The development of an HIV vaccine candidate using a phage display library
by Jon Morrell Jacobs

A dissertation submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy in Biochemistry
Montana State University

Abstract:
The development of an effective HIV vaccine is of immense importance in the long term control of HIV infection worldwide. A successful vaccine would help prevent the establishment of a chronic HIV infection which has been shown to be difficult if not impossible to eradicate from an individual. A number of neutralizing monoclonal antibodies (mAbs) are directed against the HIV glycoprotein gp120 and bind to highly conserved residues. Determining the nature of such binding epitopes would be useful in developing a vaccine candidate that would direct an immune response against these conserved residues of gp120. By using the technique of peptide phage display mapping, mAbs were used to screen a random peptide library to identify peptides which bind specifically to the mAb. A nonapeptide library was screened with six antibodies, and for three of these mAbs, sequences were determined that bound specifically to their corresponding mAbs. Synthetic peptides corresponding to two of these sequences were able to compete with gp120 for binding to the antibodies. These synthetic peptides were then coupled to carrier proteins and used to immunize mice. Sera from mice immunized with two of the peptide conjugates bound to gp120, indicating that this approach has potential for the design of novel HIV vaccine antigens. An additional consensus peptide sequence, QSYP, appeared as an artifact during the screening of one of the monoclonal antibodies. Phage bearing this peptide sequence were also selected by three other laboratories which screened the same phage library against three unrelated mAb preparations. It was determined that phage displaying the QSYP sequence were not bound by the mAb of interest, but rather bound to bovine IgG contaminating the mAb samples which was derived from the fetal calf serum present in the hybridoma growth media. Implications of this finding for interpretation of phage library screening results, and possible uses of the QSYP consensus peptide are discussed.

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APPROVAL

of a dissertation submitted by

Jon Morrell Jacobs

This dissertation has been read by each member of the dissertation committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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Date Sept 16, 2002

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ABSTRACT

The development of an effective HIV vaccine is of immense importance in the long term control of HIV infection worldwide. A successful vaccine would help prevent the establishment of a chronic HIV infection which has been shown to be difficult if not impossible to eradicate from an individual. A number of neutralizing monoclonal antibodies (mAbs) are directed against the HIV glycoprotein gp120 and bind to highly conserved residues. Determining the nature of such binding epitopes would be useful in developing a vaccine candidate that would direct an immune response against these conserved residues of gp120. By using the technique of peptide phage display mapping, mAbs were used to screen a random peptide library to identify peptides which bind specifically to the mAb. A nonapeptide library was screened with six antibodies, and for three of these mAbs, sequences were determined that bound specifically to their corresponding mAbs. Synthetic peptides corresponding to two of these sequences were able to compete with gp120 for binding to the antibodies. These synthetic peptides were then coupled to carrier proteins and used to immunize mice. Sera from mice immunized with two of the peptide conjugates bound to gp120, indicating that this approach has potential for the design of novel HIV vaccine antigens. An additional consensus peptide sequence, QSYP, appeared as an artifact during the screening of one of the monoclonal antibodies. Phage bearing this peptide sequence were also selected by three other laboratories which screened the same phage library against three unrelated mAb preparations. It was determined that phage displaying the QSYP sequence were not bound by the mAb of interest, but rather bound to bovine IgG contaminating the mAb samples which was derived from the fetal calf serum present in the hybridoma growth media. Implications of this finding for interpretation of phage library screening results, and possible uses of the QSYP consensus peptide are discussed.

CHAPTER ONE

INTRODUCTION AND REVIEW OF THE STRUCTURE AND FUNCTION OF HIV

The History of AIDS and the HIV virus

Acquired Immune Deficiency Syndrome (AIDS) is the syndrome caused by the destructive effects of the Human Immunodeficiency Virus (HIV), which ranks as one of the most important infectious diseases in the history of mankind. It is commonly believed that HIV-1 subclass M, the most widespread form of the virus, initiated infection into the human population by a cross-species transmission event, in which a human was infected from a chimpanzee, the most likely source being subspecies *P. t. troglodytes*, carrying the Simian Immunodeficiency Virus (SIV) SIVcpz (reviewed by Hahn et al., 2000). The geography points to somewhere in west equatorial Africa, and the date, due to molecular mutational clock analysis, points to a last common ancestor around the early 1930's (1915-1945), with the actual crossover event mostly likely occurring sometime before this date (Korber et al., 2000). From that point on, HIV/AIDS was essentially undetected until 1981, when doctors in California, and then later in New York, began to see patients with an increase in opportunistic infections, mostly a rare form of pneumonia, *Pneumocystis carinii*, as well as other viruses, fungi, and skin cancer, most often seen in

patients having immune deficiencies (CDC, 1981; reviewed by Grmek, 1990). These symptoms would persist in the individual until their death, which would ultimately be caused by opportunistic infections allowed to thrive, due to the patient's severe immunosuppression. Cases during this time primarily came from the male homosexual population in the U.S. but once the syndrome was reported, cases were diagnosed elsewhere in the world. In 1982 the term AIDS was coined to describe the immune deficient condition common in all patients but it was not until 1983 that the pathogenetic factor was discovered to be a previously undetermined retrovirus (Barre-Sinoussi et al., 1983; Gallo et al., 1984). After a race to isolate and characterize the previously unknown virus and a spirited debate over who was first to discover it, the virus was finally given the name Human Immunodeficiency Virus or HIV in 1986 (Grmek, 1990; Levy, 1998). HIV has since spread into the heterosexual population and has become the world-wide epidemic that we know today.

At the end of 2001, there were approximately 40 million people world-wide that were living infected with HIV, many of them unaware of their infection (WHO, 2001). Approximately 5 million people were newly infected in 2001 and 3 million deaths were associated with AIDS related diseases. In North America approximately 940,000 people now live with HIV with about 45,000 new infections per year (WHO, 2001). Out of the 40 million individuals infected with HIV, a vast majority of those cases (28 million) live in Sub-Saharan Africa. HIV/AIDS is now the leading cause of death is this area and the fourth leading cause of death worldwide. In Sub-Saharan Africa, the life expectancy is now only 47 years, compared to approximately 62 years were it not for HIV/AIDS. In the

worst-affected countries, life expectancy is even less than 40 years, due to the widespread impact of HIV/AIDS. The epidemic has risen faster in Eastern Europe and Central Asia than in any other region in 2001. In the Russian Federation, reported cases of HIV/AIDS have doubled annually since 1998, with more than 130,000 total cases now reported. Such exponential growth is also seen in most of the Central Asian republics (Kyrgyzstan, Kazakhstan, Tajikistan, and Uzbekistan) as well as parts of Central Europe (Ukraine and Estonia). These numbers are on a smaller scale compared to other areas involved in the epidemic, but considering the exponential increase in cases reported, the widespread intravenous drug use in this area (in the Ukraine, three-quarters of the current HIV infections are caused by intravenous drug use), the large population, and the disintegration of public health services, it is in Central Asia that the potential for an explosion in HIV/AIDS cases is the greatest (WHO, 2001).

HIV is divided into two main branches, HIV-1 and HIV-2, which differ in the SIV strain from which they originated (Levy,1998). The HIV-2 epidemic has been comparatively small, and has been essentially contained within Africa. The nucleotide sequence of HIV-2 is actually more closely related to SIV than to HIV-1 (Hahn et al., 2000; Levy, 1998). HIV-1 itself is divided into three groups: M, O, and N (Main, Outlier, and Non-outlier respectively), shown in Fig.1.1. HIV-1 groups N and O have also been mostly contained within Africa, with group N almost exclusively found in Cameroon. It is HIV-1 , subclass M, that is responsible for the world-wide epidemic that is seen today. Each branch, HIV-1 and HIV-2, as well as each group, M, O, and N, are believed to have originated from separate species cross-over events originating from various strains of SIV

found in either sooty mangabeys or chimpanzees. The primate natural host of SIV is not affected by infection of the virus. This is seen with all SIV primate natural hosts in regard to their respective strains of SIV, even though some natural hosts carry viral loads high enough to cause AIDS in humans (Kurth and Norley 1996). This suggests that factors other than the virus itself are involved in disease progression and it is believed that the host species response to the viral infection and genetic host factors play an important role in containment of the virus in the host (Dalgleish et al., 1999).

Fig. 1.1 shows evolution of SIV within its natural host in black, with each black/red color change representing a zoonotic transmission event. These transmission events are believed to be the result of close contacts between humans and SIV infected primates in many regions of Africa. The hunting and consumption of primates by humans is very common in these regions and it is believed that this process would provide numerous avenues for a transmission event (Hahn et al., 2000). Red lines represent evolution of HIV-1 or HIV-2 in humans. Group M is further divided into 11 clades, denoted A through K, that represent the diverse genetic sequences of HIV-1 seen throughout the world. It is clade B that is primarily found in the U.S. and within this clade, strains IIIB, MN, and SF2 are some of the more commonly used virus isolates utilized in research.

HIV Structure and Function

HIV-1 is a member of one of the five major primate lineages of the lentivirus family of animal retroviruses. Lentiviruses are capable of long-term latent infections, as

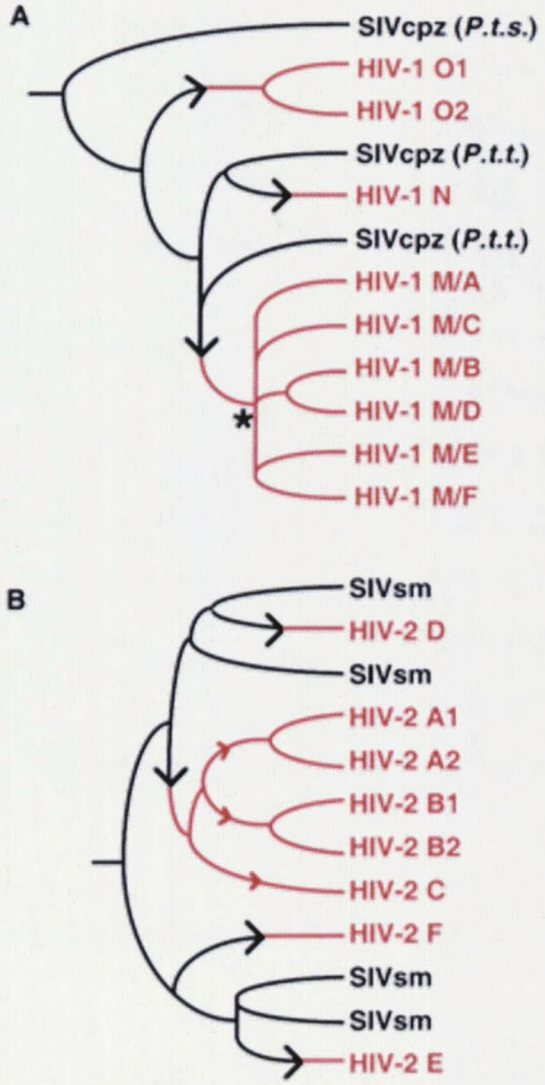

Figure 1.1 **Schematic trees of HIV-1 and HIV-2 showing the multiple independent zoonotic transmissions of SIVcpz and SIVsm to humans.** Branches in black show evolution of SIV within its natural host with black arrows indicating points of cross-species transmission into human hosts (red) and the subsequent evolution of HIV. (**A**) SIVcpz and the three main groups of HIV-1: O, N, and M. (**B**) SIVsm and HIV-2, which is comprised of six subtypes, A through F. (Reprinted with permission from Hahn et al., copyright 2000 American Association for the Advancement of Science.)

well as short term cytopathic effects, which produce slow-progressing, yet fatal diseases.

HIV particles consist of two identical strands of RNA, approx. 9.2 kb long, contained within a cone-shaped core composed of the p24 gag capsid protein and the p6 gag nucleocapsid protein (reviewed by Levy, 1998). Also contained inside the core and closely associated with the strands of RNA are the enzymes reverse transcriptase (RT), integrase, and protease. The core is surrounded by a phospholipid bilayer which is supported by a viral matrix composed of the p17 gag protein inside of the bilayer (Fig.1.2). The phospholipid bilayer envelope of the virus contains up to 72 trimers of the envelope glycoprotein gp120 non-covalently attached to the transmembrane glycoprotein gp41 in the mature virus (Levy, 1998). The RNA of HIV (Fig.1.3) has the gag, pol, and env genes characteristic of retroviruses, as well as a range of unique accessory genes labeled tat, rev, nef, vif, vpu, and vpr. The gag gene encodes for a 55 Kd protein that is cleaved to form the p24, p17 and p15 structural proteins of the virus. The pol gene products include RT, protease, and integrase enzymes. The env gene codes for the surface glycoprotein gp160, which is later cleaved by a host cell protease into gp120 and gp41 before assembly of the virion.

The life cycle of HIV begins when bodily fluids such as semen or blood from an infected individual come in contact with naive cells from another individual. The initial contact between virus particle and cell begins with the recognition by the viral gp120 glycoprotein of the CD4 molecule expressed on either T-cells, macrophages, or dendritic cells, as shown in Fig.1.2 (Dalgleish et al., 1986; Sattentau, 1998). The affinity between monomeric gp120 and CD4 is strong, between 1 and 10nM, but the affinity of soluble

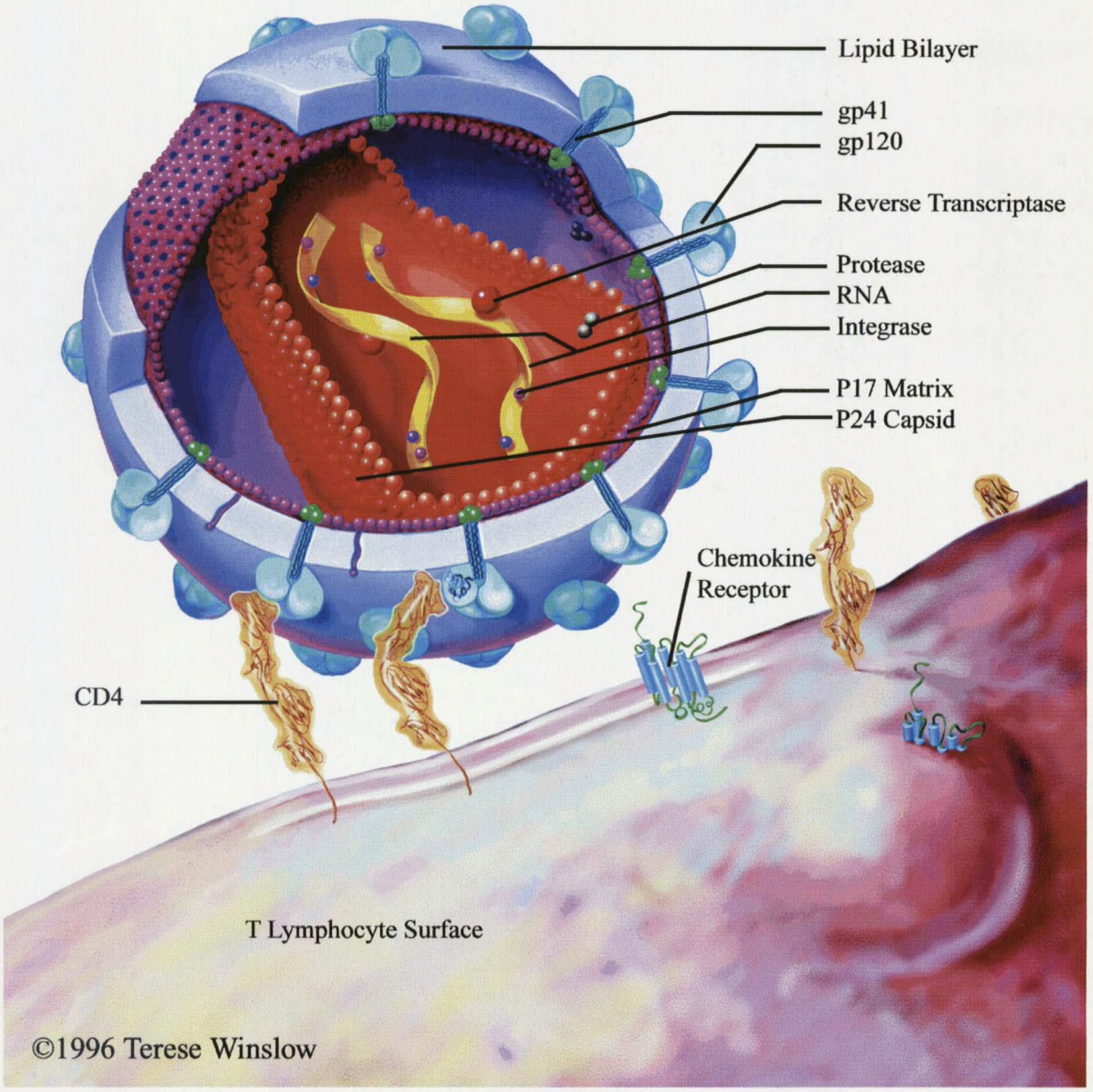

Fig.1.2 **Picture of an HIV virion.** (Used with permission from Teresa Winslow, Medical Illustration ©1996 Teresa Winslow)

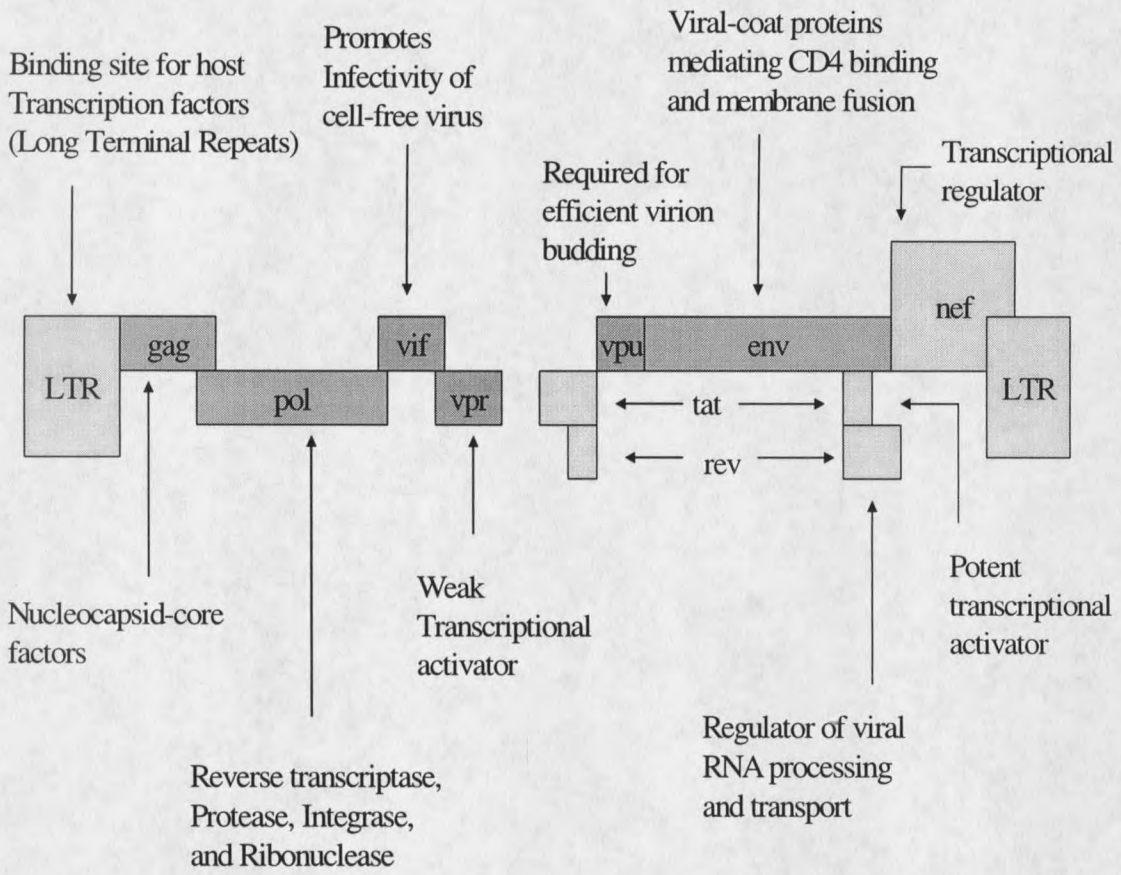

Figure 1.3. **The RNA schematic of HIV**. Genes for the linear RNA sequence of HIV are shown in blocks. Overlapping blocks indicate sharing of the same sequence between two genes but in different reading frames. A short description of each gene is also shown. (Information for figure taken from Greene, 1990)

CD4 (sCD4) to oligomeric gp120 expressed on the surface of the virion has been shown to vary a great deal between primary isolates and laboratory strains of HIV (Moore et al., 1992; Fouts et al., 1997). Binding of sCD4 to primary isolates, strains of HIV taken directly from infected patients, have been reported to be 10 to 30 fold lower than binding of sCD4 to culture-adapted laboratory isolates. The CD4 binding-site surface on gp120 is approximately 800 $Å^2$ in area, with 22 CD4 residues contacting 26 gp120 residues, which involves six different segments of gp120 (Kwong et al., 1998). These interactions include 219 Van der Waals interactions, and 12 hydrogen bonds, which are mostly contained in the core conserved regions of gp120 (see discussion of gp120 structure for more details). The CD4 binding site (CD4bs) is devoid of carbohydrate attachment sites, includes both a hydrophilic and hydrophobic pocket, and contains a relatively acidic patch of amino acids complimenting a basic patch on the CD4 molecule (Kwong et al., 1998). The CD4 residue Phe 43 plays an important role in the interaction by inserting into a hydrophobic pocket on gp120. Most importantly, the binding of CD4 induces a conformational change in gp120, allowing a previously inaccessible chemokine receptor binding site (CRbs) to be formed (Sattentau et al., 1993). The CRbs is mainly comprised of two regions on gp120, segments of the V3 loop and a group of core basic and hydrophobic residues which are thought to interact with the chemokine receptor, as determined by site-specific mutagenesis of gp120 (Rizzuto et al., 1998; Speck et al., 1997). Some monoclonal antibodies (mAbs), termed CD4-induced mAbs, are specific to the CRbs region and do not bind gp120 unless CD4 is bound; mAb 17b is an example (Thali et al., 1993). There are two chemokine receptors that are used by HIV for binding

and infection, CCR5 and CXCR4, and mutations in the CRbs of gp120 can alter the specificity of HIV between the two receptors (Moore, 1997; Feng et al., 1996; Speck et al., 1997). CCR5 is found on macrophages, and is bound by M-tropic (macrophage infecting) virus particles, which are most often involved in virus transmission. CXCR4 is specific for T-cell infecting virus particles (T-tropic) that develop in later stages of virus infection. It is believed that binding to the chemokine receptor allows positioning of the gp120/gp41 in the proper orientation for viral fusion (Wyatt et al., 1998). This binding of the chemokine receptor to gp120 induces yet another conformational shift, involving the interaction between gp120 and gp41, in which gp41 becomes exposed to interact with the membrane surface of the immune cell (Trkola et al., 1996a; Wu et al., 1996). Gp41 is an integral membrane protein containing an extra cellular coiled-coil domain, which acts very much like a loaded spring that, when released, extends outward and initiates contact with the membrane using its fusion peptide located on the extreme N-terminus (Chan and Kim, 1998; Chan et al., 1997; Weissenhorn et al., 1997). This extended transient structure of gp41 is called the prehairpin intermediate. Reassociation of the three extended helices with the three helices incorporated in the virus forms the trimer-of-hairpins structure (Chan and Kim, 1998; Furuta et al., 1998). This re-association is proposed to close the gap between virus and cell and begins the fusion of the HIV and immune cell membranes, which leads to the subsequent release of the viral core into the cell. After insertion, the viral core disseminates, leaving the strands of RNA free to be reverse-transcribed into dsDNA by the viral RT. The viral dsDNA then enters the nucleus, along with the viral enzyme integrase, which then inserts the dsDNA into the

host cell genome. The development of active virus production is not always imminent, and the ability of the virus to stay in a latent phase for long periods of time contributes to its evasion of the immune response.

Actual virus production is believed to be activated by the binding of host-cell transcription factors to the long terminal repeat region (LTR) upstream of the viral genes, as seen in Fig. 1.3. Production of virus can be divided into two phases, an early phase when regulatory genes are expressed, and a late phase, when structural genes are expressed. During early phase, the regulatory genes tat and rev are expressed, which require RNA splicing events for production of viable mRNA for translation (reviewed in Green, 1990; Pantaleo and Fauci 1995). Tat is a potent transcription activator and its binding increases transcription of the viral DNA by 1000 fold. The rev protein facilitates the transition between early and late phase by binding to viral RNA transcripts and transporting them to the cytoplasm for translation. The late phase is characterized by the production of the gag, pol, and env polypeptides needed for a complete viral assembly (Green, 1990; Pantaleo and Fauci, 1995). All gene transcripts are transported out of the nucleus and into the cytoplasm to be translated. Packaging occurs within the cytoplasm, which involves surrounding the RNA transcripts and essential enzymes needed for the next cycle with the gag core proteins. The virus is released by the cell through budding from the plasma membrane, which incorporates the host cell phospholipid bilayer on the surface of the virus as well as the critical gp120/gp41 complexes previously synthesized and located within the plasma membrane bilayer.

HIV gp120 Structure

Gp120 is the major surface-expressed glycoprotein on the HIV virion. It's discovery in 1985 (Allan et al., 1985; Montagnier et al., 1985) began a search for its 3D structure which culminated in 1998 with the publication of the crystal structure at 2.5 Å resolution of HIV-1 IIIB gp120 in complex with CD4 (Kwong et al., 1998). Before the crystal structure, the antigenic structure of gp120 had been intensely studied mostly by mutational data coupled with the binding of panels of mAbs specific for various residues on gp120 (Ditzel et al., 1997; Moore and Sodroski, 1996; Thali et al., 1992). Studies also included the antibody response against the antigenic structure of gp120 and its implications in vaccine development and in viral activity (Chamat et al., 1992; Pincus et al., 1993; Pincus et al., 1994b). This previous work had described gp120 in terms of antigenic regions which pertain to groups of mAbs that bind specifically to each region. The variable loops V3 and V2, the CD4 binding site, and the CD4-induced binding site for the chemokine receptor are some of the more important antigenic regions. The X-ray data has helped immensely in elucidating pertinent residues for the gp120/CD4 interaction, the antigenic nature of gp120, as well as providing clues on how gp120 functions.

Gp120, as its name suggests, is a 120 kD glycoprotein which is non-covalently attached to the integral membrane protein gp41. Gp120 has approximately 25 glycosylation sites with carbohydrate accounting for more than half of its molecular weight (Fig.1.4). It is composed of 492 amino acids, which have been divided into five

variable regions (V1-V5), and five constant regions (C1-C5), based upon sequence data. For the most part, each variable region forms a loop structure exposed on the surface of gp120, while the constant regions form the conserved central core. The crystal structure of gp120 published in 1998 is of the CD4-bound structure, and required some modifications to gp120 needed for crystallization purposes (Fig.1.5). These modifications include: removal of both the V1 and V2 loops (67 residues), and the V3 loop (32 residues), and replacement of each with a simple Gly-Ala-Gly linker with one residue remaining on each side of the key disulfide bonds defining the loops (Kwong et al., 1998). The protein was also enzymatically deglycosylated, leaving a "core" gp120 molecule that still bound CD4 and many mAbs with affinities comparable to the native protein (Binley et al., 1998). The structure was co-crystallized with CD4 as well as the Fab fragment from mAb 17b, which binds the CD4-induced conformation of gp120, thereby essentially flanking the gp120 structure with two other protein structures and conferring conformational stability for the formation of crystals.

The gp120 structure is divided into an inner and outer domain which is linked together by a bridging sheet. The CD4 molecule interacts with six different regions of the primary sequence of gp120 and this binding site is formed by a depression at the interface of the inner and outer domain, also including the bridging sheet (Kwong et al., 1998). More recently a refined crystal structure of the HIV-1 IIIB, at 2.2 Å resolution was obtained, as well as a 2.9 Å crystal structure of the "core" gp120 from the HIV-1 primary isolate YU2 (Kwong et al., 2000). The refined IIIB structure enhances the previous 2.5 Å structure and resolved some previously disordered residues. The YU2 structure varied

little from the IIIB structure. YU2 and IIIB have noticeably different antigenic responses; there are a number of mAbs that neutralize IIIB isolates but cannot neutralize YU2 isolates (Sullivan et al., 1995). However, their "core" structures based upon X-ray crystal data seem to be nearly identical. This lends to the hypothesis that differences in the variable region sequences result in differences in the variable loop structures, which mask many of the conserved core epitopes of gp120. Differences in the antibody response are most often amplified when directed against the trimer structure of gp120 versus the monomeric structure. The similarities in the core structure between gp120 IIIB and YU2 suggest that variable loop differences in gp120 might also have significant effects on the quaternary structure of the trimer complex, thereby changing the antigenic sites in the trimer (Kwong et al., 2000). Unfortunately, three of the variable loops (V1, V2, V3) are not contained in either of the crystal structures of the core gp120.

