

**The Antibody Response as it relates to  
Human Immunodeficiency Virus Type 1.**

**Thesis  
Presented for the degree of  
DOCTOR OF PHILOSOPHY  
by**

***Paul Roben, B.Sc.***

**Under the Supervision of  
Dr. Richard O'Kennedy**

**School of Biological Sciences,  
Dublin City University**

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**This thesis is dedicated to my parents.**

I declare that all the work reported in this thesis was performed by Paul Roben, unless otherwise stated.

Paul Roben

PAUL ROBEN

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Last, but not least, Thank God I,m finished !!

## ABSTRACT

The humoral immune system responds to antigenic challenge by producing a diversity of antibodies specific for antigenic determinants being presented by the foreign body, as is the case when a human becomes infected with the HIV virus. A large diversity of antibodies is produced against two primary epitopes of the HIV surface antigen, gp120, namely the V3 loop and the CD4-binding site. A better understanding of this, and other similar responses, has important implications for combating many diseases, including AIDS. However, the diversity of the immune response, coupled with the problems associated with producing human antibodies, has made it very difficult to study.

Phage display libraries provide a means of producing a large number of antibodies from a single individual, thereby facilitating the investigation of a complete immune response. These libraries have been utilised to study antibodies specific for the CD4-binding site of gp120, and, in conjunction with a binary plasmid system, the relative contributions of the heavy and light chains to specificity and function of the antibody were demonstrated.

Finally, Fab fragments specific for both metal ions and gp120 were produced, in an attempt to catalyse the peptide cleavage of gp120.

This work has led to an increased understanding of antibody-antigen interactions, with specific reference to neutralising the HIV virus, and has further investigated the possibility of constructing catalytic metallo-antibodies.

## ABBREVIATIONS

Ab	Antibody
Ag	Antigen
AIDS	Acquired Immunodeficiency Virus
AP	Alkaline Phosphatase
B-cell	B Lymphoblast Cell
bp	base pairs
BSA	Bovine Serum Albumin
°C	degrees centigrade
CD4	Cluster of Differentiation antigen 4
cDNA	Complimentary DNA
CDR	Complimentarity Determining Region
CMV	Cytomegalovirus
D segment	Diversity segment
dATP	Adenosine triphosphate
dCTP	Cytidine triphosphate
dGTP	Guanosine triphosphate
DNA	Deoxyribonucleic Acid
dsDNA	double stranded DNA
dsRNA	double stranded RNA
DTT	Dithiotreitol
dTTP	Thymidine triphosphate
EBV	Epstein Barr Virus
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
Fab	Fab Fragment of an antibody

GC	Germinal Center
gp120	glycoprotein 120 of HIV
HBV	Hepatitis B Virus
HCl	Hydrochloric acid
HEV	High Endothelial Vesicles
HIV	Human Immunodeficiency Virus
HPLC	High Pressure Liquid Chromatography
HRP	Horseradish Peroxidase
Ig	Immunoglobulin
IL-4	Interleukin 4
IPTG	Isopropylthiogalactosidase
J region	Joining region
M	Molar
MAb	Monoclonal Antibody
mg	milligram
mIgM	Membrane-bound Immunoglobulin, isotype M
ml	millilitre
mM	millimolar
O.D.	Optical Density
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PMSF	Propylmethylsulphonylfouride
RNA	Ribonucleic Acid
rpm	revs per minute
RU	Response units
sCD4	soluble CD4



SDS	Sodium dodecyl sulphate
ssDNA	single stranded DNA
ssRNA	single stranded RNA
TCR	T-cell receptor
T-cell	T Lymphocyte Cell
TE	Tris-EDTA
U	Units (enzyme activity)
V1/V2	Variable loop 1/2 of gp120
V3	Third variable loop of gp120
V region	Variable region
VH	Heavy Chain Variable region
VL	Light Chain Variable region
v/v	volume per volume
w/v	weight per volume
Zn	Zinc

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## INTRODUCTION

## 1.0 INTRODUCTION

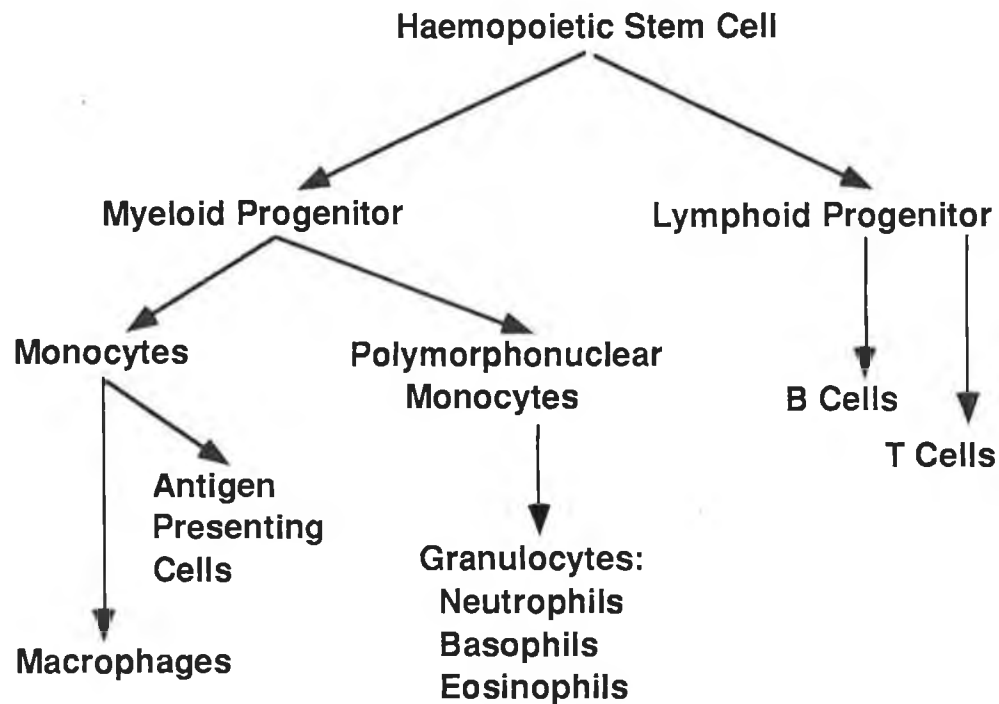
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### 1.1 THE IMMUNE SYSTEM.

One of the primary functions of all organisms is the defence of 'the self' from outside invaders. Evolution of the vertebrates over a period of millions of years has given rise to a series of protective measures known as immunity. This is a complex and elegant interaction involving a large number of cellular and molecular components, designed to effect the clearance of any foreign particles from the organism's system. This mechanism is characterised by the ability to recognise a vast array of foreign bodies while maintaining the integrity of the self and also memorises any invading body such that it can mount a quick and effective response should it meet that invader again in the future. These properties are ideal for any potential therapeutic agent that could be developed to combat many of the diseases prevalent in today's society. As a result, immunity has been the target of much research, in an attempt both to understand the mechanisms by which this system operates and also to try and harness its activities, in an effort to produce analytical, diagnostic and therapeutic agents. The experiments presented in this thesis represent one further step in the development of this area of research. However, in order to understand these studies fully, it is necessary to, first, gain a preliminary knowledge of the immune system, particularly as it relates to the human situation.

An invading body generally consists of a large number of molecules (antigens) that can be potentially targeted by the host immune system and these are dissected to yield an array of smaller molecules which can then be presented to the various components of the immune system in a way that can be recognised as foreign particles. These antigens are recognised by the cellular components of the response via cell surface receptors, and these cells produce a number of other substances to activate the complete immune response. In humans, this response is located in a number of organs: the primary organs consist of the bone marrow and the thymus, while secondary organs include the spleen, lymph nodes and tonsils. The cells of the immune response all originate from a haemopoietic stem cell (Roitt, 1985) which then differentiates to yield two major cellular classes: the myeloid and the lymphoid systems (Figure 1.1). The myeloid cells differentiate further to give monocytes and polymorphonuclear monocytes (polymorphs). The monocytes give rise to macrophages which are responsible for the endocytosis of microorganisms and other foreign bodies. Monocytes can also develop to yield antigen-presenting cells (APC) which are involved in the presentation of antigen to the other cells of the immune system. The polymorphs lead to a number of granulocytic cells all of which function in a non-specific way to counteract the affects of invaders such as microbes, viral particles and allergens.





**Figure 1.1.** Flow diagram showing the differentiation pathways of some of the cells involved in the immune system. All cells originate from a stem cell in bone marrow. From there, they enter the myeloid or lymphoid pathways. The cells then differentiate to become one of the many cells involved in regulating the immune system and controlling infection.

## **1.2 T LYMPHOCYTES AND ANTIGEN PRESENTATION.**

The lymphoid system consists of two main cellular classes; the B cells and T cells. It is the antigen-mediated activation and subsequent cooperation between these two cell lines that can activate all of the other components of the immune system and clear the foreign body from the organism. The specificity displayed by this system is characteristic of these two cell types, as is the ability to remember antigens. Therefore, these cells are of primary importance

in the overall response. The response begins with the internalisation of the foreign antigen by a variety of cells. This can include some of the cells described in Figure 1.1, such as macrophages and B cells although it is not restricted to cells of the immune response and can include any cell that has been invaded by an immunogen such as a virus. This antigen is then presented to T cells leading to a cascade of events, resulting in the destruction of the invader.

The T cell response, also known as cell-mediated immunity, originates in the thymus of humans (Finkel *et al.*, 1991; Carding *et al.*, 1991). Lymphoblasts (cells that have undergone primary differentiation from the haemopoietic stem cell) arrive in the thymus of embryos from the bone marrow or liver. These cells are then positively selected for those expressing the correct receptors on the surface, followed by negative selection of any cells that recognise self antigen. This leaves only those cells with the correct combination of surface receptors capable of specifically recognising foreign antigen and are introduced into circulation to monitor the presence of immunogenic antigen. All others are destroyed by apoptosis and subsequently removed by macrophages (Green *et al.*, 1991). The major receptor involved in antigen recognition is the T Cell Receptor complex (TCR). This consists of a number of polypeptide chains coded by an intricate set of genes, thus allowing for a high degree of genetic diversity (Rudd, 1990), which translates into a large array of diverse TCR receptors such that each T cell contains a distinct TCR and so can recognise a different antigen. It is, therefore, the TCR that confers specificity on the T cell response. The process of thymic education and the subsequent

immune responses are also mediated by a number of substances called cytokines which include interleukins, interferons, tumour necrosis factor, growth factors and many others. Each cytokine has a selective effect on a particular cell type, depending on its stage of development and it is through the use of these molecules primarily, that the cells of the immune system communicate (Beverley, 1990; Noelle *et al.*, 1990; Scott and Kaufmann, 1991). Each cell has a distinct receptor on the cell surfaces for cytokines, generally classed as 'cluster of differentiation' (CD) antigens. (T cell antigen papers, 1989; Knapp *et al.*, 1989; B cell antigens, 1989). Other receptors important for T cell antigen recognition include Major Histocompatibility Antigens (MHC). These are present on all cells capable of communicating with T cells and are classed as MHCI or MHCII (Accolla *et al.*, 1991; Germain, 1991). Both of these are used in the presentation of antigen to T cells and it is through these receptors that the T cells can distinguish between self and non-self. It is the combination of the presented antigen with the correct MHC that allows the T cell to determine if this antigen originates from a foreign source, and if so, to activate the other components of the immune system (Kelly *et al.*, 1991; Rudensky *et al.*, 1991a; Rudensky *et al.*, 1991b). Mature T cells can be classified into a number of different groupings such as helper cells ( $T_H$ ) and cytotoxic cells ( $T_C$ ) (Darnell *et al.*, 1986).  $T_H$  cells possess a surface receptor named CD4 which acts in combination with the TCR to recognise antigens that are presented on the surface of other cells in combination with MHCII (Rudd, 1990; Scott and Kauffmann, 1991). This leads to the production of cytokines by the T cell which can have a number of

effects, such as the activation of B cells, macrophages, polymorphs or a number of other cell types, all of which can bring about the destruction of the invader.  $T_H$  cells can be further classified into  $T_{H1}$  and  $T_{H2}$  which display different cell surface receptors and have slightly different functions (Mosmann *et al.*, 1986; DeKruyff *et al.*, 1989; Gajewski *et al.*, 1991).  $T_C$  cells display CD8 receptors and recognise antigen in conjunction with MHCI. This results in the destruction of the antigen presenting cell, mediated by the T cell, which would be desirable if the cell was infected by virus, for example. Finally, antigen-activated T cells can differentiate to produce memory cells which are maintained in circulation and can mount a quick response against that antigen should it be encountered by that individual in the future (Akbar *et al.*, 1991; Beverley, 1990). This is the situation when a person is said to be immune to a particular disease.

Therefore, the immune response initiates with antigen presentation to specific T cells, followed by cytokine-mediated activation of other components of the response, finally resulting in the destruction of the invader.

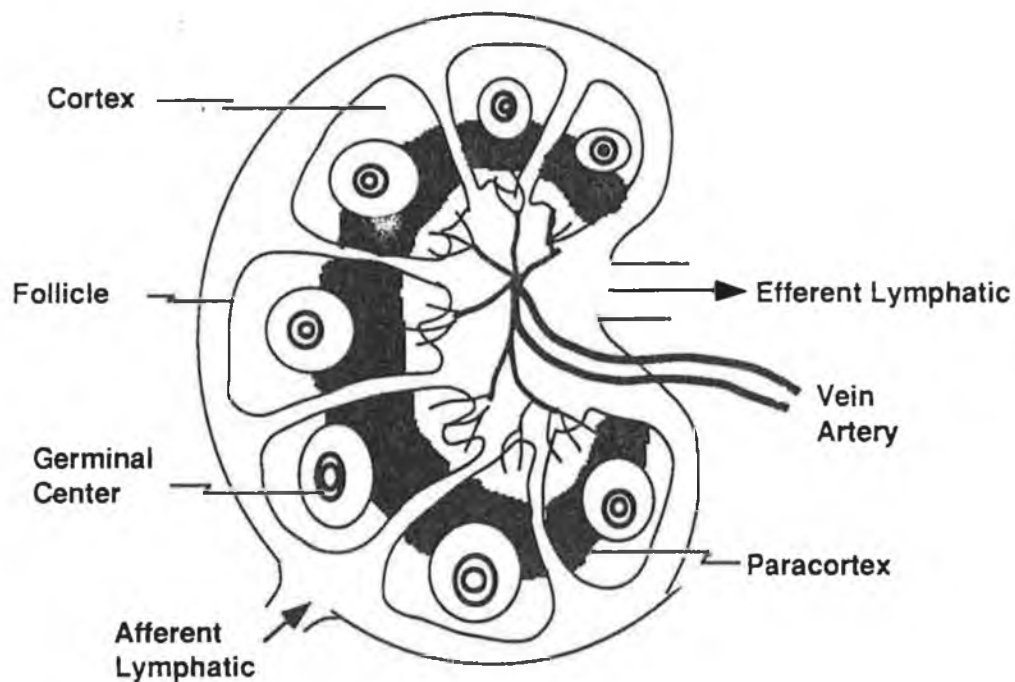
### **1.3 B LYMPHOCYTES.**

The second class of lymphocyte involved in the immune response are the B cells which generate what is known as the humoral response. This means that these cells, upon activation by antigen, produce glycoproteins of the immunoglobulin superfamily, referred to as Antibodies which are capable of mediating destruction of an immunogen (any substance capable of eliciting an immune response).

The precise mechanism of B cell activation, leading to antibody production, is not fully understood. However, there is evidence which supports one particular theory of activation. B cells originate in the bone marrow as pre-B cells that express no surface receptor capable of binding antigen (Reth *et al.*, 1985). These then differentiate to form B cell blasts, involving the expression of an antigen-specific receptor complex on the cell surface. This consists of a membrane bound Immunoglobulin class M molecule (mIgM) coupled with 2 other transmembrane molecules that provide signal transduction with biochemical cascades within the cell (Reth *et al.*, 1991; Ales-Martinez *et al.*, 1991). The complex is similar to the TCR of T cells in that it consists of members of the immunoglobulin family and it is highly antigen specific, thus conferring specificity on this aspect of the immune response also. This ensures that there is a vast array of B cells expressing highly diverse antigen-specific receptors capable of producing a similar diversity of antibodies.

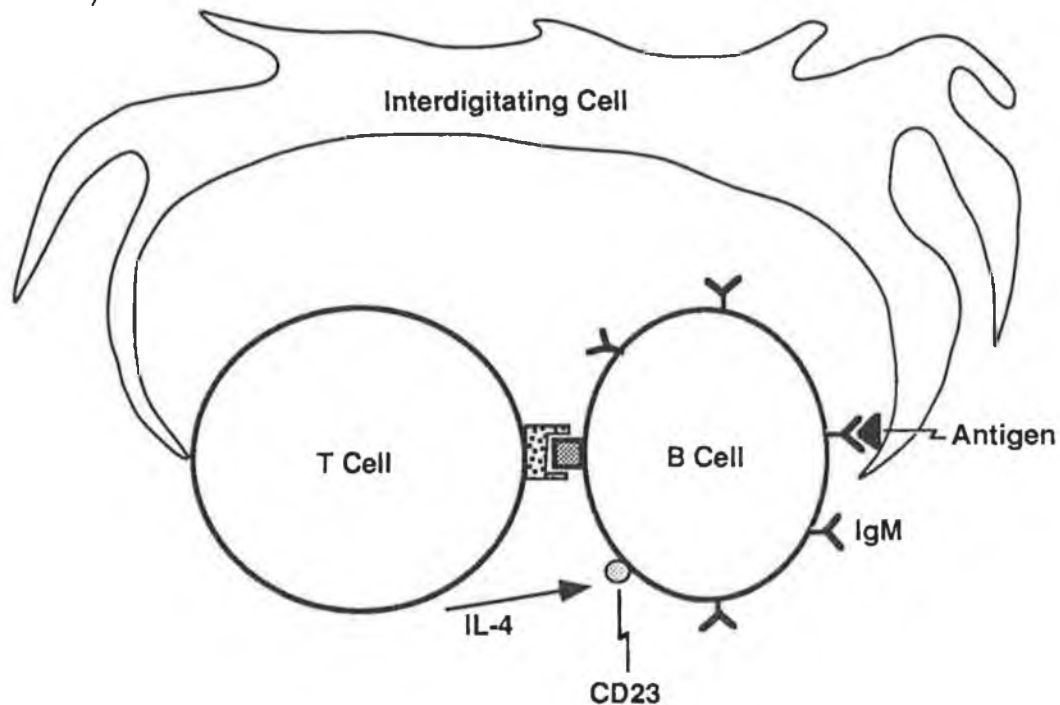
However, it is necessary to be able to select for those B cells that can produce the antibody specific for the foreign antigen at any given time (clonal selection), and to be able to induce the proliferation of that clone and effect its differentiation to produce soluble antibody. A further receptor is then expressed on the B cell surface comprising an IgD molecule which is thought to ensure the survival of the B cell on activation by antigen. This process of activation occurs in the secondary lymph organs such as the lymph nodes (Figure 1.2). The B cell blasts are transferred to the cortex of the lymph nodes where they are called germinal centre (GC) B cells (Dilosa *et al.*, 1991). These GC B cells provide a pool capable of being

activated by antigen. They enter the lymph nodes by migrating through the endothelial walls via High Endothelial Venules (HEV) using specific receptors on the lymphocyte surface. The antigen is carried to the nodes via the lymph system, where it is captured by APC, postulated to be Follicular Dendritic Cells (FDC). These cells internalise the antigen and present it as lccosomes-immune complex coated bodies (Szakal *et al.*, 1988; Kosco *et al.*, 1988; King and Katz, 1990) in the cortex of the lymph nodes. The B cells are able to bind this form of antigen via the mlgM receptor and present it on their own surfaces in conjunction with MHCII. This is the initial step in the antigen-dependent proliferation of B cells.



**Figure 1.2** The structure of a lymph node. The follicular dendritic cells (FDC) present antigen to the B cells in the cortex and they are then activated by T cells in the paracortex. Further differentiation to become memory cells occurs in the germinal centers.

However, binding of the antigen to mIgM is not sufficient to activate the cells and this requires contact with  $T_H$  cells mediated by interdigitating cells (Figure 1.3). Antigen binding merely prepares the cell for T cell interaction by altering the plasma membrane, enhancing antigen processing and upregulating the receptors necessary for T cell-mediated proliferation, eg: CD23 (Gordon *et al.*, 1989).



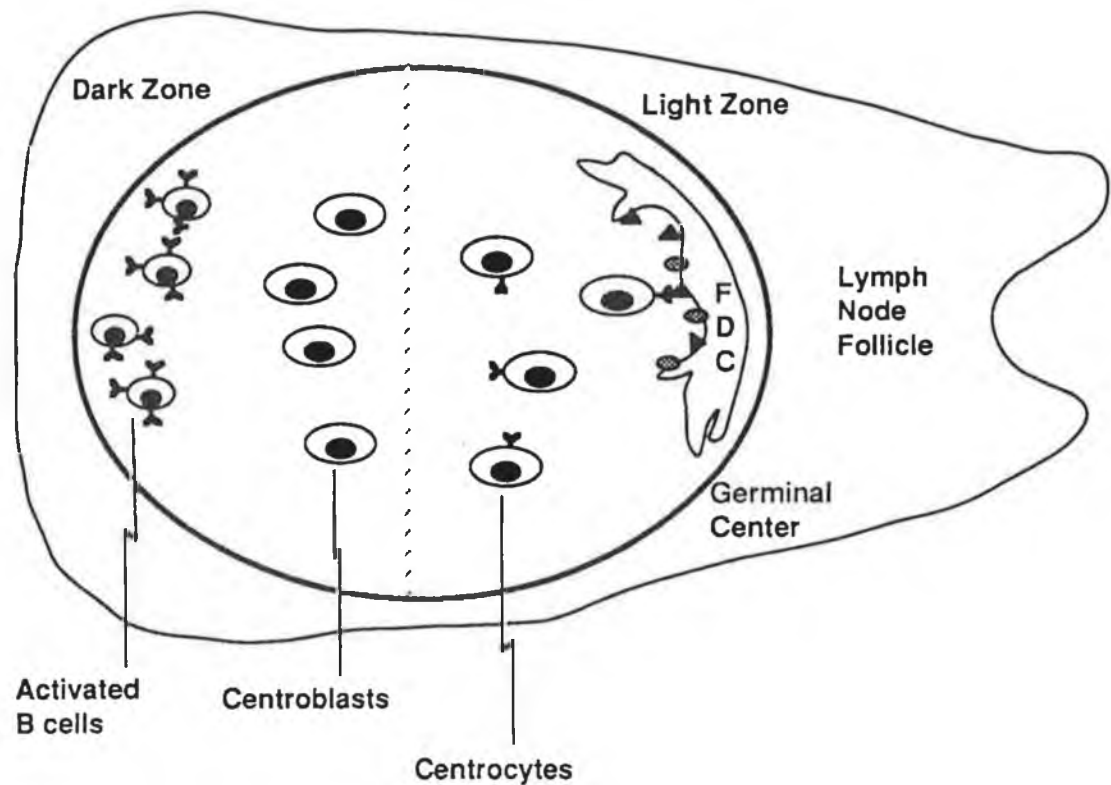
**Figure 1.3** Representation of T cell-dependent B cell activation mediated by interdigitating cells. The B cell expresses processed antigen on its surface which is recognised by the TCR complex on the T cell. This, in turn, secretes IL-4 which upregulates CD23 on the B cell, thereby activating it.

The T cell binds the B cell antigen (Ag)-MHCII complex via its own TCR-CD4 receptor, and there are probably other interactions

involved in the paracortex of the lymph node. This induces the production of interleukin-4 (IL-4) by the T cell which activates the CD23 receptor leading to the antigen specific proliferation of the B cell. It is at this stage that somatic mutation is greatest resulting in affinity maturation of the antibody (discussed later).

The B cells can then return to the bone marrow, or remain in the lymph node. Those that return to the marrow are possibly drawn there by lectin binding interactions (Dilosa *et al.*, 1991; Shieh *et al.*, 1991). Here they mature to become plasma cells that produce soluble antibody which is able to enter circulation. The maturation process is enhanced by bone marrow factors that have not yet been fully characterised. The B cells that do not leave the lymph nodes are called centroblasts. These are rapidly dividing cells lacking the mIgM and CD23 receptors and are present in the dark zone of the germinal centers in the secondary follicle (Figure 1.4). They then move to the light zone, where they become non-dividing B cells expressing high affinity mIgM, centrocytes. It is here that they once again bind to FDC expressing antigen and are subsequently returned to circulation to act as memory cells. Those that do not bind to FDC-Ag are destroyed by apoptosis (Liu *et al.*, 1992). Apoptosis, otherwise known as programmed cell death, is a distinct form of cell death, mediated through specific biochemical pathways and characterised by the condensation of chromatin, loss of nuclear structure and the formation of plasma membrane blebs (Wyllie *et al.*, 1980). This ensures a memory of the antibody response as was the case with the T cell response.





**Figure 1.4** Diagram of the secondary follicle of the lymph node with the light and dark zones of the germinal centre indicated. The differentiation of activated B cells to memory cells is also shown. B cells enter the dark zone where they rapidly divide and lose their surface slg to become centroblasts. They then move to the light zone and, once again, express slg. The specific, high affinity clones are positively selected via antigen on the surface of follicular dendritic cells.

Therefore, in summary, B cells are originally produced in the bone marrow, and transported to the secondary lymphoid tissue where they undergo T cell-dependent, antigen-mediated clonal selection and proliferation. They can then return to the bone marrow where they mature to become plasma cells secreting soluble antibody, or

they can differentiate in the lymph nodes to become memory cells for circulation.

Once the antibody has been produced, it can act in a number of ways to effect the destruction of the foreign body. Specific binding of the antibody to the surface antigen of the invader can induce the congregation of macrophages, killer cells or polymorphs that phagocytose the unwanted body. Alternatively, antibodies can induce the complement function to bring about its destruction. Complement is a collection of proteins in the blood that are capable of acting in conjunction with antibodies to cause the lysis of invaders such as microorganisms ( Kinoshita, 1991; Farries and Atkinson, 1991).

The immune system is a highly complicated protective mechanism that has evolved over millions of years to become highly effective in its operation and there probably exists greater levels of complexity that have not yet been established. However, there are instances where the system can break down. These include inflammation with the overproduction of histamines by basophils, for example, and certain autoimmune diseases such as type-1 diabetes, multiple sclerosis and rheumatism (Cohen and Young, 1991; Todd, 1990).

The remainder of the discussion of the immune system will concentrate on the antibody molecule, since this is the subject of investigation in this thesis.

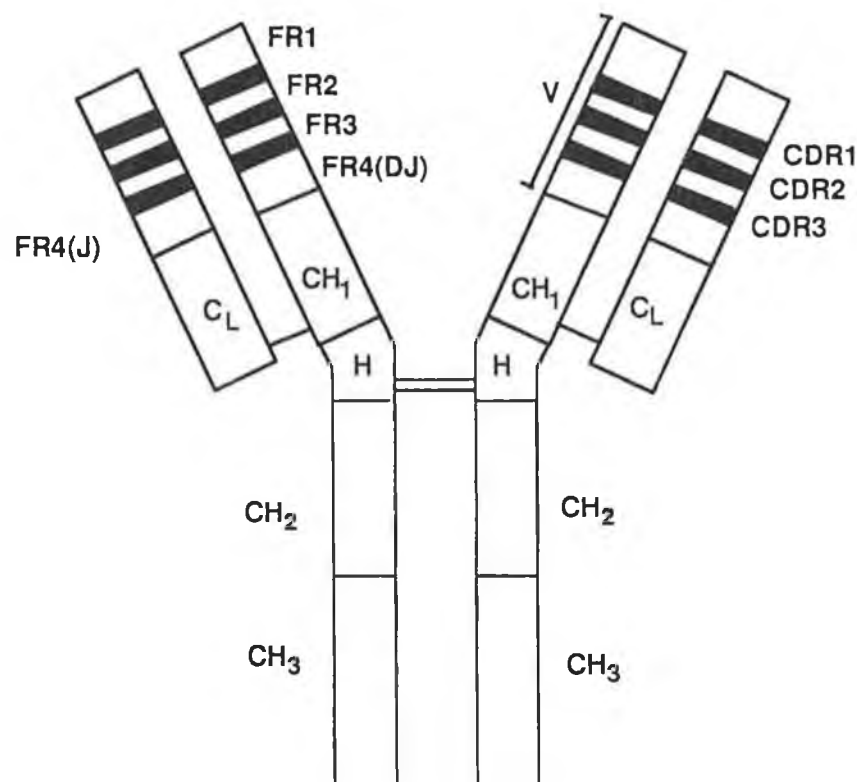
#### **1.4 ANTIBODY STRUCTURE.**

The antibody molecule consists of two heavy and two light chains, 50KDa and 25KDa, respectively (Figure 1.5). The heavy chains are made up of one variable domain and three constant domains of

approximately 110 amino acids each with glycosylation of the second and third of these constant domains. The variable region can be further subdivided into three Complementarity Determining Regions (CDR) separated by four Framework Regions (FR). The first and second constant regions are separated by a hinge region which allows flexibility of the molecule and contributes to the ability of the molecule to bind antigen. The two heavy chains are linked by disulphide bonds in the hinge region, the number of bonds depending on the class of antibody. There are five classes of heavy chain: gamma, delta, epsilon, mu and alpha, resulting in five isotypes of antibody: IgG, IgD, IgE, IgM and IgA, respectively. These differ in molecular weight, number of units, available antigen binding sites and distribution in the blood (Roitt, 1985; O'Kennedy and Roben, 1991). The light chain is divided between a variable region and a single constant region. Each light chain is joined to its complementary heavy chain by a disulphide bond in the constant regions. There are two classes of light chain: kappa and lambda which vary in the diversity of the variable regions, the sequence of the constant regions and their distribution in organisms.

The constant domains consist of seven antiparallel  $\beta$ -strands, four in one sheet and three in the other, forming a barrel with a disulphide bond between strands 2 and 6 (Branden and Tooze, 1991). The  $\beta$ -strands are joined by loops. The variable regions include two further loops between 3 and 4 named 3a and 3b meaning that there are now two sheets of five strands and four strands. The CDR1 (amino acids 21 to 33), CDR2 (49 to 55) and CDR3 (87 to 96) are formed by the loops between strands 2,3; 3a,3b and 6,7 respectively.

The variable regions associate via their five strand sheets and it is the combination of the six CDR regions, three from the heavy and three from the light chains, that primarily forms the antigen binding site and gives the antibody its specificity, commonly referred to as the idiotype of the antibody. It has been estimated that there are as many as  $10^8$  possible antibody specificities available in humans, enough for every known immunogen it may encounter.



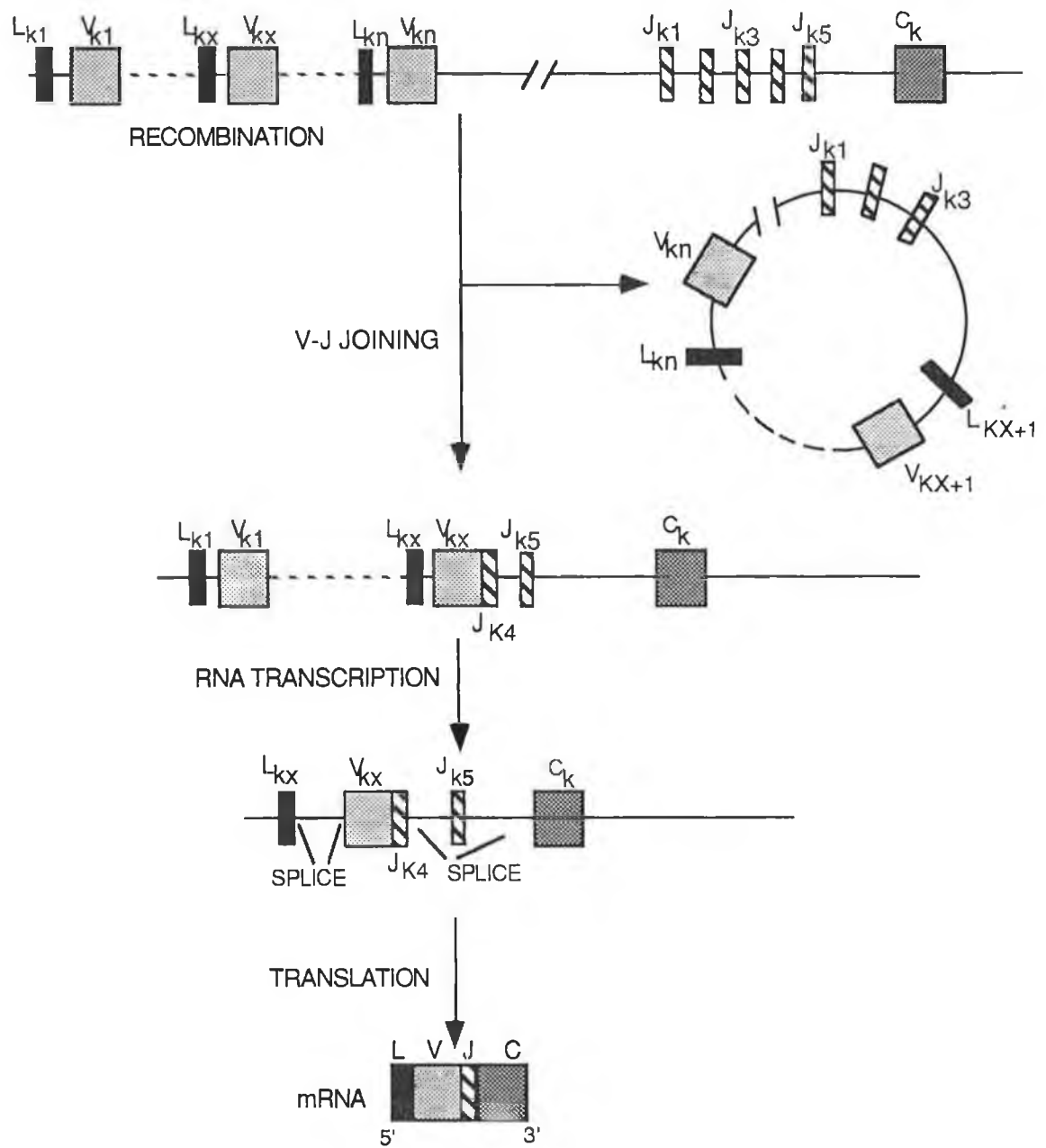
**Figure 1.5** Antibody structure. There are two heavy and two light chains joined by disulphide bonds as indicated. Each chain may be divided into variable and constant regions and the variable regions are further subdivided into three CDR and four FR regions as shown. Each of the two antigen binding sites are made up of a combination of three CDRs from the heavy chain and three from the light chain. The abbreviations are: CDR: complementarity

determining region; FR: framework region; H:hinge; V:variable; CH:constant heavy; CL: constant light; D:diversity; J:joining region.

### **1.5 GENETICS OF ANTIBODY DIVERSITY.**

The ability to produce such a diverse array of antibodies must reside in a mechanism to produce numerous different structures for the CDR regions since these are directly involved in the antigen binding site. Yet it would seem impossible to code for all the combinations that would be required for such an array. The answer lies in the organisation of the genes coding for these molecules.

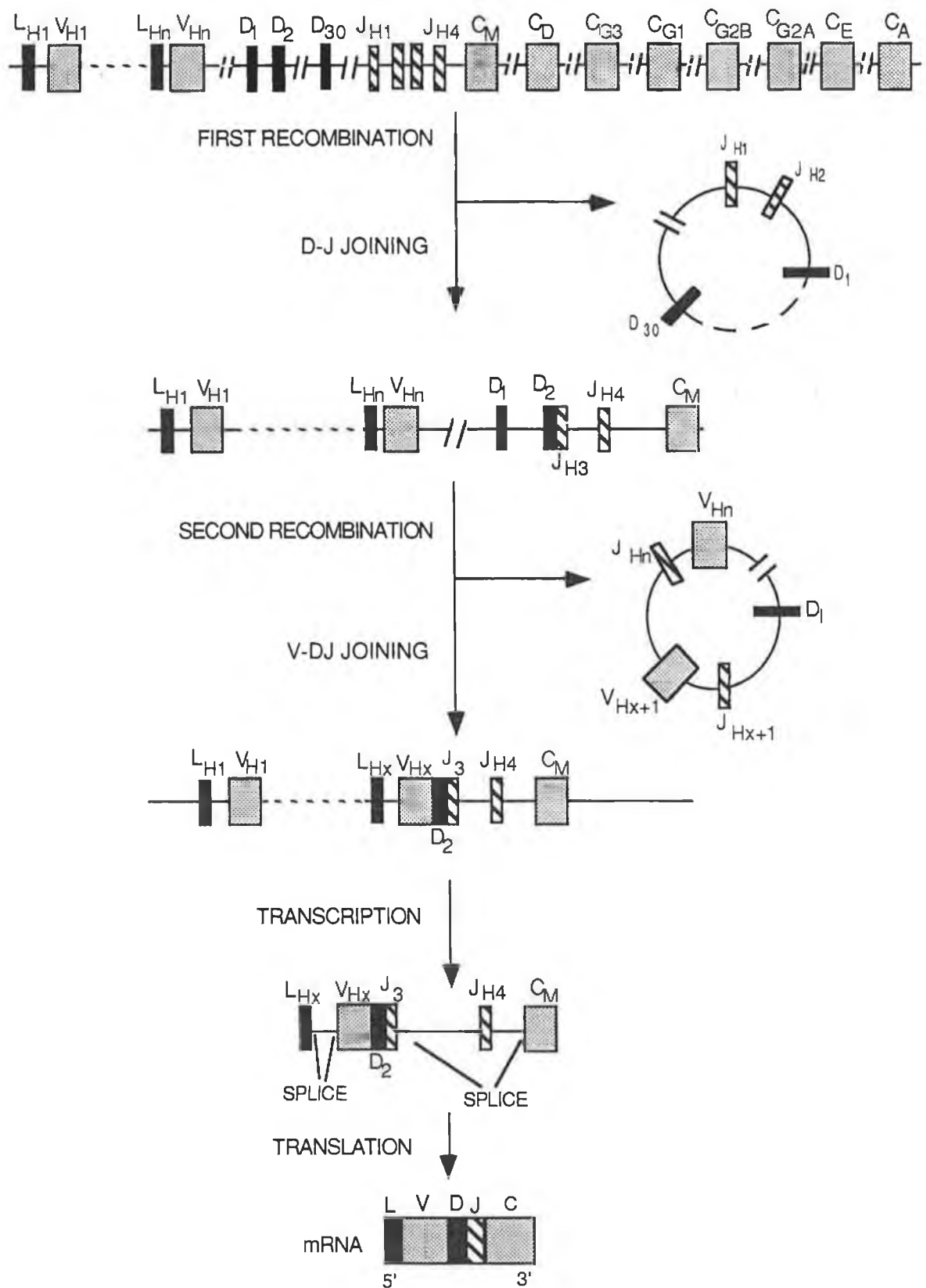
The kappa light chain locus is arranged into a number of exons. There are two or three hundred variable regions ( $V_k$ ) followed by five joining segments ( $J_k$ ) and a single constant region exon (Gough *et al.*, 1979; Watson *et al.*, 1987; Meindl *et al.*, 1990; Kabat and Wu, 1991). In any given B cell, a recombination event occurs to bring one each of the  $V_k$  and  $J_k$  exons together and it is the random combination of these exons that leads to the light chain diversity (Figure 1.6). The join between these can also vary depending on the amino acid position at the point where the two exons are joined, leading to additional diversity. It is also possible to get the insertion of N sequences between the two sequences, further increasing the diversity (Desiderio *et al.*, 1984; Decker *et al.*, 1991). This section is then transcribed along with the constant region and gene splicing occurs to bring the entire locus together which can be then translated as the light chain.



**Figure 1.6** Genetic rearrangement of the  $\kappa$  light chain locus. The recombination event brings the V and J segments together. Splicing brings about V-J-C joining and translation of the mRNA gives rise to the complete light chain. L represents the leader sequences.

The lambda gene system is similar but with less potential for diversity. There are three constant region exons but each has only one associated variable exon.

The heavy chain locus is arranged similarly with variable and joining segments (Figure 1.7). However, there is an additional D region which, together with the J region codes for the CDR3. This then requires 2 recombination events: the first brings the D and J segments together and the second adds the selected V region. This is then transcribed with the constant region and RNA splicing occurs to give the final heavy chain (Yamada *et al.*, 1991; Givol *et al.*, 1981; Menetski and Gellert, 1990; Alt *et al.*, 1992; Alt and Baltimore, 1982).





The nature of the D segments is very complex with as many as thirty genes thought to exist. There are also many possible mechanisms of recombination for these segments, which will be discussed further in chapter 4. However, it is sufficient to say that these mechanisms may have the potential to generate  $10^{14}$  different peptides in the CDR3 region and are, therefore, a major factor in the diversity of antibodies (Sanz, 1991; Matsuoka, 1988; Ichihara *et al.*, 1988). The mechanism of recombination involves two Recombinase Activating Genes, RAG-1 and RAG-2 that are known to be essential but not sufficient for recombination to occur (Chun *et al.*, 1991).

Other factors important in this event include enhancer elements which are thought to increase the likelihood of recombination occurring by causing incidental strand breaks, or by demethylating cysteine residues which may allow binding by recombination factors (Banerji *et al.*, 1983; Giles *et al.*, 1983).

Rearrangements of all these genes occur at the pre-B cell stage in the bone marrow. It is thought that heavy chains are arranged before the light chains in any one cell (Decker *et al.*, 1991) and that expansion of a clone will only ensue upon productive variable gene segment rearrangement and expression of an appropriate heavy chain. Perhaps the heavy chain is expressed on the cell surface which permits communication with other cells and clonal expansion occurs. Furthermore, productive rearrangement of the heavy chain on one chromosome prevents the rearrangement of its allele in a process known as allelic exclusion. Light chain rearrangement then follows and the generation of a complete antibody molecule switches off any further rearrangements in the cell (Ritchie *et al.*,

1984). The cell then leaves the bone marrow for the secondary lymphoid tissues. The process of recombination, therefore is highly complex and remains to be completely elucidated (Schroeder *et al.*, 1987; Mansikka *et al.*, 1990).

Another mechanism for the generation of diversity is that of somatic affinity maturation. This is a process of hypermutation (as many as  $10^{-2}$  nucleotide substitutions) acting selectively on rearranged immunoglobulin variable region genes. It has been suggested that this occurs by a process of error-prone repair of nicked DNA or mismatch repair of misaligned structural intermediates (Malipiero *et al.*, 1987). It is known to occur in the germinal centers subsequent to antigen-mediated activation of the B cell and can continue for a number of weeks following immunisation suggesting that it is developmentally controlled. The main purpose of this phenomenon seems to be to increase the affinity of the antibodies to their antigen, although decreases in affinity have also been recorded (Griffiths *et al.*, 1984; Liu *et al.*, 1989). The degree to which this affects other antibody functions has not been examined in any great detail. However, it is obviously a major contributory factor in the generation of an immune response.

It should now be evident that the generation of an antibody response is an extremely complex event involving the interplay of a large number of components of the immune system to achieve the most efficient and effective means of protecting an organism from attack by a foreign immunogen. This has lead to the evolution of an eloquent and exquisitely engineered set of antibody molecules that has addressed the problems of diversity, specificity, memory and

flexibility, reflected in the ability to mature the response as the antigenic challenge continues. A more complete understanding of the mechanisms involved would obviously be beneficial in the design of diagnostic or therapeutic agents.

## **1.6 ANTIBODY PRODUCTION.**

As the potential of antibody molecules became clear, research into the *in vitro* production of antibodies was initiated. The first major breakthrough in this area was the development of a system to produce monoclonal antibodies through the fusion of B cells from immunised mice with immortal cell lines that could be grown in flasks (Köhler and Milstein, 1975; Schulmann *et al.*, 1978). This resulted in an antibody-secreting laboratory cell line and it meant that for the first time, purified antibodies of defined specificity could be produced in relatively large quantities. This approach proved extremely useful for the production of many antibodies on a commercial level, for diagnostic (Sutherland *et al.*, 1987), analytical (Carlsson *et al.*, 1989) and therapeutic purposes particularly in the treatment of tumours (Hale *et al.*, 1988). It has also been suggested that they be used as vaccines, through the use of antiidiotypic antibodies (Nordling *et al.*, 1991). They also provided vital information about antibody structure (Chothia and Lesk, 1987), the rearrangement of antibody genes (Kabat and Wu, 1991) and about phenomena such as somatic mutation (Malipiero *et al.*, 1987). However, there were a number of drawbacks with this technology particularly in the case of human antibodies, including difficulties with immunisation, the lack of good human myeloma fusion partners,

the low secretion rates achieved and the instability of many human hybridomas.

A variety of approaches were tested to solve these problems including Epstein Barr Virus (EBV) transformation of human B cells which met with limited success, although this was improved with the use of leucyl-leucine methyl ester (Ohlin *et al.*, 1989). This substance was found to significantly increase the proliferative capability as well as immunoglobulin production of Epstein Barr virus-transformed B cells. The advent of *in vitro* immunisation permitted many more of the problems associated with human monoclonal antibody production to be overcome (Borrebaeck *et al.*, 1986; Carlsson *et al.*, 1989). However, a limited knowledge of the extent and functions of cytokines has curtailed the development of the technique.

Advances in molecular biology have revolutionised antibody production and the study of their structure/function relationships. Arguably, the single most important advancement has been the development of the Polymerase Chain Reaction (PCR) which permits the isolation and cloning of the genes coding for antibodies (Larrick *et al.*, 1989; Orlandi *et al.*, 1989; Chaudhary *et al.*, 1990).

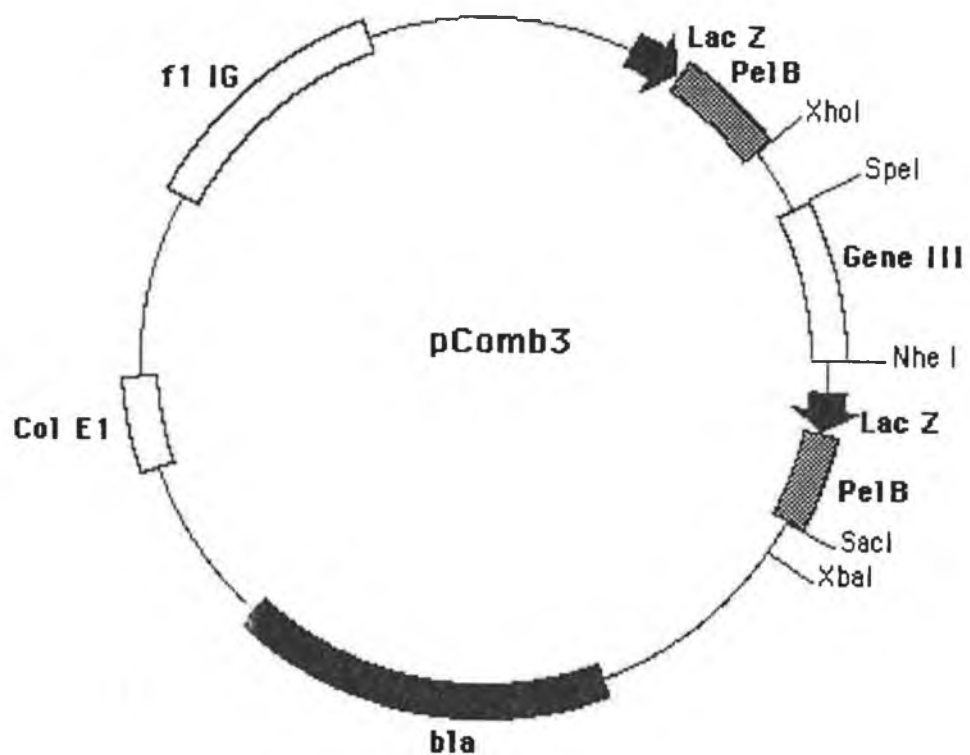
This type of technology opened up entirely new areas in terms of the manipulations which were now possible with antibody molecules. Chimaeric human-mouse antibodies were easily constructed (Morrison *et al.*, 1984) and humanisation of antibodies was also possible (Queen *et al.*, 1989). This facilitated the use of antibodies with mouse specificities (which could be produced by traditional monoclonal techniques) in human systems where the entire mouse

antibody would have previously been rejected. The fusion of antibodies with unrelated activities such as enzymes or toxins was also made easier by DNA techniques (Neuberger *et al.*, 1984; Chaudhary *et al.*, 1989). Many different antibody fragments could be produced in this way including F<sub>v</sub> (Skerra *et al.*, 1991), Fab (Better *et al.*, 1988) and even single domains (Ward *et al.*, 1989). These recent advances have resulted in associated developments in expression systems for antibodies, with a view to increasing protein yields, including lymphoid (Watanabe *et al.*, 1986), baculovirus (Hasemann *et al.*, 1990), bacterial (Plückthun, 1990), chinese hamster ovary (Wood *et al.*, 1990) and plant (Hiatt *et al.*, 1989) expression systems.

### **1.7 COMBINATORIAL PHAGE-DISPLAY LIBRARIES.**

However, many of the above techniques have relied on using mouse hybridoma or spleen cells as the starting material and so, have been limited to an investigation of the mouse response. Furthermore, none of these systems permit the study of an entire immune response, either from mice or humans. It could be very important to encapsulate the entire human antibody response to an antigen since this would provide a pool from which many different antigen-specific antibodies could be isolated. It would also facilitate the study of the complete response which could yield useful information about the immune system. It was for these reasons that the combinatorial library, phage-display system of antibody cloning was developed ( Devlin *et al.*, 1990; Huse *et al.*, 1990; McCafferty *et al.*, 1990; Burton, 1991; Burton, 1992). The system used in this thesis

was that of Huse *et al.*, which will now be examined in more detail. It is based on the vector pComb3 (Figure 1.8) which is a phagemid in that it codes for the F1 intergenic region of the M13 (fd) phage (Beck and Zink, 1981; Watson *et al.*, 1987), while the remainder is based on the pBluescript plasmid and contains the ColE1 origin of replication (Stratagene; Short *et al.*, 1988).

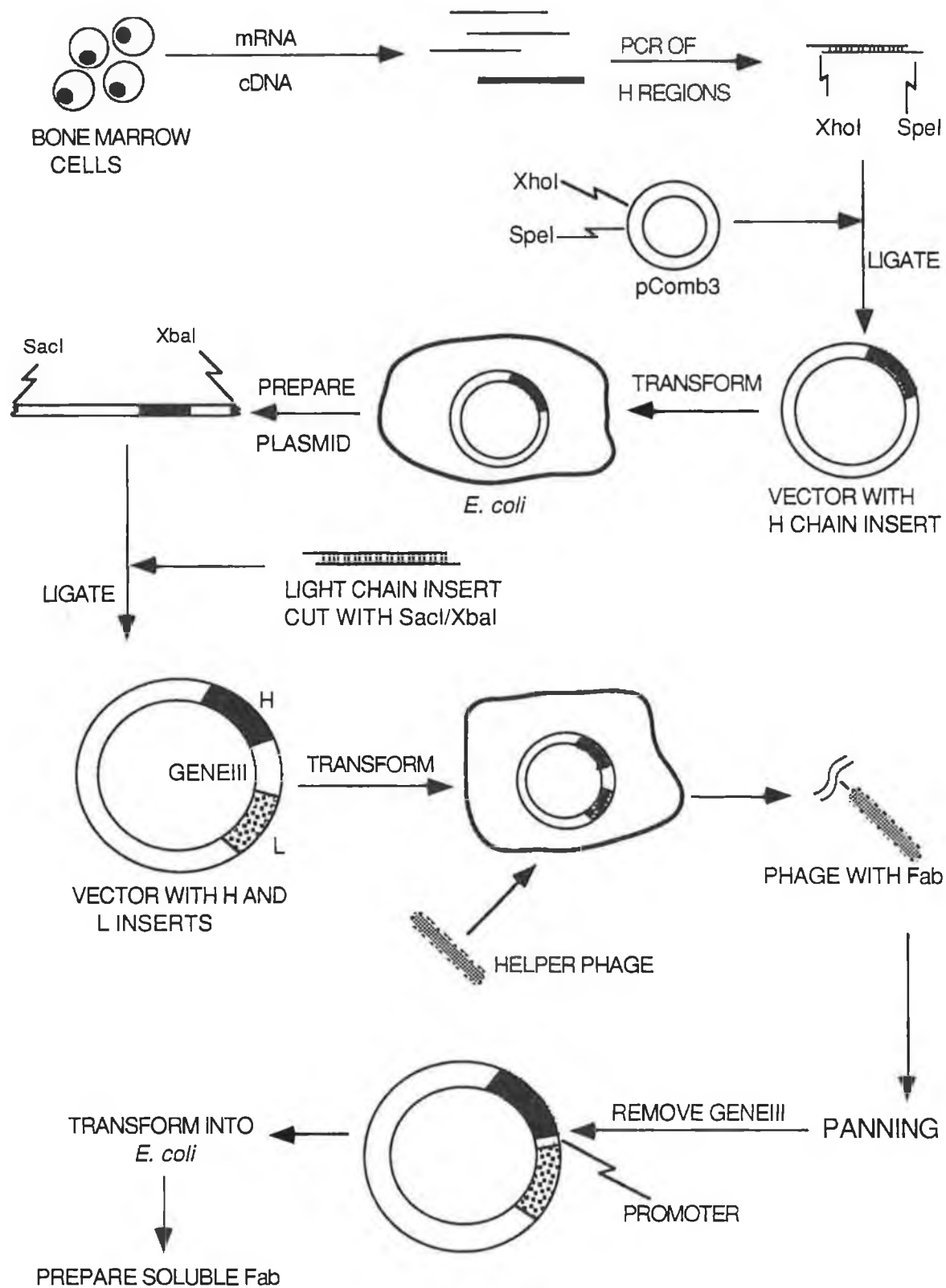


**Figure 1.8** Plasmid map of pComb3. The heavy and light chains are inserted into the sites *XhoI*/*SpeI* and *SacI*/*XbaI*, respectively. Both are under the control of the *LacZ* promoter and are preceded by a *PeIB* leader sequence to ensure transport to the periplasm. The plasmid also contains the *ColE1* origin of replication, the M13 fl intergenic region and the *bla* gene for ampicillin resistance.

The library construction (Figure 1.9) begins with a source tissue, usually from a human subject. This can be blood, bone marrow, tonsil, spleen, synovial fluid, etc. The chosen tissue depends on the type of antibodies being isolated. For example, antibodies that play a role in rheumatism could be isolated from the synovial fluid since this is where those cells are likely to have congregated. However, the bone marrow is generally a good source of cells when a diversity of antibodies is required. The RNA is isolated from the cells and the genes coding for antibodies are amplified by PCR using primers as discussed in the method. This is then cloned into a vector and transformed into bacteria to make the initial library. However, since this library could encode for as many as  $10^8$  different antibody specificities i.e.: the entire human repertoire, a prohibitively large number of clones would have to be screened before the desired antibody would be found. Therefore, a system of enriching for those antibodies before the screening process, had to be devised. The problem was to express the Fabs in a form that would make them available to antigen binding, while permitting the easy handling of extremely large numbers of clones in a way that was compatible with the cloning system. The solution was to design a phagemid vector based on the M13 phage (Persson *et al.*, 1991; Zebedee *et al.*, 1992).



**Figure 1.9** (See next page). Flowdiagram of the library construction process. The total cDNA is first isolated from the cells and the antibody-coding genes are taken from this using PCR. The heavy chain genes are first cloned into the pComb3 vector and amplified in *E. coli*. The same is then carried out for the light chain genes. Following this, the plasmid DNA is isolated from the cells and the *genIII* is removed by restriction. The resulting plasmid can be transformed into *E. coli* once again and soluble Fab can be prepared.



M13 phage is single stranded DNA (ssDNA) bacteriophage, 6400 base pairs in length and codes for 10 gene products involved in the replication, morphogenesis and capsid structure of the virus. When the virus infects the cell, it is converted to a double stranded DNA (dsDNA) molecule which is used as a template for replication. However, it is the ssDNA which buds from the cell and is once again encapsulated within the capsid. Gene product II (GP II) is expressed upon entry into a cell and this acts on the intergenic region to initiate replication. GP V terminates the process. GP VI, GP IX, GP VIII and of particular importance, GP III, are all involved in the capsid structure of the virus. The intergenic region is 508bp in length with many secondary structures in the ssDNA form. It is this region that is responsible for regulation of gene expression and also contains the origin of replication. There is also a 'packing origin' present which is required for the packaging of the DNA into the capsid.

