INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI®

Université d'Ottawa · University of Ottawa

THE EFFECT OF POINT MUTATIONS ON THE BINDING AFFINITY OF ANTI-BLOOD GROUP A ANTIBODY AC1001

Roula Thomas

Thesis submitted to the Department of Biochemistry, Microbiology and Immunology
in partial fulfillment of the requirements for the Master of Science

University of Ottawa
Ottawa, Ontario, Canada
May, 1999

ABSTRACT

The A and B blood group antigens are very similar, and differ in only one functional chemical moiety; nevertheless this single difference is enough to allow for the recognition of these carbohydrate antigens by their corresponding anti-blood group antibodies. This thesis is focussed on an anti-blood group A (BGA) antibody interaction with its A antigen. A single-chain variable-domain antigen binding fragment (scFv) gene was constructed based on the known sequence of an anti-BGA monoclonal antibody (AC1001), and the protein has been expressed in *E. coli*. The objective of this work is to improve the binding affinity of this BGA scFv for the A-antigen, and to better understand the mechanisms by which the anti-blood group antibodies can discriminate between the largely similar A and B carbohydrate antigens.

The anti-BGA antibody produced *in vivo* in the immune system does not undergo the process of affinity maturation and somatic mutation because it binds a T cell independent carbohydrate antigen. Therefore, while these anti-carbohydrate IgM antibodies are able to bind their specific antigen, they do so with a very weak binding affinity. We have mimicked the process of affinity maturation and somatic mutation *in vitro* and attempted to produce a BGA scFv with an increased affinity using two different approaches. The first approach examined the high resolution crystal structure of the BGA variable fragment (Fv), to design and produce specific mutants using site-directed mutagenesis. In tandem with this site-directed mutagenesis study, an error-prone PCR (EP-PCR) BGA scFv phagemid library was displayed on the surface of M13 phage as geneIII fusions, and screened using several panning techniques. The phage display study did not lead to the selection of a mutant BGA scFv with an improved affinity for the A antigen. However, the difficulties encountered using the standard panning procedures precipitated the development of a new panning technique that can potentially be used to pan low affinity phage displayed libraries. The site-directed mutagenesis study produced mutant BGA scFv proteins with an increased specific affinity for the A-antigen. We report here the first rationally designed single-point mutation in an anti-carbohydrate antibody that significantly increased the functional binding affinity of the BGA scFv without altering specificity.

To my husband Sam -

I love you with all my heart, and I am so grateful that God has blessed me with you. Just thinking of you makes me smile.
I will love you forever.

DEDICATION

This work is dedicated to my parents, Joseph and Georgette Wehbi.

> I love you both very much. Thank you for helping me with everything, you have work endlessly and have sacrificed your lives for me. You have always encouraged me. You have loved me more than yourselves, and have shown me this love everyday. But most importantly you have shown me how to love God, and how to pray. My love for Jesus Christ, and my faith in Him as my Saviour was a seed that you planted, and this has changed my life completely.

This work is also dedicated to my brothers and sisters: George, Nahida, Nancy, Elias and Abraham. You are my best friends. I love you all.

I also dedicate this work to my husband Sam; you have stood by me, and have helped me complete this. You are so patient and loving, and I dedicate my life to you.

ACKNOWLEDGMENTS

Over the past couple of years, I have learned a lot about science, and particularly about research in science. There have been many people who have been instrumental in this learning process. I would first like to thank my supervisors: Dr. Stephen Evans and Dr. Martin Young. Dr. Evans, you are the first supervisor that I have really looked up to. Not only do I have great respect for you, but I also trust you. Thank you for being a supervisor and a friend. Dr. Young, you are a library of knowledge, thank you for your encouragement.

I would also like to thank Dr. Roger MacKenzie, who has been an excellent research advisor. Roger has not only been a good friend; he has also played a vital role in the phage display and panning experiments, particularly in the development of the Synsorb-A panning methodology. Thank you Roger. I would also like to thank Doris Bilous. Doris has not only taught me a great deal technically, but she has been my guide through the zoo. Doris you have been like a mother to me, and have always looked out for me. Thank you for being someone I didn't have to discuss science with, and for your great stories. I am grateful for our friendship. I would also like to thank Dr. Nina Seto. Nina knew the answers when nobody else did, and had a plan if she didn't know. Nina you are what a scientist should be. I would also like to thank Tomoko Hirama for the BIACORE analysis, and for always being so helpful. I would also like to thank Mark Wood and Diana Zahab for the laughs, Rebecca To, Ginette Dubuc, Tom Devecseri, Joe Michniewicz for the DNA sequencing and Lise Bramall for printing my many diagrams, and for being so helpful.

I would also like to thank Sonia Patenaude for deducing the crystal model that much of this work was based on, and for helping me with the imaging graphics and with many of the diagrams. Sonia you have been a good friend, a source of many laughs and someone I could always talk to, you have a great heart. I would also like to thank Julie, Joanne and Crystal for keeping me in the loop, thanks Chikitas.

TABLE OF CONTENTS

CHAPTER TWO

CHAPTER THREE

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Ab	antibody
AC1001	anti-blood group A monoclonal IgM hybridoma
amp	ampicillin
amp^r	ampicillin resistance
BGA	blood group A
BSA	bovine serum albumin
BSA-A	BSA conjugated with A trisaccharide antigen
BSA-B	BSA conjugated with B trisaccharide antigen
C	constant gene of antibody variable domain
C_H	constant domain of heavy chain
C_L	constant domain of heavy chain
D	diversity gene of antibody variable domain
ddH_2O	deionized distilled water
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
ELISA	Enzyme Linked Immunosorbent Assay
EP-PCR	error-prone PCR
EP-TGASL#4	error-prone BGA short linker clones
Fab	antigen-binding fragment of an antibody
Fv	variable fragment of an antibody
g, mg,μg	gram, milligram, microgram
GalNAc	N-acetylgalactosamine
gIII	bacteriophage gene III
gVIII	bacteriophage gene VIII
h, min, s	hour, minute, second
IMAC	immobilized metal-ion chromatography
J	joining gene of antibody variable domain
K_D	equilibrium dissociation constant
k_{off}	off-rate kinetic constant

k_{on}	on-rate kinetic constant
l, ml, µl	litre, millilitre, microlitre
LB	Luria broth
M, µM	moles/litre, micromoles/litre
M13	filamentous bacteriophage
mAb	monoclonal antibody
MHC	major histocompatibility complex
NAc	N-acetyl
Ni^{2+}	nickel ion
NMR	Nuclear Magnetic Resonance
°C	degree Celsius
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pfu	plaque-forming units
pIII	bacteriophage minor coat protein III
pVIII	bacteriophage minor coat protein VIII
rpm	revolutions per minute
RU	resonance units
S.E.	standard error
scFv	single chain variable fragment
SDS	sodium dodecyl sulphate
SPR	Surface Plasmon Resonance
TGAscFv	BGA vector used to produce soluble scFv protein
TGASL#4	BGA vector used to produce scFv-pIII fusion protein
V	variable gene of antibody variable domain
V_H	variable fragment of heavy chain
V_L	variable fragment of heavy chain

OBJECTIVES

The main objectives of this work were to generate mutant blood group A (BGA) single chain antibody fragments (scFvs) with an improved binding affinity for the A carbohydrate antigen, and to ultimately better understand the interaction between this BGA antibody and its corresponding A antigen. Because the anti-carbohydrate antibodies generated in the immune system often do not undergo the process of somatic mutation and affinity maturation, they may have weak binding affinities for their corresponding antigens. Accordingly, the anti-blood group antibodies produced *in vivo* bind their corresponding carbohydrate antigens weakly. Despite the weak binding affinity of this interaction, the anti-blood group A and B antibodies are capable of discriminating between the largely similar A and B carbohydrate antigens. Therefore, the purpose of this work was to mimic somatic mutation and affinity maturation *in vitro*, and to generate mutant BGA scFvs that bind the A antigen specifically with a greater affinity. A greater understanding of the interaction of these mutant proteins with the A carbohydrate antigen may lead to a more refined understanding of the mechanisms by which these anti-blood group antibodies discriminate between the largely similar A and B carbohydrate antigens.

APPROACH

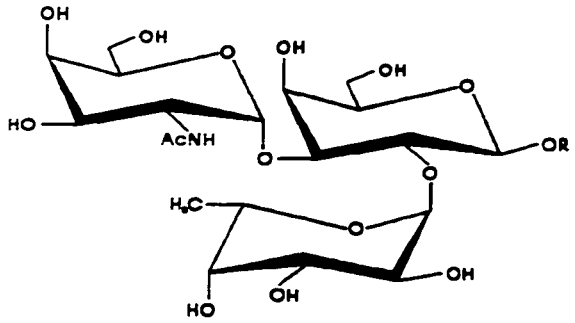
Affinity maturation of anti-carbohydrate antibodies does not occur *in vivo* in the immune system. However two different approaches were used to cause the BGA anti-carbohydrate antibody fragments to undergo affinity maturation *in vitro*. The first study produced specific BGA scFv mutants using site-directed mutagenesis after examining the binding pocket of a high resolution X-ray crystal structure of the BGA Fv. This rational design approach to mimicking the in vivo maturation process aims at producing mutant BGA scFv proteins with an improved affinity for the carbohydrate A-antigen. The second approach involved the production of random mutations in the BGA scFv gene to generate

a phagemid library of BGA mutant BGA scFv proteins. These mutants were displayed on the surface of phage, and screened for high affinity mutants using various panning techniques. The phage display approach is an alternative to the completely rational approach, however both approaches aim to overcome the immune system's obstacle and produce an anti-carbohydrate antibody with an increased affinity.

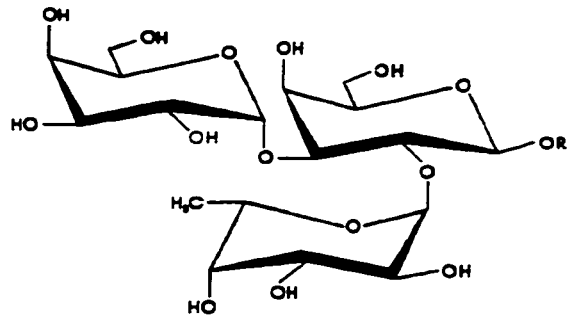
CHAPTER ONE
INTRODUCTION
1. OVERVIEW

The carbohydrate structures present on the surface of a variety of cells serve as important factors in cell-cell recognition and in the discrimination between self and non-self. The histo-blood group ABO was one of the first reported cell-surface antigens, and corresponds to the expression of A, B and H carbohydrate antigens respectively. These carbohydrate moieties were initially identified as constituents on the surface of red blood cells, however they are found on a variety of cell types throughout the body. Since carbohydrates can serve as surface markers in cellular recognition processes (Drickamer & Carver, 1992), a greater understanding of the interactions between these antigens and their corresponding antibodies would be an important contribution to the understanding of protein-carbohydrate interactions.

While the immune response to the ABO blood group antigens is mounted by serum antibodies, the A and B blood group antigens are synthesized in the body by corresponding glycosyltransferases. The A and B glycosyltransferases catalyze the addition of monosaccharides to the precursor H molecule to form the A and B antigens respectively (King, 1994). Glycosyltransferase A attaches N-acetylgalactosamine (GalNAc) in an α-1,3 linkage to the terminal galactose (Gal) of the H antigen to form the A antigen, D-GalNAcα1,3(L-Fucα1,2)D-Galβ1,3/4GlcNAc, while the 1,3 addition of Gal to the H antigen by glycosyltransferase B forms the B antigen, D-Galα1,3(L-Fucα1,2)D-Galβ1,3/4GlcNAc (Tizzard, 1984). It is important to note that the only difference between these two antigens is that the A antigen displays an N-acetyl group where the B antigen displays an hydroxyl group.



Blood group A trisaccharide



Blood group B trisaccharide

Figure 1: Schematic representation of the blood group A and B trisaccharide antigens. The only difference between these two carbohydrate antigens is that the A-antigen displays an N-acetyl (AcNH) moiety on the terminal galactose residue, whereas the B-antigen displays an hydroxyl (OH) group instead. This single difference can be recognized by corresponding anti-blood group antibodies.

This single chemical difference has serious serological impact. An individual with blood type A carries the gene for glycosyltransferase A and therefore presents the A carbohydrate antigen on the surface of their red blood cells, and consequently possesses anti-B antibodies in their serum. Accordingly, a blood type B individual carries the gene for glycosyltransferase B and presents the B antigen on the surface of their red blood cells and possesses anti-A antibodies in their serum. It is imperative in blood transfusions that donor and recipient match, since mismatched blood types can activate a full cascade of intravascular hemolysis (Seymour *et al*, 1995). Antibodies have the ability to differentiate between the A and B antigens; however, the nature of this protein-carbohydrate interaction is not understood at the molecular level.

Carbohydrate antigens and protein antigens differ fundamentally in their immunological behavior. These fundamental differences will be further discussed in the section 2.4. The immune system and the structure and function of antibodies will be discussed in greater detail in section 2.

The main objective of this work is to improve the binding affinity of an antibody fragment specific for the BGA antigen, and to gain a greater understanding of the nature of this specific protein-carbohydrate interaction at a molecular level. This was done using two different approaches in tandem. The first approach was a rationally designed site-directed mutagenesis study based on the crystal structure of the scFv, described in Part A. The second approach required the production of a BGA scFv phagemid library and applied bacteriophage display and various panning techniques to select for a BGA scFv with an improved affinity, in Part B. A single chain antibody variable fragment (scFv) consists of a V_L domain and a V_H domain joined by a peptide linker which allows these variable domains to fold on each other and form an antigen binding domain. The scFv construct maintains the original antibody's antigen binding site, as illustrated in Figure 6.

2. THE IMMUNE SYSTEM

The immune system's primary function is defense against a large variety of infectious microbial agents, such as viruses, bacteria, fungi, and parasites. Niels Jerne compared the complexity of the immune system to that of the nervous system, and recognized that the special ability of the immune system was to patrol and guard the body (Jerne 1973). The discipline of immunology grew out of the observation that individuals who had recovered from a certain infectious disease were thereafter protected or 'exempt' which in Latin is *immunis*, the source of the word immunity. Immunity as a broader term means the state of protection, and scientifically it is the protection from infectious disease and the surveillance for the detection of foreign agents.

2.1 INNATE *vs* ADAPTIVE IMMUNITY

The immune system is divided into two functional divisions: the innate immune system and the adaptive immune system. Innate or nonspecific immunity refers to various physical and cellular attributes that collectively represent the first line of defense

against infectious disease. For example, the skin and the extensive epithelial lining of mucosal tissues act as nonspecific barriers against infectious agents, preventing them from entering the body. However, if these barriers are penetrated then the adaptive immune system is initiated. The adaptive immune system produces a specific reaction to each infectious agent and recovery from the disease or infection. The primary elements of the adaptive immune system are antibodies. The production of antibodies is induced when the host's lymphoid system comes into contact with immunogenic foreign molecules, or antigens. The antibody produced, in turn, binds specifically to the antigen that induced its formation. Furthermore, the adaptive immune system establishes a specific immunological memory so that any following reinfection with the same agent results in resistance to disease development, and as a result the individual has acquired immunity against that infectious agent.

The innate and adaptive immune system consist of a variety of molecules and cells which are organized into specific tissues and organs in order to perform their functions most effectively. The cells of the immune system include: lymphocytes, macrophages, dendritic cells, epidermal and epithelial cells, which are in turn organized within the lymphoid organs and tissues: spleen, lymph nodes, tonsils, thymus and bone marrow. These structures are collectively referred to as the immune (or lymphoid) system.

2.2 IMMUNE RESPONSE

The most important cells of the immune system are the leucocytes or white blood cells. Leucocytes fall into two broad categories: phagocytes (which form part of the innate immune system) and lymphocytes (which mediate adaptive immunity). Lymphocytes are an enormously heterogeneous collection of cells, which not only differ from one another based on their surface expressed receptors, but also differ, in their

functional properties. There are two broad classes, or lineages, of lymphocytes: the B lymphocytes (B cells) and the T lymphocytes (T cells). B cells and T cells both have their origin in stem cells; however, B cells develop and differentiate in the bone marrow, while T cells differentiate in the thymus (Paul, 1989).

The bone marrow and the thymus are the primary lymphoid organs, since it is within these organs that stem cells proliferate and mature into functional effector cells. The mature B cell endogenously produces Ig molecules, which are then expressed on the B cell surface, where they act as specific antigen receptors. The majority of B cells express both surface IgM and IgD antibodies. On the surface of B cells we can also find a complex that is functionally important for the regulation of the immune response, and is known as the major histocompatibility complex (MHC). The MHC, which is found on antigen presenting cells and on various cells throughout the body, is a membrane bound protein that displays processed antigens on the surface of the B cell . If T cells encounter antigen associated with an MHC on a B cell, the T cell proliferates and differentiates into memory T cells and various effector T cells (Kuby, 1998).

2.3 AFFINITY MATURATION

The initial or primary immune response stimulates B cells to secrete antibodies of the IgM class. These primary response antibodies are generally of low affinity. In the course of the immune response, the average increase in the affinity of primary response antibodies can be as much as 100- to 10 000-fold (Kuby, 1998). This phenomenon is referred to as affinity maturation, and is the result of two processes: somatic hypermutation and antigen selection of high affinity clones (Berek and Milstein, 1987).

Affinity maturation occurs during B cells development. B cell maturation often occurs in the bone marrow, and involves the orderly rearrangement of the immunoglobulin genes; this process occurs in the absence of antigen and is referred to as

the antigen-independent phase of B cell development. These naïve B cells (which now express membrane bound IgM and IgD, have not yet encountered antigen), leave the bone marrow and are carried to secondary lymphoid organs (spleen and lymph nodes). If the naïve B cell encounters an antigen for which its membrane bound antibody is specific, the B cell proliferates and differentiates, thereby generating a population of antibody secreting plasma cells, and memory B cells. This stage of B cell development is referred to as the antigen-dependent phase. It is in this stage of the development that B cells undergo affinity maturation and class switching (Paul, 1989).

Antibody secreting plasma cells generally have short life spans. However, a small subset of these cells migrate to specialized locations in secondary lymphoid organs and are stimulated to undergo proliferation. In addition to proliferation, the antigen-activated B cells undergo extensive somatic hypermutation of the V_L and V_H chain domains. Somatic hypermutation introduces point mutations, deletions, and insertions into the V, D, and J segments of rearranged immunoglobulin genes (Kuby, 1998). Mutations can occur throughout the entire variable region, however, the majority of these somatic mutations occur in the CDRs, and as such these regions were initially referred to as hypervariable regions (Kabat, 1968). The somatic mutations that result in the process of affinity maturation occur randomly, and will generate a few cells with receptors of high affinity and many cells with receptors of low affinity. For affinity maturation to occur, not only must B cells bearing higher affinity antibodies be generated, but also cells bearing lower affinity antibodies must be eliminated.

It is not known why the CDR regions are mutation 'hot spots'; however, it could be that the enzymes that are involved in somatic mutation target these regions, or whether mutations within the CDRs are selected over other mutations because of their impact on antigen-binding affinity. For example, B cells that have undergone somatic mutation will result in B cell subpopulations with an altered antigen-binding affinity. If somatic

mutation resulted in an increase in affinity for the original antigen, re-exposure to that antigen will allow the improved subpopulation of B cells to rapidly expand and differentiate into B cells that secrete higher affinity antibodies. These higher affinity antibodies characterize the secondary immune response. While somatic mutation results in the generation of antibodies with an increased binding affinity, the majority of antibodies that arise due to somatic mutation exhibit a decreased affinity. Nevertheless, all the differences that arise due to somatic mutation contribute to the diversity of the immune systems repertoire.

2.4 IMMUNE RESPONSE TO CARBOHYDRATE ANTIGENS

A B cell can become activated when an antigen binds to the Ig receptors on its surface, thereby inducing the clonal selection of specific B cell clones which then proliferate and secrete antibody specific to that particular antigen. Depending on the nature of the antigen, B cell activation can proceed by two different routes: one that depends on T cells and one that does not. Antigens that are T cell-dependent differ from antigens that are T cell-independent, with respect to the humoral response they elicit. T independent antigens are capable of activating B cells to produce antibody without the help of T cells. The response to T independent antigens is generally weaker, no memory cells are formed, and IgM is the predominant antibody secreted, reflecting a lack of class switching (Kuby, 1998).

Since the focus of this thesis is the interaction of a carbohydrate antigen with its corresponding antibody it should be noted that unlike protein antigens, carbohydrate antigens are not processed and displayed on MHC. Carbohydrate antigens rarely induce class switching, a process where the H chain DNA can undergo a further rearrangement in the V_H-D_H-J_H unit and combine with any C_H gene segment. The inability to undergo class switching results in anti-carbohydrate antibodies being primarily IgM. The

antibody response to carbohydrate antigens is therefore a weak response, lacks affinity maturation, and does not generate memory B cells.

3. ANTIBODY STRUCTURE AND FUNCTION

Antibodies, or immunoglobulins, are glycoproteins which all possess the same basic structure (Figure 2). The basic building blocks of these molecules are two distinct types of polypeptide chains, heavy (H) and light (L) chains. The H chain is composed of 440-450 amino acids, whereas the L chain is composed of 210-220 amino acids. The H and L chains are made up of a series of domains, each of which is about 110-120 amino acids. The L chain is made up of two domains, whereas the H chain is made up of four to five domains, depending on its class.

Within any given antibody molecule there are two identical H chains, and two identical L chains, which form two identical cysteine-linked heterodimers with two identical antigen combining sites. Therefore, most antibodies are bivalent and can bind an antigen at both binding sites. IgG can serve as the prototypic antibody structure, since this abundant antibody is made up of the basic H_2L_2 unit.

In the 1950s and 1960s, Rodney Porter and Gerald Edelman elucidated the basic structure of the antibody molecule with the help of proteolytic enzymes which cleave the immunoglobulin molecule at specific points (see Figure 3). Subjecting IgG to a brief digestion with papain cleaves the IgG into two identical Fab fragments ('antigen-binding' ability was retained, each with MW 45,000) and an Fc fragment (this fragment 'crystallized' during cold storage, with MW 50,000). A similar experiment with pepsin generated only one fragment with MW 100,000 that was able to bind antigen; this Fab-like fragment was designated $F(ab')_2$. These experiments revealed that the 150,000 MW IgG molecule was composed of two 50,000 MW chains (designated heavy chains) and two 25,000 MW chains (designated light chains).

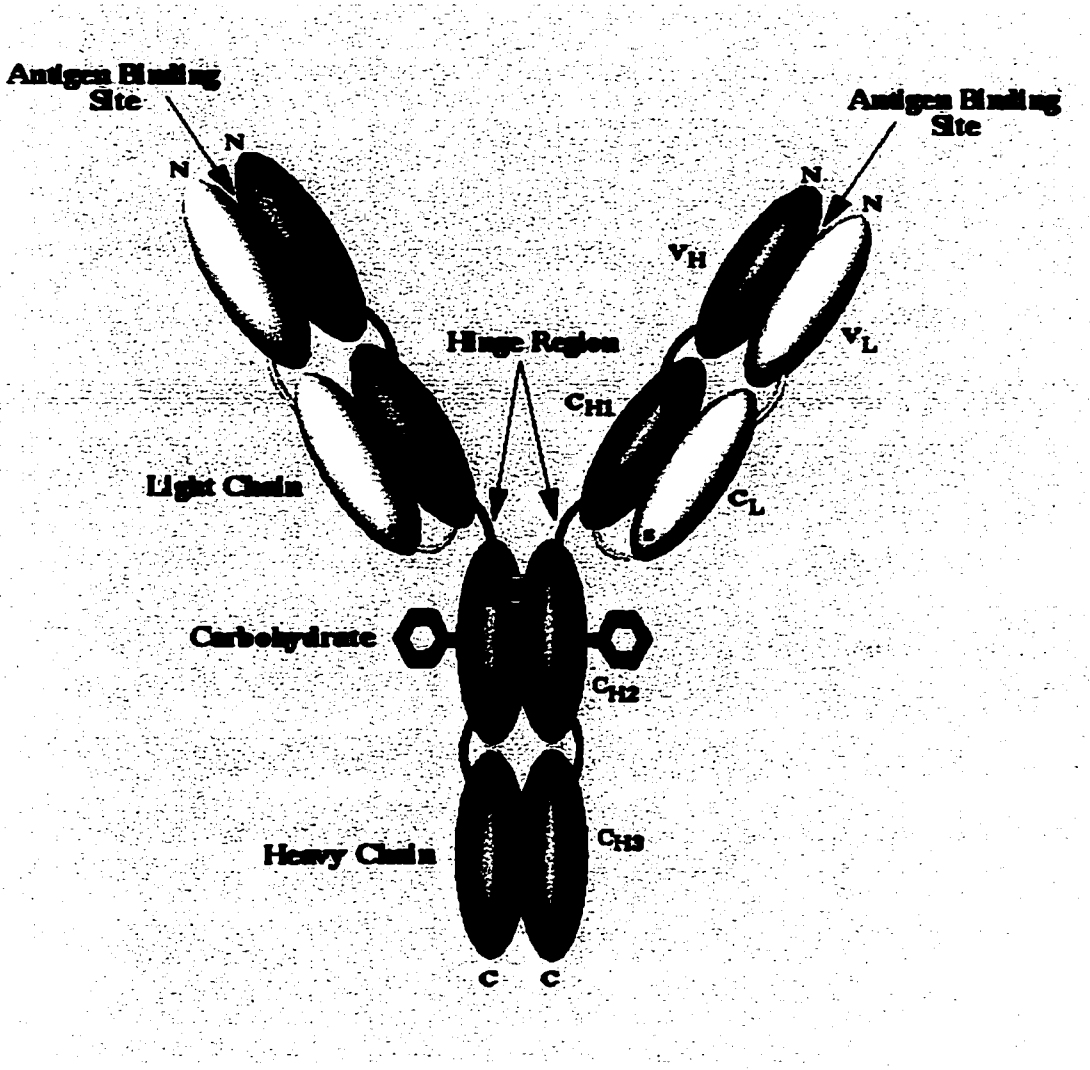


Figure 2: Schematic diagram of IgG antibody molecule. There are four polypeptide chains that make up the IgG antibody, two identical heavy chains (V_H) shown in purple, and two identical light chains (V_L) shown in white. The N-terminal portion of the antibody constitutes the variable region, and is made up of V_H and V_L domains; it is within this variable domain that the antibody binds antigen (Antigen Binding Site). There are two antigen binding sites in each IgG antibody. The constant domains contain interchain disulphide bonds (shown in yellow).

These experiments and the fragments they generated revealed important insights into the structure and function of antibodies. Antibodies are immunological proteins that exhibit two functional domains (Fleischman *et al.* 1962), the N-terminal variable domains and the C-terminal constant domains. The variable domains account for the antibodies unique binding specificities, whereas the constant domain correlates with different effector functions.

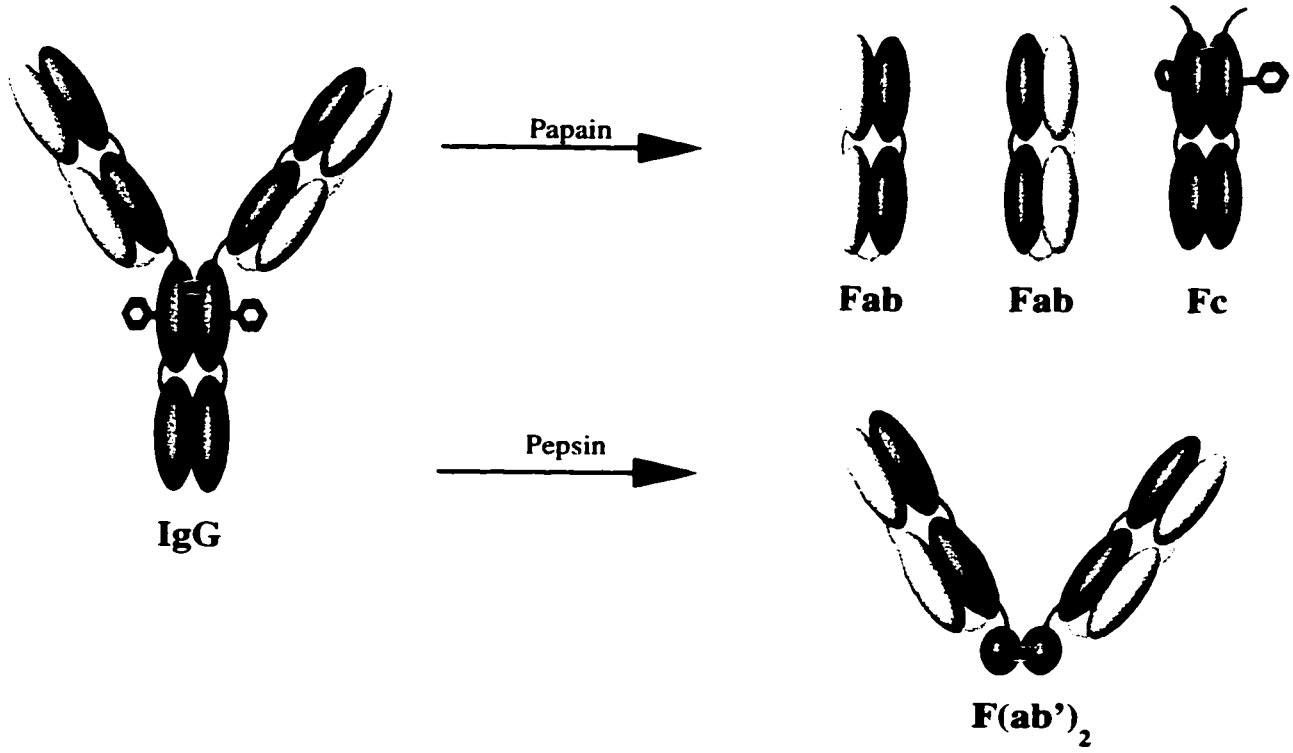


Figure 3: Enzymatic cleavage of IgG using papain and pepsin. Papain cleaves the IgG molecule in the hinge region between C_{H1} and C_{H2} to yield 2 Fab and 1 Fc fragments. Papain has nonspecific proteolytic activity and will eventually digest the entire IgG, however a brief papain digestion cleaves only the most susceptible bonds. Cleavage with pepsin generates the $F(ab')_2$ fragment.

In most higher mammals there are five distinct classes of immunoglobulins: G, A, M, D and E, which can be further divided into subclasses. The class and subclass of an immunoglobulin molecule is determined by its heavy chain type. Therefore, immunoglobulins G, A, M, D and E have heavy chains γ, α, μ, δ and γ, respectively. In some species, slight variations within a class gives rise to subclasses. In humans there are nine heavy chain isotypes: $\gamma 1$, $\gamma 2$, $\gamma 3$, $\gamma 4$, $\alpha 1$, $\alpha 2$, μ, δ and ε which give rise to the IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgM, IgD, and IgE antibody isotypes (Roitt *et al.*, 1993). The constant region of each antibody class is responsible for the distinct biological functions of that particular class. For example, IgM antibodies can activate the complement system, IgA's are secreted into a variety of bodily fluids, IgD's can act as membrane receptors, and IgE antibodies bind to specific receptors. IgG antibodies, which are the most abundant of the immunoglobulins, express a variety of functions, one of which is the ability to be transferred across the placenta (Paul, 1989).

Depending on class, the basic four chain monomeric units can form different multimeric structures (Figure 4). The H chains of all isotypes associate with one of the two L chain isotypes to form the basic H_2L_2 antibody unit seen with IgG, IgD and IgE antibodies. IgM antibodies, however, are pentameric, that is they are composed of five H_2L_2 units, thereby resulting in 10 identical binding sites (Figure 4). IgA antibodies can also be multimeric, and may consist of one or more units. Multimeric IgM and IgA antibodies contain an additional polypeptide chain, one J chain, which holds the units together.

