability and breadth of peptide-specific antibody response

Figure 2-3 is the summary of the antibody strength over time in different groups.

In our mapping studies, we showed that, in comparison to the other patient groups, the
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Figure 2-4: Summary of antibody response in serum screening with peptides.

The strength of antibody response from low to high is represented by "-", "+/-", "+", "++", and "+++". c had the broadest and strongest Ab responses. The NP group had a broader reponse compared to the SP, though this trend was not significant. This is interesting considering a previous study showed that SPs are more likely to have broader NtAbs against primary isolates (Montefiori et al. 1996). In contrast, the response against the peptides studied increased and broadened over time in the SC group. We also found that the reactivity patterns of the 13 bNt sera were not broader than those of the other groups. Our results
indicate that after ~2 years of infection, viral load is positively associated with the breadth and strength of the Ab response. While broadening of the overall Ab response is more associated with viral load, broad neutralization is associated with more restricted (i.e., oligo- or monoclonal) Abs. Further study with a larger population should help to confirm these trends.

The Ab response was investigated over time in different HIV-1-infected populations using linear peptides derived from the gp160 sequence (epitope mapping). Although polyclonal sera were used in this study, our results are discussed in light of current knowledge of the conserved regions on the envelope proteins that are involved in Ab-mediated neutralization, as defined by Nt MAbs.

Overall, vigorous Ab responses against both gp120 and gp41 proteins were produced in most individuals. Some of the regions we analyzed are also recognized by MAbs reported to have Nt activity, or bNt activity; strong reactivity was observed against many of these regions (i.e., V3, cluster I and the NHR). Due to the strength of the Ab response against these sites, and the presumed lack of Nt activity in most of the sera, it is unlikely that most of the reactivity of these sites is Nt. We show here that, the MPER was an exception, as it was weakly immunogenic through all stages of HIV-1 infection. MPER reactivity was transient, and not associated with clinical outcome. As this region is recognized by three different bNt MAbs, it may be worthwhile to investigate whether the serum Abs that bind to this region are Nt, since the titer was probably too low for their sera to be Nt. Discussion of all these regions follows.

The V3 region (6284 and 6285 peptides) was shown to be the immunodominant region on gp120, and this is consistent with most of the early HIV-1 serology studies
which have shown that more than 90% of HIV-1 infected individuals produce Abs against the V3 region (Devash et al. 1990; Zwart et al. 1991; Zwart et al. 1992). Initial studies on HIV-1 suggested V3 as a main target for vaccine design because of its immunodominance and production early after infection or brief immunizations (Javaherian et al. 1989; Putney et al. 1986; Goudsmit et al. 1988; Javaherian et al. 1990; Mascola et al. 1996). However, as it is highly variable, many V3-specific Abs are isolate-specific and most V3-specific MAbs produced by infected individuals and selected using linear V3 peptides have little Nt activity for primary isolates despite broad immunochemical cross-reactivity (Hioe et al. 1997; Gorny et al. 1997; Gorny et al. 1993). Notably, broadly reactive V3 Abs with potent Nt activity do exist. MAb 447-52D has been found to be the most broadly Nt V3-specific MAb discovered so far (Gorny et al. 2002). This MAb interacts with the 14 residues at the crown of the V3 loop; its “core epitope” is defined by the sequence GPxR, a motif that is highly conserved among clade B viruses and which exists in a minority of other HIV subtypes (Gorny et al. 1992). The presence and recognition of the arginine (R) residue in the core epitope is required for MAb 447-52D to exercise its activity (Zolla-Pazner et al. 2004). This cross-reactivity reveals the presence of features within the V3 loop that are conserved despite the sequence variation in this region. Nevertheless, most reactivity against the V3 region, while strong, is not associated with bNtAbs.

Like the V3 region on gp120, cluster I (6357 and 6358 peptides) was shown to be the immunodominant region in gp41. This finding is consistent with most of the previous studies to date (Horal et al. 1991; Chiodi et al. 1987; Gnann et al. 1987; Norrby et al. 1987; Smith et al. 1987; Wang et al. 1986). Cluster I induces high levels of Ab, most of
which are not Nt. MAb Clone 3 alone has Nt activity specific for this region. It binds to a linear epitope between aa 597 and 606 at the C-terminal end of cluster I (Cotropia et al. 1996). This epitope is quite conserved, a fact reflected by the Clone 3’s ability to neutralize three diverse lab adapted T-cell line viruses from clade B and three primary isolates from group O (Cotropia et al. 1996; Ferrantelli et al. 2004). Beyond the activity of Clone 3 against these viruses, few details are known about its functional breadth. Thus again, the cluster I epitope, while able to elicit strong reactivity, typically does not elicit bNtAbs.

Other than immunodominant regions, our epitope mapping study revealed several other regions of interest, all of which are on gp41. The first regions are the NHR (6349 peptide) and an adjacent region N-terminal to NHR (6342 peptide). In our mapping study, most sera in all five groups showed medium reactivity against the 6342 peptide and half of the serum samples in the long-term infected samples (SP, NP and RP) showed reactivity against the 6349 peptide. This is the first report of the prevalence of Abs against these regions in natural infection. As mentioned in the introduction (1.3.2), gp41 undergoes major conformational changes during membrane fusion process. Therefore, intermediate states before six-helix-bundle formation may serve as targets for Nt Abs. Furthermore, Moreno et al. (2004) showed that the inner coil of the six-helix bundle, the NHR, may play a role in virus-host membrane fusion, as peptides derived from the NHR destabilize liposomes. Recently, a Nt MAb that targets the NHR was discovered (Miller et al. 2005). MAb D5 was derived from a normal human B-cell-derived scFv-expressing phage-display library by sequential selection with two synthetic mimics of the gp41 prehairpin intermediate. Its epitope overlaps a hydrophobic pocket on the NHR.
However, MAb D5 is not very potent (high concentrations are needed to neutralize the HIV-1), and has not been shown to have bNt activity. Thus the NHR, while a good target for therapeutic drug design, may not serve as a good target for vaccine design, given the strong response typically produced against this region (which is not Nt).

Another region included in our mapping is the long C-terminal cytoplasmic domain of gp41. Conventionally, this domain is considered entirely hidden within the virion. Recently it was suggested that ~40 residues within this region are looped out on the external surface of the virion. The evidence supporting this is that some Abs specific for this site neutralize HIV-1 infection (Buratti et al. 1998; Dalgleish et al. 1988; Cleveland et al. 2000). For example, the so-called “Kennedy” epitope (PRGPDRPEGIEEGGERDPRS) is located in this external tail, which is a hydrophilic region first identified 20 years ago (Kennedy et al. 1986). It was suggested that there are three antigenic regions within this epitope, centering on PDRPEG, IEEE and ERDRD (Cleveland et al. 2003). Since Ab (IgG) theoretically cannot pass through viral membrane, it is believed that the epitope must be available on the outside of the virion (Cleveland et al. 2003). However, in our mapping study, using three peptides spanning this region (6390, 6391 and 6392), we did not detect significant reactivity in most of the sera (Figure 2-2). The function of the cytoplasmic domain in the life cycle of the virus, and its possibility as a target in disease prevention or intervention remain to be determined.