The library system exploits the M13 phage properties through the use of a helper phage, VCSM13, and a phagemid, pComb3. The helper phage is similar to M13 except that its intergenic region contains sequences that ensure it is not easily recognised by GP II for replication, and so will not be replicated or incorporated into the new phage capsid. Also, it contains a plasmid origin of replication which means that when it infects a cell, it will be converted to dsDNA and will proceed to replicate as a plasmid rather than a phage. Furthermore, it contains a gene coding for kanamycin resistance and so can be selected for in a bacterial population.

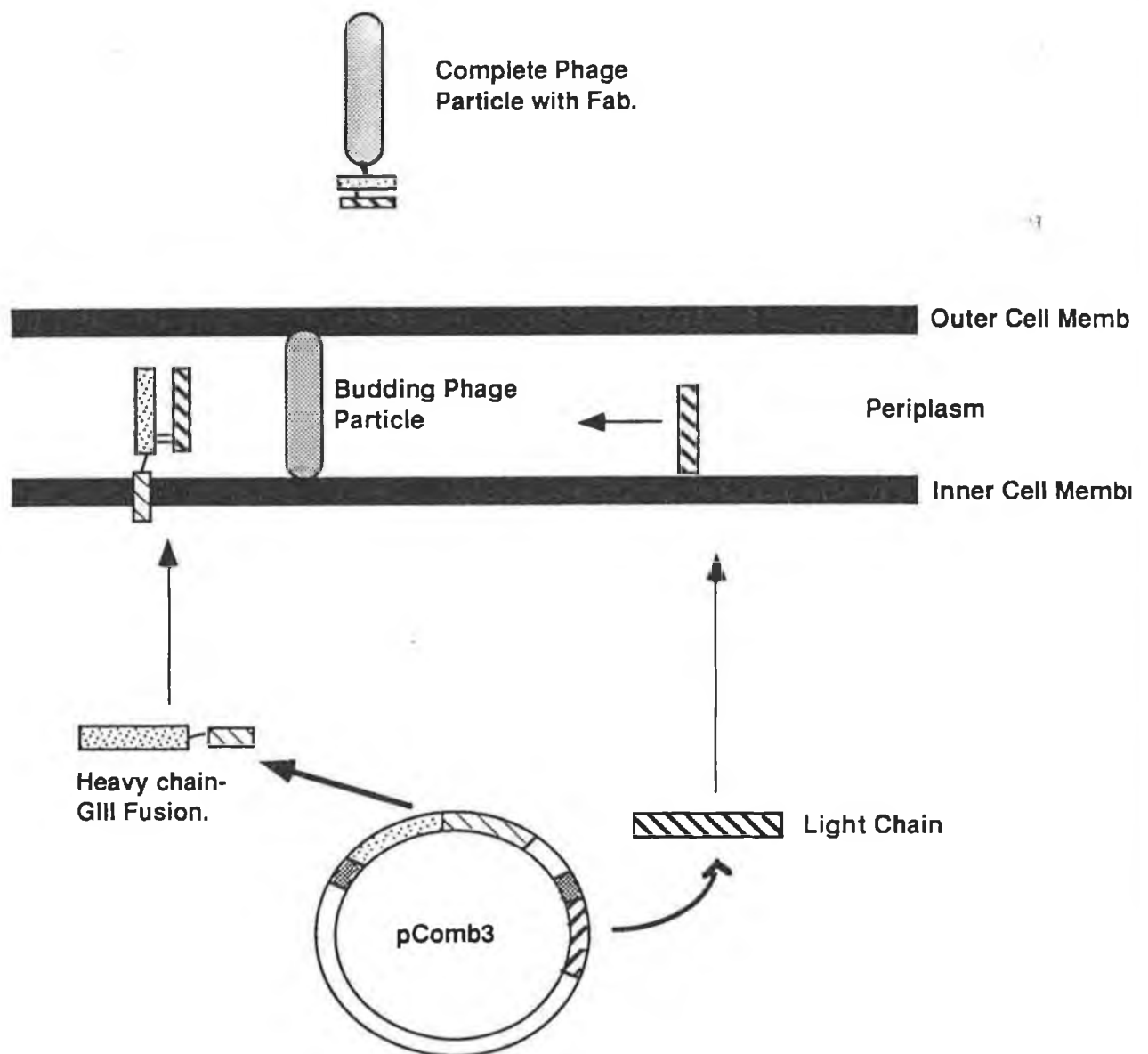
The phagemid, pComb3, codes for ampicillin resistance. It also contains a complete M13 intergenic region which can be recognised by phage gene products that will encapsulate a ssDNA form of the phagemid DNA into a viral capsid. In addition to this, the heavy chain is coded for as a fusion product with the gene III protein under the control of the LacZ promoter.

The cells used are *Escherichia coli* XL1Blue, containing an F' episome which codes for tetracycline resistance and also for a repressor of the LacZ promoter (which itself is repressed in the presence of IPTG). Therefore, if cells containing the phagemid are infected with helper phage, the GPII from the phage activates the phagemid intergenic region. This facilitates the expression of all the phage gene products and so a new viral particle can be constructed. However, it is the phagemid ssDNA that is inserted into the particle. Furthermore, the heavy chain-GPIII fusion product is expressed from the phagemid, although poorly, since it is under the control of the LacZ promoter, as is the GPIII from the phage is also expressed at approximately four times the level of that from the phagemid. Five copies of this protein are inserted into the capsid of each phage and on average, one of those copies is a fusion product with the heavy chain. This is then transported to the cell periplasm (Figure 1.10). The light chain is expressed independently and also transported to the periplasm where it combines with the heavy chain to give a complete Fab bound to the phage surface.

The recombinant phage can be then isolated from the culture supernatant and used in the panning procedure, since the Fab protein is now available for antigen binding. The panning procedure (see

methods) ensures that any Fabs specific for the antigen will be enriched for over a number of rounds. The monovalent nature of Fab expression on the phage surface means that only high affinity Fabs will be enriched since there are no avidity effects occurring. A similar system using the more prevalent gene VIII product is influenced by avidity and so can select for low affinity Fabs (Kang *et al.*, 1991b; Gram *et al.*, 1992). The enriched population of Fab-encoding phage can then be used to reinfect a bacterial culture in the absence of helper phage, thus ensuring that they will now be transmitted as plasmids. The gene III can then be removed, resulting in a library of *E. coli*. from which soluble Fab fragments can be expressed. This library has been enriched and so finding clones expressing high affinity, antigen-specific clones should not be too difficult. This method of library construction has the potential to clone the entire human repertoire of antibodies and select from that, antibodies of any specificity we may choose provided that library sizes in the order of  $10^8$  members can be constructed (Barbas *et al.*, 1992). It also opens up the possibilities of synthetic antibodies and chain shuffling (Kang *et al.*, 1991; Collet *et al.*, 1992; Marks *et al.*, 1992).

Therefore, there now exists an efficient method of cloning a large part of the human repertoire and isolating, from that, antibodies of any given specificity. It then had to be decided what specificity was required. The chosen antibodies should be relevant to the human system and serve some purpose, preferably therapeutic. Antibodies capable of neutralising viruses, notably the influenza virus, had previously been described.



**Figure 1.10** Scheme of Fab assembly and incorporation into phage. The heavy chain-genIII fusion and light chain are expressed separately from the phagemid and transported to the periplasm where the heavy chain is anchored to the inner membrane via the genIII product. The light and heavy chains assemble in the periplasm and are incorporated into the phage via the genIII, as the particle buds from the cell.

Therefore, the AIDS virus, HIV, was identified as the subject of these studies as this is a highly prevalent disease for which there are no effective therapies at present. An attempt was made to isolate antibodies from a library whose source tissue was bone marrow from an asymptomatic AIDS patient. However, before this could be done, an understanding of the structure and functions of the HIV virus was necessary.

### **1.8 ACQUIRED IMMUNODEFICIENCY SYNDROME (AIDS).**

A study isolating and characterising the first Human T cell Leukemia Virus (HTLV) was reported in 1980 (Poiesz *et al.*, 1980). The following year, the centers for disease control in America reported an unexplained cluster of immunodeficient men in Los Angeles (Blattner, 1991). The Human Immunodeficiency Virus (HIV) was subsequently discovered in 1983/84 (Barre-Sinousi *et al.*, 1983; Popovic *et al.*, 1984; Ratner *et al.*, 1985) and finally, the phenomenon of Acquired Immunodeficiency Syndrome (AIDS) was recognised in 1985 as being a completely new disease. Subsequently two distinct types of HIV have been characterised: HIV-1 and HIV-2. HIV-1 is thought to be responsible for the majority of AIDS cases worldwide while HIV-2 is probably more localised to western Africa and is less virulent (Kong *et al.*, 1988). At present, there are over 10 million cases of AIDS worldwide and that figure is increasing all the time (Blattner, 1991). The virus is transmitted through behaviour that exchanges bodily fluids and the high risk transmission groups have been identified as homosexual men, haemophiliacs and intravenous

drug users who share needles, although the entire population is potentially at risk.

The HIV virus acts by depleting the numbers of CD4<sup>+</sup> T cells in the victim thus causing immunodeficiency (Rosenberg and Fauci, 1991). This is particularly detrimental as the CD4<sup>+</sup> T cells are instrumental in inducing virtually all of the responses of the immune system as was discussed earlier in the introduction. It had been argued until recently that HIV was not the agent responsible for AIDS since it had been proving difficult to find the virus in AIDS patients (Culliton, 1992). However, recent reports finding massive infection of T lymphocytes and macrophages in the lymph nodes of patients would seem to dispel this theory (Embretson *et al.*, 1993; Panaleo *et al.*, 1993) along with studies showing the virus exists in a large proportion of T cells as integrated DNA (Schnittman *et al.*, 1989).

When an individual is initially infected with HIV, the virus attacks CD4<sup>+</sup> T cells, particularly in the peripheral blood, and macrophages and monocytes in other tissues. This induces a primary viremia, leading to the production of antibodies directed against the virus. This generally subsides giving rise to what can sometimes be a long latent period, as is the case in asymptomatic patients. As the disease progresses, the viral burden in the peripheral blood lymphocytes increases dramatically resulting in the depletion of these cells and full-blown AIDS, which can often lead to the death of the victim. The stimulus that triggers the onset of AIDS is not fully understood. However, there are a number of possibilities: a number of other viruses such as Cytomegalovirus (CMV), Hepatitis B virus (HBV) and herpes simplex virus have been shown to enhance HIV

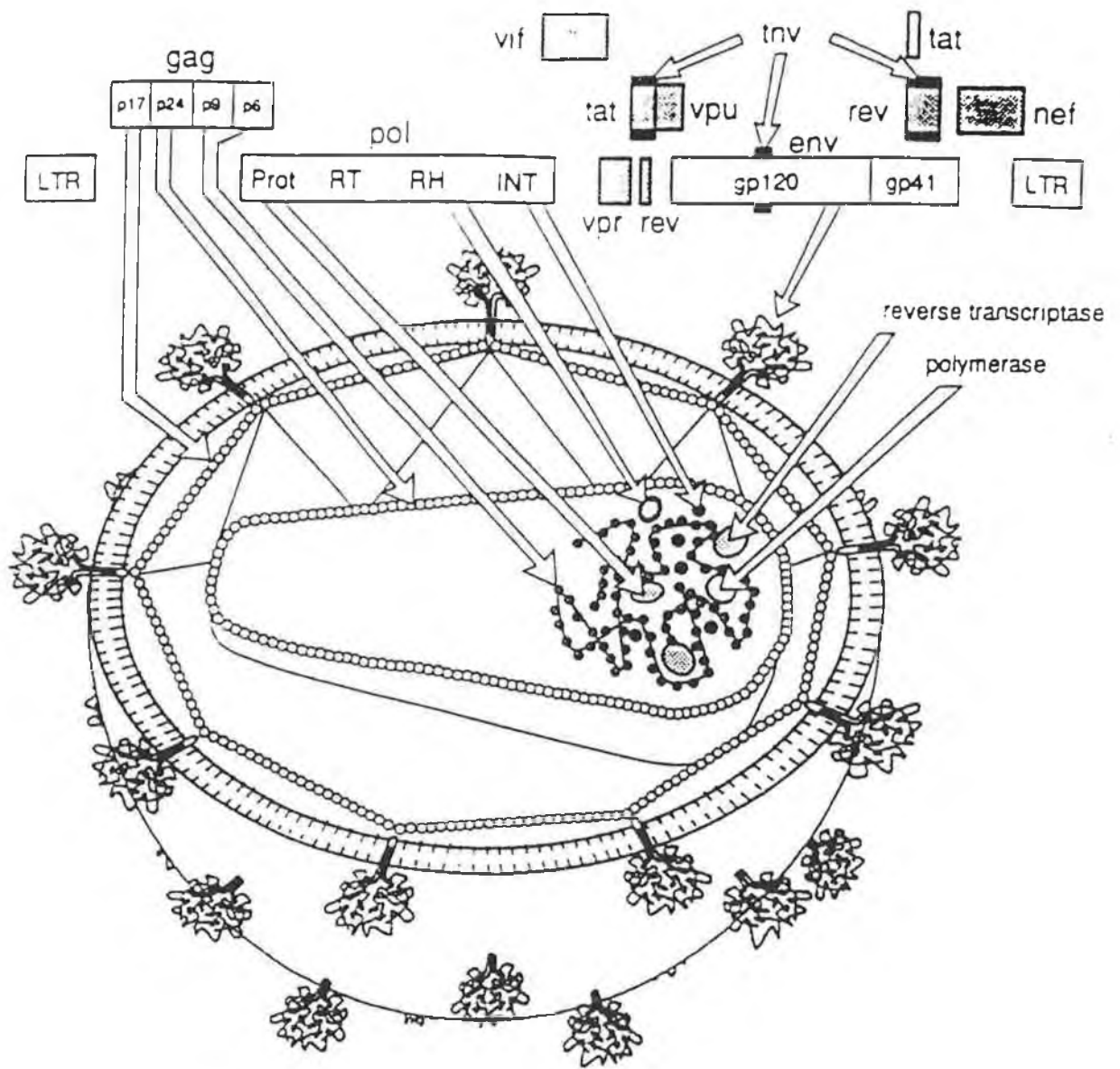


replication; certain cytokines including tumour necrosis factor- $\alpha$  are capable of up-regulating HIV expression; some physical stresses such as ultraviolet light and heat have also been implicated in the progression of the disease. More studies need to be carried out to support these theories.

The mechanism by which the virus kills the T cells also remains ambiguous (Rosenberg and Fauci, 1991). There have been a number of suggestions as to how this might occur: budding of the virus from the cell could destroy the integrity of the membrane, causing cell death. The presence of cytoplasmic viral DNA in the later stages of infection could result in the production of viral proteins which would interfere with the cellular processes sufficiently to kill the cell. An infected cell expresses viral protein on its surface which is capable of binding to the CD4 of other cells. This leads to fusion of the cells and the formation of syncytia which can often be made up of hundreds of cells. In this way, one infected cell can bring about the death of many neighbouring uninfected cells. It is also thought that viral surface glycoproteins can induce programmed cell death, apoptosis, to occur in CD4<sup>+</sup> T cells upon binding to the CD4. It may also be the case that an infected cell expressing this glycoprotein on its surface can induce apoptosis in neighboring T cells, thus killing them without necessarily infecting them (Ameisen, 1992).

Finally, the virus is thought to be capable of preventing communication between B cells and T cells, as this is achieved via CD4 (see earlier in the introduction). This would have the obvious effect of impairing the antibody response that may be generated against the virus.

The virus is also capable of altering its coat proteins such that antibodies present in the blood stream can no longer recognise it, yet the functions of these proteins remain intact. This is known as the generation of escape mutants (Nara *et al.*, 1991). The virus is thought to have other detrimental effects on the humoral response (Amadori and Chieco-Bianchi, 1990). A continuous viral load and the inability to clear it could lead to the uncontrolled expansion of HIV-specific B cell clones, leading to B cell lymphoma which appears with increased frequency in AIDS patients. Anti-CD4 antibodies could be produced through anti-idiotypic responses or by targeting to CD4 bound-gp120 which would crosslink the CD4 molecules and prevent signal transduction in the TCR complex. These cells would no longer be able to activate antibody production from B cells.



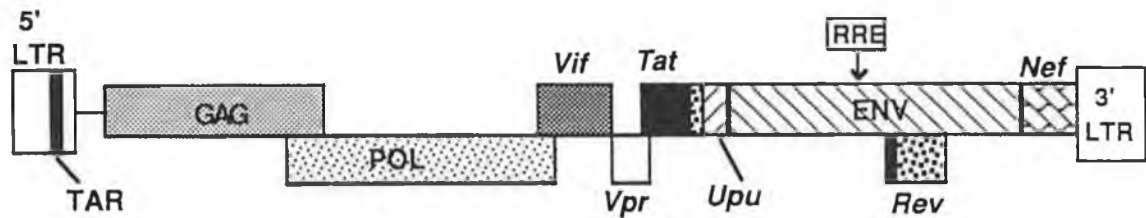
**Figure 1.11** Structure of the HIV virus, showing the inner core containing the RNA and enzymes. This is surrounded by a outer capsid, which is, in turn, enclosed by a lipid layer. The surface glycoproteins protrude from the outer capsid, through the lipid layer and are critical for the initial recognition of CD4 on cell surfaces. gag and env refer to structural genes while pol codes for the enzymes needed for viral replication and function. LTR signifies the long terminal repeats and all other genes code for regulatory proteins (see section 1.9). The diagram was adapted from Haseltine, (1991).

## 1.9 MOLECULAR BIOLOGY OF HIV.

HIV is a retrovirus of the subfamily lentivirus (Haseltine, 1991). This implies that it is a diploid ssRNA virus containing two identical ssRNA strands bound non-covalently at their 5' ends. There is an inner core containing the RNA and a number of enzymes needed for replication, including reverse transcriptase, polymerase and ribonuclease H. The inner core is surrounded by the capsid proteins which is, in turn, surrounded by a lipid membrane. Glycoproteins protrude from the capsid through the lipid, to form spikes on the viral surface (Figure 1.11). HIV belongs to the class of complex retroviruses (as opposed to simple viruses such as avian leukemia virus) in that it not only codes for the required structural proteins, but it also codes for 6 regulatory proteins, *tat*, *rev*, *nef*, *vif*, *upu* and *vpr*, all of which are necessary for the complete replication cycle (Figure 1.12). There are two long terminal repeats (LTR). The 5' LTR functions to promote viral transcription and the 3' LTR is required for efficient polyadenylation of the resultant transcripts. The *gag* gene codes for the capsid proteins, *pol* codes for the replication enzymes present in the inner core and *env* codes for the surface glycoprotein gp160. This is the protein which initially comes in contact with the cell and binds the CD4 molecule, thus facilitating viral entry.

When the virus enters the cell, a single strand of RNA is converted to dsDNA using the reverse transcriptase. This dsDNA then integrates into the cell genome to become a provirus, by means of an integrase enzyme (Gent *et al.*, 1991). A minimum basal level of transcription is facilitated by cellular transcription factors, notably

NF- $\kappa$ B (Cullen, 1991) which gives rise to fully spliced 2kB RNA transcripts in the nucleus, coding for *tat*, *rev* and *nef*.



**Figure 1.12** Representation of the HIV-1 genome. There is a long terminal repeat (LTR) at each end. The 3 main structural genes, *gag*, *pol* and *env*, are interspersed with the 6 regulatory genes. The genes coding for *tat* and *rev* exist as two exons, separated by unrelated sequences. The *tat* response element can be found in the 5' LTR, while the *rev* response element is coded by sequences within the *env* gene.

The trans-activating element, *tat*, is responsible for enhancing the transcription of viral DNA. It binds to the Tat Response Element (TAR) present at the 5' end of all transcripts and is thought to act, possibly by stabilising the transcription factors and the RNA structure (Feng and Holland, 1988; Subramanian *et al.*, 1991). However, as long as the cell remains resting, only this 2kB transcript will be present.

On initiation of viral replication, two other types of transcript appear also: a non-spliced 9kB RNA and a singly spliced 4kB structure. This single splicing can serve to bring any of the genes

immediately next to the 5' LTR in order to maximise its translation. The only two genes that cannot be spliced in this way are *pol* which is always expressed as a fusion with *gag*, and *upu* which is part of a bicistronic gene with *env*.

If the cell becomes activated in some way, the transcription and translation factors increase in quantity and this is mirrored by an increase in the amounts of *rev* protein in the nucleus. When this surpasses a threshold level, the 4kB and 9kB transcripts begin to appear in the cytoplasm. It is thought that *rev* prevents the appearance of these transcripts below a certain level, as the cell would not have enough manufacturing apparatus to support the high levels of protein production required by HIV. However, in activated cells, *rev* permits the transport of the protein-encoding transcripts to the cytoplasm, possibly by binding to the splice sites, thus preventing them from being spliced to 2kB molecules in the nucleus, or by binding to the RNA and mediating its transport through vesicles in the nuclear membrane.

The function of *nef* is not fully understood at this point in time and there is still a dispute as to whether this causes an increase or decrease in the levels of viral expression.

Translation of the *gag-pol* transcript ensues to yield a capsid polyprotein-replicative enzyme precursor which is transported, with the RNA to the cell membrane, where the viral particle is assembled. This then buds through the cell membrane. The envelope glycoprotein is produced from mRNA, glycosylated at the rough endoplasmic reticulum and passed to the golgi apparatus where it is cleaved to its two constituent subunits, gp41 and gp120. These then pass to the

outer cell surface where they are attached to the outer viral coat mediated by the *vif* protein, probably as it buds through the cell membrane. A protein U, coded for by *upu*, facilitates the transport of the virus through the cell membrane and, finally, the *vpr* gene product helps to gather the enzymes immediately after infection and so aids in the production of dsDNA.

### **1.10 HIV SURFACE GLYCOPROTEINS.**

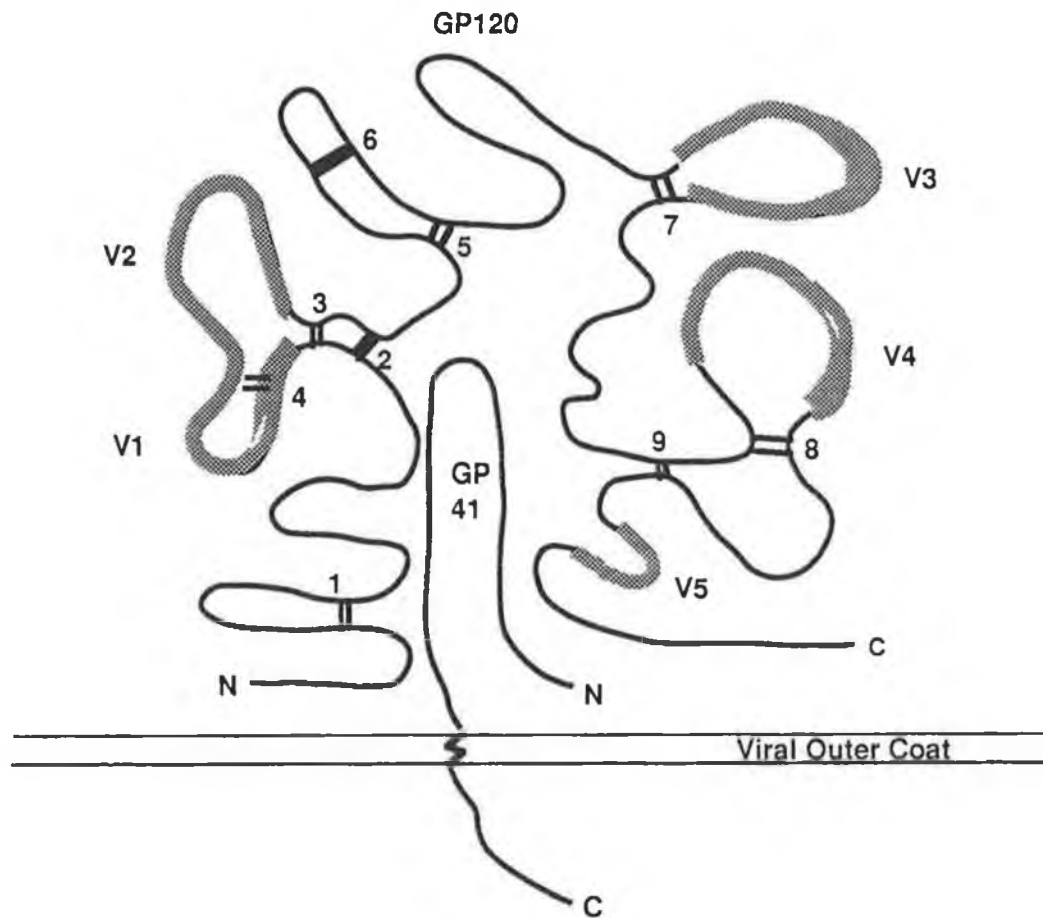
It was mentioned earlier that the surface glycoprotein, gp160, is essential in the initial binding of the viral particle to cells via CD4. It has been established that gp160 consists of 2 subunits, gp120 and gp41, connected via noncovalent bonds between amino acids in the N terminal ends of both molecules (Helseth *et al.*, 1991). The gp41 consists of an extracellular, a transmembrane and an intracellular domains while gp120 is entirely extracellular, and is connected to the viral particle via gp41 (Figure 1.13). The gp120 molecule is approximately 600 amino acids in length and is highly glycosylated, with about 55% carbohydrate and can be divided into regions determined by the variability of the amino acids such that there are 5 variable regions and 5 'constant' regions.

It has been unequivocally established that this is the viral component responsible for CD4 binding (Landau *et al.*, 1988; Moore and Klasse, 1992). Certain residues have been identified as being important in this event and this has lead to the realisation that the CD4-binding epitope is highly conformational in nature and involves amino acids from many parts of the gp120 molecule, notably 257,

368, 370 and 457 (Kowalski *et al.*, 1987; Olshevsky *et al.*, 1990; Thali *et al.*, 1992). This was established using antibodies capable of blocking the gp120-CD4 binding interaction.

Viral invasion of the cell is a highly complex set of events which is only initiated by the binding to CD4 (Bentz *et al.*, 1993). Factors that may have importance at this stage include the density of CD4 on the cell surface with increasing density resulting in a parallel increase in susceptibility. There may also be other cell surface molecules involved in the fusion event. This has been supported by the finding that CD4 is essential but not sufficient for infection to occur. Possible accessory molecules include MHCI and LFA-1. In fact, there is evidence to suggest that CD4 is not required (Bedginger *et al.*, 1988), or of lesser importance for infection in some cell lines such as glial cells or macrophages. Viral entry, in these cases, could be antibody-mediated, with viral-bound antibodies being targeted to cells containing anti-Fc receptors. The entire complex could then be internalised into the cell by endocytosis (Amadori and Chieco-Bianchi, 1990).





**Figure 1.13** Schematic diagram of gp160. It consists of two subunits, gp120 and gp41. The subunit, gp41 anchors the molecule to the viral coat. gp120 is attached to gp41 by non-covalent interactions not yet defined and consists of a series of five variable loops, as indicated by the shaded areas, interspersed with more constant regions. The 3-dimensional structure of gp160 is not known. There are nine characterised disulphide bonds, as indicated by the numbers.

The gp120-CD4 binding is not merely a process involving two single molecules, but a dimer, or perhaps a tetramer of gp120 molecules are required, and that the functional CD4 binding site may

incorporate regions of a number of gp120 molecules which complement each other. The binding to CD4 induces a number of conformational changes in the gp120. This binding may begin as a low affinity interaction with the changes leading to a higher affinity bond being formed at the expense of the gp120-gp41 interactions. The result is to expose areas usually hidden, such as the V3 loop of gp120 and regions of the gp41 molecule. The function of the V3 loop at this stage is unclear although it may bind to other accessory molecules on the cell surface (Bergeron *et al.*, 1992).

These reactions finally lead to the shedding of gp120 and although the function of this is not fully understood, it may be that it facilitates the physical contact between the viral envelope and the cell membrane (McKeating *et al.*, 1990; Moore *et al.*, 1991). This may be followed by insertion of gp41 into the membrane and fusion of the virus to the cell. There are many aspects of this response that remain to be elucidated, in particular the role of the V3 loop of gp120, the structure/function relationship of gp120-gp41 and the role of accessory cell surface molecules. It may also be possible that some of the replication regulatory genes already mentioned, such as *vif* and *upu*, may be involved in the fusion process. However, the critical role that is played by gp120-CD4 binding is well established and interference with this step is likely to effect the neutralisation of the virus. A possible approach to achieve this can be found in the natural humoral immune response mounted against the virus in infected patients.

### 1.11 NEUTRALISATION OF HIV.

When an individual becomes infected with HIV an antibody response is generated against all the various viral protein antigens. Some of these antibodies are specific for neutralising epitopes, which primarily reside in the envelope glycoproteins, gp41 and gp120 (Ho *et al.*, 1987). There are two major groups of neutralising antibodies directed against gp120 (Chamat *et al.*, 1991). The first group are specific for the V3 loop (third hypervariable domain) located between residues 300 to 328. These antibodies are generally produced early in the course of the disease and are present in high titres in the serum. They neutralise the virus by interfering with some event subsequent to binding of CD4 by gp120. The second set of antibodies appear later in the infection and are specific for a conformational, discontinuous epitope on gp120. They interfere with binding of CD4 to gp120 and are, therefore, specific for the CD4-binding site. They are present in lower titres than the other class of antibody and so it took longer to isolate and characterise these. The two groups of antibody are significantly different in that the V3 specific group tend only to neutralise a specific viral strain while the CD4 binders are capable of neutralising multiple strains which may make them more useful for use as therapeutic agents. Many antibodies have been generated from both the V3 group (Ho *et al.*, 1987; Gorny *et al.*, 1991) and the CD4 group (Ho *et al.*, 1991; Kang *et al.*, 1991; McKeating *et al.*, 1992; Thali *et al.*, 1991). These have been generated in the hope of finding a high efficiency neutralisation agent for HIV and also as a tool to investigate the mode of action of the virus in more detail. The production of an antibody capable of

neutralising the virus would obviously be highly desirable. However, there are a number of factors to consider before attempting this. Firstly, the gp120 interaction has a very high forward rate in the order of  $1 \times 10^9 \text{M}^{-1} \text{s}^{-1}$  (Nara *et al.*, 1991; Moore and Klasse, 1992). Therefore, any antibody that neutralises by reacting with the CD4 binding site must have an affinity capable of competing with the rapid rate of gp120-CD4 binding. Second, a single antibody is unlikely to be effective due to the phenomenon of escape mutants. The HIV virus has evolved so as to allow for the selection of viral epitopes capable of the conformational flexibility necessary to escape from neutralising immunoglobulin while preserving functional integrity for its survival. These mutants are antigenically close enough to the parental strain but sufficiently different in the presentation of critical epitopes to prevent the neutralisation reaction from occurring. Therefore, any therapy must consist of a number of antibodies with slightly different specificities to avoid this problem. Third, care must be taken to ensure that none of the antibodies used carry any negative effects as previously discussed (Amadori and Chieco-Bianchi, 1990).

Finally, it must be remembered that the *in vitro* and *in vivo* situations do not necessarily correlate. Hence, care must be taken when interpreting results from *in vitro* assays such as that developed by Nara (Nara and Fischinger, 1988). There have also been instances where soluble gp120 and viral gp120 do not yield the same results (Ivey-Hoyle *et al.*, 1991; McKeating *et al.*, 1991; Moore *et al.*, 1991). This must always be kept in mind.

### 1.12 CATALYTIC ANTIBODIES.

Antibodies can be employed for viral neutralisation, as was discussed previously, which may become an important mode of therapy in a number of diseases of this type. However, recent advances in technology permit the manipulation of antibody molecules so that they may be adapted for many other applications.

The immune system is capable of producing a vast repertoire of antibodies each with its own specificity and function. It is this very diversity and specificity peculiar to antibodies that makes them ideal candidates for use in the field of chemistry in that they offer the potential of producing highly specific catalysts for a vast array of chemical transformations, i.e. catalytic antibodies. Improvements in technology over the last 8 years have made it possible to generate antibodies specific for a wide range of molecules, including biopolymers, synthetic molecules and drugs. Recent advances have also allowed us to engineer antibody molecules with a high degree of specificity, thus permitting the production of designer proteins. Catalytic antibodies could prove invaluable as therapeutic agents, for example, for the break-up of blood clots in heart conditions, or vaccines could be designed to elicit antibodies capable of the cleavage of viral or cancer cell proteins. They might also prove invaluable for use in fine chemical production or in the synthesis of pharmaceuticals. These molecules could help reduce the cost and increase the safety of a number of chemical processes by replacing catalysts which require extreme and dangerous conditions to function. A further example of where they might be used would be in the synthesis of compounds whose

stereochemistry is important, such as amino acids, since antibodies are known to be highly isomer-specific (Lerner and Tramontano, 1988).

In order to be catalytic, an antibody must first have the ability to bind a component of the reaction it is trying to catalyse and secondly, must be able to carry out the conversion of the substrate to product.

A number of approaches may be taken in the design of such an antibody. An enzyme functions by stabilising the transition state thus lowering the energy of the intermediate and allowing the overall reaction to proceed. Therefore, if antibodies could be designed to bind a transition state, they might function in the same way. However, transition states are generally too unstable to exist for any period of time and so analogs must be designed which can be used to elicit an antibody response. This approach has lead to the generation of antibodies that catalyse a number of chemical reactions such as ester hydrolysis (Lerner and Tramontano, 1988) and amide hydrolysis (Janda *et al.*, 1988).

An alternative approach is to alter, chemically or genetically, the antibody in order to introduce an appropriately positioned catalytic amino acid side chain, a cofactor, or another catalytic group. This has been done to introduce a catalytic imidazole into the active site of an antibody so making it hydrolytic (Baldwin and Shultz, 1989). Metal ions can be introduced for reactions such as peptide cleavage (Iverson *et al.*, 1990; Iverson and Lerner, 1989).

Cleavable affinity labeling groups have also been used for this purpose (Pollack *et al.*, 1988; Nakayama and Shultz, 1991). This

involves coupling an affinity labeling group to an antigen such that the antibody binds the antigen, bringing the labeling group close to a residue around the active site. The residue and labeling group then bind, and the antigen can be cleaved, leaving the labeling group (e.g. thiol) which can be used to couple a number of catalytic groups or cofactors. A further approach to this problem was the production of phosphorylcholine-specific antibodies by monoclonal technology, which were found to catalyse the hydrolysis of choline carbonates (Jackson *et al.*, 1991).

Advances in combinatorial library technology have made it possible to screen a far greater number of antibodies, thus increasing the chance of obtaining a catalytic molecule. However, this increase in the numbers of antibodies can lead to problems in screening for those antibodies with the required catalytic activity, as it may be difficult to carry out the reaction and detect the products on such a large scale. The development of catELISA (Tawfik *et al.*, 1993) and other similar techniques should make it substantially easier to achieve the required selection. Finally, there are a number of new developments which should help to revolutionise this field further, in particular, the ability to solubilise antibody in reverse miscelles in order that they may be used in organic solutions (Durfor *et al.*, 1990).

## 1.12 RESEARCH PROPOSALS.

A number of areas were investigated in the course of the work presented in this thesis. Firstly, there was a study of the immune system and particularly of the antibody response generated when challenged by a specific antigen. There is, to date, very little data on the diversity of a humoral response from a single individual in terms of the types of antibody produced, their specificities, affinities and biochemical properties. This lack of information has primarily resulted from the inability to capture the response in a way that makes it accessible to analysis. The first aim of this work, therefore, was to study the feasibility of using the combinatorial library technique as a means of analysing the complete antibody response of a HIV infected individual. The extent to which the antibodies generated from this approach reflect the *in vivo* situation were determined with a view to establishing the authenticity of this approach. The antibodies were then analysed, both on a genetic and biochemical level, to ascertain what information this yielded about the overall response. The findings of this analysis were then related to the patient's situation in the hope of gaining some further knowledge as to how his immune system deals with the viral challenge. These antibodies were also investigated for neutralisation activity with the aim of finding a set of antibodies that may have potential therapeutic value. It was also hoped that these antibodies may be used to analyse further the interactions occurring between gp120 and CD4 which may be useful in elucidating the mechanism of viral-cell fusion.



Another aim of this work was to gain a better insight into the structure/function relationships intrinsic to the antibody molecule. The antibodies isolated from the library were analysed to determine the relative functions of the heavy and light chains both in antigen binding and in viral neutralisation. The contribution that each has to make to the overall specificity of the antibody and the effects of small changes in amino acid sequences was of particular interest. Specific epitope mapping experiments and mutagenesis studies were employed to establish this in more detail. The findings from these experiments were related to the neutralisation ability of the antibodies in an effort to establish a relationship between the structural features of the antibody and the function of that molecule. It was hoped that these experiments would yield valuable information, previously unknown about the evolution of antibodies and the ways in which they operate. This could prove useful in the future for designing antibodies with defined specificity and function for use as analytical, diagnostic or therapeutic agents. It may also give better insights into natural processes such as clonal selection and somatic affinity maturation.

Finally, the information obtained from the previous studies was used in an attempt to construct catalytic antibodies (Nakayama and Schultz, 1991; Lerner and Tramontano, 1988; Schultz *et al.*, 1990). These are antibody molecules capable of chemical conversions, an activity not usually found in nature. An attempt was made to design antibodies that were able to effect the peptide cleavage of the gp120 molecule with the aid of a metal cofactor. This was done in the hope of producing an antibody with improved neutralising

activity. It also served as a study on the relatively new area of catalytic antibodies. Finally, it was useful as a practical demonstration for an application of the technology being used in the other studies. The ability of these antibodies to neutralise the virus *in vitro* was also investigated.

Therefore, in conclusion, it was hoped that these studies would yield some useful information about the mode of action of the antibody response and how it adapts to antigenic challenge. The functions of the antibody molecules themselves were also investigated, particularly in relation to their structure, both at a genetic and biochemical level. Since all the antibodies to be studied were specific for the HIV surface glycoprotein, it was hoped that this would give further insights into the mode of action of this virus and especially how it infects CD4<sup>+</sup> cells. Finally, this technology was applied to the area of catalytic antibodies, both as a practical application and in an effort to find a therapeutic agent for the AIDS virus.

## MATERIALS

## 2.0 MATERIALS

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All chemicals, acids or solvents used in this thesis, unless otherwise stated, originate from:

**Sigma Immunochemicals, St. Louis, Mo 63178, USA.**

All enzymes used in molecular biology techniques, unless otherwise stated, were purchased from:

**Boehringer Mannheim, Germany.**

Following is a list of materials not included in either of the above:

### **Consumables:**

Agarose	Gibco/BRL	5510UA
Low Melting Agarose	FMG	50082
HCl	Fisher	A144
Chloroform	Fisher	C298-500
PEG 8000	Fisher	BP233-1
SDS	Gibco/BRL	5525 UB
Reverse Transcriptase	Gibco/BRL	80535A
IIIB gp120	American	
	Biotechnologies	012001
IIIB gp120	Repligen	92-57

Pfu DNA Polymerase	Stratagene	600135
Soluble CD4	American	
	Biotechnologies	3001
T4 DNA Ligase	Gibco/BRL	BNP102

### **Antibodies:**

HRP-conjugated Goat anti-Human F(ab') <sub>2</sub>	Pierce	31414X
Alkaline Phosphatase conjugated Goat Anti- Human F(ab') <sub>2</sub>	Pierce	31312X
Rabbit Anti-gp120	American Biotechnologies	402
Alkaline Phosphatase- conjugated Goat Anti- Rabbit IgG	Fisher	OB1420-ALPH

### **Kits:**

Silver Staining Kit	Pharmacia1	7-0617-01
ECL Western Blotting	Amersham	RPN 2106
Mini-Protean II System	Bio-Rad1	61-0900
Mini-Trans Blot System	Bio-Rad	170-3930
Plasmid-Prep Kit	Qiagen	
Sequencing Kit	United States Biochemicals	70775

## METHODS

### 3.1. GENERAL METHODS

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#### 3.1.1. PLASMID PREPARATION.

Two major techniques were employed for the preparation of plasmid DNA. The first, and more common technique used was Alkaline Lysis (Sambrook *et al.*, 1987) which was performed using reagents made in the laboratory, and also using the Qiagen Mini and Maxi Prep Kits. The kits were essentially the same as the lab reagents with the only difference being that gel filtration columns were supplied with the kits to further purify the DNA after extraction. The second technique used was lysis by boiling (Sambrook *et al.*, 1987). However, this was only used when the number of samples to be processed became extremely large, since the boiling method permits the simultaneous processing of many more samples than does the alkaline lysis technique.

#### **Alkaline Lysis Method of Plasmid Preparation.**

Before commencing, the bacterial culture to be used was spread on an agar plate and grown overnight at 37°C. Selection of a single colony from the plate ensured a pure culture and a homogenous plasmid preparation. The colony was used to inoculate a 10ml superbrot (10g 3-(N-morpholino) propanesulfonic acid; 30g tryptone; 20g yeast extract, pH 7.0, per litre) culture. This was grown overnight at 37°C while shaking at 250rpm. The cells were then pelleted by centrifugation at 4000rpm for 15min. The pellet was resuspended in 0.5ml buffer 1 (50mM glucose; 25mM Tris-HCl, pH 8.0; 10mM EDTA) and transferred to a 1.5ml eppendorf tube. 0.5ml

solution 2 ( 0.2M NaOH; 1 % (w/v) SDS) was then added and immediately mixed by inversion. This was left at room temperature for 10min followed by the addition of 0.5ml solution 3 (60ml 5M potassium acetate; 11.5ml acetic Acid; 28.5ml water). This was gently shaken and left on ice for 10min. The preparation was centrifuged at 13000rpm for 30min and the supernatant was removed. (In the case of the Qiagen kits, the supernatant would be passed over the gel filtration column at this stage.) 0.8 volumes of isopropanol was added to this and it was left to precipitate at -20°C for 1 hour. The DNA was recovered by centrifugation at 13000rpm for 30min at 4°C and washed once in 70% ethanol. The pellet was resuspended in TE (10mM Tris-HCl; 10mM EDTA, pH 8.0) and extracted with phenol:chloroform:isoamylalcohol; 25:24:1 followed by precipitation in ethanol. This DNA was sufficiently pure to be used for cloning or sequencing.

#### **Lysis by Boiling.**

A 10ml culture was grown as before, and the cells were recovered by centrifugation at 4000rpm for 10min. They were then resuspended in 4ml STET (0.1M NaCl; 10mM Tris-HCl, pH 8.0; 1mM EDTA; 5%(v/v) Triton-X 100) and transferred to a 15ml tube where 50µl RNase (Gibco/BRL, 1 unit/µl) was added per ml of STET . 200µl of a 50mg/ml solution of lysozyme was also added and this was incubated at room temperature for 10min. The solutions were then boiled for 2min and centrifuged at 14000rpm for 15min. The pellet was removed with a toothpick, leaving the supernatant, which was



extracted with phenol:chloroform:isoamylalcohol; 25:24:1. This was then precipitated with 2.5 volumes of ethanol at -20°C for 1 hour.

### **3.1.2 PREPARATION OF SOLUBLE Fab.**

*E.coli* XLIBLue, transformed with the appropriate Fab-encoding plasmid, were used to inoculate superbroth containing 0.5%(w/v) glucose, 10µg/ml tetracycline and 100µg/ml carbenicillin (and 30µg/ml chloramphenicol in the case of the binary plasmid system). These cultures were incubated, shaking, at 37°C for 8 hours after which time 1mM isopropyl β-D-thiogalactopyranoside (IPTG; Stratagene 300127) was added to each culture. These were then incubated for a further 12 hours at 25°C. The cells were collected by centrifugation ( 4000 rpm for 10 min at 4°C ) and the pellets resuspended in 10ml 10mM PBS, pH 7.4 containing 34µg/ml phenylmethylsulphonylfluoride (PMSF; Gibco BRL 5521UB) and 1.5%(w/v) streptomycin sulphate (Sigma S6501). This was subjected to three freeze-thaw cycles followed by centrifugation ( 17000 rpm for 30 min at 4°C ). The supernatants were collected and cleared by filtration through 0.2µm filters and were used directly for analysis, or alternatively, purified further on an affinity column as described in the next section.

### **3.1.3 PURIFICATION OF Fab.**

Soluble Fab was purified by a single pass of the supernatants over an affinity column as follows. The column consisted of goat anti-human F(ab')<sub>2</sub> antibody (Pierce) linked to Gamma Bind G sepharose™ (Pharmacia). It was equilibrated in 3 column volumes of 87.2% phosphate buffer ( 0.1M sodium phosphate, dibasic; 0.5M sodium chloride), 12.8%(w/v) citrate buffer ( 0.05M citric acid; 0.5M sodium chloride ) and the supernatants were loaded onto the column in the same buffer. The column was washed until the O.D.(280nm) (Optical Density at 280nm) of the pass-through returned to a baseline level. The Fab was then eluted in 10.8% phosphate buffer, 89.2% citrate buffer and the collected fractions were neutralised with 1M tris-HCl, pH9.0 and concentrated to a final volume of approximately 1ml.

### **3.1.4 ANALYSIS OF PURIFIED Fab BY SDS-GEL ELECTROPHORESIS.**

SDS-gel electrophoresis was used to determine the purity of the Fab after purification by affinity chromatography. The Phast™ gel electrophoresis system (Pharmacia) was used in all cases, in conjunction with silver staining of the gel.

#### **Running the gel:**

2μl of Fab sample was mixed with 2μl sample buffer and boiled for 30 seconds. A 10-15%(w/v) SDS gradient gel was removed from its package and placed on the gel bed. Impressions of the wells to be used were pressed onto parafilm using the template provided and 2μl of each boiled sample was placed in each well, with Rainbow™ markers (Amersham) in one well. These samples were picked up in

the appropriate comb, which was then positioned above the gel using the slots provided. The gel was run for 20min at 58V and then removed for staining.

### **Staining:**

Silver staining was used in the detection of Fab protein as this technique is more sensitive than Coomassie blue, and the concentration of our Fabs was generally quite low (in the order of 0.05mg/ml). The following solutions were made up and attached to the electrophoresis machine by placing a selection of tubes marked by numbers into the solutions as indicated following:

Line 3:	10% (v/v) Ethanol; 5% (v/v) Acetic Acid	300ml
Line 4:	5% (v/v) Glutaraldehyde	100ml
Line 5:	Water	500ml
Line 6:	0.45% (w/v) Silver Nitrate	100ml
Line 7:	2% (v/v) Formaldehyde in 2.5% (w/v) Sodium Carbonate	100ml
Line 8:	5g Sodium Thiosulphate + 3.7g Tris-HCl in Water	100ml
Line 9:	1% (v/v) Glycerol	500ml

The manufacturer's recommended program for silver staining was then applied and the gel was removed for examination. The gel was then dried for storage.

### **3.1.5 ENZYME-LINKED IMMUNOABSORBENT ASSAY, ELISA.**

A number of ELISAs were carried out for the analysis of the Fab fragments. The initial one was a simple spot ELISA to determine if the Fab being studied bound gp120. Competitive ELISAs were performed to determine the relative affinity of the Fabs to gp120 and to determine if the interaction was competitively inhibited by soluble CD4.

#### **Spot ELISAs on gp120.**

The wells of a Costar E.I.A./R.I.A. plate were coated with 0.1µg of IIIB gp120 (American Biotechnologies) at 4°C overnight. The plate was washed 10 times with PBS containing 0.05% Tween-20, and then coated with 3%(w/V) Bovine Serum Albumin (BSA) in 10mM PBS, pH7.4 at 37°C for 1 hour. The BSA was discarded and 50µl of Fab sample was added to each well for 2 hours at 37°C. After washing again, 50µl of a 1/500 dilution of an alkaline phosphatase-labeled goat anti-human F(ab')<sub>2</sub> antibody (Pierce 31312X) was incubated in each well for 30min. The plate was washed once more and 25µl of an alkaline phosphatase substrate (Pharmacia) was added to each well at room temperature. The O.D. at 405nm was read after approximately 20min. For each antigen, a negative control extract of XLI Blue transformed with empty plasmid was used to determine whether other components in *E.coli* had an affinity for the antigens.

in the assay. Each extract was assayed for binding activity with BSA, and albumin-binding clones were considered to be negative. Any clone showing a signal of 3 times the negative control or higher was considered positive. The titre of any positive clone was assayed by making a serial dilution of that Fab and testing each dilution on the ELISA as described. The titre was taken as that dilution which gave a signal equal to half the signal generated by the undiluted Fab, and this was the dilution used in all subsequent assays.

### **gp120 Competition ELISAs.**

These assays were used to determine the relative affinity of an Fab for gp120. The plate was coated with gp120 and blocked with BSA as before. The Fab was diluted in 1%(w/v) BSA-10mM PBS, pH7.4 to a dilution corresponding to twice it's titre and 25 $\mu$ l of this were added to each of 7 wells. IIB gp120 was serially diluted from  $2 \times 10^{-6}$  M to  $2 \times 10^{-12}$  M and 25 $\mu$ l of these dilutions were added consecutively to each of the 7 wells and mixed with the Fab. The ELISA was then performed as described for the spot ELISAs. The results for each Fab were plotted as % maximum O.D. (Maximum O.D. being the signal when only Fab was added to the well at a dilution corresponding to it's titre) versus log(gp120 concentration). The relative affinity was determined to be that concentration of free gp120 resulting in 50% of maximum O.D.

### **CD4 Inhibition ELISA.**

These assays were carried out in a similar way to the gp120 competition ELISAs except the free antigen competing with the Fab in this case was soluble CD4 (sCD4). Serial dilutions of sCD4 are made in the range  $2 \times 10^{-7}$  M to  $2 \times 10^{-12}$  M and these dilutions are co-incubated with the Fab as before. The % maximum O.D. was plotted against the log[sCD4] and the concentration of sCD4 resulting in 50% inhibition of binding was determined as the inhibition constant.

### **3.1.6 SEQUENCING OF CONSTRUCTS.**

It was crucial to determine the sequence of each Fab construct in order to be able to interpret any results obtained from assaying the soluble Fab fragments. Constructs were either sequenced from single stranded DNA, or directly from the double-stranded plasmids. Both techniques were found to be equally successful and are described below.

#### **Preparation of Single-Stranded DNA (ssDNA).**

This method was used with constructs in the phagemid pComb3 as there was a stage in the life cycle of the M13 phage (upon which pComb3 was based) where its DNA was single stranded. A 5ml culture of *E.coli* containing the construct was grown in superbroth supplemented with 100µg/ml carbenicillin, 10µg/ml tetracycline and 20mM MgCl<sub>2</sub> at 37°C overnight. 100µl of this culture was then used to inoculate 13ml of fresh medium and this was grown for 1.5 hours at 37°C. 50µl of VCSM13 helper phage at 10<sup>12</sup>pfu/ml (plaque forming units) was added and incubated for a further 2 hours, followed by the addition of kanamycin to a final concentration of 70µg/ml. The culture was then grown for a minimum of 18 hours at 37°C. The cells were removed by centrifugation and 3.7ml of 20% polyethylene glycol (PEG) 8000; 1.8ml 5M NaCl are added to the supernatant, which was chilled on ice for 30min. This was centrifuged at 11000 for 15min and the supernatant was discarded. The pellet was resuspended in 600µl TE and extracted twice with equal volumes of phenol:TE, followed by a single chloroform

extraction. The ssDNA was then be recovered by ethanol precipitation.

### **Sequencing Protocol.**

When double-stranded DNA (dsDNA) was being used, it was first denatured before annealing to the primer. However, this was not necessary in the case of ssDNA.

#### DNA Denaturation:

4 $\mu$ g of dsDNA in 8 $\mu$ l was added to 4 $\mu$ l of 2M NaOH and left at room temperature for 10min. The following reagents were then added to the reaction: 4.5 $\mu$ l of 2M ammonium acetate pH 4.5, 5.5 $\mu$ l water and 60 $\mu$ l ethanol. The mixture was then incubated at -70°C for 1 hour after which time, the DNA was recovered by centrifugation at 14000rpm for 30min. The pellet was washed in 70% ethanol and dried under vacuum. This DNA was then ready for annealing to the sequencing primer.

#### Primer Annealing.

All reagents used for the following processes were derived from the United States Biochemical Sequenase Version 2.0 DNA sequencing Kit (70770). 1 $\mu$ l primer (at 0.1 $\mu$ g/ $\mu$ l) and 2 $\mu$ l reaction buffer were added to the dried DNA, and made up to 10 $\mu$ l with water. This was heated at 65°C for 2min and subsequently let cool to 35°C over a period of 30min.



### Sequencing Reaction

The following cocktail was made while the annealing reactions are cooling:

1: 2: 0.5    0.1M DTT: 1/6 dilution of labeling mix:  $^{35}\text{S}$  ATP

A 1/16 dilution of the sequenase enzyme was made, in the enzyme buffer, and a 1/16 dilution of pyrophosphatase was made in the same solution. The pyrophosphatase permits longer incubations and prevents shadow bands appearing.

4 $\mu\text{l}$  cocktail and 2 $\mu\text{l}$  enzyme dilution were added to each annealing reaction. This was incubated for 3min at room temperature.

### Termination:

2.5 $\mu\text{l}$  of each of the dideoxynucleotides (ddNTP) were added to each of 4 wells in a 96 well plate for each clone to be sequenced. 3.5 $\mu\text{l}$  of the sequencing reaction was then added to each of the 4 wells and this was incubated at 37°C for 5min. Stop solution may be added at this stage. However, if there are problems with secondary structures, a number of additional steps are advisable:

Incubate the plate at 55°C for 10min. Add 1 $\mu\text{l}$  of Terminal Deoxynucleotide Transferase (TDT) mix (1mM dNTPs in 1X TDT buffer with 0.5 $\mu\text{l}$  TDT for every 4 wells) to each well and incubate for a further 30min at 37°C. This permitted the addition of nucleotides to the ends of all strands not terminated correctly. They then extended such that they could not enter the sequencing gel and so would not appear as unwanted artifacts.

Add stop solution and store the plate at -20°C until ready to run the gel.

### Sequencing Gel

A 6%(W/V) polyacrylamide gel was poured according to manufacturer's (Pharmacia) recommendations. 2.5 $\mu$ l of each reaction was loaded in each well in a predefined order. The gel was then run at 50°C for varying amounts of time depending on the desired area of sequence. On completion of the run, the gel was removed and dried onto a piece of filter paper in a gel dryer at 80°C for 1 hour. It was then exposed under X-ray film (Amersham) for at least 24 hours. The sequence could then be read from the autoradiograph.

### **3.1.7 NEUTRALISATION ASSAYS.**

It was not possible to conduct neutralisation assays in the laboratory, since the facilities to handle live HIV virus were not available to us. Therefore, two existing assays were used for this purpose. The first was carried out by Dr. Ewa Björling and Dr. Erling Norrby at the Karolinska Institute, Sweden, and measured the production of viral protein p24 in the supernatant (Barbas *et al.*, 1992). The second was performed by Dr. Nancy Dunlop and Dr. Peter Nara at the National Cancer Institute, Maryland and was based on syncytium formation (Nara and Fishinger, 1988).

### **p24 Neutralisation Assay.**

Diluted tissue culture supernatants of HIV-1 IIIB or MN-infected peripheral blood mononuclear cells (PBMCs) were incubated for 2 hours at 37°C with serially diluted Fab samples. 10<sup>5</sup> PBMCs were then added to the virus/antibody mixtures and incubated for 1 hour at

37°C. The cells were then washed and incubated with RPMI 1640 supplemented with 10%(v/v) foetal calf serum, 1.5mM glutamine, antibiotics and interleukin 2. These were then incubated for 7 days with medium changes at days 1 and 4. After incubation, the supernatants were analysed by HIV-1 p24 capture ELISA as described previously. (Sundqvist *et al.*, 1989). A result was determined positive if an 80% reduction in optical density occurred as compared with a negative human serum. The positive control used was a known mouse neutralising antibody and its Fab fragment (2F5).

### **Syncytium-Forming Neutralisation Assay.**

This assay used a Leu-3a positive CEM cell line (CEM-SS) that expresses CD4 on its cell surface and form syncytia on infection by HIV-1. Wells of a flat bottomed 96 well plate (Costar) were first coated with 50µl of a 50µg/ml solution of poly-L-lysine and allowed to stand at room temperature for 1 hour. The PLL was removed and the wells were coated with 50µl of  $1.0 \times 10^6$  cells/ml CEM-SS suspension. The cells had been in a logarithmically growing in RPMI 1640, 10% serum, 1% antibiotics, immediately prior to this, to ensure standardisation of the assay. The cells were allowed to attach to the plate at 37°C for 30 minutes and the supernatant was then discarded. 50µl of 100 syncytial forming units of a pretitred viral stock were mixed with 50µl of serially diluted antibody samples. 50µl of each virus-antibody mixture was divided into duplicate wells containing the CEM-SS cells and incubated for 60 minutes. The medium was replaced with 200µl of complete medium and the plate was incubated at 37°C for 5 days, with the addition of

a further 200 $\mu$ l of medium on day 3. The wells could then be examined under an inverted microscope for syncytium formation. The negative control used involved the addition of PBS in the place of virus. The positive control excluded the neutralising antibody and the number of syncytia formed in this well was designated  $V_0$ , while the number present in the antibody wells were designated  $V_n$ . The ratio  $V_n/V_0$  was calculated, and an antibody was determined to neutralise at that dilution if the ratio was 0.1 or lower (i.e.: 90% neutralisation).