Antigenic Structure of gp120

The glycoprotein gp120 has evolved several structural features to help evade the immune system due to the pressure place upon it by neutralizing mAbs. Its structure is such that the hypervariable regions or loops V1-V5 are exposed on the surface, which can support a high degree of structural polymorphism and still maintain function of the protein (Wyatt et al., 1998 and Sattentau, 1998). The high mutation rate of gp120 is caused by the high error rate of HIV reverse transcriptase (RT) converting the viral ssRNA into dsDNA for transcription and translation into protein. The variable nature of gp120 allows it to evade most specific Ab responses simply by changing enough surface

residues to elude such a response. The hypervariable loops shield the conserved, functionally necessary amino acids in the core from Abs. Gp120 is also highly glycosylated with sugar groups that resemble those expressed on proteins on the surface of the host cell. This gives the HIV gp120 protein a "self" image to the host which helps in avoiding any general recognition of the virion as foreign (Wyatt et al., 1998). The glycosylation sites are strategically placed so as to hinder recognition by Abs, but to not interfere with interaction with the CD4 molecule.

The CD4 binding site (CD4bs) has characteristics that assist immune evasion, while at the same time maintaining conserved residues needed for binding the CD4 molecule. This defense may involve partially masking the CD4bs with the V1 and V2 loops to avoid direct exposure of the conserved residues to the surface. It is thought that the V1 and V2 loops are extremely flexible and maintain a dynamic presence around the CD4bs (Wyatt et al., 1998). The CD4bs itself is somewhat recessed, hindering the ability of an antibodies' variable region to bind, as well as containing a number of residues flanking the CD4bs that are highly variable and glycosylated (Kwong et al., 1998 and Wyatt et al., 1998). Even though most residues in the binding site are well conserved, this area still contains clusters of residues that do not contact CD4 directly and are subject to mutations, such as those residues which line the hydrophilic cavity. Even some of the residues which do directly contact CD4 do so with main chain atoms allowing some variability in their sidechain atoms (Kwong et al., 1998). This also allows a certain degree of mutation even within a very conserved portion of the protein. Finally, CD4 binding confers a conformational change on gp120, which allows for the exposure of

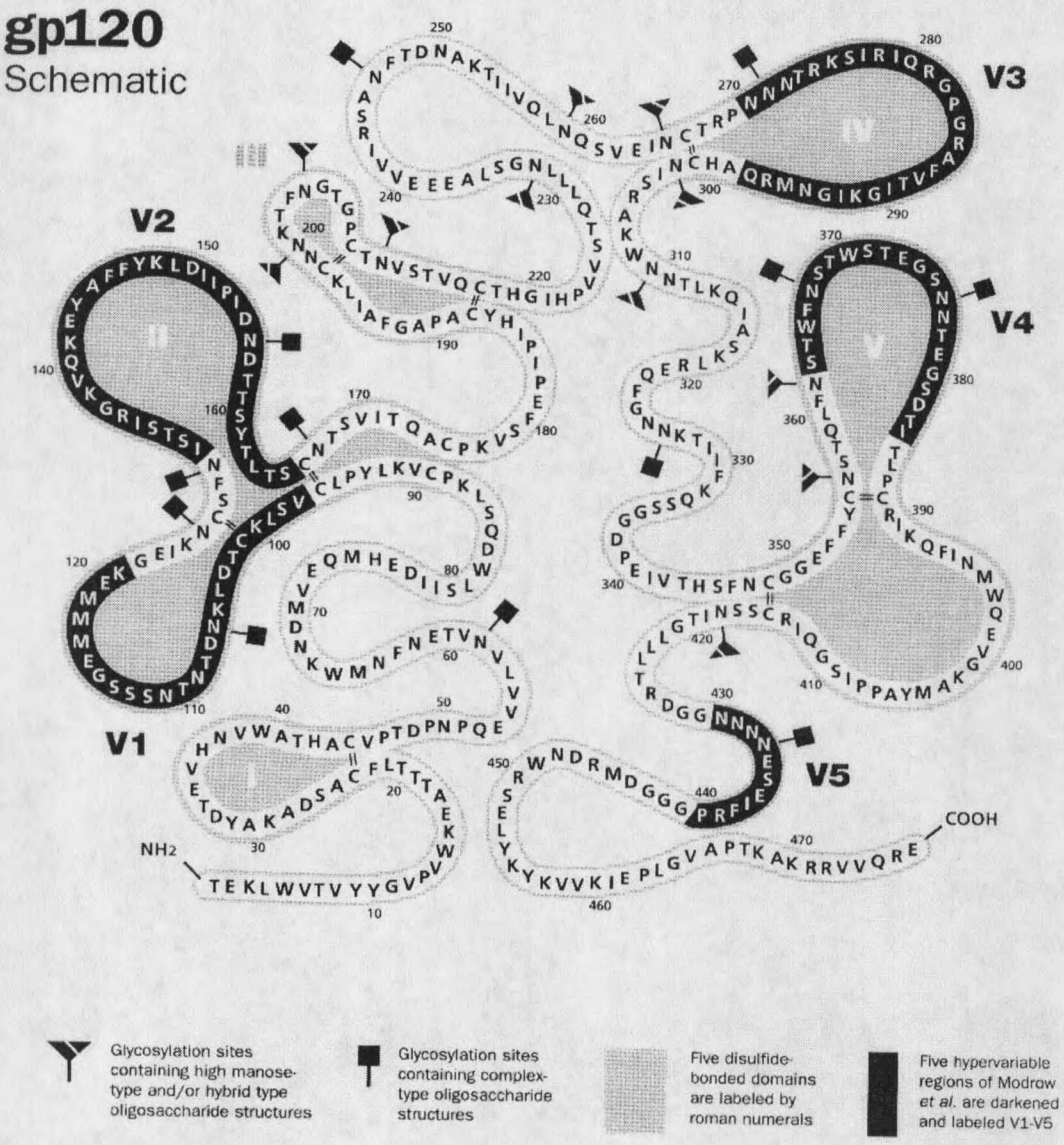

Fig. 1.4 **Amino acid schematic of gp120 structure.** Figure shows the five known variable regions as well as the many glycosylation site found on gp120.

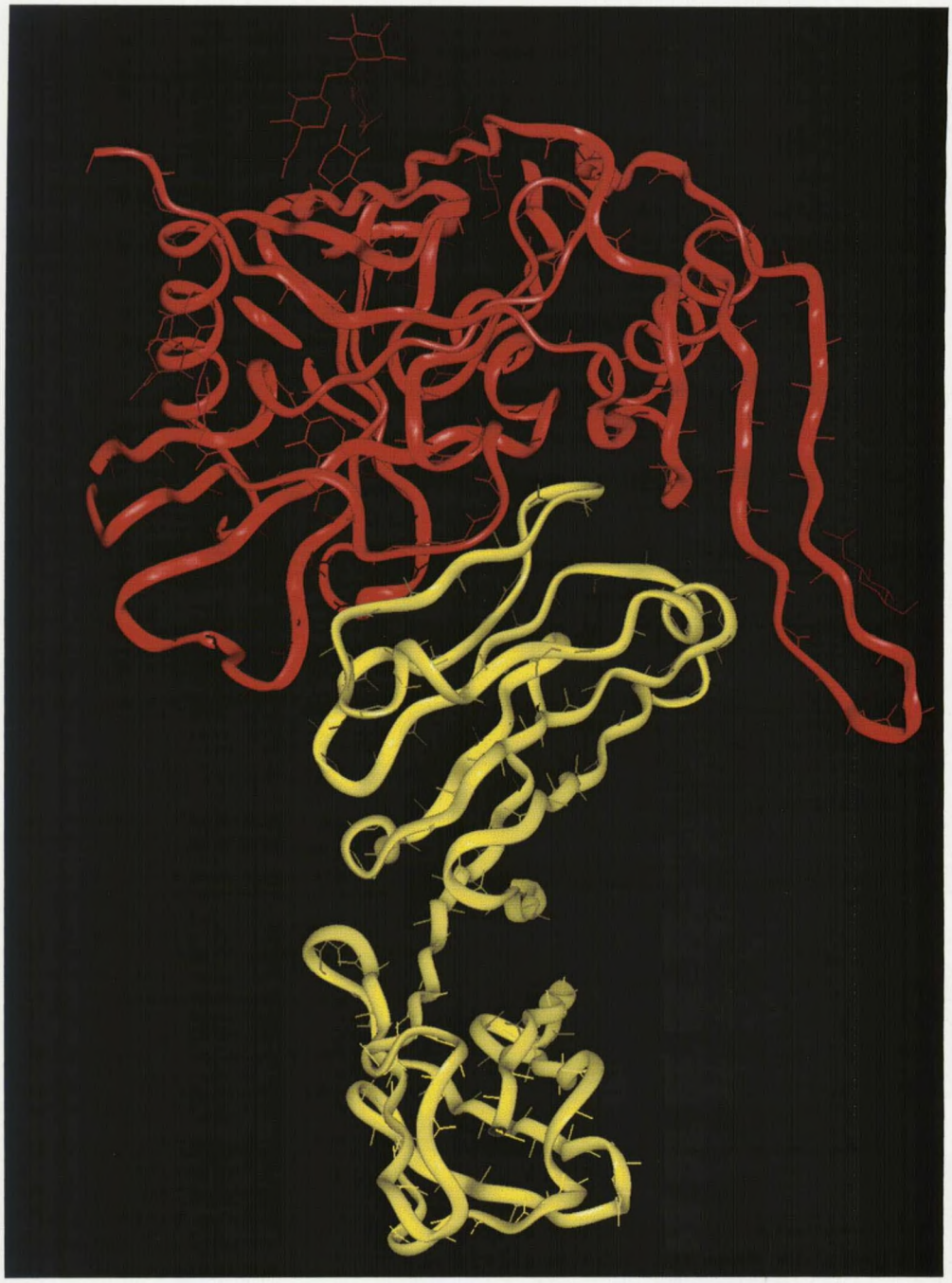

Fig. 1.5 **Crystal structure of the gp120/CD4 complex**. The gp120 molecule is shown in red with the CD4 structure in yellow. Actual structure contained the Fab of mAb 17b to facilitate crystallization but is not shown in the figure. (Kwong et al., 1998)

residues not directly exposed in the native gp120 structure. For example, a number of residues used for the chemokine receptor binding are not exposed until CD4 is bound, making it difficult for the immune system to target this area since these residues are only exposed for a short period of time (Rizzuto et al., 1998).

Despite such a tight system of defense, the immune system can develop antibodies that bind specifically to the CD4bs and other neutralizing epitopes on gp120 and are capable of neutralizing virus (Robert-Guroff et al., 1985; Weiss et al., 1986; Berkower et al., 1989). Unfortunately, these antibodies generally are not developed until later in the infection, after the virus has been able to integrate and established itself in the host. The host Ab response is often found to be directed toward a number of the more exposed variable loops of gp120 (Pincus et al., 1994a). While these Abs are effective in neutralizing their specific sequence, it is believed that changes in the amino acid structure of these regions quickly allow escape from any specific Ab response. Neutralizing Abs are developed during infection, as well as with some vaccine candidates, and are able to neutralize virus particles (Pincus et al., 1997). However, they are unable to completely eliminate the virus once it has firmly established itself by integration into the genome of the host (Pincus et al., 1994b).

Pathogenesis of HIV and Therapeutic Implications

Infection of a host by HIV begins a very complex interaction involving multiple aspects of the immune system striving to counter-balance an extremely evasive viral infection. As mentioned above, there are two different types of HIV that have

preferential affinity for two chemokine receptors. M-tropic or "R5" viruses bind specifically to the chemokine receptor CCR5 while T-tropic or "X4" viruses bind specifically to the CXCR4 chemokine receptor (Moore, 1997; Feng et al., 1996; Speck et al., 1997). R5 viruses have been implicated in the initiation of infection from one host to another. This is thought to be because sexual activity is the most common method of infection, and the population of immune cells found in the genital mucosa preferentially express the chemokine receptor CCR5 (Zaitseva et al., 1997). Generally X4 viruses do not appear until later in infection and this represents a shift in the population of cells infected by HIV (reviewed in Cohen and Walker, 2001).

HIV infection can be roughly divided into two phases, an initial acute phase and a chronic or latent phase (Fig.1.6). Initial infection by HIV can occur through different transmissions, percutaneous exposure (infected needles), or through perinatal transmission (mother to fetus), but the most common method is through the genitial mucosa via sexual activity (reviewed in Royce et al., 1997). Symptoms of acute HIV infection appear within a few days to weeks after the initial exposure and include fever, fatigue, headaches, night sweats, and a maculopapular rash that may vary in appearance. This phase is often seen as resembling symptoms of infectious mononucleosis (Cohen and Walker, 2001). There are a number of other symptoms that may be included, but are not diagnostic of HIV. These symptoms normally persist from days to weeks until the immune system of the host is able to control the initial infection and reduce the viral load present in the host. During this phase, diagnostic testing for infection is possible, if the right assay is used. In early infection, antibodies are not generated at high enough levels

to give a positive result for a standard antibody reactivity assay to gp120, but HIV RNA testing by RT-PCR has been shown to be very sensitive during this time (Cohen and Walker, 2001). Also, assays for the viral p24 antigen are also frequently positive during this acute phase and are highly specific, but it has been estimated that the p24 test results are not positive until a number of days after the virus is detected by the RNA assay. The benefit of the p24 assay would be its specificity in ruling out false positive results from a standard anti-gp120 antibody reactivity assay (Henrard et al., 1994).

As the host immune system controls the initial infection, viral titers and viral RNA levels drop significantly, which begins the chronic or persistent phase of the infection. It is believed that during the acute phase, the virus is "seeded" into select lymphoid and other organs which allows it to maintain this persistent/latent effect (Schacker et al., 2000). These reservoirs of virus are established relatively early in infection, likely within days, and are largely composed of integrated viral DNA (Bukrinsky et al., 1991). The chronic phase can be characterized as a long term chronic battle between the immune system and the virus, which can last from a few years up to and beyond twenty years. Throughout this time, the host slowly becomes depleted of its CD4+ cells, and hence its helper T cell response. Eventually viral levels begin to climb, in direct correlation with CD4+ cell depletion (Fig.1.6), which begins immunosuppression and the initiation of the opportunistic infections which eventually cause death. After the acute infection, the point at which viral levels decrease and stabilize is known as the "viral set point" and normally varies indirectly with the length of this chronic phase (Lyles et al., 2000; Mellors et al., 1997). The higher the viral levels

during the chronic phase, the shorter this phase will be and the quicker the rate of CD4+ decrease and subsequent progression of the disease. The lower the "viral set point" is, the longer the chronic phase will be with a slower rate of decrease of CD4+ cells, and a slower progression of the disease.

The most effective therapeutic treatment used to combat the chronic progression of HIV is a regimen called highly active antiretroviral therapy or HAART. This contains a group of protease and transcriptase inhibitors which often reduces the plasma viral titers to below detection limits (Markowitz et al., 1995; Collier et al., 1996). It is able to control viral progression as long as the regimen is maintained, but if treatment is suspended, the patient's virus titer will increase and the patient will continue to progress in the disease. It was initially thought that after a certain time on the regimen, the reservoir of latently infected HIV cells would die out and the virus would be eradicated from the host. However, recent estimates based upon the half life of this cell population estimate that it would take up to 60 years of treatment with this therapy for virus eradication, which does not including the possibility of re-seeding by possible periods of virus replication during the therapy (Finzi et al., 1999). It is now thought to be very unlikely that this therapy alone can eradicate the virus from a patient.

There are great differences in the progression rates of the infection in individuals during the chronic phase. There is even a subgroup of people referred to as "long-term non-progressors" or "long-term survivors" which have been observed not to manifest clinical symptoms of HIV, have viral loads at or below detection limits without the use of antiretroviral medication, and have maintained this level for a significant period of time,

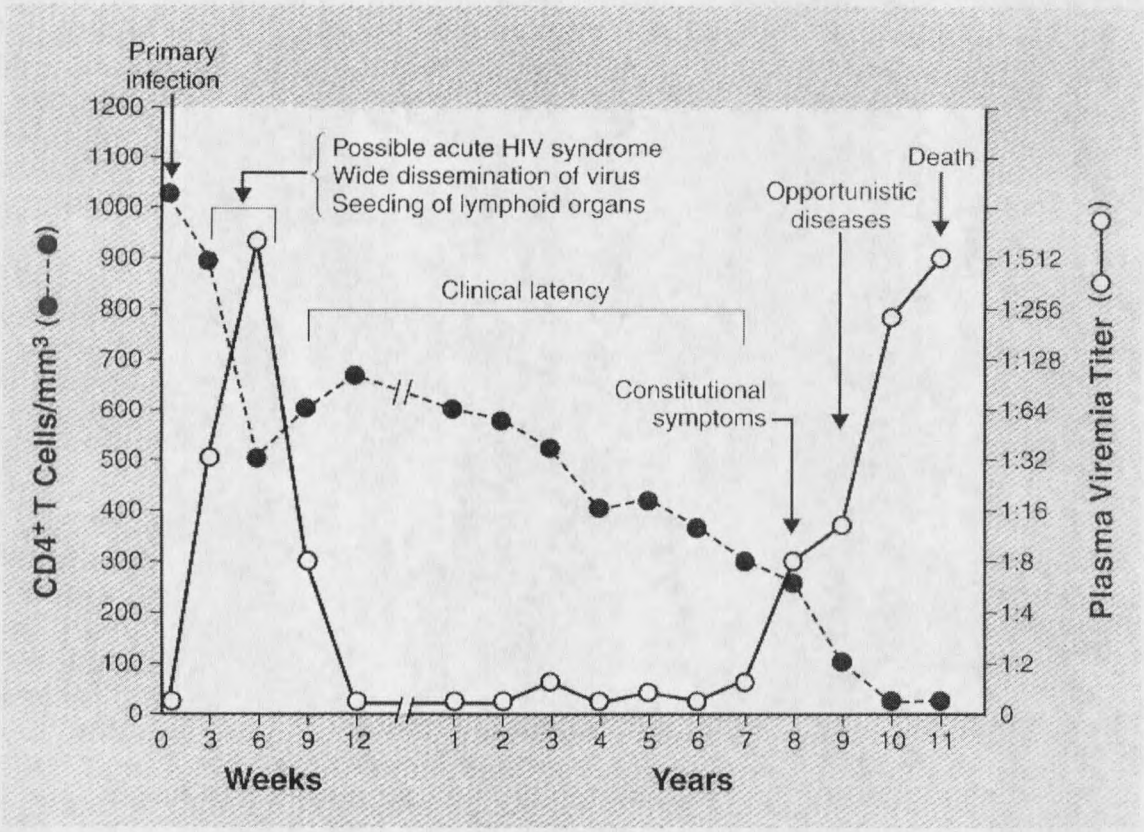

Figure 1.6 **Typical course of HIV infection.** After about 12 weeks post-infection, blood-borne virus (plasma viremia) is not detectable for many years. Nonetheless, CD4+ T cell counts steadily decline during this clinical latency period, probably because of active viral replication and T cell infection in lymph nodes. When CD4+ T cell counts drop below a critical level (about 200/ mm^3), there is a high risk of infection. (Reproduced with permission from Pantaleo et al.,1993)

sometimes up to and beyond twenty years (Harrer et al., 1996). There are many reasons for this variability in disease progression. Mutations in the virus itself can reduce its pathogenic potential, such as in regions of the nef gene (Dyer et al., 1997), and host genetic factors, such as mutations in the CCR5 chemokine receptor or certain polymorphisms in the human leukocyte antigen (HLA) types, effect the presentation of viral peptides on the cell surface for antigen recognition by a T cell response (Buchacz et al., 1998; Micheal, 1999; Magierowska et al., 1999). The most compelling reason though, is the variability of the immune system itself and its ability to combat and affect the rate of progression of the virus.

It has been know that all branches of the immune system are involved in combating an HIV infection and each has been shown to play an important role. Humoral immunity involves the production of antibodies by B-cells specific for epitopes on viral proteins and upon binding, these Abs neutralize the virus. Even though neutralizing antibodies are produced against HIV, the virus, as discussed above, has evolved several methods to evade this arm of the immune system. Broadly reactive neutralizing antibodies are generally seen later in infection (Pilgrim et al., 1997), and even though they are important in controlling the level of virus particles, they seem unable to contain viral replication alone (Poignard et al., 1999 and Carotenuto et al., 1998). A strong cytotoxic T lymphocyte (CTL) response has been widely seen as a determining factor in virus titer levels and progression of HIV (Borrow et al., 1994 and Musey et al., 1997). A cellular CTL response is responsible for specifically detecting and killing virus infected cells. They react to peptide antigen that is presented to them by MHC class I presenting

molecules expressed on the surface of these cells. The virus load in the host is in part determined by the dynamic equilibrium established by the production of virus from infected cells and the destruction of these cells by a CTL response. The loss of CD8+ cells (CTLs) anytime during SIV progression results in a dramatic increase in levels of virus in the macaque model (Schmitz et al., 1999 and Jin et al., 1999). The ability of CTLs to effectively maintain control of a viral infection is critically dependent upon a T-helper response from CD4+ cells. CD4+ cells act to coordinate an effective response against the virus infection. The helper cells specifically recognize antigen peptide much the same way CTLs do except they bind to MHC class II presenting molecules. But instead of killing the cells, CD4+ cells activate a cascade of other cells and effectors through chemokines and direct cell to cell contact to help orchestrate the immune response against the viral infection. It is the constant depletion of the CD4+ cells throughout the chronic phase that directly correlates with HIV progression. Even though all aspects of the adaptive immune response can contain HIV progression, in the end all that is done is to extend the inevitable, which is compromise of the immune system and immunosuppression.

Vaccine Development

Why work on developing a vaccine? This is a very important question and there are a number of strong arguments with regard to the nature of HIV infection that support the need for such an approach. Once the virus reaches the chronic/latent phase, it becomes very difficult to eradicate from its reservoirs, because the virus in this state is

mostly composed of integrated viral DNA. The HAART regimen has been very effective in preventing the progression of the virus but there are a number of toxicity, scheduling, and cost issues that prevent this therapy from becoming the "magic bullet". An appealing approach to this problem is to prevent the establishment of this chronic phase by not allowing the virus time to integrate into these reservoirs during the initial infection. This can be accomplished by using a vaccine to confer sterilizing immunity, meaning that when the host is challenged, it will be able to suppress the acute infection and prevent the establishment of a persistent infection. Achieving this sterilizing immunity to HIV has been elusive. A number of different approaches have been tried to confer this immunity via a vaccine and, even though much has been learned concerning HIV and its infecting mechanisms, little progress has been made in developing such a vaccine (reviewed in Klein, 2001 and Nabel, 2001).

To date there has been more than 70 phase I (dose-escalation safety and toxicity) trials, five phase II (expanded safety and dose optimization) trials, and only two phase III (efficacy) trials for HIV vaccines. These include using various gp120/gp41 envelope proteins, V3 peptides, and a combination of different viral vectors expressing various HIV protein components. Most candidates show little toxicity effects, and some neutralizing antibody responses have been seen, but their activity has been minimal and largely strain specific (Wrin et al., 1995 and Mascola et al., 1996). Some candidates have demonstrated a CTL response, but the results have not been consistent (Ferrari et al., 1997). Current opinion holds that an effective vaccine is not yet at hand, and that a great deal of work must still be done to develop and evaluate candidates that can produce not

only a neutralizing antibody response, but also a broad and long lasting CTL response (Nabel, 2001).

Despite this, some studies have shown significant protection in macaques and chimpanzees from SIV and HIV challenges. Macaques immunized with a live attenuated SIV(Δ nef) vaccine were protected against SIV challenge (Daniel et al., 1992), macaques given a group of three distinct neutralizing mAbs against the gp120/gp41 complex were protected against an SHIV (SIV/HIV hybrid) challenge (Mascola et al., 2000; Baba et al., 2000; Hofmann-Lehmann et al., 2001), and a strong T-cell response has been detected in groups of human long-term nonprogressors and highly exposed seronegative individuals (Harrer et al., 1996). Based upon these and other studies, it is believed that an effective vaccine against HIV needs to contain all the critical elements for conferring sterilizing immunity: It should elicit cross-neutralizing antibodies against wild-type R5 isolates from diverse clades, elicit strong and broad CD4+ and CD8+ T-cell responses, and confer long term memory of these responses by the production of memory cells (Klein, 2001; Nabel, 2001; Weiss, 2001). Other considerations include low cost, ease of manufacture, low toxicity to the host, and ease in administration without extensive regimens or dose schedules.

A more realistic short term goal is to develop a vaccine candidate that would merely slow the progression of the disease in order to help reduce viral transmission. It is also possible that a vaccine candidate might even be used as therapeutic agent in individuals previously infected with HIV (Cohen and Walker, 2001). Such a candidate would be used to help raise the population of specific CD4+ and CD8+

cells, and hopefully alter the rate of virus progression without the use of therapeutic drugs.

As discussed above, a number of different vaccine candidates have been tested for efficacy. The positive results shown by the macaque trials with live attenuated SIV virus have sparked interest in using a live attenuated vaccine to achieve sterilizing immunity, but the use of a live virus raises many safety concerns. The attenuated virus used was deficient in the nef gene product which down regulates CD4 and MHC class I molecules on the surface of the cell, hence helping to evade a cellular CD8+ response. The nef deficiency results in a severely replication-defective virus, but a number of monkeys immunized with such a virus have now been shown to be infected with this strain of SIV and developed disease (Baba et al., 1995; Baba et al., 1999; Wyand et al., 1999). Mutations in the nef gene are seen in HIV, and there are a small number of non-progressors infected with a nef-mutated version of HIV (Deacon et al., 1995). Replication of the virus in these individuals is reduced dramatically, but there are cases reported of AIDS in individuals infected with Δ nef strains (Greenough et al., 1999; Learmont et al., 1999). A Δ nef live virus is obviously still infective to some extent, in humans as well as in the macaque model, and appears to still be able to establish a chronic infection, which is why it is deemed unsafe as a possible vaccine for humans. Despite these results, the development of a safe live virus that is still immunogenic is one of the more promising avenues in achieving neutralizing immunity.

As indicated above, a number of vaccine trials have used recombinant viral proteins as immunogens, especially envelope-based vaccines. Gp120 seemed the obvious

choice for the development of a vaccine considering its high copy numbers, exposure on the surface of the virion, and the crucial function it plays in infection of host cells. As previously discussed, most immune responses against gp120 have been either too weak, or very strain specific thus lacking the wide neutralizing antibody effect needed for a vaccine candidate. Results showing that some protection can be achieved by passive immunization with a group of three strongly neutralizing mAbs directed against the gp120/gp41 complex gives support to the argument that protection can be achieved by the use of this structure (Mascola et al., 2000 and Baba et al., 2000). The structural features of gp120, as previously discussed, make it a difficult antigen, especially having the variable regions exposed with the more conserved regions shielded. This causes antibodies to be directed against the variable portions and hence not able to neutralize diverse strains of HIV. Also, since the gp120/gp41 heterodimer is believed to be a trimer on the virus surface, free gp120 may not have the exact structure or exposed residues as the trimer complex. Antibodies that bind to free gp120 will not always neutralize the virus with its gp120/gp41 trimer complex. The challenge is to develop an antibody response, a crucial element of a successful vaccine, to the gp120/gp41 complex that will be directed against conserved residues, thus achieving a broadly reactive, yet neutralizing response against the virion.

The two arms of the adaptive immune response, cellular-immunity and humor immunity, both have critical roles to play in any vaccine candidate. A CTL response is designed to kill those cells which are infected and may be actively producing virus particles. Such a response is even capable of killing infected cells before any active virus

are produced and hence preempting the ability of the virus to replicate itself. As previously discussed, CD8+ cells interact with the MHC class I presenting molecule and bind to antigen peptides which originate within the cell. Such a response would only be elicited by a vaccine if the candidate were able to translate a foreign protein within the cell. Common examples of this would be DNA vaccines or live attenuated viruses which would replicate viral proteins within the host cell and have the linear peptides of those proteins presented to CD8+ cells. CD8+ cells specific for these presented viral peptides would be a crucial component of a vaccine candidate. In contrast, the humoral response or antibody response is directed against free virus and virus particles. Antibodies have the ability to recognize protein structure as a whole, not just presented peptides, and are able to neutralize virus before infection of host cells. This is a crucial component of any vaccine candidate and would allow the host to eliminate virus before it is integrated into a host cell genome. Such an antibody response would be elicited against the surface exposed proteins of the virus, proteins gp120 and gp41 in the case of HIV, and its effectiveness would depend upon the ability of the specific antibodies to recognize conserved residues on the protein which would also be recognized on the infecting virus. A neutralizing antibody response would be able to recognize those conserved residues on the protein as well as bind to regions on the protein which would sterically hinder the protein-protein interactions necessary for infection of the host cell.