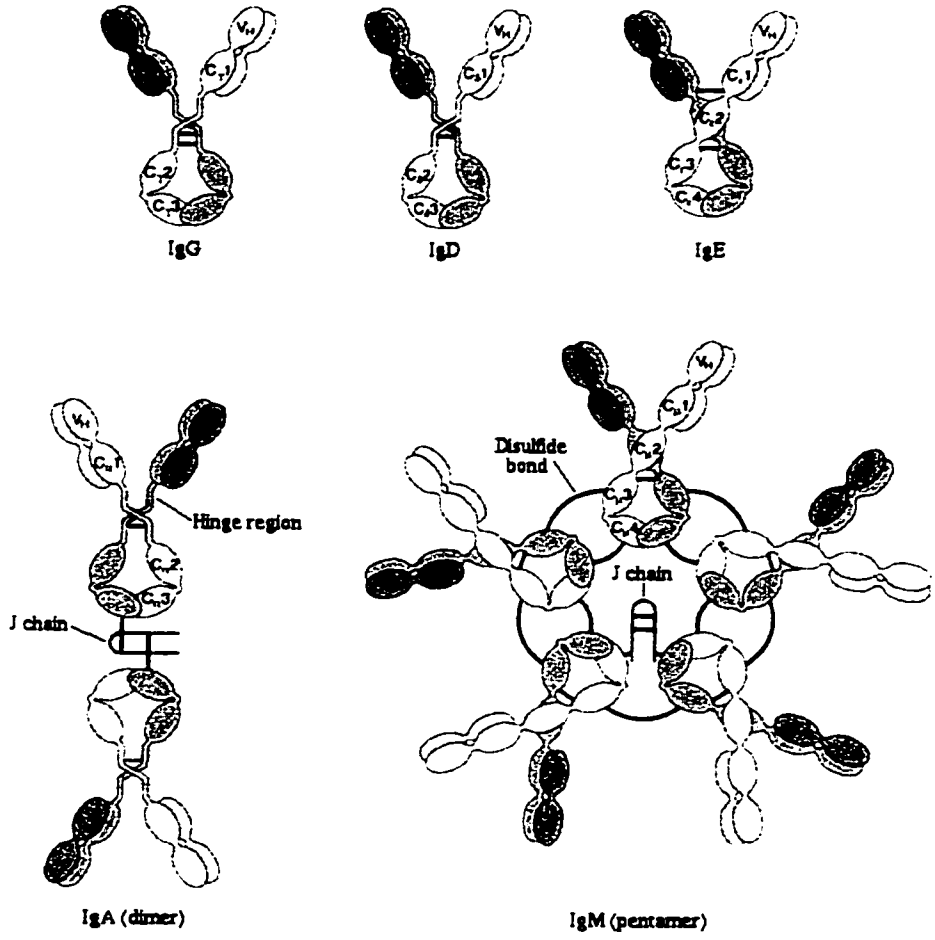


Figure 4: General structures of the five major classes of secreted antibody. Light chains are in shades of gray and heavy chains are in shades of blue; disulfide bonds are indicated by thick black lines. IgG, IgD and IgE are found as monomers, whereas IgM and IgA form multimeric structures, which contain a J chain polypeptide that is linked by two disulfide bonds to the Fc region of the individual monomers. Serum IgM is usually pentameric, and most serum IgA is monomeric but multimeric forms are sometimes present. (Kuby, 1998).

4. ANTIBODY DIVERSITY

It has been estimated that the mammalian immune system can generate more than 10^{11} different antibody sequences, allowing the ability to respond to a vast number of potential antigens. One of the early theories that tried to explain antibody diversity was the germ-line theory, which maintained that since antibodies are proteins, it follows that the genome contains a large repertoire of immunoglobulin genes sufficient to encode for such diversity. A contrasting somatic-variation theory maintained that the genome contains a relatively small number of immunoglobulin genes from which diversity is generated somatic cells by mutational and recombination mechanisms. As the amino acid sequences of many immunoglobulins were determined, it became clear that neither the germ-line nor the somatic-variation theories were sufficient to explain the diversity and the consistency that was found in the immunoglobulin sequences; however, both theories were partially correct.

In the germ-line DNA, multiple gene segments encode a single immunoglobulin H or L chain. These gene segments are carried in the germ cells but cannot be transcribed and translated into heavy and light chains until they are arranged into functional genes. During B cell maturation, these gene segments are randomly shuffled, in a carefully regulated process, and are capable of generating more than 10^8 different specificities. The germ-line genes are: V, D, J, C which encode for variable domains, diversity segments, J segments and constant domains respectively. The V_H domain is assembled by the combinatorial joining of V_H-D_H-J_H genes; the joining of these V_H genes with a C_H gene constitutes the assembly of a complete H chain. The V_L domain is assembled by the combination of V_L-J_L genes; and the attachment of a C_L to assembled V_L-J_L constitutes the assembly of a complete L chain. Since there are many V_H, D_H, J_H, C_H, V_L, J_L, and C_L germ-line genes, the rearrangement of these DNA segments is capable of generating much of the diversity observed in the antibody repertoire.

Therefore, the assembly of functional genes encoding immunoglobulin light and heavy chains involves recombinational events at the DNA level. A large part of the diversity in the antibody repertoire is due to these multiple germ-line segments and their combinatorial joining. However, there are other elements that contribute to the diversity of antibodies. Junctional flexibility occurs in the combinatorial process, when the joining of the coding sequences is imprecise, and this also contributes to diversity. Another mechanism by which diversity is generated is somatic hypermutation, which can potentially alter the specificity and affinity of the encoded antibody. And finally, the association of V_L and V_H chains to form an intact antibody also contributes to diversity since it is the association of these two chains that determines the antibodies' antigen-binding site, and therefore determines each antibody's specificity and affinity.

5. COMPLEMENTARITY DETERMINING REGIONS

Detailed comparisons of the amino acid sequences of the V_L and V_H domains revealed that the variations in the sequences are concentrated in hypervariable regions, which are separated by regions that exhibit much less variation (Kabat, 1968). These hypervariable regions are also called complementarity-determining regions (CDRs), because the antibody's antigen-binding site is complementary to the antigen in structure, and the less variable stretches between the CDRs are referred to as framework regions (FRs) (Kabat, 1968). There are six CDRs in each antigen binding site; three CDR's in the V_L and three CDRs in the V_H. These six hypervariable loops form a continuous surface that is responsible for binding antigen (Figure 5). It is the variabilities found within the CDRs that give rise to the wide range of specificities exhibited in the antigen-binding surfaces of antibodies. The intervening FRs act as a scaffold supporting the CDRs.

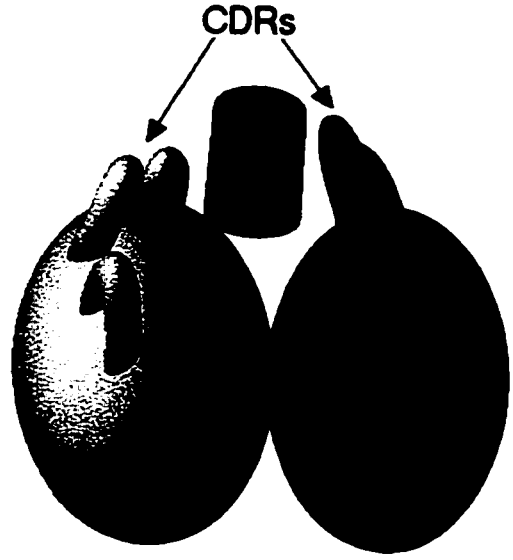


Figure 5: Schematic representation of the antibody variable fragment VL and VH domains, each of which contain three CDRs. These six hypervariable regions are responsible for specific antigen binding.

The three-dimensional structure of the antigen-binding site and the role of the CDRs in the binding of antigen have been investigated by high resolution X-ray crystallography. To date the crystallographic analyses has been completed for a number of antibody fragments; these studies have provided valuable insights into the structure of the V_L and V_H domains and the interactions between the antibodies and their corresponding antigens. We have examined the X-ray crystallographic structure of the AC1001 variable fragment and have focussed on the antigen-binding domain to select specific amino acid residues for mutagenesis. As will be explained in Part A, many of the residues selected for this mutagenesis study are residues found within the CDRs.

6. ANTIBODY FRAGMENTS

The variable domain genes of an antibody can be synthetically constructed and used to express antibody fragments that maintain the parental antibody's binding specificity. The V_L and V_H variable domain genes can be linked through a peptide linker

to form a single polypeptide chain that can be produced in *E. coli* (Skerra and Pluckthum, 1988). This single polypeptide antigen binding domain is referred to as a single chain variable fragment (scFv). We have used V_L and V_H synthetic genes based on the anti-A monoclonal antibody AC1001 (Chen and Kabat, 1985) to produce the BGA scFv construct used for the following studies. Antigen binding fragments (Fab) of the AC1001 antibody have also been produced (MacKenzie *et al.*, 1994).

Initial constructs of the BGA scFv were linked *via* a long peptide linker, which permitted intrachain V_L-V_H pairing of the variable domains and results in an scFv monomer that contains one antigen binding domain (Figure 6). However, because this BGA scFv monomer has a very weak binding affinity (K_D = 290 μM) (Patenaude *et al.*, 1998), it made kinetic analyses and phage display studies very difficult. The long linker was modified to a short linker, and the short linker construct resulted in the formation of V_L-V_H dimers. The long linker joining the V_L-V_H domains allows for a freedom of movement that allows the V_L-V_H to form intrachain pairing. The short linker does not allow these two domains to associate with each other, instead the V_L-V_H chain is forced to form dimers (Figure 6). Therefore, the short linker between the V_L and V_H domains permits interchain bonding, but not intrachain pairing of the variable domains. The inability of the V_L and V_H from one polypeptide to associate with each other forces them to associate with a corresponding V_L-V_H from a second polypeptide, thereby resulting in the formation of a bivalent fragment known as a diabody (Holliger, 1993).

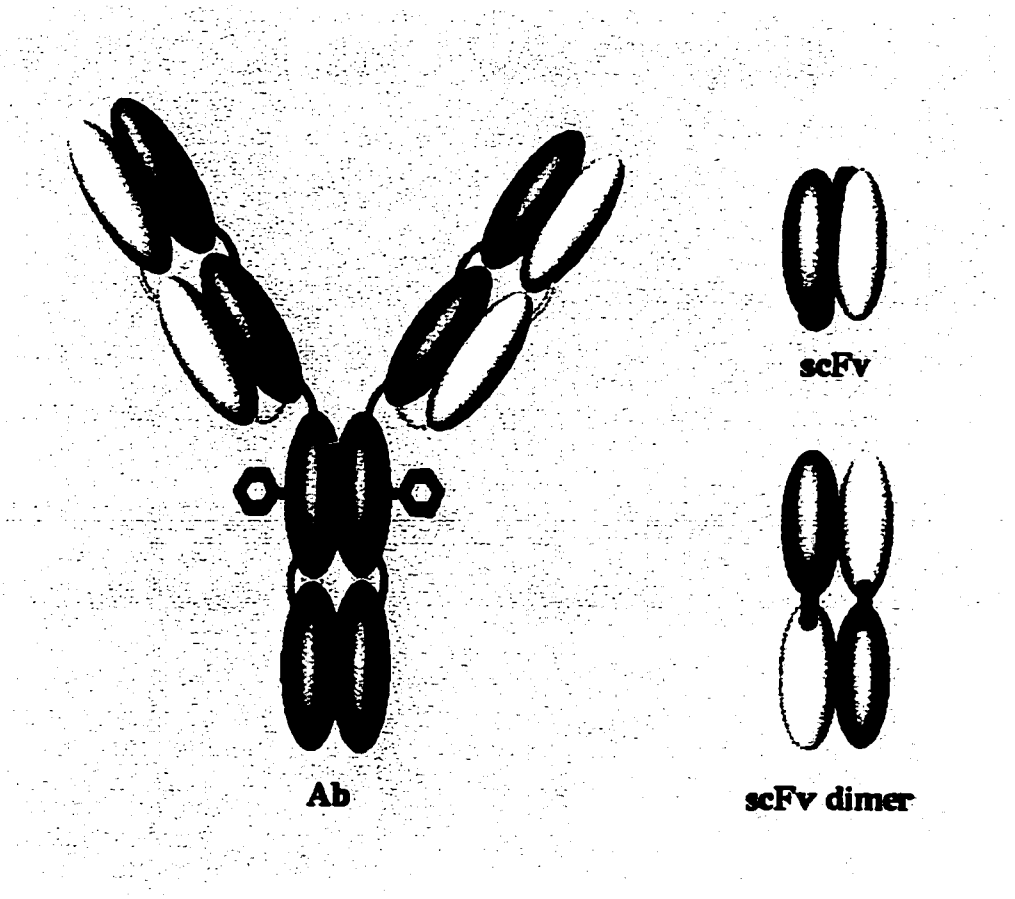


Figure 6: Schematic representation of single chain antibody variable fragment (scFv). The antigen binding domain of the IgG can be expressed as a single polypeptide chain in *E. coli*. The scFvs shown here illustrates the V_L and V_H joined by a peptide long linker and short linker, which allow the formation of monomer and dimer respectively. The scFv constructs maintains the original antibody's antigen binding ability and specificity (Bird *et al.*, 1988). However the scFv dimer has an increased avidity and therefore an increased functional affinity.

Modifying the linker from long to short, changes the scFv from primarily monomer to primarily dimer. This increase in avidity (functional affinity resulting from multiple antigen binding domains) results in an increase in antigen binding activity. The monomer to dimer change increased the binding affinity from K_D 290 μM to 26 μM, approximately a 10-fold increase in functional affinity. However, the binding affinity of the dimeric scFv is still very weak, thereby still making this work a challenge.

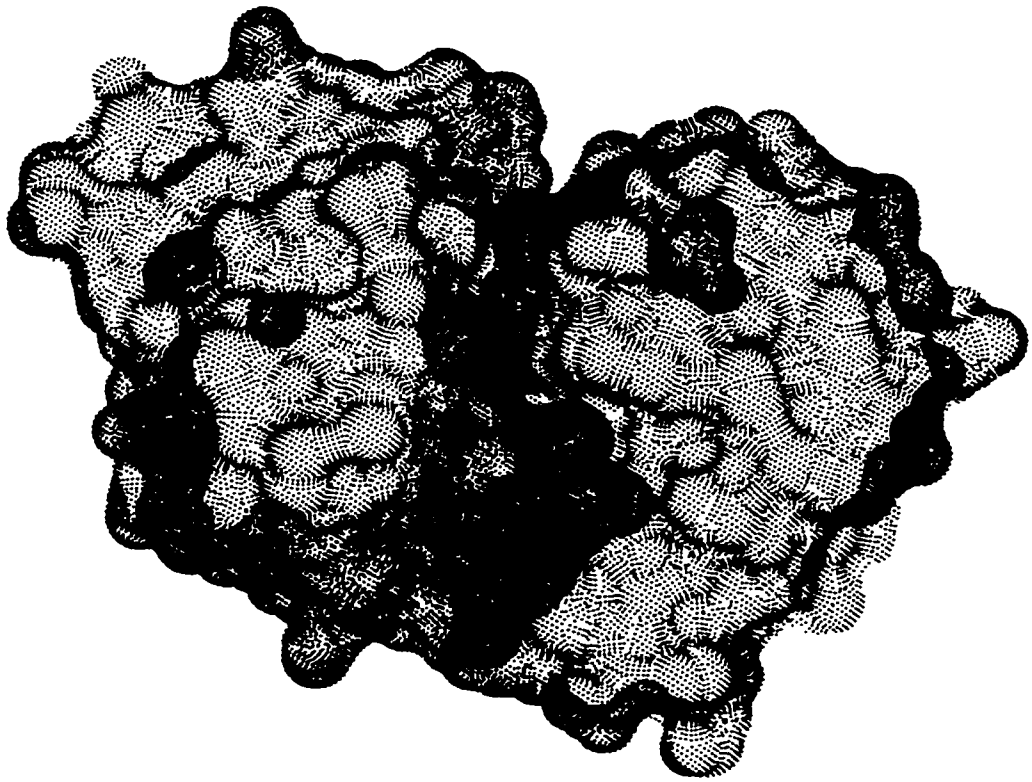


Figure 6b : Three dimension structure of the BGA variable fragment with modelled A trisaccharide antigen. This top view of the Fv (no linker) shows the V_L (blue) and V_H (green) binding the A trisaccharide antigen (red). This figure was generated using SETOR (Evans, 1993).

The scFv dimer or 'diabody' displays two antigen binding sites, and each binding site binds antigen with the same affinity and specificity as the antigen biding site on the original parental antibody from which it was derived. The scFv construct not only contains the region of interest but it is also easily produced in *E. coli* (Better *et al.*, 1988; Skerra and Plückthum, 1988). Furthermore, the scFv construct can be subtilisin digested (cleaves linker joining V_L-V_H of scFv) to form an antibody variable fragment (Fv) which is easily crystallized.

As outlined earlier, the aim of this work is to mimic *in vivo* affinity maturation *in vitro* and produce a BGA scFv with an improved affinity. This was done using two different approaches. The first approach produces specific BGA scFv mutants using site-directed mutagenesis after examining the antigen binding pocket of the BGA Fv crystal structure. The antigen binding domain contains a deep pocket, lined with various amino acid residues, and binds the A trisaccharide antigen specifically. This antigen binding pocket will be further discussed in Part A. The second approach produced mutant BGA scFv mutants randomly and used phage display and various panning techniques to isolate scFvs with improved binding affinity. The phage display study is used as an alternative to site-directed mutagenesis, and will be described in the following section.

7. PHAGE DISPLAY

Phage display technology is based on the ability to fuse foreign protein gene sequences to viral coat protein genes, and to express the resulting chimeric fusion proteins on the surface of the phage particle. In 1985, George Smith was the first to express a foreign segment of a protein on the surface of bacteriophage M13 virus (Smith, 1985). Smith's motivation was to use phage display as a method of identifying the specific epitopes of particular antibodies. Since the advent of Smith's discovery, phage display technology has not only been used to map the epitopes of various antibodies, but

has also been used to construct libraries of random peptides (Scott and Smith, 1990; Cortese *et al.*, 1995), to identify various target molecules (Lowman *et al.*, 1991), and to isolate high-affinity antibodies (McCafferty *et al.*, 1990; Barbas *et al.*, 1991).

One of the important applications of phage display technology is its ability to allow the *in vitro* evolution of proteins. For example, the coding region of a specific protein can be mutagenized to construct a library of recombinant phage expressing a large repertoire of mutant proteins. From this repertoire, higher affinity proteins can be isolated by affinity selection (to be discussed below), and DNA sequencing can identify the primary structure of the selected mutants. Part B of this thesis applies this potential of phage display, and an error-prone mutagenized BGA scFv gene library was expressed on the surface of phage. Then the mutant scFv library then undergoes various affinity selection procedures so that mutant BGA scFv clones with an increased affinity can be isolated.

7.1 M13 BACTERIOPHAGE

M13 bacteriophage are filamentous phage which are able to infect bacteria containing an F conjugative plasmid. The F conjugative plasmid encodes for an F pilus, which is required for the conjugal transfer of the viral or plasmid DNA into the recipient bacteria (Frost *et al.*, 1994). The F pilus is essentially a protein tube that can attach and detach to the bacterial membrane; the F pilus provides a tunnel in which DNA encased within the phage particle can leave the phage and be inserted into the bacteria (Frost, 1993). Therefore, M13 phage are referred to as Ff phage because to infect *E. coli* they require bacterial strains, which contain the F conjugative plasmid, and thus express the F pilus. The M13 phage particle attaches to the tip of the F pilus, and its DNA genome is translocated into the bacterial cytoplasm. The genome of the M13 phage is a circular,

single-stranded DNA molecule encased in a long cylinder, which resembles a thread-like particle; the M13 bacteriophage is therefore referred to as a filamentous phage.

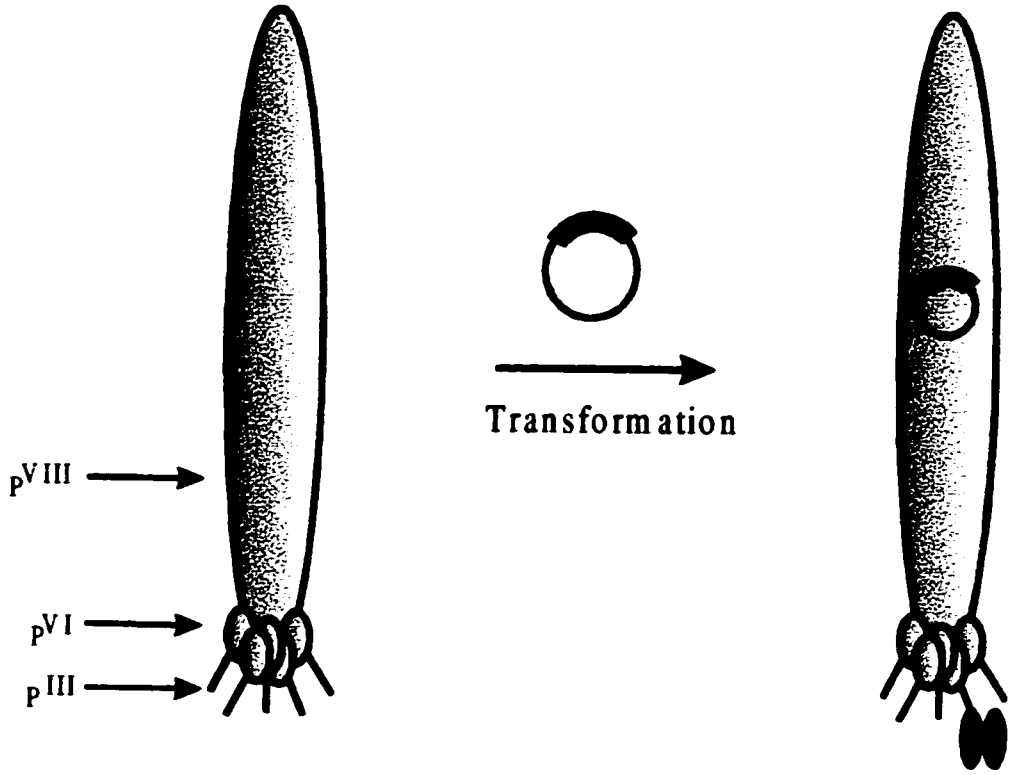


Figure 7: Schematic representation of M13 bacteriophage. The major capsid protein pVIII constitutes the bacteriophage cylinder that encapsulates the phage genome (not shown). There are generally five minor capsid proteins pIII at one end of the phage particle. The bacteriophage will display the protein encoded in the transformed plasmid on its surface through fusion to the pIII minor capsid protein.

The genome of the M13 Ff phage has 11 genes (genes I - XI), which encode for DNA replication proteins, proteins involved in the assembly of the phage, and proteins which make up the capsule of the phage particle (Webster, 1996). The genes gVIII and gIII encode for phage capsule proteins pVIII and pIII respectively. These capsule, or capsid proteins, have been particularly important in phage display studies. Peptides have been fused to minor coat protein pIII (Smith, 1985; Parmley and Smith, 1988) or to the major coat protein pVIII (Il'ichev *et al.*, 1990; Greenwood *et al.*, 1991). pVIII is referred

to as the major capsid protein, since it is the most abundant capsid protein, and largely constitutes the protein cylinder that surrounds the DNA. pIII is also a capsid protein, however it is found at one end of the phage particle, and is referred to as minor capsid protein pIII. It should be noted that the ability of the phage to be infectious appears to be a function of the pIII capsid protein. There are approximately 5 pIII minor capsid proteins located at one end of the phage particle. DNA fragments encoding a protein or a specific peptide can be fused to the pIII or pVIII genes.

Phage display technology is based on the ability of these phage genes to be fused with foreign gene sequences and to ultimately be expressed as fusion proteins on the surface of the phage particle. The foreign peptides or proteins fused to either the pVIII or pIII phage proteins must be properly folded, functional proteins that retain their normal binding ability (Bass *et al.*, 1990; McCafferty *et al.*, 1990). In the phage display study carried out in Part B, the BGA scFv gene sequence was fused to the pIII minor capsid protein gene. The mutant scFv-geneIII fusion proteins are expressed on one end of the phage particle, and constitute the library repertoire.

One of the advantages of phage display technology is that it provides a direct link between a protein's genotype with its displayed phenotype. The genotype and phenotype of the displayed protein are directly linked, since the protein displayed on the phage surface is tagged with its own DNA, which is encoded by the phagemid encased within the phage particle. Therefore, the primary structure of the attached peptide can be easily determined by determining the DNA sequence of that particular clone.

7.2 PHAGEMID VECTORS

Most standard cloning vectors contain an origin of replication, an antibiotic resistance gene, and various cloning sites. When a filamentous bacteriophage origin of replication is added to a standard vector, it is referred to as a phagemid. Phagemid

cloning vectors not only contain the above-mentioned sequences; they also carry the gIII with the appropriate cloning sites. In such a phagemid, a protein sequence may be fused to the N-terminus of the mature pIII (McCafferty *et al.*, 1990; Hoogenboom *et al.*, 1991) or to the N-terminus of a truncated pIII (Garrard *et al.*, 1991; Barbas *et al.*, 1991).

Unlike phage vectors, which contain all the phage genes necessary for phage replication and growth, phagemid vectors only contain one phage gene, gIII gene fused with a specific peptide or protein sequence. Therefore, the phagemid DNA encoding the protein-pIII fusion is packaged into particles using helper phage (Vieira and Messing, 1987), such as M13K07, which supplies all the structural proteins needed to make viable phage particles (Hoogenboom *et. al.*, 1998). However, since helper phage encode for wild-type pIII (i.e. individual pIII, not fusion protein), the great majority of phagemid particles display no fusion protein at all. Furthermore, the phage that do display fusion protein will only display a single copy (Garrard *et al.*, 1991). The valency of the fusion proteins displayed can be increased by using a phage vector (McCafferty *et al.*, 1990), which eliminates the need for helper phage, or by using a modified helper phage with the gIII deleted (Griffiths *et al.*, 1993); in both instances, every displayed pIII has protein fused to it. However, since an increase in avidity can affect the binding affinity of the phage, monovalent display of the fusion protein is desirable when selecting for higher affinity libraries.

7.3 PHAGE DISPLAY OF ANTIBODY FRAGMENTS

Antibodies were the first functional proteins to be successfully displayed on the surface of phage (McCafferty *et al.*, 1990). This was done following the initial display of peptides on the surface of phage (Parmley and Smith, 1988). The display of functional antibody proteins was achieved by fusing the coding sequence of the antibody scFv fragment to the N-terminus of pIII. The V_L and V_H domains of the scFv are correctly

folded, stabilized by an intramolecular disulphide-bridge, and are completely functional (Skerra and Pluckthun, 1988; Better *et al.*, 1988). Recall that the scFv antibody fragment contains the original antigen-binding domain of the complete antibody, and is therefore capable of binding antigen the same way its parental antibody would.

Antibody fragments have been effectively displayed on the surface of phage as scFvs (McCafferty *et al.*, 1990; Clackson *et al.*, 1991; Marks *et al.*, 1991), disulphide stabilized Fvs (Brinkmann, *et al.*, 1995), Fab fragments (Garrard *et al.*, 1991; Hoogenboom *et al.*, 1991; Barbas *et al.*, 1991), and diabody fragments (Holliger *et al.*, 1993). However, the smaller the size of the displayed fragment, the more genetically stable these libraries are; therefore, the scFv format is generally favoured over the Fab fragment. We have displayed an scFv fragment adjoined by a short linker sequence, which give rise to a pIII-scFv fusion that is able to associate with soluble scFv fragments derived from some proteolytic cleavage of the scFv from pIII. The association of the pIII-scFv fusion protein with soluble scFv protein effectively forms diabodies. The advantage of this diabody format for our phage display study was that the avidity of the displayed protein was increased (i.e. dimeric instead of monomeric). The increase in avidity consequently results in an increase in affinity per phage, and therefore makes the study of the weak BGA scFv more feasible.

7.4 PANNING

An important application of phage display is the construction of a library of fusion proteins expressed on the surface of the phage, and the subsequent use of an affinity selection process that selects for various target proteins. This process of affinity selection is known as biopanning or panning (Parmley and Smith, 1988). A panning experiment involves the capture of fusion phage with an immobilized molecule. The bound phage is recovered and amplified and again selected for binding to the

immobilized target molecule. This cycle of selection, elution and amplification is repeated, usually 3-4 rounds, thus enriching for the phage clones that bind the target molecule. Figure 10 is a schematic outline of the selection of phage antibody through panning.

Any method that separates clones that bind from clones that do not bind can be used for panning. Many different selection methods have been used, including panning on an immobilized antigen on a solid support (Griffiths *et al.*, 1994), panning on columns (Clackson *et al*, 1991), on the sensor chip of the BIAcore instrument (Malmborg *et al.*, 1996), panning on whole cells (Marks *et al.*, 1993; Bradbury, *et al.*, 1993), panning on biotinylated antigens (Hawkins *et al.*, 1992), and selection using sorting procedures (de Kruif *et al.*, 1995). All these methods effectively use a substrate (for example, an antigen), and separate phage displayed proteins based on binding selectivity. We have panned our BGA scFv-gIII phagemid library on immobilized BSA-A antigen coated microtitre wells, on the BSA-A coated sensor chips of the BIAcore, and on a Synsorb-A column. These methods, with the exception of the Synsorb-A column, have previously been used to successfully pan various phagemid libraries. However, the panning of this low affinity anti-carbohydrate BGA scFv-gIII phagemid library has been a challenge. We report here the first panning experiments on a Synsorb-A column.

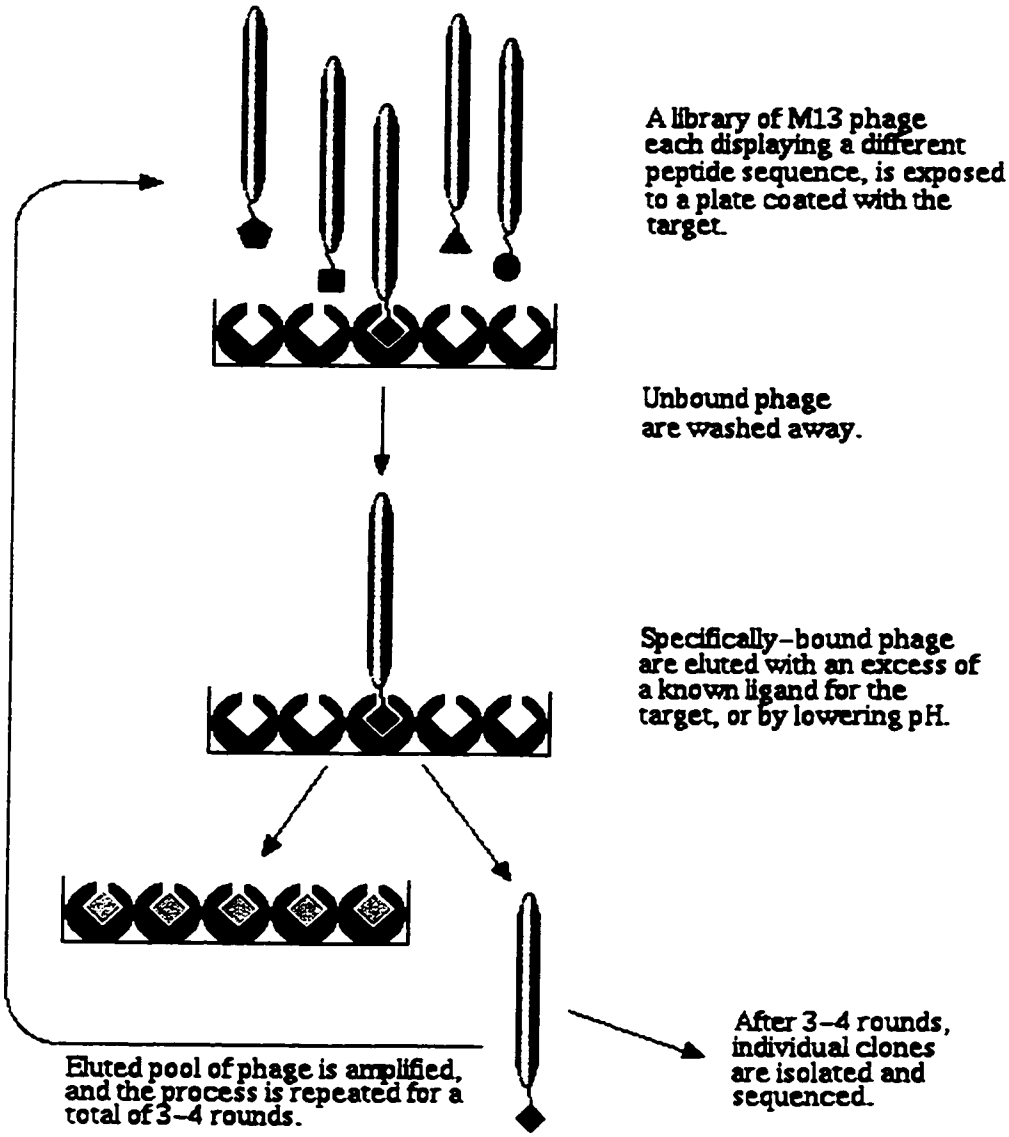


Figure 8: Schematic outline of phage selection through panning. Panning allows for selectivity by allowing phage to bind to antigen, then washing to remove unbound phage and then elution to retrieve specific binding phage. If the stringency of the washing step used to remove unbound phage is increased, then phage with an increased affinity for the antigen will be selected for in the panning rounds. The eluted phage are used to infect bacteria and are amplified prior to the next round of panning. Eluted phage are amplified to increase the better binding scFv population eluted, and to provide the next round with an increased amount of selected phage for further selection. Phage are allowed to bind to a target antigen, here it is represented as antigen immobilized on microtitre wells. However, we have panned our phagemid library on antigen immobilized onto the BIAcore sensor surface, and have also panned against antigen that is covalently linked to a column matrix (Synsorb-A). (http://www.uk.neb.com/neb/products/phd/bioflo.gif)

8. ANTIGEN-ANTIBODY BINDING AFFINITIES

The noncovalent interactions that form the basis of antibody-antigen binding include hydrogen bonds, ionic bonds, hydrophobic interactions and van der Waals interactions. The strength of these noncovalent interactions is weak (compared to covalent bonds) and a large number of interactions are required to form a strong antibody-antigen interaction. The strength of the total noncovalent interactions between a single antigen-binding site on an antibody and a single epitope is the affinity of the antibody for that epitope. Since it is these noncovalent interactions that determine the affinity of the antibody-antigen interactions, and because these interactions operate over small distances a strong antibody-antigen interaction depends on a very close fit between the antigen and antibody.

In the site-directed mutagenesis study described in Part A, the antigen binding pocket of the AC1001 variable fragment is examined with the A trisaccharide antigen modeled into the antigen binding cleft. The X-ray crystallographic model structure, specifically the antigen binding pocket, was the basis of the mutagenesis study. The aim of this study is to select amino acid residues within the binding pocket that may potentially be mutated to residues that would improve the fit between antibody and antigen and maximize the noncovalent interactions.