## **3.2 LIBRARY CONSTRUCTION**

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### **3.2.1 RNA ISOLATION.**

RNA was prepared from bone marrow cells taken from an asymptomatic AIDS patient. These cells had been rendered safe upon isolation by inactivation of any virus particles present. 10 ml of guanidium isothiocyanate were then added to  $10^8$  cells and these were homogenised for 1 minute in a thick-walled 50ml polypropylene tube. 1ml of 2M sodium acetate was added to this solution, followed by centrifugation at 1000rpm for 20min at 4°C. The supernatant was removed and added to an equal volume of isopropanol. This was allowed to precipitate for 1 hour at -20°C, and then centrifuged as above. The pellet was resuspended in 3ml guanidium isothiocyanate and the isopropanol precipitation was repeated. The final pellet was resuspended in 3ml 70% ethanol and centrifuged for 10 min. This was dried under vacuum and finally resuspended in 1ml diethyl pyrocarbonate (DEPC) treated water. The quantity of RNA was determined by reading the O.D.(260nm). An O.D. of 1.0 was equivalent to 40µg/ml RNA.

### **3.2.2 cDNA PRODUCTION.**

The primer used in cDNA production was a 24 base pair (bp) oligo dT primer, as this seemed to give better results than a specific primer (data not shown). 0.5µg RNA in 8µl were added to 2µl primer at 0.1µg/ml. 2µl RNAsin were added to this and it was incubated at 70°C for 10min. This was cooled on ice and the following additions

were made: 5 $\mu$ l 10X reverse transcription buffer (Gibco BRL); 3 $\mu$ l dNTP mix (50 $\mu$ M each of dATP, dGTP, dCTP and dTTP); 5 $\mu$ l 0.1M Dithiotreitol (DTT); 2 $\mu$ l BRL Superscript Reverse Transcriptase (200U/ $\mu$ l). This was incubated at room temperature for 10min, followed by 42°C for 50min. The reaction was terminated at 90°C for 5min and then cooled on ice for 10min. Finally, the reaction mixture was incubated with 1 $\mu$ l RNase H at 37°C for 30min. The cDNA was stored at -20°C.

### **3.2.3 PCR AMPLIFICATION OF GAMMA AND KAPPA CHAINS.**

The DNA encoding the gamma and kappa chains of the antibody were then isolated from the cDNA using the Polymerase Chain Reaction (PCR). In order to achieve this, a set of primers were designed by Burton et al. (1991) to specifically amplify different subclasses of gamma and kappa chains. The 5' primers were designed to be specific for the N'-terminus of the variable regions, and the 3' primers were designed to be complementary to the C'-terminal end of the CH<sub>1</sub> region in the case of gamma chains, or the C'-terminal end of the C<sub>K</sub> region in the case of kappa chains. The primers were also designed to incorporate restriction sites suitable for cloning the PCR fragments for library construction. The following is a list of primers used, with the restriction sites indicated in italics:

**Human kappa 3' primer. CK1z:**

*Xba 1*

5'-GCG CCG TCT AGA ACT AAC ACT CTC CCC TGT TGA AGC TCT TTG  
TGA CGG GCG ATC TCA G-3'

**Human kappa variable chain 5' primers:**

*Sac I*

5'-GAC ATC GAG CTC ACC CAG TCT CCA-3'      **VK1a**

5'-GAA ATT GAG CTC ACG CAG TCT CCA-3'      **VK3a**

5'-GAT ATT GAG CTC ACT CAG TCT CCA-3'      **VK2a**

**Human gamma chain 3' primer:**

*Spe 1*

5'-GCA TGT ACT AGT TTT GTC ACA AGA TTT GGG-3' **CG1z**

**Human gamma variable chain 5' primers:**

*Xho I*

5'-CAG GTG CAG CTC GAG CAG TCT GGG-3'      **VH1a**

5'-GAG GTG CAG CTC GAG GAG TCT GGG-3'      **VH3a**

5'-CAG GTG CAG CTG CTC GAG TCT GGG-3'      **VH1f**

5'-GAG GTG CAG CTG CTC GAG TCT GGG-3'      **VH3f**

5'-CAG GTG CAG CTA CTC GAG TCG GG-3'      **VH2f**

5'-CAG GTG CAG CTG CTC GAG TCG GG-3'      **VH4f**

5'-CAG GTA CAG CTC GAG CAG TCA GG-3'      **VH6a**

Each PCR reaction contained the 3' gamma or kappa primer plus one appropriate 5' primer. It was only after the completion of PCR that the various subclasses were mixed for library construction.

#### **PCR Reaction.**

Initially, a master PCR mix was made which was then be stored at -20°C. This comprised:

792µl sterile water.

8µl dNTP mix (50µM each nucleotide).

100µl 10X Taq reaction buffer (Promega).

Each reaction tube was then assembled as follows:

90µl PCR mix.

3µl 3' primer

3µl 5' primer

3µl DNA (approximately 1µg)

1µl Taq polymerase (2.5units/ml)



The PCR conditions were varied depending on the DNA and primer being used. However, typical conditions for library construction were:

<u>Temp</u>	<u>Time</u>
95°C	1min
52°C	30sec
72°C	1min
Followed by: 72°C	5min.

#### **Purification of PCR Products.**

The PCR reactions were directly run on a 1.5% low melting point agarose gel for 1 hour, at 80volts. The band corresponding to 700bp in size was cut from the gel and this was melted at 65°C for 10min. This was then cooled to 42°C and incubated with 1µl  $\beta$ -agarase (1unit/µl) (New England Biolabs) overnight. A 1/10 volume of 3M sodium acetate, pH5.3 was added and this was centrifuged at 13000rpm for 20min to remove any remaining carbohydrate. 2.5 volumes of ethanol was added to the supernatant and incubated at -20°C for 1 hour, followed by centrifugation as before. The pellet was washed in 70% ethanol and resuspended in 20µl TE. The DNA was quantified by O.D. at 260nm. An O.D.(260nm) of 1.0 corresponds to 50µg/ml DNA.

#### **3.2.4 RESTRICTION OF PCR FRAGMENTS.**

The following was a typical reaction mixture for the restriction of gamma chain PCR fragments:

1µg DNA in 1µl  
2µl Xho I (Boehringer Mannheim, 50U/µl)  
2µl Spe I (Boehringer Mannheim, 50U/µl)  
5µl 10 Buffer H  
40µl sterile water.

Incubate at 37°C for 2 hours.

A similar reaction was carried out for kappa chains using the enzymes Sac I and Xba I in Buffer A.

All restriction were inactivated by heating to 65°C for 5min followed by a single extraction with Phenol:Chloroform:Isoamylalcohol; 25:24:1.

### **3.2.5 PREPARATION OF THE VECTOR.**

The 4000bp vector used in the construction of combinatorial libraries was known as pComb3. This vector was constructed by Carlos F. Barbas III, and prepared by him in the following way:

*E. coli* XL1Blue were transformed with the pComb3 phagemid and grown overnight in superbrot medium supplemented with 100µg/ml Carbenicillin. A plasmid preparation was then performed, by the alkaline lysis method, on the culture to give typical plasmid yields of 1mg. 50µg of plasmid were digested with 150 units each of Xho I and Spe I restriction enzymes in a 300µl reaction at 37°C for 2 hours. This was then extracted once with phenol:chloroform:isoamylalcohol 25:24:1 and ethanol precipitated. The DNA was then treated for 1 hour at 37°C with 100 units of Calf Intestinal Phosphatase (Boehringer Mannheim) followed by gel

purification. The vector was then ready for insertion of the gamma chain inserts.

### **3.2.6 LIGATION OF THE PLASMID AND INSERT.**

It was at this stage that the different gamma chain subclass inserts obtained from the PCR reactions were mixed with the restricted vector DNA. These were then ligated to the prepared vector in the molar ratio of 2:1, insert:vector. The reaction mix used was:

2 $\mu$ g Vector DNA

0.4 $\mu$ g Insert DNA

3 $\mu$ l Ligase (Gibco BRL, 1 unit/ $\mu$ l)

30  $\mu$ l 5X buffer

2 $\mu$ l dATP(100mM)

112.6 $\mu$ l water

Incubate at 16°C overnight.

### **3.2.7 TRANSFORMATION OF LIGATIONS.**

The ligated DNA was transformed in *E.coli* XLIBlue, which could be selected, as it coded for tetracycline resistance on an F' episome. However, it was necessary to make competent cells before transformation.

### **Preparation of Electrocompetent Cells.**

A 10ml culture of *E.coli* XL1Blue was supplemented with 10µg/ml tetracycline and grown overnight at 37°C. 5 ml of each culture was then used to inoculate 500ml of superbrot, and this was grown until an O.D.(550nm) of 0.8 was reached. At this stage, the cultures were chilled on ice for 15min and then centrifuged at 4000rpm for 15min at 4°C. The cells were resuspended in 1litre 10%(v/v) glycerol and recentrifuged. This was followed by a wash with 500ml 10%(v/v) glycerol and finally a 20ml wash. The pellet was then resuspended in its own volume by stirring with a pipette, dispensed into 250µl aliquots and immediately frozen in an ethanol bath cooled with dry ice. The cells were then stored at -80°C.

### **Transformation of the heavy chain library.**

2µg of ligated DNA in water was mixed with 300µl electrocompetent cells in a 0.2cm electroporation cuvette. This was then pulsed at a resistance of 200mΩ and a voltage of 2.5Kv. This was washed immediately with 1ml SOC medium (20g tryptone; 5g yeast extract; 0.5g NaCl; 2.5mM KCl; 10mM MgCl<sub>2</sub>; 20mM glucose, pH7.0 per litre) (Sambrook *et al.*, 1989) followed by a further 2ml wash, and the cells were grown at 37°C for 1 hour. At this stage 10ml of superbrot containing 10µg/ml tetracycline and 20µg/ml carbenicillin was added and the culture was grown for an additional hour at 37°C. This was then added to 100ml superbrot supplemented with 50µg/ml carbenicillin and grown overnight. Phagemid DNA was prepared from this culture.

### **Insertion of the Kappa Light Chains.**

10µg of DNA from the above preparation was cut with Sac I and Xba I restriction enzymes, as described previously, and the kappa inserts were ligated. The vector, now contained both heavy and light chain DNA, was transformed as before and scaled- up to the 100ml culture stage. It was at this point that the panning procedure to select for gp120-specific antibodies was performed.

### **3.2.8 PURIFICATION OF PHAGE PARTICLES.**

The transformed culture was grown in 100ml superbrot h containing 50µg/ml carbenicillin and 10µg/ml tetracycline for 1 hour at 37°C. 0.5ml of VCS M13 helper phage (Stratagene) at 10<sup>12</sup> pfu/ml were added and grown for a further 1 hour at 37°C. Kanamycin was then added to a final concentration of 70µg/ml in order to select for the phage. This culture was then grown overnight. The cells were then separated from the supernatant by centrifugation, and phagemid DNA was prepared from the cells for storage. Phage particles were purified from the supernatant using the following technique:

4g of polyethylene glycol (PEG) 8000 and 2g NaCl were added to each 100ml of supernatant and chilled on ice for 1 hour. This was then centrifuged at 9000rpm for 20 min. The supernatant was discarded and the pellet was resuspended in 2ml PBS. This was cleared by centrifugation at 3500rpm for 15min followed by 14000rpm for 5min. The phage were then titred to determine the initial quantity.

### **Phage Titres.**

The phage obtained from the above procedure were serially diluted to  $10^{-12}$ . Each dilution from  $10^{-6}$  and lower were mixed with 50 $\mu$ l *E.coli* culture at 37°C for 10 min. 25 $\mu$ l of these were plated out on agar plates supplemented with 100 $\mu$ g/ml carbenicillin for counting the next day. The result could then be expressed as number of phage per ml. It was possible to titre the phage in this way, since, in the absence of helper phage they propagated as a plasmid.

### **3.2.9 PANNING.**

The wells of Costar E.I.A./R.I.A. plates were coated with 0.8 $\mu$ g/well IIB gp120 (American Biotechnologies) in 10mM PBS, pH7.4, overnight at 4°C. The wells were washed with water and coated with 3%(w/v) BSA-PBS for 1 hour at 37°C. After washing once with 1%(w/v) BSA-PBS, 25 $\mu$ l of phage were added to each well and incubated at 37°C for 1 hour. The non-specific phage were then removed and the wells were washed with 6 X 10min washes in 1%(w/v) BSA-TBS Tween, followed by a further wash in water. The specific phage were then eluted in 50 $\mu$ l of elution buffer (1.6ml 12N HCl in 150ml water was adjusted to pH to 2.2 with solid glycine; a further 200ml water was then added and the solution was autoclaved) for 10 min at room temperature. If a number of wells were used, they were combined at this stage and 3 $\mu$ l 2M Tris, pH 8.0, was added per 50 $\mu$ l eluate to neutralise the pH. This was added to 2ml of freshly grown *E.coli* XLIBLue culture and incubated at 37°C for 15min. 10ml superbrotth containing 20 $\mu$ g/ml carbenicillin and 10 $\mu$ g/ml tetracycline were then added. 20, 1.0 and 0.1 $\mu$ l volumes are

removed at this stage and plated out on carbenicillin plates to measure the input titre of phage. The 10ml culture was grown for another hour followed by the addition of 100ml of broth. This was cultured for 1 hour, and, if a further round of panning was required, helper phage were added and the panning process repeated. When the final round was reached, the cells are grown overnight in the absence of helper phage and plasmid preparations were carried out to retrieve the DNA coding for the antigen-specific antibody library.

#### **3.2.10 REMOVAL OF THE GENEIII FROM THE LIBRARY.**

The plasmid DNA prepared after panning was digested with the enzymes *Nhe* I and *Spe* I as described for vector preparation previously. The resulting 4.7Kb fragment produced was purified by gel electrophoresis and could be self-ligated in the presence of ligase, due to the compatible ends that were produced by restricting with these two enzymes. This plasmid DNA was transformed into *E.coli* and plated out on carbenicillin plates. Colonies were removed from these plates to produce soluble Fab fragments to be tested for gp120 binding on ELISA.

### **3.3 BIOCHEMICAL ANALYSIS OF Fab FRAGMENTS**

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#### **3.3.1 Fab INHIBITION ELISAs.**

Microtitre wells were coated with 0.1µg IIIB gp120 ( American Biotechnologies, Columbia MD ) Competitive ELISAs between the panel of F(ab)s used in the biochemical analysis and whole antibody 13 were performed using a similar methodology to that used for the gp120 competitive ELISAs as described in methods section 3.1.5. The following antibody dilutions were used: Whole antibody 13 was obtained by fusing the Fab with an Fc portion of IgG, and expressing the product in Chinese Hamster Ovary cells to yield a whole IgG molecule. This was then used at a constant dilution of 1:10000 in the ELISA as this was found to be the titre for this antibody. The purified Fab fragments (initially at 100µg/ml) were serially diluted from 1:100 to 1:32000 and incubated with the IgG in the ELISA plate for 2 hours. After washing, the amount of whole antibody remaining bound to the plate was detected using a peroxidase-labeled goat anti-human antibody (Pierce 31312X) specific for the Fc portion of IgG.

#### **3.3.2 DETERMINATION OF RELATIVE AFFINITIES USING BIACORE™.**

The process optimised for use with the Fab fragments involved a number of steps. Two separate channels on a biosensor chip had to be coated with IIIB gp120 (Repligen, Cambridge MA) such that one



channel could be used for the determination of association constants ( $k_{on}$ ) and the other for the determination of dissociation constants ( $k_{off}$ ).

#### **Immobilization of Antigen on the Sensor Surfaces.**

A flowrate of 5 $\mu$ l/min of 10mM PBS, pH7.4, over the biosensor chip was first established. The chip was activated by injecting 30 $\mu$ l of activation solution (Pharmacia Biosensor, 50%(v/v) 0.2M *N*-ethyl-*N*'-(3-diethylaminopropyl)-carbodiimide, 50%(w/v) *N*-hydroxysuccinimide). The flowrate was then adjusted to 10 $\mu$ l/min and the antigen was injected in 10mM sodium acetate buffer pH 4.5. A 25 $\mu$ l sample of gp120, (10 $\mu$ g/ml), was injected when association constants were to be determined (resulting in a final level of 4000 Response Units (RU)). However, 20 $\mu$ l of gp120 at 2 $\mu$ g/ml was injected for the determination of dissociation constants (giving a final level of 800RU). In both cases, a flowrate of 5 $\mu$ l/min was reestablished following the gp120 injection and the chip was blocked from any further immobilisation by injecting 30 $\mu$ l of 1M ethanolamine, pH8.5 (Pharmacia Biosensor).

#### **Determination of Association Constants ( $k_{on}$ ).**

A series of dilutions were made for each Fab to give final concentrations in the range of 1-20 $\mu$ g/ml. 30 $\mu$ l of each dilution was injected, in turn, over the immobilized antigen at a flowrate of 5 $\mu$ l/min. The change in response per unit time ( $dR/dt$ ) was plotted against time ( $t$ ) for each concentration. The slopes of each of these graphs were then plotted against their corresponding concentrations to give a final graph from which the association constant was read.

### **Determination of Dissociation Constants ( $k_{off}$ ).**

30 $\mu$ l of each Fab solution at 150 $\mu$ l/ml was injected over the immobilized antigen at a flowrate of 5 $\mu$ l/min. Once the reaction had reached equilibrium, the Fab was removed from the antigen at a constant flowrate of 50 $\mu$ l/min. A plot was then made of  $\ln(R_i/R_0)$  against  $t_i - t_0$  for the dissociation phase ( $R_i$  was the response at time  $t_i$ ;  $R_0$  was the initial response at time  $t_0$ ) and the slope of this graph was taken to be the dissociation constant. Relative affinities were then calculated as  $k_{on}/k_{off}$ .

### **3.2.3 EPI TOPE MAPPING USING BIACORE.**

A flowrate of 5 $\mu$ l/min 10mM PBS, pH7.4 was established and the biosensor chip was activated as above. 40 $\mu$ l of goat anti-human F(ab')<sub>2</sub> (Pierce) at 40 $\mu$ g/ml was injected in 10mM sodium acetate buffer, pH4.5 to give a final level of immobilization of 10000RU. The chip was then blocked as described above. The flowrate was adjusted to 1 $\mu$ l/min and 4 $\mu$ l of the first Fab at 100 $\mu$ g/ml was injected, immediately followed by 4 $\mu$ l of an anti-cytomegalovirus Fab (150 $\mu$ g/ml) to block any remaining sites on the surface. 4 $\mu$ l of IIIB gp120 (10 $\mu$ g/ml) was injected over this, followed by 4 $\mu$ l of the second Fab also at a concentration of 100 $\mu$ g/ml. The entire surface was regenerated with 25 $\mu$ l of 60mM HCl so that the next cycle could be run. Each Fab was run against all the other Fabs to give a mosaic of binding patterns and so establish an epitope map.

### **3.3.4 KNOCKOUT ASSAYS WITH HxB2 gp120 MUTANTS.**

HIV-1 envelope glycoproteins were obtained by using culture supernatants from COS-1 cells transfected with plasmids expressing either wild-type or mutant gp120. These molecules were then captured onto the surface of an ELISA plate using antibody D7324 (Aalto Bioreagents; Dublin, Ireland) which binds to the conserved 15 amino-acid peptide at the COOH terminus of gp120. The binding of a reference HIV-1 positive human serum to each mutant was assayed by incubating the serum with the immobilised gp120 in the presence of 0.5%(v/v) Tween-20, and bound antibody was detected by means of a second, enzyme-conjugated antibody. This result was taken as the reference value for each mutant. The Fabs were then assessed for binding to the mutant panel in the same way and the binding ratio of the test antibody to reference serum was determined for each gp120 mutant. The average ratio for the entire panel was calculated and any individual ratio deviating from the mean by less than 0.5 times was considered to be an inhibitory mutation, while those deviating by more than 1.5 times indicated an enhancing mutation. In this way, a map of mutations affecting the binding of the Fab to gp120 was obtained for each clone.

### **3.4 BINARY PLASMID SYSTEM**

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#### **3.4.1 PREPARATION OF EXPRESSION PLASMIDS.**

The binary plasmid system consists of two plasmids designed to facilitate separate cloning of the heavy and light chains. The light chains are cloned into plasmid pTC01, while the heavy chains are inserted into pTAC01H. The construction of these two plasmids has been described previously by Collet et al.(1992). Cloning with these two plasmids is very similar to the library construction described earlier.

#### **Construction of Light Chain-containing Plasmids**

Vector was prepared by digesting pTC01 with SacI and XbaI and treating it with 5 units of calf intestinal phosphatase (Boehringer Mannheim) for 1 hour at 37°C, followed by 10min at 65°C to inactivate the enzyme. Light chain insert was obtained by digesting the pComb3-based parent plasmid with the same combination of enzymes and isolating the 0.7 kb fragment using low melting point agarose gel electrophoresis followed by  $\beta$ -agarase digestion. Ligation was at 16° C overnight under standard conditions using a 5:1 insert-to-vector ratio. Before transformation, background was reduced by digesting the ligation mix with XhoI. Glucose was used at a concentration of 1% (w/v) in all liquid cultures to suppress expression of the cloned inserts.

### **Construction of Heavy Chain-containing Plasmids**

Vector was prepared by digesting pTAC01H with XhoI and SpeI and treating it with calf intestinal phosphatase according to the protocol above. Heavy chain insert was obtained by PCR from the pComb3-based parent plasmid: the following mixture was subjected to 30 cycles of amplification (1 min at 94° C, 0.5 min at 52° C, and 2 min at 72° C) followed by extension at 72° C for 10 min: 50 mM KCl, 10 mM Tris-HCl (pH 8.8 at 25° C), 1.5 mM MgCl<sub>2</sub>, 0.1%(v/v) Triton X-100, 200 µM of each dNTP, 1.25 units of Taq Polymerase (Promega), 3 ng of plasmid target DNA and approximately 600 nM of each primer: VH1a for the forward direction and CH1z for the reverse (supplied by American Synthesis). PCR-product of the correct size (0.7 kb) was separated by low melting point gel electrophoresis, isolated using glassmilk, and digested with XhoI and SpeI. The digested PCR-product was purified as before and ligated to the prepared heavy chain vector using a 1:2 vector-to-insert ratio. Glucose (1% (w/v)) was used at in all liquid cultures to suppress expression of the cloned inserts.

#### **3.4.2 CO-TRANSFORMATION OF THE BINARY PLASMIDS.**

Due to the very large number of transformations required to complete this experiment, it was decided not to use electroporation in this case as this would be too time consuming and would be prohibitively expensive. Instead, the technique of calcium chloride transformation was chosen. Calcium chloride- competent cells were prepared for this technique.

### **Calcium Chloride Competent Cells.**

A 10ml culture of *E.coli* XL1Blue was grown overnight in superbroth containing 10µg/ml tetracycline. This was then used to inoculate a 1litre culture (with no antibiotic) which was grown at 37°C until an O.D.(550nm) of 0.8 was reached. At this point, the cells were chilled on ice for 15min and removed from culture by centrifugation at 4000rpm for 10min. The cells were resuspended in 1litre of buffer (0.1M CaCl<sub>2</sub>; 20mM Tris-HCl, pH 8.0) and recentrifuged. Two further washes with 500ml and 25ml of buffer were carried out, and the final cell pellet was resuspended in it's own volume by stirring with a pipette. This was frozen to -80°C immediately in a dry ice bath, until ready for use.

### **Transformation Protocol.**

50 µl CaCl<sub>2</sub>-competent XL1Blue cells were incubated with 0.5 µg purified DNA of each plasmid for 30 min on ice, heat-shocked for 1 min at 42° C, and incubated for 2 min on ice. 0.3 ml SOC (Sambrook *et al.*, 1989) was added and the cultures were grown with moderate shaking at 37° C for 1 hour. The presence of both plasmids and the episome was selected for by plating 0.1 ml of these cultures on triple-antibiotic agar plates (100 µg/ml carbenicillin; 30 µg/ml chloramphenicol; 10 µg/ml tetracycline; 32 g/l LB agar) at 37° C overnight. The expression of Fab fragment in all DNA cloning experiments was suppressed by adding 1% (w/v) glucose to all media and plates.

### **3.4.3 PREPARATION OF RECOMBINANT Fab FRAGMENT**

Bacterial cultures for determination of antigen-binding activity were grown in 96 well-tissue culture plates (Costar 3596). 250  $\mu$ l Superbroth containing 30  $\mu$ g/ml chloramphenicol, 100  $\mu$ g/ml carbenicillin, and 1%(w/v) glucose were added per well and inoculated with a single double-transformant using a sterile toothpick. Plates were incubated with moderate shaking (200 rpm) on a horizontal shaker for 7-9 hours at 37° C, until the O.D.(550nm) was approximately 1-1.5. The cells were collected by centrifugation of the microtitre plate (1,500g for 30 min at 4° C), the supernatants were discarded, and the cells were resuspended in fresh media containing 1mM isopropyl-b-D-thiogalacto-pyranoside, but no glucose. Incubation was continued at room temperature overnight to induce expression of Fab fragment. Cells were then harvested by centrifugation as before, resuspended in PBS containing 34  $\mu$ g/ml phenylmethylsulfonyl fluoride and 1.5% (w/v) streptomycin sulfate, and lysed by 3 freeze-thaw cycles between -80° C and 37° C. The crude extracts were partially cleared by centrifugation (1,500g for 45 min at 4° C) before analysis by antigen-binding ELISA. These fragments were then used in ELISA analysis as described in section 3.1.5 of the methods.

#### 3.4.4 Fermentation Methods.

The following is the minimum medium designed for maximal *E. coli* growth and Fab expression:

Component	Quantity(g/l)	Concentration(mM)
$\text{Na}_2\text{HPO}_4$	20.1	75
$\text{KH}_2\text{PO}_4$	2.45	18
$\text{Na}_3\text{Citrate}$	1.21	4.1
$(\text{NH}_4)_2\text{SO}_4$	3.3	50
$\text{NaCl}$	6	8
$\text{NH}_4\text{Cl}$	2.67	50
Citrate or	10 g/l	1%
Glucose	20 g/l	2%
Casein(Difco)	12 g/l	1.2%
Thiamine(50mg/ml)	1 ml/l	
Iron Citrate(30mg/ml)	1 ml/l	
Trace Elements	1 ml/l	
$\text{NaMoO}_4$	1 ml/l	
$\text{CaCl}_2(1\text{M})$	0.5 ml/l	0.5
$\text{MgSO}_4(1\text{M})$	20 ml/l	20



10l of water were added to the fermenter vessel containing all the salts listed above and they were sterilised *in situ* according to the fermenter specifications. All other medium components were sterilised by autoclaving separately. They were then added upon completion of the fermenter sterilisation program. The vessel contents were brought to a temperature of 37°C and a pH of 7.0 (NaOH and KH<sub>2</sub>PO<sub>4</sub> were used for pH control). The oxygen meter was then calibrated and the oxygen level was adjusted to the maximum level, at an inlet pressure of approximately 1.5 Bar. 0.5l of culture had been grown up overnight, and this was then added to the vessel which was then stirred with an agitator speed of 1000rpm. Antibiotics were added along with the culture at the following final concentrations: Chloramphenicol, 30µg/ml; Carbenicillin, 100µg/ml and Tetracycline, 5µg/ml. The culture was allowed to grow under the above conditions overnight using Polypropylene glycol 2000 (PPG Industries) as antifoam. Glucose was added to the culture at intervals until the optical density had reached a value of at least 60.0, at which point no further glucose was added and the levels were monitored until they reached 0.2% or lower. The temperature was then adjusted to 30°C and IPTG was added to a final concentration of 1mM. This was cultured for a further 6 hours and then harvested.

#### **Filtration and Concentration.**

Both of these tasks could be achieved using the Pellicon filtration system (Millipore). A 0.2µM filter was used for filtration and a 10000Mw cut off filter was used for concentration, thus retaining

the Fab (Mw is 50000). The unit was assembled and run at an inlet pressure of 12 P.S.I. and an outlet pressure of approximately 3 P.S.I. The outlet pressure was controlled using the inlet pressure and the flowrate of the retentate fraction. The higher the retentate flowrate, the lower the outlet pressure. During concentration, the retentate was channeled back into the inlet sample and the process was performed continuously until the inlet sample reduced to the required volume. The supernatant from a 12l fermenter run could be reduced to a volume of 500ml in approximately 3 hours. After the completion of a run, the system was washed in water and stored in 0.05% sodium azide.

#### **3.4.5 METAL AFFINITY CHROMATOGRAPHY**

Chelating Sepharose Fast Flow (Pharmacia) was loaded with  $\text{ZnCl}_2$  according to manufacturers instructions. The antibody could be bound to the resin by either stirring the antibody and resin overnight at 4°C or by pouring the column first and loading the antibody sample over the column a number of times to ensure that the maximum amount of sample had bound. The two buffers used to elute the antibody were as follows:

Buffer A:            50mM Sodium Phosphate  
                         1M Sodium Chloride  
                         pH 7.0

Buffer B:           50mM Sodium Phosphate  
                      1M Sodium Chloride  
                      100mM Histidine  
                      0.5M Imidazole  
                      pH 6.0

The column was run in a gradient of 0-100% buffer B over a period of 6 hours at a flowrate of 8ml/min. 50ml fractions were collected and each of these were assayed for Fab content by direct ELISA. The column was washed after each run in 50mM sodium phosphate; 50mM EDTA. Any fractions containing Fab activity were pooled and a sample was assayed for purity by SDS-polyacrylamide gel electrophoresis.

## 3.6 METAL METHODS

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### 3.6.1 INTRODUCTION.

The final area studied in this thesis was that of catalytic antibodies. The type of catalysis investigated was the metal-mediated cleavage of a peptide bond. This required that a metal-binding site be engineered into the antibody. It also necessitated the development of a number of assays to facilitate measurement of metal-binding by the antibody and to measure the effects that this was having on the Fab function. This section describes the methods designed to realise those objectives.

### 3.6.2 CONSTRUCTION OF FAB FRAGMENTS.

The heavy chains used in the construction of these fragments were chosen for their ability to bind gp120, while the light chains were selected based on their ability to bind metal ions. These were then transformed and expressed in *E. coli* to yield soluble protein, as described below.

#### **Light chains.**

The light chains used in these experiments were fluorescein-specific mouse light chains specifically mutated to incorporate histidine residues, thus allowing them to bind metals. Three different mutants were produced as described previously (Sastry *et al.*, 1989). The first, entitled LCQ, was altered with the following mutations: 39 R/H, 41 Y/L, 94 S/H and 96 S/H. Residues 39 and 41 exist in the CDR1 while 94 and 96 constitute part of the CDR3.

Therefore, the metal-binding site created by these mutations resides in the inner region of the antigen-binding pocket (Branden and Tooze, 1991). The second mutant LCC was constructed with the following alterations: 54 Y/H, 55 K/H and 57 N/H. All of the above mutations are in the CDR2 region and so, this metal-binding site will reside near the outer rim of the antigen-binding pocket. A third mutant, LCQC, was constructed with all seven of the above mutations.

Each of the 3 chains were then additionally altered in order to facilitate combination of these mouse light chains with human heavy chains. It was deemed necessary to swap the mouse light chain constant regions for a human constant region from one of the most promiscuous light chains, b22. This was achieved by overlap PCR in a similar way to the mutagenesis protocol described previously (Figure 3.1). Amplification of the constant human region involved a 3' reverse primer, Ck1z, as described previously, and a 5' forward primer, ConKap. This primer coded for 4 residues at the 5' end of the constant region and 5 residues at the 3' end of the variable region immediately upstream from the constant region. These 5 residues were included because they are common to both the human and mouse sequences and, due to an error in the mouse light chain construction, they were missing from this chain. Therefore, inclusion of these amino acids in this primer provided an overlap for the PCR and permitted their reinsertion into the light chain. Amplification of the mouse variable region used the 5' forward primer, AK1 (GCC GAG CTC GTT ATG ACT CAG ACA CCA), and the 3' reverse primer VarCon. This reverse primer coded for 5 residues at the 3' end of the variable

region and the 5 missing residues, thus overlapping with the primer, ConKap. The sequences of both overlap primers are as follows:

```
ConKap:  5'   ctg gag atc aaa cga act gtg gct gca      3'
          L   E   I   K   R   T   V   A   A
          Variable(overlap) | Constant
```

```
VarCon:  5'   agt tcg ttt gat ctc cag ccg ttt ggt gcc tcc acc 3'
          Overlap          | Variable
```

The overlap PCR was carried out as described previously and the resulting fragments were purified and cloned as discussed below.

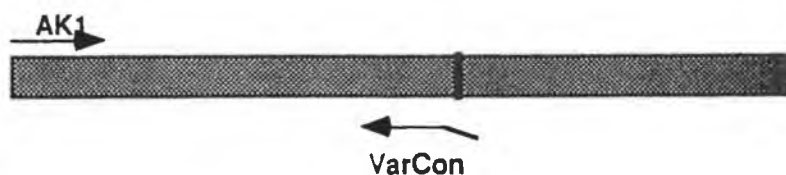
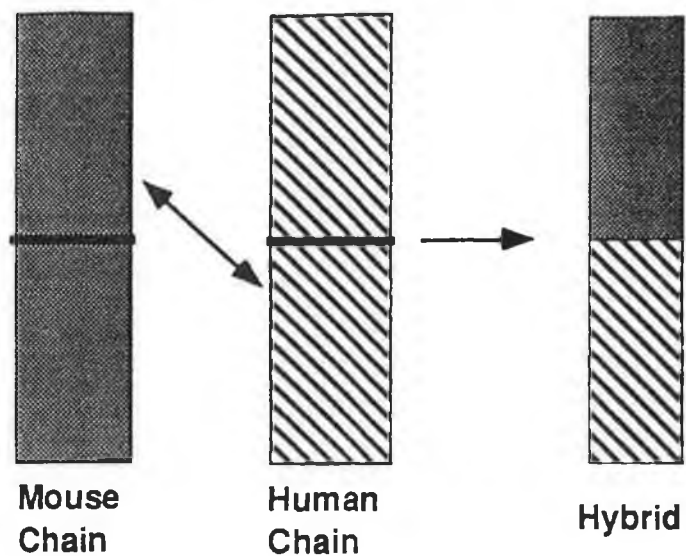
### **Heavy Chains.**

These were obtained from a library constructed by Barbas *et al.*, (1991). The material to construct this library had been taken from an asymptomatic AIDS patient and, therefore, contained a high proportion of antibodies specific for gp120. The library was obtained at the stage where only the heavy chains had been cloned into the vector pComb3, and so was devoid of all light chains.

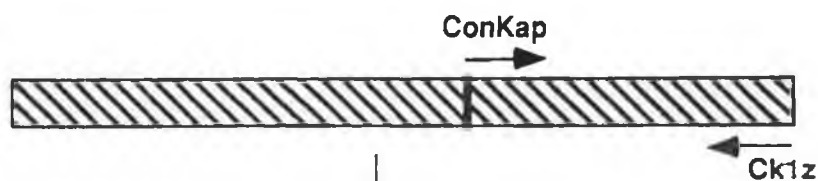
### **Construction of Expression Libraries.**

Three distinct libraries were produced by separately cloning each of the three mutant light chains into the heavy chain library as follows. The light chain inserts were prepared by digestion with the enzymes *SacI* and *XbaI* (Boehringer Mannheim) with subsequent purification of the 0.7 kilobase fragment on a low melting-point agarose gel, followed by  $\beta$ -agarase digestion (New England Biolabs). The heavy

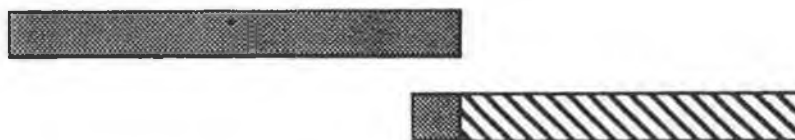
chain library in pComb3 was digested with the same combination of enzymes, and was ligated to the insert at 16°C overnight with an insert:vector molar ratio of 5:1. The three libraries were subjected to 4 rounds of panning as described previously (methods section 3.2.9). A separate panning was carried out for each library, plus one panning was performed using an equal mixture of all three libraries.



**VarCon with overhang complementary to primer 2**



**Do 2 separate PCR reactions**



**Do third, overlap PCR**



**Mutated chain comprising segments from both genes**



### **3.6.3 SCREENING OF LIBRARIES FOR gp120-BINDING CLONES.**

A total of 1000 colonies were screened from the 3 libraries. Cultures were grown in 96-well tissue culture plates (Costar 3596) and soluble Fab was expressed as described by Collet et al. (1992). The presence of gp120-specific antibodies was determined by ELISA, in wells coated with 0.1µg IIIB gp120 (American Biotechnologies 012001). Supernatant from *E. coli* transformed with empty plasmid was used as a negative control, while an *E. coli* supernatant containing a known gp120-binding antibody was employed as a positive control. A clone was determined to be positive if the O.D.(550nm) generated was at least 3 times that of the negative control.

### **3.6.4 ZINC-BINDING ASSAYS.**

Two methods for determining if the Fabs bound Zinc were used. The first method, initially developed by Johnaton Rosenblum (unpublished data), involved an ELISA technique. It was critical that ultrapure (ion-exchanged and distilled) water was used for all dilutions in this, and any other metal-binding ELISA assays, as contaminating metal ions would yield inaccurate results. The wells of amidated plates (Costar 20388) were coated with 50µl of a 1mg/ml solution of a linker molecule BS<sup>3</sup> (Pierce 21579) at 4°C for 4 hours. This solution was then discarded and 50 µl of a 10mg/ml solution of chelating agent, iminodiacetic acid (Sigma), was added to each well at 4°C overnight followed by a number of washes in 0.1M EDTA and water. 50µl of a 1mM Zinc solution was then added for

1 hour at 37°C. 0.1M EDTA in 1%(w/v) BSA-PBS was added in the case of all negative controls. The plates were washed 10 times with 0.05% (v/v) Tween-PBS. 100µl of 3%(w/v) BSA-PBS was placed in each well for 1 hour at 37°C to block any non-specific sites. Solutions of the Fabs, diluted in 1%(w/v) BSA-PBS, were then added for 2 hours at 37°C. 1%(w/v) BSA-PBS was used as a background control. The Fab was detected using an alkaline phosphatase-labeled goat anti-human F(ab')<sub>2</sub> antibody (Pierce 31312X).

#### **Zinc Western Blots.**

The second method used to detect zinc-binding by the Fabs was a western blotting technique. 5 µg of purified Fab were run on an SDS-gel (Bio-Rad mini Protean II system 165-2940) and blotted on PVDF membrane (Bio-Rad 170-3946). The blot was washed for 1 hour in metal-binding buffer (100mM Tris-HCl, pH 6.8; 50mM NaCl) at room temperature. 10µl Zn<sup>65</sup> (Amersham) were diluted to 5ml in Blotto (0.16M NaCl; 10mM Sodium Phosphate pH 7.2; 0.01%(v/v) thimerosal; 5%(w/v) non-fat dry milk) and used to probe the blot for 1.5 hours at room temperature. Three washes for 10 minutes each were then carried out on the blot and it was exposed to X-ray film (Amersham) for 2 days at -70°C in the presence of an amplifying screen. The negative controls used were other gp120 binding Fab fragments produced from combinatorial libraries in *E. coli* that did not incorporate a metal-binding site.

#### **Competition ELISA using gp120 and Zinc.**

Wells of an E.I.A./R.I.A. 96 well ELISA plate (Costar) were coated with 0.1µg IIIBgp120, as described previously. Fab and competing

zinc were then added to a series of wells, as described for other competition ELISAs, and incubated at 37°C for 2 hours. The Fab was added to all wells at a constant dilution corresponding to its titre, while the zinc was added in a series of dilutions ranging from 0.1mM to 10mM. The wells were washed and the Fab was detected using a goat anti-human F(ab')<sub>2</sub> antibody as before. Two negative controls were used in this ELISA: the first was the buffer used to dilute the antibodies and the second was a set of antibodies designed to incorporate a histidine tail of 5 histidine residues at the carboxyl end of their heavy chains. These antibodies were designed to bind metal, but would not be competed by the gp120, as their metal binding and gp120 binding sites are at opposite ends of the molecule.

#### **Bifunctional ELISA assay.**

This ELISA was designed to detect binding of the Fab to both gp120 and zinc at the same time. Amidated plates (Costar) or Covalink plates (Nunc) were coated, via a linker and chelator, with zinc, as described above. The plates were then blocked with 3%(w/v) BSA-PBS and incubated with Fab at a dilution corresponding to its titre, for 1 hour at 37°C. The plates were then incubated with gp120 at a concentration of 2µg/ml for 1 hour. A rabbit anti-gp120 antibody (American Biotechnologies 000402) was then added at a 1:1000 dilution for a further 1 hour. This was then detected with a horseradish peroxidase-conjugated goat anti-rabbit antibody (Sigma). The antibody dilution buffer and antibodies designed only to bind gp120 and not metal, were used as negative controls.

### **3.6.5 PEPTIDE CLEAVAGE ASSAY.**

0.5 $\mu$ g of purified Fab and 0.2 $\mu$ g gp120 were added together, both with and without the presence of 1mM ZnCl<sub>2</sub>. This was incubated at 37°C overnight and then run on an SDS-gel and blotted onto PVDF membrane as before. The membrane was blocked in Blotto for 2 hours at room temperature and then washed for 0.5 hours in 0.1% (v/v) Tween TBS with 2 changes of buffer. It was then probed with a 1:1000 dilution of rabbit anti-IIIB gp120 antibody (American Biotechnologies 000402) at 4°C overnight. After washing, a horseradish peroxidase-labeled goat anti-rabbit antibody (Sigma) was added for 1 hour at 37°C. Finally, 6ml of a chemiluminescent substrate (Amersham ECL kit RPN 2106) was added for 1 min. The blot was then immediately exposed to X-Ray film for 10 seconds and developed.

## CHAPTER 4

## 4.1 INTRODUCTION.

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### 4.1.1 OVERVIEW.

Animals respond to antigenic challenge by generating a diverse array of antibodies (Mayer *et al.*, 1987). The characterisation of such a response would have important implications for understanding the interplay between infectious agents and antibodies, which, in turn, could lead to the production of reagents of potential therapeutic value. This is particularly relevant with the finding that antibody is capable of providing protection against viral challenge in chimpanzees ( Berman *et al.*, 1991).

As was discussed in section 1.10, humans will respond to challenge by the HIV virus by producing antibodies directed against two major viral surface antigens, gp120 and gp41 (Kang *et al.*, 1992; Chamat *et al.*, 1992). Most of the neutralising activity is associated with the third hypervariable domain (V3) of gp120 (Freed *et al.*, 1991) and the conformationally sensitive, discontinuous CD4 binding site of gp120 (Ho *et al.*, 1991). Antibodies to this site appear later in the course of HIV infection and are capable of neutralising multiple strains of the virus. However, this form of neutralisation is only observed at low dilutions in the patient's serum, suggesting that the titre and/or affinity of the antibodies is low ( Ho *et al.*, 1987). Recently, the importance of antibodies to the V2 loop in the neutralising response has also been reported.

The complicated and diverse nature of the humoral response generated against HIV, in conjunction with the low titres, has made

it very difficult to study this response at a molecular level. It is also well known that the virus can undergo mutations which render a previously neutralising antibody response ineffective (Goudsmit *et al.*, 1991; Nara *et al.*, 1991), which further complicates an analysis of this type. However, it is particularly important in the case of HIV to understand the interactions between the virus and the immune response, in order to be able to develop a therapeutic reagent.

#### **4.1.2 COMBINATORIAL LIBRARIES.**

As was discussed earlier, antibody production at present is mainly in the realm of animal immunisation and hybridoma technology. However, both of these techniques are tedious and inefficient and the production of human antibodies is very difficult in both cases (Roben and O'Kennedy, 1991).

It was because of these limitations that we chose to use the technique of combinatorial phage-display libraries to produce a panel of Fab fragments specific for HIV surface glycoprotein (Huse *et al.*, 1989; McCafferty *et al.*, 1990). This provides a means of producing a large number of diverse antibodies, specific for a given antigen. High affinity antibodies were selected from the library using a procedure known as panning (Barbas *et al.*, 1991). Bone marrow was chosen as the source of antibody RNA for the library as this has been shown to be a major repository for differentiated B cells that spontaneously produce antibody to maintain circulating antibody titres (Lum *et al.*, 1990). The RNA levels in these cells has been shown to be elevated up to 1000-fold over resting B cells (Schiebler *et al.*, 1978). A combinatorial library was produced,

according to the methods previously described (methods section 3.2), using the bone marrow of an asymptomatic AIDS patient and a panel of thirty six Fab fragments specific for IIIBgp120, IIIBgp160, SF2gp120 and a V3 loop peptide JSISIGPGRAFYTGZ-C-NH<sub>2</sub> had been produced ( Barbas *et al* 1993). The large diversity of Fabs produced from this one individual provided an ideal opportunity for studying the humoral response of that individual when challenged with HIV-1.

#### **4.1.3 GENETIC ANALYSIS.**

The initial analysis involved sequencing the fragments and a molecular profile was then carried out on all the sequences. This led to the arrangement of all the Fabs according to heavy chain sequence. The combination of V, D and J genes used in each fragment was established and from this, a number of observations as to the clonal origin of these fragments could be drawn. A comparison was made between the diversity of heavy and light chains and the predominance of certain heavy-light chain combinations, leading to a number of conclusions about the *in vivo* distribution of these chains and the relative contribution of each to antigen specificity.

#### **4.1.4 BIOCHEMICAL ANALYSIS.**

The second half of this study involved the selection of a representative cross section of the Fabs that had previously been analysed at a molecular level and the further characterisation of these at a biochemical level. It was considered important to establish whether the diversity of antibodies seen at a genetic level was reflected in the biochemical properties of these molecules. This



could yield important information about how the immune system deals with phenomena such as the array of epitopes being presented on the viral surface, viral escape mutants and antigen shedding. Initially the epitope specificity of the Fabs was examined by competitive ELISAs with soluble CD4 antigen (sCD4). Their affinities were also established by means of inhibition ELISAs. Both of these two techniques indicated that the selected Fabs were very similar in terms of their affinity and specificity, if not identical. However, neither method was thought to be sensitive enough to detect any subtle differences in epitope specificity which may be important. Therefore, a methodology to measure the affinities of these fragments using Real-Time Biospecific Interaction Analysis (BIAcore™) was developed (Karlsson *et al.*, 1991; VanCott *et al.*, 1992). This system also provided the means to measure the epitope specificity of the Fabs in greater detail than with the ELISA, and an epitope mapping technique was developed to achieve this. Small differences in specificity then became apparent between the fragments showing they were not identical biochemically. Finally, this was confirmed by Dr. John Moore (Aaron Diamond AIDS Center, New York), using a panel of gp120 mutants in a knockout assay system. This system is capable of defining areas or residues on gp120 that directly affect the binding by the Fab, and so help define the epitope required by the antibody. Using this technique, it was possible to show significant differences in epitope specificity for the Fabs studied.

## 4.2 RESULTS

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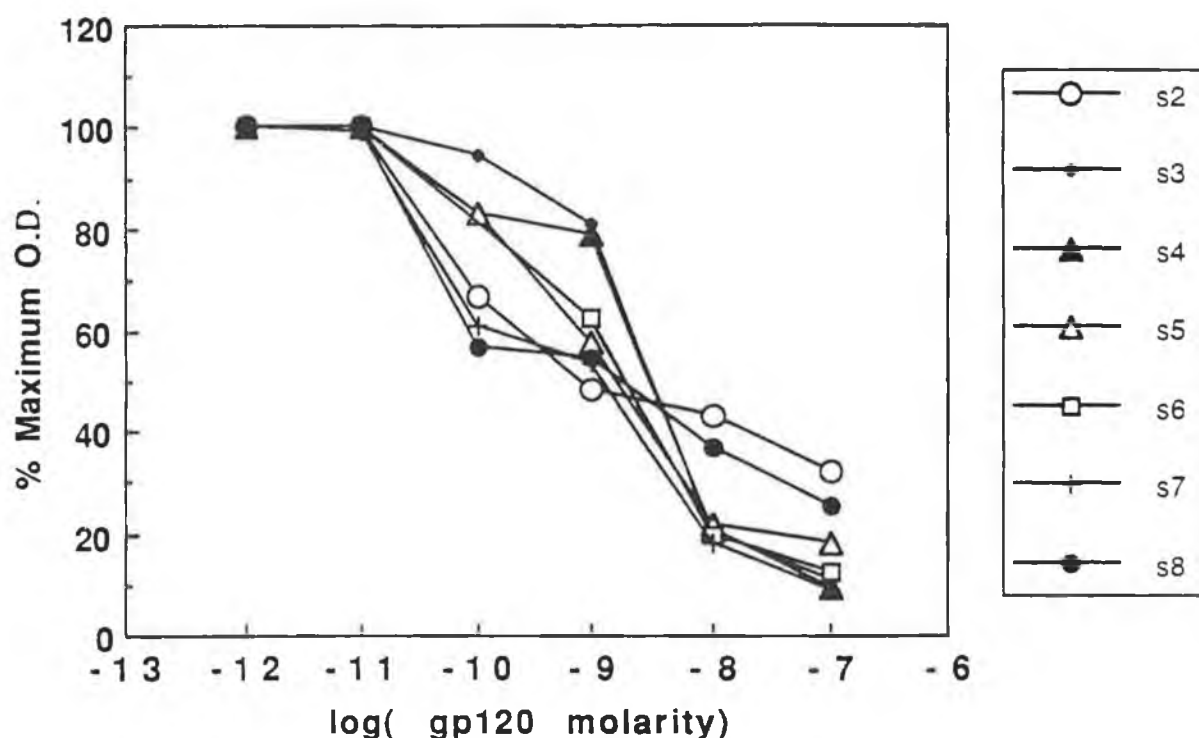
### 4.2.1 PANNING OF THE LIBRARY.

The library was subjected to four rounds of panning against the four antigens (IIIBgp120, SF2gp120, IIIBgp160 and V3 loop peptide) which produced an amplification of 100 to 1000-fold. *E. coli* XL1Blue were then transfected with the phage and the geneIII was removed from the resulting plasmid to yield a library capable of secreting soluble Fab. Forty colonies were grown from each antigen library and the supernatants were screened in ELISA assays against the antigen used in panning. Clones yielding a positive result were then taken for further analysis. The clones were assigned names according to the antigen against which they had been panned. Those panned against IIIBgp120 were called bn, where n is the clone number (e.g.: b1, b2, etc.), IIIBgp160 clones were called Bn, SF2gp120 were sn and V3 loop clones were pn. IIIBgp120 was used in all subsequent assays.

### 4.2.2 RELATIVE AFFINITIES.

The relative affinity of each clone was determined by competitive ELISA. Nineteen Fabs were derived from panning with IIIBgp120 and eight from panning with SF2gp120. These were all assayed by ELISA and all gave similar affinity constants in the range  $1 \times 10^7$  to  $1 \times 10^8$  M<sup>-1</sup>. Sixteen clones were the product of panning with IIIBgp160, but only six of these were also reactive to IIIBgp120. The other ten only reacted very weakly with IIIBgp160 and could not be assayed

further. It is possible that these Fabs were directed against gp41. The affinities of those that cross-reacted with IIIBgp120 showed similar apparent affinities to the IIIBgp120-specific Fabs. Four Fabs were derived from panning against V3 loop peptide, but only two of these showed reactivity to gp120 (SF2) with affinities values of  $10^8$   $M^{-1}$ . All relative affinity values are given in Table 4.1. The competitive ELISAs for the SF2gp120 clones are given in Figure 4.1.



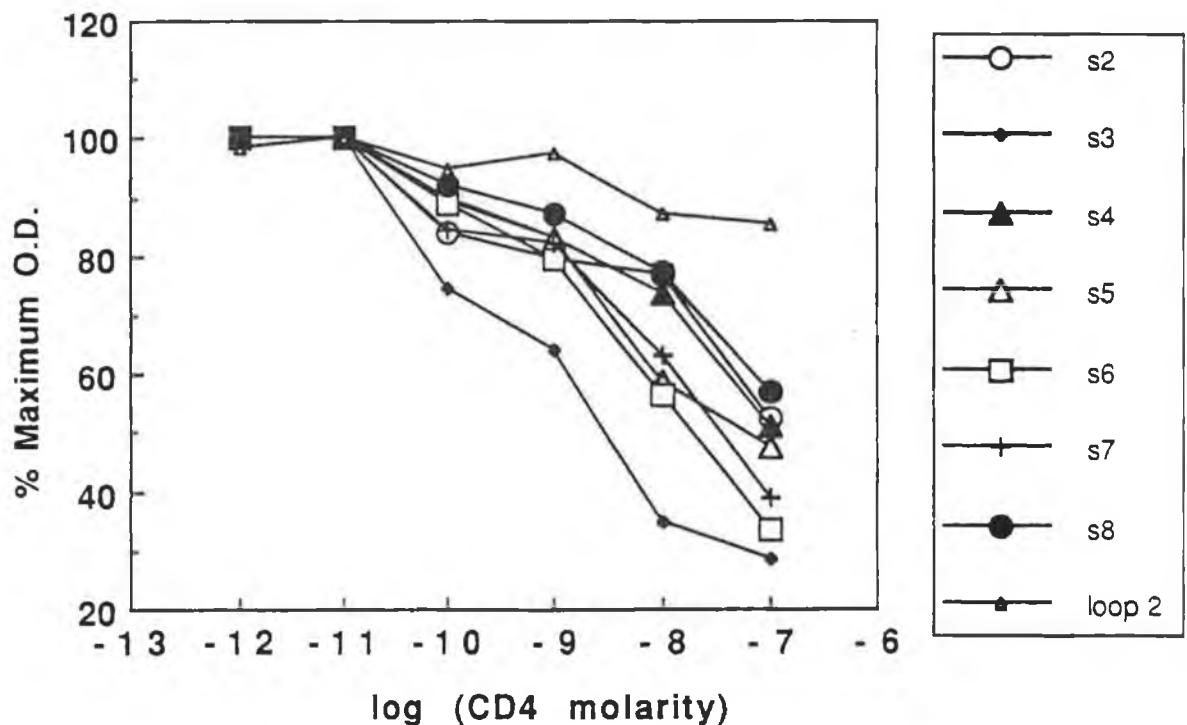
**Figure 4.1:** Specificity of antigen binding shown by competitive ELISA. The Y-axis expresses the O.D.<sub>405</sub> as a percentage of the value obtained when PBS was used in the place of competing gp120, so giving the maximum value. The relative affinity ( $K_a$ ) for each clone is determined to be the molarity of competing gp120 resulting in 50% inhibition of antibody-immobilised gp120 binding. All values are given in Table 4.1.

Those Fabs, derived from panning with IIIBgp120 and IIIBgp160, were tested for reactivity against SF2gp120, and all were found to react. Therefore, all Fabs, with the exception of those derived from panning with V3 loop peptide, reacted with gp120 of either IIIB or SF2 strains.

Panning Antigen	Clone Name	$K_a \cdot 10^8 \text{ (M}^{-1}\text{)}$	$K_i(\text{CD4}) \cdot 10^8 \text{ M}^{-1}$
gp120 IIIB	b1	5.0	6.0
	b2	7.0	0.5
	b14	5.0	1.0
	b24	2.0	5.0
gp160 IIIB	B2	3.0	0.5
	B30	0.4	4.0
gp120 IIIB	b3	3.0	6.0
	b5	4.0	5.0
gp160 IIIB	B20	0.8	0.4
gp120 SF2	s2	11.0	0.1
	s3	3.0	5.0
	s5	7.0	0.2
	s7	11.0	0.5
gp120 IIIB	b4	3.0	5.0
	b7	3.0	9.0
	b12	7.0	0.5
	b21	0.8	0.9
gp120 IIIB	b6	5.0	0.4
	b20	6.0	0.1
gp120 SF2	s6	7.0	0.8
gp120 IIIB	b8	5.0	2.0
	b13	4.0	5.0
	b18	5.0	0.5
	b22	3.0	1.0
	b27	5.0	0.9
gp160 IIIB	B8	4.0	0.3
	B26	0.8	0.8
	B35	4.0	0.1
gp120 SF2	s4	4.0	0.1
gp120 IIIB	b11	4.0	4.0
	b29	2.0	0.3
gp120 SF2	s8	11.0	0.1

#### 4.2.3 CD4 INHIBITION ELISAs.

The Fabs were screened for CD4 inhibition of their binding to IIIBgp120 and all, with the exception of the V3 loop binders, showed sensitivity to CD4 inhibition (Table 4.1). The inhibition constants were in the range  $1 \times 10^7$  to  $1 \times 10^9 \text{ M}^{-1}$  and were generally greater than the corresponding affinity constants. The CD4 inhibition curves for the SF2gp120 clones are given in Figure 4.2.