One of the more practical reasons for the development of a vaccine is the economic aspect. Anti-viral treatments such as the viral protease and RT inhibitors are costly, both for the drugs themselves and in terms of side-effects and schedules of the

treatment. The vast majority of HIV-positive patients live in underdeveloped countries, and even a steep discount in the price of these therapeutic drugs can not come close to bridging the gap financially for the majority of people infected with HIV, not to mention the rigorous schedule of treatment needed. What is needed is a one-time vaccine that can be administered on a large scale, that is not labor intensive, and that can confer immunity to the diverse strains of HIV (reviewed in Peters, 2002).

Statement of the Problem

To date, there is still no effective vaccine against HIV for humans, despite many efforts to develop one. The major problem remains the ability to develop a neutralizing vaccine that will be effective even in the face of a mutating virus. No other protein on HIV is more responsible for its polymorphism than gp120 and it is in large part its structure which enables the virus to evade the immune system. Even so, the gp120/gp41 complex still is a good choice for a vaccine candidate, but conventional mechanisms for vaccine design have not be successful in providing one. Conserved portions of the gp120 structure are partially shielded by glycosylation and variable loops, deflecting most of the antibody response away from the conserved residues. If it were possible to use the conserved residues partially shielded on gp120 to form such a vaccine candidate, it could be used to direct an antibody response toward that specific region, hence producing a broad antibody response and not allowing the virus the time needed to establish a persistent infection. This type of interaction would be contingent upon the ability of mAbs to bind to the partially masked residues. CD4 binds with high affinity to a number

of these partially masked conserved residues as do some neutralizing antibodies, as discussed previously, but they are generally slow to develop. These results give some evidence to suggest that these partially masked conserved residues would be an appropriate target of a vaccine. If such a specific antibody response could be present at the time of initial infection, the virus could be prevented from establishing a chronic infection. The epitopes of the antibodies which bind to the conserved CD4bs of gp120 would make good candidates for such an epitope-specific vaccine. A group of CD4bs mAbs are available to develop such an epitope specific vaccine, which would direct an immune response against this conserved region of gp120. By using the technique of peptide phage display mapping, it is possible to use these mAbs to screen a peptide library and isolate and characterize those peptides which bind specifically to the mAb. These peptides should mimic conserved binding epitopes on gp120 and would essentially create a representation from a group of conserved residues which can then be used to elicit an antibody response that should mimic in strength and breadth the response by the original CD4bs antibody.

A vaccine would still need to include key features in order to elicit a highly effective immune response. A strong CTL response as well as a strong and neutralizing antibody response are both crucial for prevention of virus replication. It is this latter type of response which we would hope to generate using a gp120 peptide mimetic structure. Any vaccine candidate used in clinical trials would most likely also include a component which would elicit a cellular immune response . Peptide conjugates or peptide mimetics

could be used as a component together with a DNA vaccine or some other component designed to elicit such a CTL response.

CHAPTER TWO

REVIEW OF PHAGE DISPLAY TECHNOLOGY AND ITS APPLICATION TO GP120

Phage Display Technology, History, and Review

Determining interactions between various molecular players is often the key to discovery efforts in the biological sciences. Having the ability to determine information about a specific binding site can be crucial in understanding the interaction between two molecules. The use of phage display technology has greatly increased researchers' ability to obtain information regarding a number of molecular interactions.

This technology is based upon two key concepts. First, phage display links molecular recognition by a peptide or protein to the replication and subsequent sequencing of the DNA coding for that polypeptide. Second, random libraries can be created with this phage system and can be used to selectively screen for specific molecular binding, after which phage can be isolated, sequenced, and amplified.

George Smith is the father of phage display technology, being the first in 1985 to make the connection from DNA to expression, using a foreign peptide sequence on the surface of the pIII protein of a filamentous phage (Smith, 1985). He showed that it was possible to attach a foreign sequence of residues on the N-terminus of the pIII protein by insertion of the foreign DNA into the phage genome. The phage retained its infectivity

and thereby the ability to amplify and isolate the specific foreign sequence being expressed. This idea was expanded upon by inserting a library of different (random) foreign DNA sequences at the region coding for the N-terminus of the pIII protein, which allowed the phage to express a random peptide library (Cwirla et al., 1990; Scott and Smith 1990). This library could be subsequently screened against a target molecule, yielding peptides linked to the phage that were selected to bind specifically to the target molecule. Since the peptide sequence is encoded by the phage DNA, the exact sequence of the peptide can easily be obtained by isolating the specific phage-peptide clone and sequencing the foreign DNA segment. This concept, often termed "panning", was first successfully used in screening a peptide phage library against an antibody as the target protein to map the peptide epitope of the antibody. When a random peptide library is screened against an antibody, peptides found to bind specifically to that antibody mimic the residues which comprise the binding epitope of that antibody on its target antigen. This type of screening experiment is utilized in the work described in this thesis and will be explained in detail later in the chapter. It has been shown that this type of library can be screened not only with antibodies, but with any target protein to obtain a peptide sequence specific for that target (Devlin et al.,1990). A number of variations have been made to this technique in both library construction and targets screened (reviewed in Smith and Petrenko 1997).

Variations involving the phage vector include using the pVIII major coat protein to express the peptide library. Benefits to this approach include a copy number of 2700 pVIII proteins per virion which far outnumbers the 3-5 copies of the pIII minor coat

protein. The disadvantages of this vector include the fact that the pVIII with the foreign peptide must not represent more than 20% of the total pVIII protein or the phage will lose its infectivity (Greenwood et al., 1991 and Kang et al., 1991). The size of the insert is also limited to peptides equal to or less than 6 to 8 amino acids long (Iannolo et al., 1995; Malik, 1996; Petrenko et al., 1996). The pIII protein has been extensively used to express various lengths of random peptides, cysteine loop constrained peptides, as well as randomized portions of whole proteins. A common procedure is the use of a scFv phage library which expresses on the N-terminus of the pIII protein covalently linked heavy and light chain variable regions of an immunoglobulin with randomized residues in the complementarity determining regions (Barbas et al., 1991). This library can be screened against any target protein/antigen and yield scFVs which would be specific for the target. Once selected and isolated, the scFv phage library clone can also be used to produce the scFv fragment alone. This method offers the possibility of selecting high-affinity antibodies without the use of immunization.

Similar libraries can be created from a pool of immune cell DNA specific for some target. For example, an Fab library was generated from the bone marrow of an asymptomatic HIV-1 seropositive individual (Burton et al., 1991). An Fab is similar to an scFv but instead contains the entire domain (constant and variable) of the antibody containing the heavy and light chain regions responsible for antigen recognition. Expression of the entire Fab fragment on the pIII protein involves fusion of the heavy chain to the N–terminus of the pIII protein, with the light chain linked to the heavy chain by noncovalent interactions and disulfide bonds. This random combinatorial Fab phage

library was screened with gp120 and mAb IgG1b12 was selected. This mAb is specific for HIV gp120 and has been extensively characterized (Burton et al., 1994).

Vectors other than phage have also been used to express random libraries. For example, the E. coli phage lamba receptor (Brown, 1992), fimbriae (Schembri et al.,1999), and flagellar-thioredoxin fusion protein (Lu et al., 1995) have all been used to make non-phage random libraries.

Filamentous bacteriophages are a group of viruses which contain a circular single-stranded DNA genome. The Ff class of filamentous phage (f1, fd, and M13) are virtually identical with 98% DNA sequence homology, and they use the N2 domain of the pIII protein to initiate infection of E. coli which contains the F plasmid and expresses a F conjugative pilus on its surface. The N2 domain of pIII binds to the tip of the F conjugative pilus and initiates retraction of the pilus bringing the pIII portion of the phage to the periplasm and initiating a chain of interactions which leads to the translocation of the phage DNA into the cytoplasm. Ff phage are approximately 6.5 nm in diameter and 930 nm in length. The phage particle weighs approximately 16.3 MD of which 87% is protein. The genome consists of a single-stranded circular DNA molecule 6400 nucleotides long that is stored in a flexible protein cylinder created by 2700 molecules of the 50 residue pVIII major coat protein (see Fig.2.1 for a diagram of a filamentous phage). At the head of the phage there are about 5 molecules each of the 33 residue pVII protein and the 32 residue pIX protein. In the tail, there are 3-5 copies of both the 406 residue pIII protein as well as the 112 residue pVI protein (reviewed in Barbas et al., 2001). The pIII protein itself is divided into three domains, N1, N2, and CT, separated by

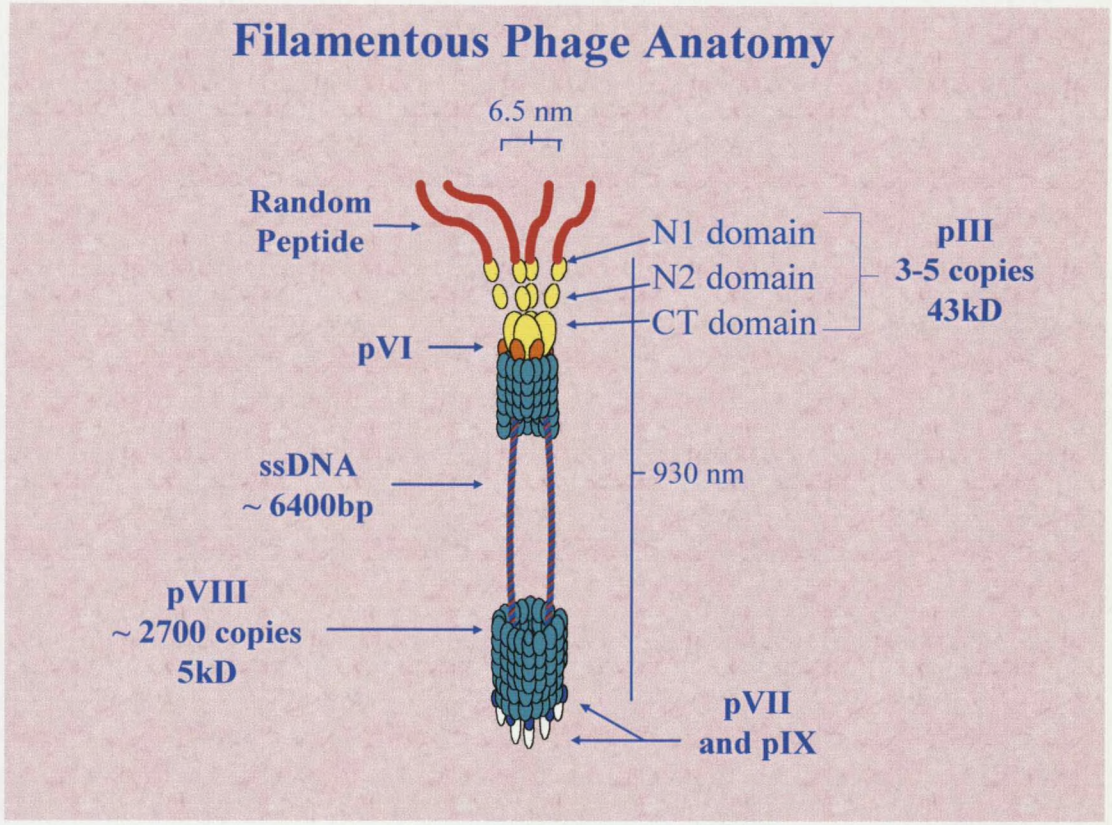

Fig. 2.1 **Cartoon of typical filamentous phage structure.** Shown is the overall size, structure, and position of the various phage proteins. Shown attached to the N1 domain of the pIII protein is the peptide insert (not drawn to scale). The peptide would be present on all the N1 domains of the pIII protein giving a total number of 3-5 peptides expressed per virion.

flexible and disordered glycine-rich regions (Fig.2.1). It is on the N–terminus of the N1 domain that the previously described pIII phage display peptide libraries are attached.

The library used in the present study is the J404 library created by Jim Burritt at the Department of Microbiology, Montana State University, Bozeman MT. This library was produced in the filamentous phage M13KBst and expresses a random 9 mer peptide sequence as an amino-terminal fusion to the phage capsid protein pIII with a complexity of ~5 X 10^8 unique phage (Burritt et al. 1996; Burritt et al. 1995). Also used was the Ph.D. -C7C Phage Display Peptide Library produced by New England Biolabs. This library expresses a loop with a 7 mer random sequence constrained by disulfide-linked cysteines fused to the amino-terminal of the phage capsid protein pIII of the M13 phage.

Methodology and Application of a Peptide Phage Display Library

This section is a discussion of the many variations that can be used while screening a random peptide phage display library against a target molecule. A number of these variations were used in this study and their results will be described in later chapters. Since it is a peptide library that was utilized in this work, the focus will be on the differences in methodology in using such a library.

The general methodology of using a peptide phage display library is to select a peptide sequence, often a consensus sequence, that binds specifically to the target molecule. A consensus sequence is defined as a sequence originating from a group of similar sequences which were combined together to form a "consensus" of like residues in the group. Once obtained, the sequence can be utilized a number of ways depending

upon the direction of the research. Common uses include mapping the sequence to the target binding site of a mAb, using the peptide in any number of activity assays (inhibition studies), or use the peptide to elicit an immune response. The screening of any peptide phage display library follows the same general procedures to obtain such a specific sequence, but within these procedures there are a number of variables that can be changed to enhance the possibility of success. The general procedures include 1. Fixing the target molecule, in this study the target molecules were mAbs. 2. Addition of the peptide phage library to the fixed mAb. 3. Extensive washing of the mAb to eliminate all phage which do not bind to the mAb. 4. An elution step to release the specific binding phage from the target protein. 5. Amplification of this pool of phage, and re-addition of this amplified pool of phage clones to the immobilized mAb for subsequent rounds of selection. Variations to these procedures can be utilized to improve specificity and to either increase or decrease phage clone diversity.

One of the most obvious variations in the procedure is the use of different libraries to obtain the peptide sequence that best fits the nature of the interaction. The types of libraries utilized can generally be divided into either linear or cysteine loop constrained peptide libraries. A linear library displays a specific number of residues in a linear fashion attached to the N-terminus of the displaying protein. The number of residues in a specific library can vary, anywhere between 6 to 21 residues (reviewed in Burritt et al., 1996). Which type of library to use would depend upon how many residues are needed for binding to the target protein. A cysteine-constrained peptide library contains an N–terminal peptide with two cysteine residues separated by a specific number of variable

residues. A disulfide bond is formed between the two thiol groups contained in the cysteine side chains forming a constrained loop expressing the variable residues in a loop conformation. Two such libraries used in our lab are a C-7-C, seven variable residue, and a C-10-C, ten variable residue library. Such libraries would be good candidates to use if it was previously known that the target molecule prefers to recognize a loop structure, or if future studies with the peptide involve display of the residues in the same constrained loop conformation.

Once a library is selected, the next variable is the method of screening. Two major options exist, either "panning" using a flat petri dish (polystyrene tubes have also been used) or by using Sepharose beads in a column. In "panning", the target molecule is attached to the petri dish, either alone or with a high affinity protein-protein linker such as Protein A. Normally this method uses less target molecule per round, so if the amount of target molecule used is limiting, this is a preferred method. The plate is generally blocked with an agent such as BSA or Blotto and after addition of the phage library, the plate is washed by simply rinsing a number of times to remove the non-binding phage.

The blocking agent used can vary but in our experience Blotto (5% non-fat dry milk, 0.5% Tween 20 in TBS) has been the agent of choice, since it has consistently given low background results in ELISA experiments. BSA (Bovine Serum Albumin) has been utilized as a blocking agent in the past but at times has resulted in high background in ELISA phage detection experiments. BSA has been utilized in the present study, but only when it was inappropriate to use Blotto (see Chapter 4, detection of bovine IgG ELISA experiments). In this instance, the presence of bovine IgG in the non-fat dry milk

component of blotto would cause a higher background in the negative control experiment, which was not seen when BSA was utilized. Other agents, such as ovalbumin have also been used with similar results as BSA.

The column method utilizes sepharose beads to attach the target molecule using either CNBr activiation, ProteinA, ProteinG, or any other high affinity protein linker. There is more time involved in preparing the beads and the amount of target molecule used is much greater, but this is also dependent upon the amount of beads used. Once prepared, the beads are pre-incubated with the phage library, placed in a column and washed with buffer. The column method is the most commonly used procedure when there is enough target molecule, normally between 300 and 1000 μg used for each screening. Either method will sometimes give better results than the other for the same target protein. The column method has the advantage of utilizing more target molecule to capture specific phage so one would suspect that this method might be more likely to elucidate a consensus sequence, but this is only speculation.

Generally the amount of peptide phage display library added to the target molecule, and the amount of washing of that library are fairly standard across most protocols. However, there is some variation in how the specific binding phage can be eluted from the target molecule. The most commonly used protocol is a non-specific low pH elution using a pH 2.2 glycine-HCl solution to wash over the plate or column. This nonspecifically disrupts any phage/molecule interactions by partially denaturing the protein involved and releases bound phage for amplification or characterization. This low pH wash is common to a number of protocols used to disrupt protein/protein

interactions, e.g. elution of mAbs from a Protein G column. A similar procedure sometimes used in phage display is the use of a high pH elution step (pH 10.5, 0.5M NH_4OH solution) to release binding phage. This type of elution might be preferred if it is believed that the interaction of the phage peptide to the target molecule involves basic residues with pKa values in this high pH range. Generally, this wash is used in conjunction with a low pH wash, to screen both ends of the pH spectrum and elute all possible phage clones. Other elution methods could be used such as various solvents and denaturants to disrupt the peptide/mAb interactions but the limiting factor in elution is to release the attached phage particle without the loss of infectivity of the virus. This could still be possible with the use of solvents and denaturants at the proper concentrations but to remove such solutions from the elution would require much more time and effort versus a pH wash where the pH level can be adjusted relatively quickly.

A different, more specific elution that is often utilized is the use of an antigen or protein that binds to the target protein with high affinity and would displace any phage binding specifically to that region. For example, if a mAb is used to screen a library to elucidate sequences which bind to its antigen recognition site, the antigen specific for that mAb could be used in the elution buffer to displace phage specifically from the mAb. This type of elution obviously is time- and concentration- dependent, but its specificity is greater than a low or high pH elution. There is the possibility, when using multiple rounds of selection, of using a low or high pH elution in one round followed by a specific protein elution for the next round. In principle, this would seem to help in selecting for specific binding phage clones, but in our experience this seems not to be the case. We

have seen that whatever elution is used in the first round influences any subsequent round, such that changing the elution method in later rounds had no real effect on the outcome of the sequences. It appears that the first round selection is the most crucial. It is the first selection step and it forms the pool of phage from which the subsequent selections are made. In these subsequent rounds, the diversity of the first biased phage pool is merely being reduced to increase the fraction of binding phage and this process may not be significantly affected by a more specific elution technique. In our experience, using one method of elution throughout the entire experiment gives the best success in elucidating a consensus sequence.

One other variable that needs to be considered is how many selection and amplification rounds are needed to elucidate a consensus sequence. There seems to be a direct correlation between the number of rounds selected versus a loss in diversity of phage in the elution. This is probably caused by both the amplification step between each round as well as the actual selection process. Three to four rounds of selection are commonly used in phage display screening mAbs, and it is thought that using fewer rounds would not bias the phage pool enough, making it difficult to isolate the specific phage clones. Using more rounds could bias the pool too much, possibly leaving very few unique clones from which to determine a consensus sequence. Having fewer unique clones could hinder determination of the pertinent residues involved in binding, because it is helpful to have phage clones with a range of binding affinities and to correlate binding strength with the presence or absence of certain residues in the peptide sequence. Theoretically though, by using more rounds of selection, even though the diversity would

decrease, the affinity of the remaining phage clones should be higher and those clones should represent a sequence which has a higher affinity to the target protein.

Application of Phage Display to the HIV Glycoprotein gp120

As previously discussed, phage display library technology can be a powerful tool in determining protein/protein interactions in biological systems. The idea behind using such technology in this study was to determine the amino acid residues on the antigen that form the binding site for the mAbs. Determining the residues on the HIV glycoprotein gp120 which interact with a number of specific mAbs would assist in determining the 3D structure of the mAb binding sites as well as show residues which are exposed and antigenic on gp120. The purpose of this study was to use a peptide phage display library to screen mAbs directed against the glycoprotein gp120 and elucidate peptide sequences which mimic structures found on gp120. Such peptide sequences would be a linear representation of at least part of the binding site of the mAb, and could then be used in future studies as a possible peptide-conjugate vaccine candidate. As previously discussed, there are a number of antigenic sites on gp120 and a number of mAbs that have been isolated against all regions of gp120. To be useful, the elucidated peptide sequence would need to mimic a region of gp120 that elicits a neutralizing Ab immune response and is relatively well conserved throughout the various strains and clades of HIV. MAbs directed against the CD4 binding site of gp120 meet these requirements and a panel of such mAbs were used in this study. All the mAbs selected also have the characteristic of binding to discontinuous epitopes on gp120. A discontinuous epitope is

defined as the binding site of a mAb that is represented by various separate regions of the protein that come together to form a binding site not represented by a single linear sequence. These mAbs do not bind denatured protein or to a panel of synthetic peptides representing overlapping linear sequences of gp120. There are a number of procedures that can be used to determine the binding site of mAbs that bind to a linear segment of a protein, but it is more difficult to determine the exact binding site of a discontinuous epitope binding mAb without a crystal structure of the bound mAb/antigen complex. Peptide sequences have been elucidated from phage display screening of discontinuous epitope binding mAbs and such linear peptides have been shown to mimic amino acids on the antigen that are comprised of non-adjacent residues (Jesaitis et al., 1999; Burritt et al., 2001; Burritt et al., 1998). The use of peptide phage display technology represents a unique opportunity to screen a panel of CD4 binding site mAbs to the HIV glycoprotein gp120 and determine a linear peptide sequence which would mimic a complex, antigenic, and conserved epitope on gp120. Such a peptide mimic could then be coupled to an appropriate carrier and used as a vaccine candidate to elicit an immune response against this specific region of gp120.

Description of mAbs Used in Study

As previously discussed, a number of mAbs have been found that are directed against the conserved CD4 binding site of gp120. Five such mAbs which were used in this study are described below as well as another mAb, 2G12, which is directed against a unique binding site separate from the CD4bs on gp120.

MAb F105 (Posner et al., 1991) is a human IgG1 which was obtained from Dr. Marshall Posner at the Department of Medicine, Dana-Farber Cancer Institute, Boston, MA. F105 originated from a seropositive AIDS patient and was derived from fusion of Epstein-Barr virus (EBV) transformants with an immortal myeloma cell line to create mAb-producing hybridoma cells. F105 has been found to bind cells infected with HIV-1 strains MN, RF, IIIB, and SF2 as well as inhibiting infection of naive cells with HIV-1 in standard neutralization assays (Posner et al., 1991). Soluble rCD4 pre-bound to infected cells inhibits F105 binding and F105 also does not react with denatured gp120 on western blots, which characterizes it as a CD4bs discontinuous epitope mAb. Significant synergistic neutralization is seen when F105 is combined with other gp120 mAbs specific for other antigenic sites of gp120 (Potts et al. 1993; Li et al., 1998). Mutations of amino acids in gp120 that abrogate binding of F105 include residues in the C2, C3, C4, and C5 regions (Thali et al., 1991). F105 is being used in animal studies and human trials of passive immunotherapy (Cavacini et al., 1998; Baba et al., 2000).

MAb 1125H is a human IgG1 which was obtained from Dr. S. Tilley at the Department of Developmental and Structural Biology, Public Health Research Institute, New York, NY. 1125H originated from an asymptomatic HIV seropositive haemophiliac and was EBV transformed into a mAb producing cell line. The mAb has potent neturalizing activity against strains MN, RF, IIIB, and SF2, as well as some primary isolates (Tilley et al., 1991). Its binding to gp120 is also inhibited by the presence of sCD4, and is totally lost with the reduction of disulfide bonds but not by deglycosylation of gp120 thus characterizing this mAb also as a CD4bs discontinous epitope mAb (Tilley

et al., 1991). Mutations of a wide number of residues abrogate binding of the mAb including Trp 427 (Thali et al. 1992).

MAb 5145A is a human IgG1 which was also obtained from Dr. S. Tilley. 5145A also originated from an asymptomatic HIV seropositive haemophiliac and was EBV transformed into a mAb producing hybridoma cell line. The mAb reacts with 14 of 15 HIV-1 isolates tested, including 6 that were not recognized by 1125H (Pinter et al., 1993). 5145A binding to gp120 is inhibited by soluble CD4 as well as mAb 1125H, and binding is lost with reduction of the gp120 disulfide bonds but not by deglycosylation. Even though it overlaps with other CD4bs mAbs, epitope mapping using gp120 mutants shows the mAb binding sensitivity contrasts with 1125H. In particular, mutations on residue Trp 427 have no effect on 5145A binding, but mutations to residues Asp 368 and Glu 370 are markedly sensitive, much like mAb F105 (Pinter et al., 1993).

MAb F91 is a human IgG1 and was obtained from Dr. J. Robinson at the Department of Pediatrics, Tulane University Medical Center, New Orleans, LA. It originated from a HIV-1 seropositive patient and also is specific for the CD4 binding site of gp120. Its binding is not effected by deglycosylation of gp120 and it has been found to have strong cross-reactivity with gp120 monomers from subtypes A-F and can neutralize the IIIB strain of HIV-1 (Moore and Ho, 1993; Moore et al., 1994).

MAb IgG_1b12 is a human IgG1 and was obtained from the NIH AIDS Reference and Reagent Program and had been donated by Dr. D. Burton at Scripps Research Institute, La Jolla, CA. B12 originated from an Fab phage display library constructed from the bone marrow of an HIV-infected donor and the b12 clone was then expressed as

a whole mAb in CHO cells (Burton et al., 1991). It has also been characterized as a CD4bs discontinuous epitope mAb and has been termed the "best" of these mAbs due to it potent neutralizing activity across a broad range of various HIV-1 clades (Burton et al., 1994; Poignard et al., 1996). The low level of mAb needed for neutralization has allowed it to be used extensively as a passive immunization candidate. B12, in combination with other neutralizing mAbs, has been shown to be effective in neutralizing a SHIV challenge to macaques (Mascola et al., 2000; Hofmann-Lehmann et al., 2001).

MAb 2G12 is a human IgG1 that was obtained from the NIH AIDS Reference and Reagent Program and had been donated by Dr. H. Katinger from the Institute of Applied Microbiology, Vienna, Austria. 2G12 originated from a HIV-1 seropositive volunteer and was fused into a myeloma cell line for production of the human mAb. 2G12 is not a CD4bs mAb but binds to a unique portion of gp120 not mimicked by any other mAb (Trkola et al., 1996b). Its binding is conformationally dependent, a discontinous epitope, but also is possibly carbohydrate dependent. 2G12 neutralizes a number of laboratory strains as well as a broad range of primary isolates (Trkola et al., 1996b), it acts in a synergistic manner when combined with other neutralizing mAbs (Li et al., 1997) and also has been shown effective in neutralizing a SHIV challenge to macaques (Mascola et al., 2000). Recently, evidence has suggested that the binding epitope of 2G12 on gp120 might be entirely composed of carbohydrate structure (Sanders et al., 2002). Since glycosylation is carried out by host enzymes in the cell, the sequence of any one carbohydrate chain is unlikely to be unique to HIV gp120 and therefore recognizable as "foreign". However, the highly glycosylated nature of gp120, as well as a high proportion

of high-mannose glycans, may facilitate formation of a unique binding epitope utilized by the mAb 2G12 that is composed entirely of carbohydrate.

Previously Work Related to gp120 and Phage Display Libraries

Even though the exact experiments described in this study have not been performed by others, there is a great deal of work that has been done that is similar in nature to the work presently described here and thus should be mentioned. Foremost is previous work describing the use of phage display technology in elucidating peptide sequences that mimic discontinuous epitopes. Jim Burritt and Al Jesaitis at the department of Microbiology at Montana State University have published a number of papers reporting phage display results that convincingly show peptide sequences which mimic discontinuous epitopes (Jesaitis et al., 1999; Burritt et al., 2001; Burritt et al., 1998). Their work has centered around various mAbs that interaction with the integral membrane protein cytochrome *b*.

The Seth Pincus lab, formerly at the department of Microbiology at Montana State University, has used peptide phage display screening to determine a peptide mimetic of a carbohydrate structure, the group B streptococcal type III capsular polysaccharide (Pincus et al., 1998). The peptide sequences were determined by screening the peptide phage library against mAbs which bind specifically to the polysaccharide. There are also other examples of peptides elucidated by phage display libraries which mimic carbohydrate structure (Grothaus et al., 2000) as well as many peptide mAb epitopes which have been determined using this method (reviewed in Burritt et al. 1996; Smith and Petrenko, 1997).