8.1. DETERMINATION OF EQUILIBRIUM CONSTANTS

In the simplest model for the interaction of an immobilized species Ag with a mobile species Ab, they react form a 1:1 complex Ag•Ab, as in reaction 1.

$$Ab + Ag \rightleftharpoons Ab\bullet Ag \qquad (1)$$

At equilibrium, the concentrations of these species are dictated by the equation:

$$K_D = \frac{[Ab][Ag]}{[Ab\bullet Ag]} \qquad (2)$$

Where K_D is the dissociation constant at equilibrium. However, because equilibrium is a dynamic state, K_D alone can not be used to characterize the reaction completely; the rates of association and dissociation are equally important. Complex association has a chemical on-rate constant, referred to as k_{on}, and a chemical off-rate constant for complex dissociation, k_{off}. The k_{on} indicates how fast the concentration of the Ab•Ag complex increases, and k_{off} indicates the fraction of Ab•Ag complexes that dissociate per second. The K_D can also be expressed as a ratio of these kinetic constants.

$$K_D = \frac{k_{off}}{k_{on}} \tag{3}$$

Two approaches are typically used to measure affinity constants:

1. determination, at equilibrium, of the ratio of the concentrations of the antibody (or antigen) free and engaged in complex and using equation (2)
2. measurement of the dissociation and association rate constants, k_{off} and k_{on}, and determination of K_D from their ratio using equation (3)

The first approach determines K_D by the analysis of the equilibrium, as is done in a Scatchard analysis. In this analysis equation (2) is linearized according to the standard protocol of Scatchard (Scatchard, 1949), and the dissociation constant is the -1/slope of the line obtained by plotting the ratios of bound to free ligand [Ab•Ag] / [Ab] versus the concentration of the bound ligand [Ab]. The second approach determines K_D by determining the kinetic rate constants, and applying equation (3). When k_{on} and k_{off} could be measured, the K_D was calculated using equation (3). However, if the k_{on} and k_{off} could not be measured on the BIACORE, (due to a weaker interaction) a Scatchard analysis of the equilibrium binding still allowed the K_D to be derived. Both approaches were used in the present study, when possible.

The binding affinity of the wild type BGA scFv and the mutant BGA scFvs for the A trisaccharide antigen were measured using SPR and the BIACORE instrument (Biacore Inc.) as outlined in the following sections.

9. SURFACE PLASMON RESONANCE

Surface plasmon resonance (SPR) (Liedberg *et al.*, 1983) is an optical phenomenon that arises in thin metal films under conditions of total internal reflection. Surface plasmons are waves of oscillating surface charge density travelling along the metal surface, and surface plasmon resonance occurs when these surface plasmon waves are excited at the metal-liquid interface. Light is directed at, and reflected from, the side of the metal film and SPR causes a reduction in the reflected light intensity at a specific combination of angle and wavelength (Figure 9).

Biomolecular binding events at the sensor surface change the refractive index and produce a shift in the resonance signal. During a binding analysis, SPR changes occur as a solution is passed over the surface of the sensor chip. The difference in the refractive index is caused by mass changes on the sensor chip surface i.e. when the analyte binds to the immobilized ligand. In our case it is when the scFv antibody fragment binds to the immobilized antigen. The changes in the refractive index are measured continuously to form a sensorgram (Figure 9 and 10), which provides a record of the progress of association and dissociation of the reactants in real time.

To perform such an analysis, one reactant must be immobilized on the sensor surface, while sample containing the other reactant is injected over this surface in a precisely controlled flow. The sensor chip is essentially a glass slide coated on one side with a thin gold film, to which a matrix of carboxymethylated dextran is covalently attached. Biomolecules are then attached to this flexible dextran surface; the sensor surface with the immobilized reactant can be regenerated, so that one sensor chip can be

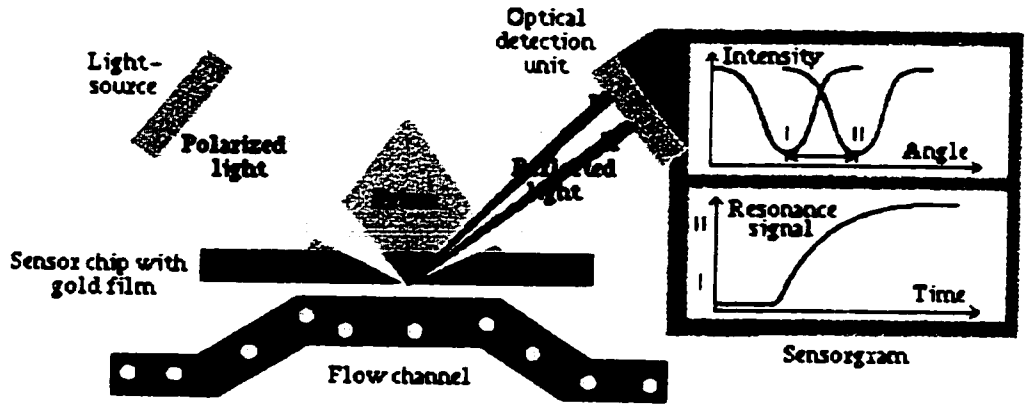


Figure 9: Real time SPR detector, sensor chip and integrated fluidic cartridge in the BIACORE system. This figure shows the optical configuration together with the sensor chip and flow channel. BIACORE places the sensor surface on a removable microchip that is optically coupled to the instrument, and the sample is supplied via an integrated computer-controlled microfluidic system (Sjolander and Urbaniczky, 1991). The strategy used in the BIACORE and other SPR biosensors involves the covalent attachment of one reactant to the sensor surface, then the association and dissociation of a second mobile reactant is monitored. The SPR response is correlated to changes in the refractive index at the sensor chip surface. The difference in refractive index is caused by mass changes on the sensor chip surface, i.e. when analyte binds to immobilized ligand. The cycle of association and dissociation is repeated using different concentration of the mobile reactant to give a sequence of binding-progress curves, called sensorgrams, which contain information regarding the rate constants and the equilibrium constants of the interaction.

used for repeated experiments. Three different surfaces were generated for the biomolecular studies reported in this thesis: a BSA control surface, a BSA-A trisaccharide antigen active surface, and a BSA-B trisaccharide antigen surface. The conjugate antigens (BSA-A and BSA-B) are essentially a BSA molecule saturated with the A-trisaccharide antigens, or the B-trisaccharide antigens. All three reactants were immobilized onto the dextran surface using amine coupling; similar surface densities were generated for all three reactants (please refer to Experimental Procedures).

9.1 BIACORE

The most frequently used commercial SPR instrument is the BIACORE™. This instrument provides a relatively new technology for real-time biospecific interaction analysis (BIA), based on the biosensor technology, in which SPR is used to monitor the binding and dissociation of biomolecules on a sensor surface. BIACORE places the sensor surface on a removable microchip that is optically coupled to the instrument at an optical interface, and the sample is supplied *via* an integrated computer-controlled microfluidic system (Sjölander and Urbancsky, 1991). This microfluidics system divides the sensor chip into four distinct flow cells, or channels, on the sensor surface that remain independent of each other and can provide four separate surfaces. Ligand may be independently and covalently coupled to each flow cell. The covalent nature of the bound ligand allows regeneration of the surface and for numerous measurements on the same surface.

10. FOLLOWING BIOMOLECULAR BINDING

To analyze the biomolecular binding in an SPR biosensor (BIACORE) the binding or dissociation of macromolecules is followed. The association and dissociation of macromolecules at the sensor surface changes the local refractive index and produces a shift in the resonance signal. This shift in the resonance signal for a given refractive index increment is proportional to the mass that is bound to the sensor surface. This output signal is measured in resonance units (RU) where one RU corresponds to an increase in surface protein concentration of approximately 1 pg/mm^2. (Stenberg *et al.*, 1991).

Therefore, in this real-time interaction analysis, the binding of the antibody to the immobilized antigen is detected as an increase in the number of RUs as a function of time, and the progress of this interaction is recorded as a sensorgram (Figure 10). A sensorgram is the recorded binding-process curve, measuring the change in RU as a function of time. The cycle of association and dissociation is repeated using different concentrations of the mobile reactant to yield a sequence of sensorgrams, which contain information regarding the rate constants and the equilibrium constant of the interaction.

Many molecular interactions have been studied using SPR in a broad variety of fields. For example, receptor-ligand interactions have been studied (Cunningham and Wells, 1993; Johanson *et al.*, 1995), as have antibody-antigen interactions (Holliger *et al.*, 1993; Kelley and O'Connell, 1993; Malmborg and Borrebaeck, 1995; Patten *et al.*, 1996), protein-carbohydrate interactions (MacKenzie *et al.*, 1996), and many more interactions including those involving DNA (Bondeson *et al.*, 1993; Buckle *et al.*, 1996; Yang *et al.*, 1995), lipid vesicles (Masson *et al.*, 1994; Ramsden and Schneider, 1993) and virus research (Glaser *et al.*, 1992; Pellquer and Van Regenmortel, 1993). We are using SPR and the BIACORE instrument to determine the binding kinetics of wild type and mutant scFvs binding to immobilized BSA-A trisaccharide surface.

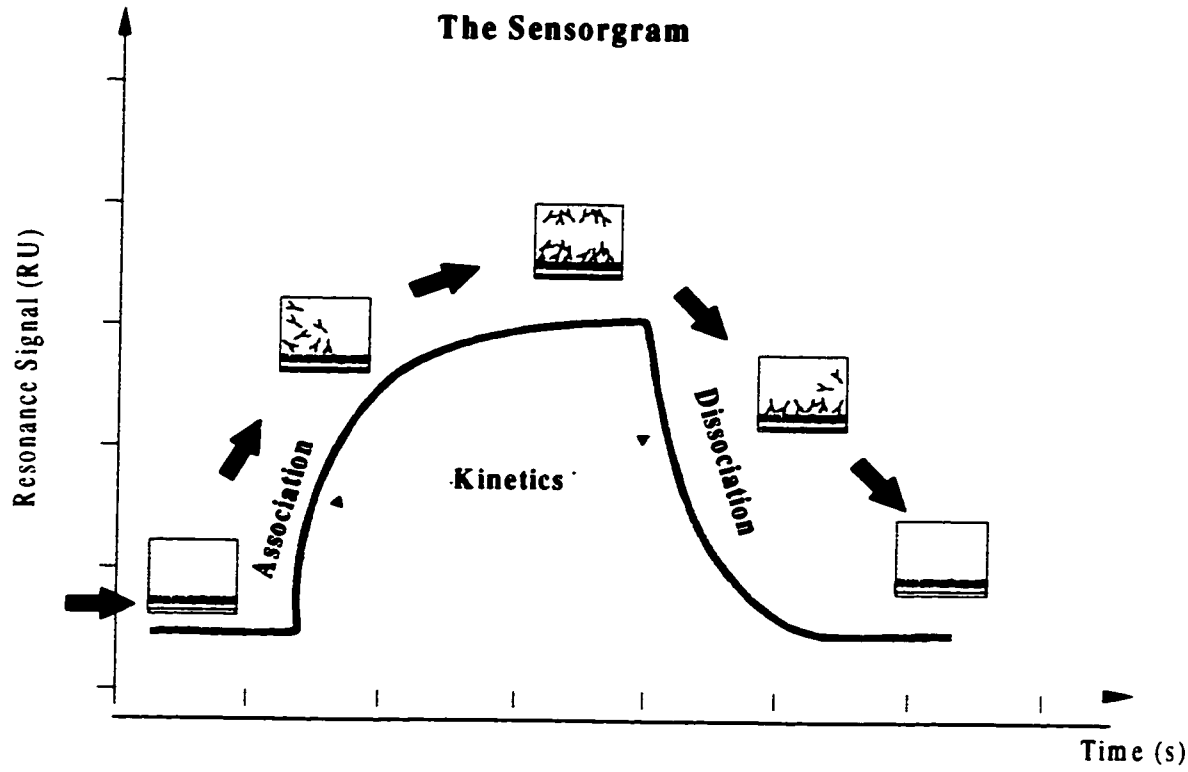


Figure 10: A sensorgram reflects the progress of an interaction. In this experiment, and in other SPR analyses, the response that is measured is the refractive index changes that are associated with the analyte (antibody) binding to the antigen (BSA-A) immobilized on the sensor surface. The change in this angle is followed as a function of time, and is expressed as arbitrary units called resonance units (RU). One RU corresponds to an increase in surface protein concentration of approximately 1 pg/mm^2 (Stenberg et al., 1991). The sensorgram contains information regarding the rate constants and the equilibrium constant of the interaction. In the first stage of the interaction the sensor surface is equilibrated with wash buffer and the baseline is determined. The analyte is injected, and the association phase of the experiment is monitored. In the association phase, the mobile analyte is introduced into the buffer flow at a constant concentration above the sensor surface, and the progress of complex formation is monitored, as is observed in the increase in the signal. During the association phase, we see a typical profile of analyte binding, and the k_{on} can be determined from this portion of the sensorgram. If conditions permit, equilibrium is reached, as it does in this sensorgram (plateau in RU) and the equilibrium binding can be determined. Once analyte is absent from the buffer flow the dissociation phase begins, and the time course of complex dissociation is recorded. The dissociation phase can be used to determine the k_{off}. Some experiments may require that the surface be regenerated, using a suitable reagent to remove the bound analyte. However, the binding illustrated above shows a dissociation rate that does not require a regeneration step; the elimination of analyte from the flow buffer was all that was needed to regenerate the sensor surface in a reasonable time. This method of measuring specific reversible binding in real-time is useful, not only because no chromophore group or labeling is needed, but also because sample volume is relatively small and a broad range of affinities can be measured; $K_D = 10^{-4} - 10^{-12}$ is the affinity range that can be analyzed on a BIACORE 2000 (Myszka *et al.*, 1997).

The BIAevaluation 3.0 software (Markley, 1997) was used to analyze the kinetic data recorded. This program allows for the analysis of complex kinetic data. One approach was to simultaneously fit the k_{on} and k_{off} from several data sets and determine the K_D for a particular interaction. This complex simultaneous curve fitting analysis is referred to as 'global fitting', or global analysis, and will be discussed below.

Equilibrium binding analyses were also carried out. Equilibrium binding analyses are generally performed on weak binding proteins that data that display rapid kinetics. An analysis of the equilibrium binding was used to determine the K_D when the rapid rise to equilibrium after injection and the rapid dissociation of bound analyte following injection do not allow for the determination of K_D from the association and dissociation phases. If the binding affinity is weak, the analytes need to present in high protein concentration in the flow buffer and this can cause bulk changes in the RUs recorded. We are measuring the functional affinity of a bivalent scFv; there are two antigen binding sites that bind two antigens. For this 2:2 reaction to occur, a high density of antigen on the sensor surface is required, and this too may contribute to non-specific binding. These bulk changes or bulk effects may occur when the flow buffer above the sensor surface has changed, or when the concentration of macromolecules above the sensor surface has increased, thereby causing an indirect RU change that is unrelated to binding. High protein concentrations in the flow buffer may also increase non-specific binding to the sensor surface, and this too will contribute to inaccuracies in the recorded RUs. Therefore the subtraction of the control surface response from the active surface response [(BSA-A) – (BSA)] in the equilibrium analysis allows for the generation of sensorgrams that represent the true biomolecular binding interaction.

10.1 GLOBAL ANALYSIS OF BIACORE DATA

Global fitting is an analysis procedure that deals with fitting experimental data from several sensorgrams simultaneously. The global fitting analysis constrains the kinetic parameters (k_{on} and k_{off}) to a single value that is the best fit to all sensorgrams simultaneously (Markley, 1997). In practice, this simultaneous evaluation of multiple data sets gives a complete and powerful evaluation, to yield better parameter estimates and extend the range of kinetic rate constants that can be accurately determined (Myszka, 1998). The global analysis of several data sets provides a non subjective analysis, and for many interactions is necessary to achieve stability in the fitting process. Using global analysis enables the analysis of complex biological interactions, and generation of a single solution from a complex data set; however the global analysis requires high quality data with low noise, good reproducibility, and pure reagents.

Global analysis is a comprehensive analysis using a large data set, and as a result global analyses lead to higher residuals. These higher residuals are a direct consequence of the global analysis, because this comprehensive analysis constrains the kinetic parameters, k_{on} and k_{off}, to a single set of values that best fit the entire data set. Residuals are differences between the observed experimental data and the calculated. Visually, the fitting process can be assessed with the overlay of the calculated data onto the observed data. However, we can plot the difference between the experimental and the fitted data for each curve and examine the scatter of the data more precisely in a residual plot. Pronounced deviation trends in the residual plot indicate poorly fit data, and may indicate that an inadequate model was used to analyze the data. Residual plots were used whenever possible to assess the goodness of fit.

Applying a global analysis to multiple data sets allows for the determination of reliable, reproducible, and non-subjective kinetic data. Global analyses provide a comprehensive and powerful means of analyzing data and allow for many experimental

limitations to be accounted for (such as mass transport, discussed below), and for the determination of an extended range of kinetic constants. A global fitting analysis was applied to the kinetic data collected whenever possible, using a 1:1 (2:2) binding model, taking into account mass transport effects.

10.2 MASS TRANSPORT

Deviations from the true K_D can result from mass transport limitations in the association and dissociation phases (Schuck, 1997). In kinetic biosensor experiments, the rate of transport of mobile reactant to immobilized ligand may be a potential limitation in the biomolecular binding experiment. The analysis of biomolecular interactions requires a direct analysis of the rate at which mobile reactant interacts with the immobilized ligand, and the rate of reactant transport to the sensor surface should not be the limiting factor in the binding experiment.

In general, the effects of mass transport can be regarded as a failure to maintain the injected concentration of free mobile reactant at the sensor surface in the vicinity of the binding sites. Thus, the effects of mass transport must be taken into account if the rate of association is higher than the rate of transport, i.e. the limiting factor is not complex formation but rather the local depletion of mobile reactant at the sensor surface, thereby limiting complex formation. Similarly, the dissociation phase can also be affected by mass transport if the rate of dissociation is higher than the transport rate, thereby allowing analyte to rebind to available antigen sites at the sensor surface. True kinetics can not be measured if transport effects limit complex association and dissociation, because the mobile reactant concentration at the sensor surface is not the same (it is less) as the injected concentration during association or zero during dissociation. Mass transport effects can be taken into account in the BIAevaluation analysis, so that true kinetic measurements can be derived.

CHAPTER TWO

STUDY A
SITE DIRECTED MUTAGENESIS STUDY OF BGA ScFv

A.1 INTRODUCTION

A.1.1 OBJECTIVE

The main objective of this work was to mimic the process of somatic mutation which accompanies affinity maturation, generate mutant anti-blood group A (BGA) scFv proteins and study the effects of these point mutations on the binding of the BGA antibody AC1001 to the blood group A carbohydrate antigen. The purpose of this work is to better understand and exploit the interaction between the blood group antigens and their corresponding antibodies, and to potentially develop a BGA scFv with an improved binding affinity for the A antigen. A greater understanding of the interaction between this BGA single chain variable fragment (scFv) and its corresponding antigen may lead to a more refined understanding of the mechanisms by which these anti-blood group antibodies discriminate between the largely similar A and B carbohydrate antigen structures. This would in turn add to the understanding of the interactions involved in cell-cell recognition and the mechanisms of viral and bacterial infections involving blood group antigens. This study sought to mimic the maturation process *in vitro* using site directed mutagenesis, thereby simulating the generation of anti-carbohydrate antibodies with a potentially higher binding affinity

A.1.2 OUTLINE

We have used the crystal structure of the AC1001 scFv to rationally design an improved binding site. The anti-blood-group-A IgM hybridoma AC1001 sequence (Chen *et al.*, 1987) was used as the basis for the construction of the AC1001 Fab (MacKenzie *et al.*, 1994). The AC1001 Fab recognizes the blood group A antigen and has been expressed in *E. coli* (MacKenzie *et al.*, 1994). We have used the V_H and V_L synthetic genes to produce an scFv form of AC1001 that displays full blood group A binding activity, and is referred to as AC1001 scFv. The subtilisin digestion of the linker that

joins the V_L-V_H domains of the AC1001 scFv yields AC1001 Fv, which has been crystallized (Patenaude *et al.*, 1999), X-ray crystallography data has been collected and the structure solved and refined to 2.2 Å resolution.

A.1.3 APPROACH

To rationally re-design the binding site we examined the high resolution (2.2 Å) X-ray crystal structure of the AC1001 variable fragment, and selected specific residues as candidates for site-directed mutagenesis (Thomas *et al.*, 1999). The features of the crystal model are described elsewhere (Thomas *et al.*, 1999) and will be referred to in the discussion. Examination of the antigen-binding pocket of the AC1001 Fv crystal allowed the rational design of these mutant proteins. The selection of candidate residues and the mutational changes that were introduced will be described in detail in this section. The mutant proteins were then cloned, expressed, purified and then analyzed using SPR. This rational design approach yielded scFv species with improved affinities for the A-antigen. Some mutations produced proteins with up to a 30-fold increase in binding affinity while still maintaining specificity for the A-antigen.

The AC1001 Fv crystal structure was derived from X-ray diffraction data, however, this report will not focus on the X-ray crystallographic techniques used to generate the BGA scFv model, but will focus on the examination of the binding pocket and the rational selection of the candidate residues for mutation. The candidate amino acid residues chosen for mutation were selected based on their presence in the binding pocket, their proximity to antigen, and on the degree of binding to the antigen. The mutation of these residues was based on producing mutations that would potentially expose the binding pocket, create greater contact with the antigen, and maximize binding interactions between the antibody and the antigen, thereby improving affinity.

A.1.4 RELATED RESEARCH

The rational design of antibodies for improved binding and the interpretation of altered binding is difficult even when the three-dimensional structure of the antigen epitope and antibody are known (Brummel *et al.*, 1993; Cygler *et al.*, 1991; Zdanov *et al.*, 1994). Furthermore, since protein-carbohydrate affinities are lower than protein-protein affinities, there are few high resolution crystal structures of anti-carbohydrate antibody complexed with their corresponding antigen.

The main objective of this work has been to rationally re-design an anti-carbohydrate antibody with an improved affinity for the BGA carbohydrate antigen through rational design. Many groups have attempted a similar approach to protein design, which usually involves the generation of a model that best describes the protein or epitope to be studied. This model can be generated using X-ray crystallography (Xiang *et al.*, 1993; Vermersch *et al.*, 1990 & 1991), by computer modelling using comparisons with related proteins (Ruff-Jamison and Gleeny, 1993; Komissarov *et al.*, 1997; Near *et al.*, 1993), and nuclear magnetic resonance (NMR) (Reichman *et al.*, 1992). Since the rational design approach is difficult, many groups have used alternate routes, such as phage display (Schier *et al.*, 1996), saturation mutagenesis (Yand *et al.*, 1995), and a combination of these various techniques in tandem (Reichmann *et al.*, 1992 & 1993; Jeffery *et al.*, 1995).

The rational design approach is difficult, particularly for anti-carbohydrate antibodies. To date there have been no reports of a single mutation in an anti-carbohydrate antibody that resulted in a specific increase in antigen binding affinity. Vermersch *et al.* (1990 and 1991) have reported two different single amino acid mutations that have caused an increase in the binding affinity in a lectin-carbohydrate interaction; however, these mutations altered specificity and did not improve the lectin's affinity for its specific sugar but for a related sugar. A single amino acid mutation that

caused a specific two-fold increase in antigen binding affinity has been reported by Xiang *et al.* (1993); however, this was a protein-protein interaction and not a protein-carbohydrate interaction. Improvements in the specific binding affinity in protein-carbohydrate interactions have been reported, but the improved mutants contained many mutations rather than a single point mutation (Thompson *et al.*, 1996; Jeffrey *et al.*, 1995). Moreover, the majority of these reports describe mutants with decreased affinities (Lavoie *et al.*, 1992; Komissarov *et al.* 1997; Korbin *et al.*, 1991). We report here the first rationally designed single point mutation in an anti-carbohydrate antibody that increased specific antigen binding affinity of a bivalent scFv 30-fold.

A.2 EXPERIMENTAL PROCEDURES

A.2.1 MATERIALS

All DNA manipulations were carried out according to standard procedures (Sambrook *et al.*, 1989). Restriction enzymes and DNA-modifying enzymes were purchased from New England Biolabs and Life Technologies, Inc. DNA sequences were performed by the dideoxynucleotide method (Sanger and Coulson, 1975) using an automated DNA sequencer model 373 (Applied Biosystems Inc.). BSA-A and BSA-B trisaccharide conjugate antigens were purchased from Chembiomed (Edmonton, Alberta) and Calbiochem-Novabiochem Corp. (San Diego, CA). Binding kinetics were determined by surface plasmon resonance using a BIACORE™ biosensor system (Pharmacia Biosensor) (Jönsson, *et al.*, 1991).

A.2.2 TGAscFv CONSTRUCT

The BGA scFv gene was cloned into the phagemid pSK4 (Deng *et al.*, 1994) at the *EcoRI-BglII* cloning sites to yield the TGASL#4 phagemid vector. The TGASL#4 scFv V_L and V_H regions are linked by a 5 amino acid short linker (Arg-Ala-Asp-Ala-Ala) to favour the formation of scFv dimers, or 'diabodies' in solution (Holliger *et al.*, 1993). The BGA scFv gene is preceded by an ompA signal peptide, and followed by a c-myc detection tag (Hoogenboom *et al.*, 1991) and a His_5 purification tail. TGASL#4 was initially cloned to produce scFv-geneIII fusion proteins, and therefore contains the geneIII phage protein sequence fused through a Gly_4Ser linker to the scFv at the *BglII* site following the His_5 tail. Since soluble BGA scFv proteins were required for this study, the TGASL#4 phagemid was modified through the insertion of a self-complementary terminator sequence (5'-AGATCTTAATAGTGATCACTATTAAGATCT-3') (Deng *et al.*, 1994) at the *BglII* site between the BGA scFv and the geneIII regions. This modified vector will

be designated TGAscFv, and will be used to produce BGA scFv mutant proteins in *E. coli.*

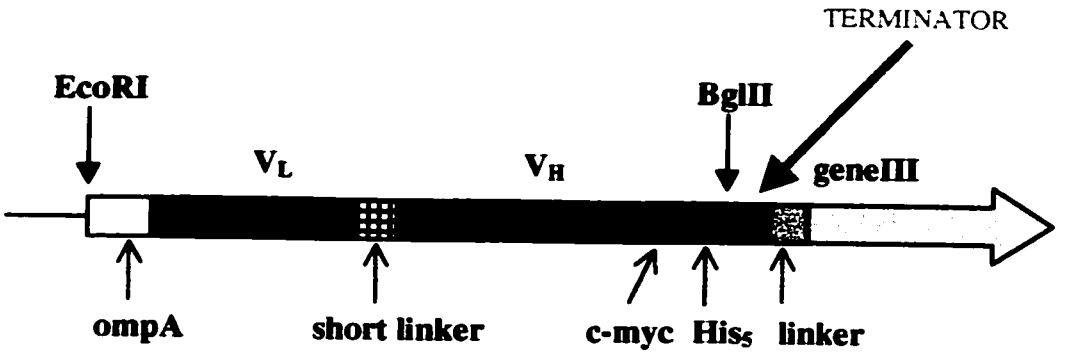


Figure 11 : TGAscFv vector segment showing BGA scFv region. A 5 amino acid linker joins the V_L-V_H scFv genes. At the BglII site a terminator sequence was inserted, so that soluble scFv fragments would be produced and not fusion proteins. The short linker forces the scFv fragments to dimerize.

A.2.3 X-RAY CRYSTALLOGRAPHY

All crystallographic and modeling experiments were carried out by Ms. Sonia Patenaude at the University of Ottawa, and will be described in her thesis.

A.2.4 SITE-DIRECTED MUTAGENESIS

PCR mutagenesis was used to construct the site-directed mutant clones. Oligodeoxyribonucleotides containing the appropriate mutations were synthesized on an automated DNA synthesizer model 394 (Applied Biosystems Inc.). The crude oligonucleotides were purified using polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (Laemmli, 1970). The slowest migrating portion of the band was removed, the DNA oligonucleotides were eluted from the gel and further purified on a Millipore C_{18} Sep-Pak cartridge (Sambrook *et al.*, 1989). Using the TGAscFv wild type plasmid as template, the purified mutant oligonucleotide primers were used to PCR

amplify the corresponding mutant fragments. Amplification was performed using 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 60 s (Perkin Elmer model 9600 thermal cycler). The PCR amplified mutant fragments were purified, digested with the appropriate restriction enzymes, and ligated into purified TGAscFv vector cut with the same restriction enzymes. The ligated products were then transformed into *E. coli* TG1 competent cells, and screened for the appropriate insert.

A.2.5 SCREENING MUTANT CLONES

The ligated TGAscFv mutant plasmid clones were transformed into *E. coli* TG1 cells. This cell line is not resistant to the antibiotic ampicillin (amp), but the TGAscFv plasmid contains the amp resistance (amp^r) gene and thereby confers resistance to amp. Therefore, TG1 cells that have been transformed with the TGAscFv plasmid have become amp^r, and will then grow on media containing ampicillin. Since the ligated clones were transformed and plated on LB amp plates, the screening process began with the random selection of a few (4-8) colonies from each transformed mutant LB amp plate. Each clone was grown in 5 ml LB ampicillin media for 12 hours at 37°C. The following morning, a glycerol stock (1 ml overnight culture + 0.2 ml sterile glycerol, stored at –80°C) for each clone was made. The remaining 4 ml of overnight culture was used to prepare plasmid DNA using the QIAprep Spin Plasmid Kit (QIAgen) and the plasmid DNA was eluted with ddH_2O and stored at -20°C.

The mutant plasmid DNA was checked for the correct plasmid size on a 1% agarose gel, and compared to the wild type TGAscFv plasmid size. Mutant plasmid clones that were of an appropriate size (i.e. appeared to contain insert) were further screened using restriction enzymes. The appropriate restriction enzymes were used to double digest each mutant plasmid, such that the correct insert length could be verified. The clones that contained the correct insert were then sequenced by the

dideoxynucleotide method (Sanger and Coulson, 1975). The sense and antisense strands for the PCR amplified insert were completely sequenced to ensure that only the desired mutation was present.

A.2.6 PROTEIN EXPRESSION, ISOLATION AND PURIFICATION

A.2.6.1 ScFv Protein Expression

Expression of the wild type and mutant BGA scFv was carried out in *E. coli* strain TG1. Clones were grown in baffled shake flasks for 24 h (140 rpm) at 25°C in 100 ml M-9 minimal media (1μM $MgCl_2$, 0.1μM $CaCl_2$, 0.0005% Vitamin B1, 0.2% glucose, 0.4% casamino acids, and 0.01% ampicillin). Thirty milliliters of this preculture was then used to inoculate 1 l M-9 minimal media and this main culture was then grown for another 24 h at 25°C prior to induction with isopropyl-1-thio-β-D-galactopyranoside (1mM IPTG /l) and supplementation with additional nutrients (12g tryptone, 24g yeast extract, and 4 ml glycerol /l) (Anand *et al.*, 1991a). After a further 48 h of growth the cells were harvested and the periplasmic fraction was obtained using an osmotic shock procedure (Anand *et al.*, 1991b).

A.2.6.2 Protein Isolation

The cells were collected by centrifugation at 7000 rpm for 20 m in a Beckman centrifuge (model J2-21M). The cell pellet was resuspended with wash buffer (0.5M Tris·HCl, pH 8.0, 0.8% NaCl), and centrifuged at 8000 rpm/40 m. The washed cell pellet was then suspended in 50 ml sucrose buffer (25% sucrose, 1mM EDTA, 10mM Tris·HCl, pH 8.0). After 10 m at room temperature, the cells were centrifuged at 7000 rpm/50 m and the sucrose supernatant was collected. The pelleted cells were then resuspended in 50 ml ice-cold Shock buffer (10mM Tris·HCl, pH 8.0, 0.5mM $MgCl_2$), centrifuged at

7000rpm/50 m. and the periplasmic extract (supernatant) was collected (Anand *et. al.*, 1991b).

A.2.6.3 Verification of Protein Production

The supernatants after each centrifugation were analyzed by SDS-PAGE (Laemmli, 1970) and Western blotting (Towbin *et al.*, 1979). SDS-PAGE (12.5% acrylamide) was performed using the buffer system described by Laemmli, and the gels were stained with Coomassie Brilliant Blue (BluePrint, Gibco-BRL). In Western blotting, the proteins were transferred to PVDF Immobilon-P membrane (Millipore) and detected using mouse monoclonal antibody to c-myc oncoprotein (Cambridge Research Biochemicals) followed by an anti-mouse IgG/alkaline phosphatase conjugate (Promega) with BCIP/NBT (BioRad) as substrate (MacKenzie *et al.*, 1994). The sucrose supernatant and the periplasmic shock extract containing protein were dialyzed in starting buffer (10mM HEPES, pH 7, 0.5M NaCl) prior to column purification.