**Figure 4.2:** Graph showing specificity of SF2 gp120 Fabs for the CD4-binding site of gp120, by competition ELISA with soluble CD4. The  $K_i$  values for these and all the other Fabs are given in table 4.1. Loop 2 is a Fab Specific for the V3 loop peptide and was included as a negative control in this experiment, since it should not be inhibited by sCD4.

#### **4.2.4 SEQUENCE ANALYSIS.**

Thirty three heavy chain variable regions (VH) and corresponding light chain variable regions (VL) were sequenced and are given in Figures 4.3 and 4.4, respectively. It was immediately obvious that the heavy chains could be separated into eight groups on the basis of close relationships in their sequences, as is shown in Fig. 4.3. The light chains were organised into the same groups as defined by their corresponding heavy chain. The predominant sequence for each of the groups is given in full, while the sequences of the remaining members of the group are given as a series of dots to indicate similarity to the predominant sequence. Only those residues that differ are shown. It is interesting to note that the members of a particular group have not necessarily been panned with the same antigen. This demonstrates that it is possible to use a number of slightly different antigens to isolates Fabs with similar primary sequences.

Clone	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4	J_gene
b1	LEESGTEFKPPGS5VKVSCASGGTFG	DYASNYAIS	WVRQAPGGQLEYIG	GITPTSGSADYAQKFQG	RVTISADRFTPILYMEI RSLRIEDTAIYYCAR	ERRERGMNPRALRGALDF	WGQGTTRVFVSP	JH3
b2	.....AAVQK.....R...Q.....D	NF.....V.	.....MM.	.....T.T.S.....	.....APL...I.....DD...V.....	.....V...V	.....T.	
b14	.....A.V.K.....I.S	.F.....	.....M.	.....	.....AA...RV.....S...V.F...	.....EV	.....T.I...	
b24	.....AAVQK.....R...Q.....D	NF.....V.	.....MM.	.....T.T.S.....	.....APL...I.....DD...V.....	.....V...V	.....T.I..S	
B2	.....AAVQK.....R...Q.....D	NF.....V.	.....MM.	.....T.T.S.....	.....APL...I.....DD...V.....	.....V...V	.....T.I..S	
B30	..Q..A.V.K.....T.....S	.....H...	.....M.	.....T.....A	.....IE...V.....SDQH.T...T	.....I	.....T.I..S	
b3	LEESGGRLVKPGGSLRLSCGSGFTFT	NAHMT	WVRQSPGKGLEWVA	SIKSKFDGGSPHYAAPVEG	RFSISRNDLEDKMFLEMSGLKAEDTGVIYCAT	KYPRYSMDVTGVRNIFYMDV	WGKGTTVIVSS	JH6
b5	..Q...G.....	.....	.....	.....	..T.....L.....	.....F..MA.....	..T.....	
B20	.....G.....	.....	.....	.....	..T.....L.....	.....MA...L...	.....	
b2	.....A.....	.S...	.....	..R.....	.....	.....M.....	.....	
a2	.....G.....S.....	.....	.....	.....	..T.....L.....	.....MA.....	.....	
a5	.....	.....	.....	.....	.....	.....M.....	.....	
a7	.....G.....A.....	.....	.....	.....S..PG...	..T...YI...L.....	.....Y..MR...Y...	.....	
b4	LEQSGAEVKKPGASVKVSCQASGYRFS	NEVIH	WVRQAPGQRFENHG	WINPYNGNKEFSAKFQD	RVTFTADTSANTAYMEI RSLRSADTAVIYCAR	VGPYSWDDSPQDNYMDV	WGKGTTVIVSS	JH6
b7	.....	.....	.....	.....	.....D.....I.....	.....	.....K.....	
b12	.....	.....	.....	.....	.....D.....T...I.....	.....T.....	.....K.....	
b21	.....	.....	.....	.....	.....D.....T...I.....	.....T.....	.....K.....	
b6	LEESGGGLVKPGGSLRLSCVSGFTFS	SAHMA	WVRQAPGKGLEWVG	LIKSKADGETTQYATPVKG	RFSISRNNLEDTVYLQMDSLRADDATVVIYCAT	QKPRYFDLLSGQYRRVAGAFDV	WGHTTIVTVSP	JH3
b20	.....A.....	.....	..G.....	.....	.....	.....	.....	
a6	.....I.....	.....T	.....K...I.	.....	..T.....N.....	...S..YN.....	.....	
b8	LEESGEAVVQGRSLRLSCAASGFIFR	NYAMH	WVRQAPGKGLEWVA	LIKYGDRNKYYADSVKG	RFTISRDNKNTLYLQMNSLRAEDTAVIYCAR	DIGLKGEHYDILTAYGPOY	WGQGTTLTVVSS	JH4
b13	..Q.....T.....T..	.....	.....	.....	..S.....E.....	.....	.....A.....	
b18	..Q.....	.....	.....	.....	..S.....E.....	.....	.....A.....	
b22	.....T.....T..	.....	.....	.....	..S.....E.....	.....	.....A.....	
b27	.....	.....	.....	.....	.....	.....	.....	
B26	..Q.....T..	.....	.....	.....	.....	.....A.....	.....	
B8	.....	.....	.....	.....	.....	.....	.....	
B35	..Q.....	.....	.....	.....	.....	.....	.....	
a4	.....	.....	.....	.....	.....	.....	.....	
b11	LEQSGGGVVKPGGSLRLSCGSGFTFP	NAHMT	WVRQSPGKGLEWVA	SIKSKFDGGSPHYAAPVEG	RFTISRNDLEDKVFLOMNGLKAEDTGVIYCAT	RYPRYSEHMGGVKRIIFYMDV	WGKGTTVSVSS	JH6
b29	..E.....	.....	.....	.....	.....	.....	.....	
a8	LEESGGGLVQGRSLRLSCAASGFIFS	SYEMN	WVRQAPGKGLEWVS	QISSSGSRTYYADSVKG	RFTISRDNKNSLYLEMTSLRVDDTAVIYCAR	GRRLVTFGGVVSOGNI	WGQGTMTVTVSS	JH3
loop35	LEQSGGGVVKPGGSLRLSCAGSGFNFS	DOTMI	WVRQAPGKGLEWVA	VISYEGSDKYYADSVKG	RFTISRDNSENTLYLQMDSLRADDATVVIYCAT	NTRENIEADGTAYYSYMDV	WGKGTTVTVSS	JH6

**Figure 4.3:** Amino acid sequences of the variable heavy (VH) regions of the Fabs binding to gp120. The predominant sequence in each group is given in full. Similarities to this in the other sequences are indicated by a dot, while differences are given.



Clone	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4	J gene
b1	ELTQSPSSLSASVGDRTITC	RASQGISNYLA	WYQOKPGKVPRLLIY	AASTIQP	GVPSRFGSGSGTDFTLTISLQPEDFATYYC	QKYNAPRT	FGQGTKVEIKRT	JK1
b2	.....I.....	.....N.....	.....R.....	.....S.....	...T.....	.....V.....	..G.....	JK4
b14	.....I.....	.....N.....	.....K.....	.....S.....	.....P.....	.....V.....	..G.....	JK1
B2	.....I.....	.....N.....	.....R...A.N.....	.....S.....	...P.....	.....V.H.....	..G.....	JK4
b24	.....GT...L.P.E.A.LS.	.....SVISNYLA	.....QA.....	GV.NRAT	.I.D.....R.E...F.V.S.	.Q.GTS.W.	.....L.....	JK1
B30	.....GT...L.P.E.A.LS.	.....SVSNNYLA	.....R...QA.....	G..NRAT	.I.D.....A.....I...	.Q.H.S.Y.	.....L.....	JK2
b3	ELTQSPGTLSPGERATLSC	RASHRVNNFLA	WYQOKPGQAPRLIIS	GASTRAT	GIPDRFGSGSGTDFTLTISRLEPDFAVYYC	QQYGDSPLYS	FGQGTKLEIKRT	JK2
b5	ELTQSPASVSASVGDVTITC	RASQDIHNLA	WYQOKPGKAPKLLIY	AASSLQS	GVPSRFGSGSGTDFTLTISLQPEDFATYYC	QQGNFSPK	FGPGTVVDIKR	JK3
B20	ELTQSPGTLSPGERATLSC	RASQSLSNNYLA	WYQOKPGQAPRLIY	GSSTRGT	GIPDRFGSGSGTDFTLTISRLEPDFAVYYC	QHYGNSVYT	FGQGTKLEIKR	JK2
#2	QSPDTLSLNPGERATLSC	RASHRISSKRLA	WYQHKRQAPRLIY	VCPNRAG	GVPRDFSGSGSGTDFTLTYSRLEPDFAVYYC	QYYGSSYT	FGQGTKVEITR	JK2
#3	QSPSHLSLSPGERATLSC	RASQVSAFYLA	WYQORPGQAPRLVIY	GASTRAT	DIPDRFGSGSGTDFTLTISRLEPDFAVYYC	QVYGQSPVL	FGQGTKLEMKR	JK2
#5	QSPGTLSPGERATLSC	RASQSLSSFLA	WYQOKPGQAPRLIY	SASMRAT	GIPDRFRGSGSGTDFTLTITRLEPDFAVYYC	QRFGTSPLYT	FGQGTKLEMKR	JK2
#7	QSPGTLSPGERATLSC	RASQSFSSNFLA	WYQOKPGQAPRLIY	VIHPNRAT	GVPRDFSGSGSGTDFTLTITRLEPDFAVYYC	QQYGASLVS	FGPGTKVHIKR	JK3
b4	ELTQSPGTLSPGERATFSC	RSSHISRSRVA	WYQHPGQAPRLVIH	GVSNRAS	GISDRFGSGSGTDFTLTITRVEPDFAVYYC	QVYGASSYT	FGQGTKLERKRT	JK2
b7	.....T.....L..	..T.....L..	...V.G.....L.Y	.....G	..P.....S.L.....V..	.Q..S.R..	.....I.....	JK2
b12	.....A.....	.....R	.....	.....	.....	.....	.....	JK2
b21	.....A.....D.....	.....N.....	.....	.....	.....L.....	.....	.....DF...	JK2
b6	ELTQSPGTLSPGERATLSC	RAGQSISNYLA	WYQOKPGQAPRLIY	GASNRAT	GIPDRFGSGSGTDFTLTISRLEPDFAVYYC	QQYGTSPYT	FGQGTQLDIKRT	JK2
#6	.....A.....	..S..L.N.....	.....	..S.T...	.....C.....T.....	.....N.V..	.....K.E...	JK2
b20	.....SS...A.V.D.V.IT.	..TS.G..NYLA	.....KV.K...	...TLQS	.G.S.....T.NS.Q...V.T.S.	.N.DSA.W.	.....KV....	JK1
b8	ELTQSPSSLSASVGDRTITC	RASQGISNYLN	WYQOKPGKAPKLLIY	AASSLQR	GVPSRFGSGSGTDFTLTISLQPEDFATYYC	QQSYSIPPLT	FGGGTKVEIKRT	JK4
b18	.....N.N.....	.....E.....H	T.FN..S	.....S	.....TA...E...T.R.....	.....T.YT	..Q.....	JK2
b22	.....S.....	.....S.....	.....S	.....S	.....T.....	.....T.YT	..Q...L.....	JK2
b27	.....S.....	.....S.....	.....S	.....S	.....T.....	.....T.YT	..Q...L.....	JK2
B35	.....S.....	.....S.....	.....S	.....S	.....T.....	.....T.YT	..Q...L.....	JK2
#4	.....T.....S.....	.....S.....	.....S	.....S	.....G.....T.....	.....T.YT	..Q...L.....	JK2
b13	.....Q...D.R.....	.....D...NSET	.....D...NSET	.....D...NSET	.....R...FT.....V.....	..HONV.LT	.....	JK4
B26	.....Q...D...H.....	.....D...NSET	.....D...NSET	.....D...NSET	.....FT.....I.....	..YDNL.LT	.....	JK4
B8	.....I.....	.....T.N.....	.....G...N..S	.....G...N..S	.....T.....F.....	.....NT...WT	..Q.....	JK1
b11	ELTQSPGTLSPGERATLSC	RASQVNSNYLA	WYQOKPGQTPRVVIY	STSRAT	GVPRDFSGSGSGTDFTLTISRLEPDFAVYYC	QQFGDAQYT	FGQGTKLEIKRT	JK2
b29	.....	.....	.....	.....	.....	.....	.....	JK2
#8	TQSPSSVSASVGDVTITC	RASQDIRNYLN	WYHQKPGKAPKLLIS	DASDLFI	GVPSRFGSGSGSATYFSTISLQPEDIGTYC	QQYADLIT	FGGGTKVEIKRT	JK4
loop15	SPGERATLSC	RASQSVGINLA	WYQOKPGQAPRLIIF	DASTIWT	YIPDTFSGSGSGTDFAITISLQSEDFGFYYC	QQYDNPPT	FGQGTKLEVKRT	JK2

**Figure 4.4:** Amino acid sequences of the variable light regions of the gp120 binders. These are arranged into groups according to their corresponding heavy chains in fig.4.3.

#### 4.2.5 Heavy Chains.

The first observation to make is that the similarity of sequence within a particular group is subject to variation. The group headed by clone b8 exhibits a very high degree of sequence-similarity between its members, with an average discrepancy between sequences of only 1.1. This figure is comparable to the typical rate of error for PCR using Taq polymerase (Saiki *et al.*, 1985; Tindall *et al.*, 1988), and so these chains may originally have been all be identical, while any differences may simply be an artifact arising from the PCR. It is also interesting to note that there is only 1 difference in the CDR3 region implying that the same V-D-J combination is used in all cases.

The b3 group display a greater diversity than the b8 group, having an average discrepancy of 3.3. This would suggest that these are all somatic variants of one particular sequence.

The b1 group show a different pattern. Clones b1 and b14 are very similar to one another, as are clones b2 and B2. However, these two pairs differ from one another by twenty three amino acids. The remaining two members of the group are quite dissimilar from each other and from the two pairs b1/b14 and b2/B2. However, all the members of this group are very similar in their CDR3 sequences, suggesting that they use the same D-J segments. This may be explained either by extensive somatic mutation, cross-over events between different germline genes and the same D-J region, or a convergent type of evolution in an effort to conserve that particular D-J region.

A further analysis of the germline genes shows that the homology between these genes and the Genbank database of VH is less than 90%, which would suggest that these are completely new germlines, or that extensive somatic mutation has occurred. The exception to this is the b8 group which shows 93% homology with germline 2P1 (Schroeder *et al.*, 1987).

All the members of a particular group use the same JH minigene (Figure 4.5b). However, the overall usage of these genes is unusual in that 4 groups use JH6, 3 use JH3 and 1 use JH4. JH6 is usually used to a lesser extent than the other 2. (Yamada *et al.*, 1991; Sanz, 1991)

Jk1: tgg acg ttc ggc caa ggg acc aag gtg gaa atc aaa cgt.

W T F G G G T K V E I K R

Jk2: tac act ttt ggc cag ggg acc aag ctg gag atc aaa ctg.

Y T F G G G T K L E I K R

Jk3: ttc act ttc ggc cct ggg acc aaa gtg gat atc aaa cgt.

F T F G P G T K V D I K R

Jk4: ctc act ttc ggc gga ggg acc aag gtg gag atc aaa cgt.

L T F G G G T K V E I K R

(A)

JH3: gct ttt gat gtc tgg ggc caa ggg aca atg gtc acc gtc tct tca.

A F T V W G G G T M V T V S S

JH4: tac ttt gac tac tgg ggc caa gga acc ctg gtc acc gtc tcc tca.

Y F T Y W G G G T L V T V S S

JH6: tac tac ggt atg gac gtc tgg ggg caa ggg acc acg gtc acc gtc tcc tca.

Y Y G M D V W G G G T T V T V S S

(B)

**Figure 4.5:** J gene sequences utilised by a) the light chains and b) the heavy chains in the Fab fragments being analysed in this study. The nucleotide sequence is given for each, followed by the equivalent amino acid sequence. All sequences taken from Kabat *et al.*, (1987).

Analysis of the D genes (Ichihara *et al.*, 1988) used has proven to be very difficult, as there is a lack of homology with known sequences. This would suggest either modification through somatic mutation, or the presence of thus-far unknown D genes. However, within the groups there is a remarkable conservation of length and sequence of the CDR3 regions, suggesting that all the members of a group use the same D-J combinations or that they are variants of one another. It is also notable that most VH use the DXP1 sequence (Figure 4.6).

Figure 4.6 shows a selection of germline D segments displaying the greatest homology to a representative member from each group (see legend, page 122). The b8 group all use the DXP1 sequence at the core, perhaps flanked by a large N segment. The b3 group may represent a fusion of the DXP1 to an inverted DK1 segment as is the case with s7, while all the other members of the group may be somatic variants of this. The b1 group do not show significant homology with any of the known D sequences and so are difficult to analyse.

**b1,b14** acggcgcggaacgaggatggaaccctagggcccttcgaggt  
 DIR2 cg\*\*g\*ga\*c\*\*g\*\*\*\*\*aa\*cc\*ag\*\*ccc\*\*c\*agg  
 DIR1 aacgag  
 DM1 tggaac  
 DM2 ggaacc

**b2,B2** acggcgcggaacgggggatggaatcctagggccctcgtcggt  
 DIR2 cg\*\*g\*ga\*c\*\*g\*\*\*\*\*aa\*cc\*ag\*\*ccc\*  
 DXP1 cgggga  
 A1 gggga\*ggaa  
 DHQ52 c\*aacgggga  
 DIR1 agggc\*c\*cgtcgg  
 DIR1/2RC ggaatcc\*a\*g  
 DN4 ctctgc

**b24** acggcgcggaacgaggatggaaccctcgggccccttcgaggt  
 DIR2 cg\*\*g\*ga\*c\*\*g\*\*\*\*\*aa\*cc\*\*g\*\*ccc\*\*c\*agg  
 AIRC accctc  
 DIR1/2 aacgag  
 DM1 tggaac  
 DM2 ggaacc

**B30** gcggcgcggaacgaggatggaatcctagggcccttcgaggt  
 DIR2 cg\*\*g\*ga\*c\*\*g\*\*\*\*\*aa\*cc\*ag\*\*ccc\*\*c\*agg  
 DFLI6 tggaatcc\*a  
 IR1/2 gaatcc\*ag

**b3** aaatatccgcgctactcggatatggtgactggtgttcgaaacc  
 DXP1 ta\*t\*\*gatat\*\*tgactggt\*\*t\*\*\*aac  
 DXP1RC aaatatc  
 DXP'1 tatggt  
 DIR1/2 ggtgttc  
 DLR1 ggatat\*gt\*actggtg  
 DLR2 gctactc

**b5** aaatatccgcgctactttgatatgatggctggtgttcgaaacc  
 DXP1 ta\*t\*\*gatat\*\*tg\*ctggt\*\*t\*\*\*aac  
 DXP1RC aaatatc  
 21.10 tatgat  
 DIR1/2 ggtgttc  
 DLR5 ctactt\*\*tatg

**B 2 0** aaatatccgcgctactctgatatgatggctgggtgttcgaaacc  
 DXP1 ta\*t\*\*gatat\*\*tg\*ctgggt\*\*t\*\*\*aac  
 DXP1RC aaatatc  
 21.10 tatgat  
 DIR1/2 ggtgttc  
 DLR2 gctactc  
 DN1/DN4 gctgggt

**s 2** aaatatccgcgctactcggatatgatgactgggtgttcgaaacc  
 DXP1 ta\*t\*\*gatat\*\*tgactgggt\*\*t\*\*\*aac  
 DLR1 ggatat\*\*t\*actgggtg  
 21.10 tatgat  
 DLR2 gctactc

**s 3** aaatatccgcgctactctgatatgatggctgggtgttcgaaacc  
 DXP1 ta\*t\*\*gatat\*\*tg\*ctgggt\*\*t\*\*\*aac  
 DXP1RC aaatatc  
 21.10 tatgat  
 DIR1/2 ggtgttc  
 DLR2 gctactc  
 DN1/DN4 gctgggt

**s 7** aaatatccgcgctattatgacatgatgagaggtgttcgaaacc  
 DXP1 g\*tatta\*ga\*at\*\*tga\*\*gggt\*\*t\*\*\*aac  
 DXP1RC aaatatc  
 22.10 tattatga  
 22.12 tattatga  
 DXP'1 tattat\*ac  
 DIR1/2 ggtgttc

**b 4** ggggccatatagttgggatgattctccccaggacaattattat  
 DK1 atatagt\*g\*\*a\*gatt  
 DLR3 catat\*gt\*g\*g\*tgatt  
 DLR1RC at\*c\*ccccag\*acaat\*at\*\*t  
 DXP'1/DXP1/23.7 ttattat  
 DIR1/2 cccagga

**b 6** cagaagcccagggtattttcgatcttttgagcgggtcaatatcgcagagttgccgggtgc  
 g  
 DXP1 gtatt\*cgat\*ttttga\*\*gggt\*a\*tat\*\*c  
 DIR1/2 ca\*aagcccag  
 DIR2  
 cgcagag\*\*\*ccg  
 DLR1/DLR4 caatatc

<b>b 8</b>	tattgggttaaagggcgagcattacgacattttgactgcttacggcccc
DXP1	g*attacga*attttgactg*tta
DA5	tattgg
DIR1/2	a*agggcg
DK1	tgg*t**a**ggc*a**attac
DN4RC	gg*cgagc
 <b>b 11</b>	 agatatccgcgctactctgaaatgatgggtggtgttcgaaaacac
DXP1	ta*t**ga*at**tg**tgg*t**t****aa
DIR1RC	aga*atccgcg
DIR2RC	tgggtgg*g
DK1	gatat****gctac
DLR1	ggtggtgt
DLR2	gcgctactc
 <b>s 8</b>	 tcgtcgggttggtcacatttgggggagttgtctcaggaggggaat
21.10	gt****tg*t*ac*tttgggggagtt*tct*a
A1	gaggg*at
 <b>p 35</b>	 aacacccgagaaaacatagaagcagatggtacggcc
DN1	atag*agcag*tggtac
DIR1/2	aacaccc

**Figure 4.6:** Analysis of known D-gene segments with greatest homology to the CDR3 regions of the gp120 binding Fabs. The CDR3 sequence of the Fabs are given as the predominant sequence in each group. All known D segments with homolgy to the predominant sequence are then listed. Identical bases are indicated, while stars represent insertions in the predominant sequence, not present in the D segment. Non-listing of the D segment sequence implies that the D region does not extend to that area of the CDR3 sequence.



#### 4.2.6 Light Chains.

Analysis of the light chains is made difficult by the fact that generally, they are more diverse than the heavy chains and it seems that a large number of different light chains are capable of combining with relatively similar heavy chains. It is also apparent that there is a remarkable bias in germline usage since thirteen of the thirty three light chains are probably related to germline Humkv325 (Meindl *et al.*, 1990).

Group b8 display a high degree of homology, with four chains being almost identical and the others could be somatic variants of this. As the heavy chains in this group are also very similar, this would seem to suggest a preference for one particular heavy-light chain combination. The light chains of group b3 are far more diverse in sequence exhibiting a high degree of promiscuity with their heavy chains. The distribution of light chains in group b1 is similar to the equivalent group of heavy chains suggesting that, as in group b8, there is a preference for particular heavy-light chain combinations. Finally, homology of light chains is generally greatest between members of the same group. However, this is not always the case. For instance, clones b6 and B20 from different groups show 99.5% homology over their entire VJ sequence.

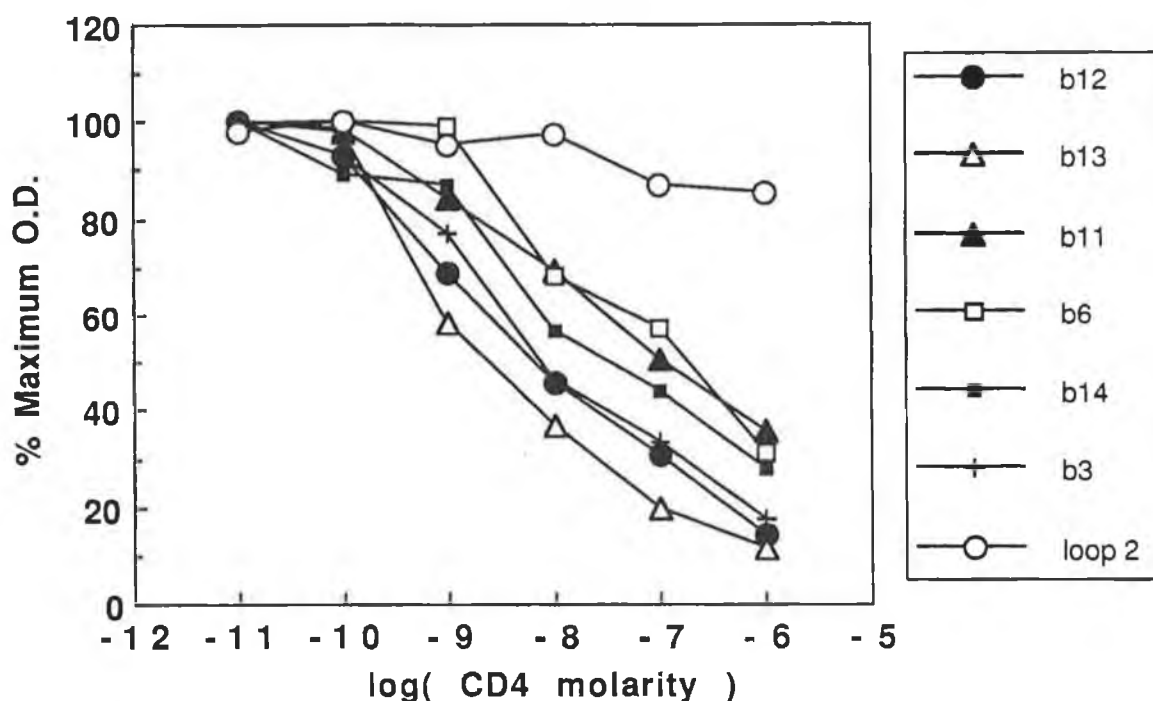
The usage of Jk minigenes (Figure 4.5a) is different from the heavy chain situation, in that not all the members of a group necessarily use the same J segment. However, there is a significant bias in the Jk usage with Jk2 occurring in 61% of sequences, Jk4 in 18%, Jk1 in 15% and Jk3 in 6%.

#### **4.2.7 BIOCHEMICAL ANALYSIS.**

Six clones were chosen to perform a biochemical analysis. These were clone numbers b3, b6, b11, b12, b13 and b14. As can be seen from figure 4.3, this includes a representative from each of six groups, excluding s8 and the V3 loop-binding Fab.

#### **4.2.8 CD4 INHIBITION ELISAs.**

Previously it has been shown by Chang-Yuil Kang *et al.*, that two major groups of neutralising anti-gp120 antibodies exist in HIV-infected patients, namely, those that react with either the V3 loop or the CD4-binding site of gp120. Once soluble Fab fragments had been made, one of the first tasks was to investigate the class of antibody to which these belong. This was done by means of a CD4 competition ELISA where the effect of serially diluted soluble CD4 (sCD4) on the binding of the Fabs to IIIB gp120 was demonstrated (Figure 4.7). As can be seen from the data, all clones are inhibited by 75% at a sCD4 concentration of  $10^{-6}$ M, while a V3 loop-binding fragment is only inhibited by 10% at this concentration. Direct ELISA also confirmed that these clones do not bind the V3 loop peptide of gp120. It can, therefore, be assumed that all the fragments are specific for epitopes that reside around the CD4-binding site of gp120.

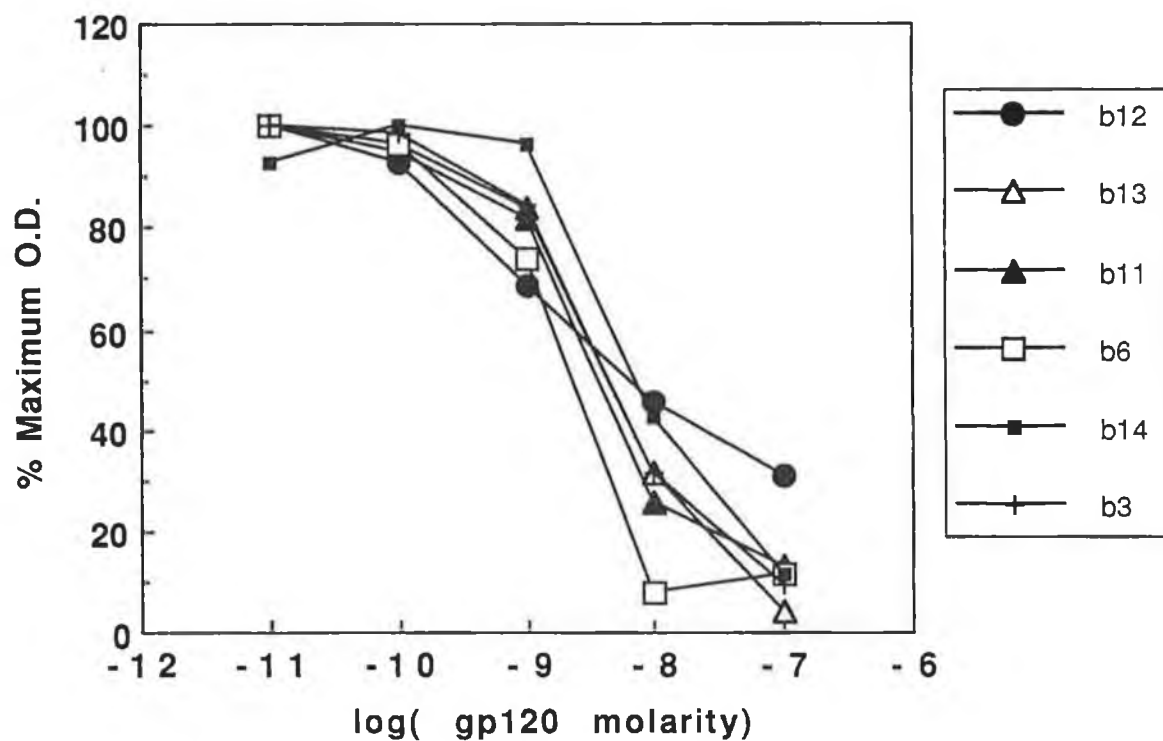


**Figure 4.7:** CD4 competition ELISAs for those gp120 binding clones used in the biochemical analysis showing that these Fabs are specific for the CD4 binding site of gp120. Loop 2 is again included as a negative control and is not inhibited by sCD4 to the same extent as the other clones.

#### 4.2.9 DETERMINATION OF RELATIVE AFFINITIES.

In order to ensure that none of the differences seen in the binding pattern of these fragments was due solely to differences in affinity, it was necessary to accurately determine the relative affinity of each. The first method used to do this was a competitive ELISA, in which soluble and immobilised IIIBgp120 competed for purified Fab preparations. The relative affinity constant was taken as the

molarity of free gp120 which inhibited binding by 50%. As can be seen from the graph (Figure 4.8), the relative affinities for all the clones are very similar, lying in the range of  $1 \times 10^7$  to  $1 \times 10^8 \text{ M}^{-1}$ . This would also seem to demonstrate the specificity of the clones, since polyspecific antibodies tend to bind with relative affinities of  $10^6 \text{ M}^{-1}$  and lower (Rosenburg *et al.*, 1991). The 50% inhibition constants for sCD4 and the relative affinities of all clones are given in Table 4.1. However, such a methodology is appropriate only to give a rough measure of affinity, which meant that it was impossible say whether the differences in relative affinities seen by ELISA were real or artificial. It was, therefore, decided to measure the affinities of these clones using real-time biospecific interaction analysis (Biacore™) in order to obtain more accurate values. The results for clone b12 are presented here to illustrate the type of data that was obtained using Biacore. The results for the other clones are not shown, but are very similar to b12. All results are given in the form of sensograms. The binding of antibody to antigen is measured by the machine as a shift in the angle of reflection of a light beam. This is converted to response units (RU) which is directly proportional to the mass of bound antibody. The response is given as a graph of RU versus time and this is represented as a sensogram.



**Figure 4.8:** Antigen specificity demonstrated by competitive ELISA with gp120. The relative affinity ( $K_a$ ) for each clone is the molarity of competing gp120 capable of inhibiting binding by 50%. It can be seen that the  $K_a$  values for all these Fabs lie in the range of  $10^8 M^{-1}$ .

#### **4.2.10 Fab BINDING TO IMMOBILISED gp120.**

An example of a sensogram showing Fab b12 binding is shown in Figure 4.9(a). The initial report point (illustrated by an 'x' on the curve) represents the baseline response to a continuous flow of PBS over a flowcell containing immobilised gp120. The subsequent rise in the response is the binding of the Fab to the gp120. Report points are taken during this phase at 10 second intervals in order to be able to calculate the association constant of this Fab. Surface regeneration is then achieved by an injection of 10mM HCl and is demonstrated by the fact that there is no difference between the first and last report points, i.e.: there is no Fab remaining bound to the gp120 once the run has been completed. This procedure is repeated for a number of different Fab concentrations and the interaction curve for each is analyzed as a  $dR/dt$  versus  $R$  plot (where  $R$  is the level of binding, given as Response Units (RU) and  $t$  is time as measured in seconds). The interaction curves for b12 at steady state binding are represented as an overlay plot in Figure 4.9(b). The slope value for each graph is then plotted versus antibody concentration, and  $K_{on}$ , the association constant, is obtained from the slope of the fitted line as shown in Figure 4.9(c).

#### **4.2.11 CALCULATION OF THE DISSOCIATION CONSTANT, $K_{off}$**

Antibody is permitted to bind to gp120 until equilibrium is reached. This is achieved by using a high Fab concentration, typically 150 $\mu$ g/ml, and by immobilising low amounts of gp120, in this case, 700RU. The antibody is then dissociated from the antigen by passing

PBS over the complex at a flowrate of 50 $\mu$ l/min. It is during this phase that report points are taken for calculations. The high flowrate ensures that there is no binding-rebinding effects, which would interfere with the calculation of  $K_{off}$ . The interaction curve for dissociation is then represented as a  $\ln(R_0/R_i)$  versus  $t_i - t_0$  plot ( $R_i$  is the response at time  $t_i$ ;  $R_0$  is the initial response at time  $t_0$ ), as in Figure 4.9(d) and the slope of this curve is taken as the dissociation constant. Relative affinity is calculated as  $K_{on}/K_{off}$ . The relative affinities for a number of clones used in this study, as calculated by BIAcore, are shown in Table 4.2. These results are very reproducible, with a standard deviation from the mean of approximately 5%, as determined by calculating a number of these constants in triplicate. They also show that there is very little difference between the relative affinities of any of these clones, which indicates that any differences identified in the binding patterns of these Fab fragments is not due to differences in affinity. All Fabs examined have affinities in the range  $5 \times 10^7$  to  $1 \times 10^8$  M $^{-1}$ . This is an accurate corroboration of the results obtained by ELISA.

<b>VALUE</b> <b>Fab</b>	<b><math>k_{on} (M^{-1} s^{-1})</math></b>	<b><math>k_{off}(s^{-1})</math></b>	<b><math>K_a (M^{-1})</math></b>
<b>b3</b>	<b><math>9.6*10^3</math></b>	<b><math>1.8*10^{-4}</math></b>	<b><math>5.1*10^7</math></b>
<b>b6</b>	<b><math>1.6*10^4</math></b>	<b><math>1.6*10^{-4}</math></b>	<b><math>9.7*10^7</math></b>
<b>b12</b>	<b><math>4.5*10^4</math></b>	<b><math>4.3*10^{-4}</math></b>	<b><math>1.1*10^8</math></b>
<b>b13</b>	<b><math>1.1*10^4</math></b>	<b><math>1.4*10^{-4}</math></b>	<b><math>7.9*10^7</math></b>

**TABLE 4.2**

**Table 4.2:** Relative affinities of the six Fabs used in the biochemical analysis obtained with BIAcore. The values given here are accurate to within +/- 5%.



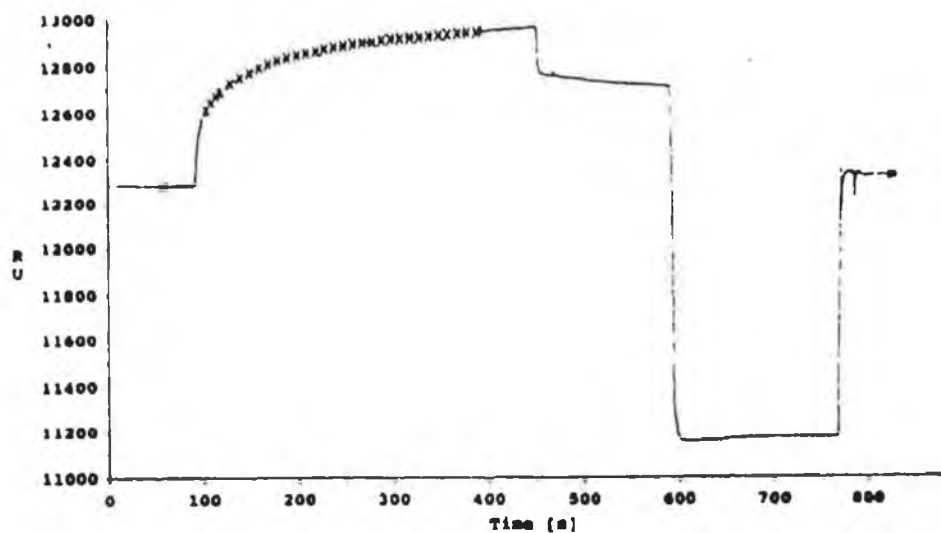


Figure 4.9(a)

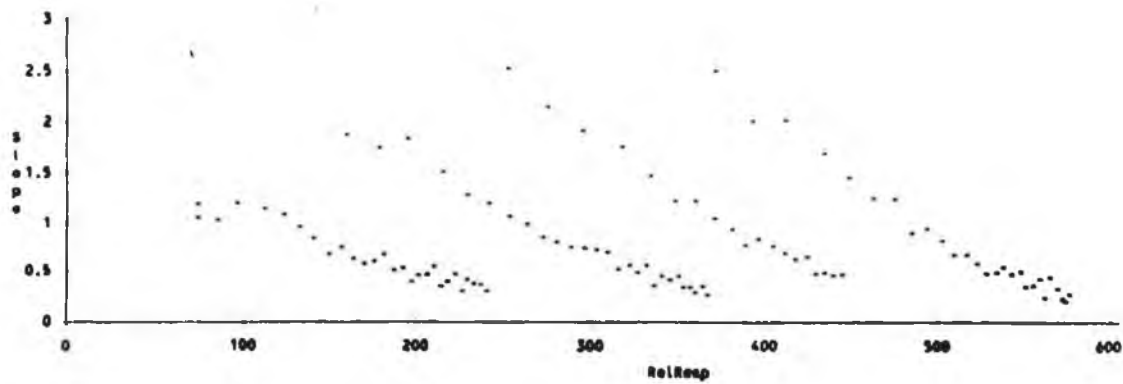


Figure 4.9(b)

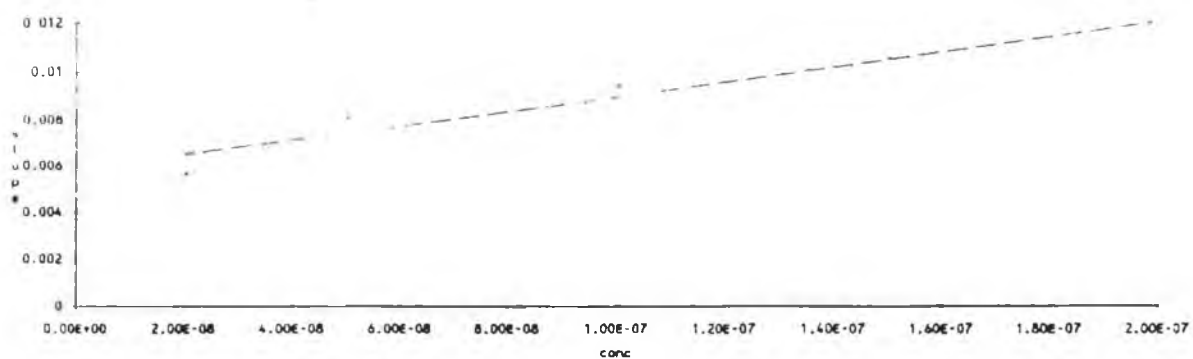


Figure 4.9(c)

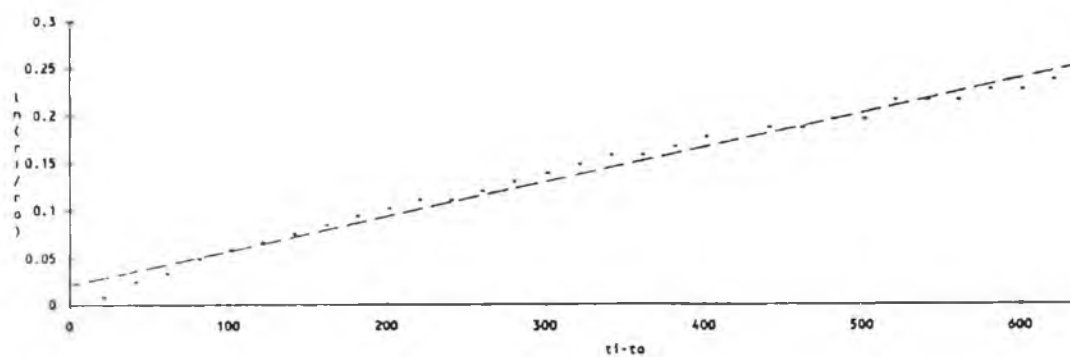


Figure 4.9(d)

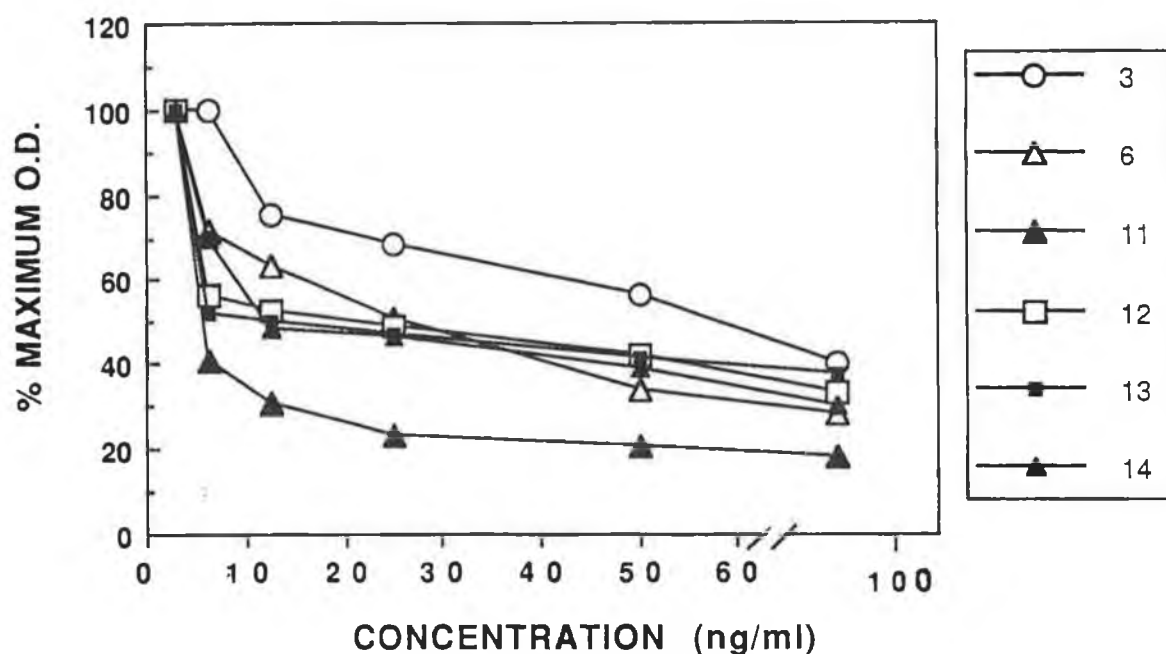
#### **4.2.12     EPITOPE MAPPING.**

Since these antibodies are all very similar in terms of affinity and inhibition by sCD4, it was sought to determine if the epitopes recognised by these Fabs on gp120 are all identical, or whether the human response we are studying involves a number of related but non-identical epitopes around the CD4 binding site of gp120. This could be important both for understanding the human response to HIV, and to understanding the mechanism by which the virus binds CD4 *in vivo*.

#### **4.2.13     COMPETITION OF ANTIBODY 13 WITH Fabs.**

The most obvious method to determine the epitope specificity of these Fabs was to perform a competition ELISA in which the fragments compete with each other for binding to the immobilised antigen. This was achieved by competing each Fab used in this study with whole antibody 13. The titre of this antibody, as previously determined by ELISA, was 1:10000, and so this dilution was used in all wells of the ELISA plate. The Fabs all had titres in the range 1:4000 (25ng/ml) to 1:8000 (12.5ng/ml), and these were diluted in a serial dilution of 1:100 (100ng/ml) to 1:32000 (3.125ng/ml). As can be seen from the graph (Figure 4.10), almost all the Fabs had inhibited the binding of b13 by 50% at a dilution of 1:5000, which is approximately equal to their titres. This would indicate that their epitopes are sufficiently similar to that of b13 to enable them to compete with it in an ELISA assay. Moreover, since all the clones compete with b13 to approximately the same extent, it may be

concluded that all the fragments bind to overlapping, but not necessarily identical epitopes.



**Figure 4.10:** Competition of whole antibody 13 (Ab13) with the Fabs. The titres of these Fabs are in the range 12.5ng/ml to 25ng/ml. It can be seen that b13 is inhibited by 50% by Ab13 within that range as expected. The other Fabs are similarly inhibited, within the error rate for this type of ELISA.

#### 4.2.14 EPITOPE MAPPING WITH BIACORE.

An alternative, and more precise method for determining similarity of epitopes is using the BIAcore. In this situation, a polyclonal anti-human  $F(ab')_2$  is immobilised to the sensor chip and used to capture

an initial Fab. This, in turn was used to capture gp120, and finally a second Fab was injected. Binding by both Fabs, as measured in response units (RU), indicates that their epitopes are distinct from one another. Conversely, a lack of binding by the second antibody indicates that the two Fabs share the same epitope.

The results of this experiment are shown in Table 4.3. 4.3(a) shows the positive and negative controls for the clones used. The positive controls are the RU levels obtained when the first Fab used is the clone as indicated and the second Fab is an anti-gp120 V3-loop Fab, which should not share any epitope with our clones since they are all CD4 inhibited. As can be seen from the table, all positive controls result in significant values of 99 or more, validating that the technique does work in the case of non-identical epitopes. The negative controls are the values obtained when both the first and second Fabs are the same in each case, as indicated. This gives the background values for each clone, and these values were subtracted from all subsequent experiments in order to give true readings.

An epitope map, Table 4.3(b), was then constructed with clones b3,b6,b11,b12,b13,and b14. It can be seen from this map that any combination of Fabs b3,b6,b11 and b14 results in no binding of the second antibody, i.e.  $RU=0$ . This would suggest that all the clones in this set share an almost identical epitope. However, Fabs b12 or b13 appear somewhat different in that they do compete with members of the above set but not as effectively as within the set. b12 and b13 are also dissimilar from one another. It could be inferred from this that the epitopes of b12 and b13 are sufficiently dissimilar from those of the other four and from each other, to allow for a certain

amount of low affinity binding when they are used in combination with any of the other clones.

It may, therefore be concluded, that clones b3,b6,b11 and b14 bind similar epitopes, with b12 and b13 showing some distinct features not found in the others. Interestingly, it is these two Fabs which have been generally shown to neutralise virus (Barbas *et al.*, 1992), while the other four display little or no neutralisation capability.

	3	6	11	12	13	14
POSITIVE	129	128	131	125	135	134
NEGATIVE	24	38	-	17	15	-

**TABLE 4.3(a)**

	13	12	6	3
14	30	24	14	0
11	54	28	14	0
3	26	29	0	0
6	21	17	0	
12	22	0		

**TABLE 4.3(b)**

**Table 4.3:** BIAcore epitope maps for the six Fabs analysed. a): Positive and negative values for each of the Fabs. b): Values obtained when the different combinations of Fabs were used. The Fabs shown on the top row represent those used as the first antibody to immobilise the gp120 initially, while those along the side, represent the secondary Fabs used in each case. The negative value subtracted in each run was that obtained for the primary Fab.

#### 4.2.15 gp120 KNOCKOUT ASSAYS.

In order to obtain a clearer picture of the specific details of each epitope, gp120 knockout assays were performed. This essentially involved examining the binding pattern of our Fabs with a panel of gp120 molecules, each of which was altered with a different, specific mutation (Olshevsky, *et al.*, 1990). This would yield information as to which residues are important for the binding of the individual clones to gp120 and would enable us to define more accurately the epitopes. The results of these assays are shown in Figure 4.11(a-f). It had previously been demonstrated that there are a number of positions very important for the binding of monoclonal antibodies (MAb) directed against the CD4 binding site of gp120, and that if these residues are mutated, this inhibits the binding of these antibodies. These mutations include residue 257 mutated from Threonine to Arginine (257 T/R), 368 D/R, 370 E/R and 457 D/A (Olshevsky *et al.*, 1990; Thali *et al.*, 1991). Inhibition of binding as a result of these mutations is a common feature of CD4 binding site antibodies and, as can be seen from the figure, are a common feature of our Fabs, showing that they are, indeed, directed against that area of gp120. The only two clones not inhibited by mutation 257 T/R are numbers b6 and b12. Fab b3 binding is not affected by mutation 457D/R. However, 457 D/A does inhibit binding by this Fab, with a binding index of 0.42 (data not shown). Therefore, b3 is also specific for the CD4-binding site.

Some of the other major features of this study are also shown in Figure 4.11. Enhancement of binding as a result of mutations at the base of the V1/V2 loop are usually accompanied by mutation 166 R/L



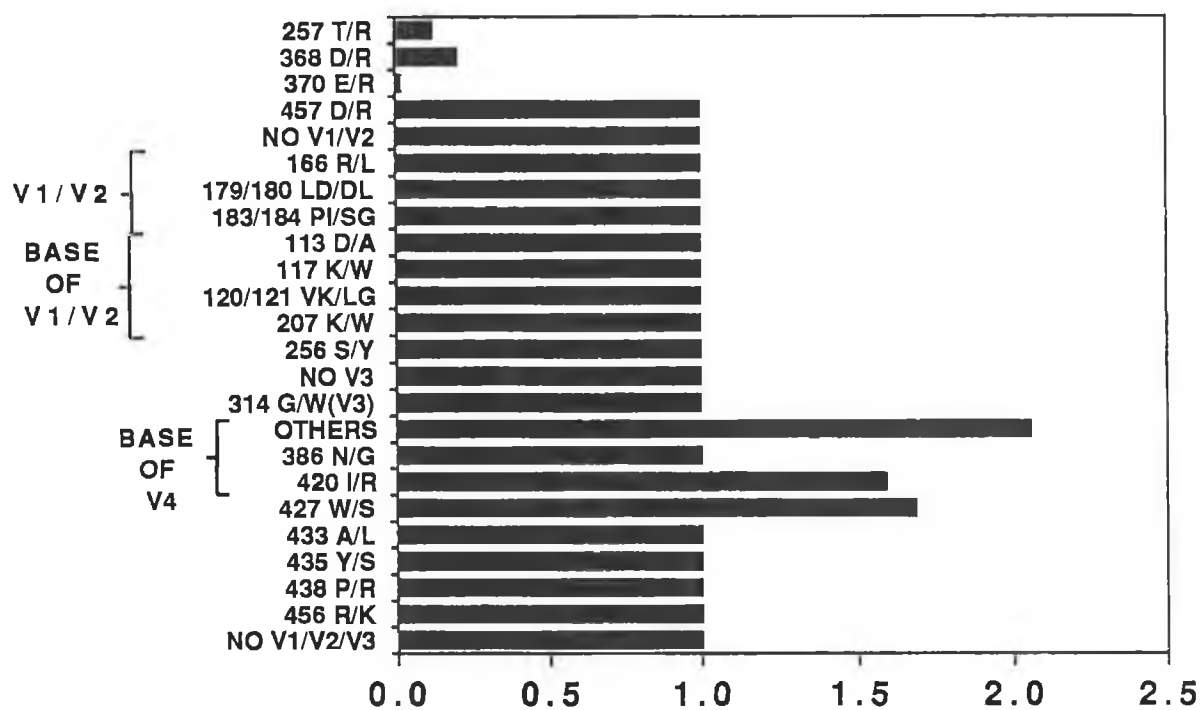
and are not uncommon with this type of antibody. This effect is seen in the case of clones b6,b11,b12 and b13. Fab b14, however, exhibits an unusual knockout effect induced by mutation 113 D/A at the base of the V1/V2 loop.

In one particular mutant of gp120, the V1/V2 loop (residues 119-205) is completely removed. It has been previously demonstrated that this type of change can actually enhance the binding of CD4 site-specific antibodies to gp120, as is the case with clones b6,b11 and b14 from our study. However, clone b12 exhibits the highly unusual effect of having its binding completely knocked-out by this mutation, suggesting that the V1/V2 loop is important for binding by this Fab.

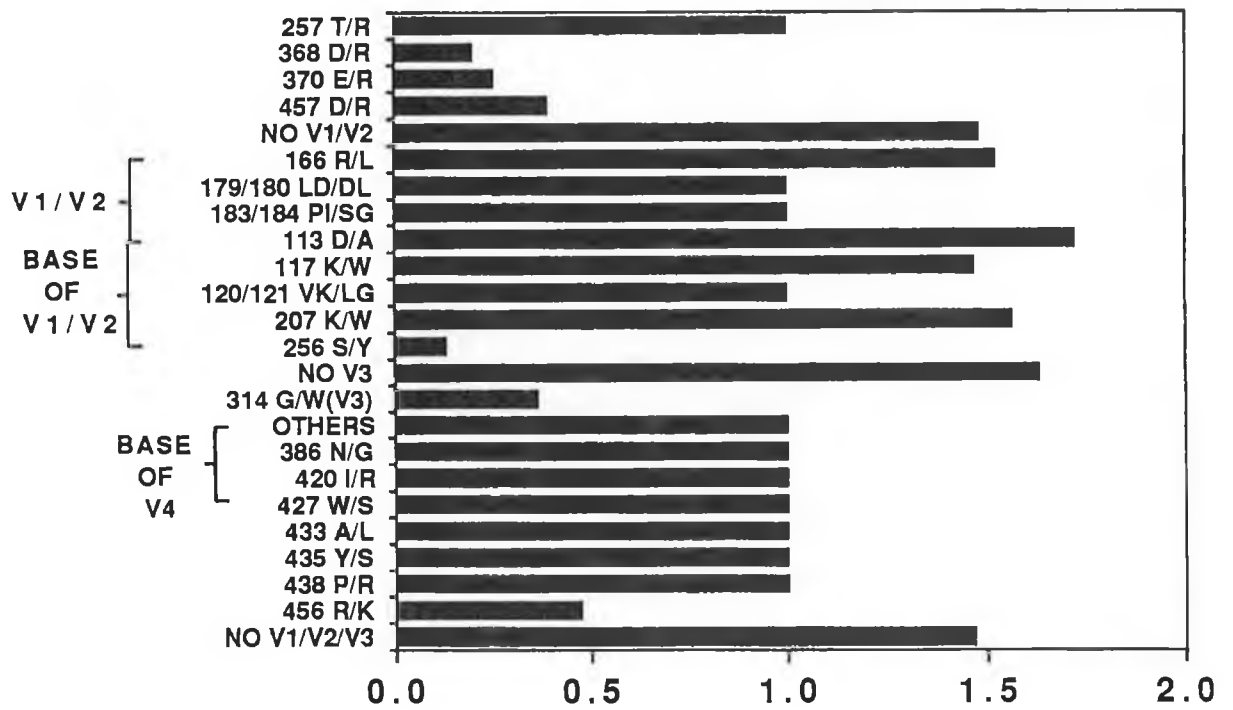
Mutations at the base of the V4 loop (residues 393 to 414) are known to affect the binding of certain MAb specific for the CD4 binding site of gp120. This usually manifests itself by enhancing binding by the antibody, as is demonstrated here in the case of clones b3 and b12. However, a more uncommon effect is apparent in the binding pattern of Fab b13. The binding of this clone is completely prevented by mutations 384 Y/E and 386 N/R, both of which are at the base of the V4 region. Other mutations in this area are also capable of inhibiting binding of clones b11 and b14, although to a lesser extent, for example 386N/G in both cases. On the other hand, some of these mutations, such as 435Y/S and 438P/R, are capable of enhancement.