In regard to gp120, the highly characterized human mAb IgG1b12 has been screened using a peptide phage display library and a constrained consensus sequence was determined, which bound to the mAb, could inhibit the mAb-gp120 interaction, and could be mapped to the structure of gp120 (Zwick et al., 2001). However, the peptide sequence did not elicit Abs that cross-reacted with gp120 in lab animals. Also, results were published regarding the phage display screening of a number of mAbs that are directed against gp120 (Boots et al., 1997). The mAbs used included two V3-loop directed mAbs (19b and 447-52D), mAb IgG1b12, mAb A32 (directed against the C2 and C4 domain), and mAb 50-69 (a gp41 binding mAb). A number of different libraries were screened with each mAb and the sequences of the isolated peptides were reported. Even though a number of phage display experiments were performed, there is very little data to support the conclusions discussed in the publication. Some of the mAb experiments isolated sequences which appeared to map to residues of gp120 or gp41, but no data was shown to determine if the phage clones actually bind to the mAbs from which they were isolated, not to mention if they would bind specifically to the antigen recognition site of the mAb. The paper describes some sequences as gp120-competing simply because they were eluted by gp120 during the elution step of the phage display experiment, not because of any gp120 binding inhibition experiment performed. Due to the lack of any binding assay data, peptide inhibition data, or any inhibition data at all, the sequence results reported in this publication are preliminary at best. There is a great deal of work to be done to show that these sequences are what they are claimed to be.

The use of a peptide as a vaccine candidate, termed an epitope vaccine, has been

suggested as a new way to neutralize HIV virus infection if it can elicit neutralizing antibodies (reviewed in Xiao et al., 2001). A peptide candidate for such work is the sequence ELDKWA, which was determined to be contained in the binding epitope of human mAb 2F5, a IgG1 mAb directed against an exposed portion of the extracellular domain of gp41 (Muster et al. 1993). 2F5 is a potent neutralizing mAb and has been used in combination with other neutralizing mAbs, F105, 2G12 and IgG1b12, for passive immunization in monkey trials (Mascola et al., 2000, Hofmann-Lehmann et al., 2001).

CHAPTER THREE

DETERMINATION AND CHARACTERIZATION OF PEPTIDE MIMETICS OF HIV-1 GP120

Introduction

This chapter contains data which has been submitted for publication under the title "Peptide Mimetics of Discontinuous Epitopes at the CD4-Binding Site of HIV-1 gp120." (Jacobs, J.M., Intamaso, U., Wilkinson, R., Pincus, S.H., Burritt, J.B., Posner, M.R., Tilley, S.A., Robinson, J., Teintze, M., 2002, submitted) All experiments described were performed by J. Jacobs in either the M. Teintze or S. Pincus laboratory except for Fig. 3.9 and Fig.3.10 which was performed by R. Wilkinson and Fig. 3.12 which was performed by A. Intamaso. Linking of the OVA and KLH conjugates in the immunization protocol was also performed by R. Wilkinson and A. Intamaso. This chapter details a more thorough description of the experiments and results which were performed to obtain peptide mimetics of the CD4-binding site of gp120 than what was previously described in the submitted article.

Background

The envelope glycoprotein gp120 is the major viral protein on the surface of HIV virions, and is therefore a logical choice for a vaccine. Unfortunately, attempts to use recombinant gp120 (rgp120), or its precursor gp160, as HIV vaccines have not been

successful (Mascola et al., 1996; Graham and Gorse, 1998; Conner et al., 1998; VanCott et al., 1995). Many of the antibodies generated by such immunizations are unable to neutralize HIV (Mascola et al., 1996; Pincus et al., 1993; Pincus et al., 1997). Many of the neutralizing antibodies that are readily generated by immunization with gp120 are directed at different epitopes of gp120 than those found after HIV-1 infection (Pincus et al., 1993; Pincus et al., 1997; Pincus et al., 1994a; Pincus et al., 1994b; Javaherian et al., 1989; Nara et al., 1988) and neutralize only a limited number of HIV strains (Palker et al., 1988); mutations can also readily generate viruses that are no longer neutralized by such antibodies (Nara et al., 1987; McKeating et al., 1989). Late in the course of HIV-1 infection of humans, however, antibodies with broader specificity for different HIV isolates appear (Berkower et al., 1989; Robert-Guroff et al., 1985; Weiss et al., 1986). Many of these recognize discontinuous epitopes at the CD4 binding site (VanCott et al., 1995; Steimer et al., 1991; Haigwood et al., 1990; Haigwood et al., 1992). The CD4 binding site on gp120 represents a conserved structure, since almost all HIV isolates are able to bind CD4. An antigen that mimics and effectively displays this conserved epitope recognized by broadly neutralizing antibodies may therefore be able to elicit a more effective immune response than vaccination with whole gp120.

The three-dimensional structure of a complex between a "core" gp120 and the N-terminal domain of CD4 has been determined (Kwong et al., 1998). Although parts of gp120 were removed to facilitate crystallization, and some regions were disordered, the gp120-CD4 interface is well resolved in this structure and the gp120 residues at the CD4 binding site are well defined. However, there is evidence for conformational changes in gp120 upon CD4 binding (Myszka et al., 2000; Denisova et al., 1997; Bachelder et al.,

1995), so one cannot presume that the CD4 binding site has the same conformation in the presence and absence of CD4. One way the CD4 binding site in free gp120 can be explored without a crystal structure, is by mapping the binding sites of monoclonal antibodies (mAbs) that bind to the same region and recognize conformation-dependent epitopes. This study includes five such mAbs: F105 (Posner et al., 1991), 5145A (Pinter et al., 1993), 1125H (Tilley et al., 1991), F91 (Moore et al., 1994), and IgG_1b12 (Burton et al., 1991; Burton et al., 1994). These mAbs were obtained from HIV-infected patients, and display neutralizing activity against a variety of HIV-1 strains; they block the binding of CD4 to gp120, and do not bind denatured gp120 or synthetic gp120 peptides. Another mAb, 2G12, was also included in this study, but is directed to another conserved, conformation-dependent epitope on gp120 that does not overlap the CD4 binding site (Trkola et al., 1996). F105, 2G12, and IgG_1b12 have been used as part of a combination of mAbs for passive immunization of primates against SHIV (Hoffmann-Lehmann et al., 2001).

A number of different methods were used to screen random peptide phage display libraries against these mAbs to identify sequences that bind with high specificity. Three such sequences were determined corresponding to three mAbs: F105, 5145A, and F91. Two of these sequences were used to immunize mice. Linear peptide sequences displayed on phage proteins have been shown to mimic discontinuous epitopes on antigen proteins in a number of other systems (Jesaitis et al., 1999; Burritt et al., 2001; Burritt et al., 1998). In our case, whether the epitope is part of the CD4 binding site on gp120 or merely close to it, using such a peptide structure as part of a vaccine antigen should elicit

antibodies with similar specificity for gp120 as the original antibodies obtained from the patients.

Materials and Methods

Materials

Recombinant gp120 ($rgp120_{SF2}$) expressed in Chinese hamster ovary cells was a generous gift from the Chiron Corporation (Emeryville, CA). MAbs F105 (from M. Posner), 2G12 (from H. Katinger), and IgG_1b12 (from D. Burton) were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. MAbs 1125H and 5145A were obtained from S. Tilley, and mAb F91 was obtained from J. Robinson. The J404 phage display library used in these studies was obtained from J. Burritt. It was produced in the filamentous phage M13KBst and expresses a random 9mer peptide sequence as an amino-terminal fusion to the phage capsid protein pIII (Burritt et al., 1996; Burritt et al., 1995). The library has a complexity of ~5 X 10^8 unique phage. Peptides were purchased from Research Genetics, Inc.(Huntsville, AL) and further purified to >90% by HPLC. Phage DNAs were sequenced using T7 Sequenase (Amersham Pharmacia, Piscataway, NJ). Polyclonal antiserum against M13 was produced by immunizing rabbits with M13KBst bacteriophage and was made in the A. Jesaitis laboratory. Alkaline phosphatase-conjugated goat anti-rabbit IgG (H+L) was purchased from BioRad (Hercules, CA). Goat anti-human IgG (Fc specific) alkaline phosphatase-conjugate was purchased from Sigma Chemical (St. Louis, MO).

ELISA Experiments

ELISA experiments were used to measure a) binding of phage to mAb, b) inhibition of phage binding to mAb by antigen or peptides, and c) peptide inhibition of mAb binding to antigen. MAb or protein was coated directly onto micro titer wells (Immulon 2, Dynatech, McLean, VA) at 0.5 to 1 μg/ml overnight at 4°C. Wells were blocked with Blotto, containing 5% nonfat dry milk, 0.5% Tween 20 in TBS (50 mM Tris, 0.15 M NaCl, pH 7.5) for 3 hours at room temperature (RT). For direct phage binding, phage was added at 100 μl per well overnight at 4°C. Plates were washed, incubated with rabbit anti-M13 serum at a dilution of 1:5,000 for 2-4 hours at RT, washed, incubated with goat anti-rabbit alkaline phosphatase-conjugated IgG at a 1:1,000 dilution for 1-2 hours at RT, washed, then developed with addition of *p*-nitrophenyl phosphate (Sigma Chemical). For addition of inhibitor, 100 μl of peptide or antigen was added per well following blocking for 1 hour at RT, followed by addition of phage at 20 μl per well for overnight incubation at 4°C. The remaining steps were as described above. For peptide inhibition of mAb binding to antigen, peptide and Ab were pre-incubated in 100 μl for 1 hour at RT, then added to wells following blocking. After incubating overnight at 4°C, plates were washed, incubated with goat anti-human IgG alkaline phosphatase at a 1:1,000 dilution for 2-4 hours at RT, washed, then developed with substrate. A_{405} was determined using one of two different micro plate readers, EL-320 (BioTek Instruments, Winooski, VT) or Safire, (Tecan, Austria GmbH).

Phage Selection Experiments

Two separate methods were utilized to select phage for binding to the mAbs. The method of "panning" involved having each mAb immobilized on 60 mm petri dishes, incubated overnight at 4°C, washed, blocked with Blotto overnight at 4°C, and washed again. Phage library was added at $1x10^{12}$ pfu, incubated overnight at 4°C, washed extensively (0.5% Tween 20 in TBS), and eluted by addition of either a specific antigen wash, rgp120 in 4X molar excess for 2 hours at RT, or by a non-specific low pH wash with 0.1M glycine, pH 2.2. The eluted phage were neutralized immediately and were then amplified in *Escherichia coli* strain K91, precipitated with 2.5%PEG/0.5MNaCl, resuspended, and then used in subsequent rounds of selection (Smith and Scott, 1993).

The column method involved immobilization of mAb on cyanogen bromide-activated Sepharose 4B (Amersham Pharmacia, Piscataway, NJ) at 2 mg of mAb/ml of beads. The beads were divided into one-third aliquots for three rounds of selection. The phage library ($1x10^{12}$ pfu) was added to the first aliquot and incubated overnight at 4°C. The bead/library mixture was placed in a column, washed extensively with ice-cold wash buffer (1% BSA, 0.5% Tween20 in TBS) and adherent phage were eluted with either the specific antigen wash, $rgp120_{SF2}$ in 4X molar excess for 2 hours at RT or the non-specific low pH wash with 0.1M glycine, pH 2.2. The eluted phage were neutralized immediately and then amplified, as described above. Amplified phage were then placed with the 2nd aliquot of the beads and the selection was repeated until three rounds of selection were completed. Each mAb was screened using a variety of the above described methods, as explained in the Results section.

Analysis of Selected Phage

After the third-round of selection, phage were diluted and plated to obtain single plaques. Phage minipreps were prepared from isolated plaques by growing infected cells in Luria broth containing 75 μg/ml kanamycin, and phage were isolated by PEG/NaCl precipitation as described above. Phage were screened by ELISA to select for clones binding to the mAb of interest. DNA from these phage clones was then sequenced to determine the amino acid sequences of the displayed peptides, as described (Burritt et al., 1996; Burritt et al., 1995).

Peptide-Carrier Conjugates

Synthetic peptides corresponding to the phage sequences with an additional C-terminal Cys residue (pep44FgC and pep19ALC) were prepared and coupled to maleimide-activated keyhole limpet hemocyanin (KLH) or ovalbumin (OVA) (Pierce Chemical) at a 1:2 ratio (wt/wt) according to the manufacturers directions. Uncoupled peptides were removed by dialysis against phosphate buffered saline (PBS).

Immunizations

Three 5-6 week old CD1 outbred mice were each injected at six or more locations subcutaneously with 50 μg KLH-peptide conjugate in 50 μl, emulsified with an equal volume of complete Freund's adjuvant. A booster immunization of 25 μg conjugate in incomplete Freund's adjuvant was given as a single intraperitoneal injection one month later, and mice were bled 10 and 17 days after the boost. Sera were tested for binding to OVA-peptide conjugate and with $rgp120_{SF2}$ in ELISA assays. Plates were coated with

OVA conjugate or gp120 at 1 μg/ml and bound antibodies were detected with goat anti-mouse alkaline phosphatase conjugate, as described above.

Results

Screening the Phage Display Peptide Library

A phage library expressing a random nine-amino acid sequence was screened against F105, 5145A, 1125H, F91, and IgG_1b12, five human IgG1 mAbs that recognize the CD4 binding site on HIV-1 gp120, as well as mAb 2G12. After three rounds of selection, at least 30 individual phage clones were screened by ELISA for specific binding to each respective mAb and sequenced.

MAb F105 screening utilized three rounds of panning, each followed by a $rgp120_{SF2}$ elution at neutral pH. Screening was initially performed using the low pH elution method, but no consensus sequence was found among the eluted phage, so the more specific gp120 elution was utilized. Phage binding to mAb F105 fell into the consensus sequence group seen in Table 3.1. Also during this screening an additional consensus sequence, QSYP, was found among the screened phage which was found to bind mAb samples F91, F105 and 1125H, as well as several irrelevant mAb samples in ELISA experiments. It was subsequently determined that this consensus was being selected by bovine IgG present in the mAb preparations as described in chapter four. MAb F105 was also used to screen a C-7-C library, with three rounds of a gp120 elution. No consensus was determined and no clones were found that would bind the mAb in ELISA experiments.

MAb F91 was also used to screen the J404 library, utilizing three rounds of panning, each followed by a rgp120$_{SF2}$ elution at neutral pH. 55 out of 58 phage clones isolated using F91 represented only one sequence, 1pF9g (See Table 3.1).

1125H was used to screen the J404 library using both panning and column methods with low pH and gp120 elutions, but no consensus sequence was found. A number of phage with sequences containing a large number of aromatic residues, Trp, Phe, and Tyr, as well as Arg and Lys were found to bind mAb1125H (Table 3.2). Also seen were phage clones which have mutations in the Gly-Pro-Pro linker region connecting the random 9 mer sequence with the pIII protein of the phage. Such mutations in this region are rare, and are due to frame shifts or deletions of the DNA which occur during the ligation and transformation steps in the production of the library.

TABLE 3.1 **Amino Acid Sequences of Phage Displayed Peptides Isolated by Screening Against mAbs F105, 5145A, and F91.**

F105 Selected Phage	
Phage Clone	Sequence
44Fg	**R** L T **P** **E** S **D** D R
9Fg	T S L R **P** **E** **P** **D** E
117Fg	N A **R** Y G E P **P** V
53Fg	Q A **R** Y A K **E** **P** **D**
97Fg	M **R** F A A **E** **P** **D** Q
34Fg	**R** W F **P** L G **D** A I
Consensus	**R** X X **P** **E** **P** **D**

5145A Selected Phage	
19AL	W K P V V I D F E

F91 Selected Phage	
1pF9g	L Y A G S W S A W

Table 3.2 **Amino Acid Sequences of Phage Displayed Peptides Isolated by Screening Against mAb 1125H.**

Phage Clone	Sequence
1HL	S I P W P A V E R A R **L E**
24HL	F S N K L Y W N G
10HL	T S V K D W R Y Y G P L **V E**
15HL	G R W N G T F I
40HL	S K G A W S W G P A **V E**
30HL	D P D S P W S W R A R **V E**
11HL	A S V Y G N W S G
29HL	D G D W L L H V G
37HL	S N S N V P N V L
21HL	W K P V V I I L R
32HL	W N N W P A **V E**
50HL	T S W M P V K V V
46Hg	G K G P W W K G L
10Hg	W I G
Normal	X X X X X X X X X G P P **V E**

These mutations, if they are to be present on viable phage virus, must be accompanied by another insertion or deletion that returns the sequence to correct reading frame before the pIII protein is coded for, so as not to alter the structure of the pIII protein. However, the sequences selected by 1125H were also found to bind non-specifically to other mAbs. Fig. 3.1 shows an example of the binding of these clones to mAb F105 and mAb 1125H. It is believed that since there is no real consensus among the sequences, that these peptides are merely "sticking" to the mAb indiscriminately and do not represent sequences which specifically bind to a constant region shared between the mAbs. Similar sequences were isolated during other phage display experiments as described below.

MAb 5145A was initially used to screen the J404 library by the panning method, using both a low pH and gp120 elution, but both methods yielded only non-specific binding phage as well as phage with mutations in the random peptide and linker region as described for 1125H above. MAb 5145A was then coupled to Sepharose beads and used for three rounds of column selection, each eluted by 0.1 M glycine, pH 2.2. After the last round of selection, thirty phage clones were assayed for mAb binding by ELISA. Nine clones were positive for specific binding to mAb 5145A, and all of these had the same sequence, 19AL (Table 3.1).

MAb 2G12 was used to screen the J404 library utilizing a combination approach of low pH elution for the first two rounds with the more specific gp120 elution for round three, also followed by a low pH elution. Phage isolated from the third round gp120 elution as well as the low pH elution showed no consensus sequence and no specific binding for 2G12. Also, mAb 2G12 cross-reacted with all phage in ELISA experiments, even the parent type M13KBst phage (Fig. 3.2). It is not known why mAb 2G12 would

cross-react with M13 phage. This was only seen with mAb 2G12 in our experiments and has not been reported by others. Nevertheless, mAb 2G12 binds with high affinity to gp120, as shown later in this chapter. Since 2G12 bound all M13-based phage, it was difficult to determine which phage, if any, were binding specifically via their displayed peptides and this most likely caused the library screening to fail. MAb 2G12 was therefore not pursued for phage library screening, but it was used in later experiments as a control mAb for binding to gp120 and did not display any cross-reactivity against blocking agents, or primary and secondary detecting antibodies during these ELISA experiments. Mab 2G12 was used as a control mAb since it was a human mAb (like F105 and 5145A) that bound to the same antigen, but bound to a region which did not overlap the CD4-binding site, and therefore did not cross-react with peptides mimicking the CD4-binding site.

MAb IgG1b12 was also used to screen the J404 library using a combination approach of a low pH elution for the first two rounds coupled with the more specific gp120 elution for round three, also followed by a low pH elution. Phage clones which were isolated from the third round of both the low pH and gp120 elutions fell into the non-specific binding category, similar to those seen for mAb 1125H. We were unable to obtain any positive results when using the combination approach of low pH and gp120 elution, discussed previously in chapter two. MAb IgG1b12 might have produced positive results if a purely gp120 elution protocol was followed for all three rounds. Other reasons for the failure to determine a consensus peptide from the IgG1b12 experiment will be discussed later in the chapter.

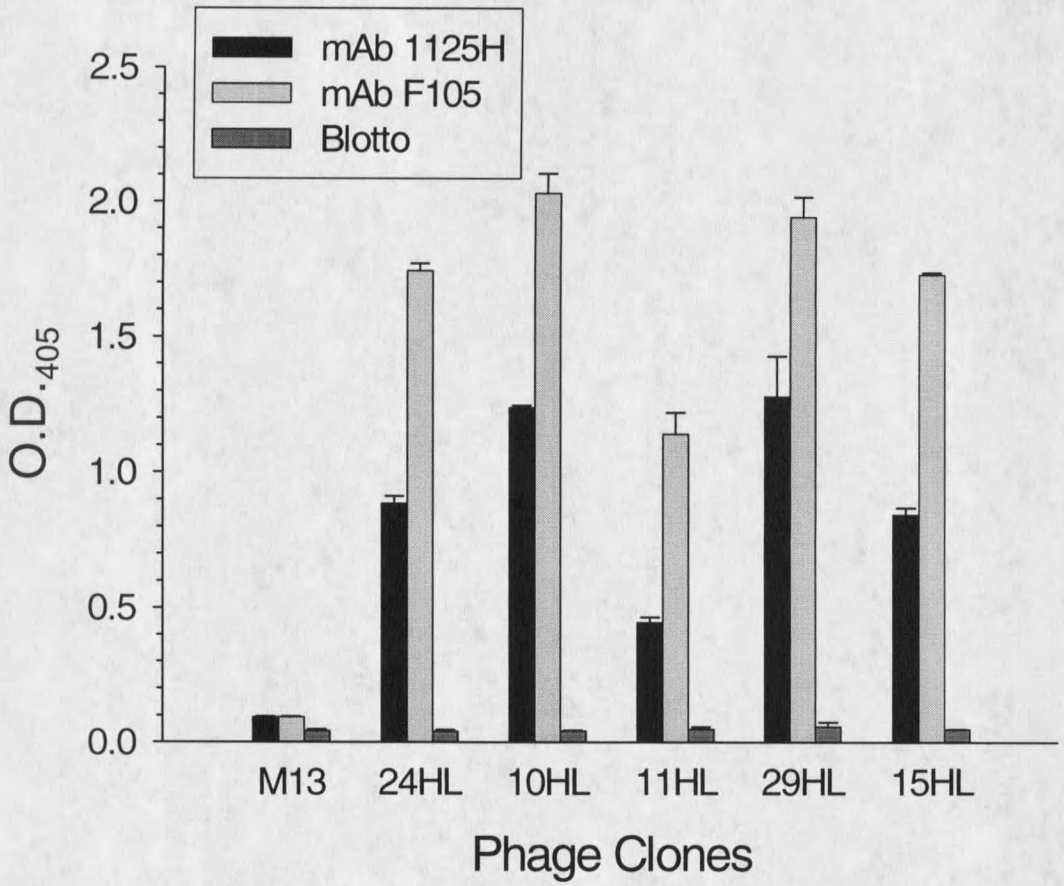

Fig.3.1 **Binding of phage clones to mAbs 1125H and F105.** mAb was used to coat wells at 1 µg/ml. Phage clones were added at $1x10^9$ pfu per well with parent phage M13KBst shown as a negative control. Control wells with only the blocking agent blotto are also shown as a negative control. Phage binding was detected by rabbit anti-M13 antiserum followed by AP-conjugate goat anti-rabbit secondary Ab. O.D. values shown are the mean of duplicate

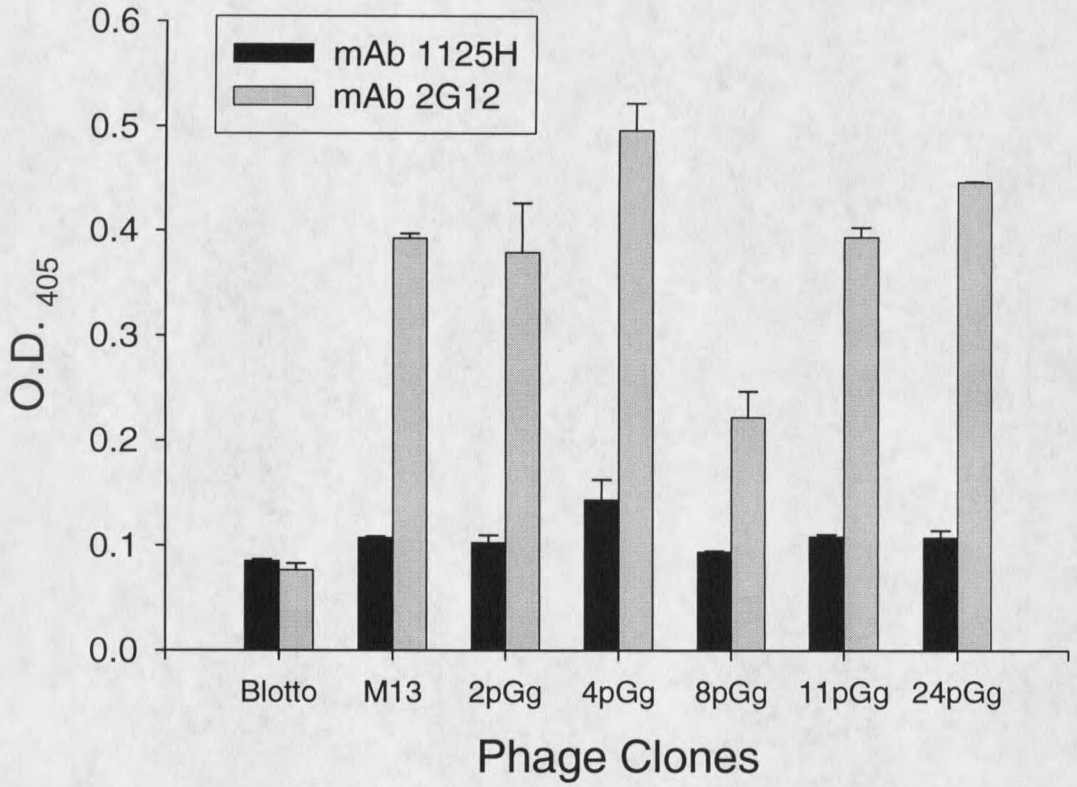

Fig.3.2 **Binding of phage clones to mAbs 1125H and 2G12.** MAb was used to coat wells at 1 μg/ml. Phage clones were added at $1x10^9$ pfu per well with parent phage M13KBst shown as a negative control. Phage binding was detected by rabbit anti-M13 antiserum followed by AP-conjugate goat anti-rabbit secondary Ab. O.D. values shown are the mean of duplicate samples with standard deviations (SD) shown as error bars. Phage clones shown are those isolated from mAb 2G12 phage library screening and are shown for comparison against the M13 background level.

In summary, out of six mAbs that were screened, some having been screened a number of times, three mAbs gave sequences which were found to bind specifically to their respective mAb. The remaining chapter describes the results obtained using these three sequences.

ELISA Binding and Competition Experiments

Figure 3.3 shows the specificity of the phage clones to their respective mAbs. Phage clones 53Fg and 44Fg showed specificity for F105 (Fig. 3.3), as did phage clones 9Fg and 97Fg (data not shown), but at a lower avidity. Phage clone 1pF9g specifically bound mAb F91, while phage clone 19AL also bound specifically to mAb 5145A (Fig.3.3).

To determine that each phage clone specifically bound to the antigen binding site on the mAb, a gp120 inhibition experiment was performed (Fig.3.4). Each mAb was coated on an ELISA plate and incubated with the corresponding phage clones in the presence of varying concentrations of $rgp120_{SF2}$. Adding gp120 greatly reduced the binding of phage 44Fg to mAb F105, phage 19AL to mAb 5145A, and phage 1pF9g to mAb F91 (Fig. 3.4).

Synthetic peptides corresponding to either sequences displayed on specific phage clones (pep19AL = WKPVVIDFE, pep1pF9g = LYAGSWSAWP), or a consensus sequence (pep44Fg = RLTPEPDD), were tested for their ability to inhibit binding of phage clones to their respective mAbs. ELISA plates were coated with mAb and incubated with phage clones in the presence of varying concentrations of peptide. At 0.5 μM concentration, pep44Fg inhibited binding of phage 44Fg to its corresponding mAb F105, and pep19AL inhibited binding of phage 19AL to mAb 5145A, while neither

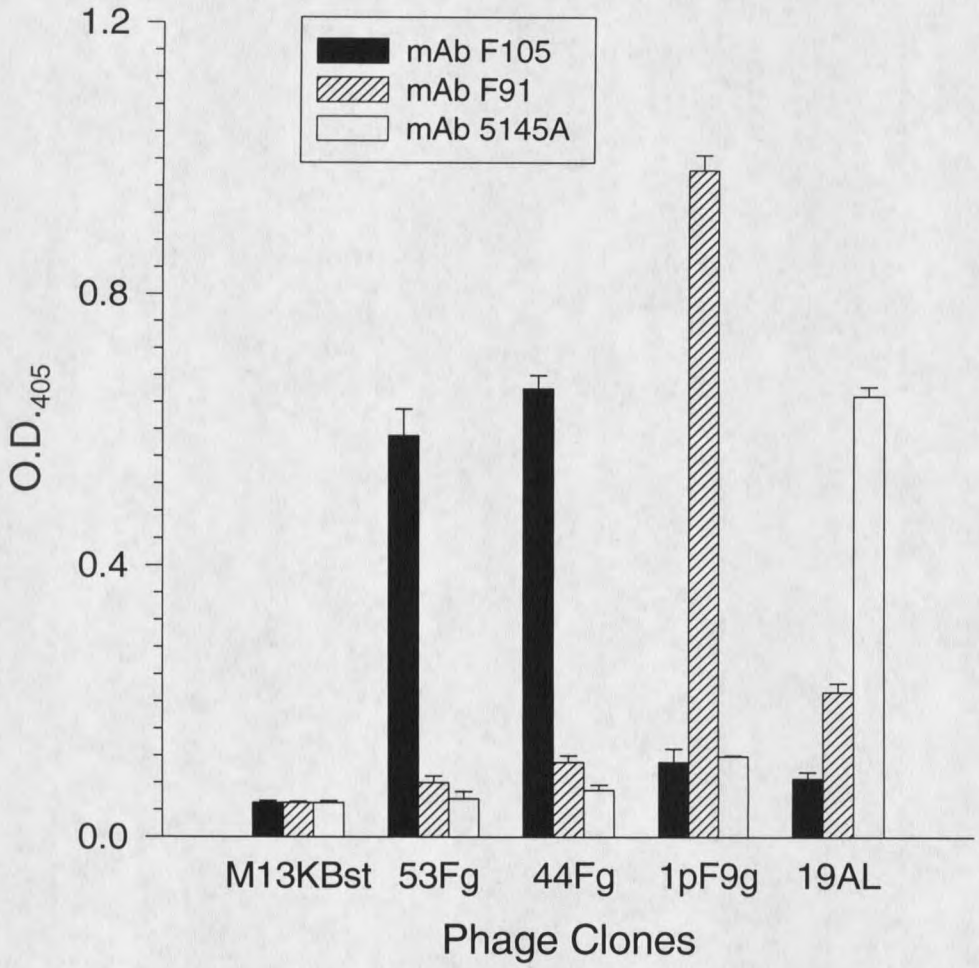

Fig.3.3 **Binding of phage clones to mAbs F105, F91, and 5145A.** MAb was used to coat wells at 1 µg/ml. Phage clones were added at $1x10^9$ pfu per well with parent phage M13KBst shown as a negative control. Phage binding was detected by rabbit anti-M13 antiserum followed by AP-conjugate goat anti-rabbit secondary Ab. O.D. values shown are the mean of duplicate samples with standard deviations (SD) shown as error bars.