A.2.6.4 Protein Purification

The histidine-tagged scFvs were purified by immobilized metal-ion chromatography (IMAC) using a Ni^{2+}-charged Chelating Sepharose Fast Flow column (HiTrap, Pharmacia). The column was equilibrated with starting buffer (10 mM HEPES, 500 mM NaCl, pH 7.0) and the bound protein was eluted with a 0-500mM imidazole gradient in the same buffer; the eluted fractions were then analyzed by SDS-PAGE and Western blotting. The pure scFv protein fractions were pooled and dialyzed in phosphate-buffered saline (PBS) and concentrated in Centricon-10 (Amicon) concentrators. The protein concentration in the dialyzate was determined by measuring the optical density at 280 nm; where an $A_{280\,nm} = 1.35$ indicates a protein concentration of 1 mg/ml.

A.2.7 SCFV DIMER SEPARATION

To ensure that the protein sample used in the kinetic analysis is homogeneous (i.e. free of aggregated protein), the dimer and oligomers were always separated using size-exclusion chromatography (Superdex 75 column, Pharmacia) and the desired protein peak was collected and subsequently analyzed. The Superdex 75 column was equilibrated in HBS (10 mM HEPES, 150 mM NaCl, 3.44 mM EDTA pH 7.4), 200 µl of concentrated (protein concentration ≥ 2 mg /ml) protein was injected into column, and the scFv dimer or 'diabody' peak (final peak) was collected.

A.2.8 BIACORE ANALYSIS

The kinetics for the interaction of the purified scFv fragments with the A antigen were determined by surface plasmon resonance (SPR) using a BIACORE™ biosensor system (Biacore Inc.). Approximately 5000 resonance units (RU) of bovine serum albumin (BSA) or BSA-A trisaccharide were immobilized on research grade CM5 sensor chips in 10 mM sodium acetate, pH 4.5, using the amine coupling kit supplied by the manufacturer. One resonance unit corresponds to an immobilized protein concentration of ~1 pg/mm^2 (Stenberg *et al.*, 1991). All measurements were performed at 25°C in 10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% P20 at a flow rate of 20µl/m. Surfaces were regenerated by washing only. Binding constants were calculated from the association rate and dissociation rate constants where possible or by Scatchard analysis of the equilibrium binding. Subtracting the control response (on BSA surface) from the active (BSA-A-trisaccharide) surface provided values for equilibrium binding. A comparison of the response on the active and control BSA surfaces allowed for subtraction of bulk effects associated with buffer changes and high analyte concentrations and the calculation of specific binding.

A.3 RESULTS

A.3.1 MUTAGENESIS OF TGASCFV MUTANT PROTEINS

The substitution of specific amino acids in the TGAscFv sequence was done using site-directed mutagenesis. PCR mutagenesis was used to construct the clones using oligodeoxyribonucleotide primers containing the appropriate mutations. These specific substitutions have been verified by DNA sequencing and all mutant TGAscFv clone sequences were identical to the wild-type TGAscFv, except for the codons that introduced the specified mutations. The wild type TGAscFv and mutant genes were cloned into the pSK4 phagemid vector (Deng *et al.*, 1994), and expressed in *E. coli* TG1. Of the 24 TGAscFv mutants that were cloned, 19 clones contained only a single amino acid point mutation, and 5 were multiple mutants (2 double mutants and 3 triple mutants) as shown in Table I.

A.3.2 SCFV EXPRESSION

All TGAscFv site-directed mutants were cloned using the same vector, and all were produced, isolated and purified using the same experimental protocols. In Table I, we can clearly see that different amino acid substitutions effected the expression levels of the various mutant proteins. Mutants Y50N, Q89E, G91S, Y32W, N34D, Q89H, N102D, L103N, W104H, and N34M all produce amounts of protein similar to or less than the amount produced from the wild-type TGAscFv (~ 7 mg/l). Mutants L46N, L46K, L46M, N102Q, N102E, L103I, L103V, L103M and G91A have a 3-4 fold increase in protein production under the same conditions. Furthermore, multiple mutants of L46N, L46M, and N102Q with L103I have a much greater protein expression than any of the other mutants produced, for example the L46M-L103I multiple mutant is produced approximately 10-fold better than the wild-type protein, yielding 65 mg/l. The amino acid mutations in the proteins with improved expression are relatively conservative

mutations; for example a leucine to isoleucine mutation yielded a protein with a 4-fold improvement in expression. The effect of mutations on expression levels will be further considered in the discussion. It should be noted that the mutations involved in the improved expression levels are not limited to a specific region in the TGAscFv sequence, they are found in both the V_L and V_H in the CDR and FR regions.

A.3.3 SHORT *vs* LONG LINKER

Initial constructs of the BGA scFv linked the V_L and V_H genes *via* a long linker, which allowed the two domains freedom of movement so that they could bind to each other and form a V_L-V_H scFv monomer (Figure 6) (MacKenzie *et al.*, 1994). However, because the BGA scFv monomer has a weak binding affinity which made phage display experiments and kinetics analysis difficult, the long linker construct was modified into a short linker, which forced the expressed protein to form a dimer (Figure 6). The conversion of BGA scFv long linker (TGASL#4) to short linker (TGAscFv) prevented the formation of V_L-V_H monomers by eliminating the freedom of movement provided by a long linker. The short linker did not allow the domains from a single polypeptide to fold and bind to each other; instead it forced the V_L and V_H domains to remain together but unassociated, forcing V_L-V_H domains to dimerize with each other, or form 'diabodies' in solution (Holliger *et al.*, 1993). This increase in avidity (change from monomer to dimer) resulted in an increase in functional affinity, and therefore made possible the study of this weak binding scFv. Since avidity can affect the binding affinity of the interaction it is imperative that the scFv population being analyzed is made up strictly of scFv dimers, and does not contain aggregates. To eliminate aggregates from the analyzed protein sample, the purified scFv dimer protein was always separated using size-exclusion chromatography immediately prior to kinetic analysis.

Table I
TGAscFv Clones Constructed and Expressed for the Site-Directed Mutagenesis Study

TGAscFv Mutant Clone	Mutant Abbreviation	Region Containing Mutation	Approximate Protein Produced[a] mg / liter
Tyr L^{50} Asn	Y50N	V_L CDR2[b]	5
Gln L^{89} Glu	Q89E	V_L FR3[c]	2
Gly L^{91} Ser	G91S	V_L CDR3	2
Tyr L^{32} Trp	Y32W	V_L CDR1	2
Asn L^{34} Asp	N34D	V_L CDR1	5
Gln L^{89} His	Q89H	V_L FR3	4
Asn H^{102} Asp	N102D	V_H CDR3	5
Leu H^{103} Asn	L103N	V_H CDR3	5
Trp H^{104} His	W104H	V_H CDR3	8
Leu L^{46} Asn	L46N	V_L FR2	26
Leu L^{46} Lys	L46K	V_L FR2	28
Leu L^{46} Met	L46M	V_L FR2	25
Asn H^{102} Gln	N102Q	V_H CDR3	17
Asn H^{102} Glu	N102E	V_H CDR3	38
Leu H^{103} Ile	L103I	V_H CDR3	30
multiple mutant	L46N-N102Q-L103I		55
multiple mutant	L46M-N102Q-L103I		30
multiple mutant	L46N-L103I		63
multiple mutant	L46M-L103I		65
multiple mutant	N102Q-L103I		51
Asn L^{34} Met	N34M	V_L CDR1	2
Gly L^{91} Ala	G91A	V_L CDR3	24
Leu H^{103} Val	L103V	V_H CDR3	27
Leu H^{103} Met	L103M	V_H CDR3	14
	TGAscFv wild-type		7

[a] Protein amount determined by measuring the optical density at 280 nm; where 1mg/ml protein has an optical density of 1.35 at 280 nm
[b] CDR, complementarity determining region (Kabat *et al.*, 1991)
[c] FR, framework region (Kabat *et al.*, 1991)

A.3.4 ANALYSIS OF BINDING INTERACTIONS

The binding kinetics of the BGA scFv proteins were determined by surface plasmon resonance (SPR) using a BIACORE™ biosensor system (Biacore, Inc.). Two approaches were used to determine the K_D, a kinetic analysis and an equilibrium analysis. The kinetic analysis was favored because it not only generated highly reliable data, but also allowed for the determination of the kinetic constants (k_{on} and k_{off}) in addition to K_D. The analysis of the equilibrium binding only determined the K_D value of the interactions; this analysis method was used when a kinetic analysis could not be done.

A.3.4.1 Kinetic Binding Studies

The first approach used to determine K_D was to measure the k_{on} and k_{off} and to determine the K_D from their ratios, using equation (3) in section 8.1 (page 30). This kinetic approach to measure K_D was used whenever the k_{on} and k_{off} could be determined from the sensorgram. Figure 12A shows the observed sensorgrams of mutant L103I at five different protein concentrations overlaid with the corresponding calculated fitted curves. The fitted curves were calculated using a global analysis and a 1:1 binding model in which mass transfer effects were taken into account. Data analysis using a 1:1 model works very well (Figure 12), but this reaction is overall a 2:2 reaction. This bivalent interaction can be analyzed using a 1:1 model only if there is a high density antigen surface on the immobilized sensor chip. Visually we can make a good assessment of the deviations between the experimental and the fitted data, and this itself is a good indicator of the validity of the fitting, as seen in Figure 12. However, a residual plot of the difference between the observed and calculated curves shows the scatter of the data more precisely, as seen in Figure 12B. This residual plot indicates that the difference between the calculated and the observed is relatively small (± 10%) for these sensorgrams. Furthermore, the random scatter of these residuals indicates that the differences observed

are due to noise in the signal and are not due to an inappropriate model or to errors in the analysis. We are using a global analysis, and would normally expect higher residuals; however, the scatter of the residuals observed for this data set and for all the kinetic data sets observed indicates an excellent fit of the calculated curve onto the observed curve. Therefore, the calculated K_D generated from this kinetic global analysis yields very reliable values.

Table II lists the calculated K_D values for the mutant proteins that were amenable to kinetic analyses. The sensorgrams and residual plots collected for all mutants in Table II are not included because the analyses for all these listed mutants resemble that illustrated in Figure 12A and B. Table II also lists the per cent standard error associated with each kinetic constant; the relatively small error associated with these values further confirms that this kinetic global analysis yields reliable K_D values. Weak binders were not amenable to the calculation of k_{on} and k_{off} because of insufficient association and dissociation data and therefore the measurement of K_D required that equilibrium binding be achieved and analyzed.

Table II

Affinities and rate constants for TGAscFv mutants on BSA-A trisaccharide surface determined using global analysis with mass transfer and a 1:1 interaction model

Mutant Clone	k_{on}	k_{off}	K_D [a]
	$M^{-1} s^{-1}$	s^{-1}	M
L103V	4.82×10^{4} (± 1.1)[b]	0.0396 (± 1.1)	0.82×10^{-6} (± 1.6)
L103I	2.57×10^{4} (± 2.8)	0.0483 (± 2.6)	1.88×10^{-6} (± 3.8)
L103M	1.96×10^{4} (± 0.6)	0.0440 (± 0.3)	2.25×10^{-6} (± 0.7)
L46N-L103I	2.68×10^{4} (± 1.5)	0.0535 (± 0.6)	2.00×10^{-6} (± 1.6)
L46M-L103I	3.13×10^{4} (± 0.7)	0.0642 (± 0.4)	2.05×10^{-6} (± 0.8)
N102Q-L103I	3.08×10^{4} (± 0.5)	0.0381 (± 0.3)	1.24×10^{-6} (± 0.6)
L46N-N012Q-L103I	2.89×10^{4} (± 0.4)	0.0365 (± 0.2)	1.26×10^{-6} (± 0.5)
L46M-N102Q-L103I	2.43×10^{4} (± 1.0)	0.0434 (± 0.6)	1.79×10^{-6} (± 1.2)
N34M	0.04×10^{4} (± 2.5)	0.0122 (± 0.2)	30.5×10^{-6} (± 2.5)

[a] k_{off} / k_{on} The wild type $K_D = 26 \times 10^{-6}$ as determined using an equilibrium analysis.
[b] Numbers in parentheses are the standard error, expressed as a %

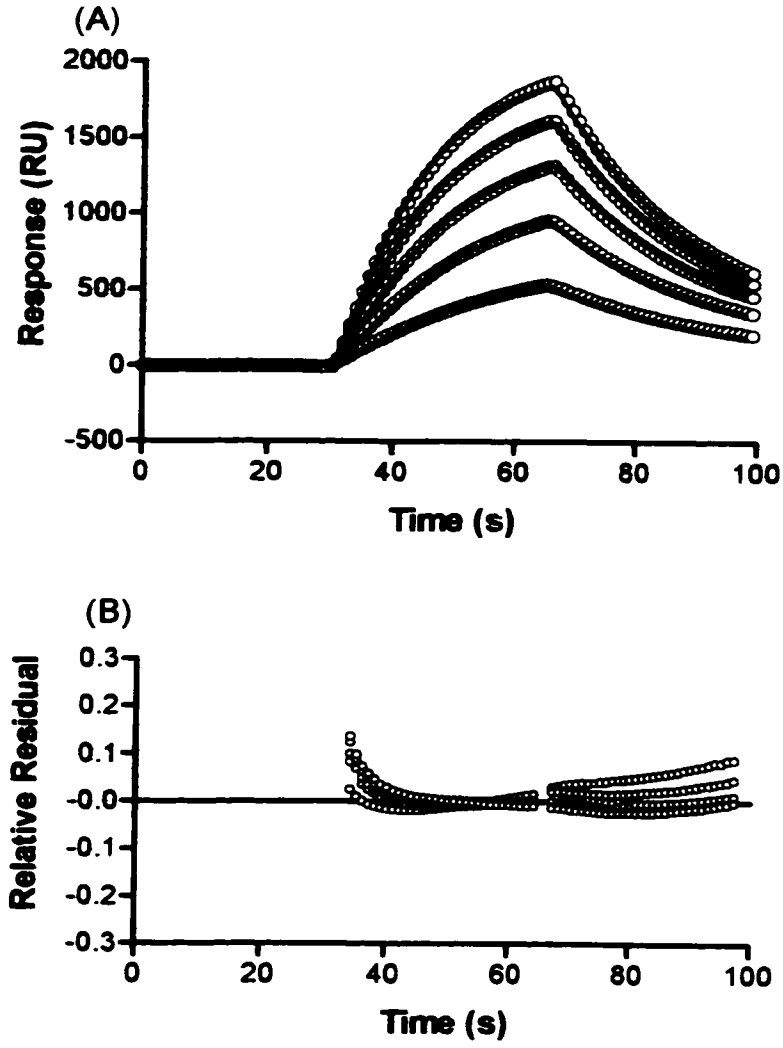


Figure 12: Global Analysis of TGAscFv mutant L103I using a 1:1 binding model with mass transfer. (A) Kinetic sensorgrams observed at protein concentrations of 0.3μM, 0.6μM, 0.9μM, 1.2μM and 1.5 μM. Overlaid on the observed data are the corresponding fitted curves for each concentration in blue. (B) Residual plots of the fitting indicate differences between the observed and the fitted data for each sensorgram.

A.3.4.2 Equilibrium Binding Studies

The analysis of the equilibrium binding to determine K_D was used for all mutants (Table III); it should be noted that for the weak binders, an equilibrium study was the only method that allowed for the determination of K_D. These poor binders have very rapid kinetics, and therefore are limited to an equilibrium analysis because the K_D can not be determined from the association and dissociation phases of their sensorgrams.

Figure 13 illustrates the sensorgrams recording the equilibrium binding of mutant Y50N to the active BSA-A trisaccharide surface (red) and the control BSA surface (black); the blue solid line is the difference between the active and control surfaces and represents the true binding, which is then analyzed.

The subtraction of the control surface from the active surface is necessary because the weak affinities of these proteins require that very high protein concentration be present in the buffer so equilibrium can be reached and analyzed. Furthermore, a high antigen density on the sensor surface is required because we are measuring a bivalent scFv interaction, and 2:2 binding will only occur on high density surfaces. Therefore, the subtraction of the control from active not only eliminates non-specific binding but also eliminates the bulk effects that may occur in an equilibrium analysis.

In this kinetic sensorgrams, equilibrium was not reached (Figure 12); however, there is a clear curve in the association and dissociation portions of the sensorgram from which k_{on} and k_{off} were determined. In the weak binding mutants we do not observe this 'curving' in the association and dissociation phases of the sensorgrams. Instead we observe a very rapid increase and decrease in the response levels (Figure 14 A). Table III lists the K_D values determined from equilibrium binding, whereas Table II lists the kinetic constants determined from a global analysis of the kinetic binding.

Table III

Dissociation constants of TGAscFv mutants to a BSA-A trisaccharide surface using a Scatchard analysis of the equilibrium binding on data collected a BIACORE™

Mutant Clone	K_D ($\times 10^{-6}$ M)
Y50N	185 (± 11) [a]
Q89E	ND [b]
G91S	Very weak (~3000) [c]
Y32W	ND
N34D	ND
Q89H	Very weak (~1000)
N102D	30 (± 19)
L103N	53 (±16)
W104H	ND
L46N	16 (± 7.5)
L46K	314 (± 51)
L46M	18 (± 5.5)
N102Q	22 (± 10)
N102E	51 (± 16)
L103I	1.49 (± 20)
L46N-N102Q-L103I	1.5 (± 20)
L46M-N102Q-L103I	1.38 (± 2.2)
L46N-L103I	2.77 (± 0.2
L46M-L103I	1.42 (± 21)
N102Q-L103I	1.23 (± 12)
N34M	47 (± 60)
G91A	ND
L103V	0.86 (± 10)
L103M	1.89 (± 10)
TGASL#4	26 (± 12)

[a] Uncertainties are given in parentheses as a % standard error
[b] ND, not determined
[c] Approximate K_D value given for a weak interaction

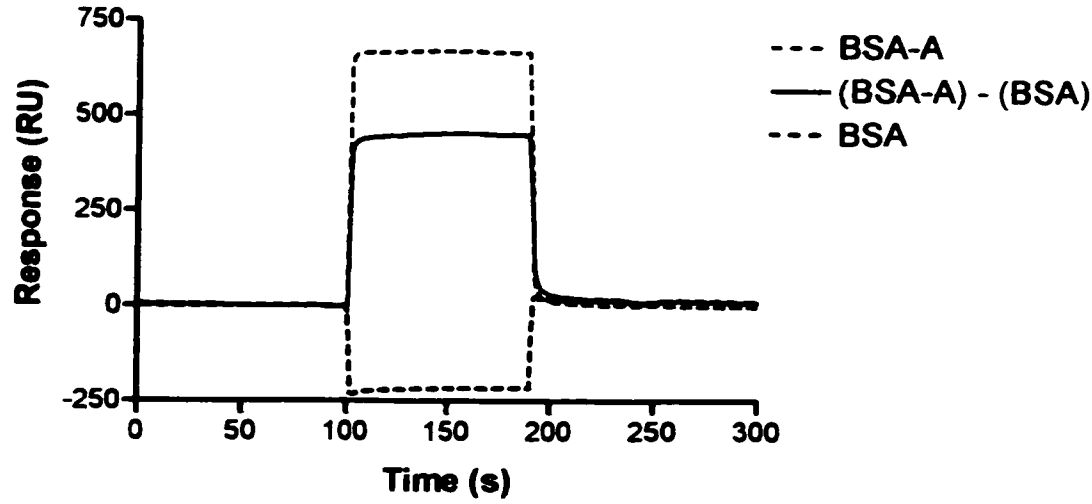


Figure 13: Equilibrium binding sensorgrams of TGAscFv Y50N mutant binding to BSA-A trisaccharide antigen active surface (red), BSA control surface (black), and the true binding (blue) which is determined from the difference between the active and control surfaces, i.e. true binding = (BSA-A active surface) - (BSA control surface).

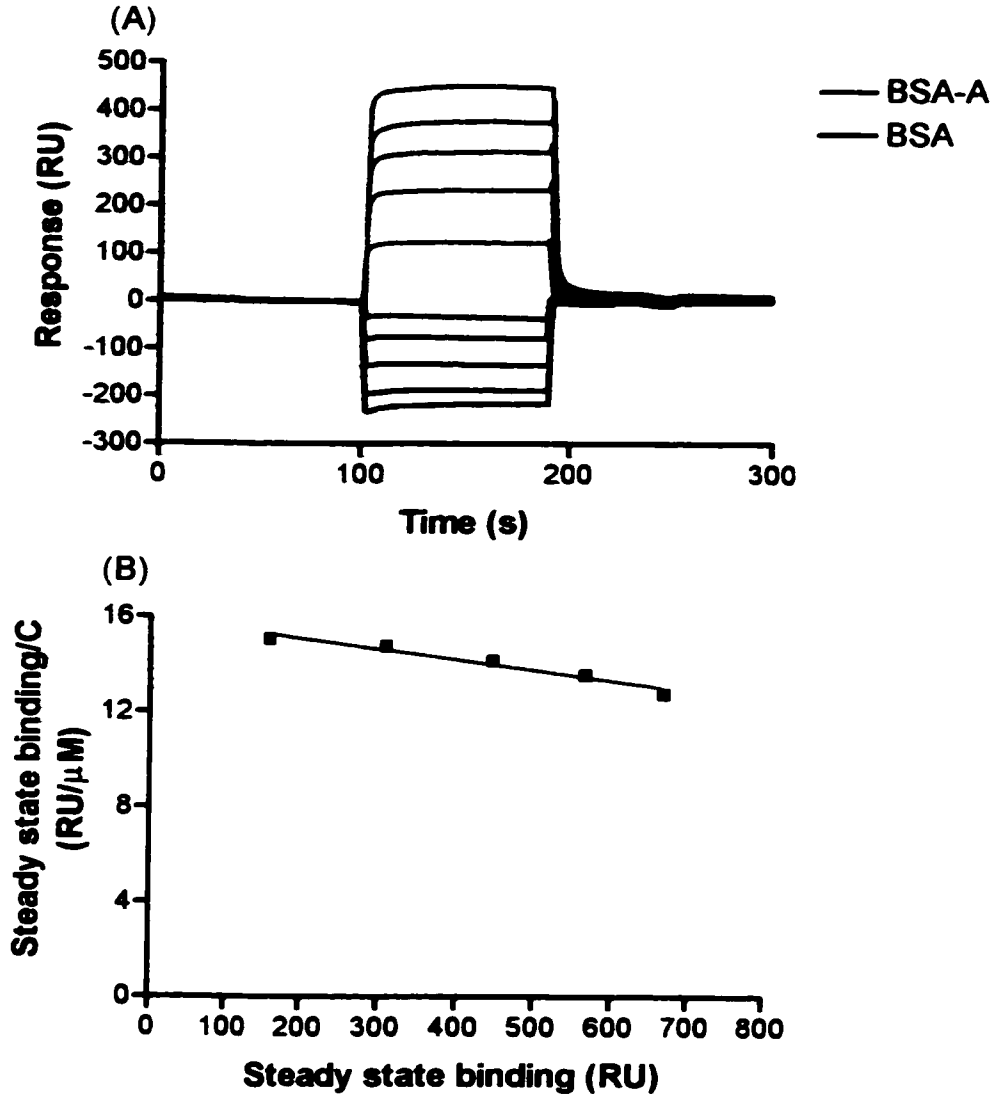


Figure 14: (A) Equilibrium sensorgram overlays of TGAscFv Y50N mutant binding to active BSA-A trisaccharide antigen surface (red) and BSA control surface (black) at concentrations of 10.4, 20.8, 31.2, 41.6 and 52 μM. (B) Scatchard plot of equilibrium binding data obtained by subtraction of control surface from active surface. K_D is -1/slope of the Scatchard.

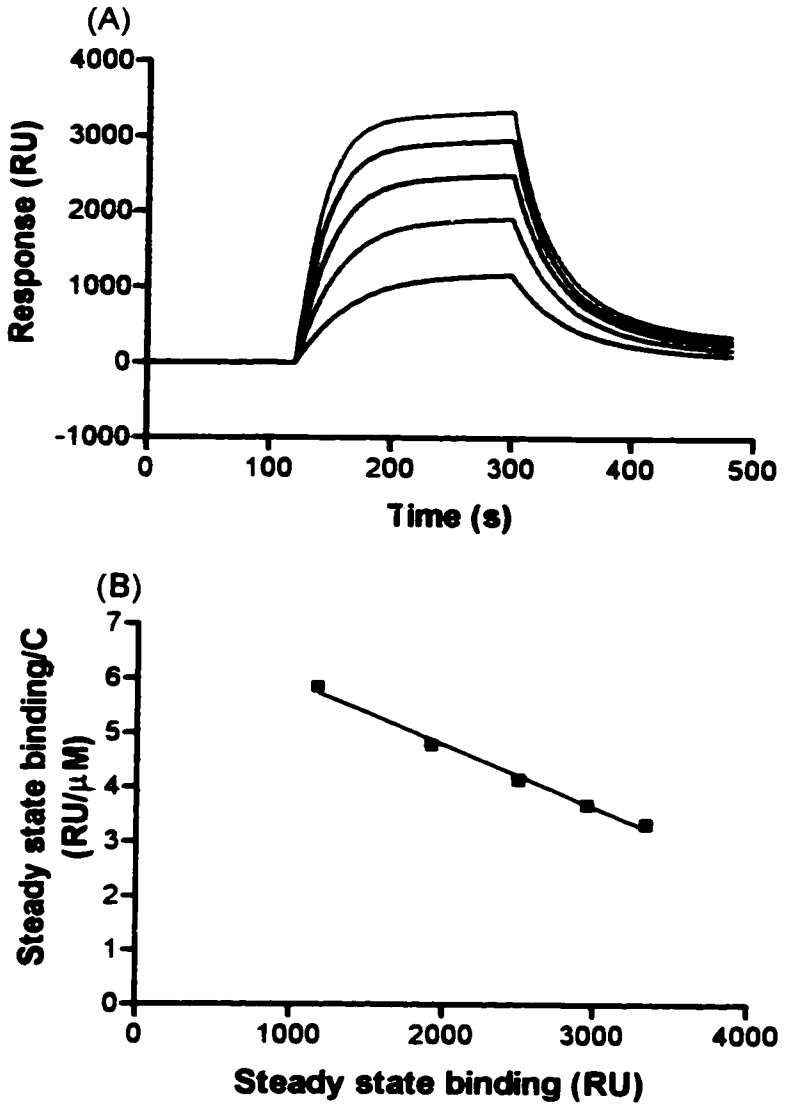


Figure 15: (A) Equilibrium sensorgram overlays of true TGAscFv L103V mutant binding to active BSA-A trisaccharide antigen surface at concentrations of 0.2, 0.4, 0.6, 0.8 and 1 μM. (B) Scatchard plot of equilibrium binding obtained from these subtracted sensorgrams. The K_D is -1/slope of the Scatchard.

To determine the K_D of the mutant proteins, equilibrium sensorgrams of the antibody at different concentrations were collected, and these sensorgrams were converted to a Scatchard plot from which the K_D was determined. Figure 14 A shows the sensorgrams of the mutant Y50N at different concentrations binding to the active BSA-A trisaccharide active surface (red lines) and the same Y50N concentrations binding to the BSA control surface (black lines). Figure 14 B shows the corresponding Scatchard plot, from which the K_D can be determined as –1/slope. The slope of this Scatchard was determined to be 1.85×10^{-4} M (±11%). Table III gives the K_D values determined using this equilibrium analysis.

Figure 15(A) shows the five sensorgrams for L103V mutant protein, and the corresponding Scatchard plot used to determine K_D. The association and dissociation phases of L103V (Figure 15) differ from the association and dissociation phases of Y50N (Figure 14). Y50N exhibits a rapid increase and a rapid decrease in the association and dissociation respectively, which is characteristic of a weak affinity. However, the sensorgram of L103V (Figure 15) reveals a progressive increase in the association phase and a slower dissociation phase, which accounts for a sharper sloping Scatchard and a smaller L103V K_D value ($K_D = 0.86 \times 10^{-6}$M). Table III lists the equilibrium K_D values determined for all the proteins examined. All equilibrium sensorgrams and Scatchards are not included in this section. Instead, two illustrative figures (Figures 14 and 15) of the equilibrium binding encountered by all the listed proteins, weak and strong binders, are shown. A comparison of the RUs at different protein concentrations further illustrates the differences in the binding affinity between the weak Y50N and strong L103V mutant proteins. In Figure 14 A, a Y50N protein concentration of 52 μM gave a response of ~490 RUs, whereas in Figure 15 A, a L103V protein concentration of 1 μM gave a response of ~3500 RUs. This observation of the sensorgrams alone can clearly indicate

that mutant L103V has a stronger interaction with the antigen surface than does mutant Y50N, since much less L103V is needed to yield a much greater response.

We can see from Table III that there is a wide range of K_D values; L103 V has K_D = 0.86 (±10%) μM, and L46K has K_D = 314 (±51%)μM. The reliability of the K_D determined and the error associated with it largely depends on the binding affinity of the mutant. For example, a very poor binder yields a shallow slope in the Scatchard plot, and therefore does not allow for the generation of reliable K_D value, as can be seen from the error associated with these K_D values. The K_D of some mutants (Q89E, Y32W, N34D and G91A) could not be determined because the Scatchard plots determined from the analysis of these mutants did not give a slope (ie. the slope was ~ 0, a flat line). Approximate K_D values were given for G91S and Q89H, but these values were determined from an almost flat slope, and thus are not very reliable. Hence, it is more accurate to say that these mutants have very weak binding. There is a range in the observed standard error associated with each K_D value; however, the K_D values were found to be reproducible in analyses of some of the mutants on different days using different antibody concentrations. The equilibrium binding analysis of the majority of the mutants was possible, and reproducible K_D values were determined; this method of analysis was used if a kinetic analysis was not possible.

Table IV

Comparison of the calculated K_D values determined from kinetic global analysis and from Scatchard analysis of equilibrium binding

Mutant Clone [a]	K_D [b]	K_D [c]
	M	*M*
L103V	0.82×10^{-6} (± 1.6)[d]	0.86×10^{-6} (± 10)
L103I	1.88×10^{-6} (± 3.8)	1.49×10^{-6} (± 20)
L103M	2.25×10^{-6} (± 0.7)	1.89×10^{-6} (± 10)
L46N-L103I	2.00×10^{-6} (± 1.6)	2.77×10^{-6} (± 7.2)
L46M-L103I	2.05×10^{-6} (± 0.8)	1.42×10^{-6} (± 21)
N102Q-L103I	1.24×10^{-6} (± 0.6)	1.23×10^{-6} (± 12)
L46N-N102Q-L103I	1.26×10^{-6} (± 0.5)	1.5×10^{-6} (± 20)
L46M-N102Q-L103I	1.79×10^{-6} (± 1.2)	1.38×10^{-6} (± 2.2)
N34M	30.5×10^{-6} (± 2.5)	47×10^{-6} (± 60)

[a] Mutant clones for which both equilibrium and kinetic data are available
[b] Determined using a global analysis with mass transfer (kinetic data)
[c] Determined using a Scatchard analysis of equilibrium binding
[d] Numbers in parentheses are the % standard error

Table IV compares the K_D values from the kinetic and equilibrium binding studies for the mutants amenable to both types of analysis. We can see that both methods gave very similar K_D values, indicating that the K_D values reported are reliable and are representative of the binding affinity of these mutants for the BSA-A trisaccharide antigen. The affinity of all the mutant proteins was also analyzed on a BSA-B trisaccharide antigen surface (data not shown); this was done to check for any alterations in the binding specificity. All mutants showed no detectable binding affinity for the BSA-B trisaccharide antigen surface, indicating that none of the specified mutations caused the binding specificity to change from A to B antigen binding.

A.4 DISCUSSION

Increasing the binding affinity of the anti-carbohydrate BGA antibody for its carbohydrate antigen was attempted using a rational design approach that examined the binding pocket of the AC1001 BGA Fv using a high-resolution X-ray crystallographic structure (Figure 16, 17, and 18). The A-trisaccharide antigen was modeled into the binding pocket of the antigen, and specific candidate residues were selected for site-directed mutagenesis. The X-ray crystallographic data of the 2.2Å high resolution AC1001 Fv structure is described elsewhere (Patenaude *et al.*, 1999). The features of this crystal model, and the amino acid residues selected for site directed mutagenesis will be described in crystallographic detail (Thomas *et al.*, 1999), but are explained in this thesis whenever necessary.

The BGA Fv antigen binding pocket contains a cleft, or deep pocket (11.1 Å deep, and 9.4 Å wide) and opens up 4.7 Å at its surface and 2.8 Å at its base. This binding pocket is of sufficient size to accommodate the A antigen, and the trisaccharide antigen was modelled into the binding pocket with a good fit. This antigen binding pocket is lined with 12 amino acid residues: Tyr^{L32}, Asn^{L34}, Tyr^{L36}, Leu^{L46}, His^{L49}, Tyr^{L50}, Gln^{L89}, Trp^{L96}, Asn^{H102}, Leu^{H103}, Trp^{H104}, Phe^{H105}. The anti-BGA antigen binding pocket is predominately made up of aromatic amino acids, similar to other anti-carbohydrate binding proteins (Quiocho, 1991; Brummel *et al.*, 1993). This binding pocket is mainly hydrophobic, but there are hydrophilic residues present that seem to help mediate antigen binding (Figure 16). For example, Gln^{L89} and Asn^{L34} directly interact with the NAc group of the GalNAc sugar residue. The residue that seemed to greatly affect binding affinity is $LeuH^{103}$; however, this residue does not seem to interact with the antigen. $LeuH^{103}$ is located in the lower portion of the antigen binding pocket, and does not seem to neighbour the GalNAc sugar residue. The GalNAc bordering residues seem to be Gln^{L89}, Asn^{L34}, and Tyr^{L32}. The mutations of these residues eliminated the binding ability

of the BGA scFv for binding the A- antigen (Table III); therefore, indicating the importance in these residues for maintaining antigen binding ability. Figure 16 is a stereodiagram of the antigen binding pocket residues with the modelled A antigen. It should be noted that the crystal structure determined was of the BGA Fv antibody, and not of the antibody-antigen complex. The A antigen was consequently modelled into the antigen binding pocket so that we could focus on the interactions of the antibody with the antigen, therefore the antibody interactions with the A antigen are putative.