Finally, we detected cross-reactivity against several peptides within the MPER of gp41 (peptides 6373-6376). This reactivity is weak and transient through all stages of infection; strong MPER reactivity was presented in only one bNt sera (#13). The MPER
has been reported to be immunogenic in several previous studies. Calarota et al. (1996) proposed that the C-terminus of the MPER is an immunodominant site on gp41. They screened 63 sera from HIV-1 infected patients using sequential overlapping peptides covering aa 647-684 (which includes the MPER, see Figure 1-3) and identified the C-terminus of the MPER as immunodominant, with residues WNWFDI (which overlaps with the epitope for bNt MAb 4E10) being most critical for Ab recognition. However, most other studies have shown that the MPER is not immunodominant (Horal et al. 1991; Srisurapanon et al. 2005; Geffin et al. 1998; Muhlbacher et al. 1999; Broliden et al. 1992; Braibant et al. 2006), especially when compared to cluster I. Although the frequencies of MPER Abs vary in different studies, our study agrees with most others, in showing that the MPER is weakly immunogenic, even within the first year after seroconversion. This is encouraging since it indicates that MPER reactivity can be produced early after infection.

Studies have been done to correlate Ab response against MPER linear peptides with serum neutralization activity or clinical outcomes. Most of these have focused on the 2F5 epitope (ELDKWA). In one study, a negative association was found between Ab reactivity to the ELDKWA epitope, and disease progression and viral load (Geffin et al. 1998). More recently, it was reported that Ab titers to the 2F5 epitope in sera from AIDS patients were significantly lower than those in sera from asymptomatic subjects which were collected in the same year (Srisurapanon et al. 2005). In our study, serum neutralization activity was not tested; therefore, we could not compare the peptide reactivity to neutralization.
2.4.2 Limitations of our epitope mapping study

Epitope mapping with linear peptides has been used early on in HIV-1 research as well as in our study to discover B cell epitopes on envelope proteins. However, there are inherent limitations of peptide-based assays (Moore et al. 1994). Most relevant is that linear peptide mapping can only reveal so called “linear” or “continuous” epitopes, yet most anti-protein Abs are against discontinuous epitopes. In addition, solid phase peptide (on ELISA plate) does not necessarily represent the conformation of the same region on the cognate protein. This is why in many cases, mapping by peptides derived from envelope proteins does not correlate with protective neutralizing Ab response. A well-studied example is V3 peptide: it was shown that the solid-phase peptide-binding assay could fail to or falsely predict reactivity of a MAb with a gp120 protein (Moore et al. 1994). In general, peptides in solution are considered more flexible and can adopt more conformations; however, peptides in solution have the drawbacks that they typically are of lower affinity than their immobilized counterparts, to some degree because of their close packing in ELISA plate wells, which allows bivalent Ab binding. Similar to our peptide mapping, a multiplexed Ab-binding and mapping assay was developed to characterize the specificity, breadth, and magnitude of the Ab response to gp41 (Opalka et al. 2004). This method is to immobilize the biotinylated peptides onto streptavidin (SA)-coated microspheres with different fluorescent colors. High-titer Ab responses were detected to the 2F5-4E10 regions by this assay using sera from HIV-1 positive individuals, but there was no direct correlation between Ab binding to these peptides and virus Nt activity.
Another limitation of our mapping study is the serum cohorts used in our study. The serum samples used from SC and most of bNt sera groups were taken from a separate cohort and in different decades compared to SP, NP and RP groups. Plus, the seroconversion times of the latter groups were not available. Although this will not impact the comparisons we did among SP, NP and RP groups, this inconsistency should be considered in the comparisons to SC and bNt groups.
CHAPTER 3: AFFINITY PURIFICATION OF PEPTIDE-SPECIFIC ANTIBODIES

3.1 Introduction

In Chapter 2, we investigated the breadth and strength of the Ab response during HIV-1 natural infection against sites where Nt MAbs bind (V3, cluster I, NHR and MPER). Strong Ab responses against V3, cluster I and the NHR were found in all or most sera, whereas weak, transient reactivity was found against the MPER through all stages of infection, as well as in some of the bNt sera. Many studies have been done to characterized Abs against some of these regions in natural infection, particularly against V3 and cluster I; most of these are not Nt or are strain specific, and have no or little Nt activity for primary isolates (Hioe et al. 1997; Gorny et al. 1997; Gorny et al. 1993). Immunization studies using the V3 region have also failed to elicit bNtAbs (Goudsmit et al. 1988; Mascola et al. 1996). Our data suggest that some of these regions (V3, cluster I and NHR) are immunodominant or strongly immunogenic. Based on the possibility that most sera are not bNt, it is not likely that the Ab response against V3 is bNt. In contrast, the MPER is weakly immunogenic, highly conserved, and is bound by three bNt MAbs (2F5, 4E10 and Z13). It may be worthwhile to investigate whether the serum Abs that bind to this region are Nt, since the low titer of these Abs in most sample is consistent with their lack of Nt activity; moreover, two of the 13 bNt sera (#6 and #13, described in Chapter 2) bound this region strongly.
The question addressed in this chapter is whether anti-MPER Abs from HIV-1 infected people, especially those Abs binding to 2F5 and 4E10 epitopes are Nt. In addition, we were particularly interested in the Abs produced early in the infection (i.e., by the SC group). The reason is that bNtAbs produced early might be easier to elicit in vivo by immunogens/vaccines, which typically expose the body to immunogens for a short period.

A common method to study NtAbs is to test the serum samples by an in vitro neutralization assay. Neutralizing antibody assays provide a measure of whether an antibody or serum sample blocks HIV entry into human T-cells. This is done by incubating virus with antibody for a set time, adding cells, incubating the mixture to allow for several rounds of viral replication and then measuring the amount of virus present, usually as a function of viral antigen expression. The traditional assay does this in peripheral blood mononuclear cells (PBMC) by collecting the cell culture supernatant and testing for p24 (viral core protein) secreted from the infected PBMCs. Other assays have also been developed. One of them is a straightforward variation of the traditional PBMC assay, using antiretroviral agents to stop viral replication after a single round. These cells are then enumerated by staining intracellular p24 and analyzed by using flow cytometry (Mascola, et al. 2002). Two other approaches both use “reporter” genes that luminesce when expressed, allowing quantification by a luminometer. One assay (Richman, et al. 2003), builds luciferase (a firefly enzyme) into an env-deleted HIV DNA construct. Target cells are co-transfected with this and an env-containing DNA construct, producing infectious virus particles that cannot produce new, functioning virus. Instead, replication stops after a single cycle, and infected cells, which luminesce, are counted.
The other assay is similar, in which the luciferase gene is incorporated into a target cell line (Wei, et al. 2003).

In all the in vitro neutralization assays, the possibility cannot be excluded that NtAbs at very low titer are not detectable in whole serum sample. In the presented study, we developed an affinity purification method to isolate peptide-specific Abs from the sera. Two different approaches to peptide-specific Ab purification were explored: one based on peptide conjugated on agarose support, and another based on biotin labelled peptide bound to streptavidin (SA)-coated magnetic beads. The goals in these methods were to isolate Abs with high yield, preserve peptide-binding activity, and produce low background in HIV-1 neutralization assay in vitro. Although the results from the neutralization assays for affinity-purified Abs were not conclusive due to very small amounts of purified Ab, the goals for this method were successfully accomplished.

3.2 Materials and methods

3.2.1 Reagents

Two different methods were explored in developing an Ab purification strategy. The SulfoLink® Coupling Gel Kit, which includes the reagents needed in the Trial 1 protocol (see below) was purchased from Pierce (Rockford, IL). The Dynabeads MyOne used in Trial 2 were from Dynal, Lake Success, NY. The magnetic apparatus (MagnaRack) was from Invitrogen (Carlsbad, CA). For both methods, biotinylated 2F5 and 4E10 peptides containing cysteine residues were purchased from NeoMPS (San Diego, CA), with more than 95% purity analyzed by HPLC, and mass spectrometry. 2F5
peptide sequence is NH$_3^+$-EQELLELDKHWASLWSGK(Biotin)GC-CNH$_2$, and 4E10 peptide sequence is NH$_3^+$-SLWNWFDTNWLWYISGK(boitin)GC-CNH$_2$.