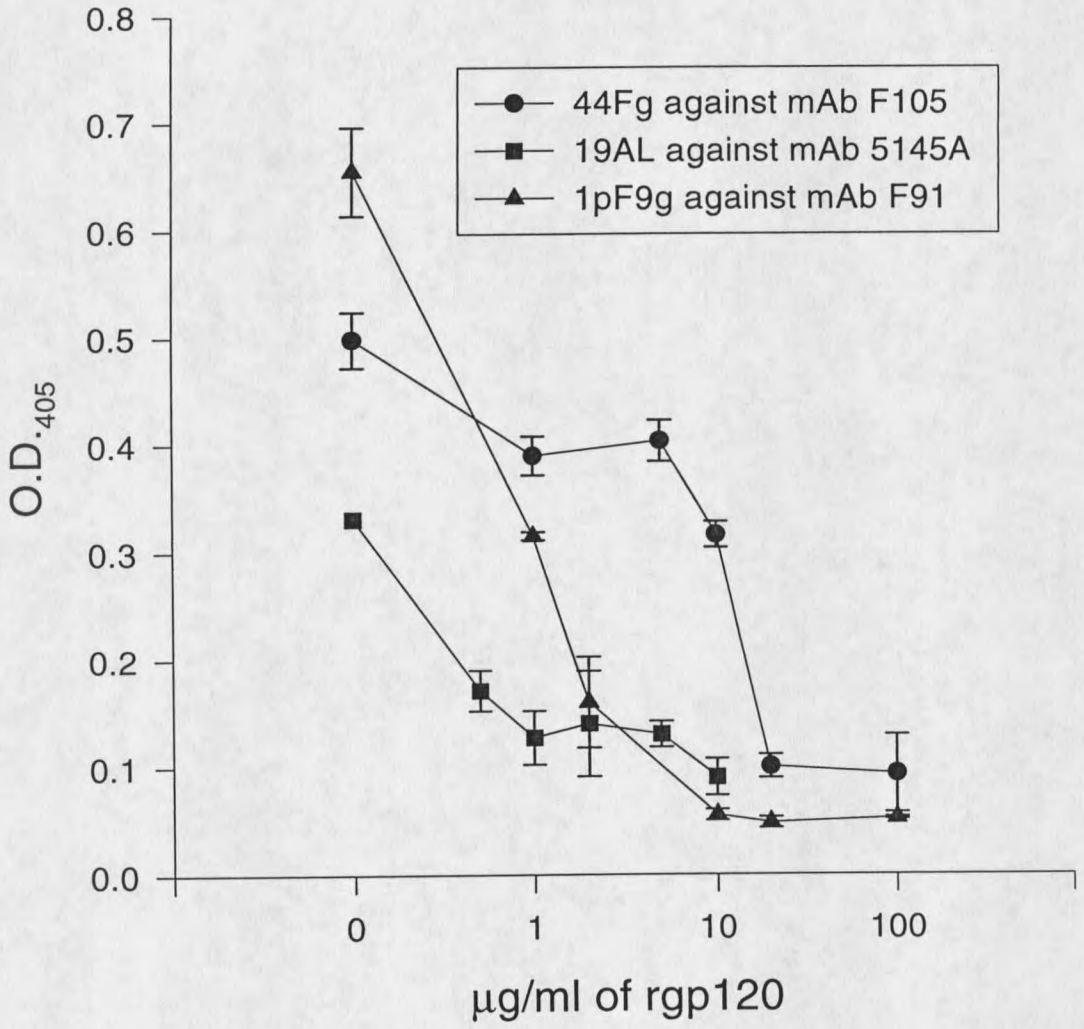

Fig.3.4 **Binding of phage clones in the presence of increasing concentrations of rgp120.** MAbs were used to coat wells at 1 µg/ml. $rgp120_{SF2}$ was added to wells in increasing concentrations followed by addition of phage clones at $1x10^9$ pfu per well to their respective mAb. Phage binding was detected by rabbit anti-M13 antiserum followed by AP-conjugate goat anti-rabbit secondary Ab. O.D. values shown are the means of duplicate samples with standard deviations (SD) shown as error bars.

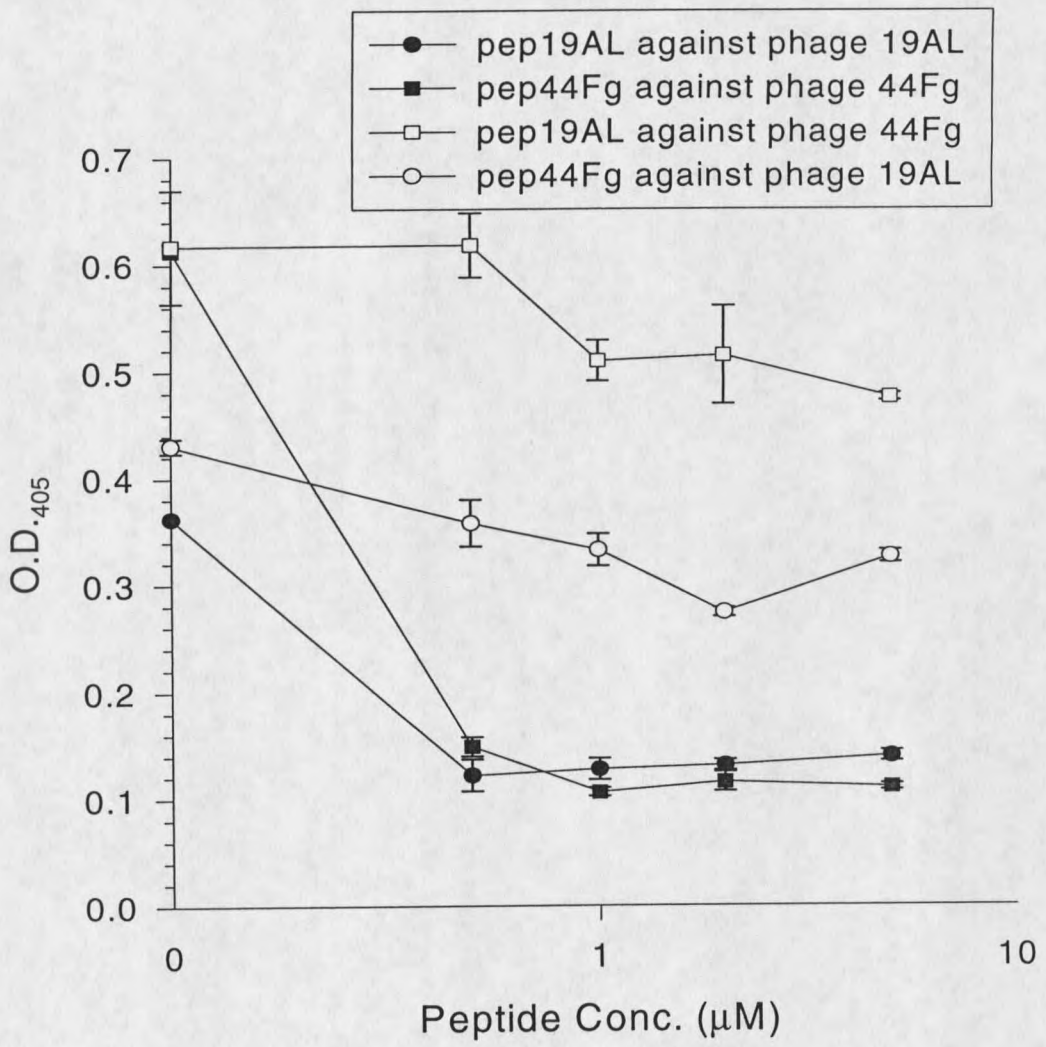

Fig. 3.5 **Inhibition of phage binding to mAbs by addition of increasing concentrations of peptide.** Mabs F105 and 5145A were used to coat wells at 1 μg/ml. Peptide was added to wells at various concentrations for 20 min. at RT followed by addition of phage clones at $1x10^9$ pfu per well for O/N incubation 4°C. Phage binding was detected by rabbit anti-M13 antiserum followed by AP-conjugate goat anti-rabbit secondary Ab. The legend shows symbols corresponding to the different combinations of peptides pep44Fg-RLTPEPDD and pep19AL-WKPVVIDFE with phage clones 44Fg and 19AL binding to their respective mAbs (F105 and 5145A) showing both a negative control and inhibition. All O.D.values shown are the mean of duplicate samples with SD shown as error bars.

peptide had any significant effect on binding of the other phage-mAb pair (Fig.3.5). Pep44Fg was also able to inhibit binding of 9Fg and 97Fg at comparable levels (data not shown). This demonstrated that the free peptides bound in the same manner as the sequences attached to the pIII protein of the phage clone. Identical results for inhibition of phage 44Fg binding to F105 were obtained with pep44FgC, which is identical to pep44Fg except for an additional Cys residue at the C-terminus for use in constructing conjugates (not shown). A possible second consensus involving residues seen in both phage clone 53Fg and 117Fg prompted the synthesis of two other peptides which corresponded to phage clone 53Fg (pep53Fg-QARYAKEPD) and a consensus sequence between phage clone 53Fg and 44Fg (pep44/53Fg-QARYAPEPD). As can be seen in Fig. 3.6, pep53Fg was only able to inhibit binding of phage clone 53Fg to mAb F105 and pep44Fg also was only able to inhibit binding of phage clone 44Fg to mAb F105. The consensus peptide 44/53Fg was unable to inhibit binding of either phage clone 53Fg or 44Fg to mAb F105. Binding of phage clone 53Fg to mAb F105 could not be inhibited by addition of gp120, at concentrations up to 100 μg/ml (data not shown) so pep53Fg was not pursued in further studies. Also, pep1pF9g did not inhibit phage 1pF9g binding to mAb F91 at concentrations up to 100 μM (data not shown) and was therefore not used in immunization studies.

Pep44FgC and pep19AL were then tested for their ability to inhibit binding of mAb to gp120. ELISA plates were coated with $rgp120_{SF2}$ and mAbs were pre-incubated with varying concentrations of peptide, then placed in the wells overnight. Figs. 3.7 and 3.8 show that both pep44FgC and pep19AL inhibit binding of their respective mAbs to gp120 while having no effect on binding of the control mAb (2G12) to gp120.

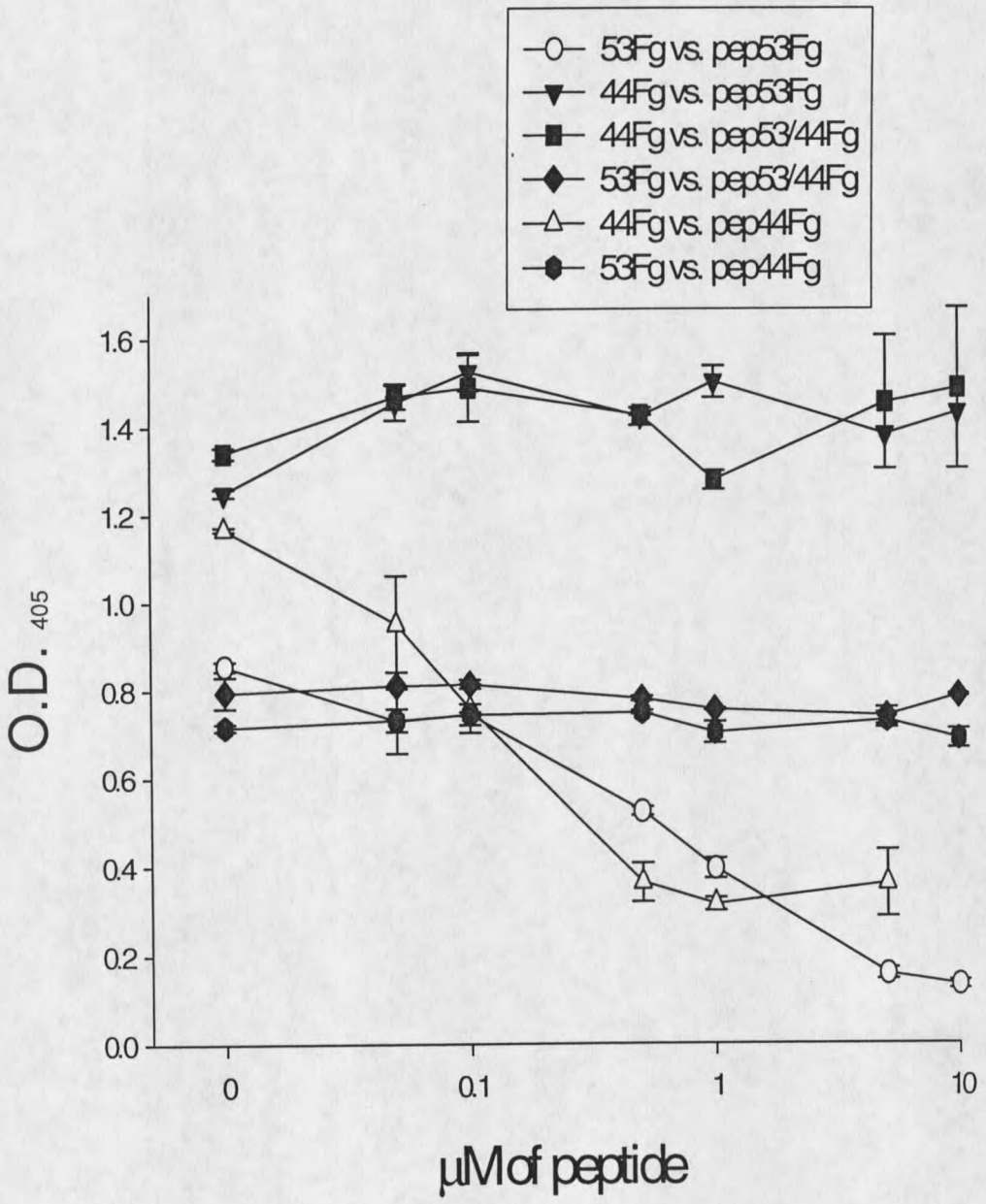

Fig.3.6 **Inhibition of phage binding to mAb F105 by addition of increasing concentrations of peptide.** Mab F105 was used to coat wells at 1 µg/ml. Peptides were added to wells at various concentrations for 20 min. at RT followed by addition of phage clones at $1x10^9$ pfu per well for O/N incubation 4°C. Phage binding was detected by rabbit anti-M13 antiserum followed by AP-conjugate goat anti-rabbit secondary Ab. The legend shows symbols corresponding to the different combinations of peptides pep44Fg-RLTPEPDD pep53Fg-QARYAKEPD and pep53/44Fg- QARYAPEPD and phage clones 53Fg and 44Fg. All O.D.values shown are the mean of duplicate samples with SD shown as error bars.

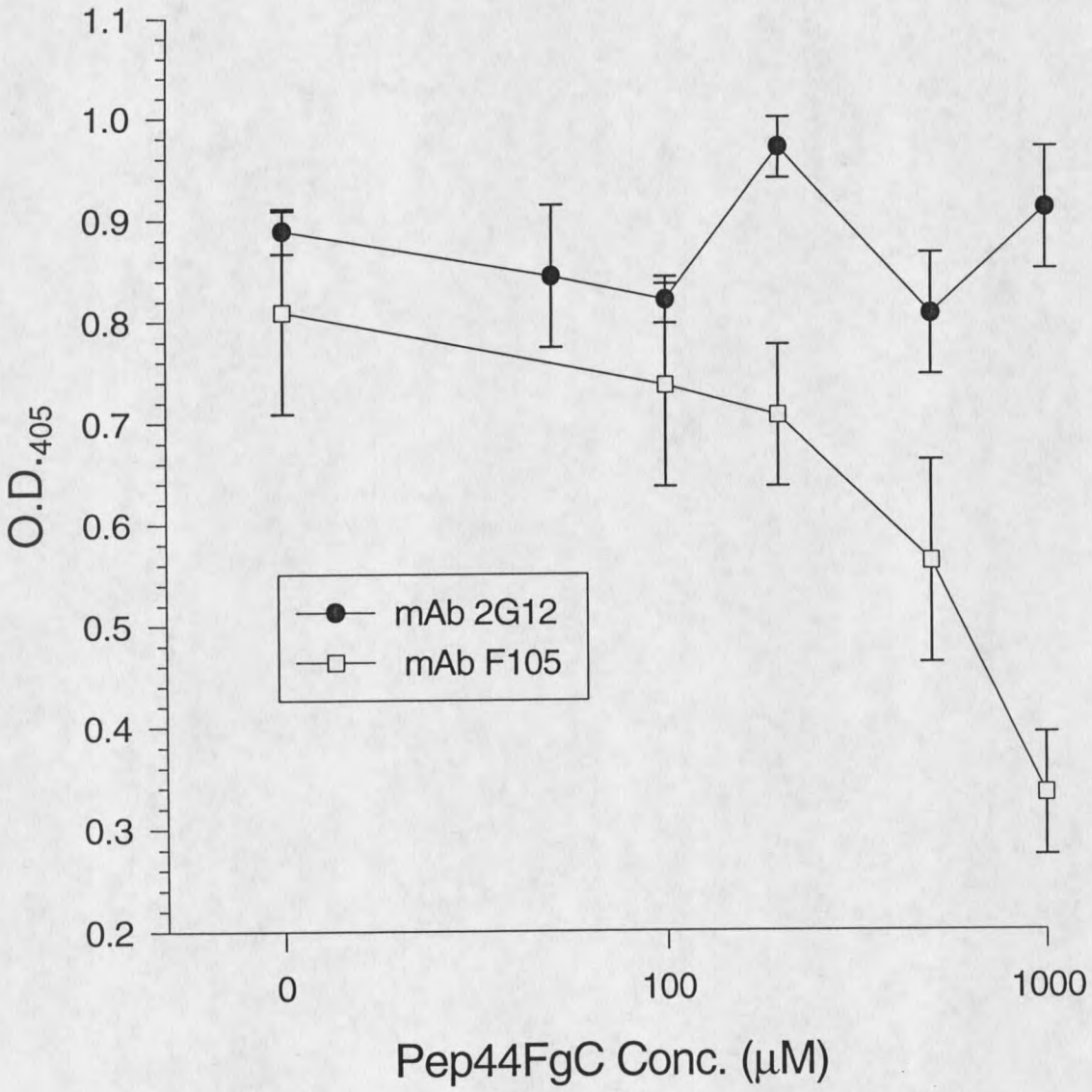

Fig. 3.7 **Inhibition of mAb F105 binding to gp120 with increasing concentrations of peptide pep44FgC.** Plates were coated with $rgp120_{SF2}$ at 0.5 μg/ml. Mab F105 at 0.2 μg/ml was pre-incubated with peptide pep44FgC for one hour at RT then placed in the well for O/N incubation. Mab 2G12 was used as a positive control. Binding of mAb was detected by goat anti-human IgG alkaline phosphatase. All O.D. value shown are the mean of duplicate samples with SD shown as error bars.

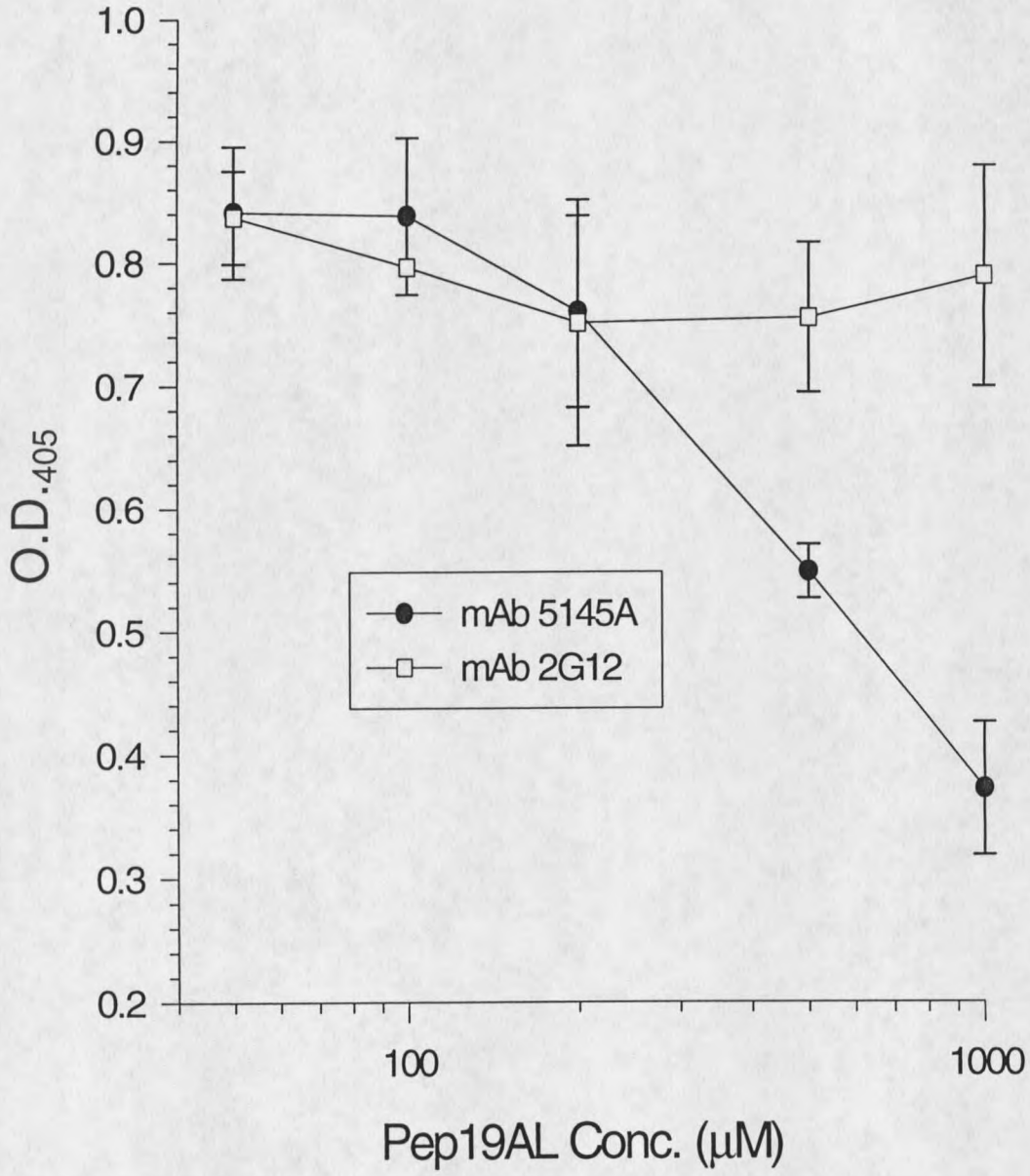

Fig.3.8 **Inhibition of mAb 5145A binding to gp120 with increasing concentrations of peptides pep19AL.** Plates were coated with $rgp120_{SF2}$ at 0.5 µg/ml. mAb 5145A at 0.2 µg/ml was pre-incubated with peptide pep19AL for one hour at RT then placed in the well for O/N incubation. mAb 2G12 was used as a positive control. Binding of mAb was detected by goat anti-human IgG alkaline phosphatase. All O.D. values shown are the mean of duplicate samples with SD shown as error bars. Pep19ALC gave similar results to pep19AL (not shown).

The concentration of the peptides needed for inhibition of mAb binding to gp120 is much greater than that seen for inhibition of phage binding to mAb. This is a result of the peptide having to overcome a very high affinity between these mAbs (F105 and 5145A) and gp120, approximately 100 nM (Posner, et al., 1991 and Pinter et al., 1993) whereas the affinity of the phage for mAb is probably much lower. The increase in the inhibiting concentration of the peptides between these two experiments reflects this change in affinity.

Immunization with Peptide Conjugates

Groups of three mice were immunized with KLH-pep44FgC and KLH-pep19ALC conjugates. Anti-peptide titers in the sera collected from these mice after the first and second boosts were determined by measuring binding of mouse Ab to OVA-pep44FgC and OVA-pep19ALC, respectively (Fig.3.9 and 3.10). All mice immunized with the KLH conjugates exhibited high titers against peptide, except mouse 2 immunized with KLH-pep44FgC.

Sera from the same mice were tested for cross-reactivity with gp120. Fig. 3.11 shows that sera from the mice immunized with KLH-pep44Fg reacted with $rgp120_{SF2}$. All three mice show reaction to $rgp120_{SF2}$ well above pre-immunization control levels, with mouse 2 showing the highest reactivity. Sera from the mice immunized with KLH-pep19AL also reacted with rgp120. Mice 1 and 3 show reactivity well above pre-bleed levels, while mouse 2 shows very little reaction above background (Fig. 3.12).

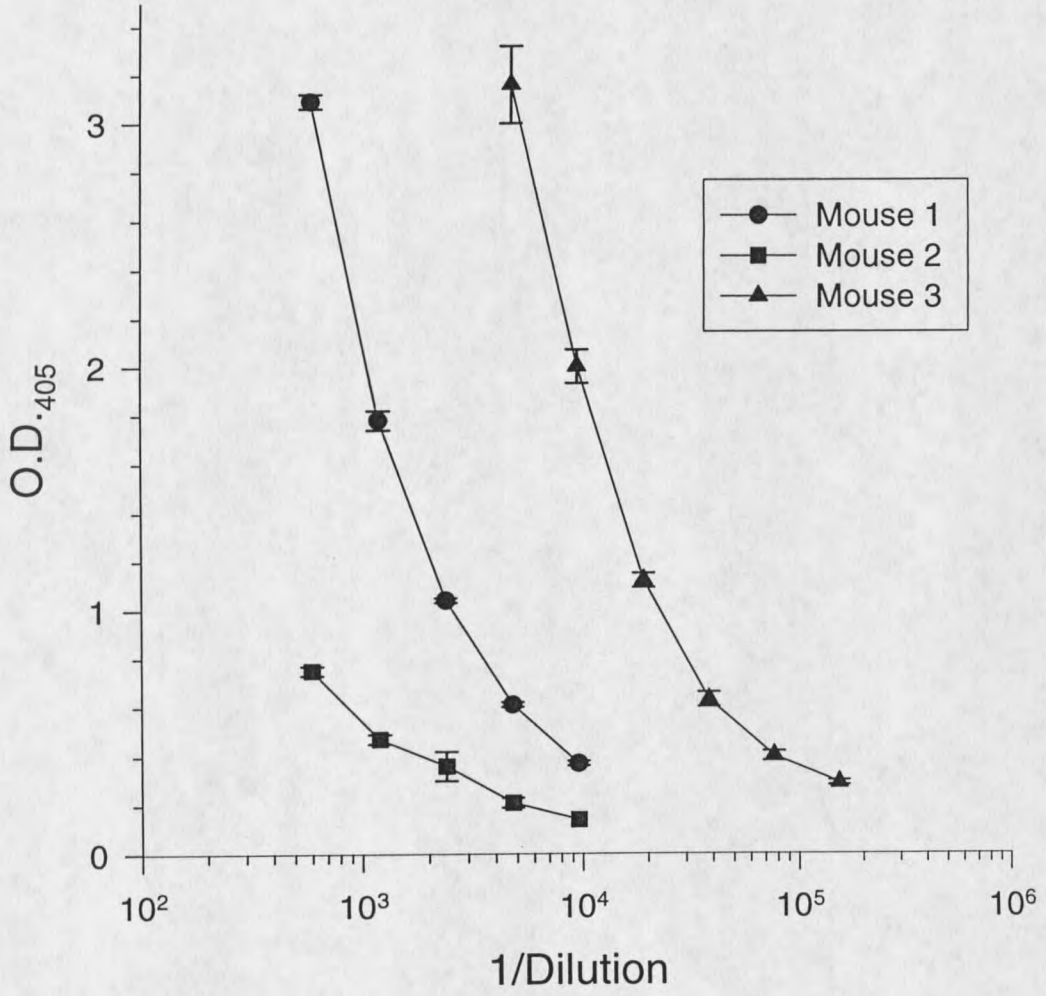

Fig.3.9 **Mouse sera derived from KLH-pep44FgC immunized mice binding to OVA-pep44Fg conjugate.** OVA and conjugates were coated in wells at 1 µg/ml. Mouse sera was added to wells in dilutions and detected by goat anti-mouse alkaline phosphatase conjugate. Unconjugated OVA controls coated on the plates and screened at each dilution of serum gave O.D. values < 0.10. All O.D. values shown are the mean of triplicate samples with SD shown as error bars. Experiment performed by R. Wilkinson.