Finally, it is interesting to note that mutations in the V3 loop of gp120 are capable of partially inhibiting binding by a number of these Fabs, namely 314 G/W which inhibits binding of clones b6,b11 and b14 and mutations in residues 308 to 310 capable of inhibiting clone b12.

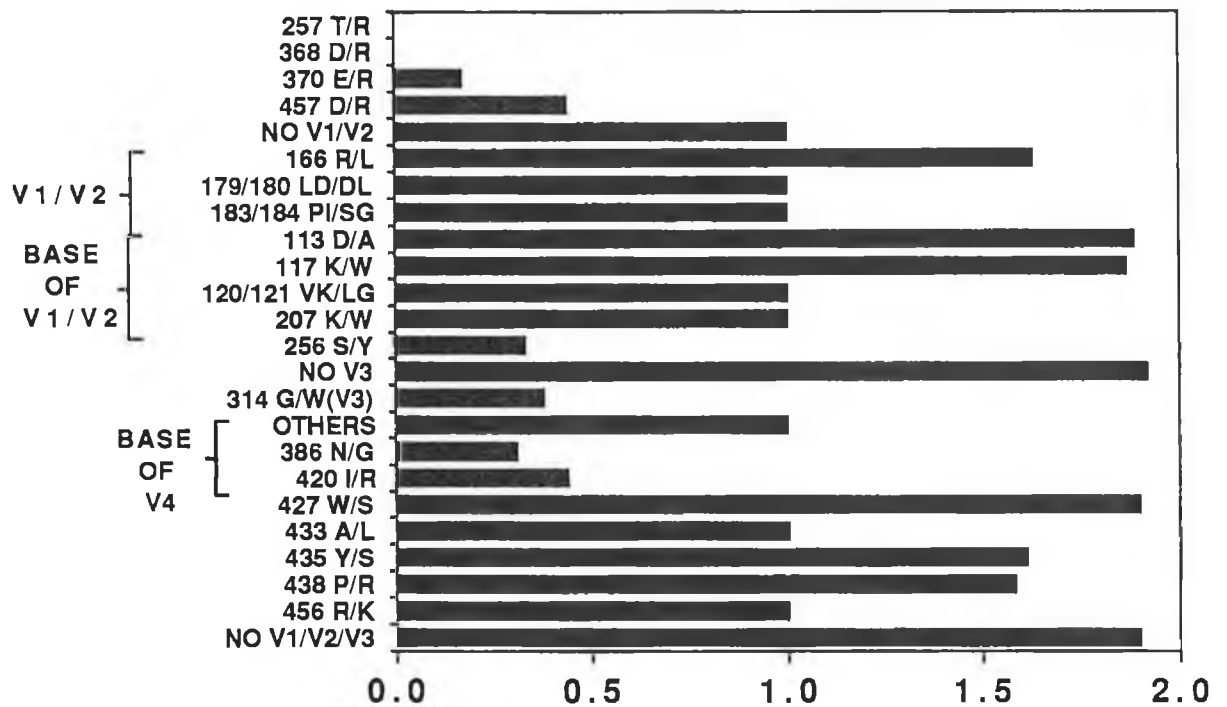
**Figure 4.11:** Graphs a-f represent the knockout patterns observed for the six clones used in this analysis. The gp120 mutations are given on the Y-axis, while the binding indices are plotted on the X-axis. A binding index of 1.0 indicates no effect; below 0.5 suggests an inhibitory mutation; above 1.5 implies an enhancing effect.



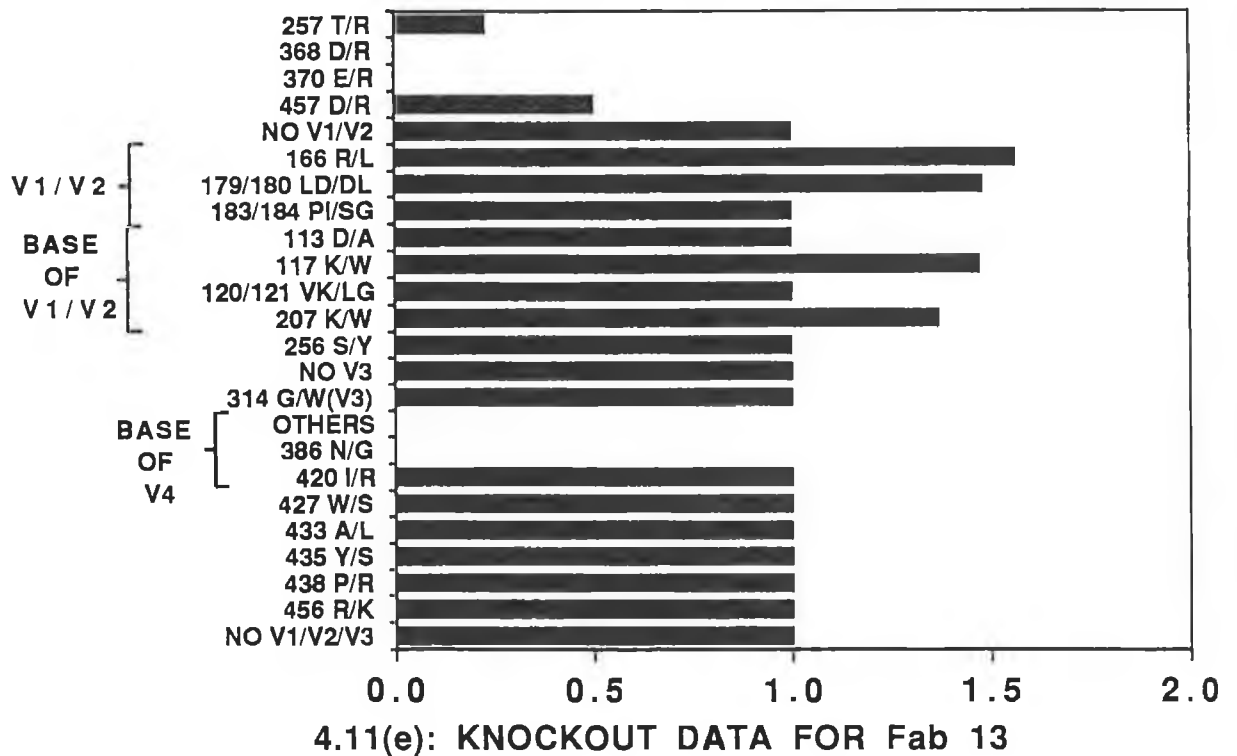
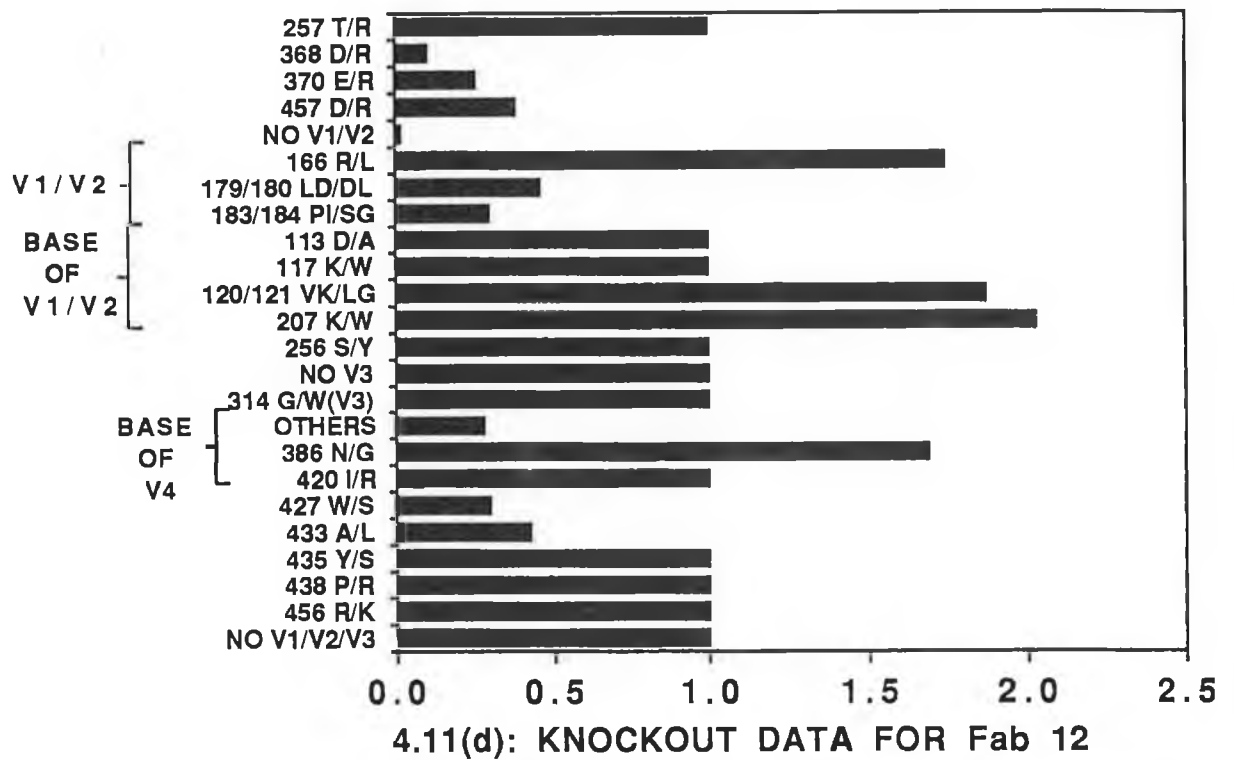
4.11(a): KNOCKOUT DATA FOR Fab 3

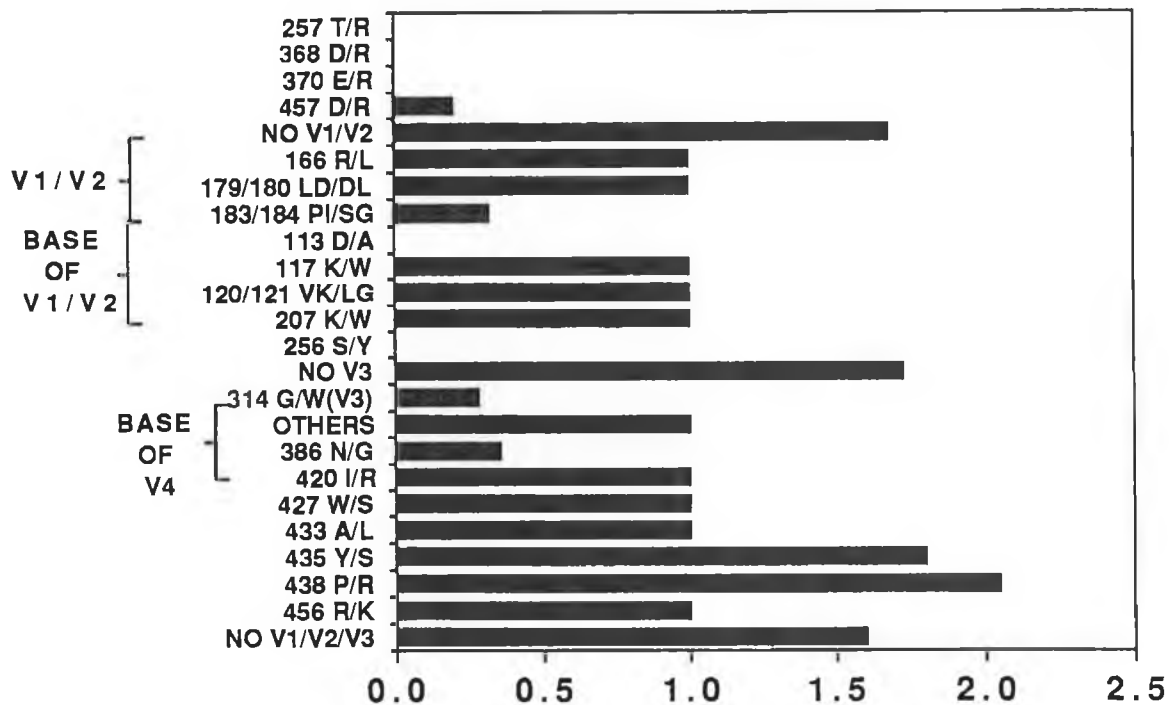


4.11(b): KNOCKOUT DATA FOR Feb 6



4.11(c): KNOCKOUT DATA FOR Feb 11





4.11(f): KNOCKOUT DATA FOR Feb 14

### 4.3 DISCUSSION.

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#### 4.3.1 VALIDITY OF THE COMBINATORIAL LIBRARY APPROACH.

The phage display library system has been shown to be very successful in isolating a large number of diverse, high affinity, antigen-specific Fab fragments (Scott *et al.*, 1991; Devlin *et al.*, 1990) as can be seen from the number of Fabs generated against gp120/gp160 in this study. Four rounds of panning lead to an enrichment of 100 to 1000-fold, which is a very significant, and greatly increases the chances of obtaining the required clones, over techniques such as plaque-lift assays described by Persson *et al.*, (1991). This study has also demonstrated that the Fabs isolated here have high relative affinities ( $10^8 \text{ M}^{-1}$ ) as a result of expressing the fragments as a fusion product with the geneIII (gIII) product coat protein. This leads to a monovalent expression of the Fabs on the phage surface ensuring that Fabs are selected on the basis of specificity and affinity during the panning process. Fusions with the gVIII product result in multivalent display on the phage surface and this can prevent the separation of phage displaying lower affinity Fabs from those displaying high affinity peptides, due to the phenomenon of avidity ( Cwirla *et al.*, 1990).

However, it can be argued that the combinatorial library approach does not reflect the *in vivo* situation, for a number of reasons. Since the purpose of this study is to investigate the antibody response of a single HIV positive patient on a molecular and biochemical levels

and to see if any conclusions can be drawn as to the nature of the response, it is, therefore, very important that the validity of the combinatorial library approach with regard to how it reflects the *in vivo* situation is first investigated. The recombination of heavy and light chains in the library is, in theory, a completely random process and therefore, any combination should be possible (It is worth noting at this point that this random nature of the combinatorial system can be taken advantage of to produce required specificities not found *in vivo*, e.g. catalytic antibodies capable of chemical transformations). In the *in vivo* situation it has been estimated that there are  $10^8$  possible antigen specificities, however, there are restrictions on the combinations that can occur as a result of phenomena such as self tolerance. This ensures that those combinations capable of harming 'self' do not occur.

Clonal selection (Liu *et al.*, 1989) is also a very important part of the *in vivo* response, not present in the construction of the libraries. This has the consequence that certain B cells expressing antibody specific for antigens to which the host has already been exposed may be present in disproportionately high numbers and may bias library construction.

Thirdly, antibodies *in vivo* are capable of undergoing affinity maturation (Griffiths *et al.*, 1984) after combination of the heavy and light chains, whereas the library combines mature chains and no further maturation is possible.

The method of library construction may also introduce artifacts. This could include the types of primers used in PCR, or the PCR itself could introduce mutations. It may also be possible that low

affinity binders will be lost through the use of the gIII fusion, which may be important in the overall immune response.

The study of this response has lead to a number of conclusions as to the validity of the combinatorial approach and about the response itself.

### **Heavy Chains.**

Firstly, the question must be answered, do the heavy chains *in vitro* reflect those found *in vivo* ? The available data suggests that they do, for a number of reasons:

First, several studies have failed to isolate high affinity binders from non-immunised libraries (Persson *et al.*,1991; Marks *et al.*,1991, Kang *et al.*, 1991). Therefore, since the patient being studied here has elevated antibody levels for gp120 in the serum and we are able to isolate antibodies from the library, there would seem to be a correlation between the two situations. Second, there are a number of heavy chains from high affinity Fabs that are highly represented in the library, suggesting that these chains were also represented in the *in vivo* situation. For example, the heavy chain from group b8 represents 30% of all heavy chains isolated. It is possible that this is just an artifact of the PCR. However, only primers for the VH1a (indicated by the N-terminal sequence LEQSG, Figure 4.3) and VH3a (N-terminal sequence LEESG) gene families were used to amplify the library, and the fact that there is an almost equal number of each family in this group would seem to argue against this possibility. It is still possible that this group arose from two genes that were disproportionately amplified, but the number of intraclonal variants present in other groups (e.g.: b3



group) would seem to suggest that this also is not the case, although it cannot be completely ruled out.

Third, it will be shown in further studies (chapter 5) that the antigen specificity of an Fab seems to be predominantly due to the heavy chain, as it can be shown that heavy chains from immunised libraries in association with unrelated light chains can bind antigen, while the converse is not true. Taken together with the other points, this is a strong indication that the heavy chains isolated from this library represent the *in vivo* situation.

### **Light Chains.**

It is more difficult to argue a similar case for the light chains as antigen specificity is not associated with the presence of a particular light chain, and so any bias could possibly be as a result of the methods used to construct the library. Since the mechanisms of heavy and light chain combinations in relation to antigen-binding and antibody function are not fully understood, it is very difficult to speculate on the validity of the light chains present in the library. However, it could be argued that the light chain must play some part in the function of the antibody, even if it is not in the initial binding of the antibody to the antigen, and that the most efficient antibody *in vivo* would consist of the best heavy and light chain combination to effect the function of that antibody.

The function of these antibodies is to neutralise virus and it will be shown later (chapter 6) that very small changes in the light chain sequence can drastically alter the ability of a particular Fab for neutralisation. It will also be shown that shuffling the chains of

Fabs within a given group can dramatically affect the neutralising capability of a Fab despite very few differences in sequence. Therefore the heavy and light chain requirements for neutralisation seem to be more stringent than for antigen binding. Bearing this in mind, and considering that a number of the library Fabs, particularly in the b4 group, neutralise, it is a strong case that the light chains found in these Fabs at least, and possibly their heavy and light chain combinations, are representative of those found in the *in vivo* antibodies.

### **Heavy-Light Chain Combinations.**

It is particularly interesting to note that there is a high degree of heavy chain promiscuity in the Fabs, i.e.: one heavy chain can pair with a number of light chains and retain binding to the antigen. This promiscuity makes it impossible to say whether the combinations of heavy and light chain seen here reflects the *in vivo* situation. However, the prevalence of certain heavy-light combinations suggest that these combinations exist *in vivo* or at least that the light chains present in these combinations are associated with antigen binding *in vivo*.

A number of approaches may be taken to study this in greater detail. For example, a given heavy chain could be crossed with a complete light chain library and panned against gp120, to see if the same combinations could be retrieved. This would indicate a preference for certain combinations *in vivo*. It could also be useful to compare

the combinatorial approach with other antibody production techniques (Canton & Koprowski, 1990; Gherardi & Milstein, 1992) such as EBV transformation and hybridoma technology, although each methodology is thought to reflect a slightly different aspect of the antibody response.

#### **4.3.2 GENETIC ANALYSIS.**

The importance of the CDR3 region of the heavy chain (HCDR3) to antigen binding has been demonstrated in this study. It has also been shown previously that this region of the antibody is probably the most diverse in terms of sequence, with the possibility of generating  $10^{14}$  different peptides at this location (Sanz, 1991). The mechanism by which such diversity has been generated has been attributed to the recombination of V, D and J germline genes (Darnell *et al.*, 1986) although the details of this process have not been fully established (Chun *et al.*, 1991; Alt *et al.*, 1992).

#### **D Segments.**

However, simple recombination is not sufficient to explain the levels of diversity observed, and so studies on large numbers of immunoglobulin genes (Ichihara *et al.*, 1988 ; Decker *et al.*, 1991; Sanz, 1991) have lead to the elucidation of a number of other mechanisms, such as DIR D genes, inverted D gene fusions, cross-over events and gene correction mechanisms including insertions, deletions and mutations. A number of these phenomena may also have been observed in the study of this panel of Fabs. The length and sequences of the HCDR3s within any given group are all remarkably

similar, suggesting that all the members of a group are derived from similar D-J combinations. The D segments of the Fabs in group b3 all seem to be related to DXP1, and it is possible that there is a D-D fusion occurring here between DXP1 and inverted DK1. The mechanisms of how this might occur are not fully understood, as it would be breaking the 12-23 spacer rule (Millner *et al.*, 1986). It is interesting to note that 13 nucleotides of the consensus sequence are homologous to a region in the D locus approximately 300bp downstream of the DLR1 gene, which is not flanked by the usual heptamers and nonamers. This may be a coincidence, or it may represent another form of recombination as yet unknown. The members of the b1 group exhibit similar V-D and D-J joins, which, in addition to the fact that they are all the same length and similar in sequence, would suggest that they all use the same D-J combination. However, the patterns of homology observed in the VH regions indicate that there may be a number of different germ lines present. This may be as a result of cross-over events during PCR, although it would require multiple, coincidental cross-overs to give the sequences observed. There may also be a form of convergent evolution occurring *in vivo* in an attempt to mix different germline VH genes with a single, beneficial, D-J combination. However, this has not been described previously. The overall lack of homology of these D regions to previously sequenced genes would argue for the existence of hitherto unknown D segments or additional somatic mechanisms that have not yet been identified. A similar conclusion was reached by Sanz in his study of CDR3-specific cDNA libraries (Sanz, 1991).

#### **4.3.3 BIOCHEMICAL ANALYSIS.**

We have used combinatorial libraries to isolate a number of antibodies specific for gp120 from a single seropositive patient.

An analysis of this panel of Fabs at a DNA level has revealed that it was possible to divide the genes into eight different groups, dependent on the sequence of the CDR3 of the heavy chain. Within these groups, there was a diversity of sequences representing distinctly different germlines. However, certain heavy chain sequences appeared in relatively high frequency, suggesting that they also appear in high frequency in the mRNA of the patient and are, therefore, representative of the heavy chains used *in vivo*. The light chains also show a diversity of sequences and there is a bias in the germline genes they represent, which also probably reflects the *in vivo* situation.

However, it was not known how this diversity of genetic sequences was related to the biochemical properties of the Fab proteins coded by these genes and so it was necessary to conduct a biochemical analysis of a representative cross section of that panel. Six Fabs were chosen for this purpose, one from each of the groups containing two or more Fabs. These were b3, b6, b11, b12, b13 and b14.

#### **CD4 Inhibition.**

Firstly, it was shown that all the Fabs used are competitively inhibited by sCD4 from binding to IIIB gp120. This categorises them in the class of antibodies that bind the CD4-binding site of gp120. This was not a surprise since the majority of antibodies found in patients in the latter stages of HIV infection, as this patient is, are

known to be of this type. However, it did mean that the analysis of specific epitopes for these Fabs would be more difficult due to the discontinuous nature of the CD4-binding site.

### **Relative Affinities.**

The second stage was to accurately measure the affinity of each clone. The ELISA method suggested that all the affinities were very similar. However, this was not sufficiently accurate, and so the affinities were measured more accurately using BIAcore, which confirmed the fact that all affinities were in the same range of  $5 \times 10^7$  to  $1 \times 10^8 \text{ M}^{-1}$ . The reproducibility of these values was found to be  $\pm 5\%$ .

### **Epitope Mapping by Competitive ELISA.**

Competitive ELISAs were performed in order to determine if the epitopes of all the fragments were similar. It was found that they were all capable of competing in an ELISA situation, suggesting that their epitopes were overlapping, if not identical. The genetic analysis of this panel of antibodies had shown that they have been encoded by distinctly different genes, yet our biochemical analysis had, thus far, not shown any differences. Therefore, more accurate methods were required to analyse this.

### **Epitope Mapping by BIAcore.**

Epitope mapping using the BIAcore, revealed certain differences between the fragments. It was possible to ascertain that the epitopes of Fabs b3,b6,b11 and b14 were similar enough as to not

allow any interactions of two clones with gp120 to occur simultaneously. However, some interaction was reproducibly detected between gp120 and any of the above four clones (b3,b6,b11 and b14), when the gp120 had already been bound by either Fab b12 or b13. This suggested that the epitopes of b12 and b13 were sufficiently different from the others to allow simultaneous low affinity binding of two clones to gp120. These two Fabs also displayed some binding characteristics dissimilar from one another. This was the first indication that the genetic diversity had translated into biochemical diversity of the Fab fragments.

### **Knockout Assays.**

It was then possible to establish, by means of knockout assays that the residues on gp120 capable of affecting the binding of the Fabs were different for each clone. All clones displayed certain knockout patterns typical of antibodies specific for the CD4 binding site of gp120, however, there were some very significant differences. Clone b12 exhibited some features not usually seen in this class of antibody, in that a deletion of the V1/V2 loops of gp120 effectively knocked out the binding by this Fab, which suggests that its epitope partially includes the V1/V2 loop. It was also inhibited by certain mutations in the V3 loop region (308-310 deletion).

Fab b13 also showed some interesting features not commonly encountered in CD4 binding site antibodies. Mutations at the base of the V4 loop were capable of knocking out the binding of this fragment to gp120 suggesting that they may be directly involved in the binding of gp120 by b13, while mutations at the base of the

V1/V2 loop strongly enhanced its binding. The knockout effect seen at the V4 base is very uncommon and suggests that the epitope of b13 is significantly different from the other Fabs. The other clones displayed interesting, though probably less significant differences in their binding patterns. This data corroborates the BIAcore evidence, in that it is Fabs b12 and b13 that are seen to be the most unique in both cases, which helps to establish BIAcore as a valid method of epitope mapping.

This result is particularly interesting in this case as it has already been demonstrated that b12 and b13 are the two Fabs best able to neutralise the virus from the panel of Fabs available to us. This ability to neutralise may be linked to the unusual binding patterns demonstrated by these Fabs. Both require residues common to most CD4 binders, while b12 also requires the V1/V2 loop and b13 is inhibited by mutations at the base of the V4 loop. This may lend credence to a theory that the V1/V2 loop, and perhaps the V4 loop fold down over the CD4 binding site, and so can be part of an epitope for this type of antibody.

Although any attempt to explain these results is merely speculation, it may be the case that the virus is capable of altering the CD4-binding region to escape being bound by antibody and that it is more limited in its ability to change the V1/V2 and V4 loop areas. Therefore, any antibody with an epitope including the CD4-binding site and one or other loop region may be a better neutraliser, since the virus would find it more difficult to produce escape mutants to prevent binding by this antibody.



The results presented here may prove useful in determining the structure of gp120 which may, in turn, yield some information as to the mode of action of the HIV virus. It was also interesting to note that mutations in the V3 loop were able to affect the binding of a number of these Fabs (b6, b11, b12 and b14), all of which were determined to be CD4 site-binding antibodies. This may be a reflection on the folding of the gp120 molecule, bringing the V3 loop in close contact with the other areas involved in CD4 binding, or it may be that changes in the V3 loop can effect changes elsewhere in the molecule, by other mechanisms. Perhaps a larger panel of antibodies such as these would yield more information about the structure of gp120.

#### **4.3.4 HIV AND THE IMMUNE SYSTEM.**

We have now established that the genetic diversity of the genes coding these antibodies can also be translated into biochemical diversity, without losing the overall specificity of the antibodies. When the immune response is challenged with the HIV virus, it must make antibodies capable of neutralising the virus. One of the most immunodominant epitopes on HIV is the CD4 binding site of gp120, which the virus uses to bind to CD4<sup>+</sup> cells and subsequently invade them (Rosenburg *et al.*,1991). Therefore, if the virus is to be neutralised, antibodies to this epitope must be produced. However, the virus is capable of altering itself in an attempt to "shake off" these antibodies. The immune system must, therefore, produce a range of antibodies capable of binding to the CD4 binding site, yet possessing slightly different specificities, so that the virus may be

neutralised even after it has changed. I believe that the subtle diversity among our panel of Fabs- all specific for the CD4 binding site of gp120, and yet with slightly different epitopes- may be an attempt by the immune system of our patient to avoid viral escape mutants.

We have been able to use the combinatorial library system in conjunction with a number of other techniques to analyse, both at a genetic and a biochemical level, the way in which evolution of the immune system has adapted the immune response of one particular individual in an attempt to fight off a virus capable of escape mutants. The information gained in this study, in conjunction with neutralisation data for these clones should prove useful in the design of antibodies capable of neutralising the HIV virus. This technique may not be limited to the particular case of the HIV virus, but may prove useful for effectively analysing the immune response of patients with a range disease states. This would allow us to obtain a better understanding of how evolution has dealt with the problems of infectious agents, and this knowledge may facilitate use in the design of better systems to combat these diseases.

## CHAPTER 5

## 5.1 INTRODUCTION

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### 5.1.1 OVERVIEW.

Combinatorial antibody libraries have been developed as a means of producing a large number of diverse antibodies (Persson *et al.*, 1991; Huse *et al.*, 1989; Marks *et al.*, 1991) both from human and murine sources (Caton & Koprowski, 1990). A large panel of Fab fragments specific for gp120 has been generated by means of combinatorial library technology, as has been discussed in chapter 4. However, it has been argued that these libraries are not a true representation of the *in vivo* response (Gherardi & Milstein, 1992) and it was not clear whether this panel of antibodies accurately reflected the *in vivo* situation, due to a number of circumstances which have previously been specified (section 4.3.1). It has already been argued that the heavy and light chains present in the panel probably represent those found *in vivo*. However, it was more difficult to say whether the same was true for the combinations of heavy and light chains in the Fabs.

### 5.1.2 REPLICON-COMPATIBLE PLASMIDS.

In order to explore this more fully, a set of two replicon-compatible plasmids were developed by Dr. Thomas Collet (Collet *et al.*, 1992; Larimer *et al.*, 1990) which enabled us to clone the heavy and light chains of these fragments separately, one into each plasmid. The plasmids were designed to be compatible with the cloning system already in place for the library construction, and so the heavy and

light chain genes could be transferred directly from one system to the other. This new binary plasmid system facilitated chain shuffling in order to study the extent to which one chain could accept a number of different complementary chains and still retain antigen-binding, i.e.: the phenomenon of chain promiscuity. This could yield information about how restricted the combinations found in the panel of Fabs were, and how likely these combinations were to exist *in vivo*. It would also be interesting from the point of view of verifying how relevant the groupings, made on the basis of heavy chain CDR3 sequence, were to antigen binding.

### **5.1.3 IMPORTANCE OF THE HEAVY CHAIN.**

A pivotal assumption made in the arguments in chapter 4, was that the property of antigen binding for any particular Fab resided mainly with the heavy chain. That assumption was made on the basis of results in this chapter; that an Fab will only retain antigen binding if it contains a heavy chain from a clone that originally bound that antigen. However, the question remained, whether or not the same was true of the light chains. This problem was examined by means of a number of directed crosses involving heavy and light chains from Fabs of two different specificities, known as interantigenic crosses. This type of chain shuffling was particularly attractive as it led to the possibility of combining two different specificities in one Fab which would be desirable for a number of applications such as, bifunctional antibodies for tumour imaging and therapy (Nolan & O'Kennedy, 1990), catalytic antibody production (Lerner &

Tramontano, 1988) and the development of metalloantibodies (Iverson *et al.*, 1990; Iverson & Lerner, 1989).

#### **5.1.4 APPLICATIONS OF CHAIN SHUFFLING.**

The use of antibodies derived from combinatorial libraries for therapy would require that they have reasonably high affinities, preferably in the same range as that of the later immune responses. Although the affinities of our Fabs are quite high ( approximately  $10^8 \text{ M}^{-1}$  ), it would be desirable to increase this value if possible. Methodologies which could be employed, include the introduction of point mutations in V genes using error-prone polymerase and the selection of clones with increased affinity (Hawkins *et al.*, 1992), or shuffling of hypervariable regions according to methods described previously (Jones *et al.*, 1986; Reichmann *et al.*, 1988). Chain shuffling also provides a means to achieve this. The implications of this technique are that we are no longer limited to the combinations of heavy and light chains obtained from the libraries, irrespective of whether or not these are the combinations present *in vivo* . It was possible that shuffling the chains could produce new fragments with increased affinity over the original antibodies. This has been done previously by Marks *et al.*, (1992). Heavy and light chains from 2-phenyloxazol-5-one (phOx)-binding clones were shuffled with light and heavy chain libraries from unimmunised donors, respectively, with the result that a new  $F_v$  fragment specific for phOx was produced upon shuffling, with an affinity 20 times greater than the original antibody.

There are a number of other reasons why it might be useful to shuffle antibody chains. It is often advantageous to produce a number of antibodies of similar specificity but of distinct idiotypic composition. For example, antibodies used for viral neutralisation can be rendered useless through the ability of the virus to form escape mutants (Blattner, 1991). This may be avoided by using a mixture of antibodies with similar, but not identical, epitope specificity. This technique may also be used to eliminate unwanted cross-reactivity while maintaining the desired specificity.

Another study (Kang, *et al.*, 1991) shuffled the chains of a panel of antibodies specific for nitrophenyl phosphoramidate (NPN) and found a very high degree of promiscuity among the chains. Therefore, the possibility of producing antibodies, with new specificities and affinities, by shuffling the chains of the panel of gp120-specific Fabs was also investigated.

Finally, it had been previously reported (Ward *et al.*, 1989) that single VH domains specific for hen egg lysozyme could be prepared with affinities only 10-fold less than the parent F<sub>v</sub> fragments. It was considered interesting to test whether this was the case with this panel of Fabs.

#### **5.1.5 LARGE-SCALE PRODUCTION OF SOLUBLE Fab.**

The final set of results presented in this chapter deal with the production of one of the Fabs on a large scale, and its purification from the culture broth. Large-scale production of purified Fab would be important if these fragments were to be used for therapeutic or diagnostic purposes. It would also be important to have a large

amount of purified protein for a number of techniques such as X-ray crystallography. A crystal structure of a gp120-antibody complex, or even a loop peptide-antibody complex could yield a great deal of information about the mechanism of action of AIDS and its neutralisation. However, as much as a 100mg of pure protein could be needed for this procedure.

A B. Braun Melsungen SG 20l fermenter was used to grow 12 litres of *E.coli* broth to an O.D.(550nm) of between 60 and 80 as described previously (methods section 3.4.4). This meant that the cells could then be separated and lysed, to release the Fab, which was concentrated on a cross-flow filtration system. First step purification of the antibody was then achieved using a zinc metal column (Skerra *et al.*, 1991), since the Fab was engineered to contain a histidine tail at the carboxyl end of the heavy chain. The tail consisted of 5 histidine residues which is sufficient for metal binding. The results of these experiments will be presented and further work in this area will be suggested.



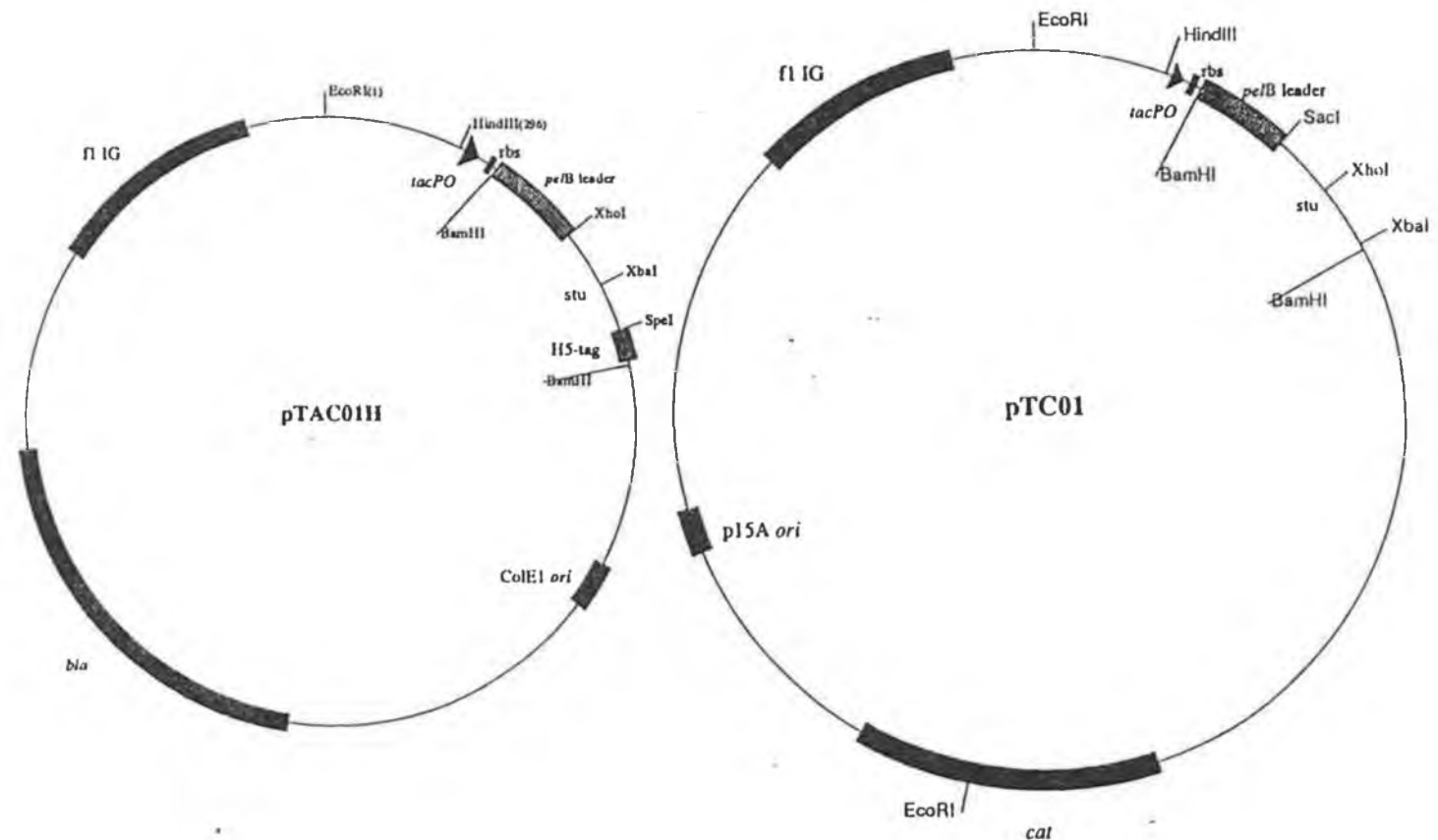
## 5.2 RESULTS

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### 5.2.1 CONSTRUCTION OF THE BINARY PLASMID SYSTEM.

A binary plasmid system consisting of two replicon-compatible plasmids was constructed previously by Dr. Thomas Collet (Figure 5.1). pTC01 was designed for cloning light chains, while pTAC01H was for heavy chain cloning. These vectors are based on the multiple cloning sites of HC2 and LC2 (Huse *et al.*, 1989), the vectors originally used in combinatorial library construction. This means that chains can be directly transferred from one system to the other by restricting with enzymes XhoI and SpeI for the heavy chains or SacI and XbaI in the case of the light chains. The leader sequences and multiple cloning sites were transferred from HC2 and LC2 to vectors pFL281 and pFL261 (Larimer *et al.*, 1990) respectively, using PCR. This provided a flush fusion of the leader sequences to the ribosome binding site (rbs) of each vector, via an internal BamHI site. pTAC01H was then further modified by insertion of a synthetic linker at the SpeI site, incorporating an oligohistidine tail followed by the stop codon TAA (Figure 5.2). This was done to facilitate the purification of the Fab by metal affinity chromatography, since a 5-base histidine sequence will bind metal ions (Skerra *et al.*, 1991). The tacPO represents a trp-lac hybrid promoter-operator, while the rbs is the consensus ribosome binding site sequence AGGAGG (as opposed to the LacZ rbs, AGGA, which resulted in much poorer expression levels). The phage f1 intergenic region was included to facilitate the production of ssDNA for sequencing (see introduction,

section 1.7). Both plasmids have selection markers allowing their maintenance in culture: pTAC01H codes for  $\beta$ -lactamase (bla) which confers resistance to ampicillin and pTC01 codes for chloramphenicol acetyltransferase (cat) conferring resistance to chloramphenicol. Both plasmids can be maintained in culture simultaneously as a result of the compatible origins of replication, ColE1 and p15A. The ratio copy number for these two replicons has been estimated to be roughly 2:1, ColE1:p15A. Expression of soluble Fab from these plasmids is partially suppressed by the addition of glucose and is induced by the addition of isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) at 30°C.



**Figure 5.1:** Plasmid maps of the replicon compatible chain shuffling vectors. pTC01 was designed for light chain cloning and pTAC01H for the heavy chains. Both plasmids contain a stuffer fragment between their respective restriction enzyme sites ready for replacement by the appropriate antibody chain. Maps are drawn approximately to scale.

tac promoter rbs  
 TGT TGACAATTAATCATCGGCTCGTATAAT GTGTGGAATTGTGAGCGGATAACAATTTACAC AGGAGGAA  
 ACA ACTGTTAATTAGTAGCCCGAGCATATT ACACACCTTAACACTCGCTATTGTTAAAGTGT TCCTCCTT  
  
 BamHI pelB leader  
           M K Y L L P T A A A G L L L L A A Q P A M A  
GGATCCATGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTTATTACTCGCTGCCCCAACCAGCCATGGC  
 CCTAGGTACTTTATGGATAACGGATGCCGTCGGCGACCTAACAATAATGAGCGACGGGTTGGTCGGTACCG  
  
           SacI XhoI XbaI  
           E L STOP  
 CGAGCTCGGTCGGTCCTCGAGGGTCGGTCGGTCTCTAGAGTTAAGCGGCCGC  
 GCTCGAGCCAGCCAGGAGCTCCCAGCCAGCCAGAGATCTCAATTCGCCGGCG

5.2(a)

tac promoter rbs  
 TG TGACAATTAATCATCGGCTCGTATAAT GTGTGGAATTGTGAGCGGATAACAATTTACAC AGGAGGAA  
 AC ACTGTTAATTAGTAGCCCGAGCATATTA CACACCTTAACACTCGCCTATTGTTAAAGTGT TCCTCCTT  
  
 BamHI pelB leader  
           M K Y L L P T A A A G L L L L A A Q P A M A  
GGATCCATGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTTATTACTCGCTGCCCCAACCAGCCATGGC  
 CCTAGGTACTTTATCCATAACGGATGCCGTCGGCGACCTAACAATAATGAGCGACGGGTTGGTCGGTACCG  
  
                   XhoI XbaI SpeI (His)5-tail  
           Q V K L L E T S H H H H H  
 CCAGGTGAAACTGCTCGAGGGTCGGTCGGTCTCTAGACGGTCGGTCGGTC ACTAGTCATCATCATCAT  
 GGTCCACTTTGACGAGCTCCCAGCCAGCCAGAGATCTGCCAGCCAGCCAGTGATCAGTAGTAGTAGTA  
  
 STOP  
 TAAGCTA  
 ATTCGAT

5.2(b)

**Figure 5.2:** Nucleotide sequence of the multiple cloning site in a) the light chain vector, pTC01 and b) the heavy chain vector, pTAC01H. Relevant restriction sites are underlined. The *tac* promoter and ribosome binding site (rbs) are indicated by boxes (Collet *et al.*, 1992). The amino acid sequence of the *pleB* leader is also given.

### 5.2.2 Direct Cross of Heavy and Light Chains.

As was previously described (chapter 4), a large panel of gp120IIIB binding Fabs had been isolated from the bone marrow of a single asymptomatic HIV patient and these could be assigned to one of seven groups based on the sequence of the CDR3 region of the heavy chains. For the purposes of the chain shuffling experiment, twenty Fabs were chosen representative of the seven gp120 binding groups (b1,b14,b24; b4,b7,b12,b21; b6;b8,b13,b18,b22,b27,B26,B8,B35,s4; b11; b3; and s8). The V3 loop peptide binding clone, p35, was also chosen. The heavy and light chains of all twenty one Fabs were cloned separately into the binary plasmids and directed crosses were carried out by co-transformation of two plasmids, resulting in 441 (21x21) shuffled fragments. The binding of these Fabs to gp120 was then analysed by ELISA and the results are given in Table 5.1. Unpurified *E. coli* supernatants were used in all cases and a clone was determined to be positive if it gave a reading of 3 times above background level or higher. The negative control used in all experiments was supernatant from *E. coli* not expressing Fab. Clones are sorted according to the groupings already established. The diagonal from top left to bottom right represents the original combinations of chains and these are all positive, as expected. The exception here is the loop binder, p35, which does not bind gp120. However, binding to V3 loop was maintained in the cross containing the original two chains of p35.

	b11	b6	b4	b12	b7	b21	b3	b13	b22	B26	b8	b18	b27	B8	B35	s4	b1	b14	b24	s8	p35
b11	+	+	w	+	+	-	+	+	-	-	+	-	-	-	-	-	+	-	-	-	-
b6	+	+	+	+	+	-	+	+	-	-	+	-	-	-	-	-	+	+	+	-	-
b4	+	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-
b12	+	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-
b7	+	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-	w	-	-	w	-
b21	+	+	+	+	+	+	+	-	-	-	+	-	-	-	-	-	+	-	-	-	-
b3	+	+	-	+	+	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-
b13	w	w	-	+	+	-	-	+	+	+	+	+	+	+	+	+	-	-	-	w	-
b22	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
B26	w	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
b8	+	+	+	+	+	-	w	+	+	+	+	+	+	+	+	+	-	-	-	-	-
b18	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	w	-	-
b27	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
B8	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
B35	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	-	w	-	-
s4	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
b1	+	+	+	+	+	-	-	+	w	-	+	w	-	-	-	-	+	+	+	-	-
b14	w	+	+	+	+	w	-	+	-	-	+	-	-	-	-	-	+	+	+	-	-
b24	+	+	+	+	+	-	+	-	-	-	+	-	-	-	-	-	+	+	+	-	-
s8	w	+	+	+	+	-	w	+	+	w	+	+	+	+	+	+	-	-	-	+	-
p35	+	+	+	+	+	-	+	w	w	w	+	w	w	w	w	+	w	-	-	-	•

**Table 5.1:** Complete set of directed crosses between heavy and light chains of all selected Fabs isolated from the original library. Heavy chains are listed horizontally and light chains are listed vertically. The clones are sorted according to the groups previously discussed, with different groups being separated by horizontal and vertical lines. + indicates a positive signal of 3 times background or greater; - denotes a negative signal and w stands for a weak positive.

### 5.2.3 HEAVY CHAIN PROMISCUITY.

An initial observation is that within any particular group, there is complete promiscuity of the heavy and light chains. In other words, any heavy chain from a group can combine with any light chain from that group and retain binding to the antigen. This lends validity to the system of grouping on the basis of H chain CDR3 sequence, at an antigen binding level at least and emphasises the importance of the heavy chain in this role. If chains from Fabs outside the group are used it can lead to a general lack of promiscuity and an inability to retain antigen binding. For example, there are very few binders amongst the crosses between the groups b1-b24 and b13-s4.

The degree of promiscuity in general was surprising, in that a given heavy chain could recombine with a number of light chains with retention of binding. This ability to productively pair with a number of light chains varied considerably, and seemed to be strongly dependent on the heavy chain sequence. Five of the heavy chains, b11HC, b6HC, b12HC, b7HC and b8HC could productively pair with any other light chain in the panel studied, including that from the loop binder. Even the least promiscuous chain from the Fabs panned with gp120IIIB, b14HC, still did so in 18% of it's crosses. An attempt was made to correlate promiscuity and amino acid sequence. However, sequence alignments between the least and most promiscuous heavy chains did not reveal any such connection (Figure 5.3). For example, b6HC, a very promiscuous heavy chain, exhibits very little sequence homology with either b7HC, also very promiscuous, or b1HC, one of the least promiscuous chains. The same can be said from a number of such comparisons considered during the analysis of these crosses.

Unlike the heavy chains, there were no light chains that would cross productively with all heavy chains, nor were there any that were distinguishable from the other light chains by unusually low promiscuity. The high degree of promiscuity among heavy chains, however, could have important implications for the combinatorial library, and how it relates to the *in vivo* situation.



CLONE	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
b6	LEESGGGLVKPGGSLRLSCVGSGETFS	SAWMA	WVRQAPGRGLEWVG	LIKSKADGETTDYATPVKG	RFSISRNNLEDTVYLQMDSLRADDVAVYYCAT	QKPRYFDLLSGQYRRVAGAFDV	WGHGTTVTVPAST
b12	..Q..AEVK...A.VKV..QA..YR..	NFVIH	.....QRF...M.	W.NPYNGNKEFSAKFQD	.VTFTADTSAN.A.MELR...SA.....R	VG.YSW.DSPQDNYYMDV	..K.....
b11	..Q...V.....E.....P	N...T	...S..K....A	S....F..GSPH..A..E.	..T...D...K.F...NG.K.E..G.....	RY...SEMMG.VKHIFYMDV	..K...S..S...

(A)

b6	LEESGGGLVKPGGSLRLSCVGSGETFS	SAWMA	WVRQAPGRGLEWVG	LIKSKADGETTDYATPVKG	RFSISRNNLEDTVYLQMDSLRADDVAVYYCAT	QKPRYFDLLSGQYRRVAGAFDV	WGHGTTVTVPAST
s8	.....Q..R...V..EA.....	.YE.N	.....K.....S	Q.S.SGSRTYYADSVKG	..T...D.AKNSL..E.T...V.....H	GRRLVTYGGVVSNGNI	..Q..M....S...
b14	.....TEFKP..S.VKV..KA..G..G	DYASNYAIS	WVRQAPGQGLEWVG	G.TPTSGSADYAQKFG	.VT...ADRFTFIL.MELR...IE...I....R	ERREKGNPNRALRGALDF	..Q..R.F.....

(B)

**FIGURE 5.3:** Amino acid comparison of the most promiscuous heavy chain, b6HC, with other promiscuous chains, b12HC and b11HC and the least promiscuous chains, b14HC and s8HC. Similarities are indicated by dots; differences are given. No sequence patterns emerge between promiscuous and non-promiscuous chains.

Most crosses using heavy chains with identical variable region sequences gave identical binding patterns. For example: those crosses involving b18HC, b27HC, B8HC, B35HC and s4HC all show identical binding patterns. However, there are some minor differences between the b12HC and b4HC (both are identical in their VH region sequences) crosses. In addition to this, all crosses involving b8HC are positive, despite the fact that b8VH is identical in sequence to b18VH, b27VH, B8VH, B26VH and s4VH. This may be due to differences in expression levels in the cell, or it could be attributed to sequence differences in the constant regions of these chains. This can not be confirmed as these regions were not sequenced. Also, it is not known if differences in the constant regions alone are capable of affecting antigen binding properties to this extent. Having said this, it should be noted that maintenance of amino acid differences in constant region alleles by selective pressure, (Schreier *et al.*, 1981) would seem to suggest that the sequence of these regions is critical for optimal antibody function.

#### **5.2.4 AFFINITY MATURATION.**

The question was then addressed as to whether or not the chain shuffling process could produce Fabs of altered affinity, and so could be used as a means of affinity maturation within a predefined set of antibodies. The affinities of a number of selected clones were measured by competitive ELISA, using gp120 as the competing antigen. A number of series of clones were selected with the same heavy chain and different light chains. The results for two of these series, b12HC and b13HC, are shown in Figures 5.4(a) and (b). No

significant differences were detected between the clones in any series tested (5 other sets were also tested, with the same result. data not shown), leading us to believe that, within this panel of antibodies, it was not possible to improve on affinity by chain shuffling.

It is also interesting to note that no single chain by itself, either heavy or light, could bind antigen, as detected by ELISA which may suggest that the phenomenon of domain antibodies (dAbs) does not exist in the context of this set of gp120 binding Fab fragments.

Figure 5.4(a)

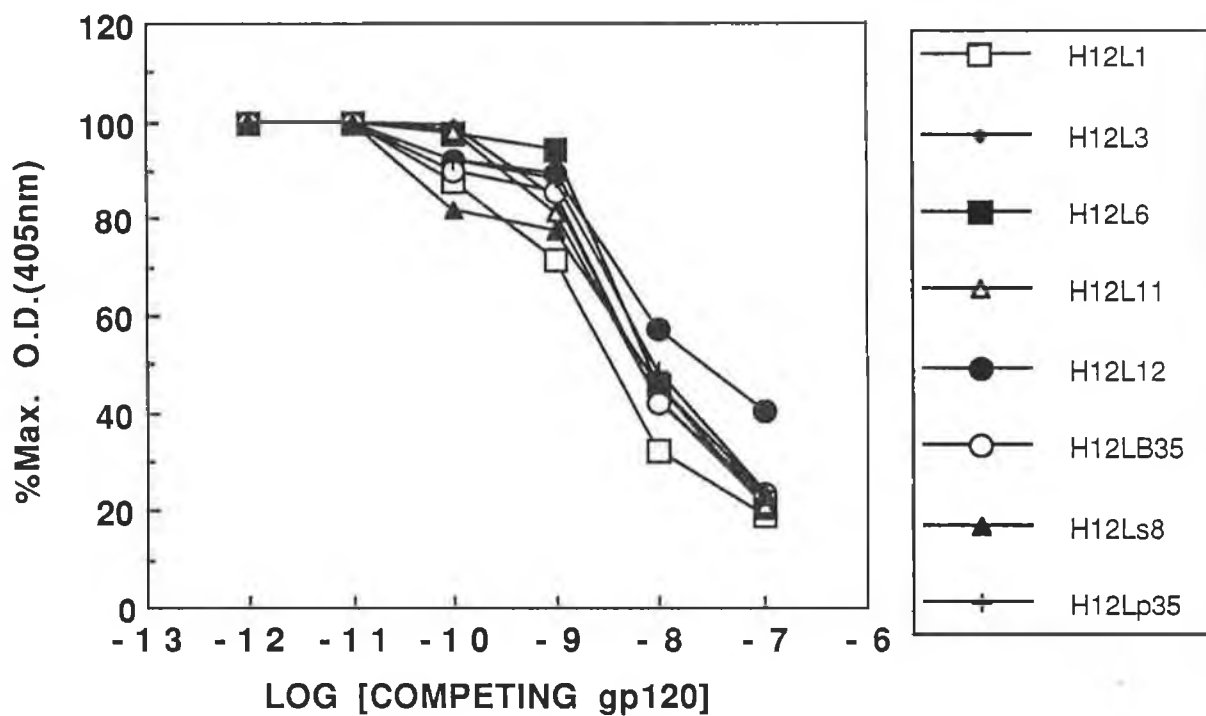
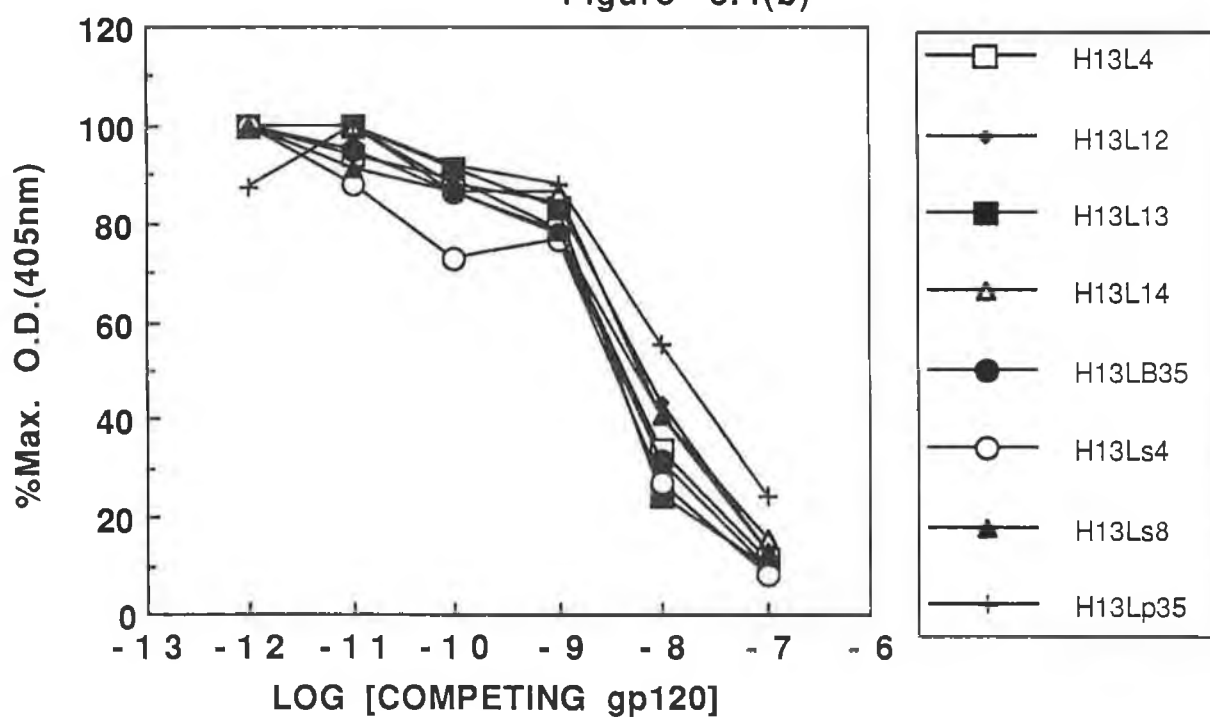


Figure 5.4(b)



### **5.2.5 INTERANTIGENIC CROSSES OF HEAVY AND LIGHT CHAINS.**

To determine if the promiscuity observed within this library of clones could be extended to other high affinity binders derived from a non-related library, an Fab fragment specific for tetanus toxoid (Persson *et al.*, 1991), P3-13, was chosen for a new set of crosses. The P3-13 heavy chain was crossed with all light chains from the gp120 library, and conversely, the heavy chains were all crossed with the P3-13 light chain. All crosses were then assayed for binding to both tetanus toxoid antigen and gp120 (Table 5.2).