### 3.2.2 Antibody affinity purification - Trial 1

Affinity purification of peptide-specific Abs in the serum samples was conducted using the SulfoLink Coupling Gel Kit. The kit enables covalent immobilization of sulfhydryl-containing peptides (i.e., containing a cysteine residue) to an agarose gel support in affinity purification procedures. A 5 ml column was loaded with 2 ml gel slurry. Sulfhydryl-containing peptide (2 mg) was dissolved in coupling buffer and added to the column. After incubating for 20 minutes (end-over-end mixing) and 30 minutes (without mixing) at RT, the top and bottom column caps were sequentially removed and the unbound peptide solution was allowed to drain from the column into a clean tube. The column was then placed over a new collection tube and washed with 3 ml coupling buffer. To block the remaining binding sites on the gel, a 1-ml solution of 50 mM L-Cysteine·HCl in coupling buffer was added to the column, and incubated first with mixing for 15 minutes, then without mixing for 30 minutes. The column was then washed with at least 6 ml of wash solution (1 M NaCl), followed by 2 ml of degassed storage buffer (phosphate buffered saline (PBS) containing 0.05% (w/v) sodium azide (NaN$_3$)). The column was then stored at 4°C.

For affinity purification of peptide-specific serum IgG, the peptide-coupled column was first equilibrated to room temperature, then washed with 6 ml PBS. Up to 500 ul of serum sample was applied to the column and allowed to run through the gel bed. (If a serum sample was > 500 ul, this step was repeated). After the serum had penetrated the gel, 200 ul PBS was added on top of the column, and the column was then
incubated at room temperature for 1 hour. The unbound serum (flow through) in the column was then washed out with 12 ml PBS and collected for later analysis. Bound Ab was eluted by flowing 8ml low pH glycine buffer (100 mM glycine, pH 2.5-3.0) through the column, then was collected into sequential 1ml fractions, and neutralized as soon as possible by the addition of 100 µl 1 M Tris, pH 7.5 (or 50 µl 1 M Tris, pH 9). Neutralized eluents were concentrated on an Amicon filter (30kD cutoff) to a volume of ~500ul.

The column was regenerated as soon as possible after chromatography to prevent damage to the immobilized peptide by the low pH elution buffer; 16 ml PBS was added to wash the column. The column was then equilibrated with 8 ml degassed storage buffer (PBS containing 0.05% NaN3) and stored upright at 4°C.

3.2.3 Antibody affinity purification - Trial 2

Biotin-containing peptides bind to SA with extremely high affinity ($10^{-15}$ $k_d$), providing another way to immobilize the peptide for purification. The following protocol is for Ab purification for 200 ul serum. First, 500 ul SA coated magnetic beads were added to a 1.5 ul Eppendorf tube, and centrifuged for 5 min at 5000 rpm. The supernatant was aspirated with the aid of the magnet apparatus to immobilize the bead pellet. Beads were washed once in 500 ul PBS, then centrifuged for 5 min at 5000 rpm, placed in the magnet apparatus, and supernatant was aspirated. 500-ul of PBS solution containing 40 ug bio-peptide was added to beads and incubated on a ferris wheel for 1 hr at room temperature. Samples were centrifuged for 5 min at 5000 rpm, and placed in the magnet apparatus; supernatants were aspirated and kept for ELISA. Subsequently, 100 ul PBS containing 40 nM biotin was added to the beads and incubated for 15 min on the rocker at RT to block remaining free biotin binding sites. Samples were then centrifuged for 5 min
at 5000 rpm, and the supernatant was aspirated. After washing once in 500 ul PBS, samples were centrifuged for 5min at 5000 rpm and supernatant was aspirated. The beads were then resuspended in 500 ul PBS and stored upright at 4°C.

For affinity purification of peptide-specific serum IgG, the SA-coated magnetic beads were first equilibrated to room temperature, then centrifuged for 5 min at 5000 rpm. After the supernatant was aspirated, 200 ul serum was added to the beads, then incubated on the rocker for 1 hour at RT. The beads were then centrifuged for 5 min at 5000 rpm, and the supernatant (unbound serum, or flow through) was collected into a separate tube. The beads were then washed 2 times with PBS. Bound Ab was eluted by adding 500 ul low pH glycine buffer (100 mM glycine, pH 2.6) to the beads, and incubated for 20 min at RT. After centrifuging the beads for 5 min at 5000 rpm, the supernatant (purified Ab) was aspirated into a separate tube. After washing 3 times by PBS, the beads were stored upright at 4°C.

To estimate the amount of Ab that was purified, the purified Ab IgG content was tested using the same method as in 2.2.3. To determine the amount of Ab removed from a serum, the binding of purified Ab and the remaining unbound serum (3.2.2 and 3.2.3) was tested for binding to specific peptide by direct ELISA, as in 3.2.4.

3.2.4 Direct ELISA for characterizing purified IgG binding to specific peptide

Initially, 400 ng peptide were coated in the wells, and dried at 37°C for 6 hours. Next, TBS containing 2%BSA, used as a negative control, was coated. The plates were incubated at 4°C overnight. Next day, after blocking with 2% BSA in TBS followed by three washes, 1:2 serial dilutions of purified Ab IgGs and unbound serum were added to
wells, starting at 1:10 dilution. The plates were incubated for 2 hours at RT, and then washed six times with TBST. HRP conjugated goat-anti-human IgG (diluted 1 in 1000) was added to the plates, and incubated for 45 minutes at RT. After six washes, plates were developed using ABTS system, and read at 30 min, as in 2.2.3.

3.2.5 Neutralization assay

Neutralization assay was performed by a recombinant virus assay as described in Richman et al. 2003. Briefly, virus particles containing envelope proteins plus an HIV genomic vector that contains a firefly luciferase indicator gene. Virus infectivity was determined by measuring the amount of luciferase activity expressed in infected cells compared with an antibody negative control after a single cycle of replication. NtAb titers were calculated as the reciprocal of the plasma dilution conferring 50% inhibition (IC50). HIV-1 MN strain was used in Trial 1 and 2, and an additional HIV-1 92HT594 strain was used in Trial 2. In both trials, amphotropic murine leukemia virus (aLMV), an unrelated retrovirus was used as negative (specificity) control.

3.3 Results

3.3.1 Ab affinity purification – Trial 1

In the epitope mapping study described in Chapter 2, MPER-specific Ab reactivity was detected in both early infection (SC group) and longer-term infection (RP group), as well as in two of the bNt sera. This was interesting because these Abs bind to the same region as bNt MAb 2F5 or 4E10. Do these Abs also neutralize HIV-1? We developed an Ab affinity purification method to answer this question. The initial purifications were performed using SC samples. The reason is that, although these MPER
signals were low, if there was any NtAb against this region in early infection, these Abs should be easier to elicit by vaccination compared to those Abs produced after long-term infection, thus an observation that MPER-specific NtAbs are in the SC sera would have greatest significance for vaccine design.

We chose the following SC samples for affinity purification based on their reactivity against the MPER: SC1.4, SC1.5, SC3.5 (specific for 2F5 epitope), SC2.8 and SC2.9 (specific for 4E10 epitope). The method used is described in section 3.2.2 (SulfoLink Coupling Gel Kit). In Ab purification, the harsh conditions used (low pH elution buffer) may have denatured Abs, which might then lose their binding property. Peptide ELISAs and IgG content ELISAs were used to determine the “specific activity” (i.e. peptide binding per unit IgG) of affinity-purified Ab preparation, and compared to the “specific activity” of the completely bNt MAb (2F5 or 4E10). Thus, samples of each affinity purified Ab, and the corresponding bNt MAb, were titrated on peptide and on protein G. This produced a rough idea of the quality of the affinity-purified Ab, in that low “specific activity” (compared to bNt MAb) indicated either denatured and/or low-affinity Ab.