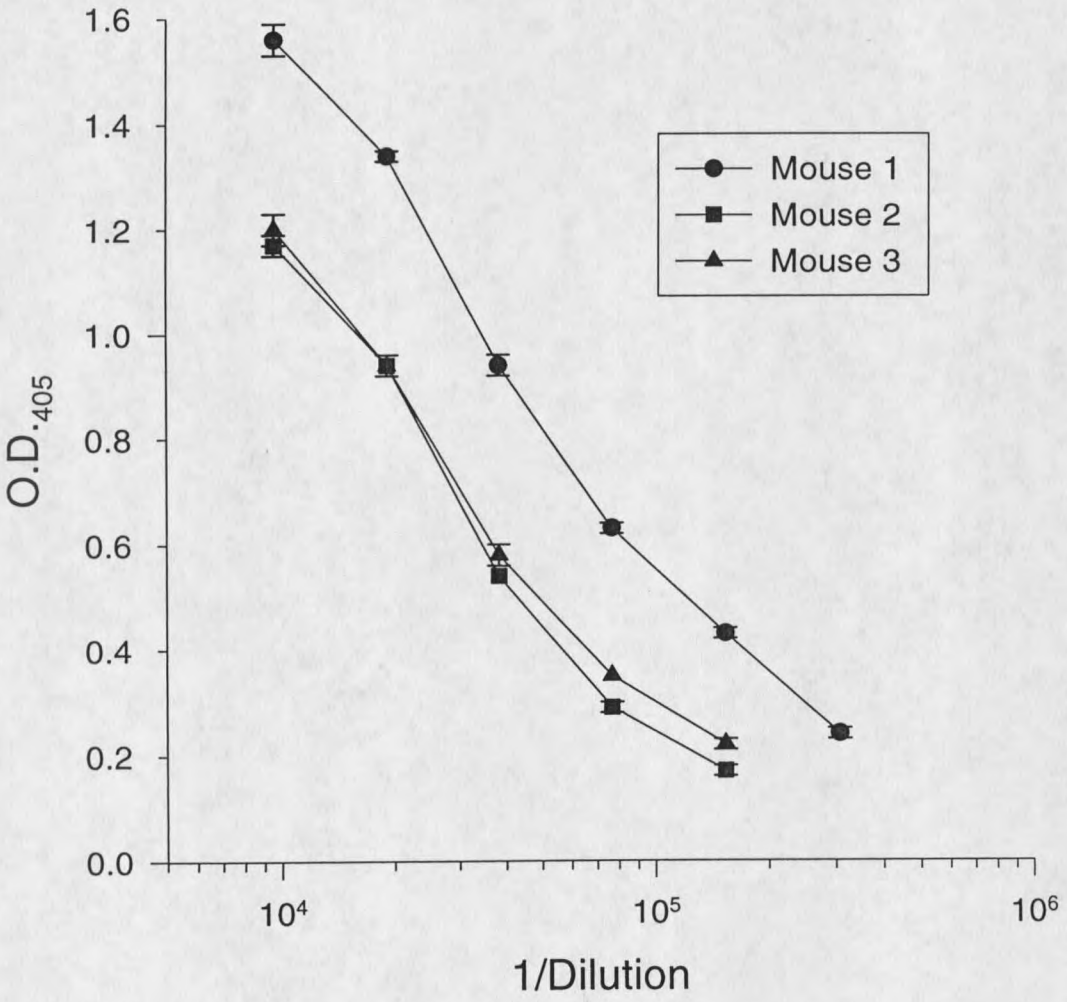

Fig.3.10 **Mouse sera derived from KLH-pep19ALC immunized mice binding to OVA-pep19AL conjugate.** OVA and conjugates were coated in wells at 1 μg/ml. Mouse sera was added to wells in dilutions and detected by goat anti-mouse alkaline phosphatase conjugate. Unconjugated OVA controls coated on the plates and screened at each dilution of serum gave O.D. values < 0.10. All O.D. values shown are the mean of triplicate samples with SD shown as error bars. Experiment performed by R. Wilkinson.

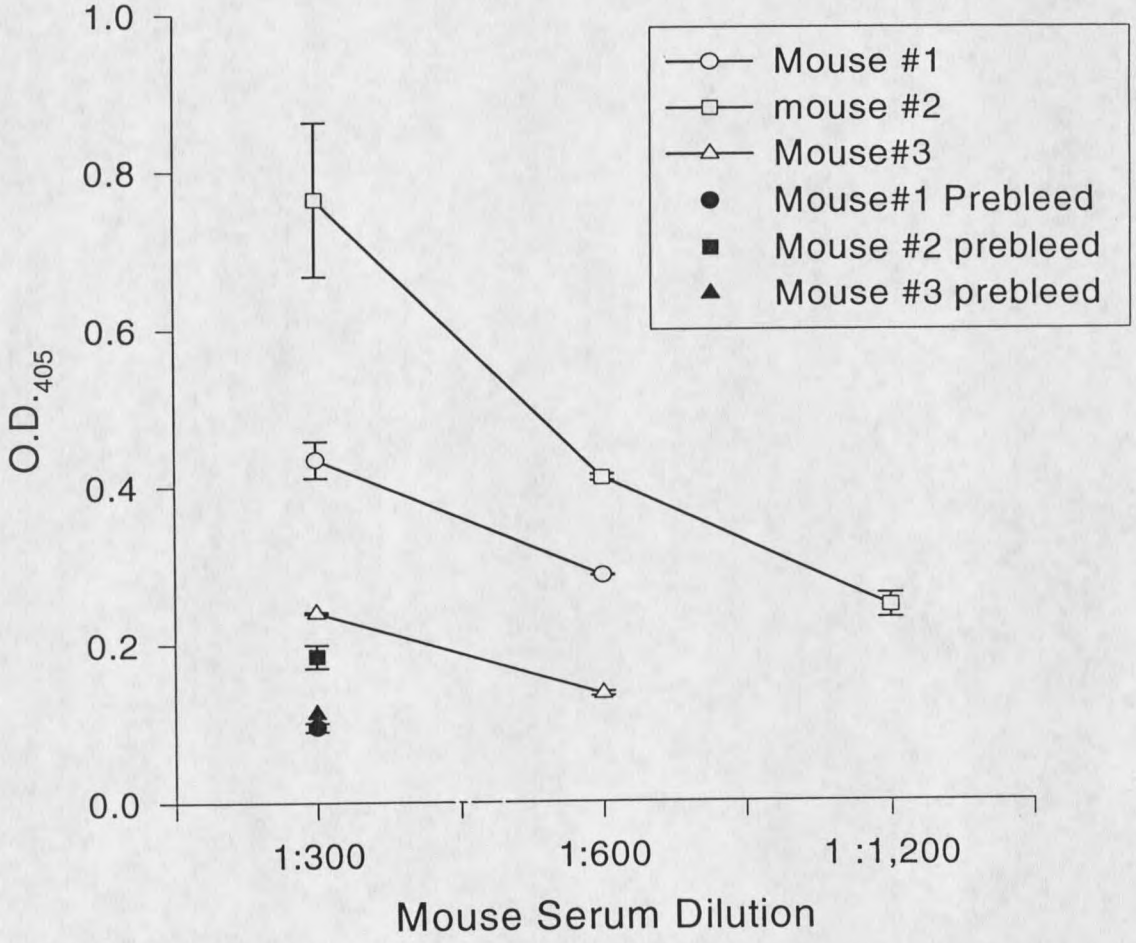

Fig. 3.11 **Mouse sera from KLH-pep44FgC immunized mice binding to gp120.** $rgp120_{SF2}$ was coated on plates at 0.5 µg/ml. Mouse sera was added to wells in dilutions and detected by goat anti-mouse alkaline phosphatase conjugate. Prebleed serum samples of each mouse are shown at the highest concentration as a negative control. All O.D. values shown are the mean of duplicate samples with SD shown as error bars.

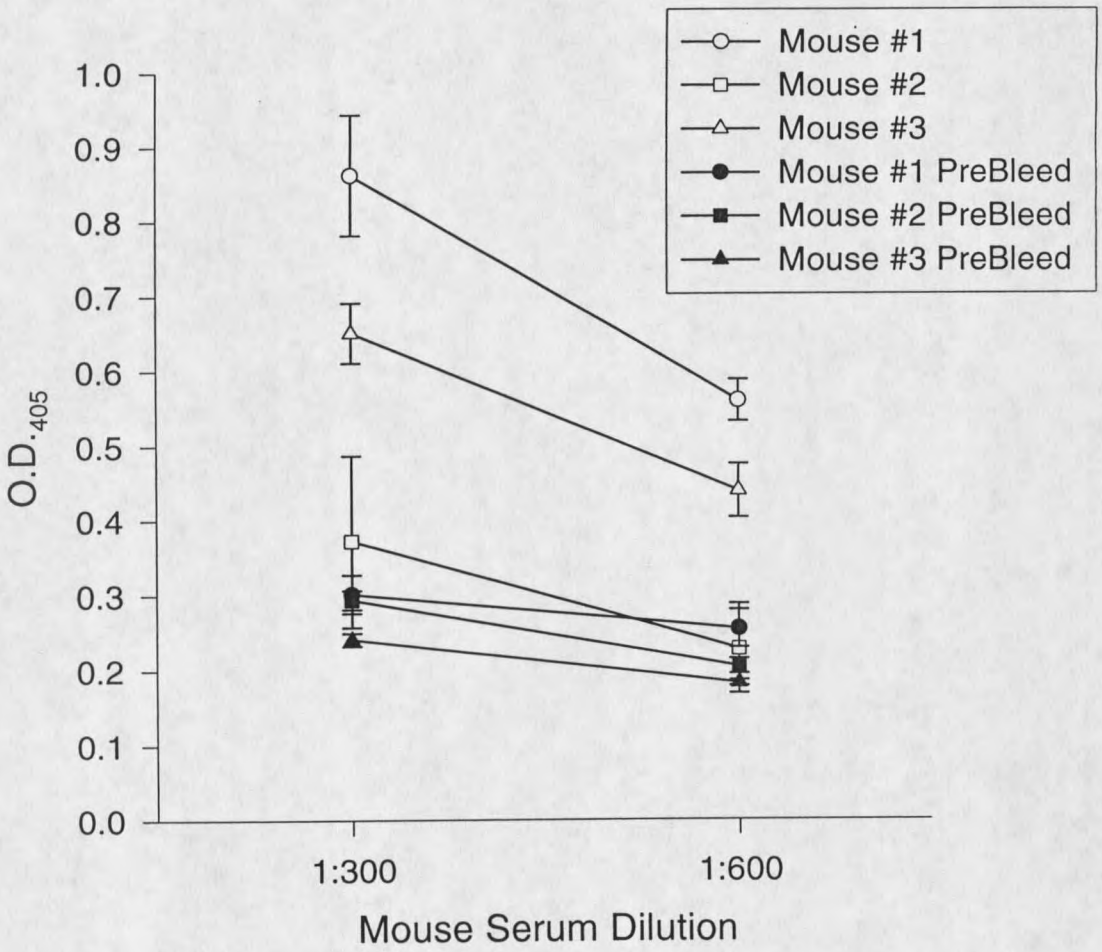

Fig. 3.12 **Mouse sera from KLH-pep19ALC immunized mice binding to gp120.** $rgp120_{SF2}$ was coated on plates at 0.5 µg/ml. Mouse sera was added to wells in dilutions and detected by goat anti-mouse alkaline phosphatase conjugate. Prebleed serum samples of each mouse are shown at the highest concentration as a negative control. All O.D. values shown are the mean of duplicate samples with SD shown as error bars. Experiment performed by A. Intamaso

Discussion

We have screened mAbs F105, 5145A, F91, 2G12, 1125H, and IgG_1b12 with phage-displayed peptide libraries and have identified sequences that bind specifically to three of these mAbs in competition with gp120. The synthetic peptides corresponding to the sequences selected by F105 and 5145A inhibited binding of the mAb both to the respective phage and to gp120. Presumably these sequences mimic residues of gp120 which interact with the mAbs, and therefore act as "mimotopes". However, since the antibodies recognize discontinuous, conformation-dependent epitopes, we did not expect to find extensive homology to a linear sequence of gp120.

The consensus sequence RXXPEPD selected by F105 can be aligned with residues found in and around the CD4 binding-site in the crystal structure of gp120 (Fig.3.13; Fig.3.14). These residues are important for interaction with F105, because mutations at these sites abrogate binding (Thali et al., 1992). Residues D368, P369, and E370 in the gp120 structure can be aligned with the peptide sequence EPD; both D368 and E370 are sites of "escape mutants" for F105 (Fig.3.14) Either residue K421, also an escape mutant site for F105, or R419 could be mimicked by the Arg in the peptide sequence. The distance between residues 368-370 and 419 or 421 in the crystal structure is within the range that could be spanned by the three residues between the Arg and the EPD in the consensus peptide, thus allowing a linear peptide to mimic a discontinuous epitope. The Pro in the fourth position of the consensus sequence may be present only to constrain its conformation rather than to mimic a specific residue on gp120. Conservation of these residues is very high through all clades of HIV-1: D368 99%, E370

99%, R419 87% (99% if substitution of Lys allowed), and K421 94% (99% if substitution of Arg allowed) using frequency data taken from the Los Alamos Env sequence database at http://hiv-web.lanl.gov. This peptide sequence obviously does not represent all residues of gp120 which are involved in binding mAb F105, but the data suggests that they do represent at least a partial epitope map. The sequence of QARY seen in phage clones 53Fg and 117Fg prompted the synthesize of the two peptides pep53Fg and pep53/44Fg for further study. Interestingly, peptide 53Fg would only inhibit binding of phage clone 53Fg while the consensus peptide pep53/44peptide was unable to inhibit binding of either phage clone 53Fg or 44Fg to mAb F105. The difference between pep53Fg and pep53/44Fg is only one residue, a Pro in pep53/44Fg which is changed to an Arg in pep53Fg. This Pro is highly conserved for phage clones with the RXXPEPD motif but the data suggests that a peptide with a proline in this position will not inhibit binding of phage clone 53Fg to mAb F105. The data also suggests that the 53Fg sequence QARY is independent of the RXXPEPD motif and may bind at a separate location on mAb F105. As previously discussed, pep53Fg seemed like an appropriate candidate for future studies but addition of gp120 was unable to inhibit binding of the phage clone 53Fg to mAb F105. This infers that the binding site of the sequence QARYAKEPD is removed from the antigen recognition site and does not mimic residues on gp120.

With mAb 5145A, it is difficult to know which residues in the pep19AL sequence are crucial for binding because it was the only sequence selected. 5145A binding is also sensitive to mutations in the 368-370 region of gp120 (Pinter et al., 1993), whose DPE sequence (shown in Fig. 3.14) can be aligned to the DFE in pep19AL.

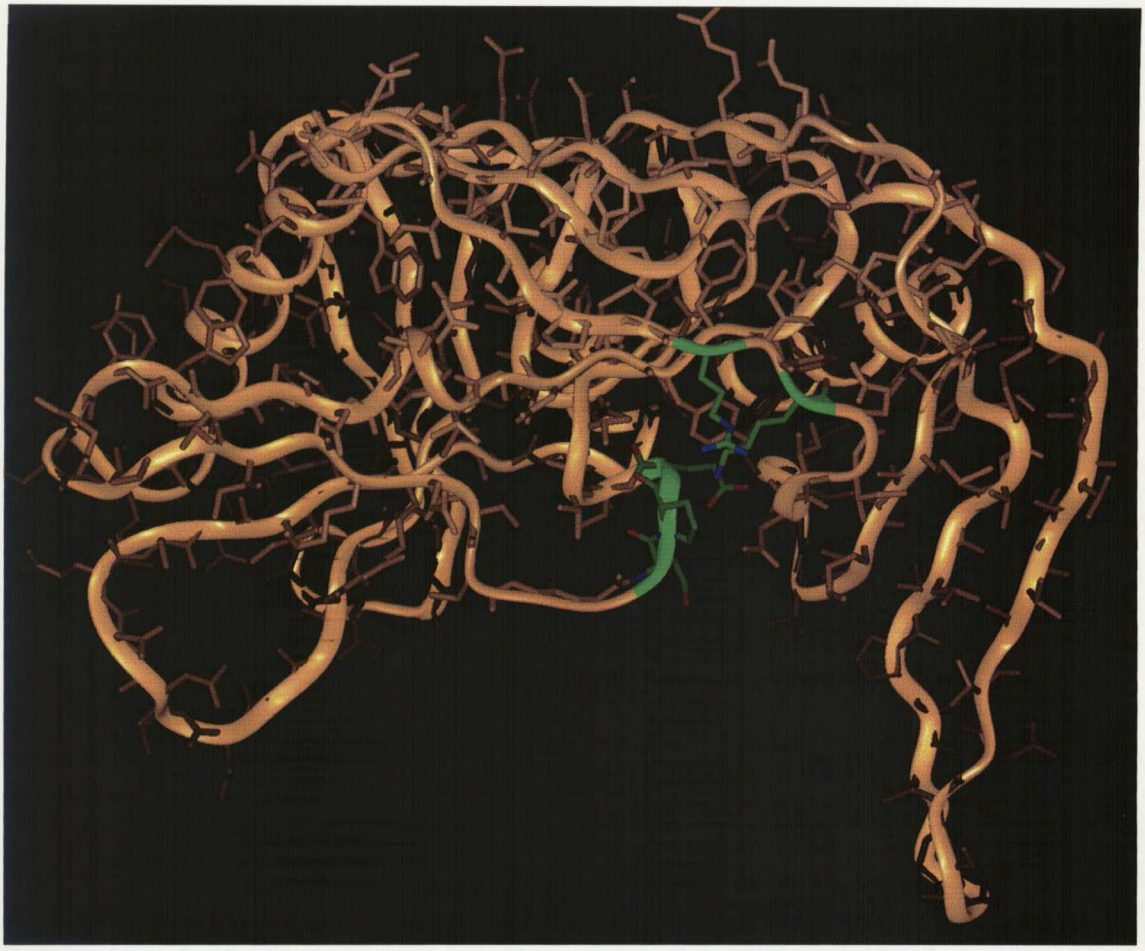

Fig. 3.13 **Crystal structure of gp120.** Crystal structure contains HIV-1 HXB2 "core" gp120 complexed to the N-terminal domain of CD4 (Kwong et al., 1998). Only gp120 is shown here, see Fig. 1.5 for comparison with CD4 bound structure. Residues highlighted in green are those which are believed to be mimicked by peptides described in the chapter. (See Fig.3.13 for a more descriptive look)

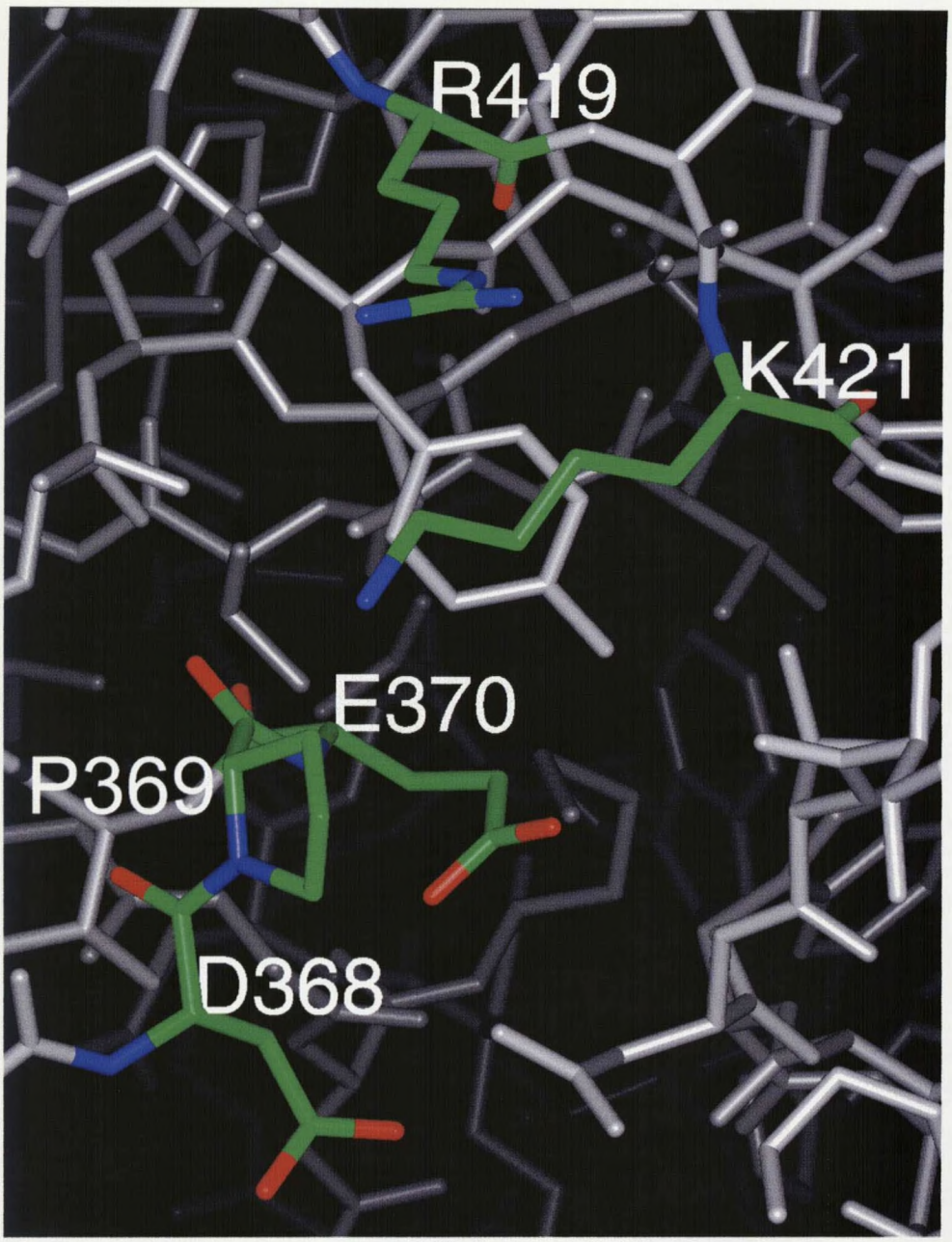

Fig.3.14 **Partial structure of the CD4 binding site on gp120 taken from the crystal structure of gp120.** (Kwong et al., 1998). The residues that can be aligned to pep44Fg are shown in color. The sequence D368, P369, E370 is in the lower left corner; either K421 or R419 can be aligned with the Arg in pep44Fg. Pep44Fg-**R**LTP**EPD**D

An appropriate experiment in this instance would be to do an alanine scan of the peptide to determine which residues are crucial for activity. This was not done at this time but would be a good experiment to perform for future studies. It was decided to continue with the active peptide pep19AL for further studies rather than use resources and time to determine what residues are important for the peptide.

The phage clone 1pF9g bound specifically to mAb F91 and this was inhibited by the addition of gp120, but the corresponding synthetic peptide had no activity. This may be due to the free peptide not assuming the same conformation as the sequence displayed on the phage.

Sera from mice immunized with both pep44Fg and pep19AL conjugates cross reacted with gp120, albeit at a lower titer than they react against the peptides themselves. The low anti-gp120 titers are not surprising, given that the peptide can adopt many conformations, of which only a few would likely mimic the three-dimensional structure of gp120. Most of the anti-peptide antibodies would therefore not be expected to recognize gp120. However, a better antigen could be developed by determining the conformations of these peptides when they are bound to their respective mAbs using X-ray crystallography on peptide-Fab complexes. For the mice immunized with pep19AL conjugates, a higher anti-peptide titer correlated with a higher anti-gp120 titer; however, there was an inverse correlation for the mice immunized with KLH-pep44FgC. This inverse correlation was not observed when mice were immunized with pep44Fg conjugated to the capsid protein of Cowpea Chlorotic Mottle Virus (A. Intamaso et al., manuscript in preparation). A mouse control group immunized with KLH only was not utilized in this study. Sera from mice immunized with KLH and KLH-S9 peptide

conjugates were obtained from the Pincus lab. These sera gave a high background in our ELISA experiments. In the case of pep19ALC, the direct correlation between anti-peptide titer and anti-gp120 titer argues against cross reaction between gp120 and KLH, but in the case of pep44FgC, an inverse correlation was seen between the anti-peptide titer and anti-gp120 titer. One reason to believe there is little cross reaction between gp120 and KLH is that when the sera from KLH-44FgC immunized mice were screened against KLH in ELISA, sera from all three mice bound equally strong to KLH (data not shown) whereas the anti-gp120 titers and anti-peptide titers varied a great deal from mouse to mouse. As stated previously, the inverse correlation between anti-gp120 and anti-peptide 44FgC titer was not seen when pep44FgC was attached to CCMV.

Soon after the panning with IgG1b12 was performed, the crystal structure of the antibody was published (Saphire, et al., 2001). The structure of the antibody revealed an extended CDR H3 region which forms a finger-like loop which is believed to extend into the CD4-binding site pocket to facilitate binding to gp120. It is believed that this extended region on the mAb is what allows IgG1b12 to bind so effectively to the somewhat recessed CD4-binding site of gp120. In retrospect, it would seem unlikely that the phage screening of mAb IgG1b12 would be successful in that screening for a peptide that binds to an extended loop structure of the mAb would probably not isolate a peptide sequence that is a mimetic of the pocket of gp120 that the finger-like loop interacts with. Determination of a peptide mimetic works best when the peptide mimics a loop structure on the antigen and binds to a cleft or pocket on the mAb. There has been a report of phage display results which selected a peptide that specifically binds the mAb IgG1b12 (Zwick et al., 2001). In this study, the phage-displayed peptide showed a high affinity for

Fab fragments of b12, comparable to that of gp120, but immunization with a synthetic peptide-ovalbumin conjugate produced only a low anti-peptide titer and no anti-gp120 cross-reactivity (Zwick et al., 2001). This peptide formed a cysteine constrained loop and was mapped to a loop structure near the CD4-binding site on gp120. These positive results suggest that mAb IgG1b12 was able to bind a phage display peptide but that the peptide needed to be cysteine loop-constrained and is probably not directly contacting the finger-like loop on the mAb (based upon the theoretical binding site of the mAb IgG1b12 loop structure on gp120 and where the phage peptide is thought to mimic gp120).

CHAPTER FOUR

SELECTION AND CHARACTERIZATION OF THE QSYP PEPTIDE SEQUENCE FROM PHAGE DISPLAY LIBRARIES

Introduction

This chapter contains data submitted for publication in the paper titled "The QSYP peptide Sequence is Selected from Phage Display Libraries by Bovine IgG Contaminants in Monoclonal Antibody Preparations"(Jacobs, J.M., Bailey, B.W., Burritt, J.B., Morrison, S.G., Morrison, R.P., Dratz, E.A., Jesaitis, A.J., Teintze, M., 2002 submitted). It is an explanation of a strong consensus sequence that was obtained while screening a peptide phage display library with mAb F105. A nearly identical consensus sequence was found in three other laboratories during peptide phage display screening with unrelated mAbs, sparking an interest in determining the origin of this consensus among the four laboratories. Peptide sequences from four phage display experiments are reported, three of which were performed by other laboratories, and one, the mAb F105 screening, by J. Jacobs in the M. Teintze laboratory. All other experiments in this chapter were performed by J. Jacobs.