Clone	P3-13HC with all light chains		P3-13LC with all heavy chains	
	TT antigen	IIIB gp120	TT antigen	IIIB gp120
b1	-	-	-	-
b3	-	-	-	6.3x10
b4	2.5x10	-	-	-
b6	-	-	-	2.5x10
b7	-	-	-	-
b8	-	-	-	-
b11	-	-	-	3.2x10
b12	6.3x10	-	-	-
b13	-	-	-	-
b14	w	-	-	w
b18	-	-	-	-
b21	3.2x10	-	-	-
b22	-	-	-	-
b24	-	-	-	-
b27	-	-	-	-
B8	-	-	-	-
B26	-	-	-	-
B35	-	-	-	-
s4	-	-	-	-
s8	-	-	-	-
p35	-	-	-	-
P3-13	6.3x10	-	-	-

It can be seen from the table that the degree of promiscuity is far less than previously observed within the set of gp120 binders, as expected, since the two chains are obtained by panning with completely different antigens. There are, however, a number of crosses that still exhibited some antigen binding. P3-13HC crossed with light chains from b4, b12 and b21 bound tetanus toxoid (TT) and similarly, heavy chains from b3, b6 and b11 crossed with the P3-13 light chain bound gp120. Fab b14 gave weak binding when crossed with P3-13 in both cases, but this was too weak to be used in any further analyses. Therefore, it is apparent that a Fab will only bind the antigen if the heavy chain of that Fab originally came from an antibody specific for that antigen. The converse is not the case: light chains do not possess the ability to confer antigen binding on an antibody. This is confirmed by the fact that none of the P3-13LC crosses bound TT and none of the gp120-bindingLC crosses bound gp120. It is also supported by X-ray crystallography data showing that it is only the heavy chain that provides the surface topography and steric complementarity for antigen binding in the active site of the antibody. Therefore, "the combination of the heavy chain with any light chain that allows the formation of the combining site without creating unfavorable steric or electrostatic interactions should preserve a significant proportion of the antibody specificity" (Sastry *et al.*, 1989).

The ability of a heavy chain to recombine with an unrelated light chain does not seem to be totally related to the promiscuity displayed by that heavy chain within a set of related clones. This is demonstrated by the fact that b3HC crosses productively with P3-

13LC and yet, within the gp120 crosses, it displays a lower rate of promiscuity (81%) than any of the clones b4HC (88%), b12HC (100%) or b7HC (100%), none of which cross productively with P3-13LC. This may be related to the fact that all three of the above chains originate from the same sequence group and are incompatible with P3-13. In addition to this, the three light chains giving rise to productive crosses with the P3-13HC all originate from the same group (b4LC, b12LC and b21LC). Therefore, it seems likely that the compatibility of a combination of heavy and light chains is highly sequence dependent and that a promiscuous sequence within a group of related clones may not be suitable to recombine with chains outside that group.

#### **5.2.6 AFFINITIES OF INTERANTIGENIC CROSSES.**

The affinities of this set of clones was also analysed (Figure 5.5(a) and (b)). Although the affinities of the interantigenic crosses were quite similar to each other, there was a significant difference between these values and the affinities of the Fabs containing the original combination of heavy and light chain. The replacement of the P3-13LC in the P3-13 Fab with another unrelated light chain, lowered the affinity of the fragment by a factor of 10-100 times (from  $6.3 \times 10^8$  to  $6.3 \times 10^6$  M<sup>-1</sup>). Similarly, the affinity of the gp120 fragments was lowered by a factor of approximately 10 times.



Figure 5.5(a)

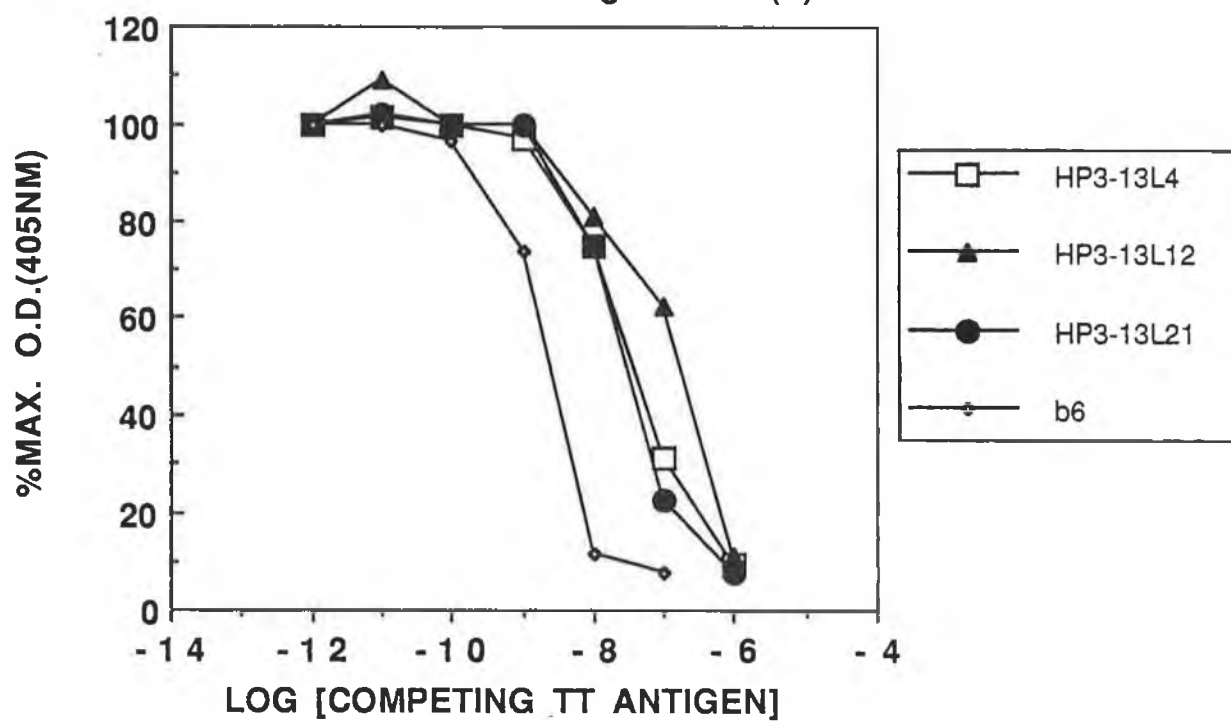
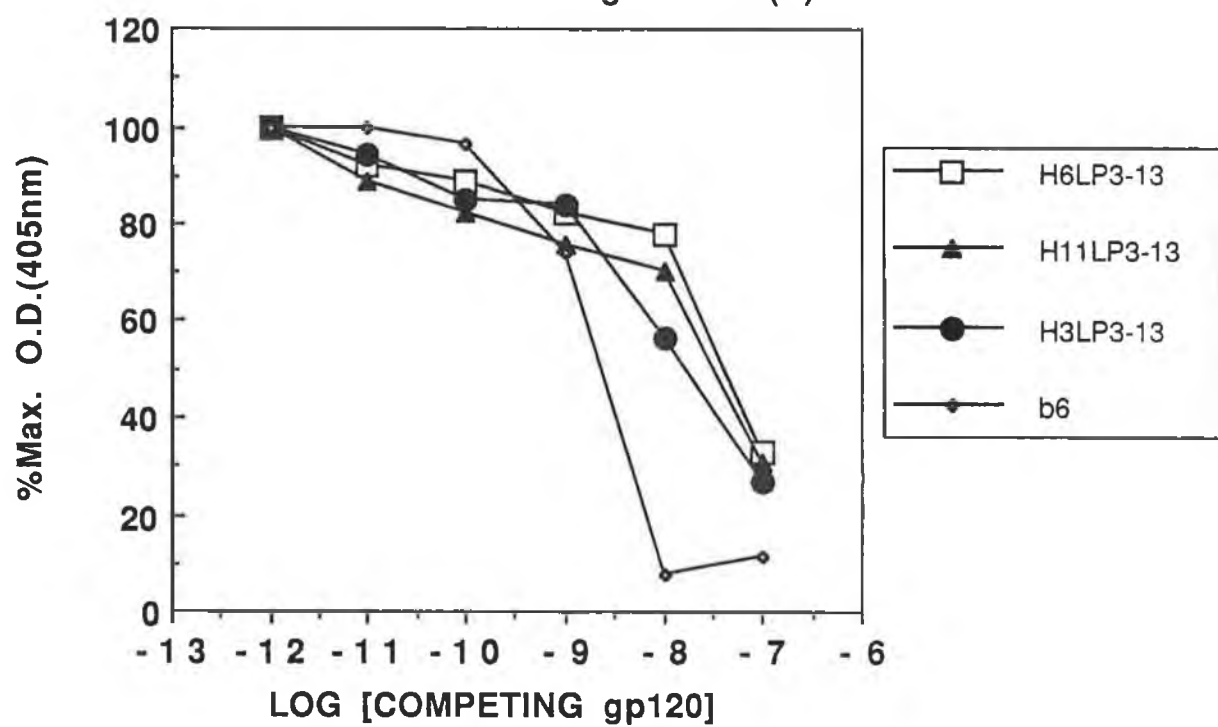
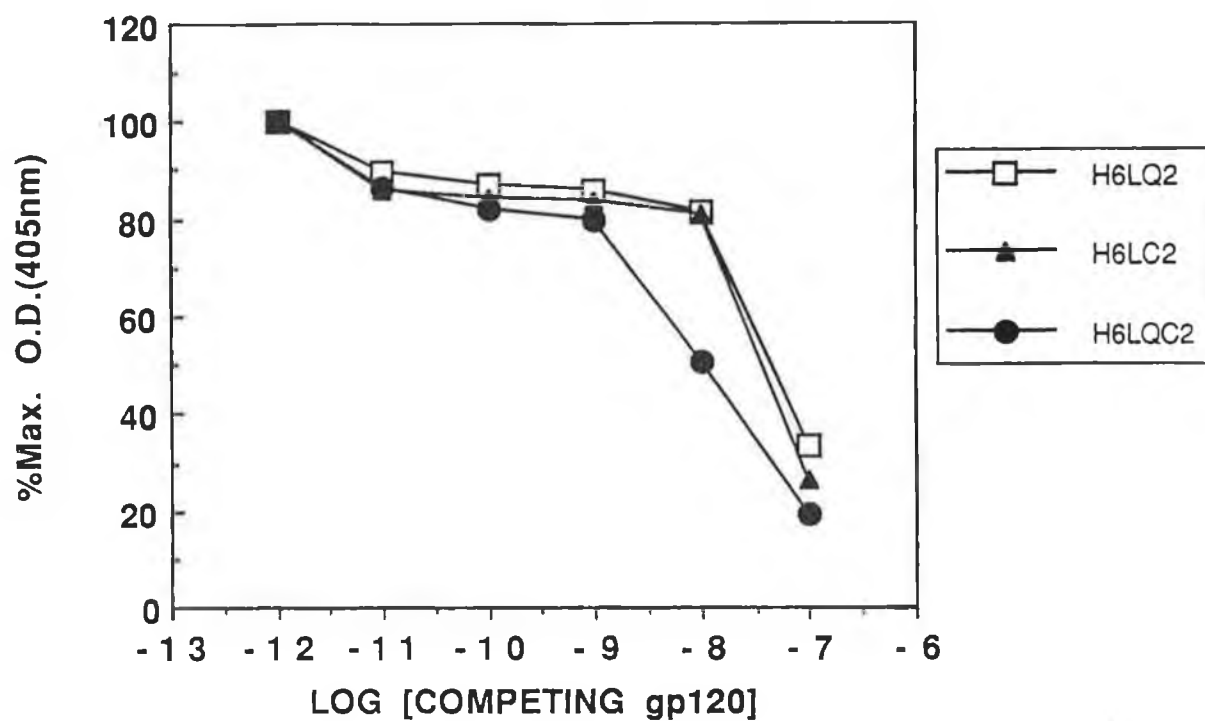


Figure 5.5(b)



A similar cross was attempted with a fluorescein-specific mouse light chain (Sastry *et al.*, 1989; Iverson *et al.*, 1990). Three mutant variants of this light chain incorporating histidine residues at 6 different locations (see chapter 7) were each combined with all of the gp120 heavy chains and binding of gp120 was tested by ELISA. The only heavy chain that showed a productive cross with these light chains was b6HC. The affinities of the 3 crosses ranged from 1 to  $6 \times 10^7 \text{ M}^{-1}$  (Figure 5.6), which is 8 to 50 fold lower than the affinity of the original b6 Fab ( $5 \times 10^8 \text{ M}^{-1}$ ). The level of expression of these Fabs in the cells was very low, however, making it extremely difficult to produce enough Fab for purification, and so further studies with these clones were discontinued in favour of another line of research (see chapter 7).

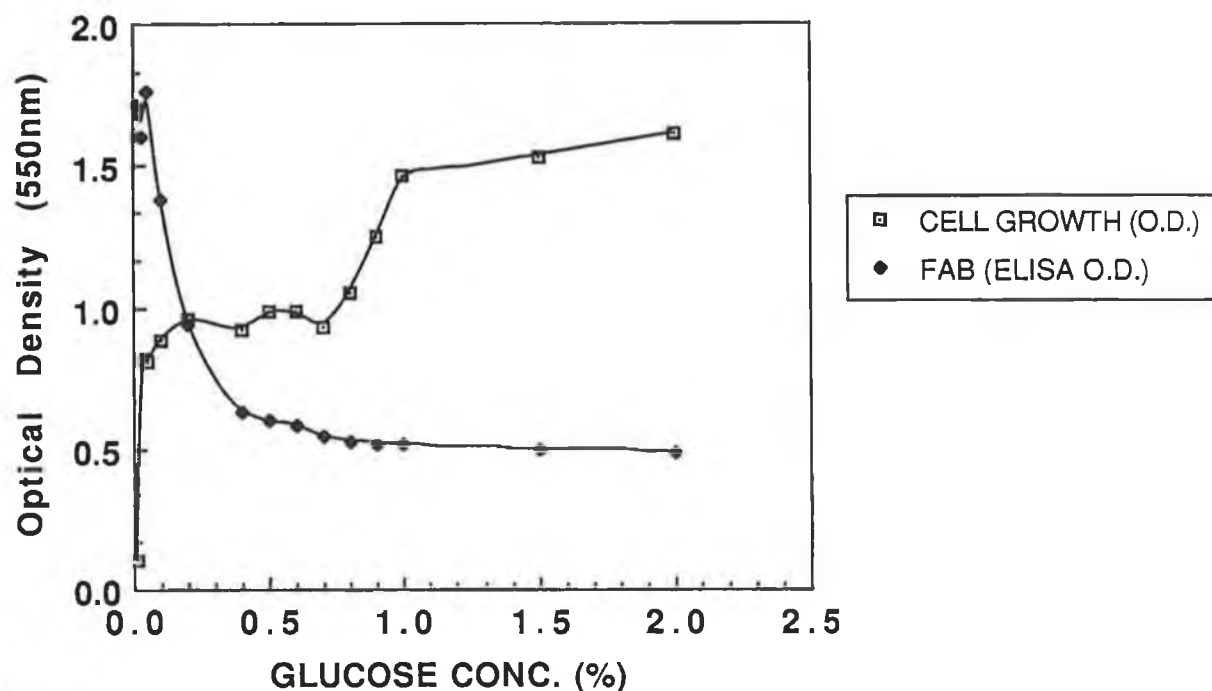


**Figure 5.6:** Affinities of the b6HC with the three fluorescein-specific light chains (Q2, C2 and QC2). All have affinities of the order of  $10^7 \text{ M}^{-1}$ . However, expression levels were too low to continue the analysis of these clones.

### **5.2.7 Large Scale Production of Soluble Fab by Fermentation.**

A minimal medium was chosen for fermentation purposes, for a number of reasons. Firstly, all the components of such a medium are added individually, and so it is completely defined. This ensures that the operator has complete control over all components of the medium and can alter each one to suit the requirements of any particular fermentation run. Secondly, commercially available media often contain ingredients which may interfere with purification protocols, especially when large volumes are concerned. Thirdly, a minimal medium is cheaper to produce. This is of critical importance when volumes of 10 to 100 litres of medium are used in each run. The medium used has been previously described in the methods section (Section 3.4.4; Dreyfus *et al.*, 1992). A number of carbon sources were assayed for support of bacterial growth and compatibility with expression of the soluble Fab. The compounds chosen were glucose, lactose, sodium citrate, succinic acid and maltose. A 2.5%(w/v) of each of the above was added to 500ml samples of minimal medium and Fab b13 was grown overnight at 37°C. The O. D. of the cultures were measured the next day. Glucose supported the highest levels of growth, resulting in O.D.(550nm) values of 10.0. Succinate and citrate gave O.D.(550nm) values between 6.0 and 8.0, while lactose and maltose proved to be unsuitable for obtaining high yields of cells. Therefore, glucose was chosen as the carbon source. However, as was mentioned earlier, glucose inhibits the expression of Fab from the plasmids, so the levels of glucose that would support adequate growth, while

permitting induction of Fab expression had to be investigated. 10ml cultures of b13 were grown for 10 hours at 37°C in the presence of varying concentrations of glucose. 5ml of these cultures were allowed to continue growth overnight while 5ml were removed and transferred to 30°C where they were induced with 1mM IPTG overnight (Figure 5.7). Glucose levels below 0.025%(w/v) did not support growth and a high level of cell lysis was obvious in these cultures. Levels above 0.2%(w/v) inhibited expression of Fab as determined by ELISA, presumably due to the presence of residual glucose at the time of induction. On the basis of this result, 2%(w/v) glucose was used in all subsequent fermenter runs during the cell growth phase and the level was then allowed to drop to below 0.2%(w/v) before induction. It was also necessary to add an alternative carbon source at this stage or cell lysis would occur. Therefore it was decided to add 1%(w/v) sodium citrate at induction.



**Figure 5.7:** Graph showing the effects of glucose levels on growth and protein expression. Glucose levels above 1.0%(w/v) resulted in maximal cell density, while levels above 0.2%(w/v) caused a significant reduction in the expression of protein. A certain minimum level of protein is always expressed as glucose does not completely suppress the *tac* promoter.

Twelve litre fermentation runs were carried out as described in the methods section 3.4.4. An example of such a run with b13 is given in Table 5.3. When the culture had reached an O.D.(550nm) of 60.0, it was decided to allow the glucose levels to fall below 0.2% and then induce Fab expression. The rate of cell growth slowed down over the next 75mins such that there was an increase of only 4 O.D. units by the time the glucose levels had dropped to 0. Glucose levels were

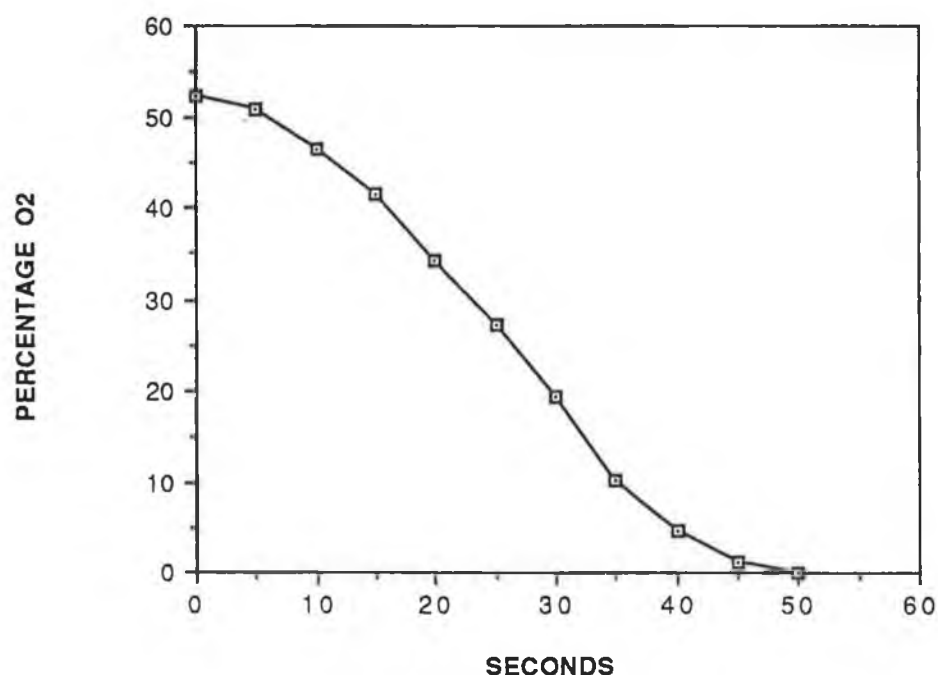
measured by means of a glucometer commercially available for diabetes patients. The culture was then induced with IPTG and 1% (w/v) citrate was also added to maintain cellular integrity. The addition of base increased until induction in order to neutralise acidic products being produced by the growing cells. After induction, there was a certain amount of cell lysis, releasing basic cellular components which were neutralised with the addition of acid.

	O.D.(550)	GLUCOSE	OXYGEN	ANTI-FOAM (SECONDS)	pH	ACID (SEC)	BASE
21:30	0.33	HIGH	100%	1	7.0	0	0
8:30	29.0	LOW	64%	49	7.0		
	ADDED 2% GLUCOSE						
9:20	33.0	HIGH	63.4%	53	7.0		
10:00	48.5	HIGH	63.4%	54	7.0	25	1912
10:30	51.5	HIGH	61.6%	57	7.0	25	1993
	ADDED 1% GLUCOSE. BOOSTED THE OXYGEN						
11:00	54.5	HIGH	64%	66	7.0	25	2054
11:30	60.0	HIGH	61.4%	78	7.0	25	2138
12:00	61.8	0.5%	61.3%	81	7.0	25	2257
12:15	62.1	0.38%	62.5%	84	7.0	25	2329
12:30	62.5	0.25%	60.5%	90	6.9	30	2428
12:45	64.0	LOW	66.4%	90	6.9	32	2507
	INDUCED WITH 1mM IPTG AND 1% CITRATE						
15:45	69.3	LOW	87.7%	228	7.0	52	2507
18:45	76	LOW	91.6%	228	7.0	62	2507

**Table 5.3:** Table of readings taken during the fermenter operation. The times each reading was taken is listed in the left hand column. 30ml samples were taken at each time point for measurement of cell density by O.D.(550nm). The glucose level of each sample was measured using a glucometer. All other parameters were measured directly from the fermenter control panel.



The factor most limiting to maximal cell growth was the dissolved oxygen content in the fermenter. Oxygen levels were maintained at approximately 60% of maximum while the cells were growing. This increased to 90% after induction as the cells were not now growing as rapidly and so, did not utilise the oxygen as quickly. The entire supply of oxygen in the vessel would be used in 40 seconds if the supply was stopped (Figure 5.8). This meant that the maximal oxygen flow had to be maintained in order to achieve sufficiently high cell numbers.



**Figure 5.8:** Graph showing the depletion of oxygen in the fermenter vessel over time, when the external oxygen supply is temporarily cut off.

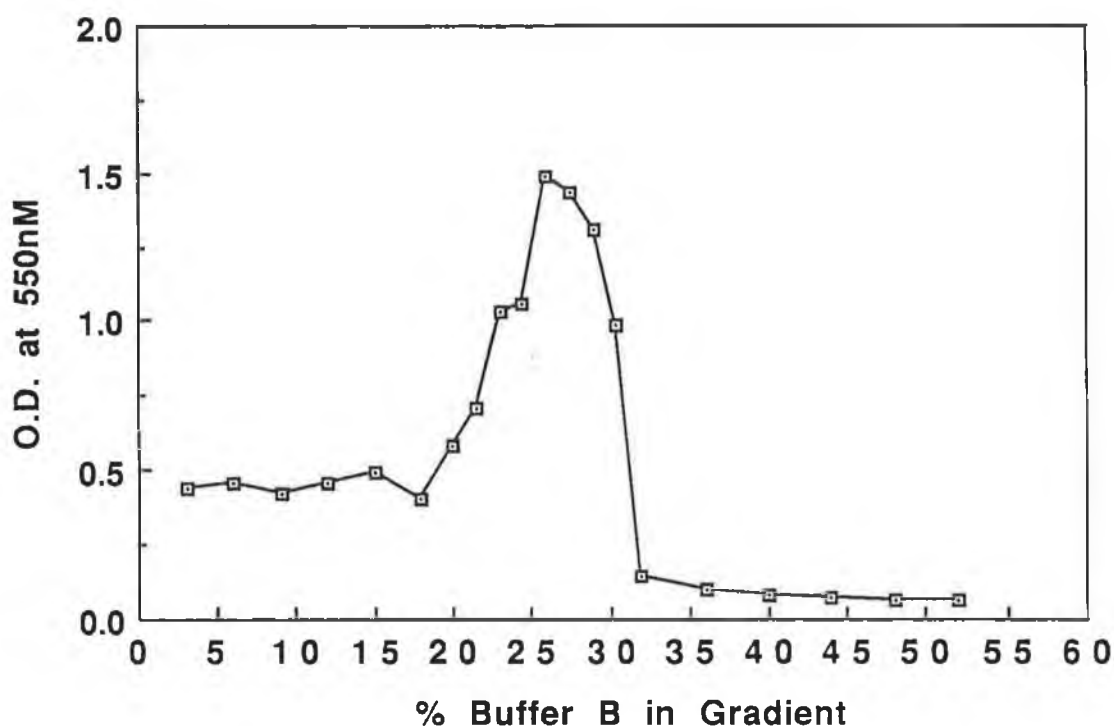
#### **5.2.8      DOWNSTREAM PROCESSING.**

Subsequent processing of the fermenter contents are shown in figure 5.9. The cells and supernatant from the broth were separated before processing. The supernatant was filtered and concentrated, while the cells were broken, first by periplasmic lysis (mixing at 4°C for 1 hour in the presence of PMSF) and then by means of a French Pressure Cell. The fermenter supernatant and lysed cell fractions were both analysed by ELISA for Fab content (fractions 1, 2, 3 and 4). It was found that the French Pressure Cell released most Fab from the cells as determined by ELISA (Table 5.4). Sonication was also an efficient technique, however, this became impractical at high volumes.

<b>FRACTION</b>	<b>O.D.(550nm)</b>
<b>FILTRATE 1 1LITRE</b>	<b>0.538</b>
<b>FILTRATE 2 1.5LITRES</b>	<b>0.594</b>
<b>CONC. FILTRATES</b>	<b>0.900</b>
<b>PERIPLASMIC LYSATE 3 (200ml)</b>	<b>0.810</b>
<b>FRENCH PRESS LYSATE 4 (300ml)</b>	<b>0.994</b>
<b>COLUMN FLOWTHROUGH</b>	<b>0.469</b>

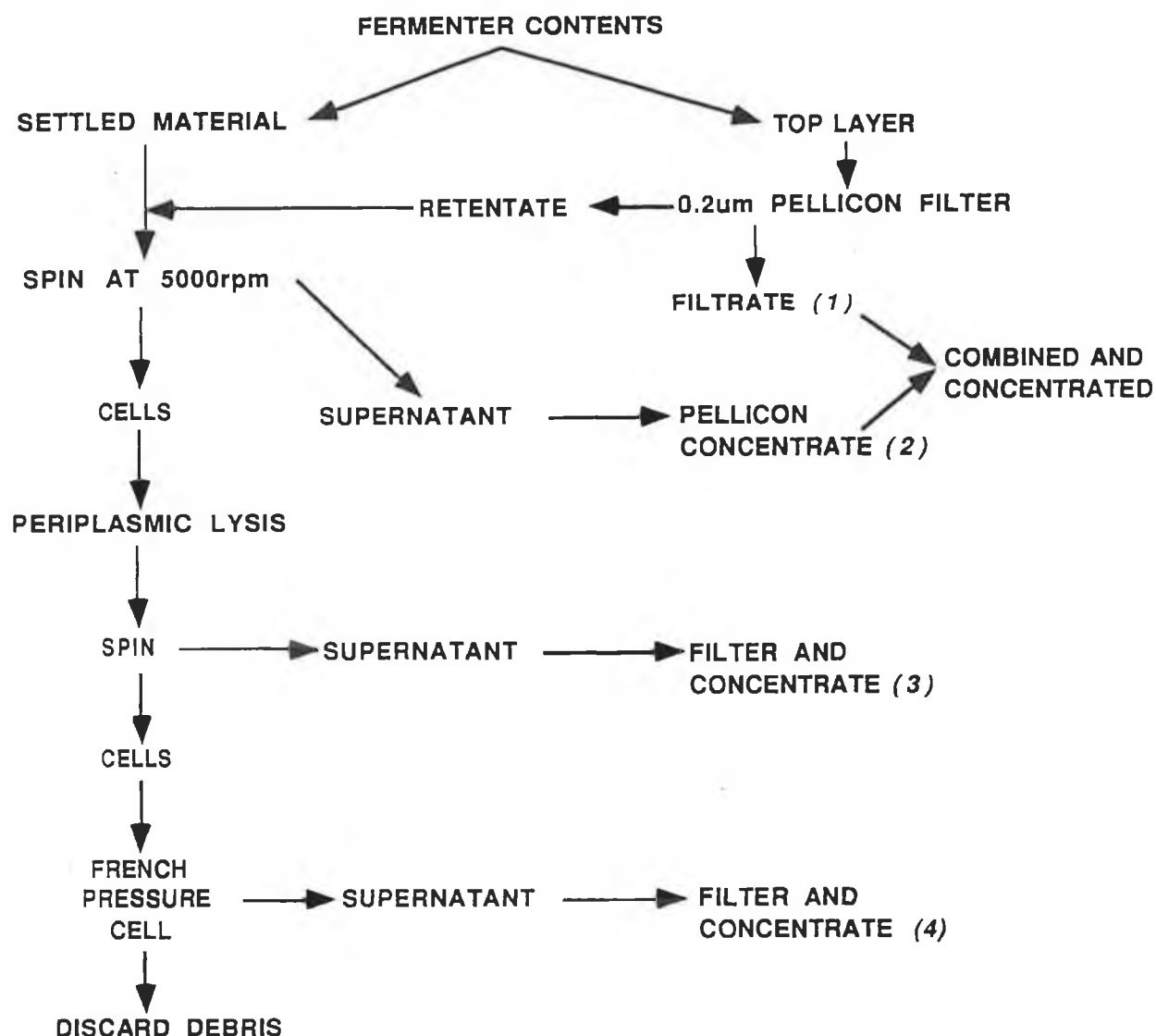
**Table 5.4:** ELISA analysis of samples taken a different stages of the downstream processing procedure. Numbers given are the O.D.(405nm) values. Filtrates 1 and 2 represent the supernatant of the fermentation broth and can be seen to contain Fab, which is retained upon concentration (row 3). The cell pellet was lysed, first by periplasmic lysis and then using a french press. It is evident from the O.D.(405nm) that the french press is more effective at releasing Fab. Finally, the metal column flowthrough also contains Fab, demonstrating that the column was overloaded.

All four fractions were combined and purified on the metal column. The fractions were eluted using a histidine/imidazole gradient and tested by ELISA. The Fab peak (Figure 5.10) was found to be eluted between 17% and 33% buffer B (Buffer B contained 100mM histidine and 0.5M imidazole).



**Figure 5.10:** Elution of Fab from the metal column as measured by performing an ELISA on each fraction. The elution was carried out in an imidazole/histidine gradient with 2 buffers: A was a phosphate buffer (50mM sodium phosphate; 1M NaCl, pH7.0) and B was the eluting buffer (50mM sodium phosphate; 1M NaCl; 100mM histidine; 0.5M imidazole, pH6.0). The Fab eluted at approximately 25mM histidine and 125mM imidazole.

These fractions were combined to give a final volume of 300ml containing 1mg/ml protein as determined by O.D. at 280nm. A sample of this was examined by SDS gel electrophoresis and was found to contain a band at 50kDa (corresponding to the size of an Fab fragment) representing approximately 30% of the overall protein content. It is likely, therefore, that this sample contained approximately 100mg of Fab protein at 30% purity. Further purification of a small fraction of this sample was carried out on a goat anti-human F(ab')<sub>2</sub> column by Diane Schoedler (Scripps Research Institute, La Jolla CA), resulting in 100% purified Fab (data not shown). However, purification of the entire sample was not performed due to the limited capacity of these columns.



**Figure 5.9:** Downstream processing of the fermenter contents after completion of the run. The supernatants were concentrated on a 10000 molecular weight cut-off membrane using the Pellicon filtration apparatus. The cell supernatants were then further concentrated on a similar membrane using a 250ml Amicon positive pressure filtration apparatus. All concentrates, labeled 1,2,3 and 4, were combined and purified by metal affinity chromatography.

## 5.3 DISCUSSION

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### 5.3.1 BINARY PLASMID SYSTEM.

The binary plasmid system presented here, in conjunction with chain shuffling, is another approach aimed at extending the range of possibilities already exploited with combinatorial library technology, and so extend antibody diversity beyond that found in nature. This type of binary system has been proven before as a means of identifying the relative contribution of two subunits of an enzyme to the active site (Larimer *et al.*, 1987). The overriding feature of these plasmids is the ability to create large numbers of directed crosses between characterised light and heavy chains without the need for recloning individual chains for each cross after the initial vector construction. In other words, each chain need only be cloned once, after which, any number of crosses can be achieved with that chain. The viability of these plasmids for the expression of a heterodimer protein has been proven by the ability to regain antigen binding with comparable levels of expression and affinity, when heavy and light chains corresponding to those in the original Fab are recombined. This was the case for all of the Fabs analysed (diagonal of Table 5.1).

### 5.3.2 HEAVY CHAINS AND ANTIGEN-BINDING.

Antibody structure has been a topic of investigation for over thirty years, and the presence of two distinct subunits was first discovered in the early sixties (Small *et al.*, 1963; Utsumi and

Karush, 1964). Since then, a great deal of progress has been made in the study of antibody structure (Chothia *et al.*, 1989; Alt *et al.*, 1992) and production (Kohler and Milstein, 1975; Huse *et al.*, 1989). However, one issue, very important for the construction of most antibody fragments (including catalytic antibodies), that has not been satisfactorily resolved, has been the relative contributions of the heavy and light chains to antigen-binding and the mechanisms by which these chains combine to produce a functional antibody. It has long been thought that the heavy chain contributes more to this function as single heavy chains in isolation have been shown to retain part of the antibody activity (Jaton *et al.*, 1968) albeit with an affinity of 10 to 100-fold less than the complete antibody. Light chains do not seem to possess this ability (Utsumi and Karush, 1964). However, the loss in affinity exhibited by single heavy chains can be restored to a certain extent by recombining these chains with a nonspecific light chain. Since then, it has been demonstrated that the majority of electrostatic and steric interactions in the antigen binding site come from the heavy chain (Sastry *et al.*, 1989), indicating that it is more important for antigen binding. This group went on to postulate that the combination of a heavy chain with any light chain which did not disrupt the interactions in the binding pocket, would preserve the specificity of the heavy chain.

This binary plasmid system provided the means to study these issues more closely. It was apparent that there was a wide diversity in the degree of promiscuity among the heavy chains, ranging from 18% to 100%, depending on the heavy chain, while the same differences were not observed among the light chains. Antigen



binding by a particular clone, therefore, seemed to be very strongly dependent on the sequence of its heavy chain. This supports the data previously published. The degree of promiscuity of a heavy chain can be more specifically attributed to the CDR3 sequence, since, when all the Fabs were arranged according to this sequence, crosses within a group were always productive, while this was not necessarily the case outside of a group. Therefore, in order to preserve a significant portion of antibody specificity, it seems that the light chain must be compatible with the CDR3 of the heavy chain. This cannot explain the variations in promiscuity completely, however, as b13HC and b8HC are far more promiscuous than other members in the same group, although they are identical in the sequence of all 3 CDR regions. Another, as yet unknown factor must also be important here.

### **5.3.3 HEAVY AND LIGHT CHAIN PAIRING.**

The heavy chains are not constrained to pairing with light chains of the same specificity, as demonstrated by the P3-13 crosses, although the limitations do seem to be greater when unrelated light chains are used. The method of library construction ensures that binders are selected from a very large pool of antibodies and probably only those with the best combinations of chains and, so, the highest affinity for antigen are selected, which could be a factor in the high degree of promiscuity observed in the gp120 crosses. It may be possible that if a library of gp120-specific heavy chains were combined with a library of TT-specific light chains, for example, that a similar number of equally promiscuous Fabs would be

generated. However, if the combinatorial library really reflects the *in vivo* situation, it is likely that clonal selection and affinity maturation (Griffiths *et al.*, 1984; Liu *et al.*, 1989) have ensured a level of promiscuity that would only be displayed in crosses between related chains. In other words, the chains represented in a panned library have already undergone a natural selection process making them more suitable for combination with related chains than with chains of a completely different specificity. This is supported by the fact that all the gp120 crosses maintained their affinities, while the interantigenic crosses all displayed affinities less than those of the original Fabs. The lack of knowledge on the exact mechanisms of chain combination make it very difficult to speculate on this. A more extensive data bank of antibody structures and more work with the libraries is needed to clarify the matter.

It can be concluded that heavy chains can combine productively with a number of light chains, presumably when unfavourable interactions in the binding pocket are not created. This seems to depend on the heavy chain sequence, and more particularly on the CDR3 sequence, although other factors must also be important here. Unrelated chains can be successfully combined, suggesting that two specificities may be incorporated into a single Fab. This could be important for the production of Fabs with beneficial activities, such as catalytic antibodies.

#### **5.3.4 LIGHT CHAINS IN ANTIBODY FUNCTION.**

What role, then, does the light chain have in the antibody, if as was suggested, any light chain will do as long as it does not interfere

with binding in the active site of the antibody? Perhaps the light chain is present merely to stabilise the heavy chain, since otherwise, large hydrophobic areas would be exposed to the surface and that, depending on sequence, certain light chains can achieve this more effectively than others, for a particular heavy chain. However, this study has confined itself to an analysis of the antigen binding properties of the Fabs, in which the light chains do seem to have a limited role to play. No attempt has been made to study the other functions of these Fabs, particularly the ability to neutralise virus (this will be done in chapter 6). It is possible that light chains are a more important for this function. This would imply that chain combination, *in vivo* at least, is not quite as random as ensuring that the specificity of the heavy chain is not impaired, but that a particular combination of chains are selected for that possess the capability to bind antigen and carry out the other functions of the antibody. Clonal selection would then be a process that selected for functionally active binders, rather than clones secreting antibody that just bound antigen.

#### **5.3.5 THE LIBRARY Fabs AND THE *IN VIVO* RESPONSE.**

A number of conclusions can be drawn from these results as to how the *in vitro* combinatorial library reflects the *in vivo* response. Firstly: the importance of the heavy chain in antigen binding has been demonstrated, supporting the arguments made in chapter 4. Secondly: it is apparent from diagrams 5.4 and 5.5 that the affinities of the gp120 crosses are all comparable to the original Fabs while those of the interantigenic crosses are as much as 100-fold lower.

This indicates that all the chains from the gp120 crosses represent a closed set, which might be explained by the fact that they all possessed the same specificity *in vivo*. If it was the case, as described above, that clonal selection *in vivo* was on the basis of both antibody specificity and functionality, then it would be reasonable to expect that both the specific heavy and light chain DNA would be highly represented in the bone marrow of our HIV positive patient, and accordingly, in the final library. It would be necessary to perform more interantigenic crosses with other Fabs of varying specificities to verify or contradict this theory.

The promiscuity of the heavy chains obviously makes it more difficult to determine if the combinations of chains observed in the libraries are present *in vivo* or not. Assuming that the chains taken separately are those found *in vivo* then there is some restriction on the possible combinations that can occur, even *in vivo* as demonstrated by the fact that chains will not always combine with others outside of their own group. For example, b21HC will only pair with chains in its own group and b22LC. It is probable, therefore that b21HC does combine *in vivo* with one of the light chains found in its own group or with a very similar light chain. Since the original b21 Fab is also a good neutraliser (Barbas *et al.*, 1992) it could be argued that this is the *in vivo* combination (however, it must be mentioned that the assays for neutralisation are, necessarily, *in vitro* assays and may not be totally representative of the true situation). It is impossible to argue this for the more promiscuous heavy chains, unless they are found to neutralise in combination with one particular light chain. Group b1 (Table 5.1) is a group whose

heavy chains in general do not combine well with light chains from outside the group. This group probably represents a set of *in vivo* antibodies, although the exact combinations are difficult to predict since there is complete promiscuity within the set. On the other hand, it is interesting to note that the pattern of sequence diversity in this group is similar for both the heavy and light chains, indicating that these represent preferred combinations. However, due to the complete promiscuity within this group and the similarity in affinities, it is difficult to draw conclusions from this study. An examination of viral neutralisation patterns for all the crosses in this group may distinguish certain heavy-light chain combinations and yield some further information about the *in vivo* situation.

In conclusion, the evidence is compelling that the chains isolated in the combinatorial library reflect those found in gp120-specific antibodies *in vivo*. The equivalent statement is more difficult to make for the combinations of heavy and light chains found, although it may be possible to limit the number of feasible combinations to certain groups. This varies for each Fab depending on the degree of promiscuity of its heavy chain, and the ability of the Fab to neutralise the HIV virus..

#### **5.3.6 Large Scale Production of Fab by Fermentation.**

The binary plasmids had been designed to incorporate a histidine tail into each Fab, so as to facilitate the purification of large quantities of antibody. Therefore, it was decided to test this system by growing large volumes of cultures using fermentation. It has been

shown that, by using the correct media components and optimising the fermentation conditions, it is possible to grow *E. coli* to a high density and to maintain the binary plasmids in the culture during growth. It is also possible to express recombinant protein, in this case Fab, from these plasmids and to extract this from the culture broth. Further optimisation of the medium conditions and possibly the reactor conditions should give rise to higher cell densities and therefore better yields of protein.

Downstream processing of the cell pellets proved to be comparatively easy, as the volumes tended to be small and, as a result, easy to handle. The supernatant, in contrast, was more difficult to process due to the larger volumes involved, which made filtration very difficult. This could be solved by using larger filters than were available to us. However, since approximately 60% of the Fab protein was extracted from the cell pellet, it was decided in further runs to discard the supernatant and concentrate solely on the pellet.

Purification of the Fab by metal affinity chromatography was quite successful, giving a product of about 30% purity. This is significant for a first step purification procedure. A similar procedure has been previously described (Skerra *et al.*, 1991) for the purification of F<sub>v</sub> fragments, with similar results to those presented here. It may be possible to increase the efficiency of this technique by altering the imidazole gradient to elute the Fab more specifically, or the use of a different metal on the column such as copper or nickel, may give higher yields. The column we used was 0.5 litres in volume and it can be seen from the flowthrough that its capacity was overloaded

by the amount of protein we applied (Figure 5.4). It would be possible to increase the yield of protein by either using a larger column or splitting the purification into a number of samples. Further purification of this sample by immuno-affinity chromatography proved to be very successful. However, this technique is very expensive and has a very low capacity in comparison to the amounts of protein being used. It may be advisable to try other methods of purification such as ammonium precipitation and ion exchange chromatography.

These results are preliminary and are open to a certain amount of optimisation to maximise the yields achievable. However, it was clearly demonstrated that it is possible to produce large amounts of pure recombinant protein from the binary plasmid system by means of fermentation. This could be an invaluable technique in circumstances where large quantities of protein are needed, such as X-ray crystallography or clinical trials.

## CHAPTER 6



## 6.1 INTRODUCTION

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### 6.1.1 OVERVIEW.

The HIV virus invades CD4-positive T lymphocytes by binding to the CD4 surface antigen via the viral surface glycoprotein, gp120 (Sattentau *et al.*, 1986; Rosenberg and Fauci, 1991). The mechanisms following this event remain to be conclusively established. However, it is thought that gp120-shedding ensues, followed by fusion of the viral and cell membranes and internalisation of the viral RNA into the cell (Moore *et al.*, 1993). This is followed by the synthesis of viral DNA and integration into the cell genome (Haseltine, 1991). Whatever the mechanisms of cell invasion, it is the binding of gp120 to CD4 that remains the most important step for initiating the fusion process and, so, inhibition of this reaction is likely to lead to neutralisation of the virus.

### 6.1.2 USE OF SOLUBLE CD4 (sCD4).

The most obvious candidate molecule for use as an inhibitory agent in this way would be soluble CD4 itself (sCD4). This form of CD4, produced in chinese hamster ovary cells (CHO) consists of the first two N-terminal domains of CD4 only. However, it has been shown previously (Landau *et al.*, 1988) that the interaction with gp120 occurs with these 2 domains. Therefore, sCD4 is a suitable candidate for neutralisation studies. The concentration of sCD4 used in neutralisation was found to be of primary importance, as low sCD4 concentrations could actually lead to an enhancement of the viral

infectivity. It is thought that sCD4 can induce conformational changes in gp120, mimicking the changes induced in the viral envelope by cell surface CD4. The sCD4-activated virions can then bind to cells and invade with higher levels of infection. Increased doses of sCD4 leads to gp120 shedding, and complete, irreversible neutralisation of the virus (Moore *et al.*, 1993). This process is highly temperature dependant and also relies on a least 50% of the total virion surface gp120 molecules being bound by the sCD4. The physical state of the virus is also crucial, with older viral isolates undergoing more gp120 shedding and are therefore more difficult to neutralise (McKeating *et al.*, 1991; Layne *et al.*, 1992).

### **6.1.3 ANTIBODIES AS NEUTRALISING AGENTS.**

A more practical approach for therapeutic purposes is the study of the humoral responses elicited in HIV-infected individuals. These patients develop antibody responses against viral proteins, some antibodies of which are specific for neutralising epitopes on gp120. As was previously discussed, there are two major groups of these neutralising antibodies: those specific for the V3 loop and those specific for the CD4-binding site of gp120 (Chamat *et al.*, 1992). A number of studies have been carried out on the V3 specific responses and particularly on the effect that this type of antibody has on the V3 loop (Gorny *et al.*, 1991). This loop is defined by a disulphide linkage between amino acids 296 to 309 and 331 to 343, depending on the viral strain. There is a significant degree of residue conservation, particularly with the central motif, GPGRF (Nara *et al.*, 1991b). Antibodies to this region tend to be viral

strain-specific and of high titre in the serum, affording neutralisation of the virus by interfering with some event subsequent to the binding of CD4. This was initially thought to be the route to finding a quick and effective therapy for AIDS. However, this optimism declined after it was learned that the V3 loop was more complex than a linear peptide epitope. It was quickly established that the gp120 molecule was capable of changing in conformation to give rise to neutralisation escape mutants (NEM), and that these could occur with the minimum of change in the primary amino acid sequence (Haseltine, 1991). These mutants would be functionally close enough to the parental strain, but sufficiently different in the presentation of antibody binding sites as to avoid being bound by these antibodies. It was also found that single point mutations in sequence are magnified through global rearrangements in the 3 dimensional structure and so, a minimum change in the primary sequence could lead to loss of recognition by an antibody. This finding lead to the formulation of theories such as Original Antigen Sin (OAS) whereby a slightly altered gp120 molecule could elicit antibodies to the unaltered form, rather than it's present conformation and so escape neutralisation (Nara *et al.*, 1988).

It was soon realised that the V3 loop could not account for the broad virus neutralising activity detected in the sera of many infected patients, particularly those in the later stages of infection (Ho *et al.*, 1991b; Kang *et al.*, 1991). A new set of antibodies was discovered that could cross-react with a number of viral strains, which was not typical of the V3 set. Also, monoclonal antibodies raised by immunisation with IIBgp120 could not compete with

known V3 loop binders and so possessed a different specificity (Ho *et al.*, 1991b). They were found to bind a conformationally sensitive epitope and inhibited binding of CD4 to gp120. The CD4 binding site has been defined in more detail through the use of these antibodies, as discussed in chapter 4 (Olshevsky *et al.*, 1990; Ho *et al.*, 1991; Thali *et al.*, 1991; McKeating *et al.*, 1992).

A number of attempts have been made to produce antibodies of either group (V3 or CD4) capable of neutralising HIV (Palker *et al.*, 1988; Ho *et al.*, 1991b). In order to be of practical therapeutic use, the antibody must be designed such that it can evade the escape mutants and so, effectively neutralise the virus even after it has altered its coat proteins. Alternatively, a cocktail of antibodies must be produced with similar but not identical epitopes such that there will always be an antibody capable of binding gp120, despite any changes made by the virus. The isolation of a panel of Fab fragments specific for the CD4 binding site of gp120 has recently been described by Barbas *et al.*, (1992). Although all of these Fabs were able to inhibit the binding of gp120 to sCD4, only a few were shown to neutralise the virus *in vitro*. This would suggest that either the process of neutralisation is more complicated than the simple blocking of receptor binding, or that the epitope specificity of these fragments is more subtle, as is discussed in chapter 4. The lack of neutralising activity in a small proportion of these Fabs could be explained by the fact that they were isolated with recombinant, soluble gp120 as opposed to virion-bound gp120. However, this could not account for all the weak or non-neutralisers found. Whatever the explanation, these Fabs provided an ideal

opportunity for the study of neutralisation as it relates to antibody structure in more detail.

#### **6.1.4 LIGHT CHAINS IN ANTIBODY FUNCTION.**

It has already been shown that antibody specificity can be attributed more to the heavy chain than the light chain within this group of Fabs. The high degree of promiscuity of the heavy chains, with regard to antigen specificity, has also been demonstrated, and it has been conjectured that heavy chain specificity would be retained in combination with any light chain that did not antagonise the thermodynamic or steric interactions in the binding pocket.

It has been postulated that the selection of the light chain *in vivo* is not as random as ensuring that the binding pocket remains viable, but that there may be a positive selection for a light chain that, in combination with a given heavy chain, can effect the desired function of the antibody. Since the antigen specificity seems to be associated with the heavy chain, the light chain must carry some other function with it.

The work in this chapter will set out to establish that the light chain, within the set of Fab fragments studied, has critical importance for neutralisation of the virus. As an extension of the directed crosses carried out in chapter 5, the neutralisation ability of a selected number of those crosses will be examined. One particular group from those crosses (the b4 group) yielded a large number of neutralisers and there was complete promiscuity within the group. These crosses will be analysed in further detail. This study will aim to establish whether the promiscuity seen for

antigen binding is translated into neutralising capability, or whether a particular heavy and light chain combination is required for this function.

It has also been previously established that each neutralising Fab is capable of acting on a different set of viral isolates. The question as to whether the isolate specificity is carried with the light chain will be addressed. If it were the case that only Fabs with a particular light chain were able to neutralise a given set of HIV isolates, this would be compelling evidence that this function was allied with the light chain. The precise epitope of a number of these mutants was also examined by gp120 knockout assays.

Finally, this study will serve as a comparison of a number of *in vitro* assays available for testing neutralisation of HIV. It is not clear whether all assays currently available give comparable results in relation to one another and so this study will examine this question.

## 6.2 RESULTS

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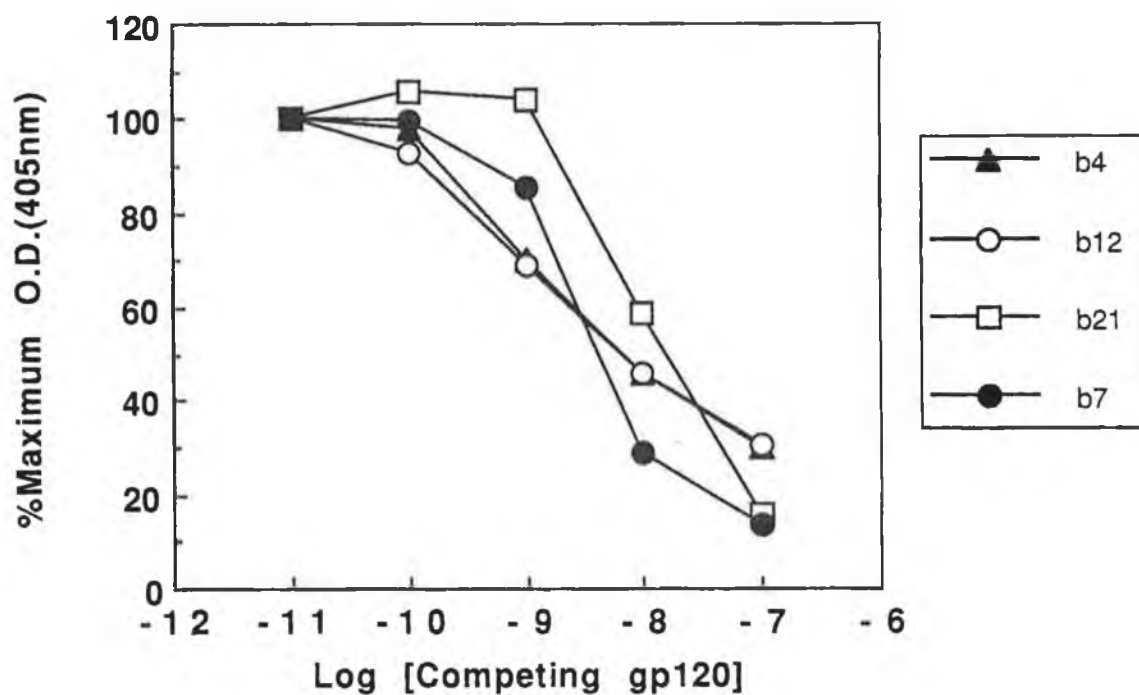
### 6.2.1 SELECTION OF Fabs.

One group in particular from the original panel of antibodies yielded a high proportion of neutralising antibodies, namely b4, b7, b12 and b21. It was shown previously (Barbas *et al.*, 1992) that the b4 and b12 displayed high levels of neutralisation in both the p24 and syncytial assays, giving rise to titres in the range 1:80 to 1:128 in the p24 and syncytial assays respectively. The Fabs b7 and b21 also neutralised, but at lower levels, typical titres being 1:20 to 1:32. Since these 4 clones exhibited a range of neutralisation titres, they provided suitable candidates for further analysis. The chain shuffling experiment presented in chapter 5 revealed complete promiscuity within this group of Fabs, i.e.: any directed cross between 2 given chains within this group yielded a productive combination in terms of antigen binding. However, in order to perform a detailed analysis, a limited number of these crosses had to be chosen. Fab b12 was the original Fab found to neutralise the virus and it was also determined to have the most unique specificity (chapter 4) which probably contributed to its high neutralisation titre. It was, therefore, decided to use the four crosses involving the heavy chain from b12 for analysis of the neutralisation patterns. This included Fabs H12L4, H12L7, H12L12 (i.e.: b12) and H12L21.