Figure 3-1 and Figure 3-2 show the peptide binding (left panels) and IgG content (right panels) results from two representative purifications; sample SC1.5 purified on bio-2F5 peptide, and sample SC2.8 purified on bio-4E10 peptide. Both results showed that by using SulfoLink Coupling Gel Kit, most peptide specific Abs were removed from the serum, indicated by the low peptide signal in unbound serum. In addition, the purified Abs maintained the peptide binding reactivity in ELISA, as shown by the titration curves in the left panels. To produce the same O.D. (i.e., O.D.=2), ~2.1 nM purified SC1.5,
compared to ~0.3 nM 2F5 MAb, and ~3.4 nM purified SC2.8, compared to ~0.2 nM 4E10 MAb were required. Thus, larger amount of “2F5/4E10-like” Abs were required to produce the same ELISA signal as compared to 2F5/4E10 MAbs. Moreover, larger amount of “4E10-like” Abs may be required, as compared to “2F5-like Abs”. 
**Figure 3-1:** Serum SC1.5 affinity purification with 2F5 peptide.

Left panel is the purified Ab (red) reactivity against 2F5 peptide compared with 2F5 MAb (green) and unbound serum (black) side by side. Right panel is the IgG content of purified Ab, unbound serum Ab compared to standard IgG.

**Figure 3-2:** Serum SC2.8 affinity purification with 4E10 peptide.

Left panel is the purified Ab (red) reactivity against 4E10 peptide compared with 4E10 MAb (green) and unbound serum (black) side by side. Right panel is the IgG content of purified Ab, unbound serum Ab compared to standard IgG.
3.3.2 Neutralization assay – Trial 1

Following affinity purification study, the purified Abs were sent to Monogram Biosciences company for neutralization assay. The results for purified Ab specific for 2F5-peptide (SC1.4, SC1.5 and SC3.5) and 4E10 peptide (SC2.8 and SC2.9) are shown in Figure 3-3 and Figure 3-4 separately. As a control for the neutralization assay, a high titer guinea pig antiserum, produced against the 2F5 peptide (Merck serum), was used. It has been shown no neutralization activity in this serum (McGaughey et al. 2003).

Significant neutralization of the TCLA strain, MN pseudovirus was observed for all of the affinity purified Abs. However, in all cases, purified Abs showed different extents of neutralization against the negative-control aMLV, indicating that something in the purified Ab preparation was affecting the assay. Although the affinity purified Abs may have Nt activity, it is difficult to draw clear conclusions without eliminating this high background.

In our purification process, a large volume (8 ml) of elution buffer containing 1% BSA was used to elute Abs from the column. Considering the concentration of BSA in purified Abs will go up to 8% after concentration, this may be one possible reason causing high background problem. Besides this, the glycerol, used to store the Abs, and/or contents eluted from the agarose support, could also have affected the assay. This result led us to try another approach for Ab purification.
**Figure 3-3:** Neutralization data for an 18-hour neutralization assay using serum Abs affinity purified on the 2F5 peptide.

The Abs were purified on a 15-mer peptide bearing the 2F5 epitope sequence from gp41 MN sequence. The upper panel represents 2F5 MAb (positive control), Merck Serum (negative control) on this page, and purified Abs (from SC1.4, and SC3.5) neutralize HIV-1 MN strain. The bottom panel represents same Abs neutralize aMLV (negative control virus). X-axis represents serial dilutions of Abs. Y-axis represents percentage of neutralization (percentage of viral infection blocked). Data courtesy of T.Wrin, Monogram Biosciences.
ICS0
64
ng/ml

IC$_{50}$ 64 ng/ml

IC$_{50}$ 102 ng/ml

ICS0
236
ng/ml

IC$_{50}$ 178 ng/ml

ICS0
236
ng/ml

IC$_{50}$ 236 ng/ml

SC1.4

SC3.5

aMLV

Figure 3-3: continued.
Figure 3-4: Neutralization data for an 18-hour neutralization assay using serum Abs affinity purified on 4E10 peptide.

The Abs were purified on a 15-mer peptide bearing the 4E10 epitope sequence from gp41 MN sequence. The upper panel represents 4E10 MAb (positive control), HIV+ plasma (positive control) on this page, and SC2.8, SC 2.9 on the next page neutralize HIV-1 MN strain. The bottom panel represents same Abs neutralize aMLV (negative control virus). X-axis represents serial dilutions of Abs. Y-axis represents percentage of neutralization (percentage of viral infection blocked). Data courtesy of T.Wrin, Monogram Biosciences.
Figure 3-4: continued.
3.3.3 Ab affinity purification and neutralization assay – Trial 2

As shown in Figure 2-2 in Chapter 2, there was strong 2F5-peptide reactivity in bNt serum #6. We thus purified this reactivity from #6 serum by Trial 2 method. As in Trial 1, Merck serum was used as negative control, and affinity purified by the Trial 2 method. To control the background problem observed with the previous SC samples, a high volume wash was performed using a 30-kD molecular-weight cut-off filter after elution of Ab. The molecular weight of IgG is ~150 kD. This may have helped remove small molecules and salt related to acid neutralization. Also, the magnetic beads are less likely to shed other contaminates as compared to the agarose-gel support used in the Trial 1 method.

Figure 3-5 and Figure 3-6 show the peptide binding (left panels) and IgG content (right panels) results of affinity purified Abs from #6 and Merck serum on bio-2F5 peptide. The IgG content for Merck serum is 223 ug/ml, whereas for #6 serum is 6 ug/ml. To produce the same O.D. (i.e., O.D.=0.6 in Figure 3-5, or 0.8 in Figure 3-6), ~8 times more affinity purified Ab than 2F5 MAb was required, and ~5.6 times more purified Ab from Merck serum was required. Figure 3-7 shows the titration curves in neutralization assay for Merck guinea pig serum (M1); purified antibody (M2, before concentrating, in 100mM glycine); purified antibody (M3, after concentrating, no glycine); #6 human serum (#6-1, Nt); #6 purified antibody (#6-2, before concentrating, in 100mM glycine); purified antibody (#6-3, after concentrating, no glycine); unbound #6 serum (#6-4). Purified Ab from Merck serum before removing acid (M2) showed neutralization activity in MN, 92HT594, and on the control virus aMLV; however after washing, there was no neutralization activity, as expected. This shows that small
molecules (either acid, glycine or Tris-HCl) in the purified Abs are causing high background, probably by compromising the cells in neutralization assay. Or, the concentrated #6 Ab after removing acid (#6-3) showed weak Nt activity, whereas the eluted Ab before washing produced high background, which obscured the Abs’ Nt activity (Table 3-1). Thus, the Trial 2 approach, using affinity purification on peptide-loaded magnetic beads, in combination with extensive washing, appears to the best approach to serum Ab purification.
Figure 3-5: Serum #6 affinity purification with 2F5 peptide.
Left panel is the purified Ab (red) reactivity against 2F5 peptide compared with 2F5 MAb (green) and unbound serum (black) side by side. Right panel is the IgG content of purified Ab, unbound serum Ab compared to standard IgG.

Figure 3-6: Merck serum affinity purification with 2F5 peptide.
Left panel is the purified Ab (red) reactivity against 2F5 peptide compared with 2F5 MAb (green) side by side. Right panel is the IgG content of purified Ab, compared to standard IgG.
Figure 3-7: Summary of neutralization assay with bNt serum #6, Merck serum (negative control).