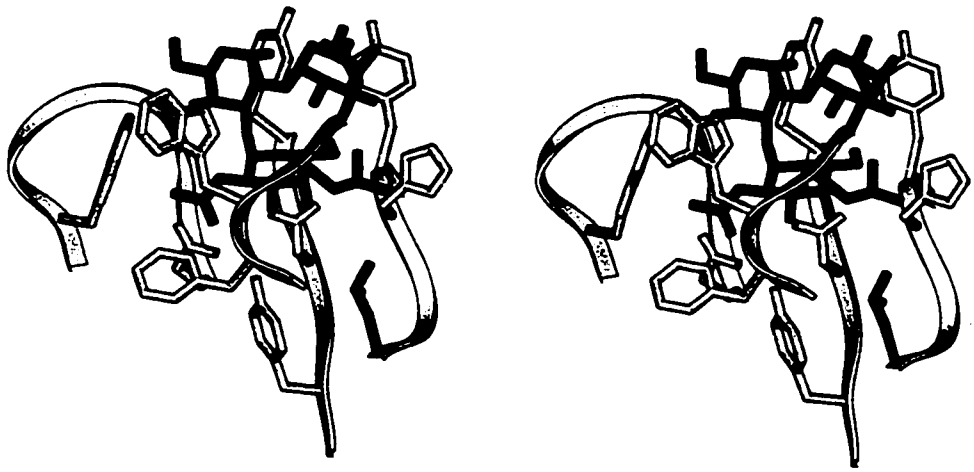


Figure 16: Stereodiagram of BGA Fv with modelled A antigen. Shown are the 12 amino acid residues lining the antigen binding pocket. Residues Asn^{H102}, Leu^{H103} and Leu^{L46} are highlighted in green. The A antigen trisaccharide is in red.

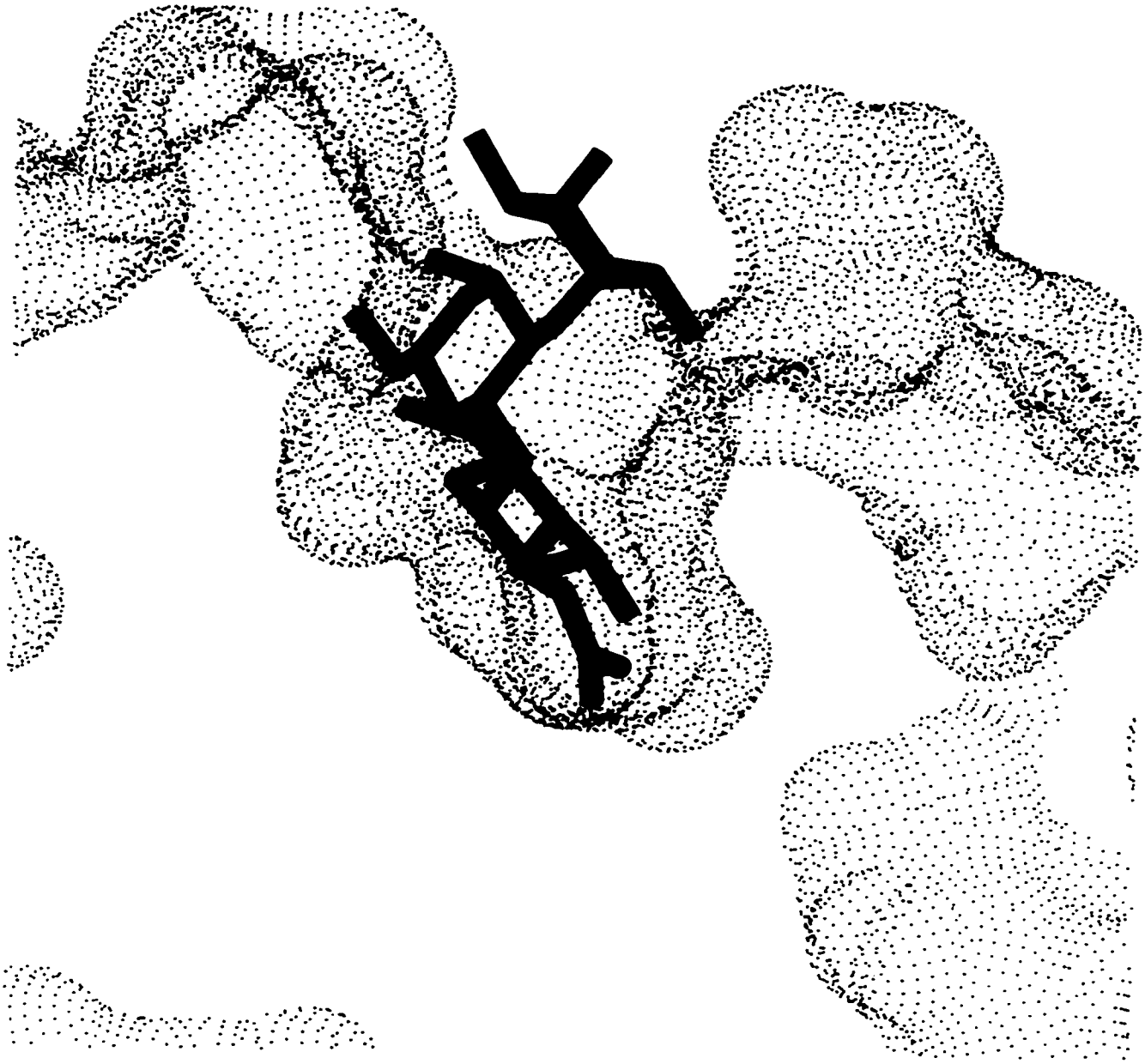


Figure 17: Three dimensional BGA Fv molecular surface at the binding pocket with the bound A trisaccharide antigen (red). This diagram was generated using SETOR programming package (Evans, 1993).

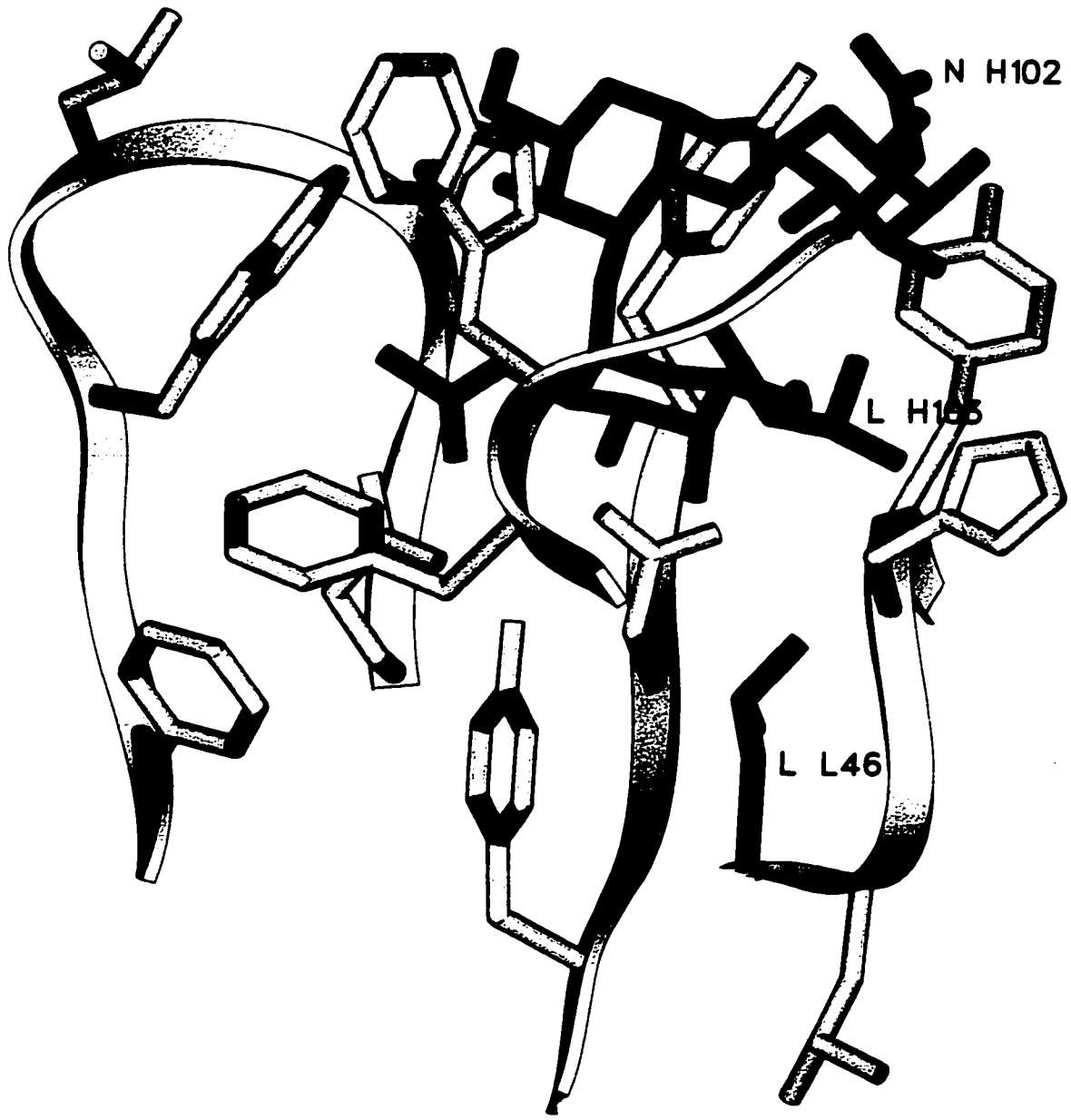


Figure 18: BGA scFv binding pocket with A-antigen (red). Protein backbone is shown as a ribbon, and only the amino acid residues lining the binding pocket are shown. Asn ^{H102}, Leu ^{H103} and Leu ^{L46} are highlighted in green. This diagram was generated using SETOR programming package (Evans, 1993)

An important study that examined the X-ray crystal structure of a germline antibody and its corresponding mature antibody (crystal of Fabs and Fab-hapten complexes determined) gives important structural insights into the evolution of an antigen combining site (Wedemayer *et al.*, 1997). A comparison of the germline and the corresponding affinity-matured antibody indicate 9 somatic mutations in the mature antibody that resulted in a 30 000-fold increase in affinity. Furthermore, significant changes in the conformation of the combining site occur upon hapten binding, whereas binding in the mature antibody proceeds *via* a 'lock-and-key' fit mechanism. Although conformational changes have been observed for other affinity matured antibodies (Wilson and Stanfield, 1994), what is important in the Wedemayer *et al.* (1997) report is that structural changes that occur in the germline-hapten complex became preorganized in the combining site of the mature antibody. This study suggested that the 30 000-fold increase in affinity may be due to a variety of factors, however they have suggested that entropic restrictions, and many small additive changes may have contributed to this significantly large increase in affinity (Wedemayer *et al.*, 1997). The crystal of the BGA Fv antigen complex has not yet been determined, and thus we can not resolve the changes that may occur upon antigen binding, if any structural changes do occur at all.

Mimicking somatic mutation using a rational design approach to improve binding affinity is difficult and often results in the production of proteins with decreased binding affinities (Lavoie *et al.*, 1992; Komissarov *et al.*, 1997; Korbin *et al.*, 1991). However, some groups have had some success in improving the binding affinity of various interactions (Xiang *et al.*, 1993; Vermersch *et al.*, 1990 and 1991). Xiang used rational design to improve the binding affinity of a protein-protein interaction 2-fold with a single point mutation (Xiang *et al.*, 1993). Others have improved the binding affinity of protein-carbohydrate interactions, but the improved mutants contained multiple mutations

(Thompson *et al.*, 1996; Jeffery *et al.*, 1995). And there have been reports of two different single point mutations in a lectin-carbohydrate interaction that increased affinity 20-fold, but altered specificity, there was no improvement in the mutant lectin for its wild type carbohydrate (Vermersch *et al.*, 1990 and 1991). None of these groups, however, have significantly improved the binding affinity of a protein-carbohydrate interaction with a single point mutation, while still maintaining binding specificity.

The binding affinity of the site-directed mutants was determined using equilibrium and kinetic binding methods. The kinetic data for the L103I mutant (Figure 12) shows that the fitted curve is almost superimposed with the observed data, therefore indicating very accurate analysis of the binding, and hence very reliable kinetic data. All kinetic data was determined by global analysis; only high quality data can be analyzed using a global analysis, and these kinetic experiments yield highly reliable global rate constants with low residuals. Table II lists the kinetic data of the BGA scFv binding to the A-antigen surface. We can see that mutant L103V binds the A-antigen with a 30-fold increase in binding affinity ($K_D = 26 \times 10^{-6}$ compared to 0.82×10^{-6} M). This increase in the binding affinity due to a single amino acid mutation is exceptional for an anti-carbohydrate antibody. There were other mutations that produced improvements in the binding affinity; Table V gives the increases in affinity with respect to the TGASL#4 wild type scFv. The reason for these improvements can not be ascertained from simply observing the binding site. However, improvements in binding affinity could be due to improved interactions, an increase or decrease in the surface bulk, or an increase in complementarity of the binding, or due to entropic changes further discussed below.

From the binding analysis, we observe that the increase in affinity can be attributed to the decrease in k_{off}. If we compare the binding of mutant Y50N (Figure 14) to the binding of L103V (Figure 15) we can see that the dissociation phase is slower in the better binding L103V mutant. According to Equation (3), a decrease in the k_{off} will

result in a decrease in the K_D, therefore indicating an increase in binding affinity. Due to their low binding affinities, anti-carbohydrate antibodies have not been extensively studied kinetically. However, mutants that have shown improvements in binding affinity, usually show decreases in k_{off} (MacKenzie *et al.*, 1996).

Table V lists the mutations that improved the binding affinity of the BGA scFv for the A-antigen. The L103I residue was the first mutant in which such a significant increase in affinity was observed. This mutant was then combined with other single mutations that exhibited an increased binding affinity, and some of these double mutants showed a further increase in affinity. H^{103} was also mutated to a Valine and a Methionine, both of which resemble Leucine. It is interesting to note that the L103V mutation resulted in a 30-fold increase in bivalent binding affinity, which is remarkable for a single point mutation, and especially remarkable for an anti-carbohydrate antibody. In addition to being a single point mutation, the Leu→Val mutation is a conservative amino acid substitution.

Table V

Mutations that exhibit an increased affinity with respect to the TGAscFv wild type

Mutant Clone [a]	Changes in affinity[b]
L46N	1.6[c]
L46M	1.4
N102Q	1.2
L103I	17.5
L46N-L103I	9.3
L46M-L103I	18.3
N102Q-L103I	21.1
L46N-N102Q-L103I	17.3
L46M-N102Q-L103I	18.8
L103M	13.8
L103V	30.2
TGAscFv wild type	1

[a] Mutant clones which exhibit an increase in binding affinity
[b] Comparison of equilibrium data (Table III)
[c] Determined by dividing with the TGAscFv wild type affinity (26 μM) For example L46N 26 / 16 = 1.6-fold increase in affinity.

The binding pocket was examined, and residues located within this cleft were selected for mutation based on: their proximity to the modelled antigen, the extent of their binding interactions with the modelled antigen, the potential of better accommodating the antigen within the binding pocket. Figure 17 and Figure 18 show the side view of the BGA binding pocket with the A-trisaccharide fitted into it. The analysis of this binding region, using various 3-dimensional images, was used to determine the candidate residues for site-directed mutagenesis. For example, a binding pocket residue that was in close proximity to the antigen, but did not seem to contribute to its binding

would be a candidate residue; and it would be mutated to an amino acid that could potentially increase the binding interactions (Deng *et al.*, 1994).

Table V lists the mutations that improved binding affinity, and how they compare with the wild type TGASL#4 scFv. Amino acid 103 in the CDRH3 was initially mutated Leu→ Ile, and resulted in a 17-fold increase in the BGA scFv's binding affinity. There were some residues that moderately improved the binding affinity of the scFv (L46N, L46K, and N102Q). Multiple mutations that combined the mutations of better binders in some cases showed further increases in binding affinity; L103I-N102Q yielded a 21-fold increase in affinity. Residue H^{103} was further mutated, and mutations Leu→ Met and Leu→ Val again showed increased binding affinity. It should be noted that all mutations bound only to the A-antigen, and none of the mutant scFv's were found to bind the B-antigen at detectable levels. Remarkably the Leu→ Val mutation at position H^{103} in CDR H3 resulted in a 30-fold increase in affinity.

The Leu^{H103} residue is a CDR H3 residue located at the lower portion of the binding pocket and does not seem to interact directly with the antigen. The increase in affinity for the Leu→ Val mutation can be a result of a reduction of the surface bulk in this region, since this decrease in the surface could possibly allow better accommodation of the antigen into the binding pocket. Alternatively, the Leu→ Val mutation may have indirectly affected other residues, thereby inducing the formation of other bonds that could potentially have increased the binding. A wide range of different changes can elicit binding to the antigen with increased affinity. An improved affinity could result from a mutant's ability to accommodate the antigen more easily, by reducing bulk in the surface of a particular region (Deng *et al.*, 1994) or by increasing surface complementarity. Mutations can be indirectly causing an increase in affinity, and the indirect influence of non-contact residues on antigen binding has been reported for several antibodies, with

these synergistic and long range effects being a common occurrence (Sharon 1990; Lavoie *et al.*, 1992).

Another explanation for the increase in affinity could be that the L103I and L103V mutations result in a lower entropic penalty upon antigen binding. If we consider the amino acids Leu, Ile, and Val, we can see that these neutral hydrophobic residues are very similar. However, these residues vary in the number of methyl groups and in the positioning of these groups. These differences confer each of these residues with a different disorder or entropy.

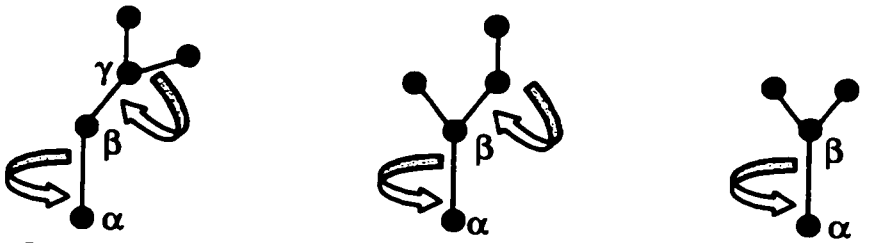


Figure 19: Schematic representation of H^{103} wild type (Leucine) and mutant amino acids. The red ball represents a carbon, or methyl groups in the amino acid residues. The branching of residue is indicated by α, β, and γ branches. Leucine contains a γ branch. Isoleucine does not have a γ branch; there is γ carbon, however there is no branching, it contains a β branch. Valine only has a β branch. Arrows indicate the freedom of movement about the bonds of these residues.

We can see that Val has the lowest entropy, since its possesses the least amount of movement about its carbon backbone. Leucine has a γ branch, isoleucine also has a β branch, and valine has a β branch. Leucine has potentially a higher conformational entropy than isoleucine and valine, because leucine has potentially more movement about its β and γ branches. Isoleucine contains a γ carbon, however there is no branching at the γ carbon, and as a result there is less bulk movement, and therefore lower entropy. Valine has the lowest entropy since it only has movement about its β branch. Therefore, the Val

residue has the conformational lower entropy, since it has the least freedom of movement, and Leu has the higher conformational entropy since it possesses more freedom of movement.

Amino acid residues that contain a high freedom of movement, or disorder have a high entropy. Whereas, residues that exhibit less movement, and as a result, less disorder, possess a lower entropy. Therefore, we can say that leucine has a higher entropy than valine, and as a result leucine is favourable to entropy. The immobilization of an amino acid residue with a higher entropy (i.e. Leu) will produce an unfavourable decrease in entropy and will result in a large entropic penalty. Conversely, amino acid residues that possess a lower entropy (i.e. Val) will yield a smaller entropic penalty upon immobilization. The Leu → Val mutation can effectively lowered the entropy of the H^{103} amino acid residue, thereby resulting in a smaller entropic penalty upon antigen binding. Since leucine has increased entropy, it will not favour antigen binding, because antigen binding would result in a decrease in disorder, and in a large entropic penalty. However, valine is favourable to antigen binding because it results in a lower entropic penalty upon antigen binding.

The ability of a conservative single point amino acid mutation to increase binding affinity 30-fold can not be explained solely from an X-ray crystallographic examination of the binding pocket. The complete characterization of a protein-carbohydrate interaction should not only include X-ray studies, but should also include a detailed thermodynamic characterization of the binding. Many thermodynamic studies have shown that most saccharide-protein interactions are accompanied by a decrease in entropy. However, the origin of this decrease in entropy has been argued, and has been suggested to be due to a loss of saccharide flexibility (Carver *et al.*, 1991), or to a reorganization of water molecules within the binding pocket (Lemieux, 1989; Lemieux *et al.*, 1991). The increase in binding affinity observed for some of our mutant scFv

proteins can not be conclusively attributed to a decrease in the entropic penalty, or due to an increase in the surface complementarity. Therefore, the precise cause of the observed increase in affinity requires further detailed analysis of this binding interaction.

It should be noted that many of the mutations that improved binding affinity were located within the CDR3 of the V_H chain (L103I, L103V, and N102Q). Functionally, the CDR H3 plays a distinct role in determining antibody specificity (Chothia and Lesk, 1987; Chothia *et al.*, 1989; Kabat and Wu, 1991). Most structural and sequence diversity is found within the CDR H3 loop, which is the most central loop of the antigen combining site. The other five CDRs seem to have limited variation (Chothia *et al.*, 1989). The CDR H3 varies most extensively in length, and can range from 2-26 amino acid residues (Kabat *et al.*, 1991), and also varies greatly in amino acid sequence. The CDR H3 of the BGA scFv is 9 amino acids long. It is interesting that the mutant that improved binding affinity 30-fold is located within this highly significant region. However, the lack of influence of the CDR H3 region of an anti-carbohydrate antibody Se155-4 is reported by Brummel *et al.* (1993); an exhaustive study of the CDR H3 region of this anti-carbohydrate antibody was unable to increase the binding affinity. However there have been many studies that indicate the importance of this highly diverse region (Chothia and Lesk, 1987; Chothia *et al.*, 1989; Kabat and Wu, 1991). Mutations to the CDR H3 region greatly effected binding in this study. The L103V bound specifically to the A-antigen with a 30-fold increase; however it did not bind the B-antigen, illustrating the importance of the CDR H3 in its ability to confer specificity to an antibody.

In addition to being better binders these mutants also had improved protein yield in *E. coli* (Table I). The wild type BGA scFv yields approximately 7 mg/l of protein, whereas mutant L46M-L103I yields approximately 65 mg/l of protein. This 10-fold increase is a very significant improvement in protein production. Higher yields were also observed for a number of other proteins, many of which contained the L103I mutation.

Moreover, it is interesting that a conservative amino acid mutation, L103I, resulted in a 3-4-fold increase in protein production.

It has been shown that single mutations can have a significant effect on the yield of active protein product (Brummell *et al.*, 1993; McManus and Reichmann, 1991), the molecular basis of which is unknown. Some mutations may result in improved folding or in better interactions between the two scFv fragments, and would in turn lead to improved production levels in *E. coli*. For example, the replacement of an Ile with an Asn or a Thr (in FR3 of V_H) resulted in an approximate 10-fold increase in protein yield, and was suggested to be due to an improvement in folding due to introduction of nonhydrophobic residues (Deng *et al.*, 1994). Moreover, some mutations may cause an overall stabilization in the scFv structure, and may thereby result in an improvement in folding and a subsequent increase in protein yields. Low expression levels have also been shown to result from secondary mRNA structure (Stemmer *et al.*, 1993), or from the domain order of variable fragment constructs i.e. V_L-V_H or V_H-V_L (Anand *et al.*, 1991b). It has also been shown that the production of functional anti-carbohydrate Fab fragments, which is often limited by V_L chain availability, can be significantly increased by the replacement of a V_L chain's C_κ domain with a $C_{\lambda 1}$ domain (MacKenzie *et al.*, 1994). This was shown for the AC1001 BGA Fab and a related anti-carbohydrate Fab, in which the V_L chain availability for both proteins was low.

A.4.1 FUTURE EXPERIMENTS

Further examination of the BGA Fv high resolution crystal with the modelled antigen, and the selection of other site-directed mutants in an attempt to further increase the antigen binding affinity for the A antigen can be continued. For example, if the increase in binding affinity for the L103V mutant is indeed driven by a reduction in the entropic penalty, it would be interesting to see if a Leu→Ala mutation could further increase binding affinity. It would also be interesting to carry out a site-directed mutagenesis study aimed at altering binding specificity from the A to the B carbohydrate antigen. Such a study could help to better understand how the anti-blood group antibodies discriminate between these highly similar antigens.

Co-crystallization of the BGA Fv with the A antigen and the determination of a high resolution crystal structure of this complex is also an important future experiment. A crystal structure of the complex would indicate the definite location of the antigen within the binding site, and would greatly simplify future site-directed mutagenesis studies. A comparison of the antigen binding pocket with and without bound antigen would elucidate any structural changes that may occur upon antigen binding, if any structural changes do occur. And finally, a complete characterization of the protein-carbohydrate interaction should include a detailed thermodynamic study.

CHAPTER THREE

STUDY B
BACTERIOPHAGE DISPLAY STUDY OF BGA ScFv

B.1 INTRODUCTION

B.1.1 OBJECTIVE

The main objective of this study was to construct a mutant BGA scFv-gIII phagemid library using error-prone PCR, and to screen this mutant library using various panning techniques for a BGA scFv clone that exhibits an improved binding affinity for the A-antigen. Contrary to the rational design of mutants in Part A, this study generates random mutants; however, both approaches aim to produce or isolate a BGA scFv with an improved affinity for the A antigen.

Phage display is a very powerful technique that allows for the display of fusion proteins on the surface of bacteriophage (Smith, 1985). Analogous to the immune system's B cells, which express antibody on their cell surface, phage display expresses antibody on the surface of phage and the phage contains the genes encoding the antibody within. Moreover, similar to the immune system's ability to produce antibodies with increased affinity through affinity maturation and somatic mutation, phage display can also produce antibodies with increased affinity through mutant libraries and affinity selection. The objective of this work is therefore to mimic the *in vivo* process of affinity maturation and somatic mutation *in vitro*, and to ultimately isolate a BGA scFv mutant with an increased affinity.

B.1.2 APPROACH

In tandem with the site-directed mutagenesis study of Part A, a phage display study was carried out as an alternative to the rational design of mutant BGA scFv proteins using X-ray crystal structure analysis and site-directed mutagenesis. In this study we have displayed a synthetic BGA scFv V_L-V_H PCR mutagenized library on the surface of phage. These synthetically produced BGA V_L and V_H genes (MacKenzie *et al.*, 1994) were based on the anti-BGA IgM hybridoma AC1001 (Chen *et al.*, 1987). The mutations

produced throughout the BGA scFv V_L and V_H genes were generated using error-prone PCR (EP-PCR). These randomly mutated genes were then displayed on the surface of phage through fusion to the pIII minor coat protein gene (pIII-scFv fusions). This library is therefore referred to as the EP-TGASL#4 phagemid library, and was panned using a variety of techniques. This study will examine these various panning methods, and will outline how these experiments led to the development of a novel panning process that can be used to pan low affinity anti-carbohydrate libraries.

The success of a phage display study largely depends on the composition and diversity of the gene library (Hoogenboom *et al.*, 1992). The initial phase of this study required the construction of a gIII phagemid library of BGA scFv mutant proteins. The EP-TGASL#4 pIII-scFv phagemid library was determined to be of adequate size (10^6 clones), and therefore includes many scFv clones of varying binding affinities. The constructed phagemid library was screened for complete scFv mutant proteins; this was done to ensure that the library to be screened contained a repertoire of complete, intact scFv mutant clones.

Equally important to the construction of the library repertoire, is the selection strategy used to select phage. Three different panning strategies have been used to select for higher affinity clones; panning in microtitre wells, panning on the BIACORE instrument, and panning on a Synsorb-A column. It should be noted that panning an anti-carbohydrate library can be challenging, particularly a low affinity anti-carbohydrate antibody library. The EP-TGASL#4 phagemid library was first panned in microtitre wells. This panning process did not select for BGA scFv clones with an improved binding affinity; instead the selected clones seemed to be predominantly deletion products, incomplete scFv fragments. It was initially thought that these deletion products could have been selected due to non-specific binding onto the plastic microtitre well surface.

The second panning approach was the use of the BIACORE instrument to affinity separate the EP-TGASL#4 library repertoire. The dynamic separation of the library into populations of varying binding affinities on the BIACORE is possible (Malmborg and Borrebaeck, 1995); however, successful BIACORE pannings have used high affinity protein-protein interactions (Malmborg *et al.*, 1996). There have been no reports of a low affinity anti-carbohydrate library being successfully panned on a BIACORE instrument. Panning on the BIACORE would essentially separate clones based on their dissociation rate constants, and the dissociating phage would then be collected as fractions. Ideally these fractions should contain phage populations of varying binding affinities, with the later dissociating fractions containing clones with lower dissociation rate constants and thus greater affinity. Four rounds of panning were completed on the BIACORE, but this initial attempt at panning our low affinity anti-carbohydrate scFv library did not select for clones with improved affinities. Similar to the microtitre well panning process, panning on the BIACORE also seemed to yield a predominance of deletion clones.

The predominance of deletion products in these panning methods necessitated the pursuit of another panning process. Panning has been carried out on column matrices (McCafferty *el al.*, 1990), in immunotubes (Pini *et al.*, 1997), and on hapten affinity columns (Clackson *et al.*, 1991). Synsorb-A beads are diatomaceous earth with covalently linked A-antigen displayed on the bead surface. A Synsorb-A column has never been used to pan a phage displayed library, however we have constructed a Synsorb-A column, and have developed a panning methodology that can be used to potentially pan a low affinity anti-carbohydrate library. Preliminary trials on this column were successful, and indicated that the Synsorb-A column could effectively separate sample based on the transient binding of the sample components as they percolate through the column. Four rounds of panning were completed on the Synsorb-A column, and the deletion products that seemed to disturb the previous panning processes did not

interfere with this panning method. Furthermore, improvements to the currently developed methodology have been initiated with success.

B.1.3 RELATED RESEARCH

Since George Smith first displayed a foreign peptide sequence on the surface of bacteriophage (Smith, 1985), *in vitro* selection technologies that generate ligands, peptides and antibodies have been transformed. Since the literature surrounding phage display is great, this section will only focus on the phage display of antibodies, and will only briefly mention other relevant technologies.

The display of antibodies on the surface of phage (McCafferty *et al.*, 1990) has led to some important advances in the study of antibodies, and protein-ligand interactions. One of the most successful applications of phage display technology is the isolation of monoclonal antibodies from large phage antibody libraries (Winter *et al.*, 1994). Furthermore, the construction of large libraries of antibodies and antibody fragments, and the use of clever selection procedures have allowed for the isolation of antibodies with specific binding affinities (Marks *et al.*, 1991) and for the selection of antibodies with improved binding affinities (Hoogenboom 1997) from these phage displayed antibody libraries.

B.1.3.1 Phage Display of Antibody Libraries

The *in vivo* affinity maturation process (Berek and Milstein, 1987) has been mimicked *in vitro* by Marks *et al.* (1991), by generating and isolating antibodies against various antigens. They prepared a diverse library of V_H, V_κ, and V_λ genes from unimmunized donors, and constructed a diverse repertoire (10^7 members) of scFvs with various binding affinities. This library was panned against three different antigens, and after four rounds of panning, the selected clones were analyzed. Some contained various

mutations, and some bound specifically to a particular antigen. This important experiment showed that the immune system can be mimicked, and that phage display can allow for affinity maturation of an antibody repertoire, and more importantly, that it has an antigen driven ability to select for antibodies to different target antigens.

Antibody repertoires can be produced naturally or synthetically. Natural repertoires can be produced from immunized and from non-immunized donors. Both theses libraries are made essentially in the same way, using the B cells from diverse lymphoid sources, peripheral blood (Marks *et al.*, 1991), bone marrow and tonsils (Vaughan *et al.*, 1996), with the only difference being the prior use of an immunogen. Vaughan *et al.* (1996) have constructed a large (10^{10} clones) primary scFv antibody repertoire from the V genes of 43 non-immunized human donors, and have selected from this library high affinity antibodies to multiple antigens. Generally the affinity of antibodies selected from these primary libraries is proportional to the size of the library; a library of 10^7 clones will have affinities ranging from 10^6 - 10^7 M^{-1} (K_D 10^{-7} - 10^{-6}) (Griffiths *et al.*, 1993), whereas a very large library of 10^{10} clones will range in affinities from 10^8 - 10^{10} M^{-1} (Vaughan *et al.*, 1996).