Background

Random peptide phage-display libraries are used to identify peptide sequences which interact with specific proteins (Smith and Scott, 1993; Burritt et al., 1996). This technique is commonly used to map epitopes bound by monoclonal antibodies (mAbs), and in cases where the epitope is comprised of discontinuous residues on the target protein, the amino acid sequences selected may also provide three-dimensional structure information about the antigen (Burritt et al., 1998; Burritt et al., 2001; Bailey, 2001). The data in this chapter lists peptide sequence results originating from four different laboratories, each of which screened a random peptide phage-display library against a different mAb. MAb F105, a human IgG_1 against the HIV envelope protein gp120 (Posner et al., 1991), was screened by J. Jacobs in the M. Teintze laboratory; mAb 4B4, a mouse IgG_3 against bovine rhodopsin (MacKenzie and Molday, 1982), was screened by B. Bailey in the E. Dratz laboratory; mAb 449, a mouse IgG_1 against human neutrophil flavocytochrome *b* (Verhoeven et al., 1989), was screened by J. Burritt in the A. Jesaitis laboratory; mAb GZD1E8, a mouse IgG_1 against the major outer membrane protein of *Chlamydia pneumoniae* (Wolf et al., 2001), was screened in the R. Morrison laboratory.

Screening the library with these mAbs selected phage with two separate consensus sequences for each mAb: One sequence that was specific to the respective mAb and could be mapped to an epitope on the antigen used to produce the mAb, and a second consensus sequence, QSYP, which was found to be selected from the library by bovine IgG contaminating the mAb preparations. The source of the bovine IgG

contamination was fetal bovine serum that was used as part of the growth medium for the hybridoma cell culture expressing the mAb of interest (Lucas et al., 1988). Purification of the mAb from the hybridoma culture supernatant is commonly accomplished by chromatography on immobilized protein G or protein A, which bind bovine IgG in addition to the mAb of interest (Bastida-Corcuera et al., 1999 and Eliasson et al., 1989). Co-purification of both the mAb and bovine IgG in this step led to the contamination of each mAb sample and hence the presence of both the mAb and bovine IgG during peptide phage display selection. Contamination of the mAb with bovine IgG led to the selection of both the mAb-specific consensus sequence as well as the bovine IgG directed QSYP consensus sequence.

Material and Methods

Materials

MAb F105 is a human IgG_1 mAb directed against the HIV envelope glycoprotein gp120 and was obtained from the NIH AIDS Research Reference and Reagent Program (donated by Dr. M. Posner). MAb F91, obtained from Dr. J. Robinson (Moore et al., 1994), is a human IgG_1 mAb directed against HIV gp120. MAb 449 (Verhoeven et al., 1989) was obtained from Dr. D. Roos, Central Laboratory of the Netherlands Blood Transfusion service in Amsterdam, The Netherlands. MAb GZD1E8 (Wolf et al., 2001) is a mouse IgG_1 that was obtained from mice immunized with the major outer membrane protein of *Chlamydia pneumoniae*. MAb 4B4 (MacKenzie and Molday, 1982) is a mouse IgG_3 directed against bovine rhodopsin and was purified from a hybridoma cell line

obtained from Dr. R. Molday. K42-41L (Adamus et al., 1991) is a mouse IgG_1 mAb directed against bovine rhodopsin and was obtained from Dr. P. Hargrave and grown by the National Cell Culture Center (Minneapolis, MN) in serum free media. The panel of mouse Ig isotypes was purchased from Zymed (San Francisco, CA). Human polyclonal IgG was purchased from Sigma Chemical Company (St. Louis, MO). Recombinant HIV gp120 (SF2) was obtained from Chiron (Emeryville, CA). The J404 phage display library used in all the experiments was produced in the filamentous phage M13KBst and expresses a random 9 mer peptide sequence as an amino-terminal fusion to the phage capsid protein pIII (Burritt et al., 1996; Burritt et al., 1995). The library has a complexity of ~5 X 10^8 unique phage. Peptide pep2Fg, SQSYPTRNS-NH_2 , was purchased from Research Genetics, Inc. (Huntsville, AL) and further purified to >90% by reverse phase HPLC. Alkaline phosphatase-conjugated goat anti-rabbit IgG (H+L) was purchased from BioRad (Hercules, CA). Rabbit anti-bovine IgG horseradish peroxidase (HRP)-conjugate was purchased from Cappel (West Chester, PA). Fetal Bovine Serum was purchased from HyClone (Logan, UT).

ELISA Experiments

The technique of ELISA was used to measure binding of phage to Ab, and peptide inhibition of phage binding to Ab. Also determined by ELISA was the direct detection of bovine IgG as well as capture and detection of bovine Ab with QSYP expressing phage clones.

For phage detection experiments, Ab was coated directly onto microtiter wells (Immulon 2, Dynatech, McLean, VA) at 1-5 μg/ml overnight at 4°C. Wells were blocked with Blotto (5% nonfat dry milk, 0.5% Tween 20 in TBS{50 mM Tris-HCl, 150 mM NaCl}) for 3 hours at room temperature (RT). Phage were added at various concentrations, $5x10^8$ to $1x10^{10}$ pfu per well, in a volume of 100 μl per well and incubated overnight at 4°C. Plates were washed, incubated with rabbit anti-M13 serum (1:5,000 dilution) for 2-4 hours at RT, washed, incubated with goat anti-rabbit alkaline phosphatase-conjugated IgG (1:1,000 dilution) for 1-2 hours at RT, washed, then developed by addition of *p*-nitrophenyl phosphate (Sigma Chemical). For peptide inhibition experiments, 100 μl of peptide at the concentrations indicated in Fig. 4.6, was added per well following the blocking step, and incubated 20 min at RT, followed by addition of 50 μl of phage at the concentrations indicated overnight at 4°C. The remaining steps were as described above.

For detection of bovine IgG, the Ab sample of interest was coated directly onto micro titer wells and incubated overnight at 4°C. Wells were blocked with BSA (0.25% BSA, 0.5% Tween 20 in TBS) for 3 hours at RT. Rabbit anti-bovine IgG HRP-conjugate was added at 1:1000 dilution either overnight or for 2-4 hours at RT. Plates were washed, then developed using a 3,3,5,5-tetramethylbenzidine (TMB)/ hydrogen peroxide solution (Sigma Chemical). For capture of bovine Ab with phage, the phage were coated directly onto microtiter wells and incubated overnight at 4°C. Wells were blocked with BSA, washed, and Ab samples were added to wells at 2 μg/ml. After overnight incubation at 4°C, rabbit anti-bovine IgG HRP was added and detected as described above. A_{450}, for

HRP, or A_{405}, for alkaline phosphatase, was determined using a micro plate reader (Safire, Tecan Austria GmbH).

Phage Selection Experiments

MAb F105 was immobilized on 60 mm petri dishes, incubated overnight at 4°C, washed, blocked with blotto overnight at 4°C, and washed again. Phage library was added ($1x10^{12}$ pfu), incubated overnight at 4°C, washed extensively (0.5% Tween 20 in TBS), and eluted by addition of rgp120 in 4X molar excess for 2 hours at RT. Eluted phage were then amplified in *Escherichia coli* strain K91, precipitated with 2.5%PEG/0.5M NaCl, resuspended, and then used in subsequent rounds of selection (Smith and Scott, 1993).

MAbs 449, 4B4, and GZD1E8 were immobilized on Protein G Sepharose beads (Amersham Pharmacia, Piscataway, NJ), using 300 µL of total beads. The beads were divided into three aliquots for three rounds of selection. The phage library ($1x10^{12}$ pfu) was added to the first aliquot and incubated overnight at 4°C. The bead/library mixture was placed in a column, washed extensively with ice-cold wash buffer (1% BSA, 0.5% Tween20 in TBS) and then eluted with 0.1M glycine, pH 2.2. The eluted phage were neutralized immediately with 2M Tris pH 10, and were then amplified as described above. Amplified phage were then combined with the 2^{nd} aliquot of the beads and the procedure was repeated until three rounds of selection were completed. The screening of the J404 library with these three mAbs (449, 4B4, and GZD1E8) was performed by the Jesaitis, Dratz, and Morrison laboratories respectively.

Analysis of Selected Phage

A dilution of the third round elution was plated and single plaques were excised. Phage minipreps were prepared by growing specific phage plaques overnight in Luria broth containing 75 μg/ml kanamycin. Phage were then isolated by PEG/NaCl precipitation as described above. Phage clones were screened by ELISA to select for phage binding to the mAb of interest. Phage clones that bound to the mAb sample shown by ELISA were then analyzed by sequencing their DNA using a primer which anneals downstream of the unique 27-mer insert in the pIII gene as described (Burritt et al., 1996; Burritt et al., 1995).

Protein G Isolation of Bovine IgG

A 2 ml slurry of Protein G Sepharose beads was placed in a column, washed with 8 ml 100 mM glycine, 0.15 M NaCl, pH=2.5, then washed with 100 mL PBS, pH=7.4. A total volume of 1L 10% fetal bovine serum in PBS was passed over the column, then washed with 100 mL PBS, and eluted with eight 1 mL aliquots of 100 mM glycine 0.15 M NaCl, pH=2.5. Eluate fractions were immediately neutralized with 1M Tris buffer, pH 8.8. Eluate protein concentration was determined by UV absorbance at 280 nm, and fractions containing the most protein were pooled. Samples were then dialyzed against PBS at 4°C using Spectra/Por membrane tubing, MWCO: 12-14,000, (Spectrum, Los Angeles, CA) and aliquots run on a reducing SDS-PAGE gel. The gel was stained with GelCode® Blue Stain Reagent (Pierce, Rockford, IL) to visualize the heavy and light

chain bands and verify the presence of bovine IgG. Bovine IgG was also assayed by reactivity with anti-bovine IgG HRP in ELISA experiments as described above.

Results

Four separate laboratories each independently screened the J404 nonapeptide phage display library with a different mAb (Human IgG_1 mAb F105, mouse IgG_3 mAb 4B4, and mouse IgG_1 mAbs 449 and GZD1E8). In each experiment, a consensus sequence of QSYP was seen in addition to a different consensus that appeared to be specific for each mAb. Phage display selection using mAb F105 resulted in a number of phage clones which matched the QSYP consensus sequence, as seen in Table 4.1. QSYP-containing phage clones bound strongly to the mAb F105 preparation and as well as to the negative control mAb F91 preparation, as shown by phage clone 2Fg in Fig.4.1. Many phage clones selected by mAb F105 also matched the consensus RXXPEPD, shown in Table 4.1, and previously discussed in Chapter 3. Two examples of phage clones representing the RXXPEPD sequence, 44Fg and 53Fg, are shown to bind specifically to mAb F105 in Fig.4.1, but not to the negative control mAb F91. The RXXPEPD sequence mapped to a possible epitope on gp120 (Chapter 3) that is consistent with the location of residues known to be escape mutations for F105 binding (Thali et al., 1993). Phage screened against the mAb 4B4 sample also resulted in selection of two consensus sequences (Table 4.2), with one consensus matching an epitope on the intradiskal face of rhodopsin (Bailey, 2001), while the other sequence (QSYP) could not be mapped to rhodopsin.

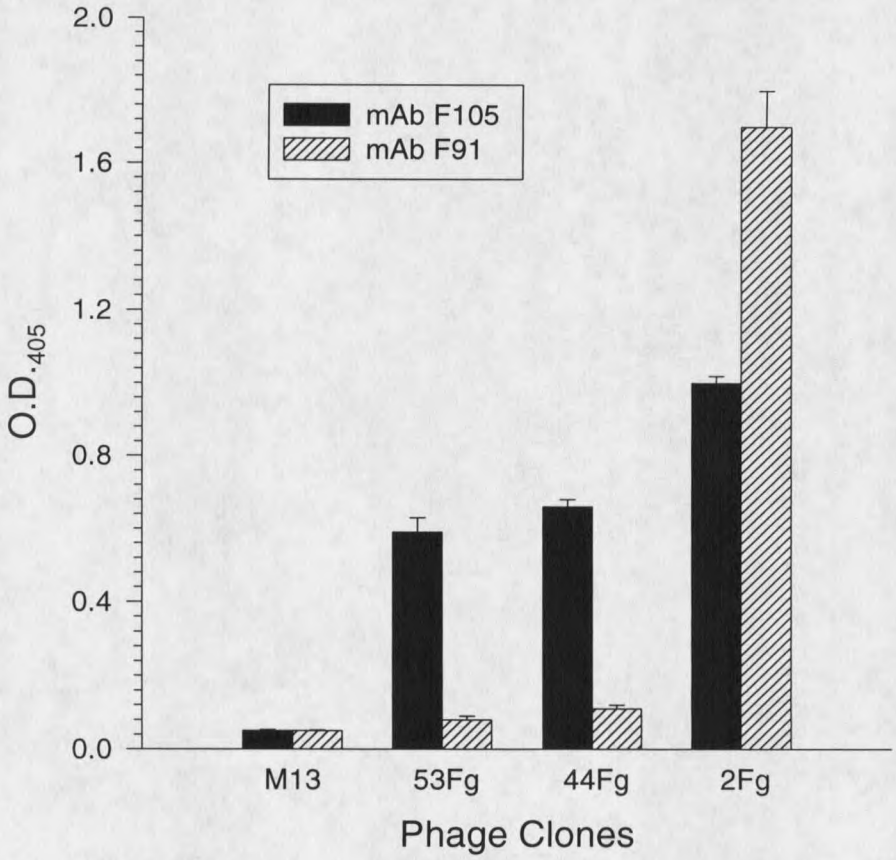

Fig.4.1. **Specificity of three phage clones isolated by selection with mAb F105**. MAb F105 was used to coat wells at 1 µg/ml. Phage clones were added at $1x10^9$ pfu per well with parent phage M13KBst shown as a negative control. Phage binding was detected by rabbit anti-M13 antiserum followed by AP-conjugate goat anti-rabbit secondary Ab. O.D. values shown are the mean of duplicate samples with standard deviations (SD) shown as error bars.

TABLE 4.1 **QSYP Containing Sequences of Phage Displayed Peptides Isolated from Screening mAb F105.**

mAb F105 Selected Phage	
Phage Clone	Sequence
2Fg	S **Q S Y P** T R N S
26Fg	D **Q S Y P** A D A N
3Fg	G G S **Q S Y P** D L
7Fg	E D G G A **Q S Y P**
108Fg	W R T A P E **S Y P**
61Fg	V Q A T L **Q S Y P**
95Fg	I H **Q S Y P** D R G
91Fg	R E G A I **Q S Y P**
Consensus	**Q S Y P**
44Fg	**R** L T **P E** S **D** D R
53Fg	Q A **R** Y A K **E P D**
Specific Consensus	**R** X X **P E P D**

TABLE 4.2 **QSYP Containing Sequences of Phage Display Peptides Isolated from Screening Against mAb 4B4.**

mAb 4B4 Selected Phage[1]
Sequence
A G P P Y **Q S Y P**
A M R D Y **Q S Y P**
S **Q S Y P** D R
S **Q S Y P** D
Y M **S Y P** N R S A
Y **Q S Y P** S R E H
Consensus **(S/Y) Q S Y P**
Specific Consensus **E Q Q V S A T A Q**

[1]Experiment was performed by B. Bailey (Bailey, 2001)

Table 4.3 **QSYP Containing Sequences of Phage Display Peptides Isolated from Screening Against mAb 449.**

mAb 449 Selected Phage[1]
Sequence
Y M S Y P
T D W H **Y Q S Y P**
T D L Q **Y Q** R **Y P**
T D W Q **Y Q S Y P**
Y Q S Y P S R E N
Y D H N **Y Q S Y P**
H M **S Y P**
Consensus **Y Q S Y P**
Specific Consensus **G P Q V**

[1]Experiment was performed by J. Burritt (Burritt, 2000)

Similarly, phage selected during screening with the mAb 449 (Table 4.3) produced a consensus sequence GPQV matching an epitope on cytochrome b, as well as producing the QSYP motif (Burritt et al., 2000). The QSYP consensus was also found when screening the phage library with mAb GZD1E8 (Table 4.4). Preliminary data suggest that mAb GZD1E8 may also have another consensus sequence, but its identity has not yet been firmly established (Morrison, et al. unpublished data). The consensus sequence (QSYP) can also be expanded to include an additional Tyr residue (YQSYP) according to the sequence data from mAbs 4B4, 449, and GZD1E8.

TABLE 4.4 **QSYP Containing Sequences of Phage Display Peptides Isolated from Screening Against mAb GZD1E8.**

	mAb GZD1E8 Selected Phage[1]
Phage Clones	
38GZ	**Y Q S** F **P**
35GZ	**Y** M **S Y P**
48GZ	**Y Q S Y** V
7GZ	S **Q S Y P** D R
43GZ	T S **Y Q S** R **P** T
37GZ	G K S Q **Y** E **S Y P**
42GZ	G A V S **Y** E **S Y P**
27GZ	N K T A **Y** E **S Y P**
39GZ	**Y** M **S Y P** N R S A
46GZ	A M R D **Y Q S Y P**
26GZ	T D W Q **Y Q S Y P**
18GZ	Q K H Y **Y** E **S Y P**
15GZ	G D V M **Y** M **S Y P**
Consensus	**Y Q S Y P**

[1]Experiment was performed in the R. Morrison Lab (R. Morrison, personal communication, 2002)

The frequency with which the QSYP sequence was selected with both human and mouse mAbs suggested that the QSYP-containing phage might bind a constant region shared by human and mouse Igs. ELISAs were used to screen a number of QSYP-containing phage clones against immunoglobulins of various isotypes, but these phage clones failed to bind either human IgG or mouse Igs (IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgA, and IgM) (data not shown). This group of Igs included all the Ig classes and isotypes represented by the various mAb samples which were used to select the QSYP phage from the library. These negative results suggested that another component contained in the mAb preparation might be contributing to the QSYP phage selection. It was determined that each mAb sample used for phage-display selection had been produced using hybridoma cells cultured in media supplemented with 5-10% fetal bovine serum, which is known to contain IgGs (Lucas et al., 1988). The mAbs were then isolated using protein G affinity chromatography, a commonly used procedure that would enrich for all IgGs, not just the expressed mAb. We postulated that the QSYP sequence was not specific for the mAb of interest, but was binding to contaminating bovine IgG. To test that possibility, we correlated the presence of bovine IgG in the mAb samples to the ability of that sample to bind phage containing the QSYP sequence. Rabbit anti-bovine IgG was used to test mAb preparations for the presence of bovine IgG. As seen in Fig.4.2, mAb samples 4B4 and F91 cross reacted well with the anti-bovine IgG, and mAb sample GZD1E8 also showed a moderate cross-reaction. MAb K42-41L (Adamus, 1991) was used as a negative control to show the background level of the experiment, since it had been prepared in serum-free media and should not contain bovine IgG.

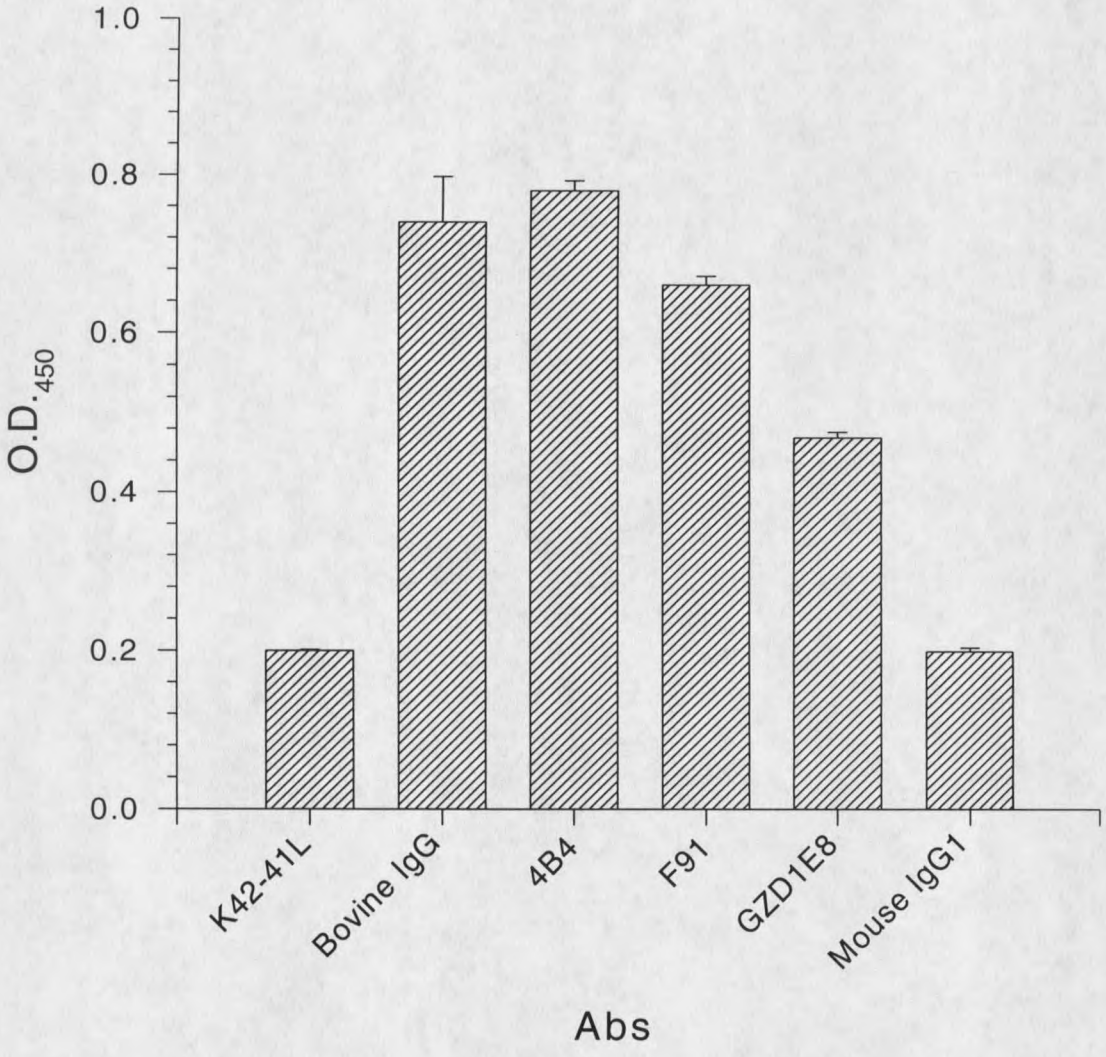

Fig.4.2 **Detection of Bovine IgG in mAb preparations**. MAb was placed in wells at 1 µg/ml and bovine IgG was detected by addition of HRP-conjugated rabbit anti-bovine IgG. MAb K42-41L was used as a negative control since it was grown in serum free medium. O.D. values shown are the mean of duplicate samples with SD shown as error bars.

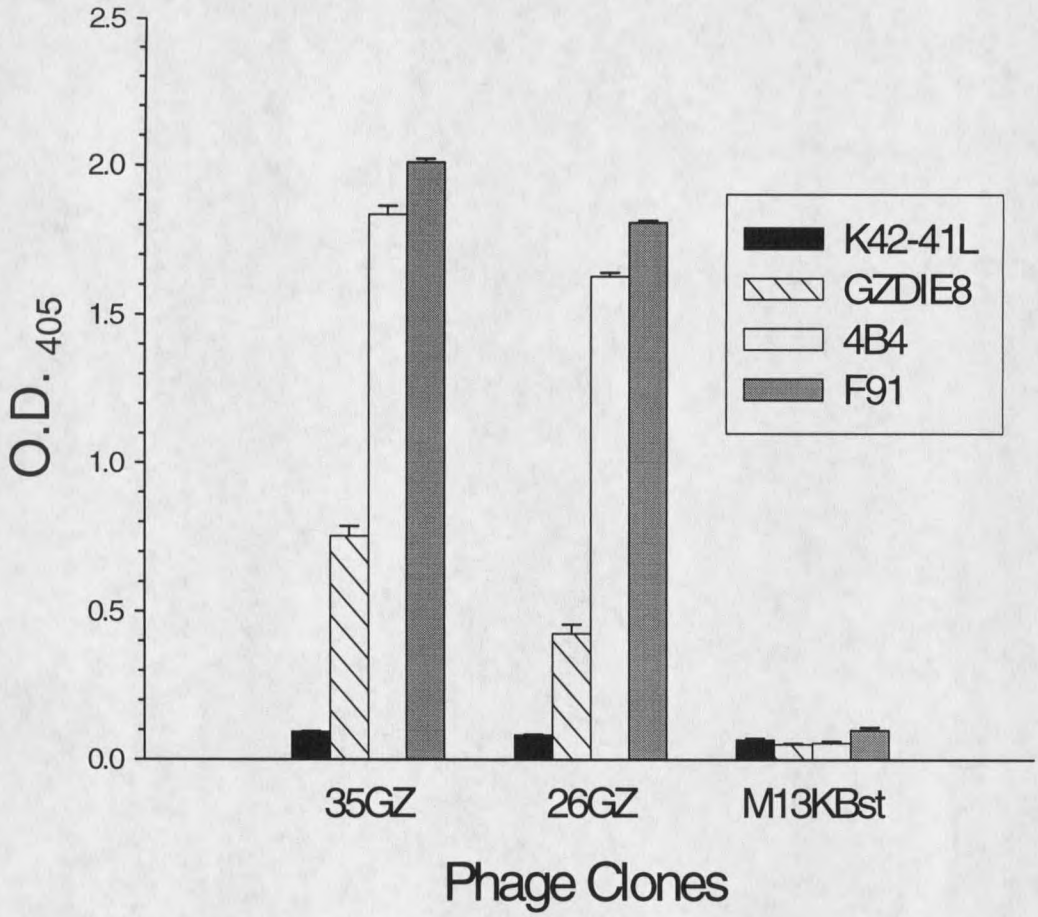

Fig.4.3 **Binding of phage clones to mAb preparations**. MAbs were placed in wells at 1 µg/ml. Phage clones were added at $5x10^8$ pfu per well with parent phage M13KBst shown as a negative control. Phage binding was detected by rabbit anti-M13 antiserum followed by AP-conjugate goat anti-rabbit secondary Ab. O.D. values are mean of duplicate samples with SD shown as error bars.

Binding of QSYP-containing phage clones 35GZ and 26GZ to different mAb preparations was measured. The results showed that both QSYP-containing phage bound mAb preparations 4B4 and F91 at a high level, mAb preparation GZD1E8 moderately, and mAb preparation K42-41L (bovine IgG free) at background levels (Fig. 4.3). These results suggest a direct correlation between the presence of bovine IgG in mAb samples (Fig.4.2) and the level of QSYP phage binding to those samples (Fig.4.3). In addition, phage clones 35GZ and 26GZ, which had originally been selected using mAb GZD1E8, showed even greater binding to the mAbs with the higher bovine IgG contamination, 4B4 and F91(Fig.4.3). These data provided strong evidence that the QSYP sequence was being selected by contaminating bovine IgG, rather than the mAbs themselves.

To confirm that the QSYP-containing phage 26GZ bound bovine IgG, this clone was bound to wells of an ELISA plate at various concentrations and used to capture bovine IgG from mAb samples, as shown in Fig. 4.4. The captured bovine IgG was detected with rabbit anti-bovine IgG HRP conjugate. The QSYP-containing phage 26GZ bound bovine IgG from mAbs 4B4 and F91, two samples contaminated with bovine IgG (see above), but control M13 phage lacking the QSYP sequence did not bind bovine IgG. It was also shown that bovine IgG was not captured from the mAb K42-41L produced in serum-free media as expected.

To demonstrate that bovine IgG contamination could have originated from serum in the culture media, one liter of a 10% fetal bovine serum solution of PBS was passed over a protein G affinity column to capture bovine IgG.