M(1): Merck Guinea pig serum, negative control, 50ul; M(2): purified antibody (before concentrating, in 100mM glycine and 1% FCS), 100ul, 14ug/ml; M(3): purified antibody (after concentrating, no glycine, ~16% FCS), 80ul, 223 ug/ml; #6 (1): #6 human serum, neutralizing, 50ul, 13mg/ml; #6 (2): #6 purified antibody (before concentrating, in 100mM glycine and 1% FCS), 100ul, 9 ug/ml; #6 (3): purified antibody (after concentrating, no glycine, ~16% FCS), 80ul, 6ug/ml; #6 (4): unbound #6 serum, should have no or very less 2F5 peptide specific antibody. The assay was done with pseudoviruses bearing the MN (TCLA), and 92HT594 (primary isolate) envelope proteins. aMLV was the negative control virus, and HIV+ plasma N16 was the positive serum sample. All Ab samples were tested in a 18-hour incubation with the viruses before the addition of cells. P/N represents positive and negative in neutralization assay. Data courtesy of T. Wrin, Monogram Biosciences
Table 3-1: Summary of IC50 in neutralization assay with bNt serum #6, Merck serum (negative control).

<table>
<thead>
<tr>
<th>sample</th>
<th>Unit (1)</th>
<th>92HT594</th>
<th>MN</th>
<th>aMLV</th>
</tr>
</thead>
<tbody>
<tr>
<td>#6-1</td>
<td>Diln/100</td>
<td>132.33</td>
<td>3637.9889</td>
<td>16.9597</td>
</tr>
<tr>
<td>#6-2</td>
<td>(ug/mL)</td>
<td>0.4248</td>
<td>0.3141</td>
<td>0.2596</td>
</tr>
<tr>
<td>#6-3</td>
<td>(ug/mL)</td>
<td>&gt;0.8000</td>
<td>0.3590</td>
<td>&gt;0.8000</td>
</tr>
<tr>
<td>#6-4</td>
<td>Diln/100</td>
<td>63.9920</td>
<td>1032.1274</td>
<td>4.6069</td>
</tr>
<tr>
<td>M1</td>
<td>Diln/100</td>
<td>&lt;12.0000</td>
<td>&lt;12.0000</td>
<td>&lt;12.0000</td>
</tr>
<tr>
<td>M2</td>
<td>(ug/mL)</td>
<td>0.7932</td>
<td>0.5543</td>
<td>0.3943</td>
</tr>
<tr>
<td>M3</td>
<td>(ug/mL)</td>
<td>&gt;24.0000</td>
<td>&gt;24.0000</td>
<td>&gt;24.0000</td>
</tr>
</tbody>
</table>

(1) For purified Abs, ug/ml concentrations were used; for sera, dilutions were used. (2) In order for a test sample to be called "positive" in the assay (the green cells) the IC50 must be at least 3X higher than the same sample when tested with aMLV. Data courtesy of T.Wrin, Monogram Biosciences.

3.4 Discussion

In this chapter, we compared methods for affinity purifying Abs that bind to the 2F5 or 4E10 epitope peptides, and their ability to produce Ab preparations that behave well in a HIV-1 pseudovirus neutralization assay. Two different purification systems were used: SulfoLink Coupling Gel Kit (Trial 1) and MyOne magnetic streptavidin beads (Trial 2). In the neutralization assay, purified Abs from SulfoLink Coupling Gel Kit showed high background (neutralized a control virus aMLV) (Figure 3-3 and Figure 3-4). The possible reason might be the agarose gel contaminated the purified Ab, though we could not prove this speculation. In contrast, using the magnetic beads, we were able to purify Ab with clean background after removing other small molecules (Figure 3-7). Other advantages of the magnetic bead purification system are listed in Table 3-2. The comparison was done by using Streptavidin Beads to affinity purify MAb 2F5, compared with the serum purification data from SulfoLink Agarose Gel.
Table 3-2:  Comparison of Two Affinity Purification Systems, SulfoLink Agarose Gel and SA Beads.

<table>
<thead>
<tr>
<th>Advantage</th>
<th>SA Beads</th>
<th>SulfoLink Agarose Gel</th>
<th>Advantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ease of Protocol</td>
<td>Very easy</td>
<td>Moderate</td>
<td>Streptavidin Beads</td>
</tr>
<tr>
<td>Peptide Binding Efficacy</td>
<td>100%</td>
<td>97%</td>
<td>Streptavidin Beads</td>
</tr>
<tr>
<td>Peptide capacity in column</td>
<td>0.056 mg/ml</td>
<td>1-10 mg/ml</td>
<td>SulfoLink Agarose Gel</td>
</tr>
<tr>
<td>Ab capture capability</td>
<td>100%</td>
<td>70-80%</td>
<td>Streptavidin Beads</td>
</tr>
<tr>
<td>Peptide-Ab Molarity ratio</td>
<td>30</td>
<td>&gt;3600</td>
<td>Streptavidin Beads</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>2.5</td>
<td>could not be optimized</td>
<td>Streptavidin Beads</td>
</tr>
<tr>
<td>Elution Buffer Volume</td>
<td>1x Sample volume</td>
<td>20x Sample volume</td>
<td>Streptavidin Beads</td>
</tr>
</tbody>
</table>

From the comparison, it is obvious that SA beads are much easier to work with and more importantly, Abs purified by this method behave well in viral neutralization assays. One disadvantage of the SA bead system is that for same amount of beads and sulfo-link gel, less peptide can be coupled to the beads, and that less Ab can be purified. The sacrifice of yield should be taken into account in future purification work since oftentimes we could not get enough purified Abs for the neutralization assay. We were encouraged by the fact that in Trial 2, 2F5-peptide specific Ab weakly neutralized a sensitive HIV-1 MN strain. Whether this Ab is indeed Nt needs confirmation. HIV-1 MN is a TCLA strain, and is relatively sensitive to Ab-mediated neutralization. In general, primary isolates are more representative of HIV-1 in vivo albeit more resistant to Ab neutralization. Thus, to purify more MPER Abs (both 2F5 and 4E10 peptide specific) and test them against more HIV-1 viral isolates is one of our future directions.

Several experimental approaches have been used to identify the epitope specificity of serum Abs of HIV-infected individuals (Berkower et al. 1991; Broliden et al. 1992; Kang et al. 1991; Matthews et al. 1986; Steimer et al. 1991; Vancott et al. 1995; Stamatos et al. 1998) (Braibant, et al. 2006). Similar to our approach (Ab
depletion), Stamatos et al. (1998) have successfully removed more than 95% of gp120 protein specific serum Abs by using Sepharose beads coupled with gp120 protein. It was shown that purified Abs neutralized HIV-1 and left over serum neutralization reactivity has mostly been removed (Stamatos et al. 1998). Therefore, although we have met technical difficulties, epitope specific Ab affinity purification is one way to circumvent the problem that NtAbs might not otherwise be detectable at extremely low titers in the serum assay.
4.1 Introduction

As mentioned above, the production of bNtAbs is likely a requirement of an effective HIV-1 vaccine. Among the bNt MAbs identified so far, 2F5 and 4E10 both recognize conserved linear epitopes on the MPER of gp41(aa 660–683). Linear epitopes have advantages over conformational epitopes in that they are easier to design as immunogens. Therefore, intensive studies have been done to elicit Abs having similar bNt properties to 2F5. However, immunization with peptide bearing the 2F5 epitope sequence (ELDKWA) expressed in a number of contexts have failed to elicit Nt Abs (Muster et al. 1994; Liang et al. 1999; Eckhart et al. 1996). Although there are no data available in the literature for 4E10 immunization studies, the possibility of reproducing 4E10-like antibody in vivo by using simple peptide bearing its core epitope (NWFDIT) is small. This has led to the hypothesis that the recognition between 2F5 or 4E10 and their linear epitopes is critically dependent on the environment in which it is present on gp41 in the trimer, and/or that these linear epitopes are not the complete epitopes.