Antibody repertoires can also be produced synthetically by an *in vitro* assembly of V-genes and D-J segments (Hoogenboom and Winter, 1992). Antibody repertoires produced synthetically are generally larger, and have more diversity. However, expression levels and *E. coli* toxicity may reduce the functional repertoire size (Griffiths and Duncan, 1998). Synthetic libraries often use the germline V genes to encode the diversity of the repertoire. The construction of these diverse synthetic repertoires is greatly facilitated by a database that lists the sequences of all the human germline V_H, V_κ, and V_λ genes and the D and J segments (Tomlinson, 1998).

Mutations in the synthetic V gene libraries can be produced by chemical mutagenesis and EP-PCR (Deng *et al.*, 1995), spiked primers (Reichmann and Weill,

1993), and other mutagenesis approaches (Glaser *et al.*, 1992) have been reported. Using these various methods, diversity can be introduced throughout the entire gene (Deng *et al.*, 1994), or diversity can be introduced in specific regions of the gene (Barbas *et al.*, 1992). The disadvantage of uncontrolled mutations throughout the entire gene sequence is that it can potentially alter residues that may confer stability to the antibody structure. However mutations can be introduced at relatively low frequencies by EP-PCR or chemical modifications, which do not induce broad structural changes, and maintain the antigen binding ability of the antibody (Deng *et al.*, 1994). Targeting diversity to specific regions has been very successful and has been done by a number of groups. Barbas *et al.* (1991) constructed a semisynthetic Fab library by replacing the V_H CDR3 gene sequence with randomly synthesized oligonucleotides of the same length. Deng *et al.* (1993) randomized the V_H CDR regions, and have simultaneously randomized all six V_H and V_L CDRs (Deng *et al.*, 1995) to create mutant scFv libraries.

From these synthetic repertoires, many antibodies to a large variety of antigens can be isolated (Hoogenboom and Winter, 1992). The largest synthetic library made to date is a 6×10^{10} clone repertoire of Fab fragments displayed on the surface of phage (Griffiths *et al.*, 1994). This library has allowed the selection of antibodies against numerous antigens, but it is difficult to re-propagate without a significant loss in diversity, and a more stable 1×10^9 scFv phagemid library has been equally effective (Hoogenboom *et al.*, 1998).

There have been many successes in the phage display of antibodies against protein epitopes. However, panning an anti-carbohydrate antibody library is difficult because of the characteristically low binding affinity of these antibodies. Nevertheless, there has been some success in the phage display of anti-carbohydrate antibodies. A mutant *Salmonella* scFv clone with a 10-fold increase in affinity has been isolated from an anti-carbohydrate scFv phage displayed library (Deng *et al.*, 1994), and mutations that

cause the dimerization of anti-carbohydrate scFvs, thereby producing an increase in the functional affinity, have been reported (Deng *et al.* 1995).

Antibodies have been displayed on phage in various formats, as Fab fragments (Garrard *et al.*, 1991; Hoogenboom *et al.*, 1991), monomeric scFv fragments (McCafferty *et al.*, 1991), dimeric scFv's or diabodies, and multimeric molecules (Holliger *et al.*, 1993). We have displayed our EP-TGASL#4 BGA scFv mutagenized library on the surface of phage, as diabodies. This was done to increase the wild type affinity of this weak binding scFv by increasing avidity. When a short peptide links the BGA V_L and V_H genes, they are prevented from pairing with each other, and are forced to pair with a soluble scFv to form a bivalent fragment known as a diabody (Holliger *et al.*, 1993). Unlike scFvs, Fabs do not have a tendency to dimerize, and as such Fabs are more easily characterized; however the small size of the scFv genes makes for more stable libraries (Plückthum and Pack, 1997).

B.1.3.2 Selection Strategies

The size and diversity of the phage display library are very important, nevertheless, the selection process used to select for the desired clones from this repertoire is equally important. Any method that allows for the separation of clones that bind from clones that do not bind can be potentially used as a selection or panning method. Panning processes allow for the enrichment of higher affinity clones from a phage antibody library, by repetitive rounds of selection against a target antigen. These repetitive rounds of selection include binding the phage library, washing the unbound phage, and eluting the bound phage for amplification. This cycle will allow for the selection and amplification of clones with an improved affinity for the target antigen, by eliminating the weaker binding clones in the panning cycles, and allowing for better binders to be enriched.

Generally, the selection of clones from a phage library can be easily and successfully performed on antigen-coated plastic surfaces such as microtitre wells, petri dishes, and immunotubes (Marks *et al.* 1991). However some disadvantages to these processes are that they require purified antigen, are unable to discriminate between clones of similar affinity, and can be affected by non-specific binding of the phage displayed library to the plastic surface. Panning on the BIACORE instrument has successfully separated phage displayed antibody libraries based on differences in the dissociation rate constants (Malmborg *et al.*, 1996); however, this successful panning process involved high affinity protein-protein interactions only (Malmborg *et al.*, 1996).

Panning on affinity purification columns has also been carried out (Clackson *et al.*, 1991), in which the target antigen is immobilized onto a column for affinity selection. Generally, this panning process selects for phage-antibodies that have bound to and then eluted from the column. However, our BGA scFv library is a very low affinity library and therefore binding of the phage-scFv to the immobilized antigen is transient, and does not require a specific elution step. The Synsorb-A panning method reported here separates a low affinity anti-carbohydrate library based on the transient binding of the phage-scFv as they percolate through the column.

There are many more panning methods that have been used to pan phage displayed antibody libraries. Some less known panning strategies include: panning directly on whole cells (Marks *et al.*, 1993), panning by cell sorting, in which antigen displaying cells are sorted by a marker that is unique for these cells, this sorting can be done by fluorescent activated cell sorting (FACS) or magnetic activated cell sorting (MACS) (Hoogenboom, 1997). Panning has also been done with biotinylated antigens, in which bound and unbound phage are separated using streptavidin coated magnetic beads (Hawkins, *et al.*, 1992). Panning using selectively infective phage (SIP) has also been done (Spada and Plückthum, 1997). Panning using SIP selects bound from unbound

phage by using non-infective phage (deleted gIII). The infectivity is restored upon binding of the phage-scFv to pIII linked antigen and as a result, phage are selected for propagation based on the binding of the scFv and its antigen.

B.1.3.3 Ribosome Display

And finally, it is worth mentioning a new display technology that does not involve the use of phage. Ribosome display was first demonstrated by Mattheakis *et al.* as a new concept for linking phenotype and genotype *in vitro* (Mattheakis *et al.* 1994). The first successful demonstration of ribosome display using proteins (Hanes and Plückthun, 1997), and the use of this technology to select antibody fragments from a mutant library (He and Taussig, 1997) seem to suggest that ribosome display is very useful for the *in vitro* identification and molecular evolution of peptides and proteins. This technology links phenotype and genotype through the association of the mRNA with its corresponding nascent protein. Essentially, in ribosome display, the polysome complex containing the encoding mRNA and the newly translated nascent protein are selected for by an immobilized monoclonal antibody. The nascent protein that binds the immobilized antibody is tagged with its mRNA. This mRNA selected from the polysomes is transcribed into DNA, amplified using PCR, and used for the next round of transcription, translation and selection. Some of the major advantages to this system is that it is very rapid, the cell-phage life cycle does not complicate the process, and the repertoire size that can be generated is potentially unlimited (Dall'Aqua and Carter, 1998).

B.2 EXPERIMENTAL PROCEDURES

B.2.1 MATERIALS

All DNA manipulations were carried out according to standard procedures (Sambrook *et al.*, 1989). Restriction enzymes and DNA modifying enzymes were purchased from New England Biolabs and Life Technologies, Inc. BSA-antigen conjugates were purchased from Chembiomed (Edmonton, Alberta). DNA sequences were performed by the dideoxynucleotide method (Sanger, 1975) using an automated DNA sequencer model 373 (Applied Biosystems Inc.). Synsorb-A and B beads were purchased from Synsorb Biotech Inc. (Calgary, Alberta).

B.2.2 TGASL#4 PHAGEMID VECTOR

The TGASL#4 phagemid vector containing the BGA scFv gene was cloned into phagemid pSK4 (Deng *et al.*, 1994) at the *EcoRI-BglII* cloning sites. As described in Part A Experimental Methods, the TGASL#4 scFv's V_L and V_H regions are linked by a short five amino acid linker, thereby favoring the formation of scFv dimers (Holliger *et al.*, 1993). The scFv gene is preceded an ompA secretory sequence, and followed by a c-myc detection tag (Hoogenboom *et al.*, 1991), and a His_5 purification tail (Skerra *et al.*, 1991), all of which are fused to the geneIII phage protein through a Gly_4Ser linker at the *BglII* site following the His_5 tail (please refer to Figure below).

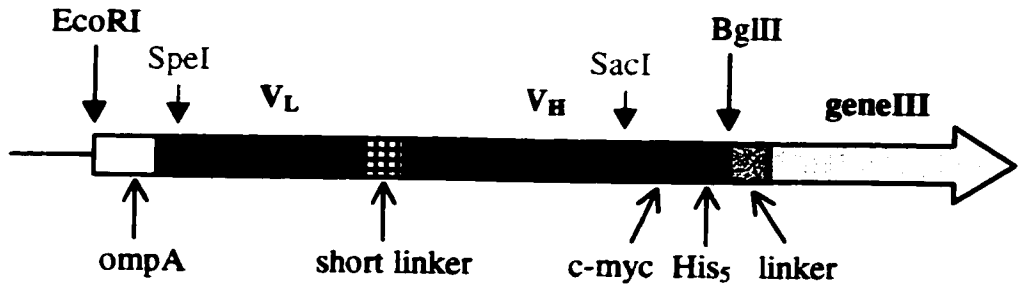


Figure 20 : TGASL#4 phagemid vector segment showing BGA scFv / geneIII fusion. The SpeI and SacI restriction sites are the sites where the forward and reverse SDMUBGUP and SDMUBGDN PCR primers amplify the scFv respectively. The 700 base pair mutagenized V_L-V_H scFv fragment was then inserted into a similarly cut TGASL#4 phagemid.

If we recall the TGAscFv construct used in Part A, there was a terminator sequence between the BGA scFv sequence and the geneIII sequence, thereby allowing the production of the soluble protein needed for the site-directed mutagenesis study. The TGAscFv construct is a modified version of the TGASL#4 phagemid vector, with the only modification being that the TGAscFv contains a terminator sequence at the *BglII* site following the scFv sequence. The TGASL#4 phagemid vector is used in this study, and since it does not contain the terminator sequence, it produces scFv-geneIII fusion proteins.

B.2.3 CONSTRUCTION OF TGASL#4 PHAGEMID LIBRARY

Mutagenesis of the TGASL#4 scFv sequence was performed using error-prone PCR (EP-PCR). TGASL#4 vector was used as template, the SDMUBGUP forward and SDMUBDDN reverse purified oligonucleotide primers were used for the EP-PCR reaction; SDMUBGUP (5'-GACCCAGACCACTAGTTCTCTGAGC-3') and SDMUBDDN (5'-CGGACGCAGAGCTCACGGTAACCAG -3') primers are found at the *SpeI* and *SacI* restriction

sites respectively. EP-PCR was performed under conditions that reduce the fidelity of DNA synthesis by Taq DNA polymerase (Berek and Milstein, 1987). The EP-PCR reaction was performed using 30 cycles of 95°C for 60 s, 50°C for 90 s, and 72°C for 90 s (Perkin Elmer model 9600 thermal cycler). The EP-PCR fragments were purified using QIAquick PCR purification kit (QIAgen), digested with *SpeI* and *SacI* restriction enzymes, and ligated into a gel purified TGASL#4 vector cut with the same restriction enzymes.

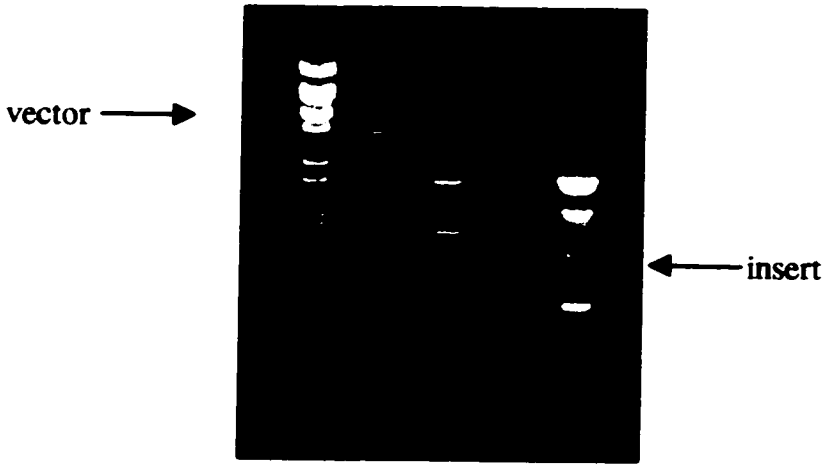


Figure 21: 1 % agarose gel of Spe I – Sac I restriction digested TGASL#4 pure vector and EP-PCR scFv insert. The purified vector and insert were run alongside size and mass standards. The amount of vector and insert used for ligation were determined from this gel. We can clearly see the 700 bp pure EP-PCR insert.

The ligation products were electroporated into electrocompetent *E. coli* XL1-Blue resulting in 10^6 transformants. The transformants are resistant to both ampicillin and tetracycline (amp^r and tet^r) since the TGASL#4 vector is amp^r and *E. coli* XL1-Blue is tet^r. The transformants were grown on LB amp plates, so that the size of this phagemid library could be determined. The plasmids of 10 randomly selected transformant colonies were prepared using QIAprep Spin Plasmid Kit (QIAgen). The plasmid's scFv sequence was then determined to confirm that the EP-PCR library produced did indeed

contain mutations. The 10 randomly selected clones contained complete, intact BGA scFv V_L-V_H inserts, and all clones contained several mutations found throughout the scFv sequence (both FRs and CDRs). A phagemid library of 10^6 intact mutant BGA scFv fusion proteins was produced.

B.2.4 PHAGE PREPARATION

After electroporation, the *E. coli* XL1-Blue transformed cells were grown in 15 ml of SOC for 1 h, 10 ml of SB (35 g/l tryptone, 20 g/l yeast extract, 5 g/l NaCl) containing 50 μg/ml ampicillin and 75 μg/ml tetracycline for 3 h; 500 μl of M13K07 helper phage (10^{12} pfu/ml) were then added and grown for 2 h. Lastly, 50 μg/ml kanamycin was added and the phage was grown overnight at 37°C. Following the overnight growth, the cultures were centrifuged and the phage present in the supernatant was harvested using the PEG precipitation protocol (Cwirla *et al.*, 1990). The precipitated phage were taken up in 2 ml of PBS and stored at -20°C, and referred to as 'unpanned EP-TGASL#4 phage' since the mutant TGASL#4 clones of this phagemid library were made using EP-PCR and had not yet undergone any panning procedures.

B.2.5 SCREENING CLONES FOR SCFV INSERT

The EP-TGASL#4 phagemid library is the repertoire of mutant BGA scFv's that have been expressed on the surface of phage for the purpose of affinity selection using phage display. Phage display is a very powerful technique because the protein expressed on the phage surface is encoded for in the plasmid encapsulated within the phage particle. Therefore the BGA scFv fusion proteins expressed in the EP-TGASL#4 phagemid library are directly linked to their plasmid DNA.

The eluted phage in the panning procedures described below, were screened for insert in the encapsulated plasmid DNA. The eluted phage were used to infect *E. coli*

XL1-Blue cells, and grown overnight at 37°C on LB amp plates. Colonies were randomly selected off the LB amp plates and their corresponding plasmid DNA was prepared using the QIAprep Spin Plasmid Kit (QIAgen). The prepared plasmids were run on a 1% agarose gel alongside the TGASL#4 wild type plasmid, so as to verify their plasmid size. The screened plasmids were also digested using *SpeI-SacI* restriction digests to check for the 700 bp scFv insert. The clones containing the scFv insert were sequenced using the dideoxynucleotide method (Sanger, 1975) on an automated DNA sequencer (Applied Biosystems Inc.).

B.2.6 PANNING

Panning is essentially an affinity selection procedure used to screen our EP-TGASL#4 scFv displayed library against the BGA A-carbohydrate antigen; this selection process allows better binding scFv clones to be selected, eluted, and amplified. This cycle is repeated (3-4 rounds) and the eluted scFv phage clones are assayed; the clones that seem to exhibit a higher affinity for the A-antigen are sequenced by the dideoxynucleotide method (Sanger, 1975) and checked for sequence homology. The EP-TGASL#4 scFv displayed library was panned using a number of techniques; bio-panning in microtitre wells, bio-panning on BIACORE™, and finally panning of this library on a Synsorb-A column.

B.2.6.1 Panning in Microtitre Wells

The immobilization of 300 µl of 100 µg/ml BSA-A trisaccharide conjugate antigen onto a microtitre well was done overnight at 4°C. The following morning the BSA-A coated well was washed 3x with PBS, blocked with 2.5% skim milk for 1 h, then washed 3x with PBS. Then 250 µl of EP-TGASL#4 unpanned phage were added to the microtitre well for 2 h so binding could occur. The unbound phage was removed and the

microtitre well was quickly washed 2× with PBS, 2× with 0.05 % Tween PBS, and the remaining bound phage was eluted from the coated well using 240 µl of phage elution buffer (0.1 M CH_3COOH, 0.15 M NaCl, pH 2.8) for 8 m. The eluted phage was removed and neutralized with 15 µl of 2 M Tris pH 9.5; this ~ 255 µl first round eluted phage was amplified, and harvested as described above (Cwirla *et al.*, 1990). The eluted phage was titred before and after amplification, and clones from the elution titre were randomly selected and screened for scFv insert.

This panning cycle was repeated four times, and in each round of panning increased the stringency of the 0.05% Tween/PBS wash was increased. This was done to promote a progressive selection of better binding scFv clones. For all cycles the panning process consisted of: coating a microtitre well with antigen, blocking with skim milk, 3× wash with PBS, adding 250 µl of phage (unpanned or amplified phage from the previous round), 2×, 3×, 4×, and 8× washes with both PBS and 0.05% Tween/PBS for the 1st, 2nd, 3rd and 4th rounds of panning respectively, neutralization of the eluted phage, and then amplification of the eluted phage.

The 4th round eluted phage (unamplified) were randomly selected from their corresponding elution titre plate and screened for insert using colony PCR (Ward, 1992). This colony PCR process is a preliminary screening of the eluted colonies, and was used to quickly check for the presence of the scFv insert. Essentially this process requires that a standard PCR reaction be 'inoculated' with an eluted colony aseptically removed from the titre plate. The DNA of the clone within this colony is used as the DNA template in this PCR reaction, and amplification of the scFv occurs using the SDMUBGUP and SDMUBGDN primers. The PCR products were then run on a 1% agarose gel, and checked for a 700 base pair insert, which indicates a complete V_L-V_H scFv sequence. Over 100 4th pan eluted clones were screened using phage ELISA (Kay *et al.*, 1996). This procedure screens the eluted phage by allowing the scFv-geneIII fusion protein to bind to

plates coated with BSA-A antigen, the binding activity was detected with an alkaline phosphatase sheep anti-M13 conjugate. The highest activity phage ELISA clones were then screened for insert and sequenced.

B.2.6.2 Testing microtitre well panning conditions

TGASL#4 wild type phage was produced so that the microtitre-well panning conditions could be tested. A single panning round was completed using this wild type phage so that two A antigens could be compared, and the stringency of the washes in this panning process could be determined. The binding affinity of the scFv fusion protein for two different A trisaccharide antigens was compared: BSA-A trisaccharide and A-polyacrylamide-biotin conjugated antigen (PA-A). Four microtitre wells were coated with the A antigen conjugates (2 with BSA-A and 2 with PA-A), washed with PBS and blocked with skim milk. Then 250 µl of TGASL#4 phage was added to each well and allowed to bind. The unbound phage was then removed, and the wells were washed with PBS and 0.05 % PBS; 10 washes and 20 washes were done for each antigen. The phage in each well was eluted and neutralized. The eluted phage after the 10 and 20 washes from both antigen surfaces was titred, and screened.

B.2.6.3 Panning on BIACORE™

The affinity selection of better binding clones from the constructed EP-TGASL#4 scFv phagemid library was also performed on the BIACORE instrument. The BSA control and BSA-A antigen active surfaces were immobilized to the dextran surface of a sensor chip as described in section 9.1 of Part A. Essentially the BIACORE panning is the affinity selection of better binding phage through the dynamic separation of phage on the BIACORE instrument.

The BIACORE panning experiment is as follows:

(1) the phage was injected into the flow buffer and equilibrium is achieved

(2) injection of phage ends

(3) the phage dissociate from the antigen surface, thereby separating phage of different affinities; the profile of the dissociation is monitored

(4) the dissociating phage fractions are collected (5 μl fractions) using an automated system within the BIACORE instrument

(5) collected fraction are titred

(6) collected fractions are amplified, and titred

(7) repeat cycle, for 4 rounds of panning on BIACORE

The measurements were performed at 25°C in 10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% P20 at a flow rate of 25 μl/min. A 15 μl aliquot of the EP-TGASL#4 phage library was injected into the flow buffer above the sensor surface, and the binding of the phage to the sensor surface was recorded as a sensorgram (Figure 22).

The sensorgram in this study was not used to determine kinetic binding constants, but was used to profile the separation of the phage as they dissociate from the sensor surface. The affinity selection that occurs in a typical panning process is mimicked in this BIACORE study; the selection of better binding phage is achieved by allowing the phage to be separated into fractions as they dissociate from the sensor surface. The fractions that are first to dissociate are the poor binders, whereas the better binding phage will not dissociate as quickly, and will be found in later fractions. The profile of the dissociation phase of the sensorgram helps to visually indicate the strength of the phage interaction with the antigen surface. An elongated profile indicates a slow dissociation of phage from the surface, thereby suggesting better binding phage. Conversely, an abrupt drop in

the dissociation profile suggests a weak interaction between the phage and the antigen surface.

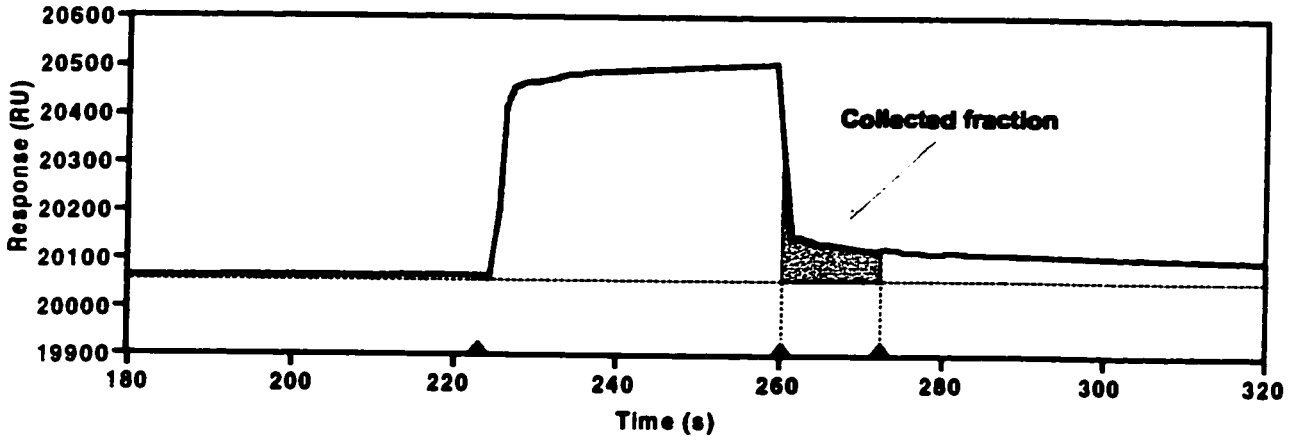


Figure 22: Sensorgram of 4th round of panning on BIACORE. Once a good baseline is achieved, 15 µl of 3rd round amplified phage is injected into the flow buffer (first arrow on x axis), and equilibrium is attained. At 260 s the injection of phage ends and we observe an abrupt decrease in the RUs, indicating the dissociation of phage from the antigen surface. Immediately after injection ends, the dissociating phage are collected as 5 µl fractions (gray shaded portion). This fraction is titred, and clones are randomly selected from this titre plate for screening.

This was the first time panning on the BIACORE was performed in our lab, and the panning conditions needed to pan this EP-TGASL#4 phagemid library had not yet been determined. Standard conditions (indicated above) were used, but there are many variables that may affect the panning process i.e. flow rate, non-specific binding. Initial trials indicate non-specific binding of phage to the sensor surface (binding on BSA control surface was observed). Attempts were made to eliminate non-specific binding through the addition of BSA (2 mg/ml), dextran, or detergents (P20 and Tween) directly to the phage sample prior to injection. However, some non-specific binding could still be observed. The optimal panning conditions needed to pan this anti-carbohydrate phagemid library on the BIACORE have yet to be determined completely.

Four rounds of panning were done on the BIACORE, and the first dissociation fraction for each round was collected, titred, amplified and used for the next round of panning. Twelve 4^{th} pan and twelve 3^{rd} pan clones were randomly selected and screened for insert. Phage ELISA was also used to screen over 120 randomly selected 4^{th} pan clones. The highest activity phage ELISA clones were screened for insert, and sequenced.

B.2.6.4 Panning on Synsorb-A column

Synsorb-A beads are diatomaceous earth with covalently linked A-antigen on the bead surface. The affinity selection, or the affinity separation, of better binding scFv-phage from the constructed EP-TGASL#4 phagemid library was explored. The use of a Synsorb-A matrix to pan a phage library has never been done, and therefore the first step required to test this proposed method was to make a Synsorb-A column, and then to perform preliminary trials to test whether or not affinity selection could be accomplished using this system.

(i) Preliminary Trials

To make the Synsorb–A column; 0.9 g of Synsorb-A beads were suspended in PBS, degassed, poured into a column, and allowed to settle overnight at 4°C. The column was attached to a pump, which controlled the flow rate of this system, and a UV detector monitored changes in the absorbance of the flow through buffer. The column was washed with PBS at 0.6 ml/min to give the baseline absorbance.

The next step was to test if the column was capable of separating a sample based on transient binding. The addition of sample to the Synsorb-A column required that the buffer solution above the matrix bed be lowered to just above the bead surface, then 200 µl of sample was applied to the bead surface and allowed to penetrate the bead

matrix. Once the sample had penetrated the matrix, the PBS buffer level above the matrix was increased, the column was washed with PBS at 0.6 ml/min, the flow through was monitored, and the eluted peaks were collected. The samples applied to the column in this preliminary trial were 200 µl of pure BGA scFv protein, a control protein *Salmonella* scFv, and TGASL#4 wild type phage. The chromatograms recorded indicate that the BGA scFv and TGASL#4 phage did bind to the column and the *Salmonella* scFv did not (please refer to results). The BGA scFv eluted peak was collected and analyzed by SDS-PAGE and Western blotting.

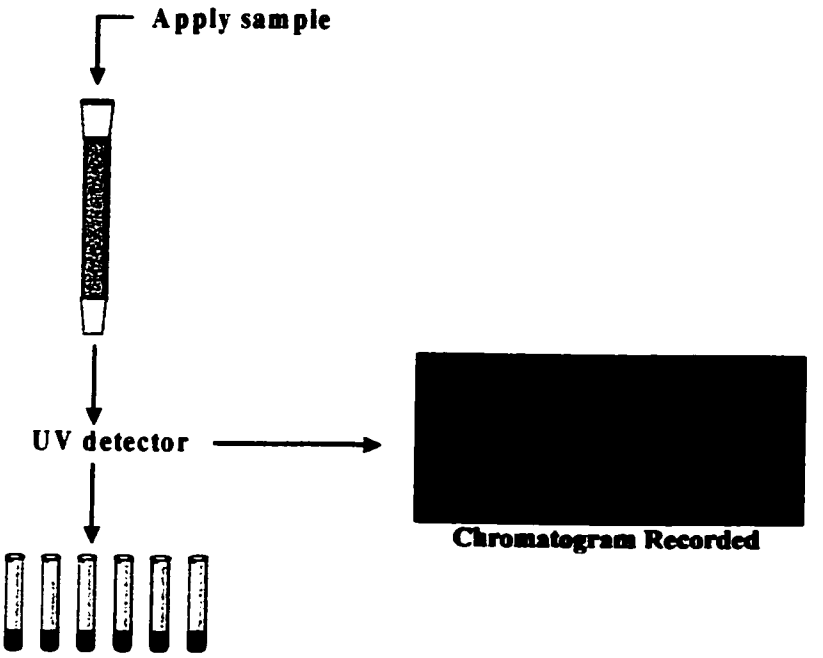


Figure 23: Initial Synsorb-A system. PBS buffer and sample were introduced into this system manually at the top of the Synsorb-A column. The flow of buffer was controlled using a pump, and the elution was analyzed in a UV detector and recorded as a chromatogram. Collected fractions were then analyzed.

(ii) Panning of EP-TGASL#4 phagemid library

The Synsorb-A column was washed with 0.2 M Glycine-HCl, and equilibrated with PBS (flow rate 0.6 ml/min, chart speed 0.1 cm/min) to baseline. The PBS buffer level above the matrix was lowered, 200 µl of unpanned EP-TGASL#4 phage was applied, and allowed to penetrate the column, and then the PBS buffer level above matrix was restored to original level. The complete entry of the sample into the matrix was noted as t=0 on the chromatogram, and is the starting, or the reference point of the recorded chromatograms. During this adsorption phase of the panning, the column was washed with PBS (0.2 ml/min, 0.5 cm/min) and 14 × 2 min (i.e. 400µl) fractions were collected. Based on the chromatogram, fractions #4 - #6 (1.2 ml) were combined, titred, and amplified. To regenerate the column, it was washed back to the original baseline with PBS (0.6 ml/min, 0.1 cm/min) and then washed with 0.2 M Glycine-HCl (0.6 ml/min, 0.1cm/min).

Prior to the addition of the 1st round amplified phage to the column, the column was washed with 2 ml BSA (1 mg/ml) to reduce non-specific binding, and then with PBS to give a stable baseline. The cycle of adsorption, fraction collection and regeneration of column was repeated as described above. Four rounds of panning were carried out; after each round of panning 9 randomly selected eluted colonies were screened for scFv insert (as described above), amplified, and used for the next round of panning. Phage ELISA was used to screen over 60 randomly selected Synsorb-A 4th pan clones. The highest activity 4th pan phage ELISA clones were screened for insert, and sequenced.

(iii) Improvements to Synsorb-A panning method

An IMAC column (Hi-Trap, Pharmacia) was emptied of its matrix, washed with water and allowed to dry. The column shell was packed with dry Synsorb-A beads and sealed with its original casing. The closed column containing the Synsorb-A beads were

attached to an HPLC controlled pump, UV detector and recorder. The closed system was washed with PBS buffer (0.5 ml/min, 0.5 ml/cm) and the sample was syringe injected into the column using the valve system of the HPLC; the injection of sample marked the reference point (t=0) of the panning. The same preliminary trials described above were repeated as a control; 200 µl of test samples were injected into this system and monitored at a flow rate of 0.5 ml/min and chart speed of 0.5 ml/cm.

200 µl of unpanned EP-TGASL#4 phage were injected into the closed system, the system was continually washed with PBS (0.25 ml/min, 0.5ml/cm), and 1 m (i.e. 250 µl) fractions were collected. Eighty fractions were collected and titred for phage. Some of the collected fractions (#3, #44, and fraction #80) were screened for scFv insert. The column was regenerated with 0.2 M Glycine-HCl, and stored at 4°C in 0.02% NaN_3.

B.3 RESULTS

B.3.1 TGASL#4 PHAGEMID LIBRARY

Error-prone PCR was used to produce random mutations within the BGA scFv V_L-V_H fragment. The plasmid clones constructed by the insertion of the EP scFv fragments into the TGASL#4 phagemid vector produced a phagemid library of various mutant BGA scFv, which were then displayed on the surface of bacteriophage. Prior to the preparation of phage displayed scFv's, the scFv clone library itself was analyzed.

The plasmid clones constructed were transformed into *E. coli* XL1-Blue, and the size of the library produced was determined to be 10^6 transformants, therefore indicating a library of adequate size. Ten clones were randomly selected from this repertoire and screened for scFv V_L-V_H inserts, and sequenced. All 10 clones contained complete scFv V_L-V_H inserts, and all the screened clones contained mutations. The EP-PCR mutations produced were randomly scattered throughout the scFv sequence, with a frequency of about 5-10 mutations/clone. This library therefore contained an acceptably-sized repertoire of complete, intact BGA scFv V_L-V_H mutants.

B.3.2 PANNING IN MICROTITRE WELLS

Four rounds of panning with the EP-TGASL#4 phagemid library were carried out in microtitre wells coated with the BSA-A trisaccharide antigen. An increasing number of washes, from round 1 to round 4, with PBS and with 0.05 % Tween/PBS was changed progressively to increase the stringency needed to select for better binding BGA scFv fusion proteins. The titre results of the eluted and amplified phage, for each panning round, are listed in Table VI below.

Table VI
Summary of EP-TGASL#4 panning in microtitre wells

Panning Round	Each number of washes with PBS and 0.05 % Tween/PBS	Titre of eluted phage[a]	Titre of amplified phage[a]
1	2	10^6	10^{12}
2	3	10^5	10^{12}
3	4	10^6	10^{12}
4	8	10^6	10^{12}

[a] phage/ml

According to the elution titre after each round of panning we can see that there seems to be no enrichment in this panning process; there is no increase in the titre of the eluted phage as the panning rounds increase. When randomly selected clones from the 4th round were screened for insert, only 2/10 clones contained an scFv. The sequences of the 8 clones with deletions between the *SpeI* and *SacI* sites showed not BGA scFv gene sequence. Since the intact scFv inserts of the unpanned EP-TGASL#4 library were generally not present in these 4th round clones, it suggests that this panning process selected for deletion clones and not for better binding BGA scFv clones from the library repertoire.