### **6.2.2 RELATIVE AFFINITIES AND CD4 INHIBITION CONSTANTS.**

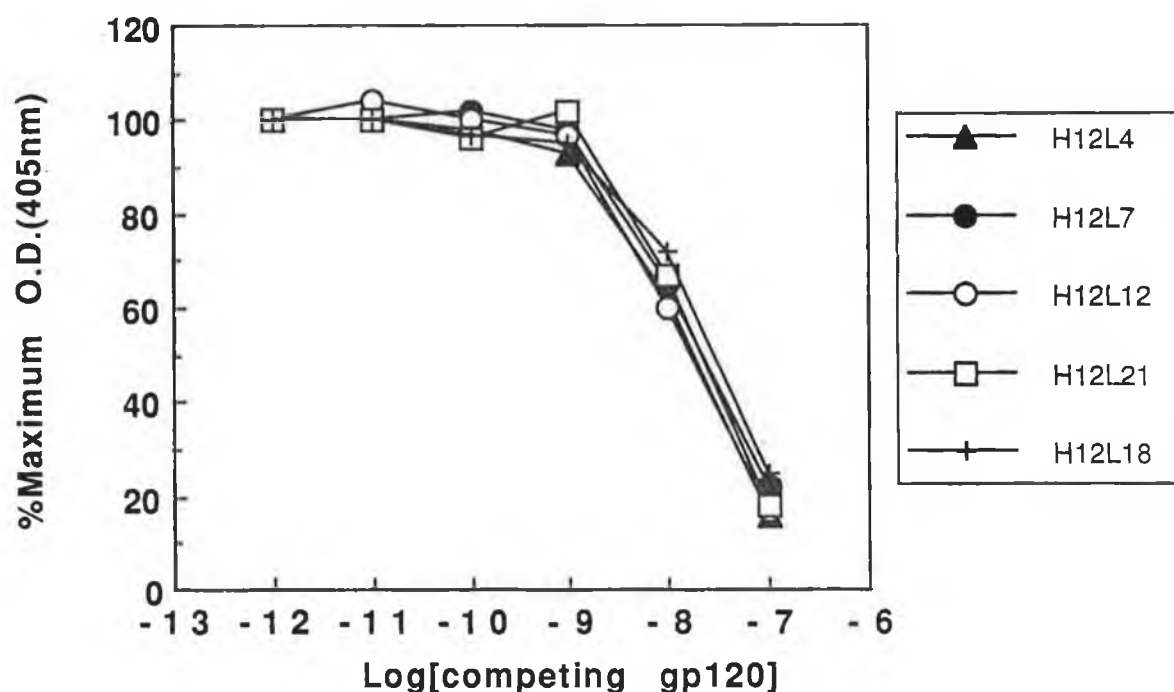
Neutralisation titres could be very sensitive to small differences in affinity and so two Fabs with slightly different affinities could demonstrate significantly different titres. Therefore, in order to determine that any effects observed during neutralisation were not merely an artifact due to affinity, it was necessary to establish whether or not the affinities of all the Fabs being used were sufficiently similar so as not to be a factor in the neutralisation patterns. This was first measured by competitive ELISA as described previously (Methods, 3.1.5). Figure 6.1 shows the relative affinities of the original 4 Fabs, b4, b7, b12 and b21. Assuming that the affinity is the concentration of competing gp120 resulting in 50% inhibition of binding, it can be seen that the values for all these Fabs are in the range  $5 \times 10^7$  to  $1 \times 10^8 \text{M}^{-1}$ .





**Figure 6.1** Relative affinities of the original 4 Fabs from group b4 as determined by competitive ELISA. The affinity value for each clone is that concentration of gp120 leading to 50% inhibition of binding.

Figure 6.2 demonstrates that the 4 crosses indicated above also yield values in the same range and that their affinities are very similar to one another.



**Figure 6.2** Relative affinities of the shuffled Fabs as determined by competitive ELISA. H12L18 was included as a Fab that was known to possess no neutralising ability.

More accurate measurements of affinity for all the Fabs were obtained using real time biospecific interaction analysis as described in chapter 4. The values obtained are shown in Table 6.1 and are accurate to  $\pm 5\%$ . The affinities of the four Fabs b4, b7, b12 and b21 are confirmed to be in the range  $7.25 \times 10^7$  to  $1 \times 10^8$ , thereby showing that they are sufficiently close as to not be a factor in the neutralisation titres. The affinities of the four crosses show a higher degree of similarity with a much narrower range of values between  $9.6 \times 10^7$  and  $1.05 \times 10^8$ . This is probably to be expected since they share a common heavy chain. The affinity of a

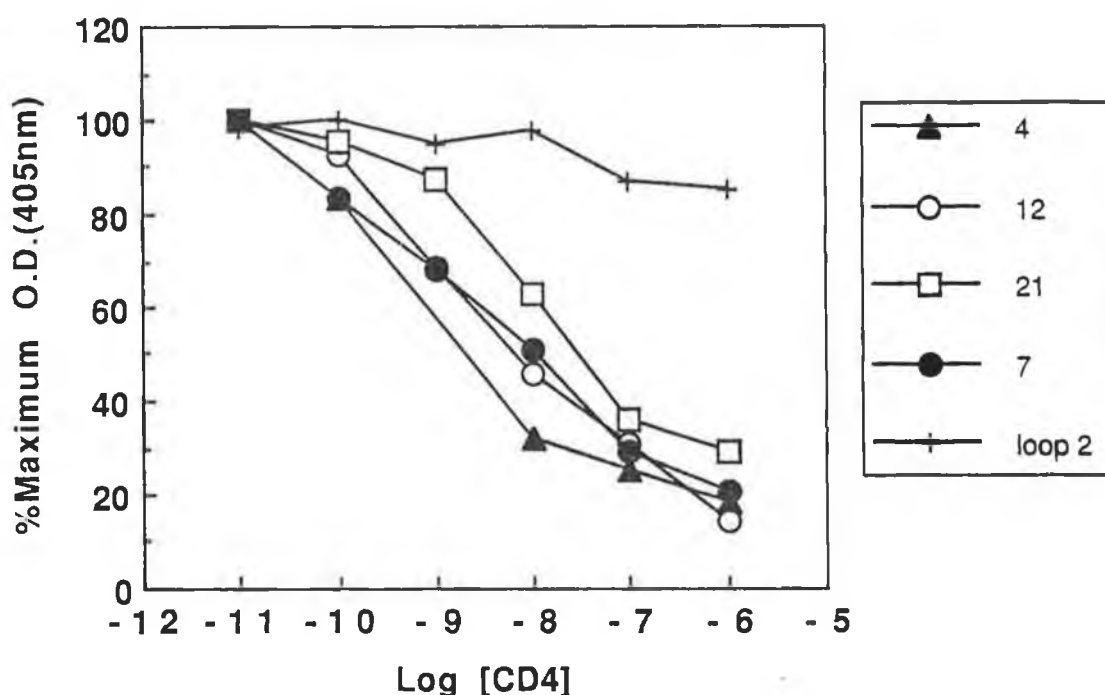
negative control for the neutralisation assays, H12L18, is also given.

<b>Value Fab</b>	<b><math>K_{on} (M^{-1} s^{-1})</math></b>	<b><math>K_{off} (s^{-1})</math></b>	<b><math>K_a (M^{-1})</math></b>
b4	$1.56 \times 10^4$	$1.74 \times 10^{-4}$	$8.97 \times 10^7$
b7	$2.35 \times 10^4$	$3.24 \times 10^{-4}$	$7.25 \times 10^7$
b12	$4.53 \times 10^4$	$4.31 \times 10^{-4}$	$1.05 \times 10^8$
b21	$3.02 \times 10^4$	$3.61 \times 10^{-4}$	$8.38 \times 10^7$
H12L4	$3.62 \times 10^4$	$3.56 \times 10^{-4}$	$1.02 \times 10^8$
H12L7	$1.78 \times 10^4$	$1.86 \times 10^{-4}$	$9.6 \times 10^7$
H12L18	$6.78 \times 10^3$	$1.43 \times 10^{-4}$	$4.73 \times 10^7$
H12L21	$1.20 \times 10^4$	$1.15 \times 10^{-4}$	$1.04 \times 10^8$

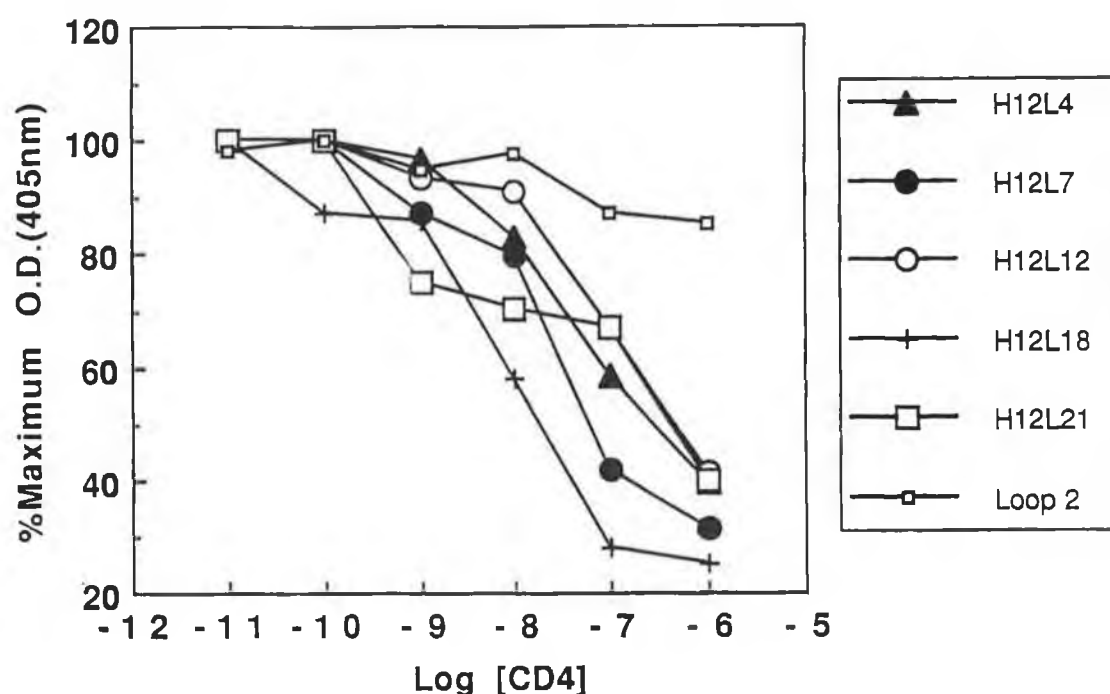
**Table 6.1** Affinities of all clones as determined using real-time biospecific interaction analysis (BIAcore). Both the 'on' and 'off' rates are given for each clone and the affinity ( $K_a$ ) is defined as  $k_{on}/k_{off}$  in units of  $M^{-1}$ . These results confirm the ELISA data insofar as the affinities are sufficiently similar not to have any significant effect on the neutralisation titres.

The 'off' rates of all the Fabs are very similar, while the 'on' rates are also quite similar except for that of H12L18 which is an average of 5 times lower than the others.

The specificity of these Fabs was determined by competition ELISA using sCD4 as the competing antigen. The results given in Figures 6.3 and 6.4 indicate that all the Fabs used in this analysis are inhibited by CD4 and are, therefore, specific for the CD4 binding site of gp120.



**Figure 6.3** CD4 inhibition curves for the original clones as determined by ELISA, using soluble CD4 (sCD4) as the competing antigen. The inhibition constant for each clone is that concentration of sCD4 leading to 50% inhibition of binding. All these constants were found to be in the range  $1 \times 10^7$  to  $1 \times 10^8 \text{ M}^{-1}$ . The loop 2 data is given as a negative control.



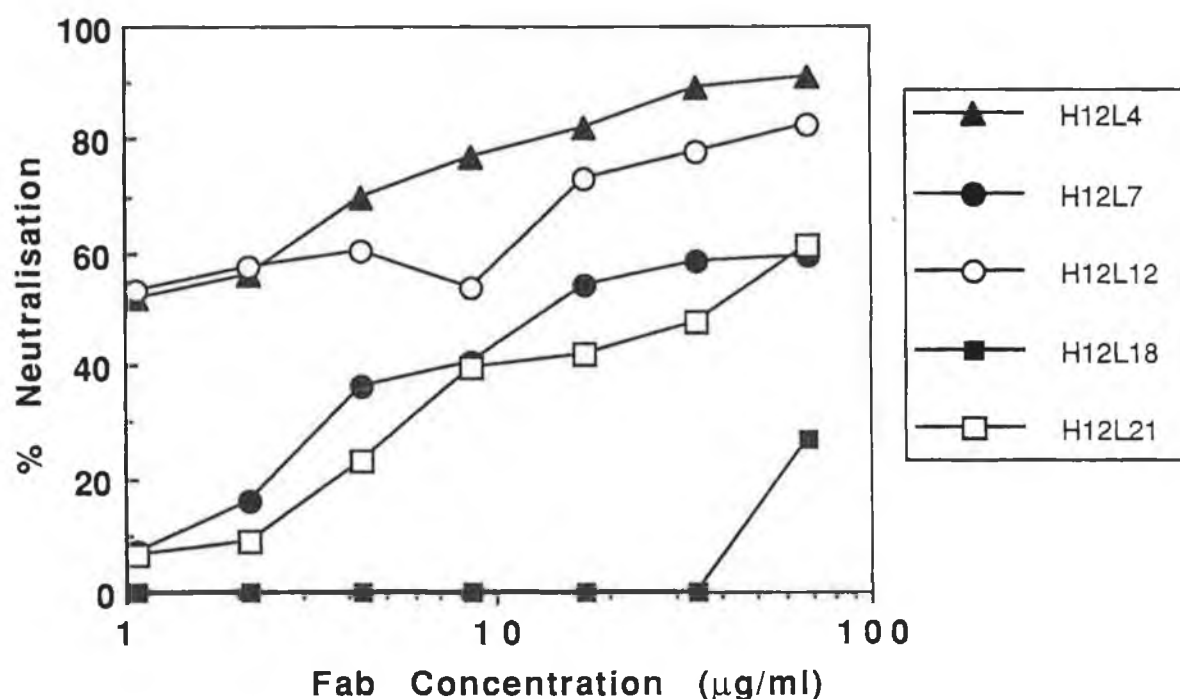
**Figure 6.4** CD4 inhibition curves for the shuffled Fabs as determined by competitive ELISA with sCD4 as the competing antigen. The constants for these Fabs all lie in the range  $1 \times 10^6$  to  $5 \times 10^7 \text{M}^{-1}$ .

### 6.2.3 NEUTRALISATION ASSAYS.

The shuffled Fabs H12L4, H12L7, H12L12, H12L18 and H12L21 were assayed for neutralisation in both the p24 assay and the syncytial assay. The results of the syncytial assay are given in Figure 6.5.

The method measures the number of syncytia formed in the presence of an MN viral strain and the Fab, in relation to the number formed when there is no antibody present, and the result is given as a percentage reduction in the number of syncytia formed at that

concentration of Fab, i.e.: the percentage neutralisation. It is evident from the graph that H12L4 and H12L12 are very good neutralisers with 50% neutralisation occurring at approximately 1 $\mu$ g/ml. However, H12L7 requires a concentration of 15mg/ml to attain the same level of neutralisation while H12L21 neutralises by 50% at 40 $\mu$ g/ml. Therefore, substitution of the light chain in b12 for LC21 leads to a 40-fold reduction in the neutralisation titre.



**Figure 6.5** Neutralisation titres for the shuffled Fabs as determined using the syncytial formation assay. The titre for a given clone is defined as the Fab concentration resulting in 50% neutralisation. H12L18 is included as a negative control and shows no signs of neutralisation except at very high concentration.

A similar result was noted when a neutralisation assay measuring the levels of p24 production was employed (Table 6.2). This assay measures the highest dilution of Fab (twofold serially diluted) that still gives rise to 80% neutralisation of the virus, as detected by p24 production. As can be seen from the figures, H12L4 and H12L12 both neutralise by 80% at maximum dilutions of 1:40 (approx. 2.5µg/ml), whereas H12L7 and H12L21 do not show any significant signs of neutralisation of either the IIB or MN strains of virus at any of the dilutions tested in this assay. It must, therefore be concluded that H12L4 and H12L12 are far stronger neutralisers than the other two. H12L18 was used in both assays as a negative control.

	MN	IIIB
H12L4	40	40
H12L7	-	-
H12L12	40	40
H12L18	-	-
H12L21	-	-

**Table 6.2** Neutralisation data for shuffled clones as determined using the p24 production assay. The assays were carried out with the MN and the IIIB strains of the HIV virus. The numbers indicate the maximum dilution for each Fab which will permit 80% neutralisation of the virus. Only H12L4 and H12L12 neutralise either strain at any of the dilutions tested in this assay.



#### **6.2.4 SEQUENCE COMPARISONS.**

The data so far seemed to indicate that Fabs with the same heavy chains but different light chains could exhibit varied neutralisation patterns, ranging from strong neutralisation to very weak titres. Since it was only the light chains that were different between these Fabs, a comparison of these chains was performed to establish how this was reflected in the amino acid sequences (Figure 6.6). LC12 was used as the predominant chain since it was clear that this was associated with neutralisation. The sequences of LC7 and LC21 are given with similarities to the predominant sequences indicated by dots and the differences are given as the single letter amino acid codes. It can be seen that LC7 differs from that of LC12 by eight residues, while LC21 only differs by four. This is remarkable that a difference of only four amino acids between two Fabs can lead to a reduction of as much as forty-fold in the neutralisation titre. The residues observed to be different between LC12 and LC21 were: an aspartate at position fifteen in LC21 was glutamate in LC12 (15D/E), 26N/S, 33A/R and 77L/V. Three of these changes are relatively conservative in both size of the residue side chains and in charge. The only one that introduces a charge difference is 33A/R, where the uncharged alanine in LC21 is replaced by a positively charged arginine in LC12.

### Comparison of the Light Chain Sequences of Clones b7, b12 and b21

Clone	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
b12	ELTQAPGTLISLSPGERATFSC	RSSHISIRSRVR	WYQHKPGQAPRLVIH	GVSNRAS	GISDRFSGSGSGTDFTLTITRVEPEDFAIYYC	QVYGASSYT	FGQG'TKLERKR
b21	.....D.....	....N.....A	.....	.....	.....L.....	.....	.....
b7	.....T.....	.T.....	...V.G.....	.....	.....V...	.Q..S.R..	.....
b4	....S.....	.....A	.....	.....	.....	.....	.....

**Figure 6.6** Comparison of the light chain sequences of clones b4,b7,b12 and b21. The predominant sequence is that of b12 since this light chain was derived from a strongly neutralising Fab. The other 3 chains are given as a series of dots to indicate similarity to the predominant sequence, while differences are shown using the single letter amino acid code.

### **6.2.5 NEUTRALISATION WITH VARIOUS VIRAL ISOLATES.**

It had been noted previously (unpublished data) that a given Fab was capable of neutralising a given set of viral isolates and that this set of isolates was different for each Fab even though all the isolates were of the same viral strain (in the case, MN). It was postulated that if the neutralisation ability of a particular Fab was associated with the light chain, then, shuffling of the chains should give rise to a situation where all the Fabs with the same light chain would neutralise a similar set of isolates. To test this, the Fabs b7 and b12 were shuffled to give the four possible combinations (H7L7, H7L12, H12L7 and H12L12). These were then tested for neutralisation of six different isolates of the MN virus using the p24 assay. The results of this experiment are given in Table 6.3. Firstly, it is evident that the shuffled Fabs neutralise fewer isolates than the original Fab containing the same heavy chain. This is expected since these shuffled Fabs do not consist of the original combination of chains and this has already been shown to drastically affect the neutralisation titre. Fab b12 (H12L12) can neutralise all six of the isolates while b7 can only neutralise four of them. Also, b12 can neutralise isolates 2815, 1702 and 1033 more strongly than b7. Therefore, b7 is a weaker neutraliser of fewer isolates. When LC12 is combined with HC7, the resulting combination can neutralise five isolates, including 4501 which was not neutralised by b7 before. Furthermore, the titres for 1702 and 1033, previously neutralised by b7, have increased, especially in the case of 1702. None of the titres have decreased from those exhibited by b7. Combining LC7 with HC12 leads to a loss of activity in all cases except that of 1702.

Therefore, the trend seems to be that the presence of LC12 confers strong neutralisation activity, while LC7 is associated with weaker, and in many cases, a complete loss of neutralising activity. This would appear to concur with results obtained previously in this study.

<b>Fab Isolate</b>	<b>H12L12</b>	<b>H7L12</b>	<b>H7L7</b>	<b>H12L7</b>
<b>2815</b>	<b>80</b>	<b>40</b>	<b>40</b>	<b>-</b>
<b>812</b>	<b>40</b>	<b>-</b>	<b>-</b>	<b>-</b>
<b>2951</b>	<b>20</b>	<b>40</b>	<b>40</b>	<b>-</b>
<b>1702</b>	<b>80</b>	<b>160</b>	<b>20</b>	<b>20</b>
<b>1033</b>	<b>40</b>	<b>40</b>	<b>20</b>	<b>-</b>
<b>4501</b>	<b>40</b>	<b>20</b>	<b>-</b>	<b>-</b>

**Table 6.3** Neutralisation data for the four Fabs indicated, on different isolates of the MN strain of virus, as determined using the p24 production assay. The isolates originated from a varied group of HIV-infected patients as described in the text. The numbers indicate the maximum Fab dilution at which 80% neutralisation of the virus occurred.

#### **6.2.6 KNOCKOUT ASSAYS.**

An epitope analysis was performed on Fabs H12L12, H12L21 and b21 in order to determine the influence that the light chain was having on the overall epitope specificity of the Fab (Figures 6.7(a), (b), (c), (d) and (e)). Firstly it can be seen that all the Fabs exhibit certain features characteristic of CD4 binding Fabs, for example the inhibitory mutations 368D/R, 370E/R and 457D/R. Binding by H12L12 is enhanced by 207K/W, 252R/W, 475W/S, 491I/F and a number of mutations around residue 430 in the fourth constant region. There are a number of inhibitory mutations at the base of the V4 loop as well as 308-310 and 298R/G in the V3 loop. It is also inhibited by loss of the loops V1, V2, and V3 and displays the highly unusual effect of being inhibited by a loss of V1/V2 loop (see chapter 4). Fab b21, on the other hand, is not affected by the V1/V2 mutation and is enhanced by mutation 477D/V rather than knocked out as is the case with b12. 384Y/E completely inhibits binding by b21 while the effect is not so pronounced for b12. 45W/S knocks out b12 and does not affect b21 and 475W/S does not enhance b21. The b12 enhancing mutations around residue 430 do not affect b21 which is also not inhibited by 298R/G.

The map for the shuffled Fab displays most of the characteristics of b12 including the inhibitory mutations due to the loss of V1/V2, 308-310 and 384Y/E and maintains the enhancing mutations at 491K/L, 207K/W, 252R/W, 475W/S and a single enhancing mutation at 429K/L. Therefore, the overall pattern of binding is reasonably conserved between the 2 Fabs b12 and H12L21, which may not be so surprising since both use the same heavy chain which is responsible

for specificity. However, there are a few mutations in H12L21 which seem to be more characteristic of b21 than of b12. These include the enhancing mutation at 477D/V which actually knocked out binding by b12. 298R/G no longer exerts an inhibitory effect which is characteristic of b21. Similarly, there is no knockout effect at 45W/S. The pattern of inhibitory mutations at the base of V4 loop has altered somewhat, although the b12 mutations 380G/F and 384Y/E had been conserved. The three enhancing mutations in the fourth constant region around residue 430 have been reduced to a single effect, 429K/L, which may be a type of compromise, since b21 did not exhibit any of these effects.

Combination of HC12 and LC7 reveals the same type of trends in binding patterns. Many of the characteristics of b12 are maintained including the inhibitory mutations 368D/R, 370E/R, 384Y/E, 457D/A and 308-310. It is interesting to note that the knockout mutation 477D/V is conserved in H12L7 and not in H12L21 and that this mutation was found to be inhibitory in one other clone studied (b11- see chapter 4). Moreover, the enhancing mutations 207K/W, 252R/W, 421K/L and 475M/S are also conserved. However, combination with LC7 introduces a number of differences. Firstly, there is a new sensitivity to mutations in the C2 region (250-270) and secondly, the Fab no longer seems to be sensitive to a loss of the V1/V2 loop, which was one of the original distinguishing characteristics of b12. Therefore, both shuffled Fabs maintain the central characteristics of the b12 binding pattern, with a number of changes, depending on the light chain being used. These changes seem to be sufficient to radically affect the neutralisation titre of a given Fab.

Figure 6.7 (a): Data for H12L12

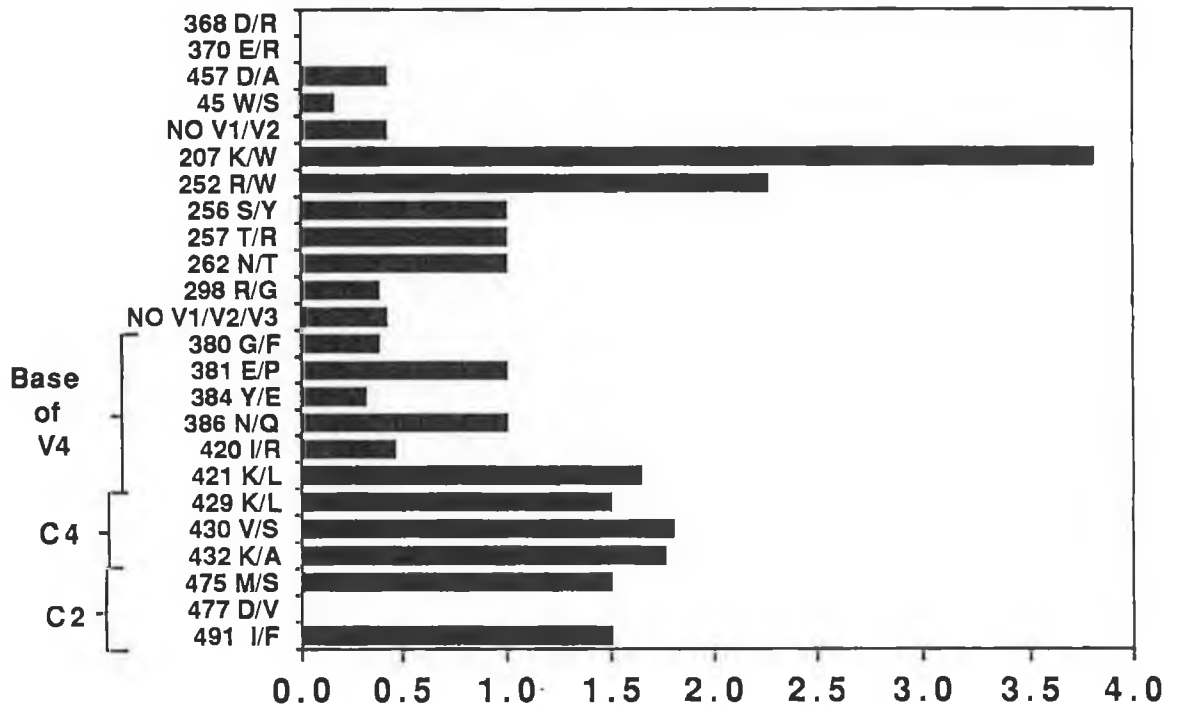


Figure 6.7 (b): Data for H12L21

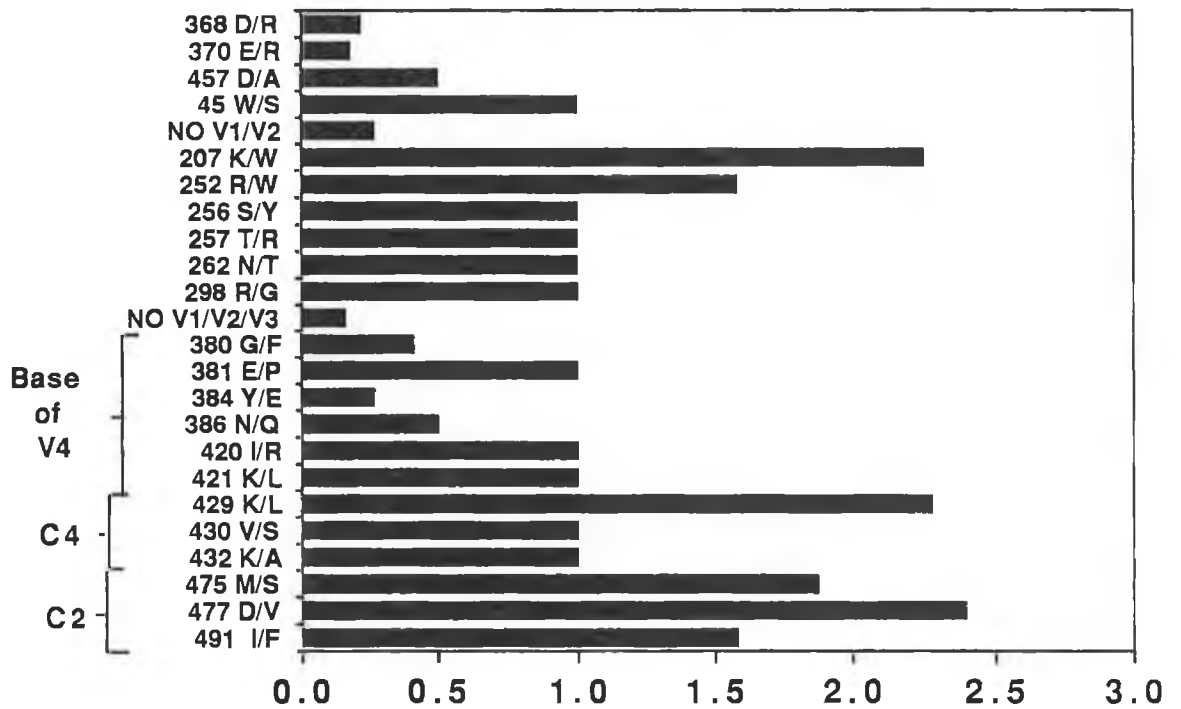


Figure 6.7 (c): Data for H21L21

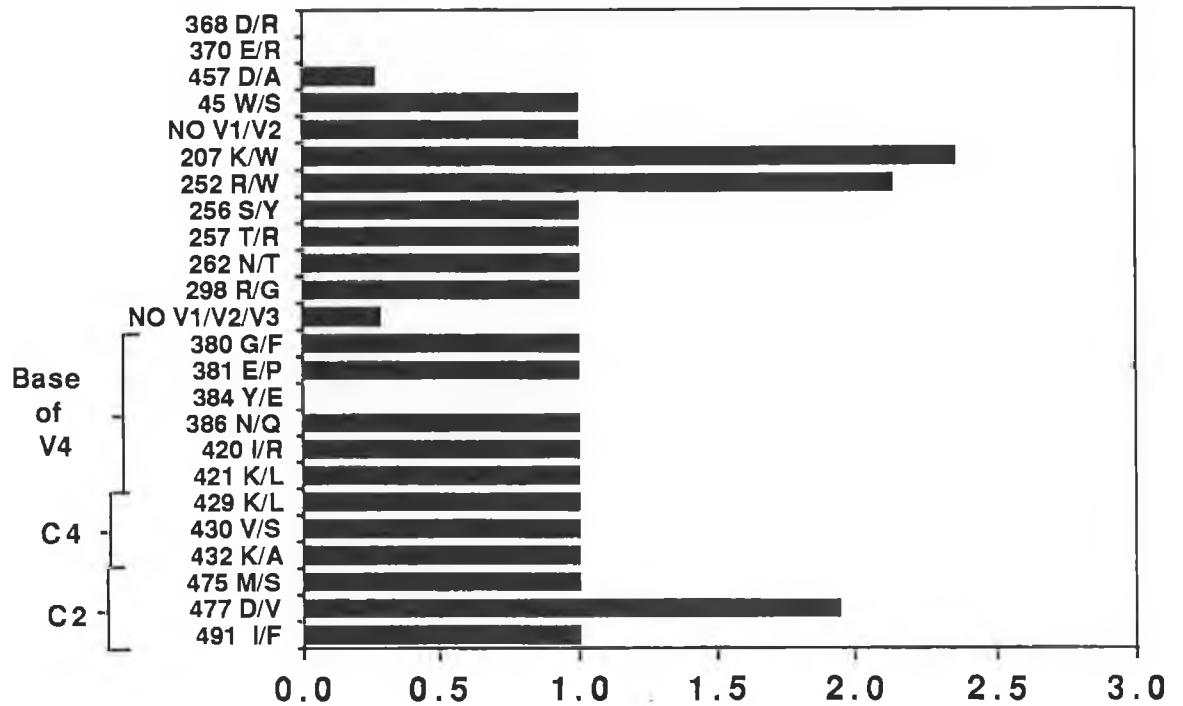
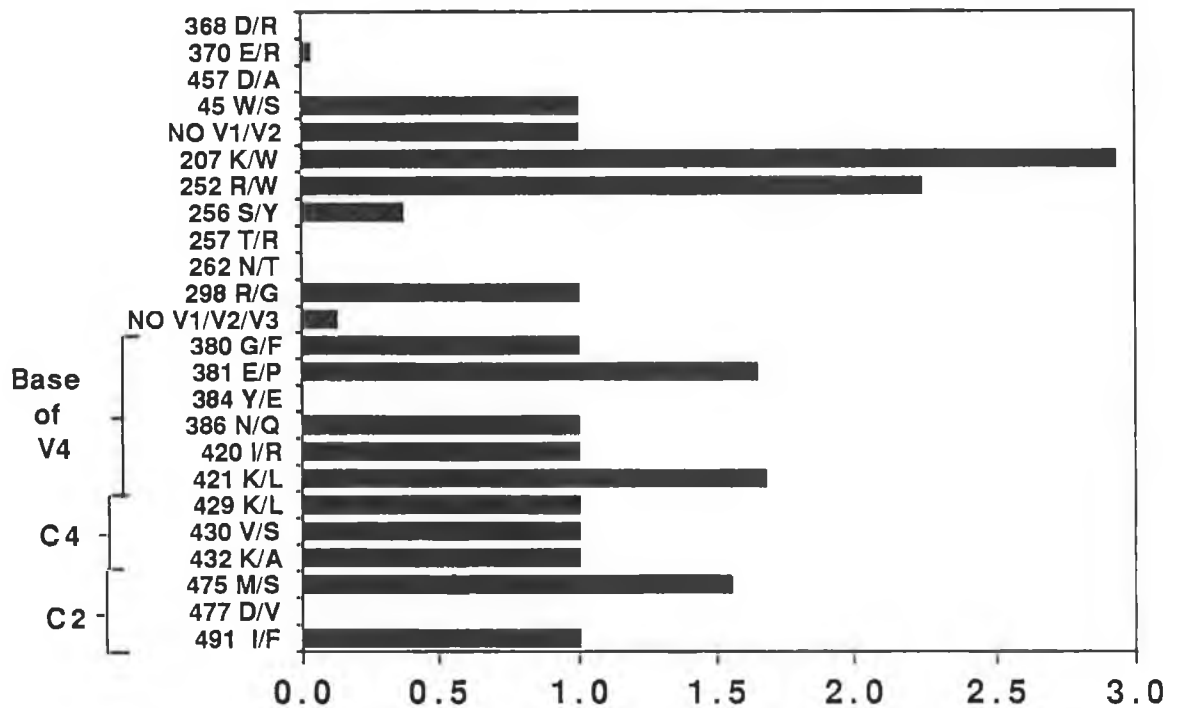


Figure 6.7 (d): Data for H12L7





**Figure 6.8** Knockout assays of the shuffled Fabs, given on a different graph for each clone. The various mutations considered are indicated on the vertical axis, while the binding index is marked on the horizontal axis. Each bar represents the binding index for that particular mutation. An index of 1 implies no effect on binding; less than 0.5 indicates an inhibitory mutation and greater than 1.5 denotes an enhancing mutation. All mutations are grouped according to their position along the secondary structure of the gp120 molecule, except for 368D/R, 370E/R and 457D/A which are shown together since they are typically inhibitory for this class of antibody.

## 6.3 DISCUSSION

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### 6.3.1 ANTIBODY FUNCTION.

It has previously been established that the specificity of a Fab is associated primarily with its heavy chain and that it is possible to use a variety of light chains while maintaining specificity. However, it is difficult to believe that the light chain is chosen at random, dependent merely on its ability not to interfere with the interactions in the binding pocket, and so the experiments in this chapter sought to define the role of the light chain more clearly.

An antibody does not just serve to bind antigen, it must effect a particular function once it has bound the antigen, depending on what that particular antigen is. In this case, the antibodies must first bind gp120 on the viral surface and then effect its neutralisation. The studies so far had only looked at antigen binding and so this series of experiments sought to bring this one step further and investigate the neutralising ability of these Fabs, and perhaps attribute this activity, in part at least, to the light chains.

### 6.3.2 THE b4 GROUP OF Fabs.

The collection of Fabs chosen all came from one particular group (as defined in chapter 4) due to the high proportion of Fabs in this group capable of neutralising virus *in vitro*. The Fabs b4 and b12 were known to be 2 of the best neutralisers isolated to date, while the remaining 2 members, b7 and b21, also neutralised, although to a lesser extent. The crosses chosen all incorporated the HC12 due to

the obvious ability of b12 to neutralise and the unique epitope specificity displayed by this Fab.

### **6.3.3 AFFINITIES.**

The initial task was to accurately measure the affinities of these Fabs in order to ensure that this was not giving rise to artifacts in the neutralisation data. All affinities were found to be sufficiently similar in both ELISA assay and on the BIAcore. It is interesting to note that the affinities of the HC12 crosses all lay within a very narrow range, even compared to the original Fabs, further supporting the theory that the binding of the Fab to antigen, in terms of specificity and affinity, are due mainly to the heavy chain. H12L18, a non-neutralising Fab, displayed an "on-rate" significantly lower than that of the other Fabs, which may be a contributory factor to its inability to neutralise.

### **6.3.4 NEUTRALISATION PATTERNS.**

Once it had been established that the affinities of all the Fabs were similar, and that they were all inhibited by sCD4, the neutralisation assays could be performed. This revealed that H12L4 and H12L12 were significantly better at neutralising, in two assays, than the Fabs H12L7 and H12L21, by factors of 15 and 40, respectively. This in itself is compelling evidence that the light chain, in combination with the heavy chain, is important to the neutralisation titre of the Fab, since these Fabs only differ in the light chains used. They did not differ in their heavy chains. A sequence analysis of the light chains revealed that there were very few differences between

chains, particularly between the neutralising LC12 and the poor neutraliser, LC21, where there were only four amino acid differences, two in the framework regions and two in the CDR1. Three of these changes were very conservative in both the size of the residue side groups and the charge they carried. The only non-conservative change was between the positively charged arginine with a large 5 carbon side chain (LC12) and the uncharged alanine consisting of a methyl group as a side chain (LC21), 33A/R. This is a very significant change especially since it resides in the CDR1 region and so is directly involved with the antigen binding site of the Fab. However, the importance of this change in relation to neutralisation titre, was not clear since an analysis of b4 and b12 showed that their heavy chains were identical and their light chains differed by 2 amino acids. The first was 5S/A in FR1 and the second was 33A/R in the CDR1, yet both of these Fabs neutralise very efficiently. Therefore, this difference, in isolation, may not be as important as it first seemed. It is apparent, however, that a small number of changes in the amino acid sequence of the light chain can radically affect the neutralisation titre of the Fab, supporting the idea that the light chain used is extremely important for this function. The criteria for neutralisation seems to be far more stringent than for antigen binding, for which there is 100% promiscuity within this group.

#### **6.3.5 VIRAL ISOLATES.**

Although it has been established that, for this set of Fabs, the light chain is critical in the context of the heavy chain combination, the

relative importance of the light chain compared to that of the heavy chain has not yet been analysed. In other words, is the light chain important only in combination with a particular heavy chain, or can it confer neutralisation ability on an antibody in the same way as the heavy chain can sometimes carry antigen specificity to interantigenic crosses?

A number of MN HIV isolates were employed to investigate this possibility. These isolates were all taken from male and female, homosexual and heterosexual patients that had become infected with the MN strain of the HIV virus. Five of the patients were European while the sixth was Ugandan and all were at various stages of the disease. The Fabs chosen for this study were b7 and b12 and the two shuffled combinations of these.

Initially it was noted that b12 neutralised more isolates with higher titres than b7, as expected. The shuffled Fab, H7L12 was able to neutralise one more isolate than b7 and the titres of two others had increased. This would suggest that LC12 had conferred an increased ability to neutralise on its Fab. This effect can be seen more clearly in the case of H12L7, which was only capable of neutralising a single isolate, that with the highest titre for b12. So HC12 with its own light chain was able to neutralise all isolates while it could only weakly neutralise one in combination with LC7. This evidence is a strong indication that the neutralising ability is more closely associated with the light chain, for this group of Fabs.

However, H7L12 was not able to neutralise all the isolates as was the case with b12 and, similarly, H12L7 could not neutralise the four isolates indicated for b7. Therefore, neutralisation is not

totally related to the light chain. The heavy chain has some role to play also and the extent to which this occurs probably depends on the sequence of the heavy chain.

It may be that the role of the heavy chain is derived from its ability to bind the antigen in combination with a given light chain in such a way that the light chain can then act to effect neutralisation. If this was the case, a light chain capable of neutralisation would always be required and the role of the heavy chain would be due mainly to the combination of chains than to specific properties encoded within its own sequence. However, this is merely conjecture and it is possible that the heavy chain has a more important role to play in neutralisation.

Having said that, the evidence does suggest that the light chain is more important in the heavy-light combination in terms of neutralisation than is the heavy chain.

Finally, it is interesting to note that most Fabs are not capable of neutralising all the isolates despite the fact that they are all of the same viral strain, the only difference being that they were all isolated from different patients. This serves to illustrate the complexity of the virus-antibody neutralisation reaction, in that it is not a simple case of binding the correct antibody to the virus to achieve neutralisation, but that there is a complex series of events taking place involving an interplay between the antibody and virus which may or may not lead to neutralisation. This must be borne in mind when investigating this interaction and it will require many more studies before it will be fully understood. One can only speculate on the mechanisms based on the results from single

studies such as the one presented here. Nevertheless, these experiments can yield vital information which may help in the final understanding of this phenomenon.

#### **6.3.6 LIGHT CHAIN AND EPITOPE SPECIFICITY.**

The next question to be addressed was: what exact effect was the light chain exerting on the antibody-gp120 interaction in order to facilitate the neutralisation of the virus? There must be a reason why one light chain in combination with HC12 leads to efficient neutralisation while another does not. It was thought that some useful information might be derived from examining the epitope specificity of a number of crosses, all involving the HC12, in more detail. This was done using the knockout assay described in chapter 4. The 3 Fabs investigated were H12L7, H12L12 and H12L21 since they all displayed different neutralisation titres. H12L12 (b12) showed many of the features common to all antibodies of this type, plus a number of characteristics specific to that antibody including the unusual V1/V2 inhibition effect which was seen to distinguish it from other Fabs in the panel. H12L21 displayed a very similar pattern of binding with the major differences being a lack of inhibition by mutation 45W/S, the loss of two enhancing mutations in C4 and perhaps the most significant change being an enhancement by 477D/V rather than the knockout effect seen with H12L12. The differences observed between H12L12 and H12L21 were seen to be characteristic of the Fab b21 and were, therefore, probably attributable to the LC21 present in Fab H12L21. Therefore, it seems that by switching the light chain in the Fab, the central

characteristics of the epitope are conserved with a small number of changes occurring around this, possibly as a result of the new light chain. This might not be particularly surprising since, if the epitope specificity resides mainly in the heavy chain as postulated, then the epitopes of these two shuffled Fabs, both having the same heavy chain, should remain essentially the same.

A similar result can be observed in the case of H12L7. The main characteristics of the H12L12 epitope are conserved, with a number of changes occurring around this. There are more changes for this Fab than was seen with H12L21, possibly because LC7 exhibits twice the number of differences that LC21 does from LC12 and six of the LC7 differences are in the CDR regions, compared to two for LC21. The presence of LC7 increases the susceptibility of the C2 region and also is no longer affected by the loss of the V1/V2 loop.

It could be postulated from these results that the heavy chain is initially responsible for binding of the Fab to gp120, which explains the conservation of the central epitope in all of the shuffled Fabs. The light chain may then be responsible for the 'fine-tuning' of this binding, hence the differences in epitopes observed around the central binding site. Perhaps it is the ability of the light chain to 'fine-tune' the binding in this way that defines whether the Fab is capable of neutralising the virus or not. This would help to explain why a particular light chain was needed for neutralisation to occur and also why the chains are not nearly so promiscuous in relation to neutralisation as they are when only antigen binding is considered. The Fab will bind the antigen as long as the correct heavy chain is present and the light chain is not obstructing the binding site.



However, neutralisation is a far more precise phenomenon possibly requiring that the heavy chain binds the antigen in the correct orientation so as to allow the light chain to 'fine-tune' the binding and effect the neutralisation. This may explain why the combination of heavy and light chain is also important in this process and why LC7 could not confer full neutralising ability in combination with HC12 when tested against the MN viral isolates.

On a more detailed level, it can be seen that there are very few differences between the binding patterns of H12L12 and H12L21 and yet the neutralisation titres differ by as much as 40-fold. The major difference is mutation 477D/V which knocks out binding by H12L12 and enhances binding of H12L21. This suggests that the presence of light chain 21 has altered the epitope in this area and that this may be contributing to the enormous difference in titres. It is also interesting to note that this mutation inhibits binding of both H12L12 and H12L7 and that H12L7 displays a higher neutralisation titre than H12L21. However, the other changes in binding pattern induced by LC7 make the effect of this mutation unclear. H12L7 is no longer inhibited by the loss of the V1/V2 loop as b12 was, which may be a factor in its weaker neutralisation titre. Whether the increased sensitivity in the C2 region augments or counteracts this effect is unknown. Further study of this area will be required to fully understand the mechanisms taking place here.

Finally, the high degree of sensitivity in the heavy and light chain combinations for neutralisation would support the theory that those Fabs isolated from the library capable of neutralising the virus, are, in fact, the *in vivo* combinations.

In conclusion, the promiscuity demonstrated with antigen binding does not translate to neutralisation, suggesting that this is a more precise phenomenon. The light chain has been shown to have primary importance in the neutralisation activity of this group of Fabs, although the combination with the heavy chain also has some role to play in this event. These experiments have lead to a theory on the mode of neutralisation of HIV by this group of Fabs, however, many more studies will have to be carried out before this event is fully understood. This type of approach could prove useful in the analysis of this and, perhaps, many other antibody-mediated viral processes.

## CHAPTER 7

## 7.1 INTRODUCTION.

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### 7.1.1 OVERVIEW.

It should be clear at this stage that there are a number of highly sophisticated techniques available, such as phage-display libraries and recombinant DNA methodologies, which permit the dissection of the antibody response, and its analysis on a very detailed scale. However, this technology was limited unless it can be applied at a practical level. The area of viral neutralisation using antibodies, which was one such application, has been previously discussed in chapter 5. Another development, which takes advantage of the diversity and specificity of the humoral response, was that of catalytic antibodies (Lerner and Tramontano, 1988; Schultz *et al.*, 1990). These are molecules capable of carrying out chemical transformations by making use of a catalytically active group in the antigen-binding site. The applications of this class of protein have been previously discussed in the introduction, section 1.11. Since antibodies with catalytic properties do not usually exist in nature, it was often necessary to use protein engineering or molecular biology techniques to introduce such groups into the molecule.

### 7.1.2 METAL-MEDIATED CATALYSIS.

Metalloantibodies have been mentioned as a means of achieving hydrolytic reactions, particularly the cleavage of a peptide bond. The study of these catalysts stems from knowledge about existing metalloenzymes such as carboxypeptidase. This consists of a  $\beta$ -

pleated sheet containing eight  $\beta$ -strands surrounded by a number of helices, folded in such a way as to bring residues His69, Glu72 and His196 in proximity to one another. These three residues bind zinc in a tetrahedral structure in the active site. The zinc then catalyses proteolytic cleavage polarising the carbonyl oxygen of its substrate (Lehninger, 1982; Branden and Tooze, 1991). Zinc was also a critical component of a similar enzyme carbonic anhydrase which catalyses the hydration of  $\text{CO}_2$  to  $\text{H}_2\text{CO}_3$ .

### **7.1.3 METAL-BINDING SITES IN ANTIBODIES.**

An antibody variable region consists of nine antiparallel  $\beta$  stands in two sheets of four and five strands arranged in a barrel, joined by loops. There are three major loops forming the CDR regions with CDRs 2 and 3 joining adjacent strands and CDR1 joining two strands across the barrel structure. This structure was compared to those of a number of metalloenzymes in order to find any structurally conserved areas and so identify possible zinc binding sites in the antibody (Roberts *et al.*, 1990). A high degree of similarity was found between the active site of carbonic anhydrase B and the area around residues 34, 89 and 91 of the light chain variable region of the antibody. It was, therefore, chosen to mutate these residues to histidines (to bind metal) in a mouse fluorescein-specific FAb as a model system, for a number of reasons: first, the 3-D structure of the binding site was known; second, this chain had already been cloned and third, there were a number of proximal tryptophan residues, so the metal could be detected by fluorescence quenching. The use of three histidines would ensure that three zinc

coordination sites would be bound, leaving the fourth free to activate a water molecule. It was found that these mutations would place the zinc in the center, at the bottom of the binding pocket, which would be ideally positioned for hydrolysis to occur. The results from this group indicated that the antibody bound both metal and fluorescein simultaneously and so acted as a noncatalytic model for this type of system. Furthermore, it was established in a separate study, that zinc was the most efficient metal cofactor for this type of catalysis. However, this engineered light chain had not been proven in a catalytic system.

#### **7.1.4 THEORY OF Fab CONSTRUCTION.**

It has already been demonstrated, in chapter 5, that it was possible to combine heavy and light chains of different specificities and retain binding to the heavy chain-specific antigen. Therefore, it should be possible to combine the functions of 2 different chains to produce an advantageous antibody. The purpose of the work presented in this chapter was to combine the gp120-specificity of the heavy chains discussed in chapter 5, with the hydrolytic capabilities of the metallo-light chains designed previously, in an attempt to produce an Fab fragment capable of cleaving gp120 on the HIV viral surface. This would validate the metal-based system designed above in a relevant, catalytic situation. It would also be highly beneficial with the obvious consequences that it may yield an antibody with equal or even improved ability to neutralise HIV over any previously observed.

Two methods for achieving this were attempted: Directed crosses with the gp120 heavy chains from chapter 5, and a cross involving the metallo-light chain and an entire gp120-specific heavy chain library. The results of both experiments will be discussed. Three different light chains were used in this study: the first was designed as shown above with histidines incorporated at positions 34, 89 and 91, named LCQ. This chain also had Tyr36 mutated to a Leu as the tyrosine would have interfered with metal binding. The second chain incorporated histidines at an alternative zinc binding site involving positions 49,50 and 53, named LCC. The third chain, LCQC, had all seven of the above changes. Western blot techniques involving radioactive zinc, and novel ELISA assays were developed to demonstrate binding of the antibody to metal. The neutralisation capabilities of the resulting antibodies were also tested both with and without zinc to determine if this was having an effect.

## **7.1 RESULTS.**

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### **7.1.1 LIGHT CHAIN.**

The sequence of the light chain is given in Figure 7.1 with the mutations indicated. The position of the five missing residues are also shown, although this was corrected during the overlap PCR (see methods section 3.6.2). The domain swapping worked perfectly, giving a chain with the mutated mouse variable region and the constant region from the human gp120 specific chain, LC22. This was confirmed by sequencing.

### **7.2.2 CROSSES WITH gp120-SPECIFIC HEAVY CHAINS.**

Initially, the three light chains (LCQ, LCC, LCQC) were cloned into the binary plasmid vector, pTC01 in order to perform directed crosses with all the heavy chains previously cloned into the complementary vector, pTAC01H (see chapter 5). Productive crosses were selected for, based on their ability to bind gp120 on direct ELISA assays. Only HC6 was found to bind productively with all three light chains. No other heavy chain gave a positive result on ELISA. The relative affinities of these three crosses were determined to be in the range  $1 \times 10^7$  -  $6 \times 10^7 \text{M}^{-1}$  (Table 7.1) which is in the range found for the interantigenic crosses presented in chapter 5.

However, the level of expression of these clones was very low, making it difficult to use them for further experiments. In addition to this, only one heavy chain had crossed productively, and so, in



order to maximise the chances of success, an alternative route was decided upon.

```

FR1                                0
GAG CTC GTT ATG ACT CAG ACA CCA CTA TCA CTT CCT GTT AGT
E   L   V   M   T   Q   T   P   L   S   L   P   V   S   9
                                CDR1
CTA GGT GAT CAA GCC TCC ATC TCT TGC AGA TCT AGT CAG AGC
L   G   D   Q   A   S   I   S   C   R   S   S   Q   S   23
                                FR2 (Y)
CTT GTA CAC AGT AAT GGA AAC ACC TAT TTA CAT TGG TTA CTG
L   V   H   S   N   G   N   T   Y   L   H   W   L   L   37
                                (R) CDR2
CAG AAG CCA GGC CAG TCT CCA AAG GTC CTG ATC CAC CAT GTT
Q   K   P   G   Q   S   P   K   V   L   I   H   H   V   51
                                FR3 (Y) (K)
TCC CAT CGA TTT TCT GGG GTC CCA GAC AGG TTC AGT GGC AGT
S   H   R   F   S   G   V   P   D   R   F   S   G   S   65
                                (N)
GGA TCA GGG ACA GAT TTC ACA CTC AAG ATC AGC AGA GTG GAG
G   S   G   T   D   F   T   L   K   I   S   R   V   E   79
                                CDR3
GCT GAG GAT CTG GGA GTT TAT TTC TGC CAT CCA CAT ACA CAT
A   E   D   L   G   V   Y   F   C   H   Q   H   T   H   93
                                FR4 (S) (S)
GTT CCG TGG ACG TTC GGT GGA GGC ACC AAA CGG GCT GAT GCT
V   P   W   T   F   G   G   G   T   K   R   A   D   A   107

GCA CCA ACT GTA TCC
A   P   T   V   S   112

```

**Figure 1:** Nucleotide and amino acid sequence of fluorescein-specific light chain mutated to bind metal. All seven mutations are included in this sequence (LCQC) shown by the underlined residues. The residue before it was mutated is given in brackets for each. The residues L,E,I,K and R are missing between R104 and A105. This was corrected during overlap PCR.(Numbering according to Roberts, 1990).

CROSS	AFFINITY (M <sup>-1</sup> )
H6LQ	1.99*10 <sup>7</sup>
H6LC	5.00*10 <sup>7</sup>
H6LQC	6.30*10 <sup>7</sup>

**TABLE 7.1:** Affinities of crosses between gp120-specific heavy chain 6 and the metallolight chains as determined by competition ELISA.

### 7.2.3 CROSSES WITH THE HEAVY CHAIN LIBRARY.

It was decided to cross the light chains with an entire library of heavy chains to try and isolate the maximum number of productive pairings possible. The heavy chain source chosen was the library used originally to isolate the gp120 binders (chapter 4) at a stage before the light chains had been inserted into the vector. It, therefore consisted of a library of pComb3 vector containing heavy chains with nothing yet inserted into the light chain site. This library was then cut with the appropriate enzymes (Xba I and Sac I) and split into three portions. One of the three metallolight chain inserts were then inserted into each portion and the three new libraries were cloned back into *E. coli*. They were then subjected to four rounds of panning against gp120 in four groups: the three

libraries and a further, equal mixture of the phage obtained from each of these. The results of the pannings are given in Table 7.2.

	PANNING 1	PANNING 2	PANNING 3	PANNING 4
LCQ	$1.46 \times 10^6$	$3.00 \times 10^6$	$4.2 \times 10^5$	$5.00 \times 10^5$
LCC	$1.09 \times 10^6$	$5.40 \times 10^6$	$2.64 \times 10^5$	$1.30 \times 10^6$
LCQC	$1.49 \times 10^6$	$2.80 \times 10^6$	$9.00 \times 10^5$	$1.00 \times 10^6$
MIXTURE	$1.12 \times 10^6$	$4.15 \times 10^6$	$2.40 \times 10^6$	$7.00 \times 10^6$

**Table 7.2.** Panning results of the metal library. The figures given are the output titres of phage (particles/ml) after each round of panning. The titres for the four rounds of panning of the 4 libraries (as explained in the text) are shown. It is notable that there is no increase in phage titre over the four rounds of panning for any of the libraries tested.

Normally, a 100 to 1000-fold increase in phage titres would be expected from the first to the fourth rounds of panning. However, as can be seen from the table, this was not the observed result in this case. No overall trend towards increasing titres can be seen, with the fourth round titre being approximately equal to the first. The reasons for this will be examined in the discussion.

This failure to increase the titre would mean that there was little or no enrichment for the required gp120 binding clones and so the frequency at which they would occur in the overall population would be extremely low. A method capable of screening a far larger number of clones was, therefore required.

A technique was developed to grow the clones and express the Fab protein entirely in 96-well plates, thus allowing 96 cultures to be screened at a time. The supernatants from these plates could be transferred directly to gp120-coated plates for the ELISA.