Crystal structures of bNt MAbs 2F5 and 4E10 revealed that both have long H3s, containing hydrophobic patches (see Introduction, 1.8). It has been suggested by Haynes et al. (2005) that such long hydrophobic H3s are generally typical of polyreactive autoantibodies (autoAbs). They tested all of the known bNt MAbs for their ability to bind a number of autoantigens, and it was found that b12 reacts with DNA, and 2F5 and 4E10
bind to cardiolipin (CL), a phospholipid found mainly in mitochondrial membranes. Serum reactivity in clinical, anti-CL assays is associated with anti-phospholipid syndrome (APS), an autoimmune, thrombogenic condition. This group concluded that 2F5 and 4E10 are polyspecific autoAbs, based on their CL reactivity, minimal extension of prothrombin time, and unusual cell-staining patterns. Furthermore, based on the hypothesis that these autoAbs originated from a pool of autoreactive B-cell precursors that are deleted during normal B-cell development, they questioned whether bNtAbs, particularly those specific for the membrane-proximal external region of gp41 (MPER), can be induced by current vaccination approaches (Haynes et al. 2005; Nabel 2005).

Our laboratory has developed specific peptide ligands for MAbs b12, 2G12, 2F5 and 4E10 (Zwick et al. 2001a; Menendez et al. 2004; Alfredo 2005; Sondra 2004). The affinity of the MAbs for these peptides is similar to that for their “cognate” antigens. Given the results of Haynes et al., several questions arose for us: How would binding of 2F5 and 4E10 to CL compare if tested side-by-side with peptides bearing their gp41 epitopes? Are 2F5 and 4E10 polyreactive in general (i.e., with non-autoantigens)? And how does this reactivity compare to that of more classical autoimmune APS MAbs?

In this chapter, the reactivity of bNt MAbs 2F5, and 4E10 were compared to that of an APS MAb, CL15. The results showed that 2F5 and 4E10 prefer their epitope peptides to CL by orders of magnitude, while the binding of CL15 to its antigen CL is not distinguishable from non-specific proteins. We then compared the specificity of these MAbs by using a panel of peptides whose hydrophobicity and charge varied. The results showed that 2F5 and 4E10 specifically bind to their cognate-epitope peptides whereas
CL15 is clearly polyspecific. Taken together, our results support the idea that HIV-1 bNtAbs are not unusually polyreactive or autoreactive.

If 2F5 and 4E10 are autoAbs as suggested by Haynes et al, there should be a repertoire of autoreactive B cells induced by HIV-1 infection and such autoreactivity should theoretically increase over time. Therefore, we further tested a panel of sera (SP, NP, RP, SC and bNt sera, see Chapter 2) for CL reactivity. Our data show no significant autoreactivity or any increase trend following increased CL reactivity in the serum samples. Possible reasons are discussed in this chapter.

4.2 Materials and Methods

4.2.1 Reagents

The NIH AIDS Research and Reference Reagent Program provided 15-mer synthetic peptides derived from HIV-1 envelope protein MN isolate (>80% HPLC purity). The sequences of the peptides used for these studies are listed in Supplement Ib. The 2F5 MN peptide (NH2-EQELLELDKWSLWSGK(BIOTIN)GC-CONH2) was provided at >95% purity by NeoMPS (San Diego, CA). CL15 IgG was kindly provided by Dr. P. Chen (University of California, Los Angeles). HIV-1(MN) recombinant gp41 (MN) and gp120 (MN) envelope proteins were purchased from ImmunoDiagnostics (Woburn, MA). HIV-1 positive human sera were provided through the MACS program (University of Pittsburgh) and the Center for AIDS Research (UCSD). AP-conjugated goat anti-human IgG (Fab specific) were from Pierce (Rockford, IL). Cardiolipin (CL), bovine serum albumin (BSA), ovalbumin (OVA), p-nitrophenyl phosphate (pNPP) disodium salt tablets were from Sigma-Aldrich (St. Louis, MO). Non-fat dried milk
(DM) was from Bio-Rad (Hercules, CA). Tween-20 was from GE Healthcare Bio-
Sciences Corp. (Piscataway, NJ).

4.2.2 Direct ELISA of 2F5, 4E10 and CL15 IgGs

The procedures for coating wells with CL, blocking and washing microtiter plates
were adapted from the optimized procedure used by Haynes et al. (2005) (pers. comm., B.
Haynes, Duke University). The order of coating plates with antigen is described for
complex ELISAs, using synthetic peptides, CL and proteins; for ELISAs using two of
these antigen types, the order of coating was the same as described. Initially, the wells of
96-well microtiter wells (Corning Inc., Corning, NY) received 400 ng MN peptides from
the NIH AIDS RRP (listed in Table 4-1) or the highly-purified 4E10 or 2F5 synthetic
peptides in 50 ul PBS and dried at 55 °C for 4 hours. Only peptides from the NIH AIDS
RRP were used for assays comparing different MN peptides (i.e., for Figure 4-1B).
Next, wells received 2 µg CL in 50 ul methanol, and were dried at 55 °C for 45 minutes.
Finally, wells were coated with proteins (50 ng gp41 in 50 µl PBS, or 50 µl PBS
containing 2% (w/v) BSA, DM, or OVA) by overnight incubation at 4 °C. Next day, all
wells were blocked at 37 °C for two hours with 100 µl blocking solution (0.05 M
carbonate bicarbonate buffer, pH 9.6 (CBC buffer), containing 3% (w/v) BSA). The
plates were washed three times with PBST (PBS, pH 7.4, containing 0.05% Tween-20).
Starting at 600 nM, 1:4 serial dilutions of 4E10, 2F5 and CL15 IgGs were prepared in
antibody diluent (PBST containing 3% (w/v) BSA and 2% (v/v) fetal calf serum (FCS));
50 µl diluted IgG were added to each well and incubated at room temperature for one
hour. The plates were washed four times with PBST, then 50 µl AP- conjugated goat
anti-human IgG (Fab-specific), diluted 1:1,000 in antibody diluent, were added to wells
and incubated for one hour at room temperature. After four washes, wells were
developed using 100 µl pNPP tablet, dissolved at 1 mg/ml in CBC buffer containing 2
mM MgCl₂. After 45 minutes' incubation, optical densities were measured at 405 nm
using a VersaMax Tunable Microplate Reader (Molecular Devices, Sunnyvale, CA).

4.2.3 Neutralization assay

Recombinant HIV-1HxB2 virions, competent for a single round of infection, were
generated using the luciferase reporter plasmid pNL4-3.Luc.R-E-, as described
previously (Connor et al. 1995; Zwick et al. 2003), and the pseudotyped virus was
assayed for neutralization using U87.CD4.CXCR4 cells as target cells (Bjorndal et al.
1997). Different concentrations of the mAbs were added (1:1 by volume) to HIV-1, and
pre-incubated 1 h at 37°C. The mixture of mAb and and HIV-1 was then added (1:1 by
volume) to the target cells, and the assay was developed using luciferase reagent
(Promega) following a 72-h incubation at 37°C. The degree of virus neutralization was
determined as a percentage reduction of viral infectivity against an antibody-free control.
The experiment was performed in triplicate.

4.2.4 Serum ELISAs

Serum ELISAs were performed as described above. Briefly, synthetic peptides,
gp41 and negative control proteins were adsorbed to microtiter wells as described. Next
day, sera, which had been previously inactivated by heating at 56 °C for 30 minutes
followed by the addition of Empigen detergent to 0.01% (v/v), were diluted 1:50 in
antibody diluent. After the blocking and washing steps, 50 ul diluted sera were added to

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each well, and incubated at room temperature for one hour. Plates were then washed, developed and read as described above.