Approximately 100 4th round clones were screened using colony PCR and phage ELISA. Over 50 4th round clones were expressed on phage and the scFv-geneIII fusion proteins were screened on BSA-A antigen coated surfaces. The DNA of the highest activity phage ELISA clones was then prepared, screened for scFv insert, and sequenced. None of these screened clones contained scFv sequences, and all seemed to be deletion clones. Over 50 4th pan clones were also screened using colony PCR, and again deletion products seemed to predominate.

During these screening processes, the plasmid size of the selected clones was compared to the wild type plasmid (on 1% agarose gel), and the DNA sequence was then verified. Restriction digests using a number of enzymes were also performed, and again there were very few clones that contained the complete scFv insert. During the screening processes, 3 populations of clones seemed to dominate. These different clone populations varied in plasmid size: slightly less than 10% of the screened clones resembled the wild type plasmid, ~ 80% were smaller than the wild type, and ~ 10% were even smaller than this abundant population. These plasmids of smaller size again indicate deletion or truncated scFv clones.

The panning of this low affinity scFv anti-carbohydrate library in microtitre wells did not yield better binding scFv clones. Instead, it appeared to be selecting for deletion products, which may be a result of this panning process, or of the amplification step of the panning cycle. These results will be further explained in the discussion.

B.3.2.1 Testing microtitre panning conditions using wild type phage

Wild type scFv was expressed on phage and panned in microtitre wells coated with BSA-A and PA-A trisaccharide conjugate antigens. The binding affinities of the scFv for the two different A trisaccharide conjugates were similar, as indicated by the similar elution titres from both surfaces (please refer to Table VII).

Table VII
Eluted phage from 1st round test panning of phage displayed wild type BGA scFv

Antigen surface[a]	# washes[b]	Titre of eluted phage[c]
BSA-A[d]	10	6×10^2
BSA-A	20	1×10^3
PA-A[e]	10	8×10^3
PA-A	20	3×10^4

[a] 300 µl of 100µg/ml antigen coating microtitre well
[b] number of washes with both PBS and 0.05% Tween/PBS
[c] phage/ml
[d] BSA-A trisaccharide antigen conjugate
[e] polyacrylamide A-trisaccharide conjugated to biotin

The stringency of the panning washes was also tested using the wild type phage. The BSA-A and PA-A antigen surfaces with bound wild type phage were washed 10× and 20× with both PBS and 0.05 % Tween PBS (Table VII). The phage eluted from both surfaces using a stringency of 10× or 20× washes yielded approximately 10^3 phage/ml. This indicates that approximately 1000 phage/ml endured the washing process. We do not know if these eluted clones are antigen-specific binders or if this is simply background. However these 10^3 phage/ml eluted clones were randomly screened for scFv insert, and all 20/20 screened clones contained a complete, intact scFv sequence. This result further validates the EP-TGASL#4 library as a repertoire of complete intact scFv clones. It consequently suggests that the deletion clones observed in the 4th round of the microtitre panning process are not a result of the constructed library, but may be a consequence of the panning cycles.

B.3.3 PANNING ON BIACORE

Four rounds of panning with the EP-TGASL#4 phagemid library against the BSA-A trisaccharide conjugate antigen were carried out on the BIACORE. In contrast to the panning process in microtitre wells, the BIACORE was used to dynamically separate phage as they dissociate from the sensor surface. A 15µl aliquot of the EP-TGASL#4 library was allowed to separate into weaker and better binders in the dynamic dissociation phase of this panning process. The dissociation fractions were collected, and the first collected fraction (5µl) for each round was amplified and used for the next round of separation (please refer to Figure 22). The titres of the eluted and amplified phage in each of the rounds of this panning process are listed in Table VIII.

Table VIII
Summary of EP-TGASL#4 panning on BIACORE

Panning Round	Titre of eluted[a] Phage[b]	Titre of amplified phage[b]
1	5×10^5	10^{14}
2	7×10^6	10^{14}
3	1×10^7	10^{14}
4	2×10^7	10^{13}

[a] First dissociating fraction is collected, 5 µl/ fraction
[b] phage/ml

The DNA of 12 randomly selected 4th pan clones was prepared and screened for insert. Only 1 clone contained the scFv insert, the remaining 11 clones were deletion products. Likewise, 12 randomly selected 3rd pan clones were screened for insert, and again only 1 clone contained a complete scFv sequence while the remaining 11 clones were deletion products. The 3rd and 4th pan phage containing scFv clones had complete scFv sequences, which were not homologous in their mutations. The dominance of

deletion products in these randomly selected clones suggested that this panning process did not select for a better binding BGA scFv population; nevertheless, the BIACORE 4th pan eluted clones were screened using phage ELISA.

Over 50 BIACORE 4th pan eluted clones were screened using phage ELISA, and the 9 highest activity clones were selected. The DNA of these 9 high activity clones was prepared, and screened based on plasmid size and restriction enzyme digestion. Only 1 of these 9 clones contained a complete intact scFv sequence (8 were missing the scFv sequence). The DNA sequence of this scFv containing clone is of a complete scFv with many mutations.

Similar to the panning in microtitre wells, this panning process yielded deletion products in the 4th pan eluted clones. The deletion products in this BIACORE panning may be a result of the conditions used to pan this library, since this is the first time a phagemid library has been panned on the BIACORE. Furthermore, the selection and amplification of the first dissociating fraction may not have allowed for the selection of better binding BGA scFv phage. These results will be expanded upon in the discussion.

B.3.4 PANNING ON SYNSORB-A COLUMN

Preliminary trials of the Synsorb-A column indicated that this column allows separation based on the transient binding of the sample as it percolates through the column. The ability of the column to separate sample is illustrated by the following test samples. IMAC purified BGA scFv should bind specifically to the column; however, *Salmonella* scFv, which is not specific for BGA-A antigen, should not bind to the column, and should essentially pass directly through the column. As indicated by the chromatograms below, the BGA scFv did interact with the Synsorb-A beads, and therefore came off later than the non-interacting *Salmonell*a scFv (Figure 24).

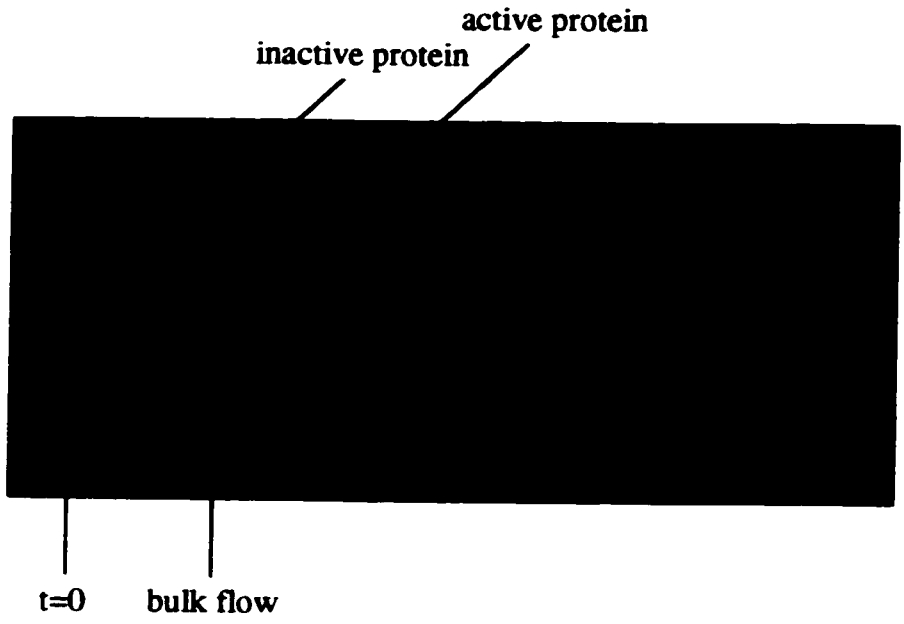


Figure 24: Chromatograms of Synsorb-A preliminary trials. Sample was applied to the column, and allowed to completely penetrate the matrix. This starting point is indicated as t=0. The *Salmonella* scFv (red) eluted out in the bulk flow, indicating that there was no interaction with the Synsorb-A column. The BGA scFv protein (blue) however did interact with the column, allowing for the separation of active and inactive protein. The first and second BGA scFv collected peaks were analyzed by SDS-PAGE and Western blotting, and determined to be inactive and active respectively.

It should be noted that because of the low affinity of the BGA scFv, a specific elution of the protein from the column was not necessary. The BGA scFv active protein did interact with the A-antigen on the Synsorb-A beads; but this interaction was a transient or temporary interaction, and does not require a specific elution step. The SDS-PAGE and Western blot analysis indicated that the first BGA scFv peak was inactive protein, either incorrectly folded or degraded protein, whereas the second BGA scFv peak was pure active protein. The ability of this column to separate a sample into inactive and active protein indicates a very promising result. It is this ability to separate sample, based on the transient binding of its constituents, that forms the basis for the affinity selection,

or affinity separation of our BGA scFv EP-TGASL#4 library into poor and strong binders.

Panning on the Synsorb-A column requires that an aliquot of the EP-TGASL#4 library be allowed to percolate through the column, thereby allowing the better binding scFv fusion proteins to separate from the poor binding scFv fusion proteins. This separation occurs since the better binders have an enhanced and therefore prolonged interaction with the column and are consequently eluted following the weak binders. The subsequent collection and amplification of these better binding scFv fusion proteins, should allow for the isolation of BGA scFv proteins with improved binding to the A-antigen.

B.3.4.1 Panning of EP-TGASL#4 phagemid library

Four rounds of panning with the EP-TGASL#4 phagemid library were carried out on the Synsorb-A column. The chromatogram profile of each Synsorb-A panning was used to select the fractions that would be combined and amplified for the next round of panning. Table IX gives a summary of the Synsorb-A panning process, and Figure 25 shows the Synsorb-A 4th round chromatogram.

Table IX
Summary of EP-TGASL#4 panning on Synsorb-A column

Panning Round	Eluted fractions[a] collected and combined	Phage[b] titre of combined fractions	Phage[b] titre of amplified fractions
1	# 8 - # 10	10^7	10^{14}
2	# 10 - # 11	10^8	10^{14}
3	# 11 - # 12	10^7	10^{14}
4	# 14 - # 16	10^6	10^{14}

[a] Fraction number listed based on chromatogram, 400µl/fraction
[b] phage/ml

Figure 25
Chromatogram of Synsorb-A 4th round panning of EP-TGASL#4 phagemid library

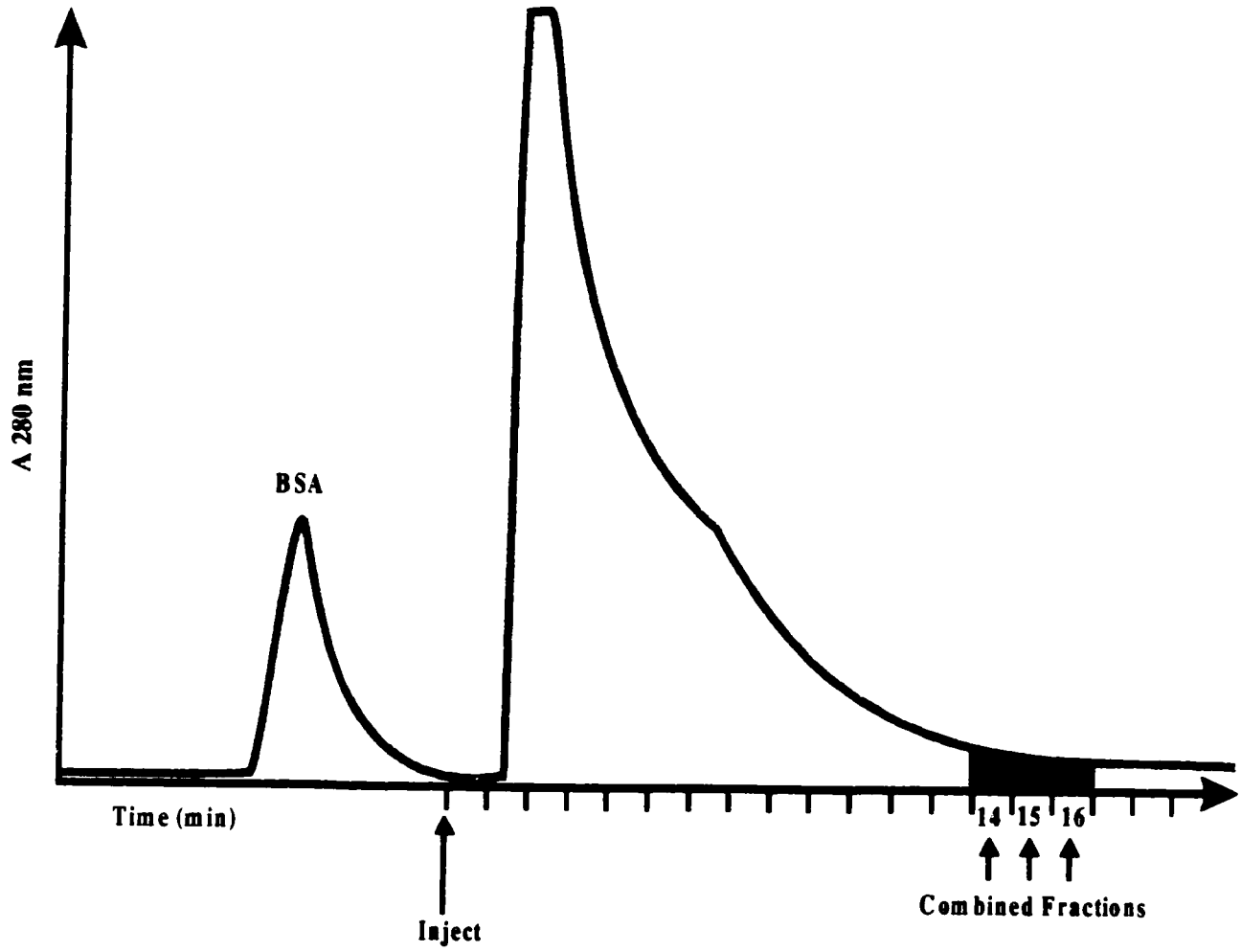


Figure 25: Chromatogram of Synsorb-A 4th round panning of EP-TGASL#4 phagemid library. 2ml (1mg/ml) BSA was initially added to a regenerated and equilibrated column to reduce non-specific binding. The column was then equilibrated with PBS to give the initial baseline. 200 µl of 3rd round eluted and amplified phage were added to the column (labeled 'inject') and eluted with PBS. Fractions # 14 - # 16 were collected, combined, titred and screened for BGA scFv insert.

The fraction(s) selected for amplification were fractions that followed the main BGA scFv peak (Figure 25); the selection of these fractions was based entirely on the chromatogram profile. Fractions were collected until the chromatogram baseline appeared to return to the initial baseline absorbance. Each round of panning had its own recorded chromatogram, but only the chromatogram of the 4th round Synsorb-A panning is illustrated in Figure 25; the 1st, 2nd, and 3rd round chromatograms resembled this profile.

Following each round of panning, clones were randomly selected from the combined fraction titre plate and screened to check for deletion products. Figure 26 shows a 1% agarose gel of the screening of the 4th round eluted phage clones.

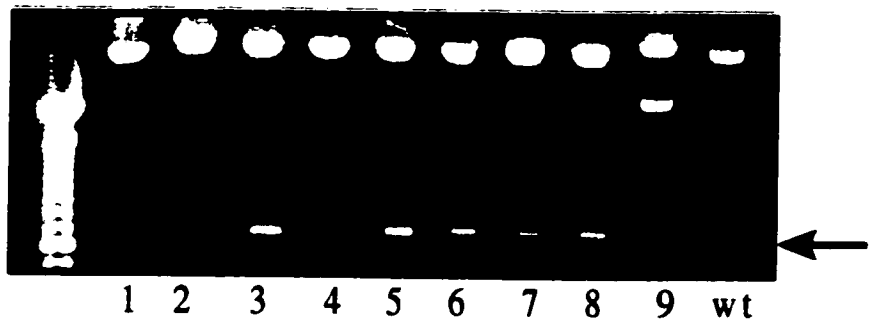


Figure 26: Random screening of nine 4th pan Synsorb-A eluted clones. The 4th round Synsorb-A collected fractions (#14 - #16) were screened for the scFv insert. This 1% agarose gel of nine randomly selected 4th pan Synsorb-A eluted clones shows that 7/9 clones contained the 700 bp scFv fragment (indicated by arrow); only 2/9 clones did not (clone 1 and clone 9 are deletions). The wild type TGASL#4 plasmid and a 100 bp DNA ladder were run alongside the screened clones.

The screening of the eluted fractions following each round of panning verified that very few deletion products were eluted in the Synsorb-A panning rounds. The plasmid size and *SpeI-SacI* restriction digestions indicated that 7/9 eluted clones

contained a complete scFv sequence for each round of panning. The 4th round Synsorb-A eluted clones were screened and only 2/9 clones were deletion products, while 7/9 clones were of complete scFv's (Figure 26). If we recall the screened eluted clones from the 4th round microtitre well panning we observed that 8/10 clones were deletion products. Furthermore, the 4th round BIACORE panning yielded 11/12 deletion clones in the screened eluted fraction. These results will discussed be in the following section.

The screening of the Synsorb-A panning rounds is encouraging because the deletion products that dominated the previous panning experiments were not prevalent in this Synsorb-A panning process. Phage ELISA was used to screen over 60 4th pan Synsorb-A clones, and the highest activity phage ELISA clones were screened for the BGA scFv. Some clones contained deletions, but the majority of the clones screened were of complete intact BGA scFvs. The DNA sequences of all the scFv containing clones were determined, and there was no sequence homology in these 4th pan eluted clones. The lack of homology in the 4th pan eluted clones indicates that a single clone population was not selected for. However, more than four rounds of panning may be required to select a better binding scFv population using this panning process.

B.3.4.2 Improvements to Synsorb-A panning

The previous Synsorb-A four rounds of panning did not select for better binding BGA scFv proteins. However this panning process can be the basis of a method that may potentially select scFv clones with improved binding affinity. Examination of the previous Synsorb-A panning rounds suggested some improvements to this panning method.

The Synsorb-A column previously used was an open system into which sample was manually introduced into the column. The system was improved so that the Synsorb-A beads were within a closed column system. Sample injection and fraction

collection were no longer done manually, the sample was syringe injected into the closed column using the valve system of an HPLC instrument, and the eluted fractions were collected using an HPLC fraction collector. This closed system ensures that the environment within the column remains constant and that buffer flow and fraction collection are consistent for all panning rounds.

One of the most important steps of the Synsorb-A panning process is the selection of specific eluted fractions for amplification. The eluted fractions selected are amplified, and used for the next round of panning; therefore, it is imperative that the fractions selected for amplification contain a population of binders from which better binding clones can be enriched and selected. Previously, the chromatogram profile of the Synsorb-A panning round was used exclusively to select the eluted fractions to be amplified. However, as can be seen from the large phage titres of the eluted combined fractions (Table IX), perhaps we are not selecting a more refined scFv population, and perhaps the better binding scFv clones would be found in later eluting fractions. Therefore, the selection of the fractions that would be amplified and used for further panning rounds should not only be determined by the chromatogram profile, but should also be determined from phage titres. Ideally the fraction selected for amplification should be a fraction that is eluted in the latter part of the chromatogram, and should have a relatively low (10^4) phage titre.

Figure 27 is a chromatogram of an improved Synsorb-A panning of the unpanned EP-TGASL#4 phagemid library. This improved panning utilized a closed system, into which the sample was injected, and fractions were collected using a fraction collector. Eighty 1 minute fractions were collected (250 µl/fraction), and fractions were randomly titred, so that the elution of phage from the column could be followed. For example, fraction # 3, # 44, and # 80 contained ~ 10^5, 10^7, 10^6 phage/ml respectively (please refer to Figure 27). Fraction # 3 was collected before the main BGA scFv-phage peak; this

fraction was screened, and all 9/9 randomly selected clones in this fraction contained the BGA scFv protein. Fraction #44 was collected after the main BGA scFv-phage peak, and 4/9 randomly selected clones contained the BGA scFv protein. Fraction # 80 was the last fraction collected, and surprisingly contained a high phage titre, the screening of this 80th fraction indicated that 4/9 randomly selected clones contained the scFv insert. These results will be further explained in the discussion; additional improvements will be suggested, and future experiments that would potentially expand this novel methodology will be addressed.

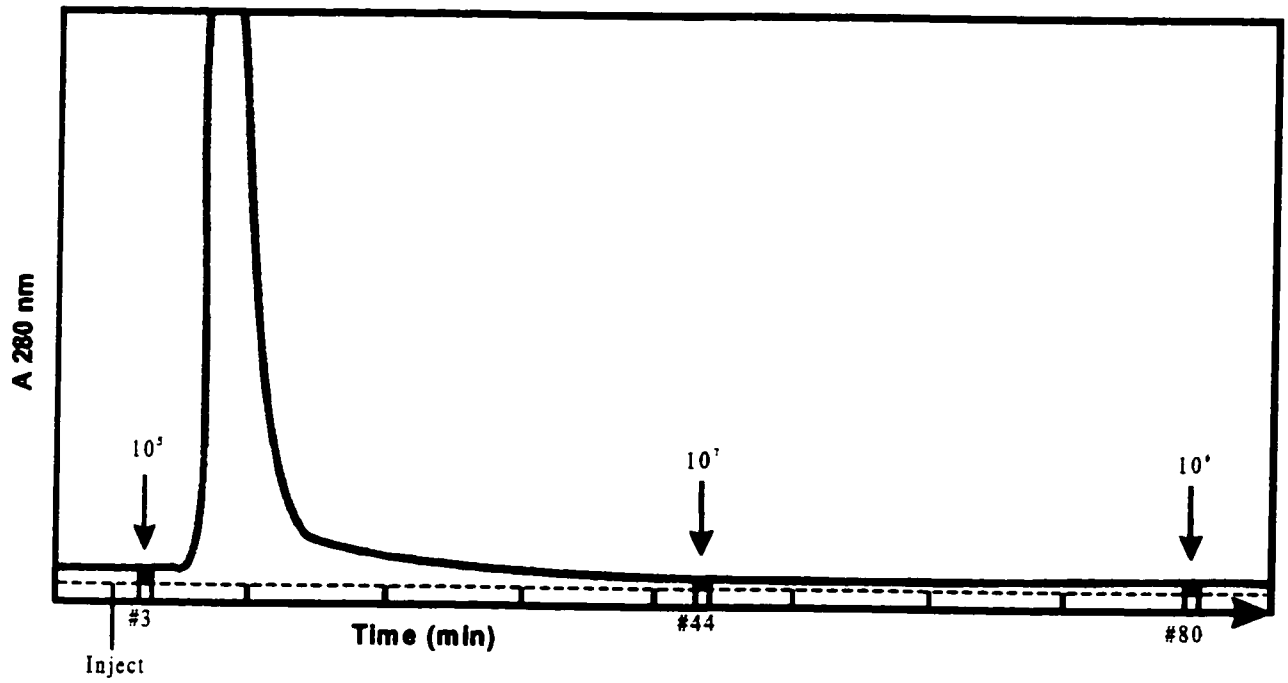


Figure 27: Chromatogram of an improved Synsorb-A panning of the unpanned EP-TGASL#4 phagemid library. The closed column system was equilibrated with PBS to give the initial baseline. 200 µl of unpanned EP-TGASL#4 phage was added to the column (labeled 'inject') and eluted with PBS. Eighty fractions were collected, and fractions # 3, # 44, and # 80 were titred and screened for BGA scFv insert. The phage titre of these fractions is indicated for each in phage/ml.

B.4 DISCUSSION

The library was constructed by using EP-PCR mutagenesis to randomly make mutations throughout the entire BGA scFv sequence. However, these mutations were made at relatively low frequencies, and as other groups have shown, such libraries can be successfully used for the isolation of clones with improved affinities (Deng *et al.*, 1994). Due to the very low binding affinity of our wild type monomeric BGA scFv, we increased avidity by reformatting this scFv, to display as diabodies (Holliger *et al.*, 1993). Phage display libraries can range in size, and can be as large as 10^{10} clones (Griffiths *et al.*, 1994), these large libraries usually being constructed for the isolation of antibodies to a variety of ligands. However, smaller libraries have been successfully panned (Parmley and Smith, 1988; Deng *et al.*, 1994; Smith and Scott, 1993), and our randomly mutated scFv repertoire of 10^6 clones is of sufficient size to contain a diversity of clones with a variety of binding affinities. A screening of randomly selected clones from this 10^6 library indicated that we had constructed a library of complete scFv proteins, all of which contained different random mutations throughout the V_L-V_H sequence. All the randomly screened clones from the unpanned EP-TGASL#4 library did not contain deletions or truncated sequences.

Selection procedures for screening a phage display library are almost as important as the library itself and therefore choosing the best or most adequate panning process can greatly affect the success of the experiment. The phagemid library described here is based on a scFv displaying a very weak antibody-carbohydrate interaction. The low affinity binding of the BGA scFv for the A-antigen has made the selection of clones from this library difficult. We began panning our EP-TGASL#4 library using standard procedures. However, these standard panning experiments often used libraries based on a stronger initial interaction than our original BGA scFv interaction. For this reason, these standard protocols did not allow for the proper screening of BGA scFv library.

Nevertheless, carrying out these typical panning process allowed for the better understanding of the limitations of these experiments, with respect to weakly-binding interactions, and precipitated the development of a new panning technique that aims at separating libraries of low affinity interactions. Furthermore, since we are using a phagemid system, the phage will only display the scFv monovalently. A disadvantage with monovalent display is that if some clones tend to dimerize, they will be selected for based solely on avidity, and will not allow for the selection of clones that exhibit increased in monovalent affinity, as was the case with Deng *et al.* (1995). Therefore, clones that tend to dimerize will always be selected over ones with slight increased in monovalent affinity (with no increase in dimerization). Using a phage system, instead of the phagemid system, will reduce the avidity advantage since the scFvs are always displayed multivalently.

B.4.1 PANNING IN MICROTITRE-WELLS

Phage display libraries have been successfully panned in microtitre wells (Deng *et al.*, 1994), but the optimum conditions for all panning processes will vary depending on the library. For example, the number of panning rounds, the stringency of panning, and the method of phage elution and amplification can be varied. Our EP-TGASL#4 library was panned according to the approved microtitre well panning. However, it was thought that our low affinity anti-carbohydrate library would not withstand a stringent panning process, and therefore the number of washes used for each panning round was small (please refer to Table I). The stringency was kept very low, because the off rate of the BGA scFv dimer is extremely rapid (Patenaude *et al.*, 1998).

The titres of the eluted phage after each panning round did not indicate enrichment for a phage population; all rounds yielding approximately 10^6 phage/ml (Table VI). There have been 20-fold (Marks *et al.*, 1991), 1000-fold (Mcafferty *et al.*,

1990), and even 1 000 000-fold (Barbas *et al.*, 1991) enrichments in bound phage, and this is a positive result with respect to the selection ability of phage display pannings. The lack of enrichment in this panning process already indicated that a better binding phage population had not been selected.

The microtitre well panning process that was used to screen our EP-TGASL#4 library seemed to select for truncated or deletion phage. Other groups have also been frustrated by the predominance of deletion products (Beekwilder *et al.*, 1999; Li *et al.*, 1998; Courtney *et al.*, 1995). Some have suggested that the displayed protein was toxic to its host, and affected the viability of the *E. coli* (Beekwilder *et al.*, 1999; Krebber *et al.*, 1996). If the protein displayed on the phage affects the viability of the *E. coli*, then the amplification process would select for deletion phage. Therefore, if the phage being selected in the amplification step are deletion phage, then the protein displaying clones would represent a very small proportion of the phage population used for the next panning rounds. This inability to select intact proteins is more difficult if the displayed protein is a weak binder.

The microtitre well panning process that was attempted to screen our EP-TGASL#4 phagemid library was dominated by deletion phage. Deletion products were not present in high concentration in the original EP-TGASL#4, since all the screened clones from this unpanned library had complete, intact scFv clones. However, deletion products could have been produced and favoured in the amplification step of the panning cycle. It is possible that deletion phage, those not hindered by an scFv, would be more infective and replicate much faster than the scFv displaying phage, and would potentially take over the amplified library, as seen by others (Beekwilder, 1999). Furthermore, since deletion phage may display only a portion of the scFv, it is possible that the exposed hydrophobic surface of the deletion scFv could potentially bind to the plastic microtitre

well surface or non-specifically bind to the coated surface, and then be eluted and amplified in the panning rounds.

B.4.1.1 Weak binding

Panning an anti-carbohydrate library is difficult, particularly because anti-carbohydrate antibodies have characteristically weak binding affinities. However, Deng *et al.* (1994) have selected an anti-carbohydrate scFv with an improved affinity using phage display. Multiple EP-PCR randomized Se155-4 scFv libraries were constructed, and five rounds of panning were carried out in microtitre wells against the *Salmonella* serogroup B *O*-polysaccharide. The majority of clones selected from these libraries did not exhibit an improved binding affinity, however, one scFv clone isolated demonstrated a 10-fold improvement in affinity (Deng *et al.*, 1994). This result is promising, and indicates that anti-carbohydrate antibodies can be panned using phage display, and that higher affinity clones can be isolated. It should be noted that the wild type *Salmonella* scFv used in this study has a 48-fold higher wild type affinity (K_D = 6 μM) than our wild type BGA scFv exhibits (K_D = 290 μM), thereby making panning with the BGA scFv more challenging. This group has also produced a phage display anti-carbohydrate library using the controlled mutagenesis of the three V_H chain CDRs (Deng *et al.*, 1995). This library was then panned against its *Salmonella* carbohydrate antigen, and clones with an improved affinity were isolated. However, these higher affinity clones were not of the wild type monomeric scFv format. Instead, the improvements in affinity were a result of mutations that caused the production of higher avidity clones, and therefore these dimeric and multimeric mutants exhibited an increase only in their functional affinity. Again, these avidity effects would be reduced with the use of a phage system.

B.4.2. BIACORE PANNING

Malmborg and Boorebaeck suggested that the BIACORE could be used to pan libraries based on their dissociation constants (Malmborg and Boorebaeck, 1995), and successfully panned a high affinity protein library on the BIACORE (Malmborg *et al.*, 1996). Panning on the BIACORE is now possible, but panning a low affinity anti-carbohydrate scFv library on the BIACORE is more involved, and more troublesome. Furthermore, the panning conditions needed to pan such a low affinity anti-carbohydrate library have not yet been determined. Control experiments using wild type phage should be used to optimize the flow rate and fraction collection of eluate, to compare various antigens, and to minimize non-specific binding. Similar to the microtitre well pannings, panning on the BIACORE also seemed to be hindered by deletion products, and again this could be a result of the amplification step of the panning cycle.

B.4.3 PANNING ON SYNSORB-A COLUMN

The standard panning experiments used above involve the binding of the phage displayed protein to an immobilized ligand, the removal of unbound phage, and the elution of the bound clones. These processes however were not capable of screening our low affinity library, therefore necessitating the pursuit of another panning process.

Panning has been carried out on affinity columns (McCafferty *et al.*, 1990; Pini *et al.*, 1997; Clackson *et al.*, 1991), the basis of these affinity column panning methods being the same as other standard panning methods. Affinity column pannings allow a phage displayed library to bind onto a column containing an immobilized ligand, then the unbound phage is washed off the column, and the bound phage is then eluted. These processes assume that the binding of the phage displayed protein is sufficiently strong that it can withstand the separation procedure that is inherent to any panning process. However, the binding affinity of our phage displayed BGA scFv is very weak, and does

not allow the effective binding, separation and elution steps of standard panning processes. We have therefore developed a panning method that allows the separation of our low affinity anti-carbohydrate antibody library based not on binding, washing and eluting, but based on the dynamic separation of a sample into its various components. The Synsorb-A panning methodology developed separated our low affinity library based on the transient binding of the phage displayed proteins as they flowed through a column containing A-antigen

Preliminary trials on this column separated an IMAC purified BGA scFv soluble protein sample into active and inactive protein. The separation of sample into its active and inactive components indicated that panning is possible on this Synsorb-A column. Separation of sample on the Synsorb-A column occurs as the components of the sample temporarily bind to the column.

To pan the EP-TGASL#4 library using the Synsorb-A column, we relied on the dynamic separation of sample as it flowed through the column. The scFv-phage with higher affinities transiently bound to the column for longer periods of time than lower affinity scFv-phage, and as a result would be retained by the column, and would be eluted in later fractions. The advantage of the Synsorb-A column is that it allows for the rebinding of sample as it percolates through the column. This therefore solves the problem of fast off rates that are characteristic to many weak binding proteins. The separation of sample was monitored by recording a chromatogram of the eluate fractions.

The selection of fractions for amplification and for subsequent use in the next panning round was initially based solely on the chromatogram profile. However, this crude method of fraction selection was shown to be inadequate. Figure 27 illustrates the chromatogram of the first round of panning of the EP-TGASL#4, and titres of the collected fraction indicate that 10^6 phage/ml remained bound on the column in the 80^{th} fraction. If we refer to Table IX and Figure 25, we can see that we were amplifying

fractions of mediocre binders, and the better binding phage in the later fractions were not collected. This was the first time panning has been performed using this methodology, and it was assumed that no phage would remain bound to the column in the more subsequent fractions, and thus only the earlier fractions were collected.