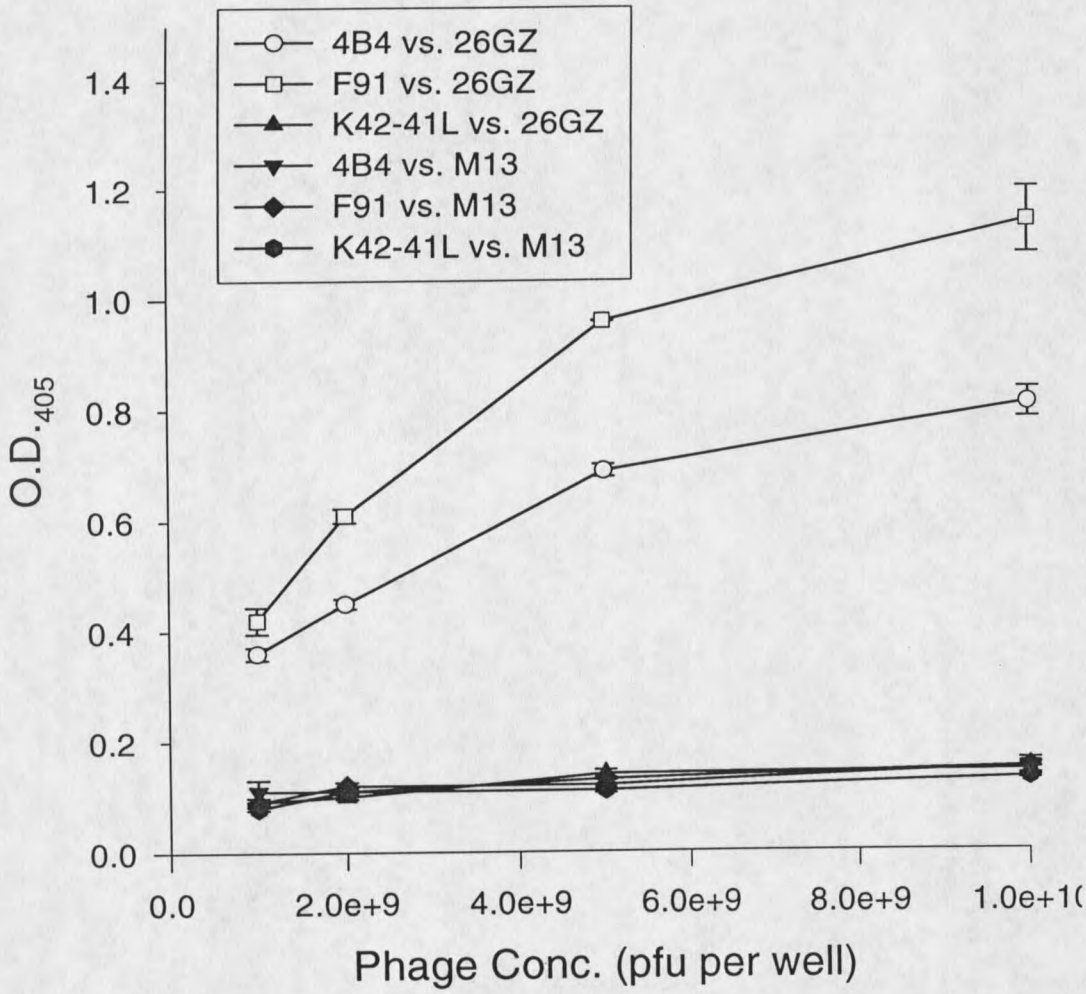

Fig.4.4. **Capture and detection of Bovine IgG contaminating mAb preparations by QSYP phage clones.** Phage clones were placed in wells at increasing concentration. MAb samples were added at 2 µg/ml and the bovine IgG in the mAb sample was captured by the QSYP-sequence containing phage and detected by rabbit anti-bovine IgG HRP. Different mAb preparations were tested against both negative control parent phage (M13KBst), and 26GZ (QSYP- containing phage clone). O.D. values shown are the mean of duplicate samples with SD shown as error bars.

Elution of the column with a 100 mM glycine 0.15 M NaCl, pH=2.5 resulted in fractions containing 4 mg of total protein, as determined by absorbance at 280 nm. When these were run on a reducing SDS-PAGE gel, only two bands were seen, which corresponded to the expected migration distances of the heavy and light chains of an immunoglobulin (Fig.4.5). This eluted bovine IgG reacted with rabbit anti-bovine IgG by ELISA (Fig. 4.2), and bound specifically to QSYP-containing phage (Fig.4.6). A synthetic peptide corresponding to the phage clone 2Fg, SQSYPTRNS, was synthesized and tested to determine its ability to inhibit binding of QSYP-containing phage to mAb samples. As seen in Fig.4.7, peptide pep2Fg inhibited the binding of QSYP containing phage clones 2Fg and 13D with a half-maximal peptide effect in the nanomolar range. The data indicate that the displayed sequence QSYP was responsible for the binding of the phage to the bovine IgG in the mAb samples, and that the activity of this sequence was not dependent upon attachment to the phage.

Discussion

Four mAb preparations with specificity for different antigens selected phage with the same QSYP consensus sequence from a random peptide phage display library. Phage clones containing this sequence bound to the mAb preparations in proportion to the amount of bovine IgG contaminating the samples. MAbs are commonly produced using hybridoma cell lines grown in media containing up to 10% fetal bovine serum, which contains significant amounts of bovine IgG (Lucas et al., 1988).

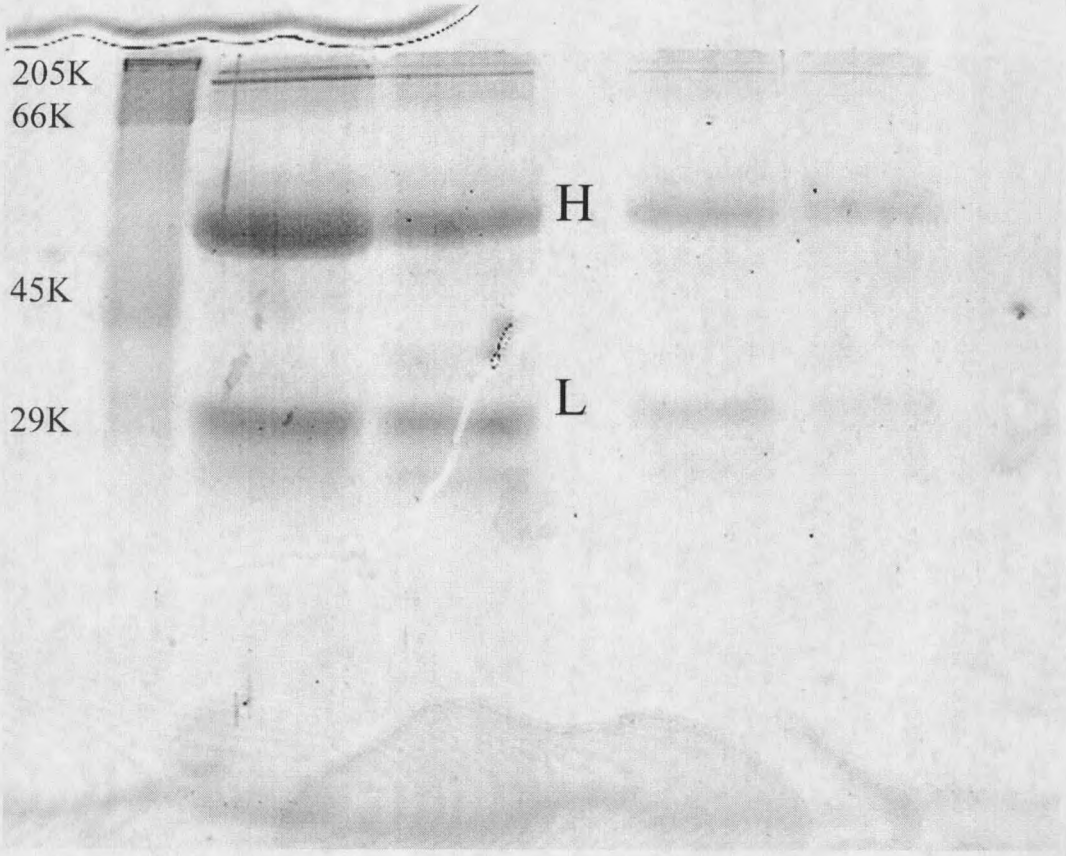

Fig.4.5 **SDS-Page reducing gel of Protein G isolated Bovine IgG.** Gel shows the presence of heavy, H, and light, L, chains of an immunoglobulin verifying the presence of an immunoglobulin in the Protein G chromatography of fetal bovine serum. Gel shows increasing concentrations of Bovine IgG, right to left, with protein standard ladder labeled on the far left.

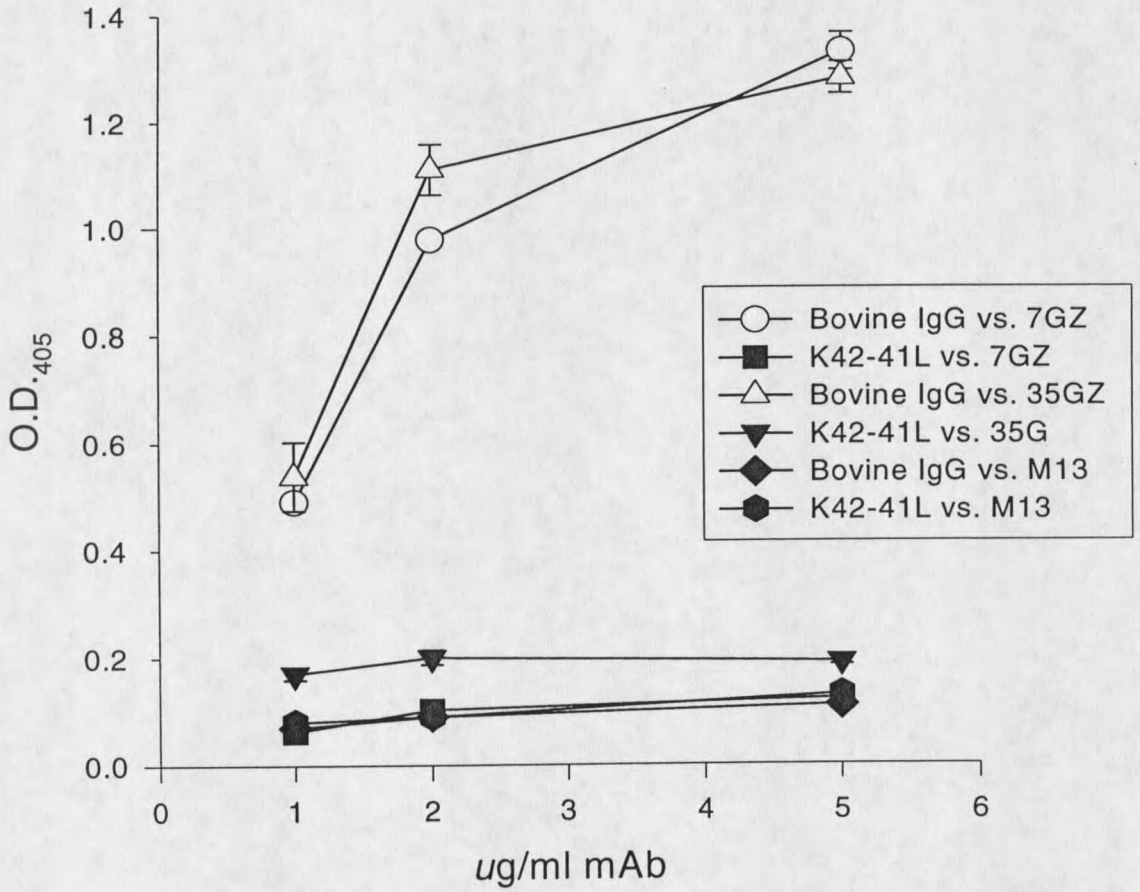

Fig.4.6 **Binding of phage clones to mAb preparations and bovine IgG.** mAbs/Abs were placed in wells at 1-5 µg/ml. Phage clones were added at a concentration of $5x10^8$ pfu per well with phage binding detected by rabbit anti-M13 antiserum followed by AP-conjugate goat anti-rabbit secondary Ab. Parent phage M13KBst was shown as a negative control. The legend shows symbols corresponding to the different combinations of mAb K42-41L and bovine IgG binding to various phage clones. All O.D.values shown are the mean of duplicate samples with SD shown as error bars.

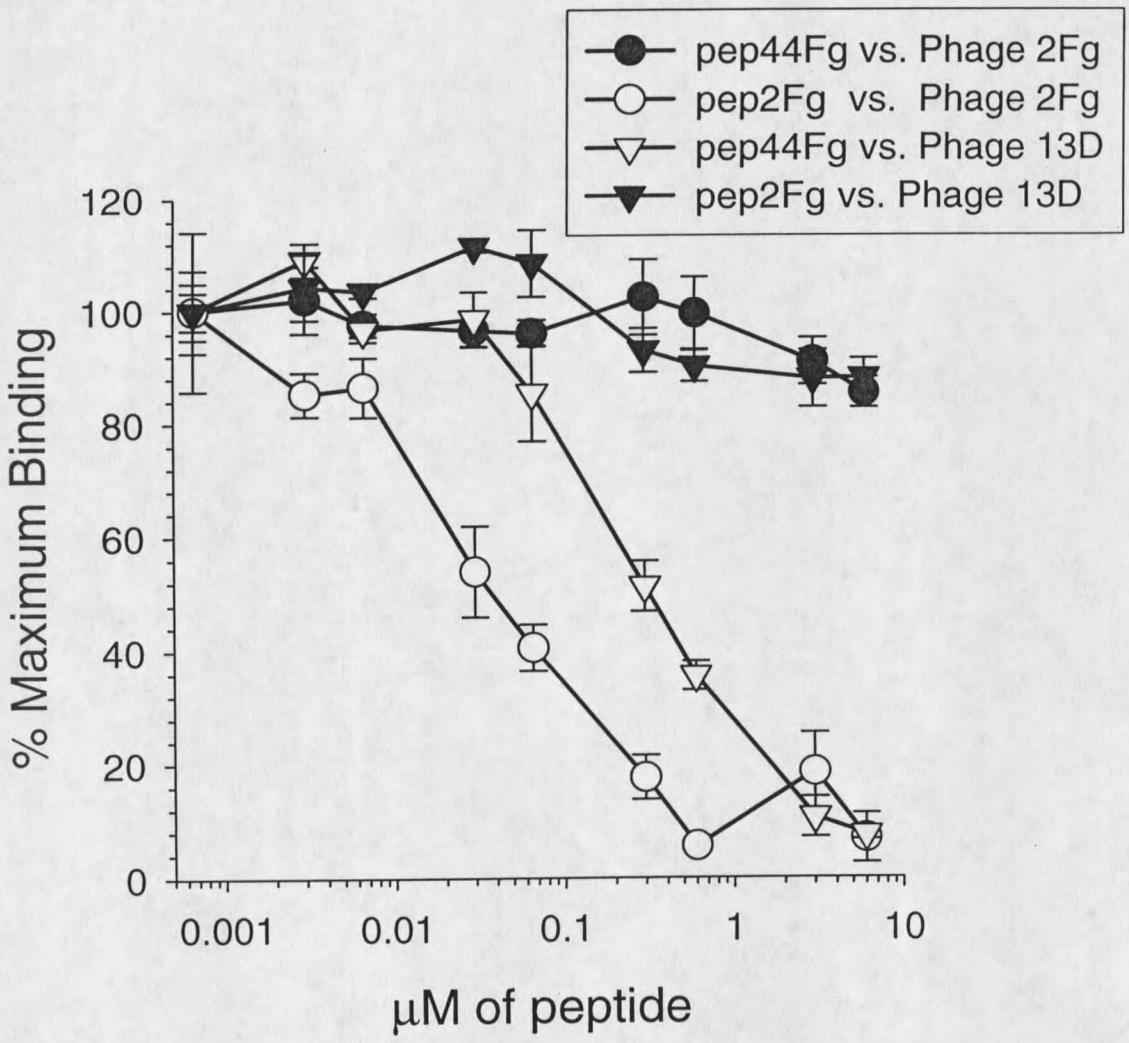

Fig.4.7 **Peptide inhibition of QSYP containing phage clones binding to bovine IgG**. Bovine IgG was coated on the plate at 3 µg/ml. Peptide was added to wells at various concentrations for 20 min. at RT followed by addition of phage clones at $5x10^8$ pfu per well for O/N incubation 4°C. Phage binding was detected by rabbit anti-M13 antiserum followed by AP-conjugate goat anti-rabbit secondary Ab. The legend shows symbols corresponding to the different combinations of negative control peptide pep44Fg-RLTPEPDD, and QSYP containing peptide pep2Fg-S**QSYP**TRNS inhibiting the binding of two QSYP containing phage clones, 13D S**QSYP**DR and 2Fg S**QSYP**TRNS. All O.D.values shown are the mean of triplicate samples with SD shown as error bars.

A widely used protocol for purifying the mAbs from the culture supernatant involves affinity chromatography on protein G beads, which will co-purify any bovine IgG in the medium.

The data presented here demonstrate that bovine IgG can select QSYP-containing phage from a peptide display library. Whether enough of such phage are selected to result in a secondary consensus sequence appearing in the alignments presumably depends on the relative amount of the bovine IgG contamination in the purified mAb preparation. A lower yield of mAb in a hybridoma cell supernatant could result in a proportionately higher fraction of bovine IgG contamination after protein G affinity chromatography purification (Mushens et al., 1993). The results shown here should be a caution to other investigators who may find such bovine IgG-selected phage sequences, and to suggest that in some instances it might be worthwhile to either use a further purification step to remove bovine IgG from the mAb preparation or grow the hybridomas in serum free or bovine IgG depleted media before using it to screen a phage display library. Bovine IgG removal might be accomplished by passing the culture supernatant or the protein G eluate over a column of immobilized anti-bovine IgG antibodies or a QSYP-containing peptide attached to beads. Alternatively, the QSYP-containing peptide could be added to the mAb sample before phage selection to block binding of phage containing the QSYP sequence to any bovine IgG that may contaminate the mouse or human mAb sample, and thus reveal more clearly the desired specific epitope.

CHAPTER FIVE

SUMMARY, CONCLUSIONS, AND FUTURE STUDIES

Summary and Conclusions

The studies described here have shown evidence that a peptide phage display library can be used to elucidate peptides which mimic discontinuous epitopes of CD4 binding site mAbs to gp120. Three peptides were shown to bind specifically to their respective mAbs and were inhibited from binding to their mAbs by addition of the antigen, gp120, providing evidence that the peptides bind to the antigen recognition sites. Two of these synthetic peptides were then able to inhibit binding of their respective phage to the mAbs, with half-maximal inhibition in the 10-100 nanomolar range, as well as also inhibit binding of the mAb to gp120, with half-maximal inhibition in the micro-molar range. Sera collected from mice immunized with KLH-peptide conjugates from both peptides bound gp120 well above pre-immunization levels suggesting that the peptides can elicit an antibodies to gp120 as a result of their mimicking its structure. Residues in both peptides can be mapped to residues on gp120 that are known sites of escape mutants for their respective mAbs (Thali et al. 1992). Based upon these results, there is reason to believe that an epitope vaccine can be developed which will allow the subject to develop an immune response that is directed against a specific, conserved region of the virus.

These results are very encouraging but at the same time there are a number of questions that arise about possible difficulties concerning this project that deserve to be mentioned and discussed here.

As previously seen and discussed in chapter three, it is somewhat difficult to pinpoint exactly how each residue in the peptides would mimic residues found on gp120. The data suggest that we have been able to map a discontinuous epitope with an active linear peptide, and there is relatively good evidence based upon the sequence of the peptides and mutational data to support this conclusion. Even so, it must be remembered that the basis for structure comparison is a snapshot picture of a highly dynamic glycosylated protein. The crystal structure of gp120 was determined bound to both CD4, which is know to induce conformational changes in the structure of gp120, as well as mAb 17b, which was used to confer stability to the structure for crystallization (Kwong et al., 1998). Mapping residues in this context must be taken as speculation. There may be a number of residues involved in binding the mAbs that are not apparent in the structure, as well as residues which we believe are important which might be inaccessible to mAb binding. Even considering this, there is a good case for these peptides mimicking a conformation of gp120 based upon their inhibition and antigenic activity. There have been more definitive examples of peptides mimicking discontinous epitopes (Jesaitis et al., 1999;Burritt et al., 1998; Burritt et al. 2001), which gives some confirmation that this is possible in the case of gp120.

In the mAb F91 phage display results, a peptide that was active when expressed on the pIII protein did not bind the mAb when it was synthesized and used alone (chapter

three). This is apparently not uncommon in phage display work (J. Burritt, personal communication) and can be explained in a number of ways. It is possible that this exact sequence, due to conformational reasons, needs to be attached via its C-terminus to the pIII protein for it to maintain its active conformation. This is a plausible explanation, especially if the sequence of the peptide has regions of charge or hydrophobicity that would bias its conformation in the free state. The sequence itself appears to have little charge (NH_3^+- LYAGSWSAW) but the peptide is very soluble in an aqueous solution, which is not suggestive of a constrained hydrophobic peptide. Also, part of the pIII protein might be involved in the interaction between peptide-phage and mAb F91. This would infer that the peptide alone would not be able to mimic the binding residues properly and lose it binding capability when used alone. Furthermore, with this particular clone, there were a number of mutations which altered the amino acid sequence of constant residues normally flanking the random nonamer in the pIII protein. Normal phage clones in the library contain a constant signal peptide sequence, which would be cleaved by the signal peptidase, linked to the nine variable residues of the library attached

Normal= KLLFAIPLVVPFYSHSXXXXXXXXXGPP-pIII
1pF9g= KLLFAIPLVVPFYSHPLAYGSWSAWPP-pIII

Fig.5.1 **Sequence of phage clone 1pF9g comparison with constant regions of a typical phage clone.** Frameshift mutations are seen in the signal peptide region as well as the GPP linker region. Overall frameshift mutations account for the loss of one codon which returned the pIII gene to the proper frame for translation of the protein.

to a constant linker region (GPP) which would attach to the N–terminus of the pIII protein of the phage (See Fig. 5.1). Mutations found in the 1pF9g clone alter both the signal peptide as well as the linker region. These mutations might allow for the use of an alternate cleavage site by the signal peptidase which cleaves the signal peptide during assembly and release of the phage outside of the cell (reviewed in Izard and Kendall, 1994). It is therefore possible that the peptide expressed on the pIII protein had a different peptide sequence than what was inferred from the DNA sequence and what was synthesized chemically.

Peptides are inherently flexible structures and, depending upon their size and sequence, can assume a multitude of different conformations while in solution. It is believed that for these peptides to bind to the mAb, they must be in a certain conformation, which is the gp120 mimetic state of that peptide. Once free in solution, or more importantly, conjugated to a carrier protein, that mimetic state of the peptide may still be possible, but might not be the preferred conformation of the peptide. The majority of the peptide conformations most likely would not be recognized as gp120 mimetics due to this flexible nature of a free peptide. Evidence of this is seen in the binding of the mouse serum directed against the KLH-peptide conjugate. Reactivity of the sera was very high against another peptide conjugate, OVA-peptide (see Fig. 3.5), but cross-reactivity against gp120 was much lower, presumably because most of the Abs were directed against conformations of the peptide other than the gp120 mimetic state (see Fig. 3.6). Even so, it appears that there is a large enough population of the peptide in the mimetic state to allow it to be recognized. There are some ways in which this conformation may

be enhanced and these will be discussed in the Future Studies section, later in this chapter.

It is known that to have an effective vaccine, both branches of the immune system should be stimulated. The work described here is not intended to be the "magic bullet" in vaccine design, but would be expected to assist in eliciting an antibody response directed against the surface exposed glycoprotein gp120. There have been some results in developing a cellular (CTL) response using phage display technology, but the displayed peptides were linear portions of various HIV proteins which would make good candidates for peptide presentation by the MHC class molecules (De Berardinis et al., 2000). Since the peptides developed in the present study are not linear representations of the protein, it would be difficult for them to elicit such a cellular response. The possible benefit of the peptides characterized in the present study would be to elicit a broadly reactive neutralizing antibody response that would be directed against a conserved region of the exposed gp120 protein. Used in conjunction with other vaccine components that elicit a CTL response, this type of vaccine could be very effective in defending against HIV.

As a side note, the results reported in chapter four represent a good example of how phage display technology works and the unexpected results that are sometimes obtained. While screening for peptides which bind to mAb F105, the elucidation of the second consensus sequence QSYP allowed for an opportunity to think "outside the box" and determine what else could be present in a mAb sample that would bind a phage display peptide sequence. The characterization of the QSYP peptide as a specific bovine IgG binding motif solved an interesting puzzle as to the origin of this sequence. The

ability to bind contaminating bovine IgG with a peptide could be used in a number of applications, either involving purification of fetal bovine serum to remove IgG, or purification of mAb samples to remove the contaminating bovine antibodies. The peptide QSYP could easily be used to produce an extremely low IgG bovine serum which can be used in hybridoma cell cultures, where the presence of bovine IgG could be detrimental. Low IgG fetal bovine serum can be currently purchased (HyClone, Low IgG Fetal Bovine Serum) but at two to three times the price of normal FBS. The peptide could also be used directly to purify mAb samples already contaminated with bovine IgG, especially in cases where a high degree of purity is needed, i.e. crystallographic studies. With regard to phage display experiments, if it is known that the screened mAb is contaminated, a preincubation of the mAb with the QSYP peptide would prevent any QSYP phage clone from binding and would guarantee that such clones would not overwhelm the population of phage specific for the mAb of interest. Put together, there are a number of very interesting applications and possibly even products which could be developed to utilize such a peptide.

Future Studies with HIV peptides

There are a number of directions which can be taken to continue to study the immunogenicity and antigenicity of the characterized peptides. Presently, Ann Intamaso, a fellow graduate student in the M. Teintze laboratory, has been working to develop an immunogenic system in which the characterized peptides, or other sequences, can be placed on the surface of the Cowpea Chlorotic Mottle Virus (CCMV). This is an RNA

plant virus whose capsid is comprised of 180 identical subunits of the outer coat protein. Ann is in the process of covalently attaching these peptides to the surface of the virus by a disulfide linkage. Cysteine residues were substituted by site-specific mutagenesis in exposed portions of the CCMV coat protein and conjugated to peptides which were synthesized with an additional cysteine residue on the C-terminus. As discussed in chapter three, previous immunization studies were done with KLH conjugated peptides, resulting in serum with cross-reactivity against gp120. The reason behind developing another conjugate such as CCMV would be to improve the immunogenicity of such a construct. CCMV, if fully conjugated, would display the peptide of interest in an ordered, 180 copy format, which might be more advantageous than the KLH presentation of the peptide. Currently, KLH conjugation is dependent upon attachment of the peptide to exposed lysine residues that have been conjugated with maleimide linkers which can vary between 100-300 linkages per molecule of KLH. Ann has results in which mouse sera to CCMV-conjugates cross-react with gp120 as effectively as the KLH-conjugates reported in chapter three (A. Intamaso, personal communication). This is encouraging since so far there has only been about a 20-30% conjugation rate associated with these constructs. If all or a higher percentage of the coat proteins of CCMV could be conjugated, this could represent a more immunogenic display system for the peptide.

One of the most important future studies which can be done is the further characterization of the anti-gp120 response of sera obtained from mice immunized with the peptide constructs. The studies so far have demonstrated that there is cross-reactivity between anti-peptide sera and gp120 but further work needs to be done to better

characterize this response. To show that the response is directed against residues conserved in the CD4bs of gp120, anti-peptide sera should be able to inhibit binding of gp120 to CD4 in ELISA experiments. Also, the sera can be used to screen a number of different strains of HIV gp120 to determine how broad the specificity of the antibody response is. Further characterization would also include neutralization studies to determine whether the antibodies in the sera will bind to gp120 on intact virions and prevent infection of naive cells by the virus. This experiment would entail preincubation of the sera with HIV after which the mixture would be placed in contact with naive cells and allowed to incubate. Rate of virus infection of the naive cells would be measured and compared in the sera treated versus untreated control samples.

A prime boost immunization experiment could be used to compare the response elicited by gp120 and peptide conjugate. This experiment would involve an initial priming (immunization) with the peptide conjugate followed by a boost using gp120. The response elicited would then be compared to a control immunization using gp120 for both the initial priming and following boost. The antibody response from this type of experiment would whether there was any benefit from using a more sequence directed peptide antigen versus the whole protein gp120.

If the mAb-bound conformation of the peptide could be determined, it would enable us to design a peptide constrained in a more gp120-mimetic conformation. This could be accomplished by X-ray crystallography of a Fab/peptide complex. This experiment is being planned using the mAb F105 Fab co-crystallized with the peptide

44Fg. Hopefully useful information will be determined that can then be used to direct future synthesis and conjugation studies.

The screening of mAb F91 selected a phage clone that bound specifically to the mAb and was competed off by the addition of the antigen gp120, but the displayed peptide was not active as a synthetic peptide. Possible reasons for this have been described previously in this chapter; one reason discussed was that the peptide expressed on the pIII protein did not have the predicted sequence. We are in the process of isolating the pIII protein from the phage and digesting it with trypsin to generate a series of peptides. These peptides would then be subject to mass spectrometry analysis to determine their identity. The mass of the peptide which contains the N–terminus would determine whether the predicted residues are attached or help to identify the peptide expressed on the protein.

There is also the possibility of screening more mAbs that bind to the CD4 binding site of gp120 to determine possible peptides which could be used as antigens. We have mainly chosen the mAbs in this study based upon their availability as well as their broadly neutralizing capability, but other broadly neutralizing mAbs could be obtained for similar studies.

Future Studies with the QSYP peptide

There are some studies which can be done that would further characterize this peptide. It is unknown exactly where this peptide binds to bovine IgG. Presumably it must bind to a constant region but whether it binds the heavy and lights chains, or a site

that includes both, is still unknown. Also in question is whether the QSYP peptide is isotype specific. Known bovine IgG isotypes include IgG1, IgG2a, and IgG2b (Tewari and Mukkur, 1975; Estes et al., 1990; Kacskovics and Butler, 1996) but these isotypes are not available commercially and few laboratories have isolated them. So far we have been unable to obtain any pure bovine IgG isotype. All three isotypes bind protein G with the approximately the same affinity, so it is difficult to tell if the QSYP peptide binds specifically to any or all isotypes (Bastida-Corcuera et al., 1999; Eliasson et al., 1989). It is also unknown which is the predominate isotype found in fetal calf serum. These are some of the more interesting questions which have potential as future studies in this area.

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