Approximately 1000 colonies from the third and fourth pannings of each library were screened in this way, giving rise to the detection of 17 clones that specifically bound gp120 (an O.D. of 3 times above the negative control). There was a bias in the clones derived from each of the light chain libraries (9 from LCQC, 6 from LCC and 1 from LCQ), with only one being isolated from the mixed panning and this used the light chain LCQ. They were divided approximately equally between the third and fourth pannings: 8 from panning 3 and 9 from panning 4. All 17 clones were sequenced for both heavy and light chains (Figure 7.2). The clones are labeled according to their light chain, the panning they were derived from and their position on the ELISA palte during screeining. Thus, C3F11 uses light chain LCC, was derived from the third panning and was isolated from well F11. None of these sequences are identical to those isolated from the original gp120-panned library discussed in chapter 4. Moreover, none of these CDR3 sequences were sufficiently similar to those from the previously isolated clones to be included in any of the seven groups

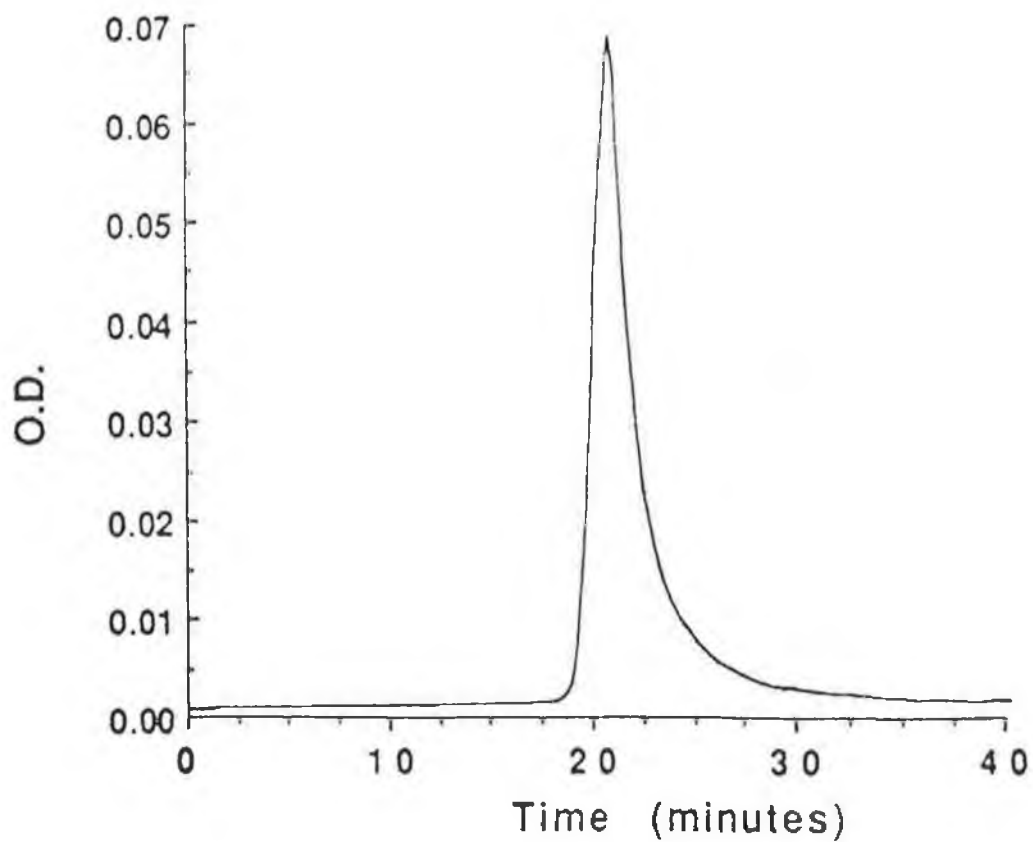
defined for those Fabs. Therefore, this was a completely new set of gp120 binding Fabs.

It was thought to be necessary to carry out all further studies with purified Fab fragments and so all of the above clones were expressed as described previously and the Fab was purified. HPLC analysis of these preparations using a size exclusion column, under the direction of Robert O'Connor, DCU, showed them to be 100% pure with no aggregates or breakdown products (Figure 7.3)

## HEAVY CHAIN SEQUENCES OF THE METALLOANTIBODIES

CLONE	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
C3F11	LEESGGGLVQPGGSLRLSCVASGFPPS	NYWLH	WVRQAPGKGLVWVA	RIHSDGSRTAYADSVQG	RFTISRDTAKITLTLQMNSLRAEDSAVYYCAK	AEDIVVVVATITTFDY	WGQGILVTVSSASTKGPS
Q4F11	LEESGGGLVKPGGSLRLSCVVGFSFS	DAWMS	WVRQAPGKGLEWVG	RIKSESEGGTIDYCAPVG	RFSISRDDSTATLYLQMNSLRSEDATVYYCTT	SPGRFSHRNY	WGQGTLVSVSSTSTKGPS
C3G1	LEESGGGVVHPGRSLTLPAAASGFSL	GYAMK	WVRQAPGKGLEWLS	VISGSGDETDYADSVKD	RFTISRDNKNTLFLQMSLRVDDSAIYYCVT	SDRPVLRFLQWFPMDV	WGKGTVTTVSSASTKGPS
QC3G7	LEESGGGLKKAGGSLRVSCEASGYTFT	DYHIH	WVRQAPGRGLEWVG	WISPDGSGTKYAKSFQD	WVTISRDTISITVYMELSRSLRSDDTAIYYCAR	THYDILTGVGTAEYFPH	WGQGTLVTVSSASTKGPS
QC3F9	LEQSGEEVKKPGESLKMSCEATSGYSFS	AYWIG	WVRQMPGKGLEWLG	IINPGDSEVIYKSSPQG	QVTISADKSISTAYLQWSSLKASDTAMYYCAR	LDSWGTLYLSGFDH	WGQGTLVTVSSASTKGPS
QC3G10	LEESGGDLVQTRSLRLSCAASGFSD	DYVMN	WVRLVPGKGLEWVS	SINWSGLNLDYADSVKG	RFTISRDNKNSLYLQMDSLRPEDSAFYFCAR	PKDYGAYQPFDL	WGRGTRVTVSSASTKGPS
C3F6	LEQSGAEMRKPGESLKISCKSGYSFS	NYWVG	WVRQMPGKGLEVMG	IIPGDSDTRYSPSSQG	QVTISVDKSINAAYLQWRSRLASDTAMYYCAR	HRAAAGGHFDY	WPGGTLVTVSSPSTKGPS
C4A5	LEESGRNLVVRPGGSLRLSCAASGFTFS	YYTMH	WLRQAPGKGLEWVA	LISSDGHRTDYADSVKG	RFTISRDNKNTLFLQVASVRFEDTAVYSCAR	DLGLPGSWYFDL	WGRGTLVTVSSASTKGPS
QC4E5	LEESGAEVKKPGESLRISCKSGYRFT	SYWIG	WVRQMPGKGLEWVG	IIPGDSDTRYSPSFQG	QVTISADKSISTAYLQWSSLKASDTAMYYCAR	HRAIQFTQIDI	WGQGTMTVTVSSASTKGPS
QC4H2	LEESGGGVVQPRSLRLSCAVSLVNFN	TYAMH	WVRQAPGKGPWVA	FISHDGKTESYADSVRG	RFSVSRDNKSNMLYLHMDRLGAEDTAVYYCAK	DFKDLVCDFWGGVYRHFDF	WGHGTLVTVSSASTKGPS
QC4A8	LEESGGGVVQPRSLRLSCAASGFIFG	RFAIH	WVRQAPGKGLEWVA	LISYDESINKFYADSVKG	RFTISRDNQYQTSWLQMNGLKADDTAVYYCAR	EHFDWRTSDYSYMDV	WGKGTVTTVSSASTKGPS
C4A1	LEESGGGVVQPRSLRLSCAASGFTFS	RYGFH	WVRQAPGKGLEWVA	VVSHDGYNEFYADSVKG	RFTVSRDDSKNTLYLQMNSLRAEDTAVYYCTR	WRRHYDFWRQYRLDF	WGQGTLLVTVSSASTKGPS
QC4C2	LEQSGGGLVQPGGSLRLSCVVGFTFS	SYDMN	WVRQAPGKGLEWVS	HVSNTGNTIYYSDSVKG	RFTISRDNKNSLYLQMNSLRADDATVYYCAR	RRQSSGWFRGWYFDL	WGRGTLVTVSSASTKGPS
QC4D2	LEQSGGGLVTPGGSLRLSCEASGFIFS	DFYMN	WIRQAPGKGLEWVA	YISSSGNIYYGDSVKG	RFIVSRDNKKSIVYLQMSDLGTEDTAVYYCAS	SSPEYNYRSGYYRWFDL	WGQGTPTVTVSSASTKGPS
Q4F11	LEQSGGDLVQPGGSLRLSCVASGFTFG	DYAMH	WVRQAPGKGLEWVS	GITWNGNSIGYVDSVKG	RFTIFRDNKNSLYLQMSDLRVEDTAFYYCAK	DKLYNSRYYGDWYFDL	WGRGTLVTVSSASTEGPS
C3F10	LEQSGAEVKKPGESLKISCKSGYRFT	SYWIG	WVRQMPGKGLEWMA	IMYPGDSHTIYSPSFQG	QITISADTSISTAYLQWSSLKASDTAMYYCVR	LSSPTVAHEPPLGY	WGQGITLVTVSSASTKGPS
QC3G6	LEESGAELVKRFGASVKLSCKASVYTST	TYDIN	WVRQIACQGLQWVG	WMNPNGVASYARKFEG	RVVMTNISTATAYMELRSLRPEDTGIYFCSR	GQKRSSSGLGS	WGQGTQVTVSSASTKGPS

**Figure 7.2.** Variable heavy chain sequences of the 17 clones found to bind gp120 when tested by direct ELISA. The clone names are given on the left and are explained in the text. None of these sequences were found to be similar to those previously isolated from the same library. Furthermore, these heavy chains cannot be organised into any of the sequence groups described in chapter 4.



**Figure 7.3.** HPLC analysis of purified clone C4A5 as an example of the results from the purification procedure. The Fab can be seen to be 100% pure with no aggregates or breakdown products. All Fabs gave a similar profile.



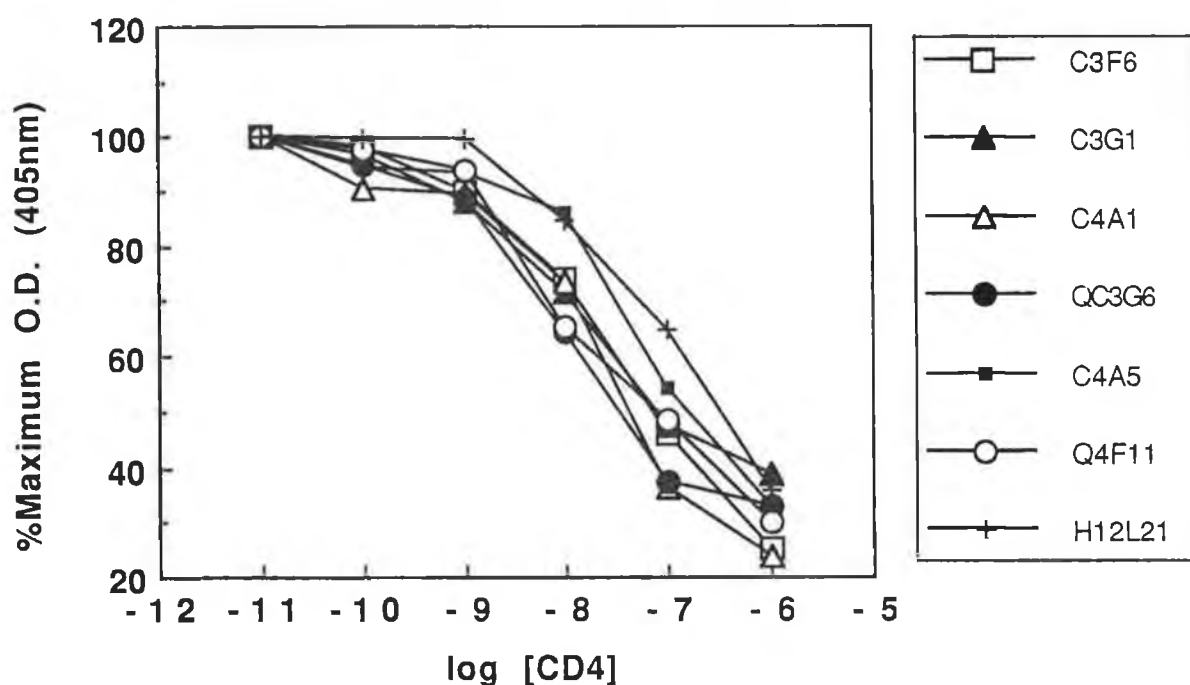
#### 7.2.4 RELATIVE AFFINITIES AND CD4 COMPETITION ELISAs.

The relative affinities of these clones were measured by competition ELISA, using gp120 as the competing antigen (Table 7.3). All the clones displayed affinities in the range  $1 \times 10^7$  to  $5 \times 10^7 \text{M}^{-1}$  which was slightly lower than the values obtained for other gp120 binders, possibly as a result of the fact that these are hybrid antibodies containing chains of 2 different specificities.

CLONE	AFFINITY ( $\text{M}^{-1}$ )	CLONE	AFFINITY ( $\text{M}^{-1}$ )	CLONE	AFFINITY ( $\text{M}^{-1}$ )
C4F11	1.0	QC4D2	1.0	C3F11	2.0
QC3G6	2.0	C4A1	2.0	QC3G7	1.0
C3G1	2.5	QC3G10	3.0	QC4E5	7.0
C4A5	1.0	QC4A8	1.5	QC4H2	5.0
C3F6	8.0	QC3F9	2.0	MQ4F11	1.0
C4A1	1.5	C3F10	6.0		

**Table 7.3.** Affinities of all gp120-binding clones isolated from the metal library. All values given are multiplied by a factor of  $10^{-7}$ .

The epitope specificity of a number of these clones were then determined by means of sCD4 competition ELISAs (Figure 7.4). It can be seen from the graph that all the clones are inhibited by sCD4 to a similar extent as a previously analysed antibody, H12L21, and therefore, it can be concluded that they are specific for the CD4 binding site of gp120. This was corroborated by the result that none of these Fabs bound to a V3 loop peptide on direct ELISA, showing that they are not specific for the V3 loop.

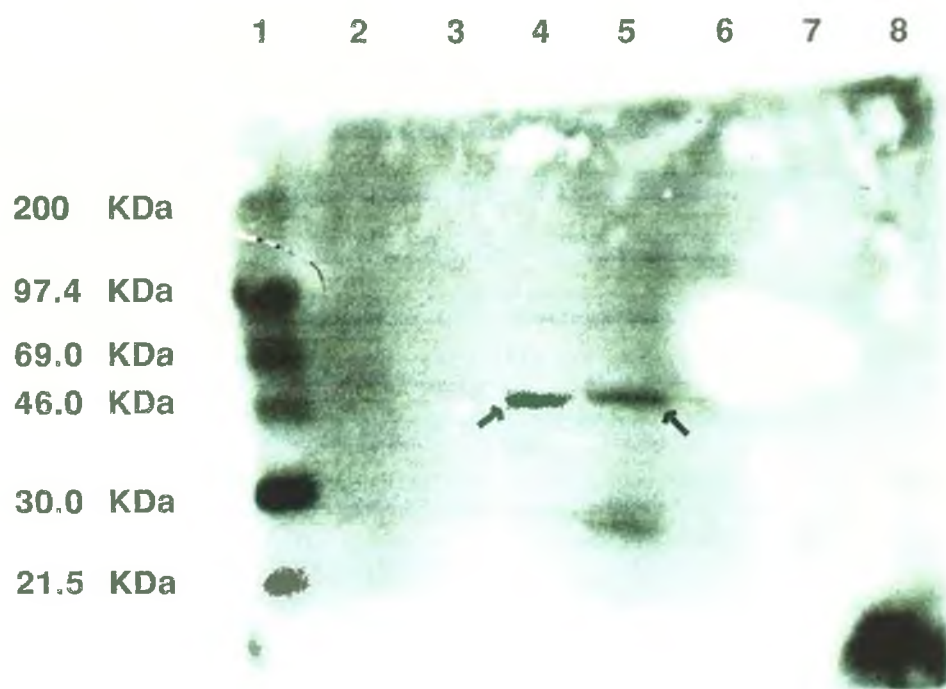


**Figure 7.4.** CD4 inhibition curves of a selection of Fabs isolated from the library. H21L21 was used as a positive control since this was known to bind to the CD4-binding site of gp120. All clones, including the positive control resulted in CD4 inhibition constants of approximately  $10^{-7}$ M.

### 7.2.5 Zinc-binding assays.

The next stage was to determine whether the Fabs bound zinc. Previously the presence of metal binding had been detected by fluorescence quenching. However, this was a relatively indirect assay and can be difficult to obtain reproducible, accurate results with. Therefore, two alternative methods for detection of zinc binding were chosen.

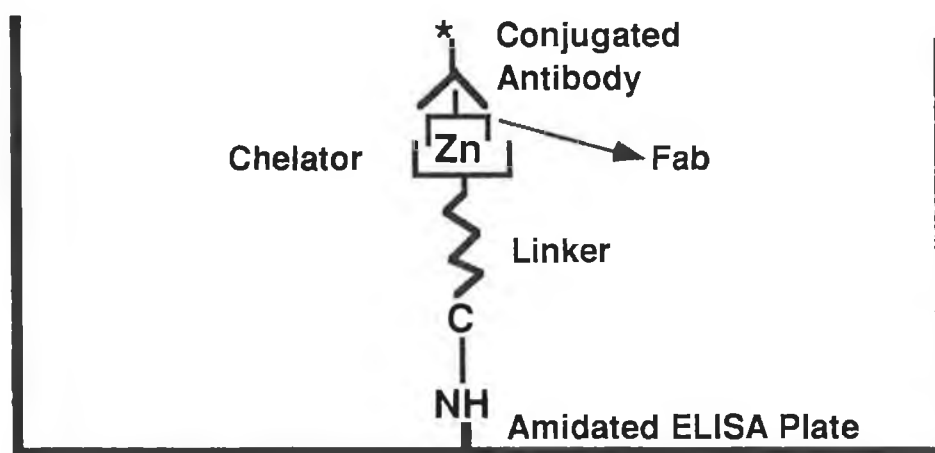
The first was a Western blotting technique (Schiff *et al.*, 1988) which involved running the antibodies on an SDS-gel, transferring them to PVDF membrane and probing this membrane with  $\text{Zn}^{65}$ . A band at 50kDa would indicate binding of the metal by the antibody. A gp120-binding Fab not engineered to bind zinc was used as a negative control. This technique met with limited success: none of the negative control antibodies ever came up positive which showed there was no cross-reactivity occurring. However, only 2 of the 17 antibodies displayed any binding by this technique, although these consistently yielded the same positive result (Figure 7.5), while all the others remained consistently negative. This result would seem to suggest that only 2 Fabs, QC3G6 and C4A5, were capable of binding both gp120 and zinc. However, it was possible that the Fabs were only being weakly expressed in culture, and that this technique was not sensitive enough to detect the metal-binding. Also, a western blot was a purely qualitative assay and did not allow any quantitation of the binding response. Furthermore,  $\text{Zn}^{65}$  is a very strong radioisotope with a relatively long half-life which made it comparatively difficult to work with.



**Figure 7.5** Western blot of Zinc-binding assays. A band at 50KDa indicates the presence of Fab, as detected with  $Zn^{65}$ . Such a band would suggest that the antibody in that lane was capable of binding metal. The sizes of the molecular weight markers are indicated to the left of the gel. 5ug of purified Fab was run in each lane, as indicated below and the blot was probed with radioactive metal as described in section 3.6.4 of the methods. The arrow indicates the position of the Fab.

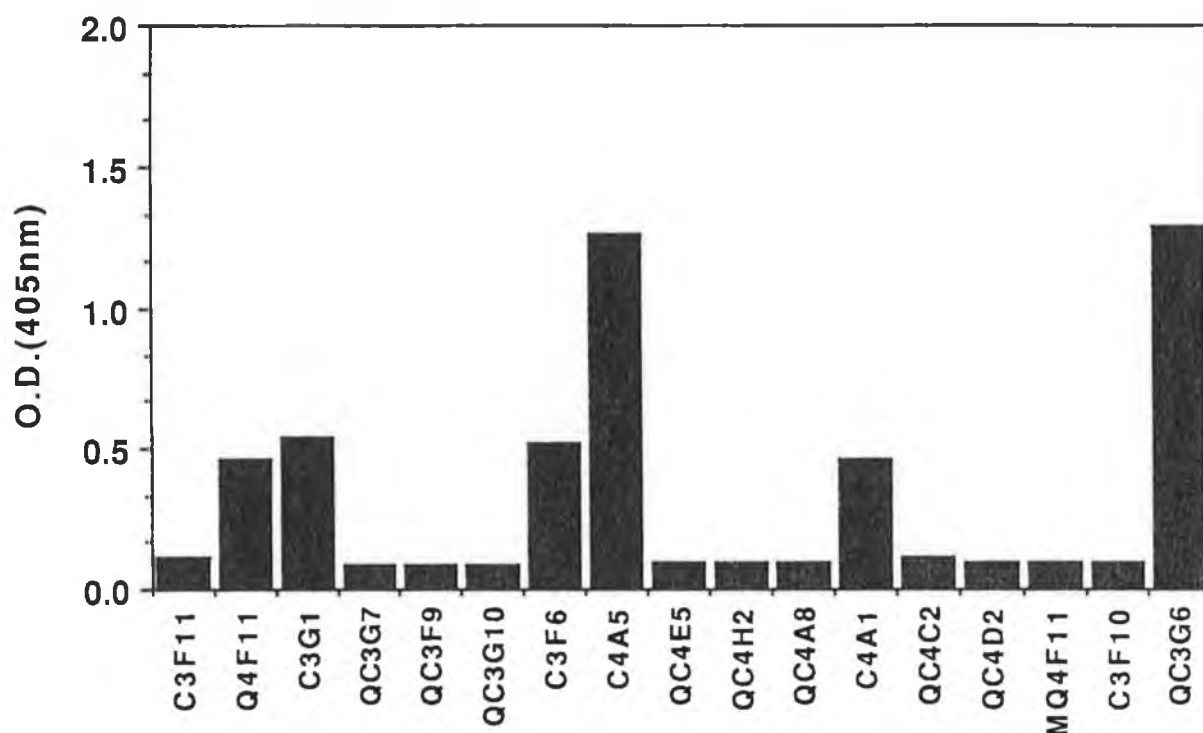
- Lane 1: Molecular weight markers.
- Lane 2: H12L12
- Lane 3: Q4F11
- Lane 4: QC3G6
- Lane 5: C4A5
- Lane 6: C4A1
- Lane 7: C3G1

Therefore, it was decided to develop an ELISA technique to detect metal binding. The main problem with this approach was to find a way of immobilising the Zn to the surface of a plate in such a way as to make it accessible to binding by the Fab. It was decided to adopt an adaptation of the technique used by J. Rosenblum, Scripps, employing amidated plates (Costar), or alternatively, Covalink plates (Nunc), both of which permit the covalent immobilisation of molecules to the plate via a surface amine group (Rasmussen, 1990). A linker molecule was immobilised in this way, followed by a chelator which could bind Zn, thus immobilising it to the plate (Figure 7.6).



**Figure 7.6.** Representation of the direct ELISA used to test for metal-binding. Commercially available amidated plates were used to immobilise a linker molecule (BS<sup>3</sup>, Pierce). This could then capture a metal chelator (Iminodiacetic acid, Sigma) which could then bind the metal ion. This provided a surface for the direct ELISA. Bound Fab could be detected using a secondary, conjugated antibody (see methods, 3.6.4).

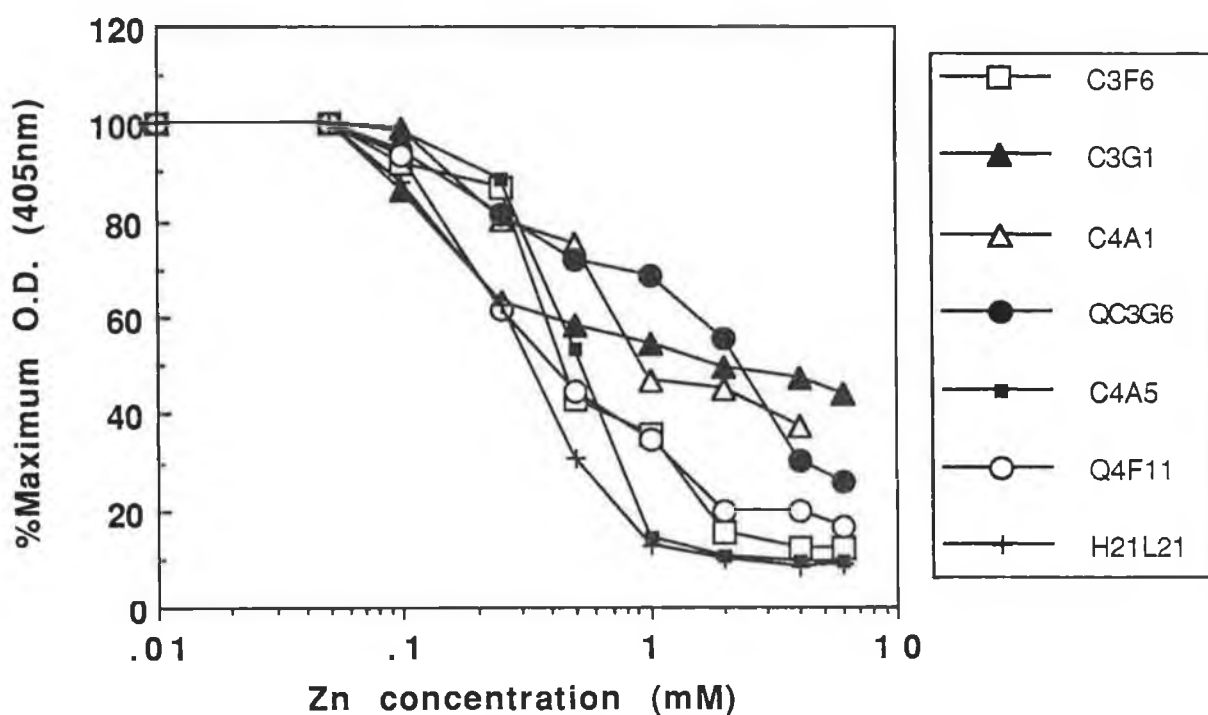
The linker molecule ensured that the Zn would be immobilised a sufficient distance from the surface to make it accessible to the Fab. The Fabs could then be tested by direct ELISA, with this plate, for Zn binding. When this was done, six of the seventeen fragments were found to bind at significantly higher levels than background (Figure 7.7). These were Q4F11, C3G1, C3F6, C4A5, C4A1 and QC3G6. It is interesting to note that The two clones resulting in the highest signal were QC3G6 and C4A5, which were the two clones that were found to be positive with the western blot technique. It seems likely, therefore, that the western blot was only sensitive enough to detect binding by these two clones and none of the others. All subsequent studies were carried out on these six positive clones only.



**Figure 7.7.** Binding of metalloantibodies to zinc as examined by direct metal-binding ELISA. 6 clones resulted in O.D. values of three times background or higher.

#### 7.2.6 Zinc binding constants.

It was necessary to measure the strength of binding of the Fabs to Zn, and so competitive ELISAs were performed using Zn as the competing molecule (Figure 7.8). It can be seen from the diagram that the Fabs were inhibited by 50% at Zn concentrations between 300 $\mu$ M and 2mM.



**Figure 7.8.** Zinc inhibition constants for metal binders. The inhibition constant is defined as that concentration of zinc resulting in 50% inhibition of Fab-binding. It can be seen that the constants for the metal binders lie in the range of 300 $\mu$ M to 2mM. The positive control used was H21L21, produced in the binary plasmid system, which had an associated constant of 200 $\mu$ M.

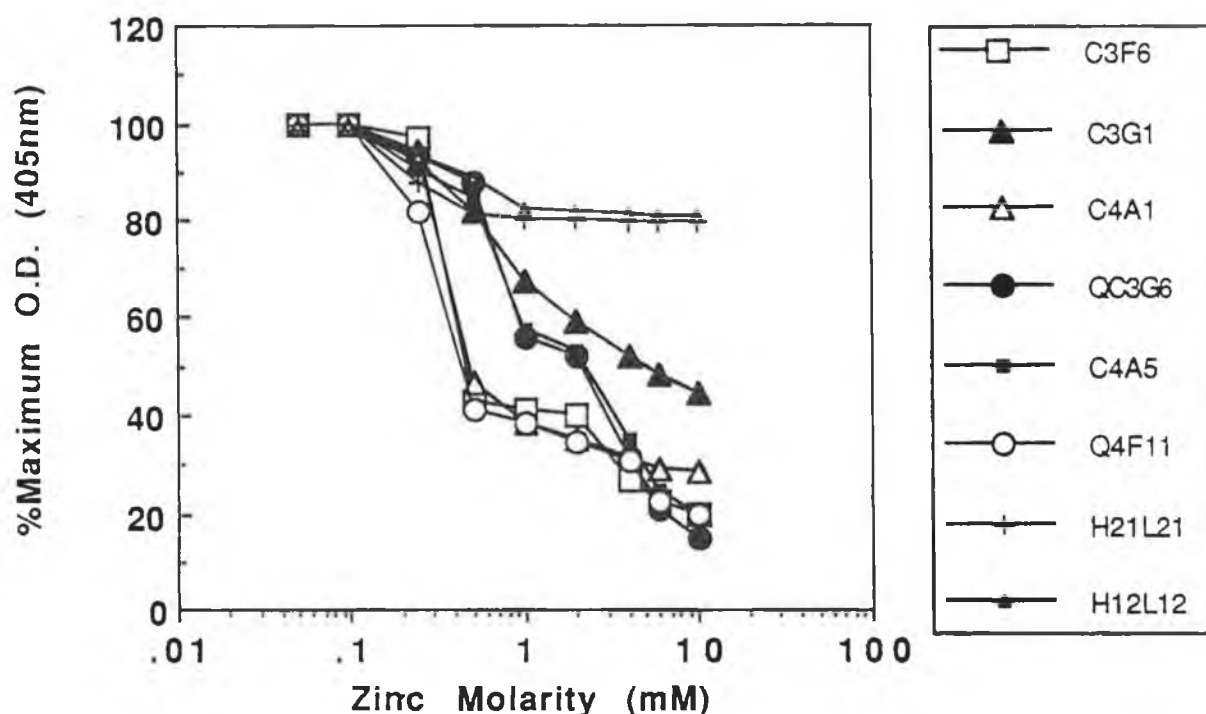
This was five to twenty times higher than values obtained previously with these light chains by fluorescence quenching techniques (Iverson *et al.*, 1990) which means that a higher concentration of Zn was required with these Fabs to reach metal



saturation of the binding site. It should be noted, however, that those studies used the complete mouse anti-fluorescein Fab, while the Fabs in this study are composed of chains with 2 different specificities. A separate study on metal antibodies (Iverson and Lerner, 1989) concluded that the Zn concentration exhibiting the highest efficiency was 1.5mM, which is well within the range found for these antibodies. Finally, H21L21 was used as a positive control in this experiment as it was produced in the binary plasmid system and so contains a His<sub>5</sub> tail at the carboxyl end of the heavy chain which was designed to bind metal ions (Regan and Clarke, 1990). It can be seen that the inhibition constant for this is the strongest of all the Fabs tested, at a concentration of 200μM. None of the negative control Fabs displayed any binding to Zn in this assay.

It was still not known if the metal was being localised to the binding site of these antibodies, or if the effect seen in ELISA was attributable to non-specific mechanisms. A further competition ELISA was designed to clarify this point. The Fabs were first bound to gp120 immobilised on a plate and this was allowed to compete with serially diluted Zn in solution. Any competition for binding would indicate that the binding sites for gp120 and metal were adjacent to one another on the Fab molecule, as Zn is very small compared to the gp120 molecule. The results can be seen in Figure 7.9. Binding by all Fabs was reduced by 50% at zinc concentrations in the range 300μM to 2mM, except C3G1 which was slightly higher. This correlates very closely with the data obtained from the previous Zn competition ELISAs. C3G1 displays the weakest binding in both cases; C3F6 and Q4F11 exhibit the strongest binding, while

C4A5, QC3G6 and C4A1 give values in the middle of the range. As all of these Fabs were inhibited from binding to gp120 by Zn at concentrations equivalent to their Zn binding constants, it would strongly indicate that the binding sites for gp120 and Zn are in close proximity on the antibody. Furthermore, two negative control Fabs, H21L21 which contained the His<sub>5</sub> tail and H12L12 which did not, were not competed by Zn to the same extent as the metal binders, suggesting that the observed inhibition was a specific reaction.



**Figure 7.9.** Zn-gp120 competitive ELISAs. The Zn was able to compete with the Fab-gp120 binding interaction in the case of the metalloantibodies, with 50% inhibition constants similar to those observed in the Zn inhibition ELISAs (Figure 7.8). Neither of the two negative controls were inhibited to the same extent.

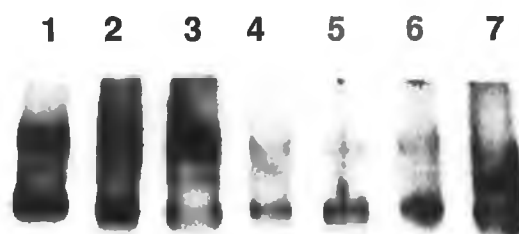
### **7.2.7 ZINC-gp120 BIFUNCTIONAL ELISAs.**

An attempt was made to establish whether the Fabs could bind both the metal and the gp120 simultaneously. This was achieved by means of a bifunctional ELISA described in the methods section 3.6.4. The Fabs were first bound to immobilised Zn and then gp120 was introduced in the hope that it would ALSO be bound by the Fab. The gp120 could then be detected by means of a series of secondary antibodies. It was not possible to bind the Fab to gp120 first and then introduce Zn, as there was no way to detect the Zn in an ELISA assay. No gp120 was detected in any of these assays, although Fab could be shown to be present suggesting that the Fab was being prevented from binding to gp120 possibly as a result of steric inhibition due to the nature of the assay. It is probable that the antigen binding site of the Fab is hidden when it binds the immobilised Zn to the extent where it can no longer bind gp120.

### **7.2.8. gp120-CLEAVING ASSAYS.**

Fab was incubated, in solution, with gp120 and zinc overnight at 37°C. The reaction was then run on a reducing SDS-polyacrylamide gel and transferred to a PVDF membrane where it was probed with a secondary, gp120-specific, antibody labeled with a chemiluminescent substance. This could then be detected with X-ray film. The objective was to determine if the Fab, in conjunction with metal, was capable of cleaving the gp120 molecule. Negative controls used were unrelated Fabs, zinc alone, unpurified metal binding Fabs and metal binding Fabs in the absence of Zn. No difference was observed between the negative and positive controls

(Figure 7.10), indicating that the Fab-Zn combination was not capable of cleaving soluble gp120 under the reaction conditions described.

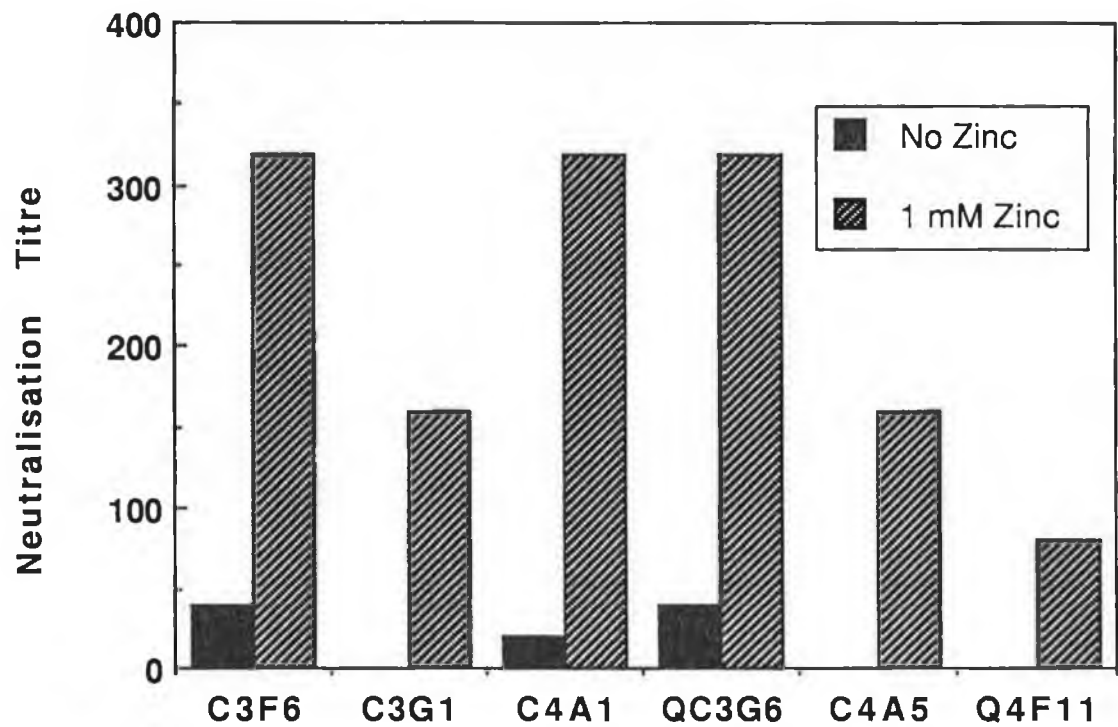


**Figure 7.10** Western blot of gp120-cleaving assays. gp120 is observed as three bands between the sizes of 110KDa and 125KDa due to differences in glycosylation patterns as a result of producing the protein in the chinese hamster ovary system. No difference can be seen between any of the lanes, suggesting that the antibodies do not cleave gp120 under these conditions. Zn was added to a concentration of 1mM in all cases; 0.2ug gp120 were used and 0.5ug of antibody was added to each tube as indicated:

- Lane 1: gp120
- Lane 2: gp120 + QC3G6
- Lane 3: gp120 + Zn.
- Lane 4: gp120 + QC3G6 (purified) + Zn.
- Lane 5: gp120 + QC3G6 (cell supernatant) + Zn.
- Lane 6: gp120 + C4A5 + Zn.
- Lane 7: gp120 + Q4F11 + Zn.

### 7.2.9. NEUTRALISATION ASSAYS.

All of the Fabs were tested for neutralisation of HIV *in vitro*, in the p24 production assay of Björling and Norrby, both in the presence and absence of 1mM Zinc. The results of these assays are given in Figure 7.11. Preliminary evidence suggested that these Fabs were good neutralisers even in the absence of metal, although further experiments need to be carried out before this can be confirmed. However, it has been established that there was a very significant increase in neutralisation titres for all six Fabs when zinc was added to a concentration of 1mM. A metal concentration of 10 $\mu$ M did not have any effect on titre, demonstrating that this is a concentration-dependent effect (data not shown). This effect was not seen in the case of antibodies not designed to bind metal. Therefore, it would seem that the metal binding Fabs bound Zn and that this was having a critical effect on the ability of these Fabs to neutralise the virus.



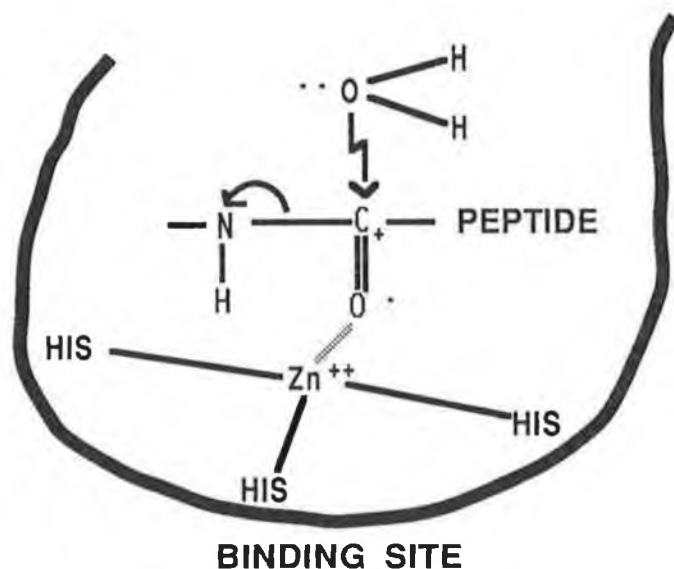
**Figure 7.11.** Neutralisation titres of metalloantibodies with and without zinc. The titres are given as the maximum dilution of antibody resulting in 80% neutralisation of the virus. The set of data presented above indicates higher titres, in all cases, when the metal is added, indicating that the zinc is having a profound affect on the ability of the Fabs to neutralise virus.

## 7.3 DISCUSSION.

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### 7.3.1 FAB CONSTRUCTION.

The work presented in this chapter was an attempt to incorporate antigen specificity and catalytic activity into a single Fab fragment. The antigen chosen was gp120 since there were many Fabs of such specificity available and this represented a relevant target with the possibility of neutralising the HIV virus. The catalytic activity decided upon was that of peptide cleavage by a metal cofactor such as zinc. The coordinated ion, when positioned close to an amide bond of the protein, polarizes the carbonyl bond for attack by hydroxide provided by an adjacent water molecule. Zn ions can also increase the nucleophilicity of the water molecule especially in the relatively non-aqueous environment of the antigen binding pocket (Figure 7.11).



**Figure 7.11.** Mode of action of Zinc in peptide cleavage. The Zn polarises the carbonyl bond, making it susceptible to attack by water, thus cleaving the peptide bond.



### 7.3.2 DIRECTED CROSSES.

Initially, directed crosses were attempted with the heavy chains previously isolated from a gp120-panned library. The only chain to result in a productive cross was HCb6. This was also one of the chains capable of crossing productively with the tetanus toxoid-specific light chain. Therefore, HC6 must have a sequence which makes it amenable to crossing with non-related chains. Perhaps the same was true for the other two members of it's group, b20 and s6, however, these clones were never tested in the binary plasmid system.

The metallolight chains were made up of the constant region from clone b22. The original b22LC was able to productively pair with 16 out of 21 of the original gp120 binding heavy chains, making it the most promiscuous of the light chains. However, swapping the variable region for this mouse fluorescein-specific variable region seems to have sufficiently interfered with the steric and electrostatic interactions in the binding site as to not allow the combination of these light chains with the heavy chains. This demonstrates the importance of the variable region in that it was not sufficient for combination just to match the constant regions, and corroborates the findings of Sastry *et al.*, (1989). The affinities of the HC6-metallolight crosses were found to be in the range  $2$  to  $6 \times 10^7 \text{M}^{-1}$  which was an order of magnitude lower than the affinity of the original b6 Fab ( $5 \times 10^8 \text{M}^{-1}$ ). This was consistent with previous results that interantigenic crosses tend to have affinities an order of magnitude lower than crosses between chains of the same specificity (chapter 5).

### 7.3.3 CROSS WITH HEAVY CHAIN LIBRARY.

A cross was then carried out with a library of heavy chains. This was the same library that had been panned against gp120 previously and used to isolate the gp120 binders (Barbas *et al.*, 1992,1993). In that case, panning had lead to a 1000-fold increase in phage titre after four rounds. However, when panning was performed on this crossed library, there was no significant increase observed, although screening of a large number of clones from this library did reveal gp120 binders with affinities between  $1 \times 10^7$  and  $1 \times 10^8 \text{M}^{-1}$ . The lack of amplification was further demonstrated by the fact that an equal number of clones were isolated from the third and fourth rounds, showing there was no increase from one round to the next. The question must, therefore, be asked why these relatively high affinity binders were not amplified in the panning process. The fact that these clones bind gp120 would indicate that the heavy chains involved originally derived from gp120-specific antibodies, yet none of these heavy chains were amplified from the original library either as can be seen from their sequences (Figure 7.2). The geneIII fusion was designed to permit the isolation of high affinity binders from a library, probably to the detriment of lower affinity Fabs. The geneVIII system was designed for the generation of low affinity Fabs. Perhaps the affinities of these crossed Fabs were too low to be amplified in the geneIII system being used here. However, this was unlikely as their affinities are comparable to Fabs previously isolated from this type of library (Persson *et al.*, 1991) and are not in the lower range of affinities ( $1 \times 10^4$  to  $1 \times 10^5 \text{M}^{-1}$ ) that it was possible to isolate from the geneVIII system (Gram *et al.*, 1992).

Nonetheless, it might be interesting to transfer this library to the geneVIII system and repeat the panning.

An alternative explanation might be that the RNA coding for these chains exists at such a low frequency in the overall population that it was not amplified during the panning process. Perhaps this was a characteristic of the geneIII fusion combinatorial library system. If it was the case, that there was a threshold level for RNA, below which, the sequence will not be amplified, then this has obvious implications for the study of an immune response using this system. It would mean that these heavy chains, which are obviously gp120 specific, were not amplified in the original library and so, the analysis of the immune response, although accurate, omits this minor part of the response and so may not be entirely complete. The importance of this facet of the response was not known. This inability to amplify rare sequences would also affect the construction of naive libraries, since it is probable that the sequence of interest in this type of library would be present at a very low frequency. This phenomenon may not apply to the geneVIII fusion since the increased levels of expression of Fab on the phage surface may, in itself, act as a form of amplification, allowing the isolation of low frequency sequences. The isolation of specific Fabs from a naive library was achieved using this system (Gram *et al.*, 1992), although this was only a single instance and may not be representative of the system in general.

In any case, screening of a larger number of clones has facilitated the isolation of a number of gp120 binding Fabs previously unidentified. There was a significant bias towards clones using the

light chains LCC(6) and LCQC(9) with only 2 fabs using LCQ. This may reflect on the structures of these chains in relation to their ability to combine with other heavy chains without disrupting the antigen binding site. Perhaps the additional histidine residues in the C mutation alter the light chain structure so as to make combination with other chain easier, or the Q mutation could have the converse effect. Whatever the case, it seems that LCC and LCQC are able to combine more easily than LCQ.

All subsequent studies were carried out with 100% purified Fab samples to ensure that no anomalous effects were occurring. ELISA results confirmed that these Fabs were of the CD4-binding site class of antibodies with affinities of  $1 \times 10^7$  to  $8 \times 10^7 \text{M}^{-1}$ . This puts them in the same category as previously isolated Fabs.

#### **7.3.4 METAL-BINDING ANALYSIS.**

The zinc-binding characteristics of these antibodies were found to be similar to Fabs studied by other groups (Iverson and Lerner, 1989; Iverson *et al.*, 1990). Their results indicated that the metal binding site their light chain (LCQ) would be fully occupied at a zinc concentration of  $100 \mu\text{M}$ . The binding constants in this study of  $300 \mu\text{M}$  to  $2 \text{mM}$  differ from their value by a factor of 3 to 20. In fact, the best value of  $300 \mu\text{M}$  was observed for clone Q4F11 which was the only clone using the same light chain as in the Iverson study. However, it may not be that surprising that the binding constants in this experiment are worse than those previously noted, since Iverson *et al.* used an Fab containing the original combination of heavy and light chain, whereas the two chains making up the Fabs in this study

originate from different sources. It has already been demonstrated that interantigenic crosses result in affinities at least one order of magnitude lower than the original Fabs. Therefore, it was reasonable to assume that mixing of chains in this case could lead to structural changes which would reduce the affinity of the light chain for metal ions by a similar factor of magnitude. Another study by Iverson *et al.*, on a set of antibodies that used a metal-trien complex as a cofactor for peptide cleavage, showed that the most efficient concentration of Zn to use was 1.5mM, which falls inside the concentration range found to be optimal for our antibodies. It should be concluded, therefore that these Fabs bind zinc with an affinity similar to that displayed by other antibodies.

Zinc-gp120 competition ELISAs were carried out in order to determine more precisely the location of the zinc-binding site in relation to the antigen binding site on the Fab. The Fab was allowed to bind immobilised gp120 first, and this was competed with zinc. Due to the small size of the zinc ion relative to the gp120 molecule, the only way the zinc would be able to dislodge the Fab would be if the binding sites for metal and gp120 were adjacent to one another. Otherwise the zinc could bind without disrupting the antibody-antigen interaction. It was found that the zinc could compete successfully with gp120 for Fab binding at concentration levels comparable to the zinc binding constants determined for each Fab in the absence of gp120. This did not occur in the case of Fabs that could not bind metal or with Fabs that bound metal via a histidine tail at the other end of the antibody molecule. This finding, coupled with the close correlation between the two ELISA assays, would

seem to indicate that the two binding sites on these Fabs are indeed proximal, and that the Fabs are binding zinc as they were designed to do, i.e.: at the engineered sites in the antigen binding pocket.

#### **7.3.5 SIMULTANEOUS BINDING OF gp120 AND METAL.**

Attempts to establish whether the Fabs could bind both metal and gp120 simultaneously necessitated that the Fab was first bound to zinc which was immobilised to a plate via a linker. It was probably the presence of the linker that prevented the subsequent binding by gp120 and so it could not be shown that both could bind the Fab simultaneously. This was quite a complex problem as it was very difficult to detect the presence of the zinc ion if the antibody was also bound to the large glycoprotein, gp120. One possible solution might be to separate the Fab on an SDS gel and transfer it to a PVDF membrane. This could then be probed first by  $\text{Zn}^{65}$  and developed under X-ray film. Then, without stripping the blot, it could be probed with gp120 followed by incubation with an anti gp120 antibody labeled with a chemiluminescent marker which could be detected in a matter of seconds with an X-ray film. The bound zinc would not interfere with this exposure since it was of such high energy that X-ray film needs to be exposed in the presence of an intensifying screen at  $-80^{\circ}\text{C}$  overnight to detect its presence. The blot could be immediately exposed with a new film to ensure the continued presence of the zinc. The detection of a correctly sized band at all three stages of exposure would be a strong indication of simultaneous binding of both the metal and the gp120.

### 7.3.6 PEPTIDE BOND CLEAVAGE.

The function of these Fabs was to cleave gp120 using zinc as a catalytic cofactor. This activity was tested for by first allowing the antibody to bind to the zinc and then adding soluble gp120 for an overnight incubation at 37°C. Detection of fragments cleaved from the gp120 was then carried out by western blot analysis.

No differences in the number or sizes of gp120 bands were observed between any of the positive or negative control samples used in this assay. There are a number of possibilities which may explain this. First, simultaneous binding of gp120 and zinc may not occur, with the binding of the zinc preventing gp120 binding. This, however, was unlikely, as a number of other studies have incorporated metals in the active sites of antibodies while maintaining antigen binding capability (Pollacket *et al.*, 1988; Iverson and Lerner, 1989; Iverson *et al.*, 1990; Nakayama and Schultz, 1991). Also, these Fabs have been shown to bind gp120, so their structure was not such that they are prevented from doing so. Second, it may be possible that the gp120 was not oriented in the correct way, upon binding by the antibody, to bring it sufficiently close to the zinc to initiate the hydrolytic reactions. The carbonyl group of an amide bond must be located proximal to the ion in order that it can be polarised, leading to cleavage of the amide bond. If this situation does not exist in the complex, then no such cleavage will occur. Therefore, it was possible that the antibody can bind both the zinc and the gp120 without hydrolysing the protein. A more detailed study of this possibility would require 3-D crystal structures for the binding sites of the antibodies. It would seem rather unusual, however, in the

light of previous studies, that this situation should exist in all six of the Fabs studied. The third possibility would be that the reaction conditions for this cleavage are not suitable for the reaction to occur. This would include the structure of the gp120 used. It was possible that soluble gp120 produced in a recombinant system, and virion surface-bound gp120, are sufficiently different in glycosylation and backbone structure, such that the soluble gp120 would not be oriented in the antigen binding site correctly, but the virion gp120 could be oriented to permit cleavage to occur. The findings from a previous study showed that the surface-bound and soluble forms of gp120 from the SF2 strain of virus were sufficiently different that antibodies bound to the surface-bound form and not the soluble (McKeating *et al.*, 1991; Ivey-Hoyle, 1991). John Moore *et al.*, (1991), also concluded that "studies with soluble *env*, glycoproteins may not mimic precisely macromolecular interactions taking place on the virion surface". Since there are no crystal structures available for gp120 (due to the high degree of glycosylation), and the exact structure was not known, it was very difficult to predict if this could be the case. It would be necessary to test these antibodies with live virus particles, and the easiest way of doing this would be to study the affect of the Fabs on viral neutralisation, since assays already exist for testing this.

#### **7.3.7 NEUTRALISATION.**

When these Fabs were tested for neutralising activity, it was found that the addition of zinc increased the neutralisation titres of all these antibodies to significantly higher values than those observed



for the same Fabs in the absence of metal. This would indicate that these antibodies, in conjunction with the zinc, are exhibiting some form of activity which was permitting the neutralisation of the virus. It was likely that this was due to the catalytic activity of the zinc, leading to the hydrolysis of a peptide bond in gp120, thus preventing invasion of the cells by the virus. It would require more experiments with the live virus to confirm that this was the case. In conclusion, this study demonstrated that it was possible to produce Fabs that are capable of binding both gp120 and zinc and the metal was capable of raising the level of neutralising activity demonstrated by these Fabs.

This has important implications for AIDS research in that it raises neutralising antibody titres and it may provide a more broad-based antibody system for combating this disease, not relying completely on the ability of the antibody to inhibit the gp120-CD4 interaction. It is also one of the first practical applications of catalytic antibody technology.

## CONCLUSIONS

## 8.0 CONCLUSIONS

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The humoral response of a single individual, when challenged by the human immunodeficiency virus, was isolated and cloned by means of combinatorial phage-display technology. An examination of the isolated Fab fragments was carried out at a genetic and a biochemical level. It was established that a large diversity of antibodies were produced by the immune system of this individual in response to the viral infection. This was observed in the large variation in sequence of these antibodies. Further analysis of these led to the postulation that a number of possible mechanisms may have given rise to such a diversity. This included somatic mutations, convergent evolution at the gene rearrangement level and diversity segment rearrangements. An analysis of the antibodies at a biochemical level revealed that, although they were very similar in specificity and affinity, there were some subtle differences in the epitopes recognised by the antibodies. Such a phenomenon could be interpreted as an attempt by the immune system to produce as many antibodies specific for the same epitope with minute differences, to counteract the possibility of escape mutants.

A binary plasmid system was developed that permitted the cloning of each heavy and light chain separately. This opened up the possibility of chain shuffling, which was carried out on a large selection of the Fabs isolated in the original study. A number of characteristics were observed from this, including the ability of heavy chains to pair with

many light chain and retain antigen-binding. Interantigenic crosses lead to the conclusion that the heavy chain was of primary importance in antigen-binding and that a variety of light chains could be used so long as they did not adversely affect the interactions occurring in the binding-site.

The validity of using combinatorial libraries for studying the *in vivo* antibody response was addressed throughout the investigations. It was argued that the isolated heavy chains were probably an accurate reflection of the *in vivo* situation, due to their prominence in antigen-binding. The array of light chains isolated and the assortment of heavy-light chains, in combination with the binary plasmid findings, suggested that the light chains represented in the library were also probably found in anti-gp120 antibodies *in vivo*.

Further studies on the ability of the shuffled Fabs to neutralise virus *in vitro* strengthened these arguments and also suggested that the light chain sequence was more important for neutralisation than for antigen-binding, within this set of antibodies. The combination of chains was also found to be crucial in this aspect of antibody function, meaning that a Fab would only neutralise virus if it consisted of the correct pairing of heavy and light chains. The lack of chain promiscuity displayed at this level and the fact that many of the original antibodies were found to possess neutralising ability, was further evidence that the library Fabs were an accurate representation of the equivalent *in vivo* antibodies.

The neutralisation studies also highlighted the complexity of the antibody-virus interaction as demonstrated by the inability of a given Fab to neutralise different isolates of the same viral strain. This emphasises the fact that there is much yet to learn about this reaction and it is more complicated than initially thought.

Finally, the interantigenic crosses performed earlier, demonstrated the possibility of combining two different functions within a single Fab. The gp120-specificity of the heavy chains was combined with light chains capable of binding metal in the hope of producing antibodies capable of metal-mediated peptide bond cleavage of gp120. This would yield Fabs with enhanced neutralising ability and would be one of the first practical applications of the field of catalytic antibodies. Six Fabs were produced that bound both gp120 and metal. The neutralisation titres of all of these were found to increase significantly with the addition of metal. This suggested that they were, indeed, capable of cleaving viral gp120.

This work has lead to a number of observations about the ability of the immune reponse to adapt to viral challenge, and has shed some light on the antibody-virus interaction. The relative contributions of the heavy and light chains within this set of antibodies has been examined and it is hoped that these studies will aid future research on the relationship between antibody structure and function. Finally a contribution has been made both to the fields of catalytic antibodies and AIDS research in the production of metalloantibodies capable of neutralising HIV.

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