A random screening of the 4th round elution clones indicated that panning on the Synsorb-A column did not select for deletion products (Figure 26). Only 2 out of the 9 clones screened were deletions in the 4th round Synsorb-A panning, compared to 8/10 and 11/12 deletions in the 4th round microtitre well and BIACORE pannings respectively. The lack of deletion products in the Synsorb-A panning indicates that this panning methodology can potentially be used to successfully pan our EP-TGASL#4 phagemid library and other low affinity libraries. The lack of or decrease in deletion products can be a direct result of the Synsorb-A column. Since the Synsorb-A column displays the A-antigen on the surface of diatomaceous earth and not on a plastic surface, there may be a reduction in the amount of non-specific binding events. This decrease in non-specific binding would therefore allow the deletion phage to quickly fall through the Synsorb-A column, as did the non-specific *Salmonella* scFv, thereby allowing the scFv containing phage to be efficiently separated.

B.4.3.1 Improvements to Synsorb-A methodology

The affinity screening of the 4th pan Synsorb-A using phage ELISA, and the subsequent DNA sequencing of the high affinity clones did not indicate sequence homology in the selected clones. However, upon examination of the titres of the collected fractions (Table IX), and the crude manner in which fractions were selected for amplification (Figure 25), this is not a surprising result. The amplification of mediocre binders in the earlier eluted fractions, and as a result the elimination of the better binders in the later fractions, would dramatically hinder the selection process. Ideally, the

fraction that contains the better binders in each round should be selected and amplified, and then be subjected to another affinity separation cycle.

Improvements to this initial panning process already seem to have refined the methodology. The four rounds of panning performed were done on an open system column, into which sample was manually injected, and fractions were manually collected. This system was modified into a closed system column so that the environment within the column and the buffer flow remain consistent for all panning rounds. Furthermore, sample was syringe injected in a more controlled manner, and fractions were more accurately collected using a fraction collector. More importantly a more accurate way of selecting fractions for amplification should allow for the successful affinity selection of BGA scFv clones on this column. The Synsorb-A panning methodology developed here has established a new panning technique that has the potential to pan a low affinity library, and can prove to be very useful for the affinity panning of weak binding anti-carbohydrate antibody libraries.

B.4.4 FUTURE EXPERIMENTS

The construction of a larger BGA scFv phagemid library should be constructed if future panning experiments are to be expanded. The library repertoires generated can be randomized or can be specific to a particular region, for example a CDR H3 library. This library can be a gIII, or gIII phagemid library. However the construction of a large gIII phage library using a phage vector would allow for the multivalent display of the BGA scFv. Multivalent display using a phage vector would reduce any avidity advantages. Furthermore, this increase in avidity may help select for clones with improved affinity. The production of a BGA scFv mutant, L103V, with a 30-fold increase in affinity in the site-directed mutagenesis study could be used in a phage display study. The L103V

stronger binding mutant can be used as the wild type clone upon which the constructed library is based.

Control panning experiments using wild type phage should be panned alongside all panning experiments, to determine if deletion products are a result of the panning cycles (specifically the amplification cycle) or if deletion clones are a result of the panning process. Eluted clones should be screened after each round of panning, and not simply following the final panning round.

An important future experiment is the panning of a BGA scFv library on the Synsorb-A column using the improvements discussed in this thesis. Fractions should be screened prior to amplification, and all fractions should be titred. Following the PBS wash to baseline, GalNAc can be added to the column to verify if sample can be eluted from the column. And finally if deletion products are observed in the eluted or the amplified fractions, the addition of Synsorb-B may help eliminate these deletion and truncated proteins through non-specific binding.

REFERENCES

Anand, N.N., Dubuc, G., Phipps, J., MacKenzie, C.R., Sadowska, J., Young, N.M., Bundle, D., and Narang, S.A. (1991) Synthesis and expression in *Escherichia coli* of cistronic DNA encoding an antibody fragment specific for a *Salmonella* serogroup B O-antigen. *Gene* **100**, 39-44.

Anand, N.N., Mandal, S., MacKenzie, C.R., Sadowska, J., Sigurskjold, B., Young, N.M., Bundle, D. R., and Narang, S.A. (1991) Bacterial expression and secretion of various single-chain genes encoding proteins specific for a *Salmonella* serogroup B O-antigen. *J. Biol. Chem.* **266**, 21874-21879.

Barbas, C., Kang, A. S., Lerner, R. A., and Benkovic, S. J. (1991) Assembly of combinatorial antibody libraries on phage surfaces: The gene III site. *Proc. Natl. Acad. Sci. U. S. A.* **88**,7978-7982.

Barbas, C. F., Bain, J. D., Hoekstra, D. M., and Lerner, R. A. (1992) Semisynthetic combinatorial antibody libraries: a chemical solution to the diversity problem. *Proc. Natl. Acad. Sci. U. S. A.* **89**, 4457-4461.

Bass, S. H., Greene, R., and Wells, J. A. (1990) Hormone phage: An enrichment method for variant proteins with altered binding properties. *Proteins* **8**, 309-314.

Bedzyk, W. D., Weidner, K. M., Denzin, L. K., Johnson, L. S., Hardman, K. D., Pantoliano, M. W., Asel, E. D., and Voss, E. W., Jr. (1990) Immunological and structural characterization of a high affinity anti-fluorescein single-chain antibody. *J. Biol. Chem.* **265**, 18615-18620.

Beekwilder, J., Rakonjac, J., Jongsma, M., and Bosch, D. (1999) A phagemid vector using the E. coli phage shock promoter facilitates phage display of toxic proteins. *Gene* **228**, 23-31.

Berek, C., and Milstein, C. (1987) Mutation drift and repertoire shift in the maturation of immune response. *Immunol. Rev.* **96**, 23-41.

Better, M., Chang, C. P., Robinson, R. R., and Horwitz, A. H. (1988) *Escherichia coli* secretion of an active chimeric antibody fragment. *Science* **240**, 1041-1043.

BIAevaluation 3.0 Software Handbook, edition July 1997.

Bird, R. E., Hardman, K. D., Jacobson, J. W., Johnson, S., Kaufman, B. M., Lee, S.-M., Lee, T., Pope, S. H., Riordan, G. S., and Whitlow, M. (1988) Single-chain antigen-binding proteins. *Science* **242**, 423-426.

Bondeson, K., Frostell-Karlsson, Å., Fägerstam, L., and Magnusson, G. (1993) Lactose repressor-operator DNA interactions: kinetic analysis by a surface plasmon resonance biosensor. *Anal. Biochem.* **214**, 245-251.

Bradbury, A., Persic, L., Werge, T., and Cattaneo, A. (1993) Use of living columns to select specific phage antibodies. *Biotechnology* **11**, 1565-1569.

Brinkmann, U., Chowdhury, P. S., Roscoe, D. M., and Pastan, I. L. (1995) Phage display of disulfide-stabilized Fv fragments. *J. Immunol. Methods* **182**, 41-50.

Brummel, D. A., Sharma, V. P., Anand, N. N., Bilous, D., Dubuc, G., Michniewicz, J., MacKenzie, C. R., Sadowska, J., Narang, S. A., Sigurskjold, B. W., Sinnott, B., Young, N. M. and Bundle, D. R. (1993) Probing the combining site of an anti-carbohydrate antibody by saturation mutagenesis: role of the heavy chain CDR3 residues. *Biochemistry* **32**, 1180-1187.

Buckle, M., Williams, R. M., Negroni, M., and Buc, H. (1996) Real time measurements of elongation by a reverse transcriptase using surface plasmon resonance. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 889-894.

Carver, J. P., Michnick, S. W., Imberty, A. and Cumming, D. A. (1991) Oligosaccharide-protein interactions: a three-dimensional view. *Ciba. Found. Symp.* **158**, 6-26.

Chen, H.-T., Kabat, E. A., Lundbald, A. and Ratcliffe, R. M. (1987) Nucleotide and translated amino acid sequences of cDNA coding for the variable regions of the light and heavy chains of mouse hybridoma antibodies to blood group A and B substances. *J. Biol. Chem.* **262**, 13579-13583.

Chothia, C. and Lesk, A. M. (1987) Canonical structures of the hypervariable regions of immunoglobulins. *J. Mol. Biol.* **196**, 901-917.

Chothia, C., Lesk, A. M., Tramontano, A., Levitt, M., Smith-Gill, S. J., Air, G., Sheriff, S., Padlan, E. A., Davies, D., Tulip, W. R., Colman, P. M., Spinelli, S., Alzari, P. M. and Poljak, R. J. (1989) Conformations of immunoglobulin hypervariable regions. *Nature* **342**, 877-883.

Clackson, T., Hoogenboom, H. R., Griffiths, A. D., and Winter, G. (1991) Making antibody fragments using phage display libraries. *Nature* **352**, 624-628.

Cortese, R., Monaci, P., Nicosia, A., Luzzago, A., Felici, F., Galfre, G., Pessi, A., Tramontano, A., and Sollazzo, M. (1995) Identification of biologically active peptides from random libraries displayed on phage. *Curr. Opin. Biotechnol.* **6**, 73-80.

Courtney, B. C., Williams, K. C. and Schlager, J. .J (1995) A phage display vector with improved stability, applicability and ease of manipulation. *Gene* **165**, 139-140.

Cygler, M., Rose, D. R. and Bundle, D. R. (1991) Recognition of a cell-surface oligosaccharide of pathogenic *Salmonella* by an antibody Fab fragment. *Science* **253**, 442-445.

Cunningham, B. C. and Wells, J. A. (1993) Comparison of a structural and a functional epitope. *J. Mol. Biol.* **234**, 554-563.

Cwirla, S. E., Peters, E. H., Barrett, R. W., and Dower, W. S. (1990) Peptides of phage: A vast library of peptides for identifying ligands. *Proc. Natl. Acad. Sci. U. S. A.* **87**, 6378-6382.

Dall'Aqua, W. and Carter, P. (1998) Antibody engineering. *Curr. Opin. Struct. Biol.* **8**, 443-450.

de Kruif, J., Terstappen, L., Boel, E., and Logtenberg, T. (1995) Rapid selection of cell subpopulation-specific human monoclonal antibodies from a synthetic phage antibody library. *Proc. Natl. Acad. Sci. U. S. A.* **92**, 3839-3842.

Deng, S.-J., MacKenzie, C. R. and Narang, S.A. (1993) Simultaneous randomization of antibody CDRs by a synthetic ligase chain reaction strategy. *Nucleic Acids Res.* **21**, 4418-4419.

Deng, S.-J., MacKenzie, C. R., Sadowska, J., Michniewicz, J., Young, N. M., Bundle, D. R., and Narang, S.A. (1994) Selection of antibody single-chain variable fragments with improved carbohydrate binding by phage display. *J. Biol. Chem.* **269**, 9533-9538.

Deng, S.-J., MacKenzie, C.R., Hirama, T., Brousseau, R., Lowary, T.L., Young, N.M., Bundle, D.R., and Narang, S.A. (1995) Basis for selection of improved carbohydrate-binding single-chain antibodies from synthetic gene libraries. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 4992-4996.

Drickamer, K., and Carver, J. (1992) *Curr. Opin. Struct. Biol.* **2**, 653-654.

Evans, S. V. (1993) SETOR: hardware-lighted three-dimensional solid model representations of macromolecules. *J. Mol. Graphics*. 11, 134-138.

Fleischman, J.B., Pain, R.H., and Porter, R.R., (1962): Reduction of gammaglobulins. *Arch. Biochem. Biophys. Suppl.*, 1:174.

Frost, L. S. (1993) Conjugative pili and pilus-specific phages. In *Bacterial conjugation*. (ed. D. B. Clewell), pp. 189-221. Plenum, New York.

Frost, L. S., Ippen-Ihler, K., and Skurray, R. A. (1994) Analysis of the sequence and gene products of the transfer region of the F factor. *Microbiol. Rev.* **58**, 162-210.

Garrard, L. J., Yang, M., O'Connell, M. P., Kelley, R. F., and Henner, D. J. (1991) Fab assembly and enrichment in a monovalent phage display system. *Biotechnology* **9**, 1373-1377.

Glaser, R. W. and Hausdorf, G. (1996) Binding kinetics of an antibody against HIV p24 core protein measured with real-time biomolecular interaction analysis suggest a slow conformational change in antigen p24. *J. Immunol. Methods* **189**, 1-14.

Glaser, S. M., Yelton, D. E. and Huse, W. S. (1992) Antibody engineering by codon-based mutagenesis in a filamentous phage vector system. *J. Immunol.* **149**, 3903-3913.

Glockshuber, R., Malia, M., Pfitzinger, I., and Pluckthum, A. (1990) A comparison of strategies to stabilize immunoglobulin Fv-fragments. *Biochemistry* **29**, 1362-1367.

Gram, H., Marconi, L.-A., Barabas, C. F., III, Collet, T. A., Lerner, R. A.and Kang, A. S. (1992) In vitro selection and affinity maturation of antibodies from a naive combinatorial immunoglobulin library. *Proc. Natl. Acad. Sci. U. S. A.* **89**, 3567-3580.

Greenwood, J., Willis, A. E., and Perham, R. N. (1991) Multiple display of foreign peptides on a filamentous bacteriophage. Peptides from Plasmodium falciparum circumsporozoite protein as antigens. *J. Mol. Biol.* **220**, 821-827.

Griffiths, A. D., Malmqvist, M., Marks, J. D., Bye, J. M., Embleton, M. J., McCafferty, J., Baier, M., Holliger, K. P., Gorick, B. D., Hughes-Jones, N. C., Hoogenboom, H. R., and Winter, G. (1993) Human anti-self antibodies with high specificity from phage display libraries. *EMBO J.* **12**, 725-734.

Griffiths, A. D., Williams, S. C., Hartley, O., Tomlinson, I. M., Waterhouse, P., Crosby, W. L., Kontermann, R. E., Jones, P. T., Low, N. M., Allison, T. J., Prospero, T. D., Hoogenboom, H. R., Nissim, A., Cox, J. P. L., Harrison, J. L., Zaccolo, M., Gherardi, E., and Winter, G. (1994) Isolation of high affinity human antibodies directly from large synthetic repertoires. *EMBO J.* **13**, 3245-60.

Griffiths, A. D. and Duncan, A. R. (1998) Strategies for selection of antibodies by phage display. *Curr. Opin. Biotech.* **9**, 102-108.

Hanes, J. and Plückthun, A. (1997) In vitro selection and evolution of functional proteins using ribosome display. *Proc. Natl. Acad. Sci. U. S. A.* **94**, 4937-4942.

Hawkins, R. E., Russell, S. J., and Winter, G. (1992) Selection of phage antibodies by binding affinity: mimicking affinity maturation. *J. Mol. Biol.* **226**, 889-896.

He, M. and Taussig, M J. (1997) Antibody-ribosome-mRNA (ARM) complexes as efficient selection particles for in vitro display and evolution of antibody combining sites. *Nucleic Acids Res.* **25**, 5132-5134.

Holliger, P., Prospero, T. and Winter, G. (1993) "Diabodies": small bivalent and bispecific antibody fragments *Proc. Natl. Acad. Sci. U.S.A.* **90**, 6444-6448.

Hoogenboom, H. R., Griffiths, A. D., Johnson, K. S., Chiswell D. J., Hudson, P. and Winter, G. (1991) Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. *Nucl. Acids Res.* **19**, 4133-4137.

Hoogenboom, H. R. and Winter, G. (1992) By-passing immunisation. Human antibodies from synthetic repertoires of germline VH gene segments rearranged in vitro. *J. Mol. Biol.* **227**, 381-388.

Hoogenboom, H. R., Marks, J. D., Griffiths, A. D. & Winter, G. (1992) Building antibodies from their genes. *Immunol. Rev.* **130**, 41-68.

Hoogenboom, H. R. (1997) Designing and optimizing library selection strategies for generating high-affinity antibodies. *Trends Biotechnol.* **15**, 62-70.

Hoogenboom, H. R., de Bruïne, A. P., Hufton, S. E., Hoet, R. M., Arends, J.-W., and Roovers, R. C. (1998) Antibody phage display technology and its applications. *Immunotechnology* **4**, 1-20.

Huston, J. S., Levinson, D., Mudgett-Hunter, M., Tai, M.-S., Novotny, J., Marjolies, M. N. Ridge, R. J., Bruccoleri, R. E., Haber, E., Crea, R., and Opperman, H. (1988) Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in Escherichia coli. *Proc. Natl. Acad. Sci. U. S. A.* **85**, 5879-5833.

Il'ichev, A. A., Minenkova, O. O., Tat'kov, S. I., Karpyshev, N. N., Eroshkin, A. M., Ofitserov, V. I., Akimenko, Z. A., Petrenko, V. A., and Sandakhchiev, L. S. (1990) [The use of filamentous phage M13 in protein engineering]. *J. Mol. Biol Mosk.* **24**, 530-535.

Jeffrey, P. D., Bajorath, J., Chang, C. Y., Yelton, D., Hellström, I., Hellström, K. E. and Sheriff, S. (1995) The X-ray structure of an anti-tumour antibody in complex with antigen. *Nature Structural Biology* **2**, 466-471.

Jerne, N. J., (1973) The Immune System, *Sci. Amer.* **229**, 52-64.

Johanson, K., Appelbaum, E., Doyle, M., Hensley, P., and Zhao, B., et al. (1995) Binding interactions of human interleukin 5 with its receptor subunit. *J. Biol. Chem.* **270**, 9459-9471.

Jönsson, U., Fägerstam, L., Ivarsson, B., Johnsson, B., Karlsson, R., Lundh, K., Löfås, S., Persson, B., Roos, H., Rönnberg, I., Sjölander, S., Stenberg, E., Ståhlberg, R., Urbaniczky, C., Östlin, H., and Malmqvist, M. (1991) Real-time biospecific interaction analysis using surface plasmon resonance and a sensor chip technology. *BioTechniques* **11**, 620-627.

Kabat, E.A. (1968). Structural concepts in immunology and immunochemistry. pp. 53-62. Holt, Rinehart and Winston, Inc., New York.

Kabat, E. A., Wu, T. T., Perry, H. M., Gottesman, K. S., and Foeller, C. (1991) Sequences of proteins of immunological interest, 5th Edition. Bethsada, MD: US Department of Health and Human Services, Public Health Service, National Institutes of Health, NIH Publication No. 91-3242.

Kabat, E. A. and Wu, T. T. (1991) Identical V region amino acid sequences and segments of sequences in antibodies of different specificities. Relative contributions of VH and VL genes, minigenes, and complementarity-determining regions to binding of antibody-combining sites. *J. Immunol.* **147**, 1709-1719.

Kay, B. K., Winter, J. and McCafferty, J. (1996) *Phage Display of Peptides and Proteins: A Laboratory Manual*, Academic Press Inc., San Diego.

Kelley, R. F. and O'Connell, M. P. (1993) Thermodynamic analysis of an antibody functional epitope. *Biochemistry* **32**, 6828-6835.

King, M.-J. (1994) *Biochm. Biophys. Acta.* **1197**, 15-44.

Kobrin, B. J., Buhl, S., Shulman, M. J. and Scharff, M. D. (1991) A V region mutation in a phosphocholine-binding monoclonal antibody results in loss of antigen binding, *J. Immunology*, **146**, 2017-2020.

Komissarov, A. A., Marchbank, M. T., Calcutt, M. J., Quinn, T. P. and Deutscher, S.L. (1997) Site-specific mutagenesis of a recombinant anti-single stranded DNA Fab. *J. Biol. Chem.* **272**, 26864-26870.

Krebber, A., Burmester J., and Pluckthun, A. (1996) Inclusion of an upstream transcriptional terminator in phage display vectors abolishes background expression of toxic fusions with coat protein g3p. *Gene* **178**, 71-74.

Kuby, Janis (1998) *Immunology*, 3rd Edition, W. H. Freeman and Co., New York.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head bacteriophage T4, *Nature* **227**, 680-685.

Lavoie, T. B., Drohan, W. N. and Smith-Gill, S. J. (1992) Experimental analysis by site-directed muatgenesis of somatic mutation effects on affinity and fine specificity in antibodies specific for lysozyme. *J. of Immunology* **148**, 503-513.

Lemieux, R. U. (1989) The origin of the specificity in the recognition of oligosaccharides by protein. *Chem. Soc. Rev.* **18**, 347-374.

Lemieux, R. U., Delbaere, L. T. J., Beierbeck, H. and Spohr, U. (1991) Involvement of water in host-guest interactions. *Ciba. Found. Symp.* **158**, 231-248.

Li, Y., Cockburn, W., and Whitelam, G. C. (1998) Filamentous bacteriophage display of a bifunctional protein A::scFv fusion. *Mol. Biotechnol.* **9**, 187-193.

Liedberg, B., Nylander, C., and Lundström, I. (1983) *Sensors Actuators* **4**, 299-304.

Lowman, H. B., Bass, S. H., Simpson, N., and Wells, J. A. (1991) Selecting high-affinity binding proteins by monovalent phage display. *Biochemistry* **30**, 10832-10838.

MacKenzie, C.R., Sharma, V., Brummell, D., Bilous, D., Dubuc, G., Sadowska, J., Young, N.M., Bundle, D.R., and Narang, S.A. (1994) Effect of C-C domain switching on Fab activity and yield in Escherichia coli: synthesis and expression of genes encoding two anti-carbohydrate Fabs. *Biotechnology* **12**, 390-395.

MacKenzie, C. R., Hirama, T., Deng, S.-J., Bundle, D. R., Narang, S. A. and Young, N. M. (1996) Analysis by surface plasmon resonance of the influence of valence on the ligand binding affinity and kinetics of an anti-carbohydrate antibody. *J. Biol. Chem.* **271**, 1527-1533.

Malmborg, A. C, and Borrebaeck, C. A. (1995) BIAcore as a tool in antibody engineering. *J. Immunol. Methods* **183**, 7-13.

Malmborg, A. C, Duenas, M., Ohlin, M., Soderlind, E., and Borrebaeck, C.,A. (1996) Selection of binders from phage displayed antibody libraries using the BIAcore biosensor. *J. Immunol. Methods* **198**(1), 51-57.

Markley, F. (1997) Catering for kinetic awarness. *BIAjournal* **1**, 5-9.

Marks, J. D., Hoogenboom, H. R., Bonnert, T. P., McCafferty, J., Griffiths, A. D., and Winter, G. J. (1991) By-passing immunization: human antibodies from V-gene libraries displayed on phage *J. Mol. Biol.* **222**, 581-597.

Marks, J. D., Ouwehand, W. H., Bye, J. M., Finnern, R., Gorick, B. D., Voak, D., Thorpe, S., Hughes-Jones, N. C., and Winter, G. (1993) Human antibody fragments specific for human blood group antigens from a phage display library. *Bio/Technology* **11**, 1145-1149.

Masson, L., Mazza, A., and Brousseau, R. (1994) Stable immobilization of lipid vesicles for kinetic studies using surface plasmon resonance. *Anal. Biochem.* **218**, 405-412.

Mattheakis, L. C., Bhatt, R. R., and Dower, W. J. (1994) An *in vitro* polysome display system for identifying ligands from very large peptide libraries. *Proc. Natl. Acad. Sci. U. S. A.* **91**, 9022-9026.

McCafferty, J., Griffiths, A. D., Winter, G., and Chiswell, D. J. (1990) Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* **348**, 552-554.

McManus, S. and Reichmann, L. (1991) Use of 2D NMR, protein engineering, and molecular modeling to study the hapten-binding site of an antibody F_v fragment against 2-phenyloxazolone. *Biochemistry* **30**, 5851-5857.

Myszka, D. G. (1997) Kinetic analysis of macromolecular interactions using surface plasmon resonance biosensors. *Current Opinion in Biotechnology* **8**, 50-57.

Near, R. I., Mudgett-Hunter, M., Novotny, J., Bruccoleri, R. and Ng, S. C. (1993) Characterization of an anti-digoxin antibody binding site by site-directed *in vivo* mutagenesis. *Molecular Immunology*, **30**, 369-37.

Parmley, S. F., and Smith, G. P. (1988) Antibody-selectable filamentous fd phage vectors: Affinity purification of target genes. *Gene* **73**, 305-318.

Patenaude, S. I., MacKenzie, C. R., Bilous, D., To, R. J., Ryan, S. E., Young, N. M. and Evans, S. V. (1998) *Acta Crystallog.* D54, 1456-1459.

Patten, P. A., Gray, N. S., Yang, P. L., Marks, C. B., and Wedemayer, G. J. (1996) The immunological evolution of catalysis. *Science* **271**, 1086-1091.

Paul, William E. (1989) *Fundamental Immunology*, 2nd Edition, Raven Press, New York.

Pecht, I. (1992) *Aspects Antibody Funct.* **6**, 1-68.

Pellequer, J. L. and van Regenmortel, M. H. V. (1993) Measurement of kinetic binding constants of viral antibodies using a new biosensor technology. *J. Immunol. Methods* **166**, 133-143.

Pini, A., Spreafico, A., Botti, R., Neri, D., and Neri, P. (1997) Hierarchical affinity maturation of a phage derived antibody for the selective removal of cytomegalovirus from plasma. *J. Immunol. Methods* **206**, 171-182.

Plückthum, A. and Pack, P. (1997) *Immunotechnology* **3**, 83-105.

Quiocho, F. A. (1991) *Curr. Opin. Struct. Biol. 1*, 922-933.

Ramsden, J. J. and Schneider, P. (1993) Membrane insertion and antibody recognition of a glycosylphosphatidylinositol-anchored protein: An optical study. *Biochemistry* **32**, 523-529.

Reichmann, L., Weill, M. and Cavangh, J. (1992) Improving the antigen affinity of an antibody Fv-fragment by protein design. *J. Mol. Biol.* **224**, 913-918.

Reichmann, L. and Weill, M. (1993) Phage Display and selection of a site-directed randomized single-chain antibody Fv fragment for its affinity improvement. *Biochemistry* **32**, 8848-8855.

Roitt, I. M., Brostoff, J., and Male, D. K., (1993) *Immunology*, Mosby, St.Louis, 5.1-6.5.

Ruff-Jamison, S. and Gleeney, J. R. Jr. (1993) Molecular modellind and site-directed mutagenesis of an anti-phosphotyrosine antibody predicts the combining site and allows the detection of higher affinity interactions. *Protein Engineering* **6**, 661-668.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sanger, F., and Coulson, A.R. (1975) A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase, *J. Mol. Biol.* **94**, 441-448.

Scatchard, G. (1949) *Ann. New York Acad. Sci.* **51**, 660-672.

Scott, J. K., and Smith, G. P. (1990) Searching for peptide ligands with an epitope library. *Science* **249**, 386-390.

Schier, R. McCall, A. Adams, G. P., Marshall, K. W., Merritt, H., Yim, M. Crawford, R. S., Weiner, L. M., Marks, C. and Marks, J. D. (1996) Isolation of picomolar affinity anti-c-erbB-2 single-chain Fv by molecular evolution of the complementarity determining regions in the center of the antibody binding site. *J. Mol. Biol.*, **263**, 551-567.

Schuck, P. (1997) Use of surface plasmon resonance to probe the equilibrium dynamic aspects of interactions between biological macromolecules. *Annu. Rev. Biophys. Struct.* **26**, 541-566.

Seymour, G. J., Savage, N. W., and Walsh, L. J. (1995) *Immunology: an Introduction for the Health Sciences*, pp. 109-110, Roseville: McGraw-Hill.

Sharon, J. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 4814-4817.

Sigurskjold, B. W., Altman, E. and Bundle, D. R. (1991) *Eur. J. Biochem.* **197**, 239-246.

Sigurskjold, B. W. and Bundle, D. R. (1992) Thermodynamics of oligosaccharide binding to a monoclonal antibody specific for a Salmonella O-antigen point to hydrophobic interactions in the binding site. *J. Biol. Chem.* **267**, 8371-8376.

Sjölander, S. and Urbaniczky, C. (1991) Integrated fluid handling system for biomolecular interaction analysis. *Anal. Chem.* **63**, 2338-2345.

Skerra, A. and Plückthum. A. (1988) *Science* **240**, 1038-1041.

Skerra, A., Pfitzinger, I. and Plückthum. A. (1991) The fundamental expression of antibody F_v fragments in *Escherichia coli*: Improved vectors and a generally applicable purification technique. *Bio/Technology* **9**, 273-278.

Smith, G. P. (1985) Filamentous fusion phage: Novel expression vectors that display cloned antigens on the surface of the virion, *Science* **228**, 1315-1317.

Smith, G. P. and Scott, K. J. (1993) Libraries of peptides and proteins displayed on filamentous phage. *Methods in Enzymology* **217**, 228-257.

Spada, S. and Plückthum, A. (1997) Selectively infective phage (SIP) technology: a novel method for *in vivo* selection of interacting protein-ligand pairs. *Natl. Med.* **3**, 694, 696.

Stenberg, E., Persson, B., Roos, H., and Urbaniczky, C. (1991) Quantitative determination of surface concentration of protein with surface plasmon resonance by using radiolabelled proteins. *J. Colloid Interface Sci.* **143**, 513-526.

Stemmer, W. P. C, Morris, S. K., Kautzer, C. R. and Wilson, B. S. (1993) Increased antibody expression from *Escherichia coli* through wobble-base library mutagenesis by enzymatic inverse PCR. *Gene* **123**, 1-7.

Thomas, R., Patenaude, S., MacKenzie, C. R., Young, N. M., and Evans, S. V. (1999) manuscript in preparation.

Thompson, J., Pope, T., Tung, J.-S., Chan, C., Hollis, G., Mark, G. and Johnson, K. S. (1996) Affinity Maturation of a High-affinity Human Monoclonal antibody against the third hypervariable loop of human immunodeficiency virus: use of phage display to improve affinity and broaden strain reactivity, *J. Mol. Biol.* **256**, 77-88.

Tizzard, I. R. (1984) *Immunology, an Introduction*, pp. 368-370. Philadelphia: Saunders College Publishing.

Tomlinson, I. M. (1998) Immunoglobulin genes. In *Encyclopedia of Immunology* 2nd Edition. (ed Delves, P. J. and Roitt, I.), Academic Press, London.
(http://www.mrc-cpe.cam.ac.uk/imt-doc/vbase-home-page.html)

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, *Proc. Natl. Acad. Sci. USA* **76**,4350-4354.

Vaughan, T. P., Williams, A. W., Pritchard, K., Osbourn, J. K., Pope, A. R., Earshaw, J. C., McCafferty, J., Hodits, R. A., Wilton, J., and Johnson, K. S. (1996) Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. *Nature Biotechnol.* **14**, 309-314.

Vermersch, P. S., Tesmer, J. J. G., Lemon, D. D. and Quiocho, F. A. (1990) A Pro to Gly Mutation in the Hinge of the Arabinose-binding Protein enhances binding and alters specificity. *J. Biol. Chem.* **265**, 16592-16603.

Vermersch, P. S., Lemon, D. D., Tesmer, J. J. G and Quiocho, F. A. (1991) Sugar-Binding and Crystallographic Studies of an Arabinose-Binding Protein Mutant ($Met^{108}Leu$) that exhibits enhanced affinity and altered specificity. *Biochemisty* **30**, 6861-6866.

Vieira, J., and Messing, J. (1987) Production of single-stranded plasmid DNA. *Methods Enzymol.* **153**, 3-11.

Ward, A. C. (1992) Rapid analysis of yeast transformants using colony-PCR. *Biotechniques* **13** (3): 350.

Wedemayer, G. J., Patten, P. A., Wang, L. H., Schultz, P.G. and Stevens, R. C. (1997) Structural insights into the evolution of an antibody combining site. *Science* **276**, 1665-1669.

Webster, R. (1996) Biology of Filamentous Bacteriophage. In *Phage Display of Peptides Proteins* (ed. Kay, B. K., Winter, J. and McCafferty, J.), pp. 1-20. Academic Press, Inc., New York.

Wilson, I. A. and Stanfield, R. L. (1994) *Curr. Opin. Struct. Biol.* **4**, 857.

Winter, G., and Milstein, C. (1991) Man-made antibodies. *Nature* **349**, 283-299.

Winter, G., Griffiths, A. D., Hawkins, R. E., and Hoogenboom, H. R. (1994) Making antibodies by phage display technology. *Ann. Rev. Immunol.* **12**, 433-455.

Xiang, J., Chen, Z., Delbaere, L. T. J. and Lui, E. (1993) Differences in antigen-binding affinity caused by a single amino acid substitution in the variable region of the heavy chain, *Immunology and Cell Biology* **71**, 239-247.

Yand, W.-P., Green, K., Pinz-Sweeney, S., Briones, A. T., Burton, D. R. and Barabas, C. F. III (1995) CDR walking mutagenesis for the affintiy maturation of a potent human anti-HIV-1 antibody into the picomolar range. *J. Mol. Biol.* **183**, 432-403.

Yang, W.-P., Wu, H. and Barbas, C. F. III (1995) Surface plasmon resonance based kinetic studies of zinc finger-DNA interactions. *J. Immunol. Methods* **183**, 175-182.

Zdanov, A., Li, Y., Bundle, D. R., Deng, S.-J., MacKenzie, C. R., Narang, S. A., Young, N. M. and Cygler, M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 6423-6427.