4.3 Results

4.3.1 Comparison of the reactivity profiles of MAbs 2F5, 4E10 and CL15

We compared the reactivity profiles of MAbs 2F5 and 4E10 to that of the APS MAb, CL15 (Chukwuocha et al. 2002). The assay conditions of Haynes et al. were used to assess binding strength and antigen specificity in direct ELISAs (see section 4.2.2).

2F5 and 4E10 were titrated on small amounts of specific antigen and on “blocking” amounts of CL and three non-specific antigens (OVA, DM and BSA) so as to gauge their level of generalized polyreactivity. Figure 4-1A shows that there is a >3-log difference between the concentration of 2F5 IgG producing a given ELISA signal on peptide bearing the 2F5 epitope (or on gp41) and that producing the same signal on CL; likewise, 4E10 produces a >2-log difference. Moreover, 4E10 reactivity for CL is similar to that for OVA. In contrast, reactivity of the APS MAb, CL15, is strong for CL, OVA and DM. Our side-by-side study shows that 2F5 and 4E10 prefer gp41 and their respective MPER peptides to CL by orders of magnitude, whereas a classic APS MAb is plainly polyreactive.
Figure 4-1: Comparison of MAbs 2F5, 4E10 and CL15.

(A) Titrations of MAb IgGs on antigens: 50 ng gp41; 400 ng 2F5 or 4E10 MN peptide; or “blocking” amounts of CL, non-fat dried milk (DM), ovalbumin (OVA) or bovine serum albumin (BSA) were used as antigens in direct ELISA following the washing and blocking procedures of Haynes et al.. O.D. values were recorded at 405 nm using an alkaline phosphatase/p-nitrophenyl phosphate developing system. (B) Antigen specificity of 2F5, 4E10 and CL15 IgG. ELISAs were performed as above, using 400 ng of each HIV-MN peptide and 10 nM MAb. The peptide sequences are shown in Table 4-1.
Figure 4-2: Regression of ELISA signal from 10 nM MAb CL15 on net charge of synthetic peptides.

Net charge, regression curve and R² values were determined by ProtParam and Microsoft Excel.

To further test the specificity of the three MAbs, we studied their binding to a panel of peptides, characterized as hydrophobic to positively charged and negatively charged (see Table 4-1). Figure 4-1B shows 2F5 and 4E10 are specific for their cognate-epitope peptides relative to CL15, which is, clearly polyspecific. Statistical analysis of CL15’s ELISA values revealed a correlation with net negative charge (R²=0.37; Figure 4-2). Thus, CL15’s extensive polyreactivity appears related to a preference for net-negative charge. This is consistent with our previous studies (Menendez et al. 2004; Bahr 2004; Visvanathan et al. 2003), in which MAbs 2F5, 4E10
<table>
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<tr>
<th>Peptide ID #</th>
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<td>CTRPNYNKRKRIHG</td>
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The peptides vary in chemical composition from hydrophobic to positively charged and negatively charged (as defined by the net charge of the peptides.) The sequences of the peptides bearing the 2F5 and 4E10 epitopes (ID # 19 and # 22, respectively) are shown in red.
and CL15 were used to screen phage-displayed, random peptide libraries. The behavior of each MAb was polyreactive to different degrees. All 2F5-selected peptides shared a consensus sequence (D(K/R)W), and half of 4E10-selected peptides shared > 3 residues in the (N/S)WF(N/D)IT core; however, CL15 selected peptides with completely unrelated sequences, indicating that it binds a very wide range of sequences.

4.3.2 CL15 neutralization assay

From above comparison studies, it is quite clear that 2F5 and 4E10 reactivity is different from CL15. Since 2F5 and 4E10 are bNt MAbs, it would be interesting to see if CL15 neutralizes HIV-1 at all. Thus, we sent out CL15 IgG to our collaborator (The Scripps Research Institute, USA) for neutralization assay. The method is described in section 4.2.3. Interestingly, although CL15 binds more strongly to the 4E10-MPER peptide than does 4E10, it does not neutralize HIV-1, as shown in Figure 4-3.
**Figure 4-3:** Neutralization of pseudotyped HIV-1HxB2 by MAbs CL15, 4E10 and 15E. The average counts represent the average number of cells that are infected in the absence of antibody. Note CL15 is relatively enhancing in this assay.

4.3.3 Serum CL reactivity analysis

It has been suggested that bNt MAbs 2F5 and 4E10 are autoAbs and produced by autoreactive B cell precursors that appear after HIV-1-mediated loss of tolerance (Haynes *et al.* 2005). By this logic, a significant pool of autoreactive, naive B cells should accumulate after tolerance loss, with HIV-1 antigens driving the subsequent selection of bNtAb-producing B cells from this pool. Thus, HIV-1-bNt sera should be expected to have increased levels of autoreactivity, and perhaps, polyreactivity.
To investigate the autoreactivity in HIV-1 infection, we used the CL assay used by Haynes et al. (2005) and CL as autoantigen to evaluate the autoreactivity in HIV-1 positive serum samples (see Chapter 2, section 2.3.1). Figure 4-4 shows the reactivity of 12 HIV+ bNt sera with small amounts of HIV-related peptides, and with “blocking” amounts of CL or “non-specific” antigens. One of the bNt sera (#6) reacted strongly with 2F5 peptide (in agreement with another, more standard, ELISA method; see Figure 2-2). Whereas many of the sera reacted with CL, reactivity was, on average, stronger with the “non-specific” antigens (except BSA).

Figure 4-4: Anti-CL Abs as a marker for autoreactivity during HIV-1 infection.

The assay is described in section 3.2.4, using sera diluted at 1:50.
To identify increases in CL reactivity over the course of HIV infection, we screened serum samples from SP, NP, RP and SC groups (Figure 4-5). No trend was observed to indicate stably-increasing CL reactivity in serum samples taken over time from the HIV+ individuals, or across groups, as compared to the non-specific antigens. Notably, at the serum dilution (1 in 50) used in the assay, high background (e.g. high signals on 4E10 and 2F5 peptides for sera that are negative in Figure 2-2) was observed. We then tried a different serum dilution, 1 in 250. As is shown in Figure 4-6, again, no significant increase of cardiolipin reactivity was observed. At the same time, peptide signals are more comparable to Figure 2-2 now. Note that 4E10 peptide used was a different batch from the one used in Figure 2-2, and the average 4E10 signal was thus higher. Taken together, our results indicate that the CL reactivity of HIV+ and uninfected people, as measured by this assay, even at dilutions that gave very high background (e.g., DM, OVA, peptides, etc.) is not significant in serum samples.
Figure 4-5: Cardiolipin reactivity in sera (1 in 50 dilution).

Serum samples from 32 HIV-1+ donors were grouped by clinical course as slow (SP), rapid (RP) and normal progressors (NP), or, covering the first 3-5 years of infection, starting at seroconversion (SC). Sera (1 in 50 dilution) were also tested from 12 HIV+ bNt donors (bNts), and 4 healthy volunteers (NEGs). Serum samples from a given HIV+ individual span over 15 years for SPs, 9 years for NPs, 4-6 years for RPs, 1-5 years for SCs, and comprise only single sample taken from the NEG.s. X-axis represents patients in each group: SC 1-5, RP 1-6, NP 1-5 and SP 1-4. Sera were tested on Peptides, cardiolipin (CL), non-fat dried milk (DM), ovalbumin (OVA) and bovine serum albumin (BSA) as described in Methods 4.3.3. The Y-axis depicts the ELISA O.D. values at 405 nm.
Figure 4-6: Cardiolipin reactivity in sera (1 in 250 dilution).

Sera used were 1 in 250 dilution. See Figure 4-6 for sample and method details.
4.4 Discussion

2F5 and 4E10 were both generated in the early 1990’s by electrofusion and Epstein-Barr virus transformation of B lymphocytes from HIV-1-positive donors (Buchacher et al. 1994). Both 2F5 and 4E10 were shown early to be able to neutralize a broad range of both laboratory-adapted and primary-isolates of HIV-1 (Conley et al. 1994; D’Souza et al. 1994). As for their neutralization capability, 2F5 is the most potent mNt Ab whereas 4E10 has the broadest cross-reactivity but is moderately potent (Binley et al. 2004). In passive transfer studies, 2F5 and 4E10 MAbs conferred at least partial protection against viral challenge in animal models (Conley et al. 1996; Gauduin et al. 1997; Baba et al. 2000; Mascola et al. 2000; Veazey et al. 2003; Haigwood et al. 2004).

In humans, the first direct evidence that bNtAbs have the capacity to contain viremia in established HIV-1 infection was shown last year in a clinic trial with a combination of 2F5, 4E10 and another bNt MAb, 2G12 (Trkola et al. 2005).

In this study, we first investigated the hypothesis that 2F5 and 4E10 are autoAbs. Our results support the idea that HIV-1 bNtAbs are not unusually polyreactive or autoreactive. The reactivity of MAbs 2F5 and 4E10 with CL was insignificant compared to their HIV-related epitopes, or to a genuine APS MAb. Moreover, elevated CL reactivity was not observed in bNt sera, nor were stable increases observed during any phase of HIV-1 infection. This low CL reactivity may reflect our CL assay (Haynes et al. 2005), which differs from the one used clinically. Similar reactivity patterns were obtained from the sera using a more standard ELISA, indicating that the high serum concentration used (1:50) was responsible for the observed polyreactivity. At higher serum dilutions (1:250), CL reactivity should not be observed, and under those
conditions, only some sera react with DM and OVA, while binding to the 4E10 and 2F5 peptides is specific, in that it can be blocked by gp41 (Figure 2-2 in Chapter 2). Firmer conclusions concerning HIV-related autoreactivity could be drawn from more comprehensive analyses (e.g., using autoantigen microarrays (Robinson et al. 2002)).

There have been studies showing NtAbs against MPER in natural infection (Broliden et al. 1992; Braibant et al. 2006). Broliden et al. first described NtAbs against MPER (Broliden et al. 1992), and found that neutralization activity in sera was inhibited when pre-incubated with a linear peptide QQEKNEQELLEDKW. In another recent study (Braibant et al. 2006), biotinylated MAb 2F5 and 4E10 were used to compete corresponding 2F5 and 4E10-like Abs (if any) in the sera. The authors stated that 2F5-like antibodies were detected in all the sera from SP, although the level is low. Note that in this study, the competition assay could only show cross-reactivity. To clearly show neutralization activity of certain serum Ab, separate the Ab from the serum, and do neutralization assay may be one of the most convincing methods. Taken together, protective NtAbs against MPER are represented in the human B-cell repertoire; future work should focus on the design of new immunogens and new approaches to induce these Abs with candidate vaccines.
CHAPTER 5: CONCLUSIONS

Developing a prophylactic vaccine offers the best hope for curbing the AIDS pandemic on a global level. Despite the success of current anti-retroviral drugs in slowing the onset of AIDS, they are not the final answer since drug-resistant isolates of HIV are becoming more and more common. In order to design a vaccine, it is important to know the type of immune response that needs to be produced for induction of protective immunity. In general, the Ab response is the predominant immunity to extracellular pathogens as opposed to cytotoxic T-cells that mediate the response against intracellular pathogens. HIV-1 is a classic intracellular pathogens, and cytotoxic T-cell responses have been shown to control the initial viremia (Pantaleo et al. 1994) (Koup et al. 1994) (Connick et al. 1996) (D'Souza et al. 1996). However, most current viral vaccines in use appear to work by eliciting NtAb responses that prevent the initial infection of cells by the virus. This may reflect a primary role for Ab neutralization in prevention of viral disease and an important role for CD8+ T cells in resolution of disease. It should be noted that most vaccines available have been developed principally by empiric methods, with an understanding of the type of effective immunity being subsequent to vaccine development.

In general, vaccines are more successful when mimicking a normally effective immune response (e.g., measles) than when trying to improve partial or absent immunity (e.g., TB, HIV) in natural infections. There is still no compelling evidence to show that the natural immune response can prevent initial HIV-1 infection. The observation that
most infected individuals maintain a healthy, asymptomatic state for many years after infection suggests that there may be immune responses that contain the infection but that eventually fail. Understanding the mechanism of such protective immunity might allow the design of a preventive vaccine.

In HIV-1 infection, Nt Abs usually appear after viremia has already decreased, suggesting that they are not responsible for the initial reduction in viral load (Pantaleo, et al. 2004). However, some studies have suggested that the antiviral humoral response may be more pronounced in individuals with long-term, nonprogressing infection (Pilgrim, et al. 1997). Neutralizing Abs mainly disrupt two stages of the HIV-1 viral life cycle, including viral attachment to target cells and membrane fusion, which are mediated by envelope proteins gp120 and gp41 separately. Specific epitopes on envelope proteins are therefore major targets for protective Abs and for epitope targeted vaccine design. One critical prerequisite for an epitope-targeted vaccine is to identify protective B-cell epitopes that are immunogenic in most humans.

In Chapter 2, the Ab response in HIV-1 infection was studied by linear epitope mapping. We showed that the polyclonal Ab response in natural infection is mostly directed against immunodominant regions, such as V3 region on gp120 protein and cluster I region on gp41. The Ab response is relatively stable during long-term infection compared with acute infection. Among groups with different clinical outcomes in long-term infection, rapid progressors had a broader and more vigorous Ab response than normal progressors and slow progressors. This may be a result from a higher load of replicating virus in the rapid progressors.
The Ab response against the MPER was detected in our study in serum samples from both acute and long-term infections. In Chapter 3, an affinity purification method was developed to purify peptide-specific Abs from sera. Unfortunately, the results from the neutralization assay for these Abs were not conclusive due to small amounts of the purified Abs. It would be interesting to purify more Abs and test for neutralization activity against more resistant primary isolates. Note that such neutralization activity in the purified Abs, if any, may represent a very small part of the neutralization activity in the serum, according to our preliminary data.

In Chapter 4, two bNt MAbs, 2F5 and 4E10 were tested for their reactivity properties based on a study that shows some evidence that these Abs are autoAbs binding to self-antigens. In our study, 2F5 and 4E10 are different from a typical autoAb, CL15, in both binding specificity and apparent affinity. As for autoimmunity during HIV-1 infection, there is no cardiolipin reactivity in general in the serum samples tested. No more conclusive results could be drawn under our assay conditions. Therefore, more comprehensive studies such as microarray could be used to detect autoimmunity in HIV-1 positive serum samples. Together with our collaborators, preliminary data in microarray have shown that MAbs 2F5 and 4E10 behave differently from CL15 on chips spotted with a variety of auto-antigens (Fathman et al. 2005) (data not shown). Therefore, bNt MAbs 2F5 and 4E10 do not seem to be autoreactive.

Combined with our data showing that the MPER is immunogenic in vivo, this highly conserved region on gp41 should be further considered as a component for an HIV-1 vaccine. As for an epitope-targeted vaccine, the MPER may need to be constrained to better mimic its conformation on the virus. Identifying new Nt or non-Nt
Abs against the MPER will help our understanding of the structure of MPER and exposure of its Nt epitopes. In addition, novel vaccination approaches such as adjuvants, prime-boost strategy and new vectors will be useful in the effort to elicit bNtAbs targeting the MPER and other Nt epitopes.
REFERENCES


